CHARACTERIZING THE ROLE OF THE CELLULAR PROTEIN SCHLAFEN 11 IN FLAVIVIRUS REPLICATION

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Dedication

Este trabajo es dedicado especialmente a mi familia. Su amor, palabras de aliento y apoyo han hecho posible este logro. Especialmente a mi madre, por ser el pilar más importante de mi vida y por siempre brindarme tu amor incondicional. A mi padre, por el modelo a seguir que eres, por lo fuerte y estoico, y por siempre hacerme sentir lo orgulloso que estás de mí. A mi hermana, que siempre ha estado junto a mi y cuyos consejos me han ayudado a afrontar los retos que se me han presentado a lo largo de mi vida. Finalmente a una de mis mayores alegrías en la vida, mi sobrina, Regina, que seas intrépida, valiente y sueñes grande.

(This work is especially dedicated to my family. Their love, words of encouragement and support have made this goal possible. Especially to my mother, for being the most important pillar of my life and for always giving me her unconditional love. To my father, for being the role model that he is, for being so strong and stoic and for always making me feel the pride that he has for me. To my sister, who has always been with me and whose advice has helped me face the challenges in life. Finally, to one of the greatest joys in life, my niece Regina, may you be bold, brave and may you dream big.)

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Abstract

Schlafen 11 (Slfn11) is a ubiquitously expressed interferon stimulating gene (ISG) that controls synthesis of proteins by regulating tRNA abundance. Through this mechanism Slfn11 has previously been shown to impair human immunodeficiency virus 1 (HIV-1) infection and the expression of codon-biased open reading frames. Because replication of positive-sense single-stranded RNA [(+)ssRNA viruses] requires the immediate translation of the incoming viral genome whereas negative sense, single stranded [(-)ssRNA] viruses carry at infection an RNA replicase that makes multiple translation competent copies of the incoming viral genome, we reasoned that (+)ssRNA viruses will be more sensitive to the effect of Slfn11 on protein synthesis than (-)ssRNA viruses. To evaluate this hypothesis, we tested the effects of Slfn11 on the replication of a panel of ssRNA viruses in the human glioblastoma cell line A172, which naturally expresses Slfn11. Depletion of Slfn11 in this cell line significantly increased the replication of (+)ssRNA viruses from the *Flavivirus* family, including West Nile (WNV), dengue (DENV), and Zika virus (ZIKV) but had no significant effect on the replication of the (-)ssRNA viruses vesicular stomatitis (VSV, *Rhabdoviridae* family) and Rift Valley fever (RVFV, *Phenuiviridae* family). Mutagenesis analysis indicated that the N-terminus of Slfn11 was necessary and sufficient to restrict WNV replication. To test the mechanism of action of Slfn11, we evaluated the effect of WNV infection on the tRNA repertoire of cells expressing or not this protein using tRNA PCR array. WNV infection down-regulated a subset of host tRNAs and this modification was opposed by Slfn11. Viral protein expression analysis suggests that the changes induced by WNV on the tRNA pool led to a decrease in viral protein translation efficiency which was accompanied by production of higher viral titers. In light of these data, we propose that WNV-induced tRNA changes enhanced co-translational protein folding. Intriguingly, overexpression of Slfn11 in HEK

293T, HeLa, and BHK-21 cells, all of which naturally lack the expression of this protein, did not impair infection by WNV or HIV-1, suggesting that other cellular protein(s) absent in these cells may be required for the antiviral activity of Slfn11. In summary, we have identified Slfn11 as an important restriction factor on the replication of flaviviruses.

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Chapter 1: Introduction

1.1 Overarching Hypothesis

Our laboratory is interested in discovering broad spectrum immune mechanisms that target viral families. These mechanisms are based on the recognition of molecular patterns or processes shared among evolutionarily distant viral families. We hypothesized that some viruses will be more sensitive to the efficiency of protein translation than the host. Therefore, mechanisms regulating protein translation efficiency will contribute to the regulation of viral replication. We consider that viruses that use a codon biased genome (i.e. lentiviruses) and the positive-sense single-stranded RNA [(+)ssRNA] viruses will exhibit a greater dependence on protein translation than the host. tRNA abundance is dictated by host codon usage, therefore, upon infection viruses with a codon biased genome will encounter a deficiency in tRNAs required for viral protein expression. In the case of (+)ssRNA viruses, the particular requirement for efficient protein translation could be determined by their strategy of replication. These viruses multiply their genome using a virally-encoded RNA-dependent-RNA polymerase that is synthesized using the incoming viral genome as mRNA. Production of this RNA polymerase triggers massive viral RNA replication providing multiple translation competent copies of the viral genome, overcoming the initial bottleneck. Therefore, the requirement of (+)ssRNA viruses to translate the incoming viral genome suggests that replication of these viruses will be particularly sensitive to the efficiency of protein translation. In support of this view, during my dissertation work it was reported that multiple ISGs controlling translation efficiency affect the replication of (+)ssRNA more than (-)ssRNA viruses, which do not require immediate translation of the incoming viral genome[1]. Flaviviruses were used as a model of (+)ssRNA viruses in order to test this overarching hypothesis of our work, with a special focus on WNV. Because of the previously established effect of Slfn11

on HIV-1 protein translation, this cellular protein was also used as a model for all of the work described below.

1.2 General Characteristics of West Nile Virus

WNV is an emerging neurotropic flavivirus transmitted primarily by the *Culex* mosquito genus. Originally identified in 1937 in the West Nile district of Uganda, it is classified as an arthropod-borne virus (arbovirus) belonging to the Japanese encephalitis serocomplex of the *Flaviviridae* family[2]. Birds are the main reservoirs for WNV[3]. The primary vector for WNV in the United States is the *Culex* mosquito species that commonly breeds in urban areas and prefers to feed on birds[4]. However at least 43 mosquito species in the U.S., including *Aedes*, *Anopheles*, and *Psorophora* mosquito species, have tested positive for WNV[5]. The mosquito is responsible for spreading the virus to humans and horses. However, since viral titers in these hosts are not sufficiently high enough for a mosquito to obtain virus in a blood meal, they are considered incidental, dead-end hosts as they do not contribute to the transmission cycle of the virus. Transmission of WNV has also been reported after blood transfusions and organ transplantation[6]. Because of this, in 2003 blood banks began to screen for the presence of WNV in donors' blood, which also allowed for better analysis of WNV activity in communities[2].

WNV clinical manifestations can range from an asymptomatic infection (70% to 80% of all infections) to a very broad spectrum of clinical illnesses such as fever, encephalitis, meningitis, meningoencephalitis, acute flaccid paralysis, an enlarged liver, splenomegaly, mild non-exudative pharyngitis and myocarditis[7]. The incubation period for the virus can range from 2-14 days[8]. IgM antibody detection to WNV in serum or cerebrospinal fluid is the most common diagnostic method[4]. The symptoms of the more severe form of the disease (encephalitis) are: headache, high fever, vomiting, photophobia, stiff neck, drowsiness, disorientation and muscle weakness

progressing to convulsions, coma and paralysis. Neuroinvasive manifestations can last several weeks and the neurological consequences may be permanent. Old age is the greatest risk factor for severe neurologic disease, disease complications or even death. The most effective form of prevention relies on comprehensive mosquito-control programs and methods to avoid mosquito bites, including the use of mosquito repellents[7].

1.3 WNV Virion Structure

WNV belongs to the genus *Flavivirus* in the *Flaviviridae* family of viruses, which consists of more than 70 members including DENV, Japanese encephalitis virus (JEV), yellow fever virus (YFV) and ZIKV. Although Flaviviruses share similar genome organization and replication mechanisms, they are able to cause a wide range of severe disease manifestations in humans including neurological disease such as meningitis or encephalitis (e.g. WNV and JEV) or vascular leak and haemorrhage (e.g. DENV and YFV)[9,10].

WNV virions are small, spherical, enveloped particles of approximately 50 nm in size[11]. The viral envelope is a host-derived membrane in which the viral membrane (M) and envelope (E) proteins are anchored. These proteins have C-terminal regions that are hydrophobic and span the membrane twice[12]. The viral capsid is approximately 30 nm in diameter and consists of C protein dimers, with the RNA binding domains located at the C- and N- termini separated by a hydrophobic region[11]. During virion maturation, the N-terminal region of the precursor M (prM) protein is cleaved by a furin-like protease inside the trans-Golgi network of the infected cell. This cleavage results in a conformational change in the associated E protein trimers that leads to the formation of E dimers that interact to form the icosahedral protein shell on the outer side of the virion envelope[13]. (Figure 1.1)

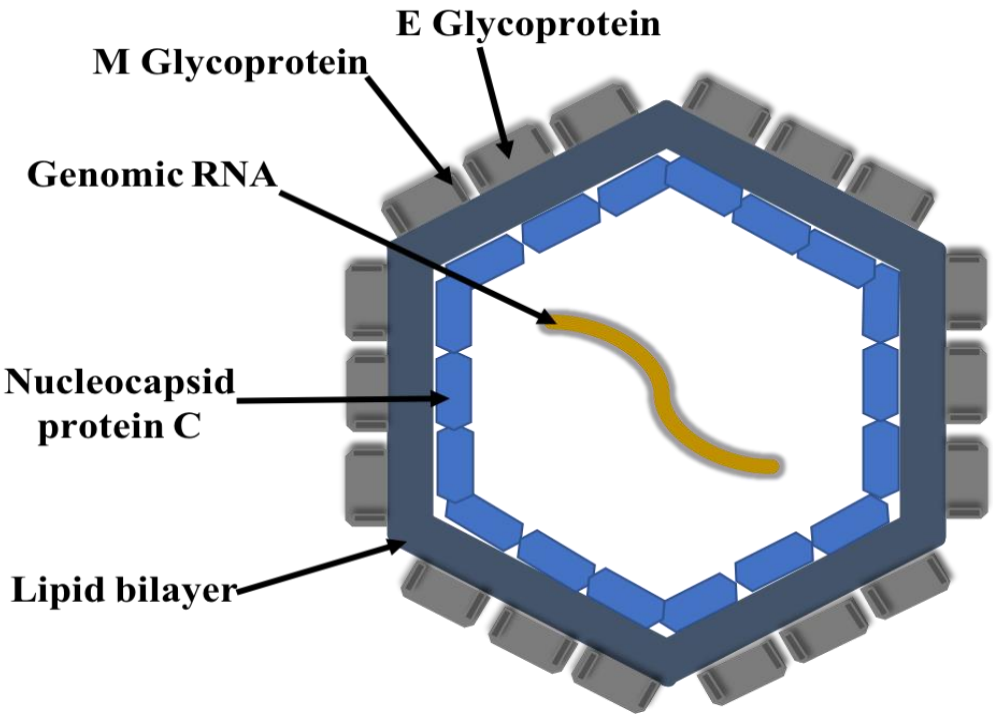

Figure 1.1 WNV virion structure.

1.4 Genome Organization

The virus contains a single-stranded, positive-sense RNA genome of ~11kb in length. The genome serves as a messenger RNA (mRNA) and also as a template for the synthesis of complementary minus-strand RNA. The non-coding region (NCR) at the 5' end of the WNV genome is ~96nts in length and contains a Type 1 cap, while the length of the 3' NCR varies from 337 to 649nts and does not contain a poly A tail[14]. The genomic RNA encodes a polyprotein that is co- and post-translationally processed by viral and host proteases to produce three structural proteins (E, M and C), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5)[13]. (Figure 1.2)

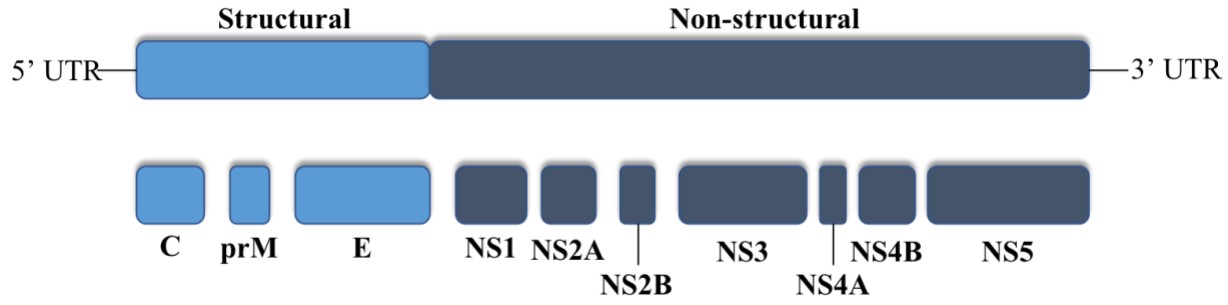

Figure 1.2 WNV genome organization.

1.5 Viral Structural Proteins

The three structural proteins, E, prM/M, and C, are encoded within the 5' end of the viral open reading frame (ORF) of the viral genome[15]. The precursor C protein contains a C-terminal hydrophobic domain that spans the ER membrane and targets prM for translocation to the ER prior to cleavage. Precursor C is cleaved at its C- terminus by the viral protease on the cytoplasmic side of the ER membrane to generate the mature C protein. The mature C protein folds into a dimer that is composed of 2 monomers each containing 4 α-helices. Internal hydrophobic regions of C protein mediate association with the ER membrane while nonspecific interactions between the charged residues at the N- and C-termini of mature C protein and the viral genomic RNA are thought to facilitate assembly of the nucleocapsid around the genomic RNA[16,13].

The M protein (~8 kDa) is a proteolytic product of its precursor glycoprotein, prM (~26 kDa). The C-terminal region of prM contains two transmembrane domains that anchor prM and M in the ER membrane, and may aid in the heterodimerization of prM and E. The prM protein maintains surface E proteins in a raised trimeric confirmation, blocking premature viral fusion with

the cellular membranes during virion trafficking through acidic compartments of the *trans*-Golgi secretory pathway[12,17].

The major viral surface protein is the E protein, which mediates viral attachment, membrane fusion as well as virion assembly[18]. In mature virions, E protein homodimers are arranged in a head-to-tail conformation and lie parallel to the lipid bilayer[19]. The E protein contains the major antigenic determinants on the surface of flavivirus particles[13].

1.6 Viral Non-Structural Proteins

The NS1 protein (~47 kDa) has been shown to exist as a monomer, homodimer and a hexamer, and is the only flaviviral nonstructural protein known to be glycosylated[13,20,21]. NS1 has been reported to play a role in viral replication, and the hexameric form is secreted from infected cells and has been reported to inhibit complement activation during WNV infection[15,22,23]. The eight C-terminal residues of NS1 and the ~140 N-terminal residues of NS2A are required for the cleavage of NS1 from NS2A by an unknown host protease[24].

The NS2A and NS2B proteins are small (23 kDa and 13 kDa, respectively) hydrophobic proteins. Mutations that prevent cleavage at the NS1/NS2A junction have proven lethal for the virus without affecting viral RNA replication, suggesting a role for NS2A in virion assembly[25,26]. In addition, mutational analysis of WNV and Kunjin NS2A demonstrated that this protein mediates attenuation of type I interferon (IFN) signaling[26,27]. NS2B is a membrane-associated protein that is required as a cofactor for the activity of the serine protease of NS3[28,29].

The NS3 protein is a large (~70 kDa) protein that is highly conserved among flaviviruses. The N-terminal region comprises the NS3 protease domain that forms an active complex with NS2B. NS3 cleaves itself from the viral polyprotein and then cleaves at multiple other sites within the polyprotein[30]. RNA-stimulated NTPase and RNA helicase activities have been mapped to C-

terminal regions of the NS3 protein. Additionally, the NS3 mediates de-phosphorylation of the 5' end of the viral genomic RNA prior to cap addition. Collectively, the data establishes the requirement of NS3 in viral RNA replication[31–33].

NS4A and NS4B are small hydrophobic proteins (16 and 27 kDa, respectively) that localize to the ER membrane. Neither of these two proteins share sequence homology with any known enzymes. The C-terminus of NS4A is cleaved by both host signal peptidase and viral serine protease[24]. NS4A has been shown to interact with NS1 and co-localizes with replication complexes within vesicle pockets, supporting a role for NS4A/NS1 in RNA replication[34,35]. Overexpression of DENV NS4A induced membrane rearrangements similar to those observed in infected cells[36,37]. Kunjin virus NS4B can induce membrane proliferation and rearrangement and perinuclear protein accumulation when overexpressed[38]. NS4A and NS4B play a role in attenuation of the IFN antiviral response to WNV infection and NS4B was shown to inhibit STAT phosphorylation and subsequent signaling[39,40]. NS2A, NS2B, NS3, NS4A and NS4B form ER membrane-associated complexes in infected cells[13].

NS5 is a large (96 kDa) multifunctional protein that is the most conserved flavivirus protein. The C-terminal region of the NS5 protein contains the viral RNA-dependent RNA polymerase (RdRp)[41]. NS5 also has an N-terminal methyltransferase domain that methylates the cap structure of flaviviral genomic RNAs[42,43]. NS5 has been shown to interact with NS3 *in vivo* as well as *in vitro*[44]. Phosphorylation of NS5 by cellular serine/threonine kinases *in vivo* has been previously reported and this phosphorylation may regulate NS3-NS5 interactions[44–46]. Although predominantly cytoplasmic, the flavivirus NS5 has also been detected in the nuclei of infected cells[44,47]. In addition to RdRp and helicase functions, NS5 plays a role in modulating the antiviral host response. In cells infected with virulent strains of WNV such as NY99, NS5 was shown to be

an antagonist of IFN signaling by preventing accumulation of STAT1 phosphorylation. Interestingly, mutation of Kunjin NS5 at S653F increased the virulence of this typically attenuated strain by making it capable of inhibiting STAT1 phosphorylation[48].

1.7 Replication Cycle

During the early stages of WNV infection, the virus typically infects keratinocytes, and skin-resident dendritic cells (DCs), which include dermal DCs and Langerhans cells[8]. Initial infection is usually followed by viral amplification within draining lymph nodes, which results in the initial viremia. In some cases, this initial infection can subsequently spread to visceral organs such as the kidney, spleen and liver. The virus is also able to cross the blood-brain barrier by stimulating the secretion of tumor necrosis factor (TNF), which induces endothelial cell permeability. Another possible mechanism by which the virus is able to enter the central nervous system (CNS) is through hijacking of immune cells able to traffic into the CNS[8].

The first step of the replication cycle starts with binding of the virion E protein to an unknown host receptor on the surface of the cell membrane. After viral attachment to the cellular receptor, it enters the cell via receptor-mediated endocytosis through the clathrin-mediated pathway[49]. After internalization, viral particles are delivered to early or intermediate endosomes, which mature to late endosomes. As the environment of the endosome acidifies, the virion E proteins undergo a conformational change that leads to fusion of the virion E protein with the endosomal membrane[50]. Viral-endosomal membrane fusion ultimately facilitates release of the viral nucleocapsid as well as the genomic viral RNA into the cytoplasm. The entering viral genome may remain associated with the capsid dimers and this association is thought to help protect the incoming viral genome from cellular nucleases and pattern recognition receptors (PRR's)[51].

Once the nucleocapsid enters the cytoplasm of the host cell, the viral genome is translated into a single polyprotein that will then be co- and post-translationally processed by the viral serine protease complex (NS2B-NS3), as well as by other cellular proteases, into a total of 10 mature viral proteins[13]. The viral genome also serves as a template for the synthesis of minus-strand RNA. Genome RNA synthesis is significantly more efficient than that of the minus-strand RNA, usually resulting in a 100:1 ratio of positive- to minus- strand RNA in infected cells[52]. The incoming viral RNA is then translated to produce the nonstructural proteins required for viral RNA replication. It is essential that efficient viral protein synthesis is established at early times during infection in order to counteract the cell's antiviral responses and to rearrange the cell in preparation for exponential genome synthesis and virion assembly[51].

During the early stages of the viral life cycle, the viral genomes in the cell switch back and forth between functioning as an mRNA for protein translation and as template for minus strand RNA synthesis[51]. Viral RNA replication primarily occurs inside perinuclear regions (replication complexes) that are located inside vesicles that form invaginations of the rough ER membranes[53]. The recently transcribed genomic viral RNA exits the replication complex through a pore and is either translated into polyprotein or binds to capsid proteins associated with ER membranes in areas where E and prM proteins are also located, leading to budding into the ER lumen[53]. Non-infectious, immature viral particles are transported through the *trans*-Golgi network where prM is cleaved into the M protein by the host protease furin to generate mature infectious particles that are transported to the plasma membrane in vesicles[54,55]. Virions are released from the cell by exocytosis as the virus-containing vesicles fuse with the plasma membrane. Typically, WNV virions begin to be released from infected cells starting at 8 to 10hrs post-infection and peak extracellular virus titers are usually observed by 24hrs[51].

1.8 Innate Immune Response to Viral Infection

The innate immune system is composed of a series of pattern recognition receptors (PRR's) that upon detection of a pathogen-associated molecular pattern (PAMP) are able to induce a strong antiviral host response, primarily characterized by the production of Type 1 interferon (IFN). Type I IFN has been found to have an important role in limiting the pathogenicity on WNV infected animals. IFN-a and IFN-b are able to reduce the viral burden by restricting cellular tropism and therefore protecting the animals from lethal WNV infection[56]. In previous animal studies, treatment of mice with type I IFN-inducing RNA transcripts protects mice challenged with lethal doses of WNV[56].

There are many PRRs encoded by mammalian cells including the Toll-like receptors (TLR), retinoic-acid inducible gene-I (RIG-I)-like receptors (RLR), and the nucleotide oligomerization domain (Nod)-like receptors (NLR). These receptors are an essential component of the antiviral defense program and each of them have been extensively defined as controllers of immunity and protection against WNV infection, although studies have shown that RLR receptors are the main regulators of innate immune responses that control WNV infection[57–60].

Detection of viral dsRNA and activation of PRRs such as RIG-I or the melanoma differentiation-associated gene 5 (MDA-5) trigger conformational changes and subcellular redistribution of these proteins to mitochondrial-associated membranes. RIG-I and MDA-5 will then interact with the mitochondrial antiviral signaling (MAVS) adaptor protein, leading to subsequent phosphorylation and nuclear translocation of interferon regulatory factors (IRFs) that drive transcription of IFN-α/β and ISGs, all essential to the cell's defense against viral infection[8,57].

IFN responses serve as an essential first line of host defense against many viruses including flaviviruses. IFNs are classified as Type I and Type II. Both types have antiviral activity[61]. The

two types of IFN are structurally unrelated and their actions are mediated by different and structurally unrelated cell-surface receptors[62]. IFN produced in an infected cell is secreted and binds to receptors on the surface of both infected cells and uninfected cells in an autocrine or paracrine manner. IFN-α/β binding to the IFN-α/β receptor (IFNAR) causes cross-activation of Jak1 and Tyk2, which phosphorylate one another along with STAT1 and STAT2. The phosphorylation of these two STATs results in the formation of IFN- stimulated gene factor 3 (ISGF3), a trimeric complex that includes STAT1, STAT2 and IFN regulatory factor (IRF) 9. The ISGF3 complex translocates to the nucleus where it binds to IFN-stimulatory response elements (ISREs) and drive transcription of IFN-b, IFN-α4, pro-inflammatory cytokines, and interferon-stimulated genes (ISGs) that aid in cellular defense against viral infection[57].

1.9 Cellular Function of the Slfn Family of Proteins

The Slfn protein family consists of 10 known or predicted murine genes clustered on chromosome 11 and 6 human genes clustered on chromosome 17[63,64]. The family is categorized into three groups, based primarily on the size of each member[65,66]. All Slfn proteins contain a Slfn box, a domain that is not found in other proteins and whose function is currently unknown[65]. This protein motif resides near a conserved N-terminus containing a putative AAA-ATPase domain. Group I Slfns (short form) range from 37 to 42 kDa in size, while group II ranges from 58 and 68 kDa. Group II and III also consists of a specific SWADL domain, defined by a Ser-Trp-Ala-Asp-Leu signature sequence. Group III Slfns (longest form) range from 100 to 104kDa in size and are additionally characterized by a C-terminal extension that is homologous to the superfamily I of RNA helicases[65,67,68], a trait they share with the nucleic acid sensors RIG-I and MDA-5[69] (Figure 1.7). Beyond that, Slfn proteins share no sequence similarity to other proteins.

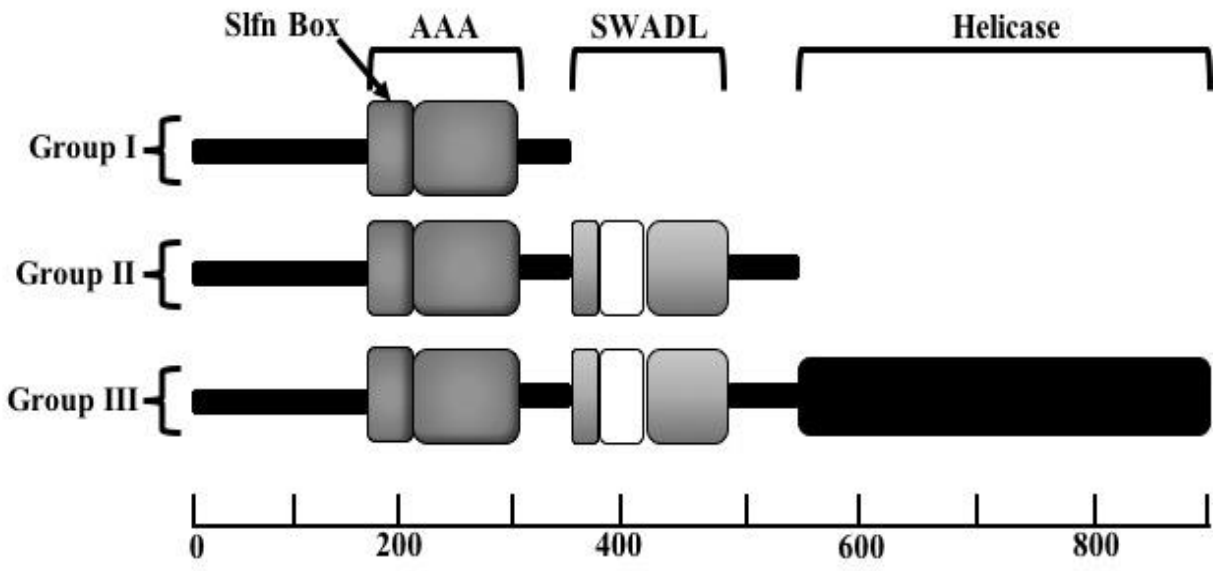

Figure 1.7 Structural model of the Slfn protein family.

Slfn genes were originally identified during screening for growth regulatory genes that are differentially expressed during lymphocyte development[70]. Although the biological role of the Slfn family remains poorly understood, some studies have implicated members of this family of proteins in specific cellular functions. Studies in mice have implicated Slfn proteins in regulation of cell growth and T-cell development[67]. Other proposed biological functions of Slfn genes include involvement in cell differentiation, inhibition of cell proliferation, inhibition of anchorage-independent growth, myeloid differentiation, sensitization to DNA-damaging agents and more recently, to inhibition of viral replication[69,71–73].

1.10 Cellular Function of the Cellular Protein Slfn11

Early work showed a strong correlation between endogenous Slfn11 expression and the response of several human cancer cell lines to Topoisomerase 1 and 2 inhibitors, as well as alkylating agents and DNA synthesis inhibitors; in other words, cancer cell lines expressing high levels of Slfn11 were significantly more sensitive to FDA-approved DNA-damaging agents (DDAs). Furthermore, since Slfn11 sensitized cells to DDAs but not to drugs with other mechanisms of action, it was suggested that Slfn11 must participate in the cell's DNA damage response by enforcing cell cycle arrest and promoting apoptosis[74]. Further studies have shown interactions between Slfn11 and the RNA helicase DHX9. This helicase is involved in multiple cellular processes such as DNA replication, mRNA translation and RNA-mediated gene silencing; as well as with a subunit of the replication protein A (RPA) protein complex, also involved in DNA replication, recombination and DNA repair[70]. High levels of Slfn11 expression is observed in monocytes and monocyte-derived dendritic cells (moDCs) as well as in $CD4^+$ T cells[75]. Intriguingly, high levels of Slfn11 has also been observed in HIV-positive individuals that are able to maintain undetectable levels of viral loads in the absence of any treatment, also known as HIV "elite controllers"[76].

1.11 Viral Regulation by Slfn Proteins

The first indication of a Slfn family member involved in viral regulation or host immune responses came in 1998, when known Slfn amino acid sequences were used to perform a BLASTp query and strong sequence similarities were observed in the right inverted terminal repeats of vaccinia, variola and cowpox viruses[77]. Further study revealed that a camelpox virus strain (176R) encoded a protein with sequence similarity with that of mouse Slfns (v-Slfn)[78]. Phylogenetic

analysis shows that the v-Slfn genes are most closely related to mouse and rat Slfn genes, suggesting that they originated by horizontal transfer from a rodent to orthopoxvirus (OPV)[68]. The camelpox v-Slfn was shown to be expressed at early and late times post-infection. Furthermore, expression of the camelpox v-Slfn in a vaccinia virus strain resulted in reduced weight loss, faster rates of recovery and earlier decay of viral titers in mice infected with the recombinant virus. Additionally, the N-terminal region of v-Slfn (encoding the AAA-ATPase domain) was found to be responsible for this phenotype[78]. Although the reason why a virus would encode a protein that decreases virulence is puzzling, the authors propose the intriguing possibility that OPVs might do so to prevent overwhelming the host, thereby impeding viral spreading throughout the host population. In any case, the link between Slfn genes and the interferon immune response clearly suggests an important interplay between these genes and the host's antiviral response.

One of the most distinct consequences of viral infection is the induction of IFNs, cytokines that have potent antiviral activity, along with the activation of interferon- stimulated genes (ISGs). Members of the Slfn family of genes are a subset of these ISGs that are also induced directly by pathogens via the interferon regulatory factor 3 (IRF3) pathway[79].

Brucella and Listeria infections have been shown to increase the expression of mouse Slfn proteins[69]. Similarly, lipopolysaccharide, poly-IC, or IFN-α/β treatment of murine macrophages resulted in induction of several mouse Slfn genes[69]. Increased expression of Slfn5 and Slfn11 was also found in human foreskin fibroblasts treated with IFN-β, poly-IC, or poly-dAdT[69]. More importantly, Slfn11 was found to significantly reduce the replication of human immunodeficiency virus (HIV-1) and equine infectious anemia virus infection[69,71]. Slfn11 binds to tRNAs and counteracts the up-regulation of the tRNA repertoire induced by HIV-1 infection that promotes translation of the codon-biased viral genome, although the exact mechanism by which this occurs

remains to be elucidated. Furthermore, the antiviral activity of Slfn11 was mapped to the N-terminal half of Slfn11, which includes the AAA+ superfamily of ring-shaped P-loop NTPases. This domain has been proposed to mediate energy-dependent remodeling or translocation of macromolecules[80].

It is possible that the antiviral mechanism of Slfn11 involves tRNA nucleolytic activity recently described in Slfn13[73]. Slfn13 was recently described as a potent tRNA/rRNA-targeting RNase able to impair HIV-I infection[73]. All the residues implicated in the tRNA nucleolytic activity of Slfn13 are conserved in Slfn11, and these two proteins share an overall homology of 83%. This enzymatic activity is required for Slfn13 to restrict HIV-1 infection. Slfn13 cleaves tRNAs close to the 3' end at the acceptor stem and also diminishes the levels of HIV-1 mRNA. The anti-HIV-1 activity of Slfn13 is specific since this protein did not affect replication of herpes simplex and only moderately impaired ZIKV replication[73].

Murine Slfn14 has recently been shown to impair replication of influenza A and varicella zoster virus. Although the precise antiviral mechanism remains unknown, it was determined that Slfn14 affects nuclear trafficking of influenza nucleoproteins. Furthermore, Slfn14 induction was revealed to be mediated by the TLR-3 and type I IFN pathways and that once induced, it enhances RIG-I-mediated IFN-β signaling[72].

In summary, Slfn11, along with other Slfn proteins, is now described as a potent, interferon-stimulated gene that responds against viral infections and regulates their replication. Slfn11 was shown to selectively inhibit the synthesis of viral proteins by exploiting the codon-usage bias of the virus. Furthermore, Slfn11 was shown to bind to tRNA and effectively counteract the changes in the tRNA repertoire triggered by HIV-1[69].

1.12 Significance and Hypothesis

The medical and veterinary relevance of lentiviruses and (+)ssRNA viruses is clearly established, as well as the need for preventive vaccines and effective therapeutics. Therefore, the discovery of viral mechanisms of replication, and in particular mechanisms of evasion of the innate immune response will pave the way for developing therapies that will synergize with the innate immune response to eradicate or control these infections. Key players in these innate immune mechanisms will also serve as biological markers of disease susceptibility, allowing for easier identification of individuals at higher risk of disease.

We focused our research on flaviviruses, a family of (+)ssRNA viruses including medically important members such as WNV, DENV, and ZIKV; as well as viruses causing hemorrhagic diseases including YFV, Omsk haemorrhagic fever virus (OHFV), and Kyasanur forest disease virus (KFDV). The mortality rate of the infections caused by these agents varies, the highest being for haemorrhagic fever viruses. None of these viral infections have a specific treatment, and an effective vaccine is available only against YFV, despite the medical and military significance of these viruses.

We chose to investigate the role of Slfn11 in flavivirus replication. Slfn11 is an ISG induced upon viral infection that regulates tRNA abundance, affecting lentiviral infection. Our knowledge of Slfn11 is extremely limited. At the beginning of this project, approximately 20 papers related to Slfn11 had been published, with only 2 of them related to viruses, and both of them reporting effects on lentiviruses. Currently, the role of Slfn11 as an antiviral protein remains poorly understood and the effect of Slfn11 on other viruses, excusing the current study, is yet to be determined.

The previously observed effect of Slfn11 on tRNA availability and consequently on protein synthesis compelled us to hypothesize that this protein would preferentially restrict the replication of viruses that require high translation efficiency. Thus, **we hypothesized that Slfn11 negatively regulates flavivirus replication.** We predicted that knockdown of Slfn11 would enhance replication of flaviviruses but would have no effect on the replication of (-)ssRNA viruses that do not have this extreme requirement for initial protein translation. Consequently, we proposed that overexpression of Slfn11 would selectively restrict replication of (+)ssRNA relative to (-)ssRNA. Since Slfn11 opposes HIV-induced changes in the tRNA pool[69], we also proposed that WNV infection would modulate the tRNA repertoire in Slfn11-deficient cells to a greater degree than in Slfn11-expressing cells.

We tested these predictions and found that flaviviruses, including WNV, DENV, and ZIKV, replicated significantly more efficiently in Slfn11-deficient than in control cells expressing this protein. As predicted, this phenotype was not observed when the replication of (-)ssRNA viruses VSV and RVFV was analyzed, highlighting the specificity of this antiviral activity. Our data also revealed that WNV infection modulates the tRNA repertoire only in Slfn11-deficient cells.

Our findings support our view that some viruses are more sensitive than the host to protein translation efficiency, further indicating that the innate immune system exploits this greater sensitivity to control infection, and that viruses have evolved strategies to counteract these mechanisms and to enhance viral protein translation efficiency. Undoubtedly, our results will encourage research directed at clarifying the exact molecular features of these mechanisms of viral replication and innate immune surveillance.

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Chapter 2: Evaluation of the Antiviral Activity of Slfn11

2.1 Introduction

Successful viral replication depends on the ability of the virus to take over the host translational machinery. The innate immune response exploits this dependency to control viral replication. Many interferon (IFN)-stimulated genes (ISGs) that regulate protein translation are well known to restrict virus replication, including Protein Kinase R, the Interferon-induced proteins with tetratricopeptide repeats family of proteins, zinc-finger antiviral protein and the 2′,5′-Oligoadenylate/RNaseL pathway[65,77]. Another family of ISGs is the Schlafen (Slfn) proteins[65,77], which were first identified as being important regulators of T cell differentiation and growth[69]. Currently, 10 mouse (Slfn1, 1L, 2, 3, 4, 5, 8, 9, 10, and 14) and 6 human (Slfn5, 11, 12, 12L, 13, and 14) Slfn genes have been identified[71,81]. Slfn11, the focus of the current study, is ubiquitously expressed and controls synthesis of proteins encoded by codon-biased open reading frames[69,71,81].

Several members of the Schlafen family have been shown to impair virus replication. Mouse Slfn14 impairs replication of influenza A and varicella zoster virus. The mechanism for this effect is unknown but Slfn14 affects nuclear trafficking of influenza nucleoproteins and enhances IFN-β signaling[72]. Human Slfn11 suppresses HIV-1 and equine infectious anemia virus infection[69,71]. Slfn11 binds to tRNAs and counteracts the up-regulation of the tRNA repertoire induced by HIV-1 infection that promotes translation of the codon-biased viral genome. The anti-viral mechanism of Slfn11 seems to involve a tRNA nucleolytic activity recently described in Slfn13[73]. All the residues implicated in the tRNA nucleolytic activity of Slfn13 are conserved in Slfn11, and these two proteins share an overall homology of 83%.

Therefore, we decided to investigate whether the previously observed antiviral activity of Slfn11 can be extended to an evolutionarily-unrelated family of viruses, as well as attempt to provide insights into the mechanisms of antiviral activity.

2.2 Materials and Methods

2.2.1 Cell and Virus Culture

HEK293T, HeLa, SupT1, LLC-MK2, BHK-21, and A172 cells were obtained from the American Type Culture Collection (Manassas, VA). HEK 293T, HeLa, BHK-21, and A172 cells were maintained in Dulbecco's Modified Eagle's (DMEM) medium, LLC-MK2 and BHK-21 cells in Eagle's Minimum Essential Medium (E-MEM), and SupT1 cells in RPMI 1640. These culture media were supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin, streptomycin, and 1% non-essential amino acids (NEAA), 1% sodium pyruvate. Maintenance media used to perform viral infections consisted of E-MEM w/L-glutamine, supplemented with 2% FBS, 1% NEAA, 1% sodium pyruvate and 1% penicillin and streptomycin.

The WNV strain TVP-7767 (Passage: Vero, #3), RVFV strain MP-12 (Passage: Vero, #3) and ZIKV strain MR-766 (Passage: suckling mice brain, #150. Vero, #3) were obtained from the World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch. DENV-2 strain 16681 (Passage: C6/36, #9) was obtained from the Navy Medical Research Center-6. VSV engineered to express eGFP has been previously described[82]. Viral stocks were prepared in Vero cells maintained in E-MEM supplemented with 2% FBS. Infectivity titers of each virus stock was determined via plaque assay as described below.

A replication-defective HIV-1 reporter virus (Hluc) was used that expressed LTR-driven luciferase from the NEF slot and contains a large deletion in ENV[83]. Hluc was generated by calcium phosphate transfection of the corresponding HIV-1 expression plasmid (pHluc, 15 ug) and the VSV-G encoding plasmid pMD.G (5 ug) into HEK293T cells, as described before[83]. In accordance with World Health Organization's and the Centers for Disease Control and Prevention's guidelines, all work involving infectious WNV was performed in a biosafety level

(BSL)-3 laboratory in accordance to biosafety practices described in the 2018 revised version of the University of Texas at El Paso's (UTEP) BSL 3 Biological Safety Manual and Standard Operating Procedures. All work involving DENV, VSV, ZIKV, HIV-1, and RVFV MP-12 was performed in BSL-2+ laboratory in accordance to biosafety practices described in the UTEP Biological Safety Manual.

2.2.2 Virus Replication Dynamics

All cell lines infected with WNV, DENV, RVFV, ZIKV and VSV were seeded in T25 cell culture flasks ($2.5x10^5$ cells in 2ml total volume) and allowed to grow overnight. The following day the cells were infected with respective viruses and incubated at 37°C for 1 hr. Cells were subsequently washed three times with serum-free medium to remove input virus, replenished with maintenance medium and incubated at 37°C. Cell culture supernatants were collected every 8hrs until experiments were stopped and stored at -80°C.

2.2.3 Plaque Assays

WNV and VSV viral titers were determined as previously described[84]. Briefly, viral supernatants were subjected to ten-fold serial dilutions and inoculated onto confluent monolayers of LLC-MK2 cells in 12-well cell culture plates and incubated at 37°C for 1 h with gentle rocking every 15 minutes. 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).

DENV titers were determined as previously described[85]. Briefly, 3 x 10^5 BHK-21 cells were seeded in 12-well cell culture plates, and then infected with viral supernatants at 37°C for 3 hrs, followed by the addition of 1ml of 3% carboxymethylcellulose overlay medium. Cells were cultured for 5 days, followed by staining and quantification as described above.

RVFV and ZIKV plaque assays were performed as described for WNV but using Vero 76 cells. RVFV- and ZIKV-infected cells were cultured at 37°C for 4 or 5 days, respectively. Staining and quantification was performed as described above.

Plaque assays for estimating viral titers were conducted in triplicate experiments with samples derived from independent viral infections.

2.2.4 Immunoblotting

Full procedures for protein detection by immunoblot have been described previously[86]. Briefly, cellular lysates were obtained by lysing cells with 2x Laemmli Buffer and boiling for 10 minutes. Cell lysates were resolved by SDS-PAGE and transferred overnight to PDVF membranes at 100 mA at 4°C. Membranes were blocked in TBS containing 10% milk for one hour and then incubated in the corresponding primary antibody diluted in TBS-5% milk-0.05% Tween 20 (antibody dilution buffer). Full-length Slfn11 and Slfn11 C-terminus mutant were detected with anti-Slfn11 antibody E-4 (Santa Cruz Biotechnology 1/500). The Slfn11 N-terminus mutant was detected with anti-Slfn11 antibody D-2 (Santa Cruz Biotechnology 1/500). WNV envelope protein was detected with antibody PA1-41073 (Thermo Fisher Scientific, 1/500). Tetherin (BST-2) was detected with anti-BST-2 antibody (Santa Cruz Biotechnology 1/500). α-tubulin was detected as a loading control with antibody from clone B-5- 1-2 (Sigma, 1/4000). Membranes were incubated overnight at 4°C with primary antibodies, whereas anti-α-tubulin Mab was incubated for 30 minutes at 25°C. Primary antibody-bound membranes were washed in TBS-0.1% Tween 20 and

bound antibodies detected with goat anti-mouse IgG-HRP (1/2000, Sigma) or a goat anti-rabbit IgG-HRP (1:4000, EMD Millipore) followed by chemiluminescence detection. Densitometry analysis of selected bands was quantified based on their relative intensities using Image Studio Software (LI-COR, Lincoln, NE).

2.2.5 Plasmids

For the generation of HIV-1-derived viral vectors, plasmids were obtained from Eric Poeschla laboratory (Mayo Clinic, Rochester, MN)[87.] These lentiviral vectors were used to express Slfn11- and control-shRNAs and Slfn11 proteins. They were generated with packaging plasmid pCMVΔR8.91, a transfer plasmid derived from pTRIP (described below), and the envelope plasmid pMD.G encoding the vesicular stomatitis virus glycoprotein G (VSV-G).

Slfn11- and SCR-shRNA plasmids: An shRNA construct (Top: 5'-GATCCGGCTCAGA ATTTCCGTACTGAATTCAAGAGATTCAGTACGGAAATTCTGAGCTTTTTTGGAAA-3', Bottom:5'-AGCTTTTCCAAAAAAGCTCAGAATTTCCGTACTGAATCTCTTGAATTCAGT ACGGAAATTCTGAGCCG-3') against Slfn11 was designed using a target sequence that has been previously described[69]. Briefly, Slfn11 shRNA construct was ligated into the pSilencer 2.1 U6 Hygro shuttle vector (AM5760, Thermo Fisher Scientific) and sequence verified. Control shRNA contains a scrambled sequence (SCR) that was obtained from the negative control plasmid provided with the kit. The Slfn11 and SCR shRNA expression cassettes were amplified by PCR and ligated into a unique PpUMI site in the HIV-1-derived transfer plasmid pTRIP-eGFP and their sequences verified.

shRNA-resistant Slfn11 expression plasmid: The shRNA-resistant Slfn11 cDNA was engineered by introducing 7 synonymous mutations within the 21nt-long shRNA target sequence of Slfn11. Plasmid pCDNA-V5-His-Slfn11 (Michael David, University of California San Diego)[69]

was used as template for the Phusion High-Fidelity DNA polymerase (ThermoFisher, F530S). Primers used to introduce mutations were forward: 5'-TCGGACCGAGGATGGGGACTGGTATGGG-3' and reverse: 5'-AAGTTTTGCGCTTCGTCAATGACG-3'. The newly created shRNA escape mutant cDNA was then amplified using the high-fidelity Deep Vent DNA polymerase (New England Biolabs), digested with SbfI and SpeI restriction enzymes, cloned into unique SbfI-SpeI sites in the pTRIP-IRES-P HIV-1-derived transfer plasmid, and the sequence verified.

N- and C-terminal Slfn11 mutant expression plasmids*:* pTRIP-IRES-P-Slfn11-shRNA-resistant plasmid was used as template to generate the Slfn11 truncated mutants using the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). The mutant expressing N-terminal Slfn11 (amino acids 1-441) was generated with primers forward: 5'-GAACAAAAACTCATCTCAGAAGAGGATCTG-3', and reverse: 5'-GAAGATCAAAATTCCCCGAAAGAAAG-3' whereas forward: 5'-TCTAGAAGTTGGGCTGTGGACC-3' and reverse: 5'-CATACTAGTGGATCCTCTAGC-3' were used to produce C-terminal Slfn11 (amino acids 442-901). Mutants were verified by DNA sequencing.

2.2.6 Production of Lentiviral Vectors

The full procedures for transfection and production of lentiviral vectors has been described previously[83,87,88]. Briefly, HEK293T cells were calcium-phosphate transfected with the corresponding transfer plasmid derived from pTRIP (15 ug), the packaging plasmid pCMVΔR8.91 (15 ug), and VSV-G envelope expression plasmid pMD.G (5 ug). The viral supernatants were harvested 48 hrs post-transfection and concentrated by ultracentrifugation at 124,750g for 2 hrs on a 20% sucrose cushion.

Expression of Slfn11- and SCR-shRNA in A172 cells. A172 cells were transduced with shRNA-, eGFP-expressing lentiviral vectors and cells expressing the highest 10% of eGFP

fluorescence were isolated by cell sorting and expanded in culture. Slfn11 levels were determined in these cells by immunoblot, as described above.

Expression of Slfn11 full-length and deletion mutants in Slfn11-dificent cell lines: A172-KD, HEK293T, BHK-21, and HeLa cells were engineered to express Slfn11 proteins by transduction with lentiviral vectors expressing Slfn11 and the puromycin resistant gene**.** Briefly, viral vectors were produced in HEK293T by transfection with the transfer plasmid pTRIP-IRES-P-Slfn11-shRNA-resistant plasmid expressing Slfn11 full-length or deletion mutants (15 ug) and the packaging and envelope expression plasmids described above. Viral supernatant was concentrated by ultracentrifugation and used to transduce cells. Three days later, transduced cells were selected in the presence of puromycin (A172-KD and HEK293T: 3ug/ml, HeLa: .375ug/ml, BHK-21: 6ug/ml). Slfn11 expression was verified by immunoblot.

2.2.7 Single-Round Infectivity Assay

HEK293T-, HeLa- and A172-derived cells were seeded onto 24-well plates ($2x10^4$ cells/well) and allowed to grow overnight. The next day, cells were infected with Hluc and 24 hrs later the cells were extensively washed to remove the input virus. Four days later cell culture supernatant was collected for HIV-1 p24 quantification and cell lysates prepared in a buffer containing 1% Triton X-100 for luciferase activity quantification (Bright-Glow Luciferase Assay System, Promega), following the manufacturer's instructions. Luciferase activity was determined in triplicate samples using a microplate luminometer reader (Thermo Scientific, Luminoskan Ascent). Luciferase and HIV-1 p24 samples were derived from at least three independent infections.

2.2.8 HIV-1 p24, IFN-α and IFN-β ELISAs

HIV-1 infection was measured by quantifying HIV-1 p24 in the supernatant of infected cells (described above) by ELISA (ZeptoMetrix Corporation, 0801008). IFN-α and β was quantified in the cell supernatant of the infected cells by ELISA (PBL Assay Science, Cat # 41115-1 for IFN-α and # 41415-1 for IFN-β). ELISAs were performed according to the manufacturer's instructions.

2.2.9 Indirect Immunofluorescence Microscopy

This technique was used for determining WNV infection in A172-derived cells and for localizing Slfn11 in A172- and HEK293T-derived cells. A172-derived cells ($1.5x10^4$/well) were seeded onto 96-well confocal microscopy plates and infected with WNV at MOI 1 twenty-four hours later. Infected cells were fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences, Cat# 554714) at 24hrs and 48hrs post-infection and then stained with an anti-Flavivirus group antigen monoclonal antibody that recognizes WNV Env (ATCC, clone D1-4G2-4-15, 1/200) for 2hrs at room temperature. Cells were then washed 3 times with PBS containing 0.1% FBS and then incubated with Alexa 568-conjugated antibody (Invitrogen, H-11004, 10ug/ml) for 1h at room temperature. Cells were again washed 3 times with PBS and cell nuclei was stained with Hoechst 33342 (Invitrogen, H-3570, 20ug/ml) for 10 min.

For subcellular localization of Slfn11, uninfected A172- and HEK293T-derived cells were staining as described above. Specifically, Slfn11 full-length and C-terminus were detected with the anti-Slfn11 antibody E-4 (Santa Cruz Biotechnology, sc-374339, 1/200) and the Slfn11 N-terminus mutant was detected with anti-Slfn11 antibody D-2 (Santa Cruz Biotechnology sc-515071,1/200).

2.2.10tRNA PCR-Array and Analysis

To determine the effects of WNV infection on the host tRNA repertoire, we quantified the tRNA pool using a PCR-based methodology previously described[89]. The procedure and data analysis were performed by Arraystar Inc. using the nrStar™ Human tRNA Repertoire PCR Array. A172-derived cells were infected with WNV at MOI 1 for 1hr at 37°C washed with fresh culture media and replenished with culture media. 8 hrs post-infection, cells were collected, and total cellular RNA was extracted using Trizol reagent. Experiments were performed in triplicate with appropriate non-infected controls. Total cellular RNA was sent to Arraystar Inc for analysis. Briefly, quality control was performed on extracted RNA samples by NanoDrop ND-1000 and RNA integrity and genomic DNA contamination were assessed by denaturing agarose gel electrophoresis. Next, RNA samples were subjected to a demethylation step, followed by first-strand cDNA synthesis (Arraystar, rtStarTM tRNA-optimized First-Strand cDNA Synthesis Kit, Cat# AS-FS-004). Real-time PCR was then performed using a proprietary human tRNA Repertoire PCR Array that is able to distinguish 66 nuclear tRNA isodecoders, covering all anti-codons available in GtRNAdb and tRNAdb databases. Three stably expressed small ncRNA genes RNU6-2 (Ref 1), SNORD43 (Ref 2), and SNORD95 (Ref 3) were included in the array as the quantification references for tRNA and data normalization. One external RNA Spike-In mix was added in the RNA sample prior to the first strand cDNA synthesis. The RNA Spike-In control assay indicated the overall success and the efficiency of the reaction beginning from the cDNA synthesis to the final qPCR. For positive PCR control, two replicates of one artificial DNA and the PCR primer pairs were used to indicate the qPCR amplification efficiency. Only Ct value greater than 35 were considered as good qPCR amplification efficiency and considered for analysis. The

positive PCR control was used as an inter-plate calibrator and a control to exclude genomic DNA contamination.

2.2.11Bioinformatics Analysis

Analysis of the impact of WNV-induced changes in tRNA on viral protein translation was performed by using a python script created to determine the differential tRNA expression values over the open reading frames within the viral genome. In short, the tRNA differential expression values were uploaded from text file into a python dictionary. For each codon not found within the tRNA expression dataset the codon value was set to 0, and for redundant codons were limited to the value of the highest expression to limit the codon to a single value. Additionally, to make visualization of the values easier, a sliding window approach was taken to average the differential expression over a desired number of amino acids. The window was then shifted by a consistent step size, and the average again determined. To allow for variation in the resolution of the graphs, the window size and step size were coded as adjustable parameters within the python script for easy adjustment. For each gene, the individual codon usage and the windowed average values were written to a CSV file. The CSV file was then imported into Excel and graphs were then created from the windowed codon usage.

2.2.12Statistical Analysis

All data used for viral replication curves were transformed to $\log_{10}$pfu/ml. Repeated-measures ANOVA was used to test the impact of different cell lines expressing or not Slfn11 on viral replication curves and the Tukey-Kramer post-hoc test was used to identify significant differences in viral titer between cell lines.

2.3 Results

2.3.1 WNV Infection Induces Expression of Slfn11

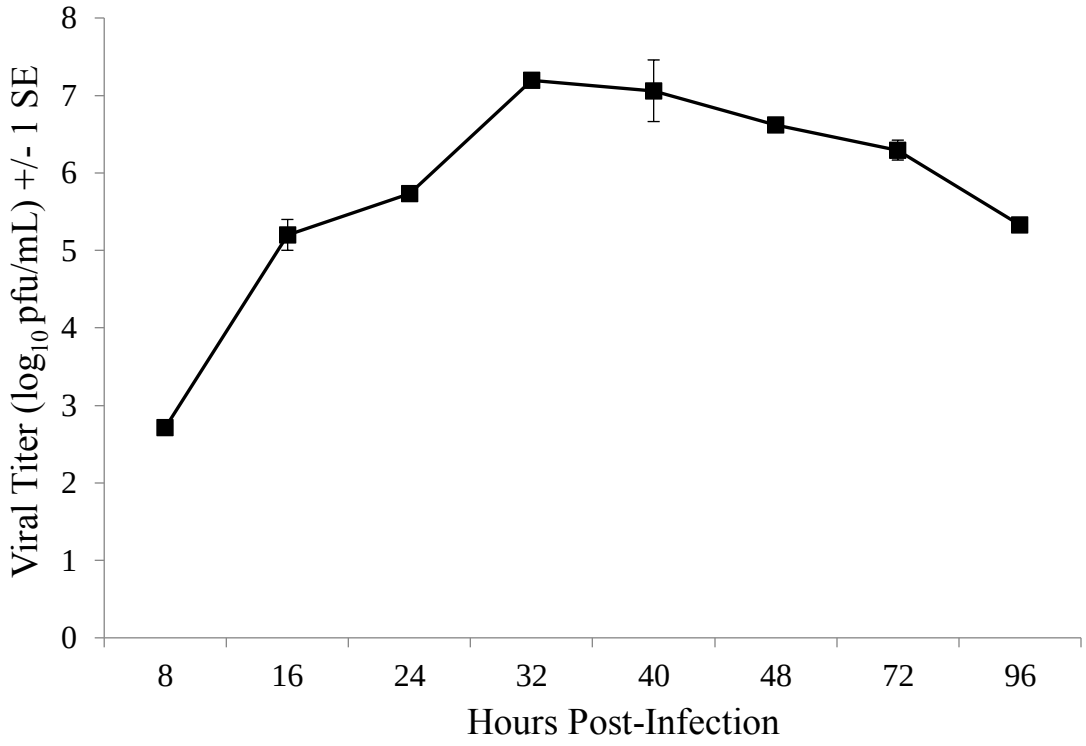
To determine whether WNV infection modulates levels of Slfn11 expression, cells of the human glioblastoma cell line A172 were infected with WNV at MOI: 0.1 and viral replication and expression of Slfn11 was determined at different times post-infection (p.i.) by plaque assay and immunoblot. Viral replication was detectable as early as 8 hrs pi and peaked by 32 hrs after infection (Fig. 2.1a). Corresponding with the peak of viral replication, we detected a sustained increase in the basal levels of Slfn11 after 40 hrs pi (Fig. 2.1b). Densitometry analysis of immunoblots from two independent infection experiments indicated that WNV infection caused 3.5-fold increase in the α-tubulin-normalized Slfn11 protein levels after 40 hrs p.i. Therefore, these data indicated that Slfn11 is up-regulated by WNV infection.

The up-regulation of Slfn11 in WNV-infected cells could be secondary to the production of type I IFN in response to the viral infection. Therefore, we evaluated the temporal sequence in production of these proteins. A172 cells were infected with WNV as described above and levels of IFN-α and -β were determined in the cell supernatant by ELISA. IFN-α and -β were undetectable, <1.95 pg/ml and <2.3pg/ml, respectively, at 8 hrs p.i even though viral replication was evident by this time (Fig. 2.1c). However, type I IFNs production was evident by 32 hrs, reaching a peak at 48 hrs post-infection. Therefore, these data indicated a temporal correspondence between type I IFN secretion and the up-regulation of Slfn11 suggesting that virus-induced type I IFN up-regulated Slfn11 expression.

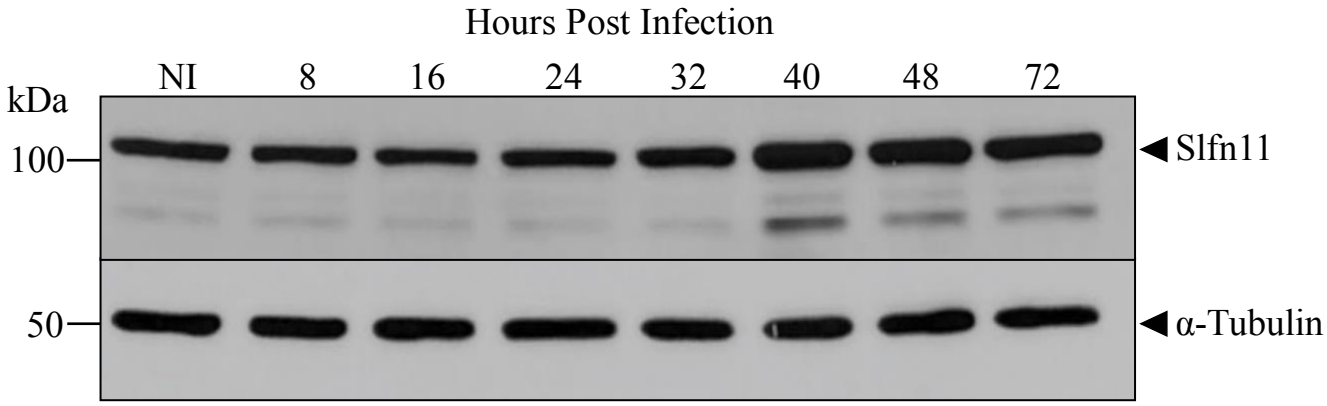
To further evaluate the role of type I IFN in the regulation of Slfn11 expression a panel of cell lines susceptible to WNV infection was treated with IFN α-1 for 24 hrs and then the levels of

Slfn11 was determined by immunoblot. Similar to WNV infection, IFN α-1 triggered a two-fold increase of Slfn11 in A172 cells (Fig. 2.1d). However, this treatment failed to induce expression of Slfn11 in HEK293T or HeLa cells, which also lack basal expression of this protein. To verify that IFN α-1 stimulated these cells, we also measured the expression of the ISG tetherin. As previously reported[90], tetherin was constitutively expressed in HeLa cells but not in HEK293T cells and the expression of this protein increased in both cell types in response to IFN α-1 stimulation (Fig. 2.1d). Tetherin was absent in untreated A172 cells but was also significantly induced after IFN α-1 treatment. Therefore, the lack of response to IFN α-1 was not the reason for the absence of Slfn11 in HeLa and HEK293T cells.

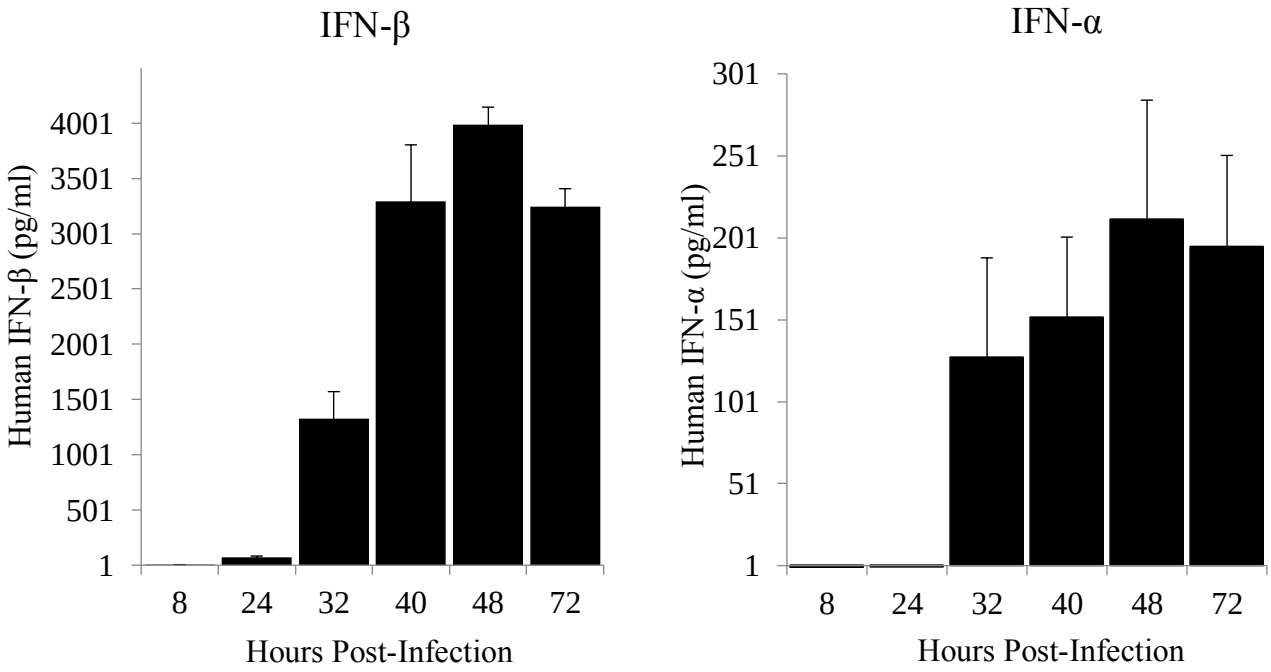
a



b


Hours Post Infection
NI
8
16
24
32
40
48
72
kDa
100
50
Slfn11
α-Tubulin

c


IFN-β
Human IFN-β (pg/ml)
4001
3501
3001
2501
2001
1501
1001
501
1
8
24
32
40
48
72
Hours Post-Infection
IFN-α
Human IFN-α (pg/ml)
301
251
201
151
101
51
1
8
24
32
40
48
72
Hours Post-Infection

d

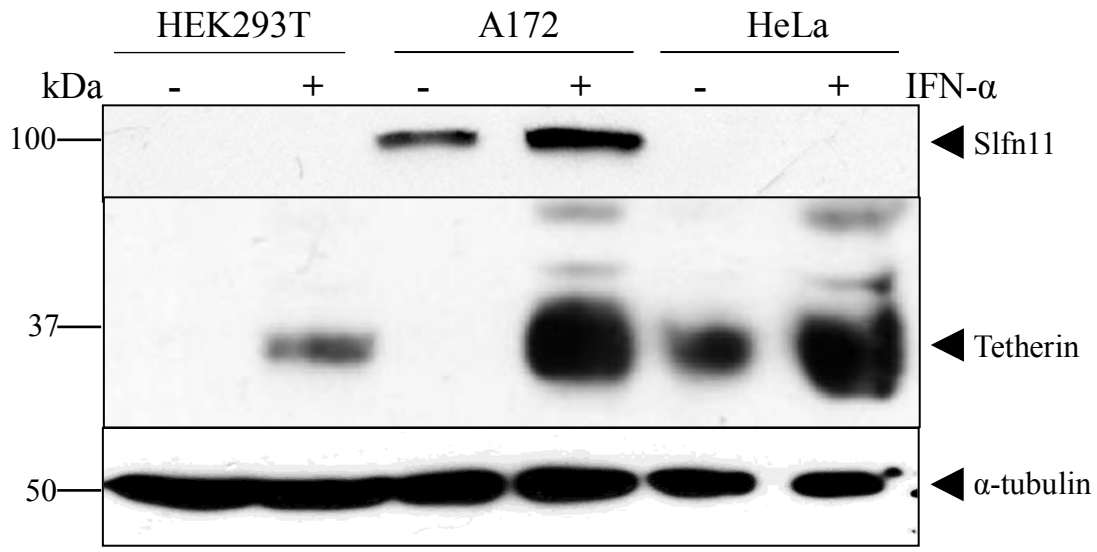


Figure 2.1 Kinetics of WNV replication, type I interferon production, and Slfn11 expression in A172 cells.

(a) WNV replication in A172 cells. Cells were infected with WNV at MOI 0.1 and viral replication was measured by titration of the cell supernatant in a plaque assay at different times post-infection. Titers were determined in triplicate experiments. Data are representative of 3 independent infection experiments. (b) Expression of Slfn11 in WNV-infected A172 cells. Cells were lysed at different times post-infection and Slfn11 and α-tubulin (loading control) were detected with specific antibodies by immunoblot. Data are representative of 2 independent infection experiments. (c) Kinetics of IFN-α and IFN-β (all subtypes) production in WNV-infected A172 cells. Culture supernatant was collected at different times post-infection and type I IFN quantified by ELISA. Data are representative of 3 independent infection experiments. (d). Effect of IFN-α1 on Slfn11 expression. HeLa, A172, and HEK293T cells were treated with 5000U/ml units of IFN-α1 for 24 hrs and the expression of the type I IFN-stimulated genes Slfn11 and Tetherin were evaluated by immunoblot.

2.3.2 Slfn11 Impairs Replication of Flaviviruses but not of (-)ssRNA Viruses

To test the relative effect of Slfn 11 expression on (+)ssRNA and (-)ssRNA viruses, A172 cells were stably transduced with lentiviral vectors expressing shRNAs containing Slfn11-specific or scrambled sequences to generate Slfn11-deficient A172 cells (A172-KD) and control cells (A172-SCR), respectively. Subsequently, A172-KD cells were engineered to over-express Slfn11 (A172-BC). Levels of Slfn11 were verified in these cells by immunoblotting (Fig. 2.2a). Then, A172-derived cell lines were infected with WNV at a MOI of 0.1 and cell culture supernatants were collected every 8 hrs to measure viral titer by plaque assay.

For all the replication curves reported in this study, we analyzed the data with repeated measures ANOVA, and we always detected a significant effect of time p.i. on virus titer; as this effect was expected and is not of interest we do not report it here. Instead we focus on whether there was a significant interaction between treatment (i.e. cell type) and time, or, in the absence of such interaction, an independent effect of treatment, and we use Tukey-Kramer post-hoc tests to elucidate the nature of these effects.

As shown in Figure 2.2b, there was a significant interaction of the effects of cell type (KD, SCR or BC) and time p.i. on WNV titer ($DF = 14$, $F = 11.1$, $P < 0.0001$). Overall, WNV replicated significantly more efficiently in A172 cells lacking Slfn11 than in either of the two cell lines expressing this protein (Fig 2.2b). Twenty-four hrs post- infection viral titers were 2 logs higher in Slfn11-deficient cells than in control cells and these differences persisted until 96 hrs post-infection, after which the experiments were terminated due to significant cytopathic effects.

We anticipated Slfn11 to impair WNV replication by targeting viral protein production. Therefore, we infected A172-derived cells with WNV at a MOI of 0.1 and measured cell-associated WNV Env at the peak of infection (40 hrs p.i.) by immunobloting analysis. As expected,

Slfn11-deficient cells expressed higher levels of WNV Env than control cells (Fig. 2.2c). Densitometry analysis of the immunoblots corresponding to three independent infection experiments indicated that α-tubulin-normalized Env levels were similar between A172-SCR and -BC cells and A172-KD cells expressed 12.3-fold more Env protein than A172-SCR cells. These results were expected based on the WNV titer reached in these cells and in the postulated mechanism of action of Slfn11.

In addition, we verified WNV Env expression by indirect immunofluorescence. A172-derived cell lines were infected with WNV at MOI of 1 and the production of WNV Env was evaluated 24 and 48 hrs after infection by indirect immunofluorescence with an anti-flavivirus antibody that reacts with WNV Env. In correspondence with data in figure 2.2c, Slfn11-deficient cells expressed higher levels of Env than A172-SCR and -BC cells at 48 hrs pi (Fig. 2.2d). As expected, analysis at 24 hrs post-infection showed similar results (data not shown). Subcellular distribution of Env was similar in all the cell lines, indicating the absence of gross defects in Env intracellular trafficking. Therefore, data in figures 2.2b-d demonstrated that Slfn11 markedly impaired the replication of WNV by impairing viral protein expression.

We next tested whether Slfn11 affected replication of two additional flaviviruses, DENV and ZIKV. Contrary to WNV, DENV does not replicate efficiently in A172 cells; however, we were interested in determining the contribution of Slfn11 to this phenotype. A172-derived cells were infected with DENV at MOI 0.1 and viral replication was followed at different times post-infection by titration of the cell supernatant by plaque assay. Data in figure 2.3a showed statistically significant interactions ($DF=8$, $F=24.4$, $P<0.0001$). Interestingly, although A172-SCR cells did not support replication of DENV (Fig. 2.3a), Slfn11-deficient A172 cells efficiently allowed DENV replication, producing 2-log higher viral titters than A172-SCR cells at the peak

of replication. DENV reached titers of 10^6 pfu/ml in A172-KD cells with a replication kinetic similar to that of WNV. In these experiments, DENV replication peaked from 24 to 32 hrs post-infection and then decayed by 48 hrs due to cytopathic effects. As expected, re-expression of Slfn11 in A172-KD cells (A172-BC cells) fully removed the permissiveness of these cells, highlighting the specificity of the effect of Slfn11 on DENV replication. Similarly to A172-SCR cells, DENV did not multiply in A172-BC cells. Therefore, basal expression levels of Slfn11 significantly contribute to the restriction of DENV replication in A172 cells.

Slfn11 and Slfn13 impair HIV-1 infection through a similar mechanism[69,73]. However, Slfn13 fails to restrict ZIKV replication[73] and the effect of Slfn11 on this flavivirus has not been explored yet. Thus, we determined whether or not Slfn11 impairs the replication of ZIKV. A172-derived cells were infected and ZIKV titer was quantified as described above. ZIKV replication peaked at 40 hrs post-infection and plateaued until 96 hrs post-infection or the end of the experiment. However, cytopathic effects were not very apparent even at these late time points of the infection. Importantly, replication of ZIKV was also significantly enhanced by deficiency of Slfn11 (Fig. 2.3b), although the intensity of the effect was less marked than for WNV and DENV ($DF=14$, $F=3.44$, $P<0.0009$). Nevertheless, a significant difference of approximately 7-fold higher titer was observed in A172-KD versus the control cells from 24 to 48 hrs post-infection. Therefore, Slfn11 restricts ZIKV replication in contrast to Slfn13[73].

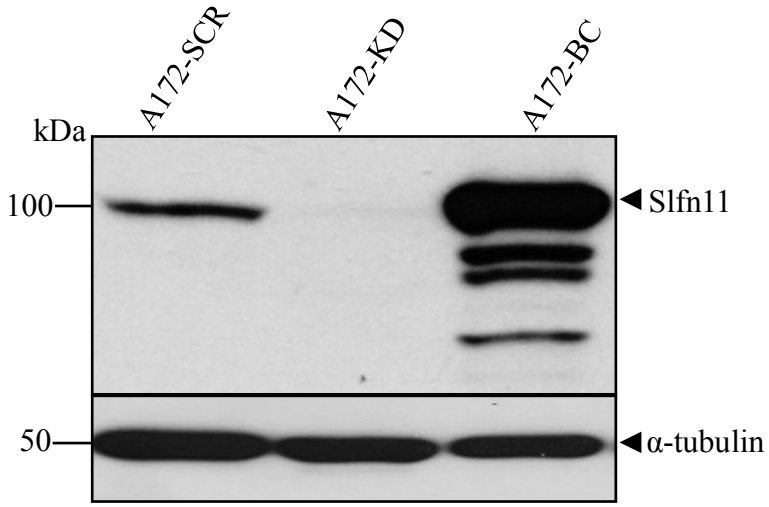
For comparison with patterns of (+) and (-)ssRNA virus replication, we also tested the impact of Slfn11 knockdown and overexpression on the replication of the (-)ssRNA viruses VSV and MP12-RVFV. Replication of both viruses was very robust in A172 cells showing a kinetic similar to that of WNV with a peak of viral replication 24 hrs post-infection (Fig. 2.3c-d). However, the replication of these (-)ssRNA viruses was very similar in terms of kinetics and titers

among the different A172-derived cell lines evaluated, despite their differences in Slfn11 expression. Therefore, there was no interaction between the effects of cell line and time p.i. on virus titer (VSV, DF=12, F=0.21, P=0.9963; RVFV, DF=12, F=0.74, P=0.6974).

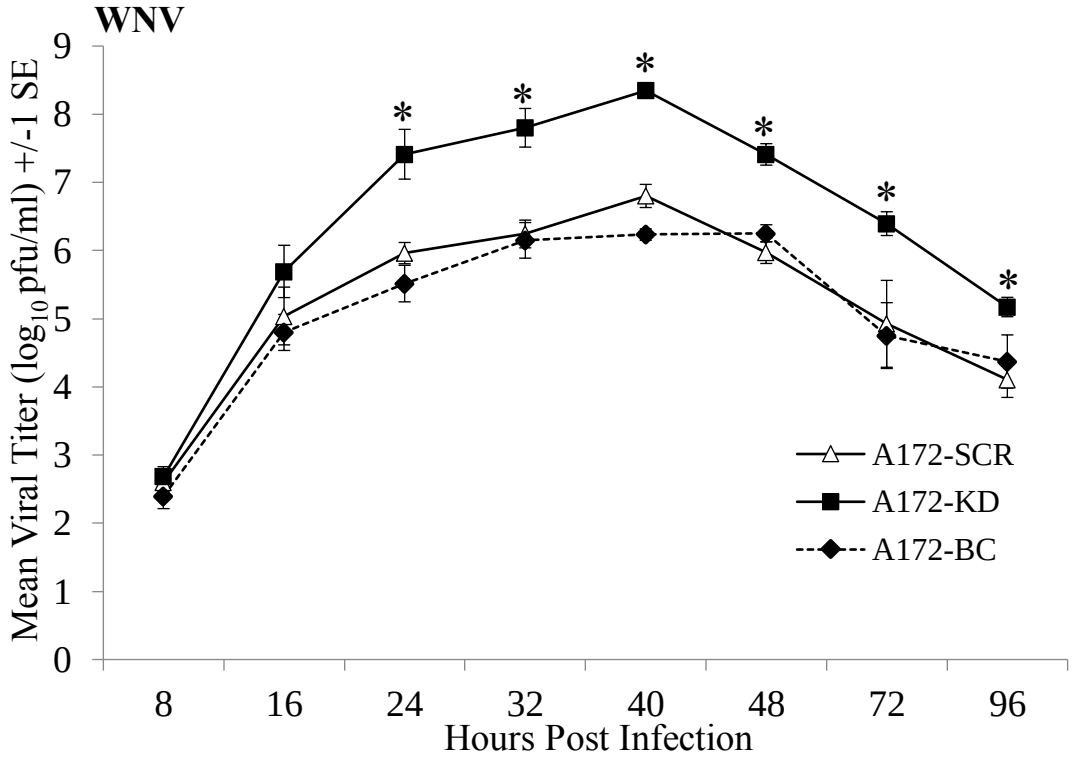
The VSV used in these experiments is a recombinant virus expressing eGFP[82]. Therefore, we also evaluated the titer of this virus obtained in the supernatant of the different A172-derived cell lines by flow cytometry analysis. A172-SCR and -KD cells were infected with three different MOIs of VSV (0.1, 0.3, and 1) in triplicates and viral supernatant was collected 24 hrs later. Then, SupT1 cells were infected with the viral supernatants and evaluated 24 hrs after infection by FACS analysis. In accordance with the plaque assay experiments, we observed a similar titer for the virus obtained from A172-SCR (8.55×10^4 +/- 0.84) and -KD (8.58×10^4 +/- 0.91) cells. In summary, data in Figs 2.3c-d indicated that Slfn11 did not influence the replication of the (-)ssRNA VSV and RVFV, highlighting the specificity of the antiviral activity of this protein for (+)ssRNA viruses and lentiviruses[69,71].

From the results presented about it is also noteworthy that despite A172-BC cells expressing markedly higher levels of Slfn11 than A172-SCR cells (Fig. 2.2a), replication of flaviviruses was similar in these two cell lines (Figs. 2.2b and 2.3a-b), indicating that above certain levels the antiviral effect of Slfn11 reaches saturation.

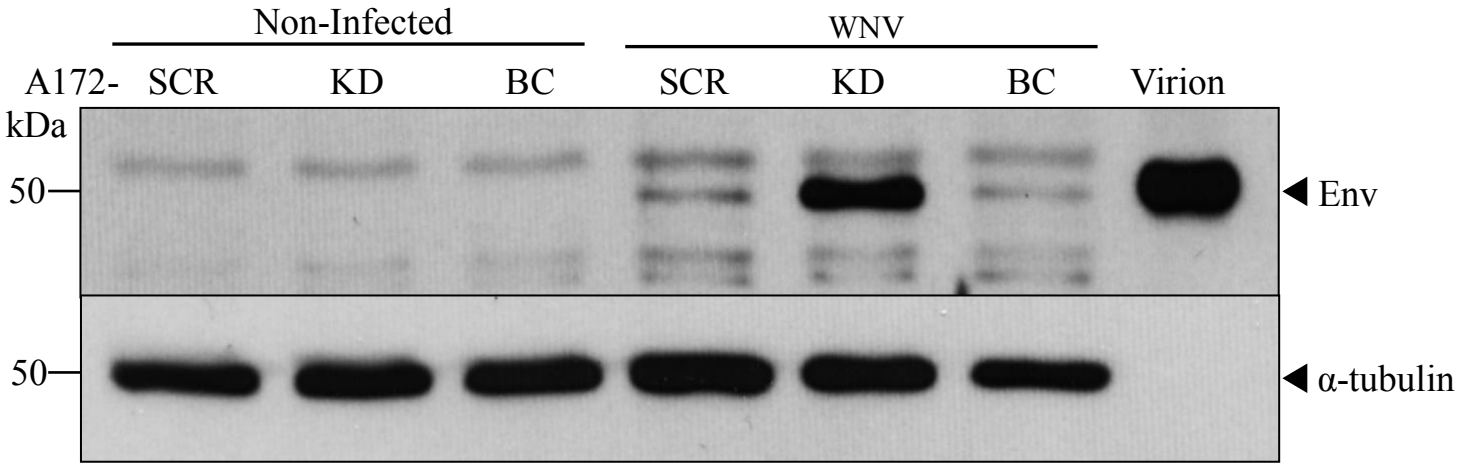
a


A172-SCR
A172-KD
A172-BC
kDa
100
50
Slfn11
α-tubulin

b


WNV
Mean Viral Titer (log10 pfu/ml) +/-1 SE
0
1
2
3
4
5
6
7
8
9
8
16
24
32
40
48
72
96
Hours Post Infection
A172-SCR
A172-KD
A172-BC
*

c


Non-Infected
WNV
A172-
SCR
KD
BC
SCR
KD
BC
Virion
kDa
50
Env
50
α-tubulin

d

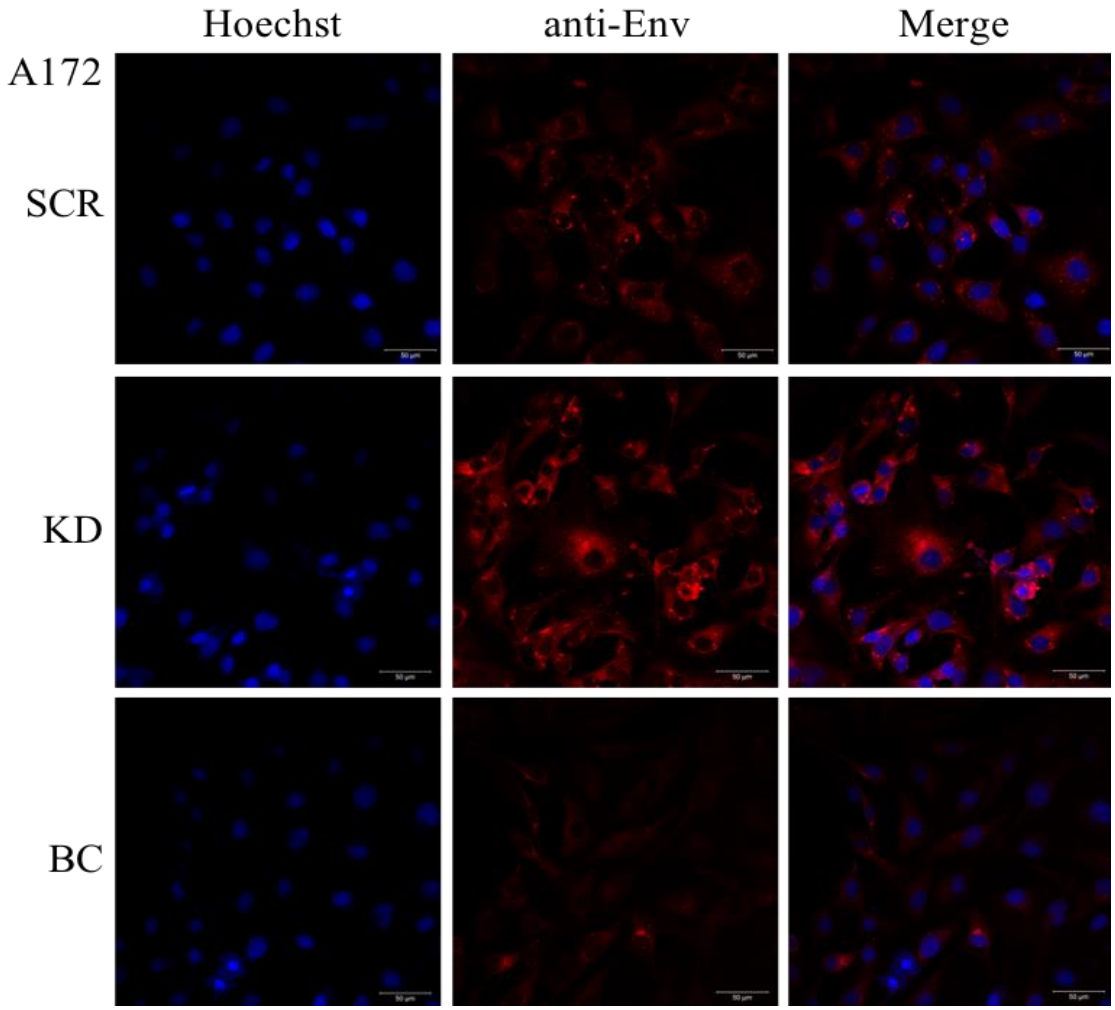
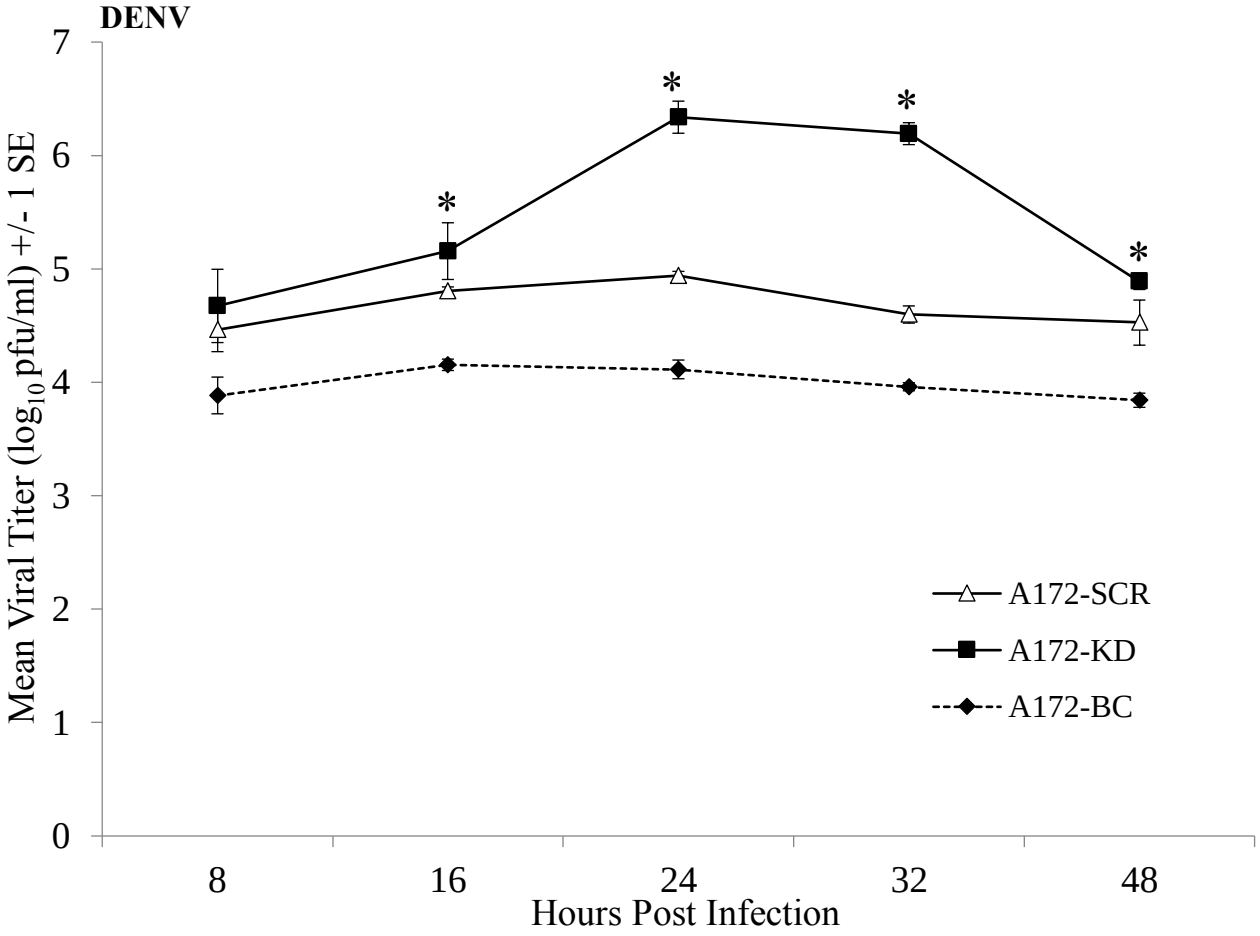

Hoechst
anti-Env
Merge
A172
SCR
KD
BC

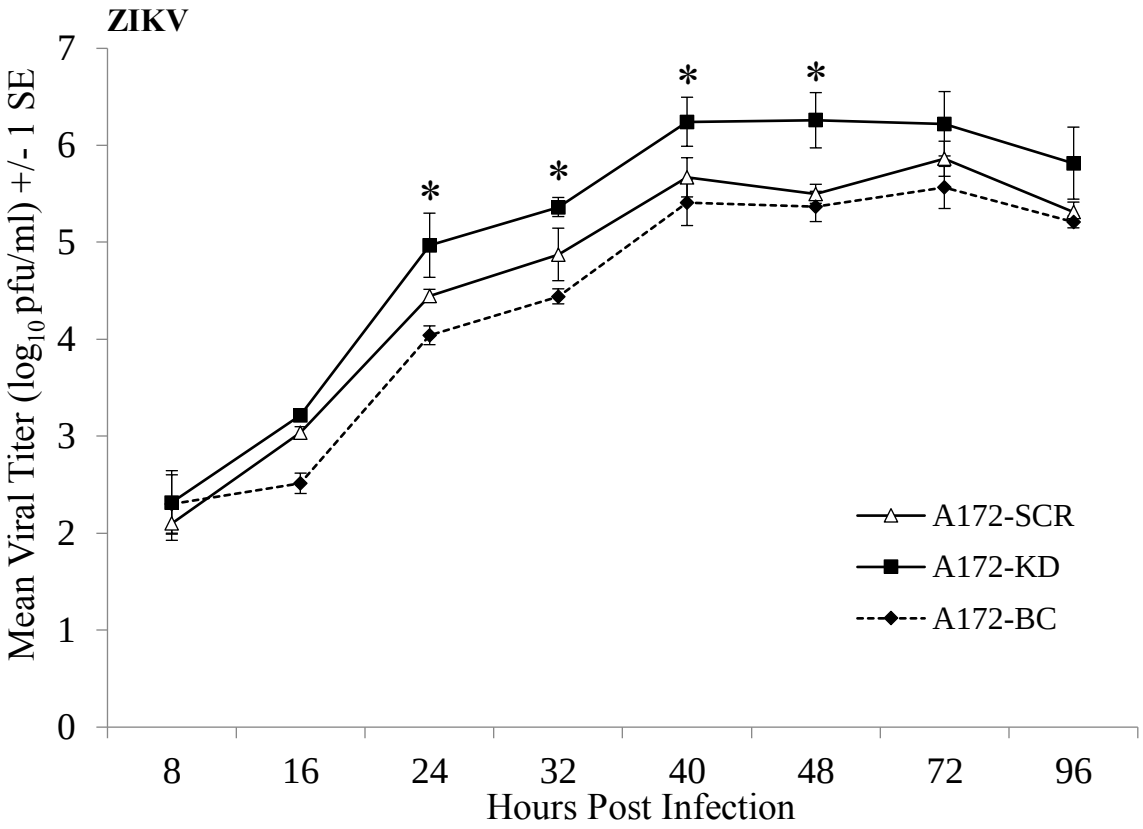
Figure 2.2 Effect of Slfn11 on WNV replication.

(a) Immunoblot analysis of the expression of Slfn11 in A172 cells stably expressing shRNAs directed against Slfn11 (A172-KD) or a scrambled (A172-SCR) RNA sequences and A172-KD cells engineered to re-express Slfn11 (A172-BC). α-tubulin was detected as a loading control. (b) WNV replication in A172-derived cells. A172-SCR (open triangles), A172-KD (filled squares) and A172-BC (filled diamonds) cells were infected with WNV (MOI 0.1) and viral replication was determined by quantification of the viral titer in the cell supernatant at different hours post-infection by plaque assay. Statistically significant differences were calculated with repeated measures ANOVA and Tukey-Kramer post-hoc tests and they are indicated with asterisks. Mean and standard deviation values represent the variability of the viral titer found in triplicate plaque assays of samples from 8 independent infection experiments performed in different days with different viral preparations. (c) Expression of WNV Env in cells infected at MOI 0.1 evaluated by immunoblot 40 hrs after infection. α-tubulin was detected as a loading control. These results are representative of 3 independent infections. (d) Expression of WNV Env (red) as detected by indirect immunofluorescence analysis of cells infected at a MOI of 1 48 hrs post-infection. Nuclei were labeled with Hoechst (blue).

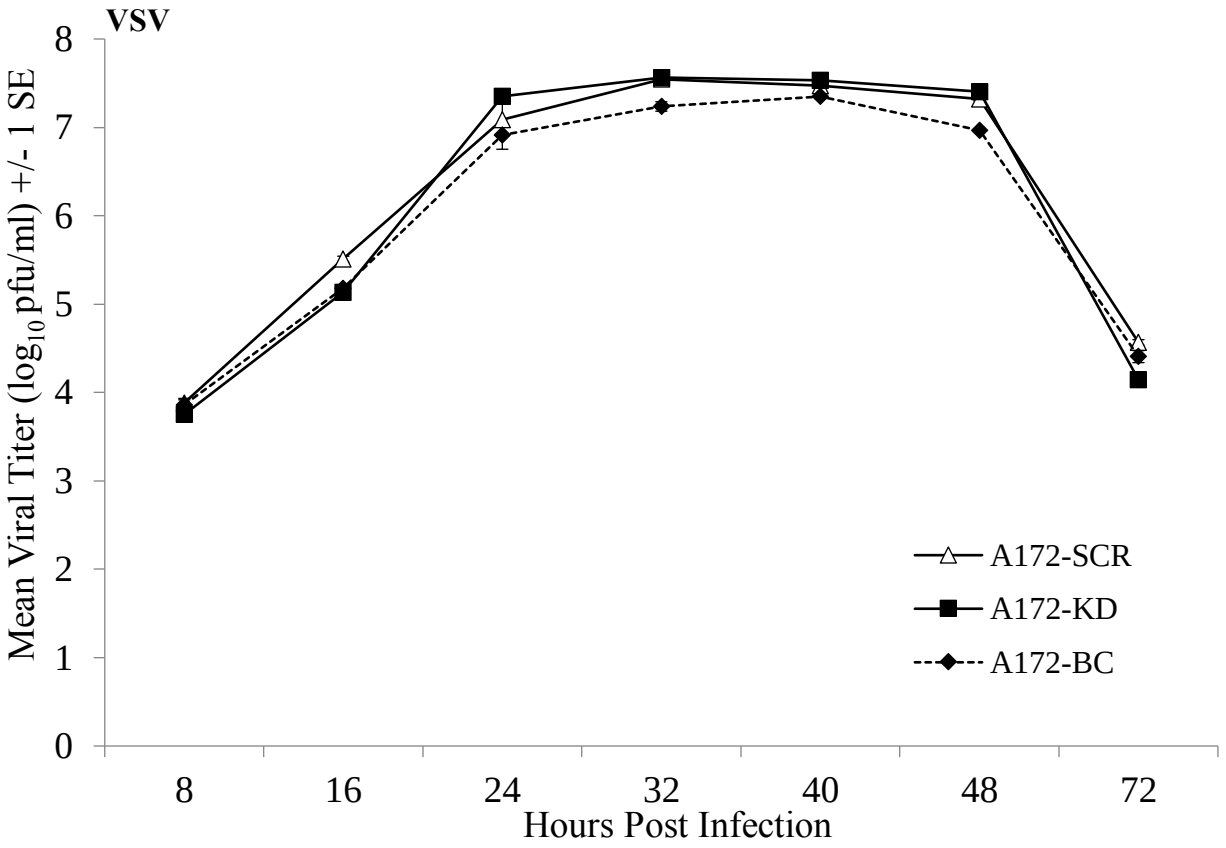
a


DENV
Mean Viral Titer (log_{10} pfu/ml) +/- 1 SE
0
1
2
3
4
5
6
7
8
16
24
32
48
Hours Post Infection
*
*
*
*
A172-SCR
A172-KD
A172-BC

b


ZIKV
Mean Viral Titer (log_{10} pfu/ml) +/- 1 SE
0
1
2
3
4
5
6
7
8
16
24
32
40
48
72
96
Hours Post Infection
*
*
*
*
A172-SCR
A172-KD
A172-BC

c


VSV
Mean Viral Titer (log_{10} pfu/ml) +/- 1 SE
0
1
2
3
4
5
6
7
8
8
16
24
32
40
48
72
Hours Post Infection
A172-SCR
A172-KD
A172-BC

d

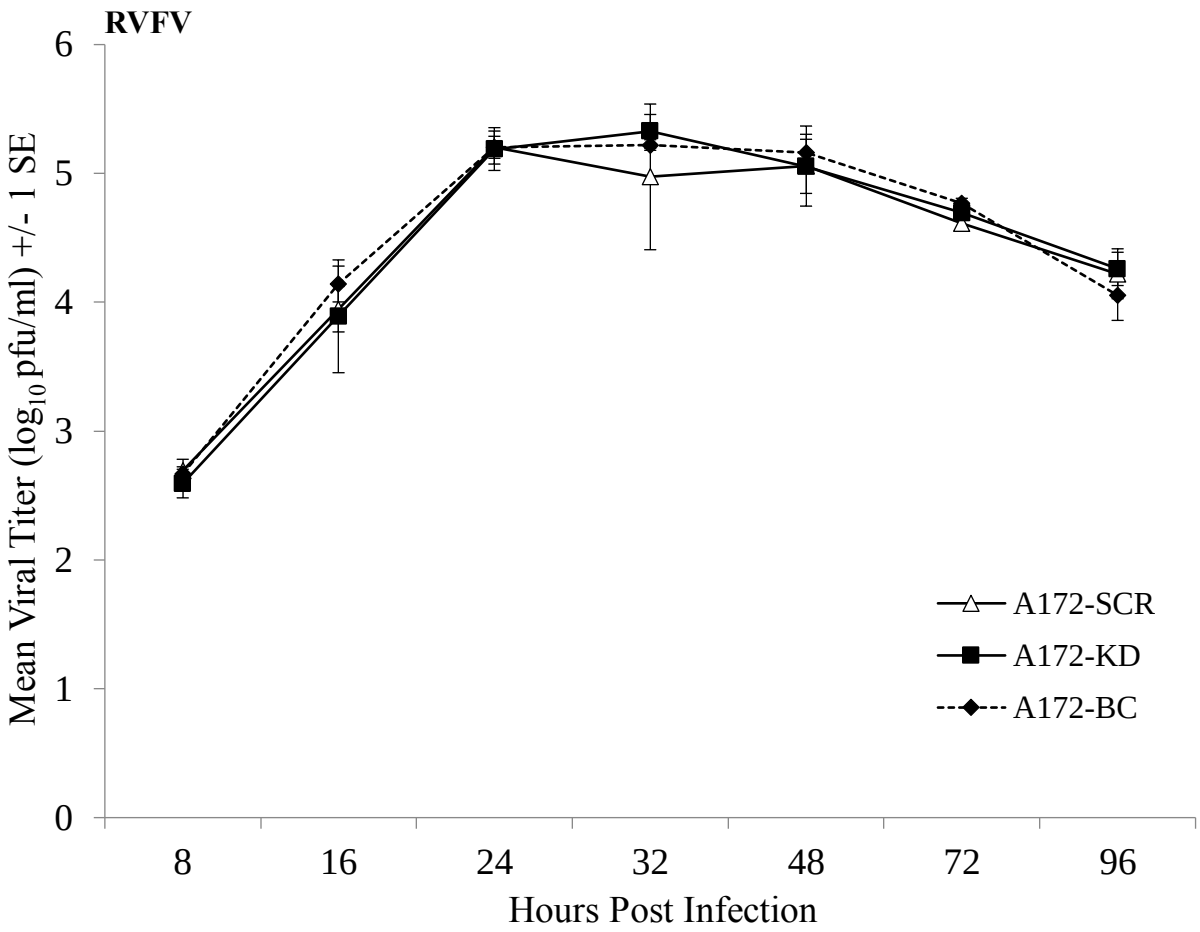

RVFV
Mean Viral Titer (log_{10} pfu/ml) +/- 1 SE
0
1
2
3
4
5
6
8
16
24
32
48
72
96
Hours Post Infection
A172-SCR
A172-KD
A172-BC

Figure 2.3 Effect of Slfn11 on viral replication.

A172-SCR (open triangles), A172-KD (filled squares) and A172-BC (filled diamonds) cells were infected with (a) Dengue Virus (DENV, MOI 0.1), (b) Zika Virus (ZIKV, MOI 0.1), (c) Vesicular stomatitis virus (VSV, MOI 0.1), and (d) Rift Valley fever virus (RVFV, MOI 0.1). Viral replication was determined by quantification of the viral titer in the cell supernatant at different hours post-infection by plaque assay. Statistically significant differences are indicated with asterisks and were calculated as described above. Mean and standard deviation values of each graphic represent the variability of the viral titer found in triplicate plaque assays of samples from 3 independent infection experiments performed in different days with different viral preparations.

2.3.3 Mutagenesis Analysis of Slfn11 Antiviral Activity

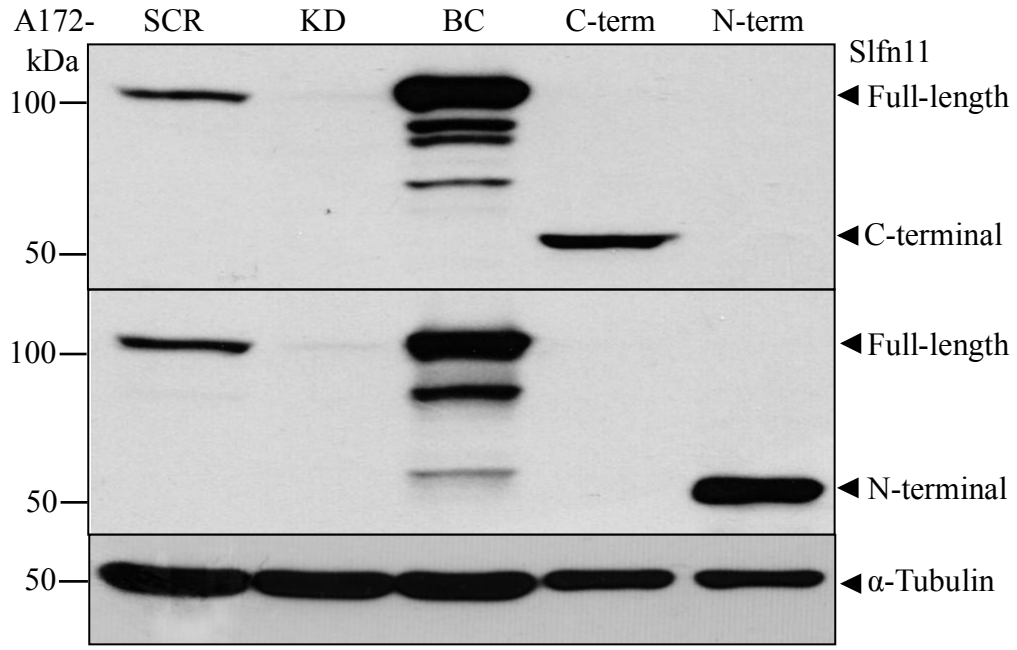
The N-terminal region of Slfn11 and Slfn13 harbors the anti-HIV-1 activity of these proteins[69,73]. Not surprisingly, in both proteins this region contains residues that are responsible for the tRNA nucleolytic activity in Slfn13[73], which is central in the anti-HIV-1 activity of these proteins. Therefore, we followed a previously described strategy[69] to determine whether the anti-WNV activity of Slfn11 also resides in the N-terminal region.

The N-terminal (amino acids 1-441) and the C-terminal (amino acids 442-901) regions of Slfn11 were expressed in A172-KD cells to generate A172-N-term and A172-C-term cell lines (Fig. 2.4a) and their susceptibility to WNV was evaluated (Fig. 2.4b). Cells were infected with WNV at MOI 0.1 and viral replication was followed by plaque assay, as described above. WNV replication was impaired in cells expressing N-terminal Slfn11. Viral replication was similar in these cells and in A172-BC cells that express full-length Slfn11. However, WNV replication was significantly higher in cells expressing Slfn11 C-terminus ($DF=14$, $F=11.69$, $P<0.0001$, Fig. 2.4b). Therefore, these findings suggest a common antiviral mechanism of action of Slfn11 against HIV-1[69] and WNV.

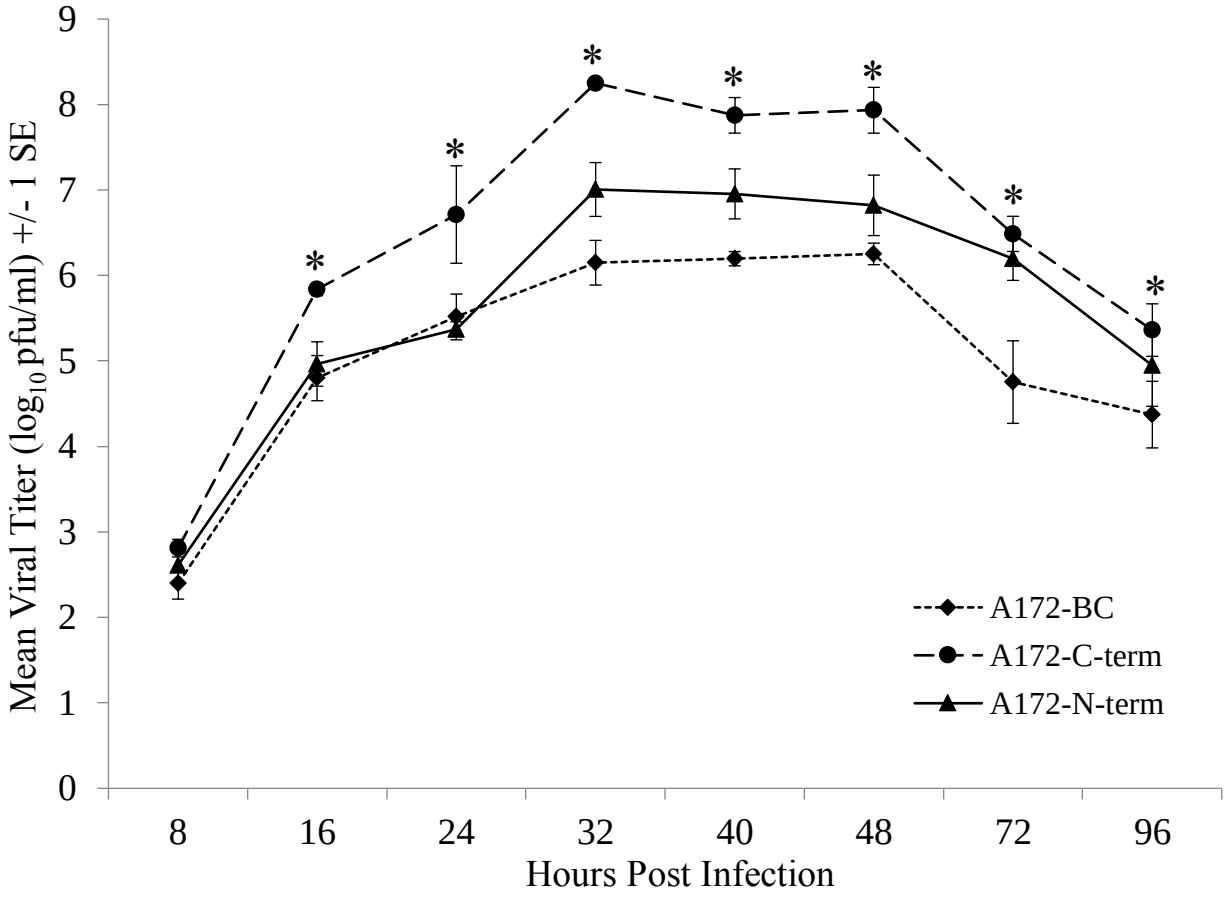
In order to define whether the subcellular distribution impacts the antiviral activity of Slfn11, we determined the localization of Slfn11 full-length and truncation mutants. A172-BC, -N-term and -C-term cells were stained with anti-Slfn11 antibodies directed against the N-terminus or the C-terminus of the protein and Slfn11 cellular distribution was determined by confocal microscopy analysis. Full-length Slfn11 accumulates in the nucleus, distributing homogenously in this organelle (Fig. 2.4c). However, both the C- and N-terminal Slfn11 proteins were uniformly distributed in the cytoplasm of the cell (Fig. 2.4c). The lack of association of antiviral activity and subcellular distribution of Slfn11 suggests that the process targeted by this protein is accessible in

both the nucleus and the cytosol. In addition, these data confirm that the inactivity of the C-terminal region of Slfn11 is not determined by miss localization or a gross defect in intracellular solubility of this protein, for example due to the formation of protein aggregates.

a



b


Mean Viral Titer (log_{10} pfu/ml) +/- 1 SE
0
1
2
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4
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8
9
8
16
24
32
40
48
72
96
Hours Post Infection
A172-BC
A172-C-term
A172-N-term
*
*
*
*
*
*
*

c

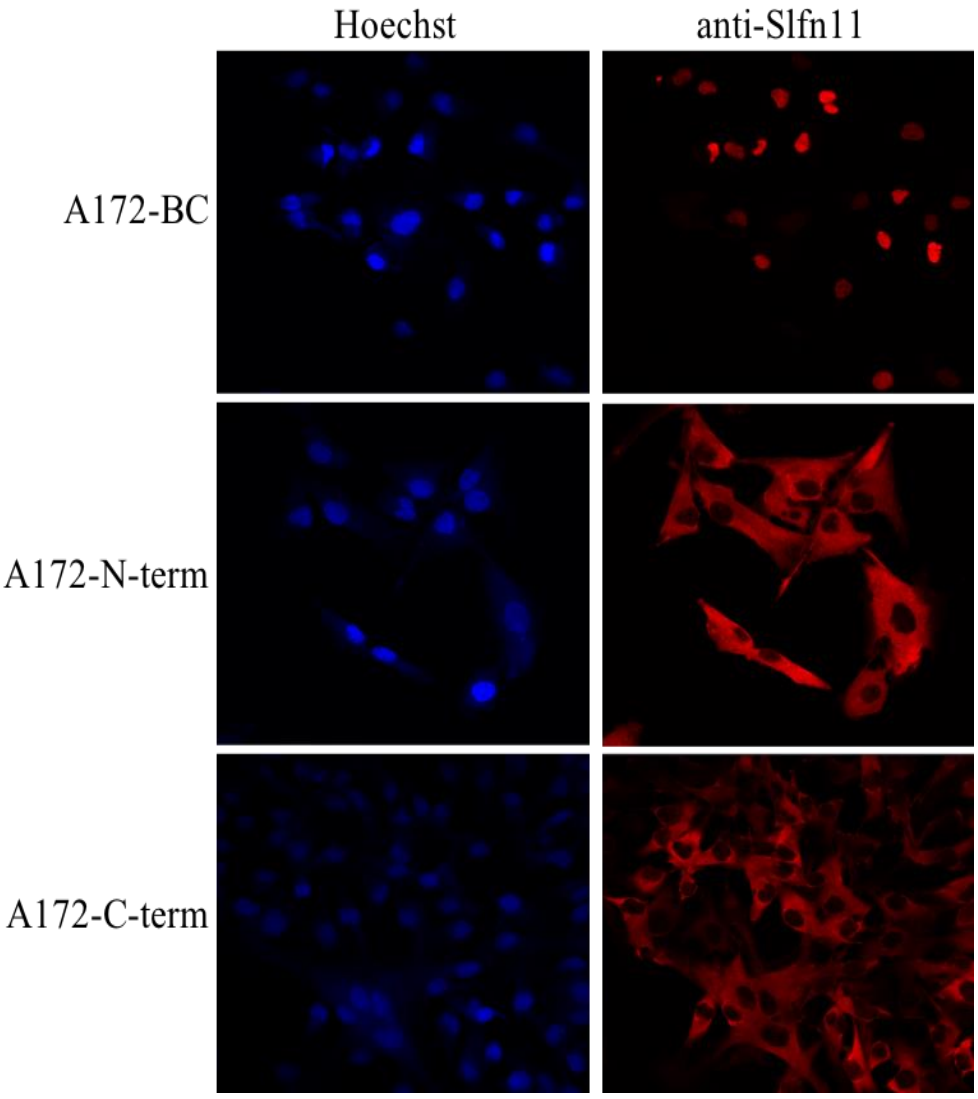


Figure 2.4 Mutagenesis analysis of antiviral activity of Slfn11.

(a) Immunoblotting analysis of the expression of Slfn11 in A172-derived cells. A172-KD cells were engineered to express the N- or C-terminus of Slfn11. A172-SCR, -KD, and BC were used as control. Different anti-Slfn11 antibodies were employed to identify the mutant proteins. α-tubulin was detected as a loading control. (b) A172-BC (filled diamonds), A172-N-term (filled triangles), and A172-C-term (filled circles) were infected with WNV (MOI 0.1) and viral replication was determined by quantification of the viral titer in the cell supernatant at different hours post-infection. Statistically significant differences were calculated as described above and are indicated with asterisks. Mean and standard deviation values represent the variability of the viral titer found in triplicate plaque assays of samples from 3 independent infection experiments. (c) Cellular distribution of Slfn11 full-length and deletion mutants. A172-BC, A172-N-term, and A172-C-term cells were fixed/permeabilized and stained with the anti-Slfn11 antibodies used in panel (a). Cell nuclei were identified with Hoechst staining (Blue staining).

2.3.4 Effect of WNV Infection and Slfn11 Expression on the tRNA Repertoire of A172 Cells

A previous report indicated that Slfn11 counteracts HIV-1-induced increase of tRNA abundance, affecting viral protein expression[69]. Therefore, we explored whether WNV infection also induces changes in the tRNA repertoire and whether these changes are opposed by Slfn11. A172-SCR, -KD, and -BC cells were infected with WNV at a MOI of 1 and their tRNA repertoire was determined 8 hrs later by tRNA PCR array, as previously described[69,89]. To ensure data robustness, the tRNA repertoire of A172-derived cells infected and non-infected was determined by using RNA obtained from three sets of independent experiments. We chose to evaluate the tRNA repertoire at 8 hrs post-infection because at this early time point of the life cycle the WNV was already replicating (Fig. 2.1a and 2.2b) and the infected cells exhibit similar viral loads (Fig. 2.2b), were not producing type I IFN (Fig. 2.1c) nor undergoing cell death (data not shown). Therefore, modifications in the tRNA pool at 8 hrs post-infection is likely be a direct consequence of the infection of these cells with similar amount of virus. Furthermore, at 8 hrs post-infection we expected WNV replication to be very sensitive to the efficiency of translation due to the low availability of translation competent viral RNA molecules caused by the low rate of synthesis of viral RNA characteristic of the early life cycle[51].

Comparison of the levels of 66 nuclear tRNAs in infected and non-infected A172-KD cells indicated that WNV infection decreased the expression of 10 tRNAs while 7 were up-regulated (Fig. 2.5a). The limited effect of WNV on the tRNA pool contrasted with the global up-regulation of tRNAs triggered by HIV-1 in cells expressing low levels of Slfn11[69]. Out of these 17 tRNAs, 9 were down-regulated and 1 was up-regulated by WNV infection in A172-KD cells (Fig. 2.5b), and none of these 10 tRNAs were modified in either in A172-SCR or -BC when infected cells and non-

infected cells were compared. Then, these WNV-induced tRNA changes were considered Slfn11-specific (Fig. 2.5b).

Notably, the tRNAs down-regulated by WNV in Slfn11-deficient cells (Fig. 2.5b) were implicated in decoding 19.2% of the viral codons, while those found up-regulated corresponded only to 7.7%. Analysis of the impact of tRNA changes on codons that WNV uses disproportionately more than human cells (rare codons, 12.3 % of the WNV codons) indicated that the cognate tRNA of 28.5 % of them were decreased by WNV infection in A172-KD cells, representing 3.5% of all the WNV codons. The codons affected by tRNA reductions were randomly distributed along the viral genome.

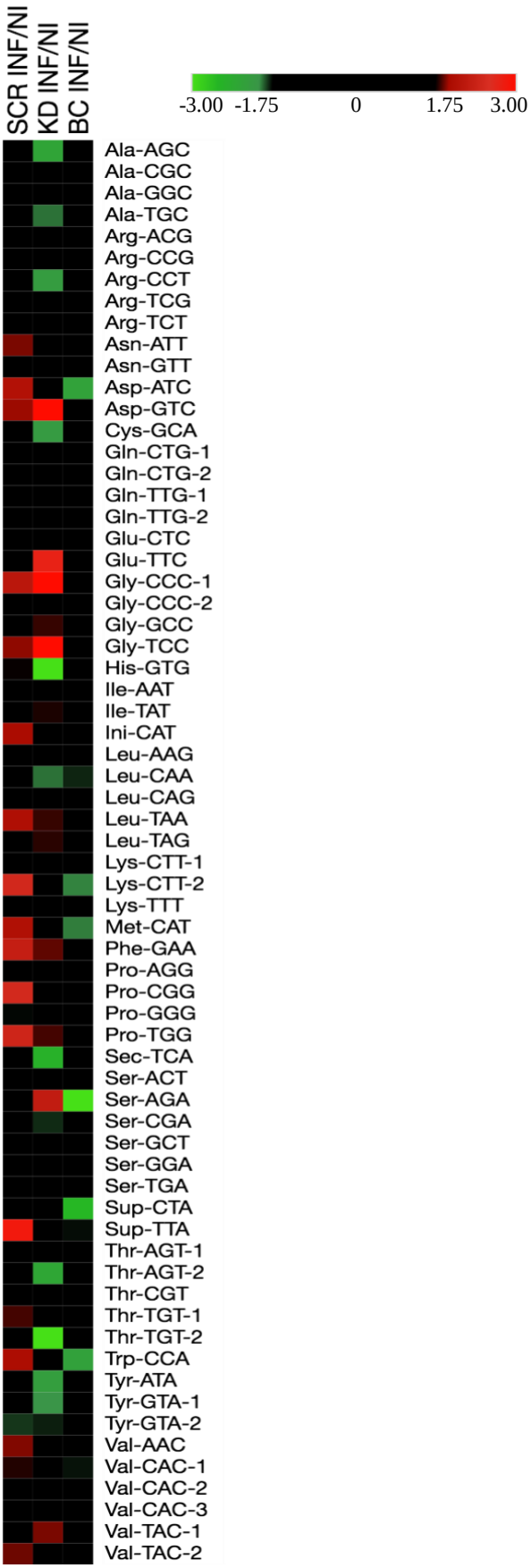
WNV infection did not consistently down- or up-regulate any of the 66 tRNAs analyzed in both A172-SCR and -BC cells (Fig. 2.5a). Instead, we noticed that tRNAs have a tendency to be up-regulated in A172-SCR and down-regulated in A172-BC following WNV infection. In A172-SCR cells 17 tRNAs increased; however, in A172-BC eight of them did not change and the other four decreased (Fig. 2.5a). This inconsistency suggests that these changes were independent of Slfn11. Alternatively; it could reflect a higher Slfn11 tRNA nucleolytic activity in A172-BC than in A172-SCR cells, as the engineered cells express more Slfn11 than A172-SCR cells (Fig. 2.2a). Nevertheless, WNV replicated similarly in A172-SCR and -BC cells, indicating that these changes in tRNA abundance did not impact WNV replication. Furthermore, analysis of the impact of tRNA down-regulation in A172-BC on the WNV polyprotein indicated that tRNAs in this group that exhibit statistically significant changes decode only common codons that represent 8.7% of the WNV genome (Fig. 2.5c).

WNV infection also modulated the expression of tRNAs that reprogram stop codons to encode specific amino acids (Fig. 2.5a). Genetic code reprogramming allows translation beyond

stop codons producing fusion proteins[91]. WNV infection decreased the levels of the $tRNA^{Sec}$(TCA) in A172-KD cells but not in cells expressing Slfn11, indicating that this change was Slfn11-specific. This tRNA incorporates selenocysteine in selenoproteins at the UGA stop codon. Furthermore, WNV infection diminished the expression of $tRNA^{Sup}$(CTA) in A172-BC cells that redefines the stop codon UAG (Amber suppressor); however, this change was not observed in A172-SCR cells that also express Slfn11.

In conclusion, the changes in the tRNA repertoire inflicted by WNV infection suggests that the virus will encounter more restrictions to protein translation in Slfn11-deficient cells than in cells expressing this protein. This seems contradictory with the more robust WNV replication observed in these cells. However, multiple lines of evidence (discussed later) indicate that a reduced availability of cognate tRNAs pauses translation at elongation favoring either protein production and/or optimal protein folding[92–100]. The later mechanism enhances viral fitness[101].

a


SCR INF/NI
KD INF/NI
BC INF/NI
-3.00 -1.75 0 1.75 3.00
Ala-AGC
Ala-CGC
Ala-GGC
Ala-TGC
Arg-ACG
Arg-CCG
Arg-CCT
Arg-TCG
Arg-TCT
Asn-ATT
Asn-GTT
Asp-ATC
Asp-GTC
Cys-GCA
Gln-CTG-1
Gln-CTG-2
Gln-TTG-1
Gln-TTG-2
Glu-CTC
Glu-TTC
Gly-CCC-1
Gly-CCC-2
Gly-GCC
Gly-TCC
His-GTG
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Ile-TAT
Ini-CAT
Leu-AAG
Leu-CAA
Leu-CAG
Leu-TAA
Leu-TAG
Lys-CTT-1
Lys-CTT-2
Lys-TTT
Met-CAT
Phe-GAA
Pro-AGG
Pro-CGG
Pro-GGG
Pro-TGG
Sec-TCA
Ser-ACT
Ser-AGA
Ser-CGA
Ser-GCT
Ser-GGA
Ser-TGA
Sup-CTA
Sup-TTA
Thr-AGT-1
Thr-AGT-2
Thr-CGT
Thr-TGT-1
Thr-TGT-2
Trp-CCA
Tyr-ATA
Tyr-GTA-1
Tyr-GTA-2
Val-AAC
Val-CAC-1
Val-CAC-2
Val-CAC-3
Val-TAC-1
Val-TAC-2

b

Amino Acid	tRNA	Codon	Fold changes	p-value	Codon Usage
Ala	AGC	GCU	-2.11	0.0256	26.5
Ala	TGC	GCA	-1.69	0.0138	19.9
		GCG			10.20
Arg	CCT	AGG	-1.92	0.0136	14
Cys	GCA	UGC	-1.88	0.0012	10*
		UGU			8.1*
Glu	TTC	GAA	2.69	0.005	30.2
		GAG			31.5
His	GTG	CAC	-8.63	0.0091	11.1
		CAU			7.5*
Thr	AGT-2	ACU	-2.16	0.0082	15.3
Thr	TGT-2	ACA	-9.8	0.0064	22.6
		ACG			11.8
Tyr	ATA	UAU	-1.96	0.003	9.6*
Tyr	GTA-1	UAC	-1.75	0.038	16
		UAU			9.6

c

Amino Acid	tRNA	Codon	Fold changes	p-value	Codon Usage
Asp	ATC	GAU	-2.74	0.0422	21.5
Lys	CTT-2	AAG	-3.30	0.0439	32
Ser	AGA	UCU	-4.91	0.0283	6.4
Trp	CCA	UGG	-2.02	0.0369	27

Figure 2.5 Effect of WNV infection on the tRNA pool of Slfn11-deficient and control cells.

(a) Heat map of fold changes of the 66 tRNA quantified. Each cell represents the tRNA ratio of infected/non-infected cells for each of the three cell lines evaluated. tRNAs that increase (red cells), decrease (green cells) or do not change (black cells) are indicated. The cut-off was set at -1.75 to 1.75-fold change. tRNAs modulated in A172-KD cells by WNV infection. (b) WNV-induced, Slfn11-specific tRNA changes. Fold differences represents the A172-KD infected/non-infected tRNA ratio. (c) WNV-induced tRNA changes in A172-BC cells. Fold differences represents the A172-KD infected/non-infected tRNA ratio. In (b) and (c) the statistical significance of the changes in tRNA levels is represented by the calculated p values. The percentage of codons decoded by each tRNA is indicated. Rare codons are marked with an asterisk.

2.3.5 Lack of Antiviral Activity of Slfn11 in HEK293T, HeLa, and BHK-21 Cells

Slfn11 has been shown to mediate antiviral activity in cells expressing endogenous Slfn11 such as CEM, HEK293[69,81], and A172 cells (Fig. 2.2b). However, the anti-WNV activity of this protein has not been evaluated in cells that naturally lack the expression of Slfn11 under basal conditions and/or following type I IFN stimulation (Fig. 2.1d). Among them, we characterized the WNV-permissive cell lines HEK293T, HeLa, and baby hamster kidney fibroblasts (BHK-21). These cell lines were engineered to express Slfn11 (HEK293T^{Slfn11}, HeLa^{Slfn11}, and BHK-21^{Slfn11} cells) (Fig. 2.6a) and then used in viral infection experiments.

HEK293T-derived cells were infected with WNV at MOI 0.1 and viral replication was determined by plaque assay as described above. WNV replicated robustly in these cells (Fig. 2.6b-1) with a kinetic similar to the replication in A172 cells (Fig. 2.1a). However, WNV replication was indistinguishable between HEK293T cells expressing or not Slfn11 (Fig. 2.6b-1).

To further corroborate the lack of activity of Slfn11 in cells that do not naturally produce it, we evaluated the susceptibility of HEK293T and HEK293T^{Slfn11} cells to a single-round infection HIV-1-derived reporter virus (Hluc). This virus expresses p24 and luciferase proteins from the viral promoter[83]. Slfn11 has been reported to affect expression of codon-biased but not unbiased open reading frames[69,81]; therefore, we expect that HIV-1 p24, but not luciferase activity, will be affected by expression of Slfn11 in HEK293T cells.

HEK293T and HEK293T^{Slfn11} cells were infected with Hluc and luciferase activity and p24 levels were measured in cell lysates and in the cell supernatant of infected cells, respectively, 4 days after infection. As expected, we did not observe any significant effect of Slfn11 on luciferase expression. Cells expressing Slfn11 exhibited 1,740.4 +/- 121 arbitrary units (AU)/ml compared

to parental cells that produced 1,188.4+/- 94.2 AU/ml. However, both luciferase-normalized (Fig. 2.6b-2) and non-normalized (data not shown) HIV-1 p24 levels were not affected by Slfn11 expression in HEK293T cells.

In contrast to HEK293T cells, the parental cell line HEK293 expresses endogenous Slfn11 and supports the anti-HIV-1 activity of this protein[69]. Therefore, we determined the effect of Slfn11 on WNV replication in HEK293. Control and Slfn11-deficient cells (Fig. 2.6a) were infected with WNV at a MOI of 0.1 and viral replication was followed by plaque assay as described above. WNV replication was very robust in HEK293 cells; however, Slfn11 knockdown significantly enhanced viral replication ($DF=7$, $F=8.11$, $P<0.0001$, Fig. 2.6c). These results demonstrated the anti-WNV activity of Slfn11 in HEK293 cells.

As an additional control, we evaluated the effect of Slfn11 on HIV-1 in A172-derived cells. Contrary to HEK293T cells, A172 cells express endogenous Slfn11 (Fig. 2.1d). A172-SCR, -KD, and -BC cells were infected with Hluc and four days later luciferase activity was measured in cell lysates and the expression of HIV-1 p24 in the cell supernatant. In these experiments we found similar levels of luciferase activity among the different cell lines evaluated indicating that Slfn11 levels did not affect the expression of a codon unbiased HIV-driven gene. A172-SCR cells produced 1,072 +/- 15 AU/ml and A172-KD cells 1.227.5+/- 6.1 AU/ml, whereas A172-BC cells exhibited 1,289.4 +/- 11.1 AU/ml of luciferase activity. In contrast to HEK293T cells, luciferase-normalized (Fig. 2.6d) and non-normalized (data not shown) HIV-1 p24 expression was 2-fold higher in A172-KD cells than in -SCR and -BC cells. Thus, these results were in agreement with previously reported observations in CEM and HEK293 cells[69], indicating that Slfn11 restricts HIV-1 in A172 cells. Furthermore, results in figure 2.6d indicate that Slfn11 similarly impaired HIV-1 infection in A172-SCR and -BC cells despite their differences in Slfn11 expression levels (Fig.

2.2a). These findings are in agreement with the saturating effect that we described above for the anti-flavivirus activity of Slfn11 (Fig. 2.2), further supporting our hypothesis that Slfn11 is not the limiting factor of the antiviral pathway.

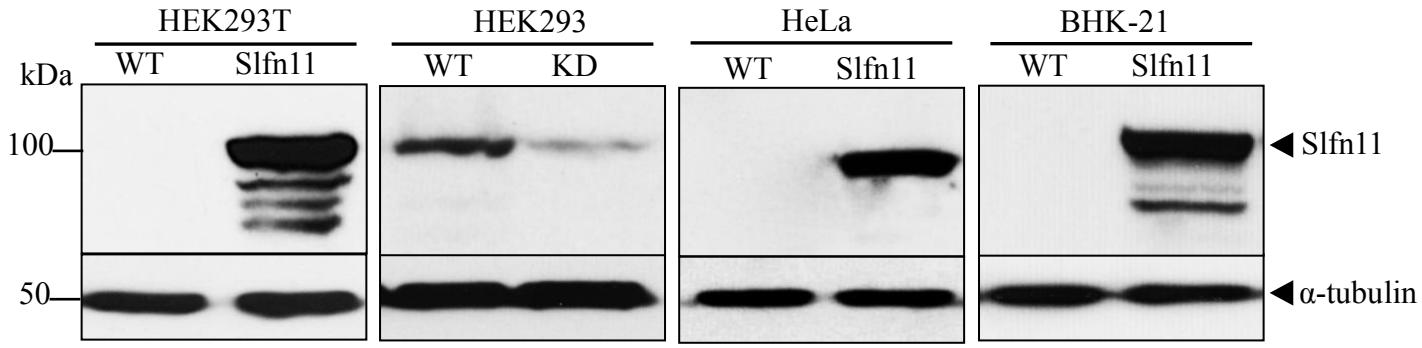
We hypothesized that the absence of antiviral activity of Slfn11 in HEK293T cells could be due to mislocalization of the exogenously expressed protein. Thus, we determined the subcellular distribution of Slfn11 in HEK293T^{Slfn11} cells by immunofluorescence analysis as described above. Similar to A172 cells (Fig. 2.4c), Slfn11 was localized entirely in the nucleus of HEK293T^{Slfn11} cells and protein aggregates were not observed (Fig. 2.6e). Therefore, these findings exclude mislocalization or protein aggregation as a cause for the lack of activity of Slfn11 in HEK293T cells.

Next, we explored the effect of Slfn11 on WNV replication in HeLa and BHK-21 cells following the procedures described above. HeLa and BHK-21 parental and cells engineered to express high levels of Slfn11 (HeLa^{Slfn11} and BHK-21^{Slfn11} cells, Fig. 2.6a) were infected with WNV at MOI 0.1 and viral replication was followed by plaque assay. Similarly to HEK293T cells, replication of WNV in HeLa and BHK-21 was very robust and exhibited a kinetic comparable to the replication in A172 cells (Fig. 2.6f). However, in contrast to A172 cells, we did not find any differences in viral replication in HeLa or BHK-21 cells expressing or not Slfn11 (HeLa: DF=6, F=2.42, P=0.0562; BHK-21: DF=7, F=0.65, P=0.711; Fig. 2.6f).

Furthermore, we determined the effects of Slfn11 on single-round HIV-1 infection in HeLa cells as described earlier. Similarly to the previous experiments we did not observe differences in the luciferase levels of HeLa cells expressing or not Slfn11. Parental cells expressed 18.73 +/- 2.63 AU/ml while cells engineered to express Slfn11 have 16.97 +/- 1.59 AU/ml. Importantly, HIV-1 p24 levels were also similar in the HeLa cell lines either prior or after luciferase normalization

(Fig. 2.6g), indicating that Slfn11 did not impair HIV-1 p24 production in HeLa cells. Therefore, our data indicated that HEK293T, BHK-21, and HeLa cells do not support the antiviral activity of exogenously expressed Slfn11. These results further support our hypothesis that Slfn11 is not the only component of this antiviral pathway.

a


HEK293T
HEK293
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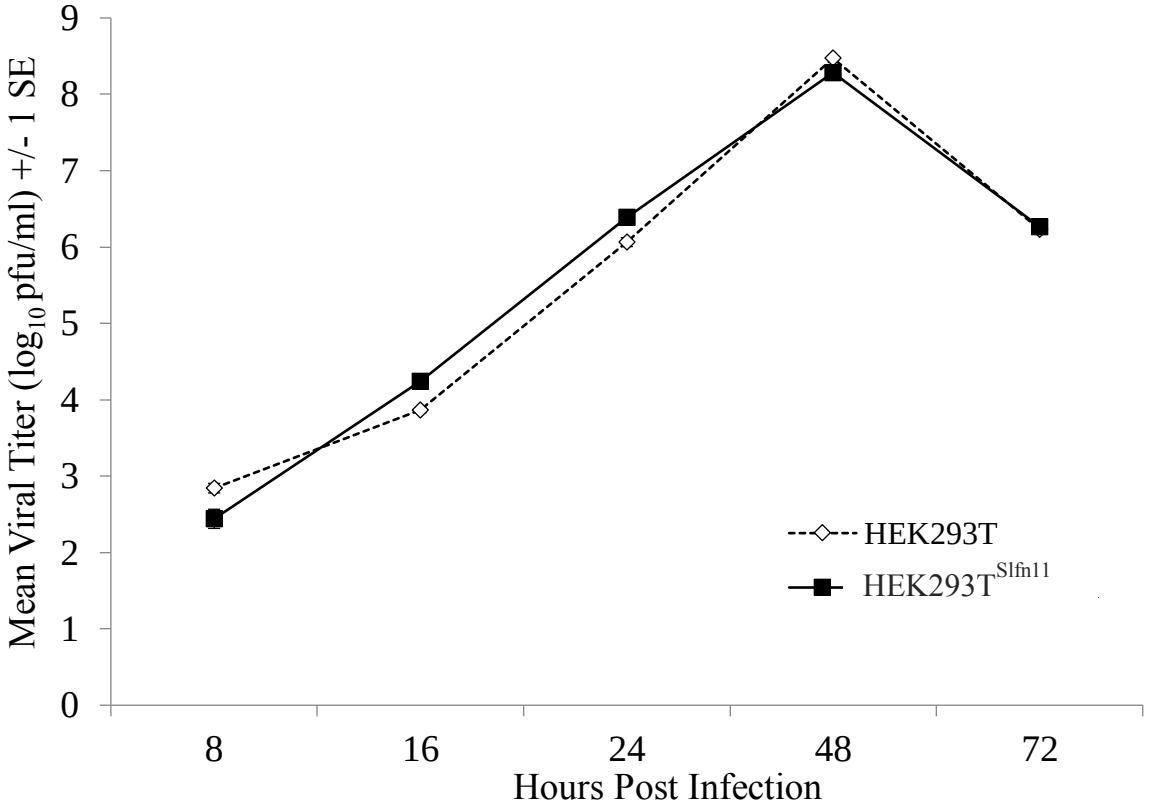
50
Slfn11
α-tubulin

b-1


Mean Viral Titer (log10 pfu/ml) +/- 1 SE
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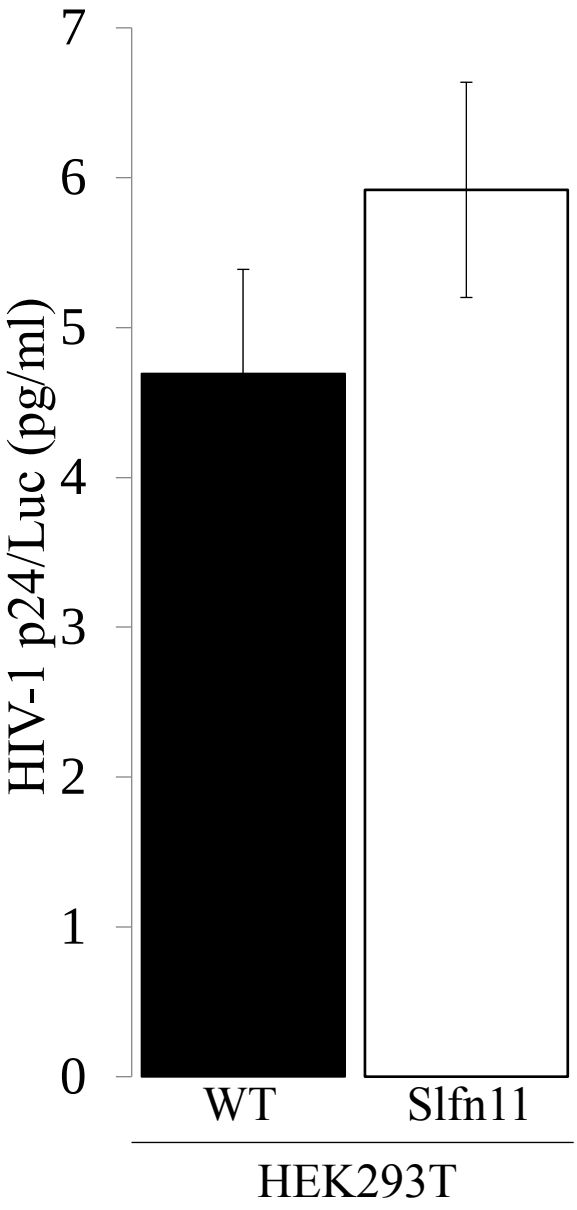
Hours Post Infection
HEK293T
HEK293T Slfn11

b-2



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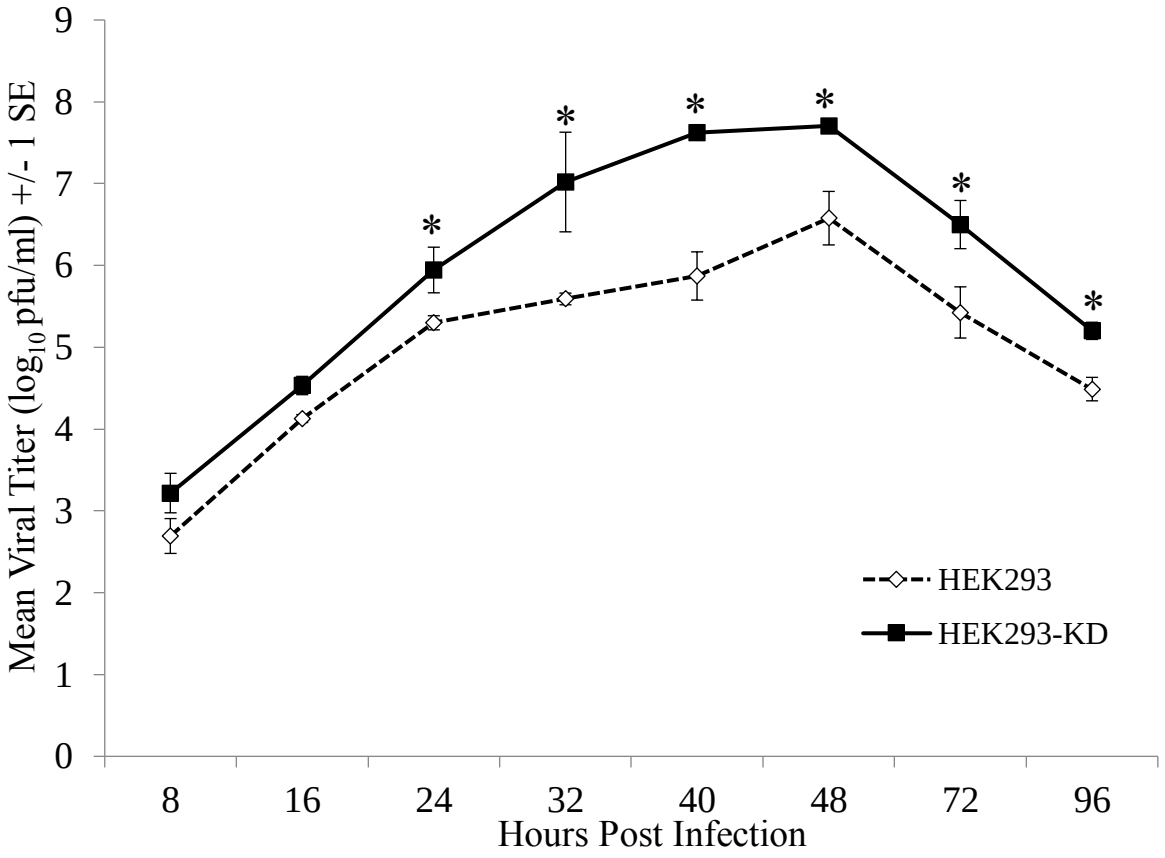
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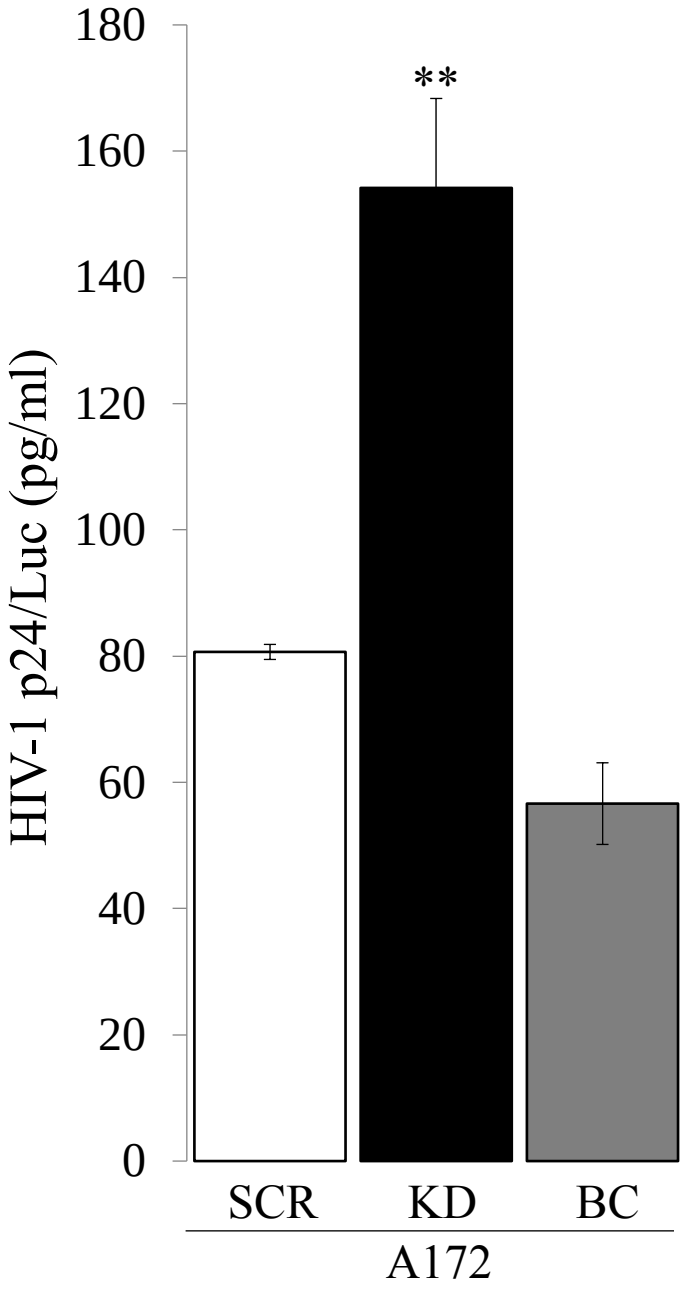

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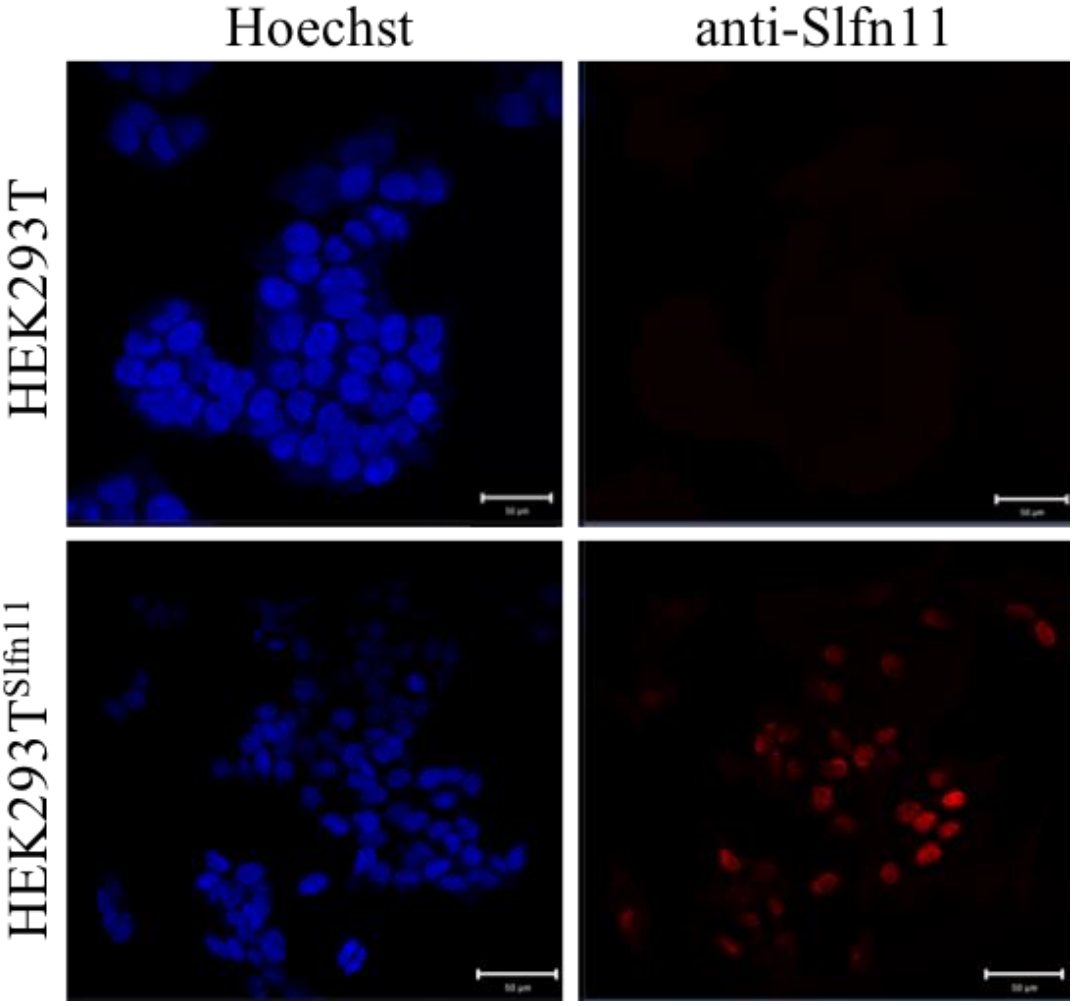
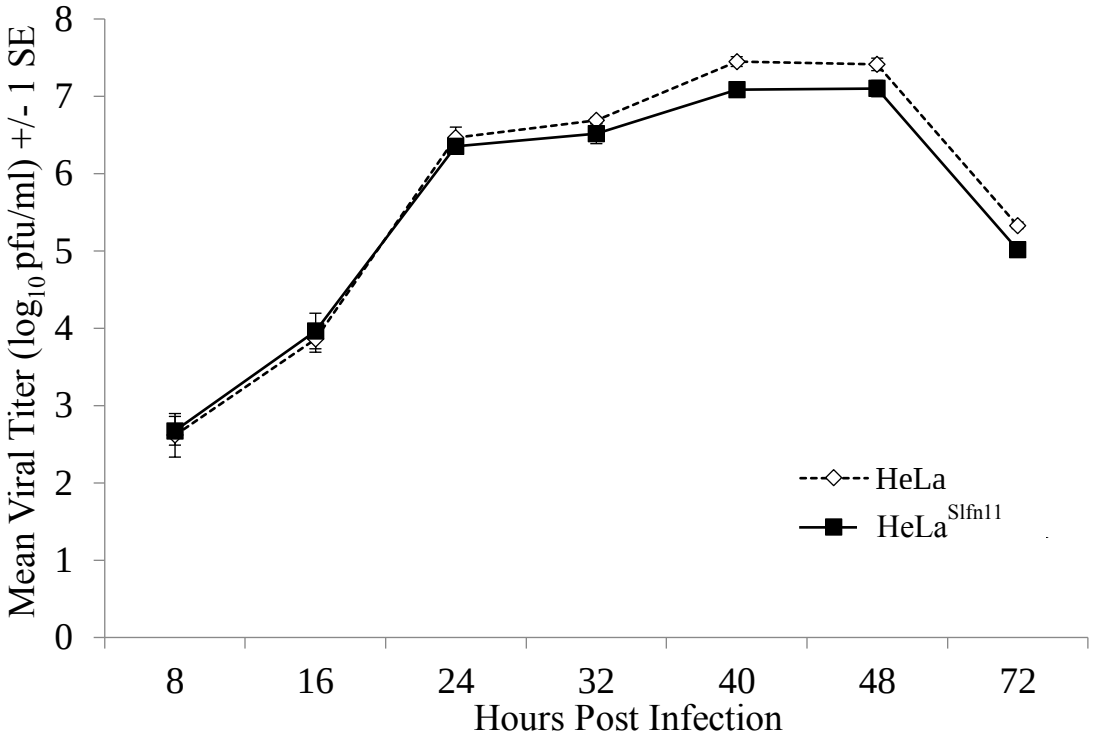


f


Mean Viral Titer (log_{10} pfu/ml) +/- 1 SE
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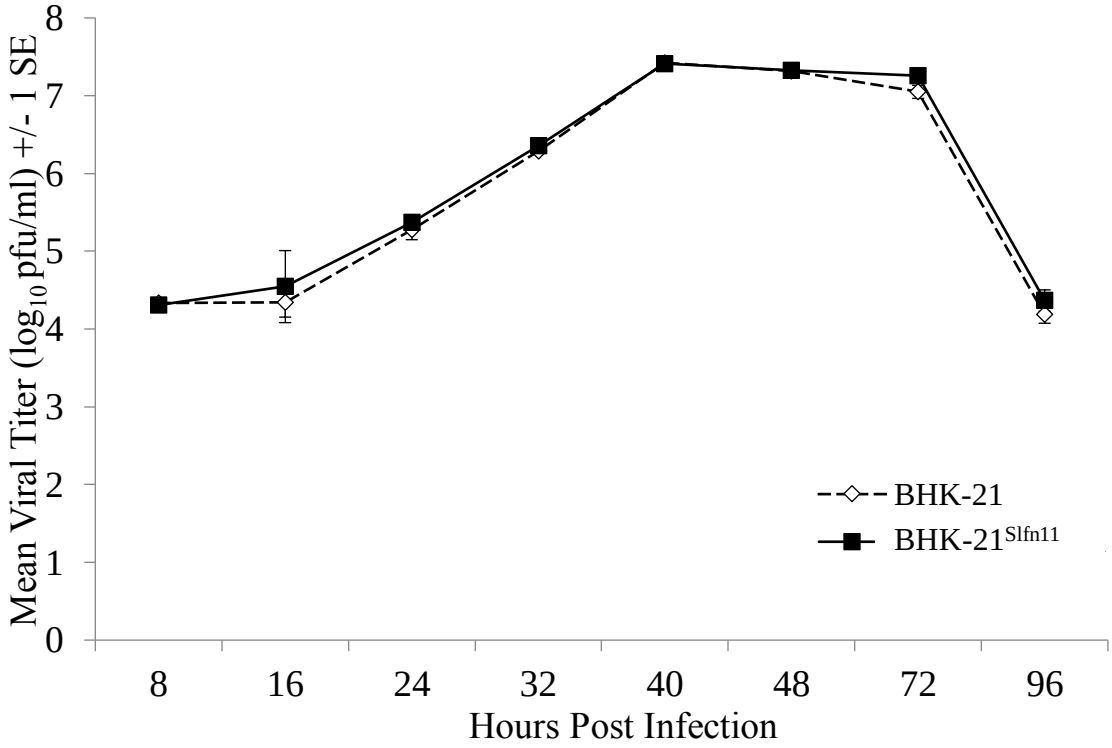
Hours Post Infection
HeLa
HeLa^{Slfn11}


Mean Viral Titer (log_{10} pfu/ml) +/- 1 SE
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Hours Post Infection
BHK-21
BHK-21^{Slfn11}

g

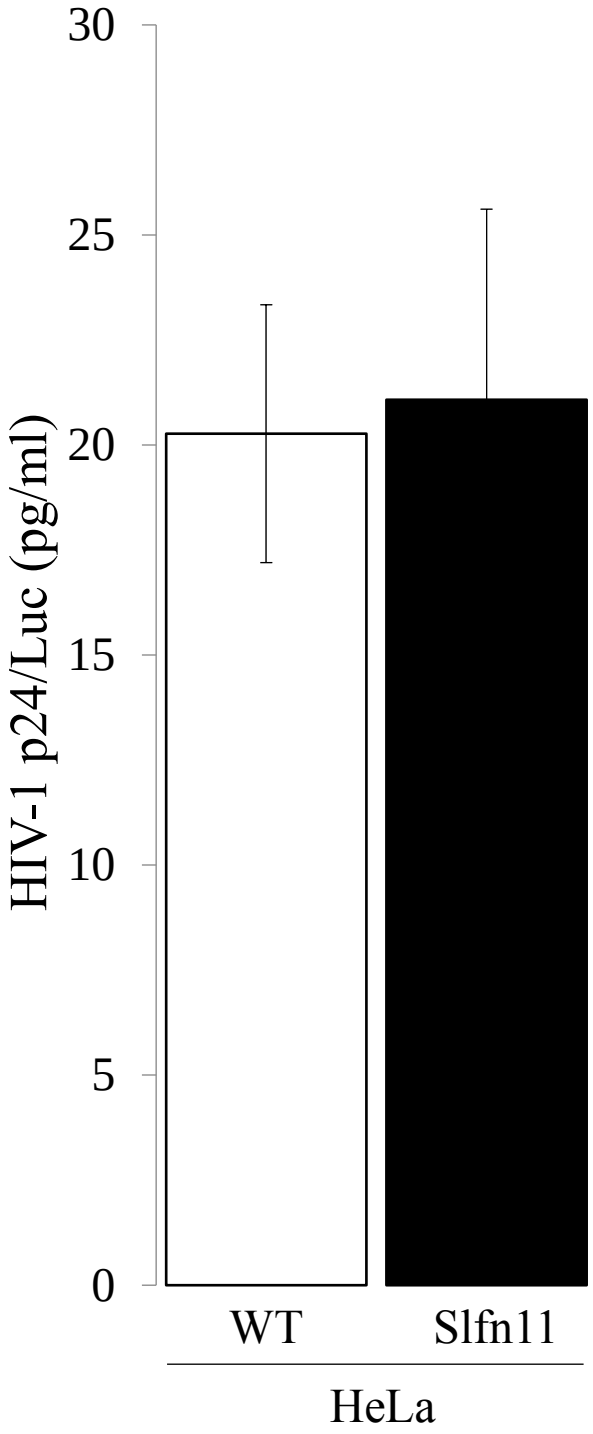

Figure 2.6 Evaluation of the antiviral activity of Slfn11 in HEK293T, HeLa, and BHK-21 cells. (a) Slfn11 expression in HEK293T, HEK293, HeLa, and BHK-21 parental and derived cell lines as detected by immunoblotting analysis. (b-1) WNV replication in control HEK293T and HEK293T^{Slfn11}. Cells were infected with WNV (MOI 0.1) and viral replication was determined by quantification of the viral titer in the cell supernatant at different hours post-infection by plaque assay. Statistically significant differences were calculated as described above and are indicated with asterisks. Mean and standard deviation values represent the variability of the viral titers found in triplicate plaque assays of samples from 3 independent infection experiments. (b-2) HIV-1 infection of control HEK293T and HEK293T^{Slfn11}. Cells were infected with a replication defective HIV-1 that expresses LTR-driven luciferase and p24. For all HIV-1 infections, mean and standard deviation values represent the variability of luciferase-normalized HIV-1 p24 levels found in 3 independent infection experiments. Statistical analysis was performed by t-test. (c) HEK293 and HEK293-KD cells were infected with WNV (MOI 0.1) and viral replication was determined as described above. (d) HIV-1 infection of A172-derived cells as described above. Statistical analysis was performed by ANOVA with Tukey HSD post-hoc test. ** Indicates $p<0.01$. (e) Cellular distribution of Slfn11 in HEK293T^{Slfn11}. Cells were fixed/permeabilized and stained with an anti-Slfn11 antibody by indirect immunofluorescence (red). Nuclei were labeled with Hoechst (blue). (f) HeLa^{Slfn11} and BHK-21^{Slfn11} were infected with WNV (MOI 0.1) and viral replication was determined as described above. Statistically significant differences were calculated as described above and are indicated with asterisks.(g) HIV-1 infection of HeLa and HeLa^{Slfn11} cells. Cells were infected with a replication defective HIV-1 as described above.

Chapter 3: Discussion

3.1 Summary and Significance of Research Performed

Our data showed that Slfn11 deficiency enhances replication of flaviviruses but not replication of (-)ssRNA viruses belonging to two different families. These findings considerably expand our knowledge of the antiviral activity of Slfn11, previously limited to retroviruses before this work was performed. Our data suggest that Slfn11 may counteract other (+)ssRNA viruses, although this remains to be tested. This broad antiviral effect is associated with viral-induced decrease of a subset of tRNAs. Our data are also novel as the effect of viral infection on the tRNA repertoire is still poorly understood. Our findings will promote research in this area; for example it is intriguing to investigate whether or not (-)ssRNA viruses change the tRNA repertoire, although our data would suggest that they do not use this strategy. The fact that Slfn11 opposes WNV-induced tRNA downregulation also shifts the paradigm of the role of Slfn11 in tRNA biology. Before this research was conducted there was only one report on the role of Slfn11 in tRNA abundance. This past work indicated that Slfn11 blocks HIV-1-induced tRNA up-regulation. In contrast, our data indicates that Slfn11 regulates changes in tRNA abundance independently of their direction. Indirectly, these findings indicate that the mechanism of Slfn11 implicated in tRNA pool stability is more complex than previously thought and that it requires other functions of this protein in addition to its tRNA degradation activity. In support of this new concept, we found that Slfn11 is required for this antiviral activity in cells endogenously expressing this protein but is not sufficient in cells that naturally lack endogenous Slfn11 expression. These observations, further support the notion that although the tRNA nucleolytic activity of Slfn11 may be necessary for the antiviral function, it is not sufficient and other host factors absent in the cells lacking endogenous Slfn11 are also required.

3.2 Mechanisms Potentially Implicated in the Antiviral Activity of Slfn11

Possible mechanisms implicated in the Slfn11 antiviral activity, and their potential relevance or lack thereof, will be discussed below. Finally, a testable working hypothesis will also be presented for consideration.

3.2.1 Why Does Slfn11 Seem to Selectively Impair Replication of Lentiviruses and Flaviviruses but not of (-)ssRNA Viruses?

Considering the lack of response of (-)ssRNA viruses VSV and RVFV to Slfn11, we propose that these types of viruses do not alter the abundance of the tRNA pool. However, we lack evidence to support this hypothesis because the effect of viral infection on the host tRNA composition is understudied. Nevertheless, it was demonstrated that influenza virus does not change the abundance of the tRNA repertoire[102], lending support to our hypothesis.

Currently the exact mechanism by which Slfn11 inhibits viral replication is poorly understood. As previously mentioned, Slfn11 impairs lentivirus infection, including HIV-1 and equine infectious anemia virus[69,71]. This protein blocks HIV-induced up-regulation of tRNAs, impairing viral protein translation, although the mechanism by which it does so remains purely speculative.

Slfn11 is able to regulate tRNA availability, which can potentially be a rate-limiting step for successful translation of host and viral proteins. Therefore, at the beginning of this project we postulated that Slfn11 could also selectively affect the replication of (+)ssRNA viruses. In contrast to other RNA and DNA viruses, (+)ssRNA viruses require the immediate translation of the incoming viral genome, making them more susceptible to changes in protein translation efficiency.

All other viruses avoid this challenge by producing multiple copies of translation competent RNA molecules by transcription or replication of the viral DNA or RNA genome. Our data support these predictions; we found that Slfn11 severely affects replication of flaviviruses, including WNV, DENV, and ZIKV; however, the replication of (-)ssRNA viruses VSV and RVFV, that belong to evolutionarily distant families, were not affected. Therefore, the antiviral activity of Slfn11 seems to be specific for (+)ssRNA viruses and lentiviruses[69]. Lentiviruses do not face the translational challenge described above for (+)ssRNA viruses, since this group transcribes the DNA copy of the viral genome producing multiple copies of mRNA. However, lentiviral protein translation is particularly sensitive to the availability of rare tRNAs in the host due to their marked biased codon usage.

Our hypothesis that (+)ssRNA viruses will be more sensitive to innate immune mechanisms targeting translation efficiency than other viruses found further support in the results of a high-throughput screening published when this thesis dissertation work was halfway to completion.

Schoggins et al. examined the effects of individual ISGs on 14 different viruses from 7 different families. From this screening 47 ISGs were identified as having antiviral effects. Interestingly, this screening also revealed a striking pattern in the differences between (+)ssRNA and (-)ssRNA viruses in terms of how ISGs mediate inhibition of infection. (+)ssRNA viruses were significantly more sensitive to ISG inhibition compared to (-)ssRNA. Furthermore, primary translation was the most common mechanism by which ISGs inhibited viral replication[1,103]. Importantly, Slfn11 was not identified in this screening.

Translational inhibition is a well-established mechanism by which ISGs mount a strong antiviral defense. Some of the best studied ISGs that use this antiviral mechanism include protein

kinase R (PKR), which undergoes autophosphorylation upon binding and activation by dsRNA. In response to viral infections, activated PKR will then phosphorylate the eIF2α translation initiation factor which results in arrest of translation of both cellular and viral mRNAs[104]. 2',5'-oligoadenylate synthetase (OAS) proteins are also activated upon detection of viral dsRNA, followed by production of 2',5'-linked oligoadenylates. These molecules will then bind to and activate the latent endoribonuclease RNase L, which proceeds to cleave both viral RNA and mRNA, leading to inhibition of protein synthesis and therefore viral replication[104]. Other examples of ISGs involved in inhibition of viral protein synthesis include the zinc finger antiviral protein (ZAP), which binds to viral RNA and targets it for degradation by recruitment of exosomes[105]. Finally, interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) also targets viruses by repression of viral protein translation. Upon induction by either dsRNA, IRF-3 or type I IFNs, IFIT1 sequesters capped RNA lacking a 2'-O-methyl group and prevents translation initiation factors from binding to the viral RNA template[8,106].

3.2.2 How Does Slfn11 Impair Viral Infection?

Similarly to other ISGs, Slfn11 also affects protein translation efficiency, in this case by regulating tRNA availability. In HIV-1-infected cells, Slfn11 globally decreases tRNA levels in the infected cells. Interestingly, a closely related member of Slfn11, Slfn13, has given us new insights into the possible mechanism of Slfn11 in downregulation of tRNAs. Recent work has shown that Slfn13 inhibits HIV-1 infection in a nucleolytic activity-dependent manner[73]. Specifically, Slfn13 cleaves 11 nucleotides from the 3' end of the acceptor stem of tRNAs, thereby inhibiting protein synthesis. It is important to note that the catalytic triad and five out of the six residues implicated in tRNA binding in Slfn13 are conserved in Slfn11, and these two proteins

share an overall homology of 83%. Therefore, Slfn11 likely opposes HIV-1-induced tRNA increase by tRNA degradation.

The enzymatic nucleolytic activity is required for Slfn13 to restrict HIV-1 infection. The antiviral activity of Slfn13 appears to be specific since this protein did not affect replication of herpes simplex or Zika viruses (ZIKV)[73]. Slfn13 seems to be unable to discriminate over tRNA types, but rather targets tRNA secondary structures. In addition, Slfn13 degrades HIV-1 mRNA but not mRNA transcribed from plasmids transfected into Slfn13-expressing cells. The effect of Slfn11 on HIV-1 mRNA has not been directly evaluated, but due to the structural homology of Slfn11 and 13 and their role in tRNA degradation, it is tempting to speculate that secondary RNA structures present in HIV-1 and flaviviruses could be targeted by Slfn11.

Similarly, Slfn14 was also recently characterized as a strong endoribonuclease that binds to ribosomes and ribosomal subunits and preferentially cleaves ribosomal as well as ribosome-associated RNA[107]. Although not studied in the context of viral infections, this work does suggest that Slfn14 could also be induced upon IFN stimulation and upon activation, target cellular RNA as well as viral RNA in a manner similar to the well characterized, IFN stimulated ribonuclease RNase L[104,108]. It is possible that upon infection, activation of ribonucleases such as Slfn13, Slfn14 and possibly, Slfn11, could induce global protein shutdown and thereby promote cell autophagy and apoptosis. However, we do not favor this possibility since Slfn11 did not affect replication of the (-)ssRNA viruses VSV and RVFV and Slfn13 failed to impair replication of ZIKV. It is also important to note that in all three Slfn proteins mentioned above, the N-terminal domain harbors the catalytically active site responsible for RNA degradation described in Slfn13. Furthermore, bioinformatics analysis indicates that the Slfn13 catalytically-active residues that mediate the ribonuclease activity are conserved in many members of the Slfn family in different species,

ranging from invertebrate to human. The characterization of the antiviral activity of these Slfn proteins deserves attention.

Our findings also revealed important differences between Slfn11 and Slfn13 in their specificity. Slfn13 failed to affect ZIKV replication[73] whereas we found Slfn11 effective against this virus. These functional differences highlight the lack of redundancy in the antiviral specificity of different members of the Slfn family. Importantly, human placenta and testes lack expression of Slfn11, otherwise, a ubiquitously expressed protein[81]. We therefore suggest the possibility that the lack of expression of Slfn11 in these tissues could significantly influence mother to fetus and sex-related transmission of ZIKV[109,110].

3.3 Slfn11 Could Affect Viral Replication Through a Translation-Independent Mechanism

3.3.1 Innate Immune Signaling

As previously mentioned, Slfn14 has also been recently shown to be an effective antiviral factor against influenza A and varicella zoster virus[72]. However, the antiviral mechanism is independent of regulation of protein translation efficiency. Importantly, this work showed that Slfn14 induction is mediated by the TLR-3 and type I IFN pathways and that once induced, it enhances RIG-I-mediated IFN-β signaling. Although not experimentally, the authors also address the peculiar similarity of the putative RNA helicase motif present in the C-terminal sections of group III Slfn proteins (Slfn5, Slfn11, Slfn12, Slfn13, and Slfn14) with that of the DNA/RNA nucleic acid sensors retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated gene 5 (MDA5). Therefore, it is possible that Slfn14 binds to viral RNA and/or DNA and upon activation participates in and enhances IFN-mediated restriction of viral replication.

Altogether, accumulating data suggests that Slfn proteins might not only play a role in the innate immune response as direct antiviral effectors, but could also be important regulators of innate immune signaling.

3.3.2 Translation-Independent Functions of tRNAs

Besides their canonical function in protein synthesis, tRNAs also participate in diverse cellular processes through the activity of tRNA-derived fragments (tRFs). These small non-coding RNA molecules influence cellular metabolism, cell death, and gene expression through different mechanisms, although the overall function of tRFs remains unknown[111]. Previous work has shown that under cellular stress, the ribonuclease protein Angiogenin (ANG) cleaves mature cellular tRNAs at the anticodon loop to produce 5′- and 3′-tRNA fragments[112]. These fragments are then able to promote translational repression by displacing eIF4G/A from capped and uncapped mRNA and eIF4E/G/A (eIF4F) from the m^7G cap, therefore impeding translation initiation[112]. Furthermore, it was shown that Respiratory Syncytial Virus (RSV) utilizes ANG to produce these tRFs, presumably to suppress antiviral responses[113]. It has been also shown that ANG inhibitors increase HIV-1 replication[114]. We verified this observation; however, we did not observe any effect of this inhibitor on WNV infection (data not reported in this work).

Modulation of tRF production could also be implicated in the antiviral mechanism of Slfn11. The work presented here indicates that similarly to HIV-1, WNV modifies the tRNA pool and Slfn11 opposes these changes. It is possible that Slfn11, by stabilizing the tRNA repertoire, also prevents changes in the host tRFs that could antagonize viral replication. However, evidence supporting this mechanism is missing.

Interestingly, our data indicate that WNV infection of Slfn11-deficient cells causes a drastic decrease of $tRNA^{His}$. Despite the fact that this tRNA is the least abundant, it contributes to

27% of the tRF pool[115]. This disproportion suggests a functional relevance of this tRF. Potentially, WNV infection could diminish production of this tRF by decreasing the levels of $tRNA^{His}$ or increase its generation causing the observed reduction in $tRNA^{His}$. In any case, it would also be interesting to evaluate this potential mechanism.

tRNA molecules are among the most stable RNAs in the cell. Unlike mRNA, which can have a half-life ranging from minutes to hours, tRNA half-lives tend to be in the order of days. This superior stability is mainly a consequence of the complex folded structure, as well as the various modifications of tRNAs[111,116]. Evidence shows that loss of function of the RNA methyltransferase NSun2, responsible for modifying cytosine-5 in tRNAs, leads to cleavage of tRNAs by ANG, and subsequent production of 5' and 3' tRFs. In the absence of NSun2, accumulation of these tRFs interferes with the cellular translation machinery and activates stress response pathways that lead to the reduction of cell size as well as increase in apoptosis in cortical, hippocampal and striatal neurons[117]. In line with the well-defined molecular mechanisms of NSun2, it is not surprising that genetic mutations or deletion of Nsun2 in the brain is linked with neuro-developmental disorders such as microcephaly[118,119], possibly as a consequence of stress-induced tRNA cleavage by ANG. As previously mentioned, our data indicates that flaviviruses decrease the tRNA repertoire of the host to enhance viral replication. Although not supported experimentally, it is intriguing to hypothesize that severe neurological consequences, such as microcephaly, could be the result of regulation or disruption of cellular processes (e.g. NSun2-ANG-mediated disruption of protein synthesis) by neurotropic viruses such as ZIKV. However, the levels of NSun2 do not change in the course of ZIKV replication in A172 cells (Data not included here).

3.4 How do Viruses Regulate tRNA Abundance?

Ours and previously published data[69,120] suggest that lentiviruses and flaviviruses have evolved different strategies to enhance viral replication by modulating the tRNA pool. Lentiviruses increase tRNA levels in the cell resulting in a higher availability of tRNAs required for the translation of their codon-biased genome. Therefore, these changes in the tRNA pool lead to enhanced viral protein translation. In contrast, flaviviruses, which lack codon-biased genomes, diminish the abundance of a subset of tRNAs, predictably reducing the efficiency of viral protein production, and nonetheless, increasing virus replication.

The mechanisms implicated in viral-induced modification of the tRNA repertoire are currently unknown. HIV-1-induced tRNA increase could be due to enhanced transcription or decreased degradation of these molecules. However, no data supporting any of these two avenues is available. The long half-life of tRNA indicates that WNV-induced tRNA downregulation should be a consequence of increased degradation rather than decreased synthesis. Nevertheless, a potential mechanism in WNV-induced downregulation of tRNAs is through the regulation of Maf1, a negative modulator of RNA polymerase III (Pol III). Maf1 is phosphorylated via protein kinase A and TOR-dependent Sch9 kinase and retained in the cytoplasm of the cells. The serine/threonine protein phosphatase 2 (PP2A) removes this phosphorylation signal inducing nuclear migration and association with Pol III. An inhibitor of PP2A, I_2^{PP2A}, prevents Maf1 dephosphorylation and therefore inhibition of tRNA transcription by Pol III[111]. Importantly, WNV capsid protein binds to I_2^{PP2A}, blocking the inhibitory effect on PP2A[121]. Potentially, WNV capsid could inhibit tRNA transcription through this non-structural function. If this pathway is implicated in the WNV-induced changes in the tRNA repertoire, it is still intriguing why only a subset of tRNAs are decreased in Slfn11-deficient cells upon WNV infection.

The Maf1/capsid pathway is predicted to affect tRNAs globally and not only a subset of them. A possibility could be that at the early time post-infection that we examined, only the less abundant tRNAs in A172 cells are down-regulated (e.g. $tRNA^{His}$). However, $tRNA^{Ala}$, which is the most abundant in cells[111,122], was also down-regulated by WNV infection. Similarly, there was no correspondence between the basal levels of tRNAs and their sensitivity to WNV infection in Slfn11-deficient cells, indicating that the sensitivity to WNV was not due the basal levels of tRNAs in human cells.

The above considerations prompt us to postulate that WNV infection triggers degradation of tRNAs in cells lacking Slfn11. In contrast to the vastly investigated processes involved in the degradation of mRNA, much less attention has been directed at the degradation of stable tRNA. Two major tRNA degradation pathways have been described as being so far. The nuclear surveillance pathway is known to affect only $pretRNA^{iMet}$. This pathway monitors pre-tRNA quality during the initial stages of tRNA biogenesis. Previous work has shown that $tRNA^{iMet}$ lacking a methylated adenine at position 58 (m^1A_{58}) will activate the Trf4 (topoisomerase 1-related 4)/Air2 (Arginine methyltransferase-interacting RING finger protein 2)/Mtr4p (mRNA transport regulator 4 protein) Polyadenylation (TRAMP) complex which polyadenylates the pre-tRNA, leading to 3'-end degradation by the nuclear exosome[111,116]. The second pathway described is called rapid tRNA decay (RTD), which unlike the nuclear surveillance pathway, primarily targets mature tRNAs and involves cytoplasmic and nuclear exonucleases. During this process, tRNAs lacking a specific methylated guanosine (m^7G_{46}) or methylated cytidine (m^5C_{49}) modification, as well as tRNAs displaying instability at the acceptor stem, are degraded by the Xrn1 or Rat1 exonucleases[111,116]. Strong evidence suggests that the RTD pathway seems to be general since various tRNAs lacking different combinations of modifications are also targeted by this pathway.

Alternatively, the cellular stress caused by WNV infection could produce changes in the tRNA repertoire. It has been demonstrated in yeast that different chemical stressors trigger specific changes in tRNA repertoire characterized by either increase or decrease of certain tRNAs[123]. Nevertheless, the degradation pathway implicated in this phenomenon is unknown.

The mechanism used by Slfn11 to prevent WNV-induced downregulation in the tRNA repertoire is also unknown. Theoretically, this could be through increasing tRNA synthesis or preventing tRNA degradation. Previous work has shown that activation of NF-kB by bacterial lipopolysaccharides (LPS) leads to upregulation of Pol III and subsequent increase in tRNA gene transcription[124]. Although no link between Slfn11 and NF-kB has been reported, Slfn2 has recently been shown to regulate NF-kB expression by interacting with PPP6R1, a subunit of protein phosphatase-6 (PP6), a member of the PPP family of Ser/Thr phosphatases, proteins found to be integral in the NF-kB signaling pathway[125]. Although no experimental evidence is available, a precedent for Slfn family members being directly involved in this signaling pathway certainly makes us consider the intriguing possibility that Slfn11 could potentially counteract WNV-induced downregulation of tRNAs through a Slfn11-NF-kB-mediated upregulation of Pol III-transcribed tRNA genes[125]. We believe that the effect of NF-kB-inhibitors on the anti-WNV activity of Slfn11 should also be evaluated.

Combined analysis of ours and previous findings[69] demonstrate that Slfn11 impairs HIV-1 and WNV through a similarly mechanism. In both cases the N-terminal region of Slfn11 is necessary and sufficient for antiviral activity and this protein blocks virus-induced changes in the tRNA repertoire of the infected cells. However, HIV-1 globally increased tRNAs in the absence of Slfn11; in contrast WNV infection only decreased a subset of tRNAs in Slfn11-deficient cells.

These findings suggest that Slfn11 opposes virus-induced changes in the tRNA pool independently of their direction.

3.5 Effect of Virus-Induced Changes in tRNA Levels on Viral Protein Synthesis

HIV-1 induces global increase of the size of the tRNA pool. This is advantageous for lentiviral protein translation because of the strong codon bias observed in these viruses as compared to their host.

Interestingly, flaviviruses do not exhibit codon bias and do not increase tRNA levels; in contrast, these viruses seem to decrease the levels of a subset of host tRNAs. This change causes pausing during translational elongation. Multiple lines of evidence indicate that translational elongation pausing at codons recognized by low abundance tRNAs might promote efficiency of protein production and/or optimal protein folding[92,94–100]. A common pattern of 30-50 rare codons clustering immediately downstream of the start codon is present in highly expressed prokaryotic and eukaryotic genes. It is thought that this "ramp" sequence increases protein synthesis efficiency by reducing ribosome stalling during translational elongation[97]. However, it is unlikely that the WNV-induced tRNA decrease will enhance viral replication by this mechanism as the codons affected do not cluster in the viral genome.

Furthermore, it has been proposed that rare codons decrease the speed of translational elongation potentially facilitating protein folding[126]. In the RNA replicase and 3C protease of foot-and-mouth disease virus[94,99] and in a *Neurospora crassa* circadian clock protein, a link between location of rare codons and protein secondary structures was described[100]. This link was also predicted by bioinformatics analysis of *E. coli, S. cerevisiae, Caenorhabditis elegans, and*

Drosophila melanogaster genomes[100]. The location of these codons in the transition boundaries of protein secondary structures suggests that the modulation of the speed of the translation elongation is necessary for proper protein folding[126]. Furthermore, replacement of native rare codons by synonymous common codons in the capsid of poliovirus[127] and hepatitis A virus[92] caused a decrease in viral fitness, suggesting that optimal protein folding due to low abundance of tRNA results in increased viral fitness. Therefore, we postulate that WNV-induced reduction in tRNA abundance in Slfn11-deficient cells leads to optimal viral protein folding and enhanced viral fitness.

3.6 The Antiviral Activity of Slfn11 Requires Other Host Cofactors

Intriguingly, we observed that exogenously expressed Slfn11 is not functional against HIV-1 or WNV infection in HEK293T, HeLa, and BHK1 cells that naturally lack endogenous production of this protein. These observations suggest the existence of a cofactor(s) required for the Slfn11 antiviral mechanism. Likely, the missing cofactor(s) is not necessary for the Slfn13-like tRNA nucleolytic activity[73] that we predict is present in Slfn11 based on the conservation of key amino acids. Purified recombinant Slfn13 cleaves purified tRNAs *in vitro*, indicating that other proteins are not required for this activity. Furthermore, co-transfection of plasmids expressing Slfn11 and codon-biased cDNAs in HEK293T cells impaired the expression of the reporter proteins suggesting conservation of the putative Slfn11 tRNA nucleolytic activity in these cells[81]. Thus, together these observations suggest that the tRNA nucleolytic activity of Slfn11, although required, is not sufficient for its antiviral activity.

Furthermore, we observed a lack of correlation between the levels of Slfn11 and the antiviral effect of this protein. Slfn11 affected HIV-1 and flavivirus infection in A172-SCR and -BC cells to a similar extension despite the fact that these cells express markedly different levels of

Slfn11. These observations indicate that above certain amounts of Slfn11, the antiviral activity of this protein reaches saturation. This phenomenon will be unexpected if the antiviral activity of Slfn11 does not require other cofactor(s). Therefore, these data also support our hypothesis that Slfn11 is not the limiting factor of the antiviral pathway.

3.7 Physiological Role of Slfn11

The proposed role of Slfn11 in maintaining the stability of the tRNA pool could also explain the direct correlation between the sensitivity of cells to genotoxic drugs and their Slfn11 levels. It has been found that cancer cells expressing higher levels of Slfn11 are more sensitive to DNA-damaging agents[70,128,129]. Therefore, it is possible that higher levels of Slfn11 could reduce the abundance of specific tRNAs in the cell affecting protein synthesis, as recently reported[81]. This effect will down-regulate the levels of DNA repair proteins encoded by codon-biased open reading frames[130], increasing the susceptibility of cancer cells to genotoxic agents.

3.8 Working Hypothesis

Although we ignore how Slfn11 affects flavivirus replication, we propose that these viruses trigger degradation of a subset of tRNAs, causing pausing of virus translation at elongation. This will lead to enhanced protein folding and increased viral fitness. In contrast, lentiviruses will increase tRNA abundance globally to overcome the tRNA functional deficiency that results from its codon bias, facilitating viral protein translation. Slfn11, in concert with other cellular protein(s), prevents these changes in tRNA abundance, impairing viral replication. It is possible that Slfn11 tRNA nucleolytic activity could be negatively regulated upon virus-induced tRNA decrease. This non-nucleolytic form of Slfn11 could still bind tRNAs, protecting them from degradation. In the case of HIV-1, virus-induced tRNA upregulation will be counteracted by the tRNA nucleolytic

activity of Slfn11. The protein(s) missing in the cells that do not support Slfn11 activity could sense the changes in tRNA levels and transduce this signal to Slfn11.

In summary, our data has revealed that Slfn11 opposes virus-induced changes in the tRNA repertoire, thus affecting evolutionarily unrelated viruses that manipulate the host tRNA repertoire to facilitate viral replication. Although the exact mechanism by which Slfn11 inhibits protein synthesis requires a considerable amount of further analysis, we believe that our findings establish Slfn11 as an important cellular protein involved in control of viral replication. Furthermore, a better understanding of the role of Slfn11 during viral replication will also improve our knowledge of flavivirus pathogenesis and possibly provide the foundation for the exploration of new therapeutic avenues with which to treat these diseases.

3.8.1 Validation of Working Hypothesis

1. We propose that flaviviruses trigger degradation of a subset of tRNAs, which causes pausing of virus translation at elongation. We need to demonstrate that flavivirus and HIV-1 viral RNA is not degraded by Slfn11. This can be done by quantifying by RT-PCR the levels of the viral RNA in infected cells expressing or not Slfn11. Furthermore, codon optimization of WNV to remove the codons read by the tRNAs that are selectively downregulated by the viral infection is expected to remove the increased viral replication in Slfn11-deficient cells.
2. We propose that pausing in translation will lead to enhanced protein folding and increased WNV fitness. To validate this hypothesis, we need to determine the viral titer normalized to the amount of viral particles. Virion quantification can be performed by measuring a virus structural protein by ELISA.

3. It is possible that Slfn11 tRNA nucleolytic activity could be negatively regulated upon virus-induced tRNA decrease. This non-nucleolytic form of Slfn11 could still bind tRNAs, protecting them from degradation. In the case of HIV-1, virus-induced tRNA upregulation will be counteracted by the tRNA nucleolytic activity of Slfn11. Therefore, we expect that catalytically-inactive Slfn11 mutants will still prevent WNV replication but will fail to impair HIV-1 infection.
4. The protein(s) missing in the cells that do not support Slfn11 activity could sense the changes in tRNA levels and transduce this signal to Slfn11. We will characterize Slfn11 interacting proteins implicated in the antiviral activity. We expect that the missing protein(s) is expressed in cells that support Slfn11 activity but not in the non-supportive cells. Therefore, we will determine the identity of this protein(s) by combining gene expression analysis of supportive and non-supportive cells with results of experiments looking at Slfn11 interactors present in supportive but absent in non-supportive cells. This protein(s) will be identified using different techniques such as BioID, immunoprecipitation followed by proteomic analysis, and yeast-two hybrid screening.

References

1. Schoggins, J. W. *et al.* Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature* **505,** 691–695 (2014).
2. Brandler, S. & Tangy, F. Vaccines in development against West Nile virus. *Viruses* **5,** 2384–2409 (2013).
3. Brinton, M. a. The molecular biology of West Nile Virus: a new invader of the western hemisphere. *Annu. Rev. Microbiol.* **56,** 371–402 (2002).
4. Huhn, G. D., Sejvar, J. J., Montgomery, S. P. & Dworkin, M. S. West Nile virus in the United States: An update on an emerging infectious disease. *Am. Fam. Physician* **68,** 653–660+671 (2003).
5. City of El Paso Department of Public Health. 2014 WNV Report. (2015).
6. Valiakos, G. *et al.* West Nile Virus: Basic Principles, Replication Mechanism, Immune Response and Important Genetic Determinants of Virulence. *Viral Replication* 43–68 (2013). doi:10.1126/science.1089316.
7. Gea-Banacloche, J. *et al.* West Nile Virus: Pathogenesis and Therapeutic Options. in *Annals of Internal Medicine* **140,** 545–553 (2004).
8. Suthar, M. S., Diamond, M. S. & Gale, M. West Nile virus infection and immunity. *Nat. Rev. Microbiol.* **11,** 115–28 (2013).
9. Sejvar, J. J. Clinical manifestations and outcomes of West Nile virus infection. *Viruses* **6,** 606–23 (2014).
10. 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).
11. Mukhopadhyay, S., Kim, B., Chipman, P. R., Rossmann, M. G. & Kuhn, R. J. Structure of West Nile Virus. **302,** 2003 (2003).
12. Zhang, Y. *et al.* Structures of immature flavivirus particles. *EMBO J.* **22,** 2604–2613 (2003).
13. Fields, B. N., Knipe, D. M. & Howley, P. M. *Fields Virology, 5th Edition*. *Fields Virology* **2,** (2007).
14. Beasley, D. W. C. *et al.* Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. *J. Virol.* **79,** 8339–8347 (2005).
15. Lindenbach, B. D. & Rice, C. M. Molecular biology of flaviviruses. *Adv. Virus Res.* **59,** 23–61 (2003).
16. Khromykh, A. A. & Westaway, E. G. RNA binding properties of core protein of the flavivirus Kunjin. *Arch. Virol.* **141,** 685–699 (1996).
17. Heinz, F. X. & Allison, S. L. The machinery for flavivirus fusion with host cell membranes. *Current Opinion in Microbiology* **4,** 450–455 (2001).
18. Mukhopadhyay, S., Kuhn, R. J. & Rossmann, M. G. A structural perspective of the flavivirus life cycle. *Nat. Rev. Microbiol.* **3,** 13–22 (2005).
19. Heinz, F. X. & Allison, S. L. Structures and mechanisms in flavivirus fusion. *Adv. Virus Res.* **55,** 231–269 (2000).
20. Brinton, M. A., Fernandez, A. V & Dispoto, J. H. The 3'-nucleotides of flavivirus genomic RNA form a conserved secondary structure. *Virology* **153,** 113–121 (1986).
21. Winkler, G., Randolph, V. B., Cleaves, G. R., Ryan, T. E. & Stollar, V. Evidence that the

mature form of the flavivirus nonstructural protein NS1 is a dimer. *Virology* **162,** 187–196 (1988).
22. Chung, K. M. *et al.* West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 19111–19116 (2006).
23. Hall, R. A. *et al.* Loss of dimerisation of the nonstructural protein NS1 of Kunjin virus delays viral replication and reduces virulence in mice, but still allows secretion of NS1. *Virology* **264,** 66–75 (1999).
24. Falgout, B. & Markoff, L. Evidence that flavivirus NS1-NS2A cleavage is mediated by a membrane-bound host protease in the endoplasmic reticulum. *J. Virol.* **69,** 7232–7243 (1995).
25. Kümmerer, B. M. & Rice, C. M. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. *J. Virol.* **76,** 4773–4784 (2002).
26. Liu, W. J. *et al.* A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. *J. Virol.* **80,** 2396–2404 (2006).
27. Liu, W. J., Chen, H. B., Wang, X. J., Huang, H. & Khromykh, A. A. Analysis of adaptive mutations in Kunjin virus replicon RNA reveals a novel role for the flavivirus nonstructural protein NS2A in inhibition of beta interferon promoter-driven transcription. *J. Virol.* **78,** 12225–12235 (2004).
28. Chambers, T. J. *et al.* Yellow fever virus NS2B-NS3 protease: Characterization of charged-to-alanine mutant and revertant viruses and analysis of polyprotein-cleavage activities. *J. Gen. Virol.* **86,** 1403–1413 (2005).
29. Chambers, T. J., Nestorowicz, A., Amberg, S. M. & Rice, C. M. Mutagenesis of the yellow fever virus NS2B protein: effects on proteolytic processing, NS2B-NS3 complex formation, and viral replication. *J. Virol.* **67,** 6797–6807 (1993).
30. Nall, T. A. *et al.* Enzymatic characterization and homology model of a catalytically active recombinant West Nile virus NS3 protease. *J. Biol. Chem.* **279,** 48535–48542 (2004).
31. Howe, A. Y. *et al.* A novel recombinant single-chain hepatitis C virus NS3-NS4A protein with improved helicase activity. *Protein Sci.* **8,** 1332–1341 (1999).
32. Wengler, G. & Wengler, G. The NS3 nonstructural protein of flavivirus contains an RNA triphosphatase activity. *Virology* **197,** 265–273 (1993).
33. Wengler, G. & Wengler, G. The carboxy-terminal part of the NS 3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase. *Virology* **184,** 707–715 (1991).
34. Lindenbach, B. D. & Rice, C. M. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. *J. Virol.* **73,** 4611–4621 (1999).
35. Mackenzie, J. M., Khromykh, A. A., Jones, M. K. & Westaway, E. G. Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A. *Virology* **245,** 203–215 (1998).
36. McLean, J. E., Wudzinska, A., Datan, E., Quaglino, D. & Zakeri, Z. Flavivirus NS4A-induced autophagy protects cells against death and enhances virus replication. *J. Biol. Chem.* **286,** 22147–22159 (2011).
37. Shiryaev, S. A., Chernov, A. V., Aleshin, A. E., Shiryaeva, T. N. & Strongin, A. Y. NS4A regulates the ATPase activity of the NS3 helicase: A novel cofactor role of the non-structural protein NS4A from West Nile virus. *J. Gen. Virol.* **90,** 2081–2085 (2009).

38. Westaway, E. G., Khromykh, A. A., Kenney, M. T., Mackenzie, J. M. & Jones, M. K. Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus. *Virology* **234,** 31–41 (1997).
39. Zheng, Y. *et al.* Gene expression profiles of HeLa Cells impacted by hepatitis C virus non-structural protein NS4B. *J. Biochem. Mol. Biol.* **38,** 151–160 (2005).
40. Muñoz-Jordán, J. L. *et al.* Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *J. Virol.* **79,** 8004–8013 (2005).
41. Koonin, E. V. Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and ??2 protein of reovirus. *J. Gen. Virol.* **74,** 733–740 (1993).
42. Zhou, Y. *et al.* Structure and function of flavivirus NS5 methyltransferase. *J. Virol.* **81,** 3891–3903 (2007).
43. Selisko, B. *et al.* Comparative mechanistic studies of de novo RNA synthesis by flavivirus RNA-dependent RNA polymerases. *Virology* **351,** 145–158 (2006).
44. Kapoor, M. *et al.* Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. *J. Biol. Chem.* **270,** 19100–19106 (1995).
45. Mackenzie, J. M., Kenney, M. T. & Westaway, E. G. West Nile virus strain Kunjin NS5 polymerase is a phosphoprotein localized at the cytoplasmic site of viral RNA synthesis. *J. Gen. Virol.* **88,** 1163–1168 (2007).
46. Reed, K. E., Gorbalenya, A. E. & Rice, C. M. The NS5A/NS5 proteins of viruses from three genera of the family flaviviridae are phosphorylated by associated serine/threonine kinases. *J. Virol.* **72,** 6199–6206 (1998).
47. Buckley, A., Gaidamovich, S., Turchinskaya, A. & Gould, E. A. Monoclonal antibodies identify the NS5 yellow fever virus non-structural protein in the nuclei of infected cells. *J. Gen. Virol.* **73 (Pt 5),** 1125–30 (1992).
48. Laurent-Rolle, M. *et al.* The NS5 Protein of the Virulent West Nile Virus NY99 Strain Is a Potent Antagonist of Type I Interferon-Mediated JAK-STAT Signaling. *J. Virol.* **84,** 3503–3515 (2010).
49. Chu, J. J. H. & Ng, M. L. Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. *J. Virol.* **78,** 10543–10555 (2004).
50. Stiasny, K. & Heinz, F. X. Flavivirus membrane fusion. *Journal of General Virology* **87,** 2755–2766 (2006).
51. Brinton, M. a. Replication cycle and molecular biology of the West Nile virus. *Viruses* **6,** 13–53 (2014).
52. Westaway, E. G., Khromykh, A. A. & Mackenzie, J. M. Nascent flavivirus RNA colocalized in situ with double-stranded RNA in stable replication complexes. *Virology* **258,** 108–117 (1999).
53. Gillespie, L. K., Hoenen, A., Morgan, G. & Mackenzie, J. M. The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex. *J. Virol.* **84,** 10438–10447 (2010).
54. Mackenzie, J. M. & Westaway, E. G. Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. *J. Virol.* **75,** 10787–10799 (2001).
55. Rauscher, S., Flamm, C., Mandl, C. W., Heinz, F. X. & Stadler, P. F. Secondary structure of the 3'-noncoding region of flavivirus genomes: comparative analysis of base pairing probabilities. *RNA* **3,** 779–791 (1997).

56. Samuel, M. A. & Diamond, M. S. Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. *J. Virol.* **79,** 13350–13361 (2005).
57. Quicke, K. M. & Suthar, M. S. The innate immune playbook for restricting West Nile virus infection. *Viruses* **5,** 2643–2658 (2013).
58. Dempsey, A. & Bowie, A. G. Innate immune recognition of DNA : A recent history. *Virology* **479–480,** 146–152 (2015).
59. Koyama, S., Ishii, K. J., Coban, C. & Akira, S. Innate immune response to viral infection. *Cytokine* **43,** 336–341 (2008).
60. Fredericksen, B. L., Keller, B. C., Fornek, J., Katze, M. G. & Gale, M. Establishment and maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1. *J. Virol.* **82,** 609–16 (2008).
61. Shtrichman, R. & Samuel, C. E. The role of gamma interferon in antimicrobial immunity. *Current Opinion in Microbiology* **4,** 251–259 (2001).
62. Sen, G. C. & Sarkar, S. N. The interferon-stimulated genes: Targets of direct signaling by interferons, double-stranded RNA, and viruses. *Current Topics in Microbiology and Immunology* **316,** 233–250 (2007).
63. Katsoulidis, E. *et al.* Role of schlafen 2 (SLFN2) in the generation of interferon α-induced growth inhibitory responses. *J. Biol. Chem.* **284,** 25051–25064 (2009).
64. Recher, M. *et al.* Genetic variation in schlafen genes in a patient with a recapitulation of the murine Elektra phenotype. *J. Allergy Clin. Immunol.* **133,** (2014).
65. Mavrommatis, E., Fish, E. N. & Platanias, L. C. The schlafen family of proteins and their regulation by interferons. *J. Interferon Cytokine Res.* **33,** 206–10 (2013).
66. Liu, F., Zhou, P., Wang, Q., Zhang, M. & Li, D. The Schlafen family: complex roles in different cell types and virus replication. *Cell Biol. Int.* **9999,** 1–7 (2017).
67. Geserick, P., Kaiser, F., Klemm, U., Kaufmann, S. H. E. & Zerrahn, J. Modulation of T cell development and activation by novel members of the Schlafen (slfn) gene family harbouring an RNA helicase-like motif. *Int. Immunol.* **16,** 1535–1548 (2004).
68. Bustos, O. *et al.* Evolution of the Schlafen genes , a gene family associated with embryonic lethality , meiotic drive , immune processes and orthopoxvirus virulence. *Gene* **447,** 1–11 (2009).
69. Li, M. *et al.* Codon-usage-based inhibition of HIV protein synthesis by human schlafen 11. *Nature* **491,** 125–8 (2012).
70. Mu, Y. *et al.* SLFN11 inhibits checkpoint maintenance and homologous recombination repair. *EMBO Rep.* **17,** 94–109 (2016).
71. Lin, Y. *et al.* Equine schlafen 11 restricts the production of equine infectious anemia virus via a codon usage-dependent mechanism. *Virology* **495,** 112–121 (2016).
72. Seong, R., Seo, S., Kim, J., Fletcher, S. J. & Morgan, N. V. Schlafen 14 (SLFN14) is a novel antiviral factor involved in the control of viral replication. *Immunobiology* **14,** 0–1 (2017).
73. Yang, J.-Y. *et al.* Structure of Schlafen13 reveals a new class of tRNA/rRNA- targeting RNase engaged in translational control. *Nat. Commun.* **9,** 1165 (2018).
74. Zoppoli, G. *et al.* Putative DNA / RNA helicase Schlafen-11 (SLFN11) sensitizes cancer cells to DNA-damaging agents. *Proc. Natl. Acad. Sci.* **109,** 15030–15035 (2012).
75. Puck, A. *et al.* Expression and regulation of Schlafen (SLFN) family members in primary human monocytes, monocyte-derived dendritic cells and T cells. *Results Immunol.* **5,** 23–

32 (2015).
76. Abdel-Mohsen, M. *et al.* Expression profile of host restriction factors in HIV-1 elite controllers. *Retrovirology* **10,** 106 (2013).
77. Schwarz, D., Katayama, C. & Hedrick, S. Schlafen, a New Family of Growth Regulatory Genes that Affect Thymocyte Development. *Immunity* **9,** 657–668 (1998).
78. Gubse, C. *et al.* Camelpox virus encodes a schlafen-like protein that affects orthopoxvirus virulence. *J. Gen. Virol.* **88,** 1667–1676 (2007).
79. Sohn, W. J. *et al.* Novel transcriptional regulation of the schlafen-2 gene in macrophages in response to TLR-triggered stimulation. *Mol. Immunol.* **44,** 3273–3282 (2007).
80. Iyer, L. M., Leipe, D. D., Koonin, E. V. & Aravind, L. Evolutionary history and higher order classification of AAA+ ATPases. in *Journal of Structural Biology* **146,** 11–31 (2004).
81. Stabell, A. C. *et al.* Non-human Primate Schlafen11 Inhibits Production of Both Host and Viral Proteins. *PLOS Pathog.* **12,** e1006066 (2016).
82. Stojdl, D. F. *et al.* VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **4,** 263–275 (2003).
83. Llano, M. *et al.* An essential role for LEDGF/p75 in HIV integration. *Science* **314,** 461–4 (2006).
84. Long, K. C. *et al.* Experimental transmission of Mayaro virus by Aedes aegypti. *Am. J. Trop. Med. Hyg.* **85,** 750–757 (2011).
85. Morens, D. M., Halstead, S. B., Repik, P. M., Putvatana, R. & Raybourne, N. Simplified plaque reduction neutralization assay for dengue viruses by semimicro methods in BHK-21 cells: Comparison of the BHK suspension test with standard plaque reduction neutralization. *J. Clin. Microbiol.* **22,** 250–254 (1985).
86. Hnasko, R. & Hnasko, T. The Western Blot. *Methods Mol. Biol.* **1318,** 87–96 (2015).
87. Garcia-Rivera, J. A. *et al.* Implication of Serine Residues 271, 273, and 275 in the Human Immunodeficiency Virus Type 1 Cofactor Activity of Lens Epithelium-Derived Growth Factor/p75. *J. Virol.* **84,** 740–752 (2010).
88. Llano, M., Gaznick, N. & Poeschla, E. M. Rapid, controlled and intensive lentiviral vector-based RNAi. *Methods Mol. Biol.* **485,** 257–270 (2009).
89. Dittmar, K. A., Goodenbour, J. M. & Pan, T. Tissue-specific differences in human transfer RNA expression. *PLoS Genet.* **2,** 2107–2115 (2006).
90. Neil, S. J. D., Zang, T. & Bieniasz, P. D. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* **451,** 425–30 (2008).
91. Namy, O., Rousset, J. P., Napthine, S. & Brierley, I. Reprogrammed Genetic Decoding in Cellular Gene Expression. *Mol. Cell* **13,** 157–168 (2004).
92. Aragonès, L., Guix, S., Ribes, E., Bosch, A. & Pintó, R. M. Fine-tuning translation kinetics selection as the driving force of codon usage bias in the hepatitis A virus capsid. *PLoS Pathog.* **6,** 1–12 (2010).
93. Goodman, B. D., Church, M. G. & Kosuri, S. Causes and Effects of N-Terminal Codon Bias in Bacterial Genes. *Science (80-.).* **342,** 475–478 (2013).
94. Ma, Z. *et al.* The effects of the codon usage and translation speed on protein folding of 3Dpol of foot-and-mouth disease virus. *Vet Res Commun* **16,** 270–274 (2013).
95. Quax, T. E. F., Claassens, N. J., Söll, D. & van der Oost, J. Codon bias as a means to fine tune gene expression. *Mol Cell.* **59,** 149–161 (2015).
96. Rodrigue, N., Philippe, H. & Lartillot, N. Mutation-selection models of coding sequence

evolution with site-heterogeneous amino acid fitness profiles. *Proc. Natl. Acad. Sci.* **107,** 4629–4634 (2010).
97. Tuller, T. *et al.* An evolutionarily conserved mechanism for controlling the efficiency of protein translation. *Cell* **141,** 344–354 (2010).
98. Zhao, F., Yu, C. & Liu, Y. Codon usage regulates protein structure and function by affecting translation elongation speed in Drosophila cells. *Nucleic Acids Res.* **45,** 8484–8492 (2017).
99. Zhou, J. H. *et al.* The effects of the synonymous codon usage and tRNA abundance on protein folding of the 3C protease of foot-and-mouth disease virus. *Infect. Genet. Evol.* **16,** 270–274 (2013).
100. Zhou, M., Wang, T., Fu, J., Ziao, G. & Liu, Y. Non-optimal codon usage influences protein structure in intrinsically disordered regions. *Mol. Microbiol.* **97,** 974–987 (2015).
101. Martínez, M. A., Jordan-Paiz, A., Franco, S. & Nevot, M. Synonymous Virus Genome Recoding as a Tool to Impact Viral Fitness. *Trends Microbiol.* **xx,** 1–14 (2015).
102. Pavon-Eternod, M. *et al.* Vaccinia and influenza A viruses select rather than adjust tRNAs to optimize translation. *Nucleic Acids Res.* **41,** 1914–1921 (2013).
103. Franz, Kate M., K. C. J. Finding a needle in a haystack of needles—A productive hunt for interferon stimulated genes with antiviral activity. *Immunol. Cell Biol.* **92,** 205–207 (2015).
104. Samuel, M. a *et al.* PKR and RNase L contribute to protection against lethal West Nile Virus infection by controlling early viral spread in the periphery and replication in neurons. *J. Virol.* **80,** 7009–7019 (2006).
105. Li, M. M. H., MacDonald, M. R. & Rice, C. M. To translate, or not to translate: viral and host mRNA regulation by interferon-stimulated genes. *Trends Cell Biol.* **25,** 320–329 (2015).
106. Schneider, W. M., Chevillotte, M. D. & Rice, C. M. Interferon-Stimulated Genes: A Complex Web of Host Defenses. *Annu. Rev. Immunol.* **32,** 513–545 (2014).
107. Pisareva, V. P., Muslimov, I. a., Tcherepanov, A. & Pisarev, A. V. Characterization of Novel Ribosome-Associated Endoribonuclease SLFN14 from Rabbit Reticulocytes. *Biochemistry* **54,** 3286–3301 (2015).
108. Scherbik, S. V *et al.* RNase L Plays a Role in the Antiviral Response to West Nile Virus. *J. Virol.* **80,** 2987–2999 (2006).
109. Govero, J. *et al.* Zika virus infection damages the testes in mice. *Nature* **540,** 438–442 (2016).
110. Ma, W. *et al.* Zika Virus Causes Testis Damage and Leads to Male Infertility in Mice. *Cell* **168,** 542 (2017).
111. Phizicky, E. M. & Hopper, A. K. tRNA biology charges to the front. *Genes Dev.* **24,** 1832–1860 (2010).
112. Ivanov, P., Emara M. mohamed, Villen Judit, Steven, G. P. & Anderson, P. Angiogenin-induced tRNA fragments inhibit translation initiation. *Mol. Cell* **100,** 130–134 (2012).
113. Wang, Q. *et al.* Identification and functional characterization of tRNA-derived RNA fragments (tRFs) in respiratory syncytial virus infection. *Mol. Ther.* **21,** 368–379 (2013).
114. Rugeles, M. T. *et al.* Ribonuclease is partly responsible for the HIV-1 inhibitory effect activated by HLA alloantigen recognition. *Aids* **17,** 481–486 (2003).
115. Casas, E., Cai, G. & Neill, J. D. Characterization of circulating transfer RNA-derived RNA fragments in cattle. *Front. Genet.* **6,** 1–7 (2015).

116. Megel, C. *et al.* Surveillance and cleavage of eukaryotic tRNAs. *Int. J. Mol. Sci.* **16,** 1873–1893 (2015).
117. Popis, M. C., Blanco, S. & Frye, M. Posttranscriptional methylation of transfer and ribosomal RNA in stress response pathways, cell differentiation, and cancer. *Curr. Opin. Oncol.* **28,** 65–71 (2016).
118. Blanco, S. *et al.* Aberrant methylation of tRNAs links cellular stress to neuro-developmental disorders. *EMBO J.* **33,** 1–20 (2014).
119. Khan, M. A. *et al.* Mutation in NSUN2, which encodes an RNA methyltransferase, causes autosomal-recessive intellectual disability. *Am. J. Hum. Genet.* **90,** 856–863 (2012).
120. Van Weringh, A. *et al.* HIV-1 modulates the tRNA pool to improve translation efficiency. *Mol. Biol. Evol.* **28,** 1827–1834 (2011).
121. Hunt, T. A. *et al.* Interactions between the West Nile virus capsid protein and the host cell-encoded phosphatase inhibitor, I2PP2A. *Cell. Microbiol.* **9,** 2756–2766 (2007).
122. Albers, S. & Czech, A. Exploiting tRNAs to Boost Virulence. *Life* **6,** 4 (2016).
123. Pang, Y. L. J., Abo, R., Levine, S. S. & Dedon, P. C. Diverse cell stresses induce unique patterns of tRNA up- and down-regulation: tRNA-seq for quantifying changes in tRNA copy number. *Nucleic Acids Res.* **42,** (2014).
124. Graczyk, D., White, R. J. & Ryan, K. M. Involvement of RNA Polymerase III in Immune Responses. *Mol. Cell. Biol.* **35,** 1848–1859 (2015).
125. M, Fischietti., A, Arslan., et al. Slfn2 Regulates Type I Interferon Responses by Modulating the NFκB Pathway. *Mol. Cell. Biol.* (2018). doi:10.1128/MCB.00053-18
126. Pechmann, S. & Frydman, J. Evolutionary conservation of codon optimality reveals hidden signatures of co-translational folding. *Nat. Struct. Mol. Biol.* **20,** 237–243 (2013).
127. Burns, C. C. *et al.* Modulation of Poliovirus Replicative Fitness in HeLa Cells by Deoptimization of Synonymous Codon Usage in the Capsid Region. *J. Virol.* **80,** 3259–3272 (2006).
128. Barretina, J., Caponigro, G. & Stransky, N. The Cancer Cell Line Encyclopedia enables predictive modeling of anticancer drug sensitivity. *Nature* **483,** 603–607 (2012).
129. Tang, S. W. *et al.* SLFN11 is a transcriptional target of EWS-FLI1 and a determinant of drug responsein Ewing sarcoma. *Clin. Cancer Res.* **21,** 4184–4193 (2015).
130. Goffena, J. *et al.* Elongator and codon bias regulate protein levels in mammalian peripheral neurons. *Nat. Commun.* **9,** (2018).

Abbreviations

AAA – ATPases associated with diverse cellular activites

Ala – Alanine

ANG – Angiogenin

ANOVA– Analysis of variance

Asp – Aspartic acid

AU – Arbitrary units

BC – Back-complement

BHK – Baby hamster kidney

BSL – Biosafety level

C – Capsid

CD4 – Cluster of differentiation 4

cDNA – Complementary DNA

CNS – Central nervous system

DC – Dendritic cell

DDA – DNA-damaging agents

DENV – Dengue virus

DMEM– Dulbecco's Modified Eagle's Medium

DNA – Deoxyribonucleic acid

dsRNA– Double-stranded RNA

E – Envelope

eGFP – enhanced green fluorescent protein

eIF2 – Eukaryotic initiation factor 2

ELISA – Enzyme-linked immunosorbent assay

ER – Endoplasmic reticulum

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

HEK293– Human embryonic kidney 293

His – Histidine

HIV-1 – Human Immunodeficiency virus 1

IFIT1 – Interferon-induced protein with tetratricopeptide repeats 1

IFN – Interferon

IgM – Immunoglobulin M

IRF – Interferon regulatory factor

ISG – Interferon stimulated gene

ISGF3 – Interferon stimulated gene factor 3

ISRE – IFN-stimulated response elements

Jak1 – Janus Kinase 1

JEV – Japanese encephalitis virus

KD – Knockdown

kDa – Kilodaltons

KFDV – Kyasanur forest disease virus

Leu – Leucine

LPS – Lipopolysaccharide

LTR – Long terminal repeats

M – Membrane

MAVS – Mitochondrial antiviral signaling

MDA5 – Melanoma differentiation-associated gene 5

moDC – Monocyte derived dendritic cell

MOI – Multiplicity of infection

mRNA – messenger RNA

NCR – Non-coding region

NLR – Nod-like receptor

NS – Non-structural

Nts – nucleotides

NY – New York

OAS – Oligoadenylate synthetase

OHFV – Omsk haemorrhagic fever virus

OPV – Orthopoxvirus

ORF – Open reading frame

PAMP – Pathogen-associated molecular pattern

PCR – Polymerase chain reaction

PKR – Protein kinase R

poly-dAdT – poly(deoxyadenylic-deoxythymidylic) acid

Poly-IC – Polyinosinic:polycytidylic acid

prM – pre-membrane

PRR – Pattern recognition receptor

RdRp – RNA-depndent RNA polymerase

RIG-I – Retinoic-acid inducible gene-I

RNA – Ribonucleic acid

RPA – Replication protein A

rRNA – Ribosome RNA

RSV – Respiratory syncytial virus

RTD – Rapid tRNA decay

RVFV – Rift valley fever virus

SCR – Scramble

SDS-PAGE– Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

Ser – Serine

shRNA– small hairpin RNA

Slfn – Schlafen

ssRNA– Single-stranded RNA

STAT1– Signal transducer and activator of transcription 1

TLR – Toll-like receptor

TNF – Tumor necrosis factor

tRF – tRNA-derived fragment

tRNA – Transfer RNA

Trp – Tryptophan

Tyk2 – Tyrosine kinase 2

vSlfn – viral Schlafen

VSV-G– glycoprotein Vesicular stomatitis virus

WNV – West Nile virus

YFV – Yellow fever virus

ZAP – Zinc Finger antiviral protein

ZIKV – Zika virus

Vita

Federico Valdez was born in El Paso, Texas on February 29, 1988 to parents Maria del Carmen Valdés and Mario Valdez. He earned his Bachelor of Science degree in Microbiology from The University of Texas at El Paso in 2012. Later that year he joined the doctoral program in Pathobiology at The University of Texas at El Paso.

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