Apoptotic Capabilities of Porcine Circovirus 1 Viral Protein 3 as a Function of Subcellular Localization

A Major Qualifying Project Submitted to the Faculty of
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirement for the
Degree of Bachelor of Science
By

Katherine Amato
Biochemistry

April 27, 2016
Approved:

Dr. Destin Heilman
Advisor
Department of Chemistry & Biochemistry, WPI

ABSTRACT

Members of the *Circoviridae* family of animal viruses encode a viral protein capable of selectively inducing apoptosis in cancer cells while leaving normal cells intact. Apoptin is nuclear in cancer cells but cytoplasmic in normal cells, whereas PCV1-VP3 exhibits cytoplasmic localization regardless of cell type. This study aimed to create a nuclear localized PCV1-VP3 mutant as well as assess the apoptotic activity of this mutant. A predicted non-classical nuclear export sequence was targeted by site-directed mutagenesis, resulting in enhanced apoptotic ability but continued cytoplasmic localization. Truncated PCV1-VP3 (105-139) was not demonstrated to retain apoptotic activity. The results described here warrant further investigation into the location of PCV1-VP3's functional NES.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Destin Heilman for his guidance and support not only on this project, but throughout my entire undergraduate career. I would also like to thank Edward Partlow and Christine Carbone for their assistance in lab, and Victoria Huntress for her assistance in using the confocal microscope.

BACKGROUND

During 2016, it is estimated that over 1.6 million new cases of cancer will be diagnosed and nearly 600,000 deaths will be attributed to cancer in the United States alone [2]. On a global scale, it is believed that 40% of people will be diagnosed with cancer at some point in their lifetime [2]. There are genetic and environmental risk factors associated with cancer, either of which ultimately results in DNA mutations involving proto-oncogenes, tumor suppressor genes, or DNA repair genes. Disruptions in these essential cellular pathways are hallmarks of cancer. As cellular abnormalities compound and cell proliferation continues, tumors can form and entire organs can be affected by the cancerous growth. The health of cancer patients deteriorates as cellular and metabolic resources are diverted from healthy cells to cancerous cells, negatively impacting the health of the patient. Radiation and chemotherapy are the standard treatment avenues for cancer patients, but unfortunately these methods have not proven successful against all forms of cancer.

Many chemotherapeutic agents attempt to destroy cancer cells via programmed cell death, termed apoptosis. This process is generally regarded as cell suicide and is a normal physiological process in living organisms, but can also be induced during times of cellular stress. Apoptosis can be triggered either by extracellular ligands binding to death receptors on the cell surface or via the release of apoptotic factors from the mitochondria, namely cytochrome c [16, 18]. Programmed cell death differs from necrosis in that it is an organized dismantling of the cell that does not result in activation of the inflammatory response [16]. Apoptosis is executed through the activation of cysteine proteases (caspases) which cleave immediately downstream of aspartic acid residues on peptide

substrates [16]. This cleavage activity not only dismantles certain cellular components, but also activates and recruits additional caspases to further commit the cell to apoptosis [16]. Furthermore, it has been demonstrated that caspase activation is regulated in a p53-dependent pathway.

p53-dependent pathways are often targeted by chemotherapeutic agents as a means of inducing apoptosis in cancerous cells. p53 is a tumor suppressor protein and widely regarded as the "guardian of the genome" for its roles in DNA damage surveillance, cell cycle regulation, and apoptosis [1]. In response to cellular stress, p53 arrests the cell cycle in G1 phase in order to initiate DNA repair pathways or commit the cell to apoptosis when damage is severe [1]. Mutations in p53 disable the cell's ability to detect DNA damage and induce apoptosis, allowing for the continued proliferation of tumors [1]. Given that many chemotherapeutic agents rely on the apoptosis-inducing ability of p53 to destroy cancer cells, cancers that lack a functional p53 gene have demonstrated resistance to traditional therapies. In fact, p53 is mutated in over half of all human cancers, demonstrating a need for treatments that do not require a functional p53 to be present.

In lieu of synthetic agents for targeting p53-independent pathways of apoptosis, cancer treatment may be achieved by the use of animal virus proteins. Members of the viral family *Circoviridae* have been demonstrated to selectively induce apoptosis in transformed (cancerous) cells while leaving primary (normal) cells intact [9]. This is true even of cell lines lacking a functional p53 gene. The *Circoviridae* family includes the Chicken Anemia Virus (CAV) as well as Porcine Circoviruses types 1 and 2. Each virus consists of a circular, non-segmented, single stranded DNA genome that produces an apoptotic protein from its third open reading frame (ORF3) [3]. For CAV, the third viral protein (VP3) resulting from

this open reading frame has been named Apoptin. The protein is well characterized and has revealed insight into p53-independent apoptotic mechanisms.

The selective apoptotic abilities of Apoptin are believed to stem from its cell-type specific localization. Apoptin has demonstrated cytoplasmic localization in primary cells, but nuclear localization in transformed cells [7]. Nuclear localization is necessary for Apoptin to induce apoptosis, and is achieved through nucleocytoplasmic shuttling via CRM1-mediated nuclear export and RanGTPase-mediated nuclear import [6, 13]. In transformed cells, a C-terminal nuclear localization signal (NLS) drives the transport of Apoptin from the cytoplasm to the nucleus. In primary cells, an N-terminal nuclear export signal (NES) retains Apoptin in the cytoplasm. This shuttling activity allows Apoptin to associate with and inhibit the anaphase promoting complex/cyclosome (APC/C), a significant regulator of the cell cycle [19]. This inhibition leads to cell cycle arrest following G_2/M phase and the eventual induction of apoptosis [9]. Apoptin also requires the formation of multimers with itself in order to induce apoptosis in transformed cells [9]. Overall, the p53-indpendent mechanism by which CAV-VP3 (Apoptin) induces cell death has been the subject of intense scrutiny and has provided insight for the development of p53-independent chemotherapeutic agents.

Closely related to the Chicken Anemia Virus is the Porcine Circovirus (PCV). Two types of PCV have been identified so far, PCV1 and PCV2, and are the smallest identified animal viruses [3]. Both types have been found endemic in pig populations, although PCV1 is nonpathogenic while PCV2 has been implicated in postweaning multisystemic wasting syndrome in pigs [5]. Like Apoptin, each PCV possesses an ORF1 and an ORF2 for the transcription of the viral replicase and capsid. Furthermore, ORF3 in each PCV encodes a

third viral protein (VP3) capable of selectively inducing apoptosis in transformed cells [10]. Despite the pathogenicity of PCV2, it has been demonstrated that PCV1 is a more potent inducer of cell death via a p53-indpendent caspase cascade pathway [3]. PCV1 and PCV2 have highly conserved N-terminal regions which are also homologous to Apoptin. Interestingly, PCV1-VP3 contains an extended C-terminal region, making it nearly double the length of PCV2-VP3 as well as CAV-VP3. It has been suggested that the conserved N-terminal region among these viral proteins responsible for the initiation of apoptosis, while PCV1-VP3's extended C-terminal region may account for enhancements in cell death [3].

While Apoptin requires nuclear localization in order to execute apoptosis within cells, PCV1-VP3 exhibits cytoplasmic localization [10]. PCV1-VP3's localization pattern is also independent of cell status, as the protein has been observed in the cytoplasm of transformed cells and primary cells alike [10]. Regardless, PCV1-VP3 is able to induce apoptosis in cells lacking a functional p53 gene, although through a mechanism that differs from Apoptin's. While Apoptin causes cell cycle arrest at G_2/M, PCV1-VP3 halts the cell cycle during G_1 phase [10]. Given the high degree of homology between the two proteins, these findings raise the possibility that protein localization within the cell is not necessarily a requirement for inducing apoptosis in transformed cells.

Scientists have experienced difficulty in trying to elucidate the necessity of Apoptin's nuclear localization. In particular, attempts to disrupt Apoptin's nuclear localization have also disrupted its ability to multimerize, and have subsequently not induced apoptosis. Research efforts have instead focused on creating a PCV1-VP3 which localizes to the nucleus of transformed cells. Creating this mutant variant of PCV1-VP3 requires the disruption of its nuclear export, which is most likely modulated by an NES.

Initial efforts identified two nuclear export sequences utilized NetNES prediction algorithms, and a third NES was discovered by CLUSTALW2 alignment [4]. NES sequences were predicted for amino acids 42-49, 134-142, and 127-136. A series of truncation mutants have since been developed in order to characterize these sequences.

The identified sequences were previously mutated by site-directed mutagenesis such that the resulting sequences would not conform to the consensus NES sequence, and thus disrupt nuclear export [14]. The consensus sequence for CRM1 mediated nuclear export is rich in leucine resides and follows an L-xxx-L-xx-L-x-L motif, where x is any amino acid [11]. In PCV1-VP3, the predicted NES regions do not strictly conform to this sequence, but are rich in leucine residues and suspected of involvement in nuclear export. The LLHL residue sequence at amino acids 46-49 of PCV1-VP3 was mutated to AAHA, and residue sequence 140-142 was mutated from LLL to AAA [4]. Analysis of localization indicated that neither of these mutants disrupted the cytoplasmic localization of PCV1-VP3. Further site-directed mutagenesis targeted additional leucine rich regions of PCV1-VP3, but point mutations to alanine were not sufficient to disrupt nuclear export and cytoplasmic localization [4, 15, 17]. This was true of mutant constructs each containing single point mutations, as well as a construct containing all of the point mutations at each leucine rich region of the protein. Based on these findings, it is possible that PCV1-VP3 achieves nuclear export via a non-classical NES that does not conform to the consensus sequence and was not predicted by computer algorithms.

Additionally, truncated mutants of PCV1-VP3 were produced to observe differences in cellular localization. PCV1-VP3 was broken into two pieces, generating the core region (residues 1-104) and the tail region (residues 105-207). It was found that the core portion

of the protein maintained a diffuse pattern throughout the cell, localizing to neither the nucleus nor the cytoplasm, while the tail region continued to exhibit strong cytoplasmic localization [10]. This implies that a very strong localization signal exists in the tail region of the protein. An even smaller portion of the PCV1-VP3 tail region, residues 105-139, continued to exhibit cytoplasmic localization [15]. Analysis of tail residues 140-207 has yet to be conducted, but could potentially serve to identify the region of PCV1-VP3 responsible for cytoplasmic localization. Furthermore, cell viability assessment of the PCV1-VP3 core and tail regions demonstrated that the tail region alone is capable of inducing apoptosis in transformed cells while the core region caused unremarkable levels of apoptosis [8].

This study aims to further explore the existence of a non-classical nuclear export sequence in Porcine Circovirus type 1 Viral Protein 3. Using site-directed mutagenesis, additional hydrophobic residues in the extended C-terminal region of the protein will be mutated, and the effects of those mutations on cellular localization of the protein will be analyzed. A PCV1-VP3 mutant containing only residues 140-207 will also be constructed and cellular localization observed. Should cytoplasmic localization be disrupted, cell viability and apoptosis assays will confirm the functionality of the protein and determine whether localization is a key component of the ability of PCV1-VP3 and its homologue, Apoptin, to selectively induce apoptosis in transformed cells. This study will also examine the apoptotic abilities of PCV1-VP3 mutants which have already demonstrated clear cytoplasmic localization in order to more accurately assess what segment of the protein is responsible for the enhancement of cell death.

METHODOLOGY

Site Directed Mutagenesis by QuikChange PCR Cloning

Primers were designed to mutate a hydrophobic region in the C-terminal domain of PCV1-VP3 suspected of nuclear export activity. Residues 113 and 114 were both mutated from phenylalanines to threonines via the Stratagene QuikChange II Protocol. The primers were designed as follows, with mutations underlined:

Forward Primer: 5' – GTG GCC TTC TTT ACT GCA GTA <u>ACC ACC</u> ATT CTG CTG GTC GTT TCC TTT - 3'

Reverse Primer: 5' – AAA GGA ACC GAC CAG CAG AAT <u>GGT GGT</u> TAC TGC AGT AAA GAA GGC CAC - 3'

In order to generate the mutant construct, PCR reactions were performed by combining dNTP mix (2.5µL), PCV1-VP3 wild type template (330ng), 10µM forward primer (2.5µL), 10µM reverse primer (2.5µL), 10x reaction buffer (5µL), PFU polymerase (1µL), and ddH_2O (35.5µL) for a total reaction volume of 50µL. A negative control was prepared as above but with the replacement of template DNA with ddH_2O. PCR was performed with an initial denaturation step of 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 3 minutes. A final extension step of 72°C for 5 minutes was performed, and samples were held at 4°C. Prior to DPN1 digestion, 2µL of PCR product was set aside and examined as a negative transformation control. The remaining PCR product was digested with 1µL of DPN1 overnight at 37°C. Final products were stored at -20°C.

Gene Truncation Amplification by GoTaq PCR

Primers were designed in order to amplify the section of the PCV1-VP3 genome corresponding to amino acids 140-207. The forward and reverse primers contained restriction endonuclease sequences on their 5' ends (EcoRI and XbaI, respectively) to allow for insertion into the multiple cloning site of the pEGFP-C1 plasmid vector.

Forward Primer: 5' – GCT CAA GCT TCG AAT TCA CTG CTT CTT AGC AAA ATT - 3'

Reverse Primer: 5' – GAT CAG TTA TCT AGA TCC TCA GTG AAA ATG CCA AGC - 3'

The PCR reaction for the generation of this truncated construct contained PCV1-VP3 wild type template (330ng), 10µM forward primer (2.5µL), 10µM reverse primer (2.5µL), 2x GoTaq Green Master Mix (10µL), and ddH_2O (4µL) for a total reaction volume of 20µL. A negative control was established by replacing the template DNA with ddH_2O. PCR was performed with an initial denaturation step of 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. A final extension step of 72°C for 4 minutes was performed, and samples were held at 4°C. PCR products were separated by 0.9% TAE agarose gel electrophoresis and visualized using ethidium bromide prepared in the gel (1µL).

GoTaq PCR Clean-Up and Gel Purification

The amplicon band containing PCV1-VP3 140-207 was excised from the agarose gel and purified using the Promega Wizard SV Gel and PCR Clean-Up System. An equal volume of Membrane Binding Solution was added to the gel slice (1µL of solution per 1mg of gel) and incubated in an Eppendorf tube at 65°C until the gel was melted. The dissolved gel was transferred to an SV Minicolumn assembly and incubated at room temperature for 1 minute. The assembly was centrifuged at 16,000 rcf for 1 minute and the flow-through

discarded. Membrane Wash Solution (700µL) was added to the column and the assembly was centrifuged again at 16,000 rcf for 1 minute. Flow-through was discarded and 500µL of Membrane Wash Solution was added. The assembly was centrifuged at 16,000 rcf for 5 minutes, and for an additional minute upon removal of the flow-through. The SV Minicolumn was transferred to an Eppendorf tube and 50µL of TE Buffer was added to the column. This was allowed to incubate for 1 minute at room temperature and then centrifuged at 16,000 rcf for 1 minute to elute the purified DNA. The purified DNA concentrations were determined using a NanoDrop Lite Spectrophotometer (Thermo Scientific) and stored at -20°C.

Restriction Digest and Ligation

Following excision and gel purification, amplicon bands were subjected to restriction digest to facilitate cloning into the desired EGFP vector. As EcoRI and XbaI were initially included in the primers, these enzymes were used to digest the PCR products. Reactions were prepared by combining 6µL PCR product, 1µL EcoRI, 1µL XbaI, 2µL Buffer H (Promega), and 10µL ddH_2O. Reactions were allowed to incubate at 37°C. An empty EGFP vector was digested under the same conditions. After 3 hours of incubation, all products were purified using the PCR Purification Protocol above. Vector and insert were combined in a 1:3 ratio, along with 1µL T4 Ligase, 1µL T4 Ligase Buffer, and ddH_2O to a final reaction volume of 10µL. Reactions were incubated at 4°C overnight to allow sufficient ligation of PCV1-VP3 (140-207) into the EGFP vector. Resulting plasmids were transformed into E. coli as described next.

Transformation of JM109 Competent E. Coli

E. coli stocks (50µL) were removed from storage at -80°C and thawed on ice. Once thawed, 2µL of PCR product was added to the cells and mixed. Cells and DNA were allowed to incubate on ice for 20 minutes. Tubes were then heat shocked at 42°C for 60 seconds and returned to ice for an additional 2 minutes. Warm Luria Bertani (LB) broth (450µL) was added to each tube and allowed to recover in a 37°C incubator shaker for 1 hour. Cells (150µL) were plated on LB agar plates containing 1x Kanamycin and incubated overnight at 37°C.

Small Scale Plasmid Purification

Following transformation, colonies were selected from plates and inoculated in culture tubes containing 3mL LB broth and 3µL of 1000x Kanamycin. Culture tubes were allowed to grow overnight in an incubator shaker at 37°C. Plasmids were then purified using the alternative protocol of the Promega PureYield Plasmid Miniprep System. Bacterial culture (1.5mL) was transferred to a sterile Eppendorf tube and centrifuged at 11,000 rcf for 30 seconds. The supernatant was discarded and the remaining 1.5mL of culture added to the Eppendorf tube. The culture was centrifuged again and resuspended in 600µL of TE Buffer. Cells were then lysed by adding 100µL of Cell Lysis Buffer and inverting the tubes six times. Neutralization Solution (350µL) was added and mixed by inverting the tube and additional six times. This lysate was centrifuged at 11,000 rcf for 3 minutes. The supernatant was transferred to the Minicolumn assembly and centrifuged for an additional 15 seconds at 11,000 rcf. Flow-through was discarded and the Minicolumns returned to the assembly. Endotoxin Removal Wash (200µL) was added to the Minicolumns and centrifuged at 11,000 rcf for 15 seconds. Column Wash Solution (400µL)

was added and the Minicolumns were centrifuged at 11,000 rcf for 30 seconds. The Minicolumns were then removed from their collection tubes and placed into sterile Eppendorf tubes. Elution Buffer (30µL) was added to the membranes of the Minicolumns and allowed to stand for 1 minute at room temperature. This setup was centrifuged for 15 seconds at 11,000 rcf, and the eluted plasmid was stored at -20°C. The purified DNA concentrations were determined using a NanoDrop Lite Spectrophotometer (Thermo Scientific) and sent to Macrogen USA for sequencing.

Medium Scale Plasmid Purification

LB broth (100mL) containing 100µL of 1000x Kanamycin was inoculated with transformed *E. coli* and incubated in an incubator shaker at 37°C overnight. The DNA plasmid was purified as described in the PureYield Plasmid Midiprep System TM253. After overnight growth, cells were transferred to conical tubes and pelleted at 5,000 rcf for 10 minutes. The supernatant was discarded and the pellet resuspended in 3mL of Cell Resuspension Solution. Cell Lysis Solution (3mL) was added and tubes inverted five times. Tubes were allowed to sit at room temperature for 5 minutes before adding 5mL of Neutralization Solution and inverting an additional ten times. This lysate was centrifuged at 15,000 rcf for 15 minutes. Clearing columns were placed into binding columns, and the column assembly attached to a vacuum manifold. The lysate was transferred to the clearing columns and the vacuum was applied until all liquid had passed through both columns. Clearing columns were removed and discarded. Endotoxin Removal Wash (5mL) was allowed to pass through the binding column, followed by 20mL of Column Wash Solution. The membrane was dried for 1 minute and the clearing column placed into a 50mL conical tube. Nuclease Free Water (600µL) was added to the membrane and the assembly was

centrifuged at 1,500 rcf for 5 minutes in a swinging bucket rotor. The eluted plasmid DNA was transferred to an Eppendorf tube, concentration determined by a NanoDrop Lite Spectrophotometer (Thermo Scientific), and stored at -20°C.

Cell Culture Maintenance

p53-null, non-small cell lung carcinoma cells (H1299) were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin/ Streptomycin/ Fungicide (D10 Medium). Cells were passed when they reached approximately 80% confluency in order to maintain proper growth conditions.

To pass cells, old D10 Medium was aspirated off cells. Cells were washed with 1x Phosphate Buffered Saline (PBS) and treated with 0.5% trypsin. The trypsin was curtained six times over the cells and immediately aspirated off. Fresh D10 Medium was added to the cells and was vigorously pipetted in order to prevent cell clumping. Cells were transferred to a new culture flask and provided additional fresh D10 Medium. Culture flasks were incubated at 37°C and 5% CO_2 humidity until cells were either required for experiments or required additional passaging.

Transient Transfections

Cells were passaged into a 6-well plate prior to transfection and allowed to reach approximately 80% confluency. For single well transfections yielding 60% transfection efficiency, an optimized amount of plasmid DNA (Table 1) was diluted in 100µL of Buffer EC (Effectene Transfection Reagent Kit, Qiagen). Enhancer (3.2µL) was added to the mixture, vortexed, and incubated for 5 minutes at room temperature. Effectene Reagent (10µL) was added and flicked to ensure mixing of components. While tubes were allowed to incubate for 15 minutes to ensure DNA complex formation, old D10 Medium was

aspirated from cells. The cells were washed with PBS and provided 1.5mL of fresh D10 Medium. D10 Medium (600µL) was also added to DNA complexes, pipetted to ensure mixing, and added to the cell well. Plates were gently swirled and returned to the incubator. As negative apoptosis controls, certain wells received pEGFP-C1 with no insert or no transfection at all.

Table 1: Optimal amount of plasmid DNA for transient transfection of H1299 cells (per single well)

EGFP-tagged construct	DNA (µg)
wt PCV1-VP3 (1-207)	0.8
PCV1-VP3 (105-139)	0.4
PCV1-VP3 ΔTT	0.9
pEGFP	0.8

In order to assess localization, cells were fixed and stained 24 hours after transfection. Cells were first washed twice with 1mL PBS, and then fixed in 1mL 4% paraformaldehyde for 15 minutes on a 3D rotator. The glass coverslips were mounted on microscopy slides in 15µL mounting media supplemented with DAPI. Fluorescent images of cellular GFP and DAPI signals were obtained using a Leica SP5 point scanning confocal microscope.

Analysis of Cell Images

Fluorescent images were analyzed using ImageJ to determine the relative GFP signal in the nucleus compared to the cytoplasm. The corrected total cell fluorescence (CTCF) for each location was calculated separately, using the equation:

$$CTCF = integrated\ density - (area * mean\ fluorescence\ of\ background)$$

Mean background fluorescence was obtained by measuring fluorescence in areas adjacent to each examined cell. The nucleus was selected for and measured as the area

within the cell indicated by DAPI staining, and the cytoplasm was selected as the area of the entire cell minus the area occupied by the nucleus. The ratio of nuclear to cytoplasmic CTCF was calculated for each of three cells containing the appropriate construct, or four cells in the case of PCV1-VP3 ΔTT.

Cell Viability Assay

Cell viability was assessed as previously described [8]. Two days post-transfection, unfixed cells were examined using a Zeiss Vert.A1 fluorescence microscope and SPOT 5.2 Basic software to detect levels of GFP-tagged protein expression. Upon confirming protein expression, cells were selected for with the antibiotic G418 (800mg/mL) for two days. This ensured that all transfected cells remaining on the plate were EGFP positive. G418 selection was continued for an additional 2 days at a higher antibiotic concentration (1200mg/mL). As controls, two wells containing EGFP-C1 transfected cells or non-transfected cells were maintained in non-selective D10 Medium.

To assess cell viability following G418 selection, cells were first washed twice with 1mL of 1% PBS and then fixed for 15 minutes with 1mL of 4% paraformaldehyde prepared in 1% PBS. Cells were washed twice more with 1mL of 1% PBS, and the PBS aspirated off. Cells were stained for 20 minutes with 0.75mL of 0.1% (w/v) crystal violet prepared in 10% ethanol, then washed with distilled water until all excess dye was removed. Plates were photographed for visual assessment of cell viability. Cells were then solubilized in 2% (w/v) sodium dodecyl sulfate and rocked for 1 hour. The optical density of the solubilized dye was measured at 590nm using a Genesys 20 Visible Spectrophotometer (Thermo Scientific). Optical density was converted to percent cell viability by the formula:

$$Percent\ Cell\ Viability\ = \frac{\text{Optical density value of experimental group}}{\text{Optical density value of control group}} \times 100\%$$

RESULTS

As more than half of human cancers contain mutations in the tumor suppressor gene p53, there is a clear need for cancer therapies which kill cancer cells independently of the p53 pathway. Interestingly, the *Circoviridae* family of animal viruses translates viral proteins which have been shown to induce apoptosis in cancer cells while leaving normal cells intact, and do so in a p53-independent manner [9]. This is promising for cancer therapies, as it would reveal a mechanism for specifically targeting cancerous cells but not interfering with the normal metabolic activities of non-cancerous cells. Two circoviruses, chicken anemia virus and porcine circovirus, have become the subjects of intense scrutiny regarding whether subcellular localization of each VP3 is a necessary component in the inducement of apoptosis. It has been demonstrated that CAV-VP3 is localized to the cytoplasm of normal cells, but undergoes nucleocytoplasmic shuttling to localize to the nucleus of cancerous cells and consequently induce apoptosis. In contrast, PCV1-VP3 exhibits cytoplasmic localization regardless of cell state, but only induces apoptosis in cancerous cells. Despite their high degree of similarity, this difference in localization status has prompted investigation into the necessity of localization as a requirement for the induction of apoptosis.

Previous attempts as disrupting the cytoplasmic localization of PCV1-VP3 have targeted leucine-rich regions of the protein that show a high degree of conformity with the established sequence for nuclear export sequences [4, 15, 17]. Microscopy following tagging with green fluorescent protein (GFP) indicated that these mutations did not disrupt localization of the protein, and indicated that alternative strategies must be employed to achieve nuclear localization of PCV1-VP3. Previous mutants also mutated leucine residues

to alanine residues, calling into the question whether more drastic changes must be made to the amino acid sequence of the protein in order to achieve a noticeable disruption of localization. Here, a mutant of PCV1-VP3 was generated that mutated two adjacent phenylalanine residues (113-114) to threonine residues in a region predicted to have transmembrane activity [12]. The sequence in question here is a structure consisting of twelve hydrophobic residues and one threonine residue. This potential transmembrane region of the protein may also explain the punctate diffusion pattern of PCV1-VP3, and mutating this sequence could have consequences on protein function beyond localization. This construct, PCV1-VP3 ΔTT, is shown in Figure 1 with sequence confirmation shown in Figure 2.

In addition to PCV1-VP3 ΔTT, a mutant construct was designed that only contained residues 140-207 of the wild type viral protein. The design of this construct stemmed from the observation that PCV1-VP3 (105-139) continued to exhibit strong cytoplasmic localization [15]. Given that the tail region of PCV1-VP3 as a whole is cytoplasmic and retains apoptotic capabilities, PCV1-VP3 (140-207) was designed to examine whether these properties are present in a small truncation of the tail region. The apoptotic abilities of PCV1-VP3 (105-139) were examined for the same reason. The individual localization patterns of these truncated mutants should indicate which half of the tail region is the cause of cytoplasmic localization, and may also reveal information about PCV1-VP3's enhanced ability over related VP3's to induce apoptosis. Construction of the PCV1-VP3 mutant containing residues 140-207 is still underway.

Localization of PCV1-VP3 ΔTT was assessed by transiently transfecting the GFP-tagged proteins into H1299 cells. Cells were allowed to express the protein for 24 hours,

and then fixed and mounted for analysis with confocal microscopy. Figure 3 shows that cells transfected with pEGFP demonstrated near equal amounts of fluorescence in both the nucleus and cytoplasm. In contrast, wt PCV1-VP3 transfected cells had considerably more fluorescence in the cytoplasm. Cells transfected with the mutated construct, PCV1-VP3 ΔTT, showed a fluorescence pattern more consistent with that of wt PCV1-VP3, localizing heavily to the cytoplasm. These visual results were quantified as corrected total cell fluorescence, shown in Figure 4. A nuclear to cytoplasmic ratio of fluorescence equaling a value of 1 indicates non-specific subcellular localization, as indicated for the pEGFP transfected cells. Ratios less than 1 indicate preferred steady-state localization to the cytoplasm, as was found for both wt PCV1-VP3 (0.12 ± 0.07) and PCV1-VP3 ΔTT (0.19 ± 0.04). Student's t test revealed no statistical significance between the localization patterns in the localization of wt PCV1-VP3 and PCV1-VP3 ΔTT, $p = 0.7633$.

Following localization analysis, mutant constructs were assessed for their ability to kill cancer cells via apoptosis-mediated cell death. Cells were plated in 6-well dishes and transfected to achieve approximately 60% transfection efficiency. All constructs were in EGFP vectors containing a gene for kanamycin resistance, making them resistant to the highly similar mammalian antibiotic Geneticin (G418). Cells underwent four days of G418 selection and were then stained with crystal violet to assess the percentage of remaining, viable cells. This was quantified by solubilizing cells in SDS and measuring optical density (Figure 6).

Figure 5 depicts the visual results of the cell viability assay. Untransfected H1299 cells were included as controls in order to demonstrate the effect of G418 on cell viability in cells containing a gene for resistance compared to cells lacking the resistant plasmid. An

empty pEGFP vector was transfected into cells and used as a negative apoptosis control. Cell viability is indicated as the number of cells that survive both G418 selection and apoptosis. As expected, cells in non-selective media grew to confluency and were viable regardless of transfection status. In the presence of G418, it was observed that untransfected cells were no longer viable and thus unable to be visualized by crystal violet staining. As expected, the well containing pEGFP under G418 selection was not completely viable, as not all cells were transfected with the construct and thus were selected against, independent of apoptosis induction.

An apoptosis baseline was established by transiently transfecting cells with wt PCV1-VP3, a construct known to induce apoptosis. As expected, the well containing wt PCV1-VP3 had less viable cells than those in the well transfected with empty pEGFP. Wells containing constructs of unknown apoptotic activity were primarily compared to the pEGFP and wt PCV1-VP3 wells also undergoing G418 selection. It was observed that the crystal violet staining and cell viability of cell wells containing PCV1-VP3 (105-139) more closely resembled the appearance of pEGFP transfected cells. The presence of more viable cells indicates that PCV1-VP3 (105-139) does not possess the same apoptotic capabilities of wt PCV1-VP3, if any apoptotic activity at all.

Also examined for the ability to induce apoptosis was the construct PCV1-VP3 ΔTT. This protein was mutated at residues predicted to coincide with a non-classical nuclear export signal and well as transmembrane activity. The point mutation introduced here did not disrupt the localization of PCV1-VP3, as the protein remained predominantly cytoplasmic. Despite being unable to disrupt localization, it was still possible that the mutation targeted a region of the protein necessary for the induction of apoptosis in H1299

cells. The results here indicate that this is the not case, as cell wells transiently transfected with this construct were almost completely unviable, visually resembling cells that received no transfection at all. In fact, the PCV1-VP3 ΔTT appears to have enhanced apoptotic activity compared to the wild type protein.

These visual results were recapitulated upon solubilizing the viable cells in SDS and quantifying cell viability in a spectrophotometer. All optical density results for cell viability were normalized to the viability of the non-transfected, non-selected for H1299 cells. Cells transfected with EGFP and selected for by G418 served as a baseline for the absence of apoptosis in transfected constructs. Figure 6 indicates that 9.95% of these EGFP transfected cells survived the selection process. When cells were transfected with wt PCV1-VP3, a construct known to induce apoptosis, only 4.50% of cells were viable. Student's t test revealed this difference in cell viability to be statistically significant, $p = 0.0102$. PCV1-VP3 (105-139) was shown to behave nearly identical to the empty EGFP vector, as 11.92% of cells were viable. Student't t test verified that there was no statistical indication of this construct's ability to induce apoptosis, $p = 0.6820$.

PCV1-VP3 ΔTT, qualitatively shown in Figure 5 to be less viable than EGFP transfected cells, was confirmed by SDS solubilisation to result in fewer viable cells. The construct was shown to significantly induce apoptosis compared to EGFP constructs by Student's t test, $p = 0.0041$. However, statistical comparison to wt PCV1-VP3 for the ability to induce apoptosis did not reveal statistical significance, $p = 0.0857$.

DISCUSSION

There is an ever growing need for cancer treatments which effectively eliminate cancerous cells from the body without having detrimental effects on the patient. Interestingly, the third viral protein of the *Circoviridae* family possesses an inherent ability to kill cancer cells via apoptosis while leaving normal cells unharmed. A better understanding of how these proteins induce apoptosis may reveal targets for drug action and lead to new cancer therapeutics. Currently, protein localization as it relates to the ability to induce apoptosis is under intense scrutiny. A well characterized VP3, Apoptin, has demonstrated nuclear localization in cancer cells but cytoplasmic localization in normal cells, and it is believed that this plays a role in the induction of apoptosis. However, the homologous protein PCV1-VP3 exhibits the same apoptotic selectively, but is exclusively localized to the cytoplasm regardless of cell status. This study aimed to build upon previous efforts to characterize localization behavior as a necessary requirement for PCV1-VP3 to induce apoptosis in cancer cells.

Previous studies which altered the localization of Apoptin were shown to disrupt a number of cellular activities required for apoptosis, making it an unsuitable candidate for localization studies [9]. As such, PCV1-VP3 has been the main mutagenesis target for localization studies that seek to assess apoptotic potential. Point mutations have been introduced into PCV1-VP3 at numerous locations, including residues 46-49, 115-117, 121-126, 134-136, and 140-142 in attempt to disrupt classically predicted nuclear export sequences [4, 15, 17]. However, none of these point mutations resulted in altered subcellular localization of PCV1-VP3, leading to the idea that PCV1-VP3 may contain a non-classical NES. It should be noted that each of the aforementioned mutations was also fairly

conservative mutation not resulting in drastic changes to the hydrophobicity of the protein. In each case, leucine or isoleucine residues were replaced with alanine residues. It is possible that the NES of PCV1-VP3 has a loose requirement for hydrophobic residues, which would account for the unaltered subcellular localization of the protein in previous studies.

Here, a more drastic point mutation was introduced into PCV1-VP3 which mutated adjacent phenylalanine residues to threonines at positions 113-114 (PCV1-VP3 ΔTT). Residues 108-120 have also been predicted to possess transmembrane activity [12], and a mutation at within this region might disrupt that supposed association as well. Even so, PCV1-VP3 ΔTT remained predominantly localized to the cytoplasm of H1299 cells, and continued to exhibit a punctate and presumably vesicular pattern when analyzed under a fluorescent microscope. In addition to remaining cytoplasmically localized, PCV1-VP3 ΔTT demonstrated enhanced apoptotic induction in transfected cancer cells when compared to wt PCV1-VP3. It may be worthwhile to introduce a more drastic mutation such as this into previously mutated regions of PCV1-VP3. Non-functionally equivalent mutations in regions of the protein classically predicted to have NES activity may in fact induce nuclear localization of PCV1-VP3. This would enforce the idea that PCV1-VP3's nuclear export mechanism only has a loose requirement for hydrophobic residues in order to function normally.

Though this mutated region of PCV1-VP3 appears to be nonfunctional in determining protein localization, it does appear to be highly involved in the induction of apoptosis. It is possible that residues 113 and 114 are components of the death effector domain of PCV1-VP3, and that the mutation from phenylalanine to threonine was a gain of

function mutation rather than a loss of function mutation. Alterations in the transmembrane activity of PCV1-VP3 ΔTT were not assessed beyond visual assessment using fluorescent microscopy, and it would be interesting to see a more in depth study that examines what effect this mutation had on the transmembrane properties of the protein. If PCV1-VP3 is in fact a transmembrane protein, this would further distinguish it from its Apoptin homologue and call into question whether these proteins induce apoptosis via the same mechanism.

In a separate attempt to locate a functional, non-classical NES sequence in PCV1-VP3, a truncated mutant was generated that contained only residues 105-139 of the full length protein [14]. This mutant was demonstrated to retain cytoplasmic localization, but has yet to be compared to its complement, a truncated protein consisting of residues 140-207. Although this previous study demonstrates that PCV1-VP3 (105-139) is likely involved in nuclear export, the same could be true of PCV1-VP3 (140-207), though this mutant is still under construction and results have yet to be obtained with regards to its localization patterns. If PCV1-VP3 contains multiple NES sequences, a comparative examination of these two mutants could reveal a more specific region to target with site-directed mutagenesis. It may also be a worthwhile study to attach an exogenous NLS sequence to the full length, wild type PCV1-VP3 to induce nuclear localization and determine the effect on apoptotic induction.

Localization aside, PCV1-VP3 (105-139) was examined for its ability to induce apoptosis in the absence of the majority of the protein. Unsurprisingly, this fragment was unable to induce apoptosis; this truncated mutant represents such a small portion of the protein that it is unlikely to have folded properly. An interesting set of experiments might

look at the core region of PCV1-VP3 (1-104) fused to either half of the tail region, be it residues 105-139 or 140-207, which may allow for proper protein folding and better assessment of each individual fragment. It has been previously demonstrated that the tail domain of PCV1-VP3 has enhanced apoptotic activity over the wild type protein, and a study such as this might reveal which portion of the tail is responsible for this enhancement.

To date, a functional NES sequence in PCV1-VP3 has yet to be found and disrupted in order to force the nuclear localization of the protein. Should nuclear localization of the protein be achieved, subsequent experiments might reveal interactions essential for the protein's induction of apoptosis. As PCV1-VP3 becomes more characterized and better understood, the possibility of identifying drug targets for the selective treatment of cancer cells becomes more attainable.

FIGURES

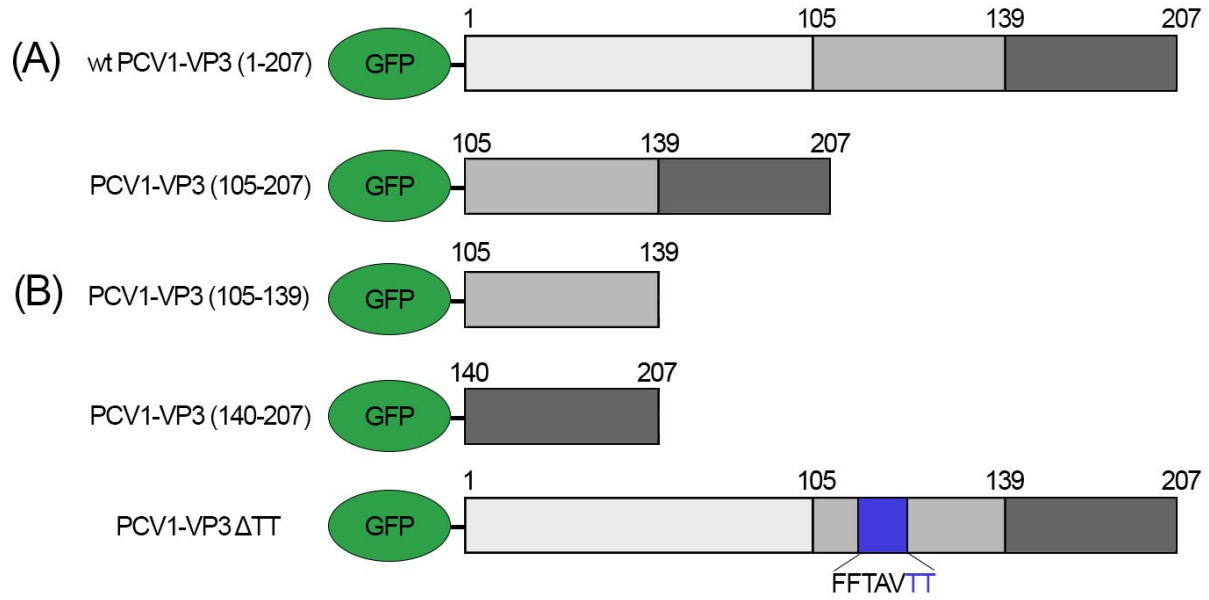

Figure 1. Schematic constructs of generated PCV1-VP3 mutants. All constructs above were cloned into pEGFP-C1 vectors in order to create N-terminally tagged EGFP constructs. (A) PCV1-VP3 constructs previously demonstrated to have apoptotic activity in p53-null cancer cells. (B) PCV1-VP3 constructs generated for further determination of NES location and relationship between subcellular localization and apoptotic activity. Construction of PCV1-VP3 (140-207) is undergoing.

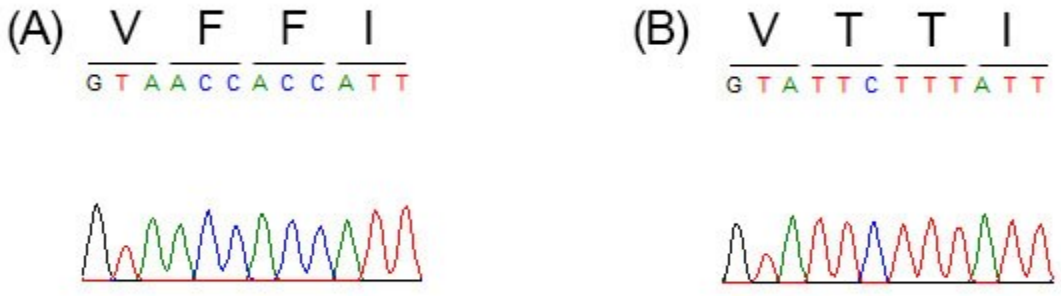

Figure 2. Sequence Analysis of PCV1-VP3 ΔTT mutant construct. Electropherograms depicting the (A) wild-type PCV1-VP3 sequence and (B) Quikchange PCR generated PCV1-VP3 ΔTT construct (Macrogen USA). Amino acid sequences are shown in black.

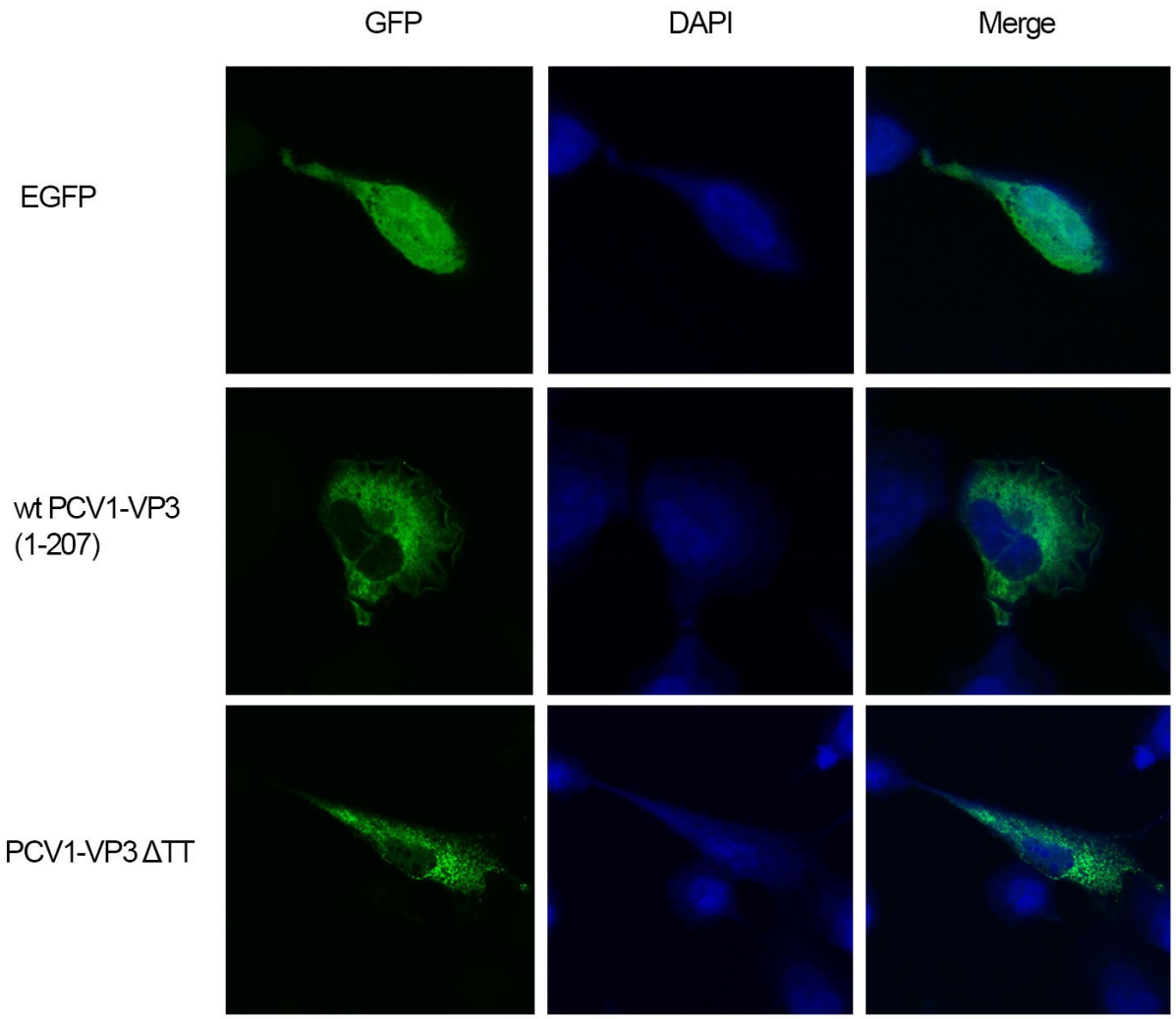

Figure 3. Confocal microscopy of cells fluorescently expressing EGFP and DAPI. Confocal fluorescence microscopy images of transiently transfected H1299 cells at 63x magnification. Localization behavior of GFP-tagged constructs is compared to DAPI nuclear staining. The pEGFP vector shows a diffuse expression pattern throughout the cell, while both wt PCV1-VP3 and PCV1-VP3 ΔTT exhibit stronger cytoplasmic localization.

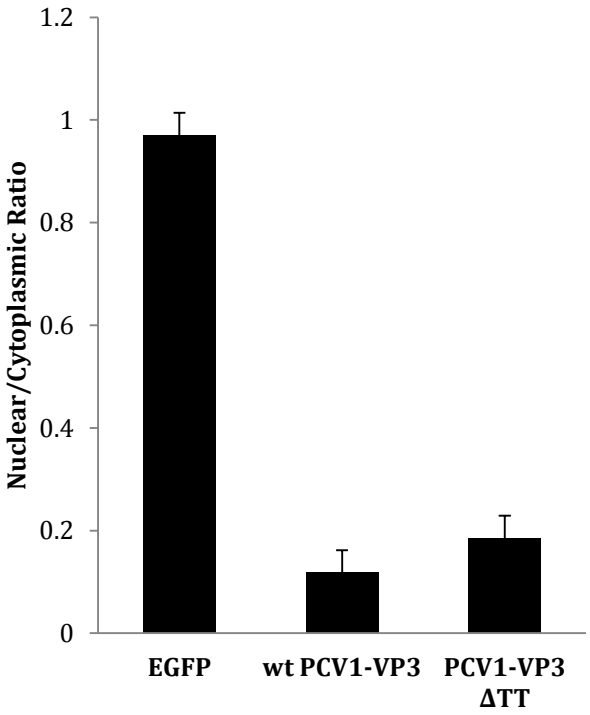

Figure 4. Calculated nuclear/cytoplasmic ratios for mutated PCV1-VP3 constructs. EGFP displays a diffuse and nonspecific localization pattern throughout the cell, represented by a nuclear/cytoplasmic ratio of approximately 1. Both wt PCV1-VP3 and PCV1-VP3 ΔTT exhibited heavy cytoplasmic localization. Student's t test revealed no statistical significance in the localization patterns in the localization of wt PCV1-VP3 and PCV1-VP3 ΔTT, $p = 0.7633$.

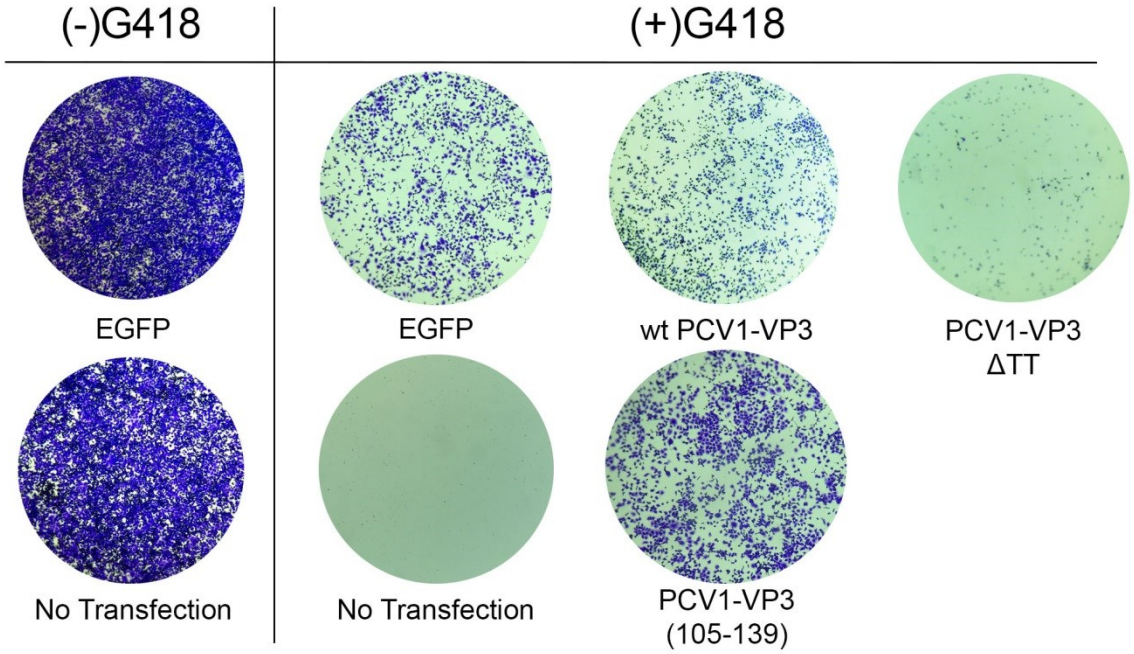

Figure 5. Viability of transiently transfected H1299 cells. Crystal violet staining was performed to determine the number of viable cells after introducing PCV1-VP3 constructs into cells, with darker staining indicating more viable cells. Cells were also exposed to the mammalian antibiotic Geneticin (G418) to select against non-transfected cells. EGFP transfections served as a transfection control and indicator that G418 selection was effective. wt PCV1-VP3 was considered to be the standard for apoptosis induction. PCV1-VP3 (105-139) shows a similar level of cell viability compared to the non-apoptotic EGFP vector, revealing that the construct is non-apoptotic. Decreased cell viability for cells transfected with PCV1-VP3 ΔTT indicates that the mutation enhanced the protein's ability to induce apoptosis.

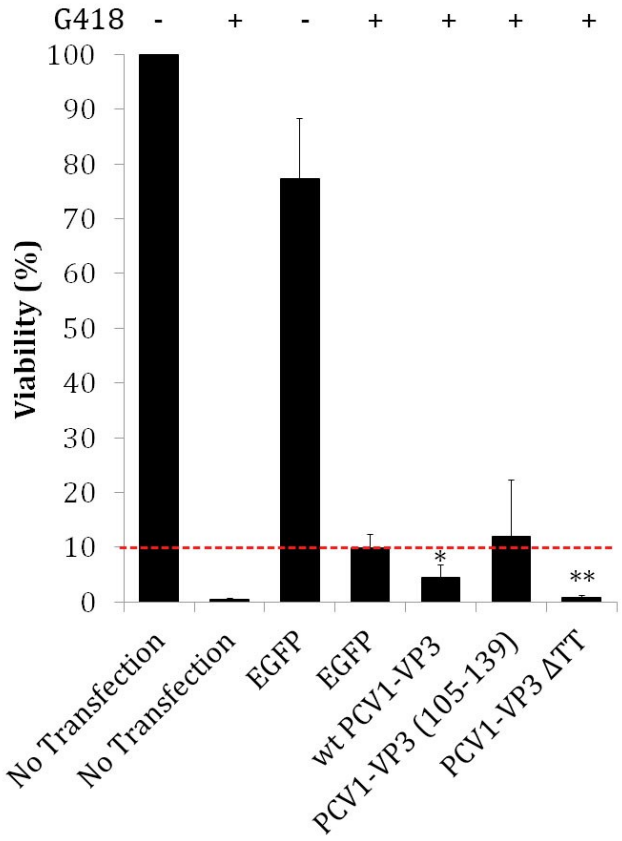

Figure 6. Quantification of cell viability. The remaining viable cells were solubilized in SDS and analyzed at 590nm using a Genesys 20 Visible Spectrophotometer (Thermo Scientific). Quantified absorbance results recapitulate the conclusions reached by visual inspection of non-solubilized cells. Transfection with wt PCV1-VP3 was shown to correlate with significantly less viable cells (Student's t test, $p = 0.0102$) when compared to empty EGFP vector. The same is true of PCV1-VP3 ΔTT when compared to empty EGFP vector (Student's t test, $p = 0.0041$).

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