Ribosomal Proteins RPLP1 and RPLP2 are Host Factors Critically Required for Flavivirus Infectivity by Promoting Efficient Viral Translation Elongation.

by

Rafael Kroon Campos

Department of Molecular Genetics and Microbiology Duke University

Date:\_\_\_\_

Approved:

Mariano Garcia-Blanco, Co-supervisor

Micah Luftig, Co-supervisor

Shelton Bradrick

Christopher Nicchitta

Debra Silver

Stacy Horner

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular Genetics and Microbiology in the Graduate School of Duke University

2018

#### ABSTRACT

Ribosomal Proteins RPLP1 and RPLP2 are Host Factors Critically Required for Flavivirus Infectivity by Promoting Efficient Viral Translation Elongation.

by

Rafael Kroon Campos

Department of Molecular Genetics and Microbiology Duke University

Date:\_\_\_\_

Approved:

Mariano Garcia-Blanco, Co-supervisor

Micah Luftig, Co-supervisor

Shelton Bradrick

Christopher Nicchitta

Debra Silver

Stacy Horner

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular Genetics and Microbiology in the Graduate School of Duke University

2018

Copyright by Rafael Kroon Campos 2018

## Abstract

The *Flavivirus* genus contains several arthropod-borne viruses that pose global health threats, including dengue virus (DENV). We identified two ribosomal proteins, RPLP1 and RPLP2 (RPLP1/2), that are crucial host factors required for translation of flaviviruses and efficient flavivirus infection of human cell lines and Aedes aegypti mosquitoes, which are natural vectors for these viruses. We hypothesized that RPLP1/2 are accessory ribosomal proteins that function to promote translation of specific cellular mRNAs sharing undefined features with the DENV genome. We found that these proteins are not broadly required for cellular translation, but are necessary for efficient accumulation of DENV proteins early in infection and ectopically expressed DENV structural proteins. The ribosome profiling technique allowed us to quantitatively map ribosomes across the transcriptome during early DENV infection in human cell lines depleted for RPLP1/2. We observed that local ribosome occupancy is altered in the viral open reading frame with RPLP1/2 knockdown, consistent with a role for RPLP1/2 in promoting translation elongation. The most prominent ribosome pausing site in the DENV RNA was in the 5' end of the E protein coding sequence which is located 210 nts downstream of two adjacent TMs. We also observed that RPLP1/2 depletion resulted in altered ribosome density in mRNAs encoding two or more transmembrane domains. This work increases our knowledge on DENV translation regulation and sheds light on the function of RPLP1/2 in translation of specific cellular RNAs.

# Dedication

To my parents and my fiancée Karen, who have always supported me.

# Contents

Abstractiv
Dedicationv
Contentsvi
List of Tablesix
List of Figuresx
List of Abbreviationsxii
Acknowledgementsxvi
1. Introduction1
1.1 Flaviviruses1
1.1.1 Attachment
1.1.2 Entry5
1.1.3 Uncoating
1.1.4 Viral Translation7
1.1.4.1. Translation Initiation9
1.1.4.2 RNA Recruitment to the ER10
1.1.4.3. Translation Elongation11
1.1.4.4 Translation Termination and Recycling12
1.1.4.5 Ribosomal Proteins
1.1.4.6 Polyprotein Biogenesis and Processing14
1.1.5 Viral RNA Synthesis
1.1.5.1 Replication Complex (RC) Formation and RNA Synthesis

1.1.5.2 RNA-Binding Proteins	21
1.1.6 Particle Assembly	22
1.1.7 Particle Egress	25
1.1.7.1 Glycosylation	26
1.1.7.2 Furin-like Protease Cleavage	27
1.2 RPLP1 and RPLP2	27
2. RPLP1 and RPLP2 are essential flavivirus host factors that promote early viral protein accumulation	n 33
2.1 Rationale	33
2.2 Results	35
2.2.1 The RPLP1/2 heterodimer is required for DENV-2 and YFV replication	35
2.2.2 Exogenous expression of RPLP1/2 rescues DENV-2 infection	38
2.2.3 RPLP1/2 are required for DENV-2 infection of Aedes aegypti mosquitoes4	40
2.2.4 The RPLP1/2 heterodimer is required for replication of ZIKV	42
2.2.5 Effects of RPLP1/2 knockdown on other positive-strand RNA viruses	44
2.2.6 The RPLP1/2 heterodimer is not required for global cellular translation	46
2.2.7 RPLP1/2 are required for accumulation of DENV-2 proteins early after infection	48
2.2.8 RPLP1/2 are required for accumulation of DENV-2 structural proteins independently of virus infection.	50
2.3 Materials and Methods	53
2.4 Discussion	60
3. Ribosomal stalk proteins RPLP1 and RPLP2 function in DENV translation elongation	۱
	65

3.1 Rationale
3.2 Results
3.2.1 RPLP1 and RPLP2 depletion cause accumulation of RPFs in the 5' end of the region of the DENV RNA encoding the E protein
3.2.2 RPLP1/2 depletion causes limited changes in ribosome occupancy of cellular mRNAs
3.2.3 Meta-gene analysis of RIBOseq reveals accumulation of RPFs towards the 5' end of the cellular mRNA ORFs in RPLP1 and RPLP2 depleted cells
3.2.4 Depletion of RPLP1/2 cause changes in ribosome occupancy of certain cellular mRNAs encoding membrane proteins
3.3 Materials and Methods91
3.4 Discussion
4. Conclusions104
References
Biography145

# List of Tables

Table 1 - Survival and blood feeding of control and RPLP1/2 knockdown mosquitoes40
Table 2 - Validation of protein accumulation changes by WB   84

# List of Figures

Figure 1: Host dependency factors and their implicated roles in the flaviviral life-cycle4
Figure 2: Flaviviral genome and polyprotein8
Figure 3 : The RPLP1/2 heterodimer and RPLP0 are required for efficient DENV-2 and YFV infection of A549 and HuH-7 cells
Figure 4: The RPLP1/2 heterodimer is required for efficient production of infectious DENV-2 and YFV
Figure 5: Exogenous expression of RPLP1/2 rescues DENV-2 infection under conditions of endogenous RPLP1/2 knockdown
Figure 6: RPLP1/2 are required for DENV-2 infection of <i>Aedes aegypti</i> mosquitoes41
Figure 7: RPLP1/2 are host factors for ZIKV43
Figure 8: Effects of RPLP1/2 knockdown on replication of CBV3 and HCV45
Figure 9: Depletion of RPLP1/2 results in cell-line-specific effects on global translation.47
Figure 10: RPLP1/2 are required for early viral protein accumulation
Figure 11: RPLP1/2 knockdown impairs accumulation of DENV-2 structural proteins expressed in stable cell lines
Figure 12: Fractionation of HeLa cells into ER and cytosol69
Figure 13– RPLP1 and RPLP2 are required for protein accumulation early in DENV life cycle
Figure 14 - Depletion of RPLP1/270
Figure 15: Ribosome occupancy of each DENV genes73
Figure 16: RPFs accumulate in DENV RNA region encoding the N-terminus of the E protein74
Figure 17: Deep sequencing counts aligned to the DENV ORF for RIBOseq and RNAseq

Figure 18: Cumulative analysis of RNAseq and RIBOseq reads76
Figure 19: Constructs containing the DENV RNA region coding for the N-terminus of the E protein have reduced protein accumulation in RPLP1/2 depleted cells
Figure 20: RPLP1/2 depletion in A549 cells causes limited changes in ribosome occupancy of cellular mRNAs
Figure 21: Assessment of protein accumulation of mRNAs identified as having differential ribosome occupancy in RPLP1/2 depleted A549 cells
Figure 22: Ribosomal proteins and eEF2K mRNAs have their ribosome occupancy and expression changed by depletion of RPLP1/2 in A549 cells
Figure 23: RPLP1/2 depletion causes accumulation of RPFs towards the 5' end of cellular mRNAs
Figure 24: Certain cellular mRNAs encoding membrane proteins have lower ribosome occupancy in A549 cells depleted for RPLP1/290
Figure 25: mRNAs encoding two or more TMs present an accumulation of RPFs towards the 5' end of cellular mRNAs

# List of Abbreviations

- aa amino acid
- ACACA- acetyl-CoA carboxylase alpha
- ATP adenosine triphosphate
- C capsid
- CBLL1 cbl Proto-Oncogene Like 1
- CBV3 coxsackievirus B3
- CFTR cystic fibrosis transmembrane conductance regulator
- COPB coatomer protein b
- CRISPR clustered regularly interspaced short palindromic repeats
- DDX56 helicase DEAD-Box helicase 56
- DENV dengue virus
- DMEM -dulbecco's modified Eagle medium
- E envelope
- eEF eukayotic translation elongation factor
- eEF2K Eukaryotic elongation factor-2 kinase
- eIF eukaryotic initiation factor
- EMC endoplasmic reticulum membrane complex
- ER endoplasmic reticulum
- eRF eukaryotic recycling factor

- ERI3 exoribonuclease family member 3
- ESCRT endosomal sorting complex required for transport proteins
- FASN fatty acid synthase
- GBF1 golgi Brefeldin A Resistant Guanine Nucleotide Exchange Factor 1
- GnRH gonadotropin releasing hormone
- GRK2 G protein-coupled receptor kinase 2
- GTP guanosine triphosphate
- HCV hepatitits C virus
- HOX group of genes containing a DNA sequence known as homeobox
- hpi hours post infection
- IFIT interferon induced proteins with tetratricopeptide repeats
- IRES internal ribosome entry site
- JEV Japanese encephalitis virus

KDELR – KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor

- LD lipid droplet
- M membrane
- MIB1 Mindbomb E3 ubiquitin protein ligase 1
- MOI multiplicity of infection
- MRTO4 -ribosome maturation factor MRTO4
- NS -nonstructural proteins

- NSC non-silencing control siRNA
- ORF open Reading frame
- OST oligosaccharyltransferase
- PABP poly(A)-binding protein
- PAIP2 PABP-interacting protein 2
- PARD6B par-6 family cell polarity regulator beta
- PRMT1 protein arginine methyltransferase 1
- PTPRO protein tyrosine phosphatase, receptor type O
- Rab ras-related in brain
- RC replication complex
- RIBOseq ribosome profiling
- RLUC Renilla luciferase
- RNAi RNA interference
- RNASEK -transmembrane protein ribonuclease kappa
- ROS reactive oxygen species
- RPF ribosome protected fragments
- RPL ribosomal protein of the large subunit
- RPS ribosomal protein of the small subunit
- RT-qPCR reverse transcription quantitative PCR
- SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

- SEMA7A semaphorin 7A
- siRNA small interfering RNA
- SPC signal peptidase complex
- SPCS1 signal peptidase complex subunit 1
- SRP signal recognition particle
- TBEV tick-borne encephalitis virus
- TCA trichloroacetic acid
- TM transmembrane domain
- TPM transcripts per million
- TSPAN 12 Tetraspanin-12
- UBA1 ubiquitin-activating enzyme
- UPR unfolded protein response
- UTR untranslated regions
- vATPase vacuolar-type H+- ATPase
- WB Western blot
- WNV West Nile virus
- XRN1 5'-3' exoribonuclease 1
- YFV -Yellow fever virus
- ZIKV Zika virus

## Acknowledgements

I would like to thank Shelton Bradrick and Mariano Garcia-Blanco, whose training and mentorship have been invaluable. I would like to express my gratitude to the past and current members from the Bradrick and Garcia-Blanco laboratory, from University of Texas Medical Branch and Duke University for their support. I thank Gaddiel Galarza-Muñoz, Stacia Phillips and Nick Barrows for their training and guidance. I would also like to thank our collaborators who made this work possible: Julien Pompon and Benjamin Wong at Duke-NUS; Premal Shah and Sagara Wijeratne at Rutgers; Chris Nicchitta and David Reid at Duke; Andrew Routh, Pei-Yong Shi and Xuping Xie at UTMB. I would also like to thank the members of my committee Micah Luftig, Stacy Horner, Debby Silver and Chris Nicchitta for their guidance and support with the move from Durham to Galveston. I would like to thank the professors at Duke MGM, who provided me with inspiring classes. I am grateful to Kimberly Kobes, Annette Kennett and Julie Quiroga for their excellent support.

## **1. Introduction**

Introductory material for this dissertation was partially adapted from the review article published by Rafael Kroon Campos, Mariano Garcia- Blanco and Shelton Bradrick, entitled "Roles of Pro-viral Host Factors in Mosquito-Borne Flavivirus Infections" (Campos et al., 2017a) and partially from the review article by Nicholas J. Barrows, Rafael K. Campos, Kuo-Chieh Liao, K. Reddisiva Prasanth, Ruben Soto-Acosta, Shih-Chia Yeh, Geraldine Schott-Lerner, Julien Pompon, October M. Sessions, Shelton S. Bradrick and Mariano A. Garcia-Blanco entitled "Biochemistry and Molecular Biology of Flaviviruses" (Barrows et al., 2018).

## 1.1 Flaviviruses

The flaviviruses comprise one of four genera within the *Flaviviridae*, a family of positive-strand RNA viruses that derives its name from the prototypical yellow (Latin: flavus) fever virus (YFV). Many flaviviruses are emerging or re-emerging global health threats (Bhatt et al., 2013, Garske et al., 2014, Weaver et al., 2016, Wilder-Smith and Byass, 2016). Prominent human pathogens among the >50 flavivirus species include dengue viruses (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), YFV and Zika virus (ZIKV). Mosquito-borne flaviviruses are distributed mainly in tropical and subtropical regions of the globe and over half of the world's population is estimated to be at risk for infection by DENV, the most widespread of the pathogenic flaviviruses (Bhatt et al., 2013). The continuing threat of DENV, the recent deadly YFV outbreaks in Africa and in Brazil (Dexheimer Paploski et al., 2018, Ahmed and Memish, 2017, Hamer

et al., 2018) and the emerging ZIKV pandemic highlight the significant burden these viruses place on humanity. Flaviviruses are transmitted mainly by mosquito or tick bites, although sexual and vertical transmission has recently been documented for ZIKV (D'Ortenzio et al., 2016, Harrower et al., 2016).

Flaviviruses are enveloped viruses with icosahedral-shaped particles of 40–50 nm in diameter. Although icosahedral symmetry is shared between flavivirus species, the surface topology varies significantly between viruses (Kostyuchenko et al., 2016, Zhang et al., 2013). The virion is composed of three structural proteins, two of which [membrane (M) and envelope (E)] are embedded within the lipid bilayer envelope. The remaining structural protein, capsid (C), is located within the interior of the viral particle, closely associated with the single-stranded RNA genome. The genomes of flaviviruses share a similar organization: all are 11 kb positive-strand RNA molecules that contain a single open reading frame flanked by 5' and 3' untranslated regions (UTRs). Flavivirus genomes are modified with a 5' m7G cap structure but lack the 3' poly-(A) tail that is characteristic of most cellular mRNAs.

To carry out all steps in the life-cycle, flaviviruses must rely on hundreds of host gene products and other factors. Pro-viral host factors are comprised of RNAs, proteins and lipids from humans and mosquitoes that are required for efficient flavivirus infection. In contrast, anti-viral host factors block infection and are often associated with innate immunity and interferon responses. I focus specifically on pro-viral host factors known to be required at various phases of the virus life-cycle and mechanisms underlying these requirements. Pro-viral host factors are sometimes termed dependency factors or simply host factors. Understanding the factors that are required for successful infection and how they assist the virus will permit a full understanding of flavivirus biology and could yield novel targets that could be exploited to treat flaviviral diseases.

### 1.1.1 Attachment

The flaviviral life-cycle (Fig. 1) starts with attachment, mediated by the viral E protein, to cellular receptor(s) on the plasma membrane (Fig. 1A). A large number of candidate receptors or co-receptors that mediate flavivirus attachment have been described in different cell types (Perera-Lecoin et al., 2013), but which of these receptors and co-receptors are used by the virus during natural infections is not known. Different flaviviruses appear to have evolved to utilize distinct receptors and co-receptors, which may partly explain the divergent syndromes associated with these viruses (Jindadamrongwech and Smith, 2004, Thepparit and Smith, 2004). It is likely that flaviviruses require multiple co-receptors to facilitate virus binding, similar to the distantly related hepatitis C virus (Zeisel et al., 2013).



**Figure 1: Host dependency factors and their implicated roles in the flaviviral life-cycle. A.** Flaviviruses bind to co-receptors which facilitate interaction of the virus with one or more receptors. The virus enters the cell by receptor-mediated endocytosis which can occur through different endocytic pathways. Uncoating of viral RNA by fusion of the virus with the endosomal membrane requires endosomal acidification which triggers a conformational change in the virus. **B.** Translation of the viral genome may initiate in the cytosol prior to ER localization where synthesis and processing of the viral polyprotein takes place. **C.** Viral RNA metabolism involves diverse host factors. Viral replication takes place in membranous vesicles that are induced by viral nonstructural proteins. **D.** Particle assembly require host factors that associate with viral RNA, capsid or are involved in ER membrane restructuring. Some viral glycosylation events take place in the lumen of the ER. **E.** Egress of the virus requires the secretory machinery of the Golgi where additional glycosylations of viral proteins take place. After transit thought the Golgi, vesicles containing viral particles become acidified, promoting furin cleavage of prM and subsequent release from the cell by exocytosis. Pro-viral factors that do not have well defined roles at a specific phase of the life-cycle include DDX6, DNAJB7, DNAJC10, SSB and NF90. Adapted from (Campos et al., 2017a).

### 1.1.2 Entry

After attachment, the virus enters the cell by endocytosis (Fig. 1A) which, depending on the virus and cell type analyzed, is either clathrin-dependent or independent (Acosta et al., 2009, Kalia et al., 2013, Smit et al., 2011, van der Schaar et al., 2007). For instance, JEV infects fibroblasts in a clathrin-dependent manner, but entry into neuronal cells is clathrin-independent (Kalia et al., 2013). Using single-particle tracking analysis of DENV in living cells, virions were noted to move along the cell surface, presumably over distinct attachment factors, until they bound to one or more entry receptors (van der Schaar et al., 2008). For DENV in mammalian cells, entry was independent of clathrin but dependent on dynamin (Acosta et al., 2009). In hepatoma cells, the G protein-coupled receptor kinase 2 (GRK2) was found to be required for efficient DENV entry independently of  $\beta$ -arrestins (Le Sommer et al., 2012), suggesting a non-canonical pathway that involves GRK2 (Evron et al., 2012). Upon clathrin-mediated entry, DENV particles are transported to an early ras-related in brain (Rab5 positive) endosomal compartment, which subsequently matures into late endosomes through acquisition of Rab7 and loss of Rab5 (Krishnan et al., 2007, van der Schaar et al., 2008). Acidification of the late endosome mediated by the vacuolar-type H+- ATPase (vATPase) complex induces conformational changes in the E protein that lead to irreversible trimerization (Luca et al., 2013) and exposure of fusion peptides that promote merging of the viral envelope and endosomal membrane. This process is likely

enhanced by the transmembrane protein ribonuclease kappa (RNASEK). RNASEK localizes to the plasma membrane and endosomes, and functionally associates with the vATPase proton pump to promote endocytosis of several diverse viruses (Hackett et al., 2015, Perreira et al., 2015). It has been proposed that virus particles fuse preferentially with small endosomal carrier vesicles and the release of the nucleocapsid into the cytosol depends on a second, cell-mediated membrane fusion event (Nour et al., 2013). In addition to low pH, phospholipid composition is also important to promote fusion with endosomal membranes. For example, anionic lipids, such as a bis (monoacylglycerol) phosphate, a lipid specific to late endosomes, are important for DENV fusion with endosomal membranes (Zaitseva et al., 2010). This highlights the role of endosomal membrane lipids and host proteins which mediate endosomal acidification as pro-viral host factors required for membrane fusion and nucleocapsid release.

Components of the endoplasmic reticulum membrane complex (EMC) were first identified in an RNAi-mediated screen for YFV host factors (Le Sommer et al., 2012) and recently confirmed in CRISPR/Cas9 screens for viral infection (DENV, WNV and ZIKV) or protection against virus-induced apoptosis (WNV) (Marceau et al., 2016, Savidis et al., 2016, Zhang et al., 2016, Ma et al., 2015). The EMC is required for DENV and ZIKV at the entry stage (Barrows et al., unpublished) (Savidis et al., 2016) and is also likely required for other early stages of the viral life cycle such co-translational folding of viral proteins (Barrows et al., unpublished)(Shurtleff et al., 2018).

### 1.1.3 Uncoating

The uncoating step is not well understood but a recent study has shed light on this phase of the life-cycle. Byk and colleagues determined that ubiquitination is crucial for DENV RNA release into the cytoplasm (Byk et al., 2016). Although ubiquitin was required, proteasome activity was dispensable for genome uncoating, and stabilization of incoming capsid protein by proteasome inhibition did not impair early viral translation (Byk and Gamarnik, 2016). These data favor a model in which capsid does not need to be degraded and may be displaced from the RNA by translating ribosomes (Garcia-Blanco et al., 2016). Inhibition of ubiquitin-activating enzyme (UBA1) was found to block uncoating of the genome during infection, preventing access of the viral genome to the translation machinery (Byk et al., 2016). The ubiquitin ligase (CBLL1) may play a similar role since it was found to be required for WNV entry (Krishnan et al., 2008).

### **1.1.4 Viral Translation**

The flaviviral open reading frame (ORF) encodes for a polyprotein that is processed by viral and host proteases into three structural (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Fig. 2). There are, however, cases described in which different proteins are produced by ribosome frameshifting. In WNV, an alternative reading frame in NS4B produces a protein named N-NS4B/WARF4 (Faggioni et al., 2009, Faggioni et al., 2012) and in Japanese encephalitis virus (JEV), a frameshift results in a C-terminally extended form of NS1 termed NS1' (Melian et al., 2010). Finally, in insect-specific flaviviruses, a protein named fifo is synthesized from an alternative reading frame spanning parts of NS2A and NS2B (Firth et al., 2010).



**Figure 2: Flaviviral genome and polyprotein. A.** Flaviviral genome. Flaviviruses have a single-stranded (+) RNA genome of approximately 11 kb. Genome is capped but not polyadenylated. It encodes three structural (blue) and seven nonstructural (red) proteins which are translated from a single ORF. In between NS4A and NS4B, the genome also encodes a small peptide of 2 kDa (2K peptide). 5' and 3' UTRs are known to have complex structure, with several hairpins, which are important for translation, RNA synthesis, and sfRNA formation. **B.** Flaviviral polyprotein topology and predicted transmembrane domains. Flavivirus polyprotein is integrated into the ER membrane. Viral proteins prM, E, and NS1 are mainly on the luminal side and C, NS3, and NS5 on the cytoplasmic side. Proteins NS2A, NS2B, NS4A, and NS4B have several transmembrane domains spanning across the ER, and thus, large parts of these proteins are on each side and on the ER membrane. 2K peptide is entirely inserted in the ER membrane. Polyprotein is cleaved co- and post translationally at multiple sites. Cleavages on the cytoplasmic side are done by the viral protease NS3 and its cofactor NS2B, and cleavages on the ER lumen side are done by the signal peptidase complex. Polyprotein also has an additional furin protease cleavage in prM that gives rise to the mature M protein in the Golgi and one additional site between NS1 and NS2A that is cleaved by an unknown enzyme. Adapted from (Barrows et al., 2016).

#### 1.1.4.1. Translation Initiation

Flaviviral translation (Fig. 1B) is likely similar to cellular cap-dependent mRNA translation, but distinct requirements for flaviviruses have not been sufficiently explored. Flavivirus genomes possess a 5' type 1 cap structure (m7GpppAm), that is required for efficient initiation of translation (Chiu et al., 2005). The viral RNA, after uncoating in the cytosol, is thought to bind the eukaryotic initiation factor 4F (eIF4F) complex via the cap structure and poly(A) binding protein via its 3' UTR (Polacek et al., 2009). In addition, 2'-O-methylation of the viral RNA masks the genome from identification by the interferon-induced proteins with tetratricopeptide repeats (IFIT) proteins as foreign RNA (Daffis et al., 2010).

Based on translation of human mRNAs, the 43S pre-initiation complex is then recruited to scan the 90–120 nts (depending on viral species) 5' UTR to identify the start codon. To correctly initiate at the first AUG, DENV requires an RNA hairpin, named the cHP element, located 14 nucleotides downstream of the AUG codon, to stall the ribosome at the correct AUG codon (Clyde and Harris, 2006). In a similar fashion to translation of human mRNAs, hydrolysis of guanosine-5'-triphosphate (GTP) bound to eIF2 and release of initiation factors is thought to allow joining of the 60S ribosomal subunit to form an intact 80S ribosome poised for elongation. The eIF4F complex is composed of eIF4E (cap-binding protein), eIF4A (helicase), and eIF4G (scaffolding protein). While flavivirus translation is generally believed to be cap-dependent, it has been reported that DENV protein synthesis remains active under conditions where cellular cap-dependent translation is repressed. Indeed, depletion of eIF4E by RNA interference (RNAi) did not affect DENV protein synthesis or infectivity (Edgil et al., 2006). Thus, it is possible that DENV initiates translation independently of eIF4E or eIF4F altogether, at least under certain physiological conditions.

Although the genomes of flaviviruses all lack a 3' poly(A) tail, the 3' UTR of the DENV genome was shown to bind poly(A) binding protein (PABP) (Polacek et al., 2009), an important trans-acting factor that regulates mRNA stability and translation (Kahvejian et al., 2005). Interaction between poly(A)-binding protein (PABP) and eIF4G is thought to circularize cellular mRNAs and stimulate multiple aspects of translation. The addition of PABP-interacting protein 2 (PAIP2; a PABP inhibitor) reduced translation of a DENV reporter, suggesting that interaction of PABP with DENV 3' UTR is important for DENV translation (Polacek et al., 2009). However, it has not been established whether eIF4G–PABP interaction is required for efficient translation of flaviviral genomes.

#### 1.1.4.2 RNA Recruitment to the ER

Little is known about how flaviviral genomes become associated with the ER membrane, but it is likely that this depends, at least in part, on the signal recognition particle (SRP) pathway (Walter and Johnson, 1994). In flavivirus genomes the first signal sequence is encoded at the C-terminal end of the capsid sequence (Lobigs et al., 2010). Once the first initiating ribosome synthesizes the signal sequence, SRP recognizes the nascent peptide and delivers the entire RNP complex to the SRP receptor and translocon embedded within ER membranes (Walter et al., 1981). While this model has not been experimentally tested for flaviviruses, and recent studies find that many cellular mRNAs are recruited to the ER independently of SRP (Ast et al., 2013, Jan et al., 2014), evidence from high-throughput screens identified components of the SRP [SRP54 (Le Sommer et al., 2012), SRP9, SRP14 (Marceau et al., 2016)], translocon-associated protein complex [SSR1, SSR2 and SSR3 (Marceau et al., 2016)] and translocon [SEC61 subunits (Le Sommer et al., 2012, Marceau et al., 2016, Sessions et al., 2009, Zhang et al., 2016), SEC63 (Zhang et al., 2016)] as important flavivirus host factors, suggesting that genome recruitment to the ER depends on the SRP.

#### 1.1.4.3. Translation Elongation

Our knowledge of translation elongation is almost exclusively based on cellular translation processes. After 60S subunit joining, the ribosome is poised for elongation and synthesis of the viral polyprotein. eEF1 $\alpha$  bound to GTP recruits the aminoacyl-tRNA and this complex binds to the A site of the ribosome, with the anticodon loop of the t-RNA binding directly to the mRNA (Dever et al., 2018). If the anticodon matches the mRNA codon, eEF1 $\alpha$  will be released once the GTP is hydrolyzed into GDP, and the eEF1 $\alpha$ -GDP will be recycled into eEF1 $\alpha$ -GTP by the exchanged factor eEF1 $\beta$  (Dever et al., 2018).

al., 2018). Then, the peptide bond formation is catalyzed between the nascent polypeptide and the new amino acid (aa) with the aid of  $eIF5\alpha$  (Schuller et al., 2017). The ribosome then undergoes a massive rearrangement in which the subunits rotate relative to each other (Lareau et al., 2014). Then, eEF2-GTP bind in the A site and promotes translocation of the tRNA in the A site to the P site. eEF2-GTP is hydrolyzed into eEF2-GDP, which releases this factor from the ribosome allowing the elongation cycle to restart (Dever et al., 2018).

Several proteins known to participate in elongation have been implicated as potent DENV (Barrows et al., unpublished) and YFV host factors based on siRNA screen data (Le Sommer et al., 2012). The elongation factor eEF2, which stimulates ribosomal translocation, was a candidate YFV host factor. Exportin-T, another putative YFV host factor, is required for tRNA export from the nucleus (Kutay et al., 1998), and thus likely maintains sufficient cytoplasmic tRNA pools to enable viral translation.

#### 1.1.4.4 Translation Termination and Recycling

Ribosomes terminate and dissociate at the stop codon, which precedes a relatively structured 3' UTR in flaviviruses. Termination factors eRF1 and eRF3 recognize ribosomes in which a stop codon is located within the A-site and catalyze the final hydrolysis reaction by the 60S subunit which releases the nascent protein (Zhouravleva et al., 1995). For cellular mRNAs this process is enhanced by PABP (Ivanov et al., 2016). Ribosomes then disassemble and are recycled back into the pool of free subunits. It is currently unknown whether translation termination on flavivirus genomes occurs by the conventional mechanism or involves distinct factors not required for most cellular mRNAs.

#### 1.1.4.5 Ribosomal Proteins

Functionality of specific ribosomal proteins (RPs) has become increasingly recognized as important for RNA virus infection. The majority of these approximately 80 proteins interact with rRNA and the functions of most RPs are not well understood. However, recent studies have revealed that, while some RPs are necessary for core ribosomal activities, others promote translation of specific subsets of mRNAs or viral RNAs. RPS25, an RP previously implicated in driving translation mediated by internal ribosome entry sites (Landry et al., 2009), was shown to be required for efficient RNA accumulation of DENV, WNV, and ZIKV, but not YFV (Marceau et al., 2016). Similarly, RPL18 was shown to be required for an early phase of the DENV life-cycle, possibly translation or RNA synthesis, but its knockdown did not affect general protein synthesis as measured by metabolic labeling (Cervantes-Salazar et al., 2015). It is important to note that RPs have been ascribed "moonlighting" roles in processes independent of the ribosome (Blumenthal and Carmichael, 1979, Friedman et al., 1981, Kim et al., 1995, Zhou et al., 2015); their role in the flaviviral life-cycle could be unrelated to translation. For example, RPSA, also known as laminin receptor 1, may be important for flavivirus attachment (Tio et al., 2005).

#### 1.1.4.6 Polyprotein Biogenesis and Processing

The viral genome has a single open reading frame that is translated into a long polyprotein; however, efficient processing by host and viral proteases ensures that the mature viral proteins are produced. Processing is sufficiently rapid that no one has reported observing the full-length polyprotein as a single product during infection. A concise characterization of YFV polyprotein synthesis and maturation was presented by Rice and colleagues in YFV-17D-infected human SW-13 cell lines (Chambers et al., 1990a). Immunoprecipitation using YFV protein-specific rabbit antisera targeting either C, prM, or E. proteins after at least 40 min of continuous radiolabeling detected specific bands that corresponded to C, prM, or E proteins but no significant precursors (Chambers et al., 1990a). Using cell-free models of mRNA translation, the flavivirus tick-borne encephalitis virus (TBEV) E protein was efficiently cleaved in reactions containing an isolated ER fraction (Svitkin et al., 1984). These data suggest that processing of structural proteins requires ER factors and that when these factors are present processing is very efficient or unprocessed fusion proteins are readily degraded. On the other hand, unprocessed intermediates of NS proteins can be observed in certain conditions. For instance, in a pulse-chase experiment, a form of NS1 with higher molecular mass accumulated first and subsequently disappeared, coincident with the accumulation of mature NS1 (Chambers et al., 1990a). This suggested that NS1-NS2A is originally produced as a metastable precursor that is subsequently processed into NS1

and NS2A. Similar analyses suggested efficient processing between NS2A and NS2B and between NS2B and NS3. Finally, a large precursor protein corresponding to NS3-NS4A-NS4BNS5 as well as a number of processed intermediates and the mature viral proteins are observed, (Chambers et al., 1990a) suggesting multiple paths to their production.

Cellular and viral proteases coordinate flavivirus polyprotein processing. The viral protease catalytically active holoenzyme is the trypsin-like NS3 protease in complex with its cofactor NS2B (Chambers et al., 1990b, Erbel et al., 2006). Sites sensitive to YFV protease have in common two basic residues (Arg being the most common) flanked by aa with short side chains (often Gly)(Rice et al., 1985). The dual cleavage between C and prM requires a coordinated two-step cleavage in which the viral protease, NS3, and cellular signalase cleave the C protein from the cytosolic and ER lumen side, respectively; releasing mature C into the cytosol and leaving a small part of the immature C protein in the ER membrane (Lobigs, 1993, Amberg and Rice, 1999, Amberg et al., 1994). Processing between prM-E and E-NS1 is mediated by the endogenous signalase and does not require the viral protease (Markoff, 1989, Ruiz-Linares et al., 1989, Svitkin et al., 1984, Falgout et al., 1989). A distinct cleavage between NS1– NS2A requires an unknown cellular protease (Falgout et al., 1989). The remaining cleavage sites, NS2A–NS2B and NS2B–NS3, are mediated by NS3 in cis (Preugschat et al., 1990) while processing between NS3–NS4A and NS4B–NS5 is mediated by NS2B and NS3 in trans (Cahour et al., 1992, Falgout et al., 1991, Zhang et al., 1992, Chambers et al., 1991). The mature NS4A and NS4B proteins are generated by cleavage at two sites by NS2B/NS3 and the cellular signalase (Cahour et al., 1992), leaving a 2 kDa protein named 2K peptide, which is inserted in the ER membrane.

Diamond and colleagues performed a screen for WNV host factors, which identified several subunits of the signalase or signal peptidase complex (SPC), of which the author followed up on the role of signal peptidase complex subunit 1 (SPCS1) (Zhang et al., 2016). In SPCS1 KO cells, WNV E protein and a high molecular weight protein reactive to anti-E antibodies is observed, suggesting that polyprotein processing is affected (Zhang et al., 2016). Using overexpression constructs, the role of the signalase at the signal sequence for prM is observed to be uniquely sensitive to SPCS1 knockout, and lack of SPCS1 causes all tested viral subunits to be reduced (Zhang et al., 2016). Also, signalase activity between NS4A and NS4B requires SPCS1, but NS1–NS2A processing is not clearly affected (Zhang et al., 2016). Surprisingly, the cleavage between E and NS1 is much more dependent on SPCS1 when prM is present in the construct. Therefore, placement of NS1 in a more internal position on the construct renders it more dependent on SPCS1 for cleavage. The signalase activity may be context dependent or the composition of the signalase may vary. Part of the maturation of viral proteins is the addition of post-translational modifications (e.g., ubiquitination) (Roby et al., 2015).

The SPC is composed of five subunits (Evans et al., 1986), three of which (SPCS1, SPCS2, and SPCS3) have been shown to be required for infection of human and

mosquito cells by DENV and WNV (Zhang et al., 2016). SPCS1 and SPCS3 have additionally been shown to be important for infection by ZIKV, JEV, and YFV. In contrast, SPCS1 knockdown caused only modest decreases in replication of chikungunya virus (Togaviridae), Rift Valley fever virus (Bunyaviridae) and vesicular stomatitis virus (Rhabdoviridae). Thus, flaviviruses, but not RNA viruses representing three different families, depend on the SPC (Zhang et al., 2016).

Protein chaperones have also been identified to be important for flavivirus infection (Das et al., 2009, Padwad et al., 2010, Taguwa et al., 2015, Ye et al., 2013). The 70 kilodalton heat shock proteins (HSP70) isoforms were shown to be important for DENV and JEV at multiple steps of the life-cycle (Das et al., 2009, Taguwa et al., 2015, Ye et al., 2013). Upon inhibition of HSP70, the levels of all DENV proteins analyzed were decreased, but NS5 and capsid proteins were disproportionally reduced (Taguwa et al., 2015). HSP70 appears to be required for proper folding of NS5, since the addition of proteasome inhibitors restored NS5 levels but did not rescue viral infection in HSP70depleted cells. In addition, HSP70 proteins also associate with capsid protein and are required for viral assembly. HSP70 substrate selection depends on DNAJ proteins, many of which (DnaJA2, DnaJB6b, DnaJB7, DnaJB11 and DnaJC10) were also found to be important for DENV infection. The EMC, a host factor for ZIKV, DENV and YFV (Le Sommer et al., 2012, Marceau et al., 2016, Savidis et al., 2016, Zhang et al., 2016), has also been suggested to be important for protein folding and stability (Jonikas et al., 2009,

Louie et al., 2012, Richard et al., 2013, Satoh et al., 2015). However, it is currently unknown whether it is necessary for viral polyprotein folding or processing.

#### 1.1.5 Viral RNA Synthesis

Although the virus encodes its own helicase (NS3), polymerase (NS5) and other NS proteins that function in RNA synthesis, several host factors were identified to be required for RNA replication (Fig. 1C). A conundrum faced by positive-strand RNA viruses is the fact that the genome must serve as a template for both translation and synthesis of negative-strand RNA. Assuming that infection begins with entry of a single genome into a cell, the virus must "switch" from protein to RNA synthesis because of the incompatibility of elongating ribosomes and viral polymerase acting on the same RNA molecule. For poliovirus, this is controlled by the viral 3CD intermediate protein which inhibits translation and facilitates negative-strand RNA synthesis (Gamarnik and Andino, 1998). For flaviviruses, I envision an oscillating system, in which concentrations of viral and host proteins determine the fate of the RNA for translation or replication (Garcia-Blanco et al., 2016).

#### 1.1.5.1 Replication Complex (RC) Formation and RNA Synthesis

Viral translation and RNA synthesis are spatially separated. Flaviviral nonstructural proteins induce extensive rearrangements of ER membranes to form subcellular factories, known as replication complexes (RCs), which are sites of vRNA synthesis (Pena and Harris, 2012). RCs are devoid of ribosomes and other translation machinery (Romero-Brey and Bartenschlager, 2014), so nascent viral genomes destined for translation must relocate from the RC to nearby sites on the ER that favor protein synthesis. The RC is formed by rearranged ER membranes containing viral nonstructural proteins necessary for replication (Romero-Brey and Bartenschlager, 2014). In addition to concentrating factors required for viral RNA synthesis, the RC has been postulated to protect dsRNA from detection by pattern recognition receptors (Uchida et al., 2014).

It is well known that diverse positive-strand RNA viruses induce significant membrane rearrangements associated with RNA synthesis (Diaz and Ahlquist, 2012, Nagy et al., 2016). Structurally, flavivirus RCs are composed of membrane invaginations into the lumen of the ER and contain a single pore that allows access to the cytosol. Once the replication complex is formed, negative-strand synthesis ensues. The negative-strand subsequently serves as template for synthesis of multiple genomes, resulting in asymmetric abundances of positive- and negative-strand RNAs. Progeny genomes go on to serve as mediators of additional viral protein and negative-strand synthesis.

RCs are enriched with cellular components necessary for RNA synthesis. Certain host lipids and enzymes, such as lysophosphatidic acid, phosphatidic acid, and flippase, have been shown to favor curvature of membranes (Devaux et al., 2008, Kooijman et al., 2003) and may possibly facilitate membrane rearrangement by NS proteins. The oligosaccharyltransferase (OST) complex, located within the ER membrane, was found to be required for DENV RNA synthesis and associated with viral NS proteins. Interestingly, the catalytic function of the OST complex is not required for DENV replication, suggesting that the complex serves a structural role in formation of RCs (Marceau et al., 2016). STT3A and STT3B, the catalytic subunits that differentiate variant OST complexes were both required for DENV replication, however, only STT3A promoted YFV, ZIKV, and WNV infectivity, highlighting differences in the requirement for OST among flaviviruses (Marceau et al., 2016).

Fatty acid synthase (FASN) and acetyl-CoA carboxylase alpha (ACACA) were found to be important for DENV RNA synthesis and FASN relocalized to sites of viral RNA synthesis after infection, likely through interaction with NS3 and Rab18 (Heaton et al., 2010). Localization of FASN to sites of viral RNA synthesis may assist in RC formation since the process of membrane invagination would be hypothetically facilitated by local de novo fatty acid synthesis. Interestingly, the requirement for FASN is conserved in mosquito cells where lipid species associated with RCs are dramatically perturbed compared to endomembranes from uninfected cells (Perera et al., 2012).

The autophagy pathway has been widely implicated as important to virus infection and innate immunity. Autophagy can be either pro- or anti-viral, depending on the virus, and can regulate different steps of the life-cycle. For DENV it has been shown that processing of triglycerides within autophagosomes increases the levels of free fatty acids that are used to generate adenosine triphosphate (ATP) through β-oxidation
(Heaton and Randall, 2010). This process is necessary for efficient viral RNA synthesis and a requirement for autophagy was obviated by supplementing cells with free fatty acids (Heaton and Randall, 2010). Another study reported that autophagy was important for DENV morphogenesis in addition to promoting RNA synthesis (Mateo et al., 2013). Interestingly, autophagy has been variously reported to restrict WNV replication (Kobayashi et al., 2014) or have no effect at all (Beatman et al., 2012). Thus, requirements for autophagy processes appear not to be conserved among flaviviruses.

#### 1.1.5.2 RNA-Binding Proteins

There are many examples of pro-viral host factors that are RNA-binding proteins and promote RNA replication. Polypyrimidine tract-binding protein was reported to be a DENV, but not YFV, host factor that interacts with NS4A and promotes viral RNA synthesis (Agis-Juarez et al., 2009, Anwar et al., 2009, Jiang et al., 2009). Exoribonuclease family member 3 (ERI3) is a host factor for both DENV and YFV. ERI3 is normally enriched in the Golgi but upon infection localizes to sites of DENV replication and enhances RNA synthesis by an unknown mechanism (Ward et al., 2016). In addition to these factors, the La protein (Garcia-Montalvo et al., 2004, Vashist et al., 2009) and NF90 (Gomila et al., 2011) have been reported to associate with flavivirus RNA, although their roles in infection are undefined. In the context of WNV, the translation elongation factor, eEF1-a, was surprisingly found to be necessary for negative strand synthesis through binding to the 3' terminal stem loop of genomic RNA (Davis et al., 2007). Another RNA-binding protein, AU-rich binding factor 1, p45 isoform (AUF1 p45), was reported to exert RNA chaperone activity that promotes WNV genome cyclization, which is important for RNA synthesis (Friedrich et al., 2014). AUF1 p45 affinity for WNV RNA is enhanced by methylation via arginine methyltransferase (PRMT1) which was shown to be important for WNV infection by stimulating RNA synthesis (Friedrich et al., 2016). DDX6 is an RNA helicase that was shown to bind the DENV 3' UTR and act as a host factor by an unknown mechanism (Ward et al., 2011). Taken these considerations together, it becomes clear that flavivirus genomes have evolved to hijack cellular RNA-binding proteins to promote multiple phases of the life-cycle.

### 1.1.6 Particle Assembly

Assembly of virus particles (Fig. 1D) initiates with association between capsid protein and the viral genome to form the nucleocapsid. The structure of the nucleocapsid remains elusive (Kostyuchenko et al., 2016, Mukhopadhyay et al., 2005, Sirohi et al., 2016, Zhang et al., 2013). Association of the nucleocapsid with E and prM heterodimers inserted into the ER membrane precedes budding of immature viral particles into the ER lumen. This is thought to occur in close proximity to the RC pore (Junjhon et al., 2014, Welsch et al., 2009). Viral particles are transported via the secretory pathway to the Golgi apparatus where maturation and N-linked glycosylation of prM and E proteins take place. Transition from the ER to the trans-Golgi network is associated with a reduction in pH, which triggers a conformational change in prM/E spikes. Furin protease cleaves prM in this acidified compartment, converting the immature viral particle into a fully infectious virus that is subsequently released from the cell by vesicular fusion with the plasma membrane (Stadler et al., 1997).

How nascent viral genomes are extruded from RCs through the pore to nearby sites of viral assembly is unknown. However, once viral genomes destined for packaging clear the RC, they associate with capsid protein (Ivanyi-Nagy et al., 2008) located on ER membranes. Capsid also localizes to the surface of lipid droplets (LDs), organelles that function in neutral lipid storage and are often associated with ER membranes (Iglesias et al., 2015, Samsa et al., 2009), and this association is possibly important for viral particle formation. On the other hand, localization to LDs may reflect a mechanism for storage of capsid protein, preventing premature association with viral RNA and/or modulation of lipid metabolism in ways that benefit virus replication (Byk and Gamarnik, 2016). Either way, mutations in capsid that disrupt targeting to LDs restrict infection (Samsa et al., 2009). Importantly, several host factors that are important for capsid localization to LDs have been identified. Golgi brefeldin A resistant guanine nucleotide exchange factor 1 (GBF1) and coatomer protein b (COPB) are two of these factors (Iglesias et al., 2015). In addition, the concerted actions of ADP-ribosylation factor 1 (Arf1) and Arf4 were also required for capsid LD localization (Iglesias et al., 2015).

These factors likely mediate localization of capsid from the site of synthesis (ER) directly to LDs.

The nucleolar helicase DEAD-Box Helicase 56 (DDX56) was found to be important for morphogenesis of WNV particles (Xu and Hobman, 2012). Knockdown of DDX56 inhibited WNV infection and this could be rescued by a siRNA-resistant DDX56 expression construct, but not by a mutant form lacking helicase function. DDX56 depleted cells produced and exported the same amount of capsid protein to the supernatant as control cells; however, the amount of viral RNA in the supernatant was lower due to DDX56 knockdown, indicating a defect in RNA packaging (Xu and Hobman, 2012). Since DDX56 also binds to the WNV capsid (Xu and Hobman, 2012), it may facilitate transfer of viral RNA from the RC to ER membranes enriched with local capsid protein.

Src kinases have also been implicated in late stages of the flavivirus life-cycle. Knockdown or chemical inhibition of c-Src inhibited the accumulation of DENV particles in the ER without affecting viral gene expression. In addition, the Src kinase, c-Yes, was reported to promote WNV trafficking through the secretory pathway (Hirsch et al., 2005). Although it is unknown how these kinases promote the late stages of flavivirus infection, they represent druggable targets that could be used as anti-viral therapies. Several endosomal sorting complex required for transport (ESCRT) proteins were found to be important for JEV and DENV infectivity (Tabata et al., 2016). The depletion of specific ESCRT factors strongly reduced the production of infectious virus but had no effect on a JEV replicon, indicating that ESCRT proteins are not required for RNA replication (Tabata et al., 2016). Microscopic analyses revealed that ESCRT proteins localize to sites of virus assembly and may promote virion biogenesis by inducing membrane deformations that enable budding of viral particles into the ER lumen (Tabata et al., 2016).

### 1.1.7 Particle Egress

Once the flavivirus particle buds from the ER, it enters the secretory pathway where final maturation steps take place (Fig. 1E). Multiple host factors are co-opted by flaviviruses at this late stage of infection. Proteasome function was shown to be important for the egress of DENV and the proteasome inhibitor, bortezomib, reduced DENV production in primary monocytes and DENV-associated pathology in mice (Choy et al., 2015). Notably, this drug was recently identified to potently antagonize ZIKV infection (Barrows et al., 2016), although the mechanism of action for ZIKV is unknown.

Rab proteins are known to play roles in vesicular trafficking and are important for egress in addition to viral entry. Rab8b was identified in a siRNA screen of 18 Rab genes to promote WNV egress (Kobayashi et al., 2016). The ADP-ribosylation proteins Arf4 and Arf5, which play important roles in endomembrane trafficking and metabolism, were found to interact with prM and stimulate DENV and YFV egress (Kudelko et al., 2012). DENV prM has also been shown to interact with KDEL receptors (KDELR), transmembrane proteins that cycle between ER and Golgi to prevent ERresident factors from "leaking" into the Golgi. Disruption of KDELR–prM interaction decreased virus egress at the stage of viral particle transport from the ER to Golgi (Li et al., 2015).

### 1.1.7.1 Glycosylation

Flaviviruses encode three proteins that are glycosylated by host machinery: prM, E, and NS1. Glycosylation of prM may promote correct folding and heterodimerization with E protein, leading to enhanced virion morphogenesis and infectivity (Courageot et al., 2000). The N-linked glycosylation of E and prM is important for viral entry (Davis et al., 2006, Dejnirattisai et al., 2011, Guirakhoo et al., 1992) and pathogenicity in animal models (Beasley et al., 2005, Kim et al., 2008). In addition, glycosylation of particular asparagine residues on E and prM differentially promotes infection of animal and mosquito cells (Hanna et al., 2005), indicating species-specific functions for viral glycosylation (Mondotte et al., 2007). For NS1, glycosylation has been shown to be important for DENV and YFV replication and pathogenesis (Crabtree et al., 2005, Muylaert et al., 1996). Furthermore, glycosylation of NS1 stimulates secretion and stabilization of the secreted hexameric form (Crabtree et al., 2005, Flamand et al., 1999, Somnuke et al., 2011). In summary, flaviviruses strongly depend on glycosylation for infection of both human and mosquito hosts.

### 1.1.7.2 Furin-like Protease Cleavage

Shortly before the virus exits the cell, the viral structural proteins undergo reversible conformational changes caused by low pH in the exocytic compartment. The vATPase, a flavivirus entry host factor, is required for egress of DENV, due to its role in acidification of exosomes (Duan et al., 2008). Structural changes that occur due to reduced pH, expose the furin cleavage site on prM (Stadler et al., 1997, Yu et al., 2008, Li et al., 2008). Cleavage by furin or furin-like proteases causes the conformational changes to become irreversible. After release from the cell by exosome fusion with the plasma membrane, pr peptides dissociate in the neutral pH of the extracellular milieu, converting the particle into a fully infectious virus (Li et al., 2008, Zybert et al., 2008).

### 1.2 RPLP1 and RPLP2

The ribosome contains approximately 80 ribosomal proteins, and two of these proteins are RPLP1 and RPLP2 (RPLP1/2), which have been previously detected as putative host factors required for DENV (Barrows et al., unpublished) and YFV infection (Le Sommer et al., 2012). Analogs of the human proteins were initially discovered in bacteria (Moller et al., 1972) and then homologs were found in yeast (Sanchez-Madrid et al., 1979) and their identification was straightforward due to their unusual biochemical properties of being acidic phosphoproteins. RPLP1/2 contain 114 and 115 aa respectively

and are the only ribosomal proteins present in more than one copy per ribosome (Choi et al., 2015). In humans, RPLP1/2 form a pentameric structure containing two copies of each protein and one copy of the ribosomal phosphoprotein P0 (RPLP0) (Choi et al., 2015). This pentameric structure forms the ribosomal stalk, which is located in close proximity with the protein RPL12 and can be visualized by electron microscopy because it protrudes from the ribosome (Strycharz et al., 1978, Marquis et al., 1981). RPLP1/2 heterodimers can also be found free from the ribosome. It is thought that this cytosolic pool of RPLP1/2 heterodimers exist as a reserve, since these proteins only assemble on the ribosome in the cytosol (Remacha et al., 1995). The pre-60S subunits are initially assembled in the nucleolus with the RPLP0 paralog MRTO4 binding in the same site as RPLP0 binds in the rRNA (Lo et al., 2010). Then, the pre-60S subunit will leave the nucleolus to the nucleoplasm, where RPLP0 will then replace MRTO4 in the 60S subunit (Lo et al., 2010). In contrast to MRTO4 and RPLP0, RPLP1/2 only bind the ribosome after its 60S and 40S subunits are exported from the nucleus to the cytosol and are two of the last proteins to assemble on the ribosome during biogenesis (Lo et al., 2010).

Unlike most RPs, RPLP1/2 are thought not to participate in ribosome biogenesis (Nicolas et al., 2016). It was found that heterodimers of the yeast homologs of these proteins only bind the ribosome after the 80S ribosome is formed and committed to translation (Bautista-Santos and Zinker, 2014), supporting a role for these proteins in translation elongation. However it is unclear if that is also the case in mammalian cells (Martinez-Azorin et al., 2008a). RPLP1/2 each contain two functional domains, the Nterminal domain and the C-terminal domain. The N-terminal domains of both proteins are important for formation of RPLP1-RPLP2 heterodimers, which stabilize RPLP1/2 proteins (Martinez-Azorin et al., 2008a), and also for binding to RPLP0 which in turn attaches directly to the 28S rRNA (Marquis et al., 1981, Camargo et al., 2011). The Cterminal domain of the protein is flexible and extremely conserved between RPLP1, RPLP2 and RPLP0. Only the C-terminal domain from either RPLP1/2 or RPLP0 are required for viability of yeast (Santos and Ballesta, 1995).

The functions of RPLP1/2 are still debated even after decades of research on this topic. *In vitro* evidence suggests the C-terminal portion of these proteins bind elongation factors with high affinity and plays a role in translation elongation (Bargis-Surgey et al., 1999, Ito et al., 2014). Furthermore, *in vitro* assays measuring eEF-2-dependent GTPase activity and eEF-1 $\alpha$ /eEF-2-dependent polyphenylalanine translation find functional importance for RPLP1/2 in translation elongation (Lavergne et al., 1987, Baba et al., 2013). On the other hand, experiments in mammalian or yeast cells using metabolic labeling or polysome profiling detected only moderate (Martinez-Azorin et al., 2008a) to no changes (Artero-Castro et al., 2015, Perucho et al., 2014) in global translation, depending on the cell line used. An attempt to address whether RPLP1/2 could function in translation elongation using a run-off assay did not detect any defect in yeast

that were null for the homologs of RPLP1/2 (Wawiorka et al., 2017). It is possible that a more sensitive technique needs to be employed to detect the activity of RPLP1/2 on elongation since it has been suggested that these proteins may assist in translation of specific mRNAs. Indirect evidence that RPLP1/2 could function in translation elongation in cells was found by Veit and colleagues (Veit et al., 2016). They found that the depletion of any one of the proteins of the stalk (RPLP0, RPLP1 or RPLP2) or depletion of RPLP12, which is located close to the base of the stalk, could partially correct the functional expression defect of  $\Delta$ F508 cystic fibrosis transmembrane conductance regulator (CFTR), which is a mutation that causes incorrect folding of the protein. In addition, they perform a run-off assay which reveals that depletion of RPLP12 causes a detectable translation elongation defect in HeLa cells (Veit et al., 2016), indicating that the CFTR folding defects were resolved by having a slower translation elongation (Veit et al., 2016). However, the runoff assay was not performed with depletion of RPLP1/2 proteins. Their findings are consistent with the fact that optimal translation elongation rates have been shown to be important for CFTR efficient folding (Kim et al., 2015). RPLP1/2 have also been implicated in playing roles in various cellular processes which may not have a connection with their possible function in translation elongation. These proteins were proposed to be regulated by anti-gonadotropin releasing hormone (GnRH) I and II, possibly playing a role in the GnRH pathway (Lee and Ge, 2010, Chen et al., 2002). They were also suggested to be required for eIF2 $\alpha$  phosphorylation in yeast

(Jimenez-Diaz et al., 2013). RPLP1/2 depletion in cells have also been shown to cause reactive oxygen species (ROS) accumulation, followed by unfolded protein response (UPR) activation ultimately leading to autophagy as a mechanism to promote cell survival. The activation of these pathways did not have any measurable impact in global cellular translation (Artero-Castro et al., 2015). The ROS accumulation was shown to be responsible for the growth defect observed when RPLP1/2 is depleted and treatment with an antioxidant was able to restore cells to normal growth rates (Artero-Castro et al., 2015). Another aspect that may play a role in the function of RPLP1/2 proteins is that they can be phosphorylated. It was shown that phosphorylation of these proteins is not required for RPLP1/2 heterodimer binding to the ribosome (Ballesta et al., 1999). Phosphorylation of RPLP1/2 on the C-termini resulted in two to four-fold increase in affinity of binding with eEF2 (Bargis-Surgey et al., 1999). In yeast, phosphorylation on the N-terminus was shown to play a role in stability of the RPLP1/2 proteins (Nusspaumer et al., 2000) and phosphorylated proteins have been found to have different localization than the unphosphorylated protein, being enriched on the cell walls (Boguszewska et al., 2002).

There is evidence that suggests that not every ribosome contains RPLP1/2 (Garcia-Marcos et al., 2008, Guarinos et al., 2003). This opens the possibility that the protein levels and interactions of these proteins with the ribosome may be regulated in different environmental conditions. Consistent with that hypothesis, there is abundant

evidence that the protein level of RPLP1/2 and their homologs are changed in a variety of conditions outlined in this paragraph. Overexpression of Gonadotropin-releasing hormone (GnRH) I or GnRH-II have been shown to downregulate RPLP1/2 mRNA and protein levels whereas not changing the expression of a control ribosomal protein (RPS14) (Chen et al., 2002). Monoclonal antibodies targeting the GnRH receptors were also shown to reduce mRNA levels of RPLP1/2 (Lee and Ge, 2010), suggesting GnRH-I/II mediate depletion of RPLP1/2 by agonist-induced internalization and downregulation of GnRH receptors (Finch et al., 2009). Capsaicin treatment was found to increase levels of RPLP2 protein in intestinal cells (Han et al., 2005). RPLP1 has been found to be downregulated in chickens subjected to energy restriction (Wang et al., 2012) and upregulated with injection of specific prebiotic and symbiotics delivered in ovo (Slawinska et al., 2016). In yeast, it has been shown that RPLP1/2 homologs have their levels reduced from exponential to stationary growth phases (Boguszewska et al., 2002), and this observation may be due to low nutrient availability. In plants, mRNAs of RPLP0 and RPLP2 homologs are found to be downregulated during salt stress (Liu et al., 2016). RPLP1/2 have also been found to have their expression upregulated in variety of cancers, including pancreatic (Gardner-Thorpe et al., 2002), gynecologic (Artero-Castro et al., 2011), lung and bone (Yang et al., 2018) and colorectal (Tian et al., 2015) tumors. Additionally, RPLP0 was observed to be released from the ribosome during nucleolar stress, which would in theory prevent RPLP1/2 binding to the ribosome

(Derylo et al., 2018). Collectively, these examples suggest that RPLP1/2 have different expression levels in response to a variety of environmental conditions, which may suggest their expression is regulated.

# 2. RPLP1 and RPLP2 are essential flavivirus host factors that promote early viral protein accumulation

This chapter is based on a research article published by Rafael Kroon Campos et al., in the Journal of Virology in 2017 (Campos et al., 2017b).

### 2.1 Rationale

While the general mechanisms of translation have been studied extensively, the specific roles of many host proteins in translation are unclear. This is the case for most of the approximately 80 ribosomal proteins (RPs), most of which interact with rRNA (Khatter et al., 2015, Shi and Barna, 2015). While some RPs are important for basic ribosomal functions, recent evidence suggests that several RPs play roles in mRNA-specific translation (Kondrashov et al., 2011, Xue et al., 2015, Lee et al., 2013b, Nguyen-Lefebvre et al., 2013, Jiang et al., 2015, Bortoluzzi et al., 2001, Ishii et al., 2006). Supporting the idea of specialized roles, many RPs are differentially expressed in mammalian tissues (Bortoluzzi et al., 2001, Ishii et al., 2006) , and mice bearing a heterozygotic or homozygotic knockout for specific RP genes display tissue-specific phenotypes during development (Shi and Barna, 2015) . For instance, ribosome protein L38 (RPL38) is required for translation of specific homeobox (HOX) mRNAs, and this

requirement is connected to the presence of a common functional element in the 5 'UTRs of some HOX mRNAs (Kondrashov et al., 2011, Xue et al., 2015).

In addition to cellular mRNAs, specific RPs have been implicated as translational enhancers for viruses, such as vesicular stomatitis virus, measles virus, rabies virus (Lee et al., 2013b), and hepatitis C virus (HCV) (Landry et al., 2009), suggesting that these viruses have evolved a dependency on ribosomal functions conferred by these RPs. Previous genome-scale small interfering RNA (siRNA) screens performed by our laboratory identified host factors required for DENV-2 (New Guinea C strain) (Barrows et al., unpublished data) and YFV (17D strain) infection (Le Sommer et al., 2012). Many of the identified host factors are components of the translation machinery, including RPs. Other research groups have also performed genome-scale screens to identify host factors for flaviviruses by using siRNA or clustered regularly interspaced short palindromic repeats (CRISPR) and identified CRISPR and identified components of the translation machinery, but follow-up experiments of these components were limited (Marceau et al., 2016, Zhang et al., 2016, Savidis et al., 2016, Krishnan et al., 2007, Yasunaga et al., 2014).

Among the top candidate proteins identified as host factors for flavivirus infection of human cells in our screens were RPLP1 and RPLP2 (RPLP1/2) (Barrows et al., unpublished)(Le Sommer et al., 2012), which form a stable heterodimer that is tethered to the ribosome by a third RP, RPLP0, to form a conserved structure known as the ribosomal stalk. The stalk acts in recruitment of elongation factor eEF2, which functions in GTP-dependent ribosomal translocation (Bargis-Surgey et al., 1999). However, previous studies have reported little (~20% reduction) to no change in global translation when RPLP1/2 are depleted in cells (Perucho et al., 2014, Martinez-Azorin et al., 2008a, Artero-Castro et al., 2015).

In this chapter, we show that RPLP1/2 are essential for DENV-2, YFV, and ZIKV infection. RPLP1/2 were also required for DENV-2 infection of *Aedes aegypti* mosquitoes, the main vector for these viruses. Investigation of the underlying mechanism revealed that RPLP1/2 promoted both the accumulation of viral protein early after infection and the accumulation of DENV-2 structural proteins in a cell-based heterologous expression assay. In contrast to viral translation, RPLP1/2 depletion resulted in milder and cell type-specific positive or negative effects on global cellular protein synthesis. Taken together, our observations suggest that RPLP1/2 are ribosomal proteins required for flavivirus translation.

### 2.2 Results

# 2.2.1 The RPLP1/2 heterodimer is required for DENV-2 and YFV replication

Previous genome-scale screens from our laboratory identified RPLP1/2 in the top 0.05% of candidate host factors necessary for infection of HuH-7 cells by DENV-2 (Barrows et al., unpublished) and YFV (Le Sommer et al., 2012). To validate the siRNA screen data, we knocked down RPLP1/2 in A549 and HuH-7 cells by using multiple

independent siRNAs and infected the cells with either DENV-2 (New Guinea C) or YFV (17D) at a multiplicity of infection (MOI) of 1 for 24 h (Fig. 3). In accordance with previous reports (Martinez-Azorin et al., 2008a), knockdown of RPLP1/2 reduced cell proliferation by about 2-fold without affecting viability as measured in a trypan blue assay (unpublished data). We noted that knockdown of either RPLP1 or RPLP2 resulted in co-depletion of the other binding partner (Fig. 3A and B), in agreement with previous observations (Martinez-Azorin et al., 2008a). Although the cells were depleted of most RPLP1/2, a fractional pool of ribosomes containing RPLP1/2 probably remains.

After infection, cells were stained for immunofluorescence using a pan-flavivirus E protein antibody (4G2) and analyzed by high-content imaging to determine infection rates (Fig. 3C). Depletion of RPLP1/2 caused a dramatic reduction in the percentage of infected cells for both viruses and in both cell lines, in comparison to control siRNA (Fig. 3D and E). Because RPLP0 bridges the RPLP1/2 heterodimer to the ribosome, we additionally, tested whether RPLP0 is required for virus replication. Knockdown of RPLP0 with two independent siRNAs strongly reduced YFV infection (Fig. 3F and G). This suggests that flaviviruses depend on RPLP1/2 bound to ribosomes to effectively replicate. In addition to measuring infection rates by immunofluorescence, we determined the titers of virus in cell supernatants under the different experimental conditions. Measurements of infectious DENV (Fig. 4A) and YFV (Fig. 4B) showed that



Figure 3 : The RPLP1/2 heterodimer and RPLP0 are required for efficient DENV-2 and YFV infection of A549 and HuH-7 cells. Cells were transfected with either a non-silencing control siRNA (NSC) or one of five independent siRNAs used to deplete RPLP1/2, three targeting RPLP1 (siP1\_1, siP1\_2, and siP1\_6) and two targeting RPLP2 (siP2\_1 and siP2\_4). After 48 h, cells were infected at an MOI of 1 and infection was assessed after 24 h. (A and B) Western blotting results show knockdown of RPLP1/2 with independent siRNAs in A549 (A) and HuH-7 (B) cells. C. Representative images showing A549 cells infected with DENV-2 (New Guinea C). Nuclei were Hoechst stained (blue), and the viral E protein was stained with 4G2 antibody (green). (D and E) Quantification of infection rates for DENV-2 and YFV (17D) are shown for A549 (D) and HuH-7 (E) cells. F. A549 cells were transfected with the indicated siRNAs against RPLP0 and infected with YFV 48 h later at an MOI of 1. Western blotting results show knockdown of RPLP0 with two independent siRNAs. G. Rates of infection are shown for cells transfected with NSC or siRNAs targeting RPLP0. The error bars represent standard deviations of three biological replicates. Statistical significance was assessed by a two-tailed Student's *t* test between NSC and experimental siRNAs. \*\* p < 0.001; \*\*\*\* p < 0.001; \*\*\*\* p < 0.001. Adapted from (Campos et al., 2017b).



**Figure 4: The RPLP1/2 heterodimer is required for efficient production of infectious DENV-2 and YFV. A.** Titers for supernatants from HuH-7 or A549 cells infected with DENV-2 were determined in Vero cells. **B.** The same experiments as show in panel A, except with YFV. Statistical significance was assessed by a two-tailed Student's t test between NSC and experimental siRNAs. \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001. Adapted from (Campos et al., 2017b).

levels of secreted virus were reduced by up to 2 orders of magnitude in A549 cells due to RPLP1/2 knockdown. Results from HuH-7 cells showed a 15- to 30-fold reduction for DENV and 10- to 50-fold reduction for YFV. These data confirmed that RPLP1/2 are required for efficient production of infectious DENV and YFV in both cell lines analyzed.

### 2.2.2 Exogenous expression of RPLP1/2 rescues DENV-2 infection

To rule out the possibility that off-target effects of the siRNAs contributed to reduction of virus infection, we performed rescue experiments using an expression construct resistant to siRNA siP1\_1 (Fig. 5A). HuH-7 cells were transfected with RPLP1



Figure 5: Exogenous expression of RPLP1/2 rescues DENV-2 infection under conditions of endogenous RPLP1/2 knockdown. HuH-7 cells were transfected with the indicated siRNAs (NSC or siP1) and subsequently transfected with either empty vector (EV) plasmid or the siRNA-resistant RPLP1 (P1) expression. plasmid. Cells were then infected with DENV-2 at an MOI of 1. **A**. Western blotting results, showing RPLP1/2 levels after transfection with the indicated siRNA/plasmid combinations. **B**. Representative immunofluorescence images of virus infection under different conditions. Nuclei were stained with Hoechst (blue), and the viral E protein was stained with 4G2 antibody (green). **C**. Quantification of virus infection rates. The error bars represent standard deviations of three biological replicates. Statistical significance was assessed by a two-tailed Student's t test between the indicated conditions. \*\*\* p < 0.001; \*\*\*\* p < 0.0001. Adapted from (Campos et al., 2017b).

siRNA and subsequently transfected with siRNA-resistant RPLP1 plasmid before DENV-2 infection. The cells transfected with non-silencing control siRNA (NSC) and empty vector had 35% infection, while transfection with siRNA against RPLP1 and empty vector reduced DENV-2- infected cells to 3% (Fig. 5B and C). Transfection of siRNA-resistant RPLP1 vector rescued DENV-2 infection to 30% (Fig. 5B and C), further solidifying RPLP1/2 as critical host factors.

# 2.2.3 RPLP1/2 are required for DENV-2 infection of Aedes aegypti mosquitoes.

*RPLP1/2* are conserved genes that have clear homologs in the main vectors for DENV-2, mosquitoes of the *Aedes* genus. Within the ORF, human (CR542209.1) and *A. aegypti* (DQ440047.1) *RPLP1* genes share 63.39% nucleotide sequence identity, whereas human (CR542212.1) and *A. aegypti* (DQ440065.1) *RPLP2* genes have 69.37% identity. We therefore, tested whether RPLP1/2 are required for DENV-2 infection of *A. aegypti*.

dsRNA target	No. of mosquitoes injected	No. of mosquitoes fed blood meal	Survival of blood-fed mosquitoes
LacZ	100	56	55/56
RPLP1	100	34	32/34
RPLP2	100	44	42/44

Table 1 - Survival and blood feeding of control and RPLP1/2 knockdown mosquitoes



**Figure 6: RPLP1/2 are required for DENV-2 infection of** *Aedes aegypti* **mosquitoes**. Mosquitoes were injected with dsRNA targeting *RPLP1*, *RPLP2*, or *LacZ* (control) and 3 days later were offered a blood meal containing DENV-2 at 1 \_ 107 PFU/ml. Six days after oral infection, mosquitoes were homogenized and virus titers were determined in a cell-based plaque assay (yielding the number PFU per mosquito). Each data point represents an individual mosquito. **A.** RNA levels of *RPLP1/2* 9 days after dsRNA injection. Statistical significance was assessed by a *t* test. \*, *P* \_ 0.05. **B**. The DENV-2 titer in each mosquito injected with dsRNA targeting *LacZ*, *RPLP1*, or *RPLP2*. The black lines represent the means and error bars represent standard errors of the means. Statistical significance was assessed by using the ranked nonparametric Mann-Whitney U test. \*, *P* \_ 0.05; \*\*, *P* \_ 0.01. N indicates the number of infected mosquitoes analyzed per condition. These experiments were performed by our colleagues Ben Wong and Julien Pompon at Duke-NUS. Adapted from (Campos et al., 2017b).

Mosquitoes were injected with double-stranded RNA (dsRNA) targeting LacZ (negative

control), RPLP1, or RPLP2 and orally infected with DENV-2 (ST strain) by blood meal 3

days later. After 6 days of infection, mosquitoes were homogenized for measurements of viral titers. Both dsRNAs targeting RPLP1/2 were effective at reducing the corresponding mRNA levels by at least 50%, and the RPLP2 dsRNA also reduced RPLP1 mRNA levels (Fig. 6A). No difference was observed in survival of the mosquitoes injected with the different dsRNAs, but the percentage of mosquitoes that fed from the blood meal was reduced in RPLP1 and RPLP2 knockdown mosquitoes (Table 1).

For at least 21 mosquitoes that fed on the DENV-2 blood meal and survived, titers were determined for each treatment group. This analysis showed a reduction of approximately 3-fold in the number of mosquitoes with detectable virus and a significant reduction in the average titer for the RPLP1/2 knockdown groups compared to the negative-control group (Fig. 6B). These data indicate that RPLP1/2 are important for productive DENV-2 infection of *A. aegypti*.

#### 2.2.4 The RPLP1/2 heterodimer is required for replication of ZIKV.

Given the urgent need to increase our understanding of ZIKV, we asked whether RPLP1/2 are also required for ZIKV infection. We analyzed ZIKV isolates of the American lineage (isolate MEX\_I\_7) (Guerbois et al., 2016) and African lineage (isolate 41525) (Ladner et al., 2016). To this end, we knocked down *RPLP1/2* in A549 and HuH-7 cells and then infected the cells at an MOI of 1. Similar to our results with DENV-2 and YFV, both lineages of ZIKV were incompetent for replication in RPLP1/2-depleted cells (Fig. 7A). We observed reductions in infection rates of up to 50-fold for ZIKV isolate



Figure 7: RPLP1/2 are host factors for ZIKV. A549 and HuH-7 cells were transfected with the indicated siRNAs and infected 48 h later with ZIKV (isolate 41525 or MEX\_I\_7) at a MOI of 1. A. Representative images of infected A549 cells are shown. Nuclei were Hoechst stained (blue), and the viral E protein was stained with 4G2 antibody (green). B. and C. Quantification of infected A549 B. and HuH-7 C. cells is shown (top graphs) as is quantifications of infectious virus in the supernatants of A549 (B) or HuH-7 (C) cells infected with ZIKV (41525 or MEX\_I\_7). Error bars represent standard deviations of three biological replicates. Statistical significance was assessed by a two-tailed student's t test between NSC and experimental siRNAs, \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.0001. Adapted from (Campos et al., 2017b).

41525 and 19-fold for ZIKV isolate MEX\_I\_7 (Fig. 7B and C), with some siRNAs reducing signal to background levels. Viral titers in supernatants were reduced by 2 orders of magnitude in A549 cells (Fig. 7B) due to the knockdown. In HuH-7 cells, titers were reduced \_20-fold for ZIKV 41525 and by 2 orders of magnitude for ZIKV MEX\_I\_7 (Fig. 7C). Thus, ZIKV was also found to be strongly dependent on RPLP1/2 for infectivity.

# 2.2.5 Effects of RPLP1/2 knockdown on other positive-strand RNA viruses

To understand whether the requirement for RPLP1/2 is generalizable to other positive-strand RNA viruses, we tested the effects of RPLP1/2 knockdown on replication of Hepatitis C virus (HCV) (JFH1 strain) (Wakita et al., 2005), a hepacivirus within the *Flaviviridae*, and coxsackievirus B3 (CBV3; strain 20) (Tracy et al., 1992), an enterovirus within the *Picornaviridae*. Notably, both of these viruses use internal ribosome entry sites (IRES) to drive viral translation, and HCV IRES activity was previously reported to be unaffected by RPLP1/2 levels (Martinez-Azorin et al., 2008b). Because RPLP1/2 are both efficiently depleted when either of them is knocked down (Fig. 3A and B), we used siRNAs against RPLP2 only. For HCV, we used HuH-7.5 cells, since the parental HuH-7 cells are less permissive for replication (Blight et al., 2002). HCV and CBV3 infection rates were reduced approximately 2-fold when RPLP1/2 were depleted (Fig. 8A to D). Infection rates with HCV and CBV3 were compared side by side with that for DENV-2, which was inhibited about 25-fold in HuH-7 and 7-fold in HuH-7.5 cells.



Figure 8: Effects of RPLP1/2 knockdown on replication of CBV3 and HCV.HuH-7 or HuH-7.5 cells were transfected with siRNAs and then infected with the indicated viruses at MOI of 1 for 8 h (CBV3), 24 h (DENV-2), or 72 h (HCV). (A) Representative images showing HuH-7 cells infected with DENV-2 or CBV3. (B) Representative images of HuH-7.5 cells infected with DENV-2 or HCV. Nuclei were Hoechst stained (blue). Shown in green are the DENV-2 E protein, CBV3 VP1 protein, or HCV core protein. (C and D) Quantification of infection rates are shown for HuH-7 (C) *and* HuH-7.5 cells (D). Quantifications of virus yields are shown for DENV-2 in HuH-7 (C) or HuH-7.5 (D) cells, for CBV3 in HuH-7 cells (C), and for HCV (D) in HuH-7.5 cells. Graphs show means and standard deviations of percentages of infected cells or viral yields from three biological replicates. Statistical significance was assessed by a two-tailed Student's t test between NSC and experimental siRNAs. \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.0001. Adapted from (Campos et al., 2017b).

We additionally determined the titers of viruses in supernatants and found that,

consistent with experiments shown above, infectious DENV was reduced at least 10-fold

in RPLP1/2-depleted HuH-7 cells (Fig. 8C) and up to 10-fold in HuH-7.5 cells (Fig. 8D).

In contrast, HCV and CBV3 titers were unaffected by RPLP1/2 knockdown (Fig. 8C and D). Thus, DENV-2 infection was particularly sensitive to RPLP1/2 depletion compared to the other positive-strand RNA viruses tested.

# 2.2.6 The RPLP1/2 heterodimer is not required for global cellular translation

We analyzed the effect of RPLP1/2 depletion on global translation in HuH-7 cells and A549 cells to determine whether the observed decreases of flavivirus infection could be explained by a global impairment of cellular translation. RPLP1/2 were knocked down (Fig. 9A), and then cells were metabolically labeled with [35S] methionine for 30 min. As a translation inhibition control, we also treated cells with cycloheximide (CHX). Incorporated label was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 9B) and liquid scintillation counting (Fig. 9C). The outcome of these experiments showed opposite trends for the two cell lines. When RPLP1/2 were depleted, incorporation of labeled [35S] methionine was reduced by 50% in HuH-7 cells, but it was increased by approximately 80% in A549 cells (Fig. 9C). It is not clear why RPLP1/2 knockdown causes different effects on cellular translation in these cell lines. However, these observations are in agreement with previous reports that showed variable effects on cellular translation upon RPLP1/2 depletion (Perucho et al., 2014, Martinez-Azorin et al., 2008a, Artero-Castro et al., 2015). Importantly, these data indicate that the potent reductions we observed in flavivirus infectivity upon RPLP1/2 knockdown cannot be explained by a generalized inhibition of cellular translation.



Figure 9: Depletion of RPLP1/2 results in cell-line-specific effects on global translation. Cells were transfected with siRNAs and 48 h later were incubated with methionine-free medium for 30 min before addition of [35S] methionine for 30 min. Cells were subsequently lysed, and proteins were precipitated using TCA. CHX was used to control for background signal after trichloroacetic acid (TCA) precipitation. (A) Western blotting results, showing knockdown of RPLP1/2 in HuH-7 and A549 cells. Only antibody against RPLP1 was used for the Western blot assay with HuH-7 cells. (B) Autoradiography of 35S-labeled proteins fractionated by SDS-PAGE. (C) Liquid scintillation counting of incorporated 35S-labeled proteins. For each cell line, the NSC results was set to 100%. The graphs show mean values of two independent experiments for HuH-7 cells and three independent experiments for A549 cells. Error bars represent standard deviation measurements. Statistical significance was assessed by a two-tailed Student's t test between NSC and other conditions. \*\* p < 0.01; \*\*\*\* p < 0.001; \*\*\*\* p < 0.001. Adapted from (Campos et al., 2017b).

# 2.2.7 RPLP1/2 are required for accumulation of DENV-2 proteins early after infection

We next used a recombinant DENV-2 virus (New Guinea C strain) engineered to express *Renilla* luciferase (RLUC) (Zou et al., 2011) to directly test whether RPLP1/2 promote early viral protein accumulation, as would be expected if these proteins are important for translation of viral RNA. Control and RPLP1/2-depleted A549 cells (Fig. 10A) were infected at MOI of 1, and viral RNA levels and luciferase accumulation were assessed at 4 h post infection (hpi). There was no effect of RPLP1/2 knockdown on viral RNA levels compared to the negative control (Fig. 10B). In contrast, we observed a strong reduction of RLUC levels at 4 hpi in RPLP1/2 knockdown cells (Fig. 10C), indicating an early defect in the virus life cycle. To ascertain whether RNA replication contributed to RLUC levels, we performed the same assay in the presence of NITD008, a potent NS5 inhibitor (Yin et al., 2009), and observed similar results (Fig. 10C), suggesting that RNA replication did not substantially contribute to RLUC levels at 4 hpi. These data are consistent with a requirement for RPLP1/2 in early DENV translation.

We next examined NS3 accumulation in control and RPLP1/2-depleted A549 cells infected with wild-type DENV-2. We chose to examine NS3 levels because of the high sensitivity achieved with the anti-NS3 antibody used (see Materials and Methods). DENV-2 NS3 protein accumulation was analyzed at 6 hpi in control and RPLP1/2depleted cells infected at a MOI of 10 (Fig. 10D).



**Figure 10: RPLP1/2 are required for early viral protein accumulation**. **A.** Knockdown of RPLP1/2 is shown by Western blotting of A549 cells with or without NITD008 treatment. **B.** Quantification of DENV-2 RNA by RT-qPCR normalized to 18S rRNA is shown under control and RPLP1/2 knockdown conditions. **C.** Luciferase measurements from infected cells harvested 4 hpi are shown for each siRNA transfection condition in the presence or absence or NITD008 treatment. **D.** A549 cells were transfected with NSC siRNA or siRNA against RPLP2 and then infected with DENV-2 for 6 h at an MOI of 10 in the presence or absence of NITD008. NS3 accumulation was detected by Western blotting, and results for triplicate samples are shown for NSC and siP2 conditions. CHX-treated and uninfected samples served as controls for background signal. **E**. Quantification of NS3 levels was calculated by normalizing results to those for actin. The NSC condition result was set to 100%. Error bars represent standard deviation measurements of three independent wells. Statistical significance was assessed by a two-tailed Student's t test between NSC and experimental siRNA conditions. \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.001. Adapted from (Campos et al., 2017b).

We used cycloheximide (CHX) treatment to control for background signal. Quantitative Western blot analysis revealed a reduction of ~85% in NS3 levels in cells transfected with RPLP2 siRNA compared to results with negative control siRNA (Fig. 10D and E). We also performed experiments in which cells were treated with NITD008 to restrict viral RNA synthesis. In the presence of NITD008, RPLP2 depletion similarly reduced NS3 levels (~75%) (Fig. 10D and E). Together with data from the luciferase reporter DENV-2 assay, these observations show that RPLP1/2 promote an early stage of DENV-2 infection and suggest that efficient DENV-2 translation requires RPLP1/2.

# 2.2.8 RPLP1/2 are required for accumulation of DENV-2 structural proteins independently of virus infection.

We next tested whether RPLP1/2 are required for accumulation of DENV-2 proteins under conditions in which viral entry is bypassed. To address this, we isolated stable HeLa and HEK293 cell lines with cytomegalovirus (CMV) promoter-driven transgenes that express DENV-2 C-prM-E structural proteins in a tetracycline-inducible manner (Fig. 11A). In these cells, the E protein is cleaved from C-prM by cellular signalase. In contrast, the capsid protein is not efficiently cleaved from prM, because this cleavage requires NS3-mediated processing of capsid on the cytosolic face of the endoplasmic reticulum (Amberg et al., 1994). Cells were transfected with control or RPLP2 siRNAs, expression of C-prM-E RNA was measured by quantitative reverse transcription-PCR (RT-qPCR), and structural proteins were measured by Western blotting with C-prM and E antibodies (Fig. 11B and C). We observed slight or no reduction in C-prM-E RNA levels upon knockdown of RPLP1/2 in HeLa or HEK-293 cells, respectively (Fig. 11B and C). For C-prM, we observed two protein bands at 33 and 37 kDa (Fig. 10A). Based on published data (Markoff, 1989), these bands are consistent with the unglycosylated and glycosylated forms of C-prM protein, respectively. In contrast to RNA levels, the 37-kDa band was reduced in RPLP1/2-depleted cells in comparison to control siRNA-transfected cells in both cell types. We also observed reduction of the 33-kDa band with RPLP1/2 depletion in HEK-293 cells (Fig. 11C), but there was no consistent reduction of the 33-kDa band in HeLa cells (Fig. 11B). For E protein, we observed a 4- to 5-fold reduction in protein levels due to RPLP1/2 knockdown in both HeLa and HEK-293 cells (Fig. 11B and C). As RPLP1/2 have been implicated in translation elongation (Bargis-Surgey et al., 1999, Veit et al., 2016), these data raise the possibility that ribosomal elongation through structural protein coding sequences may be impaired in cells with reduced RPLP1/2 levels, with the strongest effects on accumulation of full-length E protein.



Figure 11: RPLP1/2 knockdown impairs accumulation of DENV-2 structural proteins expressed in stable cell lines. Tetracycline-inducible HeLa and HEK-293 cells expressing C-prM-E were transfected with the indicated siRNAs, and 48 h later tetracycline was added to the medium for 24 h. Cell lysates were harvested for Western blot analysis 24 h after addition of tetracycline. **A**. Representative Western blots showing expression of C-prM and E proteins under NSC or siP2\_4 transfection conditions (performed in triplicate). The arrows indicate cleaved E protein, the 37-kDa C-prM minority species, and the 33-kDa C-prM majority species. Samples from uninduced cells are also indicated (-tet). **B**. Quantifications of C-prM-E RNA by RT-qPCR normalized to 18S rRNA and protein bands in the Western blot assay from HeLa cells, normalized to results with \_-actin. The NSC conditions were set to 100%. Graphs show means and standard deviations from quantification of two independent assays performed in triplicate. **C**. The same experiment in shown in panel B, except HEK-293 cells were used. Graphs show means from the quantification of three independent assay results. Statistical significance was assessed by a two-tailed Student's t test between NSC and experimental siRNA conditions. \* p < 0.005; \*\*\*\* p < 0.0001. Adapted from (Campos et al., 2017b).

### 2.3 Materials and Methods

#### Cell culture and viruses.

Vero, A549, HuH-7, and HuH-7.5 cells (Blight et al., 2002) (kindly provided by Charles Rice, Rockefeller University) were grown in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO2. C6/36 cells were grown in RPMI medium 1640 supplemented with 10% fetal bovine serum, nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml and propagated at 28°C with 5% CO<sub>2</sub>. Tetracycline-inducible cell lines were established with the Flp-In T-REx system (Thermo Fisher Scientific) according to the manufacturer's protocol. After introduction of transgenes, HEK-293 Flp-In T-REx cells were grown in medium with 100 µg/ml of hygromycin B and 15 ug /ml of blasticidin, and HeLa Flp-In T-REx cells (Kaiser et al., 2008) (kindly provided by Elena Dobrikova, Duke University) were grown in medium with 100  $\mu$ g/ml of hygromycin B and 2  $\mu$ g/ml of blasticidin. Preparation of DENV-2 (New Guinea C) and YFV (17D) stocks has been previously described (Sessions et al., 2009). The *Renilla* luciferase reporter–DENV-2 construct (Zou et al., 2011) was produced by RNA transfection as described previously and passaged once in C6/36 cells. ZIKV isolates were kindly provided by Scott Weaver (UTMB) and Nikos Vasilakis (UTMB) and produced in C6/36 (MEX\_I\_7) or Vero cells (41525). HCV (JFH1 strain) (Wakita et al., 2005) was produced in HuH-7.5 cells, and coxsackievirus B3

(strain 20) (Tracy et al., 1992) was produced in HeLa cells. The supernatants were collected at 8 hpi for CBV3, 24 hpi for DENV-2 and ZIKV 41525, and 72 hpi for ZIKV MEX\_I\_7 and HCV. Flavivirus titers were determined via focus-forming assays in Vero cells as previously described (Sessions et al., 2009). HCV and CBV3 titers were determined based on the 50% tissue culture infective dose (TCID50) using the Reed and Muench method (Reed and Muench, 1938) for HuH-7.5 or HeLa cells, respectively. **Cloning of expression constructs.** 

RPLP1 open reading frames were amplified by RT-PCR from HuH-7 cells and cloned into pcDNA5/FRT/TO plasmid (Thermo Fisher Scientific) by using BamHI and NotI. The primers used to amplify RPLP1 were as follows: forward (5′- GAGGAT CCACCA TGGCCT CTGTCTCCGAGC TCGCCC GCATCT ACTC-3′) and reverse (5′-GAGCGG CCGCTT AGTCAA AAAGAC CAAAGC CCATGT C-3′). The RPLP1 construct was mutated by overlap PCR to make it resistant to siP1\_1 siRNA. Primers used in overlap PCR were forward (5′-TCCAGC TGAGGA GAAGAA AGTGGA AGCTAA AAAAGAAGAATC CGAGG-3′) and reverse (5′-CATCAT CAGACT CCTCGG ATTCTT CTTTTT TAGCTT CCAC-3′). The C-prM-E construct, corresponding to nucleotides 97 to 2421 (AF038403.1), was cloned in pcDNA5/FRT/TO using NotI and Xhol restriction sites.

### Western blotting.

Samples were lysed in RIPA buffer (Cell Signaling Technologies) or lysis buffer [400 mM KOAc, 25 mM HEPES (pH 7.2), 15 mM Mg(OAc)2, 1% (vol/vol) IGEPAL ca-630, 1 x protease inhibitor (Roche)]. Proteins were fractionated under denaturing conditions on 4-to-12% acrylamide gels (Novex, Thermo Fisher Scientific). Antibodies used were anti-RPLP1 (Ab121190; Abcam), anti-RPLP2 (Ab154958; Abcam), anti-mouse beta-actin (sc-47778; Santa Cruz Biotechnology), anti-DENV-2 NS3 (GTX124252; GeneTex), anti-DENV-2 E (GTX127277; GeneTex), and anti-DENV-2 C (GTX103343; GeneTex).

#### Transfections.

Plasmid transfections were done using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's instructions, and medium was changed 5 h after transfection. siRNA transfections were done using RNAiMAX reagent following the manufacturer's instructions. All siRNAs used were purchased from Qiagen. RPLP1 and RPLP2 were knocked down by forward transfection using 10 nM siRNA for experiments shown in Fig. 1, 4, and 5 and using 30 nM siRNA for all other experiments.

### Immunofluorescence staining and high-content imaging.

To assess percentages of infected cells, 5 x 10<sup>4</sup> cells were plated per well in 24well plates. The next day, cells were transfected with siRNAs against RPLP1, RPLP2, or non-silencing control siRNA (siAllStars; Qiagen). Two days later, cells were infected with virus for 8 h (CBV3), 24 h (DENV-2, YFV, and ZIKV), or 72 h (HCV). After infection, cells were fixed with cold methanol and then stained using 4G2 antibody (DENV-2, YFV, and ZIKV) (Henchal et al., 1982), CBV3 VP1 antibody (Dako), or HCV core C7-50 antibody (Abcam). Secondary Alexa Fluor 488 antibody (Thermo Fisher Scientific) and Hoechst 33342 (Sigma) were used to visualize infected cells. Cells were imaged, and infection rates were quantified using a high-content imaging microscope (Opera Phenix [Perkin-Elmer] or ArrayScan VTI [Thermo Fisher Scientific]).

#### **Rescue using siRNA-resistant expression constructs.**

For the rescue assays, cells were plated as described above and 1 day later transfected with 30 nM RPLP1 siRNA. After 24 h, 800 ng of RPLP1 (resistant to siP1\_1) plasmid was transfected using 2  $\mu$ l Lipofectamine 2000 per well. After 24 h, aliquots of cells were split to a new plate and infected the following day with DENV-2 at an MOI of 1 for 24 h before fixation and staining as described above.

#### Metabolic labeling.

To assess bulk translation in RPLP1/2 knockdown cells, metabolic labeling assays with [35S] methionine were performed. A549 or HuH-7 cells were plated at 3 x 10<sup>5</sup> cells per well in 6-well plates. The following day, cells were transfected with siRNAs and after 48 h incubated in methionine-free medium (Thermo Fisher Scientific) for 20 min. Cells were then labeled with medium containing 0.2 mCi of [35S] methionine for 30 m, washed with phosphate-buffered saline (PBS) three times, and lysed in 0.2 ml lysis
buffer. We treated one sample with CHX (0.2 mM) 5 min prior and during labeling to control for background signal. Proteins were precipitated with trichloroacetic acid (TCA), homogenized in 1 x sample loading buffer, and analyzed by SDS-PAGE or liquid scintillation counting.

#### Luciferase reporter virus infections and monitoring early NS3 accumulation.

A549 cells were plated in 24-well plates at 5 x 10<sup>4</sup> cells per well. One day later, cells were transfected with siRNA as described above. After 48 h, cells were trypsinized and counted, and equal numbers of cells were plated for each infection condition. Cells were treated with 20  $\mu$ M NITD008 for 2 h before infection with an MOI of 1, and NITD008 was retained in the medium during the course of infection. After 4 h, cells were washed five times with PBS and then lysed with *Renilla* lysis buffer (Promega). Luciferase assays were performed using an EnSpire plate reader (PerkinElmer). For NS3 analysis, A549 cells were plated in 6-well plates at 3 x 10<sup>5</sup> cells/well and transfected the following day with siRNAs. After 48 h, cells were infected with DENV-2 at an MOI of 10 in the presence or absence of NITD008 or CHX, as indicated. After 6 h of infection, cells were lysed in 70  $\mu$ l lysis buffer per well and analyzed by Western blotting using NS3 antibody.

#### Viral RNA quantification by RT-qPCR.

RNA was extracted using the ReliaPrep kit (Promega) and reverse transcribed using the High-Capacity cDNA kit (Thermo Fisher Scientific). qPCR was performed with SYBR mix on a StepOne Plus instrument (Applied Biosystems) to measure DENV-2 RNA and 18S rRNA. The  $\Delta\Delta CT$  method was used to calculate relative expression levels. The primers used for DENV-2 were forward (5'-GAAATG GGTGCC AACTTC AAGGCT-3') and reverse (5'-TCTTTG TGCTGC ACTAGAGTGGGT-3), which amplify nucleotides 5755 to 5892 of the genome (AF038403.1). For amplification of C-prM-E RNA, the primers used were forward (5-'ACTGTA CAACAG CTGACA AAGA-3') and reverse (5'-TGCGTC TCCTGT TCAAGA TG-3'). For 18S amplification the primers used were forward (5'-GTAACCCGTTGA ACCCCA TT-3') and 18S reverse (5'-CCATCC AATCGG TAGTAG CG-3').

#### Mosquito experiments.

A colony of *A. aegypti* mosquitoes collected in Singapore and established in 2014 was used. Larvae were fed a mix of fish food (TetraMin Crisps Pro) and liver powder (MP Biomedicals), while adults were held in a rearing cage (Bioquip) supplemented with 10% sucrose solution. Mosquitoes were maintained in the insectary at 28°C and 50% humidity on a 12-h:12-h dark:light cycle. To deplete RPLP1 (UniProt accession number AAEL003530) and RPLP2 (accession number AAEL014583), dsRNA targeting *RPLP1* and *RPLP2* were produced from PCR-amplified fragments and using the following two primer sets, with all primers being flanked on the 5′ side with T7 promoter: dsRNA-RPLP1 forward (5′-TACTTC CGTTTT TGCGAC CT-3′) and dsRNA-RPLP1 reverse (5′- TTCAGC TTTGTTGAGAGC CA-3′); dsRNA-RPLP2 forward (5′- TGAACG TCCAAA CAAAAT GC-3') and dsRNA-RPLP2 reverse (5'-GATTTG CCCTTCAGCTCG T-3'). dsRNA targeting *LacZ* was produced from an amplicon containing the *LacZ* sequence (Fraiture et al., 2009) and the following set of primers, which were also flanked on the 5= side with the T7 promoter: dsRNA-LacZ forward (5'-TACCCG TAGGTA GTCACG CA-3') and dsRNA-LacZ reverse (5'- TACGATGCGCCC ATCTAC AC-3). dsRNA was generated and purified using the MEGAscript T7 kit (Thermo Fisher Scientific) and the EZNA total RNA kit I (Omega Bio-Tek). RNA samples adjusted to 3  $\mu$ g/ $\mu$ l were annealed by heating to 95°C and slowly cooling. dsRNAs were injected (69-nl aliquots) into the thorax of cold-anesthetized 3- to 5-dayold mosquitoes by using a glass capillary mounted onto a Nanoject II injector (Drummond). Sequence identity between human and mosquito RPLP1/2 genes was calculated using the Muscle alignment tool (version 3.8.31). To quantify the mRNA levels of *RPLP1* and *RPLP2*, total RNA was extracted from 10 mosquitoes at 9 days postdsRNA injection by using the EZNA total RNA kit I. Reverse transcription was performed with an iScript cDNA synthesis kit (Bio-Rad), and gene expression was quantified using the SensiFAST SYBR No-ROX kit (Bioline) with the following sets of primers: RPLP1 forward (5'- ACCGGG ATTACG TTGGAACC-3') and RPLP1 reverse (5'- CGAATG TGGTGC TGTTAG CG-3'); RPLP2 forward (5'-GACGAC ATGGGATTCGGT C-3') and RPLP2 reverse (5' - TATTTG GCGGAT TTTGGG CG-3'). Amplification was conducted in a CFX96 Touch real-time PCR detection system (BioRad). Actin gene (UniProt accession number AAEL011197) quantification was used for normalization and quantified following the same protocol as described above with the primers actin forward (5'- GAACA CCCAG TCCTG CTGAC A-3') and actin reverse (5'-TGCGT CATCT TCTCA CGGTT AG-3'). Mosquitoes were infected with the DENV-2 strain ST (Schreiber et al., 2009) propagated in Vero cells. Three days after dsRNA injection, 24-h-starved mosquitoes were offered a blood meal comprised of 40% washed erythrocytes from specific-pathogen-free pig's blood (PWG Genetics), 5% 5 mM ATP (Thermo Fisher Scientific), 5% human serum (Sigma), and a 50% volume of RPMI containing virus diluted to 107 PFU/ml. Blood titers were validated in a plaque assay as described previously (Manokaran et al., 2015). The infectious blood meal was maintained at 37°C using a hemotek feeder system (Discovery Workshops) covered by a stretched pig's intestine. Mosquitoes were allowed to feed for 2 h. Engorged females were selected and maintained with access to sugar in the insectary. Six days post-oral infection, individual mosquitoes were homogenized in 500 µl of RPMI, filtered through a 0.22 µm filter (Sartorius) and determined the titer of by plaque assay (Manokaran et al., 2015).

### 2.4 Discussion

Evidence has emerged that RPs may be pivotal regulators controlling translation of viral and cellular RNAs. In this chapter, we show that ribosomal stalk proteins are crucial host factors for replication of multiple flaviviruses in human cells. RPLP1/2 were also required for DENV-2 infection of *A. aegypti* mosquitoes, highlighting a conserved role for these host factors across different species. RPLP1/2 were not essential for protein synthesis in human cells, consistent with previous reports (Perucho et al., 2014, Martinez-Azorin et al., 2008a, Artero-Castro et al., 2015).

Two lines of evidence implicate RPLP1/2 as drivers of flavivirus translation: (i) the early accumulation of DENV-2-expressed luciferase and NS3 was reduced in RPLP1/2 knockdown cells after infection, and (ii) RPLP1/2 depletion reduced expression levels of structural proteins in heterologous cell-based assays that bypass viral entry. Taken together, these results are consistent with an essential role for RPLP1/2 in translation of viral RNA. Translation elongation is generally thought to be less prone to regulation than the initiation phase, although recent studies have challenged this view (Richter and Coller, 2015, Woolstenhulme et al., 2015, Gorochowski et al., 2015, Gamble et al., 2016). The observation that DENV structural proteins expressed from a chromosomally encoded, CMV promoter-driven transgene were reduced by RPLP1/2 knockdown suggests that translation elongation through the structural protein coding region could be defective when RPLP1/2 are limiting. The fact that E protein levels were more strongly reduced by RPLP1/2 knockdown than the 33-kDa C-prM protein could reflect ribosomal stalling within the E protein-coding region (Wolin and Walter, 1988). We also observed that accumulation of the 37-kDa C-prM protein species was reduced in both cell lines when RPLP1/2 were depleted. It is possible that this is due to reduced

posttranslational modification of prM as a consequence of protein misfolding or due to protein instability of the 37-kDa C-prM protein, both of which can be caused by a possible altered translation elongation (Rodnina and Wintermeyer, 2016, Tsai et al., 2008).

Diverse viruses have evolved to hijack specific RPs to achieve optimal viral protein synthesis. For example, RPL40 has been shown to promote efficient translation initiation for vesicular stomatitis virus, measles virus, and rabies virus, but is not required for global protein synthesis (Lee et al., 2013a). RPL18 knockdown was shown to inhibit an early stage of DENV-2 infection without affecting global cellular translation or cell viability (Cervantes-Salazar et al., 2015). RPS25 appears to be broadly required for IRES-mediated translation initiation of HCV, human T-cell leukemia virus 1, Cricket paralysis virus, and poliovirus, as well as ribosome shunting in adenovirus infection (Landry et al., 2009, Hertz et al., 2013, Olivares et al., 2014). For HCV, RPs of the small subunit were found to be disproportionately required for translation and virus replication compared to RPs of the large subunit (Huang et al., 2012), suggesting that the rate-limiting step of HCV translation is at the stage of 40S subunit recruitment. Interestingly, this appears to contrast with DENV-2 (Barrows et al., unpublished) and YFV (Le Sommer et al., 2012) for which we have observed an overrepresentation of RPs from the large subunit in host factors that are required for infection. This suggests that

elongation or joining of the 60S subunit during initiation is the rate-limiting step for flavivirus translation.

RPLP1/2 participate in formation of the ribosomal stalk, which has been implicated in recruitment of translation elongation factors. The stalk proteins have been shown to interact with human eEF2 (Bargis-Surgey et al., 1999) and eEF1 in other species (Huang et al., 2012), and eEF2's ribosome dependent GTPase activity has been functionally linked to RPLP1/2. Despite being implicated as constitutive drivers of translation elongation, there are a few previous studies that reported contrasting roles for RPLP1/2 in translation and/or infectivity of other RNA viruses in different model systems. L-A virus showed enhanced propagation in yeast strains lacking RPLP1/2 homologs, and this effect was hypothesized to be linked to increased translation (Krokowski et al., 2007). Foot-and-mouth disease virus, virus internal ribosome entry site (IRES) activity was elevated in cells with depleted RPLP1/2 but only in the presence of viral Lb protease (Martinez-Azorin et al., 2008b). In parallel, the HCV IRES was unaffected by RPLP1/2 knockdown (Martinez-Azorin et al., 2008b), suggesting that the reduction in HCV infection that we observed after RPLP1/2 knockdown is likely not due to defective HCV IRES function. These examples illustrate that protein synthesis may be enhanced for some viruses in the context of RPLP1/2 depletion, contrasting with effects on flaviviruses.

While some RPs are important for basic ribosomal functions and/or ribosome

biogenesis, other RPs have been shown to regulate translation of specific cellular mRNAs through promoting translation, primarily at the initiation stage. I speculate that RPLP1/2 are important for translation of a unique subset of cellular mRNAs that share currently undefined features with flavivirus genomes. 5' and 3' untranslated regions of mRNAs have been shown to play key roles in translational regulation, especially during initiation (Hinnebusch et al., 2016). Features in the ORF, such as the cHP in DENV, have also been described to regulate translation initiation (Clyde and Harris, 2006), but they appear to be less common. In contrast, translation elongation can be regulated by features contained within the ORF or nascent polypeptide (Woolstenhulme et al., 2015, Yu et al., 2015, Gamble et al., 2016). For example, codon usage (Yu et al., 2015), RNA structure (Chen et al., 2013, Mao et al., 2014), and certain nascent polypeptide sequences can alter the rate of ribosomal transit (Woolstenhulme et al., 2015). It will be of interest to identify those features of flaviviral and cellular RNAs that confer a dependency on function of the ribosomal stalk proteins.

Ribosomes with different RP compositions may help tailor protein synthesis to a given tissue or environmental condition (Mauro and Edelman, 2002). As some disorders have been linked to RP expression levels or gene mutations (Artero-Castro et al., 2009, McCann and Baserga, 2013), uncovering how each RP affects translational status of diverse cellular mRNAs should increase our grasp of underlying disease mechanisms. Relatedly, understanding how RPs interface with viruses could spur development of novel therapeutic strategies that target viral translation.

## **3.** Ribosomal stalk proteins RPLP1 and RPLP2 function in DENV translation elongation

### 3.1 Rationale

We have previously shown that infection of different human cells lines or live Aedes aegypti mosquitos by flaviviruses require the ribosomal proteins RPLP1/2 and that in RPLP1/2 depleted cells there is a defect in early viral protein accumulation, suggesting these proteins play a role in viral translation (Campos et al., 2017b). These proteins are part of a structure known as the ribosomal stalk, which contains two dimers of RPLP1/2 that interact with a RPLP0 monomer which in turn attaches to the 28S rRNA (Choi et al., 2015). Whereas in vitro evidence indicates that the stalk functions in translation elongation (Bargis-Surgey et al., 1999, Uchiumi et al., 2002), moderate to no changes were observed in global translation of yeast and mammalian cells depleted of RPLP1/2 in comparison to control cells (Campos et al., 2017b, Perucho et al., 2014, Martinez-Azorin et al., 2008a, Artero-Castro et al., 2009, Wawiorka et al., 2017), and no evidence for a function in translation elongation has been detected (Wawiorka et al., 2017, Martinez-Azorin et al., 2008a). Although the reason for the disparity between data of *in vitro* and experiments in cells is unclear, it is possible that RPLP1/2 regulate translation elongation of specific cellular mRNAs and therefore a more sensitive and specific assay is required for detection of its effects on translation elongation.

Whereas translation initiation is often considered to be the rate limiting step for most cellular mRNAs in wild type conditions, recent studies revealed that translation elongation is extensively regulated and may contribute to protein expression under certain environmental conditions (Shah et al., 2013). Translation elongation has been shown to be regulated in response to stresses such as nutrient deprivation (Leprivier et al., 2013), proteotoxic stress (Liu et al., 2013) and heat shock (Shalgi et al., 2013), and regulation of this step of translation has also been shown to play a role in many diseases (Taymans et al., 2015, Liu and Proud, 2016).

Individual mRNAs have features that shape the efficiency of their own translation (Hall et al., 1982, Tanguay and Gallie, 1996, Kudla et al., 2009), defining whether they will be more sensitive to fluctuations in the initiation or elongation rates (Acevedo et al., 2018). Alterations in the translation elongation may impact the fate of a protein, affecting not only the rate of the synthesis itself, but also its folding and stability. In the case of membrane proteins, the insertion of the transmembrane domains (TMs) into the target membrane is often co-translational, thus a slower local elongation rate may have been selected for optimal insertion of the TMs in the membrane and folding of these proteins (Ujvari et al., 2001, Jacobson and Clark, 2016). In line with that, studies using bacteria or yeast found that either the TMs or its associated features mediate ribosome slowdown at a position of the mRNA many nucleotides downstream of the region encoding the TM (Pechmann et al., 2014, Fluman et al., 2014), optimally localized to promote better association of the TM with the translocation machinery (Kepes, 1996). However, subdomains of proteins have been found to have an optimal elongation rate for efficient folding (Kim et al., 2015), and elongation rates that are slower than normal have been found to cause protein misfolding (Nedialkova and Leidel, 2015). To attune for severe translation elongation slowdown and accumulation of its resulting products, the cell has evolved mechanisms of quality control to release and recycle stalled ribosomes (Pisareva et al., 2011), degrade mRNAs with stalled ribosomes (Doma and Parker, 2006) and degrade the protein resulting from defective translation elongation (Brandman et al., 2012). To gain insights of whether translation elongation defects exist in a given system, it is necessary to determine the position of ribosomes on the mRNA.

Ribosome profiling (herein RIBOseq) is a recently developed technique in which cell lysates are treated with RNAse I, degrading the mRNAs but leaving the mRNA fragments which are protected by the ribosome. These ribosome protected fragments (RPFs) are then deep sequenced, which allows assessment of the position of ribosomes with nucleotide precision (Ingolia et al., 2009). Local accumulation of ribosomes in a region of a mRNA indicates slow ribosome movement or a stall (Ingolia et al., 2009). Here, we use RIBOseq to investigate DENV-2 (New Guinea C) translation in the context of depleted RPLP1/2, human factors which are critically important for flavivirus infectivity. We identify a role for RPLP1/2 in translation elongation of cellular mRNAs encoding 2 or more TMs and DENV RNA, being especially important in the 5' of the region of the genome coding for the envelope (E) protein, which is located 210 nucleotides downstream of two TMs.

### 3.2 Results

### 3.2.1 RPLP1 and RPLP2 depletion cause accumulation of RPFs in the 5' end of the region of the DENV RNA encoding the E protein.

We have previously identified RPLP1/2 as essential host factors for flavivirus infection of cells and *Aedes aegypti* mosquitos, the natural vectors that transmit these viruses (Campos et al., 2017b). To test whether RPLP1/2 are required for DENV RNA to reach the ER, we infected RPLP1/2 depleted A549 cells and then fractionated the cells between ER and cytosol compartments using a mild detergent as previously described (Jagannathan et al., 2011). In the control condition, we observed that at 2.5 hours post infection (hpi) approximately 60% of the DENV RNA localized to the ER, whereas the remaining 40% of the DENV RNA localized to the cytosol (Fig. 11). The localization of the DENV RNA to the ER was not decreased in RPLP1/2 depleted cells, indicating that targeting the DENV RNA to the ER does not require RPLP1/2 (Fig. 12).

To determine a time of infection that would allow us to interrogate RPLP1/2 function in viral translation, we transfected A549 cells with either NSC or siRNAs targeting RPLP1/2 (pool of siP1\_1 and siP2\_1) and infected the cells with DENV-2 at MOI of 10 for 1, 2, 3, 4, 5 or 6 h (Fig. 13).



**Figure 12: Fractionation of HeLa cells into ER and cytosol. A.** A549 cells were transfected with NSC or siP and infected for 2 h and fractionated between ER and cytosol. Then, a qRT-PCR was done of of DENV RNA normalized to 18S rRNA. **B.** WB of an ER resident protein (EMC4) and a cytosolic protein (GAPDH) to check for purity of the fractionation.



**Figure 13– RPLP1 and RPLP2 are required for protein accumulation early in DENV life cycle**. A549 cells were transfected with either non-silencing control (NSC) or a pool of siRNAs (siP) containing one siRNA against RPLP1 (siP\_1) and one siRNA against RPLP2 (siP2\_1). After 48 h, cells were infected with DENV-2 at MOI of 10 for 1, 2, 3, 4, 5 or 6 h. Cells were lysed and NS3 protein was detected by WB.

These very early timepoints attempt to capture a phase of life cycle in which the

DENV RNA is translated (Reid et al., 2018) whereas RNA synthesis is low or not yet

taking place (Edgil et al., 2003, Zanini et al., 2018). A WB against viral protein NS3

showed accumulation was reduced in RPLP1/2 depleted cells in all time points in which

it was detected (Fig. 13). NS3 accumulation was observed as early as 2 hpi, and was

noticeably increased at 3 hpi, indicating that substantial translation took place in

between these timepoints, thus we selected 2.5 hpi to perform the ribosome profiling experiment.



**Figure 14 - Depletion of RPLP1/2. A.** WB showing depletion of RPLP1/2 protein levels of the cells used for the ribosome profiling experiments. **B.** Quantification of A. All siRNAs reduced protein levels of both RPLP1 and RPLP2 by at least 70%. The error bars represent standard deviations of three biological replicates. Statistical significance was assessed by a two-tailed Student's t test between NSC and experimental siRNAs. \*\*\*, p < 0.001; \*\*\*\*, p < 0.001. **C.** Counts in each replicate of RIBOseq or RNAseq. Unlike RNAseq, RIBOseq counts present clear triplet periodicity characteristic of ribosome profiling.

We first transfected A549 cells with either a non-silencing control siRNA (NSC) in triplicates or with three independent siRNAs targeting RPLP1 (siP1\_1) or RPLP2 (siP2\_1 and siP2\_4) to deplete levels of RPLP1/2 proteins (Fig. 14A and B). Knockdown of RPLP1 or RPLP2 leads to depletion of both proteins (Fig. 14A and B) (Martinez-Azorin et al., 2008a, Campos et al., 2017b) and we reasoned that using three independent siRNAs to obtain RPLP1/2 depletion would provide a robust methodology to determine the common and thus relevant targets of this heterodimer. We performed RIBOseq in parallel with RNAseq to assess the translational landscape of A549 cells depleted for RPLP1/2 and then infected with DENV for 2.5 h at MOI of 10.

In the RIBOseq dataset, we observed the triplet periodicity that is a signature of translation of codons by the ribosome (Fig. 14C). We found that DENV RNA had an increase of approximately 50% in ribosome occupancy in RPLP1/2 depleted cells as calculated by normalizing RIBOseq to RNAseq data. This increase in ribosome occupancy could be explained either as increased initiation or defective elongation of translation. The difference between these possibilities is that increased translation initiation would have the same distribution of ribosomes increased homogeneously throughout the DENV ORF, whereas defective translation elongation would cause ribosome to unevenly accumulate in sites where the ribosome stalls. Local high RPF density has been widely shown to correlate with ribosome stalling (Zhang et al., 2017). Examples of conditions and features that have been shown to cause local accumulation

of RPFs due to translation elongation defects include cell stresses (Liu et al., 2013, Shalgi et al., 2013), polybasic aa residues (Brandman et al., 2012, Requiao et al., 2016), depletion of elongation factors (Schuller et al., 2017), tRNA modifications (Nedialkova and Leidel, 2015), known natural stalling on the XBP1 mRNA (Ingolia et al., 2011).

The increase in ribosome occupancy we observed was not equally distributed throughout the viral ORF (Fig. 15), and the RIBOseq data revealed that several regions of the DENV RNA had higher ribosome occupancy in the conditions with depletion of RPLP1/2, indicating sites in which ribosomes are prone to stall under these conditions. The most prominent increase in ribosome occupancy was observed in nucleotide (nt) position 1030 of the DENV RNA, which is located on 5' end of the region of the DENV RNA encoding the E protein (Fig. 16A-C; 17 A and B). By graphing a cumulative curve starting from the 5' end of the DENV ORF, we observed that the region around nt 1030 is an important location of accumulation of RPFs, especially in RPLP1/2 depleted cells (p < 0.0001, KS D = 0.9) (Fig. 16D, 18A and B). As this region of the DENV RNA coding for the E protein was observed to have high ribosome occupancy relative to the rest of the DENV RNA even in control conditions, it may contain features that induce ribosome stalling which are alleviated by RPLP1/2. That region of the DENV RNA is located 210 nt (or 70 aa) downstream of the 5' end of the DENV RNA region coding for the two adjacent TMs of pre-membrane (prM). Considering that the ribosome exit tunnel can accommodate 30-40 aa, and that the two adjacent TMs in prM are likely inserted co-



**Figure 15**: **Ribosome occupancy of each DENV genes.** RIBOseq reads in transcripts per million (TPM) were normalized to RNAseq reads in TPM for each viral gene on the DENV RNA and for the DENV UTRs.

translationally, a distance of 70 aa indicates that the ribosome pauses when the TMs of the prM protein are integrating in the ER membrane. In the position we observed ribosome pausing, the active site of the ribosome would be in the nt 1030 of the DENV genome (located in the 5' end of the RNA region coding for the E protein), whereas 30-40 aa of the TMs would already have left the exit tunnel of the ribosome. Since both TMs together span 34 aa (with 3 of these being in between the TMs), this would correspond to both TMs being completely or almost completely out of the ribosome.



**Figure 16: RPFs accumulate in DENV RNA region encoding the N-terminus of the E protein.** A549 cells were transfected with siRNAs and then infected with DENV at MOI of 10 for 2.5 h and RNA was extracted and prepared for RIBOSeq and RNAseq. **A.** Difference between RIBOseq data of RPLP1/2 depleted cells (siP) and NSC. B. Ribosome occupancy (RIBOseq – RNAseq) of NSC (red) or siP (cyan) is represented in transcripts per million reads (TPM) smoothed with a 50-nt running average window across the genome. Most prominently, the arrow indicates a localized increase in ribose occupancy was observed in the region of the DENV RNA encoding the initial portion of the envelope protein. **C.** Enlarged panel with ribosomal occupancy on the DENV RNA region coding for structural proteins. The arrow indicates local increase in ribosome occupancy. **D.** Difference of the cumulative plots of RIBOseq and RNAseq in the DENV ORF in NSC or siP samples. Dotted lines represent standard deviation of ribosome occupancy of three replicates in figures B, C and D.



**Figure 17: Deep sequencing counts aligned to the DENV ORF for RIBOseq and RNAseq. A.** RIBOseq counts in TPM that aligned to the DENV ORF for each replicate of NSC or RPLP1/2 depleted conditions. **B.** RNAseq counts in TPM that aligned to the DENV ORF for each replicate of NSC or RPLP1/2 depleted conditions.



**Figure 18**: **Cumulative analysis of RNAseq and RIBOseq reads.** For each replicate, counts were summed cumulatively from the start codon through the ORF and the three replicates of each condition were averaged. The y-axis shows the cumulative reads in TPM whereas the x-axis represents the nucleotide position on the DENV ORF. Dotted lines represent stand deviation of ribosome occupancy of three replicates. **A.** RIBOseq cumulative reads.

To test whether the increase in local ribosome occupancy represents ribosome stalling that could impair protein accumulation, we generated HeLa cell lines which can be induced with tetracycline to express viral structural proteins with tags on both termini, HA on the N terminus and FLAG on the C terminus (Fig. 19A). The viral UTRs were not added to these constructs because they are usually not thought to be involved in translation elongation. Using the cells which express the tagged mature capsid (HA-C-FLAG), we observed that accumulation of this protein was increased in RPLP1/2 depleted cells, as measured by either of the tags (Fig. 19B and C). Although it is not clear why the accumulation of the protein in this construct was increased, this observation could be explained by a feedback loop mechanism which in response to RPLP1/2 depletion could lead to increased global translation efficiency, as mentioned in the next

section of this manuscript. We then expressed HA-C-prM-FLAG, which remains as a fusion protein because the efficient cleavage between these proteins requires the viral protease. The tagged C-prM construct expressed two protein bands of 33 and 37 kDa, as previously observed by us and others (Campos et al., 2017b, Ruiz-Linares et al., 1989), which likely represent forms of the protein with different post translational modifications. The N and C terminal tags allow us to conclude that both of these forms of the protein had their levels increased, as seen for either of the tags (Fig. 19B and C). Next, we tested a construct containing tagged C-prM and partial E (HA-C-prM-pE-FLAG, finishing in the nt 1797 of the DENV ORF), in which the partial E (pE) protein is efficiently cleaved from C-prM by the signalase complex, resulting in expression of a N terminal protein HA-C-prM and the C terminal protein pE-FLAG. We observed that accumulation of the 33 kDa N terminal HA-C-prM protein was not statistically different between control and RPLP1/2 depletion conditions, whereas the 37 kDa band of HA-CprM was decreased in cells with reduced levels of RPLP1/2 (Fig. 19B and C). The C terminal pE-FLAG protein was also decreased in RPLP1/2 depletion condition (Fig. 19B and C). These data suggest that the levels of RPLP1/2 in the cell affect protein accumulation of constructs containing the 5' half of the E protein. Together with our RIBOseq results, these data suggest a role for RPLP1/2 in assisting translation elongation of the region of the DENV RNA encoding the N terminus of the E protein. It is possible this happens due to the presence of cis-acting features or trans-acting factors around that



Figure 19: Constructs containing the DENV RNA region coding for the N-terminus of the E protein have reduced protein accumulation in RPLP1/2 depleted cells. A. Representation of DENV structural proteins and their topology in the ER membrane. Region with faded colors indicates portion of the structural proteins not present in any of the constructs. **B.** WB in RPLP1/2 depleted cells. HeLa tetracycline inducible cells were used ectopically express DENV C-prM-E constructs with transfection of NSC or knock down of RPLP1/2. Cells were lysed and proteins were detected with Western blotting for HA or FLAG tags. Arrows indicate regions of the constructs encoding TMs. **C.** Quantification of experiments shown in B. Two independent experiments were quantified. Each graph shows mean values of two independent experiments. Error bars represents standard deviation. Asterisks represent p values: \* p< 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001. NS- not significant.

region of the DENV RNA. Possible cis-acting features capable of stalling the ribosome could include features in the RNA region coding for the E protein that cause ribosome stalling, such as rare codons, or could be due to the TMs present at the end of the prM protein which could be stalling the ribosome during integration in the ER membrane. On the other hand, several RNA binding proteins exist, some of which have been shown to bind after TMs (Hsu et al., 2018, Shurtleff et al., 2018), which could bind the DENV RNA and as a consequence stall the ribosome.

### 3.2.2 RPLP1/2 depletion causes limited changes in ribosome occupancy of cellular mRNAs.

We investigated the effect of RPLP1/2 depletion on the ribosome occupancy of cellular mRNAs. It is often considered that RIBOseq data normalized to RNAseq data equals translation efficiency of the mRNA, since under wild type conditions initiation is thought to be rate limiting (Shah et al., 2013). However, this is a misnomer since it is widely known that defects in translation elongation can cause accumulation of ribosomes towards the 5' of the mRNA on a global scale, and cause narrows peaks of accumulation of ribosomes at an individual mRNA level (Michel and Baranov, 2013). Upon depletion of RPLP1/2, we observed limited changes for cellular mRNAs in RIBOseq reads or RNAseq reads in transcripts per million (TPM), which are proxies for number of ribosomes on the mRNA and total mRNA levels, respectively (Fig. 20A). This finding corroborates previous work done in yeast, human cells and mice in which depletion of RPLP1/2 did not cause a global disruption of translation (Martinez-Azorin

et al., 2008a, Campos et al., 2017b, Wawiorka et al., 2017, Perucho et al., 2014). Unlike other approaches, RIBOseq allows us to assess the minority of transcripts with changed ribosome occupancy in an unbiased and genome-scale manner.

We classified the 11,120 genes in our dataset as having increased, decreased or unchanged ribosome occupancy (RIBOseq/RNAseq) on their mRNAs based on DEseq2 engine of the Riborex program (Li et al., 2017) using p-value cutoffs of 0.05, 0.01, 0.001. A total of 182 mRNAs were found to have increased ribosome occupancy (n = 84 with p < 1000.05, n = 40 with p < 0.01, n = 58 with p < 0.001) and 108 to have decreased ribosome occupancy (n = 87 with p < 0.05, n = 14 with p < 0.01, n = 7 with p < 0.001) (Fig. 20A). We noted that upon depletion of RPLP1/2 there was a higher number of genes with increased rather than decreased ribosome occupancy. To further assess the types of changes elicited by RPLP1/2 depletion for each mRNA, we used a log2 fold change cutoff of 0.5 and plotted the ribosome occupancy versus the RNAseq data of the 11,120 genes in our dataset (Fig. 20B). We categorized changes as having no change (gray), RIBOseq change (red), RNAseq change (blue), RIBOseq and RNAseq change in the same direction (green) or RIBOseq and RNAseq change in opposite directions (purple) (Fig. 20B). Using these parameters, there were 9314 mRNAs with no change, 1368 mRNAs with RNAseq changes (656 decreased and 712 increased), 373 mRNAs with ribosome occupancy changes (166 decreased and 207 increased), 25 mRNAs with changes in both with the same direction of change (4 decreased and 21 increased) and 40 mRNAs with

changes in both with different directions of change (15 with decreased RNAseq and increased ribosome occupancy, and 25 with increased RNAseq and decreased ribosome occupancy). These data show that RPLP1/2 depletion caused changes in mRNA levels and ribosome occupancy in both directions, with more mRNAs having increased rather than decreased ribosome occupancy. The increased ribosome occupancy in the ORF of these mRNAs could be explained as either increased initiation and/or an accumulation of ribosomes during defective elongation. To evaluate the impact of RPLP1/2 depletion on protein accumulation of mRNAs with changed ribosome occupancy, we knocked down RPLP1/2 in A549 cells in the absence of infection and carried out WB for the proteins produced by these mRNAs. This was done in uninfected cells to check whether the changes observed could be caused by RPLP1/2 depletion alone. We tested eight genes which had changed ribosome occupancy ( $\log_2$  fold change > 0.5), and in seven the protein levels were changed in the same direction as the RIBOseq data (Fig. 20C and D and Table 2) whereas in one (ZNF296) the protein levels remained unchanged. Genes whose mRNAs had increased ribosome occupancy and were validated to have increased protein accumulation were 5'-3' exoribonuclease 1 (XRN1), Mindbomb E3 ubiquitin protein ligase 1 (MIB1) and Par-6 family cell polarity regulator beta (PARD6B) (Fig. 21 A and B and Table 2), Semaphorin 7A (SEMA7A) and Protein Tyrosine Phosphatase, Receptor type O (PTPRO). Mucin 16 and TSPAN 12 were found to have lower protein accumulation as suggested by the RIBOseq data (Fig. 21 A and B and Table 2). Unlike

what was observed with DENV, all the tested genes with more ribosome occupancy either had unchanged protein accumulation or were validated as having increased protein accumulation. This suggests that many mRNAs with increased ribosome occupancy also have increased protein accumulation in RPLP1/2 depleted cells.



Figure 20: RPLP1/2 depletion in A549 cells causes limited changes in ribosome occupancy of cellular mRNAs. A. Volcano plot depicting the ribosome occupancy (RIBOseq/RNAseq) in log2 of fold change on the X axis and the Wald test p-value in log10 calculated based on triplicate experiments by the Riborex software on the Y axis. The DENV data point is black and data points for cellular genes are color coded according to their p-values, p > 0.05 (gray), p < 0.05 (blue), p < 0.01 green), p < 0.001 (red). B. XY graph showing the Fold change of ribosome occupancy (RIBOseq/RNAseq) on the y axis versus RNAseq data on the x axis. The DENV data point is black and data points for cellular genes are color coded according to their changes from the NSC compared to RPLP1/2 depleted condition. The cutoff for genes to be considered increased or decreased was 0.5 log2 fold change. Changes were observed in ribosome occupancy (red), RNAseq (blue), in both ribosome occupancy and RNAseq with the same direction of change (green) and in both with changes in opposite directions (purple). Changes were considered to be in the same direction when ribosome occupancy and RNAseq was increased and RNAseq was decreased or ribosome occupancy was decreased and RNAseq was decreased.



Figure 21: Assessment of protein accumulation of mRNAs identified as having differential ribosome occupancy in RPLP1/2 depleted A549 cells. A. Western blot of genes with differential ribosome occupancy. B. Quantification of WBs in A. Asterisks represent p values: \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001.

This increase in protein accumulation could be explained in terms of previous reports that suggest mechanistic target of rapamycin (mTOR) can sense defects in translation elongation (Liakath-Ali et al., 2018), leading to increased global translation to compensate for the deficiency (Thoreen et al., 2012). Consistent with this model, RPLP1/2 depletion resulted in a widespread increase of ribosome occupancy of ribosomal proteins and ribosomal like proteins (Fig. 22A), possibly resulting from increased initiation of translation for ribosomal protein mRNAs. It also resulted in decreased protein levels of eEF2K, a repressor of eEF2 (Fig. 22B and C).

Gene Symbol	RNAseq fold change	RIBOseq fold change	Ribosome occupancy fold change	WB fold change
SEMA7A	1.1	2	1.9	2.2
XRN1	1.3	2	1.6	1.6
MIB1	1.2	2.2	1.8	1.5
PARD6B	2.1	3.8	1.8	8.1
PTPRO	1.3	3.3	2.6	2.3
MUC16	1	0.5	0.5	0.3
TSPAN12	0.7	0.4	0.5	0.7

Table 2 Validation of protein accumulation changes by WB

Both of these phenomena have been described as a result of upregulation of mTOR pathway (Faller et al., 2015, Nandagopal and Roux, 2015). Further work is needed to determine whether mTOR1 signaling is activated in RPLP1/2 depleted cells.



**Figure 22: Ribosomal proteins and eEF2K mRNAs have their ribosome occupancy and expression changed by depletion of RPLP1/2 in A549 cells. A**. Ribosome occupancy is increased for mRNAs encoding ribosomal proteins. The y-axis shows ribosome occupancy of ribosomal protein mRNAs and the x-axis shows the log2 fold change after RPLP1/2 depletion in comparison with NSC. **B**. eEF2K but not eEF2 protein accumulation is reduced with depletion of RPLP1/2. eEF2K function to phosphorylate and inactivate eEF2. **C**. Quantification of C.

# 3.2.3 Meta-gene analysis of RIBOseq reveals accumulation of RPFs towards the 5' end of the cellular mRNA ORFs in RPLP1 and RPLP2 depleted cells.

RPLP1 and RPLP2 have been linked to translation elongation in several in vitro

experiments which showed that they bind to eEF2 (Bargis-Surgey et al., 1999) and enhance

in vitro translation of ribosomes that had been stripped of several ribosomal proteins

(Lavergne et al., 1987, Baba et al., 2013). Nevertheless, direct evidence that links RPLP1/2

to translation elongation in cells is lacking. To test whether RPLP1/2 depletion could affect

cellular translation elongation, we performed a meta-gene analysis in which the total RPF counts for each gene were normalized to the ORF length of that gene and then RPF counts on each of the first 800 nts was normalized to this value. This revealed for the first time that RPLP1/2 depletion causes a defect in translation elongation, with an accumulation of ribosomes in the first ~250 codons (Fig. 23A). This accumulation of ribosomes in the beginning of cellular mRNAs is consistent with observations of previous work reporting translation elongation defects (Liu et al., 2013, Schuller et al., 2017, Shalgi et al., 2013). This accumulation was not particularly strong compared to the published data (~10% increase at its highest point). In concert with the data suggesting that RPLP1/2 depletion reduces protein accumulation of constructs containing the 5' half of the E protein (Fig. 19A and B), these suggest RPLP1/2 assist in translation elongation of select mRNAs rather than impacting global translation elongation. To test whether distribution of RPFs is skewed to the N-terminus of the mRNAs, which was observed to happen in other cases of translation elongation defect (Schuller et al., 2017), we used a polarity score which uses the RIBOseq data (but not the RNAseq data), as previously described (Schuller et al., 2017), to provide a rough measure or ribosome positioning. Using this method, ribosomes accumulating in the middle or equally accumulating on both sides of a mRNA would both yield a score of 0. The polarity score analysis of all genes at the ORF region revealed a small but significant shift towards the 5' end of the mRNAs when RPLP1/2 were depleted (Mean of the differences = 0.013, p =  $2.2 \times 10^{-16}$  (Fig. 23B). Thus, using RIBOseq, which has high sensitivity, allowed us to detect the small differences in elongation caused by RPLP1/2. We hypothesized that this weak elongation defect could actually be caused by strong elongation defects in specific mRNAs, and the effect could be diluted when all mRNAs are averaged.



**Figure 23: RPLP1/2 depletion causes accumulation of RPFs towards the 5' end of cellular mRNAs. A.** Metagene analysis of the average ribosome occupancy from all coding sequences were aligned at the start codon for NSC (red line) or RPLP1/2 knock down (cyan line). RPLP1/2 depletion of A549 cells causes an accumulation of ribosomes in the first 100 codon typical of ribosome translation elongation defects. Dotted lines represent stand deviation of ribosome occupancy of three replicates. **B**. Polarity score showing where reads map in the cellular mRNAs of A549 cells. The large datapoints represent the DENV values. The p-value is a result of a paired t-test.

### 3.2.4 Depletion of RPLP1/2 cause changes in ribosome occupancy of certain cellular mRNAs encoding membrane proteins.

Many mRNAs that were detected to have lower ribosome occupancy in RPLP1/2 depleted cells (using the p-value < 0.05) were membrane proteins. This prompted us to assess the ribosome occupancy status of mRNAs coding for membrane proteins in the condition depleted for RPLP1/2. We found there was a significant reduction in the ribosome occupancy (RIBOseq normalized to RNAseq) for mRNAs with 2-4 TMDs (Kolmogorov-Smirnov test, D = 0.156, average  $p = 9.6 \times 10^{-9}$ ) (Fig. 24A) and this reduction was even stronger in mRNAs with  $\geq$  5 TMDs (as assessed by the D value of our Kolmogorov-Smirnov test, D = 0.242,  $p < 1 \ge 10^{-15}$  (Fig. 24A). There was also a smaller but significant reduction in mRNA levels of genes encoding TMDs in comparison with mRNAs of all genes (Fig. 23B). For genes encoding 2-4 TMDs the reduction was less prominent (Kolmogorov-Smirnov test, D = 0.087,  $p = 5 \times 10^{-3}$ ) (Fig. 24B) compared to genes encoding 5 or more TMDs (Kolmogorov-Smirnov test, D = 0.1, p =  $1.4 \times 10^{-4}$ ). Our results suggested RPLP1/2 play a role in translation elongation (Fig. 23A) which is important for folding of membrane proteins (Veit et al., 2016, Kim et al., 2015, Ujvari et al., 2001). Thus, we hypothesized that these differences in ribosome occupancy in mRNAs encoding two or more TMs could stem from a translation elongation defect, and that the differences observed in mRNA levels could be due to degradation of the mRNA by activation of the no go decay process, in which mRNAs

that have stalled ribosomes on them are cleaved and then degraded (Doma and Parker, 2006).

We performed the meta-gene analysis and polarity analysis on genes containing 2 or more TMs and we observed an increase in the RPFs in the initial ~125 codons (Fig. 25A). Unlike the analysis with all genes (Fig. 23A), after the initial increase in RPFs, there was a strong and early decrease of RPF levels in the RPLP1/2 depleted condition, whereas the decrease in the control condition was not as pronounced (Fig. 25A). This is indicative of ongoing ribosome pausing that is more pronounced in mRNAs encoding two or more TMs. The polarity score for genes encoding two or more TMs was shifted to the left more than the polarity score including all genes (Mean of the differences = 0.0167, p =  $2.14 \times 10^{-9}$ ) (Fig. 25B). The polarity score of genes encoding 5 or more TMs followed the same trend, being more shifted to the left in comparison to the other two analyses (Mean of the differences = 0.01832, p =  $2.5 \times 10^{-5}$ ) (data not shown). Considering the protein topology from Almen and collaborators (Almen et al., 2009), the median position of the beginning of the first TM was ~17 codons, which could contribute so the peak of the accumulation of RPFs is observed very early on the meta-gene analyses. Therefore, these data suggest mRNAs with more than two TMs may be differentially affected by the depletion of RPLP1/2 at the level of translation elongation.



**Figure 24: Certain cellular mRNAs encoding membrane proteins have lower ribosome occupancy in A549 cells depleted for RPLP1/2**. A. Curves of ribosome occupancy (RIBOseq/RNAseq) in TPM for mRNAs not encoding TMs, encoding 2 to 4 TMs or with more than 5 TMs for control cells and cells depleted for RPLP1/2. The y-axis shows fraction of total genes whereas the x-axis shows ribosome occupancy. B. Curves of RNAseq in TPM for mRNAs not encoding TMs, encoding 2 to 4 TMs or with more than 5 TMs for control cells and cells depleted for control cells and cells depleted for RNAseq in TPM for mRNAs not encoding TMs, encoding 2 to 4 TMs or with more than 5 TMs for control cells and cells depleted for control cells and cells depleted for RPLP1/2.



**Figure 25: mRNAs encoding two or more TMs present an accumulation of RPFs towards the 5' end of cellular mRNAs. A.** Meta-gene analysis of the average ribosome occupancy from coding sequences of genes encoding two or more TMs in A549 cells. Dotted lines represent stand deviation of ribosome occupancy of three replicates. **B.** Polarity score of genes encoding two or more TMs in A549 cells. The p-value is a result of a paired t-test. The large datapoints represent the DENV values.

### 3.3 Materials and Methods

Cell culture and viruses.

A549 and Vero cells were grown in DMEM supplemented with 10% fetal bovine

serum, non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin in a

humidified incubator with 5% CO2 at 37°C. C6/36 cells were grown in RPMI medium

1640 supplemented with 10% fetal bovine serum, non-essential amino acids, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified incubator with 5% CO<sub>2</sub> at 28°C. Tetracycline-inducible HeLa cell lines were established using the Flp-In T-REx system (Thermo Fisher Scientific) following the manufacturer's protocol. After transfection of required plasmids, HeLa Flp-In T-REx cells (Kaiser et al., 2008) (kindly provided by Elena Dobrikova, Duke University) were grown in medium with 100  $\mu$ g/ml of hygromycin B and 2  $\mu$ g/ml of blasticidin. DENV-2 (New Guinea C) was prepared as described previously (Sessions et al., 2009). The focus forming assays to determine viral titers were performed in Vero cells as described previously (Sessions et al., 2009).

### **Cloning of expression constructs.**

The double tagged constructs with HA on the N-terminus and FLAG on the Cterminus which were used to make the HeLa Flp-In T-REx cells were amplified by PCR from a HA tagged DENV construct. The same forward primer was used to amplify all the constructs (GTACCG GTACCA TGTACC CATACG A) and each construct had a specific reverse primer, mature C (ATGCGG CCGCCT ACTTGT CGTCAT CGTCTT TGTAGT CTCTGC GTCTCC TGTTCA AGATGT), C-prM (ATGCGG CCGCCT ACTTGT CGTCAT CGTCTT TGTAGT CTGTCA TTGAAG GAGCGA CAGCTG) and CprM with partial E (ATGCGG CCGCCT ACTTGT CGTCAT CGTCTT TGTAGT CCAGCC TGCACT TGAGAT GTCC).

### Transfections.
Plasmid transfections to generate Flp-In T-REx cells were done using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's instructions and medium was changed 5 h after transfection. The siRNA transfections were carried out using RNAiMAX reagent (Thermo Fisher Scientific) following the manufacturer's instructions in a forward transfection. All siRNAs used were from Qiagen, and the sequence of their sense strand is the following: siP1\_1 (5'-GAAAGU GGAAGC AAAGAA ATT-3'), siP2\_1 (5'-AGGUUA UCAGUG AGCUGA ATT-3') and siP2\_4 (5'-GCGUGG GUAUCG AGGCGG ATT. For the ribosome profiling and RNA sequencing experiments, transfections were optimized to knock down RPLP1/2 in 10 cm dishes, using a final concentration of 50nM for each siRNA and changing the media after 5 h of incubation. For downstream assays, a pool of siP1\_1 and siP2\_1 was used for a final combined concentration of 30nM.

#### DENV RNA quantification by qRT-PCR.

RNA was extracted using Trizol LS (Thermo Fisher Scientific) and reverse transcribed using the High-Capacity cDNA kit (Thermo Fisher Scientific). The qPCR was performed using SYBR green mix (Thermo Fisher Scientific) on a StepOne Plus instrument (Applied Biosystems) to measure DENV RNA and 18S rRNA. The relative expression levels were calculated using the  $\Delta CT$  method. The following primers were used to amplify nucleotides 5755 to 5892 of the DENV genome (AF038403.1): forward

# (5'-GAAATG GGTGCC AACTTC AAGGCT-3') and reverse (5'-TCTTTG TGCTGC ACTAGAGTGGGT-3).

#### Western blotting.

Cells were lysed in RIPA buffer (Cell Signaling Technologies). Proteins were loaded on 4-to-12% acrylamide gels (Novex, Thermo Fisher Scientific) under denaturing conditions and eletrophoretically fractionated. Antibodies used were anti-RPLP1 (Ab121190; Abcam), anti-RPLP2 (Ab154958; Abcam), anti-mouse beta-actin (sc-47778; Santa Cruz Biotechnology), anti-DENV-2 NS3 (GTX124252; GeneTex), anti-FLAG (F7425; Sigma-Aldrich), anti-HA (ab18181; Abcam), anti-TSPAN12 (A05472-1; Boster-Bio); anti-MUC16 (; Santa Cruz Biotechnology); anti-XRN1 (; Santa Cruz Biotechnology); anti-SEMA7A (sc-374432; Santa Cruz Biotechnology); anti-MIB1 (sc-393551; Santa Cruz Biotechnology); anti-PTPRO (sc-365354; Santa Cruz Biotechnology); anti-PARD6B (sc-166405; Santa Cruz Biotechnology); anti-eEF2K (sc-390710; Santa Cruz Biotechnology); anti-XRN1(ab70259; Abcam); anti-eEF2 (2332; Cell Signaling Technology); anti-EMC4 (ab184544; Abcam).

#### Experiments of ectopic expression of DENV constructs in HeLa and cell fractionation.

Cell fractionation to assess the relative amount of DENV RNA in the ER versus the cytosol was performed by plating  $3 \times 10^5$  A549 cells in each well of a 6 well dish, cells were infected for 2.5 h at MOI of 10 and fractionated as described previously (Jagannathan et al., 2011). Samples were then divided to perform either qRT-PCR or precipitated with TCA and resuspended with 1x buffer to concentrate the proteins for WB. In experiments of expression of DENV constructs in HeLa Flp-In T-REx cells, 3 x 10<sup>5</sup> cells were plated in in each well of a 6 well dish, cells were induced with tetracycline for 24 h, lysed and used in WB.

## **RIBOseq and RNAseq experiments.**

A549 cells were plated at 1.5 x 10<sup>6</sup> cells per 10 cm dish. Three 10cm dishes were transfected with NSC siRNA whereas three other dishes were transfected with either siP1\_1, siP2\_1 or siP2\_4 siRNAs as described in the transfections section. 48 h later cells were infected as described previously (Campos et al., 2017b) with DENV-2 (NGC strain) at MOI of 10 in a total volume of 10 ml, rocked every 15 minutes for 1 h and the infection was allowed to proceed for more 1.5 h (2.5 h total time). Cells were then flash frozen in liquid nitrogen without cycloheximide pretreatment and cold lysis buffer containing CHX was used to lyse the cells on ice. The ribosome profiling strategy was adapted from Ingolia and colleagues (Ingolia et al., 2012) with a few modifications described next, some of which were adapted from Reid and colleagues (Reid et al., 2015). After nuclease digestion, samples were run in a polysome gradient and the ribosomes fraction was collected and extracted using Trizol LS (Thermo Fisher Scientific). rRNAs were removed using the Ribo-Zero gold rRNA removal kit (Illumina) according to the manufacturer's protocol. For adapter ligation and library building we used NEBNext Small RNA Library Prep Set (Illumina).

## **RIBOseq and RNAseq analyses.**

Adapter sequences were trimmed from the reads at the 5' end using cutadapt. Reads originating from rRNAs were removed by mapping to an index of human rRNA sequences with bowtie2. Filtered reads were then mapped to the human genome using HISAT2. Reads mapping to tRNAs or noncoding RNA genes were discarded. For each CDS, counts of uniquely mapped reads whose 5'-most mapped base occupied a position in the CDS were calculated. Only reads of lengths 29-35 nucleotides were considered. For DENV analyses, reads were assigned an offset of +14 from the 5' of the read corresponding to the approximate the position of the ribosomal p-site. Transcripts per kilobase million (TPM) reads from the RIBOseq and RNAseq were calculated to assess the total RPFs and RNA accumulation, respectively. Division between RIBOseq and RNAseq were used to calculate ribosome occupancy of DENV RNA and ribosome occupancy of cellular mRNAs on figure 4B. The DESeq2 engine of Riborex was used to determine ribosome occupancy differences between NSC and RPLP1/2 depleted conditions for figure 4A. For all subsequent analyses, genes with less than 64 counts in at least one replicate were filtered out of this analysis. For the meta-gene analysis, the reads were aligned by the 5' end and for each replicate the total RPF counts for each gene were divided by the CDs length of that gene. Then, the RPF counts on each of the first 800 nts was normalized to this value as described previously (Weinberg et al., 2016). The polarity score analysis was done as described previously by Schuller and colleagues

(Schuller et al., 2017). For the multiple linear regression analysis, sequence-based parameters that may affect translation (Weinberg et al., 2016) were chosen that could explain changes caused by RPLP1/2 depletion. To identify the most informative features, we used a stepwise Akaike information criterion for model selection with both step-up and step-down model selection procedures (Weinberg et al., 2016). The model that best explained the data after penalizing for complexity included all variables, which were 5' UTR length, 3' UTR length, CDS length, 5' UTR GC content, CDS GC content, upstream AUG counts, ribosome occupancy as calculated by Riborex, folding energy of mRNA region with the start codon, folding energy of mRNA region with the transcription start site. This prediction model was used and compared to the observed data from the sequencing. For the frameshift analysis, five adjacent 30 nt windows were considered starting 30 nts upstream of the start codon. Five adjacent windows of 30 nts were also considered by the stop codon, starting 30 nts downstream of the stop codon. The percentage of RPFs mapped to the first reading frame of each window was calculated in NSC or RPLP1/2 depleted conditions. To reduce the confounding factor of irregular distribution of RPFs throughout the ORF which is observed in some genes, the percentages were then normalized by the mean of RPF counts of the five 30 nt windows, and that was done for windows at both ends of the mRNA. If the 5'UTR is less than 30 nts, the 5' most nucleotide was taken as part of the first window. If there are less than 10 windows spanning the ORF, half of the total amount of windows were taken. Then, the

normalized distributions of RPFs near the start codon were divided by that of the nucleotides near the stop codon. If there is no frameshift, the ratio will be near 1.

## 3.4 Discussion

Viruses have evolved a plethora of strategies to ensure their translation using the cell machinery. Ribosomal stalk proteins RPLP1/2 have a striking importance for flavivirus infectivity in cells and *in vivo* due to a defect in early accumulation of flaviviral proteins (Campos et al., 2017b). Conversely, depletion of these proteins has limited impact on global cellular translation or infectivity with other positive strand viruses tested (Campos et al., 2017b). Here, we discovered that RPLP1/2 promotes efficient translation elongation of the DENV RNA, showing for the first time that RPLP1/2 function in translation elongation in cells. We unearthed that RPLP1/2 are specifically important in alleviating the ribosome stall on the 5' end of the DENV RNA region coding for the E protein, which is located downstream of the two adjacent prM TMs. Our data suggests that RPLP1/2 functions not only in alleviating stalls caused by presence of DENV TMs but that this mechanism may be generalizable to cellular mRNAs encoding two or more TMs.

Although initiation is thought to be the limiting factor in translation, recent reports have shown that, under certain circumstances, translation elongation can be extensively regulated to adapt to environmental conditions (Shah et al., 2013). Translation elongation has been shown to be regulated in response to stresses such as nutrient deprivation (Leprivier et al., 2013), proteotoxic stress (Liu et al., 2013) and heat shock (Shalgi et al., 2013), and regulation of this step of translation has also been shown to play a role in many diseases (Taymans et al., 2015, Liu and Proud, 2016). Previous studies have reported several host factors and viral elements involved in translation initiation (Campos et al., 2017a), but our comprehension of flavivirus translation elongation is chiefly based on elongation of cellular mRNAs. Flavivirus RNA contains a collection of features in its coding sequence that could pose problems for ribosome movement, including many TMs, rare codons, and extensive genome length. Whereas siRNA screens to find host factors required for Hepatitis C virus found an overrepresentation of ribosomal proteins of the small subunit (Huang et al., 2012), our genome-scale siRNA screens to find host factors for Flaviviruses have previously identified an overrepresentation of ribosomal proteins of the large subunit (Le Sommer et al., 2012), suggesting that translation elongation may be the rate-limiting step for flaviviruses. The discovery of RPLP1/2 as critical host factors for flavivirus infection, (Campos et al., 2017b) which promote efficient DENV translation elongation, increases our understanding of the mechanisms of DENV translation elongation.

We found that DENV genome has an accumulation of RPFs on the 5' end of the region coding for E protein, and that this accumulation is greatly increased in cells depleted for RPLP1/2. This suggests that ribosomes move slowly or stall in that region of the genome, and that RPLP1/2 assist in the translation elongation alleviating this pause.

We found that the location of this pause (nt position 1030) is 70 aa (or 210 nts) downstream of the 5' end of the region encoding two adjacent TMs. Considering that the exit tunnel of the ribosome accommodates 30-40 aa and the lipid bilayer accommodates approximately 20 aa, this would be the approximate expected location of the RNA in which the first of the two TMs is integrated and the second is integrating into the ER lipid bilayer. This suggests that the insertion of these adjacent TMs may be causing ribosomes to pause, and that, without RPLP1/2 this pausing becomes more intense. The integration of TMs in the ER membrane is thought to be co-translational, often requiring ribosome mediated processes (Dou et al., 2014, Devaraneni et al., 2011). Exceptions include TMs of very short proteins (Johnson et al., 2012) or TM domains with short Cterminal tails (Shao and Hegde, 2011, Schuldiner et al., 2008), which have been shown to be post-translationally inserted.

We showed that in conditions in which RPLP1/2 are depleted, only the constructs containing the initial portion of the E protein had their protein accumulation decreased compared to control, indicating that RPLP1/2 function of alleviating ribosome pausing is important for protein accumulation. This is consistent with the fact that translation elongation rates are extremely important for protein expression, folding and stability (Nedialkova and Leidel, 2015, Kim et al., 2015). Slow ribosomes take longer to translate mRNA into protein whereas stalled ribosomes may in some cases be irreversibly stuck on the mRNA. Furthermore, elongation that is slower than that in wild type cells often causes misfolding and aggregation of the resulting proteins (Nedialkova and Leidel, 2015), whereas a relatively fast elongation may prevent optimal folding of some proteins or its subdomains (Kim et al., 2015, Siller et al., 2010). To attune for severe ribosome queuing and accumulation of its resulting products, the cell has evolved mechanisms of quality control to release and recycle stalled ribosomes (Pisareva et al., 2011), degrade mRNAs with stalled ribosomes (Doma and Parker, 2006) and degrade the protein resulting from defective translation elongation (Brandman et al., 2012). Although we present strong evidence that DENV translation elongation is defective when RPLP1/2 are depleted, and that this defect causes defective viral protein accumulation, more research is needed to define why the accumulation is reduced, if due to misfolding followed by degradation, triggering of ribosome control mechanisms or simply due to lower speed of elongation.

When we analyzed the RIBOseq data on cellular mRNAs, there were more mRNAs with their ribosome occupancy increased rather than decreased in RPLP1/2 depleted cells. To differentiate between the possibilities of either increased translation initiation or local increases in ribosome occupancy due to ribosome stalling, which would generate opposite effects on protein expression, we performed WBs on select proteins. This revealed that most of these mRNAs with increased ribosome occupancy also show increased protein accumulation, consistent with an increase in translation initiation. Moderately increased global translation had also been previously observed by us in RPLP1/2 depleted cells using metabolic labeling assay (Campos et al., 2017b). A recent report has suggested that mTOR can sense translation elongation defects (Liakath-Ali et al., 2018), and in turn enhances initiation and elongation of translation to attune for these defects (Liu et al., 2014). Thus, this increase in translation of several mRNAs appears to stem from a feedback loop caused due to the knockdown of RPLP1/2. In line with that, the ~80 mRNAs coding for ribosomal proteins genes had overall increased ribosome occupancy and eEF2K, which inactivates eEF2, had reduced protein levels. Both ribosomal protein levels and eEF2 activity have been shown to be regulated by mTOR, indicating a feedback loop in response to RPLP1/2 depletion, possibly caused by translation elongation defects. A signature of translation elongation defects is the accumulation of ribosomes towards the 5' of the message in meta-gene analyses (Liu et al., 2013, Schuller et al., 2017, Shalgi et al., 2013). We observed this signature in our meta-gene and polarity score analyses, which to the best of our knowledge is the first direct evidence in cells that RPLP1/2 functions in translation elongation. We also found that, similar to the viral RNA which encodes many TMs, mRNAs encoding two or more TMs are differentially affected in terms of amounts and distribution of RPFs in the message. At the individual level, it appears that some of these membrane proteins with two or more TMs did not have their RPFs affected by knockdown, and it is possible that only TMs containing certain features require assistance of RPLP1/2. It is also possible that features associated with the TMs such as

rare codons (Tsai et al., 2008) or RNA binding proteins (Hsu et al., 2018, Shurtleff et al., 2018) could be responsible for stalling the ribosome. We have thus uncovered a specific function of RPLP1/2 which resolves a long-standing discrepancy between *in vitro* results, which suggested that RPLP1/2 bind elongation factors (Bargis-Surgey et al., 1999, Ito et al., 2014) and function in *in vitro* translation system (Baba et al., 2013, Lavergne et al., 1987), and studies in cells, which have not been able to detect translation elongation defects (Martinez-Azorin et al., 2008a, Wawiorka et al., 2017) and found only moderate and cell line specific impact on global cellular translation (Campos et al., 2017b, Perucho et al., 2014, Martinez-Azorin et al., 2008a, Artero-Castro et al., 2015).

The findings of this study are generalizable to other diseases in which RPLP1/2 are considered to play important roles, such as different types of cancers (Artero-Castro et al., 2009, Artero-Castro et al., 2011, Loging and Reisman, 1999, Zhang et al., 1997); in the activity of toxins which depurinate rRNA - such as ricin (Grela et al., 2017, May et al., 2012), trichosanthin (Choi et al., 2015) and shiga toxin1 (Chiou et al., 2011) - cystic fibrosis (Veit et al., 2016, Kormann et al., 2017) and autoantibodies elicited against these proteins are often found in lupus erythematosus (Viana et al., 2017). Our findings advance our knowledge of DENV and cellular translation elongation and suggest that presence of RPLP1/2 may be a regulatory layer for translation of specific mRNAs, especially the ones encoding membrane proteins. These findings could help in the design of novel therapies that target DENV translation.

# 4. Conclusions

This dissertation describes the work leading to the discovery of ribosomal proteins RPLP1 and RPLP2 as host factors critically required for flavivirus infectivity by promoting efficient viral translation elongation. In part one, I delineate the discovery of RPLP1/2 as critical factors for infectivity of flaviviruses and early viral protein accumulation, whereas in part two I describe the function of these proteins in assisting DENV RNA translation elongation.

Previously, our group detected RPLP1/2 to be candidate host factors required for infection of DENV (Barrows et al., unpublished) and YFV (Le Sommer et al., 2012). This led us to discover RPLP1/2 as two of the most critically important host factors for flavivirus infection in human cell lines and in live infections of the mosquito *Aedes aegypti*, a natural vector of these viruses. Unlike the strong effects we observed on flavivirus infectivity, we established that depletion of RPLP1/2 only marginally affected infection of the positive strand RNA viruses HCV and CVB3, suggesting that RPLP1/2 were differentially required for flaviviruses. We also found that RPLP1/2 depletion had limited and cell type specific effects on global cellular translation. Then, we pinpointed that RPLP1/2 played a key role in early phases of the virus life cycle and found that they were required for accumulation of proteins expressed from a construct encoding DENV coding sequences, indicating that the effect observed was at least partially independent of viral entry. Additionally, our fractionation experiments suggested that viral RNA trafficking to the ER was not dependent upon the levels of these proteins.

We then assessed the role of RPLP1/2 in translation of flaviviruses by using RIBOseq, a recently developed technique that allows to infer position of the ribosomes with nucleotide resolution (Ingolia et al., 2009). This technique revealed that depletion of RPLP1/2 caused DENV RNA to have more ribosomes on its genome, which is inconsistent with a defect in translation initiation. Upon close analysis of the location of RPFs on the DENV genome, we observed that depletion of RPLP1/2 caused an intensification of RPF accumulation in the region of the viral RNA coding for the Nterminal portion of the E protein, suggesting that these proteins are important for alleviating excessive ribosome pausing in that part of the genome. We found that constructs encoding the initial portion of the E protein required RPLP1/2 for efficient protein accumulation, therefore the strong ribosome pausing observed in that location of the DENV RNA inversely correlates with protein accumulation. This ribosome pausing site was located approximately 70 aa after the beginning of a region encoding for two adjacent TMs. Considering that the ribosome exit tunnel can comport 30-40 aa and that about 20 aa are necessary for spanning the ER membrane, it is possible that the pausing of the ribosome is due to insertion of these TMs. This suggests that RPLP1/2 may assist ribosome elongation on specific region of the mRNA close to specific types of TMs, which have features that cause strong slowdown of ribosomes. Cellular mRNAs

containing only one TM did not have their ribosome occupancy distribution shifted, indicating that at least two TMs could be required, though perhaps not sufficient, to stall ribosomes. Further research is needed to understand what types of transmembrane domains, associated features or RNA binding protein could be stalling the ribosome and how RPLP1/2 are able to alleviate this stall. It is likely that RPLP1/2 act through recruitment of elongations factors, but this needs to be further confirmed as other RPs have been shown to play a variety of roles in translation as well as in functions unrelated to translation, such as a role in the immunity (Mazumder et al., 2014) or in stress response (Derylo et al., 2018). Additionally, it would be interesting to pursue what other features may require RPLP1/2 to alleviate ribosome stalls and understand how RPLP1/2 may be regulated.

Another important focus for future work is to understand the intricacies of the relationship between ribosome occupancy and protein accumulation. Whereas most of the cellular mRNAs affected by depletion of RPLP1/2 had less total ribosome occupancy, DENV RNA had an overall increased ribosome occupancy through the genome. The average ribosome occupancy on a cellular mRNA with a translation elongation defect may depend on several aspects, such as mechanism of stall, strength and location of the stall on the mRNA, time that the ribosomes remains stalled, and whether the ribosome recycling factors are activated. Relatedly, and in agreement with previous work, we also observed that many cellular mRNAs had increased ribosome occupancy and protein

accumulation. It would be valuable to understand how this is happening mechanistically. It is likely that this is caused by an increased initiation rate for these proteins triggered by the mTOR pathway, which was suggested to be able to sense translation elongation defects (Liakath-Ali et al., 2018) and to respond by enhancing efficiency of translation at the initiation and elongation levels (Thoreen et al., 2012) to compensate for the defect sensed. The hypothesis that mTOR senses translation elongation defects caused by depletion of RPLP1/2 and acts to enhance cellular translation requires further testing.

Although the function of the ribosome as a whole is known, the intricacies of the functions of the ribosomal proteins are still uncertain. Our previous genome-scale RNAi screens revealed an interesting pattern: flaviviruses are more sensitive to inhibition of ribosomal proteins of the large subunit. In contrast, HCV appears to be more sensitive to inhibition of small ribosomal subunits. This may reflect the biology of these viruses in prioritizing initiation or elongation as well as function of each ribosomal subunit. Whereas the small ribosomal subunit and other factors of the pre-initiation complex are responsible for scanning the RNA to locate the start codon, the large ribosomal subunit major role is on translation elongation. Although the large subunit cannot function without the small subunit, it may be able to function without certain ribosomal proteins. Since we found that RPLP1/2 has a function in translation elongation, it is possible that other ribosomal proteins of the large subunit have functions in elongation as well, as

that appears to be the case at least for other ribosomal proteins in the stalk region, such as RPLP0 and RPL12, and has also been found to be the case for RPL10 (Sulima et al., 2014), which drives ribosomal rotation during elongation and for RPL3, which promotes translation elongation fidelity (Al-Hadid et al., 2016).

Flaviviruses are an expanding threat to public health. We have recently seen the emergence of ZIKV, which caused several outbreaks in the Western hemisphere with many cases of fetal malformation (Aliota et al., 2017). On the other side of the spectrum, the well-established DENV continually threatens nearly 40% of the world's population (Bhatt et al., 2013), which live in DENV endemic areas. Strikingly, the risks brought by flavivirus are likely to grow in the upcoming years with the expansion of mosquito populations, which are natural vectors for these viruses (Kraemer et al., 2015). Although the life cycle of flaviviruses is relatively well studied, the knowledge of the translation step is largely based on cellular mRNA translation. This work unearths the first host factors important for translation elongation of flaviviruses and highlights that the flavivirus genome is translatable RNA with unusual characteristics that has elongation requirements different than the majority of cellular mRNAs. Furthering this knowledge could spur new countermeasure strategies to prevent and treat human infections. However, the value of studying viral infections goes beyond the goal of combating infections, as much fundamental biochemistry and cell biology were discovered by studying viruses. Viruses need to manipulate key regulatory aspects of cell biology and

reprogram them to produce viral progeny, and the understanding of how that happens can reveal important cellular processes. Ribosomal proteins are an especially attractive subject of study because little is known about their function and they play a role in vast array of diseases. Our studies have set the stage for future work on the mechanisms of RPLP1/2 function in translation elongation and how they are important for translation of mRNAs encoding membrane proteins, including flavivirus RNA. Understanding these fundamental mechanisms will lead to new insights into fundamental biology and will bolster efforts to combat diseases.

# References

ACEVEDO, J. M., HOERMANN, B., SCHLIMBACH, T. & TELEMAN, A. A. 2018. Changes in global translation elongation or initiation rates shape the proteome via the Kozak sequence. *Sci Rep*, 8, 4018.

ACOSTA, E. G., CASTILLA, V. & DAMONTE, E. B. 2009. Alternative infectious entry pathways for dengue virus serotypes into mammalian cells. *Cell Microbiol*, 11, 1533-49.

AGIS-JUAREZ, R. A., GALVAN, I., MEDINA, F., DAIKOKU, T., PADMANABHAN, R., LUDERT, J. E. & DEL ANGEL, R. M. 2009. Polypyrimidine tract-binding protein is relocated to the cytoplasm and is required during dengue virus infection in Vero cells. *J Gen Virol*, 90, 2893-901.

AHMED, Q. A. & MEMISH, Z. A. 2017. Yellow fever from Angola and Congo: a storm gathers. *Trop Doct*, 47, 92-96.

AL-HADID, Q., ROY, K., CHANFREAU, G. & CLARKE, S. G. 2016. Methylation of yeast ribosomal protein Rpl3 promotes translational elongation fidelity. *RNA*, 22, 489-98.

ALIOTA, M. T., BASSIT, L., BRADRICK, S. S., COX, B., GARCIA-BLANCO, M. A., GAVEGNANO, C., FRIEDRICH, T. C., GOLOS, T. G., GRIFFIN, D. E., HADDOW, A. D., KALLAS, E. G., KITRON, U., LECUIT, M., MAGNANI, D. M., MARRS, C., MERCER, N., MCSWEEGAN, E., NG, L. F. P., O'CONNOR, D. H., OSORIO, J. E., RIBEIRO, G. S., RICCIARDI, M., ROSSI, S. L., SAADE, G., SCHINAZI, R. F., SCHOTT-LERNER, G. O., SHAN, C., SHI, P. Y., WATKINS, D. I., VASILAKIS, N. & WEAVER, S. C. 2017. Zika in the Americas, year 2: What have we learned? What gaps remain? A report from the Global Virus Network. *Antiviral Res*, 144, 223-246.

ALMEN, M. S., NORDSTROM, K. J., FREDRIKSSON, R. & SCHIOTH, H. B. 2009. Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. *BMC Biol*, *7*, 50.

AMBERG, S. M., NESTOROWICZ, A., MCCOURT, D. W. & RICE, C. M. 1994. NS2B-3 proteinase-mediated processing in the yellow fever virus structural region: in vitro and in vivo studies. *J Virol*, 68, 3794-802.

AMBERG, S. M. & RICE, C. M. 1999. Mutagenesis of the NS2B-NS3-mediated cleavage site in the flavivirus capsid protein demonstrates a requirement for coordinated processing. *J Virol*, 73, 8083-94.

ANWAR, A., LEONG, K. M., NG, M. L., CHU, J. J. & GARCIA-BLANCO, M. A. 2009. The polypyrimidine tract-binding protein is required for efficient dengue virus propagation and associates with the viral replication machinery. *J Biol Chem*, 284, 17021-9.

ARTERO-CASTRO, A., CASTELLVI, J., GARCIA, A., HERNANDEZ, J., RAMON Y CAJAL, S. & LLEONART, M. E. 2011. Expression of the ribosomal proteins Rplp0, Rplp1, and Rplp2 in gynecologic tumors. *Hum Pathol*, 42, 194-203.

ARTERO-CASTRO, A., KONDOH, H., FERNANDEZ-MARCOS, P. J., SERRANO, M., RAMON Y CAJAL, S. & LLEONART, M. E. 2009. Rplp1 bypasses replicative senescence and contributes to transformation. *Exp Cell Res*, 315, 1372-83.

ARTERO-CASTRO, A., PEREZ-ALEA, M., FELICIANO, A., LEAL, J. A., GENESTAR, M., CASTELLVI, J., PEG, V., RAMON, Y. C. S. & LLEONART, M. E. 2015. Disruption of the ribosomal P complex leads to stress-induced autophagy. *Autophagy*, 11, 1499-519.

AST, T., COHEN, G. & SCHULDINER, M. 2013. A network of cytosolic factors targets SRP-independent proteins to the endoplasmic reticulum. *Cell*, 152, 1134-45.

BABA, K., TUMURAYA, K., TANAKA, I., YAO, M. & UCHIUMI, T. 2013. Molecular dissection of the silkworm ribosomal stalk complex: the role of multiple copies of the stalk proteins. *Nucleic Acids Res*, 41, 3635-43.

BALLESTA, J. P., RODRIGUEZ-GABRIEL, M. A., BOU, G., BRIONES, E., ZAMBRANO, R. & REMACHA, M. 1999. Phosphorylation of the yeast ribosomal stalk. Functional effects and enzymes involved in the process. *FEMS Microbiol Rev*, 23, 537-50.

BARGIS-SURGEY, P., LAVERGNE, J. P., GONZALO, P., VARD, C., FILHOL-COCHET, O. & REBOUD, J. P. 1999. Interaction of elongation factor eEF-2 with ribosomal P proteins. *Eur J Biochem*, 262, 606-11.

BARROWS, N. J., CAMPOS, R. K., LIAO, K. C., PRASANTH, K. R., SOTO-ACOSTA, R., YEH, S. C., SCHOTT-LERNER, G., POMPON, J., SESSIONS, O. M., BRADRICK, S. S. & GARCIA-BLANCO, M. A. 2018. Biochemistry and Molecular Biology of Flaviviruses. *Chem Rev*.

BARROWS, N. J., CAMPOS, R. K., POWELL, S. T., PRASANTH, K. R., SCHOTT-LERNER, G., SOTO-ACOSTA, R., GALARZA-MUNOZ, G., MCGRATH, E. L., URRABAZ-GARZA, R., GAO, J., WU, P., MENON, R., SAADE, G., FERNANDEZ-SALAS, I., ROSSI, S. L., VASILAKIS, N., ROUTH, A., BRADRICK, S. S. & GARCIA-BLANCO, M. A. 2016. A Screen of FDA-Approved Drugs for Inhibitors of Zika Virus Infection. *Cell Host Microbe*, 20, 259-70.

BAUTISTA-SANTOS, A. & ZINKER, S. 2014. The P1/P2 protein heterodimers assemble to the ribosomal stalk at the moment when the ribosome is committed to translation but not to the native 60S ribosomal subunit in Saccharomyces cerevisiae. *Biochemistry*, 53, 4105-12.

BEASLEY, D. W., WHITEMAN, M. C., ZHANG, S., HUANG, C. Y., SCHNEIDER, B. S., SMITH, D. R., GROMOWSKI, G. D., HIGGS, S., KINNEY, R. M. & BARRETT, A. D. 2005. Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. *J Virol*, 79, 8339-47.

BEATMAN, E., OYER, R., SHIVES, K. D., HEDMAN, K., BRAULT, A. C., TYLER, K. L. & BECKHAM, J. D. 2012. West Nile virus growth is independent of autophagy activation. *Virology*, 433, 262-72.

BHATT, S., GETHING, P. W., BRADY, O. J., MESSINA, J. P., FARLOW, A. W., MOYES, C. L., DRAKE, J. M., BROWNSTEIN, J. S., HOEN, A. G., SANKOH, O., MYERS, M. F., GEORGE, D. B., JAENISCH, T., WINT, G. R., SIMMONS, C. P., SCOTT, T. W., FARRAR, J. J. & HAY, S. I. 2013. The global distribution and burden of dengue. *Nature*, 496, 504-7.

BLIGHT, K. J., MCKEATING, J. A. & RICE, C. M. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol*, 76, 13001-14.

BLUMENTHAL, T. & CARMICHAEL, G. G. 1979. RNA replication: function and structure of Qbeta-replicase. *Annu Rev Biochem*, 48, 525-48.

BOGUSZEWSKA, A., TCHORZEWSKI, M., DUKOWSKI, P., WINIARCZYK, S. & GRANKOWSKI, N. 2002. Subcellular distribution of the acidic ribosomal P-proteins from Saccharomyces cerevisiae in various environmental conditions. *Biol Cell*, 94, 139-46.

BORTOLUZZI, S., D'ALESSI, F., ROMUALDI, C. & DANIELI, G. A. 2001. Differential expression of genes coding for ribosomal proteins in different human tissues. *Bioinformatics*, 17, 1152-7.

BRANDMAN, O., STEWART-ORNSTEIN, J., WONG, D., LARSON, A., WILLIAMS, C. C., LI, G. W., ZHOU, S., KING, D., SHEN, P. S., WEIBEZAHN, J., DUNN, J. G., ROUSKIN, S., INADA, T., FROST, A. & WEISSMAN, J. S. 2012. A ribosome-bound quality control complex triggers degradation of nascent peptides and signals translation stress. *Cell*, 151, 1042-54.

BYK, L. A. & GAMARNIK, A. V. 2016. Properties and Functions of the Dengue Virus Capsid Protein. *Annu Rev Virol*, 3, 263-281.

BYK, L. A., IGLESIAS, N. G., DE MAIO, F. A., GEBHARD, L. G., ROSSI, M. & GAMARNIK, A. V. 2016. Dengue Virus Genome Uncoating Requires Ubiquitination. *MBio*, 7.

CAHOUR, A., FALGOUT, B. & LAI, C. J. 1992. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. *J Virol*, 66, 1535-42.

CAMARGO, H., NUSSPAUMER, G., ABIA, D., BRICENO, V., REMACHA, M. & BALLESTA, J. P. 2011. The amino terminal end determines the stability and assembling capacity of eukaryotic ribosomal stalk proteins P1 and P2. *Nucleic Acids Res*, 39, 3735-43.

CAMPOS, R. K., GARCIA-BLANCO, M. A. & BRADRICK, S. S. 2017a. Roles of Pro-viral Host Factors in Mosquito-Borne Flavivirus Infections. *Curr Top Microbiol Immunol*.

CAMPOS, R. K., WONG, B., XIE, X., LU, Y. F., SHI, P. Y., POMPON, J., GARCIA-BLANCO, M. A. & BRADRICK, S. S. 2017b. RPLP1 and RPLP2 Are Essential Flavivirus Host Factors That Promote Early Viral Protein Accumulation. *J Virol*, 91. CERVANTES-SALAZAR, M., ANGEL-AMBROCIO, A. H., SOTO-ACOSTA, R., BAUTISTA-CARBAJAL, P., HURTADO-MONZON, A. M., ALCARAZ-ESTRADA, S. L., LUDERT, J. E. & DEL ANGEL, R. M. 2015. Dengue virus NS1 protein interacts with the ribosomal protein RPL18: this interaction is required for viral translation and replication in Huh-7 cells. *Virology*, 484, 113-26.

CHAMBERS, T. J., GRAKOUI, A. & RICE, C. M. 1991. Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. *J Virol*, 65, 6042-50.

CHAMBERS, T. J., MCCOURT, D. W. & RICE, C. M. 1990a. Production of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera. *Virology*, 177, 159-74.

CHAMBERS, T. J., WEIR, R. C., GRAKOUI, A., MCCOURT, D. W., BAZAN, J. F., FLETTERICK, R. J. & RICE, C. M. 1990b. Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. *Proc Natl Acad Sci U S A*, 87, 8898-902.

CHEN, A., KAGANOVSKY, E., RAHIMIPOUR, S., BEN-AROYA, N., OKON, E. & KOCH, Y. 2002. Two forms of gonadotropin-releasing hormone (GnRH) are expressed in human breast tissue and overexpressed in breast cancer: a putative mechanism for the antiproliferative effect of GnRH by down-regulation of acidic ribosomal phosphoproteins P1 and P2. *Cancer Res*, 62, 1036-44.

CHEN, C., ZHANG, H., BROITMAN, S. L., REICHE, M., FARRELL, I., COOPERMAN, B. S. & GOLDMAN, Y. E. 2013. Dynamics of translation by single ribosomes through mRNA secondary structures. *Nat Struct Mol Biol*, 20, 582-8.

CHIOU, J. C., LI, X. P., REMACHA, M., BALLESTA, J. P. & TUMER, N. E. 2011. Shiga toxin 1 is more dependent on the P proteins of the ribosomal stalk for depurination activity than Shiga toxin 2. *Int J Biochem Cell Biol*, 43, 1792-801.

CHIU, W. W., KINNEY, R. M. & DREHER, T. W. 2005. Control of translation by the 5'- and 3'-terminal regions of the dengue virus genome. *J Virol*, **79**, 8303-15.

CHOI, A. K., WONG, E. C., LEE, K. M. & WONG, K. B. 2015. Structures of eukaryotic ribosomal stalk proteins and its complex with trichosanthin, and their implications in recruiting ribosome-inactivating proteins to the ribosomes. *Toxins (Basel)*, 7, 638-47.

CHOY, M. M., ZHANG, S. L., COSTA, V. V., TAN, H. C., HORREVORTS, S. & OOI, E. E. 2015. Proteasome Inhibition Suppresses Dengue Virus Egress in Antibody Dependent Infection. *PLoS Negl Trop Dis*, *9*, e0004058.

CLYDE, K. & HARRIS, E. 2006. RNA secondary structure in the coding region of dengue virus type 2 directs translation start codon selection and is required for viral replication. *J Virol*, 80, 2170-82.

COURAGEOT, M. P., FRENKIEL, M. P., DOS SANTOS, C. D., DEUBEL, V. & DESPRES, P. 2000. Alpha-glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. *J Virol*, 74, 564-72.

CRABTREE, M. B., KINNEY, R. M. & MILLER, B. R. 2005. Deglycosylation of the NS1 protein of dengue 2 virus, strain 16681: construction and characterization of mutant viruses. *Arch Virol*, 150, 771-86.

D'ORTENZIO, E., MATHERON, S., YAZDANPANAH, Y., DE LAMBALLERIE, X., HUBERT, B., PIORKOWSKI, G., MAQUART, M., DESCAMPS, D., DAMOND, F. & LEPARC-GOFFART, I. 2016. Evidence of Sexual Transmission of Zika Virus. *N Engl J Med*, 374, 2195-8.

DAFFIS, S., SZRETTER, K. J., SCHRIEWER, J., LI, J., YOUN, S., ERRETT, J., LIN, T. Y., SCHNELLER, S., ZUST, R., DONG, H., THIEL, V., SEN, G. C., FENSTERL, V., KLIMSTRA, W. B., PIERSON, T. C., BULLER, R. M., GALE, M., JR., SHI, P. Y. & DIAMOND, M. S. 2010. 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature*, 468, 452-6.

DAS, S., LAXMINARAYANA, S. V., CHANDRA, N., RAVI, V. & DESAI, A. 2009. Heat shock protein 70 on Neuro2a cells is a putative receptor for Japanese encephalitis virus. *Virology*, 385, 47-57.

DAVIS, C. W., NGUYEN, H. Y., HANNA, S. L., SANCHEZ, M. D., DOMS, R. W. & PIERSON, T. C. 2006. West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. *J Virol*, 80, 1290-301.

DAVIS, W. G., BLACKWELL, J. L., SHI, P. Y. & BRINTON, M. A. 2007. Interaction between the cellular protein eEF1A and the 3'-terminal stem-loop of West Nile virus genomic RNA facilitates viral minus-strand RNA synthesis. *J Virol*, 81, 10172-87.

DEJNIRATTISAI, W., WEBB, A. I., CHAN, V., JUMNAINSONG, A., DAVIDSON, A., MONGKOLSAPAYA, J. & SCREATON, G. 2011. Lectin switching during dengue virus infection. *J Infect Dis*, 203, 1775-83.

DERYLO, K., MICHALEC-WAWIORKA, B., KROKOWSKI, D., WAWIORKA, L., HATZOGLOU, M. & TCHORZEWSKI, M. 2018. The uL10 protein, a component of the ribosomal P-stalk, is released from the ribosome in nucleolar stress. *Biochim Biophys Acta*, 1865, 34-47.

DEVARANENI, P. K., CONTI, B., MATSUMURA, Y., YANG, Z., JOHNSON, A. E. & SKACH, W. R. 2011. Stepwise insertion and inversion of a type II signal anchor sequence in the ribosome-Sec61 translocon complex. *Cell*, 146, 134-47.

DEVAUX, P. F., HERRMANN, A., OHLWEIN, N. & KOZLOV, M. M. 2008. How lipid flippases can modulate membrane structure. *Biochim Biophys Acta*, 1778, 1591-600.

DEVER, T. E., DINMAN, J. D. & GREEN, R. 2018. Translation Elongation and Recoding in Eukaryotes. *Cold Spring Harb Perspect Biol*.

DEXHEIMER PAPLOSKI, I. A., SOUZA, R. L., TAURO, L. B., CARDOSO, C. W., MUGABE, V. A., PEREIRA SIMOES ALVES, A. B., DE JESUS GOMES, J., KIKUTI, M., CAMPOS, G. S., SARDI, S., WEAVER, S. C., REIS, M. G., KITRON, U. & RIBEIRO, G. S. 2018. Epizootic Outbreak of Yellow Fever Virus and Risk for Human Disease in Salvador, Brazil. *Ann Intern Med*, 168, 301-302.

DIAZ, A. & AHLQUIST, P. 2012. Role of host reticulon proteins in rearranging membranes for positive-strand RNA virus replication. *Curr Opin Microbiol*, 15, 519-24.

DOMA, M. K. & PARKER, R. 2006. Endonucleolytic cleavage of eukaryotic mRNAs with stalls in translation elongation. *Nature*, 440, 561-4.

DOU, D., DA SILVA, D. V., NORDHOLM, J., WANG, H. & DANIELS, R. 2014. Type II transmembrane domain hydrophobicity dictates the cotranslational dependence for inversion. *Mol Biol Cell*, 25, 3363-74.

DUAN, X., LU, X., LI, J. & LIU, Y. 2008. Novel binding between pre-membrane protein and vacuolar ATPase is required for efficient dengue virus secretion. *Biochem Biophys Res Commun*, 373, 319-24.

EDGIL, D., DIAMOND, M. S., HOLDEN, K. L., PARANJAPE, S. M. & HARRIS, E. 2003. Translation efficiency determines differences in cellular infection among dengue virus type 2 strains. *Virology*, 317, 275-90.

EDGIL, D., POLACEK, C. & HARRIS, E. 2006. Dengue virus utilizes a novel strategy for translation initiation when cap-dependent translation is inhibited. *J Virol*, 80, 2976-86.

ERBEL, P., SCHIERING, N., D'ARCY, A., RENATUS, M., KROEMER, M., LIM, S. P., YIN, Z., KELLER, T. H., VASUDEVAN, S. G. & HOMMEL, U. 2006. Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. *Nat Struct Mol Biol*, 13, 372-3.

EVANS, E. A., GILMORE, R. & BLOBEL, G. 1986. Purification of microsomal signal peptidase as a complex. *Proc Natl Acad Sci U S A*, 83, 581-5.

EVRON, T., DAIGLE, T. L. & CARON, M. G. 2012. GRK2: multiple roles beyond G protein-coupled receptor desensitization. *Trends Pharmacol Sci*, 33, 154-64.

FAGGIONI, G., CIAMMARUCONI, A., DE SANTIS, R., POMPONI, A., SCICLUNA, M. T., BARBARO, K., MASUELLI, L., AUTORINO, G., BEI, R. & LISTA, F. 2009. Evidence of a humoral response to a novel protein WARF4 embedded in the West Nile virus NS4B gene encoded by an alternative open reading frame. *Int J Mol Med*, 23, 509-12.

FAGGIONI, G., POMPONI, A., DE SANTIS, R., MASUELLI, L., CIAMMARUCONI, A., MONACO, F., DI GENNARO, A., MARZOCCHELLA, L., SAMBRI, V., LELLI, R., REZZA, G., BEI, R. & LISTA, F. 2012. West Nile alternative open reading frame (N-NS4B/WARF4) is produced in infected West Nile Virus (WNV) cells and induces humoral response in WNV infected individuals. *Virol J*, 9, 283.

FALGOUT, B., CHANOCK, R. & LAI, C. J. 1989. Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. *J Virol*, 63, 1852-60.

FALGOUT, B., PETHEL, M., ZHANG, Y. M. & LAI, C. J. 1991. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J Virol*, 65, 2467-75.

FALLER, W. J., JACKSON, T. J., KNIGHT, J. R., RIDGWAY, R. A., JAMIESON, T., KARIM, S. A., JONES, C., RADULESCU, S., HUELS, D. J., MYANT, K. B., DUDEK, K. M., CASEY, H. A., SCOPELLITI, A., CORDERO, J. B., VIDAL, M., PENDE, M., RYAZANOV, A. G., SONENBERG, N., MEYUHAS, O., HALL, M. N., BUSHELL, M., WILLIS, A. E. & SANSOM, O. J. 2015. mTORC1-mediated translational elongation limits intestinal tumour initiation and growth. *Nature*, 517, 497-500.

FINCH, A. R., CAUNT, C. J., ARMSTRONG, S. P. & MCARDLE, C. A. 2009. Agonistinduced internalization and downregulation of gonadotropin-releasing hormone receptors. *Am J Physiol Cell Physiol*, 297, C591-600.

FIRTH, A. E., BLITVICH, B. J., WILLS, N. M., MILLER, C. L. & ATKINS, J. F. 2010. Evidence for ribosomal frameshifting and a novel overlapping gene in the genomes of insect-specific flaviviruses. *Virology*, 399, 153-66.

FLAMAND, M., MEGRET, F., MATHIEU, M., LEPAULT, J., REY, F. A. & DEUBEL, V. 1999. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. *J Virol*, 73, 6104-10.

FLUMAN, N., NAVON, S., BIBI, E. & PILPEL, Y. 2014. mRNA-programmed translation pauses in the targeting of E. coli membrane proteins. *Elife*, 3.

FRAITURE, M., BAXTER, R. H., STEINERT, S., CHELLIAH, Y., FROLET, C., QUISPE-TINTAYA, W., HOFFMANN, J. A., BLANDIN, S. A. & LEVASHINA, E. A. 2009. Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of Plasmodium. *Cell Host Microbe*, 5, 273-84.

FRIEDMAN, D. I., SCHAUER, A. T., BAUMANN, M. R., BARON, L. S. & ADHYA, S. L. 1981. Evidence that ribosomal protein S10 participates in control of transcription termination. *Proc Natl Acad Sci U S A*, 78, 1115-8.

FRIEDRICH, S., SCHMIDT, T., GEISSLER, R., LILIE, H., CHABIERSKI, S., ULBERT, S., LIEBERT, U. G., GOLBIK, R. P. & BEHRENS, S. E. 2014. AUF1 p45 promotes West Nile virus replication by an RNA chaperone activity that supports cyclization of the viral genome. *J Virol*, 88, 11586-99.

FRIEDRICH, S., SCHMIDT, T., SCHIERHORN, A., LILIE, H., SZCZEPANKIEWICZ, G., BERGS, S., LIEBERT, U. G., GOLBIK, R. P. & BEHRENS, S. E. 2016. Arginine methylation enhances the RNA chaperone activity of the West Nile virus host factor AUF1 p45. *RNA*, 22, 1574-91.

GAMARNIK, A. V. & ANDINO, R. 1998. Switch from translation to RNA replication in a positive-stranded RNA virus. *Genes Dev*, 12, 2293-304.

GAMBLE, C. E., BRULE, C. E., DEAN, K. M., FIELDS, S. & GRAYHACK, E. J. 2016. Adjacent Codons Act in Concert to Modulate Translation Efficiency in Yeast. *Cell*, 166, 679-690.

GARCIA-BLANCO, M. A., VASUDEVAN, S. G., BRADRICK, S. S. & NICCHITTA, C. 2016. Flavivirus RNA transactions from viral entry to genome replication. *Antiviral Res*, 134, 244-249.

GARCIA-MARCOS, A., SANCHEZ, S. A., PARADA, P., EID, J., JAMESON, D. M., REMACHA, M., GRATTON, E. & BALLESTA, J. P. 2008. Yeast ribosomal stalk heterogeneity in vivo shown by two-photon FCS and molecular brightness analysis. *Biophys J*, 94, 2884-90. GARCIA-MONTALVO, B. M., MEDINA, F. & DEL ANGEL, R. M. 2004. La protein binds to NS5 and NS3 and to the 5' and 3' ends of Dengue 4 virus RNA. *Virus Res*, 102, 141-50.

GARDNER-THORPE, J., ITO, H., ASHLEY, S. W. & WHANG, E. E. 2002. Differential display of expressed genes in pancreatic cancer cells. *Biochem Biophys Res Commun*, 293, 391-5.

GARSKE, T., VAN KERKHOVE, M. D., YACTAYO, S., RONVEAUX, O., LEWIS, R. F., STAPLES, J. E., PEREA, W., FERGUSON, N. M. & YELLOW FEVER EXPERT, C. 2014. Yellow Fever in Africa: estimating the burden of disease and impact of mass vaccination from outbreak and serological data. *PLoS Med*, 11, e1001638.

GOMILA, R. C., MARTIN, G. W. & GEHRKE, L. 2011. NF90 binds the dengue virus RNA 3' terminus and is a positive regulator of dengue virus replication. *PLoS One*, 6, e16687.

GOROCHOWSKI, T. E., IGNATOVA, Z., BOVENBERG, R. A. & ROUBOS, J. A. 2015. Trade-offs between tRNA abundance and mRNA secondary structure support smoothing of translation elongation rate. *Nucleic Acids Res*, 43, 3022-32.

GRELA, P., LI, X. P., HORBOWICZ, P., DZWIERZYNSKA, M., TCHORZEWSKI, M. & TUMER, N. E. 2017. Human ribosomal P1-P2 heterodimer represents an optimal docking site for ricin A chain with a prominent role for P1 C-terminus. *Sci Rep*, 7, 5608.

GUARINOS, E., SANTOS, C., SANCHEZ, A., QIU, D. Y., REMACHA, M. & BALLESTA, J. P. 2003. Tag-mediated fractionation of yeast ribosome populations proves the monomeric organization of the eukaryotic ribosomal stalk structure. *Mol Microbiol*, 50, 703-12.

GUERBOIS, M., FERNANDEZ-SALAS, I., AZAR, S. R., DANIS-LOZANO, R., ALPUCHE-ARANDA, C. M., LEAL, G., GARCIA-MALO, I. R., DIAZ-GONZALEZ, E. E., CASAS-MARTINEZ, M., ROSSI, S. L., DEL RIO-GALVAN, S. L., SANCHEZ-CASAS, R. M., ROUNDY, C. M., WOOD, T. G., WIDEN, S. G., VASILAKIS, N. & WEAVER, S. C. 2016. Outbreak of Zika Virus Infection, Chiapas State, Mexico, 2015, and First Confirmed Transmission by Aedes aegypti Mosquitoes in the Americas. *J Infect Dis*, 214, 1349-1356. GUIRAKHOO, F., BOLIN, R. A. & ROEHRIG, J. T. 1992. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. *Virology*, 191, 921-31.

HACKETT, B. A., YASUNAGA, A., PANDA, D., TARTELL, M. A., HOPKINS, K. C., HENSLEY, S. E. & CHERRY, S. 2015. RNASEK is required for internalization of diverse acid-dependent viruses. *Proc Natl Acad Sci U S A*, 112, 7797-802.

HALL, M. N., GABAY, J., DEBARBOUILLE, M. & SCHWARTZ, M. 1982. A role for mRNA secondary structure in the control of translation initiation. *Nature*, 295, 616-8.

HAMER, D. H., ANGELO, K., CAUMES, E., VAN GENDEREN, P. J. J., FLORESCU, S. A., POPESCU, C. P., PERRET, C., MCBRIDE, A., CHECKLEY, A., RYAN, J., CETRON, M. & SCHLAGENHAUF, P. 2018. Fatal Yellow Fever in Travelers to Brazil, 2018. *MMWR Morb Mortal Wkly Rep*, 67, 340-341.

HAN, J., AKUTSU, M., TALORETE, T. P., MAEKAWA, T., TANAKA, T. & ISODA, H. 2005. Capsaicin-enhanced Ribosomal Protein P2 Expression in Human Intestinal Caco-2 Cells. *Cytotechnology*, 49, 77-85.

HANNA, S. L., PIERSON, T. C., SANCHEZ, M. D., AHMED, A. A., MURTADHA, M. M. & DOMS, R. W. 2005. N-linked glycosylation of west nile virus envelope proteins influences particle assembly and infectivity. *J Virol*, *7*9, 13262-74.

HARROWER, J., KIEDRZYNSKI, T., BAKER, S., UPTON, A., RAHNAMA, F., SHERWOOD, J., HUANG, Q. S., TODD, A. & PULFORD, D. 2016. Sexual Transmission of Zika Virus and Persistence in Semen, New Zealand, 2016. *Emerg Infect Dis*, 22, 1855-7.

HEATON, N. S., PERERA, R., BERGER, K. L., KHADKA, S., LACOUNT, D. J., KUHN, R. J. & RANDALL, G. 2010. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. *Proc Natl Acad Sci U S A*, 107, 17345-50.

HEATON, N. S. & RANDALL, G. 2010. Dengue virus-induced autophagy regulates lipid metabolism. *Cell Host Microbe*, *8*, 422-32.

HENCHAL, E. A., GENTRY, M. K., MCCOWN, J. M. & BRANDT, W. E. 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *Am J Trop Med Hyg*, 31, 830-6.

HERTZ, M. I., LANDRY, D. M., WILLIS, A. E., LUO, G. & THOMPSON, S. R. 2013. Ribosomal protein S25 dependency reveals a common mechanism for diverse internal ribosome entry sites and ribosome shunting. *Mol Cell Biol*, 33, 1016-26.

HINNEBUSCH, A. G., IVANOV, I. P. & SONENBERG, N. 2016. Translational control by 5'-untranslated regions of eukaryotic mRNAs. *Science*, 352, 1413-6.

HIRSCH, A. J., MEDIGESHI, G. R., MEYERS, H. L., DEFILIPPIS, V., FRUH, K., BRIESE, T., LIPKIN, W. I. & NELSON, J. A. 2005. The Src family kinase c-Yes is required for maturation of West Nile virus particles. *J Virol*, 79, 11943-51.

HSU, J. C., REID, D. W., HOFFMAN, A. M., SARKAR, D. & NICCHITTA, C. V. 2018. Oncoprotein AEG-1 is an endoplasmic reticulum RNA-binding protein whose interactome is enriched in organelle resident protein-encoding mRNAs. *RNA*, 24, 688-703.

HUANG, J. Y., SU, W. C., JENG, K. S., CHANG, T. H. & LAI, M. M. 2012. Attenuation of 40S ribosomal subunit abundance differentially affects host and HCV translation and suppresses HCV replication. *PLoS Pathog*, 8, e1002766.

IGLESIAS, N. G., MONDOTTE, J. A., BYK, L. A., DE MAIO, F. A., SAMSA, M. M., ALVAREZ, C. & GAMARNIK, A. V. 2015. Dengue Virus Uses a Non-Canonical Function of the Host GBF1-Arf-COPI System for Capsid Protein Accumulation on Lipid Droplets. *Traffic*, 16, 962-77.

INGOLIA, N. T., BRAR, G. A., ROUSKIN, S., MCGEACHY, A. M. & WEISSMAN, J. S. 2012. The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. *Nat Protoc*, *7*, 1534-50.

INGOLIA, N. T., GHAEMMAGHAMI, S., NEWMAN, J. R. & WEISSMAN, J. S. 2009. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science*, 324, 218-23.

INGOLIA, N. T., LAREAU, L. F. & WEISSMAN, J. S. 2011. Ribosome profiling of mouse embryonic stem cells reveals the complexity and dynamics of mammalian proteomes. *Cell*, 147, 789-802.

ISHII, K., WASHIO, T., UECHI, T., YOSHIHAMA, M., KENMOCHI, N. & TOMITA, M. 2006. Characteristics and clustering of human ribosomal protein genes. *BMC Genomics*, 7, 37.

ITO, K., HONDA, T., SUZUKI, T., MIYOSHI, T., MURAKAMI, R., YAO, M. & UCHIUMI, T. 2014. Molecular insights into the interaction of the ribosomal stalk protein with elongation factor 1alpha. *Nucleic Acids Res*, 42, 14042-52.

IVANOV, A., MIKHAILOVA, T., ELISEEV, B., YERAMALA, L., SOKOLOVA, E., SUSOROV, D., SHUVALOV, A., SCHAFFITZEL, C. & ALKALAEVA, E. 2016. PABP enhances release factor recruitment and stop codon recognition during translation termination. *Nucleic Acids Res*, 44, 7766-76.

IVANYI-NAGY, R., LAVERGNE, J. P., GABUS, C., FICHEUX, D. & DARLIX, J. L. 2008. RNA chaperoning and intrinsic disorder in the core proteins of Flaviviridae. *Nucleic Acids Res*, 36, 712-25.

JACOBSON, G. N. & CLARK, P. L. 2016. Quality over quantity: optimizing cotranslational protein folding with non-'optimal' synonymous codons. *Curr Opin Struct Biol*, 38, 102-10.

JAGANNATHAN, S., NWOSU, C. & NICCHITTA, C. V. 2011. Analyzing mRNA localization to the endoplasmic reticulum via cell fractionation. *Methods Mol Biol*, 714, 301-21.

JAN, C. H., WILLIAMS, C. C. & WEISSMAN, J. S. 2014. Principles of ER cotranslational translocation revealed by proximity-specific ribosome profiling. *Science*, 346, 1257521.

JIANG, L., YAO, H., DUAN, X., LU, X. & LIU, Y. 2009. Polypyrimidine tract-binding protein influences negative strand RNA synthesis of dengue virus. *Biochem Biophys Res Commun*, 385, 187-92.

JIANG, N., HU, L., LIU, C., GAO, X. & ZHENG, S. 2015. 60S ribosomal protein L35 regulates beta-case in translational elongation and secretion in bovine mammary epithelial cells. *Arch Biochem Biophys*, 583, 130-9.

JIMENEZ-DIAZ, A., REMACHA, M., BALLESTA, J. P. & BERLANGA, J. J. 2013. Phosphorylation of initiation factor eIF2 in response to stress conditions is mediated by acidic ribosomal P1/P2 proteins in Saccharomyces cerevisiae. *PLoS One*, *8*, e84219.

JINDADAMRONGWECH, S. & SMITH, D. R. 2004. Virus Overlay Protein Binding Assay (VOPBA) reveals serotype specific heterogeneity of dengue virus binding proteins on HepG2 human liver cells. *Intervirology*, 47, 370-3.

JOHNSON, N., VILARDI, F., LANG, S., LEZNICKI, P., ZIMMERMANN, R. & HIGH, S. 2012. TRC40 can deliver short secretory proteins to the Sec61 translocon. *J Cell Sci*, 125, 3612-20.

JONIKAS, M. C., COLLINS, S. R., DENIC, V., OH, E., QUAN, E. M., SCHMID, V., WEIBEZAHN, J., SCHWAPPACH, B., WALTER, P., WEISSMAN, J. S. & SCHULDINER, M. 2009. Comprehensive characterization of genes required for protein folding in the endoplasmic reticulum. *Science*, 323, 1693-7.

JUNJHON, J., PENNINGTON, J. G., EDWARDS, T. J., PERERA, R., LANMAN, J. & KUHN, R. J. 2014. Ultrastructural characterization and three-dimensional architecture of replication sites in dengue virus-infected mosquito cells. *J Virol*, 88, 4687-97.

KAHVEJIAN, A., SVITKIN, Y. V., SUKARIEH, R., M'BOUTCHOU, M. N. & SONENBERG, N. 2005. Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. *Genes Dev*, 19, 104-13.

KAISER, C., DOBRIKOVA, E. Y., BRADRICK, S. S., SHVEYGERT, M., HERBERT, J. T. & GROMEIER, M. 2008. Activation of cap-independent translation by variant eukaryotic initiation factor 4G in vivo. *RNA*, 14, 2170-82.

KALIA, M., KHASA, R., SHARMA, M., NAIN, M. & VRATI, S. 2013. Japanese encephalitis virus infects neuronal cells through a clathrin-independent endocytic mechanism. *J Virol*, 87, 148-62.

KEPES, F. 1996. The "+70 pause": hypothesis of a translational control of membrane protein assembly. *J Mol Biol*, 262, 77-86.

KHATTER, H., MYASNIKOV, A. G., NATCHIAR, S. K. & KLAHOLZ, B. P. 2015. Structure of the human 80S ribosome. *Nature*, 520, 640-5.

KIM, J., CHUBATSU, L. S., ADMON, A., STAHL, J., FELLOUS, R. & LINN, S. 1995. Implication of mammalian ribosomal protein S3 in the processing of DNA damage. *J Biol Chem*, 270, 13620-9.

KIM, J. M., YUN, S. I., SONG, B. H., HAHN, Y. S., LEE, C. H., OH, H. W. & LEE, Y. M. 2008. A single N-linked glycosylation site in the Japanese encephalitis virus prM protein is critical for cell type-specific prM protein biogenesis, virus particle release, and pathogenicity in mice. *J Virol*, 82, 7846-62.

KIM, S. J., YOON, J. S., SHISHIDO, H., YANG, Z., ROONEY, L. A., BARRAL, J. M. & SKACH, W. R. 2015. Protein folding. Translational tuning optimizes nascent protein folding in cells. *Science*, 348, 444-8.

KOBAYASHI, S., ORBA, Y., YAMAGUCHI, H., TAKAHASHI, K., SASAKI, M., HASEBE, R., KIMURA, T. & SAWA, H. 2014. Autophagy inhibits viral genome replication and gene expression stages in West Nile virus infection. *Virus Res*, 191, 83-91.

KOBAYASHI, S., SUZUKI, T., KAWAGUCHI, A., PHONGPHAEW, W., YOSHII, K., IWANO, T., HARADA, A., KARIWA, H., ORBA, Y. & SAWA, H. 2016. Rab8b Regulates Transport of West Nile Virus Particles from Recycling Endosomes. *J Biol Chem*, 291, 6559-68. KONDRASHOV, N., PUSIC, A., STUMPF, C. R., SHIMIZU, K., HSIEH, A. C., XUE, S., ISHIJIMA, J., SHIROISHI, T. & BARNA, M. 2011. Ribosome-mediated specificity in Hox mRNA translation and vertebrate tissue patterning. *Cell*, 145, 383-97.

KOOIJMAN, E. E., CHUPIN, V., DE KRUIJFF, B. & BURGER, K. N. 2003. Modulation of membrane curvature by phosphatidic acid and lysophosphatidic acid. *Traffic*, 4, 162-74.

KORMANN, M. S. D., DEWERTH, A., EICHNER, F., BASKARAN, P., HECTOR, A., REGAMEY, N., HARTL, D., HANDGRETINGER, R. & ANTONY, J. S. 2017. Transcriptomic profile of cystic fibrosis patients identifies type I interferon response and ribosomal stalk proteins as potential modifiers of disease severity. *PLoS One*, 12, e0183526.

KOSTYUCHENKO, V. A., LIM, E. X., ZHANG, S., FIBRIANSAH, G., NG, T. S., OOI, J. S., SHI, J. & LOK, S. M. 2016. Structure of the thermally stable Zika virus. *Nature*, 533, 425-8.

KRAEMER, M. U., SINKA, M. E., DUDA, K. A., MYLNE, A. Q., SHEARER, F. M., BARKER, C. M., MOORE, C. G., CARVALHO, R. G., COELHO, G. E., VAN BORTEL, W., HENDRICKX, G., SCHAFFNER, F., ELYAZAR, I. R., TENG, H. J., BRADY, O. J., MESSINA, J. P., PIGOTT, D. M., SCOTT, T. W., SMITH, D. L., WINT, G. R., GOLDING, N. & HAY, S. I. 2015. The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. *Elife*, 4, e08347.

KRISHNAN, M. N., NG, A., SUKUMARAN, B., GILFOY, F. D., UCHIL, P. D., SULTANA, H., BRASS, A. L., ADAMETZ, R., TSUI, M., QIAN, F., MONTGOMERY, R. R., LEV, S., MASON, P. W., KOSKI, R. A., ELLEDGE, S. J., XAVIER, R. J., AGAISSE, H. & FIKRIG, E. 2008. RNA interference screen for human genes associated with West Nile virus infection. *Nature*, 455, 242-5.

KRISHNAN, M. N., SUKUMARAN, B., PAL, U., AGAISSE, H., MURRAY, J. L., HODGE, T. W. & FIKRIG, E. 2007. Rab 5 is required for the cellular entry of dengue and West Nile viruses. *J Virol*, 81, 4881-5.

KROKOWSKI, D., TCHORZEWSKI, M., BOGUSZEWSKA, A., MCKAY, A. R., MASLEN, S. L., ROBINSON, C. V. & GRANKOWSKI, N. 2007. Elevated copy number of L-A virus in yeast mutant strains defective in ribosomal stalk. *Biochem Biophys Res Commun,* 355, 575-80.

KUDELKO, M., BRAULT, J. B., KWOK, K., LI, M. Y., PARDIGON, N., PEIRIS, J. S., BRUZZONE, R., DESPRES, P., NAL, B. & WANG, P. G. 2012. Class II ADP-ribosylation factors are required for efficient secretion of dengue viruses. *J Biol Chem*, 287, 767-77.

KUDLA, G., MURRAY, A. W., TOLLERVEY, D. & PLOTKIN, J. B. 2009. Coding-sequence determinants of gene expression in Escherichia coli. *Science*, 324, 255-8.

KUTAY, U., LIPOWSKY, G., IZAURRALDE, E., BISCHOFF, F. R., SCHWARZMAIER, P., HARTMANN, E. & GORLICH, D. 1998. Identification of a tRNA-specific nuclear export receptor. *Mol Cell*, 1, 359-69.

LADNER, J. T., WILEY, M. R., PRIETO, K., YASUDA, C. Y., NAGLE, E., KASPER, M. R., REYES, D., VASILAKIS, N., HEANG, V., WEAVER, S. C., HADDOW, A., TESH, R. B., SOVANN, L. & PALACIOS, G. 2016. Complete Genome Sequences of Five Zika Virus Isolates. *Genome Announc*, 4.

LANDRY, D. M., HERTZ, M. I. & THOMPSON, S. R. 2009. RPS25 is essential for translation initiation by the Dicistroviridae and hepatitis C viral IRESs. *Genes Dev*, 23, 2753-64.

LAREAU, L. F., HITE, D. H., HOGAN, G. J. & BROWN, P. O. 2014. Distinct stages of the translation elongation cycle revealed by sequencing ribosome-protected mRNA fragments. *Elife*, 3, e01257.

LAVERGNE, J. P., CONQUET, F., REBOUD, J. P. & REBOUD, A. M. 1987. Role of acidic phosphoproteins in the partial reconstitution of the active 60 S ribosomal subunit. *FEBS Lett*, 216, 83-8.

LE SOMMER, C., BARROWS, N. J., BRADRICK, S. S., PEARSON, J. L. & GARCIA-BLANCO, M. A. 2012. G protein-coupled receptor kinase 2 promotes flaviviridae entry and replication. *PLoS Negl Trop Dis*, 6, e1820. LEE, A. S., BURDEINICK-KERR, R. & WHELAN, S. P. 2013a. A ribosome-specialized translation initiation pathway is required for cap-dependent translation of vesicular stomatitis virus mRNAs. *Proc Natl Acad Sci U S A*, 110, 324-9.

LEE, G. & GE, B. 2010. Growth inhibition of tumor cells in vitro by using monoclonal antibodies against gonadotropin-releasing hormone receptor. *Cancer Immunol Immunother*, 59, 1011-9.

LEE, K. M., YUSA, K., CHU, L. O., YU, C. W., OONO, M., MIYOSHI, T., ITO, K., SHAW, P. C., WONG, K. B. & UCHIUMI, T. 2013b. Solution structure of human P1\*P2 heterodimer provides insights into the role of eukaryotic stalk in recruiting the ribosome-inactivating protein trichosanthin to the ribosome. *Nucleic Acids Res*, 41, 8776-87.

LEPRIVIER, G., REMKE, M., ROTBLAT, B., DUBUC, A., MATEO, A. R., KOOL, M., AGNIHOTRI, S., EL-NAGGAR, A., YU, B., SOMASEKHARAN, S. P., FAUBERT, B., BRIDON, G., TOGNON, C. E., MATHERS, J., THOMAS, R., LI, A., BAROKAS, A., KWOK, B., BOWDEN, M., SMITH, S., WU, X., KORSHUNOV, A., HIELSCHER, T., NORTHCOTT, P. A., GALPIN, J. D., AHERN, C. A., WANG, Y., MCCABE, M. G., COLLINS, V. P., JONES, R. G., POLLAK, M., DELATTRE, O., GLEAVE, M. E., JAN, E., PFISTER, S. M., PROUD, C. G., DERRY, W. B., TAYLOR, M. D. & SORENSEN, P. H. 2013. The eEF2 kinase confers resistance to nutrient deprivation by blocking translation elongation. *Cell*, 153, 1064-79.

LI, L., LOK, S. M., YU, I. M., ZHANG, Y., KUHN, R. J., CHEN, J. & ROSSMANN, M. G. 2008. The flavivirus precursor membrane-envelope protein complex: structure and maturation. *Science*, 319, 1830-4.

LI, M. Y., GRANDADAM, M., KWOK, K., LAGACHE, T., SIU, Y. L., ZHANG, J. S., SAYTENG, K., KUDELKO, M., QIN, C. F., OLIVO-MARIN, J. C., BRUZZONE, R. & WANG, P. G. 2015. KDEL Receptors Assist Dengue Virus Exit from the Endoplasmic Reticulum. *Cell Rep*.

LI, W., WANG, W., UREN, P. J., PENALVA, L. O. F. & SMITH, A. D. 2017. Riborex: fast and flexible identification of differential translation from Ribo-seq data. *Bioinformatics*, 33, 1735-1737.
LIAKATH-ALI, K., MILLS, E. W., SEQUEIRA, I., LICHTENBERGER, B. M., PISCO, A. O., SIPILA, K. H., MISHRA, A., YOSHIKAWA, H., WU, C. C., LY, T., LAMOND, A. I., ADHAM, I. M., GREEN, R. & WATT, F. M. 2018. An evolutionarily conserved ribosomerescue pathway maintains epidermal homeostasis. *Nature*, 556, 376-380.

LIU, B., HAN, Y. & QIAN, S. B. 2013. Cotranslational response to proteotoxic stress by elongation pausing of ribosomes. *Mol Cell*, 49, 453-63.

LIU, C. H., LU, R. J., GUO, G. M., HE, T., LI, Y. B., XU, H. W., GAO, R. H., CHEN, Z. W. & HUANG, J. H. 2016. Transcriptome analysis reveals translational regulation in barley microspore-derived embryogenic callus under salt stress. *Plant Cell Rep*, 35, 1719-28.

LIU, R., IADEVAIA, V., AVEROUS, J., TAYLOR, P. M., ZHANG, Z. & PROUD, C. G. 2014. Impairing the production of ribosomal RNA activates mammalian target of rapamycin complex 1 signalling and downstream translation factors. *Nucleic Acids Res*, 42, 5083-96.

LIU, R. & PROUD, C. G. 2016. Eukaryotic elongation factor 2 kinase as a drug target in cancer, and in cardiovascular and neurodegenerative diseases. *Acta Pharmacol Sin*, 37, 285-94.

LO, K. Y., LI, Z., BUSSIERE, C., BRESSON, S., MARCOTTE, E. M. & JOHNSON, A. W. 2010. Defining the pathway of cytoplasmic maturation of the 60S ribosomal subunit. *Mol Cell*, 39, 196-208.

LOBIGS, M. 1993. Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. *Proc Natl Acad Sci U S A*, 90, 6218-22.

LOBIGS, M., LEE, E., NG, M. L., PAVY, M. & LOBIGS, P. 2010. A flavivirus signal peptide balances the catalytic activity of two proteases and thereby facilitates virus morphogenesis. *Virology*, 401, 80-9.

LOGING, W. T. & REISMAN, D. 1999. Elevated expression of ribosomal protein genes L37, RPP-1, and S2 in the presence of mutant p53. *Cancer Epidemiol Biomarkers Prev*, 8, 1011-6.

LOUIE, R. J., GUO, J., RODGERS, J. W., WHITE, R., SHAH, N., PAGANT, S., KIM, P., LIVSTONE, M., DOLINSKI, K., MCKINNEY, B. A., HONG, J., SORSCHER, E. J., BRYAN, J., MILLER, E. A. & HARTMAN, J. L. T. 2012. A yeast phenomic model for the gene interaction network modulating CFTR-DeltaF508 protein biogenesis. *Genome Med*, 4, 103.

LUCA, V. C., NELSON, C. A. & FREMONT, D. H. 2013. Structure of the St. Louis encephalitis virus postfusion envelope trimer. *J Virol*, 87, 818-28.

MA, H., DANG, Y., WU, Y., JIA, G., ANAYA, E., ZHANG, J., ABRAHAM, S., CHOI, J. G., SHI, G., QI, L., MANJUNATH, N. & WU, H. 2015. A CRISPR-Based Screen Identifies Genes Essential for West-Nile-Virus-Induced Cell Death. *Cell Rep*, 12, 673-83.

MANOKARAN, G., FINOL, E., WANG, C., GUNARATNE, J., BAHL, J., ONG, E. Z., TAN, H. C., SESSIONS, O. M., WARD, A. M., GUBLER, D. J., HARRIS, E., GARCIA-BLANCO, M. A. & OOI, E. E. 2015. Dengue subgenomic RNA binds TRIM25 to inhibit interferon expression for epidemiological fitness. *Science*, 350, 217-21.

MAO, Y., LIU, H., LIU, Y. & TAO, S. 2014. Deciphering the rules by which dynamics of mRNA secondary structure affect translation efficiency in Saccharomyces cerevisiae. *Nucleic Acids Res*, 42, 4813-22.

MARCEAU, C. D., PUSCHNIK, A. S., MAJZOUB, K., OOI, Y. S., BREWER, S. M., FUCHS, G., SWAMINATHAN, K., MATA, M. A., ELIAS, J. E., SARNOW, P. & CARETTE, J. E. 2016. Genetic dissection of Flaviviridae host factors through genomescale CRISPR screens. *Nature*, 535, 159-63.

MARKOFF, L. 1989. In vitro processing of dengue virus structural proteins: cleavage of the pre-membrane protein. *J Virol*, 63, 3345-52.

MARQUIS, D. M., FAHNESTOCK, S. R., HENDERSON, E., WOO, D., SCHWINGE, S., CLARK, M. W. & LAKE, J. A. 1981. The L7/L12 stalk, a conserved feature of the prokaryotic ribosome, is attached to the large subunit through its N terminus. *J Mol Biol*, 150, 121-32.

MARTINEZ-AZORIN, F., REMACHA, M. & BALLESTA, J. P. 2008a. Functional characterization of ribosomal P1/P2 proteins in human cells. *Biochem J*, 413, 527-34.

MARTINEZ-AZORIN, F., REMACHA, M., MARTINEZ-SALAS, E. & BALLESTA, J. P. 2008b. Internal translation initiation on the foot-and-mouth disease virus IRES is affected by ribosomal stalk conformation. *FEBS Lett*, 582, 3029-32.

MATEO, R., NAGAMINE, C. M., SPAGNOLO, J., MENDEZ, E., RAHE, M., GALE, M., JR., YUAN, J. & KIRKEGAARD, K. 2013. Inhibition of cellular autophagy deranges dengue virion maturation. *J Virol*, 87, 1312-21.

MAURO, V. P. & EDELMAN, G. M. 2002. The ribosome filter hypothesis. *Proc Natl Acad Sci U S A*, 99, 12031-6.

MAY, K. L., LI, X. P., MARTINEZ-AZORIN, F., BALLESTA, J. P., GRELA, P., TCHORZEWSKI, M. & TUMER, N. E. 2012. The P1/P2 proteins of the human ribosomal stalk are required for ribosome binding and depurination by ricin in human cells. *FEBS J*, 279, 3925-36.

MAZUMDER, B., PODDAR, D., BASU, A., KOUR, R., VERBOVETSKAYA, V. & BARIK, S. 2014. Extraribosomal 113a is a specific innate immune factor for antiviral defense. *J Virol*, 88, 9100-10.

MCCANN, K. L. & BASERGA, S. J. 2013. Genetics. Mysterious ribosomopathies. *Science*, 341, 849-50.

MELIAN, E. B., HINZMAN, E., NAGASAKI, T., FIRTH, A. E., WILLS, N. M., NOUWENS, A. S., BLITVICH, B. J., LEUNG, J., FUNK, A., ATKINS, J. F., HALL, R. & KHROMYKH, A. A. 2010. NS1' of flaviviruses in the Japanese encephalitis virus serogroup is a product of ribosomal frameshifting and plays a role in viral neuroinvasiveness. *J Virol,* 84, 1641-7.

MICHEL, A. M. & BARANOV, P. V. 2013. Ribosome profiling: a Hi-Def monitor for protein synthesis at the genome-wide scale. *Wiley Interdiscip Rev RNA*, *4*, 473-90.

MOLLER, W., GROENE, A., TERHORST, C. & AMONS, R. 1972. 50-S ribosomal proteins. Purification and partial characterization of two acidic proteins, A 1 and A 2, isolated from 50-S ribosomes of Escherichia coli. *Eur J Biochem*, 25, 5-12.

MONDOTTE, J. A., LOZACH, P. Y., AMARA, A. & GAMARNIK, A. V. 2007. Essential role of dengue virus envelope protein N glycosylation at asparagine-67 during viral propagation. *J Virol*, 81, 7136-48.

MUKHOPADHYAY, S., KUHN, R. J. & ROSSMANN, M. G. 2005. A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol*, *3*, 13-22.

MUYLAERT, I. R., CHAMBERS, T. J., GALLER, R. & RICE, C. M. 1996. Mutagenesis of the N-linked glycosylation sites of the yellow fever virus NS1 protein: effects on virus replication and mouse neurovirulence. *Virology*, 222, 159-68.

NAGY, P. D., STRATING, J. R. & VAN KUPPEVELD, F. J. 2016. Building Viral Replication Organelles: Close Encounters of the Membrane Types. *PLoS Pathog*, 12, e1005912.

NANDAGOPAL, N. & ROUX, P. P. 2015. Regulation of global and specific mRNA translation by the mTOR signaling pathway. *Translation (Austin)*, 3, e983402.

NEDIALKOVA, D. D. & LEIDEL, S. A. 2015. Optimization of Codon Translation Rates via tRNA Modifications Maintains Proteome Integrity. *Cell*, 161, 1606-18.

NGUYEN-LEFEBVRE, A. T., LEPRUN, G., MORIN, V., VINUELAS, J., COUTE, Y., MADJAR, J. J., GANDRILLON, O. & GONIN-GIRAUD, S. 2013. V-erbA generates ribosomes devoid of RPL11 and regulates translational activity in avian erythroid progenitors. *Oncogene*.

NICOLAS, E., PARISOT, P., PINTO-MONTEIRO, C., DE WALQUE, R., DE VLEESCHOUWER, C. & LAFONTAINE, D. L. 2016. Involvement of human ribosomal proteins in nucleolar structure and p53-dependent nucleolar stress. *Nat Commun*, 7, 11390.

NOUR, A. M., LI, Y., WOLENSKI, J. & MODIS, Y. 2013. Viral membrane fusion and nucleocapsid delivery into the cytoplasm are distinct events in some flaviviruses. *PLoS Pathog*, 9, e1003585.

NUSSPAUMER, G., REMACHA, M. & BALLESTA, J. P. 2000. Phosphorylation and N-terminal region of yeast ribosomal protein P1 mediate its degradation, which is prevented by protein P2. *EMBO J*, 19, 6075-84.

OLIVARES, E., LANDRY, D. M., CACERES, C. J., PINO, K., ROSSI, F., NAVARRETE, C., HUIDOBRO-TORO, J. P., THOMPSON, S. R. & LOPEZ-LASTRA, M. 2014. The 5' untranslated region of the human T-cell lymphotropic virus type 1 mRNA enables capindependent translation initiation. *J Virol*, 88, 5936-55.

PADWAD, Y. S., MISHRA, K. P., JAIN, M., CHANDA, S. & GANJU, L. 2010. Dengue virus infection activates cellular chaperone Hsp70 in THP-1 cells: downregulation of Hsp70 by siRNA revealed decreased viral replication. *Viral Immunol*, 23, 557-65.

PECHMANN, S., CHARTRON, J. W. & FRYDMAN, J. 2014. Local slowdown of translation by nonoptimal codons promotes nascent-chain recognition by SRP in vivo. *Nat Struct Mol Biol*, 21, 1100-5.

PENA, J. & HARRIS, E. 2012. Early dengue virus protein synthesis induces extensive rearrangement of the endoplasmic reticulum independent of the UPR and SREBP-2 pathway. *PLoS One*, 7, e38202.

PERERA-LECOIN, M., MEERTENS, L., CARNEC, X. & AMARA, A. 2013. Flavivirus entry receptors: an update. *Viruses*, 6, 69-88.

PERERA, R., RILEY, C., ISAAC, G., HOPF-JANNASCH, A. S., MOORE, R. J., WEITZ, K. W., PASA-TOLIC, L., METZ, T. O., ADAMEC, J. & KUHN, R. J. 2012. Dengue virus infection perturbs lipid homeostasis in infected mosquito cells. *PLoS Pathog*, 8, e1002584.

PERREIRA, J. M., AKER, A. M., SAVIDIS, G., CHIN, C. R., MCDOUGALL, W. M., PORTMANN, J. M., MERANER, P., SMITH, M. C., RAHMAN, M., BAKER, R. E., GAUTHIER, A., FRANTI, M. & BRASS, A. L. 2015. RNASEK Is a V-ATPase-Associated Factor Required for Endocytosis and the Replication of Rhinovirus, Influenza A Virus, and Dengue Virus. *Cell Rep*, 12, 850-63.

PERUCHO, L., ARTERO-CASTRO, A., GUERRERO, S., RAMON Y CAJAL, S., ME, L. L. & WANG, Z. Q. 2014. RPLP1, a crucial ribosomal protein for embryonic development of the nervous system. *PLoS One*, 9, e99956.

PISAREVA, V. P., SKABKIN, M. A., HELLEN, C. U., PESTOVA, T. V. & PISAREV, A. V. 2011. Dissociation by Pelota, Hbs1 and ABCE1 of mammalian vacant 80S ribosomes and stalled elongation complexes. *EMBO J*, 30, 1804-17.

POLACEK, C., FRIEBE, P. & HARRIS, E. 2009. Poly(A)-binding protein binds to the nonpolyadenylated 3' untranslated region of dengue virus and modulates translation efficiency. *J Gen Virol*, 90, 687-92.

PREUGSCHAT, F., YAO, C. W. & STRAUSS, J. H. 1990. In vitro processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. *J Virol*, 64, 4364-74.

REED, L. J. & MUENCH, H. 1938. A Simple Method for Estimating Fifty Per Cent Endpoints. *The American Journal of Hygiene*, 27.

REID, D. W., CAMPOS, R. K., CHILD, J. R., ZHENG, T., CHAN, K. W. K., BRADRICK, S. S., VASUDEVAN, S. G., GARCIA-BLANCO, M. A. & NICCHITTA, C. V. 2018. Dengue virus selectively annexes endoplasmic reticulum-associated translation machinery as a strategy for co-opting host cell protein synthesis. *J Virol*.

REID, D. W., SHENOLIKAR, S. & NICCHITTA, C. V. 2015. Simple and inexpensive ribosome profiling analysis of mRNA translation. *Methods*, 91, 69-74.

REMACHA, M., JIMENEZ-DIAZ, A., SANTOS, C., BRIONES, E., ZAMBRANO, R., RODRIGUEZ GABRIEL, M. A., GUARINOS, E. & BALLESTA, J. P. 1995. Proteins P1, P2, and P0, components of the eukaryotic ribosome stalk. New structural and functional aspects. *Biochem Cell Biol*, 73, 959-68.

REQUIAO, R. D., DE SOUZA, H. J., ROSSETTO, S., DOMITROVIC, T. & PALHANO, F. L. 2016. Increased ribosome density associated to positively charged residues is evident in ribosome profiling experiments performed in the absence of translation inhibitors. *RNA Biol*, 13, 561-8.

RICE, C. M., LENCHES, E. M., EDDY, S. R., SHIN, S. J., SHEETS, R. L. & STRAUSS, J. H. 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. *Science*, 229, 726-33.

RICHARD, M., BOULIN, T., ROBERT, V. J., RICHMOND, J. E. & BESSEREAU, J. L. 2013. Biosynthesis of ionotropic acetylcholine receptors requires the evolutionarily conserved ER membrane complex. *Proc Natl Acad Sci U S A*, 110, E1055-63.

RICHTER, J. D. & COLLER, J. 2015. Pausing on Polyribosomes: Make Way for Elongation in Translational Control. *Cell*, 163, 292-300.

ROBY, J. A., SETOH, Y. X., HALL, R. A. & KHROMYKH, A. A. 2015. Post-translational regulation and modifications of flavivirus structural proteins. *J Gen Virol*, *96*, 1551-69.

RODNINA, M. V. & WINTERMEYER, W. 2016. Protein Elongation, Co-translational Folding and Targeting. *J Mol Biol*, 428, 2165-85.

ROMERO-BREY, I. & BARTENSCHLAGER, R. 2014. Membranous replication factories induced by plus-strand RNA viruses. *Viruses*, *6*, 2826-57.

RUIZ-LINARES, A., CAHOUR, A., DESPRES, P., GIRARD, M. & BOULOY, M. 1989. Processing of yellow fever virus polyprotein: role of cellular proteases in maturation of the structural proteins. *J Virol*, 63, 4199-209. SAMSA, M. M., MONDOTTE, J. A., IGLESIAS, N. G., ASSUNCAO-MIRANDA, I., BARBOSA-LIMA, G., DA POIAN, A. T., BOZZA, P. T. & GAMARNIK, A. V. 2009. Dengue virus capsid protein usurps lipid droplets for viral particle formation. *PLoS Pathog*, 5, e1000632.

SANCHEZ-MADRID, F., CONDE, P., VAZQUEZ, D. & BALLESTA, J. P. 1979. Acidic proteins from Saccharomyces cerevisiae ribosomes. *Biochem Biophys Res Commun*, 87, 281-91.

SANTOS, C. & BALLESTA, J. P. 1995. The highly conserved protein P0 carboxyl end is essential for ribosome activity only in the absence of proteins P1 and P2. *J Biol Chem*, 270, 20608-14.

SATOH, T., OHBA, A., LIU, Z., INAGAKI, T. & SATOH, A. K. 2015. dPob/EMC is essential for biosynthesis of rhodopsin and other multi-pass membrane proteins in Drosophila photoreceptors. *Elife*, 4.

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., GUO, Z., GREEN, S., KOWALIK, T. F. & BRASS, A. L. 2016. Identification of Zika Virus and Dengue Virus Dependency Factors using Functional Genomics. *Cell Rep*, 16, 232-246.

SCHREIBER, M. J., HOLMES, E. C., ONG, S. H., SOH, H. S., LIU, W., TANNER, L., AW, P. P., TAN, H. C., NG, L. C., LEO, Y. S., LOW, J. G., ONG, A., OOI, E. E., VASUDEVAN, S. G. & HIBBERD, M. L. 2009. Genomic epidemiology of a dengue virus epidemic in urban Singapore. *J Virol*, 83, 4163-73.

SCHULDINER, M., METZ, J., SCHMID, V., DENIC, V., RAKWALSKA, M., SCHMITT, H. D., SCHWAPPACH, B. & WEISSMAN, J. S. 2008. The GET complex mediates insertion of tail-anchored proteins into the ER membrane. *Cell*, 134, 634-45.

SCHULLER, A. P., WU, C. C., DEVER, T. E., BUSKIRK, A. R. & GREEN, R. 2017. eIF5A Functions Globally in Translation Elongation and Termination. *Mol Cell*, 66, 194-205 e5.

SESSIONS, O. M., BARROWS, N. J., SOUZA-NETO, J. A., ROBINSON, T. J., HERSHEY, C. L., RODGERS, M. A., RAMIREZ, J. L., DIMOPOULOS, G., YANG, P. L., PEARSON, J. L. & GARCIA-BLANCO, M. A. 2009. Discovery of insect and human dengue virus host factors. *Nature*, 458, 1047-50.

SHAH, P., DING, Y., NIEMCZYK, M., KUDLA, G. & PLOTKIN, J. B. 2013. Rate-limiting steps in yeast protein translation. *Cell*, 153, 1589-601.

SHALGI, R., HURT, J. A., KRYKBAEVA, I., TAIPALE, M., LINDQUIST, S. & BURGE, C. B. 2013. Widespread regulation of translation by elongation pausing in heat shock. *Mol Cell*, 49, 439-52.

SHAO, S. & HEGDE, R. S. 2011. Membrane protein insertion at the endoplasmic reticulum. *Annu Rev Cell Dev Biol*, 27, 25-56.

SHI, Z. & BARNA, M. 2015. Translating the genome in time and space: specialized ribosomes, RNA regulons, and RNA-binding proteins. *Annu Rev Cell Dev Biol*, 31, 31-54.

SHURTLEFF, M. J., ITZHAK, D. N., HUSSMANN, J. A., SCHIRLE OAKDALE, N. T., COSTA, E. A., JONIKAS, M., WEIBEZAHN, J., POPOVA, K. D., JAN, C. H., SINITCYN, P., VEMBAR, S. S., HERNANDEZ, H., COX, J., BURLINGAME, A. L., BRODSKY, J., FROST, A., BORNER, G. H. & WEISSMAN, J. S. 2018. The ER membrane protein complex interacts cotranslationally to enable biogenesis of multipass membrane proteins. *Elife*, 7.

SILLER, E., DEZWAAN, D. C., ANDERSON, J. F., FREEMAN, B. C. & BARRAL, J. M. 2010. Slowing bacterial translation speed enhances eukaryotic protein folding efficiency. *J Mol Biol*, 396, 1310-8.

SIROHI, D., CHEN, Z., SUN, L., KLOSE, T., PIERSON, T. C., ROSSMANN, M. G. & KUHN, R. J. 2016. The 3.8 A resolution cryo-EM structure of Zika virus. *Science*, 352, 467-70.

SLAWINSKA, A., PLOWIEC, A., SIWEK, M., JAROSZEWSKI, M. & BEDNARCZYK, M. 2016. Long-Term Transcriptomic Effects of Prebiotics and Synbiotics Delivered In Ovo in Broiler Chickens. *PLoS One*, 11, e0168899.

SMIT, J. M., MOESKER, B., RODENHUIS-ZYBERT, I. & WILSCHUT, J. 2011. Flavivirus cell entry and membrane fusion. *Viruses*, 3, 160-71.

SOMNUKE, P., HAUHART, R. E., ATKINSON, J. P., DIAMOND, M. S. & AVIRUTNAN, P. 2011. N-linked glycosylation of dengue virus NS1 protein modulates secretion, cell-surface expression, hexamer stability, and interactions with human complement. *Virology*, 413, 253-64.

STADLER, K., ALLISON, S. L., SCHALICH, J. & HEINZ, F. X. 1997. Proteolytic activation of tick-borne encephalitis virus by furin. *J Virol*, 71, 8475-81.

STRYCHARZ, W. A., NOMURA, M. & LAKE, J. A. 1978. Ribosomal proteins L7/L12 localized at a single region of the large subunit by immune electron microscopy. *J Mol Biol*, 126, 123-40.

SULIMA, S. O., GULAY, S. P., ANJOS, M., PATCHETT, S., MESKAUSKAS, A., JOHNSON, A. W. & DINMAN, J. D. 2014. Eukaryotic rpL10 drives ribosomal rotation. *Nucleic Acids Res*, 42, 2049-63.

SVITKIN, Y. V., LYAPUSTIN, V. N., LASHKEVICH, V. A. & AGOL, V. I. 1984. Differences between translation products of tick-borne encephalitis virus RNA in cellfree systems from Krebs-2 cells and rabbit reticulocytes: involvement of membranes in the processing of nascent precursors of flavivirus structural proteins. *Virology*, 135, 536-41.

TABATA, T., PETITT, M., PUERTA-GUARDO, H., MICHLMAYR, D., WANG, C., FANG-HOOVER, J., HARRIS, E. & PEREIRA, L. 2016. Zika Virus Targets Different Primary Human Placental Cells, Suggesting Two Routes for Vertical Transmission. *Cell Host Microbe*, 20, 155-66. TAGUWA, S., MARINGER, K., LI, X., BERNAL-RUBIO, D., RAUCH, J. N., GESTWICKI, J. E., ANDINO, R., FERNANDEZ-SESMA, A. & FRYDMAN, J. 2015. Defining Hsp70 Subnetworks in Dengue Virus Replication Reveals Key Vulnerability in Flavivirus Infection. *Cell*, 163, 1108-23.

TANGUAY, R. L. & GALLIE, D. R. 1996. Translational efficiency is regulated by the length of the 3' untranslated region. *Mol Cell Biol*, 16, 146-56.

TAYMANS, J. M., NKILIZA, A. & CHARTIER-HARLIN, M. C. 2015. Deregulation of protein translation control, a potential game-changing hypothesis for Parkinson's disease pathogenesis. *Trends Mol Med*, 21, 466-72.

THEPPARIT, C. & SMITH, D. R. 2004. Serotype-specific entry of dengue virus into liver cells: identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. *J Virol*, 78, 12647-56.

THOREEN, C. C., CHANTRANUPONG, L., KEYS, H. R., WANG, T., GRAY, N. S. & SABATINI, D. M. 2012. A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature*, 485, 109-13.

TIAN, X., SUN, D., ZHAO, S., XIONG, H. & FANG, J. 2015. Screening of potential diagnostic markers and therapeutic targets against colorectal cancer. *Onco Targets Ther*, *8*, 1691-9.

TIO, P. H., JONG, W. W. & CARDOSA, M. J. 2005. Two dimensional VOPBA reveals laminin receptor (LAMR1) interaction with dengue virus serotypes 1, 2 and 3. *Virol J*, 2, 25.

TRACY, S., CHAPMAN, N. M. & TU, Z. 1992. Coxsackievirus B3 from an infectious cDNA copy of the genome is cardiovirulent in mice. *Arch Virol*, 122, 399-409.

TSAI, C. J., SAUNA, Z. E., KIMCHI-SARFATY, C., AMBUDKAR, S. V., GOTTESMAN, M. M. & NUSSINOV, R. 2008. Synonymous mutations and ribosome stalling can lead to altered folding pathways and distinct minima. *J Mol Biol*, 383, 281-91.

UCHIDA, L., ESPADA-MURAO, L. A., TAKAMATSU, Y., OKAMOTO, K., HAYASAKA, D., YU, F., NABESHIMA, T., BUERANO, C. C. & MORITA, K. 2014. The dengue virus conceals double-stranded RNA in the intracellular membrane to escape from an interferon response. *Sci Rep*, *4*, 7395.

UCHIUMI, T., HONMA, S., ENDO, Y. & HACHIMORI, A. 2002. Ribosomal proteins at the stalk region modulate functional rRNA structures in the GTPase center. *J Biol Chem*, 277, 41401-9.

UJVARI, A., ARON, R., EISENHAURE, T., CHENG, E., PARAG, H. A., SMICUN, Y., HALABAN, R. & HEBERT, D. N. 2001. Translation rate of human tyrosinase determines its N-linked glycosylation level. *J Biol Chem*, 276, 5924-31.

VAN DER SCHAAR, H. M., RUST, M. J., CHEN, C., VAN DER ENDE-METSELAAR, H., WILSCHUT, J., ZHUANG, X. & SMIT, J. M. 2008. Dissecting the cell entry pathway of dengue virus by single-particle tracking in living cells. *PLoS Pathog*, 4, e1000244.

VAN DER SCHAAR, H. M., RUST, M. J., WAARTS, B. L., VAN DER ENDE-METSELAAR, H., KUHN, R. J., WILSCHUT, J., ZHUANG, X. & SMIT, J. M. 2007. Characterization of the early events in dengue virus cell entry by biochemical assays and single-virus tracking. *J Virol*, 81, 12019-28.

VASHIST, S., ANANTPADMA, M., SHARMA, H. & VRATI, S. 2009. La protein binds the predicted loop structures in the 3' non-coding region of Japanese encephalitis virus genome: role in virus replication. *J Gen Virol*, 90, 1343-52.

VEIT, G., OLIVER, K., APAJA, P. M., PERDOMO, D., BIDAUD-MEYNARD, A., LIN, S. T., GUO, J., ICYUZ, M., SORSCHER, E. J., HARTMAN, J. I. & LUKACS, G. L. 2016. Ribosomal Stalk Protein Silencing Partially Corrects the DeltaF508-CFTR Functional Expression Defect. *PLoS Biol*, 14, e1002462.

VIANA, V. T., DURCAN, L., BONFA, E. & ELKON, K. B. 2017. Ribosomal P antibody: 30 years on the road. *Lupus*, 26, 453-462.

WAKITA, T., PIETSCHMANN, T., KATO, T., DATE, T., MIYAMOTO, M., ZHAO, Z., MURTHY, K., HABERMANN, A., KRAUSSLICH, H. G., MIZOKAMI, M., BARTENSCHLAGER, R. & LIANG, T. J. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med*, 11, 791-6.

WALTER, P., IBRAHIMI, I. & BLOBEL, G. 1981. Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to in-vitro-assembled polysomes synthesizing secretory protein. *J Cell Biol*, 91, 545-50.

WALTER, P. & JOHNSON, A. E. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. *Annu Rev Cell Biol*, 10, 87-119.

WANG, J. W., CHEN, W., KANG, X. T., HUANG, Y. Q., TIAN, Y. D. & WANG, Y. B. 2012. Identification of differentially expressed genes induced by energy restriction using annealing control primer system from the liver and adipose tissues of broilers. *Poult Sci*, 91, 972-8.

WARD, A. M., BIDET, K., YINGLIN, A., LER, S. G., HOGUE, K., BLACKSTOCK, W., GUNARATNE, J. & GARCIA-BLANCO, M. A. 2011. Quantitative mass spectrometry of DENV-2 RNA-interacting proteins reveals that the DEAD-box RNA helicase DDX6 binds the DB1 and DB2 3' UTR structures. *RNA Biol*, *8*, 1173-86.

WARD, A. M., CALVERT, M. E., READ, L. R., KANG, S., LEVITT, B. E., DIMOPOULOS, G., BRADRICK, S. S., GUNARATNE, J. & GARCIA-BLANCO, M. A. 2016. The Golgi associated ERI3 is a Flavivirus host factor. *Sci Rep*, 6, 34379.

WAWIORKA, L., MOLESTAK, E., SZAJWAJ, M., MICHALEC-WAWIORKA, B., MOLON, M., BORKIEWICZ, L., GRELA, P., BOGUSZEWSKA, A. & TCHORZEWSKI, M. 2017. Multiplication of Ribosomal P-Stalk Proteins Contributes to the Fidelity of Translation. *Mol Cell Biol*, 37.

WEAVER, S. C., COSTA, F., GARCIA-BLANCO, M. A., KO, A. I., RIBEIRO, G. S., SAADE, G., SHI, P. Y. & VASILAKIS, N. 2016. Zika virus: History, emergence, biology, and prospects for control. *Antiviral Res*, 130, 69-80.

WEINBERG, D. E., SHAH, P., EICHHORN, S. W., HUSSMANN, J. A., PLOTKIN, J. B. & BARTEL, D. P. 2016. Improved Ribosome-Footprint and mRNA Measurements Provide Insights into Dynamics and Regulation of Yeast Translation. *Cell Rep*, 14, 1787-1799.

WELSCH, S., MILLER, S., ROMERO-BREY, I., MERZ, A., BLECK, C. K., WALTHER, P., FULLER, S. D., ANTONY, C., KRIJNSE-LOCKER, J. & BARTENSCHLAGER, R. 2009. Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell Host Microbe*, *5*, 365-75.

WILDER-SMITH, A. & BYASS, P. 2016. The elusive global burden of dengue. *Lancet Infect Dis*, 16, 629-631.

WOLIN, S. L. & WALTER, P. 1988. Ribosome pausing and stacking during translation of a eukaryotic mRNA. *EMBO J*, 7, 3559-69.

WOOLSTENHULME, C. J., GUYDOSH, N. R., GREEN, R. & BUSKIRK, A. R. 2015. High-precision analysis of translational pausing by ribosome profiling in bacteria lacking EFP. *Cell Rep*, 11, 13-21.

XU, Z. & HOBMAN, T. C. 2012. The helicase activity of DDX56 is required for its role in assembly of infectious West Nile virus particles. *Virology*, 433, 226-35.

XUE, S., TIAN, S., FUJII, K., KLADWANG, W., DAS, R. & BARNA, M. 2015. RNA regulons in Hox 5' UTRs confer ribosome specificity to gene regulation. *Nature*, 517, 33-8.

YANG, M., SUN, Y., SUN, J., WANG, Z., ZHOU, Y., YAO, G., GU, Y., ZHANG, H. & ZHAO, H. 2018. Differentially expressed and survival-related proteins of lung adenocarcinoma with bone metastasis. *Cancer Med*, *7*, 1081-1092.

YASUNAGA, A., HANNA, S. L., LI, J., CHO, H., ROSE, P. P., SPIRIDIGLIOZZI, A., GOLD, B., DIAMOND, M. S. & CHERRY, S. 2014. Genome-wide RNAi screen identifies broadly-acting host factors that inhibit arbovirus infection. *PLoS Pathog*, 10, e1003914.

YE, J., CHEN, Z., ZHANG, B., MIAO, H., ZOHAIB, A., XU, Q., CHEN, H. & CAO, S. 2013. Heat shock protein 70 is associated with replicase complex of Japanese encephalitis virus and positively regulates viral genome replication. *PLoS One*, *8*, e75188.

YIN, Z., CHEN, Y. L., SCHUL, W., WANG, Q. Y., GU, F., DURAISWAMY, J., KONDREDDI, R. R., NIYOMRATTANAKIT, P., LAKSHMINARAYANA, S. B., GOH, A., XU, H. Y., LIU, W., LIU, B., LIM, J. Y., NG, C. Y., QING, M., LIM, C. C., YIP, A., WANG, G., CHAN, W. L., TAN, H. P., LIN, K., ZHANG, B., ZOU, G., BERNARD, K. A., GARRETT, C., BELTZ, K., DONG, M., WEAVER, M., HE, H., PICHOTA, A., DARTOIS, V., KELLER, T. H. & SHI, P. Y. 2009. An adenosine nucleoside inhibitor of dengue virus. *Proc Natl Acad Sci U S A*, 106, 20435-9.

YU, C. H., DANG, Y., ZHOU, Z., WU, C., ZHAO, F., SACHS, M. S. & LIU, Y. 2015. Codon Usage Influences the Local Rate of Translation Elongation to Regulate Cotranslational Protein Folding. *Mol Cell*, 59, 744-54.

YU, I. M., ZHANG, W., HOLDAWAY, H. A., LI, L., KOSTYUCHENKO, V. A., CHIPMAN, P. R., KUHN, R. J., ROSSMANN, M. G. & CHEN, J. 2008. Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science*, 319, 1834-7.

ZAITSEVA, E., YANG, S. T., MELIKOV, K., POURMAL, S. & CHERNOMORDIK, L. V. 2010. Dengue virus ensures its fusion in late endosomes using compartment-specific lipids. *PLoS Pathog*, 6, e1001131.

ZANINI, F., PU, S. Y., BEKERMAN, E., EINAV, S. & QUAKE, S. R. 2018. Single-cell transcriptional dynamics of flavivirus infection. *Elife*, 7.

ZEISEL, M. B., FELMLEE, D. J. & BAUMERT, T. F. 2013. Hepatitis C virus entry. *Curr Top Microbiol Immunol*, 369, 87-112.

ZHANG, L., MOHAN, P. M. & PADMANABHAN, R. 1992. Processing and localization of Dengue virus type 2 polyprotein precursor NS3-NS4A-NS4B-NS5. *J Virol*, 66, 7549-54.

ZHANG, L., ZHOU, W., VELCULESCU, V. E., KERN, S. E., HRUBAN, R. H., HAMILTON, S. R., VOGELSTEIN, B. & KINZLER, K. W. 1997. Gene expression profiles in normal and cancer cells. *Science*, 276, 1268-72.

ZHANG, R., MINER, J. J., GORMAN, M. J., RAUSCH, K., RAMAGE, H., WHITE, J. P., ZUIANI, A., ZHANG, P., FERNANDEZ, E., ZHANG, Q., DOWD, K. A., PIERSON, T. C., CHERRY, S. & DIAMOND, M. S. 2016. A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. *Nature*, 535, 164-8.

ZHANG, S., HU, H., ZHOU, J., HE, X., JIANG, T. & ZENG, J. 2017. Analysis of Ribosome Stalling and Translation Elongation Dynamics by Deep Learning. *Cell Syst*, 5, 212-220 e6.

ZHANG, X., GE, P., YU, X., BRANNAN, J. M., BI, G., ZHANG, Q., SCHEIN, S. & ZHOU, Z. H. 2013. Cryo-EM structure of the mature dengue virus at 3.5-A resolution. *Nat Struct Mol Biol*, 20, 105-10.

ZHOU, X., LIAO, W. J., LIAO, J. M., LIAO, P. & LU, H. 2015. Ribosomal proteins: functions beyond the ribosome. *J Mol Cell Biol*, *7*, 92-104.

ZHOURAVLEVA, G., FROLOVA, L., LE GOFF, X., LE GUELLEC, R., INGE-VECHTOMOV, S., KISSELEV, L. & PHILIPPE, M. 1995. Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. *EMBO J*, 14, 4065-72.

ZOU, G., XU, H. Y., QING, M., WANG, Q. Y. & SHI, P. Y. 2011. Development and characterization of a stable luciferase dengue virus for high-throughput screening. *Antiviral Res*, 91, 11-9.

ZYBERT, I. A., VAN DER ENDE-METSELAAR, H., WILSCHUT, J. & SMIT, J. M. 2008. Functional importance of dengue virus maturation: infectious properties of immature virions. *J Gen Virol*, 89, 3047-51.

## Biography

Rafael Kroon Campos was born in Wurzburg, Germany on 30<sup>th</sup> of June of 1989. He attended Pontificia Universidade Catolica de Minas Gerais, Brazil, where he obtained his bachelor's degree in biology in the year of 2011 working with natural coinfection of vaccinia viruses. He obtained his M.S. of Microbiology degree in 2013 from the Universidade Federal de Minas Gerais, Brazil, working on mimiviruses and their virophages, work which was awarded best thesis of the Microbiology Department. Rafael then began his graduate career at Duke University, where he joined the Department of Molecular Genetics and Microbiology in 2012. After joining the lab of Mariano A. Garcia-Blanco and Shelton S. Bradrick, Rafael authored and co-authored a number of scientific articles and reviews, entitled "Roles of Pro-viral Host Factors in Mosquito-Borne Flavivirus Infections", "RPLP1 and RPLP2 are Essential Flavivirus Host Factors that Promote Early Viral Protein Accumulation", "Biochemistry and Molecular Biology of Flaviviruses", "Dengue Virus Selectively Annexes Endoplasmic Reticulum-Associated Translation Machinery as a Strategy for Co-opting Host Cell Protein Synthesis" and "A Screen of FDA-Approved Drugs for Inhibitors of Zika Virus Infection". He was awarded with the "Outstanding poster Award" at the 4th Annual BMB Science Retreat at UTMB, in May of 2018.