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Localization of the Human Torque Teno Virus VP3 Protein

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Determining Localization Domains and Apoptotic Ability of the Human Torque Teno Virus VP3 Protein

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Abstract

The Human Torque Teno virus (TTV), a member of the *circoviridae* family, exhibits apoptotic activity through the expression of the VP3 protein. Closely related to TTV, the Chicken Anemia Virus (CAV) expresses the Apoptin protein, a homolog of VP3, which is also capable of inducing apoptosis. Interestingly, this induction only occurs in transformed cells while leaving primary cells intact. To achieve this specificity, Apoptin undergoes nucleocytoplasmic shuttling dictated by two localization signals: a Nuclear Export Signal (NES) and a Nuclear Localization Signal (NLS). Based on sequence similarities between Apoptin and TTV-VP3, the VP3 protein is expected to localize using a similar mechanism. Using the VP3 gene of TTV we have created two truncated mutants, each containing one of two putative localization signals (NES or NLS), which were then inserted into GFP plasmid vectors. These constructs were then transfected into transformed H1299 cells to assess functionality of the localization domains. Additionally, the complete gene encoding the VP3 protein was transfected into primary cells to determine apoptotic selectivity. Here we not only conclude the existence of the localization signals in the TTV-VP3 protein but also that the apoptosis inducing ability of the VP3 protein is cell-type specific, similar to that of CAV-Apoptin.

Introduction

The Torque Teno Virus was first discovered in 1997 from the blood serum of a Japanese posttransfusion hepatitis patient by Nishizawa *et al* [1]. The man, known only as "TT", represented a minority population of people with chronic hepatitis or cirrhosis after blood transfusion and yet tested negative for any of the known hepatitis A through E viruses. With this result, Nishizawa made the tentative conclusion that this "Transfusion-Transmitted Virus" was a likely candidate for the unknown, hepatitis causing pathogen. However, after several clinical trials, it was found that the frequencies of the TTV infection throughout the human population were widespread among several genotypes [1]. In addition, no observable correlation was ever found to link TTV to any hepatic illness or any other pathogenicity. Because of this, it is generally assumed today that TTV is a highly successful pathogen that has the ability to create a nearly perfect commensal relationship with its human host.

Initial characterization of TTV was done using the N22 viral clone, which was found to be susceptible to Mung Bean Nuclease but not to RNase A, which led to the conclusion that TTV comprised a single stranded DNA genome (ssDNA). In addition, Tween-80 treated virus was found to be unaffected in a sucrose gradient leading to the conclusion that it is non-enveloped [2]. The full genome of the TA278 strain of TTV was initially sequenced by Mushahwar and completed by Miyata *et al* [3]. The virus was shown to be a continuous circular type virus with a negative sense ssDNA genome of an average length around 3.8 kilobases. Genetic comparison to known viruses exhibited a degree of correlation with the Chicken Anemia Virus (CAV) [4]. As such, TTV was reclassified to the *Circoviridae* family of viruses which includes CAV and the Porcine Circovirus (PCV) under the "floating genus" Anellovirus. TTV was furthermore

formally renamed the Torque Teno virus. It has since been proposed that TTV, CAV, PCV, and their genetic cousins be reclassified further into a new family known as *Anelloviridae* [5].

The Torque Teno Virus, interestingly, has a great deal of genetic variance within its numerous genotypes. This high genetic variability is not characteristic of DNA-type viruses considering that the cellular polymerases used by the virus have highly accurate exonuclease activity thereby preventing an extensive mutation rate [6]. Regardless of its high mutation rate, all TTV versions have about 3.8 kilobase genomes. In addition, the Torque Teno Virus encodes for three mRNAs of length 3.0, 1.2, and 1.0 kilobases [5]. In the TA278 strain of TTV, at least three open reading frames (ORF) are known to exist.

Of particular importance to this investigation is ORF3 which is known to contain the coding region for the TTV-VP3 protein, a homologue of the Chicken Anemia Virus protein Apoptin. This protein has been widely studied for its distinct ability to cause transformed and malignant cells to undergo apoptosis and yet leave primary cells intact [7]. TTV-VP3 from the TA278 VP3 isolate of Torque Teno Virus is a non-structural protein of 105 amino acid residues [8]. While there is little sequence homology at either the nucleic acid or amino acid level between Apoptin and TTV-VP3, there are several significant structural and functional similarities that may help elucidate the apoptosis-inducing mechanism of TTV-VP3.

Relationship with the Chicken Anemia Virus

Currently, the closest known relative to the Torque Teno Virus is the Chicken Anemia Virus (CAV), a member of the *circoviridae* family. CAV is currently the only member of the family *Circoviridae*, genus *Gyroviridae*, and causes severe anemia in chicken populations via the destruction of erythroblastoid cells and thymocytes within bone marrow. CAV has a single-

stranded antisense DNA genome approximately 2.3 Kb in size. When introduced in a host population, this virus replicates as double-stranded DNA and transcribes one mRNA encoding three proteins; VP1, VP2, and VP3. Expression of the VP1 and VP2 proteins is proven to be necessary for the development of structural elements including the viral capsid, while the VP3 or Apoptin protein induces apoptosis, a process of programmed cell death [7]. CAV Apoptin is rather unique in its ability to induce apoptosis, because it employs a mechanism independent of the p53 tumor suppressor pathway. Similar to Apoptin, TTV-VP3 also induces apoptosis in a p53-independent manner [9]. P53 is a tumor suppressor protein responsible for initiating DNA repair when an external stressor creates some type of damage to the DNA. P53 also has the ability to arrest the cell cycle between the G1 and S phases in order to accomplish proper DNA repair. If necessary, the p53 tumor suppressor is capable of inducing apoptosis in permanently damaged cells. Transformed and tumorigenic cells are often p53 null, making many current cancer therapies obsolete. As CAV Apoptin acts independently of p53 regulation, it is currently of interest in anti-cancer applications. Many current cancer therapies aim to regulate the cascade of p53 DNA repair and apoptosis, and are unable to do so in malignant cells where p53 is absent [9]. Because both Apoptin and TTV-VP3 possess similar apoptosis-inducing mechanisms, it is believed that they may share other distinctive characteristics as well.

CAV is also rather unique, as it induces apoptosis via Apoptin only in transformed cells. When primary cells are transfected with Apoptin, apoptosis is not observed and long-term expression of Apoptin has not shown any detrimental effects to cell viability [7]. Accordingly, it is believed that TTV-VP3 also induces apoptosis only in transformed cell lines leaving primary cells unaffected. This highly selective mechanism of action could play a key role in the development of functional anti-cancer agents.

The highly selective nature of the apoptotic ability of Apoptin is often attributed to its ability to undergo nucleocytoplasmic shuttling. Apoptin has been shown to be nuclear localized in transformed cells and cytoplasmic in primary cells. Apoptin has been shown to associate with the APC1 subunit of the anaphase-promoting complex/cyclosome and this binding induces arrest between the Gap 2 and Mitotic phases of the cell cycle (G2/M arrest). This cell cycle arrest is believed to lead to apoptosis in a p53 independent fashion [10]. . The association of Apoptin with the anaphase-promoting complex in transformed cells leads to the assumption that Apoptin localizes to the nucleus. The binding of the APC1 subunit is not observed in primary cells transfected with Apoptin, which suggests the involvement of a shuttling mechanism that dictates the fate of Apoptin based on cell type. Conversely, TTV-VP3 localizes to the cytoplasm in both primary and transformed cell lines, though it has been suggested that the viral protein still exhibits nucleocytoplasmic shuttling in order to induce apoptosis [11]. The shuttling mechanism of Apoptin is dependent on two specific localization domains, which are believed to be present as well in the VP3 homolog.

Apoptin exhibits nucleocytoplasmic shuttling through nuclear pores via Chromosome Region Maintenance 1 (CRM1) – mediated nuclear export and Ran-dependent nuclear import. Nuclear pore complexes (NPCs) are transmembrane proteins that coordinate with freely available proteins known as importins and exportins, together known as karyopherins, which contain receptors that recognize and bind specific domains on the nuclear-transported molecules [12, 13]. The Apoptin protein expressed by CAV contains two domains, each of which resides on an opposite end of the protein. The N-terminus of Apoptin contains a putative nuclear export signal (NES) comprising amino acids 37 through 46. Nuclear export signals are commonly characterized by leucine-rich regions of protein sequences or repetitive sequences of

hydrophobic residues [12]. In the case of the NES of Apoptin, two specific leucine residues – leucine-44 and leucine-46 – display a key role in nuclear export. In contrast, the C-terminus of Apoptin contains a putative bipartite nuclear localization signal (NLS) that comprises amino acids 70 through 121 [11]. Nuclear localization signals commonly include regions characterized by a multitude of charged amino acid residues, primarily lysine and arginine. In the Apoptin protein, residues 86-88 and 116-118 are lysine and arginine rich and make up the putative bipartite NLS. Both the NES and NLS are together responsible for the nucleocytoplasmic shuttling that selectively activates the killing of transformed cells by Apoptin, binding to exportins and importins respectively. Because the shuttling mechanism of CAV Apoptin is dependent on both the NES and NLS localization domains, it is believed that TTV-VP3 also possesses these domains.

Project Overview

Previously, the VP3 gene from TTV was successfully synthesized. Laboratory synthesis was necessary because no remaining patient samples of TTV are known to exist. The homolog was created using 44-mer oligo primers that were annealed, yielding a double-stranded gene segment. The homolog was successfully cloned into a Green Fluorescent Protein plasmid vector and 3X Flag vector for subsequent study [14]. The homolog was sequenced and proven to match the expected sequence of TTV strain TA278 VP3 gene.

For this portion of the project, we wanted to investigate two potential mechanisms that could bridge the gap between CAV and TTV function. First, we set out to assess the functionality of two putative localization domains that play an essential role in nucleocytoplasmic shuttling. To confirm the existence of these two domains, we isolated the two

gene segments by effectively splitting the homolog in half. In order to visualize the putative nucleocytoplasmic shuttling of VP3, we transfected cancerous H1299 cells with VP3 truncation mutants. Our results show that TTV-VP3 does in fact undergo some sort of nucleocytoplasmic shuttling. In those transfected with the putative NES, cytosolic aggregation is observed while in NLS containing vector transfected cells, nuclear accumulation is observed. Second, we wanted to examine the potential selectivity of the VP3 apoptosis inducing mechanism. Because of the relatively high incidence of structural similarity between CAV and TTV, it is likely that TTV-VP3 also exhibits high specificity for killing only transformed cells. PFF cells were transfected with the coding region of the VP3 gene and fluorescence levels were observed. Our results allow us to conclude that both the NES and NLS localization domains are functional in the TTV-VP3 gene and that VP3 apoptosis induction is cell-type specific, inducing apoptosis in transformed cells while leaving primary cells unaffected. Both the existence of these domains and the specificity of VP3 apoptotic ability closely correlate to characteristics seen in CAV Apoptin.

Materials and Methods

PCR of Putative NES and NLS Sequence

The initial PCR was devised in order to amplify two separate halves of TTV ORF3, one containing the putative NES and the other containing the putative NLS. The template vector previously created in our lab was that of a 3xFlag vector from Sigma-Aldrich containing the full wild type sequence of TTV-VP3. Primers were designed to accomplish this while also introducing useful restriction sites (Eco R1 and Bam H1) and keeping in frame with the eventual ligation into GFP vector.

The two PCR reactions (labeled NES and NLS) were run in triplicate utilizing three different annealing temperatures: 55 C, 60 C, and 65 C. This was done in order to maximize reaction efficiency owing to the unknown effect on stringency due to primer modification. The following PCR program was used for all three PCR sets.

95 C	– 4 min	
95 C	– 30 sec	
(55/60/65)C	– 30 sec	x 30 cycles
72 C	– 30 sec	
72 C	– 5 min	
10 C	– Infinity	

The finished PCR reaction products were visualized on a 0.9 percent agarose gel electrophoresis in TAE and fluoresced with ethidium bromide. No one band pertaining to any of the three annealing temperatures had greater amplification efficiency. Therefore, all three bands were combined and the DNA isolated through the use of a GeneClean kit by MP Biomedicals and labeled NES and NLS respectively as purified insert.

Cloning and Ligation into T-vector

For greater ease in molecular cloning for both present and future use, the purified inserts for both the NES and NLS were cloned into a Promega pGEM T-Vector. The single overhanging adenosine residue left by the activity of Taq polymerase optimized the reaction and prevented self-ligation by the either the vector or the insert. The T-vector vector contains *lac* operator to allow for blue-white screening and a constitutively-expressed *amp* gene for conferring ampicillin resistance. The ligation of each insert into corresponding vectors was done according to the standard protocol provided by Promega. The newly formed full constructs were then transformed into commercially acquired competent *E. coli* cells and plated on agar plates treated with ampicillin and coated with 0.1 mM IPTG and 40 μg/ml X-gal. Positive (white) colonies were inoculated in 2 ml LB media with ampicillin and allowed to incubate overnight in a 37 C shaker.

Both sets of plasmid constructs were isolated from 1.5 mL of the *E. coli* cultures using a standard alkaline lysis protocol and resuspended in 50 μL TE. Restriction digests were performed on the diluted constructs utilizing the Eco R1 and Bam H1 sites included on the insert lengths. The restriction reactions were performed at 37 C for 1 hour using 1 μL RNAse A in order to reduce contamination by the appearance of short sequence RNA. Visualization was performed on a 0.9 percent agarose gel. After confirmation of proper transformation and ligation, the remaining 0.5 mL of *E. coli* cultures were used to inoculate 50 mL of LB media with 50 μL 100 mg/ml ampicillin and allowed to incubate overnight at 37 C. The large scale cell cultures were lysed and the DNA constructs were purified using a Promega Wizard Midipreps DNA Purification System and diluted in 300 μL TE. Again, restriction digests were performed to verify the presence of insertions. The purified NES and NLS plasmid constructs were then diluted to proper levels using UV spectroscopy and sent out for sequencing to confirm proper insertion.

Cloning and Ligation into pEGFP-C1 vector

The newly formed constructs were restricted and re-ligated into pEGFP-c1 vector by Sigma Aldrich. The pEGFP-c1 vector expresses insert proteins with an N-terminal GFP tag. This allowed for a fusion of the NES and NLS protein halves with a fluorescent GFP marker. This vector is under the control of the cytomegalovirus (CMV) promoter for mammalian expression and contains the gene for kanamycin resistance.

After verifying the two sequences in T-vector, the respective inserts were restricted with Eco RI and Bam H1 in triplicate and visualized on a 0.9 percent agarose gel. The three insert bands were purified together using a GeneClean Kit by MP Biomedicals. In addition, pEGFP-c1 plasmids from a previous study were restricted in triplicate with Eco RI and Bam H1 and purified in the same manner. This would allow for corresponding "sticky ends" on both the inserts and the GFP vector. The purified NES and NLS inserts and GFP vector were ligated at 1-to-1 (2 μL: 2 μL), 2-to-1 (4 μL: 2 μL), and 3-to-1 (6 μL: 2 μL) insert-to-vector ratios. The ligation reactions were transformed into competent *E. coli* and plated on agar plates treated with kanamycin and allowed to incubate at 37 C overnight. Colonies were harvested and inoculated in 2 mL of LB broth with 2 μL of 100 mg/ml kanamycin for incubation overnight. 1.5 mL of the *E. coli* cultures were subjected to small-scale DNA purifications as described before. The purified plasmid DNA for each both NES and NLS were again restricted by Eco R1 and Bam H1 and visualized on an agarose gel to verify transformation and ligation. The remaining 0.5 mL of the positive cultures were used to inoculate 50 mL LB media with 50 μL of 100 mg/ml kanamycin and incubated overnight. The large scale cell cultures were lysed and the DNA constructs were purified using a Promega Wizard Midipreps DNA Purification System and diluted in 300 μL TE. Again, restriction digests were performed to verify the presence of insertions. Upon confirmation, the NES and NLS constructs were labeled for eventual transfection and fluorescence microscopy experimentation.

Apoptosis Assay of Primary Cells with Wild Type TTV VP3

Previous experimentation shows that the wild type TTV-VP3 gene would encode for a protein that caused H1229 small lung carcinoma cells to undergo apoptosis. For this project, specificity was tested with non-cancerous cells. The 3xFlag vector containing the full wild type TTV-VP3 gene used as the template for the differential PCR's of the NES and NLS was used as a mammalian expression vector for primary human cells. Primary foreskin fibroblasts were transfected with the vector using a Lonza Nucleofector and incubated for 24 hours at 37 C on a 96-well plate with 200 μL media (DMEM + 10% FBS with 1x PSF). Apoptosis was measured and quantified using an Apo-One Homogeneous Caspase 3/7 Assay kit. 100 μL of the media was removed from each well and 100 μL of kit caspase substrate and buffer was added and allowed to incubate at room temperature for 18 hours in the dark. Fluorescence readings were taken on a UV/visible spectrometer at excitation 499nm and emission 521nm.

H1299 Transfection and Fluorescence Microscopy

Cancerous cell transfection was performed on H1299, a highly adherent, tumorigenic lung cancer cell line. These cells were also maintained in media consisting of DMEM + 10% FBS with 1x PSF. Cells were passed into a six well plate containing a circular glass coverslip and allowed to adhere and reach ~70% confluence over two nights of incubation at 37 C. Transfections were accomplished using a Qiagen Effectene Transfection Reagent and procedure. The pEGFP-c1 NES and NLS constructs were prepared with the transfection reagent according to the protocol and added to the correspondingly labeled wells. In addition to the NES and NLS constructs, closed pEGFP-c1 vector was transfected as a control. The newly transfected cells were provided with another 1.5 mL of media and allowed to incubate overnight at 37 C.

The next day, the plates were removed of all media and Effectene reagent, washed with PBS, fixed with a 4 percent paraformaldehyde solution in 1M PBS, and again washed with PBS. The coverslips were removed, dried, and plated face down on slides with 35 μL mounting media consisting of

50% glycerol, 100 mM Tris, 2% DABCO, and DAPI stain. The coverslips were lightly pressed to remove excess mounting media and sealed around the edge with clear nail polish. These were stored at 4 C in a dark box. Microscopy was performed on a laser confocal microscope.

Results

Based on the similarity between TTV-VP3 and CAV Apoptin, it is believed that the VP3 protein also contains two putative localization domains: both a Nuclear Export Signal (NES) and a Nuclear Localization Signal (NLS). Based on the comparison of TTV-VP3 and Apoptin, it is believed that these sequences are located on opposite ends of the peptide: NES at the N-terminus and NLS at the C-terminus. First, we set out to test the functionality of these two individual domains in TTV-VP3. A strategy was set forth to isolate the two domains and test functionality separately (Fig. 1, 2).

Rather than attempt to isolate the putative domain sequences themselves, we found it beneficial to take a conservative approach and create two adjoining constructs from the full TTV-VP3 sequence. This way, we can ensure that no portion of the full functional VP3 gene is unaccounted for. Using the full TTV-VP3 gene sequence, we identified nucleotides 1-120 (residues 1-40 in translated peptide) as the NES-containing fragment and nucleotides 121-318 (residues 41-105 in peptide) as the NLS-containing fragment (Fig. 3, 4). The result is an NES fragment of 120bp and an NLS fragment of 198bp. The gene was split at nucleotide 120 not only to easily differentiate the two constructs by size, but also to ensure that either localization signal at the ends of the peptide is properly conserved within its respective fragment.

In order to successfully isolate the two fragments from the full TTV-VP3 gene, PCR primers were designed to amplify the two fragments individually (Table 1). The reverse NES primer included sequence for stop codon creation in the NES fragment. The primers also included restriction sites; EcoRI restriction sites in both forward primers and BamHI restriction sites in both reverse primers. By incorporating these restriction sites, we allowed for the simple

excision of our truncation mutants with as little extra nucleotide sequence on either end of the fragments as possible. This also allows for a clean ligation into GFP expression vector, keeps the fragment sequence in frame with GFP vector expression, and ensures successful directional cloning. A total of 6 PCR programs were run – three for the NES fragment, three for the NLS fragment – using the respective primer sets and PCR products were then separated via agarose gel electrophoresis (Fig. 5). Each fragment PCR was run using three different annealing temperatures (55^{o}C, 60^{o}C, and 65^{o}C) in order to ensure optimal amplification efficiency.

In preparation for ligation into GFP vector, we ligated both the NES and NLS fragments into T-vector constructs. T-vector is a plasmid vector specifically designed to efficiently ligate to Taq Polymerase PCR products. Isolated Taq Polymerase PCR products contain overhanging 3' adenosine residues that efficiently and selectively ligate to 3' overhanging thymine residues found in T-vector. Rather than simply digesting our purified PCR products using Eco RI and Bam HI, ligating into and restricting the fragments out of T-vector allows for a visual representation of a successful digestion. The T-vector ligation reactions were introduced to competent E.coli. We plated the transformed E.coli onto separate LB-Amp plates. After 24 hours positive colonies were inoculated into large scale cultures. Midi preps were performed on both T-vector constructs and plasmid DNA was isolated. In order to confirm proper insertion of the NES and NLS fragments into T-vector, both purified constructs were digested using EcoRI and BamHI restriction enzymes. The restriction reaction products were then separated via agarose gel electrophoresis (Fig. 6). The dropout segments observed accurately correspond to the size of the NES fragment and the NLS fragment respectively.

In order to properly assess the functionality of these localization domains, the two truncated VP3 mutants must be attached to a reporter gene. For this we chose GFP due to its

common usage within the scientific community and commercial availability. In addition, GFP is a simple way to visualize proteins of interest due to its inherent ability to glow under specific UV wavelengths. This is easily observed using epifluorescence and laser confocal microscoscopy. By using pEGFP-C1 expression vector specifically, we are able to tag the GFP protein to the N-terminus of our truncation mutants.

We digested the pEGFP plasmid vector using EcoRI and BamHI restriction enzymes. The restriction reaction was then separated via gel electrophoresis and the cut plasmid was later purified. The two digested dropouts from the previous T-vector ligation were also purified from agarose gel. These inserts, having been restricted with Eco RI and Bam HI at opposing ends of each fragment, have "sticky ends" that will efficiently ligate to the corresponding restriction sites in pEGFP in a directionally specific manner.

The two newly ligated plasmid expression constructs were introduced to competent E.coli. Cultures were then plated on LB-kan plates, inoculated into large scale cultures, and purified via midi prep. Purified plasmid DNA for each construct was then digested with both Eco RI and Bam HI. Restriction reactions were then separated using agarose gel electrophoresis (Fig. 7). Again, fragment dropouts were confirmed for each restricted construct.

The localization and apoptotic capability of CAV Apoptin has been widely researched using a large number of cancerous, transformed, and primary cell lines. The H1299 non-small lung carcinoma cell line has become the standard cancerous cell line for Apoptin study due to its nonspecific adhesion to various surfaces, high replication rate, and ease of DNA uptake. Most importantly, the H1299 cell line demonstrates the expected behavior of CAV Apoptin localization and induction of apoptosis. For these reasons, we chose to use H1299 cells for our

TTV-VP3 truncation mutant construct transfections. Two separate transfections were performed: one using NES-pEGFP construct and the other using NLS-pEGFP construct both in H1299 cells. By performing these transfections using pEGFP constructs, we can accurately visualize the localization patterns of each fragment.

H1299 non-small lung carcinoma cells were transfected with plasmid expressing NES-pEGFP construct or NLS-pEGFP construct. After 24 hours, the cells were mounted on microscopy slides using mounting media containing DAPI nuclear stain. Distinct localization patterns were observed for cells transfected with NES-pEGFP construct (Fig. 8) and cells transfected with NLS-pEGFP construct (Fig. 9). The localization domains appear to be functional, though they do not appear to be as efficient as their CAV-Apoptin homologues. To quantify the localization of each domain fragment, representative cells transfected with either construct were chosen and GFP fluorescence was measured in both nuclear and cytoplasmic regions for all. The ratio of nuclear:cytoplasmic GFP expression was then calculated. Results were converted to percentages of cellular localization (Fig. 10). The NLS fragment displays nuclear localization of about 65% (approx. 35% cytoplasmic), while the NES fragment displays cytoplasmic localization of about 70% (approx. 30% nuclear). From this data we conclude that both the putative NES and putative NLS domains are in fact functional.

In addition to assessing the functionality of the two putative localization domains, we also set out to test the apoptosis-inducing ability of the full TTV-VP3 gene in primary cells. Induction of apoptosis in cancerous cell lines has previously been observed. To test whether TTV apoptosis induction is cell type specific, Primary Foreskin Fibroblast (PFF) cells were transfected with 3X Flag vector containing the TTV-VP3 gene insert. Additionally, PFF cells were transfected with 3X Flag vector without insert to serve as a control. Because TTV-VP3

induces apoptosis in a p53 independent manner, a caspase 3/7 assay was then performed using the transfected PFF cells. Relative fluorescence was measured in both the cells transfected with VP3-containing vector and cells transfected with plasmid vector alone. In order to normalize the obtained fluorescence data, the vector background fluorescence was subtracted from the construct fluorescence. After normalization, relative fluorescence was measured as 0.86 RFU. The results of the normalization were then compared to the relative fluorescence of H1299 cells transfected with TTV-VP3-3X Flag construct after subtraction of 3X Flag vector background fluorescence (Fig. 11). The relative fluorescence of the transfected H1299 cells was measured at approximately 150 RFU. The increased fluorescence in transfected H1299 cells corresponds to an increase in apoptosis. Accordingly, low levels of fluorescence in transfected PFF cells indicate little or no induction of apoptosis by TTV-VP3. Thus we conclude that TTV-VP3 induces apoptosis in a cell-type specific manner, only inducing apoptosis in transformed cell lines while leaving primary cell lines unchanged.

Discussion

The human Torque Teno Virus (TTV) VP3 gene is normally considered a close homologue to the widely studied Chicken Anemia Virus (CAV) protein Apoptin gene. Each encodes for a viral protein that has novel capacity to induce apoptosis in cancerous and transformed cells. It is has also been shown that the activity of CAV Apoptin is selective to cancerous cells. When expressed in primary cells, Apoptin is dormant. Unfortunately, the mechanism that it uses to carry out this task is largely unknown. It has been hypothesized in previous studies that the localization of Apoptin is imperative to its apoptotic capability [8]. In primary cells, Apoptin localizes in the cytoplasm of the cell, whereas in cancerous cells Apoptin localizes directly to the nucleus. This nuclear specificity is generally thought to be vital to its function.

Contrary to this hypothesis is the third viral protein of the Torque Teno Virus. As previously stated, TTV-VP3 has the ability to force cancerous cells to undergo apoptosis. However, while TTV-VP3 shares Apoptin's ability to kill cancerous cells, it differs on the specificity of its localization. In both primary and cancerous cell lines, TTV-VP3 localizes in the cytoplasm. This primary localization would suggest that TTV-VP3 contains a Nuclear Export Sequence (NES), much in the same way as Apoptin. In addition, prior to its partisan localization in the cytoplasm, TTV-VP3 appears to have a much more variable distribution between the cytoplasm and nucleus. The rationale for this is that the protein also has a functional Nuclear Localization Sequence (NLS). It is, however, less potent than the NES and over time the equilibrium between the two opposing sequences shifts towards the cytoplasm.

The images procured from the confocal microscopy of both the NES and NLS halves of TTV-VP3 somewhat support this hypothesis. The fluorescence imaging of the NES and NLS segments show a high degree of localization of each putative sequence to their corresponding domain. This data proves the existence of both sequences in TTV-VP3 and corroborates the evidence gathered previously. Nonetheless, the quantitative analysis of each fragment shows a nearly equivalent level of sequestering in the cell. This would oppose the hypothesis that the NLS less effective in localizing VP3 in comparison to the NES. However, this conclusion is rather premature. It is very likely that the equilibrium shuttling between the NES and NLS is specific to the state of the cell and is dependent on both being present in the cell simultaneously. Future research could focus on the relative affinities and efficacies of the NES and NLS sequences in various conditions in an effort to determine the cause of the cytoplasmic distribution.

Another interesting feature of the confocal images is the comparatively moderate level of shuttling that both the NES and NLS provide. While the NES has a clear affinity for the cytoplasm and the NLS for the nucleus, neither provides complete localization of the protein in either location. This is rather comparable to wild type TTV-VP3 which shows relatively imperfect cytoplasmic localization. If the hypothesis of varying levels of NES and NLS localization efficiency is true, one would expect the NES and NLS fragments to have a far greater level of efficiency in their respective shuttling. In other words, the competing signal of opposing direction, both the NES and NLS should be far more effective in their respective localization.

The localization of the NES and NLS of VP3 does not exhibit the same efficiency of localization as Apoptin. Fluorescent GFP tagging of Apoptin shows not only specificity of cell

type in localization, but a far greater shuttling efficiency [11]. Confocal images of Apoptin in cancerous cells show a complete localization to the nucleus of the cell. In primary cells, Apoptin shows almost no signal at all in the nucleus. The specificity for Apoptin's localization extends to its respecting NES and NLS sequences. When cloned out of the full length genome (as was done in this experiment), the results were even more pronounced, showing nearly absolute localization. As stated before, TTV-VP3 has a much more fractional representation of localization. The reasoning behind this observation most likely stands as an extension of the aforementioned hypothesis.

Previously it was thought that the consistent cytoplasmic localization of the wild type TTV-VP3 protein is due to a weaker NLS signal in comparison to the NES signal. The only amendment to this supposition is that both the NES and NLS have inferior activities in comparison to the corresponding sequences in Apoptin. These facts do not seem to affect the apoptotic capacity of TTV-VP3; in fact, cancerous cell death levels are on par with those of Apoptin [15]. However, the specificity to which TTV-VP3 produced these effects was not known. Regardless of the efficiency of localization of each domain fragment, we nonetheless conclude that the two domains are in fact functional.

With the results of the primary cell transfections with wild type TTV-VP3, it is clear that it does not induce apoptosis in normal cells. Through comparison with Apoptin, this finding is highly significant. This immediately suggests a similar mechanism through which both proteins accomplish the task of selectively killing cancerous cells. It has been previously suggested that the extremely particular cell type-specific localization of Apoptin is key to its ability to selectively control cell death. However, as TTV-VP3 does not localize differently in cancerous cells compared to primary cells, it appears unlikely that this is the case. TTV-VP3 resides in the

cytoplasm of both cell types and yet causes apoptosis in only the cancerous and transformed variety.

One could make the argument that perhaps TTV-VP3 utilizes a different mechanism than Apoptin and that evolution found another way to perform this task. However, this seems rather unlikely considering the similarities between both viruses. The primary sequences of the two viruses' DNA have little correlation, but have great similarities in the overall structure of their genomes. This indicates that the two are closely related and differ by a relatively short period of evolution. It is questionable that an entirely different mechanism would be evolved to accomplish the same task. The idea that a random evolutionary mechanism would outcompete the Apoptin mechanism is even more implausible. It is much more probable that localization has little to do with the apoptotic mechanism of both viral proteins. We propose that the cell type-specific localization is an evolutionary optimization of the original apoptotic mechanism, whatever it may be. Hopefully, future research in our lab will shed light on the similarities and differences between TTV-VP3 and Apoptin.

Future Investigations

The most obvious continued research would be a comprehensive analysis on the localization features of the NES and the NLS fragments. By varying the duration of transfections and post-transfection incubations, a comprehensive timeframe could be created to better understand the localization mechanism. This in turn could be compared to the known localization time-dependence of both wild type TTV-VP3 and Apoptin. An analysis of this type would allow for a greater understanding of the cytoplasmic specificity of TTV-VP3 and could prove the NES and NLS equilibrium hypothesis.

A further investigation into the exact sequence of the NES and NLS motifs would also be useful into a comparison into the sequence differences to the corresponding Apoptin motifs. Continual shortening of the NES and NLS clones would allow the exact sequence of each to be characterized. Once identified, the sequence similarities and differences of localization of TTV-VP3 and Apoptin would shed light onto the differing sequestration. The known sequences of the NES and NLS would then allow for controlled mutations in the sequence. Selective mutation in the NES or NLS of wild type TTV-VP3 could have a distinct effect on its apoptotic capability. On the other hand, if localization truly has little importance to VP3's function, mutating the NES or NLS could have no effect at all. Of greatest importance is a better understanding of the apoptotic mechanism of both TTV-VP3 and Apoptin. The cancer specific apoptotic capacities of each protein could provide future generations with broad-spectrum cancer therapies.

Figures

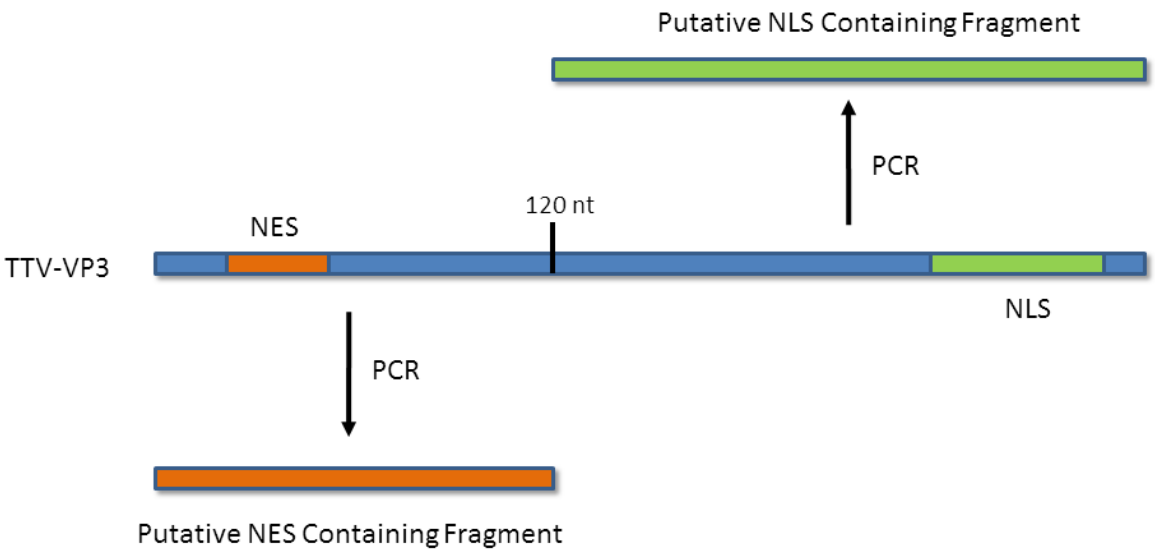

Fig. 1. Strategy for creation of truncation mutants. From the complete VP3 sequence two truncation mutants were created, each containing one of two putative localization domains (Nuclear Export Signal and Nuclear Localization Signal). Truncation mutants were synthesized via Polymerase Chain Reaction using primers listed in Table 1.

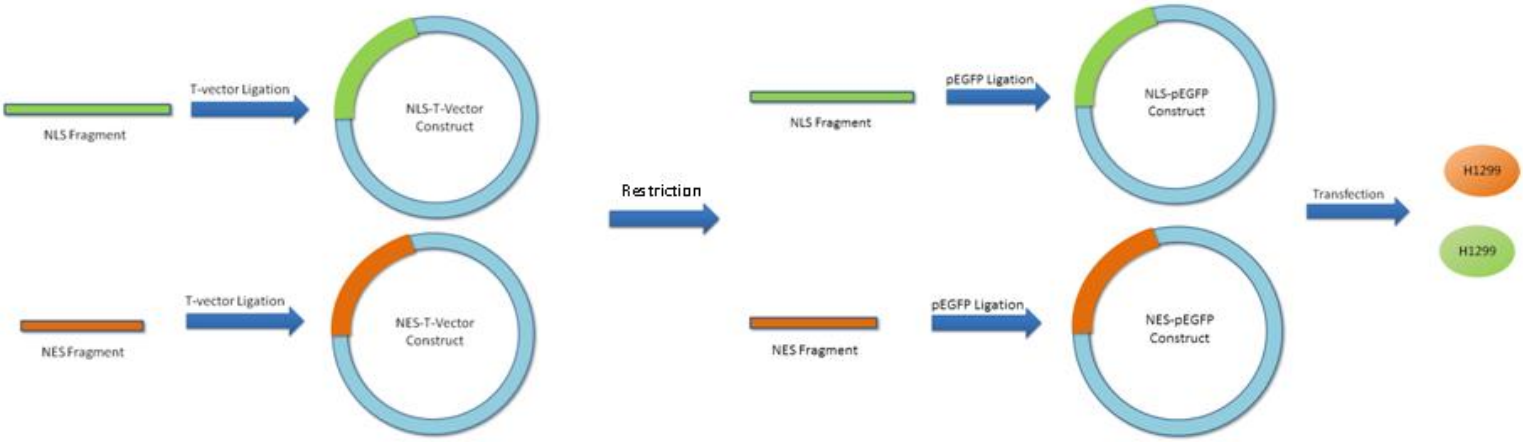

Fig. 2. Cloning strategy for truncation mutants. Both NES and NLS fragment PCR products were ligated into T-vector then digested with EcoRI and BamHI. Restricted fragments were then inserted into GFP vector and constructs transfected individually into H1299 cells.

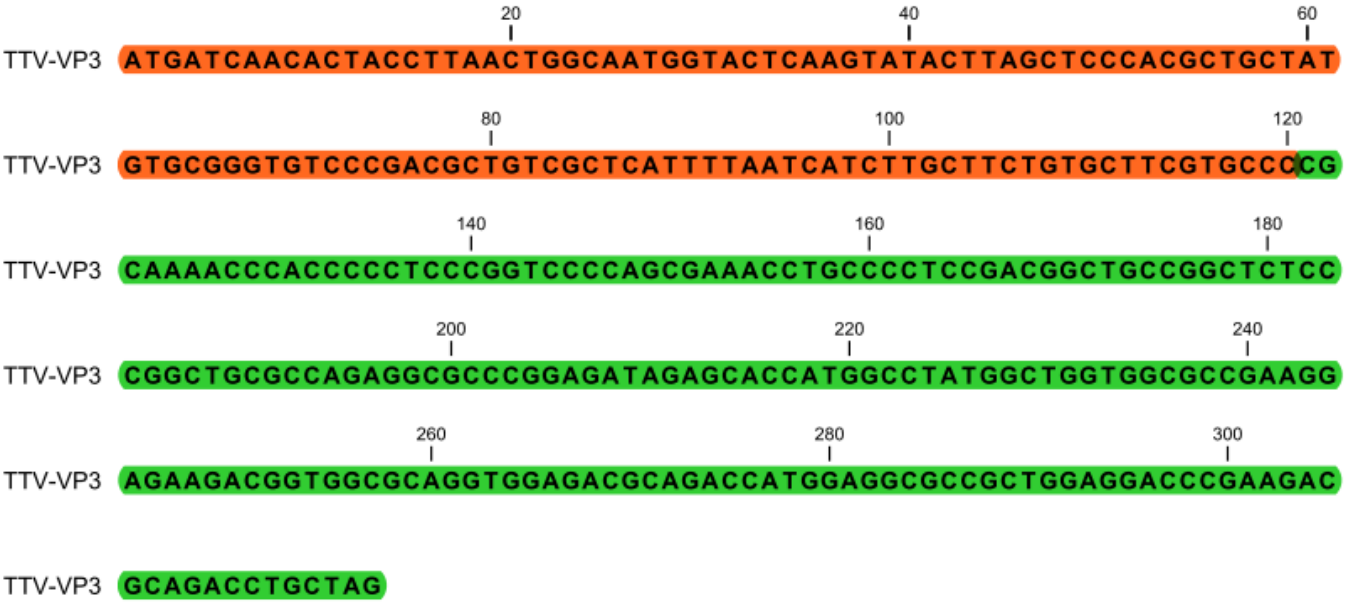

Fig. 3. Complete sequence of the TTV-VP3 gene. Section indicated in orange (1-120 bp) is the sequence of the NES-containing fragment, while section indicated in green (121-318 bp) is the sequence of the NLS-containing fragment.

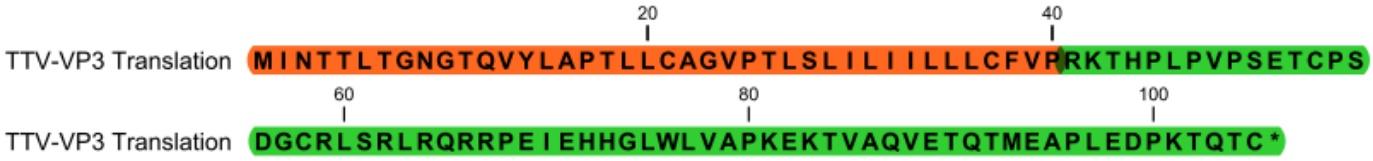

Fig. 4. Translated sequence of the TTV-VP3 protein. Section indicated in orange (residues 1-40) is the NES-containing fragment peptide, while section indicated in green (residues 41-105) is the NLS-containing fragment peptide.

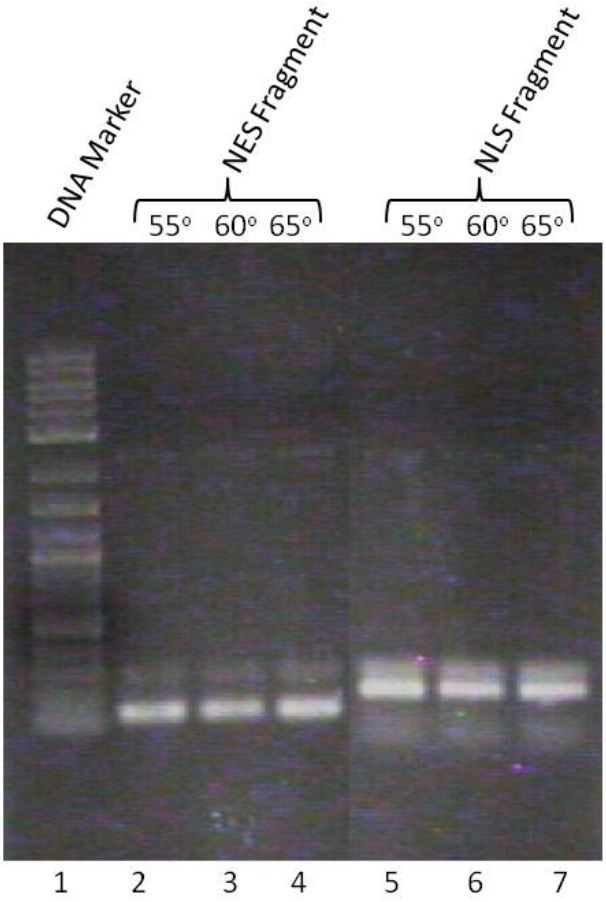

Fig. 5. Confirmation of TTV-VP3 truncation mutants. PCR products were purified via agarose gel electrophoresis for NES fragment (lanes 2-4) and NLS fragment (lanes 5-7), each utilizing three different annealing temperatures: 55, 60 and 65, respectively.

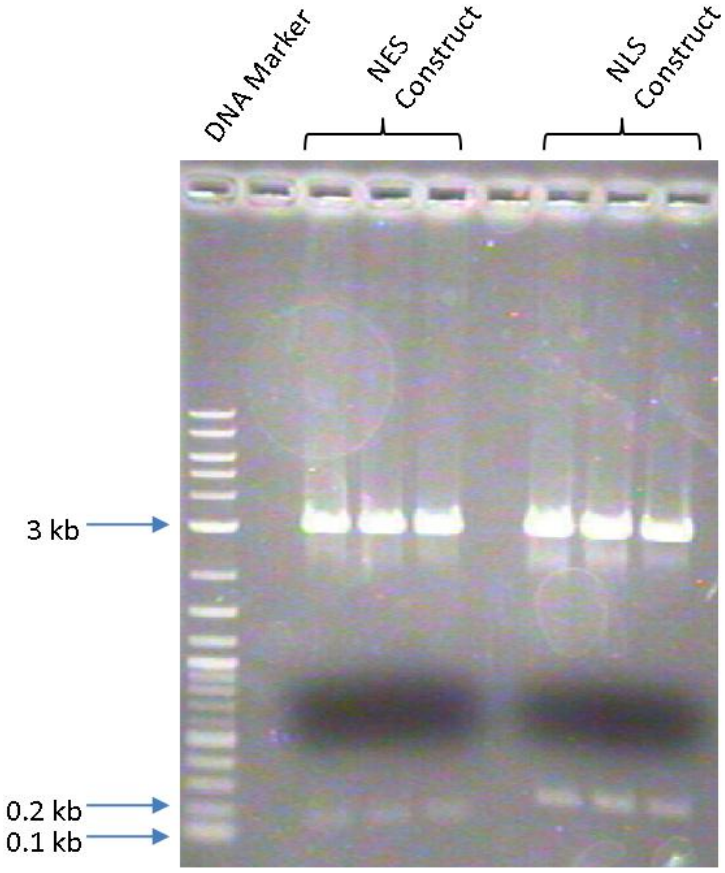

Fig. 6. Restriction of truncation mutants from T-vector. Restriction analysis of both the NES construct (lanes 3-5) and NLS construct (lanes 7-9) run in triplicate. Purified T-vector midi preps digested with EcoRI and BamHI. Positive dropout fragments are shown for both constructs.

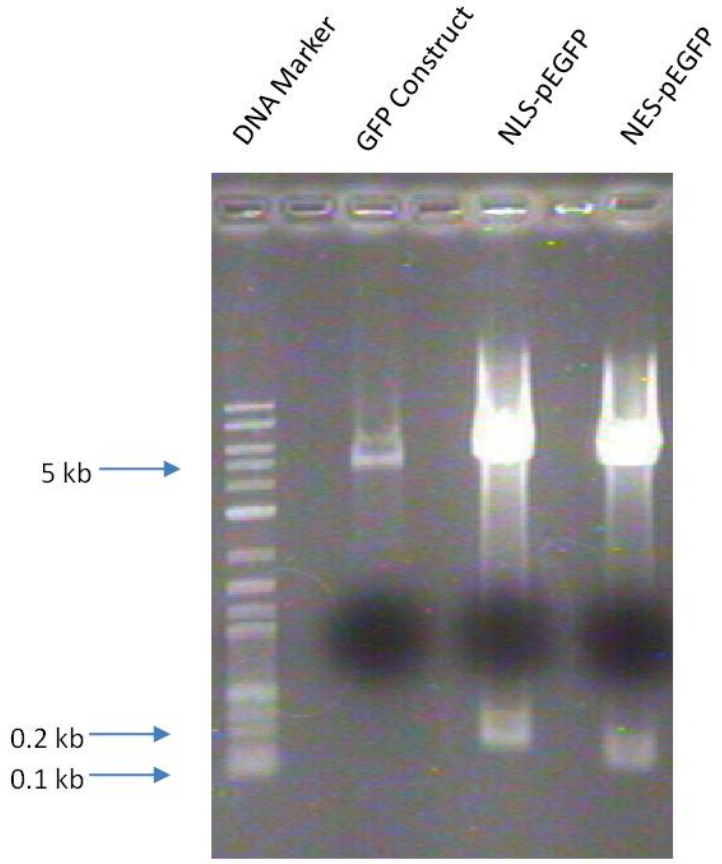

Fig. 7. Confirmation of TTV-VP3 truncation mutant cloning. Restriction analysis of GFP plasmid (lane 3, no insert), NLS-pEGFP construct (lane 5), and NES-pEGFP (lane 7) construct. Purified midi preps digested with EcoRI and BamHI. Fragment dropouts observed for both NLS-pEGFP construct and NES-pEGFP construct.

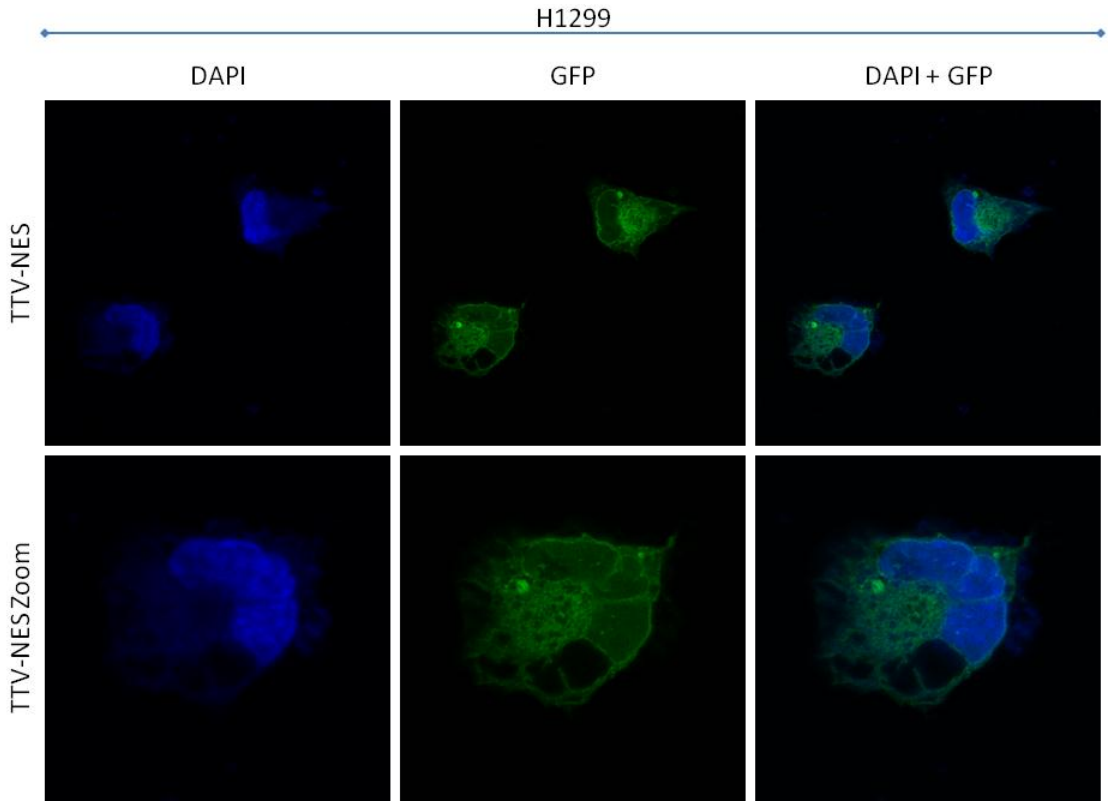

Fig. 8. Localization of TTV-VP3 NES fragment truncation mutant. H1299 cells transfected with NES-pEGFP construct. Cell nuclei were stained using DAPI. Cells observed after 24 hour transfection using laser confocal microscopy.

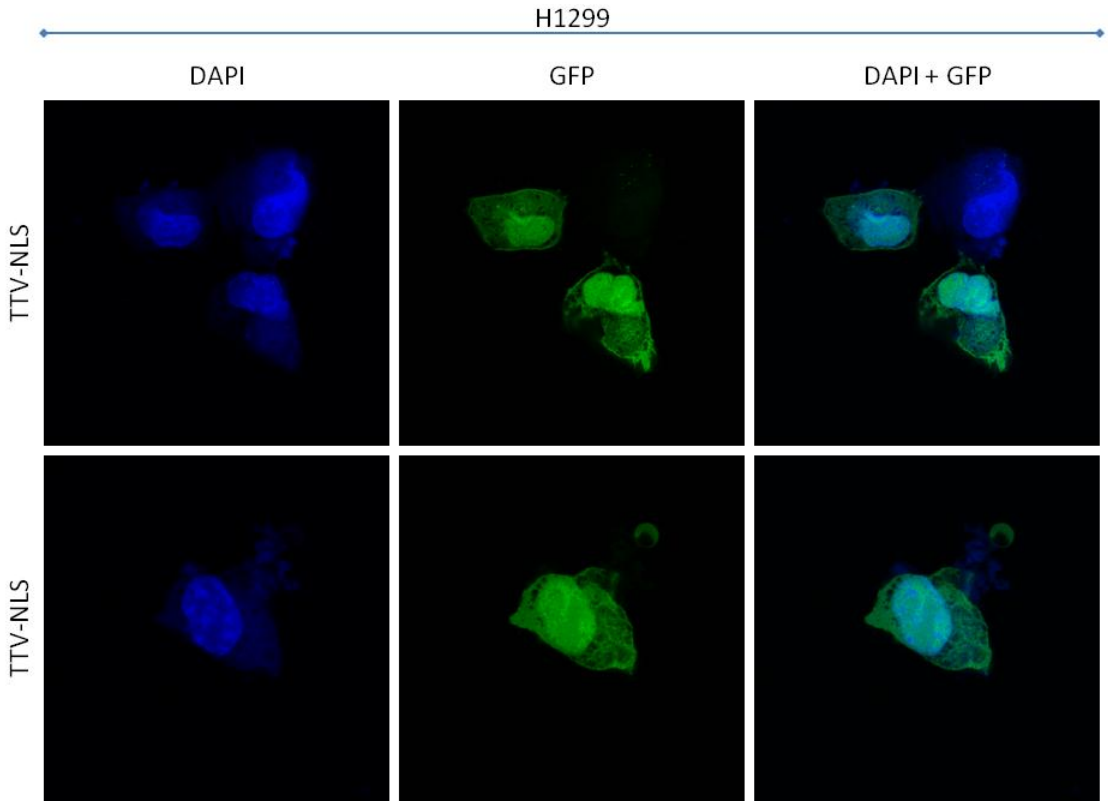

Fig. 9. Localization of TTV-VP3 NLS fragment truncation mutant. H1299 cells transfected with NLS-pEGFP construct. Cell nuclei were stained using DAPI. Cells observed after 24 hour transfection using laser confocal microscopy.

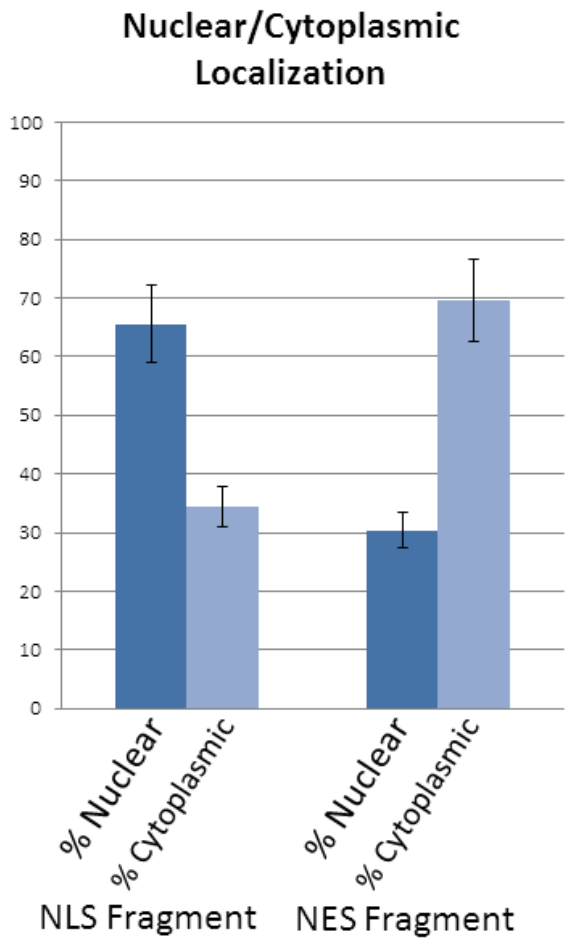

Fig. 10. Quantification of fragment localization. Representative cells were analyzed for GFP fluorescence in both the nucleus and cytoplasm of H1299 cells transfected with either NLS-pEGFP construct or NES-pEGFP construct.

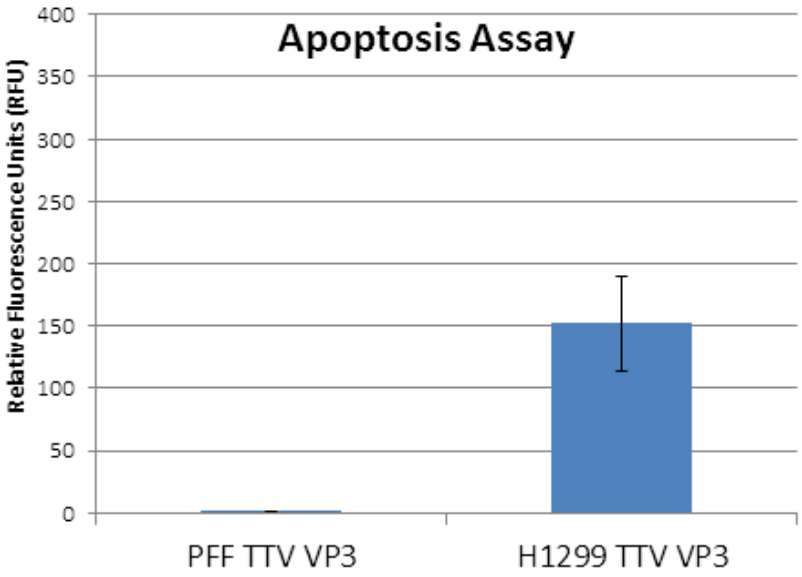

Fig. 11. Apoptosis induction via TTV-VP3 is cell-type specific. Primary Foreskin Fibroblast cells (PFF) and H1299 non-small cell lung carcinoma cells were transfected with full-length TTV-VP3 construct in flag vector and analyzed using a Caspase 3/7 assay. Induction of apoptosis causes cleavage of fluorescent substrate by caspase 3/7 interaction, yielding fluorescent signal. In PFF, TTV-VP3 does not induce apoptosis, but in H1299 apoptosis is induced.

Primer	Sequence
NES Forward	5'-GC**GAATTC**AATGATCAACACTACCTTAACTGGCAATGGT
NES Reverse	5'-GC**GGATCC**CTAGGGCACGAAGCACAGAAG
NLS Forward	5'-GC**GAATTC**ACGCAAAACCCACCCCCTC
NLS Reverse	5'-GC**GGATCC**GTCTAGCAGGTCTGCGTCTTCGGGTCCTCCAG

Table 1. Truncation mutant primer design. Primers were designed for both NES and NLS fragments. Underline indicates the position of start/stop codons in fragment sequences. Red indicates EcoRI restriction site while blue indicates BamHI restriction site.

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