VIRUS-HOST INTERACTIONS: INVESTIGATING THE INTERFERON RESPONSE ON ZIKA VIRUS REPLICATION AND ZIKA VIRUS HOST DEPENDENCIES ON METABOLISM

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Master of Science (M.Sc.)

Kavita Raniga

Department of Microbiology & Immunology

McGill University, Montreal

December 2018

Acknowledgements

First and foremost, I would like to thank my supervisor Dr Chen Liang for the opportunity to work in his laboratory and supporting me throughout my studies. His willingness to share his scientific knowledge and academically challenge me has been crucial for my scientific development. He has gone beyond the requirements of a supervisor to provide encouragement and advice. It was a real privilege to work with you and I will forever be grateful.

I would like to offer a special thank you to Quinghua Pan for training and patiently guiding me through my research project, thank you for perfecting our assays and being there in times of need. Zhen Wang, thank you for supporting me and troubleshooting, specifically related to cloning - you're a delight to work with!

In addition, I would like to thank all the lab members for providing a welcoming environment. Saina Beitari and Yimeng Wang, you both have been so kind, supportive and fun; I will never forget our morning tea (and coffee) breaks with all our fruitful discussions. Kristin Davis, your company and friendship has made my time at McGill unforgettable. Saipriya Anand, thank you for being such a wonderful friend and for sharing this journey with me.

I would also like to thank my thesis advisory committee members Drs Anne Gatignol and Selena Sagan for always having constructive criticism and excellent feedback. I would like to thank Laure Fossecave for the English to French translation of the abstract.

Finally, I express my sincere gratitude to my Mother and Father. You have been there day after day and believed in me. My growth and success are a direct product of your love, hard work and support.

Preface

This thesis conforms to the McGill University "Preparation of a Thesis" guidelines for thesis preparation and is written in the traditional monograph style. These works will not be used in any other thesis. The thesis is presented in chapter format: Chapter 1 provides the background for this research in the form of a literature review; Chapter 2 introduces the rational, hypotheses and the specific objectives of this research; Chapter 3 describes the methods and materials used in the experimental design; Chapter 4 presents the main results of the investigations and the corresponding figures; and Chapter 5 discusses the findings of the research and concludes the thesis. My supervisor Dr. Chen Liang provided editorial support in the preparation of this thesis.

The manuscripts adopted for the thesis are listed below:

Yi, D.R., An, N., Liu, Z.L., Xu, F.W., **Raniga, K.**, Li, Q.J., Zhou, R., Wang, J., Zhang, Y.X., Zhou, J.M. and Zhang, L.L., 2018. Human MxB inhibits the replication of HCV. Journal of Virology, pp.JVI-01285. DOI: 10.1128/JVI.01285-18

Raniga K, Liang C. Interferons: Reprogramming the Metabolic Network against Viral Infection. *Viruses* 2018, 10 36. DOI: 10.3390/v10010036

Table of Contents

List of Abbreviations

Abbreviation	Full Term
Ad5	Adenovirus 5
ATP	Adenosine Triphosphate
BST2	Bone marrow stromal antigen 2
CO2	Carbon dioxide
CsA	Cyclosporin A
CypA	Cyclophilin A
CZV	Congenital Zika Syndrome
DENV	Dengue virus
DMEM	Dulbecco’s modified eagle medium
ECL	Enhanced chemiluminescence substrate
EMEM	Eagle's minimum essential medium
FBS	Fetal bovine serum
GTPases	Guanosine-5'-triphosphate enzyme
HBV	hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV-1	human immunodeficiency virus type-1
hNPCs	human Neural Progenitor Cells
HPV	Human papillomavirus
IAV	Influenza A virus
IFI6	Interferon Alpha-Inducible Protein 6
IFITMs	interferon-induced transmembrane proteins
IFNAR	interferon-α/β receptor
IFNs	Interferons
IgM	Immunoglobulin M
ISGs	Interferon stimulated genes

JAK-STAT	Janus kinase - signal transducer and activator of transcription
JEV	Japanese Encephalitis Virus
MAVS	mitochondrial antiviral signalling adapter
MB21D1	Cyclic GMP-AMP Synthase
MOI	Multiplicity of infection
mRNA	Messenger RNA
mTOR	mechanistic target of rapamycin
Mx proteins	Myxovirus resistance proteins
NS	Non-structural
OCR	Oxygen consumption rate
P/S	Penicillin/Streptomycin
PCR	Polymerase Chain Reaction
PEI	polyethylenimine
PFU	Plaque forming units
prM	Pre-membrane
RLRs	Rig-like receptors
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBK1	TANK-binding kinase 1
UTR	Untranslated region
vRNA	Viral ribonucleic acid
VSV	Vesicular stomatitis virus
WNV	West Nile Virus
YFV	Yellow Fever Virus
ZAP	Zinc finger CCCH-type antiviral protein 1
ZIKV	Zika Virus

Abstract

The wide-spread outbreak of Zika virus (ZIKV) infection poses a significant threat to global health. A strong link between Zika virus and clinical birth defects, particularly microcephaly, warrants urgent study of the molecular mechanisms underlying ZIKV pathogenesis. Virus-host interactions determine the outcome of infection, thus we delved into the innate immune response against ZIKV infection and evaluated ZIKV dependency on host metabolism. Type 1 interferons (IFNs) are critical for host resistance against ZIKV. The first part of this study evaluates the effect of interferon and interferon-stimulated genes (ISGs) against ZIKV infection of Asian lineages (PLCal_ZV and PRVABC59). We have shown that type-1 IFN strongly inhibits ZIKV replication in human hepatoma Huh-7.5 cells and the ISG, IFITM3 contributes significantly to this inhibition, agreeing with published reports. We have demonstrated that cholesterol accumulation in endosomes does not restrict ZIKV replication, suggesting an alternative mechanism behind IFITM3 mediated ZIKV inhibition. To assess whether other ISGs contribute to restricting ZIKV infection, we tested a panel of ISGs. MxB, an interferon inducible myxovirus resistance protein, has been reported to restrict HIV-1 and herpesvirus infection. Interestingly, while both ZIKV Asian isolates are resistant to MxB, the African isolate MR766 is susceptible to MxB inhibition. We further identify IFI6 as a potent inhibitor of ZIKV replication for which the mechanism behind its antiviral activity remains to be elucidated.

The second part of this study addresses the dependency of ZIKV on host cellular metabolism. We investigated the effect of two ZIKV proteins, NS4A and NS4B on mitochondrial respiration in U87-MG cells, a human primary glioblastoma cell line. Our results show that NS4B significantly decreases mitochondrial respiration, while basal respiration for NS4A expressing cells are comparable to the control.

These findings expand on the role of specific ISGs in restricting ZIKV replication and highlight the need for investigating the underlying mechanisms behind viral restriction. Our results also support a model in which ZIKV infection causes an imbalance in mitochondrial homeostasis by decreasing mitochondrial respiration.

Résumé

L'éclosion répandue de l'infection au virus Zika (ZIKV) menace de façon significative la santé mondiale. L'établissement d'un lien important entre le virus Zika et certaines anomalies congénitales cliniques, et plus particulièrement la microcéphalie, presse l'étude des mécanismes moléculaires responsables de la pathogénicité du ZIKV. Les interactions entre l'hôte et le virus déterminent le cours de l'infection. Ainsi, nous avons étudié la réponse immunitaire innée au ZIKV, et évalué la dépendance du virus au métabolisme de l'hôte. Les interférons (IFNs) de type I sont cruciaux à la résistance de l'hôte contre le virus. La première partie de cette étude détermine l'effet des IFNs et des gènes stimulés par les IFNs (gènes appelés ISG pour *Interferon-Stimulated Genes*) contre l'infection au ZIKV issu de lignées asiatiques (PLCal_ZV et PRVABC59). Nous avons montré que les IFNs de type I empêchent la réplication du virus dans les cellules humaines hépatiques Huh-7.5, et que le gène IFITM3 (gène ISG) contribue significativement à inhiber le virus, ce qui concorde avec d'autres rapports déjà publiés. Nous avons également établi que l'accumulation de cholestérol dans les endosomes ne restreint pas la réplication du ZIKV, ce qui suggère un mécanisme alternatif à l'inhibition du virus induite par le gène IFITM3. Afin d'évaluer si d'autres ISGs participent à la modulation de l'infection au ZIKV, nous avons testé un panel d'ISGs. Il a été démontré que la protéine MxB dont l'expression est induite par interféron, limite l'infection au VIH-1 et à l'herpèsvirus. Étonnamment, alors que les deux lignées asiatiques du ZIKV sont résistantes à cette protéine, l'isolat MR766 d'Afrique ne l'est pas. Nous avons identifié la protéine IFI6 en tant qu'inhibiteur potentiel de la réplication du ZIKV; cependant le mécanisme de son activité antivirale demeure inconnu. La seconde partie de cette étudie aborde la dépendance du ZIKV au métabolisme de la cellule hôte. Nous avons examiné l'effet de NS4A et NS4B, deux protéines du virus, sur la respiration mitochondriale dans les cellules U87-MG, cellules humaines issues de glioblastome. Nos résultats montrent qu'alors que la respiration mitochondriale est significativement diminuée par NS4B, elle demeure inchangée dans les cellules exprimant NS4A comparativement à celle des cellules contrôle. Ces observations élargissent le rôle antiviral de certains ISGs dans la restriction de la réplication du virus Zika, et mettent en exergue le besoin d'en étudier les mécanismes. Nos données renforcent également un model selon lequel l'infection au ZIKV entraîne un déséquilibre dans l'homéostasie mitochondriale en diminuant la respiration mitochondriale.

Chapter 1 Background

This chapter is a **comprehensive literature review** regarding Zika virus and the host innate immune response elicited against ZIKV infection. Sections of this review are adapted from an article published by, Kavita Raniga and Dr Chen Liang (Raniga, K.; Liang, C. Interferons: Reprogramming the Metabolic Network against Viral Infection. *Viruses* **2018**, *10*, 36).

1.1 The Rise of Zika: From Discovery to Outbreak

In recent years, Zika Virus (ZIKV) has gained worldwide attention and emerged as a major global health challenge following outbreaks in the Americas. Despite the recent outbreaks, ZIKV was isolated in 1947 from the blood of sentinel rhesus macaques in the Zika forest region of Uganda [1]. Although serological evidence of ZIKV disease was reported from 1951 onwards, the first documented report of human ZIKV infection was not until 1964 by Simpson [2,3]. With less than 20 cases of human ZIKV infections reported within the first 60 years of discovery, ZIKV received far less attention in the medical literature and remained rather obscure [4]. This situation changed in 2007, when the virus was introduced into a naïve population on Yap Island, Federated States of Micronesia, where over 73% of the population became infected in a period of 4 months [5]. However, the epidemic subsided for a period before a new ZIKV strain emerged in French Polynesia responsible for a larger epidemic in 2013 [6,7]. Consequently, ZIKV has continued to spread its geographical range, expanding from equatorial Africa and Asia, further afield to South America, Central America, Mexico and the Caribbean including, Puerto Rico, **Figure (1)** [8]. Intriguingly, through sequencing over 100 ZIKV genomes, three collaborative studies showed the emergence of ZIKV in Brazil from the Pacific Islands and noted local transmission between 2013 and 2014, well before the first reported case in Brazil [8-10].

The majority of ZIKV symptoms vary from asymptomatic to a self-limiting febrile illness clinically resembling dengue fever. However, unlike other members of the *flavivirus* genus, ZIKV is a tetragon, epidemiologically linked to the occurrence of severe congenital malformations and neurological complications. Consequently, there is an urgent need to fully understand how ZIKV affects cellular processes to influence pathogenesis and disease.

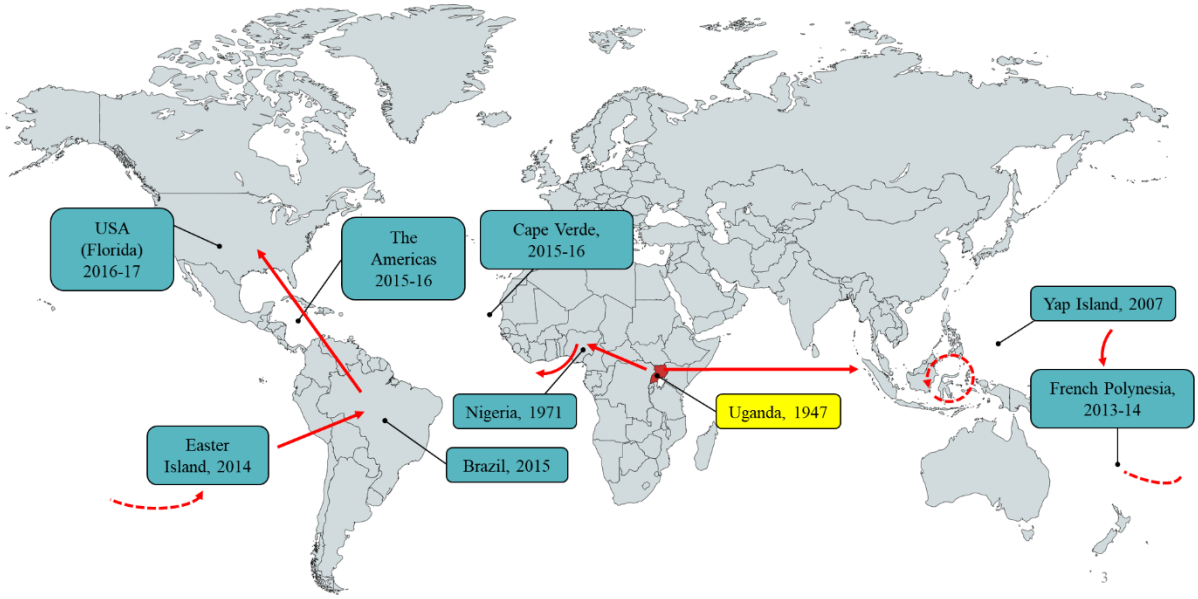

Figure 1 The emergence and global spread of ZIKV. ZIKV was first documented in Uganda with cases reported in Nigeria. Gradual expansion from equatorial Africa resulted in epidemics in Micronesia (2007), French Polynesia (2013), and Latin America (2015-2016). To date, a total of 86 countries and territories have reported evidence of mosquito-transmitted Zika infection.

1.2 Zika Virus Phylogenetics

ZIKV, an emerging arbovirus, belongs to the genus *Flavivirus* within the family *Flaviviridae*, which includes several pathogenic viruses such as Dengue virus (DENV), Japanese Encephalitis virus (JEV), Yellow fever virus (YEV) and West Nile virus (WNV). Multiple phylogenetic analyses of ZIKV genomes have revealed the presence of two distinct ZIKV lineages: the African lineage and the Asian Lineage, **Figure 2** [11-13]. Moreover, ZIKV sequence alignments revealed that ZIKV formed an independent clade together with Spondweni virus (SPOV) among mosquito-borne flaviviruses. ZIKV showed highest identity with SPOV, with 68.6% identity in nucleotides compared to 58%-60% nucleotide identity with JEV, DENV and WNV [14,15]. Fascinatingly, all the contemporary human isolates have greater sequence homology to the mosquito isolate P6-740 (Malaysia/1966) than IbH-30656 (Nigeria/1968), suggesting that the current strains responsible for the recent outbreaks evolved from the Asian lineage, anchored by P6-740 [16]. The human strains responsible for the epidemic in the Americas (2015-2016) are more closely related to the H/PF/ 2013 strain (French Polynesia/2013) than the FSM strain (Micronesia/2007), indicating these two variants evolved independently from a common ancestor and later dispersed to South America [16,17].

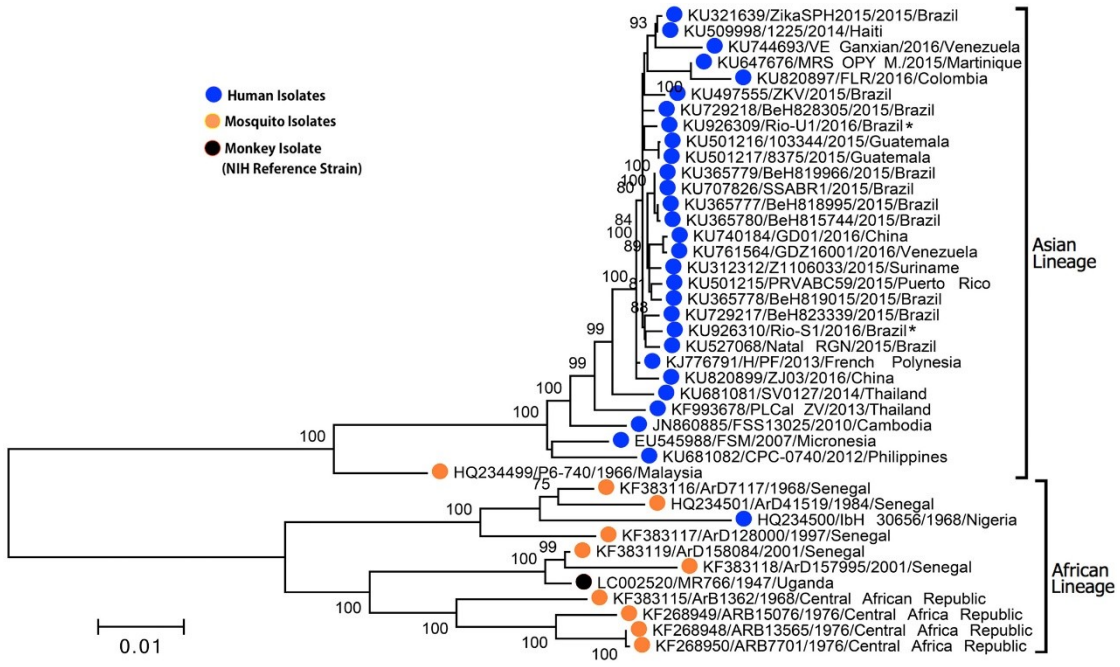

Figure 2 Evolutionary Relationships of Zika Virus. Phylogenetic tree constructed from nucleotide data from 41 viral complete ORF sequences of ZIKV strains by the maximum-likelihood-method logarithm in MEGA7 based on the Tamura-Nei model. A bootstrap percentage for 1,000 replicates was shown on the left. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates are not shown. Strains isolated from human, mosquito, and monkey (NIH) reference strains were labelled with blue, orange, and black circles, respectively. The two subtypes were labelled on the right side of the tree. The new strains Rio-U1 and Rio-S1 were highlighted using (*). Adapted from Wang *et al*, From Mosquitos to Humans: Genetic Evolution of Zika Virus, Cell Host & Microbe, Volume 19, Issue 5, 2016. Copyright (2016), with permission from Elsevier.

1.3 Disease & Transmission

ZIKV is primarily spread by Aedes species mosquitos (including *Aedes aegypti, Aedes albopictus* and *Africa aedes)*. ZIKV circulates between these sylvatic mosquitoes and non-human primates (apes and monkeys) that inhabit the forest canopy, not excluding other possible transmission cycles. Additionally, ZIKV has evolved the ability to circulate in an urban transmission cycle, allowing for humans to be both carriers, and suppliers of ZIKV for uninfected mosquitoes. Furthermore, non-vector borne transmission (sexual and blood transmission and intrauterine transmission) was confirmed during the outbreaks in the Americas [18] .

1.3.1 Sexual & Blood Transmission

Approximately 3% of blood donations from the French Polynesian outbreak tested positive for ZIKV RNA by PCR [19]. Given that the majority of ZIKV infected individuals are primarily asymptomatic and blood transfusion has been reported as a mode of transmission for other Flaviviruses, donated blood particularly from endemic regions, must be screened for ZIKV.

Both African and Asian ZIKV isolates have been reported to be sexually transmitted, implying this mode of transmission was plausible early in evolution prior to viral divergence [20,21]. Additionally, multiple cases have been reported for the presence of ZIKV RNA in semen and testicular tissue from 30-50% of infected men in the first month following disease onset [22,23]. Further, mouse models of sexual transmission have shown the presence of infectious ZIKV in 60-70% of ejaculates [21,24,25]. Interestingly, one case of female-to-male transmission of ZIKV has also been reported however, further investigations into the underlying mechanism behind the sexual transmission of ZIKV are warranted. One study signified the importance of sexual transmission during pregnancy and the development of congenital abnormalities [25]. The group found that sexual transmission of ZIKV to pregnant female mice significantly increased the infection rate and viral titres in developing foetuses compared to other inoculation routes [25].

1.3.2 Congenital & Perinatal Transmission

Of great concern is the congenital (viral transmission during pregnancy) and perinatal transmission (infection around the time of birth) of ZIKV, which is responsible for devastating birth defects including, congenital ZIKV syndrome. The pandemic in the Americas positively correlated with a dramatic upsurge in cases of central nervous system malformations in neonates specifically, microcephaly, of note disease outcomes do vary in

severity in foetuses or infants. ZIKV has been detected in the placenta and amniotic fluid of pregnant women [26,27]. Although, the mechanism behind ZIKV transmission across the placental barrier has yet to be established, compelling arguments have been made for the ability of ZIKV to infect different human placental cells including, trophoblasts and placental macrophages leading to the infection of fetal neuronal cells [28,29]. To date, no cases have confirmed the transmission of ZIKV through breast-feeding however, infectious ZIKV has been detected in breast milk indicating a potential route of transmission warranting further studies [30].

1.4 Clinical manifestations of Infection

Zika virus displays broad tropism and persistence in body tissues and fluids. Human primary dermal fibroblasts, epidermal keratinocytes and immature dendritic cells are among the first cell types to be infected [31]. Subsequently, ZIKV spreads to the draining lymph nodes and is disseminated through the blood stream reaching the peripheral tissues and visceral organs. The incubation period is typically between 3 to 12 days and ZIKV can be detected by RT-PCR and/or ELISA within the first 10 days of infection [31,32]. Based on clinical presentation alone, ZIKV cannot be reliably distinguished from other pathogens that cause a systemic febrile illness, this includes dengue virus and chikungunya virus. This is particularly important in regions where there is endemic circulation of numerous mosquito-borne pathogens. Notably, ZIKV caught people's attention during recent epidemics in the south Pacific and South and Central America as accumulating evidence linked ZIKV to serious neurological complications including congenital ZIKV syndrome and Guillain-Barré syndrome [33]. The factors for the sudden rise in these complications have yet to be explained and current research efforts are striving to understand host-pathogen interactions and environmental factors linked to these diseases.

1.4.1 Asymptomatic ZIKV Infection

Approximately 80% of people with Zika virus infection are asymptomatic, based on a household survey on Yap Island, 2007 [5]. However, mild self-limiting symptoms are present in up to 20% of cases. These symptoms include fever, maculopapular rash, headaches and fatigue [5,34]. Importantly, a lack of symptoms does not imply protection from potential complications, such as fetal microcephaly and Guillain-Barré syndrome in adults. Indeed, sexual transmission of ZIKV between two asymptomatic persons has been reported [35]. Furthermore, an analysis on a group of completed pregnancies reported that among women positive for ZIKV infection, there was no difference in the prevalence of birth defects in foetuses born to asymptomatic or symptomatic women [36].

1.4.2 Congenital ZIKV Syndrome

There is mounting evidence to conclude that ZIKV infection is the cause of microcephaly (a neonatal malformation in which the circumference of the head is much smaller than normal) and severe brain abnormalities. During the wide-spread epidemic in Brazil 2015, microcephaly cases were on the rise (a 20-fold increase in cases), in which the Brazilian Ministry of Health declared a state of health emergency [37]. Viral RNA in placenta and amniotic fluid along with ZIKV-specific IgM antibodies in the cerebrospinal fluid of infected neonates further link ZIKV infection to microcephaly [38,39]. Furthermore, it has now been established that ZIKV can attenuate the growth of human neural progenitor cells (hNPCs) and induce premature differentiation of hNPCs; the resulting depletion of the pool of neural progenitors leads to cortical thinning which is phenotypic of microcephaly [40,41]. However, microcephaly is now just one of the many ZIKV-related birth defects, known as congenital Zika syndrome (CZV) and is specific to foetuses and new-borns infected with ZIKV before birth. Approximately 20% of babies born with CZV have normal head size [27]. A case of a newborn who displayed no obvious signs of ZIKV-associated symptoms was later found to

have developed brain damage and developmental problems [42]. CZV includes a range of symptoms varying at differing degrees of severity: severe microcephaly, decreased brain tissue, damage to the back of the eye and hypertonia [43]. Although the full spectrum of ZIKV-associated reproductive outcomes is not yet determined, the recognition of CZV by healthcare providers for infants and children can help ensure appropriate etiologic evaluation, as well as clinical investigations to define the range and degree of anomalies in an affected infant [43].

1.5 Virology

1.5.1 ZIKV Life Cycle

The name 'flavivirus' is derived from the Latin *'flavus'* meaning yellow in Latin to signify the hallmark jaundice caused by infection with YFV. The primary mode of transmission is through the Aedes mosquitos (sylvatic life cycle) with humans being incidental hosts (urban life cycle), **Figure 3**. In this regard, the life cycle of Zika virus resembles that of other flaviviruses.

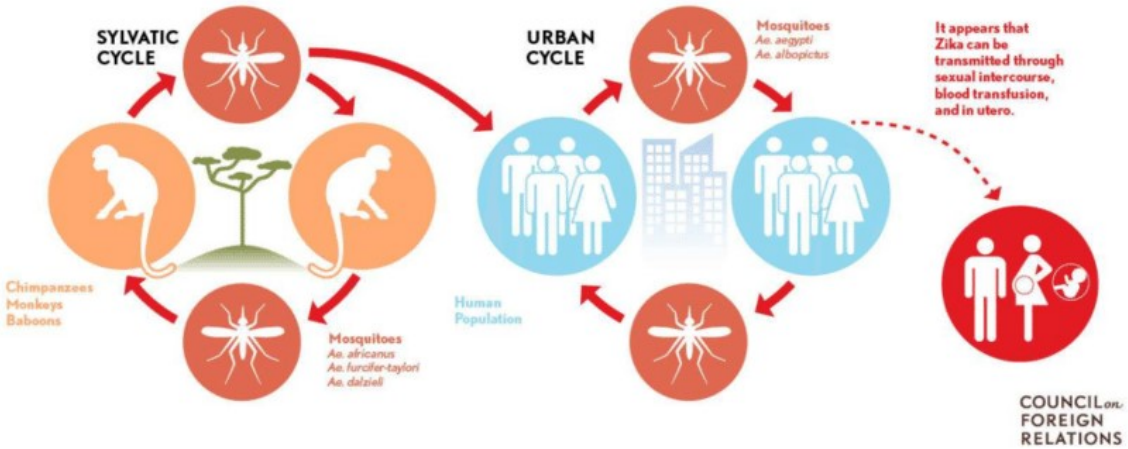

Figure 3 Routes of ZIKV transmission. A graphical representation of Zika virus transmission including, sylvatic and urban life cycles, sexual transmission and in utero transmission. Source: CDC, PLOS, Reuters; Credits: David Foster, Laurie Garrett, Doug Halsey and Gabriela Meltzer.

The mature ZIKV virion is composed of a single-positive strand RNA genome that is packaged by virus capsid protein [44]. These virions attach to the surface of host cells and enter the cell by receptor-mediated endocytosis. The acidic environment of the endosome triggers trimerization of the E protein which results in viral fusion with the cell membrane. Multiple primary receptor candidates for ZIKV have been identified however, there is debate amongst different groups [31,45-49] Subsequently, viral RNA is released into the cytoplasm and the positive-sense RNA is translated into a single polyprotein which is processed co- and post-translationally by viral ad host proteases on the surface of the endoplasmic reticulum (ER). Viral structural proteins and newly synthesized RNA buds into the lumen of the ER to form immature viral particles [50]. As these immature particles egress through the trans-Golgi network, changes in pH and cleavage by the host protease furin results in the formation of mature, infectious virions which are released from the host cell by exocytosis [51], **Figure 4.**

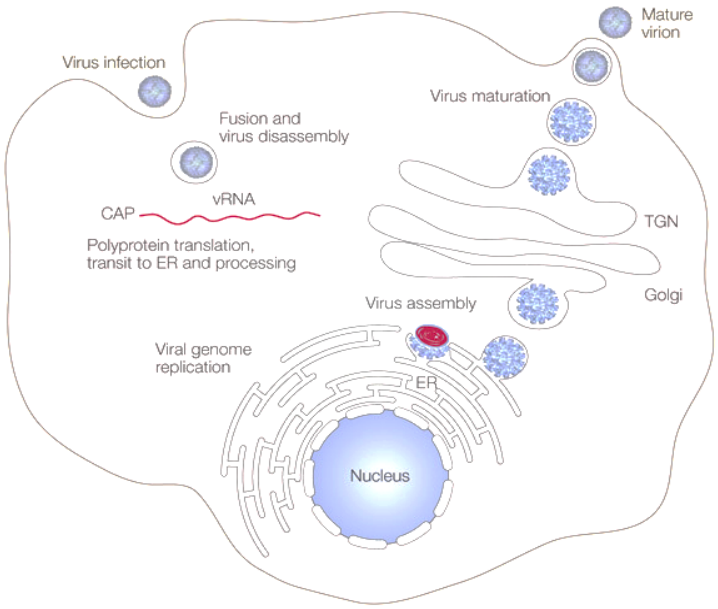

Figure 4 ZIKV replication cycle. Zika virus attaches and enters the host cell by receptor-mediated endocytosis. The virus fuses with the endosomal membrane and is released into the cytoplasm. The viral RNA (vRNA) is translated into a single polypeptide, which is subsequently cleaved by host and viral proteases into structural and non-structural proteins. Viral non-structural proteins form the replication complex and progeny viral RNAs are packaged into virions by the structural proteins. The immature viral particles are transported through the trans-Golgi network (TGN), where mature infectious virions are formed. These virions are released from the cell. Reprinted with permission from Nature Publishing Group.

1.5.2 ZIKV Genome

ZIKV, a single-stranded positive-sense RNA arbovirus belongs to the *Flavivirus* genus of the *Flaviviridae* family. Members of this family, including yellow fever virus, dengue viruses and West Nile virus are known to cause widespread morbidity and mortality throughout the world [52]. To note, viruses in the Flaviviridae family maintain an evolutionary conserved genome organisation of the structural and non-structural proteins. The entire genome of the African ZIKV strain (MR766) was first sequenced in 2007, and contemporary ZIKV strains are currently being sequenced. The ZIKV positive-sense RNA is located between two noncoding regions (5' and 3' untranslated regions) that flank a single open reading frame of ~10kb [53]. The open reading frame encodes a single polyprotein which is subsequently processed by host and viral proteases into three structural proteins, the capsid (C); the pre-membrane (prM); the envelope protein (E); and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), **Figure 5**.

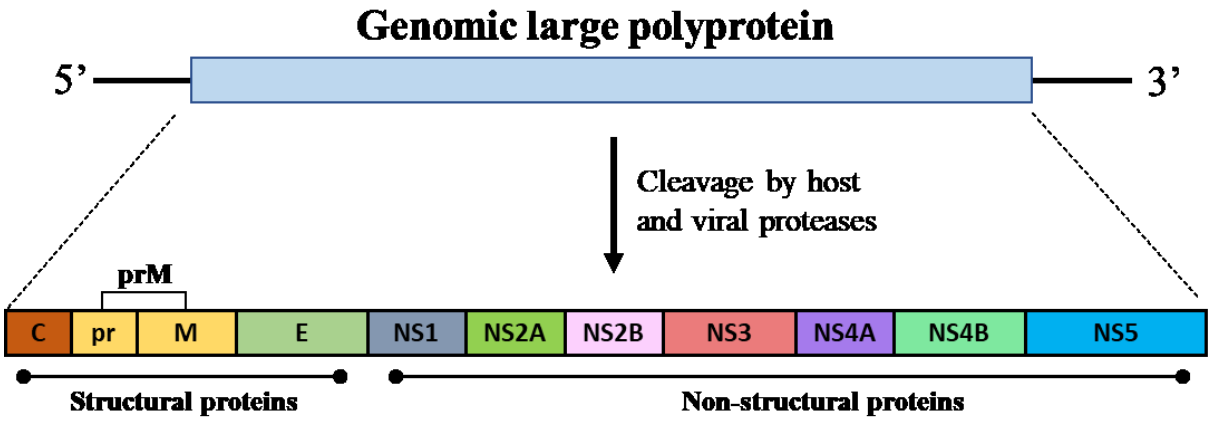

Figure 5 Schematic diagram of the ZIKV genome. ZIKV has a single-stranded positive-sense genome of approximately 11Kb. The polyprotein encodes three structural proteins (capsid C, pre-membrane prM, envelope, E), and seven non-structural proteins (NS1, NS2A, NS3, NS4A, NS4B and NS5).

Together the structural proteins, together with viral RNA, orchestrate the formation of virions: a collection of C protein subunits assemble to form nucleocapsids containing viral RNA; the prM protein assists in chaperone-mediated folding of the E protein and viral maturation; the E protein is the main structural [54] competent of the virus particle responsible for virus fusion and entry in susceptible cells [55-57]. Whilst the primary functions of these structural proteins are characterised, other functions unique to each virus are less explored.

The non-structural proteins have essential roles in viral replication and assembly [58]. The majority of *flavivirus* non-structural proteins are multifunctional. Additionally, these proteins are directly and intimately involved in the evasion of the host immune response [37,59-63].

1.5.3 ZIKV Non-Structural Proteins

NS1

The NS1 glycoprotein plays an important role in viral replication and immune invasion and evasion strategies. NS1 along with other non-structural proteins are translocated to the ER lumen, where the NS1 homodimer plays a critical role in the formation of replication complexes (RC), which facilitate viral replication. Moreover, the dimeric form of NS1 gets trafficked to the plasma membrane through the endocytic or secretory pathways or can fuse with *trans*-Golgi network for secretion in infected cells in the form of hexamers (Sns1) [64]. In the related Dengue virus, sNS1 circulates in the blood at high concentrations, therefore sNS1 is often used as a diagnostic marker for flavivirus infection [65]. The inner surface of the ZIKV NS1 protein shares high similarity (~50% sequence identity) with DENV-2 NS1, implying that both these proteins function in a similar manner when interacting with the ER membrane to form RCs. Despite their overall similarity, studies comparing ZIKV NS1 with West Nile and Dengue virus NS1 revealed different electrostatic properties on the outer face

of the hexamer which may contribute to the differing pathogenesis between these viruses [66,67]. Furthermore, secreted NS1 proteins interacts with various components of the complement system which contribute to overall pathogenesis [64]. With regards to the role of ZIKV NS1 in overcoming host antiviral immunity, it has been demonstrated that NS1 and NS4B inhibit IFN-β signalling by binding TANK-binding kinase 1 (TBK1) [68].

NS2A

Flavivirus NS2A is a small hydrophobic membrane-assoociated protein involved in RNA replication and the host-antiviral interferon response [69-72]. NS2A binds to the 3' UTR of viral RNA and associated with other components of the RC [73]. Interestingly, a study showed that ZIKV-NS2A, not DENV-NS2A reduced the proliferation and differentiation of radial glial cells in the embryonic mouse cortex [74]. Furthermore, the group showed that ZIKV-NS2A interacts with multiple adherens junction complex (AJ) components impairing its formation in human forebrain organoids. Another study with Kunjin virus (a subtype of WNV) confirmed the role of NS2A in virus assembly, specifically in the biogenesis of virus-induced membranes [75]. These results begin to uncover the distinctive mechanisms responsible for ZIKV-associated congenital disorders.

NS2B/NS3 protease

Flavivirus NS2B is a cofactor for the protease activity of NS3 [76,77]. The NS2B/NS3 protease is a highly conserved large multifunctional protein with the activities of a serine protease, nucleotide triphosphatase, and a helicase [78,79]. Not only does the NS2B/NS3 protease mediate polyprotein processing and RNA synthesis, the protease also plays an important role in inhibiting the induction of IFN and downstream ISGs. NS2B/NS3 impairs the JAK-STAT signalling pathway by degrading JAK1 and reducing IFN-induced apoptotic

cell death [80]. ZIKV employs a novel antagonistic system whereby, multiple non-structural proteins orchestrate to dampen the innate antiviral response.

NS4A & NS4B

The NS4A and NS4B proteins of several flaviviruses are known to play multiple roles in viral replication and virus-host interactions including, the remodelling of ER membranes to form the viral RC [81]. While WNV NS4A regulates the ATPase activity of NS3, DENV NS4B interacts with the helicase domain of NS3, suggesting that these two proteins modulate viral assembly and replication via distinct mechanisms in different flaviviruses [82,83]. In virus-infected cells, NS4A interacts with NS4B which facilitate in viral replication [84]. The NS4A protein has further been shown to induce autophagy to prevent cell death and enhance viral replication [85]. The mTOR kinase plays a crucial role in autophagy induction, whereby activation of mTOR and downstream signalling leads to the suppression of autophagy. Recently, ZIKV NS4A and NS4B have been shown to supress the host Akt-mTOR signalling leading to the upregulation of autophagy in human fetal neural stem cells (fNSCs) [86]. In relation to the host immune response, the NS4A and NS4B proteins of multiple *flaviviruses*, including ZIKV are known to inhibit the JAK-STAT and RLR signalling pathway via multiple mechanisms [59,62,87]. While much is not known about the specific function of the NS4B protein, DENV NS4B induces mitochondrial elongation via direct contact with ER-derived convoluted membranes [88]. The restructuring of the mitochondria has critical downstream implications in the regulation of cellular metabolism and innate immune signalling at the ER-mitochondria interface. It has yet to be discovered whether ZIKV NS4A and NS4B also alter mitochondrial morphology.

NS5 (RNA-dependent RNA polymerase)

The highly conserved *flavivirus* NS5 protein encodes both a methyltransferase (required for viral RNA capping and 2'-O-methylation) and the RNA-dependent RNA polymerase which is critical for viral RNA synthesis. As discussed above, multiple flavivirus non-structural proteins contribute to preventing IFN induction or inhibiting IFN signalling however, NS5 is a specific antagonist of IFN signalling and exerts its activity via different mechanisms depending on the *flavivirus* [61,89-91]. For example, WNV NS5 prevents the expression of IFNAR1 on the cell surface while DENV NS5 recruits cellular factors to degrade STAT2 [92,93]. Like DENV NS5, ZIKV NS5 targets human STAT2 for degradation (not in mouse) however, unlike DENV the E3 ligase UBR4 is not required for ZIKV mediated STAT2 degradation [94]. Intriguingly, ZIKV-mediated degradation of STAT2 was shown to promote the formation of STAT1-STAT1 protein complexes leading to the activation of IFN-γ-stimulated genes (Type II IFN) [95].

The orchestration of multi-functional viral proteins during the flavivirus life cycle makes them key determinants of viral fitness and we can begin to unravel the causes of ZIKV-related birth defects and complications which are not observed for other *flaviviruses*.

1.6 The Innate Immune Response Against ZIKV Infection

1.6.1 The Type 1 Interferon Response

As key cytokines with multifaceted antiviral and modulatory properties, the interferon (IFN) system is the first line of defence against pathogens. Based on their receptor usage, IFNs are divided into three classes: Type I (IFN-α/β), II (IFN-γ) and III (IFN-λ) IFNs. Although all IFNs are key mediators of the antiviral response their roles in antiviral defence vary. The major features of the IFN family members are described in **Table 1.** Of note, 13 subtypes of IFN-α have been identified in humans [96]. Over the past decade, our knowledge concerning

the involvement of type 1 IFNs in a variety of viral infections and inflammatory disease has rapidly expanded. The recognition of viral pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRR) trigger signalling pathways that induce the production of type 1 IFNs. For example, activation of RIG-I-like receptors (RLRs), which recognise cytosolic viral RNA, including ZIKV, leads to the interaction with mitochondrial antiviral signalling adapter (MAVS) [97]. Subsequently, type 1 IFN transcription is initiated and these IFNs signal through common heterodimeric receptors, IFNAR1 and IFNAR2, initiating a signalling cascade to drive the synthesis of hundreds of ISGs [98].

Table 1 Classification of IFNs based on their receptors, sources and effects.

IFN Type	Interferons	Receptors	Sources	Effects
I	IFNα IFNβ IFNκ IFNω IFNε	IFNAR1, IFNAR2	Ubiqitous	Proinflammatory response Immune cell recruitment IFNγ production IL12 production Enhanced septic shock Bacterial clearance Immunosupressive cytokine production Activation & maturation of dendrtic cells.
II	IFNγ	IFNGR1, IFNGR2	T cells, activated NK cells	Proinflammatory response Induction of MHC I and II antigen expression Increases the activity of macrophages Immune response to bacterial infection
III	IFNλ1 IFNλ2 IFNλ3 IFNλ4	IFNLR1, IL10R2	Dendritic cells, macrophages Epithelial cells	Limiting viral infections Defence at the mucosal barrier Development of adaptive immune response Immune regulation

What are Interferon Stimulated Genes (ISGs)?

ISGs are a diverse group of cellular proteins, whose expression is transcriptionally up-regulated by interferon signalling. ISGs take on a large number of diverse roles as well as acting as key modulators of the interferon response. Many ISGs control viral, bacterial and parasite infection by targeting different stages of the pathogen's life cycle. These ISGs serve as key antiviral effectors, to varying degrees, during the host innate immune response and play an important role in limiting viral spread. For example, viperin, a highly conserved

protein across species, exerts potent antiviral activity against ZIKV by interfering with ZIKV RNA replication [99]. Furthermore, interferons and their ISGs have been influenced by strong selective pressures, due to the evolutionary arms race between host and viruses, indicative of the important and non-redundant roles that they play in host defence [100]. Since the discovery of ISGs more than 35 years ago [101,102], genome-scale cataloguing of ISGs had led to a better understanding in their effector functions and mechanisms. Here, we review the current literature on several well-known ISGs and summarise our current understanding of their antiviral activity.

1.6.1.1 The IFITM Family

The IFITM (interferon-induced transmembrane) proteins are mainly distributed at the plasma membrane and endosomal membranes. IFITM1, IFITM2 and IFITM3 are highly upregulated by both Type 1 and Type 2 IFNs. IFITMs are ubiquitously expressed at basal level and characterized by C- and N-terminal variable regions, two-membrane associated regions and a conserved intracellular loop [103]. In the case of IFITM3, the N-terminal domain is linked to its antiviral activity, while in IFITM1 the C-terminal domain modulates its antiviral function [104,105]. While IFITM2 and IFITM3 both inhibit viral entry, their efficacy is dependent on the virus and viral strains. These proteins are well known to inhibit a broad range of RNA viruses including ZIKV, DENV, WNV, HCV and Influenza A virus, from traversing the lipid bilayer of the cell [106-110]. It has been recently reported that IFITM3, and to a lesser extent IFITM1 inhibits ZIKV replication and can prevent ZIKV-induced cell death [107]. Another study extended on these findings showing that low IFITM3 levels human epithelial cells, primary skin fibroblasts and astrocytes increased vacuolization and ZIKV-mediated cell death [111].

With few studies exploring the function of IFITM proteins *in vivo,* Gorman *et al* demonstrated that IFITM3 supressed WNV replication in non-neuronal cells *in vivo*, while

ifitm3-/- mice were vulnerable to lethal injection [110]. In contrast, a recent study using a murine CMV model of infection showed that IFITM3 controlled murine CMV pathogenesis without directly inhibiting viral replication. In their study, IFITM3 established an antiviral cellular state through the restriction of virus-induced lymphopenia and T cell survival [112]. Interestingly, the entry process of mouse leukaemia virus (MLV), Lassa virus (LASV) and lymphocytic choriomeningitis virus (LCMV) were not inhibited by IFITMs [113]. Moreover, less is known regarding IFITM restriction of DNA viruses. Specifically, the replication of human papillomavirus (HPV), human cytomegalovirus (HCMV) and adenovirus 5 (Ad5) is not restricted by IFITM1, 2 and 3 expression [114]. These recent studies begin to elucidate into the function and multiple mechanisms of IFITM3-mediated restriction of viral pathogenesis *in vivo*.

1.6.1.2 The myxovirus resistance (Mx) proteins

Mx proteins comprise a small family of GTPases and are key components of the cellular antiviral state induced by IFNs. Mx proteins were first discovered in mice that showed a high degree of resistance against influenza A virus infection [115,116]. Humans express two Mx genes, called MxA and MxB, located on the long arm of chromosome 21 [117]. MxA has long been known for inhibiting a variety of RNA and some DNA viruses, notably influenza A virus (IAV), vesicular stomatitis virus (VSV) and hepatitis B virus (HBV) [116,118-121]. However, the antiviral function of MxB was not identified until recent years where MxB was shown to inhibit human immunodeficiency virus type 1 (HIV-1) [122-124]. Additionally, it has been shown that MxB plays a critical role as a potent restriction factor for a range of herpesvirus infections including, herpes simplex virus 1 and 2 (HSV-1 and HSV-2) [125].

MxB is found to be enriched on the cytoplasmic face of nuclear membranes and in the nucleus [124,126]. MxB also contains an N-terminal tri-arginine motif which has been found

to interact with cyclophilin A (CypA) on HIV-1 capsid [127,128]. Studies have known that MxB binds to the HIV-1 core and prevents the uncoating process of HIV-1 [129-131]. This interaction was found to be dependent on the HIV-1 capsid protein host factor cyclophilin A [122,128]. However, this mechanism alone does not suffice for the full extent of the antiviral activity of MxB. The antiviral activity of MxB is dependent on both the amino-terminal region of MxB and protein oligomerization [132]. Studies have identified that the GTP hydrolysis of MxB is required for its antiviral activity against HIV-1 [123,124,133]. Beyond lentivirus restriction, the antiviral activity of MxB is confirmed against HSV-1, HSV2 and Kaposi's sarcoma-associated herpesvirus [125,134]. As with HIV-1, the studies showed that herpesviral capsids are also targeted at the nuclear pore and the uncoating of viral DNA is blocked. Remarkably, examination of structural features of MxB revealed that both GTP binding and hydrolysis were essential for its antiviral effect against herpesvirus infection, showing resemblance to the function of MxA. Although the molecular details of MxB inhibition of herpesviruses remain to be clarified, it can be said that MxB targets an early post-entry step, during genome uncoating for both HIV-1 and herpresviruses.

1.6.1.3 Tetherin/BST2

Bone marrow stromal antigen-2 (BST-2), also called 'tetherin' by a number of retro virologists as it represents the protein's unique antiretroviral properties, is a potent antiviral protein known for 'tethering' mature Vpu deficient HIV-1 virions, thereby preventing their release [123,135]. Tetherin is an IFN inducible type II membrane protein, constitutively expressed in mature B cells, bone marrow stomal cells, monocyte-derived macrophages and dendritic cells [136,137]. The function of Tetherin is attributable to its unique topology. Tetherin contains an N-terminal transmembrane domain and a C-terminal GPI-anchor [138,139]. Once incorporated into nascent virions via one of its membrane anchors, the

second anchor remains attached in the plasma membrane, thus preventing the release of virions.

Tetherin was first described as a marker for B cell maturation and was later found to be a ligand for immunoglobulin-like transcript 7 (ILT7) on plasmacytoid dendritic cells, thus acting as a modulator for the IFN response [136,140,141]. Tetherin has also been further characterised to restrict other enveloped viruses including, MLV and Chikungunya virus [142-146]. The antiviral activity of Tetherin likely depends on a few factors: its ability to bind specific viral proteins, the degree of IFN induced protein synthesis and the role of tetherin in promoting the immune response. Despite the role of Tetherin in preventing the release of viruses from infected cells, the retention of virus particles during viral replication also has considerable indirect antiviral effects, including, the stimulation of proinflammatory signalling pathways [147,148], enhancement of antiviral immune responses [149-151], and enhancement of ADCC [152]. Several possibilities have been suggested for the fate of the tethered virions, the most likely being virion internalization resulting in lysosomal degradation, however it remains unclear if internalized virions can be cycled back to the cell surface [153,154]. Further investigation involving *in vivo* studies are needed to clarify the physiological role of Tetherin in other enveloped viruses and its role in cell-to-cell virus transmission.

1.6.1.4 IFI6 (IFI-6-16)

IFI6 is a type I ISG, which encodes for a small, 130 amino acid protein in the FAM14 family. Interestingly, *IFI6* is identified only in higher-order mammals and appears to have arisen through divergent inter and intra-chromosomal duplication events [155]. IFI6 acts as anti-apoptotic in myeloma, breast and ovarian cancers [156-158]. Several reports have shown IFI6 is localised in the mitochondria and ER; IFI6-overexpressing cells play a vital role in

antagonising TRAIL-, IFNs-and chemotherapeutic-induced apoptosis by stabilizing the mitochondrial membrane potential ($\Delta\Psi$) [159,160]. While the functional role of IFI6 in the immune system is well known, emerging studies are beginning to uncover its antiviral effects. IFI6 strongly regulates flaviviruses Dengue 2 virus, WNV and HCV infection [110,160-162]. Several reports have attempted to reveal mechanistically the antiviral function of IFI6. Qi et al showed that IFI6 inhibits DENV2-induced vascular endothelial cell apoptosis via the caspase dependent pathway implying that IFI6 may function as a protective gene during DENV infection. With regards to HCV infection, IFI6 was shown to enhance the antiviral effect of IFN-α and inhibit HCV entry rather than directly inhibiting HCV RNA replication [162]. A recent study, demonstrating IFI6 localisation in the ER suggested that IFI6 prevents the formation of virus-induced ER membrane invaginations, thus prophylactically protecting uninfected cells [160]. Together, these studies highlight the critical role of IFI6 during virus infection through multiple mechanisms.

The host has evolved multiple mechanisms to inhibit viral replication, one of them being the induction of hundreds of ISGs. However, for many of these ISGs their antiviral function, mechanism and potential are just beginning to be tapped into. Not surprisingly, viruses have developed elaborate strategies to escape or inhibit the IFN response.

1.6.2 ZIKV Evasion of Host Innate Immunity

Recent studies have demonstrated that certain ZIKV non-structural proteins can antagonize type 1 IFN responses. The NS1 and NS4B proteins of ZIKV have been shown to target TBK1, thereby preventing the activation of RLR signalling and subsequently supressing IFN production [80]. Also, studies have shown that the NS4B protein of dengue virus (DEN-2) strongly blocks type 1 IFN induction by inhibiting STAT1 phosphorylation [72].

Additionally, research groups have demonstrated that the ZIKV NS5 protein can supress type 1 IFN signalling by specifically targeting STAT2 for degradation [94,163]. Fascinatingly, one group showed that compromising STAT2 led to the promotion of STAT1 homodimerization, thus activating type II IFN signalling, resulting in IFN-γ production and differential ISG expression [95]. This phenotype, not yet observed in other flaviviruses, reveals another layer of complexity between the interaction of viruses with the host immune system. Contrary to these findings, an independent study observed no significant degradation of STAT2, even though the phosphorylation of these proteins were blocked in ZIKV infected monocyte-derived dendritic cells [164]. Discrepancies regarding the function of the ZIKV NS5 protein in inhibiting IFN signalling could very well be cell-type specific. Other studies have demonstrated that ZIKV NS2B-NS3 proteins impaired JAK-STAT signalling by promoting the degradation of Jak1 [80]. The dengue virus NS2B-NS3 protease complex has been shown to inhibit type 1 IFN signalling via degrading the stimulator of interferon genes (STING) in human dendritic cells [165].

Future research will shed new light on mechanisms by which ZIKV non-structural proteins work synergistically to antagonise the type 1 IFN response. Deepening our understanding of how ZIKV proteins interact with not only type II and III IFN signalling, but also the adaptive arm of the immune system, will elucidate the complex interactions behind the immune evasion strategies adapted by ZIKV.

1.6.3 Type II and III IFN Responses to ZIKV Infection

Type II interferon (IFN-γ) displays some antiviral properties of type I IFN, however IFN-γ is uniquely expressed by cells of the immune system and the dominating role of IFN-γ is in the stimulation of the adaptive immune system [166]. IFN-γ was first described merely as an antiviral factor, however it has since been demonstrated to be a broad-spectrum antimicrobial

agent and an important player in overall inflammatory responses, including macrophage activation and driving leukocyte migration to sites of infection [167-169]. Binding of IFN-γ to the type II IFN receptors (IFNGR1 & IFNGR2) leads to the activation of JAK1 and JAK2 which regulate the downstream phosphorylation of STAT1. Subsequently, STAT1 homodimers translocate to the nucleus and induce the transcription of IFN-γ-stimulated genes. The transcription of these genes is regulated by GAS elements[170-174]. When comparing type I and type II IFN signalling during ZIKV infection, studies have shown that type II IFN signalling increases ZIKV replication in placental and glioblastoma cell lines [95,175]. However, another study demonstrated that type II IFNs strongly and dose dependently inhibit viral replication in primary skin fibroblasts [31]. Given these conflicting findings, further research is required to detail the specific interactions of type II IFN signalling during ZIKV infection.

Type III IFNs are co-expressed with type I IFNs during viral infection and may induce a similar set of ISGs [98]. Both Type I and type III IFNs activate STAT1 and STAT2, which complex with IRF-9 and lead to the transcription of ISGs. However, unlike type I and II IFNs, type III IFNs bind to a unique heterodimeric receptor (IFNLR1) [176]. In addition to the role of IFNs in establishing antiviral immunity, IFNs also have important roles in supporting pregnancy and protecting the developing fetus from viral infections [177]. For example, specific ISGs are up-regulated during embryo implantation, and human syncytiotrophoblasts highly express type III IFNs, making them resistant to viral infection, including ZIKV infection [178,179]. Moreover, type III IFNs play a critical role in increasing the antiviral capacity of the placental barrier to restrict ZIKV vertical transmission in mice [180]. Paradoxically, one study in mice showed that foetuses with a functional copy of IFNAR are resorbed after ZIKV infection, which highlights the determinantal role of fetal IFNAR signalling. Moreover, their results showed that type I IFN, but not type III IFN,

treatment led to robust ISG induction in human midgestational chorionic villous explants [181]. Importantly, beyond viral infections, the effects of IFNs on the developing placenta have critical implications in pregnancy complications.

Many congenital infections (Toxoplasmosis, Human Cytomegalovirus and Herpes viruses) have common presentations of microcephaly and other birth defects. Collectively, these studies highlight the importance of type III IFNs in restricting ZIKV related pathogenesis and future studies will surely provide important insights into the function of type III IFNs in defending the developing fetus against the vertical transmission of ZIKV and other viruses associated with congenital disorders.

1.7 Striking a Balance in Host-Virus Interactions: Modulating Metabolism

We have discussed to great detail the host response elicited against ZIKV infection, we now consider the dependencies of the virus on the host. The opposing goals of host and viral factors have resulted in an evolutionary arms race that has been illuminated by evolutionary and computational methods [182]. The limited coding capacity of many viruses makes them fully dependent on the host for its viral life cycle. Viruses are known to exploit the host and induce drastic metabolic changes to ensure an optimal environment for replication and the production of viral progenies. While not covered in the chapter, the host has evolved countermeasures to virus-induced metabolic changes, reviewed in our recent paper [183].

The effects of viruses on cellular metabolism were already described in studies from the 1950s, when it was observed that addition of glucose into the minimal media significantly increased the yield of polioviruses from the infected HeLa cells [184]. The first large-scale study investigated over 60 metabolites during HCMV and inspired a renewed focus on the critical role of metabolism in virus infection [185]. Extensive metabolic studies have now been performed on a variety of viruses, including DENV [186], HCV [113], HIV-1 [187],

IAV [188] and HSV-1 [189]. Like these viruses, ZIKV is no exception in this regard. **Figure 6** illustrates the core metabolic pathways which are significantly altered by multiple viruses.

Glucose metabolism is the primary source of energy to support cell proliferation and survival. In the presence of sufficient oxygen, glucose is metabolised to pyruvate, translocated to the mitochondria and oxidized to produce ATP. In anaerobic conditions, pyruvate is converted to lactate and glycolysis functions as the main source of ATP production. Most cancer cells and viruses utilize glucose for the production of lactic acid even in the presence of abundance oxygen (the Warburg effect) [190]. It is now known that viral infection can induce the 'Warburg effect' to generate a rapid source of ATP, nucleotides, amino acids and fatty acids that are needed to assemble a large number viral progenies. However, even in cancer cells or virus-infected cells, where a large proportion of ATP is produced during glycolysis, oxidative phosphorylation is still a major source of ATP production and the balance between glycolysis and oxidative phosphorylation is virus-dependent.

Many viruses have distinct mechanisms to modulate cellular metabolism. Fascinatingly, Dengue virus, hepatitis C virus and ZIKV hijack autophagy to control the processing of lipid droplets and triglycerides to support viral replication [29,31,191]. Furthermore, ZIKV reprograms the host metabolic machinery to support ATP production and glycolysis to allow for sustained infection [192,193]. Not only do these viruses modulate glycolysis, recent research has demonstrated that DENV and potentially ZIKV alter mitochondrial morphology to induce mitochondrial elongation [88,194]. DENV-induced mitochondrial elongation was shown to alleviate RIG-I-dependent innate immunity as well as increasing oxidative phosphorylation. However, the modulation of metabolism by ZIKV remains unknown. In particular, the ZIKV non-structural proteins which participate in altering the host metabolic network remains to be elucidated.

As viruses have evolved distinct mechanisms to target host cell metabolism, simultaneously, the hosts have accordingly evolved antiviral mechanisms to counteract these changes. While not investigated in this research, several ISGs and restriction factors are now known to interfere with cellular metabolism to ensure cell survival and restrict viral replication, extensively reviewed in [183].

In recent decades, emerging and re-emerging pathogens have become an increasing concern for global public health, as highlighted by the Ebola outbreak in West Africa and the Zika virus outbreak in the Americas. Thus, understanding the viral life cycle, host defences and pathogenesis increases our ability to develop novel therapeutics to limit these infections. Studies on the effects of viruses on cellular metabolism during replication have provided novel insights into these complex networks and helped to identify specific molecules that may contribute to disease pathogenesis and act as new targets for therapy and biomarkers.

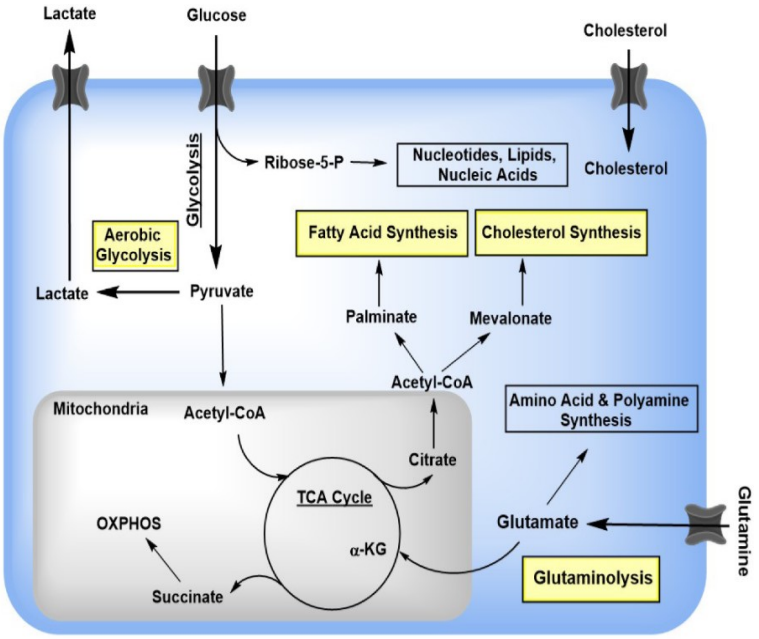

Figure 6 Host Cell Metabolism. Glucose is taken up by specific transporters (GLUT family), where it is converted to pyruvate in the cytoplasm, generating two ATP molecules (glycolysis). In the presence of oxygen, pyruvate is transported into the mitochondria and oxidized into acetyl coenzyme A (acetyl-CoA), which enters the tricarboxylic acid (TCA) cycle. Intermediates of the TCA cycle feed off for fatty acid and cholesterol synthesis. Viruses are known to alter these key cellular metabolic pathways (highlighted in yellow).

Chapter 2 Rationale, Hypotheses and Objectives

This chapter outlines the **rationale, hypotheses** and the **key objectives** of the following research. Together with the principle investigator, Dr. Chen Liang, we formulated the research question, the specific aims, and the experimental design of the project.

2.1 Rationale and Hypotheses

The emergence and geographical expansion of ZIKV has become a major burden on global public health [195]. The spread of ZIKV throughout the Americas saw concurrent increases in reports of Guillain-Barré syndrome and neonatal malformations notably, microcephaly [27,196]. Multiple factors including the abundance of the *Aedes* species mosquitoes, rapid population mobility of infected individuals, and the genetic diversity of the circulating ZIKV strains likely contribute to ZIKV pathogenicity and the rapid emergence of recent ZIKV epidemics [197] . In addition to these factors, immunological naivety of the human population has facilitated rapid viral evolution and there is major concern regarding the infection of pregnant women who are immunologically naïve to ZIKV and intrauterine infection of their babies. Therefore, infected individuals are dependent on their immune response to control and counteract ZIKV infection. Innate immune responses, in particular type I IFNs, represent the first-line of defence against a plethora of pathogens. They primarily function by upregulating ISGs that either directly interfere with the viral life cycle or subsequently induce downstream intermediate effectors to invoke a potent antiviral state in the cell. Murine models of ZIKV pathogenesis have shown IFN signalling-deficient mice are highly susceptible to ZIKV infection compared to wild-type mice, signifying that ISGs can prevent or suppress increased levels of infection [198]. While the antiviral role of IFNs has long been known, the complete spectrum of ISGs has not been identified, thus ISGs with potential antiviral effect remain unknown. In particular, the ISGs that limit ZIKV replication

at the molecular level are ill-defined. **Therefore, we raise the question of which ISGs contribute to the restriction of ZIKV replication *in vitro* and seek to uncover the underlying mechanisms behind their antiviral activity.**

As with identifying specific ISGs which can restrict ZIKV replication, equally important is the characterisation of host factors which are modulated during viral infection. Flaviviruses have complex life cycles which rely on host cell resources. A recent genomic screen identified multiple host factors in ZIKV infection [193]. One major host resource exploited by viruses is the host cellular metabolic network. Viruses induce drastic metabolic changes to ensure for optimal replication and the production of viral progenies [199]. While ZIKV non-structural proteins assist in viral replication and immune evasion, they also play a role in modulating cell survival. A recent study found that ZIKV NS4A and NS4B work cooperatively to supress the Akt-mTOR pathway leading to cellular dysregulation [29]. While the focus of this study was directed at autophagy, PI3K/AKT/mTOR signalling contribute to a variety of processes including cellular metabolism. **We hypothesised that ZIKV NS4A and NS4B plays a role in altering cellular metabolic state for successful viral replication.**

To successfully establish an infection, all viruses must overcome host defence mechanisms at the site of infection, infect cells which are both susceptible and permissive to producing infectious virions and these released virions must be sufficient in numbers to travel and again infect a susceptible host cell. Analysing the course of ZIKV infection in terms of this model (both host response and viral countermeasures) can shed light on the possible mechanisms of viral dissemination, and importantly help to address the current lack of antiviral treatment and therapies for ZIKV infection. The specific objectives for this research are:

2.2 Objectives

Aim 1: To identify and investigate the effect of ISGs on ZIKV replication using gene overexpression in Huh-7.5 cells (hepatocarcinoma cell line).

Aim 2: Determine and characterise the role of ZIKV NS4A and NS4B in modulating cellular metabolism in U87-MG human astrocytoma cells.

Chapter 3 Materials & Methods

3.1 Antibodies

The primary antibodies used for immunoblotting are: mouse monoclonal anti-Flag (Sigma-Aldrich) (1:5000), mouse monoclonal anti-β-Tubulin (Santa Cruz Biotechnology) (1:3000). The secondary antibodies used for immunoblotting are: horseradish peroxidase-linked donkey anti-rabbit IgG (GE Healthcare) (1:10000) and horseradish peroxidase-linked sheep anti-mouse IgG (GE Healthcare) (1:10000). The antibodies used to stain cells for flow cytometry are: antibody for the detection of FLAG™ conjugated proteins (MOUSE) Monoclonal Antibody (Rockland) (1:1000).

3.2 Cell lines

Human embryonic kidney (HEK 293T) cells, Huh-7.5 cells and African green monkey epithelial vero cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Fisher) and 1% Penicillin/Streptomycin (P/S) (Thermo Fisher).

U87MG cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Wisent Bio Products) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher), 1% Penicillin/Streptomycin (P/S) (Thermo Fisher).

Huh-7.5 ISG overexpressing cell lines were maintained in DMEM supplemented with 10% FBS, 1% P/S and 1 µg/ml puromycin.

NS4A-U87MG, NS4B-U87MG and U87MG control cell lines were maintained in EMEM supplemented with 10% FBS, 1% P/S and 1 µg/ml puromycin.

Cells were grown in CO2 incubators at 37°C with 5% CO2 concentration.

3.3 ZIKV strains and amplification

The Canadian imported Thai ZIKV strain PLCal_ZV (Genbank accession KF993678.1), was passaged 4 times as previously described and kindly provided by Dr Wainberg's lab at McGill University, Montreal, Canada [200]. ZIKV strains MR766 (Uganda, 1947, Genbank accession: HQ234498.1) and PRVABC-59 (Puerto Rico, 2015, Genbank accession: KU501215) were supplied by ATCC.

ZIKV stocks were propagated in Vero cells after infecting at a multiplicity of infection (MOI) of 0.5. Supernatants were harvested after 72 h post-infection and viral stocks were titrated by plaque assay on Vero cells.

3.4 Plasmids & Transfection

Human ISGs were cloned into pQCXIP (Clontech) retroviral vector, see Table 2 for the list of ISGs. HEK293T cell transfections were conducted using polyethlenimine (PEI) transfection reagent as per the manufactures protocol.

Briefly, HEC 293T cells were seeded in 6-well plates 20 hours before transfections. 0.8µg plasmids, 5.1µl PEI (1µg/µl), 0.8µg of MLV-gag/pol, 0.1µg VSV-G and 100µl Opti-Modified Eagle Medium (opti-MEM) (Invitrogen) were mixed and incubated in room temperature for 20 minutes. The mixtures were then added into the wells. The media was changed 4 to 6 hours following transfection.

3.5 Transduction

Huh-7.5 or U87-MG cells were seeded 6-well tissue culture plates with 1.5ml of drug-free media 16 hours before transduction. Supernatant containing the retroviral vectors were added to each well with 2µl of polybrene (5mg/µl). The cells were spinoculated at 1800rpm, room temperature (25°C) for 45 minutes. The media was changed 48 hours following transduction followed by selection with puromycin (2 µg/mL).

3.6 Plaque assay

The ZIKV plaque assay protocol was kindly provided by Dr Selena Sagan at McGill University in Montreal, Canada. Five different 10-fold dilutions of purified virus were spread onto monolayers of Vero cells (ATCC) at 37°C for 2 hours to initiate attachment to cells. A mixture of nutrient solution containing carboxymethyl cellulose (Sigma-Aldrich), DMEM, 2% FBS and 1% penstrep was then added. The cells were incubated at 37°C for 3 days before the plaque assay. For plaque counting, cells were incubated with 10% formaldehyde for 1 hour at room temperature, followed by adding staining solution (10% ethanol and 0.1% crystal violet, in water). Viral titres were calculated as follows:

$$\frac{No.\,of\ plaques}{dilution\ factor\ X\ infection\ volume}$$

3.7 Zika Virus Infection of Huh-7.5 cell lines

Huh-7.5 cells were seeded in 6-well plates at 0.2 x 106 cells per well for 24hr after which they were infected with ZIKV (MOI: 0.02) for 4 hrs at 37°C/5% CO_2, the viral inoculum was removed, and 8ml of fresh culture medium was added. Supernatants were harvested 48 hr and 72 hr post infection. Viral titres were determined by plaque assay.

3.8 ZIKV RNA extraction and RT-PCR

Total RNA was extracted from Vero cells infected with PLCal_ZV at MOI 0.02 for 24hr with Tizol reagent according to the manufacturer's instructions and RNA concentration was determined using a NanoDrop 2000 spectrophotometer. Complementary DNA was reverse transcribed from µg of total RNA….

ZIKV expression constructs were amplified by PCR (see Table 3 for primers) and cloned into the retroviral vector PQCXIP with an N-terminal Flag tag. All constructs were sequenced to verify 100% correspondence with the original sequence. U97-MG stable cell lines were generated using a standard selection protocol with puromycin (2 µg/mL) for individual expressions of ZIKV genes NS4A and NS4B.

Table 2 ZIKV primers used for PCR amplification

ZIKV NS4A-Forward	5'-gtcgcgcggccgcatggtgatggaagccctgggaac-3'
ZIKV NS4A-Reverse	5'-agctggatccttacttgtcgtcatcgtctttgtagtctctttgcttctctggctcagg-3'
ZIKV NS4B-Forward	5'-gactcgcggccgcatgaatgaactcggatggttggaa-3'
ZIKV NS4B-Reverse	5'-cgcaggatccttacttgtcgtcatcgtctttgtagtcacgtctcttgaccaagccag-3'

3.9 Flow Cytometry Intracellular staining

Cell samples were fixed in 2% PFA at 4°C for 1 hr and were washed once with 1ml of PBS containing 2% of FBS. Cells were washed twice with the 1× Permeabilization buffer. Antibodies were diluted in the 1× Permeabilization buffer according to the manufacturer's instructions. The cells were resuspended with 50µl of the diluted antibodies and were incubated on ice for 1 hour in the dark. After the incubation, the cells were washed three times with the 1× Permeabilization buffer and were then resuspended in 300µl of PBS containing 2% of FBS. Subsequently, the samples were analyzed by flow cytometry.

3.10 Western Blot

Huh-7.5 and U87-MG cell lines were harvested and washed once in cold PBS. The cells were then lysed for 30 minutes on ice in radioimmunoprecipitation assay (RIPA) buffer supplemented with complete protease inhibitor. Cell lysates were purified by centrifuging the lysed cells at 32000rpm, 4°C for 20 minutes. 75µl of the purified cell lysates were mixed with 25µl 4×loading buffer and 10µl of the mixed sample was loaded and run on SDS-polyacrylamide gels (SDS-PAGE). Proteins were transferred on PVDF membranes (Roche) and the membranes were blocked in 5% non-fat milk dissolved in PBST (0.05% Tween-20 in PBS) at RT for 1 hour. The membranes were then incubated with the primary antibody at RT for 2 hours or at 4°C overnight, washed 5 times with PBST solution and then incubated with the secondary antibody for 1 hour. The signals were visualized by applying enhanced chemiluminescence (ECL) substrate (PerkinElmer).

3.11 Immunofluorescence

Vero cells were fixed in 4% paraformaldehyde in PBS for 1 hour, permeabilization with 0.4% Triton-X 100 in PBS for 10 min and blocked with BSA in PBS for 1 hour. Cells were stained with Filipin III (Sigma-Aldrich), mounted and visualised by fluorescent microscopy using equal exposure times.

3.12 Mitochondrial Functional Profile

Oxygen consumption rate (OCR), an indicator of mitochondrial respiration, was measured using a Seahorse Bioscience XF96 extracellular flux analyzer (Seahorse Bioscience). U87-MG cell lines were seeded in a XF96 microplate using complete growth media. The day before the assay, the cartridge sensor was hydrated overnight with Seahorse Bioscience XF96 Calibration Buffer at 37°C without CO_2. On the day of the assay, the growth medium was replaced with XF96 Assay medium and cells were incubated at 37°C in a non-CO_2 incubator

for 1h. OCR values were monitored under basal condition and measured after sequential injection of oligomycin (1 μM), FCCP (1 μM), and antimycin A/rotenone (0.5 μM) using XF Cell Mito Stress kit (Seahorse Bioscience). Mitochondrial function parameters were analyzed according to Seahorse Bioscience instructions.

Statistical Analysis

Data were analysed using GraphPad Prism7 software. Growth curves were normalized by taking the log10 of each measurement. P values of less than 0.05 were considered statistically significant.

Chapter 4 Results

This chapter presents the results and corresponding figures for this research project.

4. Interferons and ISGs

4.1 Type 1 Interferon strongly inhibits ZIKV replication

As an early response to viral infection, the production of type 1 IFNs by mammalian cells exerts potent antiviral activity. In mice, peripheral ZIKV replication is limited therefore, murine models of ZIKV pathogenesis require the absence of type 1 IFN signalling, highlighting the importance of early immune responses against viral infection [198]. Further evidence for the protective role of IFNs comes from studies showing that placental cells can resist ZIKV infection due to the production of IFN-λ, and the rapid production of type 1 IFN in astrocytes limits viral spread [178,201]. In light of these data, we set out to determine the sensitivity of the contemporary ZIKV isolate, PLCa1_ZV, to interferon-α in Huh-7.5 cells. We pre-treated Huh-7.5 cells with different concentrations of interferon-α (0, 1, 10, 100, 500 IU/ml) for 24 hr and infected cells with a MOI of 0.02. When Huh-7.5 cells were pre-treated with increasing concentrations of interferon-α and infected with ZIKV, viral titres significantly reduced, even with 1U/ml of IFN, **Figure 7A.**

At 500 U/ml interferon treatment, very few plaques are formed indicative of low viral titres, **Figure 7B**. Overall, the results show dose-dependent inhibition of ZIKV replication by pre-treatment with IFNα-2b in Huh-7.5 cells. This result is in consistence with previous studies showing that type I IFN inhibits Zika virus infection by upregulating ISGs [107,163].

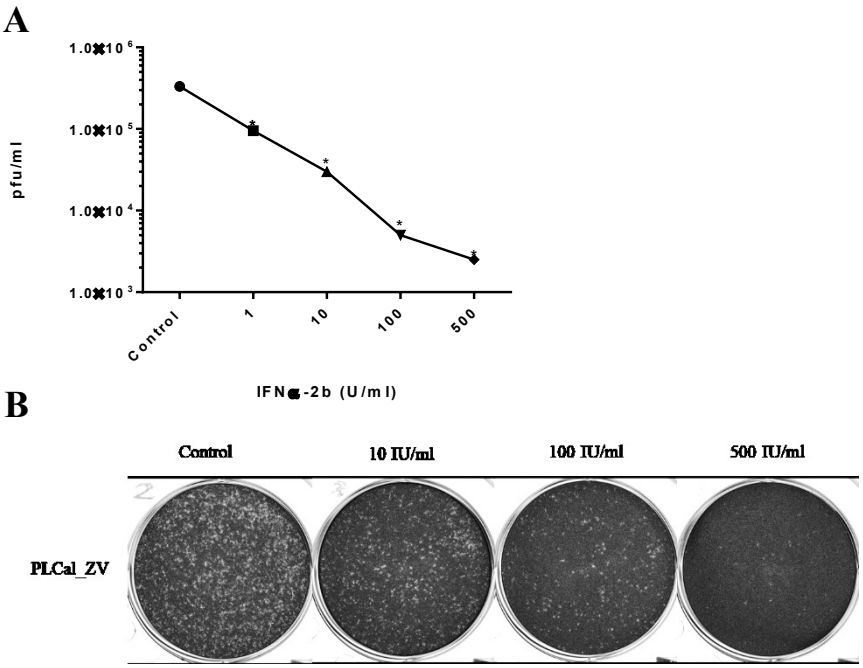

Figure 7 Type 1 IFN inhibits Zika virus replication in Huh-7.5 cells. (A) Following ZIKV (PLCal_ZV, MOI: 0.02) infection of Huh-7.5 cells, cell culture supernatants were collected at 48 h to determine viral titres by plaque assay. (B) Representative images of Vero cell plaque assays. Values represent mean ± SEM of at least three independent experiments. *$p < 0.05$.

4.2 ISGs as potent inhibitors of ZIKV replication

As we have shown that IFNα inhibits ZIKV replication in Huh-7.5 cells, we next wanted to identify which ISGs contribute to the inhibition of viral replication. In order to test this, we chose a panel of ISGs **(Table 3)** known for their ability to inhibit the replication of several important human viruses including, hepatitis C virus, West Nile virus, chikungunya virus and HIV-1 [146,202]. These ISGs act on different stages of the viral life cycle, from entry and replication to assembly and release: Tetherin, also known as

bone marrow stromal antigen 2 (BST2), is a lipid raft associated protein, is known to prevent virus release from infected cells [135]; Cytosolic DNA sensor cyclic GMP-AMP synthase, also known as MB21D1, broadly inhibits several RNA viruses [203]; Heparanase (HPSE) regulates the availability of heparan sulfate chains on the extracellular matrix and plays an integral role in regulating the life cycle of many viruses [204]; ZAP (Parp13.1) is an antiviral factor against RNA viruses which binds viral RNA and targets it for degradation by recruiting mRNA decay factors [205,206]; and IFI6, a minisatellite protein, has proposed functions in inhibiting apoptosis and preventing formation of virus-induced membrane invaginations [160,161].

To examine the effect of these ISGs on ZIKV, Huh-7.5 cells were transduced to overexpress a single flag-tagged ISG. Cells were kept in DMEM containing puromycin in order to maintain the expression of these ISGs. ISG expression levels were measured by flow cytometry or western blot**, Figure 8.** MxB and BST2 protein levels in lentivector transducing Huh-7.5 cells can be clearly seen by western blot. Huh-7.5 transduction with a lentivector resulted in about 90% of the cells expressing IFITM proteins and MxB, and 65% of the cells expressing MxA and ZAP (Parp13.1). For some of these ISGs (Parp4, Parp9, Parp13.2 and viperin) there was no flag expression after transduction and selection with puromycin, hence we did not continue further with these ISGs. Of note, even though we did not detect flag expression of IFI6 we continued on to infect with ZIKV as previous literature has described an antiviral effect of IFI6 specifically on flaviviruses (please refer to literature review).

Table 3 The characteristics of ISGs used in our study.

Gene	Targeted Viruses	Virus life cycle	Mechanism related to antiviral activity
C6orf150(MB21D1)	CHIKV, VEEV, WNV, YFV	Translation	unknown
HPSE	CHIKV, VEEV, WNV, YFV	unknown	unknown
IFI6	HCV, YFV	Replication	Inhibition of apoptosis & viral replication
IFITM1/2/3	DENV, HIV-1. SARS-CoV, VSV, WNV, YFV	entry	unknown (endocytic pathway?)
MXB	HIV-1, VSV, LACV	unknown	unknown
C19orf66 (RyDEN)	DENV	Translation?	unknown
BST2 (Tetherin)	DENV, HIV-1, HBV, CHIKV	Entry - Budding	Tethers budding virons on cell surface
ZAP (PARP13)	EBOV, FLUAV, retro viruses	Post-entry, translation	Target viral RNA, promote RIG signalling

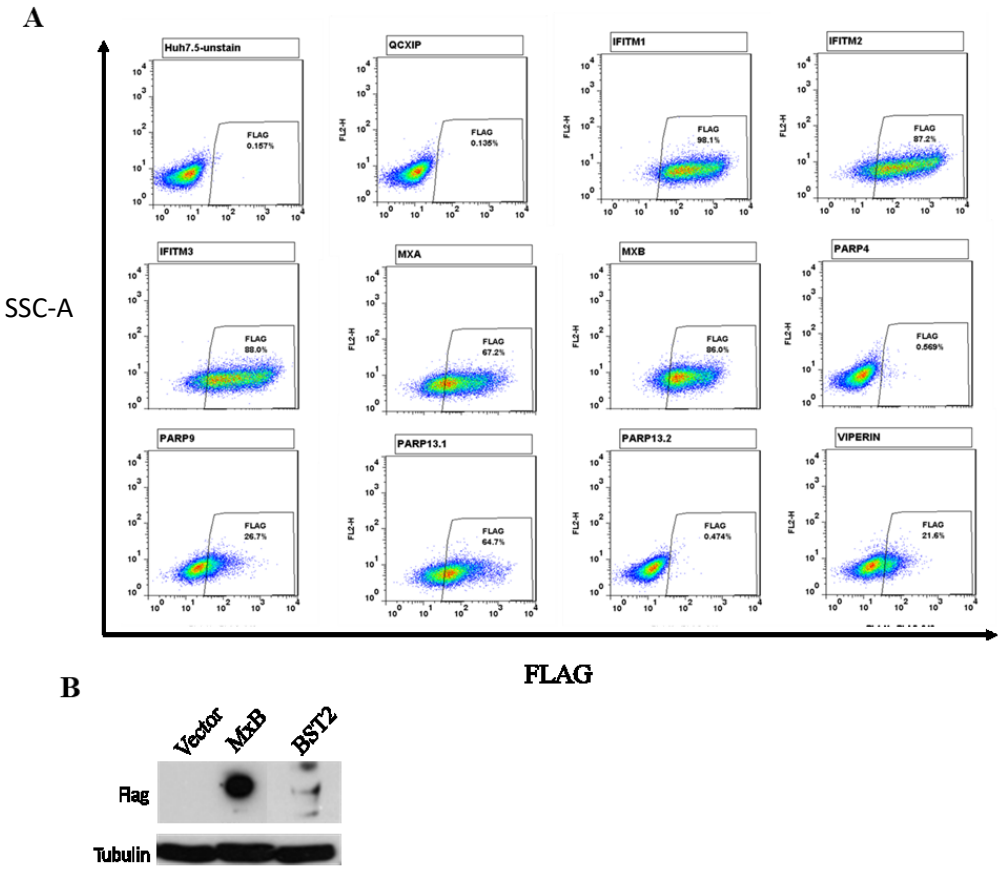

Figure 8 ISG expression levels in Huh-7.5 cells. Huh-7.5 cells were transduced with 500µl of retroviral vectors accommodating the genes of interest. 500µl blank vector was transduced in the control group. Cells were kept in DMEM with puromycin for selection. After two weeks of selection, the cells were harvested for flow cytometry (A) and western blot (B). Percentage of positive cells are indicated.

To investigate the potential antiviral effects of these ISGs on ZIKV replication, transduced Huh-7.5 cell lines were infected with ZIKV for 48 hrs or 72 hrs and viral titres were measured by plaque assay. At 48hrs post infection, in control cells (PQCXIP), productive ZIKV infection was established, with viral titres rising to 8.85 x 10^4 pfu/ml. The effect of BST2, MB21D1, HPSE, and ZAP (Parp13.1) on ZIKV replication 48hrs post infection was minimal, with only a maximum viral inhibition of 2-fold, **Figure 9A**. Interestingly, we see dramatic decrease in ZIKV titres (up to 10-fold inhibition) when IFI6, a type 1 ISG that plays a central role in regulating apoptosis and immunomodulation, is overexpressed in Huh-7.5 cells. Of note, we were unable to detect Flag-tagged IFI6 levels by western blot or flow cytometry, most likely due to the sensitivity of the Flag tag.

As overexpression of these ISGs, in particular IFI6, supressed ZIKV infection, we tested whether there would be any differences in the degree of antiviral activity at 72hrs post infection. At 72hrs post infection we see a modest increase in productive ZIKV infection as viral titres rise to 1.22833×10^5 in control cells. The antiviral effect of BST2, MB21D1, HPSE, and ZAP are increased at 72hrs post infection as the production of new infectious ZIKV virions continues to reduce. The robust antiviral effect of IFI6 seen 48hrs post infection is still maintained at 72hrs post infection. These data indicate that IFI6, a poorly characterized ISG, is necessary for a robust anti-ZIKV IFN response.

Furthermore, it has been recently reported that IFITM proteins, a family of broad-spectrum antiviral factors, inhibit ZIKV replication and ZIKV-induced cell death [107]. In particular, IFITM3 was shown to be the most potent restriction factor against ZIKV infection. Here, we confirmed these initial observations using transformed Huh-7.5 cells. In control cells, ZIKV establishes a productive infection, as seen by the high number of plaques forming units within 48hr of infection, **Figure 9B.** When IFITM3 is overexpressed, productive ZIKV infection is severely impaired, with up to a 20-fold inhibition of viral titres 48hr post infection. We show that IFITM3 is an essential component of the type-1 IFN response against ZIKV infection. Given the potent antiviral activity of IFITM3, we wanted to shine light upon the antiviral mechanism against ZIKV infection (discussed below).

Regarding the human Mx genes, MxA and MxB, the antiviral activity of MxB appears to be much more limited, known to be restricted to anti-HIV-1 activity. Recent research has elucidated the role of MxB in other viruses [125,134]. Nevertheless, much of our knowledge of the antiviral function of MxB has been limited to its inhibition of HIV-1 and a few SIV strains. As such, we sought to investigate the effect of MxB on ZIKV. We chose a panel of ZIKV isolates including: The African strain isolated in Uganda 1947 (MR766); an early Asian lineage strain, isolated from a Canadian traveller who returned from Thailand in 2013

(PLCal_ZV); and an Asian isolate from the 2015 outbreak in Puerto Rico (PRVABC-59). These strains isolated between 1947 to 2015 are spread across different geographical regions. Interestingly, our results show that while both the Asian isolates are resistant to MxB, the African isolate MR766 is somewhat susceptible to MxB inhibition, **Figure 9C.** This suggests that the main difference in susceptibility to MxB is between the African and Asian lineages. The ability of MxB to inhibit HIV-1 has been extensively studied, however little is known about the mechanisms beyond lentiviral restriction. Thus, we investigated the dependency of ZIKV on CypA.

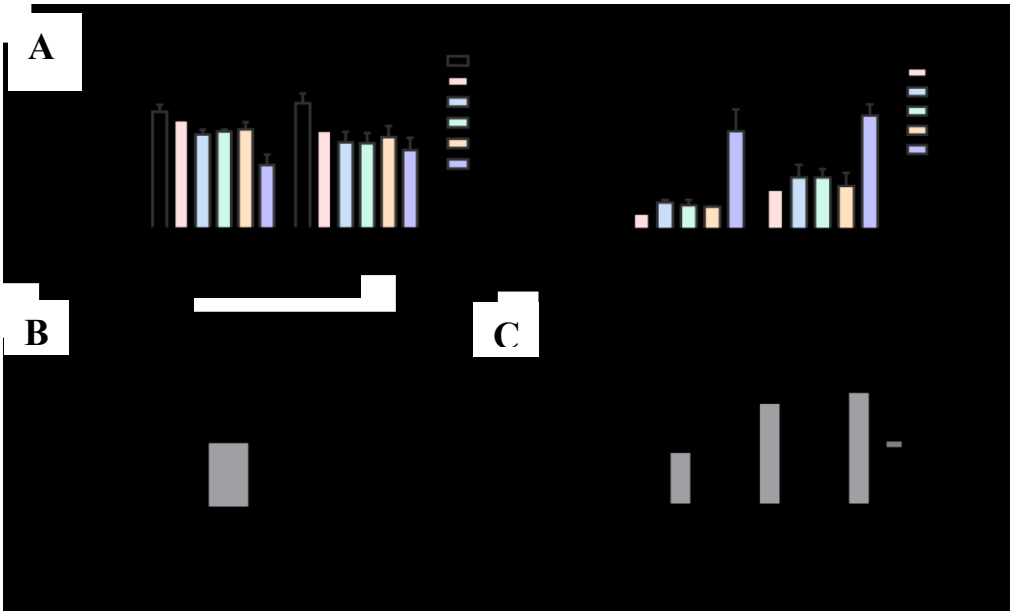

Figure 9 ZIKV infection in Huh-7.5 cells transduced with ISGs. IFITM3 and IFI6 are shown to drastically inhibit ZIKV replication. (A) Huh-7.5 cell lines overexpressing each of the ISGs were infected with ZIKV (PLCal_ZV, MOI: 0.02). Viral supernatants were harvested at the indicated time points and viral titres was quantified by plaque assay. (B) Huh-7.5 cells overexpressing IFITM3 were infected with ZIKV as described above. Viral titres were quantified by plaque assay 48hr post infection. (C) MxB moderately inhibits the historical African isolate MR766. ZIKV Huh-7.5 cells overexpressing MxB were infected with ZIKV (PLCal_ZV, PRVABC-59, MR766, MOI: 0.02) and viral titres were quantified by plaque assay 48hr post infection. The fold change of inhibition elicited by each ISG is presented on the right-hand side. These results shown are a summary of 3 independent experiments. $p^* < 0.05$

4.3 ZIKV is not dependent on CypA

It is known that MxB inhibition of HIV-1 is CypA dependent [122]. CypA is a ubiquitously distributed host peptidylprolyl isomerase that binds to cyclosporin A (CsA). It was previously found that depletion of CypA impairs MxB inhibition of HIV-1 [122]. Given that our previous results showed MxB inhibited ZIKV replication (African isolate), we wanted to investigate whether ZIKV was dependent to CypA. We treated Huh-7.5 cells with 5µM CsA for 24hrs and infected cells with three strains of ZIKV for 48hrs. An increase in viral titres for the African strain (approx. 15-fold increase), and not the Asian stains were observed, **Figure 10.** This may suggest that the historical African strain is susceptible to CypA. Interestingly, one study found that while CsA enhanced infection at 1µM compared to their control, at 10µM concentration CsA reduced the virus infection rate. As CsA is an FDA-approved drug it these results warrant further investigation into the use of CsA to treat ZIKV infection.

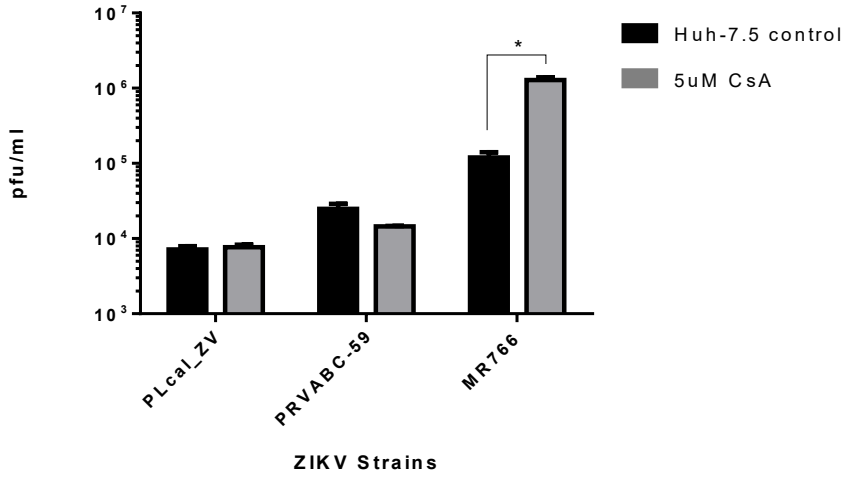

Figure 10 CsA increases viral titres for the African isolate. Huh-7.5 cells were treated with a CypA inhibitor, cyclosporine A (CsA, 5µM) for 24hr and infected with three ZIKV strains (MOI:0.02) for 48hr. Viral titres were quantified by plaque assay. These results shown are a summary of 3 independent experiments. $p^* < 0.05$

4.4 Cholesterol accumulation does not inhibit ZIKV replication

Cellular lipid membranes tightly regulate the entry of multiple viruses. Cholesterol is an essential component of lipid raft membranes. These lipid rafts are involved in entry, assembly and budding of a diverse number of viruses including, influenza A virus, HIV-1 and HSV [207,208]. As previously shown, IFITM3 exerts potent antiviral activity against ZIKV infection. One of the proposed mechanisms by which IFITM3 exerts its antiviral activity is through the interaction with vesicle-associated membrane protein (VAMP)-associated protein A (VAPA), resulting in substantial cholesterol accumulation in late endosomes and multi-vesicular bodies [209]. We, therefore, investigated whether ZIKV entry is differentially susceptible to inhibition by drugs that induce cholesterol accumulation. Several proteins are required for the function and maturation of late endosomes including, Niemann-Pick type C protein (NPC1). When NPC1 is dysfunction, cholesterol accumulates in late endosomes [210]. In order to test this, we treated Vero cells with three different drugs, at varying concentrations, which leads to the accumulation of cholesterol levels within late endosomes: a selective oestrogen receptor modulator, Clomiphene; a cationic amphiphilic drug Terconazole; and the Niemann-Pick type C protein inhibitor, U18666A. These drugs were shown to inhibit Ebola pseudovion entry in target cells [211,212]. To confirm these drugs were accumulating intracellular cholesterol levels, we stained with filipin III, a naturally fluorescent antibiotic which binds to unesterified (free) cholesterol. We can see a clear increase in cholesterol in Vero cells pre-treated (24hrs) with Clomiphene, Terconazole and U18666A compared to DMSO control. Our results show that while pre-treatment with Clomiphene at 0.01 μg/ml increased viral titres by 1.5-fold, concentrations of 0.1 and 1 μg/ml resulted in viral titres similar to that of DMSO control cells, **Figure 11A.** Pre-treatment with increasing concentrations of terconazole had very minimal effect on viral titres, **Figure 11B**. Fascinatingly, cells pre-treated with increasing the concentration of U8166A led to a gradual

decrease in ZIKV titres, resulting in an overall 2-fold decrease by 0.5 µg/ml of U81666A, **Figure 11C**. Taken together, these results suggest an alternative mechanism for IFITM3 mediated ZIKV inhibition, considering the degree of IFITM3-mediated ZIKV inhibition, and that increasing intracellular levels of cholesterol had no major effect.

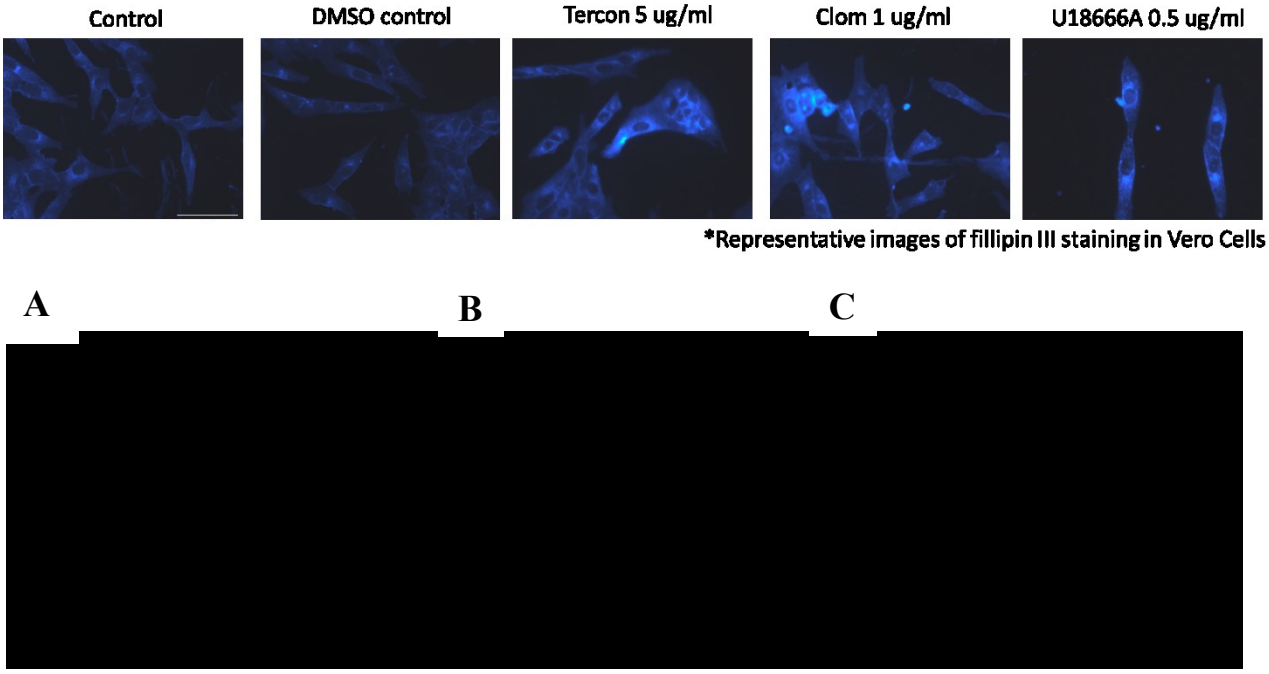

Figure 11 Addition of drugs resulting in intracellular cholesterol accumulation show no effect on ZIKV replication, despite the modest effect seen by U18666A. The top panel shows Filipin III staining of Vero cells pre-treated with three drugs for 24hr. (A-C) Vero cells were pre-treated with increasing clomiphene, terconazole and U18666A concentrations for 24hr, and subsequently infected with ZIKV (PLCal_ZV, MOI: 0.02). Viral titres were measured by plaque assay. These results shown are a summary of 3 independent experiments.

4.5 U87-MG cells ectopically expressing ZIKV NS4B display decreased mitochondrial respiration

The positive-sense-single stranded RNA genome of 10.8 kb encodes for 10 viral proteins. This limited coding capacity makes ZIKV fully dependent on the host for its life cycle. Like other viruses, ZIKV needs to induce large-scale alterations in host cell metabolism for its own replication. A recent report showed that ZIKV NS4A and NS4B inhibit Akt-mTOR signalling which results in an increase in autophagy [29]. The Akt-mTOR signalling pathway has broad

cellular effects with regard to cell proliferation and survival; it is also an important mediator of metabolism. Furthermore, there is increasing evidence that Dengue virus alters mitochondrial morphology resulting in elongated mitochondria, which has a direct effect on mitochondrial function, namely oxidative phosphorylation [88,194]. The researchers also suggested that ZIKV infection induces similar mitochondrial elongation. Given these reports, we wanted to assess whether ZIKV NS4A and NS4B affect the function of mitochondria. To analyse the function of ZIKV NS4A and NS4B, we transduced U87-MG cells (human primary glioblastoma cell line) with lentivirus containing each flag-tagged ZIKV gene (strain: PLCal_ZV) and examined for changes in mitochondrial function. Expression of ZIKV NS4A and NS4B was detected by western blotting and flow cytometry, **Figure 12.** The flow cytometry results show almost 100% flag expression in U87-MG cells expressing NS4A or NS4B, corresponding with proteins levels observed by western blot. In U87-MG cells transduced with both NS4A and NS4B we clearly see flag protein expression by western blot.

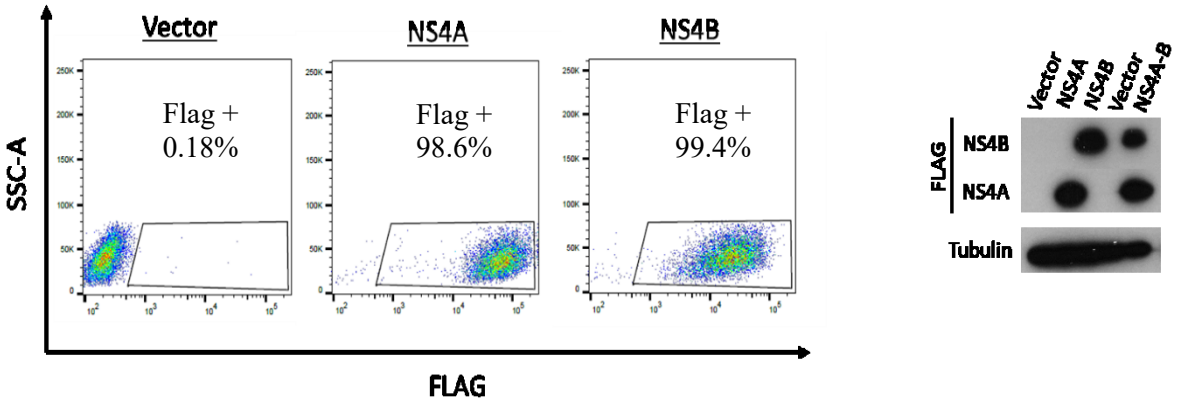

Figure 12 The expression of ZIKV NS4A and NS4B in U87-MG cells. U87-MG cells were transduced with 500µl NS4A and/or NS4B retroviral vectors. 500µl of blank vector was transduced for the control. Cells were kept in EMEM/10% FBS containing puromycin (2µg/mL) for individual expressions of ZIKV gene, or puromycin with hygromycin (200µg/mL) for the co-expression of NS4A-NS4B. The percentage of ZIKV protein-expressing cells were measured by flow cytometry and protein levels were measured by western blot using an anti-flag antibody.

Mitochondria are key organelles involved in energy metabolism and antiviral sensing. To assess whether ZIKV NS4A and/or NS4B affect the function of mitochondria, the OCR was measured in control U87-MG cells and NS4A and NS4B expressing cells. Sequential addition of mitochondria perturbing agents, oligomycin, FCCP and rotenone/antimycin A to the cell culture medium was used to assess the function of each complex of the electron transport chain, **Figure 13A.** When compared to the control, expression of NS4B decreased the mitochondrial respiration rate, a measure of oxidative phosphorylation. The addition of oligomycin, which inhibits ATP synthase (Complex V), demonstrated that ATP-linked production was significantly decreased in NS4B expressing cells, as compared with control cells, but proton leak was unchanged. Upon the addition of FCCP, which forces the mitochondria to work at maximum efficiency, we see similar rates of oxygen consumption of control cells and NS4A cells, however maximal mitochondrial respiration in NS4B cells is significantly lower, indicating decreased mitochondrial efficiency, **Figure 13A-B.** Co-expression of NS4A and NS4B in U87-MG cells resulted in the decrease of ATP production and the basal respiration rate, similar to that observed for U87-MG cells expressing NS4B, **Figure 14A-B**. Interestingly, we see no significant change in the rate of maximal respiration however, a substantial decrease in proton leak is observed **Figure 14B**. Overall, these results indicate that U87-MG cells expressing NS4B decrease the efficiency of the mitochondria, resulting in reduced basal respiration and less ATP production when compared to control cells.

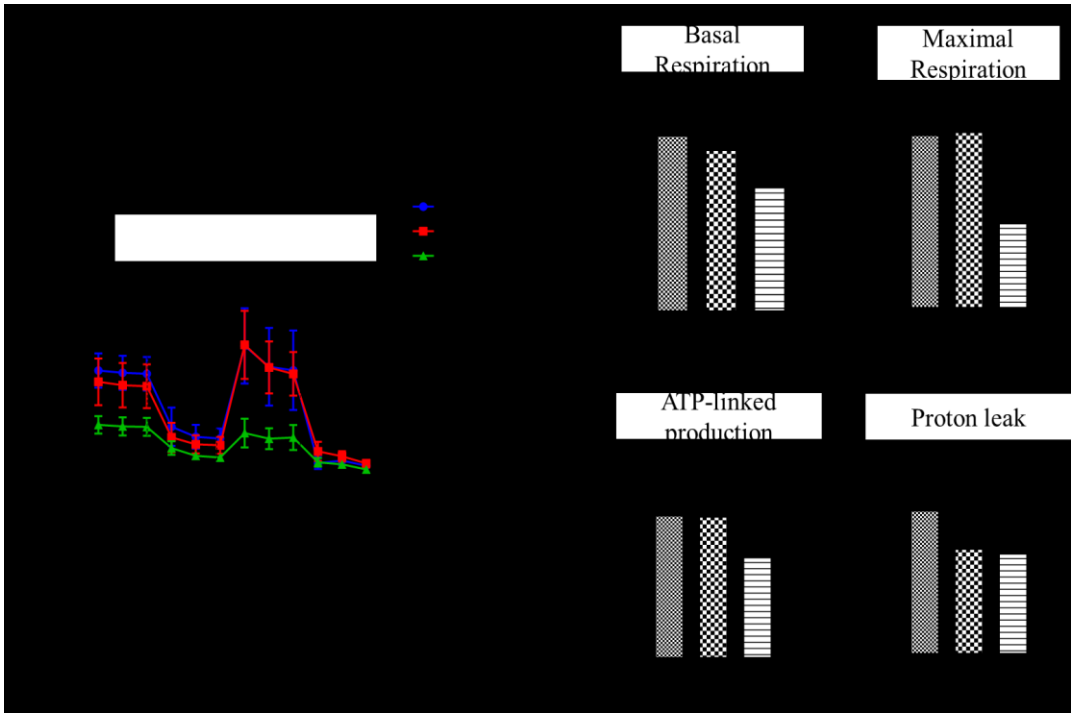

Figure 13 ZIKV NS4B decreases mitochondrial respiration. (A) Oxygen consumption rate (OCR) in U87-MG cells expressing ZIKV NS4A or NS4B was measured in real time under basal conditions and in response to injections of oligomycin (1µM), FCCP (1µM) and antimycin A/rotenone (1µM) using XF96 Seahorse analyser. Data were normalized by protein concentration. (B) Mitochondrial functional parameters were analysed according to Seahorse Bioscience instructions. Data are shown as mean +/- SD and analysed with One-way ANOVA.

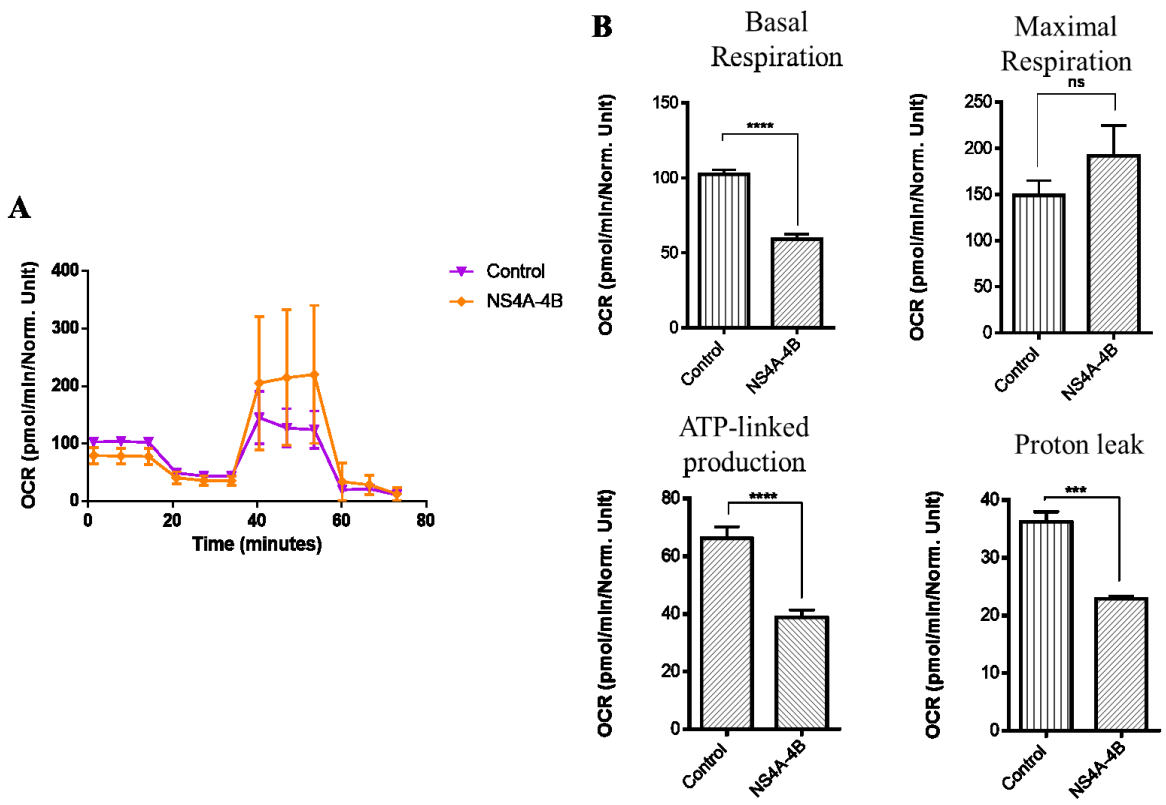

Figure 14 Co-expression of NS4A-NS4B results in decreased mitochondrial respiration. (A) Oxygen consumption rate (OCR) in U87-MG cells expressing ZIKV NS4A or NS4B was measured in real time under basal conditions and in response to injections of oligomycin (1µM), FCCP (1µM) and antimycin A/rotenone (1µM) using XF96 Seahorse analyser. Data were normalized by protein concentration. (B) Mitochondrial functional parameters were analysed according to Seahorse Bioscience instructions. Data are shown as mean +/- SD and analysed with Student t-test.

Chapter 5 Discussion and Conclusion

The first part of this chapter discusses the results regarding the innate immune response against ZIKV infection. The second part of this chapter discusses the results in the context of ZIKV host dependency on metabolism. The reader is encouraged to refer to Chapter 1 for more information on the interferon response and host metabolism. Following the discussion, the conclusion summarises the key findings and the possibility of therapeutic intervention is explored.

5.1 Investigating the effect of ISGs on ZIKV replication

The recent expansion of ZIKV represents an emerging global health threat, with the most severe effects observed in resource-limited areas. Certainly, congenital abnormalities and neurological disorders have triggered an increasing interest in ZIKV. The relative contribution of ISGs to the innate antiviral immune response may vary among viral species and tissue cell types. Therefore, it is important to gain an understanding of which ISGs participate in restricting ZIKV infection. Towards this goal, we have tested the effect of several antiviral proteins on ZIKV replication.

As a first step toward understanding the innate immune response elicited against ZIKV, we determined the sensitivity of ZIKV to IFN-mediated antiviral signalling. Huh-7.5 cells were treated with increasing doses of IFN-α prior to infection and then replication was assessed, **Figure 7.** Our results showed that ZIKV was highly sensitive to pre-treatment with IFN-α. These results are consistent with previous observations with other flaviviruses including ZIKV and DENV [31,163,213]. Despite the sensitivity of ZIKV to IFN-α, ZIKV replication is only moderately affected by IFN-λ [163]. Therefore, the pattern of ISG induction will be triggered by type-1 IFN subtypes.

Subsequently, we ectopically expressed the following ISGs: MB21D1, HPSE, IFI6, IFITM3, MXB, RyDEN, TETHERIN and Parp13.1/2 (ZAP) to investigate the effect of these proteins on ZIKV replication in the human hepatocyte derived carcinoma (Huh-7.5) cell line (Figure). We used the Asian lineage isolate (PLCal_ZV) and compared viral titres at two time points (48-hour post infection and 72-hour post infection). Interestingly, among the ISGs we tested our study revealed IFI6 as a key effector of the IFN response to ZIKV infection. Time courses (48 h.p.i and 72 h.p.i) revealed potent and sustained IFI6-mediated inhibition of ZIKV. We were unable to detect the overexpression of IFI6 by flow cytometry, this could very well be due to the sensitivity of the system (we attached Flag not 3X flag to the C-

terminus of IFI6). To confirm the anti-ZIKV activity of IFI6, the infectivity of cells could be monitored by fluorescence-activated cell sorting (FACS) and the suppression of viral replication could be observed by staining for double-stranded RNA (dsRNA) replication intermediates.

The antiviral role and function of IFI6 has only recently begun to emerge. *IFI6* encodes a 130 amino acid protein in the IFI-6-16 family and is only present in higher-order mammals [155]. Within this family, only IFI6 and IFI27 are IFN inducible [214]. A current perspective on the antiviral mechanism of IFI6 suggests that IFI6 is an ER integral membrane protein which prevents the formation of virus-induced ER membrane invaginations, however having little effect on viruses which form ER-derived double-membrane vesicles, for example HCV [160]. Conversely, other groups have reported that IFI6 inhibits HCV and via alternate mechanisms [162,215]. Reports have suggested that IFI6 is a mitochondria-targeted protein and inhibits HCV infection by inhibiting the function of EGFR kinase [156,162]. Future studies will be needed to verify the localisation of IFI6 and expand on how viruses are able to overcome IFI6 mediated inhibition for productive replication.

Before the reveal of the anti-HIV-1 activity of MxB, the protein was believed to be non-antiviral over the decades. Only recently has the antiviral role of MxB been extended beyond retroviruses, we now know that MxB is a key restriction factor for human herpesviruses and HCV [125,134,216]. Thus, we investigated the effect of MxB on three different ZIKV isolates, one isolate from the 2015-2016 in Puetro Rico (PRVABC-59), an earlier Asian lineage isolate (PLCal_ZV), and the historical Uganda 1947 isolate (MR766). We found no significant difference in viral titres between the contemporary strains however, the African isolate is slightly susceptible to MxB inhibition, **Figure 9C.** This may reflect the differences between the pre-epidemic and epidemic strains however, future studies on pre-epidemic strains and epidemic strains would be needed to confirm if this phenotype is due to

differences between viral genomes. Interestingly, a recent paper showed that cyclophilin A (CypA) dependent viruses, HCV, DENV and JEV, were inhibited by MxB [216]. CypA is a peptidyl prolyl isomerase which is recruited by HIV-1 and HCV as a co-factor to promote viral infection [217,218]. The depletion of CypA or treatment with Cyclosporin A (CsA), an inhibitor of CypA, alleviates MxB inhibition of HIV-1 and HCV [122]. This, we set out to test whether Zika Virus was dependent on CypA. We noticed that the two Asian stains resist CsA which may imply a potential link between viral dependence on CypA, **Figure 10.** Surprisingly, viral titres of the African strain are significantly increased with pre-treatment of CsA. It should be noted that this finding is contradictory by investigations suggesting that CsA is had antiviral effects on several viruses including, HSV, vaccinia virus, HIV-1 and HCV [219-222]. Nevertheless, this could indicate that the African isolate is not dependent on CypA or that CypA hinders productive viral production.

To further understand the role of IFITM3 during ZIKV infection we explored one potential mechanism proposed for IFITM3-mediated viral inhibition. This is of particular interest because it has been published that IFITM3 can prevent ZIKV-induced cell death by restricting viral access [107,111]. In accordance with these reports, we show that IFITM3 potently inhibits ZIKV replication, **Figure 9B.** Of interest, a rare single nucleotide polymorphism (SNP rs12252-C) allele of IFITM3 is predicted to form a truncated version of IFITM3, which has been associated with severe clinical outcomes for patients with IAV infection [223,224]. Given this, it would be worth investigating the severity of ZIKV infection with polymorphisms in *IFITM* genes.

The exact mechanism of IFITM-mediated ZIKV restriction is unknown; however, results from experimental approaches suggest two possibilities: IFITMs alter properties of the endosomal lumina thereby restricting viral fusion [225-227] or that IFITMs alter physical properties of cellular membranes to prevent the formation of fusion pores

[209,228]. In line with the theory that IFITMs induce the accumulation of cholesterol in the endosomal membrane, we treated Huh-7.5 cells with the compound's clomiphene, terconazole, and U18666A and measured viral titres. Clomiphene and terconazole are cationic amphiphilic drugs which induce cholesterol accumulation in late endosomes. U18666A is an inhibitor of lysosomal cholesterol export by directly inhibiting the NPC1 protein. Our results showed that these three drugs had no significant effect in reducing ZIKV titres even with pre-treatment at higher concentrations, **Figure 11**. It is tempting to speculate that cholesterol accumulation has no effect on ZIKV infection. However, it must be noted that viral entry was not directly measured and the mechanism behind IFITM-mediated ZIKV inhibition has not been thoroughly investigated to date.

5.2 Exploring the role of ZIKV NS4A and NS4B on mitochondrial metabolism

The virus life cycle is a dynamic process that involves different organelles. Viruses are completely dependent on host metabolic functions for efficient replication, and hence all viruses manipulate metabolic pathways, but to varying degrees. Mitochondria actively participate in the viral replication process. In addition to meeting the cellular energy demand, mitochondria serve as an antiviral signalling platform and contribute to the activation of apoptosis and cell death.

Previous reports have established that viruses change mitochondrial bioenergetics and induce functional alterations to drive replication. Although these reports signify the importance of the mitochondria during viral infection, the underlying mechanisms that link mitochondria to ZIKV replication in cells is poorly defined. Here, we investigated the role of ZIKV proteins NS4A and NS4B on mitochondrial respiration. These two proteins are involved in viral replication and are known to inhibit neurogenesis and induce autophagy via Akt-mTOR signalling [29]. Moreover, NS4A has been recently discovered to antagonize the

RLR-mitochondrial antiviral-signalling protein (MAVS) interaction and subsequent induction of the immune response [62]. Given these reports, we ectopically expressed flag-tagged ZIKV NS4A and NS4B in U98-MG cells (**Figure 12)** and measured key parameters of mitochondrial function. Our results indicate that ZIKV NS4B decreases mitochondrial respiration associated with ATP production with little to no change in protein leak, **Figure 13**. Co-expression of NS4A and NS4B lead to a similar decrease in mitochondrial respiration as observed with NS4B expression alone, **Figure 14B**. Our data show a significant decrease in proton leak when cells are expressing both viral proteins, reflective of the decrease in the rate of oxygen consumption. These findings suggest that ZIKV NS4B decreases mitochondrial energy production, which is in accordance with previous observations indicating depression of oxidative phosphorylation by other members of the Flaviviridae family, for example HCV [229]. It would be interesting to investigate whether the decrease in mitochondria-linked ATP production is compensated by an increase in glycolysis. This would not be surprising considering many viruses are known to induce a metabolic shift form oxidative phosphorylation to glycolysis [199].

Particularly for ZIKV, a change in mitochondrial function not only reflects a decrease in cellular energy production but it can also contribute to neurological disorders, notably the progression of microcephaly. Mitochondrial dysfunction has been associated with many diseases, particularly neurodegenerative disorders [230]. New research has demonstrated the importance of mitochondria and metabolism in the regulation of stem cell function and differentiation, including neural stem cells [231]. The adult mammalian brain contains self-renewable, multipotent stem cells that are critical for neurogenesis and plasticity [232,233]. Neural stem cells give rise to neurons, astrocytes and oligodendrocytes. Stem cells are predominantly glycolytic in nature and as they commit and differentiate, there is a metabolic switch leading to the activation of oxidative phosphorylation. Of note, this reliance towards

glycolysis is not due to the presence of dysfunctional mitochondria [234]. Stem cells with a defect in oxidative phosphorylation are unable to undergo differentiation. It is well known that ZIKV infects and inhibits neural stem cell differentiation [29,40,41,235]. ZIKV potentiates human neural stem cell growth inhibition and strongly induces death in early differentiating cells [236]. Li and colleagues showed that ZIKV infection of neural stem cells led to attenuated neural progenitor cell expansion through virally induced apoptosis and cell-cycle dysregulation [235]. Thus, it is reasonable to hypothesise that ZIKV NS4B may induce alterations to mitochondrial metabolism in stem cells and shed light onto the neurodegenerative disorders linked with the virus. Future studies should now be aimed at elucidating the underlying mechanisms behind NS4B mediated repression of mitochondrial respiration. While viral proteins play key roles in deterring the host immune response, we must not neglect the effect of these proteins on mitochondrial dysfunction which could aid in understanding the progression of ZIKV associated birth defects. Given that mitochondria are vital in the process of neural development and regeneration, this places it as an attractive therapeutic target. Diseases defined by mitochondrial dysfunction has led to the search for new therapeutic and preventive strategies targeting mitochondrial function, reviewed by Sorrentino and colleagues [237]. Whether such treatments could be transferred to treating ZIKV infected cells remains to be seen, nevertheless exploiting key components of mitochondrial metabolism and dynamics represents a novel treatment strategy for improving stem cell function.

Conclusion

The sudden emergence and spread of ZIKV along with the unexpected neurological complications, have made ZIKV a public health emergency of international concern. As a poorly defined virus with no antiviral treatments or vaccines, scientific research is critical.

Our study centres on understanding host -virus interactions which ultimately determine the outcome of infection. Our data suggests that upregulation of the interferon stimulated genes, IFITM3 and IFI6 strongly supresses ZIKV replication. We found that IFITM3-mediated ZIKV inhibition was not via the accumulation of intracellular cholesterol levels. Further research is needed to identify the underlying mechanism of IFITM3 antiviral activity.

The second part of the study investigated the role of ZIKV non-structural proteins on mitochondrial respiration. We show that ZIKV NS4B decreases mitochondrial respiration and ATP production. This work can be translated to examine the effect of NS4B in neural progenitor cells, which will be clinically relevant given the association of ZIKV with devastating birth defects. Taken together, this work advances our understanding of the host response to ZIKV infection and provides a foundation for understanding the consequences of mitochondrial dysfunction.

References

1. Dick, G.W.A.; Kitchen, S.F.; Haddow, A.J. Zika virus (i). Isolations and serological specificity. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **1952**, *46*, 509-520.
2. Dick, G.W.A. Zika virus (ii). Pathogenicity and physical properties. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **1952**, *46*, 521-534.
3. Simpson, D.I. Zika virus infection in man. *Trans R Soc Trop Med Hyg* **1964**, *58*, 335-338.
4. Filipe, A.R.; Martins, C.M.; Rocha, H. Laboratory infection with zika virus after vaccination against yellow fever. *Archiv fur die gesamte Virusforschung* **1973**, *43*, 315-319.
5. Duffy , M.R.; Chen , T.-H.; Hancock , W.T.; Powers , A.M.; Kool , J.L.; Lanciotti , R.S.; Pretrick , M.; Marfel , M.; Holzbauer , S.; Dubray , C.*, et al.* Zika virus outbreak on yap island, federated states of micronesia. *New England Journal of Medicine* **2009**, *360*, 2536-2543.
6. Musso, D.; Nilles, E.J.; Cao-Lormeau, V.M. Rapid spread of emerging zika virus in the pacific area. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **2014**, *20*, O595-596.
7. Cao-Lormeau, V.M.; Roche, C.; Teissier, A.; Robin, E.; Berry, A.L.; Mallet, H.P.; Sall, A.A.; Musso, D. Zika virus, french polynesia, south pacific, 2013. *Emerging infectious diseases* **2014**, *20*, 1085-1086.
8. Metsky, H.C.; Matranga, C.B.; Wohl, S.; Schaffner, S.F.; Freije, C.A.; Winnicki, S.M.; West, K.; Qu, J.; Baniecki, M.L.; Gladden-Young, A.*, et al.* Zika virus evolution and spread in the americas. *Nature* **2017**, *546*, 411-415.
9. Faria, N.R.; Quick, J.; Claro, I.M.; Theze, J.; de Jesus, J.G.; Giovanetti, M.; Kraemer, M.U.G.; Hill, S.C.; Black, A.; da Costa, A.C.*, et al.* Establishment and cryptic transmission of zika virus in brazil and the americas. *Nature* **2017**, *546*, 406-410.
10. Grubaugh, N.D.; Ladner, J.T.; Kraemer, M.U.G.; Dudas, G.; Tan, A.L.; Gangavarapu, K.; Wiley, M.R.; White, S.; Theze, J.; Magnani, D.M.*, et al.* Genomic epidemiology reveals multiple introductions of zika virus into the united states. *Nature* **2017**, *546*, 401-405.

11. Haddow, A.D.; Schuh, A.J.; Yasuda, C.Y.; Kasper, M.R.; Heang, V.; Huy, R.; Guzman, H.; Tesh, R.B.; Weaver, S.C. Genetic characterization of zika virus strains: Geographic expansion of the asian lineage. *PLOS Neglected Tropical Diseases* **2012**, *6*, e1477.
12. Faria, N.R.; Azevedo, R.d.S.d.S.; Kraemer, M.U.G.; Souza, R.; Cunha, M.S.; Hill, S.C.; Thézé, J.; Bonsall, M.B.; Bowden, T.A.; Rissanen, I.*, et al.* Zika virus in the americas: Early epidemiological and genetic findings. *Science* **2016**, *352*, 345.
13. Shen, S.; Shi, J.; Wang, J.; Tang, S.; Wang, H.; Hu, Z.; Deng, F. Phylogenetic analysis revealed the central roles of two african countries in the evolution and worldwide spread of zika virus. *Virologica Sinica* **2016**, *31*, 118-130.
14. Ye, Q.; Liu, Z.-Y.; Han, J.-F.; Jiang, T.; Li, X.-F.; Qin, C.-F. Genomic characterization and phylogenetic analysis of zika virus circulating in the americas. *Infection, Genetics and Evolution* **2016**, *43*, 43-49.
15. Gupta, A.K.; Kaur, K.; Rajput, A.; Dhanda, S.K.; Sehgal, M.; Khan, M.S.; Monga, I.; Dar, S.A.; Singh, S.; Nagpal, G.*, et al.* Zikavr: An integrated zika virus resource for genomics, proteomics, phylogenetic and therapeutic analysis. *Scientific Reports* **2016**, *6*, 32713.
16. Wang, L.; Valderramos, Stephanie G.; Wu, A.; Ouyang, S.; Li, C.; Brasil, P.; Bonaldo, M.; Coates, T.; Nielsen-Saines, K.; Jiang, T.*, et al.* From mosquitos to humans: Genetic evolution of zika virus. *Cell Host & Microbe* **2016**, *19*, 561-565.
17. Rajah, M.M.; Pardy, R.D.; Condotta, S.A.; Richer, M.J.; Sagan, S.M. Zika virus: Emergence, phylogenetics, challenges, and opportunities. *ACS Infectious Diseases* **2016**, *2*, 763-772.
18. Cauchemez, S.; Besnard, M.; Bompard, P.; Dub, T.; Guillemette-Artur, P.; Eyrolle-Guignot, D.; Salje, H.; Van Kerkhove, M.D.; Abadie, V.; Garel, C.*, et al.* Association between zika virus and microcephaly in french polynesia, 2013–15: A retrospective study. *The Lancet* **2016**, *387*, 2125-2132.
19. Musso, D.; Nhan, T.; Robin, E.; Roche, C.; Bierlaire, D.; Zisou, K.; Shan Yan, A.; Cao-Lormeau, V.M.; Broult, J. Potential for zika virus transmission through blood transfusion demonstrated during an outbreak in french polynesia, november 2013 to february 2014. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* **2014**, *19*.

20. Foy, B.D.; Kobylinski, K.C.; Chilson Foy, J.L.; Blitvich, B.J.; Travassos da Rosa, A.; Haddow, A.D.; Lanciotti, R.S.; Tesh, R.B. Probable non-vector-borne transmission of zika virus, colorado, USA. *Emerging infectious diseases* **2011**, *17*, 880-882.
21. McDonald, E.M.; Duggal, N.K.; Brault, A.C. Pathogenesis and sexual transmission of spondweni and zika viruses. *PLoS Negl Trop Dis* **2017**, *11*, e0005990.
22. Paz-Bailey, G.; Rosenberg, E.S.; Doyle, K.; Munoz-Jordan, J.; Santiago, G.A.; Klein, L.; Perez-Padilla, J.; Medina, F.A.; Waterman, S.H.; Gubern, C.G.*, et al.* Persistence of zika virus in body fluids — preliminary report. *New England Journal of Medicine 0*, null.
23. Musso, D.; Richard, V.; Teissier, A.; Stone, M.; Lanteri, M.C.; Latoni, G.; Alsina, J.; Reik, R.; Busch, M.P. Detection of zika virus rna in semen of asymptomatic blood donors. *Clinical Microbiology and Infection* **2017**, *23*, 1001.e1001-1001.e1003.
24. Sheng, Z.Y.; Gao, N.; Wang, Z.Y.; Cui, X.Y.; Zhou, D.S.; Fan, D.Y.; Chen, H.; Wang, P.G.; An, J. Sertoli cells are susceptible to zikv infection in mouse testis. *Frontiers in cellular and infection microbiology* **2017**, *7*, 272.
25. Duggal, N.K.; McDonald, E.M.; Ritter, J.M.; Brault, A.C. Sexual transmission of zika virus enhances in utero transmission in a mouse model. *Scientific Reports* **2018**, *8*, 4510.
26. Mlakar, J.; Korva, M.; Tul, N.; Popović, M.; Poljšak-Prijatelj, M.; Mraz, J.; Kolenc, M.; Resman Rus, K.; Vesnaver Vipotnik, T.; Fabjan Vodušek, V.*, et al.* Zika virus associated with microcephaly. *New England Journal of Medicine* **2016**, *374*, 951-958.
27. França, G.V.A.; Schuler-Faccini, L.; Oliveira, W.K.; Henriques, C.M.P.; Carmo, E.H.; Pedi, V.D.; Nunes, M.L.; Castro, M.C.; Serruya, S.; Silveira, M.F.*, et al.* Congenital zika virus syndrome in brazil: A case series of the first 1501 livebirths with complete investigation. *The Lancet* **2016**, *388*, 891-897.
28. Aagaard, K.M.; Lahon, A.; Suter, M.A.; Arya, R.P.; Seferovic, M.D.; Vogt, M.B.; Hu, M.; Stossi, F.; Mancini, M.A.; Harris, R.A.*, et al.* Primary human placental trophoblasts are permissive for zika virus (zikv) replication. *Scientific Reports* **2017**, *7*, 41389.
29. Quicke, K.M.; Bowen, J.R.; Johnson, E.L.; McDonald, C.E.; Ma, H.; O'Neal, J.T.; Rajakumar, A.; Wrammert, J.; Rimawi, B.H.; Pulendran, B.*, et al.* Zika virus infects human placental macrophages. *Cell host & microbe* **2016**, *20*, 83-90.
30. Sotelo, J.R.; Sotelo, A.B.; Sotelo, F.J.B.; Doi, A.M.; Pinho, J.R.R.; Oliveira, R.d.C.; Bezerra, A.M.P.S.; Deutsch, A.D.; Villas-Boas, L.S.; Felix, A.C.*, et al.* Persistence of

zika virus in breast milk after infection in late stage of pregnancy. *Emerging infectious diseases* **2017**, *23*, 854-856.

31. Hamel, R.; Dejarnac, O.; Wichit, S.; Ekchariyawat, P.; Neyret, A.; Luplertlop, N.; Perera-Lecoin, M.; Surasombatpattana, P.; Talignani, L.; Thomas, F.*, et al.* Biology of zika virus infection in human skin cells. *Journal of Virology* **2015**, *89*, 8880-8896.
32. Elisabeth, R.K.-L.; Brad, J.B.; Staples, J.E. Estimated incubation period for zika virus disease. *Emerging Infectious Disease journal* **2017**, *23*, 841.
33. Petersen, E.; Wilson, M.E.; Touch, S.; McCloskey, B.; Mwaba, P.; Bates, M.; Dar, O.; Mattes, F.; Kidd, M.; Ippolito, G.*, et al.* Rapid spread of zika virus in the americas--implications for public health preparedness for mass gatherings at the 2016 brazil olympic games. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases* **2016**, *44*, 11-15.
34. Chang, C.; Ortiz, K.; Ansari, A.; Gershwin, M.E. The zika outbreak of the 21st century. *Journal of autoimmunity* **2016**, *68*, 1-13.
35. Freour, T.; Mirallie, S.; Hubert, B.; Splingart, C.; Barriere, P.; Maquart, M.; Leparc-Goffart, I. Sexual transmission of zika virus in an entirely asymptomatic couple returning from a zika epidemic area, france, april 2016. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin* **2016**, *21*.
36. Honein, M.A.; Dawson, A.L.; Petersen, E.E.; Jones, A.M.; Lee, E.H.; Yazdy, M.M.; Ahmad, N.; Macdonald, J.; Evert, N.; Bingham, A.*, et al.* Birth defects among fetuses and infants of us women with evidence of possible zika virus infection during pregnancy. *Jama* **2017**, *317*, 59-68.
37. Kleber de Oliveira, W.; Cortez-Escalante, J.; De Oliveira, W.T.; do Carmo, G.M.; Henriques, C.M.; Coelho, G.E.; Araujo de Franca, G.V. Increase in reported prevalence of microcephaly in infants born to women living in areas with confirmed zika virus transmission during the first trimester of pregnancy - brazil, 2015. *MMWR. Morbidity and mortality weekly report* **2016**, *65*, 242-247.
38. Calvet, G.; Aguiar, R.S.; Melo, A.S.O.; Sampaio, S.A.; de Filippis, I.; Fabri, A.; Araujo, E.S.M.; de Sequeira, P.C.; de Mendonça, M.C.L.; de Oliveira, L.*, et al.* Detection and sequencing of zika virus from amniotic fluid of fetuses with microcephaly in brazil: A case study. *The Lancet Infectious Diseases* **2016**, *16*, 653-660.

39. Cordeiro, M.T.; Pena, L.J.; Brito, C.A.; Gil, L.H.; Marques, E.T. Positive igm for zika virus in the cerebrospinal fluid of 30 neonates with microcephaly in brazil. *The Lancet* **2016**, *387*, 1811-1812.
40. Tang, H.; Hammack, C.; Ogden, S.C.; Wen, Z.; Qian, X.; Li, Y.; Yao, B.; Shin, J.; Zhang, F.; Lee, E.M.*, et al.* Zika virus infects human cortical neural progenitors and attenuates their growth. *Cell stem cell* **2016**, *18*, 587-590.
41. Gabriel, E.; Ramani, A.; Karow, U.; Gottardo, M.; Natarajan, K.; Gooi, L.M.; Goranci-Buzhala, G.; Krut, O.; Peters, F.; Nikolic, M.*, et al.* Recent zika virus isolates induce premature differentiation of neural progenitors in human brain organoids. *Cell stem cell* **2017**, *20*, 397-406.e395.
42. Oliveira, D.B.L.; Almeida, F.J.; Durigon, E.L.; Mendes, É.A.; Braconi, C.T.; Marchetti, I.; Andreata-Santos, R.; Cunha, M.P.; Alves, R.P.S.; Pereira, L.R.*, et al.* Prolonged shedding of zika virus associated with congenital infection. *New England Journal of Medicine* **2016**, *375*, 1202-1204.
43. Moore, C.A.; Staples, J.E.; Dobyns, W.B.; Pessoa, A.; Ventura, C.V.; da Fonseca, E.B.; Ribeiro, E.M.; Ventura, L.O.; Neto, N.N.; Arena, J.F.*, et al.* Congenital zika syndrome: Characterizing the pattern of anomalies for pediatric healthcare providers. *JAMA pediatrics* **2017**, *171*, 288-295.
44. Lindenbach, B.; Thiel, H.; Rice, C. Flaviviridae: The viruses and 602 their replication. *DM Knipe, and PM Howley,(ed.)* **2001**, *683*, 991-1041.
45. Nowakowski, T.J.; Pollen, A.A.; Di Lullo, E.; Sandoval-Espinosa, C.; Bershteyn, M.; Kriegstein, A.R. Expression analysis highlights axl as a candidate zika virus entry receptor in neural stem cells. *Cell stem cell* **2016**, *18*, 591-596.
46. Hastings, A.K.; Yockey, L.J.; Jagger, B.W.; Hwang, J.; Uraki, R.; Gaitsch, H.F.; Parnell, L.A.; Cao, B.; Mysorekar, I.; Rothlin, C.V.*, et al.* Tam receptors are not required for zika virus infection in mice. *Cell reports* **2017**, *19*, 558-568.
47. Wells, M.F.; Salick, M.R.; Wiskow, O.; Ho, D.J.; Worringer, K.A.; Ihry, R.J.; Kommineni, S.; Bilican, B.; Klim, J.R.; Hill, E.J.*, et al.* Genetic ablation of axl does not protect human neural progenitor cells and cerebral organoids from zika virus infection. *Cell stem cell* **2016**, *19*, 703-708.
48. Li, F.; Wang, P.R.; Qu, L.B.; Yi, C.H.; Zhang, F.C.; Tang, X.P.; Zhang, L.G.; Chen, L. Axl is not essential for zika virus infection in the mouse brain. *Emerging microbes & infections* **2017**, *6*, e16.

49. Tabata, T.; Petitt, M.; Puerta-Guardo, H.; Michlmayr, D.; Wang, C.; Fang-Hoover, J.; Harris, E.; Pereira, L. Zika virus targets different primary human placental cells, suggesting two routes for vertical transmission. *Cell Host Microbe* **2016**, *20*, 155-166.
50. Lindenbach, B.D.; Rice, C.M. Molecular biology of flaviviruses. *Advances in virus research* **2003**, *59*, 23-61.
51. Elshuber, S.; Allison, S.L.; Heinz, F.X.; Mandl, C.W. Cleavage of protein prm is necessary for infection of bhk-21 cells by tick-borne encephalitis virus. *The Journal of general virology* **2003**, *84*, 183-191.
52. Huang, Y.J.; Higgs, S.; Horne, K.M.; Vanlandingham, D.L. Flavivirus-mosquito interactions. *Viruses* **2014**, *6*, 4703-4730.
53. Kuno, G.; Chang, G.J. Full-length sequencing and genomic characterization of bagaza, kedougou, and zika viruses. *Archives of virology* **2007**, *152*, 687-696.
54. Zai, J.; Mei, L.; Wang, C.; Cao, S.; Fu, Z.F.; Chen, H.; Song, Y. N-glycosylation of the premembrane protein of japanese encephalitis virus is critical for folding of the envelope protein and assembly of virus-like particles. *Acta virologica* **2013**, *57*, 27-33.
55. Roby, J.A.; Setoh, Y.X.; Hall, R.A.; Khromykh, A.A. Post-translational regulation and modifications of flavivirus structural proteins. *Journal of General Virology* **2015**, *96*, 1551-1569.
56. Bos, S.; Viranaicken, W.; Turpin, J.; El-Kalamouni, C.; Roche, M.; Krejbich-Trotot, P.; Desprès, P.; Gadea, G. The structural proteins of epidemic and historical strains of zika virus differ in their ability to initiate viral infection in human host cells. *Virology* **2018**, *516*, 265-273.
57. Konishi, E.; Mason, P.W. Proper maturation of the japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. *J Virol* **1993**, *67*, 1672-1675.
58. Fields, B.N.; Knipe, D.M.; Howley, P.M. *Fields virology*. 5th ed. ed.; Wolters Kluwer Health/Lippincott Williams & Wilkins: Philadelphia, 2007.
59. Dalrymple, N.A.; Cimica, V.; Mackow, E.R. Dengue virus ns proteins inhibit rig-i/mavs signaling by blocking tbk1/irf3 phosphorylation: Dengue virus serotype 1 ns4a is a unique interferon-regulating virulence determinant. *mBio* **2015**, *6*.
60. Munoz-Jordan, J.L.; Laurent-Rolle, M.; Ashour, J.; Martinez-Sobrido, L.; Ashok, M.; Lipkin, W.I.; Garcia-Sastre, A. Inhibition of alpha/beta interferon signaling by the ns4b protein of flaviviruses. *J Virol* **2005**, *79*, 8004-8013.

61. Ashour, J.; Laurent-Rolle, M.; Shi, P.Y.; Garcia-Sastre, A. Ns5 of dengue virus mediates stat2 binding and degradation. *J Virol* **2009**, *83*, 5408-5418.
62. Ma, J.; Ketkar, H.; Geng, T.; Lo, E.; Wang, L.; Xi, J.; Sun, Q.; Zhu, Z.; Cui, Y.; Yang, L.*, et al.* Zika virus non-structural protein 4a blocks the rlr-mavs signaling. *Frontiers in Microbiology* **2018**, *9*.
63. Best, S.M. The many faces of the flavivirus ns5 protein in antagonism of type i interferon signaling. *Journal of Virology* **2017**, *91*.
64. Muller, D.A.; Young, P.R. The flavivirus ns1 protein: Molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. *Antiviral research* **2013**, *98*, 192-208.
65. Paranavitane, S.A.; Gomes, L.; Kamaladasa, A.; Adikari, T.N.; Wickramasinghe, N.; Jeewandara, C.; Shyamali, N.L.; Ogg, G.S.; Malavige, G.N. Dengue ns1 antigen as a marker of severe clinical disease. *BMC infectious diseases* **2014**, *14*, 570.
66. Xu, X.; Song, H.; Qi, J.; Liu, Y.; Wang, H.; Su, C.; Shi, Y.; Gao, G.F. Contribution of intertwined loop to membrane association revealed by zika virus full-length ns1 structure. *The EMBO journal* **2016**, *35*, 2170-2178.
67. Hilgenfeld, R. Zika virus ns1, a pathogenicity factor with many faces. *The EMBO journal* **2016**, *35*, 2631-2633.
68. Xia, H.; Luo, H.; Shan, C.; Muruato, A.E.; Nunes, B.T.D.; Medeiros, D.B.A.; Zou, J.; Xie, X.; Giraldo, M.I.; Vasconcelos, P.F.C.*, et al.* An evolutionary ns1 mutation enhances zika virus evasion of host interferon induction. *Nature Communications* **2018**, *9*, 414.
69. Chambers, T.J.; McCourt, D.W.; Rice, C.M. Yellow fever virus proteins ns2a, ns2b, and ns4b: Identification and partial n-terminal amino acid sequence analysis. *Virology* **1989**, *169*, 100-109.
70. Liu, W.J.; Wang, X.J.; Mokhonov, V.V.; Shi, P.-Y.; Randall, R.; Khromykh, A.A. Inhibition of interferon signaling by the new york 99 strain and kunjin subtype of west nile virus involves blockage of stat1 and stat2 activation by nonstructural proteins. *Journal of Virology* **2005**, *79*, 1934-1942.
71. Liu, W.J.; Chen, H.B.; Wang, X.J.; Huang, H.; Khromykh, A.A. Analysis of adaptive mutations in kunjin virus replicon rna reveals a novel role for the flavivirus nonstructural protein ns2a in inhibition of beta interferon promoter-driven transcription. *Journal of Virology* **2004**, *78*, 12225-12235.

72. Muñoz-Jordán, J.L.; Sánchez-Burgos, G.G.; Laurent-Rolle, M.; García-Sastre, A. Inhibition of interferon signaling by dengue virus. *Proceedings of the National Academy of Sciences* **2003**, *100*, 14333-14338.
73. Mackenzie, J.M.; Khromykh, A.A.; Jones, M.K.; Westaway, E.G. Subcellular localization and some biochemical properties of the flavivirus kunjin nonstructural proteins ns2a and ns4a. *Virology* **1998**, *245*, 203-215.
74. Yoon, K.-J.; Song, G.; Qian, X.; Pan, J.; Xu, D.; Rho, H.-S.; Kim, N.-S.; Habela, C.; Zheng, L.; Jacob, F.*, et al.* Zika-virus-encoded ns2a disrupts mammalian cortical neurogenesis by degrading adherens junction proteins. *Cell Stem Cell* **2017**, *21*, 349-358.e346.
75. Leung, J.Y.; Pijlman, G.P.; Kondratieva, N.; Hyde, J.; Mackenzie, J.M.; Khromykh, A.A. Role of nonstructural protein ns2a in flavivirus assembly. *Journal of Virology* **2008**, *82*, 4731-4741.
76. Arias, C.F.; Preugschat, F.; Strauss, J.H. Dengue 2 virus ns2b and ns3 form a stable complex that can cleave ns3 within the helicase domain. *Virology* **1993**, *193*, 888-899.
77. Chambers, T.J.; Nestorowicz, A.; Amberg, S.M.; Rice, C.M. Mutagenesis of the yellow fever virus ns2b protein: Effects on proteolytic processing, ns2b-ns3 complex formation, and viral replication. *J Virol* **1993**, *67*, 6797-6807.
78. Brecher, M.; Zhang, J.; Li, H. The flavivirus protease as a target for drug discovery. *Virologica Sinica* **2013**, *28*, 326-336.
79. Li, Z.; Zhang, J.; Li, H. Chapter 7 - flavivirus ns2b/ns3 protease: Structure, function, and inhibition. In *Viral proteases and their inhibitors*, Gupta, S.P., Ed. Academic Press: 2017; pp 163-188.
80. Wu, Y.; Liu, Q.; Zhou, J.; Xie, W.; Chen, C.; Wang, Z.; Yang, H.; Cui, J. Zika virus evades interferon-mediated antiviral response through the co-operation of multiple nonstructural proteins in vitro. *Cell discovery* **2017**, *3*, 17006.
81. Miller, S.; Kastner, S.; Krijnse-Locker, J.; Buhler, S.; Bartenschlager, R. The non-structural protein 4a of dengue virus is an integral membrane protein inducing membrane alterations in a 2k-regulated manner. *The Journal of biological chemistry* **2007**, *282*, 8873-8882.
82. Shiryaev, S.A.; Chernov, A.V.; Aleshin, A.E.; Shiryaeva, T.N.; Strongin, A.Y. Ns4a regulates the atpase activity of the ns3 helicase: A novel cofactor role of the non-structural protein ns4a from west nile virus. *The Journal of general virology* **2009**, *90*, 2081-2085.

83. Umareddy, I.; Chao, A.; Sampath, A.; Gu, F.; Vasudevan, S.G. Dengue virus ns4b interacts with ns3 and dissociates it from single-stranded rna. *Journal of General Virology* **2006**, *87*, 2605-2614.
84. Zou, J.; Xie, X.; Wang, Q.-Y.; Dong, H.; Lee, M.Y.; Kang, C.; Yuan, Z.; Shi, P.-Y. Characterization of dengue virus ns4a and ns4b protein interaction. *Journal of Virology* **2015**, *89*, 3455-3470.
85. McLean, J.E.; Wudzinska, A.; Datan, E.; Quaglino, D.; Zakeri, Z. Flavivirus ns4a-induced autophagy protects cells against death and enhances virus replication. *Journal of Biological Chemistry* **2011**.
86. Liang, Q.; Luo, Z.; Zeng, J.; Chen, W.; Foo, S.-S.; Lee, S.-A.; Ge, J.; Wang, S.; Goldman, Steven A.; Zlokovic, Berislav V.*, et al.* Zika virus ns4a and ns4b proteins deregulate akt-mtor signaling in human fetal neural stem cells to inhibit neurogenesis and induce autophagy. *Cell Stem Cell* **2016**, *19*, 663-671.
87. Ding, Q.; Cao, X.; Lu, J.; Huang, B.; Liu, Y.J.; Kato, N.; Shu, H.B.; Zhong, J. Hepatitis c virus ns4b blocks the interaction of sting and tbk1 to evade host innate immunity. *Journal of hepatology* **2013**, *59*, 52-58.
88. Chatel-Chaix, L.; Cortese, M.; Romero-Brey, I.; Bender, S.; Neufeldt, C.J.; Fischl, W.; Scaturro, P.; Schieber, N.; Schwab, Y.; Fischer, B.*, et al.* Dengue virus perturbs mitochondrial morphodynamics to dampen innate immune responses. *Cell Host & Microbe* **2016**, *20*, 342-356.
89. Laurent-Rolle, M.; Boer, E.F.; Lubick, K.J.; Wolfinbarger, J.B.; Carmody, A.B.; Rockx, B.; Liu, W.; Ashour, J.; Shupert, W.L.; Holbrook, M.R.*, et al.* The ns5 protein of the virulent west nile virus ny99 strain is a potent antagonist of type i interferon-mediated jak-stat signaling. *J Virol* **2010**, *84*, 3503-3515.
90. Best, S.M.; Morris, K.L.; Shannon, J.G.; Robertson, S.J.; Mitzel, D.N.; Park, G.S.; Boer, E.; Wolfinbarger, J.B.; Bloom, M.E. Inhibition of interferon-stimulated jak-stat signaling by a tick-borne flavivirus and identification of ns5 as an interferon antagonist. *J Virol* **2005**, *79*, 12828-12839.
91. Laurent-Rolle, M.; Morrison, J.; Rajsbaum, R.; Macleod, Jesica M.L.; Pisanelli, G.; Pham, A.; Ayllon, J.; Miorin, L.; Martínez-Romero, C.; tenOever, Benjamin R.*, et al.* The interferon signaling antagonist function of yellow fever virus ns5 protein is activated by type i interferon. *Cell Host & Microbe* **2014**, *16*, 314-327.
92. Lubick, Kirk J.; Robertson, Shelly J.; McNally, Kristin L.; Freedman, Brett A.; Rasmussen, Angela L.; Taylor, R.T.; Walts, Avram D.; Tsuruda, S.; Sakai, M.;

Ishizuka, M., *et al.* Flavivirus antagonism of type i interferon signaling reveals prolidase as a regulator of ifnar1 surface expression. *Cell Host & Microbe* **2015**, *18*, 61-74.

93. Morrison, J.; Laurent-Rolle, M.; Maestre, A.M.; Rajsbaum, R.; Pisanelli, G.; Simon, V.; Mulder, L.C.; Fernandez-Sesma, A.; Garcia-Sastre, A. Dengue virus co-opts ubr4 to degrade stat2 and antagonize type i interferon signaling. *PLoS Pathog* **2013**, *9*, e1003265.
94. Grant, A.; Ponia, S.S.; Tripathi, S.; Balasubramaniam, V.; Miorin, L.; Sourisseau, M.; Schwarz, M.C.; Sanchez-Seco, M.P.; Evans, M.J.; Best, S.M., *et al.* Zika virus targets human stat2 to inhibit type i interferon signaling. *Cell Host Microbe* **2016**, *19*, 882-890.
95. Chaudhary, V.; Yuen, K.S.; Chan, J.F.; Chan, C.P.; Wang, P.H.; Cai, J.P.; Zhang, S.; Liang, M.; Kok, K.H.; Chan, C.P., *et al.* Selective activation of type ii interferon signaling by zika virus ns5 protein. *J Virol* **2017**, *91*.
96. Gibbert, K.; Schlaak, J.F.; Yang, D.; Dittmer, U. Ifn-α subtypes: Distinct biological activities in anti-viral therapy. *British journal of pharmacology* **2013**, *168*, 1048-1058.
97. Seth, R.B.; Sun, L.; Ea, C.K.; Chen, Z.J. Identification and characterization of mavs, a mitochondrial antiviral signaling protein that activates nf-kappab and irf 3. *Cell* **2005**, *122*, 669-682.
98. Schneider, W.M.; Chevillotte, M.D.; Rice, C.M. Interferon-stimulated genes: A complex web of host defenses. *Annual review of immunology* **2014**, *32*, 513-545.
99. Van der Hoek, K.H.; Eyre, N.S.; Shue, B.; Khantisitthiporn, O.; Glab-Ampi, K.; Carr, J.M.; Gartner, M.J.; Jolly, L.A.; Thomas, P.Q.; Adikusuma, F., *et al.* Viperin is an important host restriction factor in control of zika virus infection. *Scientific Reports* **2017**, *7*, 4475.
100. Manry, J.; Laval, G.; Patin, E.; Fornarino, S.; Itan, Y.; Fumagalli, M.; Sironi, M.; Tichit, M.; Bouchier, C.; Casanova, J.L., *et al.* Evolutionary genetic dissection of human interferons. *The Journal of experimental medicine* **2011**, *208*, 2747-2759.
101. Larner, A.C.; Jonak, G.; Cheng, Y.S.; Korant, B.; Knight, E.; Darnell, J.E., Jr. Transcriptional induction of two genes in human cells by beta interferon. *Proceedings of the National Academy of Sciences of the United States of America* **1984**, *81*, 6733-6737.

102. Knight, E., Jr.; Korant, B.D. Fibroblast interferon induces synthesis of four proteins in human fibroblast cells. *Proceedings of the National Academy of Sciences of the United States of America* **1979**, *76*, 1824-1827.
103. Bailey, C.C.; Zhong, G.; Huang, I.C.; Farzan, M. Ifitm-family proteins: The cell's first line of antiviral defense. *Annual review of virology* **2014**, *1*, 261-283.
104. Jia, R.; Pan, Q.; Ding, S.; Rong, L.; Liu, S.L.; Geng, Y.; Qiao, W.; Liang, C. The n-terminal region of ifitm3 modulates its antiviral activity by regulating ifitm3 cellular localization. *J Virol* **2012**, *86*, 13697-13707.
105. Jia, R.; Ding, S.; Pan, Q.; Liu, S.-L.; Qiao, W.; Liang, C. The c-terminal sequence of ifitm1 regulates its anti-hiv-1 activity. *PLoS ONE* **2015**, *10*, e0118794.
106. Brass, A.L.; Huang, I.C.; Benita, Y.; John, S.P.; Krishnan, M.N.; Feeley, E.M.; Ryan, B.J.; Weyer, J.L.; van der Weyden, L.; Fikrig, E.*, et al.* The ifitm proteins mediate cellular resistance to influenza a h1n1 virus, west nile virus, and dengue virus. *Cell* **2009**, *139*, 1243-1254.
107. Savidis, G.; Perreira, J.M.; Portmann, J.M.; Meraner, P.; Guo, Z.; Green, S.; Brass, A.L. The ifitms inhibit zika virus replication. *Cell reports* **2016**, *15*, 2323-2330.
108. Wilkins, C.; Woodward, J.; Lau, D.T.; Barnes, A.; Joyce, M.; McFarlane, N.; McKeating, J.A.; Tyrrell, D.L.; Gale, M., Jr. Ifitm1 is a tight junction protein that inhibits hepatitis c virus entry. *Hepatology (Baltimore, Md.)* **2013**, *57*, 461-469.
109. Chan, Y.K.; Huang, I.C.; Farzan, M. Ifitm proteins restrict antibody-dependent enhancement of dengue virus infection. *PLoS ONE* **2012**, *7*, e34508.
110. Gorman, M.J.; Poddar, S.; Farzan, M.; Diamond, M.S. The interferon-stimulated gene ifitm3 restricts west nile virus infection and pathogenesis. *Journal of Virology* **2016**, *90*, 8212-8225.
111. Monel, B.; Compton, A.A.; Bruel, T.; Amraoui, S.; Burlaud-Gaillard, J.; Roy, N.; Guivel-Benhassine, F.; Porrot, F.; Génin, P.; Meertens, L.*, et al.* Zika virus induces massive cytoplasmic vacuolization and paraptosis-like death in infected cells. *The EMBO Journal* **2017**, *36*, 1653-1668.
112. Stacey, M.A.; Clare, S.; Clement, M.; Marsden, M.; Abdul-Karim, J.; Kane, L.; Harcourt, K.; Brandt, C.; Fielding, C.A.; Smith, S.E.*, et al.* The antiviral restriction factor ifn-induced transmembrane protein 3 prevents cytokine-driven cmv pathogenesis. *The Journal of Clinical Investigation* **2017**, *127*, 1463-1474.
113. Diamond, D.L.; Syder, A.J.; Jacobs, J.M.; Sorensen, C.M.; Walters, K.-A.; Proll, S.C.; McDermott, J.E.; Gritsenko, M.A.; Zhang, Q.; Zhao, R.*, et al.* Temporal

proteome and lipidome profiles reveal hepatitis c virus-associated reprogramming of hepatocellular metabolism and bioenergetics. *PLOS Pathogens* **2010**, *6*, e1000719.

114. Warren, C.J.; Griffin, L.M.; Little, A.S.; Huang, I.C.; Farzan, M.; Pyeon, D. The antiviral restriction factors ifitm1, 2 and 3 do not inhibit infection of human papillomavirus, cytomegalovirus and adenovirus. *PLoS One* **2014**, *9*, e96579.
115. Lindenmann, J. Inheritance of resistance to influenza virus in mice. *Proceedings of the Society for Experimental Biology and Medicine* **1964**, *116*, 506-509.
116. Horisberger, M.A.; Staeheli, P.; Haller, O. Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. *Proceedings of the National Academy of Sciences* **1983**, *80*, 1910-1914.
117. Aebi, M.; Fäh, J.; Hurt, N.; Samuel, C.E.; Thomis, D.; Bazzigher, L.; Pavlovic, J.; Haller, O.; Staeheli, P. Cdna structures and regulation of two interferon-induced human mx proteins. *Molecular and Cellular Biology* **1989**, *9*, 5062-5072.
118. Mänz, B.; Dornfeld, D.; Götz, V.; Zell, R.; Zimmermann, P.; Haller, O.; Kochs, G.; Schwemmle, M. Pandemic influenza a viruses escape from restriction by human mxa through adaptive mutations in the nucleoprotein. *PLOS Pathogens* **2013**, *9*, e1003279.
119. Staeheli, P.; Pavlovic, J. Inhibition of vesicular stomatitis virus mrna synthesis by human mxa protein. *J Virol* **1991**, *65*, 4498-4501.
120. Deeg, C.M.; Hassan, E.; Mutz, P.; Rheinemann, L.; Gotz, V.; Magar, L.; Schilling, M.; Kallfass, C.; Nurnberger, C.; Soubies, S.*, et al.* In vivo evasion of mxa by avian influenza viruses requires human signature in the viral nucleoprotein. *The Journal of experimental medicine* **2017**, *214*, 1239-1248.
121. Gordien, E.; Rosmorduc, O.; Peltekian, C.; Garreau, F.; Bréchot, C.; Kremsdorf, D. Inhibition of hepatitis b virus replication by the interferon-inducible mxa protein. *Journal of Virology* **2001**, *75*, 2684-2691.
122. Liu, Z.; Pan, Q.; Ding, S.; Qian, J.; Xu, F.; Zhou, J.; Cen, S.; Guo, F.; Liang, C. The interferon-inducible mxb protein inhibits hiv-1 infection. *Cell Host Microbe* **2013**, *14*, 398-410.
123. Goujon, C.; Moncorge, O.; Bauby, H.; Doyle, T.; Ward, C.C.; Schaller, T.; Hue, S.; Barclay, W.S.; Schulz, R.; Malim, M.H. Human mx2 is an interferon-induced post-entry inhibitor of hiv-1 infection. *Nature* **2013**, *502*, 559-562.
124. Kane, M.; Yadav, S.S.; Bitzegeio, J.; Kutluay, S.B.; Zang, T.; Wilson, S.J.; Schoggins, J.W.; Rice, C.M.; Yamashita, M.; Hatziioannou, T.*, et al.* Mx2 is an interferon-induced inhibitor of hiv-1 infection. *Nature* **2013**, *502*, 563-566.

125. Crameri, M.; Bauer, M.; Caduff, N.; Walker, R.; Steiner, F.; Franzoso, F.D.; Gujer, C.; Boucke, K.; Kucera, T.; Zbinden, A.*, et al.* Mxb is an interferon-induced restriction factor of human herpesviruses. *Nature Communications* **2018**, *9*, 1980.
126. Melen, K.; Julkunen, I. Nuclear cotransport mechanism of cytoplasmic human mxb protein. *The Journal of biological chemistry* **1997**, *272*, 32353-32359.
127. Goujon, C.; Greenbury, R.A.; Papaioannou, S.; Doyle, T.; Malim, M.H. A triple-arginine motif in the amino-terminal domain and oligomerization are required for hiv-1 inhibition by human mx2. *Journal of Virology* **2015**, *89*, 4676-4680.
128. Liu, Z.; Pan, Q.; Liang, Z.; Qiao, W.; Cen, S.; Liang, C. The highly polymorphic cyclophilin a-binding loop in hiv-1 capsid modulates viral resistance to mxb. *Retrovirology* **2015**, *12*, 1.
129. Fribourgh, J.L.; Nguyen, H.C.; Matreyek, K.A.; Alvarez, F.J.D.; Summers, B.J.; Dewdney, T.G.; Aiken, C.; Zhang, P.; Engelman, A.; Xiong, Y. Structural insight into hiv-1 restriction by mxb. *Cell Host Microbe* **2014**, *16*, 627-638.
130. Schulte, B.; Buffone, C.; Opp, S.; Di Nunzio, F.; De Souza Aranha Vieira, D.A.; Brandariz-Nunez, A.; Diaz-Griffero, F. Restriction of hiv-1 requires the n-terminal region of mxb as a capsid-binding motif but not as a nuclear localization signal. *J Virol* **2015**, *89*, 8599-8610.
131. Fricke, T.; White, T.E.; Schulte, B.; de Souza Aranha Vieira, D.A.; Dharan, A.; Campbell, E.M.; Brandariz-Nunez, A.; Diaz-Griffero, F. Mxb binds to the hiv-1 core and prevents the uncoating process of hiv-1. *Retrovirology* **2014**, *11*, 68.
132. Alvarez, F.J.D.; He, S.; Perilla, J.R.; Jang, S.; Schulten, K.; Engelman, A.N.; Scheres, S.H.W.; Zhang, P. Cryoem structure of mxb reveals a novel oligomerization interface critical for hiv restriction. *Science Advances* **2017**, *3*.
133. Goujon, C.; Moncorge, O.; Bauby, H.; Doyle, T.; Barclay, W.S.; Malim, M.H. Transfer of the amino-terminal nuclear envelope targeting domain of human mx2 converts mx1 into an hiv-1 resistance factor. *J Virol* **2014**, *88*, 9017-9026.
134. Schilling, M.; Bulli, L.; Weigang, S.; Graf, L.; Naumann, S.; Patzina, C.; Wagner, V.; Bauersfeld, L.; Goujon, C.; Hengel, H.*, et al.* Human mxb protein is a pan-herpesvirus restriction factor. *Journal of Virology* **2018**, *92*.
135. Neil, S.J.; Zang, T.; Bieniasz, P.D. Tetherin inhibits retrovirus release and is antagonized by hiv-1 vpu. *Nature* **2008**, *451*, 425-430.
136. Ishikawa, J.; Kaisho, T.; Tomizawa, H.; Lee, B.O.; Kobune, Y.; Inazawa, J.; Oritani, K.; Itoh, M.; Ochi, T.; Ishihara, K.*, et al.* Molecular cloning and chromosomal

mapping of a bone marrow stromal cell surface gene, bst2, that may be involved in pre-b-cell growth. *Genomics* **1995**, *26*, 527-534.

137. Blasius, A.L.; Giurisato, E.; Cella, M.; Schreiber, R.D.; Shaw, A.S.; Colonna, M. Bone marrow stromal cell antigen 2 is a specific marker of type i ifn-producing cells in the naive mouse, but a promiscuous cell surface antigen following ifn stimulation. *Journal of immunology (Baltimore, Md. : 1950)* **2006**, *177*, 3260-3265.
138. Rollason, R.; Korolchuk, V.; Hamilton, C.; Schu, P.; Banting, G. Clathrin-mediated endocytosis of a lipid-raft-associated protein is mediated through a dual tyrosine motif. *Journal of Cell Science* **2007**, *120*, 3850-3858.
139. Masuyama, N.; Kuronita, T.; Tanaka, R.; Muto, T.; Hirota, Y.; Takigawa, A.; Fujita, H.; Aso, Y.; Amano, J.; Tanaka, Y. Hm1.24 is internalized from lipid rafts by clathrin-mediated endocytosis through interaction with α-adaptin. *Journal of Biological Chemistry* **2009**.
140. Cao, W.; Rosen, D.B.; Ito, T.; Bover, L.; Bao, M.; Watanabe, G.; Yao, Z.; Zhang, L.; Lanier, L.L.; Liu, Y.J. Plasmacytoid dendritic cell-specific receptor ilt7-fc epsilonri gamma inhibits toll-like receptor-induced interferon production. *The Journal of experimental medicine* **2006**, *203*, 1399-1405.
141. Cao, W.; Bover, L.; Cho, M.; Wen, X.; Hanabuchi, S.; Bao, M.; Rosen, D.B.; Wang, Y.H.; Shaw, J.L.; Du, Q.*, et al.* Regulation of tlr7/9 responses in plasmacytoid dendritic cells by bst2 and ilt7 receptor interaction. *The Journal of experimental medicine* **2009**, *206*, 1603-1614.
142. Matsuda, K.; Chen, C.Y.; Whitted, S.; Chertova, E.; Roser, D.J.; Wu, F.; Plishka, R.J.; Ourmanov, I.; Buckler-White, A.; Lifson, J.D.*, et al.* Enhanced antagonism of bst-2 by a neurovirulent siv envelope. *J Clin Invest* **2016**, *126*, 2295-2307.
143. Jones, P.H.; Mehta, H.V.; Maric, M.; Roller, R.J.; Okeoma, C.M. Bone marrow stromal cell antigen 2 (bst-2) restricts mouse mammary tumor virus (mmtv) replication in vivo. *Retrovirology* **2012**, *9*, 10.
144. Liberatore, R.A.; Mastrocola, E.J.; Powell, C.; Bieniasz, P.D. Tetherin inhibits cell-free virus dissemination and retards murine leukemia virus pathogenesis. *J Virol* **2017**, *91*.
145. Mahauad-Fernandez, W.D.; Jones, P.H.; Okeoma, C.M. Critical role for bone marrow stromal antigen 2 in acute chikungunya virus infection. *The Journal of general virology* **2014**, *95*, 2450-2461.

146. Weidner, J.M.; Jiang, D.; Pan, X.-B.; Chang, J.; Block, T.M.; Guo, J.-T. Interferon-induced cell membrane proteins, ifitm3 and tetherin, inhibit vesicular stomatitis virus infection via distinct mechanisms. *Journal of Virology* **2010**, *84*, 12646-12657.
147. Galão, Rui P.; Le Tortorec, A.; Pickering, S.; Kueck, T.; Neil, Stuart J.D. Innate sensing of hiv-1 assembly by tetherin induces nfκb-dependent proinflammatory responses. *Cell Host & Microbe* **2012**, *12*, 633-644.
148. Tokarev, A.; Suarez, M.; Kwan, W.; Fitzpatrick, K.; Singh, R.; Guatelli, J. Stimulation of nf-κb activity by the hiv restriction factor bst2. *Journal of Virology* **2013**, *87*, 2046-2057.
149. Li, S.X.; Barrett, B.S.; Heilman, K.J.; Messer, R.J.; Liberatore, R.A.; Bieniasz, P.D.; Kassiotis, G.; Hasenkrug, K.J.; Santiago, M.L. Tetherin promotes the innate and adaptive cell–mediated immune response against retrovirus infection in vivo. *The Journal of Immunology* **2014**, *193*, 306-316.
150. Li, S.X.; Barrett, B.S.; Guo, K.; Kassiotis, G.; Hasenkrug, K.J.; Dittmer, U.; Gibbert, K.; Santiago, M.L. Tetherin/bst-2 promotes dendritic cell activation and function during acute retrovirus infection. *Scientific Reports* **2016**, *6*, 20425.
151. Urata, S.; Kenyon, E.; Nayak, D.; Cubitt, B.; Kurosaki, Y.; Yasuda, J.; de la Torre, J.C.; McGavern, D.B. Bst-2 controls t cell proliferation and exhaustion by shaping the early distribution of a persistent viral infection. *PLOS Pathogens* **2018**, *14*, e1007172.
152. Alvarez, R.A.; Hamlin, R.E.; Monroe, A.; Moldt, B.; Hotta, M.T.; Rodriguez Caprio, G.; Fierer, D.S.; Simon, V.; Chen, B.K. Hiv-1 vpu antagonism of tetherin inhibits antibody-dependent cellular cytotoxic responses by natural killer cells. *Journal of Virology* **2014**, *88*, 6031-6046.
153. Sato, K.; Yamamoto, S.P.; Misawa, N.; Yoshida, T.; Miyazawa, T.; Koyanagi, Y. Comparative study on the effect of human bst-2/tetherin on hiv-1 release in cells of various species. *Retrovirology* **2009**, *6*, 53.
154. Miyakawa, K.; Ryo, A.; Murakami, T.; Ohba, K.; Yamaoka, S.; Fukuda, M.; Guatelli, J.; Yamamoto, N. Bca2/rabring7 promotes tetherin-dependent hiv-1 restriction. *PLoS Pathog* **2009**, *5*, e1000700.
155. Parker, N.; Porter, A.C. Identification of a novel gene family that includes the interferon-inducible human genes 6-16 and isg12. *BMC genomics* **2004**, *5*, 8.
156. Cheriyath, V.; Glaser, K.B.; Waring, J.F.; Baz, R.; Hussein, M.A.; Borden, E.C. G1p3, an ifn-induced survival factor, antagonizes trail-induced apoptosis in human myeloma cells. *J Clin Invest* **2007**, *117*, 3107-3117.

157. Bani, M.R.; Nicoletti, M.I.; Alkharouf, N.W.; Ghilardi, C.; Petersen, D.; Erba, E.; Sausville, E.A.; Liu, E.T.; Giavazzi, R. Gene expression correlating with response to paclitaxel in ovarian carcinoma xenografts. *Molecular cancer therapeutics* **2004**, *3*, 111-121.

158. Cheriyath, V.; Kaur, J.; Davenport, A.; Khalel, A.; Chowdhury, N.; Gaddipati, L. G1p3 (ifi6), a mitochondrial localised antiapoptotic protein, promotes metastatic potential of breast cancer cells through mtros. *British journal of cancer* **2018**, *119*, 52-64.

159. Cheriyath, V.; Leaman, D.W.; Borden, E.C. Emerging roles of fam14 family members (g1p3/isg 6-16 and isg12/ifi27) in innate immunity and cancer. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research* **2011**, *31*, 173-181.

160. Richardson, R.B.; Ohlson, M.B.; Eitson, J.L.; Kumar, A.; McDougal, M.B.; Boys, I.N.; Mar, K.B.; De La Cruz-Rivera, P.C.; Douglas, C.; Konopka, G.*, et al.* A crispr screen identifies ifi6 as an er-resident interferon effector that blocks flavivirus replication. *Nature Microbiology* **2018**.

161. Qi, Y.; Li, Y.; Zhang, Y.; Zhang, L.; Wang, Z.; Zhang, X.; Gui, L.; Huang, J. Ifi6 inhibits apoptosis via mitochondrial-dependent pathway in dengue virus 2 infected vascular endothelial cells. *PLOS ONE* **2015**, *10*, e0132743.

162. Meyer, K.; Kwon, Y.C.; Liu, S.; Hagedorn, C.H.; Ray, R.B.; Ray, R. Interferon-alpha inducible protein 6 impairs egfr activation by cd81 and inhibits hepatitis c virus infection. *Sci Rep* **2015**, *5*, 9012.

163. Kumar, A.; Hou, S.; Airo, A.M.; Limonta, D.; Mancinelli, V.; Branton, W.; Power, C.; Hobman, T.C. Zika virus inhibits type-i interferon production and downstream signaling. *EMBO reports* **2016**, *17*, 1766-1775.

164. Bowen, J.R.; Quicke, K.M.; Maddur, M.S.; O'Neal, J.T.; McDonald, C.E.; Fedorova, N.B.; Puri, V.; Shabman, R.S.; Pulendran, B.; Suthar, M.S. Zika virus antagonizes type i interferon responses during infection of human dendritic cells. *PLOS Pathogens* **2017**, *13*, e1006164.

165. Aguirre, S.; Maestre, A.M.; Pagni, S.; Patel, J.R.; Savage, T.; Gutman, D.; Maringer, K.; Bernal-Rubio, D.; Shabman, R.S.; Simon, V.*, et al.* Denv inhibits type i ifn production in infected cells by cleaving human sting. *PLOS Pathogens* **2012**, *8*, e1002934.

166. Muller, U.; Steinhoff, U.; Reis, L.; Hemmi, S.; Pavlovic, J.; Zinkernagel, R.; Aguet, M. Functional role of type i and type ii interferons in antiviral defense. *Science* **1994**, *264*, 1918-1921.
167. Wheelock, E.F. Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. *Science* **1965**, *149*, 310-311.
168. Johnson, H.M. Gamma interferon: From antimicrobial activity to immune regulation. *Frontiers in Immunology* **2014**, *5*, 667.
169. Billiau, A.; Matthys, P. Interferon-γ: A historical perspective. *Cytokine & Growth Factor Reviews* **2009**, *20*, 97-113.
170. Stark, G.R.; Kerr, I.M.; Williams, B.R.; Silverman, R.H.; Schreiber, R.D. How cells respond to interferons. *Annual review of biochemistry* **1998**, *67*, 227-264.
171. Darnell, J.E., Jr. Stats and gene regulation. *Science* **1997**, *277*, 1630-1635.
172. Aaronson, D.S.; Horvath, C.M. A road map for those who don't know jak-stat. *Science* **2002**, *296*, 1653-1655.
173. Boehm, U.; Klamp, T.; Groot, M.; Howard, J.C. Cellular responses to interferon-gamma. *Annual review of immunology* **1997**, *15*, 749-795.
174. Platanias, L.C. Mechanisms of type-i- and type-ii-interferon-mediated signalling. *Nature Reviews Immunology* **2005**, *5*, 375.
175. Barnard, T.R.; Rajah, M.M.; Sagan, S.M. Zika virus infection: Induction, restriction and evasion of host interferon responses. *Future Virology* **2017**, *12*, 627-630.
176. Pagliaccetti, N.E.; Robek, M.D. Interferon-λ in hcv infection and therapy. *Viruses* **2010**, *2*, 1589-1602.
177. Racicot, K.; Aldo, P.; El-Guindy, A.; Kwon, J.-Y.; Romero, R.; Mor, G. Cutting edge: Fetal/placental type i ifn can affect maternal survival and fetal viral load during viral infection. *The Journal of Immunology* **2017**, *198*, 3029.
178. Bayer, A.; Lennemann, Nicholas J.; Ouyang, Y.; Bramley, John C.; Morosky, S.; Marques, Ernesto Torres De A., Jr.; Cherry, S.; Sadovsky, Y.; Coyne, Carolyn B. Type iii interferons produced by human placental trophoblasts confer protection against zika virus infection. *Cell Host & Microbe* **2016**, *19*, 705-712.
179. Corry, J.; Arora, N.; Good, C.A.; Sadovsky, Y.; Coyne, C.B. Organotypic models of type iii interferon-mediated protection from zika virus infections at the maternal–fetal interface. *Proceedings of the National Academy of Sciences* **2017**, *114*, 9433-9438.

180. Jagger, B.W.; Miner, J.J.; Cao, B.; Arora, N.; Smith, A.M.; Kovacs, A.; Mysorekar, I.U.; Coyne, C.B.; Diamond, M.S. Gestational stage and ifn-λ signaling regulate zikv infection in utero. *Cell Host & Microbe* **2017**, *22*, 366-376.e363.
181. Yockey, L.J.; Jurado, K.A.; Arora, N.; Millet, A.; Rakib, T.; Milano, K.M.; Hastings, A.K.; Fikrig, E.; Kong, Y.; Horvath, T.L.*, et al.* Type i interferons instigate fetal demise after zika virus infection. *Science Immunology* **2018**, *3*.
182. Daugherty, M.D.; Malik, H.S. Rules of engagement: Molecular insights from host-virus arms races. *Annual review of genetics* **2012**, *46*, 677-700.
183. Raniga, K.; Liang, C. Interferons: Reprogramming the metabolic network against viral infection. *Viruses* **2018**, *10*, 36.
184. Eagle, H.; Habel, K. The nutritional requirements for the propagation of poliomyelitis virus by the hela cell. *The Journal of experimental medicine* **1956**, *104*, 271-287.
185. Munger, J.; Bajad, S.U.; Coller, H.A.; Shenk, T.; Rabinowitz, J.D. Dynamics of the cellular metabolome during human cytomegalovirus infection. *PLOS Pathogens* **2006**, *2*, e132.
186. Fontaine, K.A.; Sanchez, E.L.; Camarda, R.; Lagunoff, M. Dengue virus induces and requires glycolysis for optimal replication. *Journal of Virology* **2015**, *89*, 2358-2366.
187. Hegedus, A.; Kavanagh Williamson, M.; Huthoff, H. Hiv-1 pathogenicity and virion production are dependent on the metabolic phenotype of activated cd4+ t cells. *Retrovirology* **2014**, *11*, 98.
188. Ritter, J.B.; Wahl, A.S.; Freund, S.; Genzel, Y.; Reichl, U. Metabolic effects of influenza virus infection in cultured animal cells: Intra- and extracellular metabolite profiling. *BMC Systems Biology* **2010**, *4*, 61.
189. Vastag, L.; Koyuncu, E.; Grady, S.L.; Shenk, T.E.; Rabinowitz, J.D. Divergent effects of human cytomegalovirus and herpes simplex virus-1 on cellular metabolism. *PLOS Pathogens* **2011**, *7*, e1002124.
190. Pavlova, Natalya N.; Thompson, Craig B. The emerging hallmarks of cancer metabolism. *Cell Metabolism* **2016**, *23*, 27-47.
191. Sir, D.; Kuo, C.-f.; Tian, Y.; Liu, H.M.; Huang, E.J.; Jung, J.U.; Machida, K.; Ou, J.-h.J. Replication of hepatitis c virus rna on autophagosomal membranes. *Journal of Biological Chemistry* **2012**, *287*, 18036-18043.
192. Tiwari, S.K.; Dang, J.; Qin, Y.; Lichinchi, G.; Bansal, V.; Rana, T.M. Zika virus infection reprograms global transcription of host cells to allow sustained infection. *Emerging Microbes &Amp; Infections* **2017**, *6*, e24.

193. Savidis, G.; McDougall, W.M.; Meraner, P.; Perreira, J.M.; Portmann, J.M.; Trincucci, G.; John, S.P.; Aker, A.M.; Renzette, N.; Robbins, D.R.*, et al.* Identification of zika virus and dengue virus dependency factors using functional genomics. *Cell reports* **2016**, *16*, 232-246.

194. Barbier, V.; Lang, D.; Valois, S.; Rothman, A.L.; Medin, C.L. Dengue virus induces mitochondrial elongation through impairment of drp1-triggered mitochondrial fission. *Virology* **2017**, *500*, 149-160.

195. Heymann, D.L.; Hodgson, A.; Sall, A.A.; Freedman, D.O.; Staples, J.E.; Althabe, F.; Baruah, K.; Mahmud, G.; Kandun, N.; Vasconcelos, P.F.C.*, et al.* Zika virus and microcephaly: Why is this situation a pheic? *The Lancet* **2016**, *387*, 719-721.

196. Calvet, G.A.; Santos, F.B.; Sequeira, P.C. Zika virus infection: Epidemiology, clinical manifestations and diagnosis. *Curr Opin Infect Dis* **2016**, *29*, 459-466.

197. Tripathi, S.; Balasubramaniam, V.R.M.T.; Brown, J.A.; Mena, I.; Grant, A.; Bardina, S.V.; Maringer, K.; Schwarz, M.C.; Maestre, A.M.; Sourisseau, M.*, et al.* A novel zika virus mouse model reveals strain specific differences in virus pathogenesis and host inflammatory immune responses. *PLOS Pathogens* **2017**, *13*, e1006258.

198. Lazear, H.M.; Govero, J.; Smith, A.M.; Platt, D.J.; Fernandez, E.; Miner, J.J.; Diamond, M.S. A mouse model of zika virus pathogenesis. *Cell Host Microbe* **2016**, *19*, 720-730.

199. Goodwin, C.M.; Xu, S.; Munger, J. Stealing the keys to the kitchen: Viral manipulation of the host cell metabolic network. *Trends in microbiology* **2015**, *23*, 789-798.

200. Fonseca, K.; Meatherall, B.; Zarra, D.; Drebot, M.; MacDonald, J.; Pabbaraju, K.; Wong, S.; Webster, P.; Lindsay, R.; Tellier, R. First case of zika virus infection in a returning canadian traveler. *The American journal of tropical medicine and hygiene* **2014**, *91*, 1035-1038.

201. Lindqvist, R.; Mundt, F.; Gilthorpe, J.D.; Wölfel, S.; Gekara, N.O.; Kröger, A.; Överby, A.K. Fast type i interferon response protects astrocytes from flavivirus infection and virus-induced cytopathic effects. *Journal of Neuroinflammation* **2016**, *13*, 277.

202. Schoggins, J.W.; Wilson, S.J.; Panis, M.; Murphy, M.Y.; Jones, C.T.; Bieniasz, P.; Rice, C.M. A diverse range of gene products are effectors of the type i interferon antiviral response. *Nature* **2011**, *472*, 481-485.

203. Schoggins, J.W.; MacDuff, D.A.; Imanaka, N.; Gainey, M.D.; Shrestha, B.; Eitson, J.L.; Mar, K.B.; Richardson, R.B.; Ratushny, A.V.; Litvak, V.*, et al.* Pan-viral specificity of ifn-induced genes reveals new roles for cgas in innate immunity. *Nature* **2014**, *505*, 691-695.
204. Thakkar, N.; Yadavalli, T.; Jaishankar, D.; Shukla, D. Emerging roles of heparanase in viral pathogenesis. *Pathogens* **2017**, *6*, 43.
205. Gao, G.; Guo, X.; Goff, S.P. Inhibition of retroviral rna production by zap, a ccch-type zinc finger protein. *Science* **2002**, *297*, 1703-1706.
206. Zhu, Y.; Chen, G.; Lv, F.; Wang, X.; Ji, X.; Xu, Y.; Sun, J.; Wu, L.; Zheng, Y.T.; Gao, G. Zinc-finger antiviral protein inhibits hiv-1 infection by selectively targeting multiply spliced viral mrnas for degradation. *Proceedings of the National Academy of Sciences of the United States of America* **2011**, *108*, 15834-15839.
207. Veit, M.; Thaa, B. Association of influenza virus proteins with membrane rafts. *Advances in virology* **2011**, *2011*, 370606.
208. Gianni, T.; Campadelli-Fiume, G. Alphavbeta3-integrin relocalizes nectin1 and routes herpes simplex virus to lipid rafts. *J Virol* **2012**, *86*, 2850-2855.
209. Amini-Bavil-Olyaee, S.; Choi, Y.J.; Lee, J.H.; Shi, M.; Huang, I.C.; Farzan, M.; Jung, J.U. The antiviral effector ifitm3 disrupts intracellular cholesterol homeostasis to block viral entry. *Cell host & microbe* **2013**, *13*, 452-464.
210. Lloyd-Evans, E.; Platt, F.M. Lipids on trial: The search for the offending metabolite in niemann-pick type c disease. *Traffic* **2010**, *11*, 419-428.
211. Fan, H.; Du, X.; Zhang, J.; Zheng, H.; Lu, X.; Wu, Q.; Li, H.; Wang, H.; Shi, Y.; Gao, G.*, et al.* Selective inhibition of ebola entry with selective estrogen receptor modulators by disrupting the endolysosomal calcium. *Scientific Reports* **2017**, *7*, 41226.
212. Shoemaker, C.J.; Schornberg, K.L.; Delos, S.E.; Scully, C.; Pajouhesh, H.; Olinger, G.G.; Johansen, L.M.; White, J.M. Multiple cationic amphiphiles induce a niemann-pick c phenotype and inhibit ebola virus entry and infection. *PLoS One* **2013**, *8*, e56265.
213. Diamond, M.S.; Harris, E. Interferon inhibits dengue virus infection by preventing translation of viral rna through a pkr-independent mechanism. *Virology* **2001**, *289*, 297-311.

214. Gjermandsen, I.M.; Justesen, J.; Martensen, P.M. The interferon-induced gene isg12 is regulated by various cytokines as the gene 6-16 in human cell lines. *Cytokine* **2000**, *12*, 233-238.

215. Itsui, Y.; Sakamoto, N.; Kurosaki, M.; Kanazawa, N.; Tanabe, Y.; Koyama, T.; Takeda, Y.; Nakagawa, M.; Kakinuma, S.; Sekine, Y.*, et al.* Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis c virus replication. *Journal of Viral Hepatitis* **2006**, *13*, 690-700.

216. Yi, D.-r.; An, N.; Liu, Z.-l.; Xu, F.-w.; Raniga, K.; Li, Q.-j.; Zhou, R.; Wang, J.; Zhang, Y.-x.; Zhou, J.-m.*, et al.* Human mxb inhibits the replication of hcv. *Journal of Virology* **2018**.

217. Luban, J.; Bossolt, K.L.; Franke, E.K.; Kalpana, G.V.; Goff, S.P. Human immunodeficiency virus type 1 gag protein binds to cyclophilins a and b. *Cell* **1993**, *73*, 1067-1078.

218. Chatterji, U.; Bobardt, M.; Selvarajah, S.; Yang, F.; Tang, H.; Sakamoto, N.; Vuagniaux, G.; Parkinson, T.; Gallay, P. The isomerase active site of cyclophilin a is critical for hepatitis c virus replication. *The Journal of biological chemistry* **2009**, *284*, 16998-17005.

219. Vahlne, A.; Larsson, P.-A.; Horal, P.; Ahlmén, J.; Svennerholm, B.; Gronowitz, J.S.; Olofsson, S. Inhibition of herpes simplex virus production in vitro by cyclosporin a. *Archives of Virology* **1992**, *122*, 61-75.

220. Damaso, C.R.A.; Keller, S.J. Cyclosporin a inhibits vaccinia virus replication in vitro. *Archives of Virology* **1994**, *134*, 303-319.

221. Franke, E.K.; Luban, J. Inhibition of hiv-1 replication by cyclosporine a or related compounds correlates with the ability to disrupt the gag–cyclophilin a interaction. *Virology* **1996**, *222*, 279-282.

222. Watashi, K.; Hijikata, M.; Hosaka, M.; Yamaji, M.; Shimotohno, K. Cyclosporin a suppresses replication of hepatitis c virus genome in cultured hepatocytes. *Hepatology (Baltimore, Md.)* **2003**, *38*, 1282-1288.

223. Zhang, Y.-H.; Zhao, Y.; Li, N.; Peng, Y.-C.; Giannoulatou, E.; Jin, R.-H.; Yan, H.-P.; Wu, H.; Liu, J.-H.; Liu, N.*, et al.* Interferon-induced transmembrane protein-3 genetic variant rs12252-c is associated with severe influenza in chinese individuals. *Nature Communications* **2013**, *4*, 1418.

224. Everitt, A.R.; Clare, S.; Pertel, T.; John, S.P.; Wash, R.S.; Smith, S.E.; Chin, C.R.; Feeley, E.M.; Sims, J.S.; Adams, D.J.*, et al.* Ifitm3 restricts the morbidity and mortality associated with influenza. *Nature* **2012**, *484*, 519-523.
225. Bailey, C.C.; Kondur, H.R.; Huang, I.C.; Farzan, M. Interferon-induced transmembrane protein 3 is a type ii transmembrane protein. *The Journal of biological chemistry* **2013**, *288*, 32184-32193.
226. Li, K.; Markosyan, R.M.; Zheng, Y.M.; Golfetto, O.; Bungart, B.; Li, M.; Ding, S.; He, Y.; Liang, C.; Lee, J.C.*, et al.* Ifitm proteins restrict viral membrane hemifusion. *PLoS Pathog* **2013**, *9*, e1003124.
227. Feeley, E.M.; Sims, J.S.; John, S.P.; Chin, C.R.; Pertel, T.; Chen, L.M.; Gaiha, G.D.; Ryan, B.J.; Donis, R.O.; Elledge, S.J.*, et al.* Ifitm3 inhibits influenza a virus infection by preventing cytosolic entry. *PLoS Pathog* **2011**, *7*, e1002337.
228. Lin, T.Y.; Chin, C.R.; Everitt, A.R.; Clare, S.; Perreira, J.M.; Savidis, G.; Aker, A.M.; John, S.P.; Sarlah, D.; Carreira, E.M.*, et al.* Amphotericin b increases influenza a virus infection by preventing ifitm3-mediated restriction. *Cell reports* **2013**, *5*, 895-908.
229. Ripoli, M.; D'Aprile, A.; Quarato, G.; Sarasin-Filipowicz, M.; Gouttenoire, J.; Scrima, R.; Cela, O.; Boffoli, D.; Heim, M.H.; Moradpour, D.*, et al.* Hepatitis c virus-linked mitochondrial dysfunction promotes hypoxia-inducible factor 1 alpha-mediated glycolytic adaptation. *Journal of virology* **2010**, *84*, 647-660.
230. Itoh, K.; Nakamura, K.; Iijima, M.; Sesaki, H. Mitochondrial dynamics in neurodegeneration. *Trends in cell biology* **2013**, *23*, 64-71.
231. Chandel, N.S.; Jasper, H.; Ho, T.T.; Passegue, E. Metabolic regulation of stem cell function in tissue homeostasis and organismal ageing. *Nature cell biology* **2016**, *18*, 823-832.
232. Massirer, K.B.; Carromeu, C.; Griesi-Oliveira, K.; Muotri, A.R. Maintenance and differentiation of neural stem cells. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine* **2011**, *3*, 107-114.
233. Sanai, N.; Tramontin, A.D.; Quinones-Hinojosa, A.; Barbaro, N.M.; Gupta, N.; Kunwar, S.; Lawton, M.T.; McDermott, M.W.; Parsa, A.T.; Manuel-Garcia Verdugo, J.*, et al.* Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature* **2004**, *427*, 740-744.
234. Khacho, M.; Slack, R.S. Mitochondrial activity in the regulation of stem cell self-renewal and differentiation. *Current Opinion in Cell Biology* **2017**, *49*, 1-8.

235. Li, C.; Xu, D.; Ye, Q.; Hong, S.; Jiang, Y.; Liu, X.; Zhang, N.; Shi, L.; Qin, C.F.; Xu, Z. Zika virus disrupts neural progenitor development and leads to microcephaly in mice. *Cell stem cell* **2016**, *19*, 120-126.
236. Devhare, P.; Meyer, K.; Steele, R.; Ray, R.B.; Ray, R. Zika virus infection dysregulates human neural stem cell growth and inhibits differentiation into neuroprogenitor cells. *Cell Death &Amp; Disease* **2017**, *8*, e3106.
237. Sorrentino, V.; Menzies, K.J.; Auwerx, J. Repairing mitochondrial dysfunction in disease. *Annual Review of Pharmacology and Toxicology* **2018**, *58*, 353-389.