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Characterization Of The Anti-Viral Activity Of Schlafen13

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CHARACTERIZATION OF THE ANTI-VIRAL ACTIVITY OF SCHLAFEN13

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CHARACTERIZATION OF THE ANTI-VIRAL ACTIVITY OF SCHLAFEN13

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ABSTRACT

Schlafen13 (Slfn13) is an enzyme that belongs to the Schlafen family whose expression and function is not very well characterized. The N-terminal has a pseudo dimer structure that contains its catalytic site. There is no characterization functionally or structurally of the C-terminal of Slfn13 other than the prediction of a region with helicase activity. **The objective of my thesis was to increase our understanding of the Slfn family of proteins**. Currently Slfn13 is reported to play a role in the differentiation of monocytes and to function as an endoribonuclease that cleaves tRNA and rRNA molecules in a site dependent sequence independent manner. Further analysis of the anti-viral activity is hampered by the poor expression of the full-length protein. We have been unable to detect Slfn13 by western blot using different antibodies in different cell lines which were successfully transfected either stably or transiently. Our data indicates that regulation of Slfn13 expression is at a post-transcriptional level. We demonstrated the robust expression of endogenous and exogenous mRNA encoding Slfn13 in different cell lines by means of RT-PCR. This suggests a potential instability and/or toxicity of Slfn13. We investigated whether regulation is at the post translational level by using compounds that inhibit proteasomes and lysosomes. Despite obstructing these major pathways of protein degradation, we were unable to rescue Slfn13 expression. It is reported that type I-interferons play a role in the induction of Slfn proteins. Intrinsically, we theorized that perhaps an interferon-induced chaperon was required for Slfn13 expression but interferon α failed to stabilize this protein. In contrast to Slfn13, the family member Slfn11 is widely expressed at very high levels in multiple cell lines. We took advantage of the evolutionary conservation between these two proteins and swapped the last third residues of Slfn13 with those of Slfn11. This chimera was readily expressed indicating that the regulation was post-translational. Furthermore, this indicates that the last third of Slfn13 is responsible for the instability of this protein. Since the Slfn11 portion in the chimera does not have anti-viral activity, we also investigated the activity of Slfn13/11 Chimera against HIV-1 and flaviviruses. Our findings indicate that the Slfn13/11 chimera lacks anti-viral activity. However, we cannot ascertain the presence or absence of Slfn13 through normal methods. We believe that localization plays a role in the expression of this protein as the c-terminal of region of Slfn11 in the Slfn13/11 contains a predicted nuclear localization sequence. This led us to probe which of the last third of the c-terminal is responsible for the instability of this protein and how we could exploit this feature for molecular biology purposes. **In summary, because the Slfn13/11 Chimera is 80 percent identical to Slfn13 and it is known that c-terminal has no activity; we can say that Slfn13 also lacks anti-viral activity.**

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

ABSTRACT v

TABLE OF CONTENTS vi

LIST OF FIGURES vii

INTRODUCTION 1

HYPOTHESIS 18

SPECIFIC AIMS 19

MATERIALS AND METHODS 21

RESULTS 30

CONCLUSIONS 43

DISCUSSION 45

BIBLIOGRAPHY 49

VITA 54

LIST OF FIGURES

Figure 1.HIV-1 Genome Structure.....6

Figure 2. HIV-1 Life Cycle…….……....…...6

Figure 3. Flavivirus Genome Structure……..…..…..…8

Figure 4. Flavivirus Life Cycle…….……...…..11

Figure 5. Structure of Schlafen 13……..…15

Figure 6. Reverse Transcriptase PCR of Endogenous and Exogenous Slfn13 mRNA.……...…33

Figure 7. 293T MG132 ……...…..34

Figure 8. 293T IFN alpha …….....35

Figure 9. A172-KD 13/11 Chimera …….....…36

Figure 10. Cellular distribution of Slfn13/11 Chimera..……...….37

Figure 11. 293T NH4Cl ……..……...…….38

Figure 12. A172-KD NH4Cl ……..……..…….....…….38

Figure 13. 293T 13/11 Chimera and 13/11 Chimera 2……..……..……...…….39

Figure 14. HIV-1 p24 Analysis……..……...…….....…….40

Figure 15. WNV Viral Titer Analysis……..…….....…….41

Figure 16. VSV Viral Titer Analysis……..……..…....…….42

INTRODUCTION

Viruses require exploiting cellular resources for replication and the immune system thwarts these strategies by targeting these resources. In this study I will focus on two group of evolutionarily distant viruses, HIV-1 and *Flaviviruses*, and the activity of members belonging to a family of type I interferon-induced proteins called Schlafen. The relevance of this study resides on the medical significance of these pathogens that I describe in the next pages.

Medical Relevance of HIV-1 and *Flaviviruses*. Presently, there is no vaccine for HIV. Anti-retroviral therapy is very efficient at controlling viral replication[1]. These drugs are categorized by inhibition of integration, reverse transcription, and protease cleavage[1]. However, there are concerns about toxicity and side effects related to required long term use and drug-drug interactions[2,3]. Individuals receiving anti-retroviral therapy are often administered a cocktail of drugs that must be administered for the remainder their lives[2,3]. Studies illustrate that a combination of drug interactions and overall toxicity from long term use lead to a variety of disorders[2,3]. These disorders are generally associated with co-morbidities of HIV infected individuals and are in the realm of metabolic complications but can also be neurological and cardiovascular[2–4]. The adverse effects of nucleoside reverse transcriptase inhibitors (NRTI) are attributed to these anti-viral drugs blocking mitochondrial DNA polymerase leading to mitochondrial toxicity that induce metabolic disorders[5]. Integrase strand transfer inhibitors (ISTI) induce a decrease in insulin sensitivity certain individuals[2,3]. Lipodystrophy and type 2 diabetes is a common indication of the adverse metabolic effect of antiviral drugs[1-3]. In addition, it is reported that protease inhibitors not only contribute to the same metabolic disorders as NRTIs and ISTIs but also increases rate of heart failure mortality[4]. Antiretroviral treatment is also associated with increased risk of neuropathy that is present in the form of decreased motor

function and neurocognitive impairment[6].

Regarding *Flaviviruses*, there are no anti-viral options in clinical use to combat infection of any viruses belonging to the genus[7]. There are currently vaccines for several including yellow fever, Japanese encephalitis, and Dengue virus[7]. While the vaccines for yellow fever and Japanese encephalitis appear to be efficient and safe, administration of the dengue virus vaccine has been limited due to complications in certain groups of individuals[7]. Studies demonstrate that the vaccine protects against dengue virus for an average of five years in individuals with prior exposure to the pathogen[8]. However, vaccinated individuals with no prior exposure are at higher risk of being hospitalized for dengue than those who have not been vaccinated[8]. As for West Nile virus (WNV) and Zika virus (ZKV), no vaccines exist[7]. Treatment for those infected with these viruses is limited to pain management and intravenous fluids[7]. The medical implications relating to HIV and flaviviruses demonstrate the need for further research relating to these pathogens.

HIV Pandemic. Acquired Immunodeficiency syndrome (AIDS) is a global threat[9]. It was classified as a new disease in 1981, after a rise in individuals dying due to unusual circumstances[10]. Human Immunodeficiency Virus (HIV), the virus responsible for the disease was later identified and has become one of the fast spreading infectious diseases[10]. Global spread of HIV has led to its classification as a pandemic with more than 75 million individuals that have been infected since its classification and 37 million infected individuals that live with the virus today[1,10]. Areas with the highest population of infected individuals are seen in third-world and developing nations; the highest of which are located in sub-Saharan Africa[10]. There are two species of HIV, HIV-1 and HIV-2[1,10,11]. Both species belong to the subgroup lentivirus genus of the retrovirus family of positive sense single strand RNA viruses[1,10,11]. The two species of HIV differ only in one of the accessory proteins[11]. It is reported that origins both HIV-1 and HIV-2

can be traced back to simian immunodeficiency virus, with HIV-1 being the more virulent of the two and responsible for the current pandemic[10].

Flaviviruses are Emerging Pathogens. *Flavivirus* infection rates are rising globally. In 2016 there were 4 million ZIKV infections in the Americas alone[12]. DNV infections have increased six-fold between 2010 and 2016[13]. There has been a similar rise in WNV infections between 2014 and 2018. Areas with the highest rate of flavivirus infections much like HIV are developing countries. Rates of infection are particularly the highest in African countries[14]. It is believed that these rates are higher than reported due to the Pathogenesis of flaviviruses where 80 percent of infected individuals are asymptomatic[15,16]. However, there are variances in pathogenesis that lead to different disease prognosis[17,16]. Microcephaly was not reported to be associated with ZIKV until 2013 despite being first discovered in 1947[12,18]. Regarding WNV, 1% of those infected develop neuro-invasive disease[14,15]. The emergence of *Flaviviruses* and the variance in their pathogenesis illustrates the relevance for researching these pathogens

Strategies of Viral Replication. The Baltimore classification system of viruses was characterized by David Baltimore in 1971[19]. This classification also acknowledges the significance of the central dogma of molecular biology in that viruses only carry out two processes[19]. Generating genetic material and mRNA to be used for protein synthesis[19]. As such, it classifies animal viruses based on genome and their mechanisms of replication[19,20]. Class I viruses are classified by having a double strand DNA genome[19,20]. These viruses are much like hosts and give rise to mRNA through transcription of their DNA genome[19,20]. Class II are single stranded DNA genome viruses and produce mRNA in the same manner as Class I viruses[19,20]. Class III viruses consists of double stranded RNA genome viruses[19,20]. They have multiple copies of double stranded RNA and usually produce a single protein from each piece of genetic

information[19,20]. Class IV viruses are single stranded positive sense viruses (ssRNA (+)), their genomes are translated directly to protein[19,20]. These viruses tend to produce a polyprotein from their mRNA that is cleaved into several proteins[19,20]. Some also produce different proteins through sub-genomic mRNA and ribosomal frameshifting[19,20]. It is worthy to note that the mRNA used to produce proteins is identical in base sequence to the viral genome[19,20]. Thus, for these viruses to replicate their genomes they must first produce a negative sense strand of RNA from their genome[19,20]. Class V is comprised of single stranded negative sense RNA genome viruses ssRNA(-)[19,20]. These viruses have a genome that is complementary to the mRNA required to produce viral proteins[19,20]. They require an RNA dependent RNA polymerase to produce a complementary strand of RNA that is positive sense[19,20]. The complementary strand serves as a template for viral genome and produces viral protein[19,20]. Class VI viruses are ssRNA (+) that have a DNA intermediate. They do not use RNA as a template to produce copies[19,20]. The RNA genome is reverse transcribed into DNA which is integrated into the host and then transcribed using host polymerases[19,20].

HIV-1 Structure. The HIV-1 genome consists of two identical copies of 9.7kb single strand positive sense RNAs[21](Fig. 1). There are nine gene products obtained from splicing that code for 9 different proteins, 3 of which are cleaved into several different protein products[11,22]. The genome is divided into structural genes, essential regulatory genes, and accessory genes enclosed by a three prime and five prime long terminal repeats[21,23]. The structural genes of the HIV-1 genome are gag, pol, and env. The envelope gene codes for glycoprotein gp160 which is cleaved to form two glycoproteins gp120 and gp41. The glycoprotein gp41 is a transmembrane protein that spans the lipo-protein bilayer of the envelope made as new virions are budding from infected host cells. A non-covalently bonded heterodimer is formed between gp41 and trimers of gp120.

The gag gene codes for structural proteins p6, p7, p17, and p24[24,25]. Proceeding inward from the viral envelope is the matrix made of p17 followed by the viral capsid made of p24 and the nucleocapsid made of p17. Reverse transcriptase (RT), integrase (IN), RnaseH, and protease (PR) are all proteins that are coded for by the pol gene[11]. PR cleaves protein products, RT's function is to transcribe viral RNA to double stranded DNA, and IN facilitates the integration of viral DNA into the host genome. These structural genes are hallmarks of all retroviruses. Moving on to the essential regulatory elements, the genes tat and rev code for the tat and rev proteins respectively. Tat is said to be involved in the expression of the viral genome while rev functions in exporting messenger viral RNA from the nucleus after processing. The accessory genes are vif, vpu, vpr, and nef. They code for proteins of the same name respectively. Viral infectivity factor protein coded for by the vif gene is involved in the infectivity of HIV-1 in specific cell lines. It is required for the infectivity in some cell lines but not others. Vpu is exclusive to HIV-1 and is not present in HIV-2. This protein's function is involved with the release of viral particles from infected host cells. Vpr assists with the movement reverse transcribed viral DNA into the nucleus and is also involved in cell cycle arrest. Nef, or negative factor is associated with viral budding, and infectivity.

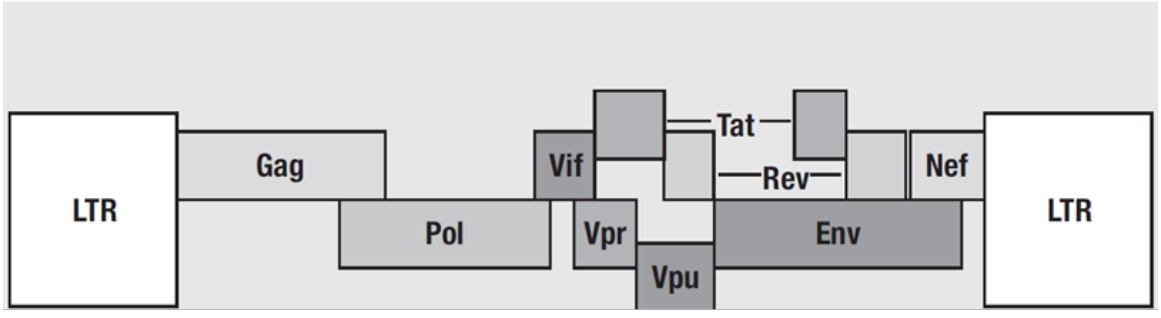

Fig. 1 HIV-1 genome structure.
Emanuele Fanales-Belasio(a), Mariangela Raimondo(b), B. S. and S. B. HIV virology and pathogenetic mechanisms of infection: a brief overview. Clin. Ter. 46, 5–14 (2010)

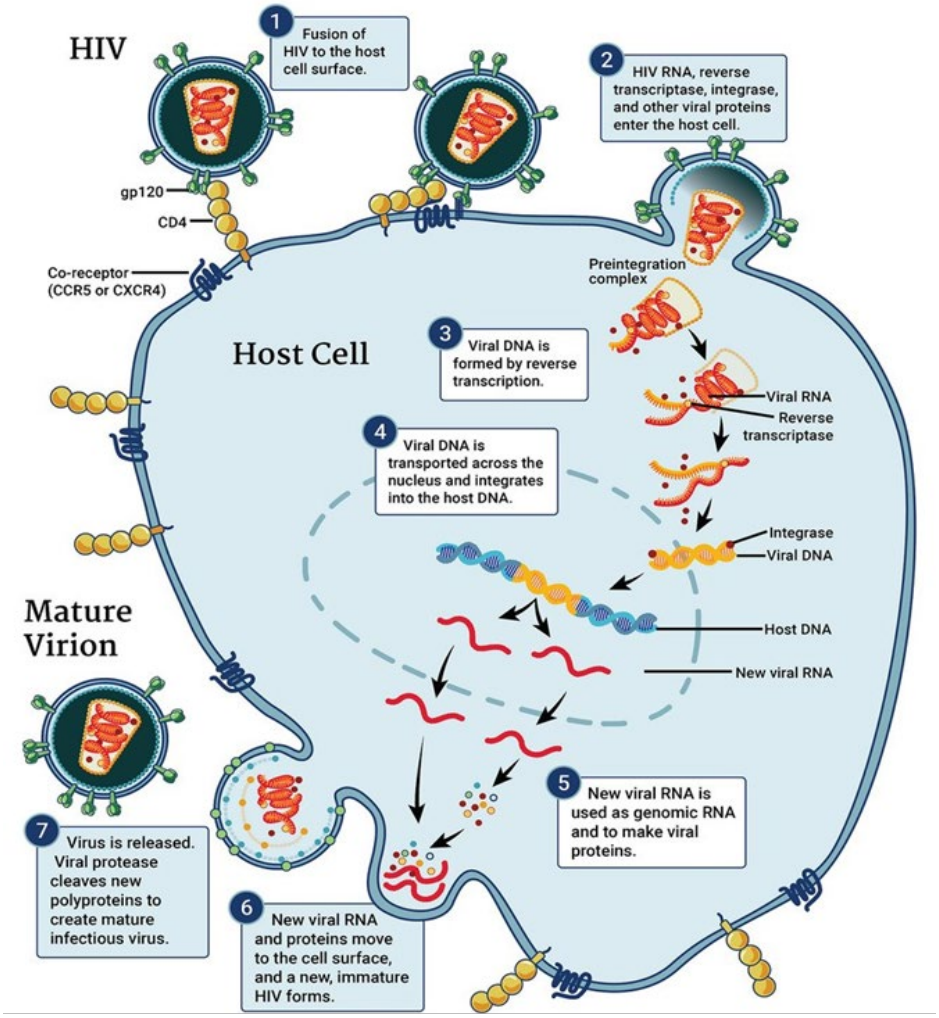

Fig. 2 HIV life cycle

HIV-1 Life Cycle. The primary target of HIV-1 are T-lymphocytes which are circulating but also T-cell precursors located in the bone marrow and thymus. HIV-1 can also target other cells such as macrophages, eosinophils, dendritic cells and microglial cells[11,26]. These mentioned cell types are known to express the HIV-1 target receptor CD4[11,26](Fig. 2). First, the viral envelope glycoprotein gp120 recognizes and binds to the CD4 receptor and the coreceptor CXCR4 or CCR5 of the host cell[26]. This binding activates a conformational change that allows the gp41 subunit of the envelope glycoprotein heterodimer to penetrate the cell membrane and facilitate fusion[27]. The Accomplishment of fusion reveals the viral core comprising the capsid containing

two copies of positive sense single strand RNA viral genome, reverse transcriptase, integrase, vpr, vif and matrix protein[26,27]. Microtubules are used to transport the virion core to the nuclear envelope avoiding premature uncoating[11,23]. Reverse transcriptase then produces a cDNA/RNA double strand hybrid from viral RNA via its ribonuclease H active site. This is accomplished using tRNA Lys3 as a primer for the synthesis of negative strand DNA[28]. Its ribonuclease H active site subsequently breaks down the RNA and it uses its polymerase active site to enzymatically produce a complementary strand of DNA[28]. The result is a double stranded DNA molecule[28]. A pre-integration complex is formed with integrase and viral DNA that travels into the nucleus assisted by the host protein transportin 3[29]. Once inside the nucleus, Integrase cleaves the three-prime end of both molecules resulting in sticky ends and integrates the viral DNA into the host genome[29]. Viral mRNA is then transcribed by host RNA Pol II and differentially spliced via host cell machinery[29]. In this early phase the transcripts produced are completely spliced and contain the essential regulatory elements Tat and Rev as previously described[30]. Late transcripts are un-spliced and transported out of the nucleus via Rev and include structural genes and accessory genes[30]. These un-spliced transcripts are either packaged into new viral progeny as genomic RNA or produce structural proteins. The structural proteins are translated from the gag gene on viral mRNA as a polyprotein and cleaved to form their respective proteins as earlier mentioned[25,31]. Once produced envelope gp120 and p41 make their way to the plasma membrane and from an immature virion containing all elements of a viral particle[31]. Protease then facilitates the release of the virial particle and the virion rearranges into a mature infectious viral particle[31].

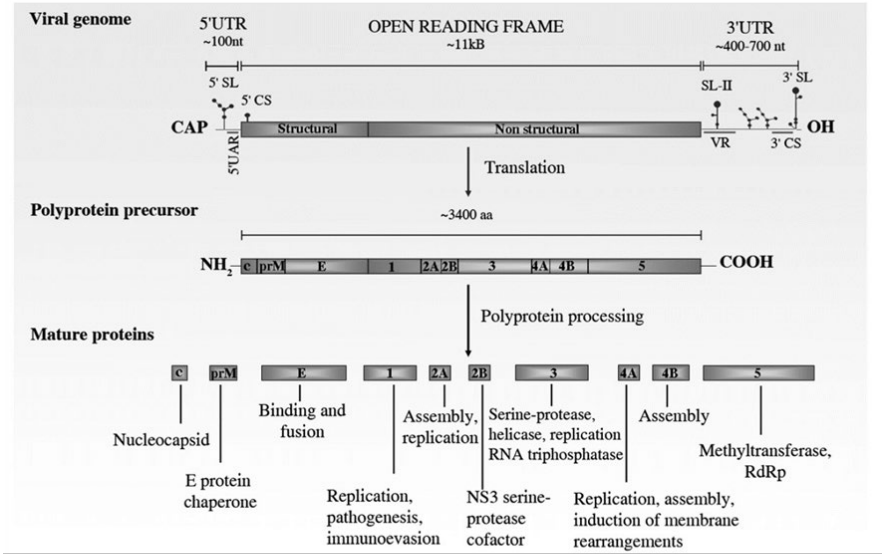

Fig. 3 Flavivirus genome structure and proteins.
Fernandez-Garcia, M. D., Mazzon, M., Jacobs, M. & Amara, A. Pathogenesis of Flavivirus Infections: Using and Abusing the Host Cell. Cell Host Microbe 5, 318–328 (2009).

Flaviviruses. *Flaviviruses* are a genus of viruses belonging to the family Flaviviridae[17,32]. Within the genus are more than 70 different species of viruses[17]. The name *Flavivirus* is comes from the Latin word for yellow as yellow fever virus was the first of the genus to be discovered and characterized by jaundice exhibited by infected individuals. Generally speaking, *Flaviviruses* infect vertebrate hosts through arthropod vectors such as ticks and mosquitoes and so are considered arboviruses[16]. Viruses in this genus share the same genome and replication but can cause a variety of distinct disease[16,17] (Fig.3). Regarding those that are pathogenic to humans, *Flaviviruses* can be separated into two groups. Viruses such as dengue virus and yellow fever virus that cause vascular leak and hemorrhagic fever form one group[17,33]. The other being viruses that can lead to encephalitis such as (WNV) and Japanese encephalitis virus. However, many individuals that become infected by *Flaviviruses* are asymptomatic[33]. Despite having the same genome these viruses can produce structurally varied viral particles resulting in a range of molecular pathogenesis that is difficult to predict or comprehend[16,34]. *Flaviviruses* are made of a

lipid bi-layer envelope with 180 copies of two surface glycoproteins and a capsid containing a single stranded positive sense RNA viral genome[35]. The RNA genome of *Flaviviruses* is around 10.8kb and codes for ten proteins. Three of the proteins coded for are structural proteins C, E and prM/M[18]. They are found at the five-prime end of the genome[18,35]. Structural protein C is the viral capsid protein. Structural protein E is the surface glycoprotein found on the lipid bilayer[18,36]. prM is found on noninfectious particles and is a precursor of the M protein, found on infectious particles[18,36]. The other seven proteins are non-structural and play a role in replication, assembly, and host cell response to the virus[16,35,36]. NS1 is involved with replication, pathogenesis, and immune system invasion. N2A, NS4A, and NS4B facilitate replication, assembly and membrane rearrangements[16,35,36]. The NS2B protein is a serine protease cofactor while the NS3 protein functions as a serine protease, helicase and RNA triphosphatase[16,35,36]. The NS5 protein is a methyltransferase but also functions as an RNA dependent RNA polymerase[16,35,36]. Although *Flaviviruses* have the similar genomes coding for structurally similar proteins our lab focuses primarily on WNV.

West Nile Virus Replication Cycle. WNV shares the same genome configuration coding for structural and nonstructural proteins in the same manner as other viruses in the *Flavivirus* genus[32,37](Fig. 4). Regarding human infection, WNV targets include monocytes, macrophages, dendritic cells, endothelial cells and neurons[32,37]. Virial entry into the cell is not very well characterized but the cell surface protein DC-SIGN has been illustrated to be a prominent receptor involved in viral fusion and entry into the cell[32,37]. Viral E protein is responsible for binding to the cell surface receptor[32,37]. The viral particle is internalized by means of receptor mediated endocytosis[32,37]. Within the endosome the E protein changes conformation in response to the acidic pH of the endosome and fusion occurs releasing the nucleocapsid into the

cytoplasm[32,37]. After which, the single strand positive sense RNA is translated into a polyprotein. This polyprotein will be processed by cellular proteases as well as viral serine protease combination of NS2B and NS3 to produce all the structural and non-structural proteins associated with the virus[32,37]. Negative sense RNA is produced from the positive sense viral genome which in turn produces more copies of the positive sense genome to be packaged into viral particles, this is associated with the endoplasmic reticulum[32,37]. There is an asynchronous shift between producing viral RNA used for the translation of protein and producing negative sense viral RNA[32,37]. Viral assembly and encapsidation occurs that produce non infected particles and are shuttled to the trans-Golgi network[32,37]. At this point host protease furin cleaves viral protein prM to produce viral M protein and produces an infectious viral particle that is released from the cell by means of exocytosis[32,37].

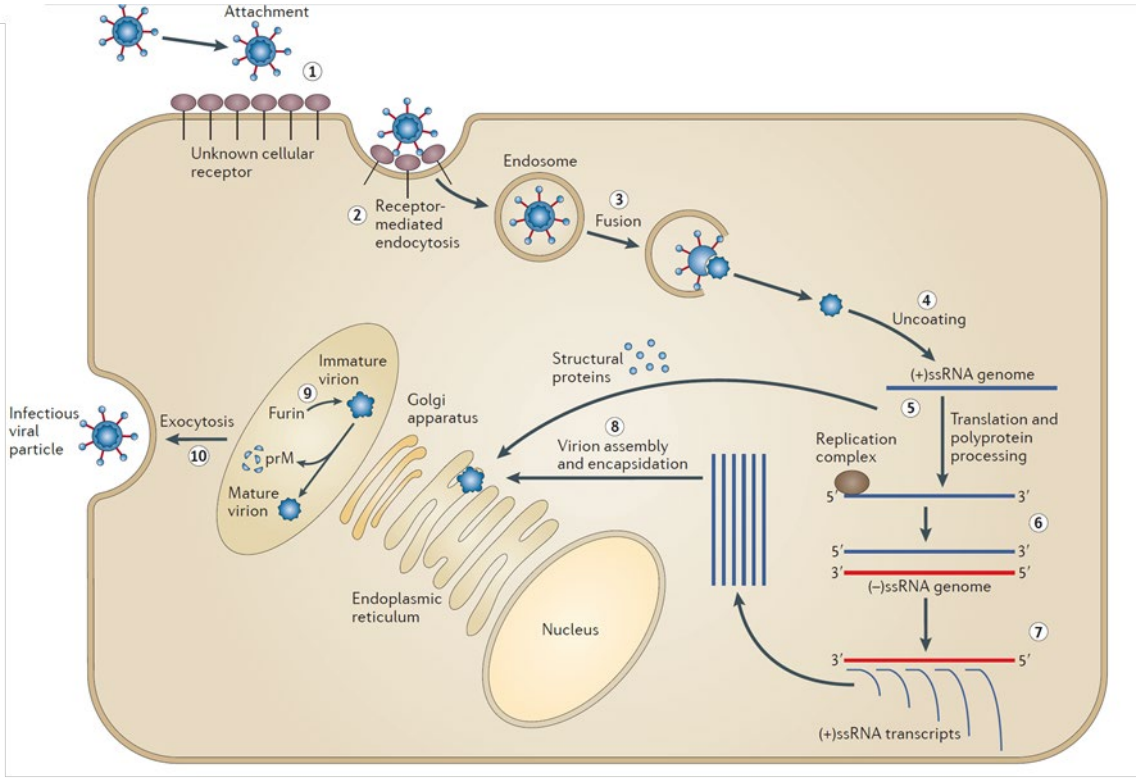

Fig. 4 West Nile Virus life cycle
Suthar, M. S., Diamond, M. S. & Gale, M. West Nile virus infection and immunity. Nat. Rev. Microbiol. 11, 115–128 (2013).

Schlafen Family of Proteins. Schlafen (Slfn) family of proteins are coded by interferon stimulated genes discovered to play a role in thymocyte development[38,39]. They were thought to only be present in mammalian cells but have been discovered in other types of chordates cells[40]. It was demonstrated that that some members of the schlafen family can block cell growth by keeping the cells in S phase of mitosis[38]. As a result, the family of proteins were given the name schlafen, German for sleep[38]. The schlafen family of proteins is distributed into three subgroups based on protein structure. Subgroup I consist of mouse Slfn 1 and 2 and contains the least members of the subgroups[41]. The proteins in subgroup I are cytoplasmic and contain AAA_4 domain region and a schlafen box sequence which is unique to Slfn proteins[41-43]. There is no known function of the schlafen box; however, the AAA_4 domain is associated with transcriptional regulators and helicases[44]. These domains are located at the N-terminal[40]. Both

domains are conserved in subgroup II and subgroup III[40,42]. Subgroup II is comprised of mouse schlafen 3 and 4 and human Slfn12 both of which are cytoplasmic[41,42]. This subgroup contains an additional SWADL sequence not found in subgroup I[41-43]. Subgroup III is made of the largest proteins of the family and has the most members. Its members are mouse schlafen 5,8,9,10,14 and human Slfn(hSlfn) 5,11,13,14[40,41]. All of which have the AAA-4 domain, schlafen box, and SWADL sequence in addition to a C-terminal domain that contains a helicase motif ruminant of superfamily I RNA helicases[41,45]. The proteins in this subgroup are said to be nuclear localized and thus contain a nuclear localization sequence in the C-terminal apart from hSlfn13 that is said to be cytoplasmic[42]. The unique structure of schlafen family of proteins allows them to have a variety of cellular functions such as programing cell death, controlling cell cycle and differentiation[46-49]. Schlafen proteins are also said to play a role in cancer development and viral replication as a result of these cellular functions[50-54]. Here we are focused more on members of the schlafen family that play a role in rRNA and tRNA regulation and those that have illustrated the capability to inhibit viral replication[44,53-56]. hSlfn11 is said to inhibit HIV-1 through a codon bias manner[57]. Furthermore, human schlafen 11 has also illustrated antiviral activity against not only HIV but also other positive sense single strand RNA viruses in the *Flavivirus* family such as WNV, Dengue, and Zika virus[50,57]. hSlfn13 and hSlfn11 share sequence homology and structure, both being in Subgroup III of the schlafen family[56]. It is suggested that human schlafen13 has some antiviral activity against HIV and minimal antiviral activity against Zika virus by means of tRNA regulation, but this suggestion is not completely confirmed[56]. As such, we are sincerely interested in its expression and antiviral activity against positive sense single strand RNA viruses like those of the flavivirus family and HIV.

Schlafen13 Structure. Slfn13 is not a very well characterized protein. Although it is mentioned vaguely in a few publications, there exist only one publication focusing on Slfn13. Slfn13 is said to be an interferon stimulated gene located on chromosome 17. There are two confirmed transcript variants which produce two isoforms of the protein[58,59]. The full-length protein has a transcript of 8.39kb that produces an 897 amino acid protein[58,59]. The second transcript produced by alternate splicing is 5.92kb[58]. This transcript produces an isoform that is 579 amino acids and is missing amino acids 9-326[58,59]. Furthermore, there are seven other computationally predicted transcripts and four other predicted isoforms of the protein[58]. hSlfn13 belongs to subgroup III of the schlafen family of proteins previously discussed and is said to likely be an ortholog of mouse schlafen 8 and rat schlafen 13[56,60]. Rat Slfn13 N-terminal crystal structure containing amino acids 14-353 was solved and allows us to speculate on the structure of that same region of hSlfn13[56]. The N-terminal of Slfn13 is said to be in a pseudo-dimeric structure consisting of and N-lobe and C-lobe. Both lobes are made of four stranded β-sheet surrounded by three α-helices[56]. Two bridging domains made of a β-sheet and an α-helix is joins the two lobes giving the N-terminal a U-pillow structure[56](Fig. 5). Hydrogen bonding occurs between each lobe of the N-terminal and their corresponding bridging domain[56]. Regarding the C-lobe, a zinc finger motif was identified that is a result of the hydrogen boding between it and the corresponding bridging domain[56]. Features of the Slfn13 include two positively charged patches that is said allows the protein to bind to base-paired RNA. Furthermore, there is a negatively charged patch located on the C-lobe of the N terminal[56]. This negatively charged patch, only found on the C-lobe consisting of Glu205, Glu210, and Asp248 is conserved and said to be the active site of Slfn13[56]. Regarding the C-terminal of Slfn13 there is little known other than the presence of a helicase

domain[56]. Whether this helicase domain is functional remains to be determined. Slfn13 is said not to contain a nuclear localization sequence thus remaining in the cytoplasm[56].

Cellular Role of Schlafen13. According to literature the cytoplasmic location of Slfn13 gives it access to rRNA and tRNA where its cellular role is determined to be an endoribonuclease[56]. Slfn13 preferably cleaves tRNA versus rRNA with hSlfn13 demonstrating more activity than its mouse or rat counterpart[56]. Slfn13 is said to bind to the acceptor stem of tRNA and cleaves elven nucleotides from the three-prime end[56]. Regarding rRNA, Slfn13 can elicit its activity on base paired rRNA subunits because of their similar structure to tRNA[56]. This endoribonuclease activity is said to be site-specific and sequence independent[56]. A significant factor in the selection of rRNA and tRNA cleavage by Slfn13 is acceptor stem length[56]. hSlfn13 prefers acceptor stem length less than nine nucleotides whereas rat schlafen13 prefers tRNA molecules with longer acceptor stems[56]. This activity was also said to be concentration dependent as increase in Slfn13 leads to a second cleavage site at the T-loop of tRNA[56]. Furthermore, it is said that this activity led to global reduction in translation and cellular protein synthesis as the number of tRNA and rRNA cleaved increases with increase in Slfn13 as well[56].

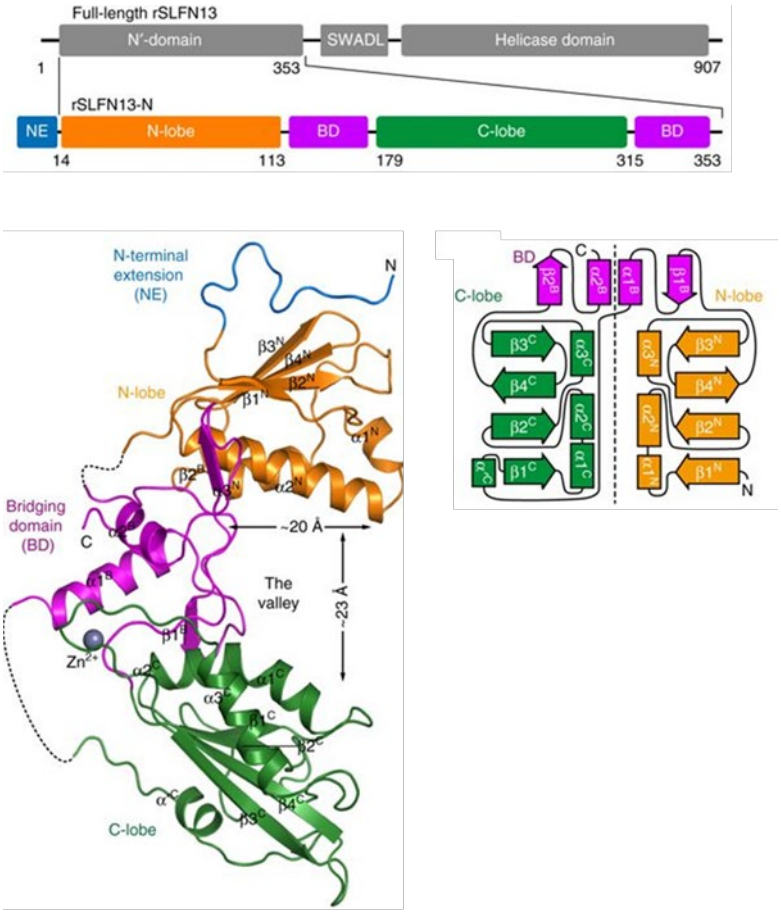

Fig. 5 Structure of Slfn 13 N-terminal is pseudo-dimeric U pillow-shaped

Yang, J. Y. et al. Structure of Schlafen13 reveals a new class of tRNA/rRNA- targeting RNase engaged in translational control. Nat. Commun. 9, (2018).

tRNA Relevance. tRNA are comprised of around 76 nucleotides and are RNA molecules that help drive the translation of mRNA to protein[61,62]. Their function consists of aminoacylation and peptide bond formation[61,62]. They are said to have originated by ligation of hairpin RNAs and may have played a role in the composition of ribosomes[61,62]. tRNAs were some of the first RNA discovered that did not code for protein[61,62]. tRNA have conserved sequences and motifs which make up its three-dimensional L-shaped tertiary structure[61,62]. This structure is comprised of the acceptor stem, T-loop, D-loop, variable loop, and anticodon loop[61,62]. It obtains this L-shape

through stacking of the helices that make up the D-loop and the T-loop of its cloverleaf appearing secondary structure[61,62]. The acceptor stem consists of base-paring between the 5' end containing a terminal phosphate group and the 3' end contains a CCA terminal sequence that attaches to amino acids[61,62]. The anticodon loop is five base pairs where the anticodon is found in reverse order because it must be read from 5' to 3' by the mRNA during translation[61,62]. Both the T-loop and D-loop are around four to six base pairs and poses modifications to the tRNA after processing[61,62]. Processed tRNA molecules are very stable and have a longer half-life than other RNA molecules[63]. Although most modification promote stability and longer half-life, others serve in significant roles such as codon-anticodon recognition and other smaller biological functions[63-65]. Of the modifications present in tRNA molecules methylation of pseudoruridine and adenosine are the most prominent as they facilitate the stability of its tertiary structure[64,65]. However, there modifications that lead to degradation as well[64]. Mutations in the tRNA gene could hinder its exportation out of the nucleus by the protein exportin where modifications that stabilize the molecule occur[65]. Under-modified tRNA are targeted for degradation and half a very short half-life[64,65]. Studies show that tRNA cleavage is a host defense mechanism used to regulate the rate and efficiency of translation[64,66]. For example, tRNA cleavage has been shown to be involved in cancer proliferation, inhibition of apoptosis, and promotion of ribosome biogenesis[64,66]. Furthermore, cleavage and regulation of tRNAs is relevant to our investigation of viruses because it is also involved in regulation of retroviral elements, modulation of translation and immune response to infection[64,66]. It has been suggested that cells under stress regulate the abundance and repertoire of their tRNA to manage translation rates of transcripts needed to survive[64,66]. Regarding viral infection, this mechanism is also used to avoid producing viral proteins. It is reported that viruses tend to possess biased nucleotides in their genomes and this

bias differs from human genes[57]. Lentiviruses such as HIV have genomes that possess a high occurrences of adenine nucleotides[57]. Host cells can be generally characterized by contain high guanine and cytosine content in their sequences[57]. In contrast HIV-1 has low guanine and cytosine content resulting in inferior usage of tRNA with the codons needed for translating viral proteins[57]. Adenine or uracil in the third position of the codon is the result of this codon bias[57]. This facilitates ribosome pausing and suboptimal translation[57]. Reports indicate that HIV-1 alters cellular tRNA levels during infection to promote the translation of its proteins[57]. However, there are several viral restriction factors exhibited by host cells that modulate changes tRNA repertoire and abundance such as Dicer, Angiogenin, Slfn11, and Slfn13[57,56]. These host viral restriction factors are expressed in response to viral induced changes to tRNA and translation of viral proteins[57,56]. It is reported that Slfn11 mediates antiviral effects on HIV-1 in a codon usage discriminatory manner[57]. Our lab is interested in host factors that restrict viral replication and so focus on the Slfn family of proteins.

HYPOTHESIS

ssRNA (+) RNA viruses are unique in that they have their genomes directly translated to protein during infection. They rely heavily on efficient translation of one or a few copies of viral genome by the host machinery upon infection. HIV-1, like other pathogens produce multiple copies of mRNA through transcription of a host integrated DNA intermediate in an attempt surpass this obstacle. However, the transcripts produced are not well suited to be translated by the host translation machinery. Here we focus on the Slfn family whose expression is regulated by type I interferon. A subgroup of which degrade tRNAs, and some exhibit anti-viral activities against these two group of viruses. More specifically we will evaluate the anti-viral activity of Slfn13 against HIV-1 and flaviviruses. **We hypothesize that the innate immune system exploits this limitation in the viral life cycle of ssRNA (+) viruses through a mechanism involving Slfn13 that negatively regulates translation efficiency.**

SPECIFIC AIMS

1. **Determining mechanisms of regulation of Slfn13 protein expression.** In order to evaluate the antiviral activity of Slfn13 we needed to modulate the expression of this protein. However, Slfn13 expression is tightly regulated in cell lines susceptible to HIV-1 and/or flaviviral expression. Therefore, we first determined mechanisms of regulation of Slfn13 to be able to generate cell lines expressing this protein. Then we developed Slfn13/11 Chimera cell lines in cell types that are relevant for the in vivo infection of these viruses. In the case of HIV-1 we studied a panel of human CD4+ T cell lines. With respect to *Flavivirus* replication, we will characterize A172 cells. A172 cells are a glioblastoma cell line that has been used as a model for flavivirus replication and have also been used to characterize the anti-flavivirus activity of Slfn11.

2. **Determine role of Slfn13 on HIV-1 and flavivirus replication.**

We established the role Slfn13/11 Chimera plays in the in vitro infection of these viruses. By extension, these findings also pertain to Slfn13 due to the sequence conservation between these two proteins.

The objective of my thesis was to increase our understanding of the Slfn family of proteins. Specifically, the mechanisms regulating the expression of Slfn13 and to discover whether this protein has antiviral activity towards HIV-1 and *Flaviviruses*. Despite the emerging role of these proteins in diverse immunological functions, our knowledge of this family is still very limited. There is only one report indicating that Slfn13 impairs translation of HIV-1 proteins in non-infected cells and that fails to impair the replication of the flavivirus Zika. These studies are very limited for several reasons. The impact of Slfn13 on HIV-1 was evaluated by co-transfecting

plasmids expressing HIV-1 proteins and Slfn13 in cells not relevant for the replication of these viruses. These experimental conditions do not allow evaluation of the impact of infection on the activity of Slfn13 and limit the study to the late step of viral replication cycle. Similarly, the functions discovered with over-expressed Slfn13 are difficult to extrapolate to physiological protein levels. Here we provide further verification of these findings by evaluating the role of physiological levels of full-length Slfn13 in the replication of HIV-1 and a group of flaviviruses including WNV, dengue and Zika viruses.

MATERIALS AND METHODS

Cell Culture. A172 and HEK293T cells were obtained from the American Type Culture Collection (Manassas, VA). Dulbecco's Modified Eagle's (DMEM) medium was used to maintain A172 and HEK293T cells. 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin-Glutamine (PSG) were used to supplement the culture media.

RNA Extraction. One million cells were harvested, washed with phosphate buffer saline, and placed in TRIzol Reagent (Ambion). Chloroform was then added, and nucleic acids separated by means of centrifugation. Equal volume of seventy percent ethanol was added RNA eluted using PureLink RNA Mini Kit (Invitrogen/Thermofisher).

Reverse Transcriptase PCR. RNA extracted from cells were transcribed into cDNA with primer 5'-AAGGCCTCAGCAAAGTCTGGAGG-3' targeting and internal sequence for Slfn13 or 5'- CTTATCGTCGTCATCCTTGTAATC-3' targeting the flag- tag on the expression vector using iTaq Universal SYBR Green One Step Kit (Bio-RAD). Subsequently, this cDNA was amplified using Gotaq PCR Master Mix (Promega) to verify the transcripts are present. Forward primers: 5'-GACGCAGATCCAGAGTTTCC-3', 5'-CAAAATCGTAGAAGTGTTTTGTG-3', 5'- GAGGCAAATCACTGCTCCCTG-3', and 5'-CATTCAACACATCGTCATTGACG-3' were used. Reverse primers: 5'-AAATGTCCTGGTGGAACTGG3', 5'-AAGGCCTCAGCAAAGTCTGGAGG-3', 5'- CTTATCGTCGTCATCCTTGTAATC-3' were used.

Plasmids. Eric Poeschla laboratory (Mayo Clinic, Rochester, MN)87 provided plasmids used for the generation of HIV-1-derived viral vectors. The pCMVΔR8.91 plasmid was used for packaging, and the pMD.G encoding the vesicular stomatitis virus glycoprotein G (VSV-G)

plasmid was used to produce envelope to generate viral vectors. These lentiviral vectors were used to express Slfn11 shRNAs, Slfn13 and Slfn13/11 Chimera proteins.

Slfn11-shRNA plasmids: An shRNA construct against Slfn11 was designed using a target sequence that has been formerly described[57]. The Sequence is (Top: 5'-GATCCGGCTCAGA ATTTCCGTACTGAATTCAAGAGATTCAGTACGGAAATTCTGAGCTTTTTTGGAAA-3', Bottom:5'-AGCTTTTCCAAAAAAGCTCAGAATTTCCGTACTGAATCTCTTGAATTCAGT ACGGAAATTCTGAGCCG-3'). The Slfn11 shRNA construct was ligated into the pSilencer 2.1 U6 Hygro shuttle vector (AM5760, Thermo Fisher Scientific) and sequence verified. This construct was used to produce the A172 Slfn11-KD cell line (A172-KD).

Slfn13 expression plasmid: pcDNA3.1+/C-(K)-DYK Slfn13 expression plasmid (OHu32094D, GenScript). This plasmid was used to produce stable cell lines in A172-KD cells via lipofectamine transfection using Lipofectamine 3000 reagent (Thermofisher) previously described[67,68,69]. Lipofectamine reagents were mixed with DNA and incubated at room temperature for 15 minutes. The mixture was then added to the cells and incubated for 4-hours. After the incubation, the media was supplemented with fresh media. Transfected cells were selected in the presence of G418 (A172-KD 750 ug/ml). It was also used for transfection of HEK293T cells via calcium-phosphate transfection[70,71]. The calcium-phosphate reagents were mixed with DNA and incubated for 15 minutes. The mixture of reagents and DNA were then added to the cells. 24-hours later the media was supplemented with fresh culture media.

C-terminal Slfn11 expression plasmids: pTRIP-IRES-P-Slfn11-shRNA-resistant plasmid was used as template to generate the Slfn11 truncated mutants using QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). The forward primer: 5'-TCTAGAAGTTGGGCTGTGGACC-3' and reverse primer: 5'-C

ATACTAGTGGATCCTCTAGC-3' were used to produce C-terminal Slfn11 (amino acids 442-901). This was verified by DNA sequencing.

N-terminal Slfn13/C-terminal Slfn11 expression plasmid: pcDNA3.1+/C-(K)-DYK Slfn13 expression plasmid (OHu32094D,GenScript) was used as a template to generate the N-terminal Slfn13 using Phusion Site Directed Mutagenesis Kit (Thermo Scientific) with forward: 5'-CCGACTCTAGCTAGAGGATCCACTAGTATGGAGGCAAATCACTGCTCCC-3' and reverse: 5'-CCACAGCCCAACTTCTAGAGAGGATCACAATTCCCTGGG-3' primers. This produced the N-terminal Slfn13 (amino acids 1-441). Production of the C-terminal Slfn11 plasmid was described previously. The NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs) was used to join the two DNA fragments of N-terminal Slfn13/C-terminal Slfn11. This was verified by DNA sequencing. This construct was transfected into HEK293T or A172-KD cells using calcium chloride or lipofectamine as previously described[67-71].

Slfn13 Cat Mut: Slfn13/11 sequence in pTrip Ires P Slfn13/11 plasmid described previously was removed and placed in pUC19 plasmid (New England BioLabs). A catalytic mutant was produced using QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies) with forward 5'-ATCCATAGCGTTTAAACAGTTCTCTAC-3' and reverse 5'-ATCCATAGCGTTTAAACAGTTCTCTAC-3' primers. This mutation occurs at the catalytic site of the N-terminal of Slfn13 changing both cytosines at bp-623 and bp-638 to adenine. This results in changing both amino acid residues from Glutamic Acid to Alanine. The sequence was verified and the N-terminal of Slfn13 pcDNA3.1+/C-(K)-DYK Slfn13 expression plasmid (OHu32094D, GenScript) was with replaced with this construct. This construct was transfected into HEK293T or A172-KD cells using calcium chloride or lipofectamine as previously described[67-71].

Slfn13/11 Chimera 2: Slfn13/11 pUC 19 sequence previously described was used as a template for PCR via Phusion Site Directed Mutagenesis Kit (Thermo Scientific) with forward: 5'- CTC TTGAAAGCAATGAGGAAG-3' and reverse 5'-GGTCTCTGCTCGGCAGATATT-3' primers. This was to remove a 525nt sequence from Slfn13/11 chimera. The pcDNA3.1+/C-(K)-DYK Slfn13 expression plasmid (OHu32094D, GenScript) was used as a template to amplify a 525nt sequence of Slfn13 using forward 5'-AATATCTGCCGAGCAGAGACCGAAACTTTCCTAAGAGAAAAATTTG-3' and reverse 5'-CTTCCTCATTGCTTTCAAGAGCTTAGACTGATACTGCTCCAC-3' primers via QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). The NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs) was used to join the two DNA fragments. The sequence was verified, and the construct was moved into pTrip Ires P. This construct was transfected into HEK293T or A172-KD cells using calcium chloride or lipofectamine as previously described[67-71].

Production of Lentiviral Vectors The full lentiviral vector transfection and production procedures of has been described previously[72-74]. HEK293T cells were transfected with calcium-phosphate using the transfer plasmid derived from pTRIP (15 ug), the packaging plasmid pCMVΔR8.91 (15 ug), and VSV-G envelope expression plasmid pMD.G (5 ug). 48-hours post-transfection, the viral supernatants were harvested and concentrated by ultracentrifugation at 124,750g for 2 hrs on a 20% sucrose cushion.

Expression of Slfn11-shRNA in A172 cells*:* A172 cells were transduced with shRNA-, eGFP-expressing lentiviral vectors and cells expressing the highest 10% of eGFP 26 fluorescence were isolated by cell sorting and expanded in culture. Immunoblot was used to determine the level of Slfn11 expression.

Expression of Slfn11 full-length and Slfn13/Slfn11 chimera cell lines: Transduction with lentiviral vectors expressing Slfn proteins and the puromycin resistant gene was engineered in A172-KD**.** Viral vectors were produced in HEK293T by transfection using the transfer plasmid pTRIP-IRES-P-Slfn11-shRNA-resistant plasmid expressing Slfn11 full-length or Slfn13/Slfn11 chimera (15 ug) and the packaging and envelope expression plasmids as previously described. In order to transduce cells, viral supernatant was concentrated by ultracentrifugation. After Three days, puromycin (A172 and HEK293T: 3ug/ml) was used to select transduced cells. Expression was verified by immunoblot.

Proteasome Inhibitor Analysis. HEK293T or A172-KD cells were transfected via calcium-phosphate with Slfn13 expression plasmid and EGFP plasmid as previously described. After 24-hours 6 microliters of 50 micromolar MG132 (Sigma) protease inhibitor was added to the media. DMSO was used as a vehicle. 18-hours later the cells were harvested and analyzed via immunoblotting.

Interferon α Analysis. HEK293T cells were transfected via calcium-phosphate with Slfn13 expression plasmid or Slfn11 expression plasmid. Interferon α was added to culture media in the concentration of 500 units/ml immediately after cells were transfected and 24 hours post transfection. Cell lysates were collected 48 hours after transfection and analyzed via immunoblotting.

Lysosome Inhibitor Analysis. Aluminum chloride (Sigma) was used to inhibit lysosomes at a 20mM concentration in culture media. HEK293T or A172-KD cells were plated on a 6-well plated ($0.45x10^6$ cells/well) and allowed to grow over night at 37°C. 24-hours later the cells were then transfected via calcium-phosphate with different Slfn13 expression construct plasmids or Slfn11 and EGFP plasmid as previously described. 18-hours later cells were treated with 20mM

Aluminum chloride culture media containing 20mM aluminum chloride. 48-hours later the cells were harvested and analyzed via immunoblotting. Anti-Slfn11 antibodies and anti-flag antibody were used to evaluate protein expression plasmid.

Viral culture. For HIV-1 viral assays we produced a replication-defective HIV-1 reporter virus (Hluc). $3x10^6$ HEK293T cells were plated in a T-75 flask and allowed to grow overnight at 37°C. They were subsequently transfected using calcium phosphate with Hluc (5ug) and VSV-G encoding plasmid pMD.G (5 ug) as previously described[70-73]. 48-hours post-transfection, the viral supernatants were harvested and concentrated by ultracentrifugation at 124,750g for 2 hrs on a 20% sucrose cushion. This Hluc virus is missing a large section of ENV and expresses a LTR-driven luciferase from the NEF slot[73].

The World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch provided the WNV strain TVP-7767 (Passage: Vero, #3). The VSV used which was designed to express eGFP has been previously described[75]. E-MEM supplemented with 2% FBS was used to maintain Vero cells where Viral stocks were made. Described below are materials and methods regarding infectivity titers of each virus stock by way of plaque assay.

Research conducted with VSV and HIV-1, was performed in BSL-2+ laboratory adhering to the UTEP Biological Safety Manual. Regarding WNV research, a (BSL)-3 laboratory was used adhering to the biosafety practices described in the UTEP BSL 3 Biological Safety Manual and Standard Operating Procedures.

Single-Round Infectivity Assay. A172-derived cell lines were plated onto 24-well plates ($2x10^4$ cells/well) and allowed to grow overnight at 37°C. The following day, cells were infected with Hluc virus previously described. 24 hrs post infection the cells were washed with 1X sterile phosphate-buffered saline to remove the input virus. Supernatant was collected for HIV-1 p24

quantification four days post infection and cell lysates were prepared for luciferase assay analysis as well as immunoblot analysis.

HIV-1 p24 ELISAs. Supernatant of infected cells were used to measure and quantify HIV-1 infection via p24 (described above) by ELISA (ZeptoMetrix Corporation, 0801008). ELISAs were performed according to the manufacturer's instructions.

Virus Replication Dynamics. Every cell line infected with VSV or WNV were plated in T25 cell culture flasks (2.5x105 cells in 2ml total volume) and allowed to grow overnight. 24-hours later the cells were infected with VSV or WNV and incubated at 37°C for 1 hr. To remove the input virus, they were then washed with serum-free medium three times. After which, the culture media was replaced with maintenance medium and incubated at 37°C. Every 8hrs supernatant was collected until experiments were stopped and stored at -80°C.

Plaque Assays. The methods previously described[76] give more detail about how WNV and VSV viral titers were determined. In summary, viral supernatants were inoculated onto confluent monolayers of LLC-MK2 cells. This was conducted in 12-well cell culture plates and incubated at 37°C for 1 h with gentle rocking every 15 minutes. The viral supernatants used for this assay were subjected to ten-fold serial dilutions prior to inoculation of the LLC-MK2 cells. The cells were then overlaid with 1 ml of 0.5% agarose in E-MEM maintenance medium. Cells were incubated at 37°C for 3 days and then stained with 1g/L of Napthol Blue Black, 13.6g/L of Sodium Acetate Anhydrous, 60ml/L glacial acetic acid to visualize plaques. Plaque formation on each cell line was quantified and viral titers were expressed as plaque-forming units per milliliter (PFU/ml). The estimation of viral titers via the plaque assay was carried out in triplicate experiments and each of samples was produced from independent viral infections.

Statistical Analysis. All data used for viral replication curves were transformed to log10pfu/ml. To test the effect of different cell lines expressing or not Slfn13/11 Chimera on viral replication curves repeated-measures ANOVA was used. To discover significant differences in viral titer between cell lines the Tukey-Kramer post-hoc test was used. Regarding p24 production in cells infected with HIV-1 analysis was executed by ANOVA with Tukey HSD post-hoc test.

Immunoblotting. The complete procedures for detection of protein by immunoblot have been formerly described[77]. To summarize, 2x Laemmli Buffer was used to lyse the cells followed by boiling for 10 minutes to obtain cellular lysates. SDS-PAGE was used to resolve the Cell lysates which were then transferred overnight to PDVF membranes at 100 mA at 4°C. TBS containing 10% milk was used to block the membrane for an hour. The primary antibody was diluted in TBS-5% milk-0.05% Tween 20 (antibody dilution buffer) and added to the membrane. To detect Slfn13, anti-Slfn13 antibody (Santa Cruz Biotechnology 1/250), anti-Slfn13 antibody (Thermofisher 1/500) and anti-flag antibody (Sigma, 1/500) were used as primary anti-bodies. Anti-Slfn11 antibody E-4 (Santa Cruz Biotechnology 1/500) was used to detect Slfn11 C-terminus of Slfn11 and anti-Slfn11 antibody D-2 (Santa Cruz Biotechnology 1/500) was used to detect the N-terminus of Slfn11. As a loading control, clone B-5- 1-2 (Sigma, 1/4000) antibody was used to detect α-tubulin. Incubation of primary antibodies were overnight at 4°C, with an exception being anti-α-tubulin which was incubated for 30 minutes at 25°C. TBS-0.1% Tween 20 was used to wash membranes containing bound primary antibody. The bound antibodies were then detected with secondary antibody, goat anti-mouse IgG-HRP (1/2000, Sigma), goat anti-rabbit (1/4000, Santa Cruz), or a donkey anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology) followed by chemiluminescence detection.

Immunofluorescence Microscopy. HEK293T cells were plated onto a 96-well confocal microscopy plate ($5x10^4$/well). Using Cytofix/Cytoperm buffer, the cells were fixed and permeabilized after 24 hours. They were then stained with either anti-Flag (1/500) or anti-Slfn11 antibody E-4 (1/200) primary antibody buffer at room temperature for 2 hours. Cytofix/Cytoperm buffer was then used to wash the cells 3 times. Following the 3 washes they were placed in secondary antibody Alexa goat anti-mouse 568-conjugated antibody (1/200) for 45 minutes at room temperature. Cytofix/Cytoperm buffer washes were repeated 3 times after the secondary antibody incubation. They were then incubated with DAPI (1/100) for 10 minutes at room temperature to stain cell nuclei followed by one wash with PBS. For imaging the cells were placed in 100uL of PBS.

RESULTS

Specific aim 1. Determining mechanisms of regulation of Slfn13 protein expression.

Endogenous Slfn13 is not expressed in a panel of cell lines. In order to select as a cellular model representative of cell types implicated in the in vivo replication of HIV-1 and *Flaviviruses*, specifically WNV, we determined the expression of Slfn13 by immunoblot. The glioblastoma cell line A172 relevant for WNV, and a panel of CD4+ T cells (SupT1, Jurkat, MolT-4, and CEM) and myeloid cells (K-562), representative of the main in vivo targets of HIV-1 were evaluated. Other cell types including epithelium-derived cells (HeLa, A549), and cells of undefined origin (293 and 293T) that allow infection of WNV and replication incompetent HIV-1 were included in our study. Two commercially available anti-Slfn13 antibodies (Santa Cruz and Thermofisher) were used in these experiments but Slfn13 was not detected. Importantly, one of these antibodies recognizes amino acids 632-663 of the C-terminal and the other a region that is unspecified by the manufacturer.

Exogenous Slfn13 is not expressed in a panel of cell lines. Since we could not detect endogenous levels of Slfn13 using an anti-Slfn13 antibody via immunoblot, we opted to express Slfn13 from a transiently and stably transfected plasmid to verify that our experimental conditions to detect Slfn13 were optimal. Furthermore, this plasmid expressing Slfn13 was flag tagged Slfn13, allowing the use of a third antibody to detect this protein. Cell Lysates were obtained from HEK293T cells transfected with this expression plasmid 48 hours post transfection and evaluated via immunoblot with anti-Slfn13 antibody (Santa Cruz), anti-Slfn13 antibody (Thermofisher), and anti-Flag antibody. There was no detection of Slfn13 expression with any of the antibodies used. However, the anti-Flag antibody recognized other tagged proteins and eGFP. The latter reactivity is hard to explain but it was recurrently verified in

multiple experiments. The Slfn13 plasmid was also stably transfected in A172-KD cells using Lipofectamine. Cell lysates were made and evaluated in the same manner as transiently transfected cells using anti-Flag and anti-Slfn13 antibodies. The results were identical to previous findings. We then had the transcription unit cloned in the expression vector sequenced by the genomic core at UTEP and by the company. No mutations were found.

Expression of endogenous and exogenous mRNA is present. Due to lack of protein expression we chose to assess if the messenger was present. Different cell lines were cultured, and RNA extracted using Trizol reagent and PureLink RNA Mini Kit (Invitrogen/Thermofisher). RT-PCR was done using the RNA from each of the different cell lines. A schematic of the different primers and which isoforms they amplify are provided (Fig. 6-A). A 207bp and 1091bp product was amplified in A172-KD cells indicating the presence of isoform 2 (Fig. 6-B). A 207bp product was amplified in Hela cells also indicating the presence of isoform 2 (Fig. 6-C). 293 cells also revealed the presence of isoform 2 by amplification of a 207bp PCR product (Fig. 6-D). 207bp and 137 bp bands were present in 293T illustrated the presence of isoform 1 and isoform 2 (Fig. 6-E). These results indicated that endogenous Slfn13 mRNA is present in these cell lines but is not translated efficiently.

Furthermore, we decided to probe if exogenous Slfn13 transcript was being produced in stably transfected A172-KD cells. RNA was extracted from these cells and evaluated by RT-PCR using a reverse primer specific to the flag-tag sequence of the Slfn13 and a forward primer that hybridize in Slfn13 (Fig. 6-F). This strategy also identified robust levels of Slfn13 mRNA, excluding a cellular regulation affecting only endogenous mRNA. Therefore, these results demonstrate that defect in expression of Slfn13 protein is at the post-transcriptional level.

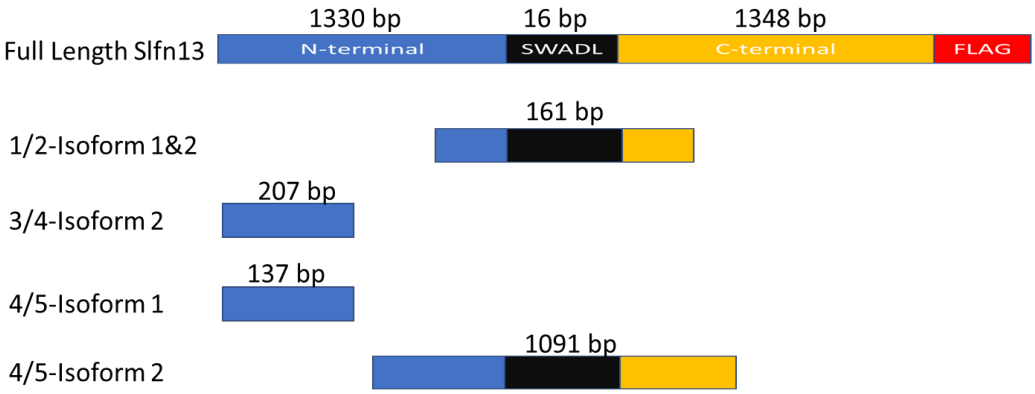

Fig. 6-A Slfn13 RT-PCR Schematic of the primers recognizing different isoforms of Slfn13 used for PCR analysis.

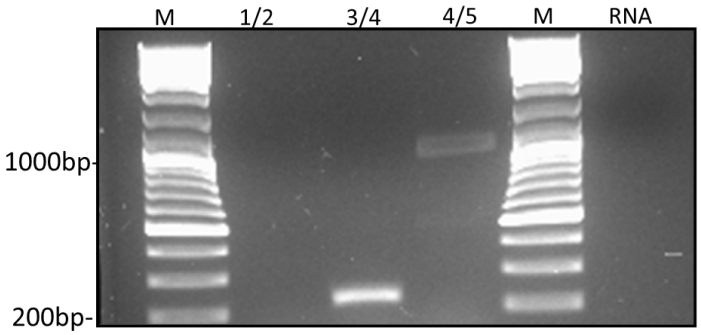

Fig. 6-B A172 kD Endogenous Slfn13 RT-PCR Expression analysis of endogenous Slfn13 in A172-KD cells. RNA was extracted from the cells and evaluated with primers detecting the sequences of different Slfn13 isoforms by RT-PCR.

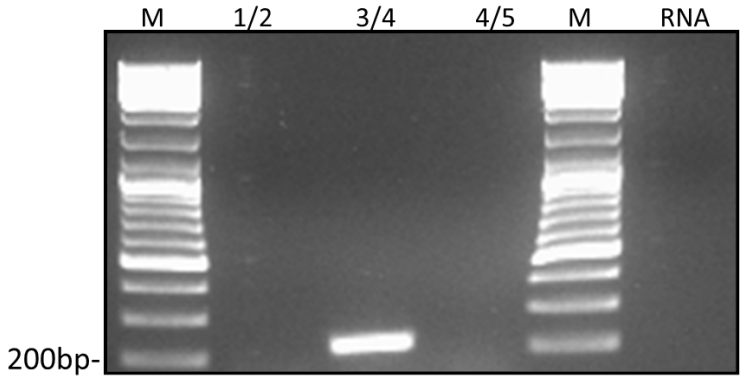

Fig. 6-C Hela Endogenous Slfn13 RT-PCR Expression analysis of endogenous Slfn13 in Hela cells. RNA was extracted from the cells and evaluated with primers detecting the sequences of different Slfn13 isoforms by RT-PCR.

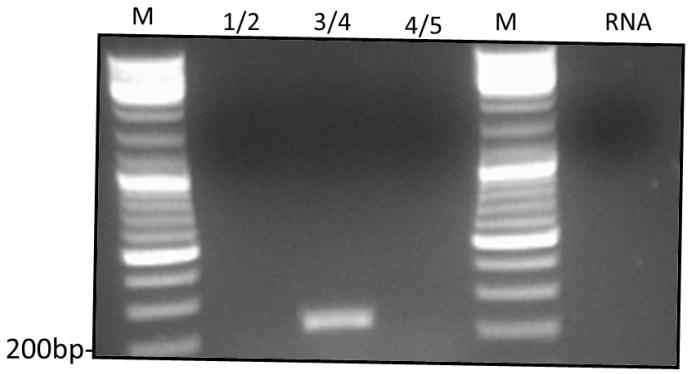

Fig. 6-D 293 Endogenous Slfn13 RT-PCR Expression analysis of endogenous Slfn13 in 293 cells. RNA was extracted from the cells and evaluated with primers detecting the sequences of different Slfn13 isoforms by RT-PCR.

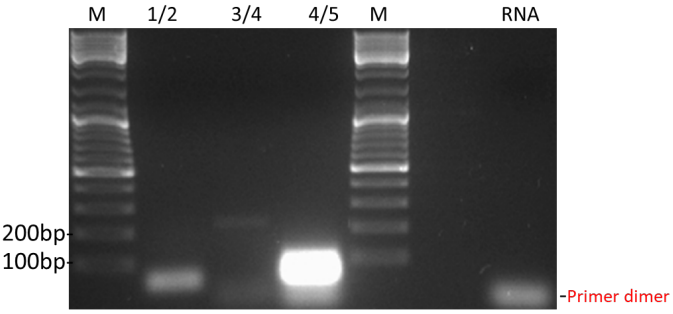

Fig. 6-E 293T Endogenous Slfn13 RT-PCR Expression analysis of endogenous Slfn13 in 293T cells. RNA was extracted from the cells and evaluated with primers detecting the sequences of different Slfn13 isoforms by RT-PCR.

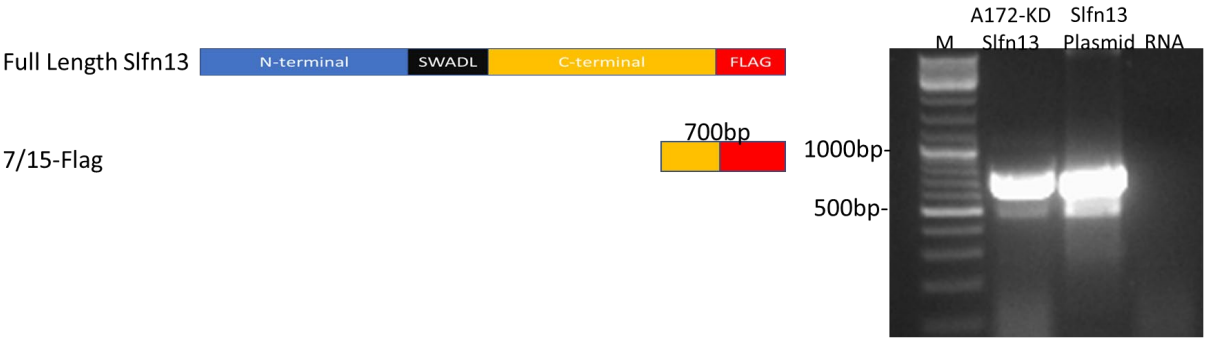

Fig. 6-F A172-KD STABLE SLFN13 RT-PCR Expression analysis of flag-tagged Slfn13 in A172-KD cells. Cells were stably transfected with Slfn13-Flag. Then, RNA was extracted from the cells and evaluated with primers detecting Flag sequence by RT-PCR.

Effect of MG132 on Slfn13 protein expression. To eliminate the possibility that protein expression is regulated at the post-translational level we determined the effect of a proteasome inhibitor on Slfn13 expression. HEK293T cells were transiently transfected with flag tagged Slfn13 plasmid using calcium phosphate and treated with MG132 24 hours later. 48 hours post

transfection the cells lysates were made and evaluated by immunoblot using anti-flag antibody. A plasmid expressing eGFP was co-transfected to evaluate transfection efficiency and to verify the activity of the anti-Flag antibody. DMSO was used as a vehicle control. Results in (Fig. 7) illustrate that we were unable to rescue expression of Slfn13 using MG132.

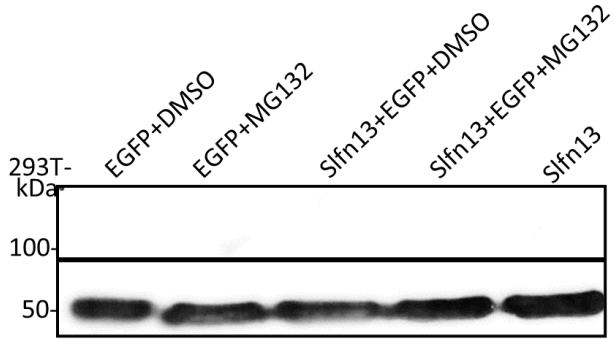

Fig.7 293T MG132 Expression analysis of flag-tagged Slfn13 in HEK 293T cells. Cells were transiently transfected with Slfn13-Flag treated with MG132, a proteasome inhibitor. Then, cell lysates were evaluated with anti Flag antibody by immunoblot. Tubulin was detected as loading control.

Effect of type I interferon on Slfn13 protein expression. We demonstrated that cells that do not express Slfn13 have robust levels of mRNA indicating a post-transcriptional block. We postulated that co-factors required for Slfn13 protein expression could be induced by type I interferon since this cytokine increases the levels of Slfn13 mRNA and the expression of Slfn11 mRNA and protein. To evaluate this hypothesis, we transiently transfected HEK293T cells with a plasmid expressing flag tagged Slfn13 and treated them with IFN alpha the same day and 24 hours later. Cell lysates were obtained 24 and 48 hours after treatment and evaluated by immunoblot with an anti-Flag antibody (Fig. 8). IFN alpha did not rescue the expression of Slfn13, indicating that this cytokine only controls Slfn13 expression at a pre-translational level.

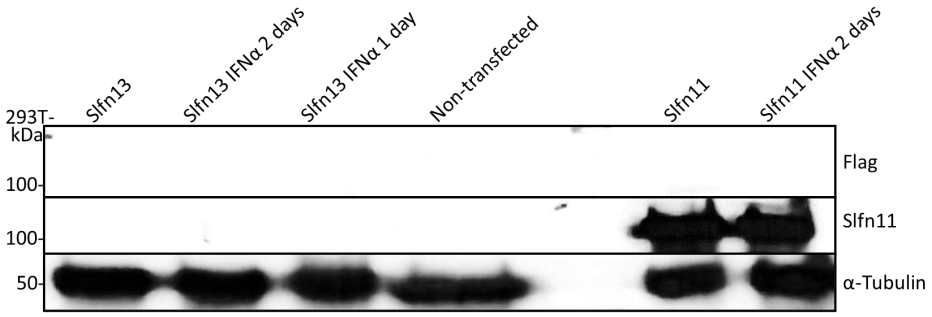

Fig. 8 293T IFN alpha Expression analysis of flag-tagged Slfn13 and Slfn 11 in HEK 293T cells. Cells were transiently transfected with Slfn13-Flag or 11 and treated for 1-2 days with IFN alpha. Then, cell lysates were evaluated with anti Flag or Slfn11 antibodies by immunoblot. Tubulin was detected as loading control.

Effect of replacing the C-terminal of Slfn13 with that of Slfn11 on protein stability. In contrast to Slfn13, Slfn11 is robustly expressed. These proteins are 71% conserved at the amino acid level and have a similar protein architecture. Therefore, we postulate that chimeras between these two proteins will facilitate mapping the instability regions of Slfn13. Previous studies have indicated that the anti-viral activity of Slfn11 resides in the N-terminus of the molecule and similar results were proposed for Slfn13 whereas the C-terminus is dispensable[50,56]. Therefore, we generated a chimera Slfn13/11 containing the Slfn13 in the N-terminus and the C-terminus of Slfn11 in (Fig. 9). Expression of this chimera was evaluated by immunoblot in transiently transfected HEK293T cells. Cell lysates were evaluated with anti-Flag or Slfn11 antibodies by immunoblot (Fig. 9). Tubulin was detected as a loading control. Our results in (Fig. 9) illustrate that the Slfn13/11 Chimera is detectable by anti-Slfn11 C-terminal antibody but not by anti-Slfn11 N-terminal antibody, as expected, indicating that this chimeric protein was readily expressed. Therefore, these results demonstrated that the C-terminus of Slfn13 plays a significant role in the expression of this protein. Furthermore, we stably expressed the chimera in A172-derived cell knockdown for Slfn11 (A172-KD cells) and again verified the expression by immunoblot. A172-KD 13/11 cells allowed us to determine the role of Slfn13 in WNV and HIV-1 infection in aim 2.

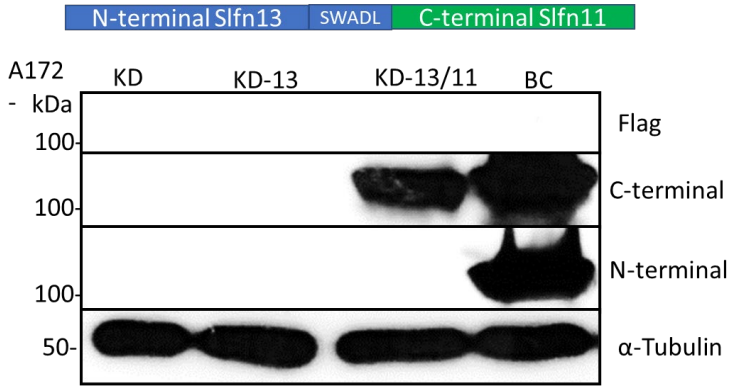

Fig. 9 A172-KD 13/11 Chimera Expression analysis of flag-tagged Slfn13, Slfn13/11, Slfn11 in A172-KD cells. Cells were stably transfected with Slfn13-Flag or transduced with Slfn13/11. Then, cell lysates were evaluated with anti Flag or Slfn11 antibodies by immunoblot. Tubulin was detected as loading control.

Slfn13/11 Chimera cellular distribution. We decided to investigate the localization of the Slfn13/11 Chimera now that we were able to detect the protein via western blot. Slfn11 has been reported to reside in the nucleus; however. no functional NLSs has been formally mapped in this protein and deletion of the N-terminus or the C-terminus move the protein into the cytoplasm suggesting that at least two NLSs located in each half of the protein cooperate in its nuclear import[50]. In contrast, Slfn13 localization has never been defined although images deposited in https://www.proteinatlas.org suggest that in A549 the protein is cytoplasmic whereas in U-2 OS is pancellular. Nevertheless, the expression levels of Slfn13 in these images were very low raising additional doubts of the accuracy of these findings. To determine the subcellular distribution of the chimeric protein, A172 Slfn11 KD cells stably expressing Slfn13/11 Chimera were stained with anti-Slfn 11 C-terminal and DAPI and cells evaluated in a fluorescence confocal microscope (Fig. 10). The chimeric protein was completely localized to the nucleus of the cell suggesting that like Slfn11 this chimera could have NLSs located in the N- and the C-terminal of the protein. *In silico* analysis of the sequence of the chimera and Slfn11 indicated the presence of N-terminal and C-terminal NLSs. In the case of the chimera amino acids 158-178 (Slfn 13 N-terminus) and 831-859 (Slfn11 C-terminus) contain high score bipartite NLSs

whereas that in Slfn11 wild type the N-terminal NLS although differs in primary sequence is localized to the same segment of the protein (amino acids 151-172) and predicted with high score. Importantly in Slfn13 are conserved the same NLSs predicted n the chimera, suggesting that this protein could be nuclear. In summary these findings, demonstrated that fusing the N-terminal of Slfn13 to the C-terminal of Slfn11 results in the localization of the protein in the nucleus.

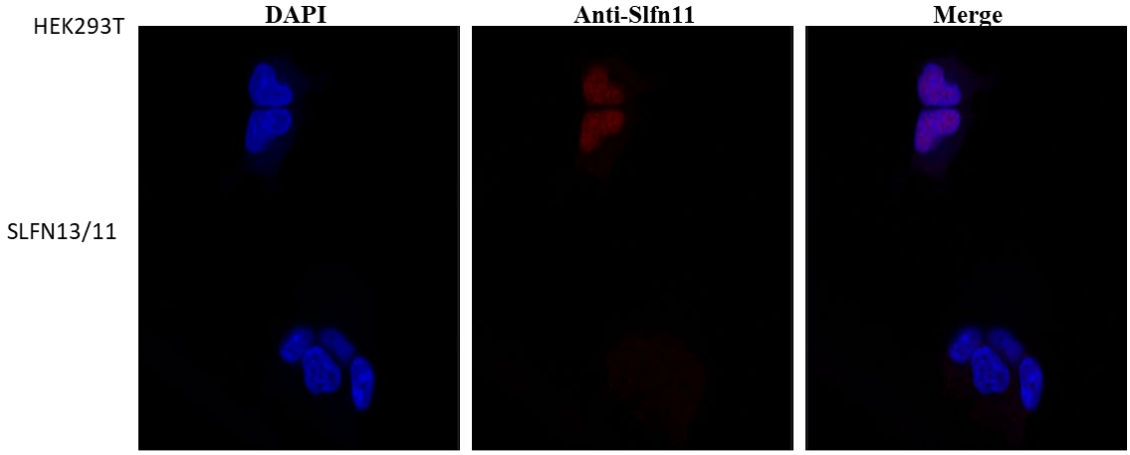

Figure 10. Cellular distribution of Slfn13/11. Nuclear localization. HEK293T cells were fixed/permeabilized and stained with anti-Slfn11 E-4 antibody (red). Cell nuclei were identified with DAPI dye (blue).

Effect of NH_4Cl on Slfn13 protein expression. We also decide to evaluate the role of the lysosome on Slfn13 instability. This is the second most important pathway implicated in protein degradation. Then, HEK293T or A172-KD cells were transiently transfected with Slfn13-flag, Slfn13/11 Chimera, Slfn13CatMut, or Slfn11 plasmid using calcium phosphate and treated with NH_4Cl 24-hours later. 48-hours post transfection the cells lysates were made and evaluated by immunoblot using anti-flag antibody. A plasmid expressing eGFP was co-transfected to evaluate transfection efficiency and to verify the activity of the anti-Flag antibody. Results in (Fig. 14-15) illustrate that we were unable to rescue expression of Slfn13 using NH_4Cl. Furthermore, the Slfn13 catalytic mutant was not detected in either cell line (Fig. 11-12). Once again, the C-

terminus Slfn11 antibody demonstrates the expression of both Slfn11 and Slfn13/11 Chimera. However, the N-terminus Slfn11 antibody is only able to detect Slfn11. The ability to express Slfn13/11 Chimera illustrates that the endo-nucleolytic activity of Slfn13 plays minimal to no role in its expression.

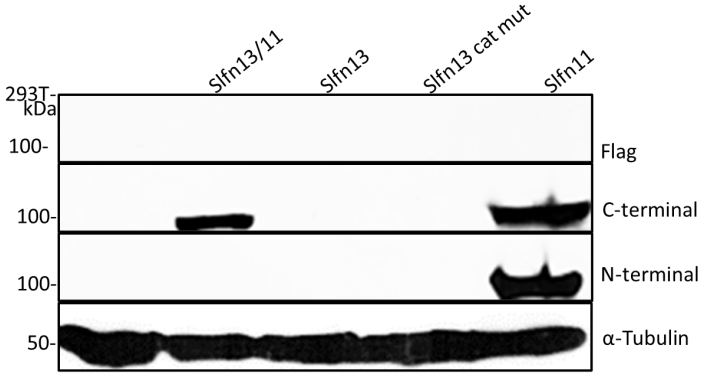

Fig.11 293T NH_4Cl Expression analysis of flag-tagged Slfn13 in HEK 293T cells. Cells were transiently transfected with different Slfn13 constructs or Slfn11 and treated with NH_4Cl, a lysosome inhibitor. Then, cell lysates were evaluated with anti Flag or Slfn11 antibodies by immunoblot. Tubulin was detected as loading control.

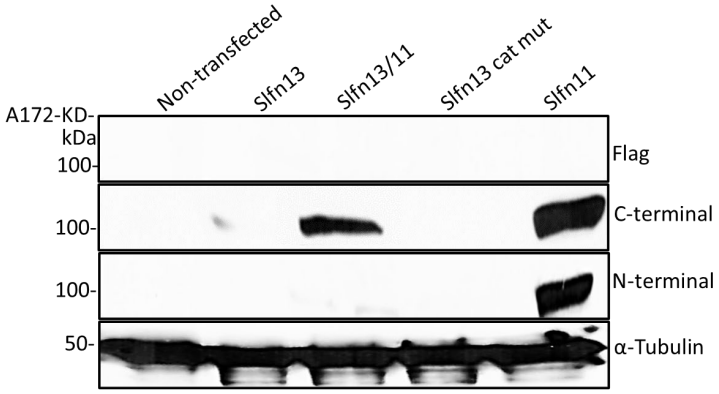

Fig.12 A172-KD NH_4Cl Expression analysis of flag-tagged Slfn13 in A172-KD cells. Cells were transiently transfected with different Slfn13 constructs or Slfn11 and treated with NH_4Cl, a lysosome inhibitor. Then, cell lysates were evaluated with anti Flag or Slfn11 antibodies by immunoblot. Tubulin was detected as loading control.

Effect of increasing Slfn13 sequence on the stability of the Slfn13/11 Chimera. The Slfn13/11 Chimera 2 was constructed by replacing 174 amino acids of Slfn11 in the C-terminal region with the corresponding 174 amino acid from Slfn13 C-terminus. When this region is swapped, Slfn13/11 Chimera 2 fails to be detected by Anti-Slfn13 that successfully detect Chimera 1 (Fig. 13). α-tubulin was detected as a loading control. These findings demonstrated

that this region of 174 amino acids of Slfn13 contains a protein instability region. Moreover, comparison of Slfn13 with Slfn11 in this region identifies 37 residues which are not conserved.

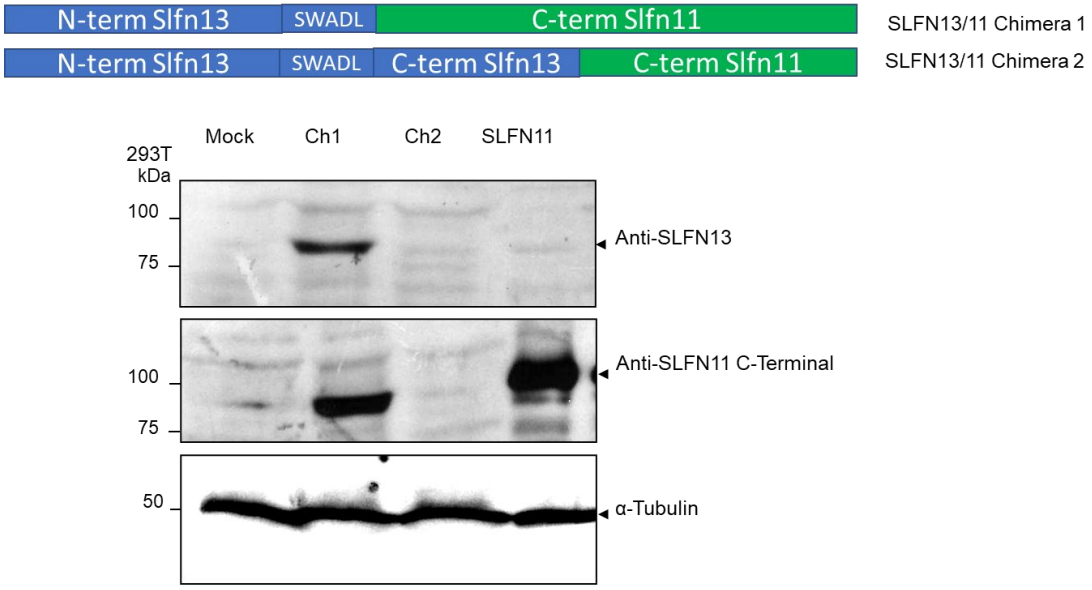

Fig. 13 293T 13/11 Chimera and 13/11 Chimera 2 Expression analysis of Slfn13/11 Chimera, Slfn13/11 Chimera 2, and Slfn11 in 293T cells. Cells were transiently transfected and cell lysates were evaluated with anti-Slfn13 or Slfn11 antibodies by immunoblot. Tubulin was detected as loading control.

Specific aim 2. Determine role of Slfn13 on HIV-1 and flavivirus replication.

Slfn13 does not appear to be active against HIV-1. The inability to detect the expression of Slfn13 leads to speculation of the absence or presence of the protein. Innately, any results gathered related to HIV-1 anti-viral activity would be dubious at best. The endoribonucleolytic activity Slfn13 is located on the N-terminus of the protein. The Slfn13/11 chimera was a suitable candidate to test our hypothesis regarding Slfn13 antiviral activity because it can be detected and still contains the N-terminus of Slfn13. A172 derived cell lines were infected with HIV-1 made of Hluc and VSV-G as previously described. 48 hours post infection the supernatant was collected. ELISA was used to measure and quantify p24 to look at the difference in infectivity between these cell lines. Statistical Analysis of the results (Fig. 14) shows there is no significant difference between A172-Cat Mut and A172-KD or A172-SCR and A172-BC. There are

significant differences when comparing the other A172 derived cell lines to each other **p<0.01. However, the results (Fig. 14) indicate that A172-KD Slfn13/11 Chimera cell line produced more p24 than both the A172-SCR and A172-BC lines. Both of which are expressing Slfn11 that is known to have anti-viral activity against HIV-1.

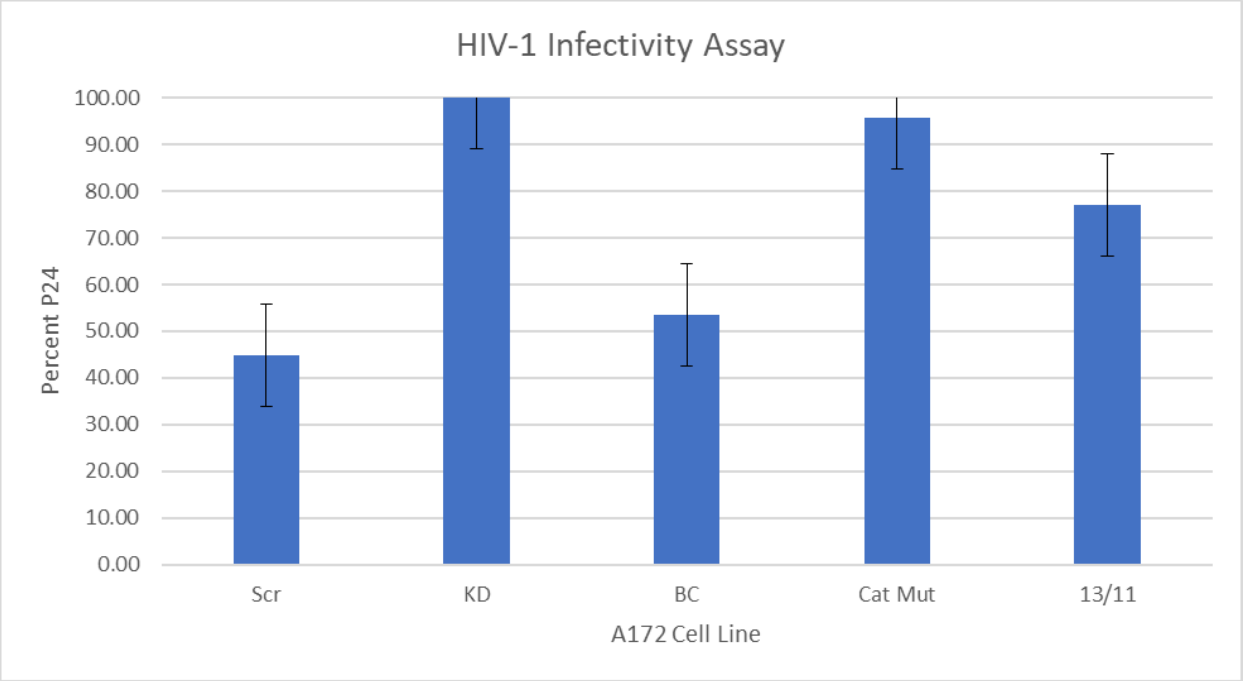

Fig.14 HIV-1 p24 Analysis Percent p24 of A172 derived cell lines infected with HIV-1. Supernatant was collected from each cell line and p24 was measured using ELISA to quantify HIV-1 infectivity.

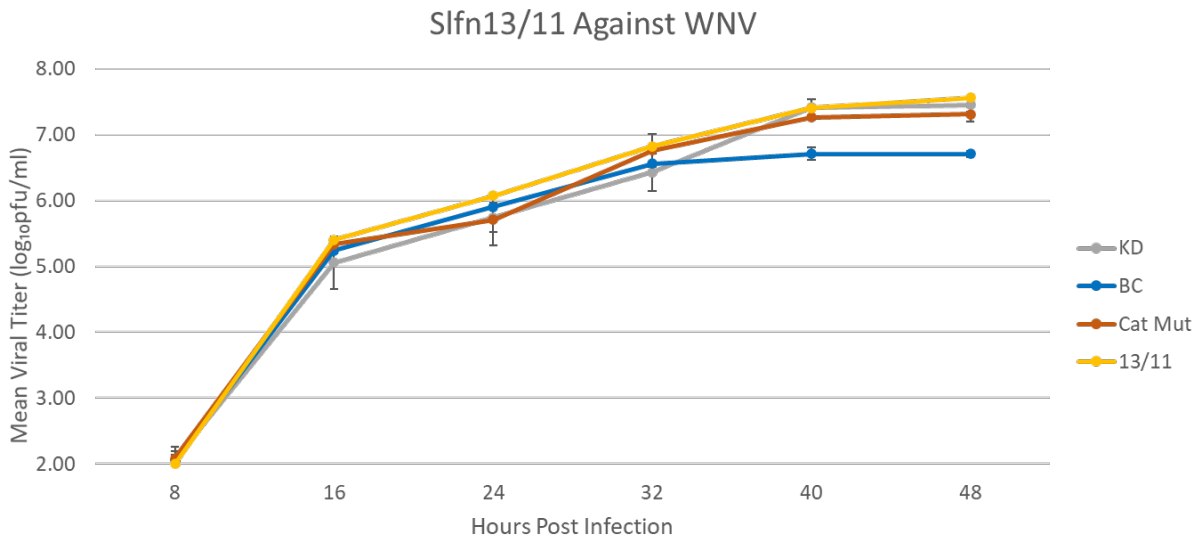

Fig.15 WNV Viral Titer Analysis Mean viral titer of A172 derived cell lines infected with WNV. Plaque formation on each cell line was quantified and viral titers were expressed as plaque-forming units per milliliter (PFU/ml). These experiments were conducted in triplicate.

Slfn13 WNV Infection Results. Monolayers of LLC-MK2 cells were inoculated with supernatants collected from A172 derived cell lines were infected with WNV to ascertain the impact of Slfn13/11 Chimera on WNV replication. Statistical analysis shows there is no significant difference in the mean viral titer between different A172 derived cell lines, **p value<0.01. The results were reminiscent of the results involving HIV-1 infection (Fig. 15). The consensus is that Slfn13/11 Chimera is not active against WNV.

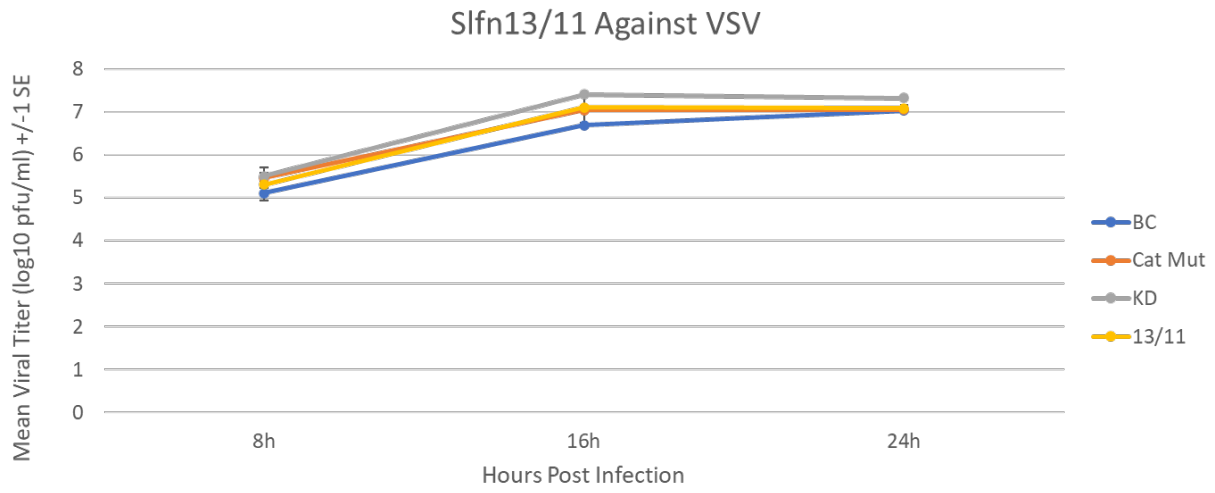

Fig.16 VSV Viral Titer Analysis Mean viral titer of A172 derived cell lines infected with VSV. Plaque formation on each cell line was quantified and viral titers were expressed as plaque-forming units per milliliter (PFU/ml). These experiments were conducted in triplicate.

Slfn13 does not appear to be active against VSV. To Assess whether Slfn13 has anti-viral activity against ssRNA(-) viruses we decided to investigate the effect the Slfn13/11 Chimera has on VSV. A172 derived cell lines were infected with VSV and the supernatant collected in 8-hour intervals. Serial dilutions of the viral supernatant for each time point were used to inoculate LLC-MK2 cells. Plaque formation on each cell line was quantified and viral titers were expressed as plaque-forming units per milliliter (Fig. 16). Statistical analysis shows there is no significant difference in the mean viral titer between different A172 derived cell lines, **p value<0.01. These results demonstrate the same trend we saw regarding the HIV-1 infectivity. The mean viral titer obtained from the A172-KD Slfn13/11 Chimera cell line at all time points are the same as the A172-KD Cat Mut but lower than the A172-KD which lack Slfn11. The A172-BC which expresses Slfn11 has the lowest mean viral titer at all time points.

CONCLUSIONS

Specific aim 1. Determining mechanisms of regulation of Slfn13 protein expression. To conclude, expression of Slfn13 is not detectable by western blot with neither anti-Slfn13 antibody nor anti-flag. The transcripts are proven to exist by means of RT-PCR meaning regulation of Slfn13 is post transcriptional. Furthermore, in the presence of a proteasome inhibitor MG132 Slfn13 is still undetectable. This proves that the protein is not being degraded through proteasome pathway and suggest that regulation of Slfn13 is through some other means. The Slfn13/Slfn11 Chimera was able to be detect by western blot using an antibody against Slfn11 C-terminal and Anti-Slfn13 but not detectable using an antibody against Slfn11 N-terminal. This result solidifies the correct architecture of the chimera. Based on preliminary confocal microscopy results we determined that the Slfn13/11 Chimera is localized in the nucleus as is the case with Slfn11. The results of the lysosome proteolytic pathway inhibition using NH_4Cl suggest that this pathway is not involved in the regulation of this protein. It also suggests that mutating the catalytic domains of Slfn13 has no effect on expression. However, with Slfn13/Slfn11 chimera 2 where we extend the region of the C-terminal in the initial chimera that is exclusive to Slfn13 we lost expression of the protein. This construct reduced the sequence that is exclusive to Slfn11 while increasing the sequence that is exclusive to Slfn13. This suggest that this region of the C-terminal plays a significant role in expression.

Future Directions. Now that we have determined which region of Slfn13 that we believe is responsible for lack of protein expression we can produce other constructs to reaffirm this finding. The first of which is Slfn13-NLS where we introduce a nuclear localization sequence in order to illustrate the effects of localization on protein expression. This nuclear localization sequence would be placed at the C-terminus end of the sequence prior to the flag-tag. Two other

construct we would like to produce are Slfn13-N and Slfn11/13 which are tagged versions of the of the N-terminus of Slfn13 and a Chimera of composing the N-terminal of Slfn11and C-terminal of Slfn13 respectively. We would also like to create constructs where this sequence is added to proteins that are well expressed and detected to investigate its effect on protein degradation/half-life. Furthermore, we would like to explore the use of this sequence as a biotechnology tool where it's application can be used to knockdown or knockout protein expression.

Specific aim 2. Determine if Slfn13 possesses anti-viral activity against HIV-1 and flavivirus replication. Our findings illustrate that the anti-viral activity of Slfn13 against HIV-1 is minimal if present at all. The ELISA quantifying p24 in A172 derived cells which were infected show that the controls behave as expected. The A172-BC which over express Slfn11 show the least amount of p24 while the A172-KD which lacks Slfn11 show the most amount of p24. The Slfn13/11 Chimera was used in lieu of Slfn13 due to our ability to detect the presence of the protein. Furthermore, this trend was also seen when A172 derived cell lines were infected with WNV and VSV which is a ssRNA(-) virus. Our overall perspective is that Slfn13 has no anti-viral activity.

Future Directions. Our plan is to use the new constructs previously described that we are producing to further investigate any potential anti-viral activity that Slfn13 may possess. We will use the new constructs to produce cell lines in cell types that are relevant for the in vivo infection of these viruses. Regarding HIV-1, this will be a panel of human CD4+ T cell lines. With respect to *Flaviviruses*, we will once again choose to characterize A172 cells.

DISCUSSION

The initial focus on Slfn13 was to investigate its potential anti-viral activity[56]. Slfn proteins are not very well characterized but there were some studies that pointing to specific members of this family demonstrating anti-viral capabilities[39-41,56]. Furthermore, our lab was successful in proving that Slfn11 was active against HIV-1 and flaviviruses[50]. The over 65% sequence identity between Slfn13 and Slfn11 was enough motivation to interrogate Slfn13 for Slfn11-like anti-viral functions. We were not anticipating the expression problems of Slfn13 that precluded its analysis. These problems were not observed in Slfn11 or the other mouse members of the group III of this family Therefore, we were forced first to understand and solve the stability problems to be able to study the anti-viral activity of the protein. Our findings give some clue as to how this protein is regulated but there are still some areas unaccounted for. Our future works are focused on protein degradation, a post translational method of protein regulation. We are also interested in determining whether subcellular localization is an aspect to consider regarding the regulation of the expression of Slfn13.

Regulation of Slfn13 at the transcriptional level. We were able to show that lack of protein expression was not due to lack of transcription of the endogenous gene or the plasmid expressing this protein in several cell lines. Not only was endogenous mRNA present in cells but also exogenous mRNA from a transfected plasmid. Furthermore, we sequenced the entire transcription unit of the Slfn13 plasmid purchased finding no mutations in agreement with the manufacturer. Therefore, if the mRNA is present and the protein is absent the protein is either not being translated or is being degraded, indicating that the regulation of Slfn13 expression was at a post-transcriptional level. *Cis* acting sequences located at the 3' untranslated region of mRNAs transcribed from endogenous genes are central in the translational regulation of gene

expression. However, these sequences are expected not to be present in the cDNA cloned in the plasmid that also failed to express this protein. Then, we reasoned that it was more likely that Slfn13 was being degraded early during translation and decided to evaluate first the potential role of protein degradation.

Post-translational regulation of Slfn13 expression. Intracellular proteins are degraded mainly by the ubiquitin proteasome and the lysosomal pathways, being the first one the most common[78]. In the ubiquitin proteasome pathway, ubiquitin is attached to a lysine residue of the protein marking it for degradation by the proteasome[78]. The other pathway, lysosomal proteolysis; occurs to a lesser extent and is achieved by lysosomes digesting proteins that are typically cytoplasmic with longer half-lives[78]. We tried to inhibit both pathways, hoping that we could rescue protein expression, but these attempts were unsuccessful.

Expression of Slfn13/11 Chimera. We knew that Slfn11 is detectable by several antibodies and the expression levels are significant. Furthermore, the amino acid sequence and protein domain conservation suggest that chimeric molecules between these two Slfns will allow evaluating large portions of the protein for a role in expression without significantly altering their architecture. Therefore, we established a chimera by fusing the N-terminus of Slfn13 to the C-terminus of Slfn11. This chimera was readily expressed and detectable with antibodies against the C-terminal of Slfn11 and the middle regions of Slfn13. Furthermore, we were able to discover that Slfn13/11 Chimera is localized in the nucleus. Initially we concluded that nuclear localization inhibits the catalytic activity of Slfn13 as an endoribonuclease thus allowing Slfn13/11 Chimera to be expressed. However, producing the Slfn13 tRNase catalytic mutant did not rescue expression. Therefore, these findings indicate that the C-terminus of Slfn13 is responsible for the lack of expression of this protein which is independent of its catalytic activity.

Slfn13/11 Chimera 2 loss of expression. To further map the region implicated in the lack of expression of Slfn13, we generated Slfn13/11 Chimera 2 that reduces the amount of Slfn11 sequence present in the C-terminal of Slfn13/11 Chimera there by increasing the sequence of Slfn13 present. Importantly, this protein was not expressed as determined by immunoblotting with an anti-Slfn13 antibody that recognized the Slfn13/11 Chimera. Analysis of the predicted nuclear localization signals (NLSs) indicate that these chimeras share the same NLSs suggesting that is unlikely that they have a different subcellular distribution. Therefore, likely, amino acids 652-826 are responsible for the lack of expression of Slfn13.

Chaperone mediated autophagy. Lysosome proteolysis can occur in two manners[78]. Autophagy is the method mostly seen where vesicles containing proteins or even organelles form an autophagosome fuses with the lysosome to facilitate protein digestion[79,80]. This manner is non-discriminative[79,80]. The other method involves recognition of the consensus sequence KFERQ[79,80]. This sequence targets proteins to be chaperoned by a member of Hsp70 proteins, Heat Shock Cognate or Hsc70[79,80]. This manner of lysosome degradation is referred to as chaperone-mediated autophagy (CMA). Prediction of CMA sequences in Slfn13 amino acids 652-826 identified two potential targets that are absent in Slfn11 and all the other murine Slfns belonging to the group III of this family that can be expressed very efficiently. However, this mechanism is not supported by the results of our experiments of lysosomal inhibition by alkalinization. An explanation could be that the concentration used was not high enough[79,80]. Also, inhibiting lysosomal degradation via chaperone mediated autophagy increases levels of proteins degradation by other autophagic pathways and by endocytosis[79,80]. Evaluation of other methods of inhibition of CMA including removing the motifs from Slfn13[79,80] should be conducted. The future of the Slfn13 project looks promising.

Our data also demonstrated that the N-terminus of Slfn13 does not have the anti-viral activities evidenced in Slfn11. The N-terminal region of Slfn13 and 11 was shown to be necessary and sufficient for their antiviral activities[50,56]. Therefore, analysis of the anti-viral activity of the 13/11 Chimera, which can be efficiently expressed, was possible. In contradiction with the proposed anti-HIV-1 activity we did not observe any effect on this virus with this chimera[56]. A possible explanation is that the reported anti-HIV-1 activity was evidenced in an experimental system that does not involve viral infection. These authors transiently co-transfected uninfected HEK 293T with the plasmids encoding Slfn13 and HIV-1; whereas we infected with HIV-1 cells stably expressing the 13/11 chimera[56]. It has been previously demonstrated that Slfn proteins do not specifically inhibit protein synthesis in the co-transfection model[81,82]. We also found that the chimera did not affect WNV replication in agreement with a lack of activity on the related *Flavivirus* Zika virus[56]. Therefore, our findings indicate that Slfn11 and 13 lack overlapping functions.

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VITA

My name is Jordan Winfield and was born in St. Catherine, Jamaica. I have served in the United States Army from March 2013-Present. I am currently enrolled in a Master of Science program in Biological Sciences at the University of Texas of El Paso. I began the program in August of 2018 and completed my thesis titled "Characterization of the anti-viral activity of Schlafen13" in May 2020.

During my time as a master's student, I was as a Teaching Assistant teaching for several different biology labs including Microbiology, Microbial Physiology, and General Biology. My duties included preparing lab, giving weekly lectures and grading the students. Throughout my master's I was also a Graduate Research Assistant, here my focus was assisting in laboratory experiments to investigate a family of proteins that have potential antiviral activity against HIV and Flaviviruses. During this time, I obtained several different skills involving molecular biology techniques. These include but are not limited to RNA and DNA extraction, tissue and cell culture, ELISA, molecular cloning, immunoblotting, polymerase chain reaction assays, and many more.

My previous degree is a Bachelor of Science in Cellular and Molecular Biochemistry from the University of Texas at El Paso in December 2017. I have recently been admitted the Doctor of Pharmacy program at the University of Texas at El Paso where I plan to earn my PharmD and continue research in virology.