PULSe

Preliminary Examination
Written Research Proposal Cover Sheet

Complete this form and submit an electronic copy with your Preliminary Examination Written Research Proposal to the PULSe office by your assigned due date. These materials will be forwarded to your exam committee members.

STUDENT: Jasmine Moore

Training Group: Immunology and Infectious Diseases

Preliminary Examination Title:

CCHFV NSs Induces Cell Death Through Interactions with Mitochondrial Machinery

Preliminary Examination Committee:

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Oral Preliminary Examination Date: September 24th, 2018
Exam Deadline: October 15th, 2018

Date Written Proposal Submitted: August 27th, 2018

The Chair of the examination committee is responsible for providing the student and the PULSe office with the results of the written proposal evaluation within no more than two weeks from the date of submission for review.

Written Research Proposal Basic Requirements

Refer to the PULSe program "2018 Prelim Guidelines" for further explanation of the written proposal sections to be included.

- Expected length may vary, but not to exceed 10-12 single-spaced pages (excluding title page, references, figures and figure legends). Use 11 pt. Arial or 12 pt. Times New Roman font size.
- Abstract
- Background and significance
- Specific aims
- Research designs and methods
- Discussion of expected results
- References in bibliography with full titles

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Abstract

Cell death mechanisms are regulatory functions of host cells that can kill or neutralize invading pathogens through self-destruction. While many pathogens have largely evolved strategies to improve the likelihood of successful infection and survival, some alternatively induce the host immune response instead. Initiating cell immunity can benefit the viral life cycle or infection process because it results in enhanced replication. One pathogen that employs this tactic is Crimean-Congo Hemorrhagic Fever Virus (CCHFV). CCHFV expresses a non-structural protein from the small genomic segment (NSs) that has been shown to induce apoptosis. Furthermore, two leucine residues—referred to as L127A and L135A—have been found to be necessary for this apoptotic induction. The mechanisms behind this approach have not been identified and would greatly enhance understanding of the CCHFV life cycle. This project seeks to further investigate the mechanisms behind NSs-mediated apoptosis by characterizing interactions that occur in the intrinsic pathway. Our overarching hypothesis is that NSs enters the mitochondrial matrix via interactions with the translocases of the outer and inner mitochondrial membranes where it then binds to the mitochondrial transcription factor A (mtTFA), resulting in respiratory chain failure and subsequent cell death. Completion of this project will provide insight into the pro-apoptotic mechanisms of NSs, and shed more light on the CCHFV life cycle and the function of these events. Identifying the interactions behind apoptotic induction may result in new therapies for CCHFV infection where there are currently none.

Specific Aims

Crimean-Congo Hemorrhagic Fever Virus (CCHFV) proves catastrophic in endemic regions like the Middle East, Asia, the Balkans, and all of Africa. Infections from the tick-borne virus produce febrile symptoms that escalate to fatality rates of 10-40% are further compounded by a lack of therapies. As a member of the *Orthonairoviridae* family in the *Bunyavirales* order, CCHFV has a tripartite genome, with the segments named for their respective sizes of small, medium, and large. The non-structural protein in the small segment (NSs) has been shown to be critical for inducing apoptosis[3]. Furthermore, two specific mutations in the C-terminal domain of NSs disrupt apoptotic induction[2]. The identities of these interactions and how they affect CCHFV pathogenesis are still unknown.

Evading the host immune system is a common survival strategy among pathogens. Some viral approaches include negative modulation of cytokine responses via neutralization, inhibition of antigen presentation by interfering with the endocytic pathway, and suppressing cell death. Similarly, previous studies have shown that in early CCHFV infection, apoptosis is suppressed by the nucleoprotein, but then becomes activated in late infection (48 hours) in NSs-transfected cells[2,4]. Furthermore, removing the N-terminus of NSs causes an increase in viral progeny. Although there is evidence of apoptosis following transfection of CCHFV NSs, there are other pathways that may also explain cell death. Of these, necrosis can occur via ATP depletion caused by a lack of blood flow or deficiencies in the electron transport chain. In both apoptosis and necrosis, mitochondrial permeabilization and other dysfunctions are common events, even though there mechanisms of action are very different.[5] By completing this project, we expect to impact current understanding in both the CCHFV life cycle and the mechanisms behind pro-apoptotic induction by viruses. Through these aims, prospective treatments may be designed to suppress the severity of CCHFV infection.

Aim 1: Quantify Effects of NSs on Cell Death in CCHFV-Permissive Cells

Our hypothesis is that NSs promotes death in cell types that are both susceptible and permissive to CCHFV infection. We intend to compare different cell lines with assays that gauge the rates of activated cell death pathways in CCHFV infection or NSs transfection. CCHFV is known to mainly infect blood peripheral macrophages and dendritic cells, along with tissues such as the epithelium and liver. Hepatocytic necrosis is a major hallmark of CCHFV infection, yet the reason for these pathways being activated are unknown. By quantifying the percentages of cell death mechanisms in these cell lines, we expect to broaden present information on how CCHFV induces damage to host cells and potential routes to reduce tissue damage.

Aim 2: NSs interacts with the TOM and TIM Complexes to Enter the Mitochondrial Matrix

Our hypothesis is that NSs interacts with the translocases of the outer and inner mitochondrial membranes—TOM and TIM, respectively—to enter the mitochondrial matrix. Most mitochondrial membrane proteins are encoded in the nucleus, synthesized by cytosolic ribosomes, then guided to the outer mitochondrial membrane (OMM) for import. Protein transport pathways in the TOM and TIM complexes are dependent on the intended destination of each protein[6]. For example, the Influenza A protein, PB1-F2 interacts with the channel of the TOM complex (TOM40) to enter the inner membrane space[7]. We will complete co-immunoprecipitation assays to identify interactions between NSs and TOM or TIM subunits. We will also include the L127A and L135A mutants to determine their relevance in protein import.

Aim 3: The NSs—TFAM Interaction is Detrimental to Respiratory Chain Function

Our hypothesis is that the interaction between NSs and mtTFA (also known as TFAM) results in deficiencies in the respiratory chain, resulting in apoptosis in infected cells. A previous report showed that transfection of NSs resulted in its colocalization with TFAM[1]. We plan to verify the interaction between NSs and TFAM and quantify the effects the interaction has on electron transport chain (ETC) function through measuring light chain transcription levels and respiratory chain activity.

Research Strategy

Background and Significance

Crimean-Congo Hemorrhagic Fever Virus (CCHFV) is spread via tick bites and transmission of bodily fluids. After an initial incubation period of 3-7 days, a sudden onset of symptoms occurs, including high fever, rashes, hemorrhaging, and headaches. An increasingly hemorrhagic state ensues throughout a period of roughly 10-14 days, with elevated liver enzymes and low platelet/WBC counts as major hallmarks of infection. The case fatality rate spans about 10-40%, with those who succumb to the disease experience major blood loss and organ failure[8,9]. No therapies have yet been developed for CCHFV; although the antiviral drug ribavirin has been beneficial to some patients, a consistently therapeutic drug is crucial to combat arising epidemics.

Completely treating the hemorrhagic symptoms of CCHFV infection will require more knowledge on the viral life cycle. The genome consists of three single-stranded RNA segments. These segments are encapsulated by the nucleoprotein and polymerase, forming the ribonucleoprotein complex, a structure necessary for the replication and transcription of genomic viral RNA[3]. In the nucleoprotein, there is a 4-residue motif comprised of amino acids Asp-Glu-Val-Asp (DEVD). This sequence is recognized by the host apoptosis mediator, caspase-3, which cleaves the motif at the 3' end[2–4]. Furthermore, inhibiting apoptosis increases production of novel virions up to 90%, and mutating the DEVD motif results in enhanced CCHFV polymerase activity during transcription[2]. The nucleoprotein DEVD motif is highly conserved among all strains of CCHFV, as sequence comparisons have shown[2,10]. This suggests that cleavage of the nucleoprotein may be valuable to the viral life cycle or that the virus has evolved another tactic to survive caspase cleavage[1]. The nucleoprotein has also been found to inhibit apoptosis in the early stages of CCHFV infection[4], yet transfecting cells with the non-structural protein results in apoptosis late in infection. The exact mechanisms and reasons behind these events in CCHFV infection are still unknown. *This project will significantly increase understanding of the role that the CCHFV NSs protein plays in host cell death.* Information on virally exploited host immunity continues to develop, yet much is still unknown. Discovering the function of NSs will escalate current understanding of the role that inducing cell death has in the viral life cycle.

Previous studies discuss the role of NSs proteins in other bunyaviruses. La Crosse encephalitis virus, from the *Orthobunyavirus* genus, causes severe encephalitis in children. Its NSs protein has been shown to induce caspase activation and apoptosis [11]. From the *Phlebovirus* genus, Punta Toro virus induces apoptosis in mammalian cells *in vitro* and in hepatocytes *in vivo*. Subsequently, Punta Toro virus NSs has been shown to induce hepatocyte apoptosis by activating the extrinsic and intrinsic pathways[12]. Following these studies, CCHFV NSs has also been shown to induce apoptosis via both apoptotic pathways, although the exact mechanisms of action are currently unknown. Cell imaging experiments have revealed that NSs co-localizes with the mitochondrial transcription factor A (mtTFA, or TFAM), and accumulation of NSs in mitochondria corresponds with membrane potential disruption[1]. How the internal localization of NSs disrupts mitochondrial membrane potential is presently unknown, and we seek to uncover the cause in this proposal.

Apoptosis is a vital part of the overall immune defense of host cells. In addition to the cascade of proteolytic activity of cellular components, caspases have been shown to affect transcription of viral genes like those of bunyaviruses. The induction of caspase cascades often results in activation of transcription factors—such as AP-1 and NF-κB—which in turn regulate the expression of cytokines and subsequent inflammation. Caspase activation can also result in pro-viral gene expression. When apoptosis occurs via B-cell receptor activation in Epstein-Barr Virus (EBV) infection, caspase cascades destabilize the transcriptional regulators that inhibit expression of viral lytic genes[13]. While most apoptotic viral genes aim to suppress the cellular

immune response, there are examples of viral proteins that in fact induce apoptotic pathways. More specifically, there are studies detailing the pro-apoptotic properties of other viral NSs proteins. Because the NSs protein localizes in mitochondria and induces membrane permeabilization, this proposal focuses on its effect on the intrinsic pathway[1]. Identifying the specific mechanisms in CCHFV-mediated apoptosis or necrosis would shed more light on the potential purpose for inducing cell death.

In order to enter the mitochondrial matrix, proteins must have a targeting sequence that is recognized by the mitochondrial transport machinery. There are multiple pathways that are used to import cytosolic proteins and result in proteins localized to areas of the mitochondria (inner membrane space, matrix, imbedded in membrane, etc.). The CCHFV NSs protein contains a putative amphipathic alpha helix at its carboxyl terminal which may be the binding site in its mitochondrial interactions. Barnwal, et al used alanine scanning and caspase assays to show that two residues in the C-terminus of NSs, leucine-127 and leucine-135 (L-127 and L-135, respectively) are necessary for apoptosis induction. Furthermore, live imaging has revealed that NSs co-localizes in mitochondria and disrupts the mitochondrial membrane potential[1]. By identifying the mechanisms behind NSs entry and subsequent apoptotic activation, we may uncover prospective therapies that inhibit or neutralize CCHFV activity in these occurrences[2].

Innovation

An emerging topic in host-pathogen interactions is the revelation that some viruses exploit apoptosis in host cells as a part of their life cycle. Contradictory to pathogens that inhibit or evade host apoptosis as survival tactics, some viruses like CCHFV induce apoptosis for increased dissemination or to kill off uninfected immune cells[14]. CCHFV is already known to induce apoptosis in host cells late in the infection cycle[1], despite this process resulting in decreased viral titers and cleaved nucleoprotein[2,3]. In conjunction with the nucleoprotein (NP) containing the caspase-3 recognition motif DEVD[2], as well as the same NP inhibiting apoptosis during the early steps of the CCHFV replication cycle, there is reason to suspect that NSs-mediated cell death serves as a regulatory factor in CCHFV replication. This project has the potential to shift current research towards a better understanding of pro-apoptotic viral proteins. This research would be the first time that the mechanisms behind NSs-mediated cell death are examined. Revealing these mechanisms offers further insight into virus-induced apoptosis or other forms of cell death. Previous studies have provided insight on related viral proteins from cytomegalovirus, hepatitis C, and coronavirus[15–17], but such analyses have not yet been conducted on the CCHFV NSs protein. Uncovering the apoptotic mechanisms of NSs would further clarify the system CCHFV employs to create a more favorable environment for viral replication. This may occur by CCHFV utilizing both inhibitive and inductive means on the host cell to its own benefit.

Experimental Design: Overview

We will complete infections and transfections on CCHFV-supportive cell types with variations of CCHFV and the NSs protein (fig. 1). The cell lines that will be used are ones that (1) support CCHFV infection and (2) have been discovered as sites of infection. In order to study infection in dendritic cells and macrophages, a human monocytic cell line (THP-1) must be cultured and stimulated with growth factors to differentiate into those cell types[18,19]. Infection efficiency will be quantified by qPCR and the transfection efficiencies will be measured using a reporter system in the luciferase-pcDNA3 plasmid. There are many mechanisms of cell death that may explain the caspase activity found in NSs transfection, and this aim is intended to explore the potential pathways in CCHFV infection.

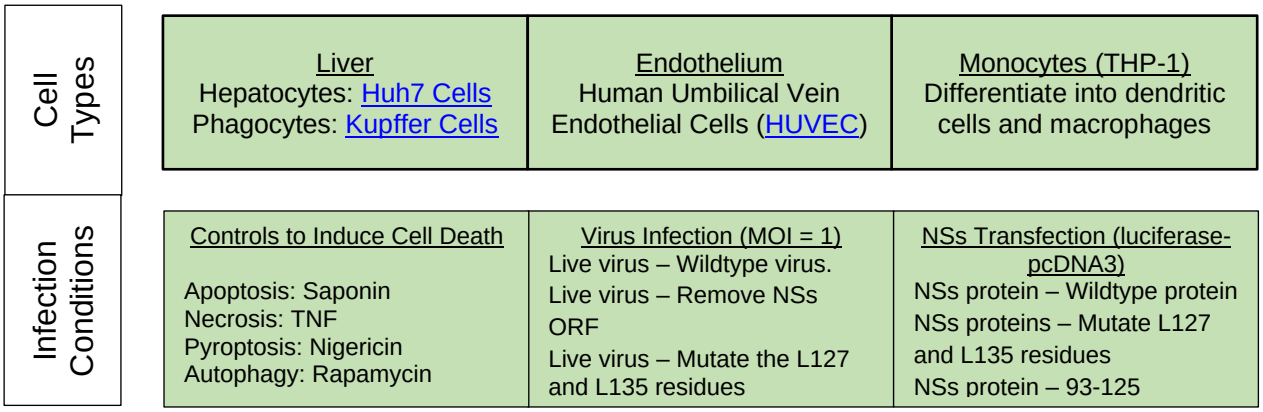


Figure 1: Infection and transfection conditions. Highlights the cell types and conditions to be used in this proposal. Every cell line will undergo viral infection with CCHFV and transfection with both wildtype and mutant NSs. Controls will be introduced to each cell type to compare results of assays listed in Table 1. *TNF*, Tumor Necrosis Factor*, MOI,* multiplicity of infection.

THP-1 can be differentiated into mature dendritic cells (DCs) when cultured in serum-free medium containing GM-CSF, TNF-alpha, and ionomycin[18]. The same cell line can also be differentiated into macrophages by incubating with 150 nM phorbol 12-myristate 13-acetate PMA for 24 hours, then incubated in RPMI medium for another 24 hours. To polarize the cells into M1 macrophages, they will be incubated with 20ng.mL of IFN- γ and 10 pg/ml of LPS. To differentiate into M2 macrophages, the cells must incubate with 20 ng/ml of interleukin 4 and 20 ng/ml of interleukin 13[20].

Experimental Design: Specific Aims

Aim 1
Quantify Effects of NSs on Cell Death in CCHFV-Permissive Cells

Rationale: Multiple cell lines have been transfected with the CCHFV non-structural protein (NSs), resulting in increased caspase activity (caspases-3, -8, and -9) and PARP cleavage, signifying apoptotic induction. This activity was blocked when two leucine residues located in the C-terminus of the NSs protein were mutated[1,21], pointing to the importance of this protein in promoting apoptosis. However, the three cell lines utilized in this study – VeroE6, HEK293, and HeLa – are not representative of the pathogenesis often seen in CCHFV infection. Furthermore, massive liver necrosis is a hallmark of severe CCHFV infection[8,22]. Hepatocytes can be killed via apoptosis or necrosis following the opening of permeability transition pores in mitochondria after a period of oxidative stress or damage. Ubiquitous expression of death receptors in the liver results in hepatocytes being susceptible to death receptor-mediated apoptosis, resulting in mitochondrial permeabilization[5]. This aim sets out to examine NSs-mediated cell death in cell types related to those affected by disease. Our hypothesis is that most infected cell types undergo apoptosis, and this process is mediated by the NSs protein. In hepatocytes, a different mechanism occurs to induce massive liver necrosis.

There are known hallmarks of the major cell death pathways that can be quantified through various assays. Measuring DNA fragmentation and caspase activation, as well as noting morphological changes such as cell shrinkage, chromatin condensation and cleavage, and formation of apoptotic bodies can be used as characteristics of apoptosis. Necrosis, another major cell death pathway, can be triggered by extreme temperature changes, physical trauma, or components of the immune system, such as complement or natural killer cells. Morphological characteristics of necrosis include swelling in cell volume and organelles,

ruptures in the plasma membrane and the release of intracellular substances. While not considered a programmed cell death pathway like apoptosis, necrosis is regulated through signal transduction pathways and other mechanisms[23]. Other forms of cell death also utilize caspases, such as pyroptosis. When pyroptosis occurs, inflammatory caspases like caspase-1 trigger the lytic pathway resulting in inflammation. Another programmed cell death is autophagy, a lysosome-dependent process. In cells with defective proteins or organelles, the cell forms an autophagosome to engulf the offending materials, then fuses with the lysosome for degradation and recycles the molecules for other uses. While autophagy is often used as a survival mechanism in response to stress, high levels of autophagic degradation can result in death[24].

Experimental Design: To measure the effects of NSs on cell death during CCHFV infection, we will use a combination of cell imaging and assays that determine qualitative and quantitative measurements of different modes of cell death. Because the apoptotic pathways are well known, many assays that have been developed to measure throughout the pathway. Focusing on the intrinsic pathway, we can use immunofluorescence staining to visualize when proteins in the inner membrane space (IMS), like cytochrome c, Smac/Diablo, and AIF are either inside or outside of the mitochondria by determining their colocalization with mitochondrial markers imbedded in the outer mitochondrial membrane. (HSP-60 or VDAC-1). Utilizing other antibodies for active caspase-3 will provide additional information about the apoptotic induction[25]. Titon X-100 disrupts OMM and can be used as a control to specifically induce MMP. We will perform immunoblotting with antibodies against the specific conformations of the proapoptotic group of Bcl-2 proteins (Bax, Bak, Bid). These conformation-specific antibodies allow us to detect their activation in the presence of NSs.

There is an assay that can be used to compare apoptosis and necrosis. In healthy cells, phosphatidylserine (PS) is located on the inside of the lipid bilayer of the plasma membrane. When apoptosis occurs, the phosphatidylserine gets translocated to the external layer and serves as a recognition signal for phagocytes. The ubiquitous protein Annexin V can strongly bind PS, allowing us to detect early apoptosis. However, when necrosis occurs, the plasma membrane becomes permeabilized, and Annexin V can then bind to the PS that is still located in the internal side of the lipid bilayer. To get around this, propidium iodide will be used to detect necrotic cells as PI not permeable to apoptotic and live cells.

Colorimetric/fluorogenic substrate-based assays of cell lysates in microtiter plates will allow us to measure the activation of calpains and cathepsins seen in necrotic death. Some literature has suggested that cellular ATP levels dictate whether cells die by apoptosis or necrosis, where low levels of ATP with an increase in reactive oxygen species (ROS) result in cell death by necrosis. An assessment of the ATP/ADP ratio will be completed. Necrosis induced by heat shock in the HL60, CEM7, Jurkat, and U937 cell lines resulted in ADP:ATP ratios in excess of 15.0[26], so heat shock will be used as a positive control along with TNF.

For both pyroptosis and apoptosis, colorimetric assays and immunoblotting can be used to quantify the activity and cleavage of their respective caspases.

Finally, there is autophagy. Modulating the interactions between Beclin 1 and members of the anti-apoptotic Bcl-2 family (Bcl-2, Bcl-X_L, etc.) is critical in controlling autophagy. Enhancement of the Bcl-2 and Beclin 1 interaction can repress autophagy, whereas inhibition would lead to autophagic activity[27]. Co-immunoprecipitation studies can aid us in determining if autophagic modulation occurs in CCHFV infection. When autophagosomes engulf cytoplasmic components, the cytosolic form of LC3 (LC3-I) is bound to phosphatidylethanolamine to form the conjugate LC3-II, which is then recruited to autophagosomal membranes. Autophagy completes, and LC3-II is degraded. LC3-II can be used as an autophagosomal marker of starvation-induced autophagic activity. Immunoblotting against LC3 can then be used to monitor all autophagy processes[28].

Type of Cell Death	Assay	Measurement	Controls
Apoptosis	IF microscopy colocalization studies of IMS proteins (Cyt c) with sessile mitochondrial proteins (e.g., VDAC1)	Mitochondrial Membrane Permeabilization	
Apoptosis	Immunoblotting with conformation-specific antibodies	Activation of Bcl-2 apoptotic proteins	
Apoptosis/Necrosis	FACS quantification with Annexin V and propidium iodide	PS exposure/PI uptake	
Necrosis	Colorimetric/fluorogenic substrate-based assays of cell lysates in microtiter plates	Activation of calpains and cathepsins	
Necrosis	Luminometric assessments of ATP/ADP ratio	Drop in ATP levels	
Apoptosis/Pyroptosis	Colorimetric caspase assays (caspase-3 for apoptosis; caspase-1 for pyroptosis)	Caspase activity	
Apoptosis/Pyroptosis	Immunoblotting	Assess caspase cleavage products	
Autophagy	Co-immunoprecipitation studies	Beclin-1 dissociation from Bcl-2/XL	
Autophagy	Immunoblotting with LC3-specific antibodies	LC3-I to LC3-II conversion	

Table 1. Assays to characterize cell death in CCHFV-supportive cell types.

We expect that apoptosis will be the primary mode of cell death in all cell lines but liver-derived cells, which may result in higher levels of necrosis. However, it is possible that NSs is not directly inducing cell death, but rather initiates a pathway to lead to apoptosis or necrosis. To test this, we could conduct a label transfer reaction to determine what proteins NSs is interacting with.

Aim 2

NSs interacts with the TOM and TIM Complexes to Enter the Mitochondrial Matrix

Rationale: An immunofluorescence assay of NSs-transfected VeroE6 cells revealed that the protein co-localizes with the endogenous transcription factor TFAM[1], showing that NSs translocates into the mitochondrial matrix by some unknown mechanism. In light of the newly discovered activity and location of NSs during transfection, other viral apoptotic proteins were examined. All currently known viral proapoptotic proteins contain amphipathic helices, and the predicted 2nd structure of NSs revealed an amphipathic alpha helix where the minimal catalytic residues are located[1,29]. Both pro- and anti-apoptotic proteins can have mitochondrial targeting sequences (MTS) at the C-terminus, with a similar homology to bcl-2 family proteins. Because NSs contains a putative amphipathic helix, parallels to other viral apoptotic proteins can be inferred in structure and entry into mitochondria. The voltage-dependent anion channel allows diffusion of solutes up to about 5kDa in size through the outer mitochondrial membrane[25]. The NSs protein is 16.3 kDa in size, and would have to enter via the general transport machinery—the TOM and TIM complexes.

The Influenza A viral protein PB1-F2 is known to enter the inner membrane space via an interaction with the TOM complex. Because this channel is also the entry point of matrix- bound proteins, we hypothesize that NSs also enters through this channel. The hypothesis of this aim is that the NSs protein interacts with the TOM and TIM complexes to enter the mitochondrial matrix, and this entry is required for apoptotic induction. In this aim, we will investigate the translocation of NSs into the mitochondrial matrix by utilizing similar experiments used to deduce the mechanisms of other viral proteins.

The TOM and TIM complexes are the major import machinery of mitochondria. While human mitochondria contain about 1,500 proteins, only one percent of those proteins are encoded by the mitochondrial genome[6]. Instead, the majority of mitochondrial proteins are encoded in the nucleus and synthesized by cytosolic ribosomes. These proteins are then guided to the outer

mitochondrial membrane for transportation. There are different pathways in the TOM and TIM complexes dependent on the intended destination of each protein (fig. 2)[6]. After entering the inner membrane space, proteins may either be inserted into the outer membrane via the sorting and assembly machinery (SAM) complex, or it interacts with different components of the TIM complex. These proteins are divided into carrier proteins, which interact with TIM9/TIM10 complex in the inner membrane space, or matrix proteins, which interact with the TIM23 and PAM complexes to transport protein through the inner mitochondrial membrane (IMM) (fig. 3). Because the CCHFV NSs protein has been localize inside mitochondria and specifically co-localizes with the transcription factor TFAM, we hypothesize that the protein NSs travels through the TOM/TIM complexes to enter mitochondrial matrix. Another viral protein, Influenza A PB1-F2 interacts with Tom40 to enter the inner membrane space. In order to enter the matrix, CCHFV NSs must also pass through the TIM complex. We expect that NSs interact TIM channel TIM23 to enter the matrix.

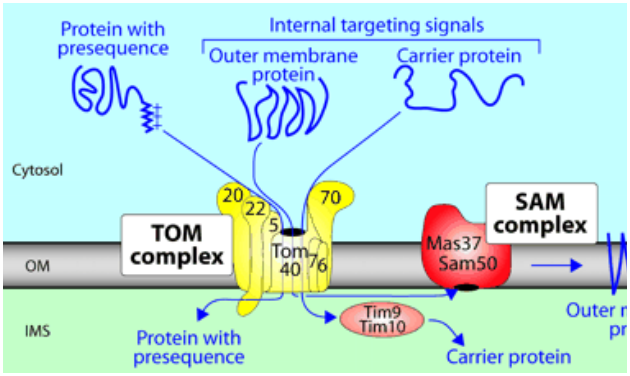


Figure 2: Protein translocation across the outer mitochondrial membrane.
Mitochondrial precursor proteins possess either an N-terminal presequence (positively charged amphipathic α-helix) or contain internal targeting signals. The presequence gets recognized by import receptors Tom20 and Tom22, and the protein is transferred with the help of Tom5 to the pore formed by Tom40. The presequence proteins bind to Tom22 before being transferred to the TIM proteins. Carrier proteins with internal targeting signals are first recognized by the receptor Tom70, then Tom20, Tom22, and Tom5 translocate the carrier protein through Tom40 and bind it to the Tim9-Tim10 complex of the intermembrane space (IMS). Outer membrane proteins depend on Tom20 and Tom22 for entry, then the SAM complex inserts the β-barrel proteins into the outer membrane (OM)[6].

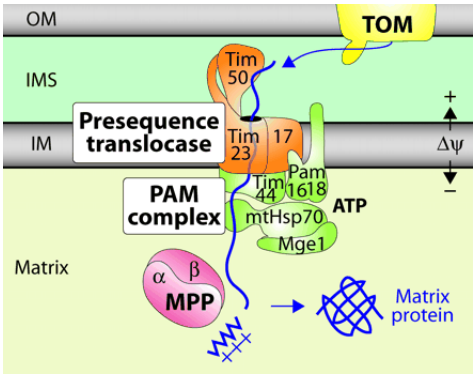


Figure 3: Import of presequence proteins into the mitochondrial matrix.
After passing through the channel of the TOM complex, presequence-carrying preproteins are recognized by an intermembrane space (*IMS*) domain of Tom22. From here, they are brought into contact with Tim50, which then assists in guiding them to the presequence translocase (TIM23 complex). Their subsequent insertion into the channel, formed by Tim23, requires the membrane potential (Δψ) across the inner membrane (*IM*). A further driving force is provided by the PAM in the matrix. The central component of this motor, mtHsp70, is transiently anchored at the translocase by Tim44 and requires assistance from Pam18, Pam16, and Mge1 for promotion of the reaction cycle. The presequences of the precursor proteins are cleaved off by the MPP in the matrix[6].

In order to find potential mechanisms that NSs may employ to enter mitochondria, the CCHFV NSs sequence was run in BLAST against eight known proapoptotic viral proteins (fig. 4). However, there was no homology in the gene blast, and small protein homology among four out of eight sequences. Of the four homologous sequences, all are known to localize in mitochondria. The hepatitis B X protein (HBX) is known to colocalize with HSP60 and VDAC3, proteins located in the matrix and outer mitochondrial membrane, respectively. Bovine leukemia virus (BLV), a retrovirus similar to human T leukemia virus (HTLV), has the G4 protein which localizes with HTLV p13 II in the inner membrane space. The walleye dermal sarcoma virus (WSDV) Orf C and avian encephalomyelitis virus (AEV) VP3 proteins are known to both localize

in mitochondria, but there precise locations are still unknown[29]. The NSs sequence was also run through a sequence predictor to find potential for targeting by the translocase complexes (fig. 5).

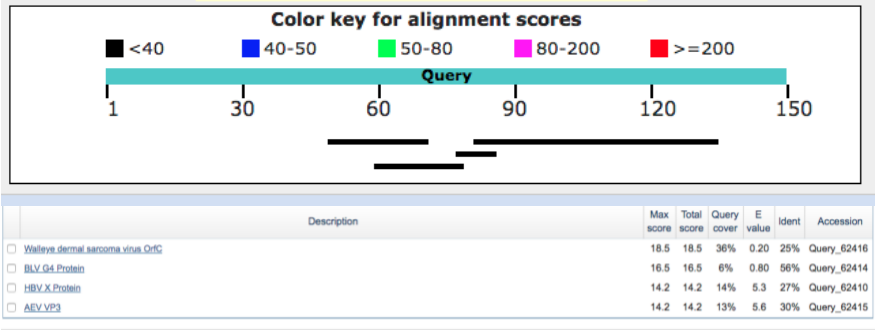


Figure 4: Results of BLAST search. CCHFV NSs sequence was aligned with eight known pro-apoptotic viral sequences. These were walleye dermal sarcoma virus (WDSV) Orf C, hepatitis B X (HBX), bovine leukemia virus (BLV) G4, and avian encephalomyelitis virus (AEV) VP3.

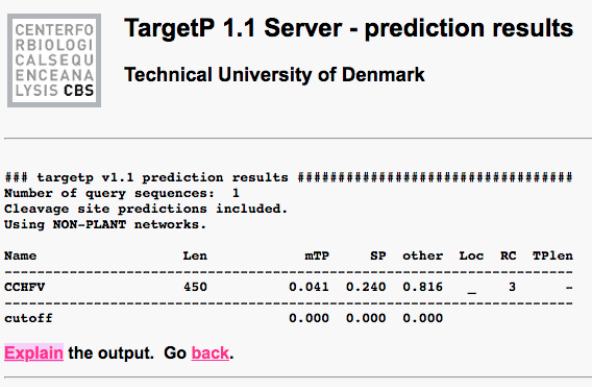


Figure 5: Target sequence predictions for NSs. The NSs sequence was also run through a target sequence predictor. There were predicted mitochondrial targeting and signal peptide sequences.

Due to previous examples of viral proapoptotic proteins interacting with mitochondria, we expect that NSs protein is entering mitochondria via the TOM and TIM complexes and that the L127A and L135A residues are not necessary for entry.

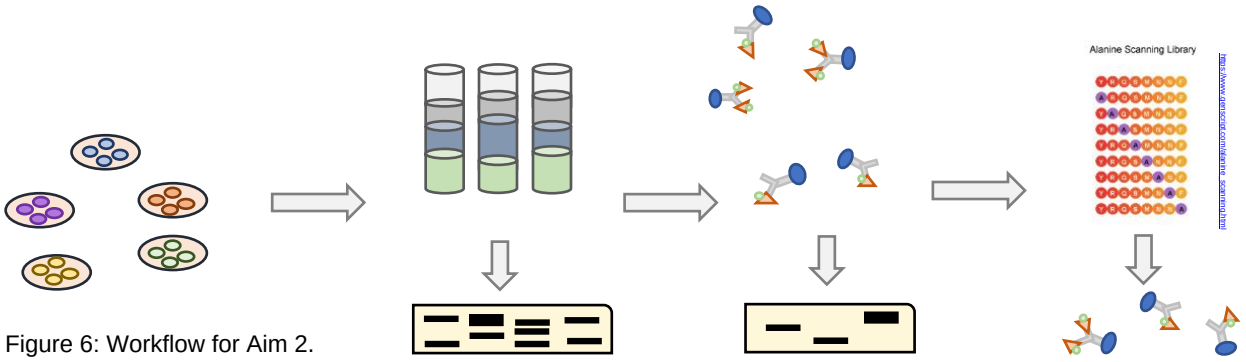


Figure 6: Workflow for Aim 2.

1. Transfect with NSs (WT and mutant)	2. Mitochondrial fractionations and immunoblotting	3. Co-IPs with NSs and TOM/TIM	4. Alanine scanning; repeat Co-IPs

Experimental Design: Mitochondrial fractionations and immunoblotting can be utilized to identify which mitochondrial compartment a particular protein is in. Mitochondrial markers include antibodies against VDAC (outer membrane), COXIV (inner membrane), CytochromeC (intermembrane space), and Manganese superoxide dismutase, MnSOD (matrix). The Influenza A PB1-F2 protein may be used as a control for TOM interactions, as it gets transported to the inner membrane space, and the polymerase basic 2 (PB2) subunit of the Influenza A RNAP

can be used as a positive control for matrix-bound proteins. This experiment will allow us to detect the NSs protein in the matrix, where it is known to colocalize with the mitochondrial transcription factor TFAM. PB1-F2 should be found with COXIV and both the matrix control PB2 and the NSs proteins will be with MnSOD upon blotting. As both of the NSs mutants were present in mitochondria, it is unlikely that those residues are necessary for entry and the proteins are expected to be found in the matrix as well.
We will also run co-immunoprecipitation assays order to verify that NSs interacts with the TOM and TIM complexes. This will allow us to determine which subunits of the translocase complex interact with NSs. Again, the Influenza A PB1-F2 and PB2 proteins can be used as controls for the TOM and TIM interactions, respectively. We will also use alanine scanning to define the binding site of NSs and the translocase complexes. Because the NSs mutants also enter mitochondria, we do not know what residues of NSs may be critical in binding. We will run co-immunoprecipitations again with the results of alanine scanning; this will serve to identify the binding region of NSs by detecting interactions with the TOM and TIM complexes. We expect that both the wildtype and mutant NSs transfections will result in matrix localization and that the C-terminus of the NSs protein is needed for entry, but the leucine residues are not themselves crucial for interacting with the TOM and TIM proteins. If the alanine scanning does not produce any interactions, it may be that the interaction is too transient to be picked up. Thus, we may also use a label transfer reaction if the interaction is not initially detected. This way, we will be able to detect any interactions with NSs.

Aim 3

The NSs—TFAM Interaction is Detrimental to Respiratory Chain Function

Rationale: NSs was revealed to co-localize with TFAM, the mitochondrial transcription factor. TFAM binds to the promoter for the light strand of mitochondrial DNA and regulates transcription by being a requirement of promoter recognition by the RNAP. The light DNA strand carries the gene for MT-DN6, a core subunit of the respiratory chain. In addition to this, TFAM is critical in regulating respiratory chain function. One study investigated the role of TFAM by inducing apoptosis in TFAM-KO mouse embryos. The results showed that the heart tissue had an increase in apoptotic damage and respiratory chain failure[30], suggesting that a lack of TFAM results in deficiencies in respiration and initiates intrinsic apoptosis (fig. 6). Defects in respiratory chain function result in either decreased or complete loss of electron flux, which leads to impaired respiration and adenosine triphosphate (ATP) synthesis. The depletion of ATP in turn can induce apoptosis with the remaining ATP or necrosis with total depletion.
The co-localization of NSs with TFAM as discovered by Barnwal, et. al may be a crucial component of the induction of apoptosis. We hypothesize that NSs may inhibit TFAM's function in regulating the respiratory chain. In this aim, the interactions between NSs and TFAM will be further characterized by investigating the transcription levels of MT-DN6 and activity of the mitochondrial respiratory chain complexes. Our hypothesis is that upon entering the mitochondrial matrix, NSs binds to transcriptional factor TFAM, inhibiting its regulatory activity and causing respiratory chain failure, resulting in apoptotic induction.

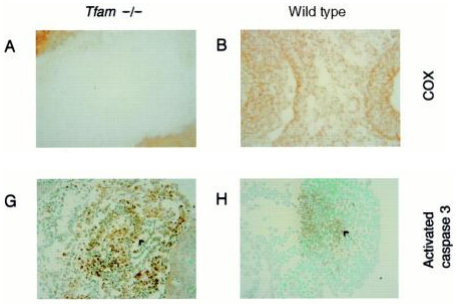
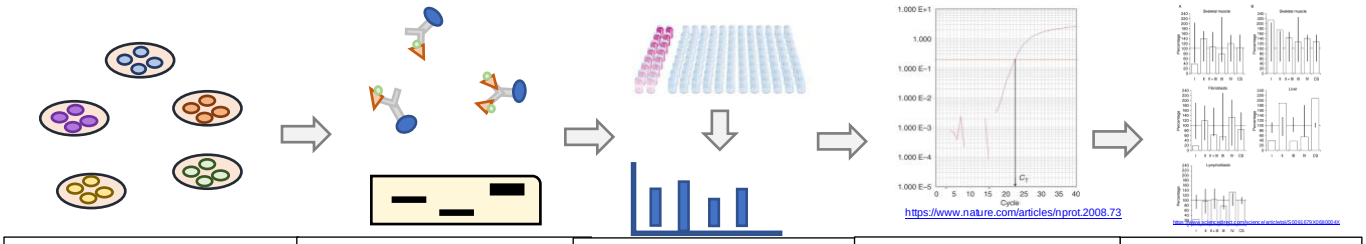


Figure 7: Massive apoptosis occurred in Tfam knockout (Tfam−/−) mouse embryos[31].

A and B: Cytochrome *c* oxidase (COX) activity. Mosaic loss in KO hearts, normal levels in the controls. The lack of cytochrome c is the result of its release during apoptosis.
G and H: Activated caspase 3. Abundant in Tfam knockout embryos and occasional in control embryos.
Caspase 3 activation is a hallmark of apoptosis.



1. Transfect with NSs (WT and mutant)	2. Co-IP with TFAM and NSs	3. Test activity levels of TFAM	3. Measure mt transcription levels	5. ETC activity *in vitro*.

Figure 8: Experimental workflow for Aim 3.

Experimental Design: We will first assess the binding ability of NSs-TFAM by co-immunoprecipitations, using antibodies against TFAM. This way, we will determine whether an interaction is capable between the proteins. As a positive control, we will include ARL6IP1 as a binding partner to TFAM and PB1-F2 as a negative control. We expect to find an interaction with wildtype NSs, but not the mutants. It is our hypothesis that it is at this point that the leucine residues in the C-terminus of NSs are imperative for binding. Thus, we expect that the NSs mutants will be similar to the PB1-F2—TFAM non-binding result. If our hypothesis regarding the mutants being unable to bind to TFAM is true, then we can use those constructs in our future experiments.

We would next measure the transcriptional activity of TFAM in NSs-mediated apoptosis by detecting binding of TFAM to DNA response elements to be bound on a microplate. A primary antibody against TFAM will be added, then a secondary antibody that is conjugated to HRP and the colorimetric readout will be read at OD450. This will let us determine how active TFAM is when exposed to NSs. We expect that TFAM activity will decrease in cells containing wildtype NSs, but not with the mutants. This would be due to inhibition on TFAM activity from the interaction with wildtype NSs.

We will then measure the transcriptional activity of TFAM by RNA extraction, mRNA purification, separated by gel electrophoresis and run on a Northern Blot. We will include probes against MT-DN6 and cytochrome *b* (both mitochondrially encoded), TFAM and cytochrome *c* (nuclearly encoded). The housekeeping gene 18S rRNA will be used as a reference, and we plan to use the Comparative Ct method to quantify our results. We will include both the CCHFV-infection and NSs-transfections in order to measure RNA expression for those four proteins. We expect that in conditions containing the wildtype NSs protein, there will be decreased MT-DN6 and cytochrome *b* mRNA expression, but not of TFAM or cytochrome c. The mutants, we suspect, would result in no difference between them because the NSs-TFAM interaction inhibits TFAM activity.

Once the NSs-TFAM interaction has been established, we will then measure respiratory chain enzyme activity via assays against the respiratory chain complexes. This will be used to establish a link between NSs-TFAM binding and respiratory chain failure. We expect that respiratory chain activity will be disrupted with NSs-TFAM binding, but that activity would be retained when NSs is inhibited or absent.

It is highly possible that the NSs mutants will bind to TFAM. We would then suggest that there may be a secondary interaction *in vivo* that inhibits the interaction between mutant NSs proteins and TFAM.

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