Localization Mechanics and Multimerization Ability of Human Torque Teno Virus Viral Protein 3: A Comparison of the Mechanistic Actions of TTV VP3 and Apoptin

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Abstract

Cancer affects millions of people worldwide each year. Although effective treatment courses have been established for many forms of the disease, these therapies are not effective on all cancers. In particular, cancers in which the tumor suppressor p53 is knocked down or down regulated do not respond to conventional chemotherapies. It is therefore critical to seek novel treatment methods that can affect these types of cancers. One group of homologous viruses found across multiple species code for a set of homologous proteins that have been found to selectively induce apoptosis in transformed cells in a p53 independent manner. Apoptin, produced by Chicken Anemia virus, has been extensively characterized; however, little is known about the human homolog, Torque Teno Virus (TTV), which codes for Viral Protein 3. TTV has no known toxicity, is found in more than 90% of the adult population worldwide, and most importantly evolved in humans. Several experiments were thus performed to determine if TTV VP3 and Apoptin follow similar apoptotic pathways. Confocal microscopy of H1299 cells, a non-small cell lung carcinoma, transfected with wild type TTV VP3 demonstrate the characteristic punctate expression pattern and follow a 48 hour apoptotic time course. Additionally, cells treated with the CRM1 export inhibitor leptomycin B show a marked increase of nuclear localization of VP3. These results support that TTV VP3 and Apoptin follow a similar apoptotic pathway, and also provide grounds for future study of TTV VP3 and its potential as a cancer therapeutic.
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Introduction

Characterization and Genealogy of Torque Teno Virus

The Torque Teno Virus (TTV) is a nonenveloped virus with a genome of length 3.8kb, though the prototypic TA278 virus is reported to have a 3.852kb sequence. The “torque teno” name given to the virus in 1999 translates to “thin necklace” in Latin owing to the virus’s small circular single stranded DNA. The seemingly simple virus contains immense genomic diversity with over 5 taxonomic groups and 40 genotypes spanning numerous species. TTV exhibits broad infection diversity as well as an immense genomic diversity with only 60% homology across all of the 20 known human TTV genotypes.\(^1\) Phylogenetic analysis of full length or near full length sequences reveals a division into five main groups based upon order of discovery, genetic structure and relative homology. Group 1 is represented by the prototypical virus TA278, group 2 by US35, group 3 by JA10, group 4 by TUS01, and group 5 by SANBAN.\(^2\)\(^3\) Sequence variation occurs mostly in the coding regions due to high levels of recombination that can allow for a number of pathological differences among different groups.\(^4\) This genomic recombination accounts for the genetic diversity that contrasts with the fairly conserved genotype of the homologous Chicken Anemia Virus (CAV).\(^5\)

Originally part of the circoviridae genus due to structural similarities, the virus has been compared to the genetically similar Chicken Anemia Virus (CAV) and Porcine Circovirus (PCV). However, TTV’s genome is much larger than most viruses of circoviridae. Also, most circoviridae are ambisense, or transcribed on both strands, whereas TTV has antisense open reading frames (ORF). CAV’s genome is likewise antisense.\(^6\) TTV is currently classified into the genus anellovirus but not into a taxonomic family. Other families of viruses share the same characteristics of TTV including the unique single-stranded circular genome. These families
include inoviridae and microviridae for bacterial viruses and geminiviridae and nanoviridae for plant viruses. However, TTV is the first human virus found with these characteristics. [5]

Three polycistronic transcripts, produced from the TTV genome, encode three putative proteins. These proteins are poorly characterized due to TTV’s ubiquitous presence in all infected species and lack of known pathogenicity. No culture system has been developed yet for growing TTV in the laboratory, leaving the biological function of the virus unknown. [7] Researchers postulate on the role of each viral protein by comparing the relatively unstudied human TTV to the homologous CAV.

**TTV in the Human Population**

TTV was initially discovered in the blood serum sample of a non-A-E hepatitis patient, but since its discovery very little has been determined regarding its biological significance in humans. However, TTVs are ubiquitously present in the human population, as various strains of the virus are found in more than ninety percent of adults worldwide. [8] In 2000, Springfield et al. posed three questions to influence the direction of TTV research: “What are the exact molecular mechanisms of viral replication?, What is the origin and molecular relatedness of TTV?, and What is the significance of TTV as a human pathogen?”. In the time since, only the second question has been significantly explored, and will be discussed in later sections [9].

Hino and Miyata site two main obstacles for this lack of information in their review of the current status of TTV. The first is the lack of a culture system to support viral replication, which makes it difficult to study the virus in a laboratory setting. The second is the lack of a significant link to human disease and pathogenicity, which results in the question of why one would want to pursue research on TTV. Although no human pathogenicity of TTV has been fully established, and all known strains of the virus are currently characterized as harmless to humans,
many people record moderate to high levels of TTV in their systems. In addition, TTV is unique in that it is the first known human virus with a single stranded circular DNA genome. Viral transmission modes are also known. Okamoto et al. cite widespread transmission throughout the population by parental transmission via blood, blood transfusion, and fecal-oral infection. Deng et al also characterize viral transmission in saliva.

Additionally, multiple different groups have noted TTV infection as a possible factor in the diseases they have studied. Most notably, TTV has been implicated in worsened conditions of hepatitis, liver disease, and liver cancer. Hino reviews a series of cases in which hepatitis was seen in the acute infection phase of TTV primary infection in infants. Research has shown that recovery for patients with alcoholic liver disease have slower and less significant improvement rates when infected with TTV, and that patients with high TTV loads and hepatitis C have an increased risk of developing hepatocellular carcinoma. Due to this high incidence and apparent effect on liver diseases, TTV has also been postulated as a possible novel hepatitis virus, hematogenously spread.

Further, a higher viral load has been documented in patients with varied conditions, including idiopathic inflammatory myopathies, cancer, and lupus. Idiopathic pulmonary fibrosis patients with TTV were more symptomatic and had a reduced 3 year survival rate. Similar results were also found in acute respiratory diseases in children aged two years and under, suggesting that TTV might replicate in inflammatory tissue. In hematologic diseases, TTV infection could reduce platelet count in hepatitis patients. Viral load is also higher in patients with compromised immune systems.

Overall, these studies show that TTV has a very broad tropism, with higher concentrations in bone marrow, spleen tissue, lung tissue, and liver tissue and bile, suggesting
that these tissues are possible target cells for the virus\textsuperscript{[8]} The virus has also been shown to replicate well in pulmonary epithelial tissue. Additionally, it has been postulated that TTV targets inflammatory tissue, although little concrete evidence exists to support this. One group did show active replication of the virus in infants with inflammatory disease\textsuperscript{[8]} Clearly, there are still many more questions than answers regarding TTV in humans, and further study will be required in order to further illuminate these interesting correlations.

\textbf{Comparison of TTV VP3 to Known Homologs}

Chicken anemia virus (CAV) is a member of circoviridae that is not only homologous to the enigmatic TTV, but is also highly characterized. CAV VP3 codes for the extensively studied protein, Apoptin, which has been shown to selectively induce apoptosis in transformed, or tumorigenic, cells but leave primary cells intact.\textsuperscript{[7]} Due to the similarities between TTV and CAV in regards to structure and ORF orientation, TTV is thought to express a protein similar to Apoptin with a possible cancer killing capability. As described earlier, each mRNA transcribed by TTV is polycistronic, with one ORF in each of its reading frames. The TTV and CAV share a

\textbf{Figure 1: Genealogy of Torque Teno Virus.} (a) Electron micrographs of TTV-associated particles with a diameter of 30-32 nm in aggregated form. Serum samples with TTV DNA titer of 108 copies/ml obtained from HIV-infected patients.\textsuperscript{[5]} (b) Human TTV Genomic Map showing positions of overlapping open reading frames.
similar genomic distribution of the putative ORFs, suggesting that their viral proteins share a
common function. \[^5\]

Unlike TTV, the extensively characterized CAV is known to have three viral proteins,
VP1, VP2 and VP3 with established functions. VP1 exists exclusively in the capsid of the virus
and is believed to form the capsid. \[^15\][\[^16\]\] VP2 has been speculated to serve as a scaffold protein
that aids in the assembly of the virus due to its dual specificity phosphatase (DSP) activity. \[^15\]\ Research has shown that the expression of both VP1 and VP2 is required to generate the fully
functional capsid and therefore VP2 is thought to play a role in the assembly of VP1 into a capsid
structure. \[^16\]\ VP3, the viral protein of interest, is the only non-structural protein of 121 residues
that can induce apoptosis within varied cells after infection. Apoptin, CAV VP3, induces
apoptosis independently of p53, a key protein in tumor suppression and cellular growth, which is
often overridden by transformed growth. \[^5\]\ Apoptin is comprised of 121 amino acids and
contains two proline rich regions and two basic regions. \[^17\]\

The variance across TTV’s many genotypes is between 3.6 and 3.8 kb. \[^18\][\[^19\]\] One third of
TTV’s genome is comprised of untranslated DNA including a GC-rich 113 nucleotide region. \[^5\]\ Within this GC-rich region is the origin of replication, a 36 nucleotide stretch that forms a stem-
loop structure. This origin of replication is found in CAV as well, sharing over 80% homology
with TTV for this region. \[^20\]\ All genotypes that have been studied show a similar set three
conserved spliced mRNAs of lengths 3.0, 1.2 and 1.0kb. Three open reading frames are known
to exist in all genotypes, ORF1, ORF2 and ORF3. Though ORF1 is present in every genotype of
TTV, it is only partially conserved across the more distally related genotypes. ORF2 consistently
appears with similar variability and overlaps with ORF3, which is present in all mRNA
transcripts. \[^19\]\ In the prototypic TA278, all transcribed mRNAs use the same TATA box and
poly-A signal found on the antisense strand at bases 86-92 and 3037-3079. Critical regulatory elements at -76 and -154 nucleotides govern the TTV promoter, thereby controlling transcription regulation. [5]

The similar ORF distribution and genomic structure of TTV and CAV led researches to hypothesize that TTV expresses three main viral proteins like CAV. However, TTV has been reported to produce greater than three proteins. One study claims that TTV genotype 6 expressed up to seven distinct proteins. The four proteins not coded by TTV’s 3 ORFs are coded by the alternatively spliced mRNAs of lengths 1.0 and 1.2kb and are not expressed significantly enough to warrant further investigation. [21] Analysis using circular dischroism revealed that Apoptin lacks alpha-helical structure and resembles proteins with unordered structure. [22]

TTV ORF1 codes for a protein of 770 amino acids and contains several conserved replicase protein motifs as well as a highly basic region near its N terminus. These discoveries led researchers to believe that ORF 1 codes for the major structural protein of TTV because both properties are qualities present in circoviral capsid proteins. The protein coded by ORF2 has been studied less but is thought to play a structural role similar to the CAV VP2 due to the similar orientations of ORFs. [7]

TTV VP3, the protein for ORF3, is coded for by all three mRNAs. Like Apoptin, the 105 amino acid protein has been reported to be nonstructural. VP3 shows more structural similarity to Apoptin than the other TTV viral proteins. Both contain a high level of proline residues, 10.5% in TTV VP3 and 11.5% in Apoptin, which are highly dispersed. This distribution suggests that these residues contribute to structural function in their respective viral proteins. A high concentration of threonine, 12.4% in TTV VP3 and 11.5% in Apoptin, likewise suggests a structural function or these residues in both proteins. Finally, a section of hydrophobic amino
acids near the N-terminus of Apoptin forms the site of multimerization and nuclear export. This leucine-rich region is found in TTV in a similar location, suggesting that the section serves a multimerization and export function. \[^{[5]}^{[20]}\]

Noteborn et al. published a study in which researchers expressed the protein of ORF3 from the prototypic TA278 and transfected the TTV protein in hepatocellular carcinoma cells (HCC). The report claimed that the functions of TTV ORF3 and CAV Apoptin were similar. TTV VP3 was shown to induce apoptosis in a similar manner to Apoptin but on a more limited scale. The foundation for their work was based on a comparative analysis of TTV and CAV in terms of their overall genomic framework. The researchers concluded that TTV VP3 was homologous to CAV Apoptin and a potential cancer therapeutic, referring to the protein as “TTV Apoptosis-inducing protein” (TAIP). \[^{[15]}\]
Figure 2: Sequence Alignment of TTV VP3, PCV1 VP3, and CAV VP3 (Apoptin). CLC Main Workbench software was used to compare homologous viral proteins to our protein of interest. The consensus sequence under each VP3 sequence shows regions of the three proteins that contain 100% homology of residue classifications (i.e. hydrophobic, aliphatic, polar, basic, etc.) Beneath is the conservation graph that shows degree of residue agreement for specific amino acids. The bottom row of the chart shows specific amino acid symbols where the size represents the degree of homology.
Apoptotic Ability of TTV

Apoptosis refers to the process by which a cell induces its own death. Cellular localization of Apoptin has been identified as the key to its apoptotic capability; it localizes in the cytoplasm in primary cells while localizing in nucleus in cancerous cells. However, since TTV is a more recent discovery, significantly less work has been done in studying the activity and functional characteristics of TTV in comparison to CAV. Still, the studies that have been performed have highlighted some key similarities between the two proteins. Specifically, the apoptosis-inducing ability of the TTV VP3 protein is cell-type specific, similar to that of CAV Apoptin, in that both proteins only target cancerous cells in vitro.

Importantly, TTV VP3 and Apoptin both induce apoptosis in cancer cells in a p53-independent manner. This fact is critical in the future use of these protein products as potential cancer therapies. The p53 gene codes for the p53 protein, which is a widespread and important tumor suppressor in normal body cells. As a tumor suppressor, it functions to prevent uninhibited proliferation.
Figure 3: p53 Involvement in MAP Kinase Signaling Pathways.
p53 plays a major role in preventing the development of cancer through its involvement in the MAP Kinase signaling cascade. This flow chart demonstrates the complex nature of the regulatory pathways required to maintain normal cellular growth.\textsuperscript{[23]}

When a mutation occurs in p53, cells are able to divide uncontrolled, and malignancies develop. The p53 gene is implicated in approximately 50% of human cancers. Additionally, many cancers are caused by mutations in genes that code for proteins that interact with p53 or are components in the p53 pathway. Due to this, the majority of chemotherapies that specifically target cancer today rely on p53 activation in the cancer cells. However, many cancers knock out or down regulate the expression of p53, rendering these therapies ineffective. Since TTV VP3 and Apoptin rely on a p53 independent manner, understanding the mechanism by which they
induce apoptosis is critical to the possible development of therapies to target cancers in which p53 activation is not possible. \[^{23}\]

However, differences are also present in the apoptotic abilities of the two proteins. Results of the studies that have been performed on TTV indicate that TTV VP3 appears to induce apoptosis preferentially in HCC (human hepatocellular carcinoma) cells. \[^{15}\] This finding contrasts the findings from similar studies of CAV, which showed that Apoptin induces high levels of apoptosis independent of the tumor origin. \[^{25}\]

**Localization and Multimerization of CAV Apoptin and TTV VP3**

The ability of a protein to localize within the cell is critical to effectively perform its designate function. Apoptin contains a nuclear localization sequence (NLS) located at the C terminus of the protein between residues 80-21. The protein also contains a leucine-rich nuclear export sequence (NES) located at the N terminus between residues 33-46. \[^{26}\] In a 2006 study conducted by Heilman, et. al, researchers concluded that the bipartite NLS was functional through a point mutation. \[^{27}\] The presence and of these functional sequences indicate the protein must be localizing to either the nucleus or cytoplasm depending on its role. Nuclear import and export is mediated by nuclear pore complexes (NPCs) and requires karyopherins (kaps). Kaps include importins, exportins, and transportins that are required for molecules larger than 50 kDa to traverse the NPC. \[^{28}\]

A 2006 study proved Apoptin can be imported into the nucleus via the IMP1 karyopherin through the Ran-GTP cycle. IMP1 binds to Apoptin’s NLS in the cytoplasm, allowing VP3 to dock to the nuclear pore complex. Apoptin will then be translocated into the nucleus via the NPC. Ran-GTP binds to the importin, releasing Apoptin. The exportin, CRM1, will facilitate nuclear export using the same Ran-GTP cycle. CRM1 binds to the NES of Apoptin, causing VP3
to dock to the NPC, which will allow the translocation from the nucleus to the cytoplasm. During this cycle, the GTP is hydrolyzed to GDP which will allow CRM1 to release Apoptin \cite{28}.

The apoptotic capability of the protein is regulated by this localization process. In normal cells, Apoptin is found scattered within the cytoplasm. In transformed cells, however, Apoptin is found in the nucleus prior to inducing apoptosis. Tumor cells were treated with Leptomycin B, a nuclear export inhibitor, in the aforementioned 2006 study by Heilman et al. With nuclear export inhibited, Apoptin localized in the nucleus but its apoptotic ability was removed. Similarly, when Apoptin was artificially localized in the nucleus of normal cells, its apoptotic ability was removed. These results suggest that the expression of the nuclear export sequence correlates with the protein’s apoptotic ability.\cite{27} However, the localization of the protein cannot be definitively determined to be the sole inducer of apoptosis due to multiple domain overlap within the NES.

Nucleo-cytoplasmic shuttling regulation in tumor and normal cells cannot induce apoptosis on its own. The formation of soluble 30-40 subunit Apoptin multimers is thought to be required to induce apoptosis. The multimerization domain, between amino acid residues 33-46, overlaps with the NES. Lilivelt et al. hypothesized in a 2003 study that residues in the NES between Glu32 and Leu46 could fold as an anti-parallel beta sheet with Ala38 and Gly39 to form a beta-turn or a hairpin. This folding would result in hydrophobic and hydrophilic residues oriented away from each other which would aid in the formation of Apoptin multimers \cite{22}.

It has been shown that multimerization directly correlates with localization of Apoptin in primary and transformed cells. In primary cells, Apoptin will form large insoluble aggregates within the cytoplasm but smaller soluble multimers will localize in the nucleus in tumor cells. Though TTV VP3 has been shown to have different distributions within transformed cells than Apoptin, the remarkable homology between the two proteins suggests similar behavior. In 2009,
Evan-Browning and Orme-Johnson sought to examine whether TTV VP3 would localize and multimerize in a fashion similar to CAV Apoptin. TTV VP3 was tagged to GFP in order to visualize the localization of VP3 within transformed cells. At 24 hours, the protein existed seemingly without preference in both the nucleus and the cytoplasm. At 48 hours, the protein was free of nuclear localization, appearing as punctate aggregates around the nuclear and cytoplasmic periphery. These results suggest that TTV VP3 may induce apoptosis in a pathway different from the CAV Apoptin homolog. However, VP3 appeared to have a functional NES and the resulting punctate foci suggest that it may form soluble multimers in transformed cells, the two aspects of Apoptin’s apoptotic mechanism described. [29]

These findings complicate the apoptotic capability of TTV VP3. One would expect the protein to localize in the nucleus in order to induce apoptosis in transformed cells, similarly to Apoptin, but the protein localizes in the cytoplasm. In addition, VP3 may forms soluble multimers in transformed cells instead of forming insoluble aggregates as predicted. Since TTV VP3 seems to deviate from the expected apoptotic pathway established in Apoptin, more research about TTV VP3 method of inducing apoptosis is needed. The enigmatic apoptotic mechanism of TTV VP3 warrants elucidation in the form of experiments revolving around its cell-specific localization patterns and multimerization ability. The following study seeks to uncouple these two criteria of Apoptin’s selective induction of apoptosis in cancer cells within the homologous TTV VP3. Inhibition of nuclear export by an established method, shown effective in Apoptin, provides insight on the localization of TTV VP3 as a function of apoptotic capability. Furthermore, time-course and densitometric analysis of TTV VP3 within cancer cells allows for multimerization assessment in comparison to Apoptin.
Materials and Methods

Site-Directed Mutagenesis of NES Sequence

The megaprimer PCR approach was employed in order to mutate and amplify the third reading frame of TTV. The initial PCR was developed in order to mutate the appropriate bases on each strand of the TTV VP3 gene. In two separate reactions, the forward mutagenic primer was combined with the TTV VP3 C-terminal primer and the reverse mutagenic primer was combined with the TTV VP3 N-terminal primer. A TTV VP3 GFP backbone previously constructed in the lab was used as the template, and Go Green Taq Master Mix (Promega, Catalog #M7122) was used as a source of polymerase and nucleotides. Nuclease-free water was used to bring the reactions up to a volume of 20 µl. A subsequent PCR was run to anneal and amplify the full length mutated gene. The two products resulting from the first PCR were diluted 1:100, mixed together, heated to 95°C for 5 minutes, and allowed to cool to room temperature to anneal. This mixture was then used as the template in the reaction, along with the terminal primers and Go Green Taq Master Mix as before.

Both PCR reactions were carried out according to the following conditions: a 4 minute 95°C denaturing step, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and a final 4 minute step at 72°C. Each of the reactions was run in duplicate to ensure an ample amount of product.

Visualization and Purification of PCR Products

After PCR, products were confirmed by agarose gel electrophoresis in TAE. Ethidium bromide was used as an intercalating agent to fluoresce the DNA on the gels. Bands obtained from the first PCR were used for confirmation of appropriate amplification, and were not isolated.
The second PCR product was purified using the protocol from the Wizard SV Gel and PCR Clean-UP System (Promega, Catalog #A9281). The elution volume was reduced from 50 µl specified in the protocol to 20 µl in order to increase concentration. After this initial purification, the PCR product was restricted with Eco RI and Bam HI to cut the excess small fragments off both ends of the product. The product was then gel purified using the Promega kit to remove these fragments. An equal volume of NaI was added in the binding step to assist with agarose degradation. Constructs were labeled TTV VP3 NES Mutated Insert.

**Molecular Cloning and Selection**

For construct cloning, a GFP vector with a PCV 1 insert was selected because it was a recent construct prepared in the lab, and had recently been proven to be effective. The vector was prepared by restriction with Eco RI and Bam HI, and then subsequent gel purification as described above.

Ligation was performed using a 3:1 ratio of purified TTV VP3 insert to purified cut vector, ligase buffer, and T4 ligase enzyme. The reaction was allowed to proceed at room temperature for 1 hour.

To amplify the cloned constructs for further experimentation, the ligated vector products were inserted into competent JM109 *Escherichia coli* cells through bacterial transformation. Upon thawing of the cells on ice, 3 µl of the ligated product was added to 50 µl of cells and allowed to incubate for 15-20 minutes. The mixture was then heat shocked at 42° C for 60 seconds and allowed to recover on ice for 3 minutes before addition of 450 µl of LB media. Tubes were placed in a 37° C incubator to recover for 1 hour. 150 µl of each tube was then plated on LB kanamycin plates and incubated 18-24 hours at 37° C. Presence of colonies indicated a successful transformation.
In addition to the ligated vector products of interest, positive controls with the addition of supercoiled vector product and negative controls in which the ligation reaction did not contain any of the purified TTV VP3 insert were run in parallel with the ligation transformations. These controls were employed both as a test of the integrity of the plates and as a comparison tool for colony number obtained for the desired TTV VP3 ligation.

**Preparation and Amplification of DNA Constructs**

Colonies resulting from transformation were individually selected and inoculated into 50 ml LB. Flasks were allowed to incubate overnight at 37°C until they were observed to be visibly turbid. Plasmid preparation was then performed using the PureYield Plasmid Midiprep System (Promega, Catalog #A2492). The result of the preparation was 600 µl samples of purified plasmid from each isolated colony. After preparation samples were quantified using a UV spectrophotometer at a wavelength of 260 nm. Samples were then diluted to appropriate concentrations and sent out for sequencing analysis.

**Transfection of TTV VP3 Constructs into H1299 Cell Line, Leptomycin B Experiments, and Microscopy**

Transfection of wild type TTV VP3 was performed on the cancerous cell line H1299, a non-small cell lung carcinoma. Cells were maintained between 30-80% confluence in D10 media (DMEM + 10% FBS with 1x PSF) at 37°C. For transfections, cells were passed into sterile 6 well plates, with each well containing a glass coverslip previously stored in ethanol. Cells were allowed to adhere 24 hours or until they reached approximately 70% confluence before transfection. The Effectene Transfection Protocol (Qiagen, Catalog #301425) was used to perform transfections.

Approximately 24 hours after transfection, cells were fixed using the following procedure. All media was aspirated from the plate, each well was washed with 1x PBS, cells
were fixed with 4% paraformaldehyde solution, and allowed to incubate on a gentle shaker for 15 minutes. After this time, the paraformaldehyde was aspirated off, another 1x PBS wash was performed, and 70% ethanol was added to the wells until slide mounting was performed. To mount cells the coverslips were removed and placed cell-side down on slides using approximately 30 µl of prepared mounting media (50% glycerol, 10mM Tris, 2% DABCO, and DAPI stain), and sealed with commercial clear nail polish. Slides were stored at 4° C and in the dark to prevent photobleaching. Transfection results were viewed using a Leica SP5 laser confocal microscope with an inverted Leica DMI 6000 CS microscope base.

For transfection experiments using leptomycin B, the cells were treated with the addition of liquid leptomycin B in methanol at a concentration of approximately 10 ng/ml. The drug was added directly to the media 3 hours before fixation. The ΔΔ NLS Apoptin mutant previously prepared in the lab was used as a positive control for these experiments because it is known to show normal cytoplasmic localization and nuclear localization with LMB treatment.

**Image J Software Analysis**

Image J Software was used for analysis of confocal images in punctate assessment and Leptomycin B experiments. For densitometric analysis, punctate assessment was performed using the line tool. A line was drawn through a region of the selected cell image where punctate clusters were observed. Analytical plots were created by selecting plot profile under the analyze bar. Leptomycin B analysis employed several different tools. To create the surface plots, the image was first adjusted to 8 bit color by selecting that option under Image and then Type. The cell was then selected using the rectangle tool and then the surface plot button was selected under the analyze bar. To obtain useable plots, some images had to be adjusted for brightness. To create the nuclear and cytoplasmic region fluorescence intensity plots, the rectangle tool was
used to select a region in the cell that contained both a part of the nucleus and a part of the cytoplasm. Plots were created by selecting analyze and then plot profile, as before. Approximate distances were noted so as to distinguish the cutoff on the plots produced. Finally, to obtain data required to calculate nuclear to cytoplasmic ratio, the following steps were taken. Initially, the set measurements button was selected under the analyze bar. Boxes for area, integrated density, and mean gray value were checked. For each cell analyzed, the rectangle tool was used to select first an area in the nucleus and then an area in the cytoplasm. For these selections, the measure button was selected under the analyze bar, and the values were recorded in excel. To process this raw data, the following equation was used: CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings), where CTCF represents total fluorescence. Values obtained for the nucleus were divided by values obtained for the cytoplasm to obtain the nuclear to cytoplasmic ratio. Values were then normalized to an eGFP ratio standardized to 1.
Results

In Silico Nuclear Export Sequence Analysis

Apoptin has been demonstrated to selectively induce apoptosis based on its localization to either the nucleus or the cytoplasm. The nucleo-cytoplasmic shuttling of Apoptin is dependent on competing signals: the nuclear localization sequence (NLS) and nuclear export sequence (NES). Previous studies have shown that, in early stages of transfection, TTV VP3 localizes seemingly without preference in the cytoplasm and nucleus but eventually localizes primarily in the cytoplasm of both primary cells and transformed cells. The homology between the two apoptotic proteins suggests that TTV VP3 would localize using the same mechanism as Apoptin. Therefore, in silico analysis of the NES of TTV VP3 was performed in order to prove this hypothesis.

The NES is a short region of an amino acid sequence that targets the protein for export from the cell nucleus to the cytoplasm through the nuclear pore complex (NPC). This sequence is recognized by the karyopherin CRM1 which binds the NES-containing protein and consequently shuttles it out of nucleus. The Center for Biological Sequence Analysis (CBS) Software, NetNES 1.1, was used to analyze the third ORF of TTV. This machine learning prediction method is considered to be a significant improvement over the generally used consensus patterns because the software employs both neural networks and hidden Markov models in the prediction algorithm and verify the method on the most recently discovered NESs. Figure 4 shows the resulting NES prediction. The NES score is comprised of the HMM score, calculated based on the hydrophobicity of the region, and the NN score, which determines the likelihood of a specific amino acid to be the last residue of the NES. If the NES score for a series of residues exceeds the threshold, this region is predicted to be the NES.
Nuclear export sequences are 8-15 amino acids that generally contain at least 4 hydrophobic residues separated by a consistent number of nonspecific amino acids. These hydrophobic residues are usually leucine so that region is said to be the leucine-rich NES. The resulting sequence from the prediction software was compared to the consensus sequence determined from in silico analysis in Figure 5. The consensus sequence is defined as $\varphi$-$X_{2,3}$-$\varphi$-$X_{2,3}$-$\varphi$-$X_{2,3}$-$\varphi$ where $\varphi$ is L, V, I, F or M and X is any amino acid. The predicted sequence and consensus sequence are in agreement, therefore, amino acid residues 29-38 (LILIILLLCF) were determined to be the optimal NES and used for mutation design.

**Mutagenesis of the Nuclear Export Sequence**

Once the location of the NES was determined, mutations within the sequence were designed to eliminate nuclear export function. If export capability was eliminated and apoptotic ability was hindered, one could determine a definite relationship between export and apoptotic ability. Furthermore, it has been shown that multimerization correlates with nuclear export and apoptotic ability but the relationship remains obscure. The two domains regulating multimerization and nuclear export overlap. Uncoupling the two functions could elucidate the pathway by which TTV VP3 selectively induces apoptosis. Therefore, two critical residues within the NES were chosen for mutation in order to explore the resulting apoptotic relationship.

Two cytosine to guanine point mutations resulted in two consecutive leucine to alanine changes of residues 35 and 36 of TTV VP3, as shown in Figure 6. Leucine is classified as a hydrophobic amino acid due to its aliphatic isobutyl side chain. Alanine contains a methyl group side chain and is therefore classified as a non-polar, aliphatic amino acid with minor hydrophobicity. Since the NES is recognized in the karyopherin-dependent nuclear export mechanism by its hydrophobicity, the consequence of this standard residue change to alanine is
loss of export without serious misfolding of the protein. The mutations were incorporated into designed primers in order to form the mutated construct. Primer design is represented in Figure 7.

With the design in place, three PCRs were then carried out in order to accomplish this mutagenesis. Figure 8 shows this megaprimer PCR approach. Initially, two separate PCRs were run in parallel. Conditions were identical except for the primers used. In one reaction, the primers were the N terminal primer and the reverse mutagenic primer, while in the other reaction the primers were the C terminal primer and the forward mutagenic primer. This design is shown in Figure 8, panel A. This created two overlapping products, with each reaction amplifying a different half of the TTV VP3 gene, where the overlap occurred in the mutated NES. The two products can be visualized in panel B of Figure 8.

These two overlapping products were then diluted, mixed, denatured, and re-annealed to use as a template in the final PCR step. N and C terminal primers were used to extend and complete the TTV VP3 gene. This design is depicted in Figure 8; panel C, with the final product shown on an agarose gel in panel D. The final result was a complete, mutated TTV VP3 gene. Experiments to amplify and express the mutated gene via transformation into competent *e.coli* are underway. Two other approaches were employed in order to examine the similarities between the apoptotic pathway of TTV VP3 in comparison to CAV Apoptin. Unlike the mutagenesis to uncouple multimerization and localization, the following experiments sought to look at each of Apoptin’s criteria for apoptotic capability independently.

**Leptomycin B Treatment of Nuclear Export**

The nucleo-cytoplasmic shuttling pathway of TTV VP3 was studied in order to determine the mechanism by which the protein localizes in cancer cells. H1299 cells, a human non-small
cell lung carcinoma, were treated with Leptomycin B (LMB), a secondary metabolite produced by Streptomyces spp that inhibits the CRM1 export receptor by glycosylating a critical cysteine residue. The altered CRM1 export receptor cannot bind the NES-containing protein and therefore cannot shuttle the protein out of the nucleus via the nuclear pore complex (NPC). This receptor has been proven to be critical for Apoptin shuttling out of the nucleus; since Apoptin and TTV VP3 are close homologs, if both proteins are employing the same receptor for export, this evidence would support the hypothesis that they follow similar apoptotic mechanisms. Cells were transfected with either wild type TTV VP3 tagged to GFP or an Apoptin mutant, ΔΔ NLS, also tagged to GFP. The Heilman lab has previously established that ΔΔ NLS infected cells respond to LMB treatment, and thus the mutant was used as a positive control to test the viability of the drug. Twenty four hours after transfection, cells were given one of two treatments: no treatment or addition of LMB to the media. Both populations were allowed to incubate for an additional three hours before fixation. After fixation, fluorescence microscopy was employed to image both populations, with results depicted in Figure 9. Images were analyzed visually for nuclear and cytoplasmic expression of protein. Observations were similar for both cells transfected with TTV VP3 and cells transfected with ΔΔ NLS; Samples not treated with LMB displayed cytoplasmic localization of the protein, whereas samples treated with LMB were observed to have nuclear protein localization. This data indicated that inhibition of the CRM1 export receptor prevented nuclear export of TTV VP3, causing it to accumulate in the nucleus. Therefore, TTV VP3 appears to rely on the CRM1 export receptor for cellular shuttling, as does Apoptin.

Although these visual observations clearly indicated the increased presence of TTV VP3 in the nucleus after LMB treatment, further analysis was conducted to confirm these results. The
images were further analyzed using ImageJ software. Initially, three dimensional surface plots of each sample were created, shown in Figure 10, panel A. The presence of green color indicated protein expression in that region of the cell, with white representing no protein expression. The height of each pixel column represents the intensity of expression. The surface plots supported the conclusion that TTV VP3 relies on the CRM1 exporter as they also showed high cytoplasmic expression and low nuclear expression in LMB- cells, with clear nuclear expression in LMB+ cells. Additionally, ImageJ was used to create nuclear to cytoplasmic intensity of expression plots, depicted in Figure 10, panel B. The plots were created by selecting an area of interest containing nucleus and cytoplasm in each cell, and measuring the fluorescence gradient throughout using the program. These plots showed little to no fluorescence for protein expression in the nuclear region of cells without LMB treatment. With LMB treatment, nuclear expression was significantly increased with increased fluorescence levels measured in the nuclear regions of the cells. Again, this data indicates accumulation of protein in the nucleus after CRM1 inhibition, suggesting that TTV VP3 relies on the receptor for export.

Finally, a quantitative analysis was performed to quantitate the nuclear to cytoplasmic ratio of protein expression, normalized to GFP. ImageJ was used to measure the ratio of protein expression in the nucleus compared to the cytoplasm for each cell type and treatment. An increase in ratio was observed for both the ΔΔ NLS and TTV VP3 transfected cells, as shown in Figure 11. As expected for ΔΔ NLS, the nuclear to cytoplasmic expression ratio increased from 0.073 for LMB- cells to 0.75 for LMB+ cells. TTV VP3 showed a similar pattern with an increase in nuclear to cytoplasmic expression ratio of 0.043 in LMB- cells to 0.288 in LMB+ cells. This quantitative analysis confirmed that nuclear export was prevented with LMB treatment, supporting that CRM1 is required for TTV VP3 shuttling. Raw data for this analysis is
provided in Table 1. The change in nuclear protein expression within the entire transfection populations after LMB treatment was also quantified and summarized in Table 2. 12.2% of untreated ΔΔ NLS cells showed nuclear expression (n=41) and 13.8% of untreated TTV VP3 cells showed nuclear expression (n=58). Upon treatment with LMB, 28% of ΔΔ NLS cells (n=50), and 76% of TTV VP3 (n=50) cells showed nuclear expression. Figure 12 shows similarly, that LMB treatment results in increased percentage of cells showing nuclear protein expression for both TTV VP3 and ΔΔ NLS. These results further support the finding that TTV VP3 export is CRM1 dependent. Once the localization pattern of TTV VP3 was shown to mimic the export pathway of Apoptin, the multimerization of the two viral proteins in regards to apoptotic capability were compared.

**Densitometric Analysis of Punctate Expression Pattern**

Multimerization, like nucleo-cytoplasmic shuttling, has been shown to correlate with apoptotic capability in CAV Apoptin. Apoptin forms soluble multimers within normal cells but large insoluble aggregates within tumorigenic cells. TTV VP3 shares significant homology with Apoptin and is therefore hypothesized to behave similarly within transformed cells. To investigate this possible similarity, wild type TTV VP3 DNA, fused to GFP for visualization, was transfected into H1299 cells, a human non-small cell lung carcinoma, and examined for aggregate formation. A scanning confocal microscope was used to photograph cells 24 and 48 hours after transfection with wt-TTV VP3 which was compared to expression of eGFP. Confocal images were analyzed using ImageJ software to determine expression patterns of wild type TTV VP3 DNA.

Protein multimerization is indicated by a punctate expression pattern. Figure 13 shows the confocal images of eGFP transfection and wt-TTV VP3 transfection at two time points.
eGFP is known to be expressed in a non-punctate pattern and can be seen as a continuous gradient within transfected cells. The 24 hour wt-TTV VP3 shows a clear punctate pattern, with sharp green clusters separated by black space. After 48 hours, these aggregates become larger. The expression pattern is likewise demonstrated in the adjacent fluorescence graphs by characteristic peaks. Isolated peaks with large gaps between them indicate a punctate expression pattern. Broader peaks correlate with larger punctate clusters, while narrower peaks indicate small clusters. eGFP expression demonstrates a non-punctate pattern, seen clearly in the plot with no isolation of peaks. 24 hours after transfection with wild type TTV VP3 DNA, H1299 cells demonstrated a clear punctate expression pattern. 48 hours after transfection with wild type TTV VP3 DNA, punctate clusters appeared to become larger and an increase in apoptotic cells was observed. This increasing aggregation over time could contribute to more potent apoptotic ability of the viral protein observed after 48 hours. Protein multimerization has thus been shown, not unlike CAV Apoptin, to correlate with the apoptotic ability of TTV VP3. Both multimerization and localization patterns of TTV VP3 parallel those of Apoptin, providing significant evidence that the two share a common apoptotic time course and pathway.
Discussion

The primary focus of our project was to examine two major criteria of Apoptin’s selective induction of apoptosis within transformed cells in relation to TTV VP3. The leptomycin B (LMB) treatment of wild type TTV VP3 and ΔΔ NLS DNA transfection into H1299 cells has elucidated the export pathway of TTV VP3. ΔΔ NLS is an Apoptin mutant that is known to respond to LMB and was thus used as a positive control for LMB viability. LMB inhibits the CRM1 export receptor by alkylation which prevents binding of the nuclear export sequence (NES)-containing protein to the CRM1 receptor, thus eliminating protein export from the nucleus. CAV Apoptin dependence on CRM1 export in primary cells has been previously established. The homology between Apoptin and TTV VP3 suggests similar export behavior, which our treatment with LMB has successfully concluded. Since TTV VP3 nucleo-cytoplasmic shuttling is likewise depending on the CRM1 karyopherin, the apoptotic pathway between the two viral proteins must be similar. Further exploration of this pathway in regards to multimerization yielded a comparable conclusion.

Multimerization, like localization patterns, has been shown to correlate directly with apoptotic capability within Apoptin. In CAV Apoptin, large insoluble aggregates are present within the cytoplasm of primary cells but small soluble multimers form within the nucleus of transformed cells. Our densitometric analysis of wt-TTV VP3 transfections showed an increase in aggregation alongside an increase in apoptotic cells over time. This apoptotic and multimerization time course of TTV VP3 mimics that of CAV Apoptin. We’ve successfully established similarities in multimerization and localization patterns in relation to apoptotic capacity in TTV VP3 and CAV Apoptin. Therefore, TTV VP3 must follow the same pathway as Apoptin to selectively induce apoptosis. However, many aspects of this pathway beg to be further elucidated.
In terms of future experiments, one major question for scientists is why to pursue TTV research when more information is currently available concerning other homologs, particularly Apoptin. It is critical for researchers to continue to study Torque Teno Virus Viral Protein 3 for multiple reasons. Most importantly, however, is the fact that Torque Teno Virus evolved in humans. Based on the protein’s ability to selectively kill cancer cells, which has been demonstrated in vitro repeatedly, TTV VP3 has the potential to act as or influence future p53 independent cancer therapeutics. This potential could lead to the development of a treatment course which targets the approximately 50% of cancers that currently do not respond or respond poorly to classical chemotherapies that rely on p53 activation. Because it evolved in humans, as opposed to its homologs Apoptin and PCV, which evolved in chickens and pigs respectively, TTV VP3 is a better candidate as a potential drug candidate. This is due mainly to the fact that xenogeneic viruses pose much more of a threat to public health and safety; having evolved independent of the human population, they have the potential to be much more dangerous and unpredictable than intraspecies viruses. In addition to the fact that TTV VP3 originates in a virus that infects more than 90% of tested humans, this virus has been largely found to be non-pathogenic, which further identifies it as a possible drug candidate.

Understanding that further study of TTV is critical to the development of VP3 as a cancer therapeutic, there are multiple different experiments that represent the next logical steps in evaluation of the protein. Initially, it is critical to study the effects of uncoupling the multimerization ability of the protein from its nuclear export functionality. In Apoptin, the nuclear export sequence and predicted multimerization domain cannot be uncoupled. This is due to the fact that they overlap to such a great extent that mutations required to knock out the NES also knock out multimerization. Therefore, the two cannot be studied separately. However, it is
currently not known whether this is the case for TTV VP3. The mutagenic PCR described in the results section established the procedure to obtain the appropriate mutated construct required to study this domain uncoupling. However, no conclusive results have been obtained from transfection of this mutated construct. Therefore, we suggest that transfection experiments be performed using this construct. These experiments will hopefully shed light on the apparent requirement for TTV VP3 to multimerize to cause apoptosis in transformed cells.

Though TTV VP3 export has been shown to be CRM1 dependent, like Apoptin, there is a significant difference in the actual localization patterns of both viral proteins. Apoptin is a primarily cytoplasmic protein that only localizes to the nucleus when inducing apoptosis in transformed cells. TTV VP3, however, localizes primarily in the cytoplasm in transformed cells. It is unknown why TTV VP3 remains in the cytoplasm within cancer cells and how the viral protein is able to induce apoptosis from this position. We suggest that future experiments examine the apoptotic pathway of Apoptin and look for the same known protein interactions within TTV VP3. Since Apoptin is known to induce apoptosis within cells via direct signal transduction, we suggest future researchers employ experiments directed towards detecting caspases, highly conserved, cysteine-dependent aspartate-specific proteases that play a central role in DR apoptotic signals. Such experiments could include immunoprecipitation, western blots, northern blots to detect caspase mRNA, etc. of TTV VP3 transfected into primary and transformed cells at different time points. The results of such experiments could further relate the two viral proteins or provide insight as to why TTV VP3 deviates from the expected localization.

Additionally, it will be critical to demonstrate that the observed multimerization of TTV VP3 is in fact true multimerization of the protein, as opposed to simple clustering, such as in a vesicle, lysosome, or other organelle. The punctate expression pattern we observed with
transfection of GFP-[wt-TTV VP3] usually allows researchers to conclude one of two things. Either the protein is forming insoluble multimers, which we’ve assumed, or the protein is clustered within an endosome. One way we suggest future researchers to definitely determine whether the latter is true is by co-localization studies. Researchers could conclude if the viral protein is, in fact, trapped within vesicles by tagging wt-TTV VP3 to GFP and staining cells to visualize the endoplasmic reticulum (GRP94), Golgi (GM130), and early endosomes (EEA1). If the merged images don’t show expression within stained regions, researchers could further support out multimerization findings.

Finally, we observed a similar apoptotic time course in TTV VP3 transfected cancerous cells as has been established for Apoptin. More specifically, a significant percentage of cells were observed to be apoptotic 24 hours after protein transfection, and nearly 100% of cells were apoptotic 48 hours after transfection, which is concurrent with results obtained for Apoptin. Apoptotic time course is an important piece of evidence in elucidating whether Apoptin and TTV VP3 follow similar apoptotic pathways; if the two proteins are able to induce apoptosis in similar time frames it further suggests that they follow similar pathways. In contrast, if very different time frames are observed, that is strongly indicative that different pathways may be involved. Our observations were made using fluorescence microscopy, which provides clear, visual results, as apoptotic cells are easily distinguished by their characteristic spherical appearance. However, to further support these observations, we suggest that biochemical studies be performed to measure apoptosis. These types of studies are the current standards for measuring apoptosis, and are considered more reliable than simply counting apoptotic cells under microscopy.
Appendix of Figures

Figure 4: Prediction of Nuclear Export Sequence. (a) Plot of NES compatibility score by amino acid position. The NN recognizes the last hydrophobic residue within the NES motif. The HMM recognizes regions of hydrophobic residues that could potentially belong to the NES. The prediction server calculates the NES score from the HMM and NN scores. If the calculated score exceeds the threshold, then that particular residue is expected to participate in a nuclear export signal. (b) The predicted NES of TTV VP3 compared to the consensus NES where φ is a hydrophobic residue and X is any amino acid. The consensus NES contains a series of hydrophobic residues separated by a consistent amount of nonspecific residues. The predicted NES from the NetNES software directly correlates with the consensus NES.
Figure 5: Identification and Analysis of TTV Nuclear Export Sequence. The predicted NES of TTV VP3 compared to the in-silico derived consensus NES where φ is a hydrophobic residue and X is any amino acid. The consensus NES contains a series of hydrophobic residues separated by a consistent amount of nonspecific residues. The predicted NES from the NetNES software directly correlates with the consensus NES.
Two point mutations within the region of the TTV VP3 gene that codes for the predicted nuclear export sequence (NES) results in two leucine to alanine mutations in amino acid residues 35 and 36. These minor mutations are accepted as the conventional method employed for NES knock out within a protein; it is therefore assumed that these mutations consequently eliminate the nuclear export functionality of TTV VP3.

**Figure 6: Design of Mutagenic Parameters to Knock Out TTV VP3 NES Functionality.**
Two point mutations within the region of the TTV VP3 gene that codes for the predicted nuclear export sequence (NES) results in two leucine to alanine mutations in amino acid residues 35 and 36. These minor mutations are accepted as the conventional method employed for NES knock out within a protein; it is therefore assumed that these mutations consequently eliminate the nuclear export functionality of TTV VP3.
Figure 7: Primer Design for use in Mutagenic PCRs.

C and N terminal primers were obtained from previous experiments within the Heilman lab group. Terminal primers represent the extreme end of the TTV VP3 gene, with the N terminal primer complimentary to the region of the gene that codes for the N terminus of the protein and the C terminal primer complimentary to the region of the gene that codes for the C terminus of the protein. Forward and reverse mutagenic primers were designed to incorporate the specified NES mutations, with the critical codons bolded. Mutagenic primers extended approximately twenty bases beyond the point of mutation both upstream and downstream of this point in order to ensure appropriate annealing and extension.
Figure 8: Incorporation of Mutation into the TTV VP3 Template Gene by PCR. (a) Two separate PCRs, each involving a terminal primer coupled with a mutagenic primer yield two overlapping products. The N terminal primer was coupled with the reverse mutagenic primer to produce a top strand mutation, whereas the C terminal primer was coupled with the forward mutagenic primer to produce a bottom strand mutation (b) Agarose gel electrophoresis showing two resulting strands from first PCR of different lengths. Lane designations are 1: Marker; 2: Forward Mutagenic Primer + TTV VP3 C Terminal Primer; 3: Reverse Mutagenic Primer + TTV VP3 N Terminal Primer. (c) N terminal and C terminal primer extend overlapping strands to yield full double stranded product. (d) Agarose gel showing resulting strand from second PCR in which the products from the first PCR are annealed together. Lane designations are 1: Marker; 2: Amplified TTV VP3 Insert with point mutations incorporated.
Figure 9: Localization Patterns of TTV VP3 in H1299 Cells Treated with Leptomycin B. Scanning confocal microscope images were recorded after transfection and 3 hour treatment with LMB. The first row shows eGFP control, which was recorded for comparison of GFP normal expression and expression patterns of transfected proteins. The second and third rows show ΔΔ NLS mutants (positive control for LMB viability) LMB- and LMB+, respectively. The fourth and fifth rows show wild type TTV VP3 transfections, LMB- and LMB+, respectively. Cytoplasmic localization was observed for both protein transfections in LMB- cells. Clear nuclear accumulation was observed for both protein transfections in LMB+ cells.
Figure 10: Qualitative Analysis of Nuclear and Cytoplasmic Expression Levels of TTV VP3 in LMB- and LMB+ Cells. Images of cells obtained from Figure 9 were analyzed qualitatively to measure protein expression levels in the nuclear and cytoplasmic regions (a) Three dimensional surface plots of TTV VP3 Expression in LMB- and LMB+ Cells. Green color indicates expression and relative height of each column indicates level of expression in that area of the cell. (b) Qualitative assessment of nuclear and cytoplasmic expression before (left column) and after (right column) LMB treatment. Red dotted line indicates the division between nuclear and cytoplasmic cellular regions.
Figure 11: Quantitative Analysis of Nuclear and Cytoplasmic Expression Levels of TTV in LMB- and LMB+ Cells. Quantification of Nuclear to Cytoplasmic Expression Ratio Normalized to eGFP. eGFP ratio of nuclear to cytoplasmic expression was normalized to value of 1:1. Ratios for all other conditions were then adjusted accordingly.
Figure 12: Percentage of Cells with Visible Nuclear Expression of Protein

Cells in both the ΔΔNLS and TTV VP3 transfection populations were observed using fluorescence microscopy for protein localization. (a) ΔΔNLS cell population with and without LMB treatment. A marked increase was observed in the percentage of cells expressing protein in the nucleus with LMB treatment, from 12.1 to 28. (b) TTV VP3 cell population with and without LMB treatment. A marked increase was observed in the percentage of cells expressing protein in the nucleus after LMB treatment, from 13.8 to 76.
Figure 13: Punctate Assessment of TTV VP3 Expression in H1299 Cells 24 and 48 Hours after Transfection. A qualitative evaluation of the punctate nature of expression over time was completed using ImageJ software. (a) eGFP cellular expression was evaluated for punctatedness as a control. (b) WT TTV VP3-GFP expression was evaluated for punctate quality 24 and 48 hours after transfection of TTV VP3 DNA. Plots assess punctatedness in terms of peak patterns, where isolated peaks indicate a punctate spot. Narrow peaks indicate smaller clusters, while broader peaks represent larger punctate clusters.
Appendix of Tables

Table 1: Nuclear to Cytoplasmic Expression Ratio Data

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Table 2: Apoptotic Time Course Analysis

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References


