

# Apoptin and PCV1-VP3 Selectively Induce Apoptosis in Transformed Cells via a Conserved Mechanism Mediated by the Anaphase Promoting Complex

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By

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

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## Abstract

The *circoviridae* family contains viruses that produce proteins capable of selectively killing cancerous cells in mammals, yet little is known about the mechanism of these proteins. Cell cycle analysis and localization studies of cells expressing these proteins reveal phenotypic differences, suggesting that different homologues may induce apoptosis via distinct mechanisms. Prior research has yielded little information to support or refute said assumption. Our experiments have yielded evidence that suggests Chicken Anemia Virus ORF 3 (apoptin) and Porcine Circovirus Type 1 ORF 3 (PCV1-VP3) both interact with the anaphase promoting complex subunit cdc27, supporting a hypothesis that the mechanism of induced cell death is conserved among the species, and that the observed phenotypic differences between cells expressing these proteins are ancillary to the effect. This implies that the extraneous phenotypes do not necessarily need to be addressed in the development of a small molecule drug that recapitulates the effect of the circovirus ORF 3 proteins.

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## Introduction

Viruses are a fascinating class of microorganisms that employ diverse strategies as part of their development and survival. Interestingly, viruses can only divide inside of a host cell, causing much debate about whether they are organisms at all. Because of the direct dependence of a virus on its host, viruses evolve along with the host in a way that promotes proliferation of the virus. Sometimes this occurs in a symbiotic way, but more commonly, viruses infect their host in a way that is not necessarily essential or beneficial to the host, and may even be harmful. A prevalent example of this type of virus is Herpes Simplex Virus 1. This is the virus responsible for cold sores in humans, and has been evolving along with humans for at least 60,000 years<sup>[1][6]</sup>. Very rarely will viruses evolve such that they are fatal to the host. The host is essential to the survival of the virus, thus killing the host would also extinguish the virus. Usually viruses that are lethal, such as Ebola, are viruses that evolved to be nonlethal in one host (believed, in the case of Ebola, to be bats), but then mutate in a way that allows them to become infectious, and potentially lethal, to another species<sup>[3]</sup>.

Because animal viruses often evolve along with the host organism, the life cycle of the virus is often intrinsically tied to the organism and cell type that it infects. The life cycle of the virus can be broken into three main stages. The first stage is the entry of the virus into the cell, the second stage is the replication of the virus, and finally, the newly synthesized virus exits the cell and is able to infect more cells. One of the mechanisms by which viruses exit cells is by inducing apoptosis, which is a cellular process resulting in programmed cell death<sup>[9]</sup>.

In order to fully understand the evolved strategies of viruses, it is important to understand the behavior of the host cell. Similar to viruses, cells also have a life cycle,

nominally the cell cycle. Immediately after a cycle of division, a nascent cell enters G<sub>1</sub> phase, which is a period of cellular growth before the synthesis of DNA. Partway through G<sub>1</sub> phase, if a cell is not going to divide again, it will enter a phase called G<sub>0</sub>, which can be considered outside of the traditional cell cycle. For a cell that is intended to continue dividing, it will remain in G<sub>1</sub>. The amount of time spent in G<sub>1</sub> is in part regulated by the anaphase promoting complex, or APC. The APC is an ubiquitin ligase that targets certain proteins for degradation in order to regulate cellular processes. One class of proteins that serve as substrates for the APC are the cyclins, which are produced and degraded temporally so the relative level of various cyclins at a given time provides the cell with information about what functions should be occurring. The cyclin environment of the cell ultimately produces a complex feedback network that is interactive with cellular processes and guides the timing of the cell cycle. In G<sub>1</sub>, the APC is active as a complex with cdh1 (APC<sup>cdh1</sup>), which holds the cell in G<sub>1</sub> until the cell has grown sufficiently to support cell division. This is achieved through ubiquitination and subsequent degradation of cyclins A and B, as well as the cell division cycle protein cdc20. When the cell is ready to divide, APC<sup>cdh1</sup> becomes inactive and the cell enters S phase, which is the phase of the cell cycle where DNA synthesis occurs. Once DNA synthesis is complete, another period of growth occurs, called G<sub>2</sub>. During this period of growth, extensive processes occur to ensure that DNA replication has occurred with fidelity. Excessive errors will result in recruitment of DNA damage response mechanisms which, if the damage is severe enough, will induce cell cycle arrest in G<sub>2</sub>. If the damage is completely irreparable, a pathway mediated by a protein called p53 will result in apoptosis. Once the DNA checkpoint is complete, the cell progresses into metaphase (M phase) where the chromosomes are aligned and divided between the two daughter cells. This is initiated in

part by the APC complexed with cdc20 (APC<sup>cdc20</sup>), which initiates ubiquitination and degradation of securin and allows separation of chromosomes. Finally, the two nuclei are formed, the actual fission of the cells occurs (cytokinesis), and the two new cells enter G<sub>1</sub>. Sometimes viruses will contain proteins that will alter the cell cycle in some way to encourage their own replication<sup>[10]</sup>. Such is the effect of a protein encoded by some viruses in the family *circoviridae*.

*Circoviridae* are small, non-enveloped viruses with 1-4kb single stranded DNA genomes. These are animal viruses that are known to infect pigs, ducks, chickens, dragonflies and cockroaches<sup>[8]</sup>. The most studied virus in the family is the chicken anemia virus (CAV), which is studied for two major reasons. One is that this virus is responsible for death of young chickens and results in severe impacts on farming. In this context, the actual pathogenesis is not studied as much as development of treatments and vaccinations. The other area of study of CAV is in cancer research. This is because the ORF-3 protein of CAV, called apoptin, has a remarkable ability of selectively arresting and killing human cancer cells, while leaving normal cells unaffected<sup>[1]</sup>. As mentioned above, viruses that evolve in one species yet infect another have drastically different effects. Although CAV does not infect human cells, expressing the third viral protein manifests a novel effect not necessarily present in infected chickens. This is the property of most interest to our studies. What is particularly intriguing is that apoptin achieves this property in the absence of the tumor suppressor protein p53, which is generally required to be present for the efficacy of most common chemotherapeutics<sup>[11]</sup>. It is important to note that CAV did not evolve to produce this activity in human cells, and the fact that it harbors this activity is likely explained by some behavior of the virus as it occurs naturally in chicken cells. Unfortunately, very little



study has been done on the evolutionary push for this behavior, rather it has been almost exclusively studied in human cells.

One of the foundational studies of the mechanism by which apoptin induces apoptosis showed an interaction with the anaphase promoting complex (APC) to cause cell cycle arrest in the G<sub>2</sub>/M phase<sup>[4]</sup>. The researchers show that apoptin coprecipitates with cdc27, a subunit of the APC. The researchers hypothesize that the association of apoptin with the APC leads to its destabilization, preventing degradation of securin and inducing the G<sub>2</sub>/M arrest, although no conclusions are drawn on the molecular mechanism responsible for apoptosis.

Another characteristic property of apoptin that accompanies the apoptotic selectivity is a localization response. While primary cells express apoptin as an aggregated cytoplasmic protein, transformed and cancer cells express apoptin in their nucleus<sup>[4]</sup>. It has often been assumed that this localization is directly related to the apoptotic mechanism. Another group's research showed that the localization of apoptin is dependent upon the DNA damage response mechanisms, specifically responding to the proteins Ataxia Telangiectasia Mutated Kinase (ATM) and DNA dependent protein kinase (DNA-PK)<sup>[7]</sup>. It is interesting to note that *in vivo*, the chicken anemia virus infects T cell progenitors, in which V(D)J recombination production of antibodies occurs, which involves a DNA intermediate with a double strand break and DNA-PK activity, suggesting that the mechanism by which apoptin is selective to cancer cells relates to the mechanism by which the virus is selective to these immune cells<sup>[12]</sup>.

Although the research alluded to thus far show a direct correlation between the localization behavior and apoptotic activity, more recent studies of a homologous protein

challenge the relation. The third ORF protein from another member of *circoviridae* called porcine circovirus 1 (PCV1-VP3) also exhibits cell type specific apoptosis, yet always exists in the cytoplasm<sup>[5]</sup>. Although PCV1-VP3 is approximately twice the length of apoptin, the N-terminal portion has significant sequence homology. Because of the similar phenotype as well as sequence homology, it is easily assumed that the two proteins induce apoptosis via a similar or shared mechanism, and the localization change in apoptin is ancillary. However, cell cycle analysis experiments conducted with PCV1-VP3 shows that the cell is arrested in G<sub>1</sub> phase, instead of G<sub>2</sub> phase as with apoptin<sup>[5]</sup>. This leaves an unanswered question as to the relationship of the mechanisms of apoptin and PCV1-VP3.

The project described below aims to address the question of the relationship between the two proteins by comparing their molecular behaviors *in vivo*, as well as the behavior of a mutant created by recombinantly adding the non-homologous C-terminal region of PCV1-VP3 to the C-terminus of apoptin, we named this mutant mut-AP. The purpose of mut-AP is to isolate any differences in activity of apoptin and PCV1-VP3. If there is a difference, the behavior of mut-AP would give us evidence as to whether the difference is due to the differences in the core, or the presence of the tail.

Ideally, the information gained from this project can serve to advance our lab's research into how these fascinating viral proteins can be used to fight cancer. A conserved mechanism between apoptin and PCV1-VP3 would be a promising result, suggesting a simpler activity that may be recapitulated by a small molecule, ultimately becoming a chemotherapeutic.

## Materials and Methods

### Overlap Extension PCR Cloning

Primers were designed that would amplify the non-homologous C-terminal domain of the PCV1-VP3 gene while adding overhangs. The 5' overhang consisted of an 18 nucleotide sequence complementary to the final 18 nucleotides of apoptin, minus the stop codon. The 3' overhang consisted of an 18 nucleotide sequence complementary to the stop codon in the apoptin plasmid and the subsequent 15 nucleotides of the plasmid. An initial PCR was performed. Gotaq master mix was used in the reaction. The sample was first heated to 95°C for 2 minutes. A three stage cycle, 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 68°C, was cycled 30 times. Finally, the sample was held at 68°C for 2 minutes, and then stored. The PCR product was then purified with the Promega Wizard SV PCR and gel cleanup kit. The DNA content was quantified with spectroscopy. In the second reaction, Q5 DNA polymerase was used. The product of the first reaction was used as the primer in this reaction. The template was Apoptin in pEGFP-C1. The reagents were mixed such that the primer was at ~250x the concentration of the template. The reaction was cycled similarly to the first stage PCR, except the 68°C step was adjusted to 72°C and lasted for 6 minutes, 40 seconds. Additionally, the reaction was only cycled 17 times. 1 unit of *dpnI* restriction endonuclease was added to the product to degrade the template DNA. The sample was incubated at 37°C for 4 hours and transformed into JM109 competent E. Coli.

### Wizard SV PCR and Gel Cleanup

Equal volume of Promega Membrane Binding Solution was added to the PCR product. The sample was transformed to a Promega Minocolumn. The column was incubated at room

temperature and centrifuged in a microcentrifuge at maximum speed for one minute. The supernatant was discarded and 700µL Promega Column Wash Solution was added. The column was centrifuged at maximum speed for one minute. The supernatant was discarded and 500µL Column Wash Solution was added. The column was centrifuged at maximum speed for five minutes. The supernatant was discarded and the column was centrifuged at maximum speed for an additional one minute with the microcentrifuge lid off. 50µL ultrapure water was then added to the column. The column was incubated at room temperature for one minute and transferred to a new microcentrifuge tube. The assembly was centrifuged at maximum speed for one minute, the flowthrough was stored at -20°C.

### **Transformation of Competent E. Coli**

A tube containing 50µl competent E. Coli was thawed on ice. 2.5µL of product was added to the tube, and it was gently flicked to mix. The tube was incubated on ice for 15 minutes. The sample was then heat shocked at 42°C for exactly 60 seconds and returned to ice for 2 minutes. 450µL warm LB media was added and the sample was incubated at 37°C for one hour with vigorous shaking. 150µL was then plated on an LB agar plate containing kanamycin antibiotics for selection.

### **Cell Passage**

*(25cm<sup>2</sup> flask)*

Media was aspirated from the cells and 1.5mL dilute (0.05%) trypsin protease solution was added to the flask and it was rocked several times. The excess trypsin was immediately aspirated, and the flask was allowed to incubate at room temperature for about

a minute for the cells to detach. A bright field microscope was used to ensure efficient lifting of the cells. 5mL of DMEM with 10%FBS and 1% penicillin/streptomycin/fungicide was added to the flask. Media was forcefully pipetted up and down several times to prevent clumping of cells. 1mL of the cells were transferred to a new flask, and 4mL of fresh media was added. For 75cm<sup>2</sup> flasks and 100mm dishes, volumes of reagents were adjusted appropriately.

### **Effectene Transfection**

*(100mm dish format)*

3µg DNA was diluted to 300µL with EC buffer, 16.0µL enhancer was added and the tube was vortexed and incubated at room temperature for 2-5 minutes. 60µL of effectene reagent was added and the tube was gently flicked to mix, the tube was again incubated at room temperature for 10-15 minutes. During this incubation, growth media was aspirated from the cells in a 100mm dish. Cells were washed once in 5mL of filter sterilized PBS, and 7mL fresh media was added to each well. Once finished incubating, 3mL growth media was added to the effectene complex, the solution was pipetted up and down twice to mix and immediately the solution was added drop wise to the cells. The dish was gently swirled and returned to the incubator at 37°C and 5% CO<sub>2</sub>. For transfections in 6-well dishes, reagent volumes were adjusted appropriately

## Cell Lysis for Immunoprecipitation

*(100mm dish)*

Cells were washed once in PBS and then 1.5mL fresh PBS was added. Cells were harvested by removing them from the surface with a sterile plastic cell scraper and then transferring the suspension to a tube. Samples were centrifuged for 5 minutes at 1500g, the supernatant was aspirated, and the cells were lysed by suspension in 500 $\mu$ L Buffer X and incubation on ice for 20 minutes. Cell extracts were centrifuged for 30 minutes at 9000g and the supernatant was transferred to a new tube. For experiments conducted in 6-well dishes, volumes were adjusted appropriately.

## Immunoprecipitation of Cell Extract

10 $\mu$ L mouse M2 conjugate EZ view beads were added to the cell extracts and incubated with agitation overnight at 4°C. Sample was centrifuged at 1000g for 60 seconds and supernatant was removed and sample was resuspended in 500 $\mu$ L Buffer X. This was repeated five times to wash the samples. After the last centrifugation, the supernatant was removed and the beads were resuspended in 50 $\mu$ L gel loading buffer. The samples were then boiled for 5 minutes at 95°C.

## SDS PAGE

A 12 % resolving gel was prepared in a 15mL conical tube by mixing 3.4mL ddH<sub>2</sub>O, 4.0mL 30% Bis/Acrylamide, 2.5mL 4x resolving gel buffer (1.5M Tris-HCl, pH 8.8), and 0.1mL 10% w/v SDS. To this formulation, 50 $\mu$ L 10% w/v ammonium persulfate (APS) and

7 $\mu$ L tetramethylethylenediamine (TEMED) was added to catalyze jellification and the solution was poured into the gel assembly until it reached approximately 15mm from the top. Approximately 1mL isopropanol was placed on top to prevent inhibition by oxygen gas. The stacking gel was then prepared in a 15mL conical by mixing 3.05mL ddH<sub>2</sub>O, 0.65mL 30% Bis/Acrylamide, 1.25mL 4x stacking gel buffer (0.5M Tris-HCl, pH 6.8), and 0.05mL 10% w/v SDS. Once the resolving layer solidified, the isopropanol was decanted out of the gel assembly. To the stacking gel formulation, 25 $\mu$ L 10% w/v APS and 5 $\mu$ L TEMED was added to begin catalysis. The stacking gel was poured on top of the resolving layer, and a comb added to establish wells. The stacking gel was given 20 minutes to set. The gel was then transferred from the pouring cassette into the running cassette and was immersed in running buffer (25mM Tris, 20mM glycine, pH 8.3 with 1.0% SDS). The samples were loaded into the wells and electric current was applied to produce a constant 20mA until the bromophenol indicator from the loading buffer ran off the end of the gel.

## Western Blot

A transfer assembly was built such that the electric field would draw the protein from the gel to a nitrocellulose blot. The transfer assembly was submerged in transfer buffer and surrounded by ice. Constant 200mA current was applied for one hour. The membrane was removed from the assembly and cut based on which lanes would be blotted with each antibody. Membrane was placed in blocking buffer with shaking at 4°C overnight. The membrane was washed in TBS-T for 5 minutes with shaking five times. The primary antibody was diluted 1:5000 in TBS-T (or blocking buffer) and added to blot. The blot was

incubated with the primary antibody with shaking for one hour. The membrane was again washed in TBS-T for 5 minutes with shaking five times. The secondary antibody was diluted 1:5000 in TBS-T and added to the blot. The blot was incubated with shaking for one hour. The membrane was washed again in TBS-T for 5 minutes with shaking five times, and then in TBS for 5 minutes with shaking twice.

### **Developing Western Blot**

One method of develop the western was with a goat anti-mouse ig secondary antibody conjugated to alkaline phosphatase. Alkaline phosphatase substrate (BCIP tablet) was dissolved in 10mL ddH<sub>2</sub>O and added to the membrane. The membrane was incubated with the substrate with shaking for about 2 minutes or until bands were clearly visible.

The other method was using a horse anti-mouse ig secondary antibody conjugated to horseradish peroxidase. Development of the blot was conducted with Pierce ECL Western Blotting Substrate. 1mL of reagent 1 was added to the blot drop wise, then 1mL of reagent 2 was added. The blot was then imaged with long exposure.



## Results

### Recombinant DNA cloning is reliable with overlap extension PCR

To study not just PCV1-VP3 as a whole, but its individual parts, a mutant was generated that would allow isolation of any differences between apoptin and PCV1-VP3. This mutant was a chimera containing the entirety of apoptin with the C-terminal non-homologous region of PCV1-VP3 fused to the end. If apoptin and PCV1-VP3 behave differently due to the homologous region, then the mutant should behave like apoptin, but if the difference is due to the non-homologous region, the mutant should behave like PCV1-VP3. At the time that this construct was needed, a novel method of cloning was under development in our lab that did not require the use of restriction enzymes. This method would be particularly useful not just for this construct, but for many applications on other projects in our lab that would normally be addressed with restriction ligation.

This method of cloning is PCR based, and uses primer design to insert the gene of interest specifically into a vector. The method is very similar to that used to introduce point mutants in quick change PCR. The cloning intermediates are diagrammed in Figure 1. Besides the fact that this method of cloning requires fewer enzymes and much less incubation time than traditional restriction ligation cloning, this method is sequence directed, allowing direct linking of sequences. If restriction sites are used, the recognition sequences of the endonucleases persist in the final product, and several random amino acids are inevitable. With the proposed method, the first codon of the gene of interest can occur immediately after any point in the destination vector. This was particularly desirable to generate the aforementioned chimera protein because in order to retain homology, the first

codon of the non-homologous region should occur immediately after the penultimate codon of the apoptin gene (excluding the stop codon).

Initial experiments attempted to complete the entire reaction in a single tube. A highly processive polymerase was used (*pfu*) that would complete the cloning process in a single reaction. The gene of interest (non-homologous region of PCV1-VP3) is replicated with primers that add the destination specific floppy ends to the gene of interest. As this is produced, it begins serving as a primer for the template apoptin vector, effectively stitching the gene of interest to the end of apoptin. Results were not fruitful and modifications of primer and template concentrations were made that did not result in success. It was then decided that a 2 step process would be more successful. In the first step, the gene of interest is replicated and the floppy ends added. This is then purified and used as the primer in the second PCR, with the destination vector containing apoptin as the template. The primer is included at molar concentrations approximately an order of magnitude greater than the template. The final product is then treated with *dpnI*, a methylated DNA specific endonuclease that degrades any template in the tube. At this point, analysis of the final product at varying number of cycles produces gels showing consumption of the insert, as well as the appearance of a high molecular weight band, suggesting that the gene of interest was in fact being extended around the template vector, yet the result still did not produce colony forming units upon transformation.

At about this time, our lab found a previously published paper describing the process that was independently under development in our lab<sup>[13]</sup>. The major difference between our attempted methodology and the one described in the published paper was the processivity of

the polymerase. The polymerase used in the paper, *phusion*, was several orders of magnitude more processive than *pf $\alpha$* . We then attempted the process once more following the methods described in the paper, while using the polymerase *Q5*, a polymerase that has comparable processivity to *phusion*, with about double the fidelity. The first attempt by this method produced viable colony forming units upon transformation. The colonies were inoculated and scaled up, and upon DNA purification and sequencing, it was shown that the chimeric construct was successfully produced.

### PCV1-VP3 and mut-AP coimmunoprecipitate with *cdc27*

Previous research has shown that apoptin is capable of coimmunoprecipitating with *cdc27*, a core subunit of the anaphase promoting complex. It is hypothesized that the mechanism of apoptin is to bind to and inactivate the anaphase promoting complex, leading to cell cycle arrest and apoptosis. To determine whether the apoptotic mechanism is shared between apoptin and PCV1-VP3, an assay was performed to see if PCV1-VP3 and mut-AP also associate with the APC *in vitro*. Flag tagged versions of apoptin, PCV1-VP3, and mut-AP were transfected into H1299 cells. 24 hours later, the cells were collected, lysed, and an immunoprecipitation was performed. The product was separated via SDS-PAGE and a Western blot was performed using an anti-*cdc27* antibody. For all three proteins, with normalized DNA content and identical transfection conditions, *cdc27* bands were present on the Western blots (See Figure 2).

These results lead to several plausible possibilities on the nature of association of the proteins with the APC. One possibility is that the homologous region of PCV1-VP3 retains the ability to bind to the APC. It is also possible that the homologous regions of

PCV1-VP3 is incapable of binding to the APC, but the effect is rescued by the non-homologous C-terminal domain. Finally, it is possible that both the homologous region and the non-homologous C-terminal domain are capable of associating with the APC.

It is worth noting that the strongest band for cdc27 is seen for apoptin, followed by PCV1-VP3, with mut-AP showing the weakest band. This may suggest that it is unlikely that both the homologous region and the non-homologous C-terminal region of PCV1-VP3 are capable of associating with the APC, or mut-AP or PCV1-VP3 would be expected to show the strongest band. It is worth noting that we assume consistent expression of the three constructs based on the identical transfection conditions, but western blots testing for flag expression should be conducted to confirm these results.

## Discussion

The findings above provide intriguing insight on the behaviors of the proteins apoptin and PCV1-VP3. It has long been assumed that the localization of apoptin is an integral part of its cancer killing abilities until the discovery of the exclusively cytoplasmic protein PCV1-VP3. It was then concluded that the localization of apoptin is unimportant. The recent findings by KP Hough *et al.* and the varying cell cycle arrest phenotypes suggested the possibility of unique mechanisms, in which case the localization may in fact play a role. Based on the results described above and the past research conducted on these proteins, a more complete hypothesis may be formed as to how these proteins behave in transformed cells. Both apoptin and PCV1-VP3 induce apoptosis in transformed cells and associate in some way with the APC. I propose that apoptin may associate with cdc20, another subunit of the APC that is active in G2 and, when incorporated in the APC, causes the cell to divide and enter anaphase. Apoptin may bind to cdc20 in a way that lowers the ability of APC to advance the cell, causing G2 arrest and inducing apoptosis. I further suggest that PCV1-VP3 also binds to cdc20, and the phenotypic differences of the proteins are due to localization. PCV1-VP3, while it exists primarily in the cytoplasm, is transiently present in the nucleus, where it can bind cdc20 and possibly sequester it in the cytoplasm. This would result in an apparent stability of the other isoform of the APC, which incorporates cdh1 instead of cdc20. The APC<sup>cdh1</sup> complex is responsible for sustaining the cell in G1 phase until it is ready for division. The increased presence of the APC<sup>cdh1</sup> over APC<sup>cdc20</sup> would result in the G1 arrest observed in cells expressing PCV1-VP3 and subsequently apoptosis (See Figure 3). Yet despite the differences in cellular localization

and cell cycle arrest, the findings of this paper also suggest that the apoptotic selectivity is dependent solely on the interaction with the APC, and that the localization and cycle arrest effects are peripheral. Although the precise mechanisms of these proteins is still unknown, if it is confirmed that the mechanism is conserved, then these proteins may serve well as a model for a small molecule drug to recapitulate the effect. Proteins are complex, particularly virus proteins, and often the many functions of a protein cannot be modeled by a drug. If the cancer killing abilities of these proteins depended on localization and cell cycle arrest, as well as other functions, a small molecule drug may not be feasible. If the localization and arrest are not integral to the selective induction of apoptosis, however, it may be simpler to develop such a drug as we learn more about the mechanisms of these viral proteins. Further research on these proteins may focus on the molecular mechanisms either upstream, in how the DNA damage response leads to their activation, or downstream, how the binding of cdc27 ultimately leads to apoptosis. In order to confirm the above stated hypothesis, association assays can be performed for cdc20 and for cdh1. If these proteins associate with the APC through cdc20, cdh1 should never coprecipitate. These assays, as well as cell cycle and localization assays should also be performed for the homologous region of PCV1-VP3 and the non-homologous C-terminal domain independently to further isolate the source of the phenotypic differences.

## Figures

Figure 1: Primer Annealing Intermediate of OE-PCR

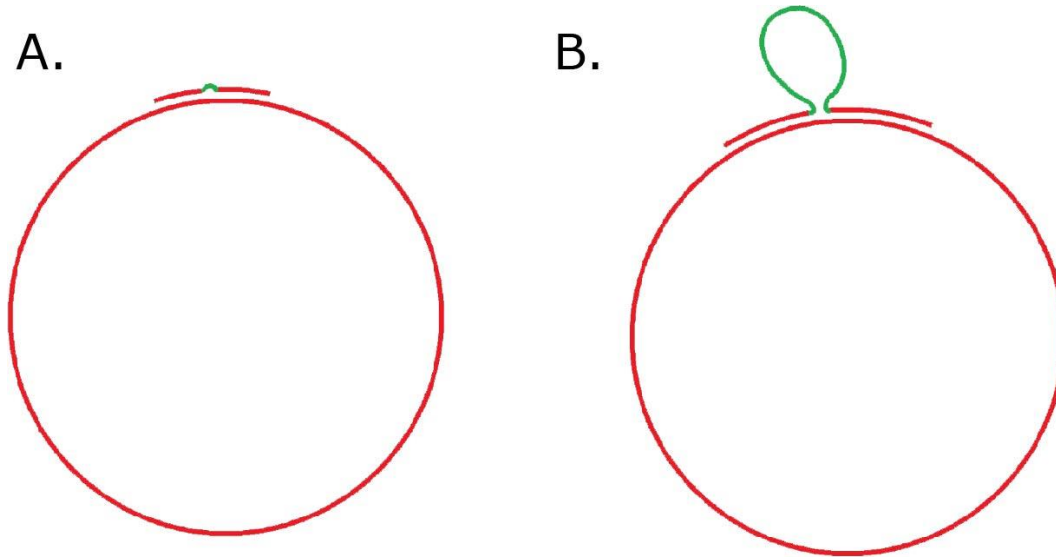
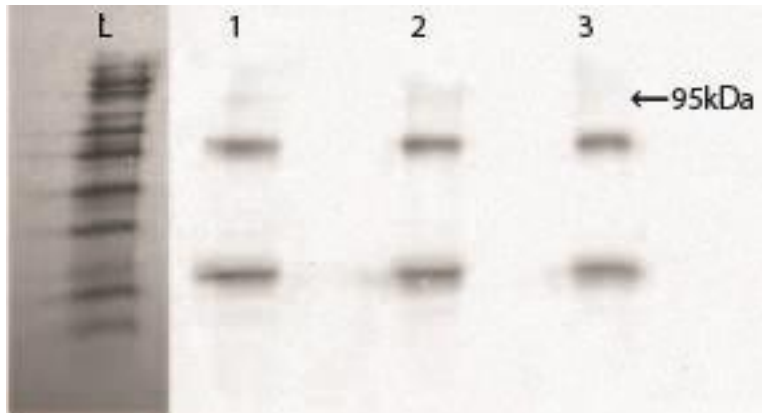


Figure 1: A) Primer annealing intermediate of quick-change PCR to introduce mutations. B) Primer annealing intermediate of Overlap Extension PCR Cloning. A mutant construct was constructed to add the non-homologous C-terminal domain of PCV1-VP3 to the C terminus of apoptin. In this case, the non-homologous C-terminal region was amplified and served as the primer (green) that annealed to the apoptin vector (red).

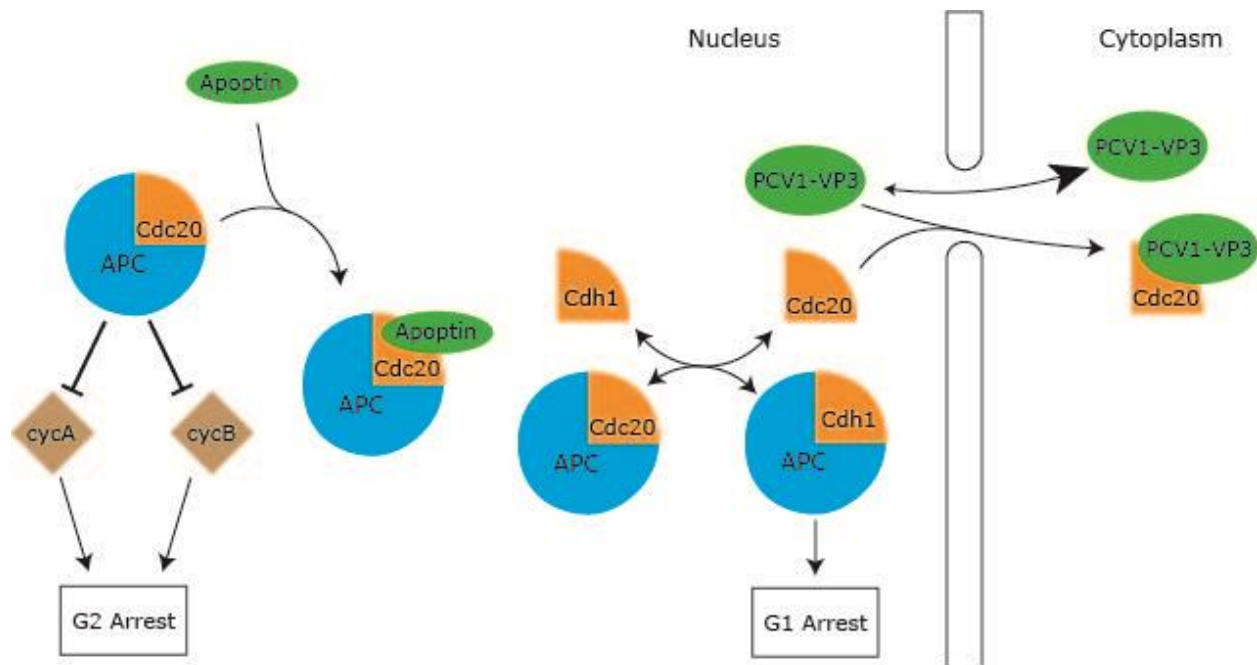
**Figure 2: Western for Association with cdc27**



*Figure 2: Long exposure image of a developed membrane with luminol based substrate. Samples are immunoprecipitations of flag-tagged proteins blotted for cdc27 L) Molecular weight marker. 1) 3x-flag Apoptin 2) 3x-flag PCV1-VP3 3) 3x-flag Apoptin-PCV1-VP3 chimera. All three proteins produced a positive signal for association with cdc27. Because both apoptin and PCV1-VP3 produced positive results, there was no additional information gained by including the chimera protein in the test. The observed results show that both apoptin and PCV1-VP3 are capable of associating with the anaphase promoting complex.*



**Figure 3: Proposed Activities of Apoptin and PCV1-VP3**



*Figure 3: Proposed schematic of apoptin and PCV1-VP3 activities in the cell. In this model, apoptin and PCV1-VP3 associate with cdc20, another subunit of the anaphase promoting complex that is active in G2 and, when incorporated in the APC, signals the degradation of cyclin proteins A and B, causing the cell to divide. Apoptin may bind to cdc20 in a way that lowers the ability of the APC to advance the cell cycle, resulting in the observed G2/M arrest. PCV1-VP3 would also bind to cdc20 in this model, and the phenotypic differences of the proteins are due to the localization. PCV1-VP3 binds cdc20 like apoptin, but the cytoplasmic localization results in the sequestering of cdc20 and inactivity. This sequestering would result in the apparent stabilization of the other isoform of the APC, which incorporates cdh1 instead of cdc20. The APC<sup>Cdh1</sup> complex is responsible for sustaining the cell in G1 phase until it is ready for division. The inability of cdc20 to outcompete cdh1 would result in increased activity of the APC<sup>Cdh1</sup> complex, explaining the G1 arrest observed with PCV1 expression.*

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## Appendices

### Appendix A: DNA Damage Response Inhibition

The first experiments conducted to mechanistically compare apoptin and PCV1-VP3 were to see if the presence of caffeine, a DNA damage response pathway inhibitor, would change the localization or apoptotic effects of PCV1. This was based on prior experiments conducted by Kucharski *et al.* that showed variable localization of flag-tagged apoptin in the presence of caffeine. To do this, an EGFP tagged version of apoptin and PCV1-VP3 were transfected into H1299 cells and treated with caffeine containing media. Surprisingly, the apoptin still appeared to be expressed in the nucleus, contrary to the findings of the Kucharski paper. Because Kucharski *et al.* conducted their experiments by using a flag tag and immunoblotting, it was concluded that the presence of the EGFP tag on apoptin interfered with the normal response to caffeine. Correspondence with a coauthor on the Kucharski paper, Jose Teodoro, confirmed that the experiment does not work with GFP tagged apoptin constructs. Our lab does not have the resources to perform immunoblotting experiments, so this approach was dropped and apoptosis assays were not conducted.