DENGUE VIRUS AND THE SUPPRESSION OF THE CELLULAR INNATE IMMUNE RESPONSE: ANTIVIRAL MICRORNAS AS A TOOL FOR PROBING HOST-VIRUS INTERACTIONS

By Ashleigh R. Murphy

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Abstract

The flaviviruses are a genus of enveloped, positive-strand RNA genome viruses that includes the dengue viruses (DENV) and West Nile virus (WNV). Transmitted by the bite of an infected tick or mosquito, many flaviviruses cause severe disease in humans. The dengue viruses, in particular, are thought to infect 100 million people a year and can be found in tropical and subtropical environments worldwide. Primary infection by any of the four DENV serotypes can be asymptomatic or cause a mild, but painful febrile illness; however, heterologous secondary infection has been linked to severe, life-threatening disease characterized by vascular disruption leading to systemic shock. In the face of the disease risk associated with endemic and emerging flaviviruses, research to further our understanding of the interactions between virus and host may open avenues toward developing new therapeutics and improved techniques for patient care.

Our lab has previously demonstrated the utility of a class of small regulatory RNAs called microRNAs (miRNAs) in elucidating virus-host interactions at the cellular level. In brief, by identifying a miRNA that alters flavivirus infection and the mRNAs regulated by that miRNA, cellular factors directly involved in viral infections can be revealed. In this thesis, I will describe two miRNAs that regulate cellular proteins that alter flavivirus replication. The first study characterizes the cellular miR-424, which was observed to restrict DENV infection by inhibiting the E3 ubiquitin ligase SIAH1. Further analysis demonstrated that the cell's unfolded protein response (UPR), triggered by DENV infection, induces transcription of SIAH1 that results in ubiquitination and degradation of the innate immune signaling molecule MyD88.

These observations revealed a unique pathway of UPR-mediated innate immune inhibition induced by DENV infection that is regulated by miR-424.

In the second study, I investigated the mechanism of miR-526b inhibition of flavivirus infection. I observed that miR-526b greatly enhanced intracellular immune signaling when cells were stimulated with a pathogen recognition receptor agonist. However, a change in cytokine response was not observed during flavivirus infection, suggesting that interference with innate immunity by the virus overcomes the enhancing effect of miR-526b. Based on a time-dependent reduction in viral genome replication, I propose a role for miR-526b in regulation of the endoplasmic reticulum (ER) or ER-resident proteins required for genome replication and virion assembly.

In conclusion, this dissertation describes the implementation of a technique for using cellular miRNAs as tools for the discovery of virus-host interactions. With this methodology, I discovered a new function for the UPR during DENV2, namely inhibition of innate immune signaling, as well as identifying a miRNA that impedes flavivirus infection in spite of failure to enhance cellular immunity.

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Selected Abbreviations

ADE: Antibody dependent enhancement

Ago: Argonaute

ATF: Activating transcription factor

BDV: Border disease virus

BVDV: Bovine viral diarrhea virus

C: Capsid

CSFV: Classical swine fever virus

DC: Dendritic cell

DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DENV: Dengue virus

DHF: Dengue hemorrhagic fever

dsRNA: Double-stranded RNA

DSS: Dengue shock syndrome

E: Envelope

eIF2α: Elongation initiation factor 2α

ER: Endoplasmic reticulum

GADD34: Growth arrest and DNA damage inducible 34

GAS: Gamma-activated sequence

HBV: Hepatitis B virus

HCMV: Human cytomegalovirus

HCV: Hepatitis C virus

HECT: Homologous to E6-AP COOH terminus

HIV: Human immunodeficiency virus

hnRNP: Heterogeneous nuclear ribonucleoprotein C1/C2

HPV: Human papillomavirus

IFN1: Type 1 interferon

IFNAR: Interferon-α/β receptor

IκB: Inhibitor of kappa B

IL: Interleukin

IRAK: Interleukin 1 receptor associated kinase

IRE1: Inositol-requiring protein-1

IRF: Interferon-regulated factor

ISG: Interferon stimulating gene

ISRE: Interferon-stimulated response element

JEV: Japanese encephalitis virus

JNK: c-Jun N-terminal kinase

kb: Kilobase

kDa: KiloDalton

KSHV: Kaposi's sarcoma-associated herpesvirus

LGP2: Laboratory of genetics and physiology 2

M: Membrane

MAVS: Mitochondrial antiviral-signaling protein

MDA5: Melanoma differentiation-associated gene 5

miRNA: MicroRNA

mRNA: MessengerRNA

MTase: Methyltransferase

MyD88: Myeloid differentiation primary response 88

NC: Non-coding

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHP: Non-human primate

NLR: Nod-like receptor

NS: Nonstructural

nt: Nucleotide

NTPase: Nucleoside 5'-triphosphatase

ORF: Open reading frame

PAMP: Pathogen-associated molecular pattern

PAR-CLIP: Photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation

pDC: Plasmacytoid dendritic cell

PERK: Protein kinase RNA-like ER kinase

poly(I:C): Polyinosinic:polycytidylic acid

POWV: Powassan virus

pre-miRNA: Precursor miRNA

pri-miRNA: Primary miRNA

prM: Precursor membrane

PRR: Pattern recognition receptor

RdRp: RNA-dependent RNA polymerase

RIG-I: Retinoic acid-inducible gene I

RING: Really interesting new gene

RTPase: RNA triphosphatase

SBD: Substrate-binding domain

siRNA: Small interfering RNA

STAT: Signal transducer and activator of transcription

STING: Stimulator of IFN genes

SUMO: Small ubiquitin-like modifier

TBEV: Tick-borne encephalitis virus

TIRAP: TIR domain containing adaptor protein

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TRAF: TNF receptor-associated factor

TRIM: Tripartite motif protein

Ub: Ubiquitin

UPR: Unfolded protein response

UPS: Ubiquitin-proteasome system

UTR: Untranslated region

VEEV: Venezuelan equine encephalitis virus

VSV: Vesicular stomatitis virus

WHO: World Health Organization

WNV: West Nile virus

XBP1: X-box binding protein 1

YFV: Yellow fever virus

ZIKV: Zika virus

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Chapter 1: Introduction

1.1 Flaviviruses

1.1.1 Classification

The flaviviruses are a genus of positive-stranded RNA viruses primarily transmitted by the bite of a hematophagous arthropod and are capable of causing serious disease in humans and animals. Flaviviruses are one of four genera of the family *Flaviviridae*, which also includes the hepaciviruses, the pestiviruses, and the pegiviruses (Figure 1.1)[1]. The *Flaviviridae* have spherical particles consisting of an icosahedral nucleocapsid enclosed in a host cell membrane-derived lipid bilayer containing two to three viral proteins[2]. Their genome is a single strand of positive sense RNA 11 to 12 kilobases (kb) long with extensive secondary structure in the 5' and 3' end non-coding (NC) regions[3]. The genome consists of a single open reading frame (ORF) that is translated into a large polyprotein and proteolytically cleaved into the individual viral proteins, both structural and nonstructural (NS). Although the *Flaviviridae* share general similarities between genera and species in regards to their virion structure, genome replication, and general protein function, the transmission cycles, host species, cell tropism, and disease manifestations vary greatly between the genera and species of *Flaviviridae.*[3]

The flaviviruses comprise the largest genus of the *Flaviviridae*, representing 53 species of viruses[1]. Most flaviviruses are transmitted horizontally between a vertebrate host species and a hematophagous mosquito or tick species. The mosquito-borne viruses include the dengue viruses (DENV), West Nile virus (WNV), yellow fever virus (YFW), Zika virus (ZIKV), and Japanese encephalitis virus (JEV), all sources of significant human disease globally [3,4]. Likewise, the tick-

borne flaviviruses, such as tick-born encephalitis virus (TBEV) and Powassan virus (POWV), pose a significant threat to human life[5].

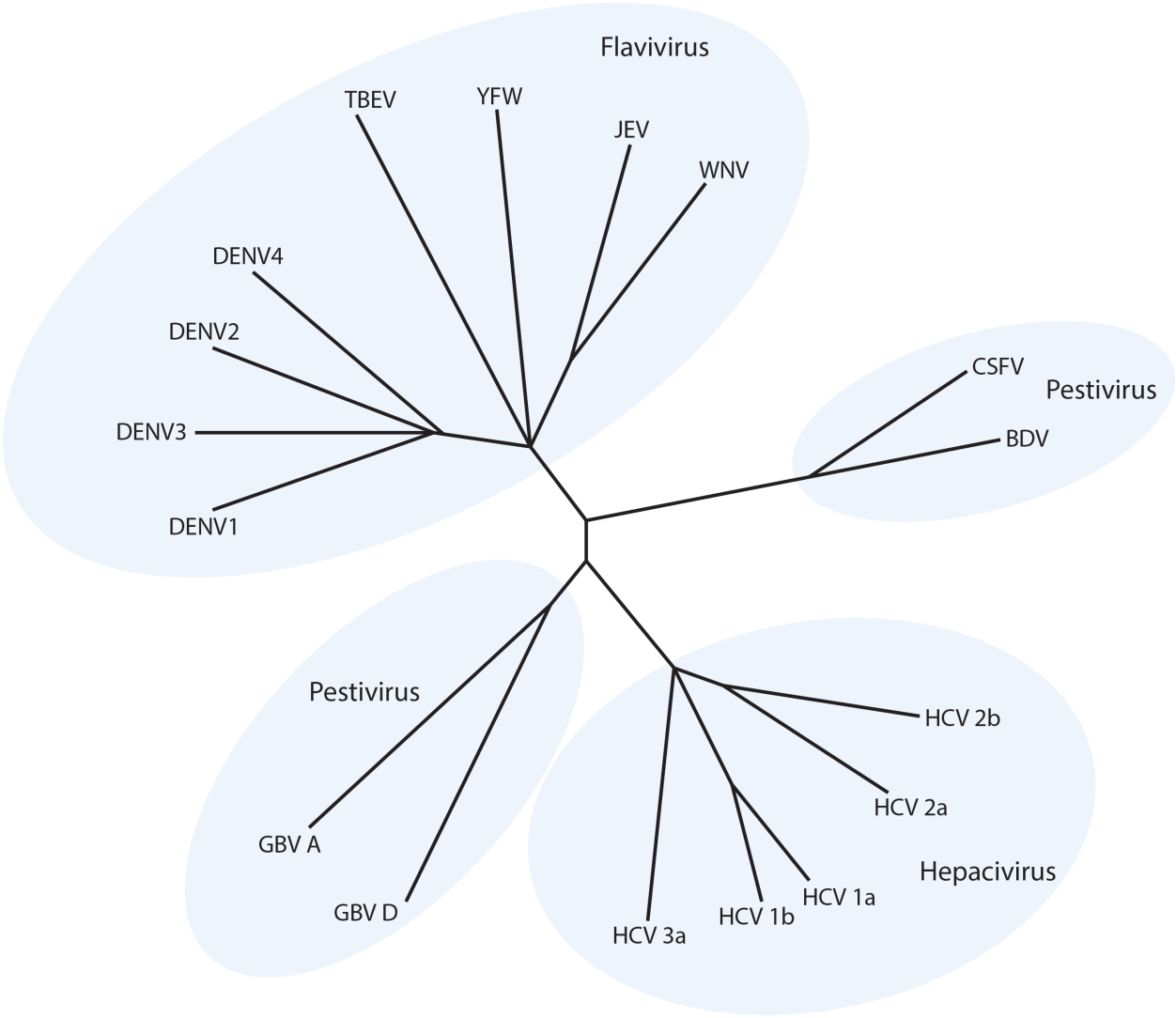


Figure 1.1 *Flaviviridae* phylogeny

Phylogenetic relation of representative members of the *Flaviviridae* genera.

Hepatitis C virus (HCV) is the only known human pathogen in the hepacivirus genus, although similar hepaciviruses have been identified in a variety of wild and domesticated animal species, including bats, primates, horses, and cows[6]. HCV is transmitted person-to-person, typically hematogenously, and infects 185 million people globally[7]. Infection with HCV often causes a lifelong chronic hepatotrophic infection, leading to liver fibrosis, cirrhosis, and cancer[3]. However, the recent advent of potent antiviral drugs for treating HCV infection may significantly reduce global HCV morbidity when made broadly available[8].

The best studied pestiviruses are pathogens of livestock as pestiviral morbidity and mortality is an important factor in the economics and stability of the global livestock industry[3,9]. Common pestiviruses, such as classical swine fever virus (CSFV), bovine viral diarrhea virus types 1 and 2 (BVDV-1, BVDV-2), and border disease virus (BDV), are shed in high amounts and are readily transmitted through all bodily fluids as well as vertically from mother to fetus. In addition, pestiviruses can establish asymptomatic persistent infection, making the virus difficult to detect and eliminate in spite of vaccination and well-established elimination protocols in practice in most countries[9] .

In 2013, the International Committee on Taxonomy of Viruses officially recognized the genus pegivirus as a distinct genus within the *Flaviviridae*[1,10]. Originally designated the GB viruses, based on the initials of the surgeon from whom the first virus was thought to be isolated, the pegiviruses were placed in the *Flaviviridae* due to their genomic similarity to HCV[11-13]. Subsequent amino acid analysis of the viral polymerase and the identification of GB viruses in tamarins, other New World monkeys, and bats led to the establishment of the pegivirus genus[12,14-16]. Pegiviruses have also been identified in Old World, as well as New World,

primates, rodents, horses, pigs, and although the viruses can cause prolonged, even life-long, infection, they have not been associated with any disease manifestation[14,17].

1.1.2 Host-vector cycle

The genus flavivirus can be subdivided into four clusters based on mode of transmission and host species. The mosquito-borne and tick-borne clades are the only two that contain viruses that have been found to cause human disease; the remaining two clades contain viruses transmitted only between arthropods and those viruses of unknown vector (typically identified in bats and rodents)[18-20]. Among the mosquito-borne flaviviruses for which humans and primates as the primary vertebrate hosts, such as DENV, ZIKV, and YFV, two geographically distinct transmission cycles have been observed: an enzootic, sylvatic cycle and an epizootic, urban cycle (Figure 1.2)[20,21]. In forest environments, these viruses circulate between arboreal mosquitoes and non-human primates; in contrast, in regions of dense populations, the viruses cycle between humans and domestic or peridomestic species of mosquitoes such the *Aedes aegypti* and *Aedes albopictus*[22]. In addition to infection via mosquito, non-vector transmission can occur—most often through percutaneous or mucosal exposure to infected blood, although ZIKV has also been shown to be transmitted sexually as well as vertically from mother to fetus[23-26].

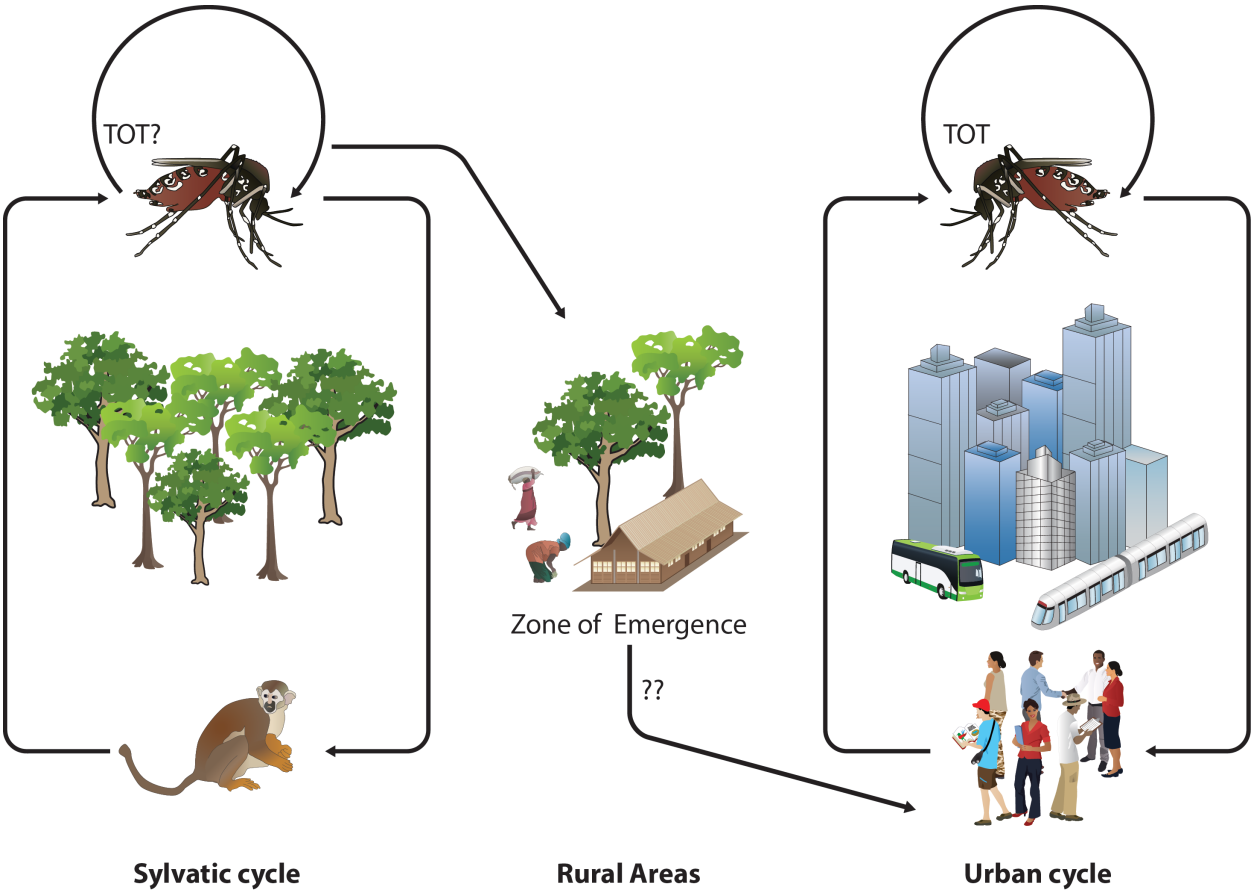


Figure 1.2 DENV transmission

DENV, as well as YFW and ZIKV, exist in a sylvatic cycle between arboreal mosquitoes and non-human primates and an urban cycle between domestic mosquito species and humans. (TOT = transovarial transmission) Images courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

In contrast to DENV, YFV, and ZIKV, humans are only incidental hosts of WNV. Rather, the natural reservoir of WNV appears to birds, with over three hundred species vulnerable in the United States alone[27]. The virus is maintained in an enzoonotic bird-mosquito-bird cycle by the ornithophagic species of *Culex* mosquitoes (Figure 1.3)[28]. Transmission of WNV amongst birds can also occur through exposure to infected avian fecal matter or the consumption of infected mosquitoes[29]. Birds typically survive WNV infection, developing transient viremia sufficient to infect naïve mosquitoes, followed by recovery and development of permanent immunity[30-32]. However, some avian species, often corvids in North America, appear particularly vulnerable to WNV, resulting in large die-offs[30,33]. Both humans and horses may develop encephalitic disease when infected with WNV but do not appear to transmit the virus to naïve mosquitoes, presumably because circulating viral titers in the blood are insufficient to infect a feeding mosquito[29,34]. Non-vector modes of human transmission include blood transfusion, organ transplant, transplacentally, or through breast milk[35-39].

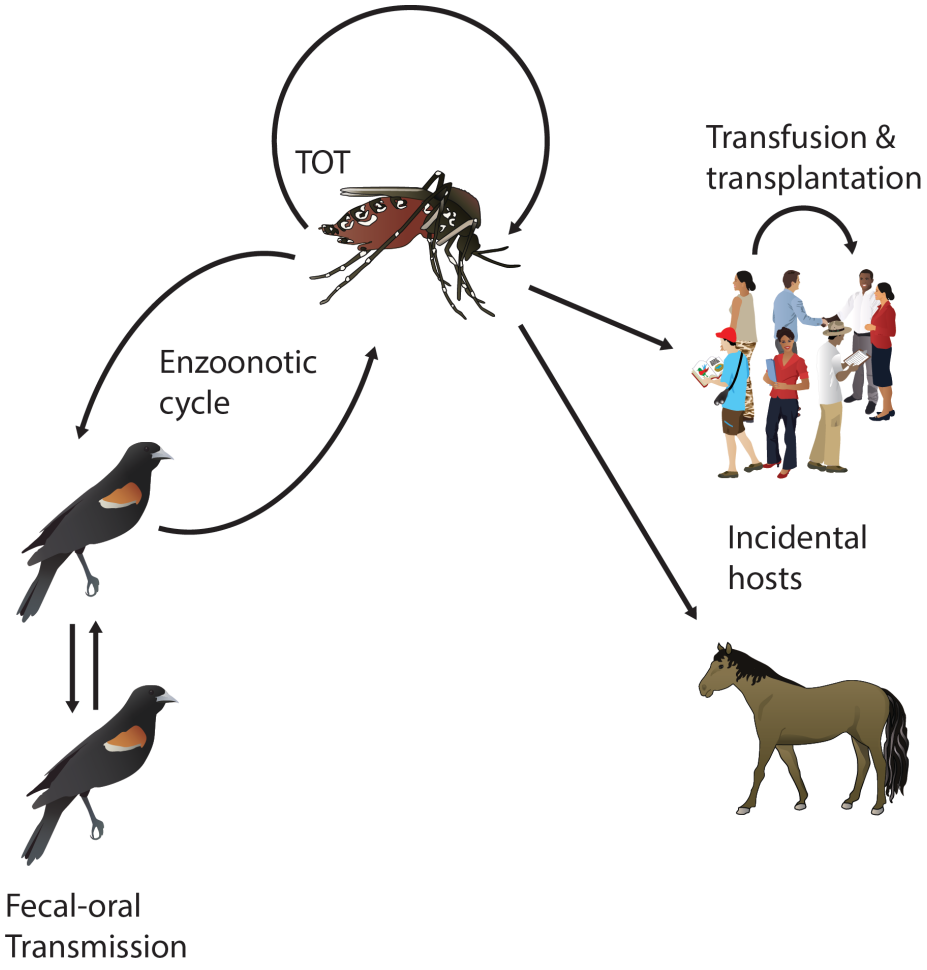


Figure 1.3 WNV transmission

WNV infects birds and *Culex* mosquitoes through the enzootic cycle and through exposure to infected fecal material. Infected *Culex* mosquitoes can also transmit the virus to offspring. Horses and humans are dead-end hosts of the virus but infection can be transmitted via blood transfusion and organ transplantation. (TOT = transovarial transmission) Images courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

Similar to WNV, the tick-borne flaviviruses appear to infect humans only incidentally, cycling primarily through either rodents or birds[40]. Due the prolonged attachment of the tick during feeding and the tendency of ticks to cluster in groups on the host animal, such as on the ears or near the beak, tick-borne flaviviruses do not appear to require viremia in the host in order to infect naïve ticks[41-43]. Rather, infection of migratory Langerhans cell, neutrophils, and macrophages in the skin region infested by co-feeding ticks appears sufficient to transmit the virus to uninfected ticks[43-45]. Humans do not produce high enough viremia to transmit the virus nor are they often subject to clustered tick infestation, and therefore, no do not represent an amplifying host to the virus[40,42]. However, infection with TBEV or another tick-borne flavivirus can cause severe disease, including encephalitis and hemorrhagic fever[40].

1.1.3 Serology

Prior to the advent of gene sequencing and genome analysis, the classification of arthropod-borne viruses, or arboviruses, relied on serologic reactivity, determined by the degree to which antibodies raised against one virus would neutralize another[3]. Jordi Casals and Leslie Webster first demonstrated the cross-reactivity between antibodies raised against some arboviruses in 1944: whereas sera from mice infected with either Russian spring-summer encephalitis virus or Louping ill virus (both now classified as tick-borne flaviviruses) would protect naïve mice from infection with the other virus, such sera would not protect mice from infection with rabies or poliomyelitis[46,47]. Furthermore, while both the tick-borne flaviviruses cross-reacted with the mosquito-borne WNV, JEV, and St. Louis encephalitis viruses, the neutralization was stronger amongst the members of the tick-borne or mosquito-borne groups

than between viruses of differing vector groups, suggesting that viruses within each vector group were more closely antigenically related to one another[47,48]. These observations provided a template on which the structure of arbovirus classification was formed, driven by three guidelines developed by Casal and supported by antigen modeling and genome sequencing decades later: in his words, "'(1) No virus can belong to two antigenic groups. (2) If two viruses cross-react antigenically, they are related. (3) If viruses of different groups cross-react, they do not belong to different groups'"[47].

Further advancement in techniques for growing large amounts of virus and for collecting antigens and antibodies, as well as the rapidly expanding catalogue of virus isolates sampled from around the world, allowed more and more detailed serologic classification of the arboviruses as well as provided a much needed tool for diagnosis of arbovirus infection using patient sera[47]. These studies into the interrelationships of viruses led to the categorization of arboviruses into groups based on antigenic cross-reactivity: the Group A arboviruses, which is now the genus alphavirus of the *Togaviridae* family, and the Group B arboviruses, which now compose the genus flavivirus. Further work reclassified the flaviviruses, from the *Togaviridae* to an independent family, *Flaviviridae*[1,47]. Studies of arboviral particle structure by electron microscopy revealed morphological characteristics common among antigenically-related virus groups, providing a practical explanation of the cross-reactivity of antibodies between virus groups[47,49,50].

Work by Calisher, et al. published in 1989 demonstrated that the flaviviruses could be further subdivided in eight serologic groups, termed serocomplexes[51]. These groupings corresponded not only to serologic cross-reactivity but also correlated to the transmission vector

of the virus[2]. For example, members of the Japanese encephalitis virus serocomplex, such as JEV, WNV, and Murray Valley encephalitis virus, are transmitted by *Culex* species mosquitoes. The International Committee on the Taxonomy of Viruses now recognizes 14 flavivirus serocomplexes: nine mosquito-borne, two tick-borne, two with unknown vectors, and one nonvectored, arthropod serocomplex[1]. Epitope mapping, amino acid sequencing, and molecular modeling of the exterior of the virus particle, in particular the envelope protein E, has shed light on the source of the antigenic relationships within and between serocomplexes. About 40% amino acid identity is conserved between E proteins of the 14 serocomplexes[52]. This variation is concentrated in the portions of E on the outer surface of the virus particle, resulting in a significant variation in exterior epitopes and explaining the poor cross-reactivity of antibodies from one serocomplex to another[52]. Antigenic similarity within serocomplexes varies as well; while WNV and JEV in the Japanese encephalitis complex share approximately 80% amino acid sequence identity, within the dengue serocomplex, amino acid identity of E diverges by up to 37%[52]. The divergence between the four members of the dengue serocomplex (DENV1-4) provides an explanation for the temporary cross-reactivity and the rapid loss of protection from heterologous infection after DENV infection.

1.2 The dengue viruses

1.2.1 Introduction and history of DENV

The earliest records of a disease matching the clinical description of dengue infection come from a Chinese medical encyclopedia printed and reprinted several times between 265 AD and 992 AD[53,54]. The book refers to the disease as "water poison" and draws a connection between instances of sickness and flying insects near bodies of water[54]. Seven centuries later, reports from the French West Indies and Panama describe outbreaks of disease matching the symptoms of dengue occurred in 1635 and 1699, respectively[54]. A series of epidemics of dengue-like disease in Indonesia, Egypt, Spain, and the United States late in the 18th century suggest a global distribution of dengue virus, although the true etiological source of the outbreaks cannot be determined definitively[54,55]. Until the onset of World War II, a century and a half later, large but infrequent epidemics of dengue-like disease occurred in tropical and subtropical regions worldwide, becoming endemic in urban centers and frequently causing illness in nonimmune travelers[54].

Although the causative agent of dengue was identified as filterable (and therefore, a virus) in 1907 and transmission by *Aedes aegypti* confirmed in 1926, the virus was not isolated until 1943 when the Japanese and US militaries independently formed scientific commissions to determine the etiologic agent of the dengue epidemics sweeping through vulnerable populations displaced by World War II[56-58]. The ecologic changes and mass troop and refugee movement wrought by the war expanded the geographic distribution of both mosquito vector and virus, leading to epidemics throughout East Africa, the Caribbean, and the Pacific theater of operations[58]. Following the cessation of World War II, no epidemics of dengue occurred outside

of Southeast Asia for nearly two decades. The absence of dengue in the Americas may be attributed to *Aedes* mosquito eradication programs instated throughout the region to eliminate the *Aedes*-transmitted YFV, although the mosquito quickly rebounded upon discontinuation of the eradication measures[58].

Dengue returned to the Americas in 1963, emerging in Puerto Rico and Jamaica, and reappeared in Oceania in 1964[59-61]. A surveillance program in Nigeria in 1964 detected endemic DENV amongst the human population[62]. Dengue outbreaks continued to increase in frequency and intensity worldwide through the 1990s as urban populations expanded, international and intercontinental air travel increased, and vector mosquito control programs failed[58]. The first two decades of the 21st century have witnessed further spread of DENV, which is now present on all continents save Antarctica[63]. Epidemics causing confirmed infections in tens of the thousands to hundreds of thousands of people have occurred in the Americas, Southeast Asia, India, and Africa[2,64]. The World Health Organization (WHO) estimates 100 million DENV infections occur globally each year, leading to 20,000 deaths[65]. Mortality from severe dengue ranges from 1-5%, dependent heavily upon a patient's access to hospitalization and advanced supportive care[66,67]. Approximately 3 billion people are at risk of dengue infection, particularly in the world's urban and periurban areas, and as global temperatures rise and new regions become hospitable for vector mosquito habitation, the at-risk global population is expected to grow[65,68].

1.2.2 Serotypes

The dengue virus serocomplex consists of four antigenically-distinct but symptomatically-similar viruses, designated serotypes 1-4 (DENV1, DENV2, DENV3, and

DENV4)[51]. Albert Sabin first noted the existence of immunologically-discrete species of DENV when he observed that serum against a strain of DENV from New Guinea cross-reacted against a strain from Hawaii if collected 4 to 8 weeks after the infection with the New Guinea strain but not with sera collected after more than 8 weeks[69]. The discovery of the two serotypes represented by the Hawaii and New Guinea DENV, dubbed DENV1 and DENV2, was soon followed by the identification of DENV3 and DENV4 in the Philippines in 1956[70]. Sylvatic and urban strains of DENV1, -2, and -4 have been isolated and seroconversion of sentinel monkeys suggests a sylvatic DENV3 strain, indicating that the 4 DENV serotypes likely diverged and were maintained in sylvatic cycles separately, before independently emerging into the human population between 1,000 and 1,500 year ago [71-74].

The twentieth century post-World War II witnessed the explosive spread of DENV across the globe; however, most endemic areas reported the presence of one or rarely, two DENV serotypes circulating in a given region[75]. In the 1980s, the number of serotypes reported in many regions began in to increase. The rise may be partially attributed to advances in diagnostic techniques at the time, but the trend of multiple serotypes becoming endemic (hyperendemicity) in a given region has continued through the beginning of the twenty-first century. As of 2013, all 4 serotypes had been observed on all afflicted continents save Africa[75].

Dengue viruses vary significantly at the genetic level between serotypes as well as between genotypes within a single serotype. For example, the 5 genotypes of DENV2 that circulate amongst humans differ in the E protein amino acid sequence by an average of 7.3%[76]. Whether the differing genetics of individual species between and within serotypes correlate to virulence is not well understood, but evidence suggests that disease outcome may be somewhat

dependent on the serotype and genotype of the infection[58]. A study of patient samples collected in Thailand between 1994 and 2006 found that DENV1 and DENV3 were more likely to cause severe disease in patients who had not been infected with DENV before; in patients who had previously been infected with DENV, DENV2 and -3 more often cause severe disease[77]. Evidence also suggests that the Asian strains of DENV2 cause severe disease more often than the endemic American DENV2 strains and that a subgroup of a DENV3 genotype in Sri Lanka causes severe disease more often than the other members of that genotype[58,78]. The diversity of DENV serotypes, genotypes, and strains not only complicates understanding and predicting DENV ecology and epidemiology but also the study of DENV pathogenesis and the clinical manifestations of DENV infection.

1.2.3 Disease

Infection with any of the four DENV serotypes can cause a range of disease manifestations, from asymptomatic infection to painful febrile illness to potentially lethal shock from plasma leakage and internal hemorrhage[79]. DENV infection begins when an infected mosquito blood-feeds on a human, injecting virus-filled saliva, containing vasodilating and anticoagulating factors, into the skin to facilitate feeding[80]. In the skin, DENV infects local mononuclear phagocytic cells such as monocytes, macrophages, and dendritic cells (DCs)[81,82]. Infected cells transit from the site of infection to the lymph nodes, where the virus infects additional monocytes and macrophages, disseminating the virus throughout the body[83]. Symptoms appear 5 to 7 days post-infection, beginning with a sudden onset of high fever, accompanied by an intense headache associated with pressure behind the eyes, muscle and joint

pain, a distinctive skin rash, nausea, and vomiting[2,79]. During this time, patients are viremic and capable of spreading the virus to naïve mosquitos (although evidence shows that asymptomatic people with DENV can also spread the virus)[84,85]. The fever and associated symptoms typically abate after 3-7 days, and in most cases, patients recover completely; however, a fraction of patients develop severe, life-threatening symptoms concordant with the time of defervescence[2].

Between the 1970s and 2008, the febrile illness caused by DENV was referred to as dengue fever, while the less common but more dangerous DENV diseases were categorized as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)[2]. However, in 2009 the WHO adopted a new classification system intended to better illustrate the range of disease DENV may cause and aid in identifying patients most likely to experience serious complications, now referred to as severe dengue [2,79]. Dengue fever was reclassified as dengue with and without warning signs, allowing physicians to more accurately triage patients that appear to be at risk of developing severe dengue. Warning signs of severe dengue, such as mucosal bleeding, abdominal pain, swelling of the liver, and decreased platelet count, occur near the end of the febrile stage of disease and when carefully monitored, can indicate a patient is developing severe dengue. Severe dengue lasts 2 to 3 days and is characterized by the patient experiencing at least one of the following: plasma leakage or fluid accumulation sufficient to cause shock or respiratory distress, severe hemorrhage, or severe organ impairment (often of the liver). Due to the risk of catastrophic vasculopathy, severe dengue patients require hospitalization, careful observation, and supportive care to recover[2].

Prior to the global resurgence of DENV, reports of severe or life-threatening cases of DENV-associated disease were rare[2,58]. Concordant with the rapid rise in DENV

hyperendemicity and frequency of epidemics during the twentieth century, however, the incidence of severe dengue-associated disease also increased, becoming a major cause of hospitalization and death among residents of endemic areas[58,86]. Careful epidemiological investigation and surveillance revealed that the majority of cases of severe dengue occurred in people older than 1 year who were experiencing a secondary infection with DENV[86]. Although primary infection results in immunity to reinfection by that DENV serotype, infection with a heterologous serotype was much more likely to culminate in severe disease. In addition, infants borne to dengue-immune mothers were vulnerable to severe disease due to the transient passive immunity transferred from mother to child[86,87]. A variety of mechanisms have been proposed to explain the correlation between secondary DENV infection and severe dengue disease: cross-reactivity of antibodies against DENV NS1 protein with blood clotting factors, platelets, or vascular endothelium; prematurely activated T cells; "original antigenic sin" in reactivated T cells; activation of the complement system[86,88]. The most widely accepted explanation is that of antibody dependent enhancement (ADE): neutralizing antibodies raised against the primary infection serotype fail to neutralize heterotypic virus, resulting in infection of Fc-receptor-expressing cells and increased viral replication, increased viremia and release of cytokines, and culminating in an intensified disease state[86,89,90].

1.2.4 Antibody Dependent Enhancement

The mechanism of antibody dependent enhancement proposes that antibodies produced against the primary infecting serotype will lack sufficient avidity to completely neutralize heterotypic virus particles due to the sequence diversity of DENV serotypes[2,90]. Fc-receptor

bearing cells, such as macrophages and monocytes, will internalize an uninhibited but antibody-bound virus, leading to viral replication and increased overall viral load in the body[86]. Evidence supporting ADE has been found in both cell culture and *in vivo*. When Fc-bearing cells in culture were inflected with DENV in the presence of sera from DENV-infected patients or anti-DENV monoclonal antibodies, the infected cells released both more virus and more cytokines than the cells with immune sera or antibodies[86,91,92]. Similarly, mice and monkeys passively transferred anti-DENV antibodies showed increased viremia and disease burden[93,94].

Primary infection with DENV results in production of IgG antibodies against E and the membrane protein prM[86]. These antibodies provide lifelong protection from reinfection by the primary serotype and protection from the other serotypes for several months following primary infection. However, as antibody titers wane with time, the concentration of cross-reactive antibodies becomes too low to neutralize heterotypic infection (Figure 1.4) [95,96]. The pattern of severe dengue incidence by age in infants borne to dengue-immune mothers supports this explanation—severe dengue occurs rarely in infants less than 3 months old, peaks at 6-8 months as the passively inherited maternal immunity wanes, then falls again once the maternal antibodies disappear entirely[86,97]. Low concentration of cross-reactive antibodies appears insufficient to neutralize heterotypic DENV and instead, increases the vulnerability of Fc-receptor-bearing cells to DENV infection[2].

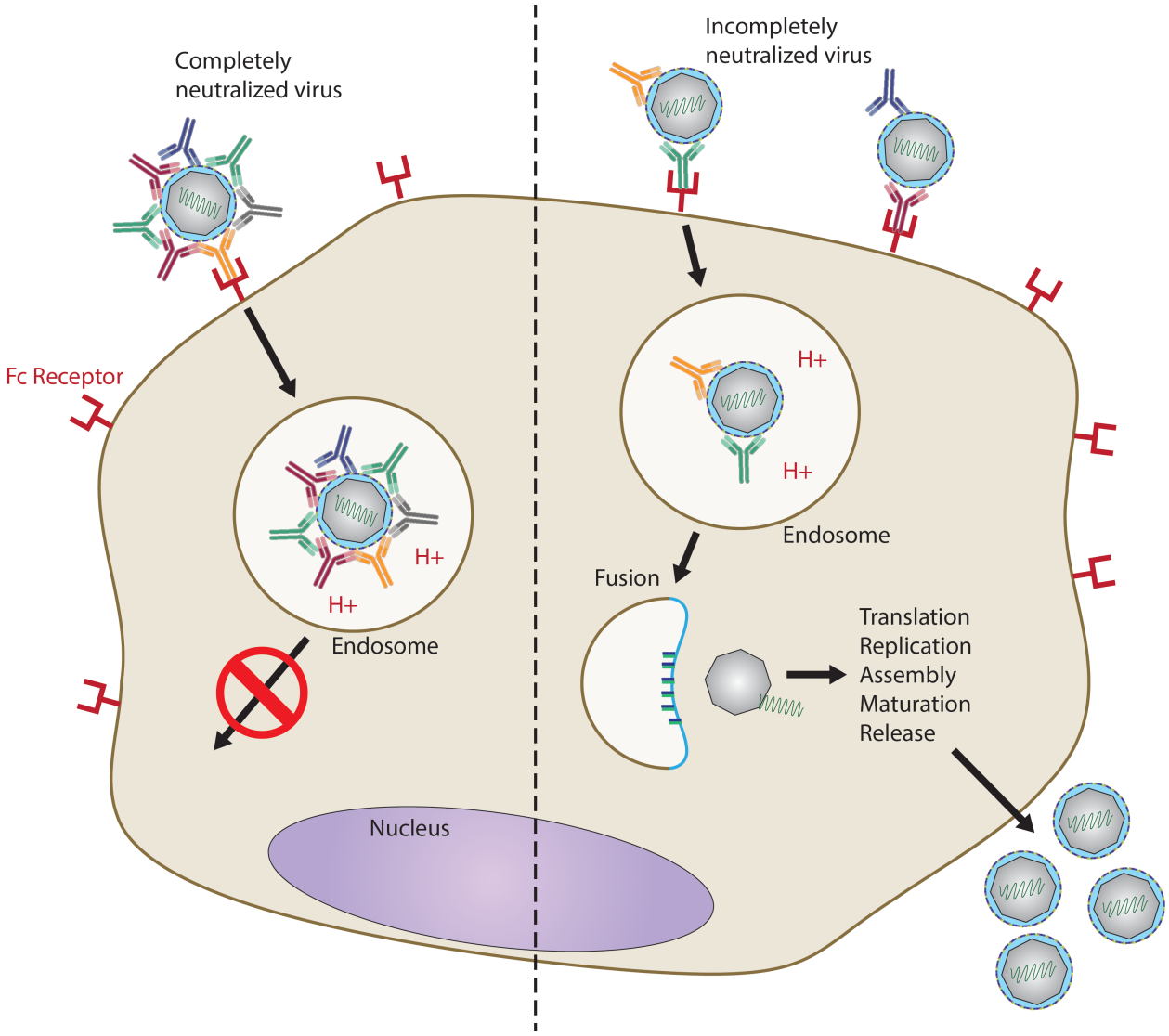


Figure 1.4 Model of antibody dependent enhancement

Left panel: Opsonized virus cannot infect. *Right panel*: Subneutralizing concentrations of antibody result in infection of Fc-receptor bearing cells.

The increased infectivity of monocytes and macrophages in the presence of antibody-bound virus has been to attributed to a variety of mechanisms, including improved attachment to the cell surface, increased internalization of virus, delivery of the antibody-bound virus to a more favorable endocytic environment, and improved fusion of the viral particle to the endocytic membrane[86]. Infection of these cells by antibody-bound virus not only results in greater viral yields but also suppresses some portions of the innate immune response to DENV[98-101]. *In vitro*, cells infected via DENV-antibody complexes downregulate the retinoic-acid gene I (RIG-I) signaling pathway and produce less type I interferon (IFN1) and interferon-activated genes[102,103]. ADE models in cell culture have also revealed negative regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Toll-like receptor (TLR) pathways, culminating in suppressed of innate immune responses and higher viral titers[104].

1.2.5 Vaccine development

Vector control for the prevention of DENV infection has been only moderately successful; therefore, vaccination is the viable alternative to preventing dengue disease[65]. Of great concern in the development of a DENV vaccine is the risk of predisposing a vaccinated person to ADE upon infection with a serotype heterologous to the vaccinating serotype[105]. The challenge, then, has been to identify an antigen shared between serotypes sufficient for protection or a combination of serotype-specific antigens capable of producing an immune profile that adequately protects against each serotype.

Pre-clinical trials of candidate DENV vaccines are difficult because the available animal models do not fully replicate the course of DENV infection and disease in humans. Wild type

mice may develop neurological symptoms but do not develop classical human dengue disease[106]. Mice lacking the interferon-α/β receptor (IFNAR) or lacking both IFNAR and the interferon-λ receptor develop dengue-like disease when infected in the present of non-neutralizing DENV antibodies (mimicking ADE)[107]. However, the absence of a complete immune response makes extrapolating vaccine efficacy to human infection difficult. The same is true of humanized mice, produced by engrafting immunodeficient mice with human cells or tissue such as hematopoietic stem cells or fetal thymus or liver tissue[105,108-110]. DENV infection of non-human primates (NHPs) has presented similar problems: NHPs develop viremia and immune protection but do not develop dengue disease symptoms[105]. In spite of the complications cause by animal modeling of DENV disease, numerous groups are working to produce a DENV vaccine on a variety of platforms.

The largest DENV vaccine trial to date was of Sanofi Pasteur's CYD-TDV—created by replacing the YFV E and prM genes in the successful yellow fever cDNA vaccine YF 17D with the E and prM genes of DENV[111]. A separate chimera was produced for each serotype, which were combined into a single preparation and administered in three subcutaneous injections scheduled six months apart. The vaccine was tested in Thailand, Malaysia and Latin America, but in spite of strong antibody responses after inoculation and 100% tetravalent neutralization after 3 doses, CYD-TDV failed to produce equal protection from infection by all DENV serotypes, although a reduction in severe dengue incidence was observed among the inoculated[112,113]. Possible complications to the tetravalent technique include misfolding of the chimeric virus particles, a requirement for T cell immunity for DENV protection, and interference between the four serotype-specific vaccines[114,115]. Nevertheless, CYD-TDV has

been licensed for use in five DENV endemic countries in Latin America and Asia in light of the ongoing risk of severe dengue in those regions[111,116].

A number of other vaccines are also in clinical trials. Takeda Pharmaceuticals has produced a tetravalent chimeric vaccine composed by substituting prM and E of DENV1, -3, and -4 into a live attenuated DENV2[117]. The Whitehead group has attenuated serotype vaccines for DENV1, -3, and -4 by deleting sections of the 3' untranslated region (UTR) of the viral genome[118]. The Walter Reed Army Institute of Research in collaboration with GlaxoSmithKline developed a multivalent live vaccine through serial passages of DENV but further work is on hiatus in lieu of pursuing a formalin-inactivated DENV-based vaccine[119,120]. Other candidate vaccines include one composed of a recombinant E protein produced in Drosophila S2 cells in development by Merck & Co. and a tetravelent DNA vaccine produced by the U.S. Naval Medical Research Center[121,122].

Substantial challenges still exist that must be overcome before global dengue disease can be effectively controlled through vaccination. As demonstrated by the CYD-TDV trial, post-vaccination antibody titers do not predict protection from specific DENV serotypes, meaning future trials must identify an alternative method for measuring vaccine efficacy[113]. The duration of protection from DENV is another important factor that must be well understood, particularly in light of the two cohorts of people requiring vaccination: travellers from non-endemic regions visiting DENV-endemic areas and residents of endemic regions who are likely to be infected as children and to experience infection with more than one serotype over their lifetime[105]. In addition, vaccinating individuals with pre-existing DENV serotype immunity may result in a difference in duration and composition of the immune response compared to the immunity

developed by individuals naïve at vaccination[105]. Finally, although no increase in severe dengue has been observed in clinical trials, the possibility of predisposing vaccinated populations to severe disease via ADE remains of great concern.

1.2.6 Genome

The DENV genome consists of a single-stranded, positive-sense RNA 11 kilobases (kb) long and composed of a single open reading frame bordered by UTRs on the 5' and 3' ends[123]. The two UTRs have highly structured secondary folding and inverted complementary sequences important for viral replication and maintenance of the genome[2]. The genomic RNA serves as both messenger RNA (mRNA) and template for synthesis of negative-stranded RNA. The negative-strand, in turn, guides synthesis of new RNA genomes for protein translation or encapsidation in new viral particles[124]. The DENV ORF contains ten genes: at the 5' end the structural genes capsid C, envelope E, and the membrane protein precursor prM, followed by seven nonstructural genes NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Figure 1.5)[125,126]. All ten genes are translated into a single large polyprotein, then cleaved into individual proteins at conserved sites by the viral serine protease complex NS2B/NS3 or a host cell signalase.

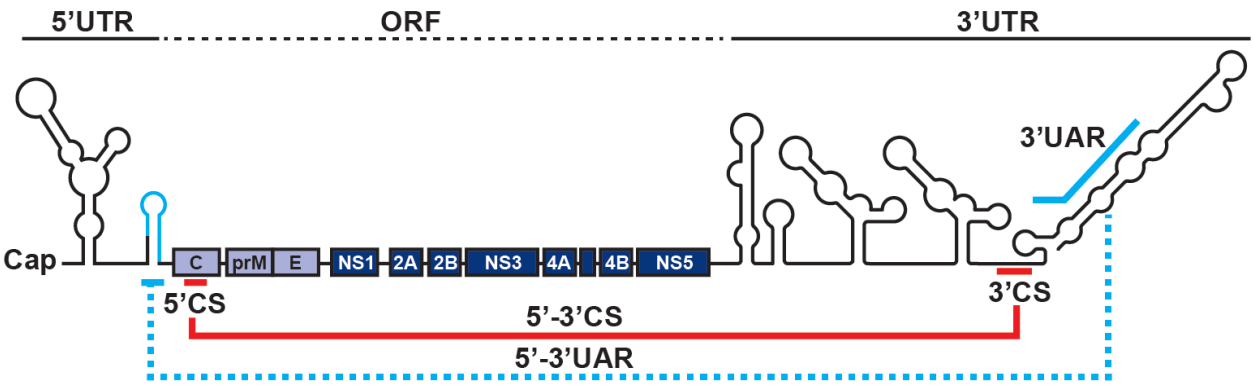


Figure 1.5 DENV genome

The DENV genome comprises a single ORF and complex secondary structure in the 5' and 3' UTRs. The complementary cyclization sequences are identified by the solid red and hashed blue lines. Image courtesy of Andrew Townsend.

In contrast to other genera of the *Flaviviridae*, the flaviviruses, including DENV, have a type I cap (m7GpppAmpN2) at the 5' end of the genome that allows for cap-dependent translation initiation[127]. The 5' and 3' UTRs of DENV fold into large stem-loops, pseudoknots, and other secondary structures that have been attributed to a variety of essential functions. Complementary sequences in the two UTRs allow the ends of the genome to hybridize[128]. Cyclization of the genome is vital to replication as the viral polymerase NS5 binds to a stem loop in the 5' UTR and is proposed to transfer by long-range RNA-RNA interaction to the replication initiation site in the 3' end of the genome[128,129]. The genome lacks a 3' terminal polyadenylate (poly(A)) tail; the poly(A) binding protein PABP that plays key roles in protein translation initiation, instead, binds the 3' UTR farther upstream[130,131]. An additional stem loop in the coding region just downstream of the polyprotein start codon has been shown to be required for both genome replication and protein translation[132,133].

1.2.7 Proteins

Post-translational cleavage of the DENV polyprotein produces the three structural and seven nonstructural proteins of the virus. The DENV capsid protein C, a highly charged basic protein 12 kilodaltons (kDa) in size, is found both in the cytoplasm and the nucleus[2,134]. In the cytoplasm, the capsid assembles the virus particle, coordinating interaction between the viral RNA genome and the membrane of the endoplasmic reticulum (ER)[135]. The nuclear role of C is unclear, although interactions between C and nuclear proteins such as histones have been shown, and presence of C in the nucleus can trigger apoptosis of the cell[136,137].

The DENV E protein is an elongated protein that assembles into head to tail dimers at neutral pH and covers the surface of the virion in a distinctive "herringbone" pattern[138]. E is composed of three domains: a central domain or domain I; domain II, the dimerization domain, that also contains a hydrophobic fusion loop; and the receptor binding domain III[139]. At low pH in the endosome, the E protein rearranges into trimers, exposing a fusion loop and precipitating membrane fusion and escape of the nucleocapsid into the cytoplasm[139,140]. E is the most exposed and antigenic of the DENV proteins[141].

The DENV virion exists in an immature and mature form, dependent on the cleavage state of the prM protein on the surface of the viral particle (Figure 1.6)[2,142]. The primary role of the 91-amino acid pr domain is to cap the fusion domain of E during transit of the new virus particle through the acidic environments of the trans-Golgi network[143,144]. While the particle is still in the trans-Golgi, the cellular protease furin cleaves between pr and M, allowing pr to disassociate from the virion in the pH neutral extracellular environment[145].

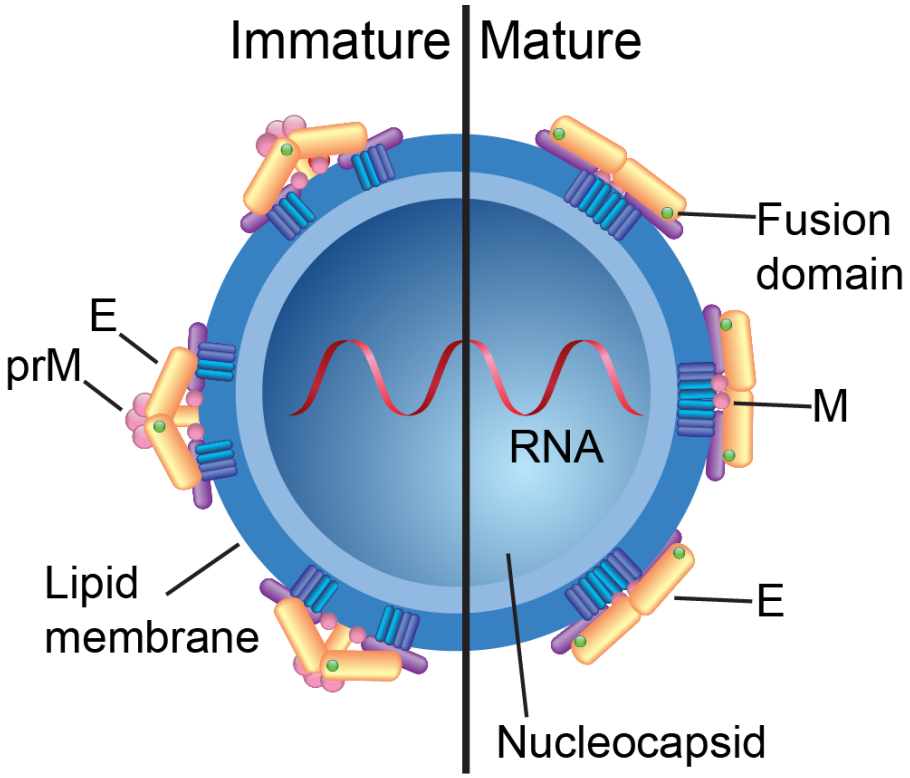


Figure 1.6 DENV particle composition

The immature DENV virion consists of a lipid bilayer containing the E (orange) and prM (pink) proteins surrounding the nucleocapsid. After cleavage of pr from M, the mature virion is formed and the fusion domain (green) is hidden in a hydrophobic pocket. Image courtesy of Andrew Townsend.

The 46-55kDa glycosylated nonstructural protein NS1 has a variety of functions in the infected cell and exists in several oligomeric forms—some membrane-associated, some soluble[146-148]. Monomeric NS1 is glycosylated after translation and cleavage from the DENV polyprotein into the ER lumen[149,150]. Glycosylated NS1 dimerizes and associates with ER invaginations induced by DENV, called vesicle pockets, where replication of the viral genome occurs[151]. The function of NS1 in the vesicle pockets is unknown although NS1 does colocalize with double-stranded RNA (dsRNA) of the virus, suggesting NS1 may play a role in anchoring and stabilizing the replication complex across the ER membrane[151-153]. Dimeric NS1 can also transit to the cell surface through the trans-Golgi network where dimers may remain bound to the extracellular face of the cell or may form soluble hexameric complexes that are secreted from the cell[148,154]. Soluble NS1 can be detected in the sera of dengue patients and has been linked to disease pathogenesis including complement binding, innate and adaptive immune activation, and induction of vascular permeability[155-160].

NS2A is a small transmembrane protein that is an essential component of the DENV replication complex[161,162]. NS2A localizes to the ER-derived membranous vesicle pockets and binds the 3' UTR of the DENV genome, although the function of the protein in replication is as yet unknown[163]. Two other functions for NS2A have been described: playing a role both in virion assembly and in downregulating IFN-β signaling[161,164]. However, the mechanism of NSA participation in these two processes is also unknown.

NS2B is another transmembrane protein and is required for function of the viral protease NS3. The hydrophilic region of NS2B must be present for catalytic function of the NS2B/3 protease complex as this region appears to contribute to the substrate binding domain of the

complex[165-167]. NS2B/3 autocatalyzes the viral polyprotein in several locations, releasing viral proteins[168]. In addition, NS2B/3 has been shown to cleave various innate immune signaling proteins as a mean of impairing the cellular antiviral response[169-171].

The NS3 protein contains two domains, each with enzymatic activity and both required for DENV replication: an N-terminal domain composed of a serine protease and a C-terminal domain containing an RNA helicase, nucleoside triphosphate (NTPase), and an RNA triphosphatase (RTPase)[172,173]. The NS3 protease is responsible for all polyprotein proteolytic cleavage on the cytoplasmic side of the ER membrane (Figure 1.7)[2]. The helicase domain of NS3 is responsible for unwinding RNA secondary structures and duplexes during genome synthesis; energy for the unwinding is provided in part by the activity of the NTPase domain[174-176]. The RTPase activity of NS3 initiates the addition of the methylated cap to the 5' terminus of the genome[177].

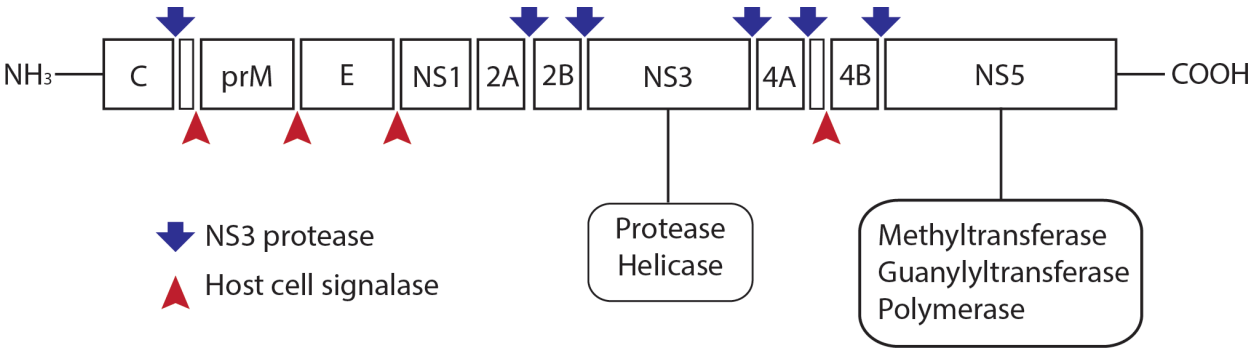


Figure 1.7 Polyprotein cleavage sites

The viral protease NS3 cleaves the DENV polyprotein at six sites on the cytoplasmic side of the ER membrane. Host cell proteases cleave sites within the ER lumen.

Another component of the DENV replication complex is NS4A, a transmembrane protein with ER membrane restructuring capabilities[178]. A 23-amino acid signal sequence at the C-terminus of NS4A appears to direct translocation of NS4B into the ER lumen. Subsequent cleavage of the signal sequence from NS4A by the NS2A/3 protease induces ER membrane restructuring, suggesting that NS4A plays a role in the formation of membranous replication pockets[178]. In addition, NS4A appears to inhibit IFN-β signaling and induces autophagy, which has been found to enhance DENV replication[179-181].

NS4B is also a member of the DENV replication complex and appears to assist in the membrane rearrangements of the ER[182]. DENV NS4B forms homodimers that may be required for the formation of the membranous replication pockets; disrupting NS4B dimerization of HCV has been shown inhibit membrane rearrangements and genome replication[183-185]. Like NS2A and NS4A, NS4B inhibits IFN-β signaling, as well as suppressing antiviral RNA interference (RNAi) by the cell[164,186].

NS5 is the largest of the DENV proteins and the mostly highly conserved between serotypes. The C-terminal domain comprises the viral RNA-dependent RNA polymerase (RdRp) and the N-terminal domain bears the RNA methylatransferase (MTase) responsible for completing the addition of the 5' methylated terminal cap after the process is initiated by the RTPase of NS3[187,188]. Binding to the circularized RNA genome, NS5 *de novo* synthesizes a negative-strand RNA, which in turn serves as the template for the semi-conservative synthesis and 5' capping of new copies of the positive-strand RNA genome to be translated into viral protein or packaged into new viral particles[189]. NS5 also plays an important role in downregulating the cellular antiviral response by binding and inducing the degradation the

signaling molecule signal transducer and activator of transcription (STAT)2[190,191]. NS5 also contains two nuclear localization signals; however, the function of NS5 in the nucleus is unknown[192,193].

1.2.8 Life Cycle

DENV particles adhere to the surface of a target cell through the binding of the virus surface proteins to a host cell receptor (Figure 1.8). Binding between DENV virions and a number of receptors has been observed, and no single, unique molecule has been definitively identified as the DENV receptor, suggesting that DENV binds a diverse group of surface molecules, consistent with the variety of cell types DENV infects in both mammals and mosquitoes[194]. Amongst the candidate receptors, DENV has been observed to bind cells via the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-DIGN), the mannose receptor of macrophages, glycosaminoglycans such as heparin sulfate and lectins, stress-induced proteins such as heat-shock proteins 70 and 90, and the ER chaperone GRP78[195-201]. Like cell receptor binding, the type of internalization by which the virion enters the cell appears to depend on cell type as well as DENV serotype: DENV-2 enters HeLa, A549, and Huh7 cells via clathrin-coated endocytosis but enters Vero cells in a clathrin-, caveolae-, and lipid raft-independent pathway[202-204]. In contrast, DENV-1 enters Vero cells in a clathrin-mediated manner[203].

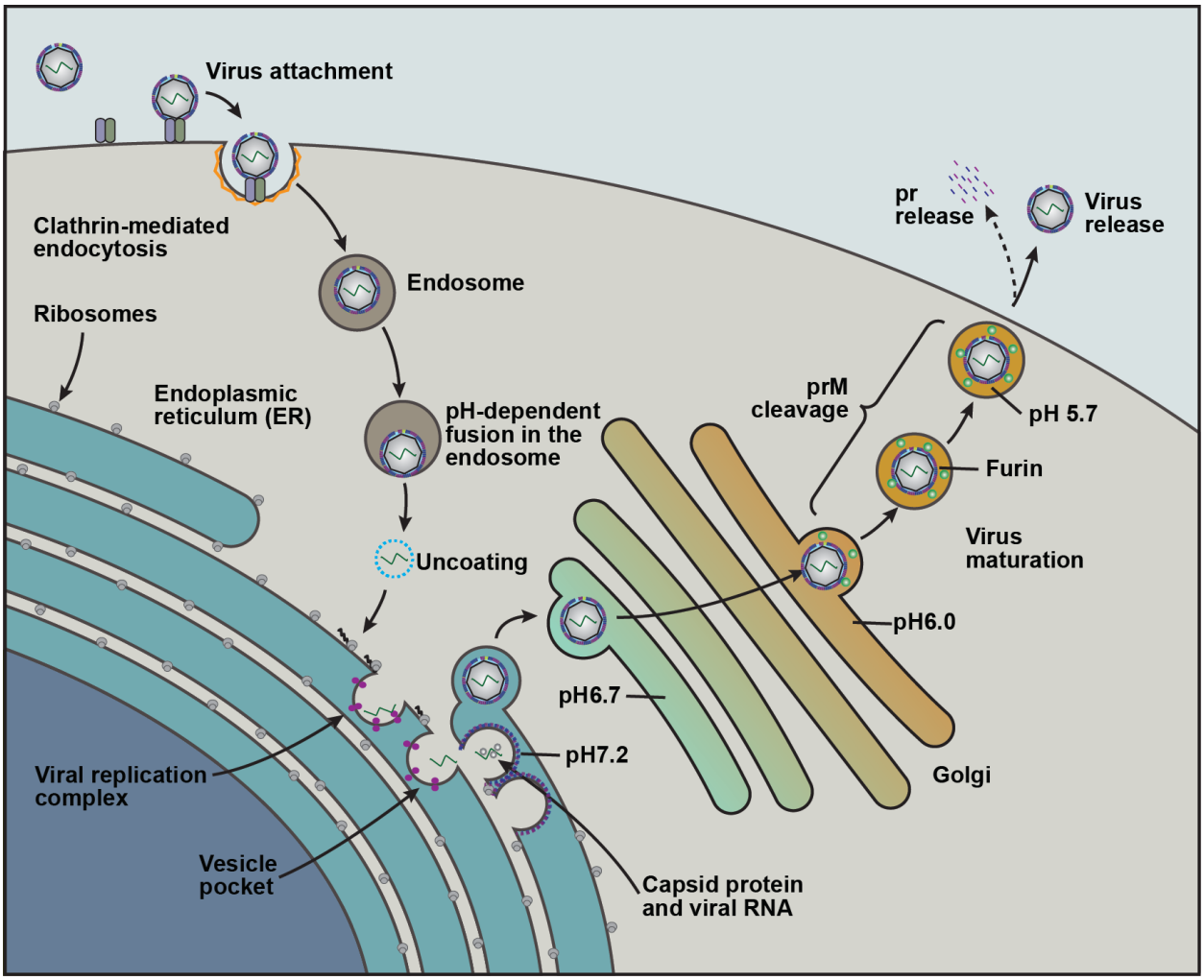


Figure 1.8 DENV life cycle

Image courtesy of Andrew Townsend.

The dengue virion transits into the cell enclosed within an endosomal vesicle. As the endosome traffics through the cells, the pH of the internal endosomal environment drops to allow for degradation of endosomal cargo. The acidic environment also induces a series of conformational changes in the surface of the DENV particle[205]. The E protein dimers rearrange into trimers and expose the hydrophobic fusion loop domains[139,206]. The fusion loops become buried in the endosomal membrane[207]. Additional conformational changes fold E back upon itself and drive the viral membrane and endosomal membrane into close proximity where fusion of the two membranes occurs. Fusion allows a pore to develop in the surface of the endosome through which the DENV nucleocapsid escapes into the cytoplasm of the cell.

The disassembly of the DENV nucleocapsid and release of the viral genome into the cytoplasm is not well characterized; although, recent work suggests that disassembly is a ubiquitin-dependent, but proteasome-independent process[208]. In the cytoplasm, the positive-stranded RNA genome serves as the mRNA for the translation of the DENV polyprotein. The polyprotein contains many transmembrane sequences in addition to cytoplasmic and ER luminal domains; the viral protease complex, composed of NS2B and NS3, autocatalyzes the cytoplasmic side of the polyprotein, while cellular signalases cleave the ER luminal side, to produce the ten DENV proteins[209,210].

The newly synthesized viral proteins induce rearrangements of the ER membrane into structures known as convoluted membranes and vesicle pockets[182,211]. Viral replication complexes, composed of the seven NS proteins, form within the vesicle pockets and recruit the RNA genome[178]. NS5 interacts with secondary structures at the 5' end of the genome, and genome circularization allows NS5 to access the promoter and initiate negative-strand

synthesis[212]. RNA synthesis results in a linear double-stranded RNA (dsRNA) that is predicted to be unfavorable for re-circularization[213]. Binding to the 3' UTR of the new negative-strand RNA and transcribing additional positive genome copies is thought to be more energetically favorable—providing an explanation for the high positive-strand to negative-strand ratio observed in cells and likely required to provide sufficient supply of RNA for both translation and for packaging into new virus particles[213].

Little is known about recruitment of the RNA genome and assembly of the nucleocapsid following genome replication in the vesicle packets, although coupling of the processes of RNA synthesis and virion assembly has been demonstrated[214]. The C protein accumulates in ER-adjacent lipid droplets, suggesting that these lipid droplets may serve as sites of nucleocapsid assembly[215]. Experiments have shown that mutations to C that disrupt lipid droplets localization inhibit virion formation[215]. However, the precise role of lipid droplets in nucleocapsid assembly is not clear, as observing capsid protein-RNA interactions in infected cells has presented significant challenges[216,217].

Budding of DENV virions into the ER membrane opposite the vesicle packet pore has been noted, resulting in the virion acquiring an ER-derived, lipid bilayer envelope containing E and prM[182]. Immature virions transit from the ER lumen through the increasingly acidic compartments of the secretory pathway to be released into the extracellular matrix[3]. In the low-pH environment of the trans-Golgi network, the pr portion of the prM protein lies exposed on the surface of the virion[145]. The cellular serine protease furin, located in secretory vesicles, cleaves pr from the rest of the M protein to produce the mature virus particle. However, pr does not dissociate from the virion until in the neutral pH of the extracellular environment to prevent

fusion of the new virion with the vesicle membrane[145]. Cleavage of pr is an inefficient process and immature or partially mature virions can be observed outside of cells and can be infectious in some circumstances, such as ADE[218-222].

1.3 Innate immune response to DENV

To protect against infecting pathogens, humans—as with all jawed vetebrates—possess two separate but synergistic systems of defense: the innate and adaptive immune systems[223]. Comprised of antigen-specific effector cells produced by receptor gene rearrangement, the adaptive immune system represents a highly specific and long-term memory response to pathogens of past exposure[224]. The adaptive immune system takes time to become effective, however, as cells specific to the current infection must be developed or recalled from immune memory and reproduced to sufficient levels to repel infection. The innate immune system, therefore, serves as the first line of defense against infection: recognizing common pathogen characteristics, rapidly creating an unfavorable environment for pathogen reproduction, and signaling for the activation for adaptive immune responses.

1.3.1 Pattern recognition

In order to detect the presence of a pathogen as early as possible, many cells express a number of proteins intended to interact with conserved motifs common amongst pathogens—such as the distinctive lipopolysaccharides of gram-negative bacteria—or aberrant molecular arrangements, such as the dsRNA present in the cytoplasm during flavivirus replication[224]. These cellular pathogen detectors, called pattern recognition receptors (PRRs), are highly conserved, binding a specific molecular motif and do not undergo gene rearrangement in the manner of adaptive immune receptors. When bound to the appropriate ligand, the pathogen-associated molecular pattern or PAMP, PRRs initiate a series of signaling cascades that

culminate in the transcription of infection-responsive genes, including immunomodulatory cytokines and anti-pathogen effector molecules[225].

The cell encodes several classes of PRRs that surveil for PAMPs in different compartments of the cell. TLRs are transmembrane receptors located at the cell surface and within intracellular compartments. The ectodomains of TLRs contain leucine-rich repeat segments to which PAMP ligands bind; the TLR cytoplasmic domains are composed of Toll-interleukin 1 (IL-1) receptors (TIRs) that initiate a downstream signaling cascade in response to ligand binding[226]. Humans encode ten TLRs, each binding a specific PAMP motif: TLR1, -2, -4, -5, -6, and -11 are located on the cell surface and detect microbial membrane components such as lipids and proteins. TLR3, -7, -8, and -9 bind microbial nucleic acids located in intracellular vesicles such as endosome and lysosomes (Figure 1.9).

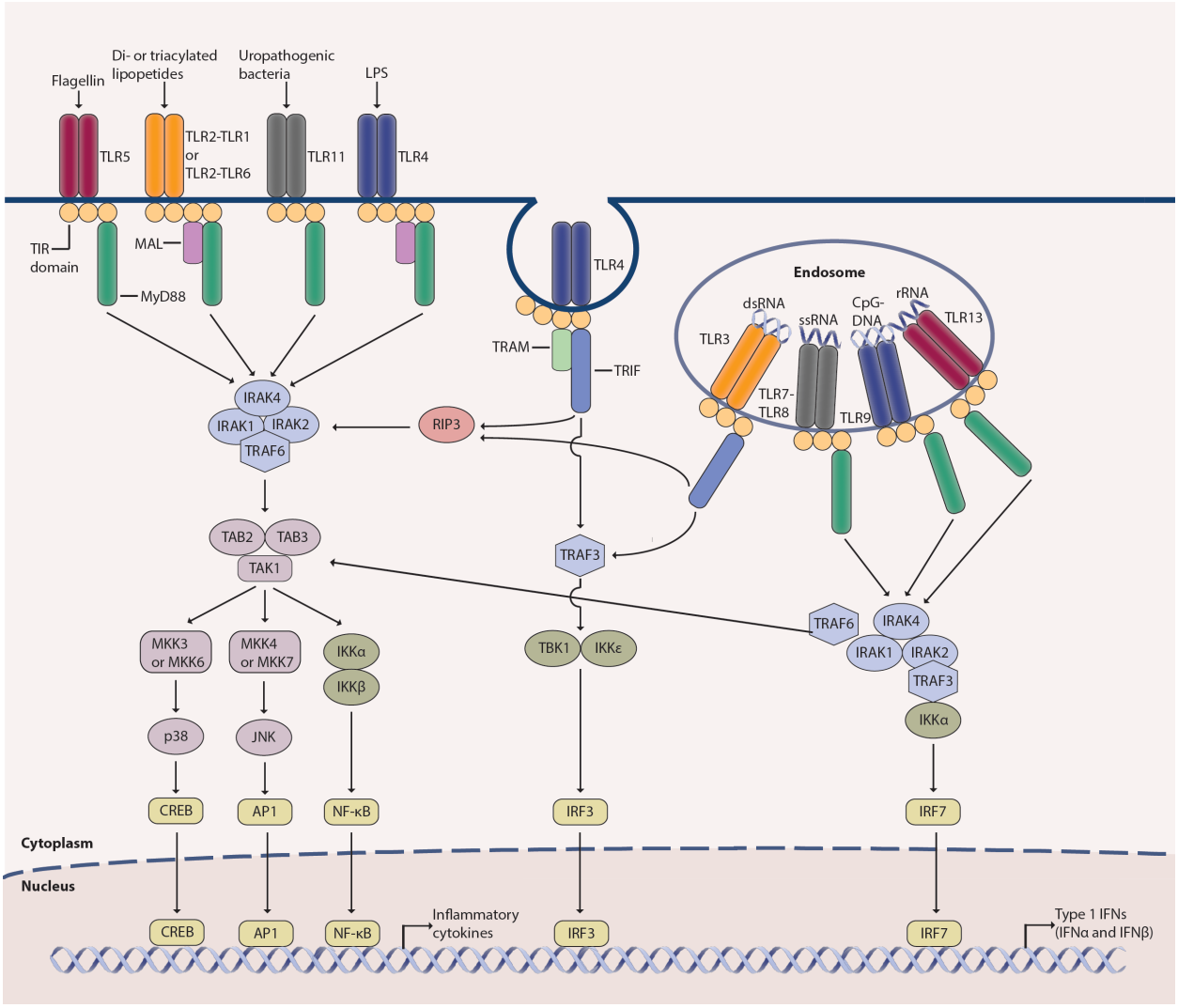


Figure 1.9 Mammalian TLRs

Ligands and signaling pathways of cell surface and endosomal TLRs.

Binding of a ligand to a TLR triggers dimerization—some TLRs homodimerize, while others heterodimerize with another TLR[227]. Dimerization brings the cytosolic TIR domains into close proximity, where they recruit additional TIR domain-containing adaptor molecules and serve as a scaffold for the assembly of a signaling complex. The downstream response to TLR activation is determined by which of four adaptor molecules binds the TLR TIR: myeloid differentiation primary response 88 (MyD88), TIR domain containing adaptor protein (TIRAP or Mal), TLR adaptor molecule (TRIF or TICAM1), or TLR adaptor molecule 2 (TRAM or TICAM2)[226]. All human TLRs, save TLR3, engage MyD88, which in turn recruits several more adaptor and kinase proteins, leading to a series of ubiquitination and phosphorylation steps that result in the activation of NF-κB-directed transcription. In plasmacytoid DCs (pDCs), TLR7 and -9 signaling through MyD88 also activates interferon-regulated factor (IRF) 7, leading to IFN1 activation[228,229]. TLR3 binds TRIF exclusively, which also binds endosomally localized TLR4. Utilizing a set of signaling cascades distinct from those triggered by MyD88, TRIF activates both the transcription factors IRF3 and NF-κB, leading to the induction of IFN1 and inflammatory cytokines, respectively[226]. TRAM and TIRAP serve as sorting mechanisms for proper targeting of MyD88 and TRIF to certain TLRs.

Several families of cytoplasmic, rather than membrane-bound, PRRs have also been identified. The RIG-I-like receptors (RLRs)—RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2)—are RNA helicases that detect RNA structures not found in cellular RNA, including dsRNA and single-stranded RNA (ssRNA) lacking a 5' methylated cap structure[230-232]. Ligand bound RIG-I and MDA5 associate with the membrane-associated mitochondrial antiviral-signaling protein (MAVS), which serves as an

adaptor and scaffold for the recruitment of a series of proteins, sequentially activating pathways leading to induction of NF-κB and IRF directed transcription[233].

The Nod-like receptors or NLRs are a large family of PRRs that recognize a wide range of ligands—PAMPs as well as cellular stress signals and other molecules indicating cellular distress[226]. Some NLRs are responsible for induction of the inflammasome, a complex of proteins that coordinates inflammatory cytokine expression in response to pathogen detection[234]. Through the robust but diverse assortment of responses to PRR activation, the cell can mount the immune response best suited to the nature of the threat based on recognition of a conserved, non-self motif.

1.3.2 Interferon response

When PRR-stimulated signaling pathways activate interferon-regulated factors such as IRF3 and IRF7, these transcription factors translocate from the cytoplasm to the nucleus of the cell, where they bind and induce transcription of IFN1[226]. Once translated, IFN1 is secreted from the cell and serves as an autocrine and paracrine signal of cellular distress[235]. IFN1 binds to a cell surface receptor dimer composed of IFNAR subunits alpha and beta, triggering structural rearrangements in the cytoplasmic domains of the IFNAR dimer[236]. These dimer rearrangements trigger autophosphorylation by two kinases associated with that domain, Janus kinase (JAK)1 and tyrosine kinase (TYK)2, which in turn recruit and phosphorylate the STAT1 and -2 transcription factors (Figure 1.10)[237]. Once phosphorylated, STAT1 homodimerizes or heterodimerizes with STAT2[236]. STAT1/2 dimers attract an addition partner IRF9, translocate to the nucleus and bind DNA transcription promoter motifs known as IFN-stimulated response

elements (ISREs), thereby initiating transcription of IFN stimulated genes (ISGs). The STAT1 homodimers bind a separate promoter sequence known as gamma-activated sequence (GAS) to produce an additional set of ISGs. The proteins encoded by ISGs have a variety of functions to protect the cell from infection: molecules for increased pathogen detection; cytokines and receptors for cell-to-cell communication; proapoptotic factors if the infection cannot be resolved and antiapoptotic factors to restore homeostasis if the infection is cleared[238]. Many ISGs target cellular pathways that viruses require—shutting down protein translation, expressing proteins that prevent membrane fusion and budding, restricting resources such as lipids.

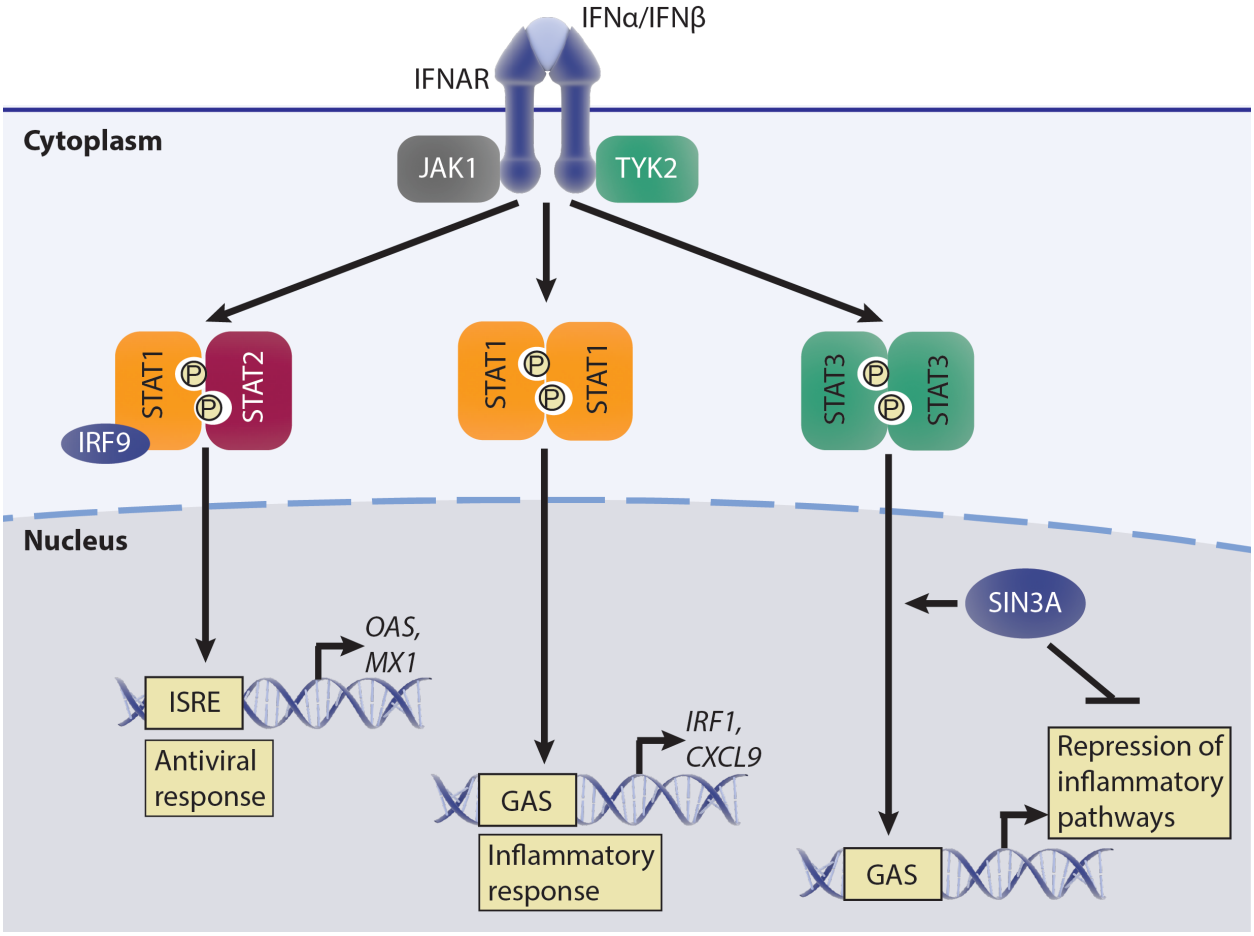


Figure 1.10 IFN1 signaling

IFN1 binding to IFNAR initiates STAT signal transduction and result in ISG gene expression.

1.3.3 NF- κB response

NF-κB is kept in an inactive state in the cytoplasm by binding partner inhibitor of kappa B (IκB)[239]. Activation of MyD88- or TRIF-dependent signaling pathway leads to phosphorylation of IκB by the IκB kinase (IKK) complex; phosphorylated IκB is rapidly ubiquitinated and proteasomely degraded, freeing NF-κB to translocate to the nucleus[240,241]. Nuclear NF-κB is a transcription factor and binds to gene promoter regions containing κB consensus sequences[242]. The degenerate nature of the κB motif results in a great number of genes susceptible to NF-κB-stimulated transcription, further dictated by posttranslational modification of NF-κB, the presence of additional transcription coactivators, and cell type. Among the genes expressed as a result of NF-κB activation are many proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8, that are secreted from the cell and serve a variety of signaling functions. IL-8 is a chemoattractant, recruiting neutrophils to sites of injury or infection; TNF-α and IL-1β induce expression of cell surface adhesion molecules on endothelial cells to assist with leukocyte infiltration into damaged or infected tissue[243,244]. In addition, NF-κB self-regulates by promoting transcription of IκB, effectively replacing the degraded inhibitor and curtailing NF-κB signaling[245,246].

1.3.4 Innate immune evasion by DENV

Both membrane-bound and cytosolic PRRs detect the presence of DENV, in particular those sensors that detect non-cellular nucleic acids motifs—dsRNA, ssRNA, and unusually modified RNA termini. TLR3, TLR7/8, RIG-I, and MAVS have all been linked to antiviral responses to DENV RNA, inducing IFN-α/β and other chemokines including ISGs and

inflammatory cytokines[247-249]. The expression of IFN-α/β is a powerful inhibitor of DENV infection, and pretreatment of cells with IFN-α/β can completely inhibit DENV infection[250]. The RNA interference (RNAi) pathway also appears to serve as an anti-DENV immune response, although the mechanism of inhibition is not known. When major components of the pathway such as Dicer, Drosha, and the Argonaute (Ago) protein are absent, DENV replication is enhanced[251].

In order to avoid attempts by the cell to circumvent or alleviate infection, DENV employs a variety of methods for inhibiting and hiding from the antiviral immune response. The DENV genome is capped at the 5' end, avoiding recognition by the RLR MDA5[252]. The virus also directly inhibits RLR signaling farther downstream through NS2B/3 cleavage of the signaling modulator stimulator of interferon genes or STING[171,253,254]. Of note, NS2B/3 recognizes and cleaves human, but not murine, STING[170,254]. The mouse STING homolog does not contain the NS2B/3 cleavage site, which may contribute to the inability of DENV to effectively infect immunocompetent mice[170]. In addition to minimizing the induction of IFN-α/β by the cell, DENV interferes with the function of IFN-α/β by targeting the signaling pathway downstream of IFNAR. The DENV proteins NS2A, NS4A, and NS4B associate with cellular membranes and inhibit the phosphorylation of STAT1, preventing activation and translocation to the nucleus (Figure 1.11)[179]. NS5 binds to STAT2 and recruits the E3 ubiquitin ligase UBR-4, leading to the ubiquitination and proteasomal degradation of STAT2 and depletion of the pool of STAT2 available for IFN-α/β-induced signaling[190,255]. Interestingly, NS5 fails to bind mouse STAT2, suggesting that—as with NS2B/3 and STING—the intact IFN1 signaling pathways help protect mice from DENV[256].

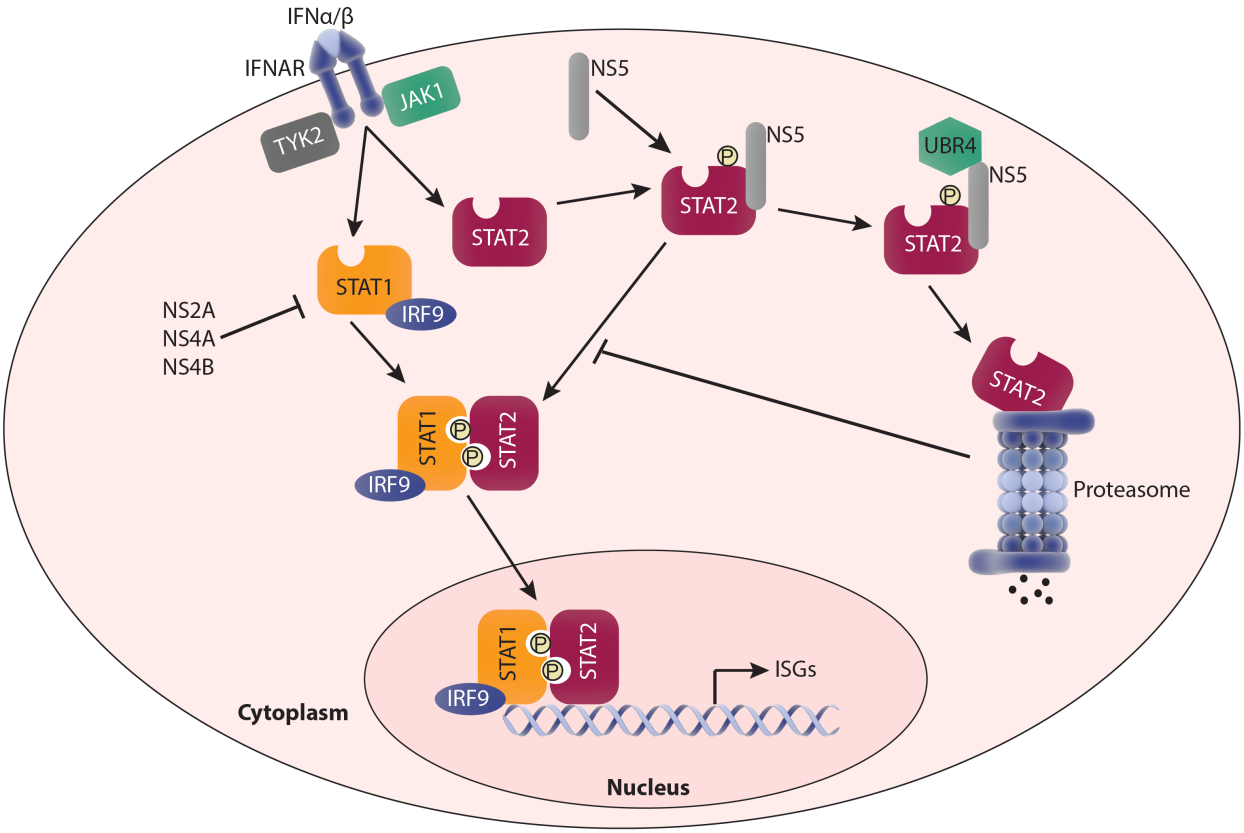


Figure 1.11 DENV inhibition of IFN1 signaling

Nonstructural proteins of DENV inhibit ISG induction by inhibiting STAT signaling.

In the context of ADE, when antibody-bound virus infects cells via the Fc-receptor, the innate immune response appears to be dampened. Cells infected with antibody-bound virus downregulate TLRs and increase expression of negative regulators of NF-κB, RIG-I, and MDA5[102,104,257]. In some cell types, infection by antibody-bound DENV also increases expression of the anti-inflammatory cytokine IL-10[102,257].

DENV NS4B has been found to inhibit the cellular RNAi pathway by preventing the production of small inhibitory RNA fragments such as small interfering RNAs (siRNAs) and microRNAs (miRNAs), but the mechanism of the inhibition is unknown[251]. In addition, DENV produces a subgenomic strand of DENV RNA, composed of the 3' UTR of the viral genome and produced by incomplete degradation of the genome by cellular exonucleases[258,259]. This subgenomic RNA fragment prevents RNAi-mediated cleavage of dsRNA by saturating the RNase domain of the RNAi protein Dicer.

Infection with DENV triggers extensive rearrangements and expansions the ER compartment, including the development of vesicle pockets proposed to hide viral RNA from cytosolic PRRs[260]. These manipulations to the ER membrane result in the induction of the unfolded protein response or UPR, a group of stress response pathways responsible for restoring homeostasis to the ER[261]. DENV appears to carefully manipulate the UPR pathways to modulate immune signaling, translation inhibition, and minimize proapoptotic signaling, often by controlling one arm of the UPR with another. For example, the inositol-requiring protein-1 (IRE1) pathway can produce both pro- and antiapoptotic signals but DENV infection appears to bias expression toward antiapoptic signaling[262]. In addition, IRE1 can downregulate the protein kinase RNA-like ER kinase (PERK) UPR pathway, allowing protein translation to continue in

spite of ER stress. Prolonged activation of the UPR also appears to result in decreased NF-κB activation, although the molecular basis of this inhibition is unknown[263].

1.4 Unfolded protein response in DENV infection

1.4.1 Unfolded protein response

Misfolded proteins accumulate in the ER when the cell experiences stressors such as nutrient starvation, ion fluctuation, or infection by a pathogen[264]. The unfolded protein response is a group of signaling pathways that work in parallel to sense and counteract ER stress and restore homeostasis to the cell. Failure of the UPR to alleviate ER stress leads to apoptosis of the cell.

Three transmembrane proteins are responsible for initiating the UPR—IRE1, PERK, and activating transcription factor (ATF)6—and are maintained in inactive states in the ER membrane by the chaperone binding immunoglobulin protein or BiP[265,266]. BiP preferentially binds misfolded proteins, freeing IRE1, PERK, and ATF6 to initiate the UPR. Activated IRE1 homodimerizes and autophosphorylates, resulting in activation of the cytosolic RNase domain[265]. The IRE1 RNase cleaves the mRNA of X-box binding protein 1 (XBP1) to excise an unconventional intron, resulting in translation of XBP1s[267,268]. XBP1s is a potent transcriptional activator, upregulating a variety of genes involved in protein entry, folding, and degradation as well as membrane lipid biogenesis and ER expansion. Recent work has also shown IRE1-dependent splicing of mRNAs and some miRNAs, leading to inflammatory responses and apoptosis[269,270]. Additional pro-apoptotic IRE1 signaling arises from IRE1 binding TNF receptor-associated factor (TRAF) 2 and activating the c-Jun N-terminal kinase (JNK) pathway[264].

Like IRE1, activation of PERK results in homodimerization and phosphorylation as well as phosphorylation of eukaryotic initiation factor 2α (eIF2α)[267]. When phosphorylated, eIF2α

reduces global protein translation by interfering with the assembly of the protein translation complex[271,272]. When eIF2α is phosphorylated, ATF4 is among the mRNAs that can still be translated, leading to induction of ER stress response genes controlling ER chaperone and foldase enzymes, amino acid metabolism, and redox processes[273]. The third UPR pathway results from the cleavage of activated ATF6 to produce the transcription factor ATF6p50 that regulates expression of genes controlling surveillance for and degradation of misfolded proteins in the ER[274]. ATF6p50 also promotes the transcription of XBP1 to be cleaved for translation by IRE1.

The UPR is also tied to the innate immune response to viral infection. TLR signaling can induce IRE1 activation through reactive oxygen species as well as contribute to continued IRE1 signaling by recruiting TRAF6 to interact with IRE1 and prevent dephosphorylation and deactivation of the protein[275-277]. Downstream of IRE1 activation, both XBP1s and JNK induce cytokine expression, either directly or through activation of NF-κB[265,278]. The inhibition of protein translation by PERK results in greater NF-κB relative to the inhibitor IκB, resulting in greater NF-κB activity[279]. Tandem activation of the TLR and UPR pathways can amplify the innate immune response of the cell, but many viruses, including DENV, have developed mechanisms for manipulating the UPR to augment infection in spite of the innate immune response.

1.4.2 DENV infection and the UPR

In addition to activation of the UPR through TLR-ligand binding, the rapid production of viral proteins during infection leads to accumulation of aberrant protein in the ER that also induces the UPR pathways[267]. DENV infection induces all three arms of the UPR, albeit to

different degrees and durations[262]. The PERK pathway is activated early in infection but is rapidly suppressed, allowing for unimpeded viral protein translation. The proposed mechanism for DENV inhibition of PERK is through protein growth arrest and DNA damage inducible 34 (GADD34), which mediates the dephosphorylation of EIF2α and restores translation complex formation[262,280](Figure 1.12). Like PERK, the ATF6 pathway is active only transiently during DENV infection, appearing late in infection. Infection of ATF6-deficient cells is unimpeded, suggesting that ATF6 activation and the downstream induction of XBP1 transcription is dispensable for DENV infection[262].

The IRE1-XBP1 pathway becomes active about midway through DENV infection, as the virus transitions from genome replication to production of infectious progeny[262,281]. Inhibition of XBP1 expression by siRNA during DENV infection reduced ER compartment expansion and increased DENV cytopathic effects on the cells[281]. While IRE1 can direct either pro-survival or pro-apoptotic signaling, induction of genes that reduce ER stress and promote protein translation, such as GADD34 and BiP, suggests that DENV skews the pathway toward host cell survival[262]. A role for IRE1-activated JNK function in regulation of cholesterol distribution and lipid availability during DENV infection has also been proposed[262,282].

Autophagy is a cellular process by which material is degraded and recycled in membranous vacuoles. Often an antiviral response that removes viral protein from the cell and facilitates antigen presentation, autophagy is modulated by DENV to benefit infection by reducing apoptotic signaling and providing additional membrane for the formation of replication pockets[180,181,283,284]. PERK-directed induction of autophagy early in DENV infection may assist in the availability and accessibility of membrane for viral genome replication[284]. In addition,

suppression of innate immunity through UPR-autophagy manipulation by HCV has been well studied. In HCV infected cells, UPR-directed autophagy strongly reduces RIG-I signaling and as a result, IFN1 expression[285]. The same group demonstrated that induction of autophagy greatly reduced IFN1 expression in cells exposed to a DENV PAMP. By modulating cellular responses to infection-related ER, DENV takes advantage of the cell's own regulatory mechanisms to optimize the environment for viral replication and protein translation and to dampen the immune response.

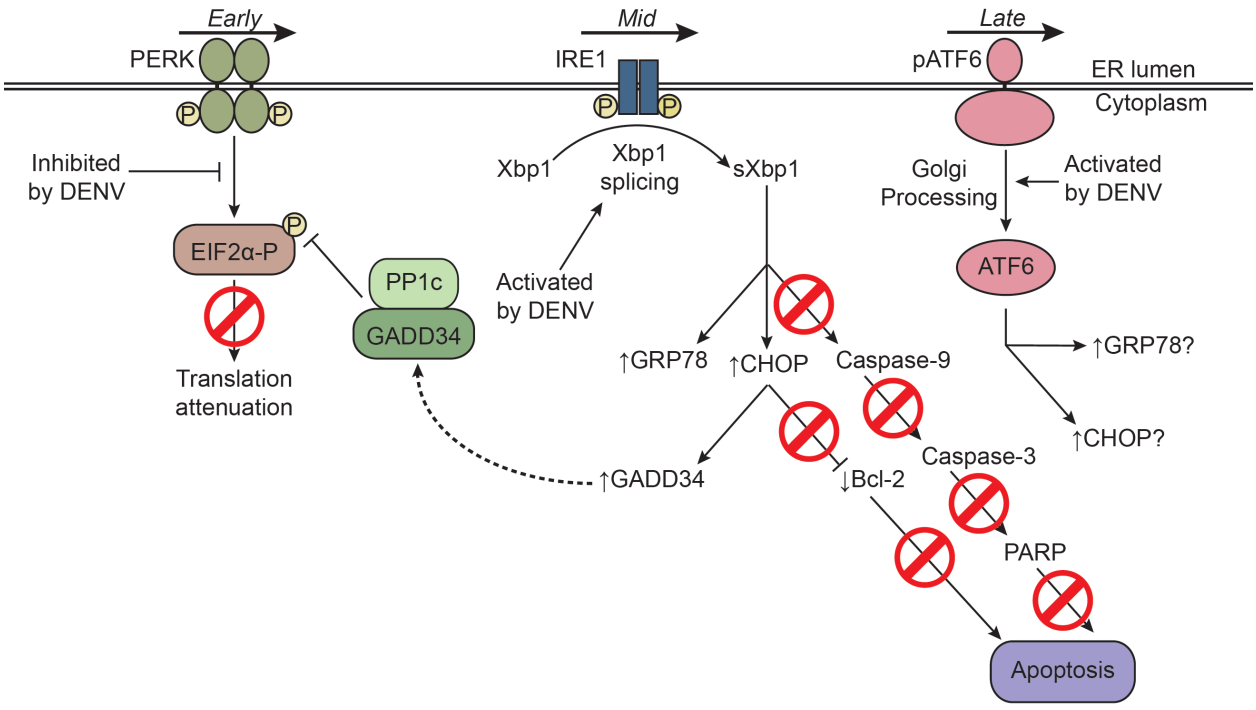


Figure 1.12 UPR pathways during DENV infection

Temporal induction of the PERK, IRE1, and ATF6 pathways of the UPR by DENV.

1.5 Ubiquitin-proteasome system (UPS) in DENV infection

1.5.1 Protein degradation by the UPS

To dispose of misfolded or obsolete proteins, the cell employs a highly regulated and complex cascade of protein modifications and proteolysis, leading to the ultimate destruction of the protein and recycling of the constituent amino acids[286]. Proteins destined for degradation are decorated with chains of ubiquitin (Ub) molecules that are recognized and cleaved by the 26S proteasome, a large cytosolic protease. Conjugating ubiquitin to a target protein occurs in three steps: (1) a ubiquitin-activating enzyme E1 binds the C-terminus of the 76-amino acid Ub peptide and replaces the C-terminal hydroxyl with a thioester moiety. (2) The Ub passes from the active site of E1 to the Ub-conjugating enzyme E2. (3) The E3 Ub ligase mediates the transfer of Ub from E2 to the target substrate, either by serving as a scaffold to bring E2-Ub and the substrate into proximity or by E2 transfer of Ub to E3, which directly catalyzes the transfer to the substrate (Figure 1.13A). After the initial Ub has been bound to a target protein (monoubiquitination), additional Ubs may be added to the first or any subsequent Ubs, creating linear or branching polyubiquitin chains. Ub-Ub bonding usually occurs at lysine 48, although any of the seven lysines and the N-terminus of a Ub peptide are potential linkage sites[287-289]. Not all ubiquitination results in proteasomal targeting of the substrate; mono- and polyubiquitination have been associated with a variety of protein interactions, including signaling and stabilization. However, polyubiquitination at lysine 48 is the most commonly recognized marker of a proteasome-destined protein[290]. Once conjugated to a chain of at least four Ub proteins and released from the E3 ligase, the ubiquitinated protein transits to the proteasome for degradation[291].

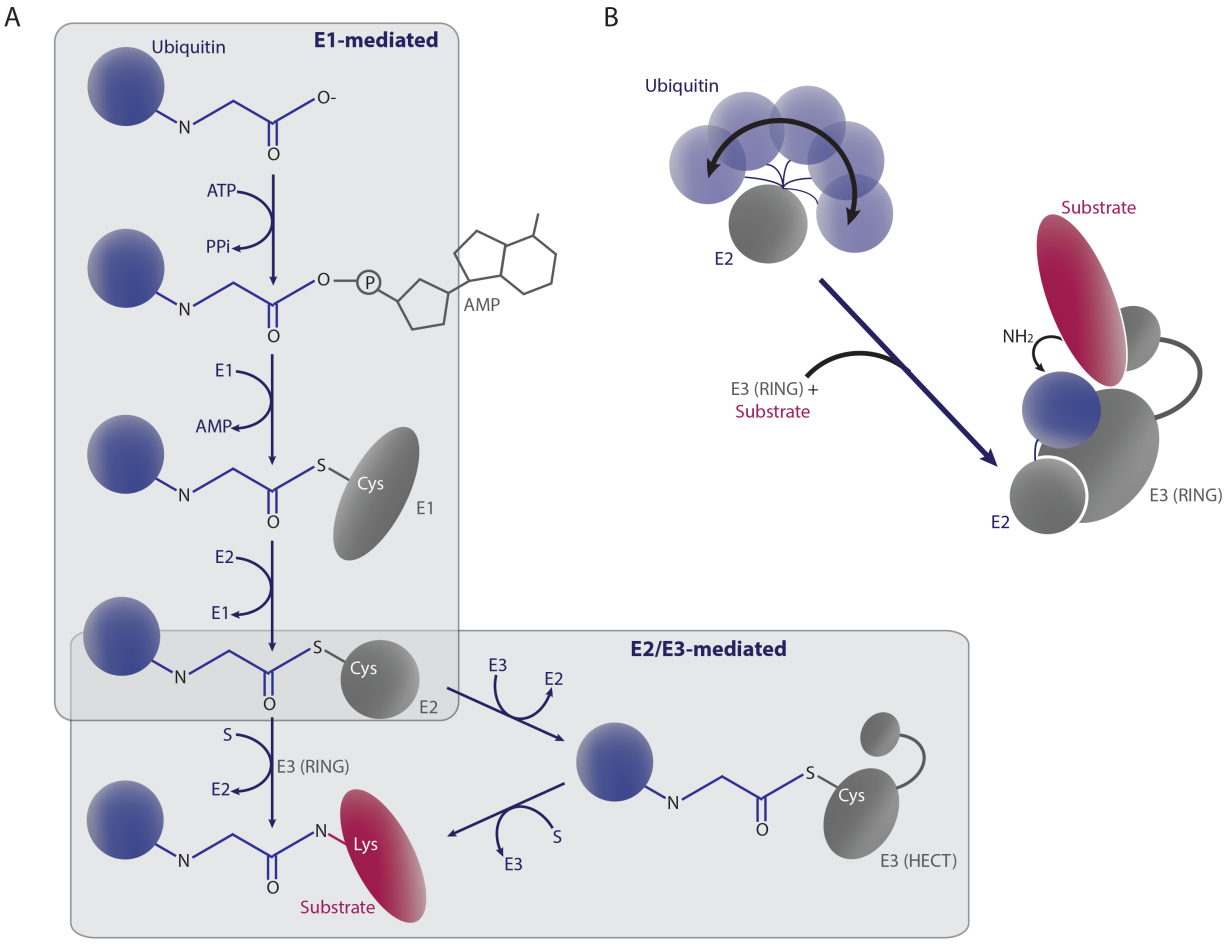


Figure 1.18 Ubiquitin transfer from conjugating enzymes to substrate

A) Modification and transfer of ubiquitin from E1 to E2. E2 transfer of Ub to E3 (HECT, U-box) or directly to substrate mediated by E3 (RING). **B)** Model of RING E3 ligase binding of substrate and E2-Ub.

The 26S proteasome is composed of a large, barrel-shaped 20S core subunit and two 19S regulatory subunits[292]. The interior of the 20S core is a narrow, proteolytic tunnel, open at each end. The 19S subunit caps the tunnel and regulates entrance and exit of proteins and peptides. A polyubiqutinated protein binds the 19S regulatory particle via interactions between the ubiquitin chain and ubiquitin receptor subunits of the 19S particle[293,294]. Another 19S subunit, a metalloprotease, cleaves the Ub chain intact from the substrate, allowing the polyubiqutin to exit the proteasome to be reused[295,296]. Freed from the Ub chain, the substrate protein is progressively unfolded and threaded into the 20S core by ATP hydrolysis-driven cycles of substrate-19S particle binding and release[292]. Within the proteasome core, the 20S subunits β1, β2, and β5 cleave the polypeptide with caspase-like, trypsin-like, and chymotrypsin-like activity, respectively, producing peptides with a median length of seven to nine amino acids[297,298]. Once released into the cytosol, these peptides are quickly degraded and the amino acids available for incorporation into new proteins[292].

1.5.2 E3 ubiquitin ligases

Mammalian cells encode two E1 Ub activating enzymes, nearly 40 E2 Ub conjugating enzymes, and over 600 E3 ubiquitin ligases[299]. The diversity of E3 ligases arises from the variety of protein substrates subject to ubiquitination. Most E3 ligases belong to a family defined by presence of the really interesting new gene (RING) finger domain, which binds E2-Ub. Interaction between the E3 RING finger and E2 brings the Ub in close proximity to the target substrate already bound to the E3, prompting the transfer of Ub from E2 to substrate (Figure 1.13B)[300]. In contrast, the homologous to E6-AP COOH terminus or HECT family of E3 ligases

have an internal cysteine residue; the E2-Ub transfers the Ub to the HECT E3, which catalyzes the binding of Ub to the substrate[301]. A third family, the U-box domain E3 ligases, also called E4 ligases, serve primarily as a scaffold for E2 to conjugate ubiquitin to an already ubiquitinated protein, producing polyubiquitin chains[302].

1.5.3 DENV and the UPS

Ubiquitination and the proteasome appear to play vital roles in the DENV life cycle: blood samples collected from DENV infected patients as well as infected primary cells reveal strong induction of UPS components, and inhibition of the UPS using pharmaceuticals or siRNA severely inhibit DENV production[303-306]. DENV capsid protein is degraded by the proteasome after virion entry and disassembly; however, while nucleocapsid disassembly does not require the function of the proteasome, inhibition of ubiquitination prevented uncoating of the viral genome and blocked translation and replication of the RNA[208]. Inhibition of the proteasome also inhibits egress of DENV from cells, although virion assembly and furin-mediated maturation are not affected[307]. Another post-translational modification—the addition of the small ubiquitin-like modifier or SUMO—is catalyzed, like ubiquitination, by E1, E2, and E3 enzymes. SUMOylation of DENV NS5 stabilizes the protein, inhibiting proteasome-independent degradation and increasing viral replication[308]. DENV further utilizes the UPS to downregulate the antiviral response of the cell. NS5 coordinates targeting of the E3 Ub ligase UBR-4 to STAT2, resulting in the UPS-mediated degradation of STAT2 and reduction in the IFN1 response to infection[190,255]. Subgenomic DENV RNA binds the E3 ubiquitin ligase tripartite motif protein (TRIM)25, inhibiting RIG-I ubiqutination required for downstream signaling by RIG-I[309]. DENV

exploits the diverse and complex mechanisms of ubiquitin conjugation and proteasome-dependent degradation for both viral reproduction and to evade antiviral responses produced by the host cell.

1.6 Human miRNAs

1.6.1 Expression and Function

miRNAs are short, non-coding RNA segments that, in coordination with an RNA-binding protein complex, regulate translation of target mRNAs in a sequence-specific manner. Potent and flexible regulators of gene expression, many miRNAs are highly conserved between distantly related species. Several thousands miRNAs have been identified in the human genome, and altered miRNA expression has been linked to a variety of disease states[310].

miRNAs have been identified on all human chromosomes save the Y chromosome and can occur between gene coding regions as well as within introns and rarely, exons[311]. The primary transcript of the miRNA or pri-miRNA is made up of a long, capped, and polyadenylated transcript containing an imperfect stem-loop of approximately 80 nucleotides (Figure 1.14)[312,313]. Cleavage by nuclear resident RNase III enzyme Drosha excises most of the stem-loop from the flanking sequences, producing an ~65-nt hairpin with a 2-nt overhang on the 3' end[314,315]. This precursor miRNA (pre-miRNA) is bound by Exportin 5 and transported to the cytosol for further processing[316,317]. The cytoplasmic RNase Dicer removes the terminal loop of the pre-miRNA to produce an RNA duplex, ~22-24-nt to a side and with 2-nt 3' overhangs; one strand of the duplex contains the mature miRNA, often called the guide strand[310,318,319]. The miRNA duplex is loaded into the RNA-induced silencing complex (RISC), a ribonucleoprotein complex composed of Argonaute (Ago) 2, GW182, and a variety of accessory proteins. Degradation of the passenger strand results in the mature miRNA-RISC.

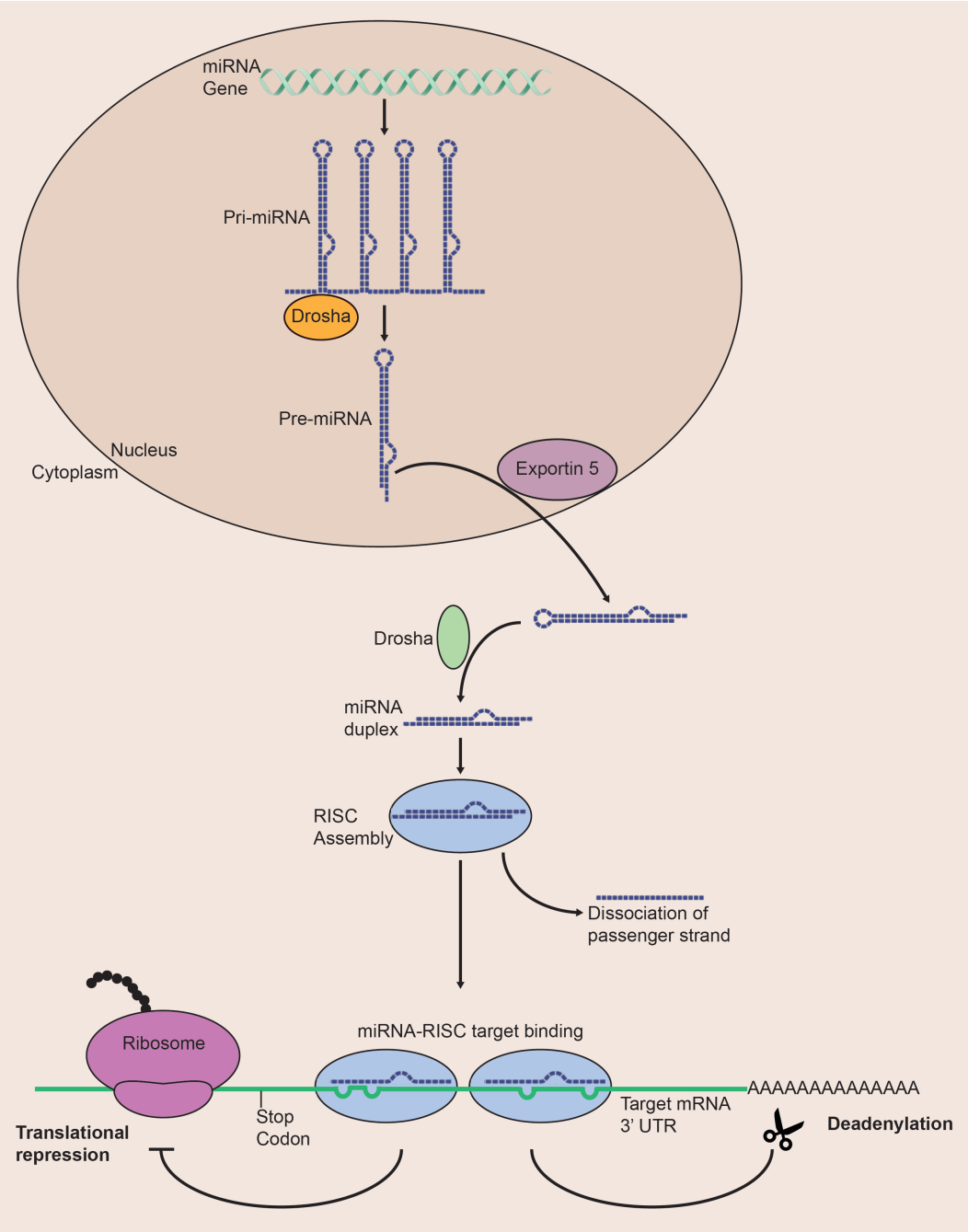


Figure 1.14 miRNA biosynthesis

Cellular miRNA transcription and initiation processing by Drosha occurs in the nucleus. Post-nuclear export, further processing by Dicer produces the miRNA duplex, which is loaded into the RISC. The mature miRNA-RISC binds complementary sequences in the 3' UTR of mRNA.

miRNA-RISC recognizes target transcripts based on partial complementarity between the miRNA and the 3' UTR of the target mRNA (binding sites in the 5' UTR have also been described, albeit rarely)[320-323]. Although the mature miRNA is between 20 and 24-nt long, only nucleotides 2-8, referred to as the seed sequence must pair exactly with the mRNA. In contrast, siRNA, which also function as a transcript silencing mechanism when incorporated into the RISC, binds with a high degree of complementarity along the entire length of the siRNA[324]. The tight binding between siRNA and target mRNA draws the target nucleotides bound to residues 10 and 11 of the guide siRNA within range of the hydrolase domain of Ago, leading to cleavage of the target mRNA and subsequent endonucleic degradation of the resulting 3'-hydroxyl and 5'-phosphate termini[324,325]. Unlike siRNA binding, up- and down-stream of the miRNA seed sequence may have varying degrees of complementarity with the target and as a result, do not induce the hydrolytic activity of Ago2 but repressof mRNA translation by the miRNA-RISC occurs by several alternative mechanisms. As miRNAs fail to inhibit 5' cap-independent translation, miRNA binding may inhibit mRNA cap recognition and assembly of the ribosome, preventing translation initiation[326-330]. During translation elongation, the miRNA-RISC induces the premature dissociation of the ribosome from the mRNA or may induce degradation of the polypeptide co-translationally[331-333]. miRNA can direct transcript degradation in spite of incomplete complementarity through deadenylation of the target: a miRNA-bound mRNA is deadenylated by the PAN2-PAN3 and Ccr4-Not complexes, decapped by DCP2, and finally degraded by the cytoplasmic exonuclease XRN1[334-338]. The factors that determine fate of a miRNA-RISC bound mRNA are unclear, although evidence suggests that the location, length, frequency, and type of mismatches between miRNA and mRNA 3' UTR play an important role

in the association of proteins regulating translation and degradation at the site of miRNA-RISC binding[324]. The flexibility in target binding provided by the incomplete complementarity required for a miRNA to bind and the various consequences of miRNA binding make miRNAs powerful regulators of cellular function; a single miRNA may regulate dozens of individual gene transcripts, restricting translation of some transcripts while mediating destruction of others.

1.6.2 Cellular miRNAs during viral infection

Viral infection requires a great many cellular resources, inevitably bringing the virus in contact, and often conflict, with miRNA regulation of gene expression. While many DNA virus genomes encode viral miRNAs, both DNA and RNA viruses have evolved mechanisms for counteracting or capitalizing on cellular miRNA functions[339]. miRNAs may function antivirally—augmenting the immune response, inducing apoptosis, or targeting the viral genome. Conversely, many viruses exploit cellular miRNAs for replication, latency, oncogenesis, and to avoid immune surveillance.

miRNAs that obstruct viral infection do so by a variety of mechanisms. Direct inhibition of viral RNA—whether genomic or mRNA—has been observed for several viruses. The liver-specific miRNA miR-122 binds a highly conserved sequence in the pregenomic mRNA of the hepadnavirus hepatitis B virus (HBV). Binding the 3' UTR of the viral polymerase ORF, miR-122 inhibits viral replication and protein expression[340]. HBV-infected cells and samples from patients have greatly reduced expression of miR-122, suggesting that HBV has evolved mechanisms to dampen expression of the antiviral miR-122[341]. The cellular miRNAs, miR-24 and miR-93, target the negative strand RNA genome of vesicular stomatitis virus (VSV) while

miR-29a binds the 3' UTR of human immunodeficiency virus (HIV) and represses viral replication[342,343].

Antiviral miRNA regulation of cellular proteins and pathways may be specific to a single virus or broadly antiviral across genera and families. Our lab observed induction of miR-6124 (née Hs_154) by WNV infection but not other flaviviruses; miR-6124 was found to repress the prosurvival protein CTCF and ECOP, contributing to WNV-induced apoptosis[344]. In contrast, the Hirsch group also found that miR-34a and other members of the miR-34 family inhibited not only the flaviviruses tested, but also alphaviruses and herpesviruses, by preventing downregulation of IFN1 induction[345]. Similarly, JEV infection results in miR-15b preventing expression of a negative regulator of RIG-I, leading to increased innate immune induction[346]. Another broadly antiviral miRNA, miR-199a-3p, also appears to contribute to innate immune signaling, although the exact targets of miR-199a-3p repression are not known[347]. miRNA antiviral effect is not restricted to influencing innate immune responses; during poliovirus infection, miR-555 inhibits the translation of heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP), an important cellular component for viral RNA replication, and consequently severely reduces viral RNA replication and viral yield[348].

Exploitation of cellular miRNAs for viral benefit is also often tied to the antiviral immune response. DENV, as well as the paramyxovirus Hendra virus, induce expression of miR-146a, which targets TRAF6, a signaling molecule downstream of TLRs, and causing decreased antiviral cytokine production[349,350]. Likewise, several herpesviruses upregulate miR-132 during infection; miR-132 represses translation of the transcription co-activator p300 and consequently, attenuates induction of ISGs and NF-κB responsive genes[351]. miR-21, found in high abundance

in serum of chronically infected HCV patients, targets both MyD88 and interleukin 1 receptor associated kinase (IRAK) 1 to reduce IFN signaling [352-354]. HCV also requires the cellular miRNA miR-122 to protect the viral genome from decay, in contrast to the antiviral effect of miR-122 on HBV infection[355,356]. miR-122 binds two seed sites in the 5' UTR of the HCV genome, one of which produces an overhang that masks the 5' terminus of the genome and protects the viral RNA against exonuclease activity and PRR recognition[356-358]. Furthermore, the interaction of HCV RNA and miR-122 contributes to ribosome recruitment and enhances HCV genome translation[359]. Clinical trials of a miR-122 inhibitor showed effective reduction in circulating viral RNA levels[360].

Various cellular miRNAs have been found to play a role in inducing and maintaining latency in herpesvirus infection. The miR-200 family of miRNAs binds and inhibits the translation of human cytomegalovirus (HCMV) immediate early protein and transcription factor UL122, consequently preventing expression of lytic genes[361]. In HCMV infection, as well as herpesvirus such as Kaposi's sarcoma associated virus, Ebstein-Barr virus, and the herpes simplex viruses, a delicate balance of regulation, directed by both viral and cellular miRNAs, controls the latent and lytic phases of the viral life cycle.

1.7 Hypothesis

In light of the serious global health threat posed by DENV and the absence of targeted therapeutics, efforts towards elucidating the mechanisms by which the virus subverts host cell processes may reveal options for drug development and disease reduction. The importance of such cellular pathways as the UPR, UPS, and intracellular pathogen sensing during infection are well established, but the many roles such processes play in the viral life cycle remain to be fully investigated. We have developed a pipeline using genome-wide miRNA screens to identify individual regulators of viral infection. I hypothesize that miRNAs that inhibit flavivirus infection identified by high-throughput screening can be used to examine cellular pathways subverted by the virus to enhance infection and in particular, elucidate additional proviral functions of pathways known to be required for infection such as the UPR and UPS. In this dissertation, I will discuss two studies concerning anti-flaviviral miRNAs, miR-424 and miR-526b, to determine the proviral function of the cellular mRNA targets repressed by said miRNAs. Collectively, the work presented here validates the use of cellular miRNA for identifying virus-host cell interactions required for flavivirus replication. These results not only further our understanding of the myriad interactions between the virus and the host during infection, but also suggest potential targets for therapeutic development.

Chapter 2: Cellular microRNA miR-424 inhibits dengue virus infection by preventing virally-induced degradation of MyD88

Ashleigh R. Murphy[1], Jessica L. Smith[1], and Alec J. Hirsch[1]

[1]Vaccine and Gene Therapy Institute, Oregon Health & Science University, Beaverton, OR 97006

Manuscript written by Ashleigh R. Murphy and Alec J. Hirsch.

All experiments were conducted by Ashleigh R. Murphy, with the exception of the miRNA library screen and analysis executed by Jessica L. Smith, Alec J. Hirsch, Sophia Jeng, and Shannon K. McWeeney. The MyD88-deficient HeLa cell line was produced in association with Kara M. Wishart and Victor R. DeFilippis.

2.1 Abstract

The dengue viruses present a serious threat to human health globally and can cause severe, even life-threatening illness. DENV is endemic on all continents except Antarctica, and it is estimated that more than 100 million people are infected each year. DENV induces the unfolded protein response in a time-dependent manner, inhibition of which severely impairs infection. Although the UPR appears to mitigate translation attenuation and to skew stress-induced transcription towards prosurvival factors, additional functions of the UPR during DENV infection remain to be elucidated. In this study, we observed that the stress responsive E3 ubiquitin ligase SIAH1 is induced by DENV infection. The SIAH1 mRNA is targeted by the cellular miRNA miR-424, which we found to inhibit flavivirus infection. Knockdown of SIAH1 also inhibited infection and increased expression of MyD88 and downstream innate immune gene expression. We show that MyD88 degradation during DENV2 infection is SIAH1-dependent and that induction of SIAH1 via the UPR results in MyD88 degradation. These observations elucidate a new pathway for innate immune regulation by the UPR during DENV2 infection.

2.2 Introduction

The dengue viruses (DENV) consist of four antigenically distinct seroptypes (DENV1-4) that together present considerable risk of infection in tropical and subtropical regions worldwide[362]. DENV is a positive-stranded RNA genome flavivirus, primarily transmitted to humans by the bite of an infected *Aedes aegypti* mosquito. Infection with any of the DENV serotypes can result in a self-limiting febrile illness called dengue fever, often characterized by severe myalgia and a distinctive cutaneous rash. Life-threatening severe dengue illness, often associated with secondary infection by a heterologous DENV serotype, can cause thrombocytopenia, vascular leakage, and hypotensive shock. DENV-related disease results in millions of hospitalizations and thousands of deaths each year[363]. Currently, antiviral therapeutics are not available for DENV infection, and the vaccine currently licensed in some countries offers less than optimal protection[111,364].

Infection with DENV induces an antiviral immune state in the host cell through various pattern recognition receptors (PRRs), among them Toll-like receptor (TLR)3, TLR7, retinoic acid-inducible gene I (RIG-I), and stimulator of interferon genes (STING). Binding of pathogen-associated molecular patterns (PAMPs), such as double-stranded RNA, to a PRR triggers a signaling cascade resulting in transcription of a multitude of infection response genes. DENV, like many viruses, employs a variety of direct and indirect methods to reduce or avoid the intracellular immune response. The viral nonstructural protein NS5 binds directly to the interferon-stimulated cellular transcription factor signal transducer and activation of transcription (STAT)2, leading to the proteasomal degradation of STAT2 and a reduction in type 1 interferon signaling[190,255]. Other DENV proteins also target immune signaling pathways: the DENV NS2B3

protease complex cleaves STING, and NS4A and NS4B inhibit phosphorylation of STAT1[170,171,179]. DENV also indirectly avoids detection by coopting structures and pathways of the cell. Infection by DENV results in extensive restructuring of the endoplasmic reticulum (ER), leading to the development of invaginations of the ER membrane, which may allow viral replication to occur without detection by dsRNA-sensing PRRs[182,261]. DENV infection also triggers portions of the unfolded protein response (UPR), mitigating proapoptotic stress signaling and ensuring continuous phospholipid and protein production[262].

We have shown that microRNAs (miRNAs) are useful tools for identifying cellular interactions with viruses[344,345]. A miRNA has the potential to regulate expression of over 100 different genes, and a single miRNA may alter viral infection by targeting several different transcripts relevant to infection. However, as many miRNAs target genes with similar functions or that are members of the same cellular pathway, the study of miRNAs that inhibit virus growth provides an avenue to examine cellular proteins and pathways necessary for viral infection.

In this study, we observe that expression of a cellular miRNA, miR-424, inhibits DENV2 infection by repressing translation of the E3 ubiquitin ligase SIAH1. We show that DENV2 infection induces SIAH1 expression, resulting in SIAH1 binding and ubiquinating the immune signaling adaptor MyD88. Inhibition of the ubiquitinating activity SIAH1 and the subsequent proteasomal degradation of MyD88 results in significantly increased expression of MyD88 and MyD88-dependent genes. These observations suggest a mechanism by which DENV2 counters cellular defenses through the induction of negative regulators of immune signaling.

2.3 Results

2.3.1 miR-15/16 miRNA family members inhibit flavivirus replication

To identify miRNAs that inhibit replication of flaviviruses, a high-content assay was conducted using a library of double-stranded miRNA mimics representing all humans miRNAs annotated in miRBase v16.0[345]. HeLa cells in 96-well culture plates were transfected, one miRNA mimic per well, and subsequently infected with DENV2, West Nile virus (WNV), or Japanese encephalitis virus (JEV) 2 days post-transfection. 24 h (WNV, JEV) or 48 h (DENV) p.i., cells were fixed and stained for the viral envelope (E) protein. Cell nuclei were stained with DAPI. Nine fields per well were imaged for E and DAPI staining using an Opera LC high-content imaging system, and for each image, Acapella software was used to measure pixel intensity in each image after subtraction of background (obtained from an image of a field devoid of cells). The pixel intensity measurements were quantile normalized across all plates for each infection. To examine changes to viral protein expression, the ratio of viral protein to cell nuclei was calculated for each well and compared with control values obtained from wells transfected with nonsense or nonfunctional miRNA mimics. Results were fitted to a linear model for each miRNA, and significant effects on viral protein expression determined with p-values for each well calculated using a one-sided *t*-test, null values equal to 1, and false discovery rate adjusted for multiple comparison of biological replicates[365]. Image sensor constraints limiting maximum pixel intensity detectable precluded identification of proviral miRNAs that would result in increased viral E protein expression and therefore, increased fluorescence. Inhibitory miRNAs were assessed as causing a greater than 60% decrease in fluorescence with a raw p-value of <0.05. However, wells with less than 80% DAPI staining compared to control were

considered cytotoxic and not included in further analysis. Of approximately 1,200 miRNAs screened, 45, 93, and 204 miRNAs were identified as meeting these criteria for DENV2, WNV, and JEV, respectively, and 24 miRNAs inhibited more than one of the viruses tested[345].

Of note, several miRNA families—generally defined by similarity within the seed sequence—were found to have multiple members with anti-flavivirus activity in this assay[345]. One such miRNA family was the miR-15/16 family, which consists of ten miRNAs with similar sequences [366]. Eight of the ten miRNAs have the same 6mer in positions 2-7 of the seed sequence (Figure 2.1A), while the remaining two have the sequence offset by one nucleotide. We re-examined the ability of miR-15/16 family members to inhibit DENV replication; HeLa cells were transfected with the duplex mimic of a miR-15/6 family members and infected with DENV2 (Figure 2.1B). Supernatants collected three days p.i. contained viral titers that were reduced 15-70 fold in cells transfected with miR-424, miR-15a, miR-15b, miR-16, miR-195, miR-497, miR-503, and miR-646, all of which share identical seed sequences. The remaining two members of the miR-15/16 family, miR-103 and miR-107, did not appear to inhibit DENV2 infection. While miR-103 and miR-107 are similar in sequence to the rest of the family, the miRNAs do not share the same seed sequence, changing the predicted mRNA targets and demonstrating that the AGCAGC seed sequence is crucial for the antiviral activity of the miRNA family.

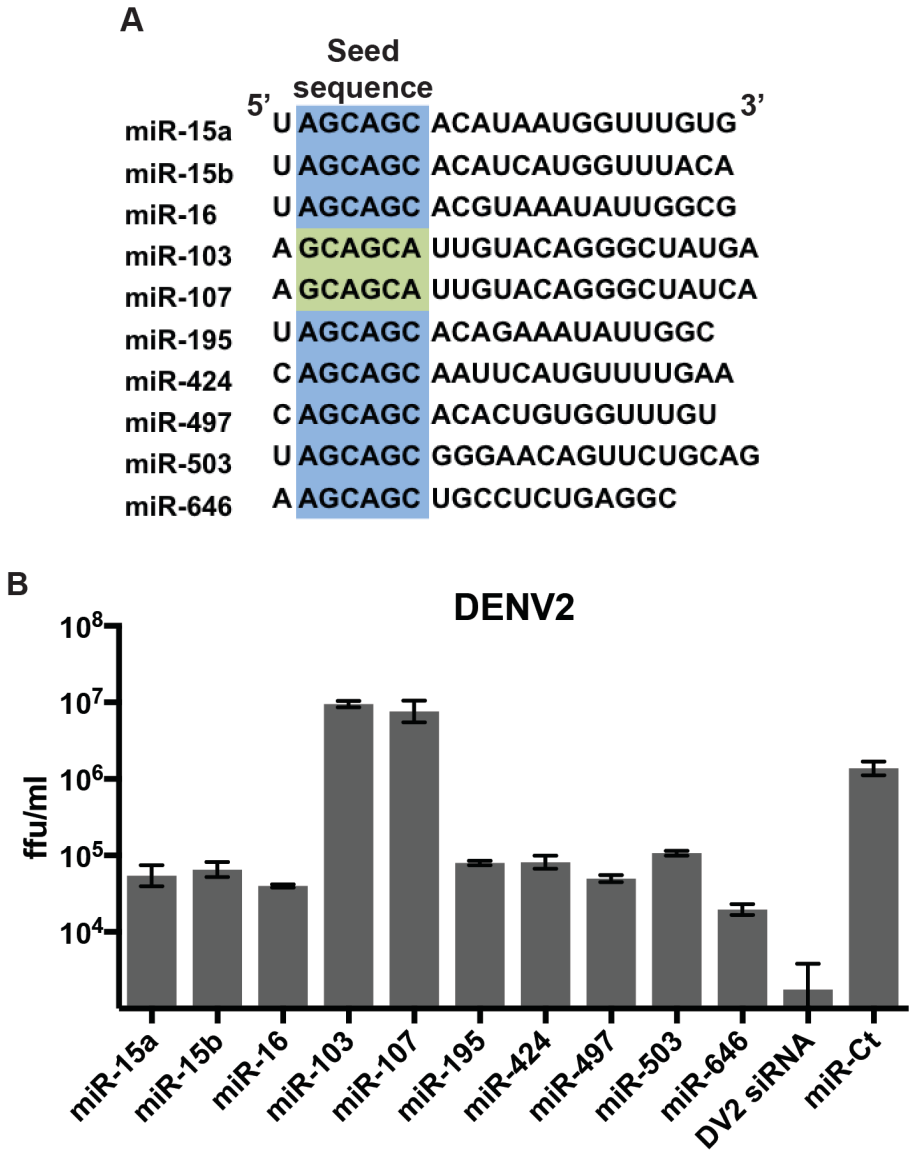


Figure 2.1 miR-424 and members of the miR-15/16 family inhibit DENV2

A) Comparison of the seed sequences of miR-424 and members of the miR-15/16 family of miRNAs. Identical seed sequences (nucleotides 2-7) are highlighted in blue; the seed sequences of miR-103 and miR-107 (in green) are similar but offset by one nucleotide. **B)** Cells were transfected with the indicated duplex RNA mimics or DENV2 genome siRNA (DV2 siRNA) and infected with DENV2 (MOI=3 ffu/cell) 48 h post-transfection. Supernatants were collected 3 days p.i. and titered on Vero cells.

We next focused on one member of the miR-15/16 family that had not previously been studied during RNA virus infection, miR-424, in order to elucidate its activity against other flaviviruses as well as other virus families. HeLa cells were transfected with a double-stranded RNA mimic of miR-424, infected with DENV2 or WNV and fixed at 3 (DENV2) or 2 (WNV) days post-transfection. Expression of the flavivirus E protein was detected by indirect immunofluorescence. A DENV2 genome-specific siRNA or the broadly antiviral miRNA miR-199a-3p were used as positive controls for viral inhibition[347]. Cells transfected with the miR-424 mimic showed inhibition of E protein expression (Figure 2.2A). When supernatants were collected and titer determined by focus forming assay (ffa), viral titers from cells transfected with the miR-424 mimic were at least a ten-fold lower than control cells when infected with DENV2 or WNV (Figure 2.2B).

However, when total RNA was collected from DENV2 infected cells and viral genome copy number quantified using qPCR, replication of the viral genome was not reducted in the presence of miR-424, suggesting that the inhibition of DENV2 and WNV infection resulted from the regulation of a cellular process required for viral replication by miR-424 rather than the miRNA binding directly to the viral RNA (Figure 2.2C). Neither of the strains of DENV2 and WNV used in this study (New Guinea C and 385-99, respectively) encode sequences complementary to the miR-424 seed sequence in the 3' UTR of the viral RNA, and only WNV has a potential binding site elsewhere in the genome: specifically, within the protein coding region corresponding to NS2A. We next examined the effect of miR-424 on infection with several RNA and DNA viruses to determine if the antiviral effect of miR-424 extended to additional virus families. We found that miR-424 reduced the infectious titer produced when

cells were infected with Chikungunya virus (CHKV), a member of the positive-strand RNA alphavirus genus (Figure 2.2C). In contrast, miR-424 did not impair infection by a negative-stranded RNA virus (Vesicular Stomatitis virus, VSV) or by the DNA viruses, herpes simplex virus (HSV), and vaccinia virus (VacV), suggesting that miR-424 regulates a cellular target or targets required for some but not all viral infections.

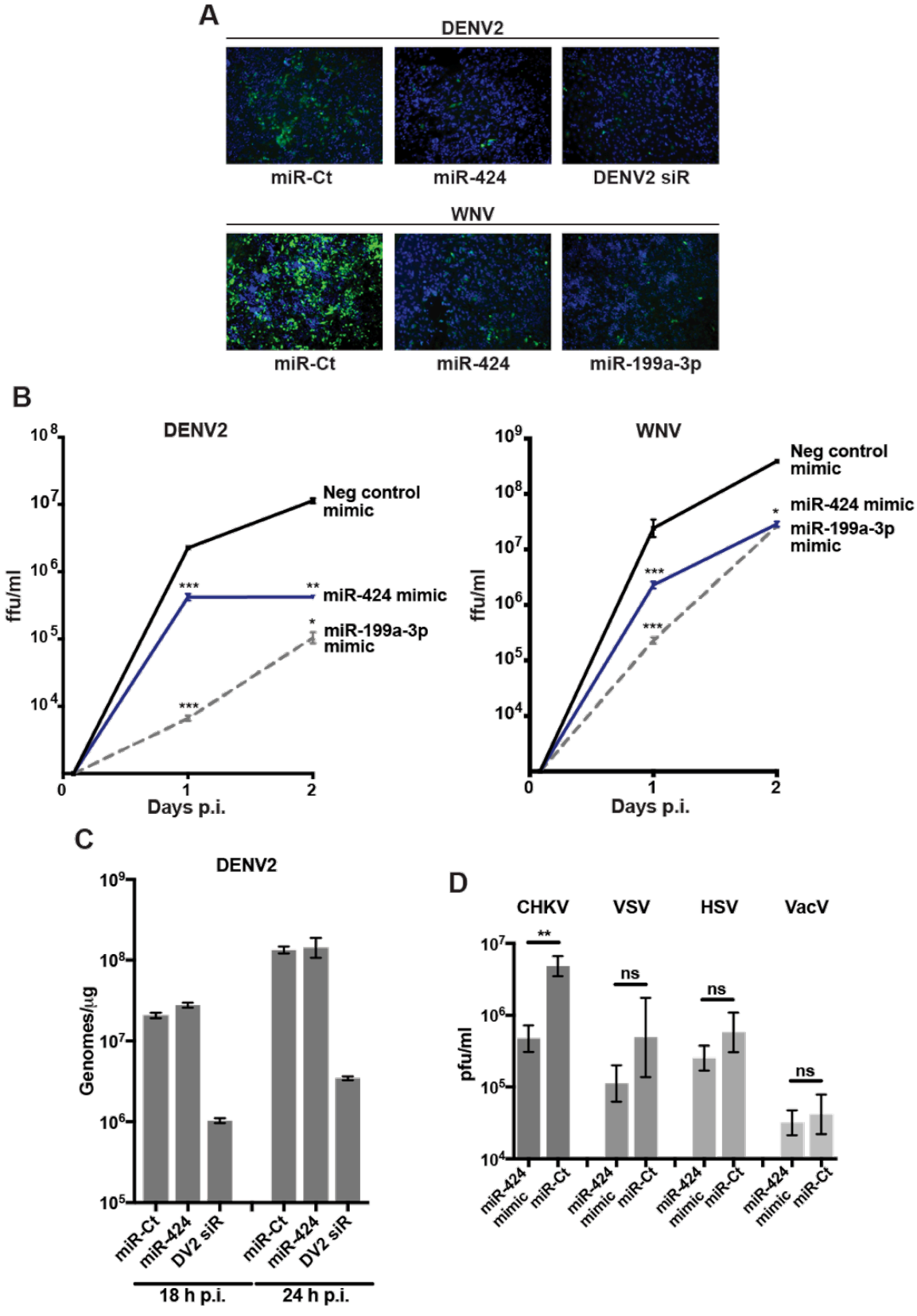

A
DENV2
miR-Ct
miR-424
DENV2 siR
WNV
miR-Ct
miR-424
miR-199a-3p
B
DENV2
ffu/ml
Neg control mimic
miR-424 mimic
miR-199a-3p mimic
Days p.i.
WNV
ffu/ml
Neg control mimic
miR-424 mimic
miR-199a-3p mimic
Days p.i.
C
DENV2
Genomes/µg
miR-Ct
miR-424
DV2 siR
18 h p.i.
24 h p.i.
D
CHKV
VSV
HSV
VacV
pfu/ml
ns
miR-424 mimic
miR-Ct

Figure 2.2 Expression of a miR-424 mimic inhibits RNA virus infection.

A) HeLa cells were transfected with a duplex RNA mimic of miR-424, the broadly antiviral miR-199a-3p, an siRNA against the DENV2 genome, or a negative control miRNA mimic. 48 h post-transfection, cells were infected with DENV2 (MOI=10 ffu/cell) or WNV (MOI=3 ffu/cell). 2 (WNV) or 3 (DENV2) days p.i., cells were fixed and immunofluorescently probed for the flavivirus E protein (green), and cell nuclei stained with DAPI (blue). **B)** Supernatants were collected from the above infected cells at 2 h, 1 day, and 2 days p.i., and titers determined by focus-forming assay on Vero cells. Significance determined by two-way ANOVA with Bonferroni's test for multiple comparisons to control mean value (Neg control mimic). Adjusted p-values reported (* p-value<0.05, ** p-value<0.01, *** p-value<0.001, **** p-value<0.0001). **C)** Total RNA was collected from cells transfected with the miR-424 mimic, negative control mimic, or DENV2 siRNA 18 h or 24 h p.i. with DENV2 (MOI=10 ffu/cell). Genome equivalents were determined by qPCR using a primer-probe set amplifying the DENV 3' UTR. **D)** HeLa cells transfected with the miR-424 mimic or negative control mimic miR-Ct were infected with CHKV, VSV, HSV, or VacV (MOI=0.5pfu/cell). Supernatants were collected 3 days p.i., and titers determined by plaque-forming assay on Vero cells. Significance determined by Student *t* test.

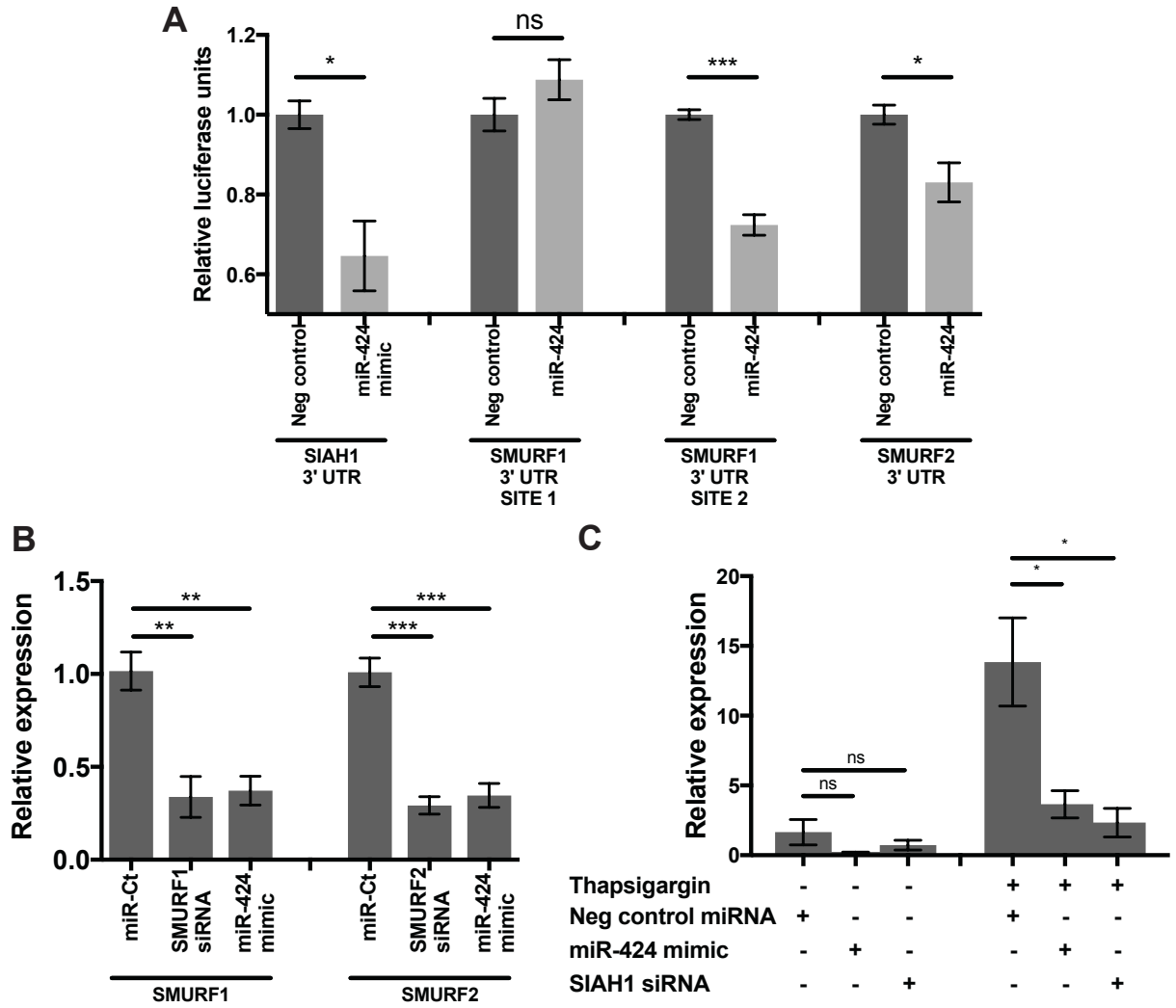
2.3.2 miR-424 targets several cellular E3 ubiquitin ligases

miR-424 target predication via *in silico* analysis and experimental evidence revealed that genes related to ubiquitin conjugation, and in particular, E3 ubiquitin ligases, were highly represented suggesting a role for miR-424 in regulating protein stability via control of ubiquitination[367]. Notably, the ubiquitin-proteasome system (UPS) is essential for DENV and WNV infection, and inhibition of the UPS severely impairs replication [208,306,368]. Therefore, we hypothesized that perturbation of the UPS via miR-424-mediated inhibition of expression of one or more E3 ubiquitin ligases may contribute to inhibition of DENV and other flaviviruses. We initially examined repression of the E3 ubiquitin ligases SIAH1, SMURF1, and SMURF2, having been previously identified to be targets of miR-424[369,370]. To verify modulation of SIAH1, SMURF1, and SMURF2, the 3' UTR of each gene, containing the predicted binding site of miR-424, was cloned into the dual luciferase reporter system psiCHECK-2. SMURF1 contains two sites complementary to the miR-424 seed sequence, which were each cloned into separate plasmids. Cells were cotransfected with the reporter plasmid and the miR-424 mimic, and luciferase expression measured (Figure 2.3A). Reporter expression was reduced 35% and 15% when miR-424 was present in cells cotransfected with the SIAH1 or SMURF2 3' UTRs, respectively. Of the two miR-424 binding sites in the SMURF1 3' UTR, only the downstream site resulted in reduced luciferase expression when miR-424 was present, consistent with the binding site identified by Xiao, et al [111,370].

To examine the effect of the miR-424 mimic on expression of the endogenous mRNA transcripts of SIAH1, SMURF1, and SMURF2, RNA was collected from cells transfected with the miR-424 mimic and transcript expression measured by qPCR. The miR-424 mimic reduced

the endogenous transcript expression of both SMURF1 and SMURF2 to a similar extent as siRNAs specific for those transcripts (Figure 2.3B). SIAH1 is expressed at very low constitutive levels, but expression can be induced by the unfolded protein response (UPR) [190]. As expected, we detected very low levels of SIAH1 mRNA in untreated HeLa cells, but much higher levels in cells treated with thapsigargin, which inhibits calcium exchange in the endoplasmic reticulum (ER), causing ER stress and inducing the UPR (Figure 2.3C). In cells transfected with the miR-424 mimic, SIAH1 induction by thapsigargin was blocked.

Flavivirus infection has been shown to activate several arms of the UPR, suggesting that SIAH1 expression may be induced in cells infected with DENV2 [261,262]. To examin this possibility, RNA was isolated from DENV2-infected cells at 24 h and 48 h p.i. and SIAH1 expression quantified by qPCR (Figure 2.3D). SIAH1 mRNA was detectable at 24 h p.i. and increased more than 20-fold above constitutive levels by 48 hours post-infection. As expected, transfection of the miR-424 mimic blocked SIAH1 expression in infected cells. SMURF1 and SMURF2 transcripts were detected prior to infection and showed moderate induction during DENV2 infection, although SMURF1 expression dropped back to near constitutive levels by 48 h (Figure 2.3D). Like SIAH1, miR-424 inhibited expression of SMURF1 and SMURF2 during DENV2 infection.


A
Relative luciferase units
1.2
1.0
0.8
0.6
*
ns
***
*
Neg control
miR-424 mimic
miR-424
SIAH1 3' UTR
SMURF1 3' UTR SITE 1
SMURF1 3' UTR SITE 2
SMURF2 3' UTR
B
Relative expression
1.5
1.0
0.5
0.0
**
**
***
***
miR-Ct
SMURF1 siRNA
SMURF2 siRNA
miR-424 mimic
SMURF1
SMURF2
C
Relative expression
20
15
10
5
0
*
*
ns
ns
Thapsigargin
Neg control miRNA
miR-424 mimic
SIAH1 siRNA

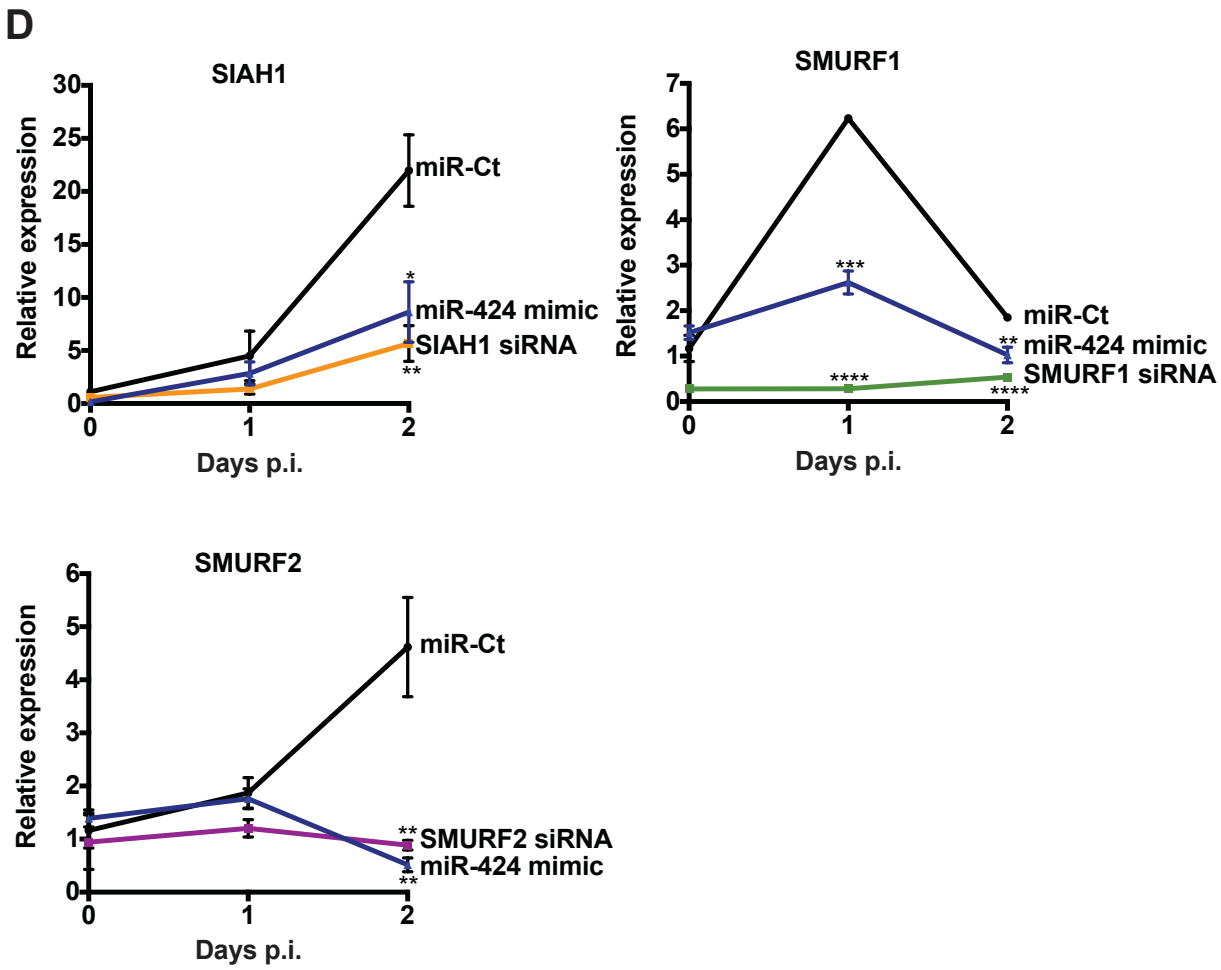


Figure 2.3 miR-424 targets the 3' UTR of SIAH1, SMURF1, and SMURF2, inhibiting transcript expression during DENV2 infection

A) Dual luciferase reporter plasmids were constructed containing the 3' UTRs of SIAH1, SMURF1, or SMURF2. The 3' UTR of SMURF1 contains two putative miR-424 binding sites, prompting the construction of two plasmids containing one binding site each. Cells were transfected into HEK293 cells with the reporter plasmid and the miR-424 mimic or control small RNA. *Renilla* (test) and firefly (control) luciferase activity was measured 48 h post-transfection. Activity is represented as relative luciferase units (RLU), calculated as test normalized to control. Significance determined by Student *t* test (* p-value<0.05, ** p-value<0.01, *** p-value<0.001). **B)** Total RNA was collected in Trizol from HeLa cells transfected with siRNA against SMURF1

or SMURF2, the miR-424 mimic, or a control mimic. SMURF1 and SMURF2 mRNA expression was determined by qPCR and normalized to β-actin. Significance determined by one-way ANOVA with Bonferroni test for multiple comparisons. Adjusted p-values reported. **C)** Cells were transfected with the SIAH1 siRNA, the miR-424 mimic, or control mimic, and 48 h post-transfection, treated with thapsigargin (1μM) for 6 h. Total RNA in Trizol was collected and relative mRNA levels of SIAH1 determined as described above. Significance determined by one-way ANOVA with Bonferroni's test for multiple comparisons. Adjusted p-values reported. **D)** Cells were transfected as indicated and infected with DENV2 (MOI=5 ffu/cell). Total RNA was collected in Trizol at the indicated time points and relative mRNA levels determined as described above. Significance determined by one-way ANOVA with Bonferroni's test for multiple comparisons. Adjusted p-values reported.

2.3.3 Knockdown of SIAH1 and SMURF2 E3 ubiquitin ligases inhibits DENV2 infection

To determine if SIAH1, SMURF1, and SMURF2 are required for DENV2 infection, cells were transfected with siRNA against one of the three ligases prior to infection (Figure 2.4). Knockdown of SIAH1 reduced viral titer released from infected cells at 48 h p.i., coinciding with the induction of SIAH1 gene expression. The extent to which DENV2 infection was inhibited by the SIAH1 siRNA was similar to the inhibition seen in miR-424 mimic transfected cells. Infection was also inhibited by SMURF2 knockdown, although to a lesser extent than the SIAH1 knockdown or miR-424 expression. In contrast, the SMURF1 siRNA appeared to slightly increase (2-fold) viral titers at 48 h p.i. (Figure 2.4). These results suggest that repression of SIAH1 expression by miR-424 plays an important role in the antiviral effects of miR-424, although other genes regulated by miR-424 also contribute to the overall inhibition of DENV2 infection.

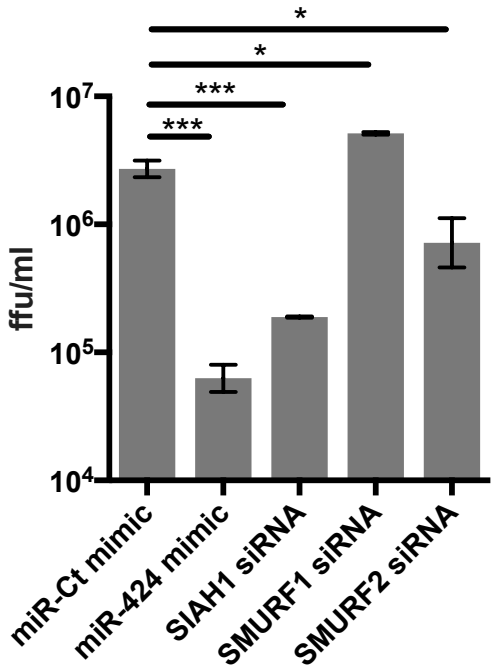


Figure 2.4 Inhibition of SIAH1 expression reciprocates the antiviral effect of miR-424 during DENV2 infection.

Cells were transfected as indicated and infected with DENV2 (MOI=5 ffu/cell) 48 h post-transfection. Supernatants from infected cells were collected 48 h p.i. and titered on Vero cells.

2.3.4 SIAH1 knockdown inhibits proteasome-dependent degradation of MyD88

Many of the miRNAs identified as inhibitors of flaviviruses act through modulation of cellular innate immune pathways[345,unpublished data]. Notably, previous studies have identified SIAH1 as a potential binding partner of the immune signaling adaptor MyD88, suggesting that SIAH1 may play a role in regulating MyD88 through ubiquitination [371,372].To determine if MyD88 protein expression is altered during DENV2 infection, cell lysates from infected cells were collected, subjected to SDS-PAGE, and MyD88 protein visualized by western blotting. At 48 h p.i., expression of MyD88 was much lower (73% reduction in band intensity) compared to uninfected cells (Figure 2.5A, lanes 1-2). However, in cells that were transfected with the SIAH1 siRNA or miR-424 mimic prior to infection, MyD88 protein expression was restored, suggesting that the presence of SIAH1 is responsible for the decrease in MyD88 protein in DENV2 infected cells (Figure 2.5A, lanes 3-4). Inhibition of the proteasome using the drug MG132 also restored MyD88 expression, indicating that the loss of MyD88 during DENV2 infection is a result of proteasomal degradation (Figure 2.5A, lane 6). Our data suggest that SIAH1 mediates proteasome-dependent degradation of MyD88 during DENV2 infection.

To address the effect of SIAH1 inhibition on infected cells in the absence of MyD88 expression, we generated a HeLa cell line deficient in MyD88, using the CRISPR/Cas9 gene editing system[373,374]. As expected, viral titers collected from cells with intact MyD88 were 14- or 44-fold reduced in cells transfected with the SIAH1 siRNA or miR-424 mimic (respectively) prior to infection (Figure 2.5B). In contrast, the strong reduction in viral titer was not observed in the MyD88-deficient cells. These results suggest that the inhibition of DENV infection we observed results from the inhibition of SIAH1-dependent degradation of MyD88.

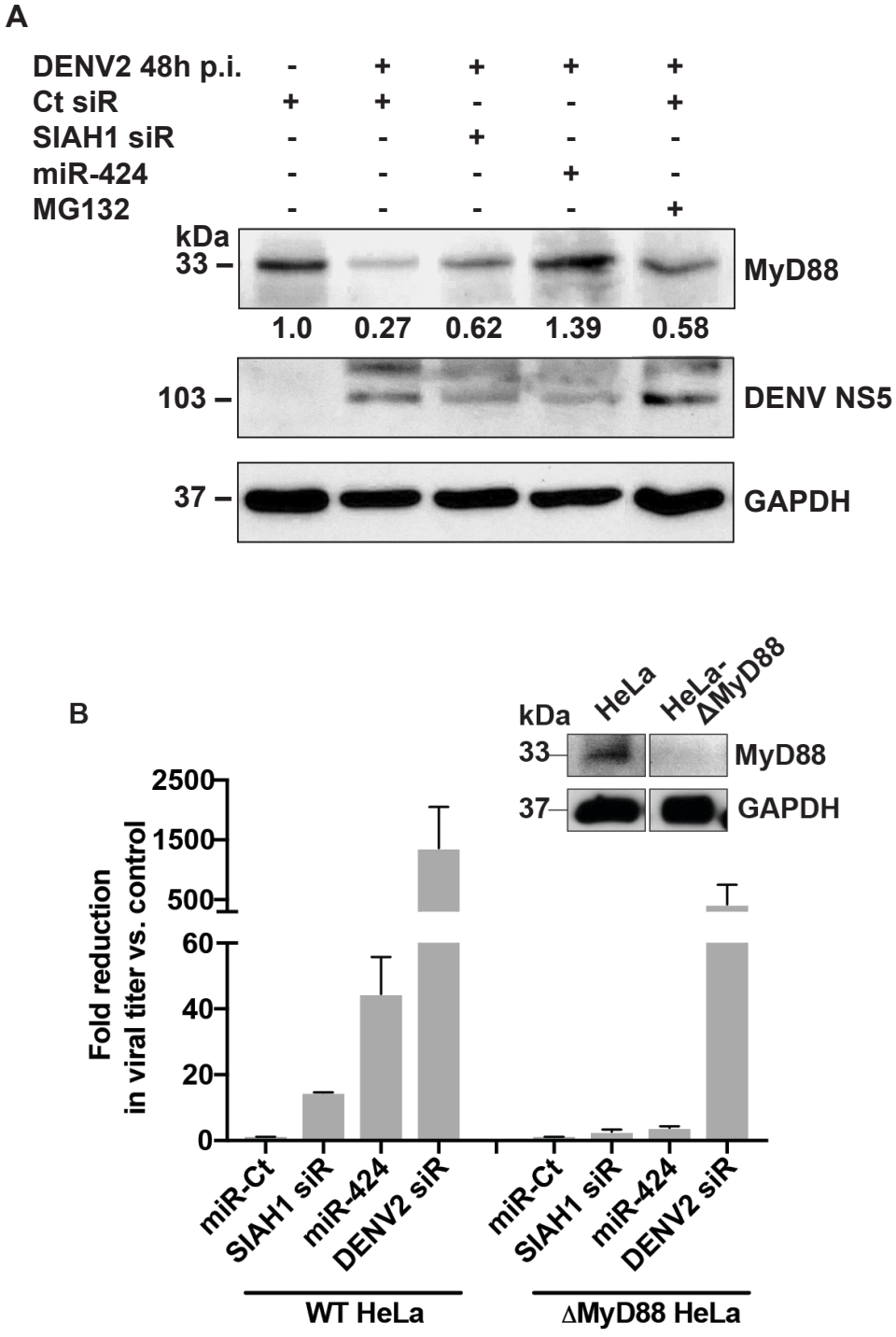

A
DENV2 48h p.i. - + + + +
Ct siR + + - - +
SIAH1 siR - - + - -
miR-424 - - - + -
MG132 - - - - +
kDa
33 –
MyD88
1.0 0.27 0.62 1.39 0.58
103 –
DENV NS5
37 –
GAPDH
B
kDa
HeLa
HeLa-ΔMyD88
33
MyD88
37
GAPDH
Fold reduction in viral titer vs. control
2500
1500
500
60
40
20
0
miR-Ct
SIAH1 siR
miR-424
DENV2 siR
WT HeLa
ΔMyD88 HeLa

Figure 2.5 SIAH1 knockdown inhibits proteasome-dependent degradation of MyD88.

A) Cells were transfected with as indicated and infected with DENV2 (MOI=5 ffu/cell) 48 h post-transfection. 42 h p.i. a cohort of siRNA control transfected wells were treated with MG132 to inhibit the proteasome. Cell lysates were collected 48 h p.i. and analyzed by western blot for MyD88 expression. **B)** A HeLa cell line deficient in MyD88 was produced using the CRISPR/Cas9 gene editing system (inset). WT and MyD88-deficient HeLa cells were transfected as indicated, infected with DENV2 (m.o.i.= 5ffu/ml) 48 h post-transfection. Supernatants were collected 48 h p.i.

2.3.5 SIAH1 binds and ubiquitinates MyD88

Because we observed that degradation of MyD88 during infection occurs in a proteasome-dependent manner, we next examined the physical interaction between MyD88 and SIAH1. Cells were co-transfected with plasmids encoding MyD88 with a C-terminal myc epitope and SIAH1 with a C-terminal FLAG epitope tag. 48 h post-transfection, cell lysates were collected and immunoprecipitated with a FLAG antibody or an isotype control. Western blotting revealed MyD88 in samples immunoprecipitated with FLAG but not in the control samples, indicating physical interaction between SIAH1 and MyD88 (Figure 2.6A).

We next examined ubiquitination of MyD88 in the presence of SIAH1. Cells were transfected with plasmids encoding MyD88 (not epitope tagged) and ubiquitin bearing a myc epitope tag. SIAH1 expression was modulated by co-transfection of a SIAH1 siRNA or negative control small RNA. 24 h post-transfection, thapsigargin was added to the media to induce expression of SIAH1 via the UPR. After overnight treatment with thapsigargin, the media was replaced and cells treated with MG132 for 6 h to allow accumulation of ubiquitinated proteins. Cell lysates were collected and subjected to immunoprecipitation with an anti-myc antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and MyD88 detected by western blotting with a MyD88-specific antibody. In control cell lysates, we detected mono- and poly-ubiquitinated MyD88 immunoprecipitated with the myc-tagged Ub (Figure 2.6B). In contrast, when SIAH1 expression was inhibited with the siRNA, little MyD88 ubiquitination was observed. SIAH1, therefore, not only binds MyD88 but ubiquitinates the protein, in agreement with the observation that inhibiting SIAH1 during DENV infection increases MyD88 expression.

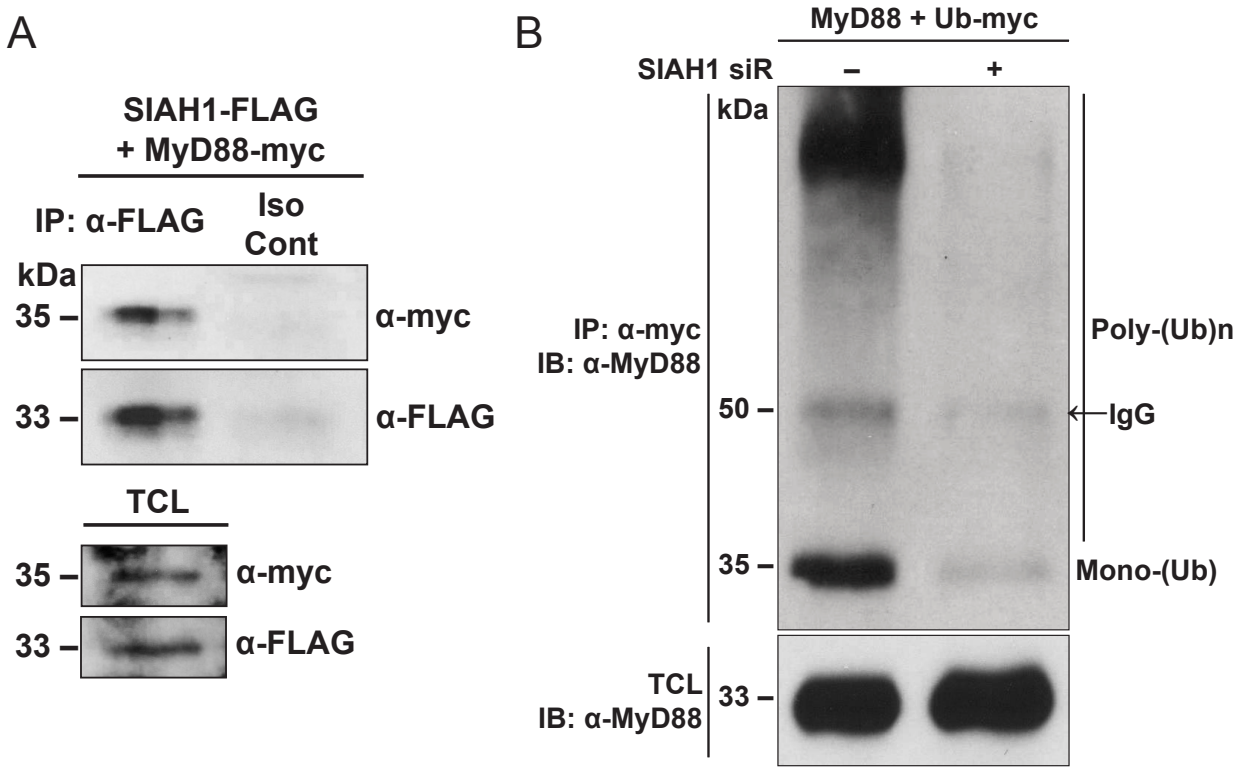


Figure 2.6 SIAH1 binds and ubiquitinates MyD88.

A) Cells were transfected with plasmids expressing SIAH1-FLAG and MyD88-myc. 24 h post-transfection, cells were treated with thapsigargin (1μM) overnight, then treated with MG132 for 6 hr and cells lysates collected. Total cell lysates were immunoprecipitated with α-FLAG or an isotype control antibody and analyzed by western blot. (TCL= total cell lysate) **B)** Cells were transfected with or without SIAH1 siRNA as well as with plasmids expressing MyD88-FLAG and Ub-myc. 24 h post-transfection cells were treated with thapsigargin to induce SIAH1 expression, and 2 d post-transfection with MG132 to inhibit the proteasome. Total cell lysates were immunoprecipitated with α-myc and analyzed by western blot.

2.3.6 Inhibition of SIAH1 increases NF-κB signaling during DENV2 infection

MyD88 functions downstream of TLR and interleukin receptor 1 signaling, resulting in induction of the NF-κB transcription factor complex, which is requires for multiple genes involved in the innate immune response, including interferon β[375]. To determine if the antiviral effects of SIAH1 knockdown and miR-424 expression are a result of increased MyD88-dependent intracellular immune signaling during infection, we examined the induction of genes activated by NF-κB and interferon signaling during DENV2 infection. We used qPCR to measure the gene expression of the NFκB-activated genes IL-8 and IL-6 as well as the interferon-stimulated genes IFIT1 and IFIT2 in the CRISPR/Cas9 MyD88-deficient cells and MyD88-intact cells during DENV2 infection. In cells with intact MyD88, expression of IL-6 and IL-8 was increased 10-15-fold and IFIT1 and IFIT2 5-10-fold after infection with DENV2 as expected. However, induction of all four genes was substantially higher in cells transfected with the SIAH1 siRNA or the miR-424 mimic (Figure 2.7A-D). In contrast, when the MyD88-deficient cells were infected with DENV2, IL-8 induction was strongly inhibited regardless of the presence of the SIAH1 siRNA or the miR-424 mimic. The increased expression of IL-6, IFIT1, and IFIT2 resulting from SIAH1 knockdown was largely lost in the MyD88-deficient cells, although the three-fold induction of IL-6 in infected control cells was unchanged in the absence of MyD88.

DENV2 infection induces NF-κB signaling pathways through a variety of cellular sensing mechanisms, including PRRs such as TLRs and RIG-I-like receptors (RLRs). To determine if the increased induction of NF-κB-activated genes in the presence of miR-424 or knockdown of SIAH1 could be observed by activating a single receptor pathway, cells were

treated with the drug imiquimod, a guanosine analog that specifically activates TLR7, which signals in a completely MyD88-dependent manner (Figure 2.7E). As observed during DENV2 infection, IL-8 expression was increased 26- and 82-fold when SIAH1 siRNA or miR-424 mimic transfected cells (respectively) were treated with imiquimod for 24 h. In contrast, imiquimod treatment induced only a 9-fold increase in IL-8 over untreated cells. These data demonstrate that inhibition of SIAH1 expression results in increased NF-κB-regulated cytokine expression in a MyD88-dependent manner.

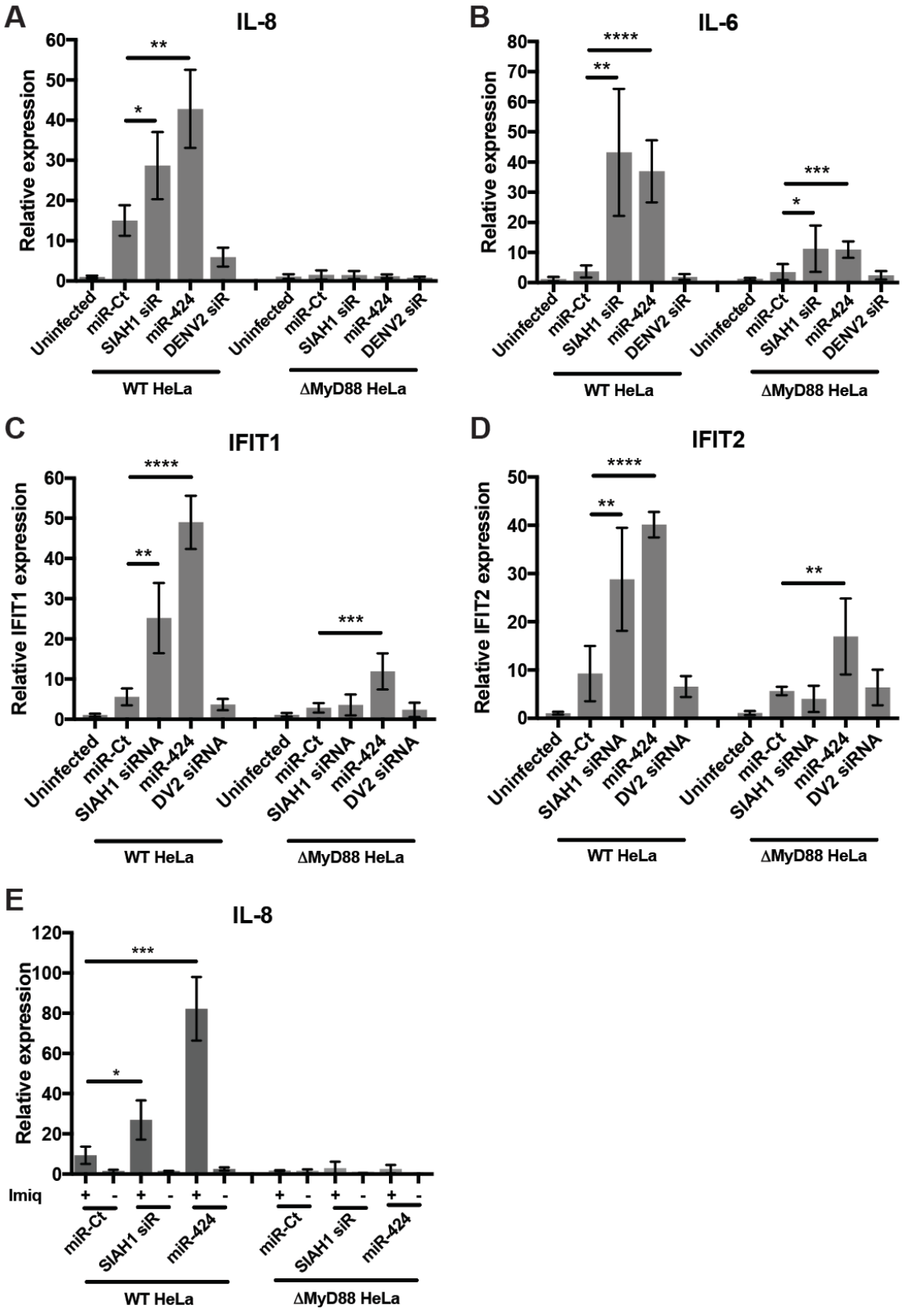

A
IL-8
Relative expression
Uninfected
miR-Ct
SIAH1 siR
miR-424
DENV2 siR
WT HeLa
ΔMyD88 HeLa
B
IL-6
C
IFIT1
Relative IFIT1 expression
SIAH1 siRNA
DV2 siRNA
D
IFIT2
Relative IFIT2 expression
E
Imiq

Figure 2.7 Inhibition of SIAH1 expression increases NF-κB-induced genes IL-6 and IL-8

A-D) Cells were transfected with the indicated siRNA or miRNA mimic and infected with DENV2 (m.o.i.=5ffu/ml) 48 h post-transfection. Total RNA was collected in Trizol 48 h p.i., and relative transcripts levels determined by qPCR normalized to β-actin and uninfected control mRNA levels. **E)** 48 h after transfection, cells were treated with imiquimod (10μg/ml) for 24 h. Total RNA was collected and relative mRNA levels determined as described above. Significance determined by one-way ANOVA with Bonferroni's test for multiple comparisons. Adjusted p-values reported (* p-value<0.05, ** p-value<0.01, *** p-value<0.001).

2.4 Discussion

In this study, we report the induction of the E3 ubiquitin ligase SIAH1 by DENV2 infection. Mediated by the miRNA miR-424, SIAH1 binds and ubiquitinates MyD88. Inhibition of SIAH1 during infection results in increased innate immune signaling and inhibits viral replication. SIAH1 expression is dependent on the UPR, and as DENV infection also requires activation of the UPR, our findings suggest that SIAH1 induction and the subsequent degradation of MyD88 play an important role in avoiding the antiviral immune response during DENV infection[262,376]. In contrast to the requirement for the UPR and associated pathways for infection by flaviviruses as well as CHIKV, VSV infection is not disrupted by UPR induction, and HSV and vaccinia virus encode proteins that inhibit UPR signaling, a possible explanation for absence of inhibition of these viruses by miR-424[344,377-381].

DENV infection causes extensive rearrangements to the host cell ER, including membrane restructuring and assembly of numerous replication complexes composed of viral proteins in both the ER lumen and associated with the cytoplasmic side of the ER membrane[178,182,382]. Rapid accumulation of misfolded cellular and viral proteins within the ER as well as TLR signaling in response to DENV infection may be responsible for induction of the UPR, although the specific mechanisms are currently unknown[267,275-277]. Studies have reported activation of multiple arms of the UPR during flavivirus infection. However, the observed effect of the UPR on replication varies—possibly depending on the virus, viral strain, or cell culture conditions[262,280,281,383]. The UPR is made up of several signaling pathways activated in response to cellular stressors that disrupt protein translation and folding in the ER. Of the three arms of the UPR, the protein kinase RNA-like ER kinase (PERK) and activating transcript 6 (ATF6)

pathways are active only transiently during DENV infection [262]. In contrast, evidence of activation of the inositol-requiring protein-1 (IRE1) pathway appears about mid-way through DENV infection, as well as in JEV and WNV infections[262,281,282,384]. Activation of the IRE1 pathway typically leads to expression of both pro-survival and pro-apoptotic proteins, but in the case of DENV infection, IRE1-directed gene expression appears to favor pro-survival ER homeostasis and protein production[262]. Expression of pro-survival, UPR-stimulated proteins—such as the chaperone GRP78 that aids viral protein translocation to the ER lumen—assists the virus in ensuring continued viral protein translation and RNA replication in spite of cellular defenses[262,282,384]. In addition to augmenting viral translation and replication, activation of the IRE1 pathway appears to play a regulatory role in inflammatory signaling during prolonged ER stress, promoting NF-κB activation when the UPR is initially induced but suppressing NF-κB function during prolonged ER stress[263]. In WNV (Kunjin) infected cells, the UPR also attenuates the innate immune response through inhibition of type I interferon receptor signaling[385-388]. In this study, we showed that the UPR induced by DENV infection also results in SIAH1 expression and the inhibition of NF-κB–signaling via the degradation of MyD88. MyD88 has also been shown to contribute to IFN signaling through a direct interaction with IRF7, so SIAH1-mediated degradation of MyD88 may also contribute to modulation of IFN signaling in infected cells, in conjunction with previously described mechanisms of flavivirus regulation of innate immunity[228,229].

The ubiquitin-proteasome system (UPS) also plays a vital role in flavivirus infection: inhibition of the UPS strongly inhibits DENV and WNV RNA replication, and infection with DENV induces expression of several key components of the UPS in DENV-infected persons as

well as in cell culture[303,304,306,389]. The ubiquitination and degradation of MyD88 as a result of flavivirus infection that we describe in this study is consistent with the appropriation of the UPS by a variety of virus families[301,390-395]. In some cases, the virus itself encodes a ubiquitin ligase that targets cellular proteins, as with the rotavirus nonstructural protein 1 (NSP1), which appears to function as an E3 ubiquitin ligase targeting IRF3, IRF5, and IRF7 and a subunit of the complex responsible for activation NF-κB via IκB degradation[390,391,395,396]. Likewise the replication and transcription activator (RTA) protein of Kaposi's sarcoma-associated herpesvirus (KSHV) serves as an E3 ubiquitin ligase, binding and mediating the poly-ubiquitination of IRF7, culminating in the proteasomal degradation of IRF7 and blocking IFN induction downstream of IRF7[392]. In contrast, the human papillomavirus (HPV) E6 protein does not have ubiquitin ligase activity; rather, E6 binds the cellular ubiquitin ligase E6-associated protein (E6AP), turning the cellular protein against the cell by increasing the ligase's substrate specificity for the tumor suppressor protein p53[301,393]. Polyubiquitination of p53 by E6AP targets p53 to the proteasome for degradation, reducing pro-apoptotic signaling and improving survival of the infected cell[301,393]. Similarly, the NS5 protein of DENV binds to STAT2 and recruits the E3 ubiquitin ligase UBR4, culminating in poly-ubiquitination and degradation of STAT1 and reduction of the type 1 interferon response[190,191,256]. The study described in this chapter demonstrates that in addition to direct interaction between viral proteins and cellular targets of ubiquitination, infection indirectly stimulates proteasomal degradation of cellular proteins through induction of cellular stress responses. Coxsackievirus also indirectly utilizes the UPS in a proviral manner, by inducing the cellular relocation of a subunit of the proteasome responsible for preferentially degrading p53[397]. JEV appears to abrogate antiviral innate immune signaling by triggering the

proteasomal degradation of viperin, although whether the ubiquitination is through a direct, viral protein-mediated mechanism or through indirect means is unknown[398]. Our investigation into the antiviral effects of miR-424 revealed an additional mechanism of viral manipulation of the host cell UPS for reduction of the host innate immune response by degradation of PRR-activated signaling molecules.

miRNA families are known to regulate the function of entire cellular networks, a result of the cumulative effect of individual family members targeting various components of the same pathway[366]. The miR-15/16 family, which includes miR-424, may regulate cellular innate immunity during flavivirus infection as additional miR-15/16 family members that target ligases responsible for degradation of antiviral signaling molecules have been identified[346,399-401]. Work by Zhu, et al. demonstrated that cells infected with JEV express the miR-15/16 family member miR-15b, which in turn inhibits translation of the ubiquitin ligase RNF125, responsible for targeting RIG-I for proteasomal degradation[346]. Although miR-15b shares a similar seed sequence with miR-424, our work did not support inhibition of RNF125 translation by miR-424 (unpublished data). Another member of the miR-15/16 family, miR-497, represses expression of the E3 ubiquitin ligase SMURF1, and although we focused primarily on SIAH1 in this study, we also showed that inhibition of both SMURF1 and SMURF2 resulted in increased MyD88 protein expression, suggesting that total MyD88 degradation during DENV2 may be a cumulative result of these three E3 ubiquitin ligases[399, unpublished data]. Interestingly, while transfection of miR-497, like miR-424 and other miR-15/16 family miRNAs, impaired DENV2 infection, siRNA knockdown of SMURF1 slightly increased viral titers, suggesting an additional antiviral role for SMURF1 in DENV2 infection, perhaps as a result of SMURF1-mediated degradation of the

TGFβ receptor and other members of the TGFβ signaling pathway, which appears to play an important proviral role in DENV2 and other flavivirus infections[402-406]. Nonetheless, the inhibition of E3 ubiquitin ligases by miR-15/16 family members and the concordant enhancement of innate immune signaling suggests that positive regulation of innate immune pathways, particularly through inhibition of pathway component degradation, may be a conserved trait amongst miRNA of the miR-15/16 family.

A recent study revealed that in mouse embryonic fibroblasts, the murine ortholog of miR-424, miR-322, is downregulated by activation of the UPR and can attenuate the activation of the IRE1α and ATF6 pathways, suggesting that the induction of the UPR DENV infection may repress miR-424 expression as well as upregulating SIAH1[407]. miR-424 is not constitutively expressed in the cell lines chosen for this study, nor does the miRNA appear to be induced by infection of these cells with DENV2 or WNV [408, unpublished data]; however, in humans, DENV2 infects several cell types that do express mir-424, including monocytes, macrophages, dendritic cells, and endothelial cells[200,409-411]. Induction of the UPR by DENV may represent an additional mode of immune suppression during DENV infection of these cell types. Examining miR-424, SIAH1, and MyD88 expression in primary cells over the course of DENV infection may provide more insight into the role of the UPR in DENV pathogenesis, particularly in light of the caveats associated with the use of immortalized cell lines. For example, the tumor-derived HeLa cell line expresses an integrated human papillomavirus gene, E1, that represses interferon signaling and may predispose the cell line to infection with other viruses[412]. Although in this study, expression of miR-424 in HeLa cells resulted in increased innate immune gene expression, including interferon-dependent genes, in spite of E1 integration, studying the effect of miR-424 and SIAH1

on innate immune expression in primary cells targeted during human infection may provide a more thorough understanding of the role of the UPR in DENV infection. The expression and role of miR-424 varies between cell types and in the context of viral infection, UPR-mediated repression of miR-424 may have significant effects on the expression of other pathways mediated by miR-424. For example, miR-424 appears to play an essential role in directing monocyte differentiation into macrophages. Should induction of the UPR by DENV infection of monocytes result in downregulation of miR-424, differentiation of infected monocytes may be altered, an additional potential mechanism of DENV regulation of the antiviral immune response.

Our work and that of other groups has demonstrated the utility of cellular miRNAs as tools for identifying interactions between an infecting virus and the protein expression of host cells[344-346,413]. In this study, we identified an antiviral miRNA, miR-424, that represses a UPR-dependent E3 ubiquitin ligase, SIAH1, resulting in loss of SIAH1-mediated degradation of the immune signaling molecule MyD88. Although we did not examine the role of endogenous miR-424 in flavivirus infection, but rather utilized the miRNA as a tool for identifying cellular actors required for viral replication, further studies probing the interactions between miR-424, other miR-15/16 family members, and the impact of these miRNAs on innate immune signaling may reveal a cellular defense mechanism against the virus and the cellular pathways subverted by infection. One such pathway, the UPR, serves multiple functions in DENV infection, including maintaining the host cell's protein translation mechanisms, producing prosurvival signals, allocating lipid resources, and as shown in this study, moderating the cellular antiviral response. Several inhibitors of UPR pathways have been approved for use in humans by the United States

Food and Drug Administration, and use of these drugs to inhibit components and individual pathways of the UPR may have the potential to serve a therapeutic role in humans infected with dengue virus[414].

2.5 Materials and methods

Cell culture and virus strains

HeLa (ATCC) cells were grown in Dulbecco modified Eagle medium (Thermo Fisher) supplemented with 5% fetal bovine serum (FBS; HyClone), 2 mM L-glutamine (Thermo Fisher), 100 U penicillin G sodium/ml, and 100µg of streptomycin sulfate/ml. HEK293 (Microbix) cells were grown in modified Eagle medium (Thermo Fisher) supplemented with 5% FBS, 2 mM L-glutamine, 100 U penicillin G sodium/ml, and 100µg of streptomycin sulfate/ml.

DENV2 (New Guinea C) was obtained from ATCC. WNV (385-99) has been previously described[415]. DENV2 and WNV were passaged twice on C6/36 insect cells and purified by centrifugation as previously described[416]. DENV2 and WNV virus titers were determined by focus forming assay: virus was serially diluted and plated on Vero cells. Following 1 h incubation with rocking to allow adsorbtion, a 0.5% carboxymethyl cellulose (CMC; Sigma-Aldritch) overlay was added. 48 h p.i., the cells were fixed in 4% paraformaldehyde (PFA; Thermo Fisher), washed 3 times with phosphate-buffered saline (PBS), then blocked and permeabolized in PBS containing 2% normal goat serum (NGS; Sigma-Aldritch) and 0.4% Triton X-100 (Thermo Fisher). The cells were then incubated for 2 h with 2µg/ml anti-flavivirus envelope antibody 4G2 in PBS containing 2% NGS and 0.4% Triton X-100. Cells were then washed with PBS three more times and incubated with secondary antibody, anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotech), at 0.4µg/ml in PBS, 2% NGS, and 0.4% Triton X-100. Foci were visualized using the Vector VIP peroxidase substrate (Vector labs) as directed by the manufacturer.

HSV 1 (F1 strain) was a gift from A. Hill of Oregon Health and Science University. VacV (Western Reserve strain) was a gift from M. Slifka of Oregon Health and Science University. CHIKV (MH56 strain) was a gift of D. Streblow of Oregon Health and Science University. CHIKV, HSV, and VacV titers were determined by plaque forming assay. Serial dilutions of virus were added to Vero cells, and incubated for 1 h, before overlaying with 0.5% CMC. 72 h p.i., cells were fixed in 4%. Cells were stained with crystal violet.

VSV-GFP was a gift of V. DeFilippis of Oregon Health and Science University. VSV-GFP titers were determined by fluorescent plaque: 24 h p.i. with serially diluted virus, Vero cells were fixed in 4% PFA and plaques examined using fluorescent microscopy.

In all infections, cells were incubated for 1 h with virus at the indicated MOI in a low volume of medium containing 2% FBS, washed with PBS, and refed with medium containing 2% FBS, 2 mM L-glutamine, 100 U penicillin G sodium/ml, and 100µg of streptomycin sulfate/ml.

Reagents

Where indicated, cells were incubated with 1µM thapsigargin (Sigma Aldritch) in complete medium for 6 h unless otherwise noted. Cells treated with MG132 (Thermo Fisher) were incubated for 6 h in medium containing 5µM MG132 for 6 h unless otherwise noted. In experiments employing imiquimod treatment, cells incubated with 1µg/ml imiquimod (Enzo Life Sciences) for 24 h in complete medium. The antibodies used were: anti-MyD88 (Cell Signaling Technology), anti-myc tag (Life Technology), anti-FLAG tag (Sigma Aldritch), anti-GAPDH

(Abcam), anti-flavivirus E antibody 4G2 (hybridoma purchased from ATCC and maintained by the Vaccine and Gene Therapy Institute monoclonal antibody core facility (OHSU)).

Plasmids

The 3' UTRs of SIAH1, SMURF1, and SMURF2 were amplified by PCR (SIAH1-A, 5'-CTCGAGCACCCATCTGTCTGCCAACC-3'; SIAH1-B, 5'-GCGGCCGCCTGTGCATGACGATGCCTTCTTC-3'; SMURF1-1-A, 5'-CTCGAGGCTGCTCTCCAATGCCATCAG-3'; SMURF1-1-B, 5'-GCGGCCGCCGTAGCCTTCGGGCAGTTC-3'; SMURF1-2-A, 5'-CTCGAGGTTCTACTTTGGGTCCGCG-3'; SMURF1-2-B, 5'-GCGGCCGCCCAGCCTGCTGTACAATCAC-3'; SMURF2-A, 5'-TTGCGGCCGCCCAGCCTGCTGTACAATCAC-3'; SMURF2-B, 5'-TTGCGGCCGCGAGATGGGTCTGCAACCAG-3'). PCR products were digested and ligated into reporter plasmid psiCHECK2 (Promega). Full-length SIAH1 and MyD88 were amplified by PCR (SIAH1-A, 5'-GCGGCCGCCCACCATGAGCCGTCAGAC-3'; SIAH1-B, 5'-CTCGAGACACATGGAAATAGTTACATTGATGCC-3'; MyD88-A, 5'-GGATCCCCACCATGCGACCCGACCG-3'; MyD88-B, 5'-GCGGCCGCTGGGCAGGGACAAGGCCTTGGC-3'). PCR products were digested and ligated into pcDNA4 (Thermo Fisher) or pEF1/myc-His B (Thermo Fisher). The Ub-myc plasmid was a kind gift A. Moses of Oregon Health and Science University.

miRNA, siRNA, and plasmid transfections

miRIDIAN miRNA mimics and controls (Dharmacon) and siRNAs (Thermo Fisher) were transfected using Lipofectamine RNAiMAX reagents (Thermo Fisher) according to manufacturer protocols. Plasmids expressing SIAH1, SMURF1, and SMURF2 cDNA or 3' UTRs were transfected using Lipofectamine 3000 (Thermo Fisher) according to manufacturer recommendations. Cotransfections of plasmids and miRNA duplexes or siRNAs were also performed with Lipofectamine 3000.

Immunofluorescence

Infected cells were fixed in 4% PFA and washed two times with a wash buffer consisting of PBS containing 0.2% BSA and 0.2% Triton X-100, then incubated for 1 h in blocking buffer composed of 2% BSA and 0.2% Triton X-100 in PBS. Cells were next incubated in 2µg 4G2 antibody/ml wash buffer, washed 3 times with additional wash buffer, and incubated 1 h with 1µg goat α-mouse Alexa Fluor 488 (Thermo Fisher)/ml wash buffer. Cells were washed several times, with one wash containing 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher) to stain cell nuclei, and visualized by fluorescent microscopy.

Luciferase assays

HEK293 cells were transfected with psiCHECK2 reporter plasmids and either the miR-424 mimic or negative control mimic using. Cells were lysed 48 h post-transfection, and luciferase assays performed using the Dual Luciferase Reporter Assay System (Promega) according to manufacturer's protocol.

qPCR

100ng of total RNA were reverse transcribed and amplified using SIAH1 (Hs02339360_m1)-, SMURF1 (Hs00410929_m1)-, SMURF2 (Hs00224203_m1)-, IL-6 (Hs00985639_m1)-, IL-8 (Hs00174103_m1)-, IFIT1 (Hs01911452_s1), IFIT2 (Hs00533665_m1), or actin (Hs99999903_m1)-specific Taqman gene expression probes (Thermo Fisher) and Taqman RNA-to-CT 1-Step reagents (Applied Biosystems) on a StepOne Plus real-time PCR instrument (Applied Biosystems) based on manufacturer's recommendations. Relative transcript expression of the gene of interest was calculated using ΔCt with actin as the endogenous control transcript.

General statistical analysis

Experiments were performed at least three times unless otherwise indicated in the figure legends. Data are presented as mean ± standard error of the mean (SEM). Student *t* test was used for single time point viral titers and luciferase assays when two sets were compared. For data containing more than two sets or more the a single time point, a one-way ANOVA analysis was conducted with Bonferroni's correction for post hoc multiple comparisons. A p-value < 0.05 was considered significant.

Chapter 3: Unpublished observations

3.1 Summary

In this chapter, I present unpublished results pertaining to the inhibition of flavivirus infection by the cellular miRNA miR-526b. I show that in addition to restricting DENV2 and WNV infection, miR-526b inhibits several other RNA and DNA viruses. Based on functional analysis of mRNAs predicted to be subject to miR-526b-mediated repression, I examined the effect of miR-526b on expression of genes induced by innate immune activation. Interestingly, while miR-526b profoundly enhanced expression of ISGs and NF-κB-activated genes when cells were stimulated with a TLR agonist, innate immune gene expression was unaltered in WNV infection of miR-526b expressing cells. Furthermore, miR-526b exhibits a time-dependent inhibition of DENV genome replication, suggesting that miR-526b may regulate expression of a cellular factor or factors associated with the ER and necessary for viral replication.

All experiments presented in this chapter were conducted by Ashleigh R. Murphy, with the exception of the miRNA library screen and analysis executed by Jessica L. Smith, Alec J. Hirsch, Sophia Jeng, and Shannon K. McWeeney. The IRF3-deficient HeLa cell line was a gift of Victor R. DeFilippis.

3.2 Introduction

As described in the previous chapter, a high throughput screen was undertaken in order to identify cellular miRNAs that restricted flavivirus infection[345]. Subsequent study of individual anti-flaviviral miRNAs revealed the miR-424-mediated regulation of innate immune signaling by the UPR discussed in the previous chapter as well as the miR-34a-mediated induction of interferon through the Wnt signaling pathway discussed in Smith, Parkins, and Hirsch 2017[345]. This chapter will address an additional antiviral miRNA identified by the screen, miR-526b, a poorly characterized member of the miR-515 family of miRNAs.

miR-526b, as well as the other members of the miR-515 family, is encoded in the C19MC cluster of miRNAs—the largest miRNA cluster in the human genome[417,418]. miRNAs of the C19MC cluster are found highly expressed in embryonic stem cells and the placenta and circulating in the blood of pregnant women, as well as in various cancers, such as hepatocellular carcinoma[418-421]. The role of the miR-515 family and of its individual members has not been defined, although functions such as control of cell differentiation and growth development and protection of the maternal-fetal interface from viral infection have been proposed[422,423]. Endogenous expression of the C19MC cluster miRNAs in placental trophoblasts and exogenous expression of the cluster as a whole in several other cell types protects against infection with a variety of RNA and DNA viruses[423]. As the role of miR-515 family members in viral inhibition has not been investigated, I chose to investigate the mechanism of miR-526 inhibition of flavivirus infection.

3.3 Results

3.3.1 miR-526b inhibits DENV2 and WNV infection

To identify human cellular miRNAs that inhibit flavivirus infection, a library of small RNA duplex mimics corresponding to human miRNAs were transfected into HeLa cells. Following infection with DENV2, WNV, or JEV, the effect on viral E protein expression was determined by indirect immunofluorescence and quantification of fluorescent intensity[345]. Among the anti-flaviviral miRNAs identified by the screen was miR-526b, a member of the miR-515 family of miRNAs. To confirm the results of the screen, we tested the effect of miR-526b on viral protein expression and infectious viral titer. Cells were transfected with the miR-526b mimic, a control mimic, or a mimic of the broadly antiviral miRNA miR-199a-3p and 2 d post-transfection, infected with either WNV or DENV2. 2 d (WNV) or 3 d (DENV2) p.i., the cells were fixed and probed for E protein by indirect immunofluorescence. miR-526b strongly reduced the expression of E in both WNV and DENV2 infections (Figure 3.1A). Likewise, when supernatants were collected during infection and viral titer determined by focus forming assay (ffa), cells transfected with the miR-526b mimic produced 10-20-fold less infectious virus than control cells (Figure 3.1B).

I next examined whether the antiviral effects of miR-526b on flavivirus infection extended to additional virus families. Cells transfected with miR-526b were infected with one of the following: the plus-strand RNA alphaviruses CHKV or Venezuelan equine encephalitis virus (VEEV); the minus-strand RNA rhabdovirus VSV; the DNA viruses HSV or vaccinia virus (VacV). Supernatants were collected from infected cells 2 d p.i. and titered by plaque assay on Vero cells. As with the flaviviruses, alphavirus titer was reduced about 2-log by miR-526b

expression. Infection by either of the DNA viruses was also significantly inhibited. In contrast, no significant change in infectious titer was observed in VSV cells in the presence of miR-526b compared to control infection. These results indicate that miR-526b alters expression of one or more of the transcripts that play important roles in the life cycle of a range of viruses.

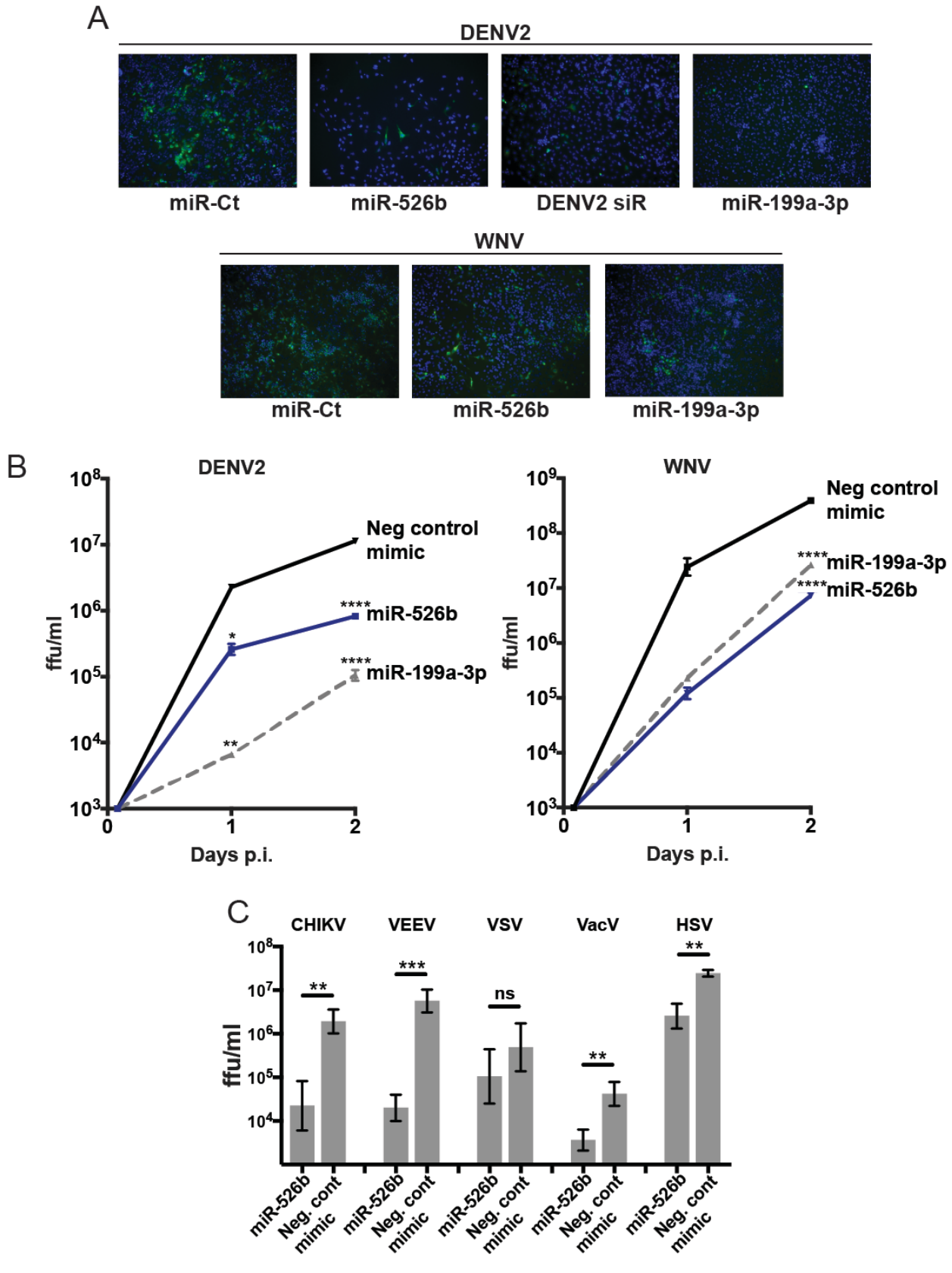

A
DENV2
miR-Ct
miR-526b
DENV2 siR
miR-199a-3p
WNV
miR-Ct
miR-526b
miR-199a-3p
B
DENV2
ffu/ml
Neg control mimic
miR-526b
miR-199a-3p
Days p.i.
WNV
ffu/ml
Neg control mimic
miR-199a-3p
miR-526b
Days p.i.
C
CHIKV
VEEV
VSV
VacV
HSV
ffu/ml
ns
miR-526b
Neg. cont mimic

Figure 3.1 miR-526b inhibits flavivirus infection

A) HeLa cells were transfected with the miR-526b duplex mimic, miR-199a-3p mimic, control mimic, or an siRNA directed against the DENV genome. 2 d post-transfection, cells were infected with DENV2 (MOI=10 ffu/cell) or WNV (MOI=3 ffu/cell). 2 (WNV) or 3 (DENV2) days p.i., cells were fixed and immunofluorescently probed for the flavivirus E protein (green). Cell nuclei were visualized with DAPI (blue). **B)** Supernatants were collected from the above experiment at 2 h, 1 d, and 2 d p.i., and serially diluted on Vero cells. Infectious viral titer was determined by ffa. Significance determined by one-way ANOVA with Bonferroni's test for multiple comparisons to control mean value (Neg control mimic). Adjusted p-values reported (* p-value<0.05, ** p-value<0.01, *** p-value<0.001, **** p-value<0.0001). **C)** Cells were transfected with the miR-526b mimic or control and 2 d post-transfection, infected with CHKV, VEEV, VSV, VacV, or HSV (MOI=0.5pfu/cell). Supernatants were collected 2 d p.i. Titers were determined by plaque forming assay on Vero cells. Significance determined by Student *t* test.

3.3.2 Other members of the miR-515 family do not inhibit WNV

miR-526b belongs to the miR-515 family of miRNAs. Although the 28 members of the miR-515 family are expressed from the large miRNA cluster C19MC and share some sequence similarity, the seed sequences are not conserved across the family [417]. The four members of the miR-515 family with the most seed sequence conservation to miR-526b—miR-526a, miR-515, miR-516a, and miR-518a—were tested for inhibition of WNV (Figure 3.2A). Cells were transfected with a miR-515 family member, the negative control mimic, or miR-199a-3p mimic and infected with WNV 2 d post-transfection. Focus forming assays were conducted to determine viral titer in supernatants collected 2 d p.i. Of the four miR-515 family members examined, only miR-526a inhibited WNV infection, though not to the extent of the miR-526b inhibition. The high throughput screen for antiviral miRNAs that identified miR-526b also suggested antiviral activity by several other members of the miR-515 family: miR-517a, -517b, and -519e. However, miR-526b shares no seed sequence conservation and only about 20% sequence similarity with each of these miRNAs, suggesting that the mRNA target sites bound by miR-526b are not shared with miR-517a, -517b, and -519e, and the antiviral effect of these miRNAs may occur through regulation of different cellular targets.

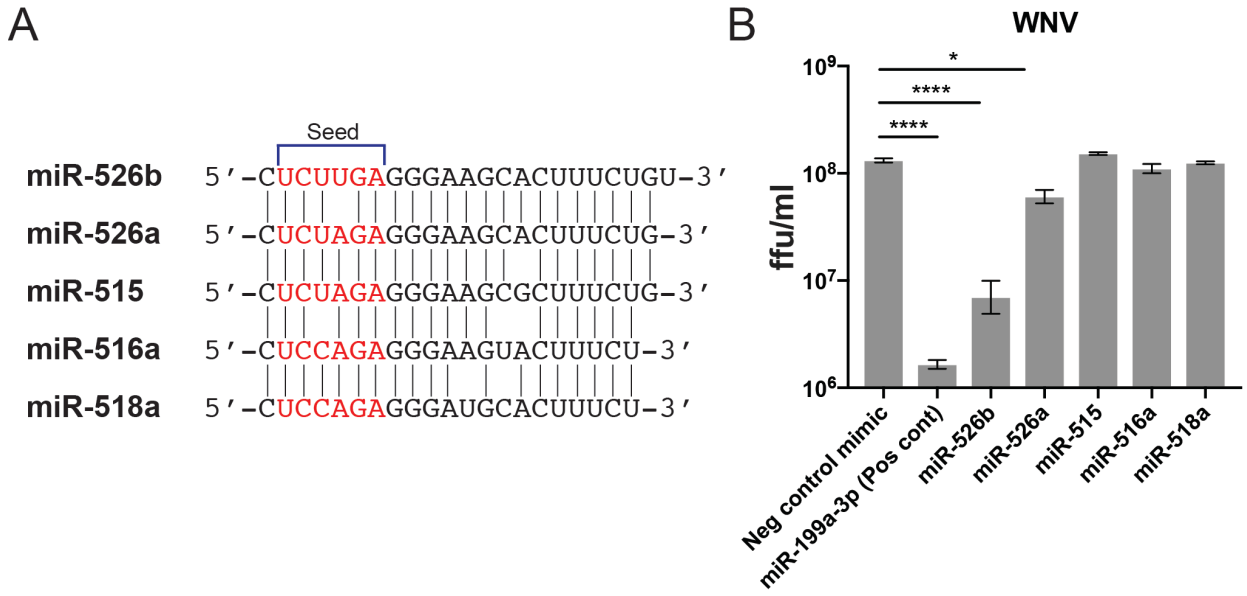


Figure 3.2 Inhibition of WNV is not conserved across the miR-515 family

A) Alignment of five members of the miR-515 family. Seed sequences are indicated in red. **B)** Cells were transfected with the indicated miRNA mimic or control and infected with WNV (MOI=3 ffu/cell) 2 d post-transfection. Supernatants were collected 2 d p.i. and titered on Vero cells. Significance determined by one-way ANOVA with Bonferroni's test for multiple comparisons. Adjusted p-values reported (* p-value<0.05, ** p-value<0.01, *** p-value<0.001).

3.3.3 miR-526b strongly induces innate immunity but not in the context of flavivirus infection

Although miR-526a did not inhibit WNV infection to the same extent as miR-526b, miR-526a has been shown to have an strong antiviral effect on macrophage infection by enterovirus 71 through prolongation of innate immune signaling[424]. To determine if miR-526b has a similar enhancing effect on innate immunity, gene expression after innate immune stimulation was examined in the presence of miR-526b. The synthetic dsRNA analog polyinosinic:polycytidylic acid or poly(I:C) activates the TLR3 signaling cascade, resulting in IFN1 and ISG expression as well as NF-κB-activation. To measure the effect of miR-526b on poly(I:C)-induced innate immunity, miR-526b or the control mimic transfected cells were incubated overnight in media containing poly(I:C). Total RNA was isolated, and qPCR used to measure expression of NF-κB-stimulated genes IL-8 and TNFα (Figure 3.3A,B) and IFN-stimulated genes IFIT1 and IFIT2 (Figure 3.3C,D). In both poly(I:C)-treated and untreated cells, innate immune gene expression was higher in the miR-526b transfected cells than the untransfected. However, the effect was much more pronounced in the cells exposed to poly(I:C). Similar results were observed during infection with the murine paramyxovirus Sendai (unpublished data), suggesting that miR-526b strongly enhances the innate immune response to some stimuli.

I next investigated the effects of miR-526b on the innate immune response to flavivirus infection. Cells transfected with miR-526b or a control were infected with WNV, and 24 h p.i. total RNA was collected. In contrast to the results in poly(I:C)-treated cells, IL-8, TNFα, IFIT1, and IFIT2 expression showed little difference between control cells and miR-526b expressing cells (Figure 3.4A). While infection activates the IFN1 and NF-κB responses as demonstrated by

upregulation of downstream genes, the enhancing effect of miR-526b is absent. In contrast to the murine Sendai virus, flaviviruses have a variety of methods for impeding the antiviral response of human cells, including blocking signaling pathways and cleaving or degrading receptors and signaling molecules[425]. One or more of these mechanisms may serve to block the enhancement of signaling that otherwise results from miR-526b expression.

Although flaviviruses appear to escape miR-526b-mediated immune enhancement, miR-526b nonetheless exerts an inhibitory effect on viral infection (Figure 3.1B). To confirm that the effect of miR-526b on infection is not dependent on the IFN1 transcription factor IRF3, a deficient HeLa cell line (ΔIRF3) was produced using the CRISPR/Cas9 system as described previously[345]. IRF3-deficient cells were transfected with the miR-526b mimic, the control, or an siRNA directed against the DENV2 genome. 24 h post-transfection, cells were infected with DENV2, and supernatants collected. Viral titers showed that IRF3-deficient cells expressing miR-526b produced 10-fold less infectious virus than the control transfected cells (Figure 3.4B), comparable to the inhibition of infection in wild type cells (Figure 3.1B). The IRF3-independent inhibition of flavivirus infection by miR-526b further supports a mechanism of inhibition distinct from the enhanced innate immune signaling observed when cells were exposed to alternative stimulators of immunity.

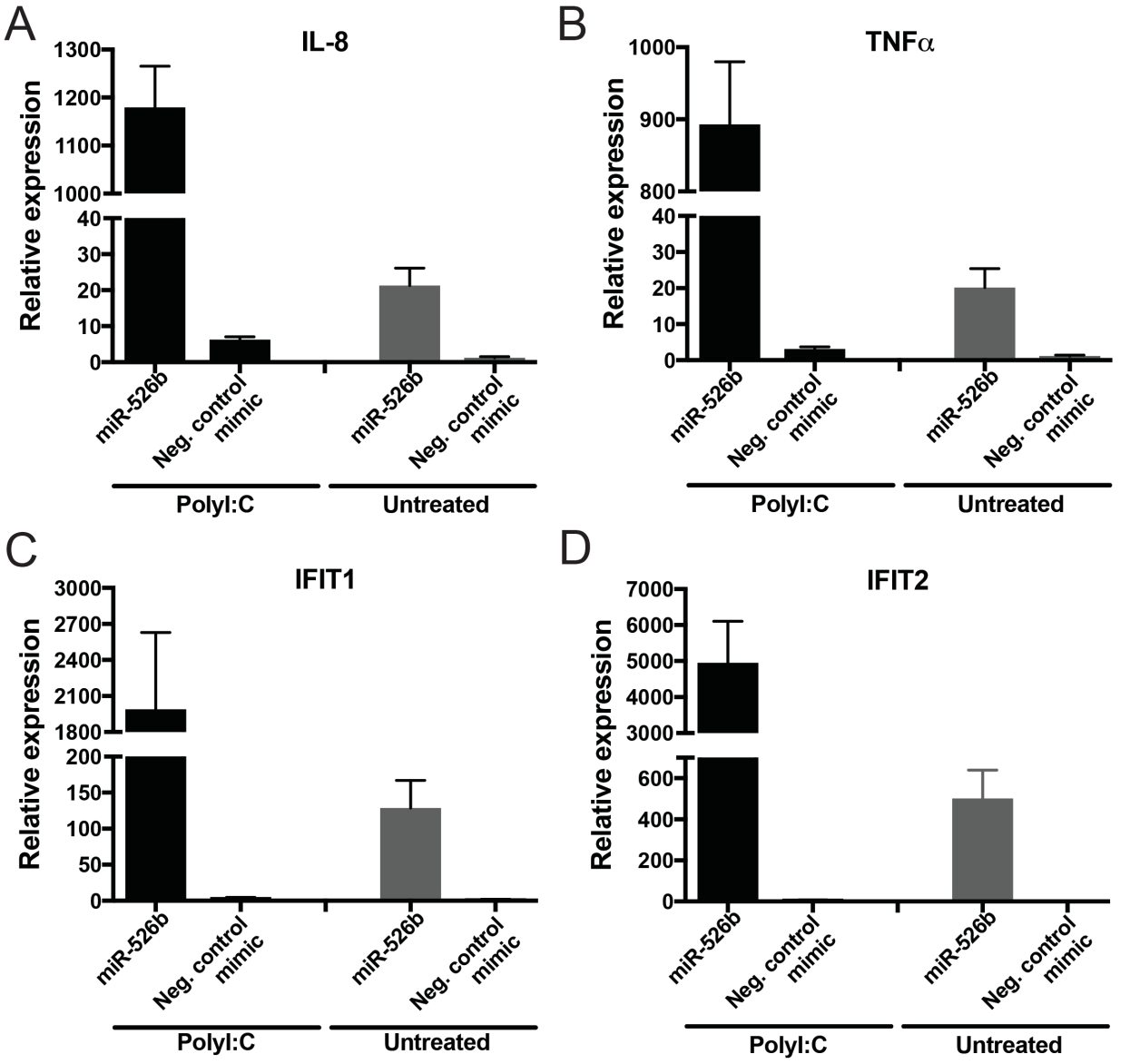


Figure 3.3 miR-526b greatly enhances IFN1 and NF-κB signaling in poly(I:C)-stimulated cells

Cells were transfected with the miR-526b duplex mimic or control mimic for 2 d, then refed with medium containing 5μg/ml poly(I:C) overnight. Total RNA was collected in Trizol, and relative gene expression for **A)** IL-8, **B)** TNFα, **C)** IFIT1, and **D)** IFIT2 determined by qPCR, normalized to β-actin.

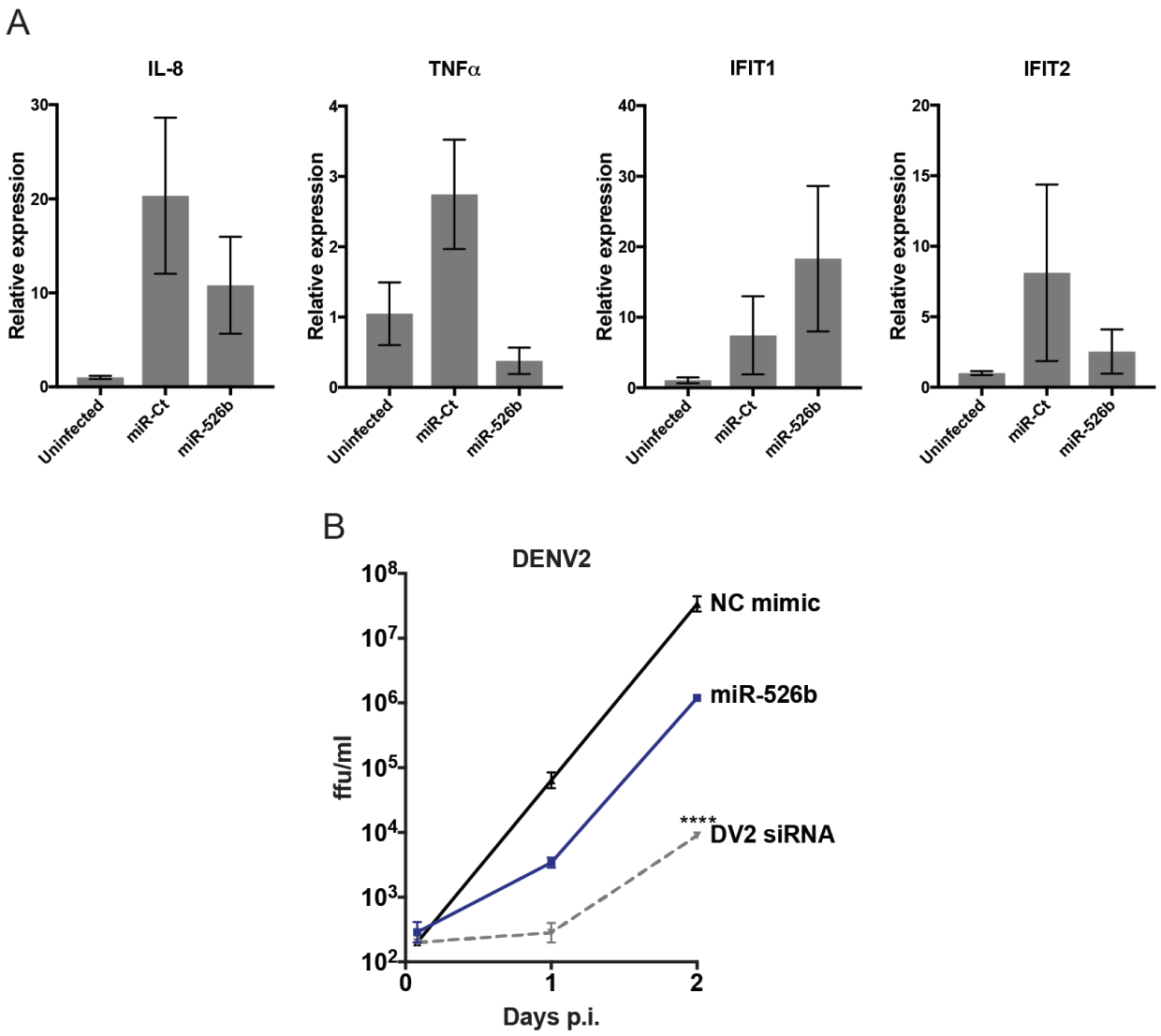


Figure 3.4 miR-526b does not enhance innate immunity during flavivirus infection

A) Cells were transfected with the miR-526b mimic or control for 2 d, then infected with WNV (MOI=3 ffu/cell). Total RNA was collected in Trizol 24 h p.i. Relative gene expression was determined by qPCR and normalized to β-actin. **B)** IRF3-deficient HeLa cells were produced using the CRISPR/Cas9 system. 2 d post-transfection with the miR-526b mimic, control, or the DENV genome siRNA, cells were infected with DENV2 (MOI=5 ffu/cell). Supernatants were collected at 2 h, 1 d, and 2 d p.i. and titered on Vero cells. Significance determined by one-way ANOVA with Bonferroni's test for multiple comparisons. Adjusted p-values reported (* p-value<0.05, ** p-value<0.01, *** p-value<0.001).

3.3.4 miR-526b inhibitions DENV2 genome replication in a time-dependent manner

Although miR-526b strongly enhances innate immune signaling in response to some stimuli, flavivirus infection does not appear to be susceptible to these effects; therefore, I turned to other pathways required for flavivirus replication that could potentially be regulated by miR-526b expression. To determine if miR-526b restricts genome replication, miR-526b-transfected cells were infected with DENV2, and total RNA collected 18 h and 24 h p.i. to ensure measurements were of a single round of infection. Viral genomes were detected using qPCR and a primer-probe set that amplifies the DENV 3' UTR. At 18 h p.i., no difference in genome copy number was detected between miR-526b transfected and control cells (Figure 3.5). In contrast, at 24 h p.i., a five-fold reduction in genome production was detected in cells transfected with miR-526b. These results suggest that miR-526b may regulate cellular factors important to replication of the viral genome.

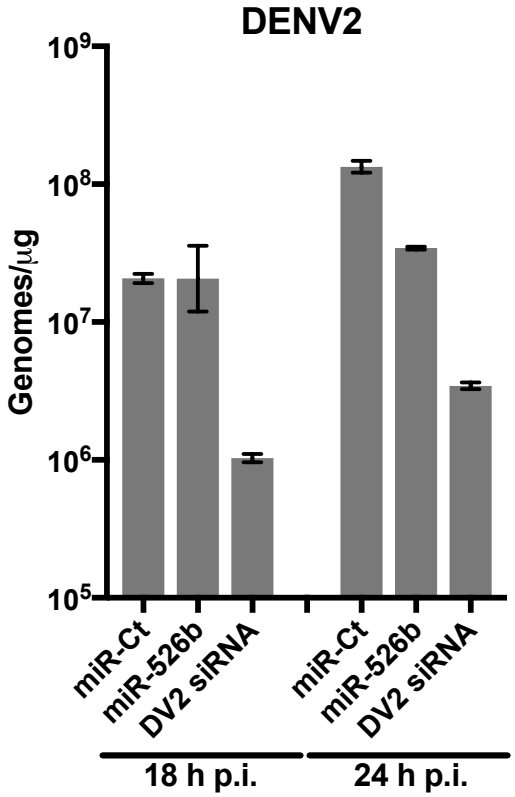


Figure 3.5 miR-526b does not impede DENV2 genome replication early in infection

Cells transfected with the miR-526b mimic, control mimic, or DENV genome siRNA were infected with DENV2 (MOI=10 ffu/cell). Total RNA was collected at 18 h and 24 h post-infection and genome equivalents determined by qPCR using a primer-probe set amplifying the DENV 3' UTR.

3.4 Discussion

In this chapter, I present results of experiments examining the inhibition of flavivirus infection by the cellular miRNA miR-526b. Interestingly, although the presence of miR-526b in cells treated with the TLR3 agonist poly(I:C) or infected with the murine Sendai virus enhanced the expression of NF-κB- and IFN-stimulated genes, infection with WNV did not result in greater expression of these genes in cells transfected miR-526b prior to infection. Both DENV and WNV disrupt or impede the antiviral immune response of the cell through a variety of mechanisms during infection. The NS3 proteins of both viruses competitively bind 14-3-3ε, a crucial RIG-I chaperone protein, and inhibit RIG-I signaling by preventing relocation of RIG-I to the mitochondria[426]. Likewise, WNV and DENV NS4B, as well as DENV NS2A, inhibit auto-phosphorylation-dependent activation of TANK binding kinase 1 (TBK1) and along with DENV NS2B/3 binding and inhibition of IKKε, block activation of transcription factors such as IRF3[427,428]. Furthermore, type 1 IFN signaling during DENV and WNV infection is restricted by the NS5 proteins of both viruses, albeit via differing mechanisms[190,191,429,430]. Additional anti-immune functions have been traced to nonstructural and structural proteins of both viruses[253,254,431,432]. The innate immune enhancement observed in miR-526b transfected cells treated with poly(I:C) may be counteracted by one or a combination of these flaviviral immune restriction mechanisms, resulting in the unaltered immune gene induction during WNV infection and IRF3-independent inhibition of DENV2. As miR-526b expression inhibited several other viruses of different families, all with alternative modes of avoiding or inhibiting the antiviral response of the infected cell, an examination of IFN- and NF-κB-activated gene expression

during infection with these viruses may provide further insight into the immune modulatory functions of miR-526b [433-436].

Having observed that miR-526b inhibits flavivirus replication in spite of not contributing to an enhanced immune response by the infected cell, I examined the effect of miR-526b on geneome copy number of the replicating virus, noting a time dependent reduction in viral replication in cells transfected with miR-526b. Replication of the flavivirus genome takes place at the cytoplasmic surface of the ER and requires extensive remodeling of the ER membrane[260]. Following several rounds of translation of the infecting genome, replication complexes are assembled adjacent to the ER membrane and induce invaginations called vescicle pockets. These membranous pockets house the replication complex where new copies of the viral genome are produced. As positive-sense RNA viruses, the flaviviruses must first produce negative-sense copies of the viral genome to serve as the template for synthesis of the positive-sense strand, although the signal that initiates the transition from negative-sense to positive-sense genome production is unknown[213]. The techniques used to probe genome copy number discussed in this chapter do not differentiate between positive- and negative-strand synthesis. However, by using Northern blotting to examine RNA collected from infected cells at the appropriate time points, whether miR-526b specifically inhibits synthesis of either strand of the viral genome could be determined. The host factors associated with synthesis of the flavivirus genome are not well-defined and determining the role of miR-526b in the regulation of one or both of these processes may provide insight into the host cell's contribution to viral genome synthesis.

The transition from negative-strand to positive-strand RNA synthesis of the flavivirus genome has been proposed to be coupled with progressive restructuring of the ER membrane[213].

A group of host proteins associated with ER membrane restructuring have been previously identified as required for flavivirus infection through knockout screens using CRISPR or siRNA[202,437-439]. The 3' UTR of several of these ER resident proteins contain miR-526b seed sequence matches and are among the predicted targets of miR-526b produced by TargetScan analysis. Regulation of ER restructuring by miR-526b may restrict replication of either strand of the viral genome, resulting in the time-dependent reduction in genome copy number I observed.

Inhibition of genome replication may not occur directly through miR-526b restriction of ER membrane restructuring and vesicle pocket formation, however. TargetScan prediction of miR-526b binding sites also suggests host genes associated with protein translation, translocation, and glycosylation as well as induction of the unfolded protein response and resource allocation to the ER. Failure to properly fold or localize viral proteins may prevent the formation of functional replication complexes and reduce the infected cell's capacity for viral genome replication. Similarly, miR-526b inhibition of the IRE1α arm of the UPR, an essential pathway for flavivirus infection, may result in failure to restore EIF2α-independent protein translation or to maintain sufficient phospholipid availability in the vicinity of viral replication and assembly[262].

To examine the role of these predicted miR-526b targets during infection, the link between miRNA, targets, and virus must be established. In order to determine if miR-526b targets the transcripts of ER proteins, the 3' UTR of each putative target gene—containing the predicted miR-526b binding site or sites—will be cloned into a reporter plasmid expressing luciferase. Inhibition of luciferase expression by miR-526b would indicate binding of miR-526b to the predicted binding site and miR-526b-mediated repression of luciferase translation.

Mutation of the binding site and restoration of luciferase expression would further confirm the interaction between the target 3' UTR and the miRNA. Reduction of the target mRNA by miR-526b, particularly in the context of viral infection, would demonstrate that the 3' UTR binding site is functional and that miR-526b is responsible for regulating expression of the transcript in the cell. Inhibition of translation of the target of interest, usually by siRNA, followed by infection will determine if the target is required for viral infection. Although inhibition of a single miRNA target may not entirely recapitulate the antiviral effect of the miR-526b—owing to the promiscuous nature of miRNA targetting—a reduction in viral titer would serve to indicate the target is of importance to viral infection. Further characterisation of the function of a given target in the life cycle of the virus can then be conducted. In this manner, miRNAs that regulate cellular gene expression can serve as a unique tool for identifying proviral factors.

3.4 Materials and methods

Cell culture

HeLa (ATCC) and HEK293 (Microbix) cells were grown in Dulbecco modified Eagle medium (Thermo Fisher) or modified Eagle medium (Thermo Fisher), respectively. Media was supplemented with 5% fetal bovine serum (FBS; HyClone), 2 mM L-glutamine (Thermo Fisher), 100 U penicillin G sodium/ml, and 100µg of streptomycin sulfate/ml.

Virus strains

DENV2 (New Guinea C) was obtained from ATCC. WNV (385-99) has been previously described [415]. DENV2 and WNV were passaged twice on C6/36 insect cells and purified by centrifugation as previously described [416]. HSV 1 (F1 strain) was a gift from A. Hill of Oregon Health and Science University. VacV (Western Reserve strain) was a gift from M. Slifka of Oregon Health and Science University. CHIKV (MH56) and VEEV (TC83) was a gift of D. Streblow of Oregon Health and Science University. VSV-GFP was a gift of V. DeFillipis of Oregon Health and Science University.

Growth curves

DENV2 and WNV virus titers were determined by focus forming assay. Foci were resolved using the anti-flavivirus envelope antibody 4G2 (hybridoma purchased from ATCC and maintained by the Vaccine and Gene Therapy Institute monoclonal antibody core facility (OHSU)), anti-mouse IgG-horseradish peroxidase (Santa Cruz Biotech), and the Vector VIP peroxidase substrate (Vector labs) as directed by the manufacturer. CHIKV, HSV, and VacV

titers were determined by plaque forming assay. VSV-GFP titers were determined by fluorescent plaque.

miRNA and siRNA transfections

miRIDIAN miRNA mimics and controls (Dharmacon) and siRNAs (Thermo Fisher) were transfected using Lipofectamine RNAiMAX reagents (Thermo Fisher) according to manufacturer protocols.

Immunofluorescence

Infected cells were fixed, permeablilized, and probed with the 4G2 antibody and goat α-mouse Alexa Fluor 488 (Thermo Fisher). 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher) was used to stain cell nuclei.

qPCR

Total RNA was reverse transcribed and amplified using Taqman gene expression probes (Thermo Fisher) and Taqman RNA-to-CT 1-Step reagents (Applied Biosystems) on a StepOne Plus real-time PCR instrument (Applied Biosystems) based on manufacturer's recommendations. Relative transcript expression was calculated using ΔCt with actin as the endogenous control transcript. The Taqman gene expression probes used were: IL-8 (Hs00174103_m1), TNFα (Hs01113624_g1), IFIT1 (Hs01911452_s1), IFIT2 (Hs00533665_m1), and actin (Hs99999903_m1) (Thermo Fisher). DENV genome equivalents were detected using a primer probe set specific for the DENV 3' UTR.

General statistical analysis

Experiments were performed at least three times unless otherwise indicated in the figure legends. Data are presented as mean ± standard error of the mean (SEM). For viral growth curves containing more than one time point or treatment, a one-way ANOVA analysis was conducted with Bonferroni's correction for post hoc multiple comparisons. Student *t* test was used for single time point viral titers containing only two sets. A p-value < 0.05 was considered significant.

Chapter 4: Discussion and future directions

Flaviviruses are responsible for significant morbidity and mortality worldwide[440]. In spite of the health risk that infection with these viruses can pose, much remains to be learned about the interactions between the host and the virus. Flavivirus infection must balance the needs of the virus to consume cellular resources and co-opt cellular pathways with preventing or hiding from attempts by the cell to clear the infection or undergo apoptosis. We have demonstrated the utility of small regulatory RNAs for demarcating points of interaction between the virus and host cell[344,345]. The promiscuous but predictable targeting of cellular gene transcripts by miRNAs provides a platform for examining the gene expression essential for flavivirus infection, even if the miRNA itself is not a factor during infection. Our pipeline for identifying cellular factors the virus requires to maximize viral procreation and minimize the cellular antiviral response is as follows: 1) Identify miRNAs that positively or negatively affect flavivirus infection—often by screening large miRNA libraries[345] or miRNA expression profiles during infection[344]. 2) Produce a dataset of genes potentially regulated by the miRNA of interest using a combination of target prediction software, sequencing of transcripts bound to the RISC-miRNA of interest, and literature review of past studies. 3) Functionally confirm one or more targets of the miRNA of interest. 4) Characterize the role of the target or targets during flavivirus infection. In this thesis, I discuss two projects conducted using this system. The first, examining miR-424, revealed an additional immune regulatory role for the UPR during DENV2 infection, and the second demonstrated that although flaviviruses are not vulnerable to the immune enhancing effects of miR-526b, the miRNA nonetheless restricts viral infection. The two miRNAs were chosen for

study from a large screen of human cellular miRNAs exogenously expressed in cells subsequently infected with DENV2, WNV, or JEV[345]. In the screen, both miR-424 and miR-526b were found to reduce E protein expression by minimum 70% in at least two infections, but neither had previously been studied in the context of flavivirus infection.

4.1 miR-424 inhibition of UPR-mediated MyD88 degradation

Dengue virus infection of host cells and production of viral progeny relies heavily on the unfolded protein response, absence of which severely inhibits DENV replication[262]. Peña and Harris demonstrated that DENV2 induces the three arms of the unfolded protein response in a precise and time-dependent manner: the PERK pathway transiently early in infection, the IRE1-XBP1 pathway midway through the virus life cycle as genome replication and protein translation escalate, and the ATF6 pathway late and dispensibly[262]. Although the UPR functions as a crossroads between prosurvival and proapoptotic mechanisms of the cell, Peña and Harris showed that DENV2 infection drives expression of prosurvival factors, restores eIF2α-inhibited protein translation, and manages lipid redistribution. In addition, the UPR is closely tied to innate immunity in the cell—triggered by TLR activation and able to induce cytokine transcription[275-279]. However, just as the UPR manages both pro- and anti-apoptotic signaling, based on our work, the UPR also appears able to both positively and negatively regulate innate immunity.

As described in Chapter 2, I identified an anti-flaviviral miRNA, miR-424, that inhibits the translation of a cellular transcript, SIAH1. Transcription of this E3 Ub ligase is mediated by XBP1s, and I found that upregulation of SIAH1 gene expression coincided with the timing of IRE1-XBP1 activation by DENV2 infection observed by Peña and Harris[262,376]. SIAH1 has been linked to modulation and maintenance of the UPR, promoting apoptosis as well as ubiquitinating several proteins capable of NF-κB activation[376,441,442]. Observing that knockdown of SIAH1 using siRNA resulted in increased IFN1 and NF-κB signaling, I examined the possibility that SIAH1 regulates degradation of MyD88, as suggested by immunoprecipitation and yeast two-hybrid screens conducted independently by Wang, et al. and De Arras, et al[371,372]. I demonstrated

that SIAH1 binds and ubiquitinates MyD88 and that in the absence of SIAH1, both MyD88 and downstream cytokines are more highly expressed in DENV2 infection. Use of the small molecule ER stressor thapsigargin yielded the same results, suggesting that SIAH1 upregulation by the UPR is a negative regulator of innate immune signaling (Figure 4.1). The immune inhibitory function of SIAH1 may serve as a negative feedback loop, shutting down UPR-associated cytokine expression. These results suggest a novel mechanism by with DENV coopts the UPR to favor viral replication.

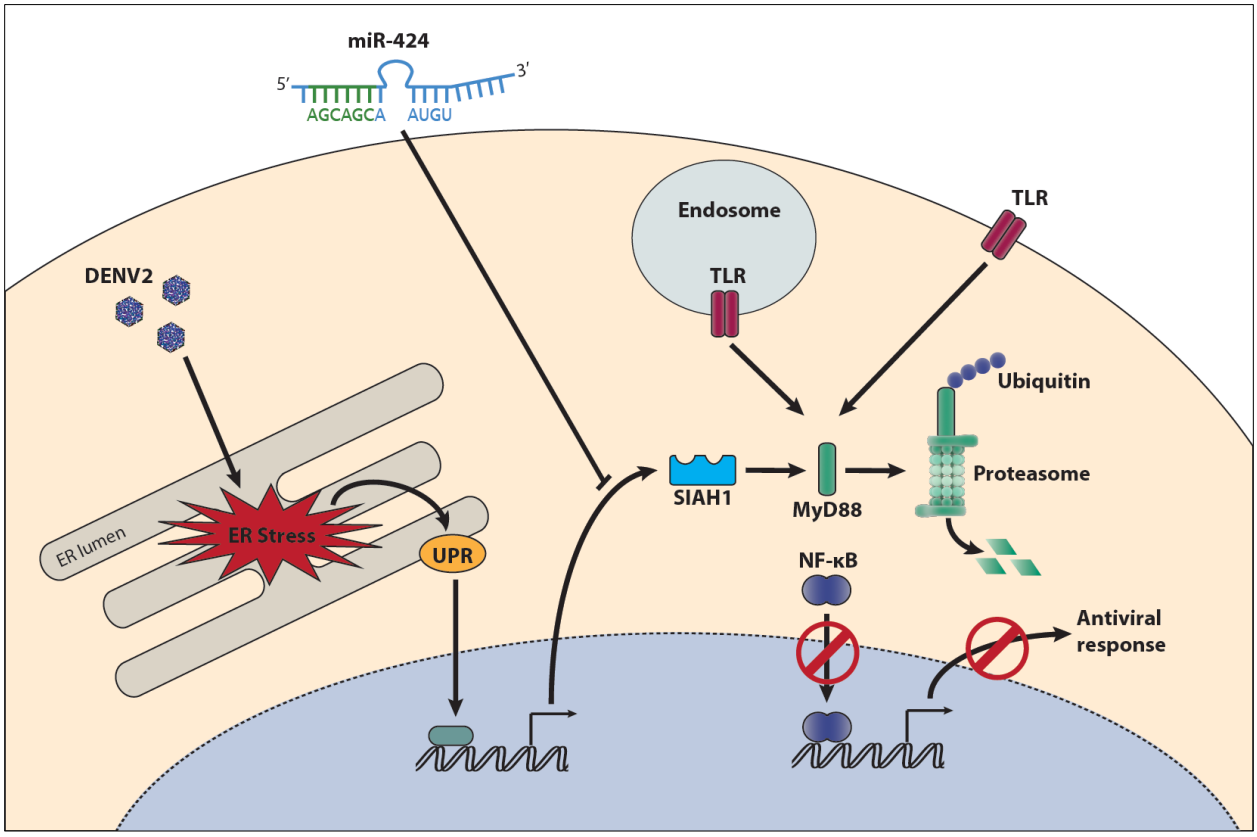


Figure 4.1 Model of SIAH1 induction and effect during DENV2 infection

DENV2 infection induces the UPR via IRE1/XBP1, resulting in SIAH1 transcription. miR-424 represses SIAH1 translation. SIAH1 ubiquitinates MyD88, resulting in degradation of MyD88 and reduces antiviral immune signaling.

Post-transcriptional regulation of SIAH1 by miR-424 was first described by Imig, et al[369]. However, this study is the first to examine the effects miR-424 and SIAH1 during flavivirus infection. Although the HeLa and HEK293 cell lines used in this study do not appear to express miR-424 constitutively or during flavivirus infection, cells vulnerable to DENV infection *in vivo*, such as endothelial cells and monocytes, are reported to express miR-424[408]. Furthermore, work by Gupta, et al. reveals PERK-mediated downregulation of miR-424 following UPR induction by thapsigargin in mouse fibroblasts[407]. These findings suggest that the antiviral activity of miR-424 may be mitigated early in DENV infection by the transient activity of the PERK pathway, allowing the unimpeded expression of SIAH1 following the mid-infection induction of the IRE1α pathway. To investigate this scenario, the expression profiles of miR-424 and SIAH1 as well as markers of both PERK and IRE1α pathway activation would be tracked over the course of DENV infection in primary human cells such as human foreskin fibroblasts. Together, the results of this experiment would map the proposed correlation between temporal induction of PERK and IRE1α by DENV infection and the respective inhibition and expression of miR-424 and SIAH1, as well as the subsequent effects on downstream innate immune signaling. Using the CRISPR/Cas9 system to produce of stable cell lines deficient in key aspects of these pathways, such as PERK, IRE1α, or MyD88, would further assist in establishing UPR-mediated innate immune expression during DENV infection. The use of primary cells that constitutively express miR-424 would provide the opportunity not only to examine the relationship between DENV2 and endogenous miR-424 but also the innate immune reaction of cells physiologically relevant to DENV2 infection of a human host, including autocrine and paracrine signaling, which may be absent in immortalized cell lines derived from tissue not typically subject to DENV2 infection.

The study discussed in Chapter 2 demonstrated that SIAH1 activity results in the reduction of the MyD88-mediated innate immune response to DENV infection; however, the interaction between SIAH1 and MyD88 and the resulting addition of a poly-ubiquitin chain to MyD88 may be further probed by disrupting the interactions and functions of the two proteins. SIAH1 is composed of a C-terminal substrate-binding domain (SBD), two Zinc finger motifs, and an N-terminal RING-finger domain to which the ubiquitin-bearing E2 ligase binds[443]. Amino acid substitution has been used to identify the molecular interactions required for SIAH1 SBD binding to substrate proteins[441,444]; however, the SBD consists of nearly 200 amino acids and may require extensive individual or combined mutations to fully disrupt MyD88 binding[443]. Alternatively, a small peptide, referred to as PHYL, derived from the *Drosophila* protein phyllopod, has been shown to interact with the SIAH1 SBD and when a plasmid coding for this fragment is transfected into cells, prevents binding of other SIAH1 target substrates[445]. Introduction of a plasmid encoding PHYL into cells should inhibit MyD88 binding to SIAH1, decreasing ubiquitination and proteasomal degradation of MyD88. An inactive SIAH1 mutant containing two serine substitutions in the RING finger domain fails to bind ubiquitin-bearing E2 ligases and therefore, cannot ligate ubiquitin to bound substrate proteins[446]. Co-expression of MyD88 and the inactive SIAH1 should also result in reduced ubiquitination of MyD88, although co-immunoprecipitation should still reveal interaction between the two proteins.

Determining the residues of MyD88 to which SIAH1 binds presents similar obstacles as mutating individual amino acids in the SIAH1 SBD, although the general region of binding may be determined by co-immunoprecipitating SIAH1 with truncated fragments of the MyD88 protein, rather than individual and combinations of single amino acid substitutions. Removing

the ubiquitination sites on MyD88, however, presents a less daunting task. Poly-ubiquitination signaling for proteasomeal degradation typically occurs at a lysine residue; MyD88 contains 15 lysine amino acids that can be replaced with alanines to curtail the isopeptide binding of the C-terminus of ubiquitin to the substrate lysine[447]. Substitution of a lysine on MyD88 and subsequent loss of SIAH1-mediated ubiquitination would further support the functional interaction between MyD88 and SIAH1 that I demonstrated in Chapter 2.

While the expression of miR-424 and siRNA knockdown of SIAH1 inhibited DENV2 infection (Figure 2.3.2B), replication of the viral genome was not affected (Figure 2.3.2C), suggesting that the disruption of the viral life cycle resulting from repressed SIAH1 translation and enhanced MyD88 expression occurs at a stage downstream of RNA synthesis. The positive-stranded RNA genome of DENV2 serves both as genome to be packaged into progeny virions as well as the mRNA for translation of viral protein[3]. When cells infected with DENV2 for three days were immunofluorescently probed for the DENV2 envelope protein, cells transfected with miR-424 prior to infection contained less viral protein than control cells (Figure 2.3.2A); to determine if the increased expression of MyD88 in the absence of SIAH1 impairs translation of DENV proteins, cells can be infected a DENV containing a luciferase gene and luciferase expression examined at time points over the course of infection[448,449]. Reduction in luciferase expression in the presence of miR-424, a SIAH1 siRNA, or overexpression of MyD88 would indicate that DENV requires the SIAH1-mediated degradation of MyD88 for translation of viral proteins. Alterations in the expression of individual viral proteins during infection can be tracked by western blot and could indicate failure of cellular or viral proteases to cleave the viral polyprotein or other steps in protein maturation such as glycosylation of prM/M, E, and NS1[450].

Viral protein localization post-maturation may also play a role in the MyD88-mediated inhibition of DENV infection; C and NS5 can be found in the nucleus as well as cytoplasm, and hexameric NS1 is secreted from the cell[136,148,193]. Western blotting of nuclear, cytoplasmic, and extracellular fractions for these viral proteins may reveal MyD88-signaling mediated failure to localize to the appropriate subcellular compartment or rapid degradation of the viral proteins in those locations. Although the reduction in viral protein indicates inhibition of viral protein translation resulting from reduced MyD88 degradation and the subsequent enhanced innate immune signaling, multiple stages in the viral life cycle post-RNA synthesis, including virion assembly, trafficking, and budding may also be impaired. However, identifying the source of the MyD88-mediated reduction in viral protein expression will provide a point of reference from which to examine known MyD88-mediated pathways of translation regulation.

In addition to inhibiting innate immune signaling through MyD88, SIAH1 activity influences a variety of other pathways that may also play a role during viral infection[451,452]. For example, SIAH1 has been shown to interact with POSH (plenty of SH3s), a multidomain scaffolding protein important for signaling by the c-Jun N-terminal kinase (JNK) stress response pathway[446,453,454]. The JNK pathway can be activated by a variety of stimuli, including DNA damage, oxidative stress, and the IRE1 pathway of the UPR[455-457]. The repercussions of JNK signaling depend on cell type as well as the nature of the pathway stimulus and may be either pro-survival or pro-apoptotic[458]. Infection with DENV induces JNK signaling, possibly through activation of IRE1, and inhibition of JNK strongly inhibits DENV infection[262,459]. Although the role of JNK signaling during DENV infection is not known, possible functions include assisting in redistribution of cholesterol and other lipids in the cell, autophagosome formation, and the

production of anti-apoptotic proteins[262,459,460]. Although the function of the SIAH1-POSH interaction in JNK signaling is unknown, inhibition of SIAH1 strongly reduces JNK signaling, suggesting that DENV induction of SIAH1 may contribute to the JNK signaling required for infection[446]. To investigate if activation of IRE1 by DENV infection results in both SIAH1 expression and JNK signaling, phosphorylation of JNK (indicating activation) can be monitored during infection in the presence of a SIAH1 inhibitor as well as in cells deficient in IRE1 expression. A demonstration of IRE1-mediated JNK signaling activation that also requires IRE1-induced SIAH1 expression would further our understanding of the role of the UPR in DENV infection.

SIAH1 also binds to *S*-nitrosylated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) during times of cellular stress, but rather than resulting in the ubiquitination and degradation of GAPDH, SIAH1 appears to serve as a chaperone for nuclear localization of the two proteins as a result of a nuclear localization signal located in the SBD of SIAH1[461,462]. In the nucleus, the GAPDH-SIAH1 complex appears to regulate gene transcription pathways through trans-nitrosylation by GAPDH or ubiqutination by SIAH1[452,461]. Although overexpression of the complex results in cell death, the targets and downstream effects of nuclear signaling by GADPH-SIAH1 activity are unclear[452,461,463]. Interestingly, GAPDH also binds to the 3' termini of both the positive and negative strands of the JEV RNA genome[464]. GADPH binds preferentially to the minus strand, a phenomenon also observed during infection with a positive-strand RNA genome plant virus, Tomato bushy stunt virus, and is believed to regulate asymmetric RNA synthesis by retaining the negative-strand within the replication complex to serve as the template for positive strand genome synthesis[464,465]. Between 12 and 24 h post-

infection with JEV, GAPDH concentrations in the nucleus increase, correlating with the point in the JEV life cycle when, as with DENV, the IRE1 pathway of the UPR is activated[384,464,466]. Induction of SIAH1 during infection may mediate the translocation of GAPDH to the nucleus observed during JEV infection, and a transcriptome analysis after GAPDH and SIAH1 co-expression may provide clues to the cell's gene expression response to JEV-induced ER stress. In addition, although a decrease in cytosolic GAPDH was not observed during infection regardless of nuclear concentration, even a small shift in available GAPDH may influence the bias of RNA strand synthesis[464]. Although GAPDH association with the RNA genome of other flaviviruses has not been studied, infection with both DENV and HCV is strongly restricted in GAPDH-deficient cells[467]. Inhibition of SIAH1 expression and analysis of the ratio of positive to negative genome copies may hint at a mechanism through which flaviviruses produce sufficient negative strand copies for efficient positive strand production.

Using miR-424 as a tool for identifying cellular factors subverted by flavivirus infection revealed the induction of the E3 ubiquitin ligase SIAH1 by DENV2, which results in the proteasomal degradation of the innate immune signaling molecule MyD88. The unfolded protein response plays a variety of roles in DENV2 infection, including restoration of protein translation through GADD34 inhibition of EIF2α phosphorylation, prosurvival signaling through via CHOP expression, and as demonstrated in Chapter 2, inhibition of innate immune activation through MyD88 degradation[262]. By reducing the MyD88 in the cell available to transduce PRR activation signals, DENV2 defends against the antiviral responses of the host cell and likely reduces the secretion of cytokines responsible for paracrine signaling to neighboring cells. In additional to the other mechanisms of immune evasion utilized by DENV, restriction of innate immune

signaling through MyD88 degradation likely increases the production of DENV progeny within the host cell and the susceptibility of adjacent cells to infection by the virus.

4.2 The innate immunity-independent antiviral effect of miR-526b

Because viral infection requires numerous host resources, the variety of cellular transcripts by which cellular miRNAs inhibit viral infection is vast. Beyond targeting the viral genome or messenger RNA, antiviral miRNAs have been found that restrict infection by downregulating host factors required for viral replication, assembly, and egress, by enhancing the antiviral immune response, and by contributing to apoptosis. Having established the antiviral effects of miR-526b, we sought to determine the mechanism of inhibition. miR-526a—which within the miR-515 family, shares the closest sequence similarity to miR-526b—has been shown to target CYLD, a deubiquitinase responsible for inhibiting NF-κB signaling by preventing degradation of IκB[424]. Based on the innate immune enhancing function of miR-526a and the sequence similarity between miR-526a and -526b, I examined expression of several transcripts representative of ISGs and NF-κB-stimulated genes, all of which were potently induced by miR-526b expression[468,469]. Surprisingly, during WNV infection, miR-526b did not significantly alter expression of any of these genes. In contrast, infection with the murine Sendai virus also showed enhanced innate immune induction in the presence of miR-526b. While flaviviruses inhibit the innate immune response in human cells through both viral proteins and manipulation of cellular genes, the murine Sendai virus cannot, suggesting that the antiviral mechanisms of WNV inhibit miR-526b enhancement of innate immunity. In the interest of investigating the mechanism of miR-526b innate immune enhancement, cells created by the CRISPR/Cas9 system to be deficient in individual innate immune PRRs, signaling adaptors, and immune effectors would be screened for loss of miR-526b enhancement after innate immune stimulation by Sendai virus infection or poly(I:C). Ablation of innate immune enhancement in the presence of miR-526b would aid in the

identification of the innate immune pathway and cellular factors contributing to the effects of miR-526b expression. Furthermore, the mechanisms for innate immune evasion vary extensively between viruses, and the expression of innate immune stimulated genes was not examined in the other virus families inhibited by miR-526b—CHKV, VEEV, HSV, and VacV. An examination of the effect of miR-526b on the immune response during infection of those viruses may also provide guidance toward identifying the role of miR-526b in innate immunity and how flaviviruses, and perhaps other families, avoid the immune enhancement.

In regards to the inhibitory effect of miR-526b on flaviviruses, having demonstrated that the antiviral activity of miR-526b does not derive from innate immune enhancement, other pathways of infection restriction must be considered. miR-526b displays a temporal effect on viral genome replication, suggesting a target associated with the replication process or the ER vicinity where replication, translation, and assembly occur in close proximity. Remodeling of the ER membrane is a requirement for flavivirus replication but the cellular factors involved not only in the restructuring but also membrane composition and lipid transport during infection are not well defined[260,261]. The cellular proteins necessary for genome replication, viral protein translation, folding, and localization, and virion assembly are likewise incompletely understood. A screen for cellular proteins required for WNV infection using the CRISPR/Cas9 system to knockout individual genes identified a set of ER-associated proteins essential for viral replication, including several ER stress regulated protein translocases as wells as proteins mediating glycosylation and cleavage of the flavivirus polyprotein[437]. Interestingly, several of the genes identified in this screen have potential miR-526b binding sites. Inhibition of proper protein translation, folding, or glycosylation would impede viral genome replication as the needs

for additional replication complex proteins could not be met as infection progresses—a possible explanation for the time-dependent inhibition of WNV genome replication we observed.

However, in order to verify that expression of these ER proteins is responsible for the antiviral effect of miR-526b, binding and inhibition of translation of the gene transcripts by miR-526b must be tested. In the event that miR-526b cannot be demonstrated to target and regulate the candidate genes identified by the CRISPR screen that bear putative miR-526b binding sites or that regulation of these transcripts by miR-526b does not appear to be responsible for the reduction in viral titer or genome copy number, additional candidate transcripts can be identified using miRNA target prediction software such as TargetScan or through immunoprecipitation of RISC-miRNA-mRNA complexes and sequencing of the bound transcript. These techniques, such as photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation or PAR-CLIP, identify mRNAs physically bound to the miRNA-RISC, providing a more succinct list of potential miRNA targets producing the phenotype of interest[470]. As with the targets suggested by the CRISPR screen referenced above, confidence in a miRNA target requires functional testing: confirmation of miRNA binding and inhibition as well as recapitulation of the antiviral effect, if possible. The numerous options for identifying, testing, and studying miRNA targets during virus infection reiterates the power of miRNAs as a tool for studying virus-host cell interactions.

Expression of miR-526b and the other miRNAs of the C19MC cluster is restricted to a specific set of cells: placental cells, embryonic stem cells, and some types of cancer[418,420,421]. The C19MC miRNA cluster appears to provide strong protection against viral infection of placental trophoblasts[423]. Primary human trophoblasts appear resistant to infection by a variety of RNA and DNA viruses, including poliovirus, HSV, VSV, vaccinia virus, and human cytomegalovirus

(HCMV)[423]. In addition, placental trophoblasts secrete exosomes containing high levels of C19MC miRNAs that appear to serve an antiviral role in nearby non-trophoblastic cells of the placenta[423,471]. Media collected from primary human trophoblasts in culture confers similar resistance to viral infection to HeLa cells as well as physiologically-relevant primary cells such as uterine endothelial cells and placental fibroblasts[423]. Although autophagy appears to be required for the antiviral activity of the C19MC cluster, neither the mechanism of this inhibition of infection nor the individual gene transcripts regulated by the miRNAs of the C19MC are known[423].

Evidence indicates that infection with the flavivirus ZIKV during pregnancy, particularly during the first trimester, can result in a placental inflammatory response adversely affecting fetal development or transplacental infection of the fetus[472]. Although ZIKV was not one of the viruses examined in the study discussed in Chapter 3, one would predict that, like DENV2 and WNV, ZIKV infection would be restricted in the presence of miR-526b. Investigation into the targets of miR-526b during flavivirus infection may reveal details of the flavivirus life cycle that can be exploited for drug development or disease treatments. Furthermore, inhibition of ZIKV by a member of the C19MC miRNA cluster may suggest that studies of ZIKV infection during pregnancy using a mouse model may benefit from the use of transgenic mice expressing the C19MC cluster, which is found only in primates. Although the influence of the C19MC miRNA cluster has not been examined during ZIKV infection, these facts suggest that ZIKV may not be vulnerable to miR-526b-mediated inhibition. Should DENV and WNV, but not ZIKV, be inhibited by miR-526b, the opportunity to study the differing cellular effects of related viruses

may provide insight into the varying cell and organ tropism and disease states cause by the flaviviruses.

4.3 Cellular miRNAs as a tool for studying virus-host interactions

The human genome encodes thousands of miRNAs, each one potentially regulating expression of hundreds of protein-coding genes. The mRNAs subject to translation-suppression by a given miRNA is based on perfect complementarity between the miRNA seed sequence and mRNA 3' UTR binding site and a variable degree of base pairing to the remainder of the miRNA sequence. The known parameters for miRNA targeting allow for prediction of the set of mRNAs vulnerable to regulation by a particular miRNA, and the interaction can be functionally confirmed by demonstrating miRNA-mRNA binding and restriction of transcript expression. The work presented in this dissertation is grounded in the use of miRNAs as a tool for screening for cellular proteins that produce a given phenotype—in this case, restricting flavivirus infection. After identifying a miRNA that inhibits infection, a trail of breadcrumbs can be followed from miRNA to the cellular transcripts inhibited by miRNA to the role the transcript-coded proteins play in promoting flavivirus infection. In this dissertation, I have demonstrated the efficacy of this pipeline and presented new information regarding the interactions between the host cell and the virus.

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