A Role of Unfolded Protein Response in the Cellular Response to Flavivirus Infection

Thesis

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A role of Unfolded Protein Response in the cellular response to Flavivirus infection

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A thesis submitted in fulfillment of the requirements of the Faculty of Life Sciences of the Open University for the degree of Doctor of Philosophy

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ABSTRACT

Flaviviruses are responsible for a myriad of epidemics globally. Factors like movement and climate change perpetuate their spread making control rather challenging. The understanding of host-virus interactions is therefore cornerstone in attempts to produce efficient vaccines and/or therapies. The host immune system is indispensable in fighting infections but viruses have developed evasion strategies which abrogate the response that would otherwise function in virus clearance.

The unfolded protein response (UPR), an intrinsic cellular proteostasis pathway is increasingly associated with innate immunity in response to cellular insults including viral infection. UPR activation by external stimuli elicits an earlier induction of IFNβ and antiviral ISGs suggesting UPR as a priming event that potentiates a more robust response against flaviviruses.

Prerequisite to the core of this study, an analysis of the effects of TM and TG (UPR chemical inducers) as used in our current experimental settings show that they are only cytostatic but not cytotoxic.

Afterwards, I delve into the search for alternative UPR inducers as potential alternatives for the ones currently in use. I show that E, NS1 and NS2B proteins of TBEV can induce UPR and several antiviral ISGs when ectopically expressed, an effect that inhibits TBEV in infection.

Of the three proteins studied, NS2B seems to be the most potent inducer of UPR and antiviral ISGs. Furthermore, expression of NS2B together with RIG-I augments the IFNβ promoter activity suggesting a possible role in innate signaling independent of infection.

The findings of this work open up possible avenues that require further investigation particularly in the case of NS2B, which is not a well-documented flavivirus protein apart from the context of its function as a protease cofactor. The mechanism by which it induces ER stress is especially intriguing because unlike E and NS1 proteins, it is not a glycoprotein.
DEDICATION

In loving memory of the late Mrs. Musanna. Thank you for shining a light and planting a seed of belief in my academic path. May your soul find eternal peace.
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My three-year journey would not have been possible without many people I’ve met along the way. Firstly, I thank God for the strength and courage when I needed it the most especially in the few months just before my PhD defense when both my parents fought serious medical battles so far away from me.

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LIST OF ABBREVIATIONS & ACRONYMS

AAT/α1AT: Alpha-1-antitrypsin
AMP: Ampicillin
ANOVA: Analysis of Variance
AP-1: Activator protein 1
ATF4: Activating transcription factor 4
ATF6: Activating transcription factor 6
ATP: Adenosine triphosphate
BiP: Binding Immunoglobulin protein
bZIP: Basic Leucine Zipper Domain
CARD: Caspase activation and recruitment domain
CD: Cluster of differentiation
cGAS: cyclic GMP-AMP Synthase
ChIP-Seq: Chromatin Immunoprecipitation DNA Sequencing
CHOP: C/EBP homologous protein
CMC: Carboxymethyl cellulose
CPE: Cytopathic effects
CRISPR-Cas9: clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9
DDIT3: DNA damage inducible transcript 3
DENV: Dengue virus
DHX58: DExH-Box Helicase 58
DMEM: Dulbecco’s Modified Eagle Medium
DMSO: Dimethylsulfoxide
DNA: Deoxyribonucleic acid
dsRNA: double-stranded RNA
DTT: Dithiothreitol
E (protein)/Env:Envelope protein
EDEM1: ER Degradation Enhancing Alpha-Mannosidase Like Protein 1
EDTA: Ethylenediaminetetraacetic acid
eIF2α: Eukaryotic translation initiation factor 2 alpha
ER: Endoplasmic Reticulum
ERAD: ER-associated degradation
ERK: Extracellular signal–regulated kinases
FACS: Fluorescence-activated cell sorting
FBS: Fetal bovine serum
FC: Flow cytometry
GADD34: Growth arrest and DNA damage-inducible protein
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GRP78: Glucose-Regulated Protein, 78kDa
GRP94: Glucose-Regulated Protein, 94 KDa
h.p.i/t: hours post infection/transfection
HA: Hemagglutinin
HAV: Hepatitis A virus
HSPA5: Heat Shock Protein Family A (Hsp70) Member 5
HCMV: Human cytomegalovirus
HCV: Hepatitis C virus
HEK cells: Human embryonic kidney cells
HIV: Human Immunodeficiency virus
HRP: Horseradish peroxidase conjugate
HSV: Herpes simplex virus
IAV: Influenza A virus
ICAM3: Intercellular Adhesion Molecule 3
ICTV: The International Committee on Taxonomy of Viruses
IFI44: Interferon Induced Protein 44
IFI44L: Interferon Induced Protein 44 Like
IFI6: Interferon Alpha Inducible Protein 6
IFIH1: Interferon Induced with Helicase C Domain 1
IFIT1: Interferon Induced Protein with Tetratricopeptide Repeats 1
IFITM: Interferon-induced transmembrane protein
IFNα/β: Interferon alpha/beta
IFNAR: Interferon-α/β receptor
IFNLR: Interferon Lambda Receptor
IKKε: Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IPS-1: IFNβ promoter stimulator
IRE1: Inositol-requiring enzyme 1
IRES: internal ribosome entry site
IRFs: Interferon regulatory factors
ISG: Interferon-stimulated gene
ISGF3: Interferon-stimulated gene factor 3
JAK: Janus Kinase
JEV: Japanese encephalitis virus
JNK: c-Jun N-terminal kinase
Kana: Kanamycin
kDa: kilo Dalton
KUNV: Kunjin virus (West-Nile subtype)
LB: Lysogeny broth/ Luria broth/ Lennox broth/ Luria-Bertani medium
LGP2: Laboratory of Genetics and Physiology 2
LPS: Lipopolysaccharides
LTR: Long terminal repeats
MAM: Mitochondria-associated membrane
MAPK: Mitogen-activated protein kinase
MAVS: Mitochondrial antiviral-signaling protein
MDA5: Melanoma Differentiation-Associated protein 5
mRNA: messenger RNA
MyD88: Myeloid differentiation primary response 88
NCR: Non-coding region
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NHK: Null Hong Kong
NLRs: Nod-like receptors
NS: Non-structural
NY99: New York 1999 (West-Nile virus subtype)
O/N: Overnight
OASL: 2'-5'-Oligoadenylate Synthetase Like
PAMPs: Pathogen-associated molecular patterns
PBS: Phosphate-Buffered Saline
PCR: Polymerase chain reaction
PDI: Protein disulfide isomerase
PERK: Protein kinase R (PKR)-like endoplasmic reticulum kinase
PFA: Paraformaldehyde
PI3K: Phosphoinositide 3-kinase
prM: premembrane
PMSF: Phenylmethylsulfonyl fluoride
PRRs: Pattern recognition receptors
qRT-PCR: Real-Time Quantitative Reverse Transcription PCR
RIG-I: Retinoic acid-inducible gene I
RIP1: Receptor interacting protein 1
RIPA: Radioimmunoprecipitation assay
RLRs: RIG-I like receptors
RNA: Ribonucleic acid
RT-PCR: Reverse Transcription PCR
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA: small interfering RNA
S1/2P: site 1/2 protease
ssRNA: single-stranded RNA
STAT1/2: Signal transducer and activator of transcription ½
STING: Stimulator of interferon genes
SV40: Simian VIRUS 40
TBEV: Tick-borne encephalitis virus
TBK1: TANK Binding Kinase 1
TG: Tharpsigargin
TGN: Trans-Golgi Network
TLRs: Toll-like receptors
- TM: Tunicamycin
- TRAF3: TNF receptor-associated factor
- TRIF: TIR-domain-containing adapter-inducing interferon-β
- TRIM56: Tripartite Motif Containing 56
- TYK2: Tyrosine-protein kinase
- U20S: Human Bone Osteosarcoma
- UPR: Unfolded Protein Response
- UTR: Untranslated region
- VISA: Virus-induced signaling adapter
- WB: Western blot
- WNV: West-Nile virus
- WT: Wild type
- XBP1(s): X-Box Binding Protein 1 (spliced)
- YFV: Yellow fever virus
- ZIKV: Zika virus
1. INTRODUCTION
1.1 Family Flaviviridae

Flaviviridae is a large family of small (40 to 60 nm diameter), enveloped viruses with a linear, non-segmented, single-stranded RNA of positive polarity. The genome of members in this family ranges from 9 to 13kbp and their surface proteins are arranged in an icosahedral-like symmetry (Lindenbach and Rice, 2001; Wengler et al., 1978; Zhang et al., 2003). The word Flaviviridae is derived from “Flavus” which is Latin for “yellow”, this came due to the importance of the Yellow Fever virus, the prototype virus in this family and the genus Flavivirus; that was the first proven human pathogen, isolated in 1927 in the Rhesus monkey (Macaca mulatta), through the inoculation of blood from an African patient (Stock et al., 2013, Strode G. 1951)

There currently exist four genera in this family (Fig. 1).

1.1.1 Flavivirus

This genus is largely made up of arboviruses, that is, their transmission to vertebrate hosts is through arthropod vectors mainly mosquitoes and ticks. However, there also exist viruses in this genus with no known vectors. The 5’ end of the genome possesses a type I cap (m7GpppAmp) not seen in the other genera and the 3’ end lacks terminal polyadenylation (Simmonds et al., (ICTV), 2017). The genus Flavivirus will be discussed in more detail in the following paragraphs.

1.1.2 Hepacivirus

This genus contains both primate (Hepacivirus C) and non-primate viruses. Non-primate viruses have been described in horses, donkeys, colobus monkeys as well as in rodents and bats. Hepacivirus C has been widely characterized virologically and clinically more than all its counterparts in the genus. A general distinctive feature of members of this genus as compared to other members in the family is the limited propagative ability of Hepaciviruses in cell culture. The 5’ UTR of Hepacivirus C contains a type IV IRES structural element that directs cap-independent translation. (Simmonds et al., (ICTV), 2017)
1.1.3 Pegivirus
This genus in contrast to the Hepaciviruses does not encode a core protein and instead of the type IV IRES structural element, it employs a type III IRES in the cap independent translation of the genomic RNA. Pegiviruses can be found in a wide variety of humans, non-human primates, pigs, horses as well as rodents and bats (Simmonds et al., (ICTV), 2017).

1.1.4 Pestivirus
Translation of the Pestivirus genomic RNA is cap-independent and initiated by a type IV internal ribosomal entry site of about 370-385 nucleotides within the 5’-non-coding region of the virus genomic RNA and the 3’-NCR, with about 185–273 nucleotides, is complex and contains a region with variable sequences and a highly conserved terminal region. The genome encodes for four structural and 8 non-structural proteins. Pestiviruses infect pigs and ruminants but have also been detected in wild ruminants and wild boar (Smith et al., 2017, Simmonds et al., (ICTV), 2017)
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1.2 Flaviviruses

Majority of viruses in the genus flavivirus are arthropod-borne pathogens that is, transmitted between arthropods (mainly ticks and mosquitoes) and vertebrates also referred to as dual-host viruses (Blitvich and Firth, 2015). With over 40 human-tropic viruses most of which are mosquito-borne, Flavivirus is an important genus in the family Flaviviridae because of its members’ global distribution, most of them considered important human and veterinary pathogens responsible for a myriad of haemorrhagic fevers and encephalitides that have been considered global public health threats. One example is the dengue virus (DENV), reported to cause approximately 390 million cases globally.
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per year (Bhatt et al., 2013). In addition to ticks and mosquito-borne viruses, there exists viruses with no-known vectors also termed vertebrate-specific viruses, these are isolated from rodents and bats, as well as insect-specific flaviviruses which have only been known to infect insect cells but not mammalian cells (Kenney et al., 2014; Blitvich and Firth, 2015; Bolling et al., 2015). Some of the most important flaviviruses include Yellow fever virus (YFV), dengue virus (DENV 1-4), Japanese encephalitis virus (JEV), West Nile virus (WNV), Zika virus (ZIKV) and Tick-borne encephalitis virus (TBEV), which is endemic in Europe and parts of Asia.

There is a constant spread of flaviviruses to new geographical areas where they never existed before such as the recent re-emergence of WNV and Zika virus in the Americas in 1999 and 2015, respectively (Asnis et al., 2000; Nash et al., 2001; Fauci et al., 2016; Petersen et al., 2016).

Despite wide deployment of the YFV-17D vaccine in many African countries since 2006, in 2016, new YFV epidemics occurred in Angola and DR Congo resulting to about 965 confirmed cases and 400 mortalities. According to WHO, about 27 countries in Africa remain at risk of epidemics despite the current efforts (https://www.afro.who.int/health-topics/yellow-fever)

This like many other epidemics are highly contributed by factors such as urbanization, transportation, poor public health infrastructure especially in the developing world, land use changes as well as natural factors, such as genetic changes in the viruses, host-vector relationships, bird migration and global climate changes (Mackenzie et al., 2004).

Due to the close relationships among flaviviruses and the general rapid mutation rates of RNA viruses, there is a general problem in specific diagnosis, as well as a general lack of approved and/or efficient treatments and/or vaccines.
There exist effective, licensed vaccines against JEV, TBEV and the 17D vaccine against Yellow fever virus (YFV), which is the “gold standard” of successful vaccines and has severally been declared as the most efficient live attenuated vaccines ever generated to date (Fernandez-Garcia et al., 2016)

1.2.1 Flavivirus life cycle (Fig. 3)
Most of the flavivirus life cycle is largely inferred to from collective studies done on multiple flaviviruses especially Dengue virus

1.2.1.1 Attachment and entry
Binding of the virus is mediated by the viral large surface E glycoprotein and putative receptors on target cells. Additionally, some attachment factors such as the heparin sulphate, a factor present in many cell types have been shown to be involved in the initial stages of virus attachment in several flaviviruses. TIM and TAM proteins are coreceptor families identified to function as entry factors in DENV (Meertens et al., 2012). Other attachment factors documented to be utilized by flaviviruses include the ER chaperone protein GRP78/BiP which facilitates entry of JEV (Nain et al., 2017), heat shock proteins 70 and 90, laminin, ICAM-3 and CD14 (Smit et al., 2011). The virus is then taken up by receptor-mediated endocytosis via clathrin-coated pits and then virions are trafficked to prelysosomal endocytic compartments of the host cell. The acidic pH in these vesicles induces a conformational change and reorganization of the viral envelope protein E from dimers, dissociation into monomers and then reformation into trimers (Allison et al., 1995). Some identified receptors and entry strategies of DENV, YFV, ZIKV and JEV have been reviewed by Laureti et al., 2018

While not much is known about the uncoating process, it is thought that the conformational change at this stage is key in the fusion of the viral proteins and endosomal membrane, a process that results in the uncoating and release of the viral nucleocapsid into the host cytoplasm (Heinz and Allison, 2003).
1.2.1.2 Translation and polyprotein processing

Translation is cap-dependent, occurs in the ER, is coupled with replication and initiates by ribosomal scanning. After uncoating, the positive, capped RNA viral genome (being an mRNA) allows for direct translation of the single open reading frame in the Endoplasmic reticulum to produce a single precursor polyprotein of approximately 3400 amino acids that is further co- and post-translationally processed by both virus-encoded NS2B/3 protease and host signalases into 3 structural (Capsid, Envelope and the pre-Membrane which matures into the Membrane) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Fig. 2).

The 5’ end contains the structural proteins followed by the non-structural proteins stretching out until the 3’ end. It has been previously suggested that translation competency may determine infectivity in flaviviruses and they therefore use several mechanisms to facilitate that with some specialized structures within the 5’ and 3’ non-coding regions, mutations the 3’ UTR in DENV2 has been shown to influence the RNA structure and changes the translation efficiency and the ultimate infectivity phenotype (Edgil et al., 2003)
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1.2.1.3 Replication

The process of replication occurs within invaginations of the ER membrane called replication vesicles. Replication is catalyzed by the virus-encoded RNA dependent RNA polymerase activity of NS5 and is typically benchmarked by the production and accumulation of double-stranded RNA (dsRNA) intermediates that are classical markers of flaviviral replication (Paul and Bartenschlager, 2015). The process begins with the production of first negative strand copies that are further used as templates in proceeding rounds of replication yielding new positive strands that serve as the genome packaged into newly formed viral particles but also for more rounds of replication. During polyprotein synthesis, the surface proteins prM and E are translocated into the ER lumen and released by host cell signalase (Mandl, 2005).

NS1 also translocates into the ER lumen once cleaved from the polypeptide and undergoes dimerization there, this dimeric form is ER-associated while the hexameric form, whose synthesis mechanism is unclear moves further along the
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Golgi secretory pathway and is the circulating form. (as reviewed by Yap et al., 2017)

1.2.1.4 Assembly and Budding

The process of replication is closely related and somewhat intertwined with that of virus assembly both spatially and temporally. Additionally, it has been suggested that only actively replicated RNA gets packaged and this plays a role in the infectivity of the virus (Khromykh et al., 2001) The RNA genome is packaged by the Capsid protein in nucleocapsids on the cytoplasmic side of the ER membrane, a process coordinated with the viral envelope assembly that is acquired by budding of the nucleocapsid into the ER. Nucleocapsids are then transported into the lumen of the endoplasmic reticulum at the replication vesicle pore during formation of the prM-E lipid envelope (Roby et al., 2012).

1.2.1.5 Maturation and virion release

Assembled, immature virions are trafficked to the Trans-Golgi Network (TGN) where they undergo reversible conformational changes due to the low pH rendering them accessible to furin, a cellular protease abundant in the TGN and responsible for the proteolytic cleavage of the prM and the ultimate dissociation of its pr peptide. This event is the hallmark of virion maturation and marker for infection-competent virions. Mature virions finally bud out of the cell by exocytosis (Yu et al., 2008).

Only properly cleaved progeny virions, with a nucleocapsid containing a full genome copy can successfully infect other cells in proceeding rounds of infection (Chambers et al., 2003)
1.2.2 Flavivirus structural Proteins

Throughout replication, post-translational control of flaviviral structural proteins is strictly controlled to regulate efficient virion assembly, secretion and infectivity. These proteins and some of their known functions are highlighted in Figure 2.

**Capsid protein**

This is a small (approximately 11kDa), highly basic, dimeric cytosolic flaviviral protein that is translated at the N-terminus of the polyprotein. It has four structurally conserved α-helices and an internal hydrophobic domain that presumably interacts with viral membranes while the other side interacts with the viral RNA. Maturation to a soluble protein happens after cleavage from the prM signal sequence by the viral encoded NS2B/3 protease (Lindenbach et al., 2007; Chambers et al., 2003)
**Introduction**

**prM glycoprotein**

This is an approximately 26kDa glycoprotein which is the precursor of the membrane protein. It folds quite rapidly and acts as the chaperone of the Envelope that is, it assists in the proper folding and secretion of the E protein. The N-terminal region of the prM contains one or three N-linked glycosylation sites and six conserved cysteine residues all of which are disulfide linked. The prM protein has been implicated together with several host factors in the flavivivirus life cycle specifically in virus entry, secretion and egress from the cell. The prM prevents the Envelope from undergoing rearrangements to a fusogenic form during transit in the secretory pathway and the dissociation of its pr peptide by furin yields mature virions (Duan *et al.*, 2008; Lindenbach *et al.*, 2007).

**Envelope glycoprotein**

The envelope (E) is a protein of approximately 53kDa and the major surface glycoprotein in flaviviruses. It contains 12 conserved cysteins forming disulphide bonds. The E protein is the viral hemagglutinin and induces a protective immune response and the domain III of this protein is the target of neutralizing antibodies.

Its major function in the flaviviral life cycle is mediating binding of the virus to the host receptors and the fusion of the virus onto the host cell membrane. The proper folding and secretion as well as its stability in low pH conditions depend on its co-expression with the prM protein (Heinz *et al.*, 1991; Lindenbach *et al.*, 2007).

**1.2.3 Flavivirus non-structural proteins**

**NS1 glycoprotein**

The flavivirus NS1 is a highly conserved glycoprotein, approximately 46 to 55kDa depending on its glycosylation status. It is localized in the ER lumen by a signal sequence located at the C-terminus of the E protein. This glycoprotein contains two or three N-linked glycosylation sites and 12 conserved cysteines that form disulfide bonds. NS1 is thought to be involved in the viral RNA replication.
process and the development of disease. NS1 is considered a major viral immunogen and found in the blood during early stages of infection as a viral marker of infection (Avirutnan et al., 2007; Lindenbach et al., 2007).

**NS2A**

This is an approximately 22kDa, hydrophobic membrane non-structural protein that is a product of the processing of the NS2 portion of the flaviviral polyprotein. The actual function of NS2A is not entirely known but has been suggested to function as a scaffold protein that organizes the replication complex (Barrows et al., 2018). It has been suggested that NS2A, alone or in complex with NS3, may be involved in genome transport (reviewed by Apte-Sengupta et al., 2014).

**NS2B**

This is a small membrane associated protein of approximately 14kDa. Its main known function is to serve as a cofactor of the viral protease NS3 forming the NS2B3 complex. This interaction between the two proteins is absolutely essential for optimal protease activity during infection. The cofactor activity of NS2B is encoded in a conserved hydrophilic region of 40 residues flanked by hydrophobic regions that mediate membrane association, suggesting a possible role of the protein in modulation of membrane permeability during infection (Lindenbach et al., 2007). NS2B of JEV and DENV have been shown to be associated with lipid rafts, a feature which makes them possess membrane-destabilizing properties (Cordero et al., 2014; Gopala Reddy et al., 2018). NS2B of JEV has been shown to contribute to replication and virion assembly through interaction with NS2A, and its transmembrane domains playing a fundamental role in both (Li et al., 2016).

**NS3**

This is a multifunctional, modular protein of approximately 70kDa involved in processing of the polyprotein and also in viral RNA replication. NS3 serves as the viral protease in the complex NS2B3 by processing the precursor polyprotein and therefore in the maturation of viral proteins. However, it harbors an NTPase-dependent RNA helicase activity in its C-terminus that functions may play a role.
in genome replication and vRNA synthesis. (Barrows et al., 2018; Li et al., 2014; Lindenbach et al., 2007).

**NS4A**

This is a small (≈16kDa), hydrophobic transmembrane protein and one of the products of the processing and cleavage of the NS4 portion of the polyprotein that yields NS4A and NS4B proteins. The two proteins are linked by a fragment of approximately 23 amino acids that form the 2K-linker peptide. NS4A has been suggested to be a major driver of ER rearrangements in DENV infection (Miller et al., 2007) where the replication factories are assembled, and has a role in replication (Lindenbach et al., 1991). NS4A may also help in the organization of luminal, transmembrane and cytoplasmic components of replication complexes (Barrows et al., 2018)

**NS4B**

This is a transmembrane protein of approximately 27kDa linked to NS4A by the 2K-linker peptide. The linker specifically leads NS4B into the ER membrane where it has been shown to localize at sites of replication as well as in the nucleus (Barrows et al., 2018). It has also been shown to interact with NS3 suggesting an interaction with the replication complexes, and probably its role in replication. NS4B has also been implicated in ER rearrangements (Kaufusi et al., 2014).

**NS5**

This is the largest (≈ 103kDa), most conserved multifunctional protein that is indispensable in viral replication. It contains at its C-terminal the RNA dependent RNA polymerase and a methyltransferase at its N-terminus that catalyzes the methylation in the genome capping process (Lindenbach et al., 2007). These processes are vital for viral genome replication (suggested to occur in complex with NS3 by Barrows et al., 2018) as well as viral genome stability and are therefore central to the regulation and coupling of RNA synthesis and virion morphogenesis (Murray et al., 2008)
**1.2.4 Virus–induced membrane rearrangements**

A vast majority of viruses from different families have been shown to induce extensive membrane rearrangements in the cells they infect and more specifically in the endoplasmic reticulum that has been described as a favorite niche for most viruses including flaviviruses as the site of replication and assembly of viral particles. This is of a particular interest due to different families of viruses converging at this particular organelle in the course of infection and even incorporating some of the ER intrinsic functions to exert a promotive role in almost every step of the life cycle (reviewed by Ravindran et al., 2016).

The morphological changes are nonetheless quite distinct among DNA and RNA viruses as well as among virus families (Fig 4). Studies done in flaviviruses have shown dramatic morphology changes of ER membranes in infected cells and more importantly the formation of ER-derived invaginations or vesicle packets or replication vesicles. This vesiculation of the ER lumen results in the membrane sheets become dilated giving rise to swollen ER sacs. The vesicles serve as physical support where the replication factories/complexes are anchored for the coordinated accumulation of viral and cellular components required for efficient replication. Moreover, membranes ensure minimal or no exposure of viral nucleic acids to the host immune system by shielding the viral genome from cellular pattern-recognition receptors and nucleases (Mackenzie et al., 1999, 2005; Miorin et al., 2012, 2013; Overby et al., 2010).

These invaginations of ER membranes retain an open connection to the surrounding cytoplasm via a pore that may be involved in the import of host factors and nucleotides required for efficient RNA replication and the export of the newly synthesized viral genomes (Welsch et al., 2009; Miorin et al., 2013). While the actual mechanism of membrane remodeling is unknown, it is thought to be a collective output of modulation of host lipid metabolism, influence of integral membrane proteins, activity of cytoskeleton components and microtubule motors, scaffolding by peripheral and integral membrane proteins (McMahon and Gallop, 2005). More importantly is the primal involvement of
some viral proteins such as the NS4A and NS4B proteins that have been implicated in remodeling of membranes, in DENV and the Kunjin strain of WNV infections (Miller et al., 2007; Kaufusi et al., 2014). NS4A and NS4B of DENV contain central helices assumed to lie in plane on the ER-luminal membrane leaflet, this arrangement is thought to locally increase the membrane surface area and induce invaginated vesicles. The oligomerization of NS4A and its interaction with NS4B may be the sustenance force to membrane rearrangements (Miller et al., 2006, 2007; Stern et al., 2013; Zou et al., 2015).

However, it has been suggested that additional viral proteins may also play a role in the rearrangements that are induced in the morphogenesis of replication factories as expression of these proteins alone did not resemble those observed in infected cells. One such protein may be NS1 that has been shown to dimerize and bind to and remodel membranes in vitro (Akey et al., 2014) and likely interacts with the NS4A-NS4B complex (Lindenbach and Rice, 1999; Youn et al., 2012). In addition, there is a possibility that NS2A might also be involved in the induction of flavivirus replication organelles (Chang et al., 1999; Xie et al., 2013; Kummerer and Rice, 2002).

ER invaginations result in vesicle packets, elongated vesicles or tubular structures that arise in the ER during DENV and TBEV infection of mammalian cells. The abundance of these structures differs between acute and persistent infections, with the latter showing more remodeling. These tubules have been suggested to be replication factories that are products of anomalous remodeling or represent an antiviral cellular defense mechanism restricting infection (Welsch et al., 2009; Offerdhl et al., 2012).
Figure 4: Different ER morphological changes owed to different families of DNA and RNA viruses (Romero-Brey and Bartenschlager, 2016) A) Membrane Inclusions; viral genome replication occurs in the cytoplasm in close-association with ER-derived membranes B) ER-derived invaginations/vesicles or spherules curving towards the ER lumen, what is referred to as negative curvature C) Single membrane tubules and Double Membrane Vesicles (DMVs); ‘exvaginations’ derived outwardly from the ER (positive curvature manner) D) Zippered ER; tethered spherules lacking luminal space due to zippering of ER cisternae E) ER-derived convoluted membranes, devoid of ribosomes, probably derived from smooth ER membranes F) Membranous inclusions; also called viroplasm, of cellular origin and closely surrounded by rough ER cisternae, both ER elements and virus particles are in contact with the cytosolic face of the plasma membrane that are part of the replication and assembly factory.
Figure 5. Representative images of membrane rearrangements induced by different members of the family Flaviviridae (Romero-brey and Bartenschlager, 2014). Slices through tomograms of cells infected with A) Dengue Virus (DENV); B) West Nile Virus (WNV); C) Tick-borne Encephalitis Virus (TBEV); D) Hepatitis C Virus (HCV) showing characteristic virus-induced structures.
1.3 Host innate sensing and resistance to flaviviral infection

1.3.1 Pattern Recognition Receptors
To establish a potent antiviral state upon infection, target cells possess germ-line encoded receptors that recognize distinct signatures from invading pathogens known as pathogen-associated molecular patterns (PAMPs), these are conserved molecular motifs unique to specific pathogens that are essential for survival, and cannot be altered by the pathogen (Nazmi et al., 2014), the PRR-PAMP interactions are therefore specific depending on PRR localization and PAMP specificity, this is also how the host cells that respond to viral invasion differentiate self from “non-self “molecules.

The identification of these signatures is the core event in the initiation of the antiviral signaling cascade which culminates in the production of numerous host cell defense molecules including type I and type III interferons that promote an antiviral state. These receptors are known as pattern recognition receptors (PRRs) and they are located on both the cytoplasm and the cell surface (Cumberworth et al., 2017). There are different classes of PRRs but the most important as far as flaviviral infections are concerned are the Toll-like receptors (TLRs) and the cytoplasmic sensors, retinoid acid- Inducible gene I (RIG-I) like receptors (RLRs) and Node-like receptors (NLRs) (Munoz - Jordan and Fredericksen, 2010; Takeuchi and Akira, 2010; Suthar et al., 2013).

1.3.2 Toll-like Receptors
These are membrane-bound sensors, a family of about 11-12 members that detect specific PAMPs. Specific in flavivirus infections, TLR7 and TLR8 identify single-stranded RNA (ssRNA) and TLR3 identifies double-stranded RNA (dsRNA) produced during viral infection. TLRs sense invading pathogens from both outside the cell and in intracellular endosomal compartments, they do so through their leucine-rich ectodomains that mediate PAMP recognition. Signaling
of all TLRs except TLR3 is mediated by the adaptor protein MyD88 whereas TLR3 does so through the adaptor protein TIR-domain containing adaptor Inducing Interferon β (TRIF) (Kawai and Akira, 2010). The recruitment of these intermediate proteins in the course of the cascade leads to activation of the NF-κB, MAPK, ERK, and JNK pathways. TLR3 through TRIF signals through the TRAF3 and RIP1 signaling pathways to activate the transcription factors IFN - regulatory factor, IRF3, NF-κB, and AP-1 to stimulate the IFN-I pathway (Akira et al., 2006).

1.3.3 RIG-I like receptors

The RLR family of receptors is made up of three members namely; retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and Laboratory of genetics and physiology 2 (LGP2; also known as DHX58). These sensors are localized in the cytoplasm and trigger antiviral responses including type I interferon (IFN) production and pro-inflammatory signaling following viral invasion (reviewed by Pichlmair and Caetano e Sousa, 2007, Rehwinkel and Caetano e Sousa, 2010). RIG-I and MDA5 contain a DExD/H box RNA helicase domain that hydrolize ATP and binds and possibly unwind the dsRNA in the cytoplasm, two caspase-recruitment domains (CARDs) important in signaling and carboxyl-terminal repressor domain, which in RIG-I is involved in autoregulation (Takeuchi and Akira, 2010; Kato and Fujita, 2015). LGP2 lacks the CARD domains and is thought to positively regulate RIG-I and MDAS (Satoh et al., 2010).

![Figure 6: RIG-I like receptors RIG-I, MDA5 and LGP2 highlighting the distinctive features of each receptor](Kato and Fujita, 2015)
1.3.4 RIG-I signaling in flaviviral infection

The RIG-I cytosolic sensor specifically recognizes ssRNA molecules containing free 5’triphosphate (5’ppp) moieties (Cui et al., 2008; Hornung et al., 2006; Spiegel et al., 2005; Takahasi et al., 2008), 5’ diphosphate (5’pp) such as those found in genomes of some viruses (Pichlmair et al., 2006; Goubau et al., 2014). Reportedly, RIG-I has been triggered experimentally by a variety of double-stranded RNAs (dsRNA) in a length-dependent manner, regardless of the 5’ end modifications (Kato et al., 2008).

In non-infected cells where RIG-I is in a resting state, the CARD domains are folded over one another and the Repressor domain is folded over the helicase and RNA binding domains (Saito et al., 2007).

In the course of infection, when the pathogenic signature is recognized, RIG-I hydrolyzes ATP and undergoes a conformational change that triggers the interaction between the RNA binding domain with the pathogenic RNA. At the same time, the CARDs are released for interaction with the adaptor protein mitochondrial antiviral signaling (MAVS, also known as IPS-1, VISA, and Cardif), located in the mitochondrial-associated membrane (MAM) (Liu et al., 2012).

MAVS relays the signal to kinases such as TANK-binding kinase 1 (TBK1) and IκB kinase (IKKε), which phosphorylate interferon regulatory factors (IRF3, IRF7) and nuclear factor-kappa beta (NF-κB), to initiate downstream signaling (Hou et al., 2011) through the translocation to the nucleus to evoke the transcription and secretion of Type I interferon, pro-inflammatory cytokines and the establishment of an antiviral state within the cell.

1.3.5 Type I IFN signaling

The cell intrinsic immunity against cellular insults including viral invasion is a conserved entity in nucleated vertebrate cells. It is primarily mediated through a signaling cascade that culminates in the production of IFN, virus-induced cytokines that are produced by both immune and non-immune cells (de Weerd et al., 2013; Chen K. et al., 2017). The first part of this cascade involves the PRR-PAMP interaction that has been previously explained (Fig. 6A), while the
secondary signaling involves the use of the secreted interferon molecules that
bind to specific receptors on target cells (Fig. 6B).

The initial production of interferon serves as a “warning/alarm signal” of
infection but the secondary signaling is what is indispensable in the antiviral
activity because, IFNs themselves are not antiviral effectors. Rather, they are
secreted by virally infected cells and act in an autocrine and paracrine
amplification loop, binding to IFN receptors that signal to induce interferon-
stimulated genes (ISGs) (Caetano Reis e Sousa, 2017).

The type one interferons IFNα and IFNβ bind to an ubiquitously expressed
receptor(s) known as IFNAR1/2 which associates with Janus kinase 1(JAK1) and
Tyrosine kinase 2(TYK2) kinases which phosphorylate Signal transducer and
activators of transcription 1 and 2(STAT1 and STAT2) transducer proteins which
then associate with IRF9 to form the ISGF3 complex that culminates in the
expression of ISGs, which are the actual antiviral effectors (Stark and Darnell, Jr.,
2012; Ivashkiv and Donlin., 2014). ISGs encode a variety of effector proteins that
restrict virus propagation by shutting down cell translation, cleaving cellular and
viral RNAs and blocking virion replication, assembly and/or release (Caetano Reis
e Sousa, 2017). In addition to antiviral functions, ISGs play a role in the
modulation of the IFN (Kumar et al., 2003). Several ISGs desensitize cells to IFN
stimulation, and the ability of the IFN subtypes to signal can be differentially
affected.

The type I and type III interferons are both known as the antiviral cytokines but
the type III interferons signal through a heterodimeric receptor complex IFNLR1
and is largely restricted to epithelial cells. Even though they bind different
receptors, both type I and type III interferons regulate similar sets of genes and
display similar activities (Sommereyns et al., 2008)
**Figure 7**: The IFN pathway of cell intrinsic antiviral Immunity (Caetano Reis e Sousa, 2017). Depiction of A) primary and B) secondary type I interferon signaling highlighting the various viral PAMPs, their respective PRRs/sensors (e.g. RIGI, MDA5), adaptor proteins (e.g. MAVS) and several kinases (e.g. IKKe, TBK1) that phosphorylate regulatory proteins (e.g. IRF3 in the case of TBEV), the translocation of which culminates in the secretion of type I IFN genes that facilitate the secondary cascade which involves the IFNAR receptors which bind the IFN molecules, followed by the phosphorylation of STAT transducer proteins and nucleus translocation. These events culminate in the production of ISGs in the nucleus which are in essence the actual antiviral effectors.
1.3.5.1 Interferon Stimulated genes

Following initial discoveries of IFN and its role in cell-intrinsic antiviral immunity, the role of ISGs became more apparent with their function as the actual effectors of antiviral signaling. Simply defined, an Interferon-Stimulated Gene is any gene that is induced during the IFN response. However, some of these genes are direct targets of Interferon regulatory factors (IRFs), NF-κB and can thus be induced even in the absence of IFN signaling. Additionally, some ISGs exist basally but are also IFN-inducible. Factors such as these make the classification of ISGs not as simplistic as one would hope (as reviewed by Schoggins, 2019). Other ISGs are themselves IFN signaling promoters while others are negative regulators targeting PRRs and IRFs. A set of ISGs have also been characterized to be pro-viral in some infections (Fig. 8, Chen et al., 2010, Randall et al., 2006).

Initially, ISGs were identified by the approach of associating their expression with virus suppression during infections and functional testing usually followed this. Ectopic ISG expression as a gain-of-function approach has also been used. However, more recently there has been large-scale screening of ISGs credit to development of techniques such as RNA-Seq, CHIP-Seq and Microarray technologies as well as the revolutionary gene editing technology, CRISPR-Cas9.

Some of the mostly characterized antiviral ISGs include those of the Interferon-induced proteins with tetratricopeptide repeats (IFIT) family, RSAD2 commonly known as Viperin, Interferon-inducible transmembrane (IFITM) family and the IFI-6-16 family. Some analyses have also been done to identify further the steps in the virus life cycle that these ISGs target as well as their mechanism of action (as reviewed by Schoggins, 2019).
Figure 8: Interferon Stimulated Genes. A simplified depiction of the primary and secondary IFN signaling (left panel, Tanguy et al., 2017) and a general categorization of ISGs (right panel). Most characterized ISGs are antiviral (red blocked lines), some IFNs are induced independently of IFN (Thin blue line), some are themselves IFN signaling promoters (dotted red lines), Others are negative regulators targeting PRRs, IRFs (thin, red blocked lines) and some are proviral (green lines) (Schoggons’ lab, UT SouthWestern web page)

1.3.6 Virus innate immunity evasion strategies

To ensure productive, potent infection cycles, viruses have evolved some strategies through which they use to evade the host cell innate immunity. They do so by hijacking some of the intrinsic cellular functions and using them to their advantages and/or using their proteins to inhibit different parts of the innate immune signaling cascade thereby defeating the host cell in eliciting an antiviral response.

Flaviviruses are no exception and some members have been shown to successfully subvert the innate antiviral signaling and promoting their replication. As described earlier, some flaviviruses such as TBEV, DENV and WNV replicate in modified ER membranes, which not only serve as replication sites but a way to evade PRR-sensing by delaying the host cell immunity (Frederickson and Gale, 2006; Mackenzie et al., 1999, 2005; Miorin et al., 2012, 2013) which offers a replication advantage during early stages of replication.

DENV suppresses the IFN signaling cascade by blocking TYK phosphorylation, impairing STAT1 phosphorylation, and it targets STAT2 by its NSS protein for proteosomal degradation (Ashour et al., 2009; Mazzon et al., 2009; Green et al., 2014). NS2A of DENV has also been shown to be important in the RNA synthesis and plays a role in the viral evasion of innate immunity by inhibiting the RIG-I/MAVS signaling by inhibiting TBKI/IRF3 phosphorylation in DENV and HCV
**Introduction**

infections (Kaukinen et al., 2013; Dalrymple et al., 2015) and the NS1 of WNV has been shown to target RIG-I and MDA5 to antagonize IFNβ production (Liu et al., 2004, 2005; Zhang et al., 2017). Additionally, the NS2B3 complex of DENV2 has been shown to target the human cGAS-STING pathway and thus impeding type 1 interferon signaling (Aguirre et al., 2012, 2017) same as the NS2B3 of WNV subtypes NY99 and KUN that prevent the translocation of STAT proteins to the nucleus in response to IFN-α treatment (Liu et al., 2005). The roles of NS5 proteins of different flaviviruses in the antagonism of type I Interferon signaling have been reviewed by Best, 2017.

Even when the primary IFN signaling has been activated, some viruses are able to inhibit some ISG effectors from eliciting their antiviral activity for example the methylation and capping of WNV RNA, there may be some modulation of the activity of the ISG IFIT1 (Daffis et al., 2010; Szretter et al., 2012)

DENV and JEV block caspase-dependent apoptotic cell death by activating PI3K signaling at early stages of infection, which initiates survival signaling to maintain the cells in a favorable condition for sustained virus production (Lee et al., 2005)

1.4 ER stress and the Unfolded protein response (Fig. 9)

1.4.1 ER stress

The ER is the designated cellular organelle for protein folding and maturation of nascent secretory and transmembrane proteins. To ensure that these functions are carried out effectively, the ER lumen maintains a homeostasis in balancing the protein load entering the ER and its capacity to handle this load.

In case of ER perturbations by physiological and pathological insults such as high protein demand, viral infections, environmental toxins, inflammatory cytokines, and mutant protein expression resulting to an accumulation of misfolded and unfolded proteins in the ER lumen, ER stress occurs (Oslowski and Urano, 2011). The chronic induction of ER stress by accumulation of misfolded and unfolded proteins without the activation of the proceeding remedial pathway is characteristic of some disease pathogeneses (as reviewed by Lin et al., 2008)
1.4.2 Unfolded Protein response

The unfolded protein response (Figs. 9, 10) is a cellular response to ER stress, a remedial process to restore protein synthesis regulation that is highly conserved in vertebrates (Schroder and Kaufmann, 2005).

The activation of UPR is mediated by three transmembrane proteins Inositol Requiring kinase I (IRE1), Activating transcription factor 6 (ATF6) and PKR-like endoplasmic reticulum kinase (PERK) that are held in place by the chaperone protein BiP/HSPA5/GRP78 in their inactive form. Upon ER perturbation and the induction of ER stress, BiP dissociates from these proteins rendering them active (Lee, 2005).

The UPR involves up-regulation of chaperone proteins such as BiP, GRP94 and PDI to promote protein folding, translational attenuation to reduce the load of proteins within the ER, preventing further accumulation of misfolded proteins, and up-regulation of ER-associated protein degradation (ERAD) as well as autophagy to promote degradation of misfolded proteins (Zhu and Lee, 2015). In circumstances of prolonged ER stress, however, the UPR becomes pro-apoptotic and triggers cell death (Perri et al., 2015).

The unfolded protein response as the name suggests is almost always assumed to be in response to the presence of unfolded proteins that create ER stress. However, it has been shown in yeast to also be due to changes in the ER lipid composition, in essence a more specific stress on the membranes with little or no involvement of unfolded proteins. This was performed in IRE1 mutants that were deficient in their ability to bind unfolded proteins. Other forms of stress stimuli culminating in membrane and/or lipid composition changes may be detected by the UPR sensors (in this case IRE1) in a different manner (Promlek et al., 2011). The mechanism that IRE1 employs has been further expounded on that the sensing mechanism relies on a juxta-membrane amphipathic helix that causes local membrane compression and acyl chain disordering. This way, the amphipathic helix can sense aberrant physical membrane properties. IRE1 is therefore able to combine both stress due to unfolded proteins as well as lipid
bilayer stress (Halbleib et al., 2017). In mouse cells, both IRE1α and PERK can be activated by luminal-independent signals in cells with enhanced lipid saturation, this is to say lipid perturbation may also be such a stimulus that can invoke UPR in the absence of unfolded proteins Volmer et al., 2013.

The plasticity nature of the ER allows for its dilation in conditions of stress. This is true for both ER stress due to external stimuli but also due to different cellular requirements such as B-cell differentiation that is typically accompanied by expansion of the ER (van Anken et al., 2003).

ER expansion/dilation can be measured by estimating the amount of phospholipid biosynthesis that occurs during UPR in response to ER stress. This is done by labelling cells with fluorescent dyes that can bind to lipid droplets and then analysed by flow cytometry and/or fluorescent microscopy (Yu et al., 2006).

More elaborate morphological changes occur during viral infection as a result of emergence of replication vesicles where viruses exploit the ER during the different steps of their life cycle. The morphology of these vesicles is due to changes in ER shaping proteins such as a family of Rab GTPases (Mateus et al., 2018) and tubule formation proteins such as the Reticulon family of proteins. ER membrane rearrangements appear to be specific among viruses as discussed in section 1.2.4 and summarised in Figure 4. ER dilation and/or remodelling in this context, i.e. during virus infection can be visualized by electron microscopy which has been extensively used to study replication vesicles in multiple viruses (Oslowski et al., 2011, Romero-Brey and Bartenschlager, 2016).

1.4.2.1 ATF6

ATF6 is a type II transmembrane protein with two isoforms ATF6α and ATF6β. ATF6 has its carboxyl terminus on the luminal side and a basic leucine zipper (bZIP) transcription factor in its amino terminus (Yoshida et al., 1998; Haze et al., 1999). In response to ER stress, ATF6 is trafficked to the Golgi apparatus, where it undergoes regulated intramembrane proteolysis by sequential cleavage by site 1 and site 2 proteases (S1P and S2P, respectively) (Hetz et al., 2011). This releases a 50kDa fragment that translocates to the nucleus and there it functions
as a transcriptional activator that upregulates many UPR genes related to protein folding. (Haze et al., 1999; Lee et al., 2002; Yamamoto et al., 2007).

1.4.2.2 IREI
IREI is an approximately 110kDa type I transmembrane protein with a serine-threonine kinase domain and a RNase domain. There exist two isoforms of the protein: IREIα and IREIβ but the earlier is the ubiquitously expressed form. Upon activation, IRE I oligomerizes and subsequently allowing transphosphorylation of juxtaposed kinase domains. This activates the RNase domain, which cleaves XBP1 mRNA (encoding ‘X-box-binding protein 1’, a transcription factor of the bZIP family) at two discrete stem-loop structures through an unusual cytoplasmic splicing reaction. The resulting fragments are ligated by an RNA ligase, yielding the active transcription factor XBP1s (Cox et al., 1996; Yoshida et al., 2001). XBP1s translocates to the nucleus and induces the expression of chaperones, molecules involved in lipid biosynthesis and ERAD.
IREI is the most conserved arm of the UPR and appears to respond to a vast majority of UPR inducers, possibly an evolutionary consequence as a sole ER stress sensor (DuRose et al., 2006)

1.4.2.3 PERK
PERK is an ER-localized type I transmembrane protein. When UPR is activated, oligomerization of PERK in the ER membranes occurs inducing its trans-autophosphorylation and kinase domain activation (He, 2006; Kim et al., 2008). PERK further phosphorylates the α subunit of eukaryotic translation initiation factor 2(εIF2α) on Ser51, resulting in the shutdown of mRNA translation and therefore reducing the load of newly synthesized proteins entering the ER lumen (Hetz et al., 2006). An exceptional case to this general response is that certain mRNAs gain a selective advantage for translation under conditions in which εIF2α is phosphorylated (Lu et al., 2004), most common is the transcription factor ATF4 which activates the transcription of prosurvival genes as well as pro-apoptotic genes such as CHOP. Additionally, ATF4 is a co-factor to GADD34,
whose role is to dephosphorylate eIF2α reversing protein synthesis attenuation with a negative feedback mechanism (Novoa et al. 2001).

**Figure 9: Unfolded Protein Response** (Janssens et al., 2014)

### 1.4.3 Chemical vs Physiological Induction of UPR (Fig. 10)

There are several pharmaceutical UPR inducers with different modes of action available and routinely used in cell culture systems to induce ER stress and activate the UPR. These include Tunicamycin, Thapsigargin, Brefeldin A, dithiothreitol (DTT), and MG132. Oxidative cellular stress associated with changes in the translocon complex results to a global protein translation halt, a typical event in UPR induction through the PERK pathway. Compounds such as puromycin and aminoacid analogues lead to the aggregation of misfolded polypeptides in the ER, eliciting stress and ultimately induce UPR (Hightower, 1980., Hammadi et al., 2013, Druelle et al., 2016, Schwenzer et al., 2019).

There are also some physiological UPR induction methods that have been reported to induce mild ER stress such as the deprivation of glucose to cells. The concentrations used and duration of induction are predetermined based on cell systems used and generally, the kind of output expected from an experiment (Oslowski and Urano, 2011; Bergman et al., 2018). ER stress and UPR induction
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by either chemical or physiological inducers differs from cell to cell and activate the UPR in a specific manner. However, while these agents act in different ways, they all converge in their ability to induce an accumulation of unfolded glycoproteins in the ER, activating ER stress and ultimately UPR.

Figure 10: ER stress and Unfolded Protein Response induction (So, 2018)

Other methods of physiological induction of ER stress and UPR involve different strategies that amount to the accumulation of misfolded or unfolded proteins in the ER and by doing so achieving a more organelle-specific induction (protein overload which in theory is confined to the lumen of the ER) as opposed to general perturbation of cellular homeostasis. The strategies investigated in this study are reported herein.

siRNA interference of BiP

Apart from being a major chaperone in the ER, BiP binds the three UPR transducers in non-stressed cells and dissociates from them under conditions of ER stress and in doing so, activates the UPR. In line with this and evidence from
previous reports in HEK293 and HeLa cells (Suzuki et al., 2007), the depletion of BiP by siRNA transient knockdown has been shown to induce ER stress and activating UPR.

**Overexpression of BiP ATPase mutants**

BiP (also known as HSPA5, GRP78) is a chaperone protein in the ER with numerous functions including folding of nascent proteins, targeting misfolded proteins for degradation (ERAD) and regulating the UPR. BiP has two major domains; the N-terminal domain contains an ATP catalytic site and the C-terminal domain contains the substrate-binding site. The two domains communicate by binding and releasing ATP and unfolded peptides respectively to regulate activity and most of the activities of BiP are dependent on its ATPase activity for proper function (Mayer et al., 2005)

BiP ATPase mutants have amino acid substitutions within the nuclear binding domain, which renders the protein unable to carry out its activities. Some of these mutants include G227D, a non-ATP binding mutant and T37G, an ATP-conformational change induction mutant (Hendershot et al., 1996). The overexpression of the ATPase mutants will impair the folding capacity of the ER, thereby inducing ER stress and UPR.

**Overexpression of ectopic proteins such as α1-antitrypsin mutants**

The alpha 1 anti-trypsin (α1AT) is a member of the serpin family of protease inhibitors that inhibit proteases and protect tissues from damage (Fregonese et al., 2008).

α1AT mutants may induce either an overload of proteins in the ER (polymer aggregation) or accumulation of misfolded proteins. A common and widely studied mutant of α1AT is the null Hong Kong (NHK) mutant, a truncated variant that does not fold properly. While the wild type (α1AT) is completely secreted, only a fraction of the NHK mutant can be secreted thus inducing ER stress and activate the UPR (Ordóñez, et al., 2013; Ferris et al., 2013).
1.4.3.1 Cancer as a physiological ER stress inducer

As shown in figure 10, physiological stressors include cancers and viral infections. In general, the elicitation of UPR induction relies on the sustenance of the inducing stimuli in exerting ER stress. Cancer-induced ER stress is implicated in the initiation of transformation, unrestricted cell division, invasion and spread of tumorigenic cells. In most cancers, ER stress is accompanied by hypoxia and nutrient deprivation especially in the early stages of tumorigenesis (Chipurupalli et al., 2019; Madden et al., 2019). Transformed cells take advantage of the pro-survival mechanisms of UPR to sustain growth and proliferation of tumorigenic cells and meeting the vast protein folding demands accompanied by the progression of cancer. However, unlike in viral infections (next section, 1.4.3.2) where ER stress can be a direct result of accumulation of viral proteins as well as those of the host, the process of tumorigenesis ultimately results to a protein load, almost always solely belonging to the host cells. And depending on the circumstances and cell’s condition, UPR activation may also help the resistance to treatment and production of clones less sensitive to chemotherapy. In most cases, UPR activation is a vital step for oncogenic transformation, as UPR signaling molecules interact with well-established oncogene and tumor suppressor gene networks to modulate their function during cancer development (Walczak et al., 2019).

1.4.3.2 Virus-induced ER stress and UPR

As previously discussed, virus replication and/or assembly of newly synthesized viral progeny for most flaviviruses occurs in the ER. This implies a large accumulation of viral proteins within the ER resulting into a protein overload in the ER lumen during infection, the essence of ER stress and subsequently UPR activation. Several studies have suggested a connection between UPR activation and either the process of viral replication (herpes simplex virus (HSV) 1, JEV, and HCV (Su et al., 2002; Cheng et al., 2005; Tardif et al., 2005) or the production of a specific viral protein in the ER (Zhang and Wang, 2012). For example, the ectopic
expression of the E2 protein, but not E1, core and NS3 proteins of Hepatitis C virus (HCV), has been shown to activate the expression of BiP (Liberman *et al.*, 1999). HCV replicons expressing only non-structural proteins are also capable of stimulating BiP expression (Tardif *et al.*, 2002).

Like innate signaling, viruses have evolved to use the UPR signaling to their advantage throughout the life cycle as reviewed by Ambrose and Mackenzie, 2013b and Green *et al.*, 2013, suggesting in some instances a pro-viral function. This selective manipulation of the UPR is also different when the infection is acute or chronic.

Any viral infection whether acute or chronic is an arms race between the host cell and the virus with the host attempting to eradicate the virus and the virus trying to take advantage of the various responses including the UPR, manipulating them to its advantage.

While pro-survival is a common goal in any virus infection, as far as the UPR is concerned, the major difference is that chronic infections are skewed in that the virus targets some parts of the stress response with an aim of promoting persistence and/or latency. Indeed, such is the case in Hepatitis C which activates all three arms of the UPR but after this initial upregulation and peaking, there follows only milder stress that is long-lived. This is made possible by increased eIF2α phosphorylation alleviating the protein translation halt thus supporting the HCV chronic infection and later with diminished CHOP levels avoiding immediate cell death (Mercuiol *et al.*, 2011; reviewed by Chan *et al.*, 2014). HCMV is the largest human herpesvirus encoding at least 200 proteins. Its replication is relatively slow and due to its size, it is understandably stressful for the cell and particularly the ER to endure the slow replication. For this reason, HCMV has evolved to mitigate this by an upregulation of BiP throughout its cycle. BiP helps to control the prevailing UPR due to infection by protein folding, and has been shown to play some roles in virus assembly and egress (Buchkovich *et al.*, 2010). In other cases, viruses may decide to only utilize certain arms of UPR and inhibit others such as HSV that strongly activates GADD34 to relieve the protein...
translation inhibition while WNV activates only ATF6 and XBP1 but not PERK so as to bypass the translation halt. Different viruses and how they may manipulate the UPR to their advantage have been reviewed by Dash et al., 2016.

1.4.4 The UPR and antiviral immunity
The role of UPR in the remedial of ER stress has increasingly been associated with modulation of immunity and inflammation activities revealing more about functions other than ER stress management. The UPR has also now been recognized for its role in immune cell differentiation and function, and in regulating immune and inflammatory responses, including those associated with infections, tumors and autoimmune responses (reviewed by Grootjans et al., 2016).

When cells undergoing an acute UPR upon treatment with Thapsigargin were treated/transfected with TLR and MDA5 agonists, there was an augmentation in TLR-induced cytokines and IFNβ production, a log-fold or more over the PRR agonist alone. In dendritic cells, this synergistic effect was shown to be dependent on the transcription of XBP1 (Smith et al., 2008; Hu et al., 2011). This synergistic effect was not only observed upon pre-treatment with pharmacologic agents, cells expressing the misfolding α1AT mutant responded to LPS with greater cytokine production (Caroll et al., 2010). This synergistic effect has been observed in multiple culture cell types, human and mouse macrophages as well as dendritic cells as shown.

There is also evidence that UPR sensing of Influenza hemaglutinin (HA) triggers a direct anti-IAV response by targeting HA degradation via the ER associated degradation pathway (ERAD). The host cells detect HA as a misfolded or “nonself” protein and ER mannosidases target HA to ERAD for degradation and limit IAV replication (Frabutt et al., 2018).

There is increasing data showing that the UPR may be a danger signal that synergizes with PRR-sensing to elicit an antiviral state or may do so via activation of intrinsic pathways such as ERAD to limit infection (as reviewed by Janssens et al., 2014; So, 2018).
1.4.5 ER stress, UPR and interplay with autophagy

While the last resort for cells undergoing prolonged ER stress is cell death, autophagy can help cells to cope with mild ER stress by its contribution to elimination of misfolded and unfolded proteins suggesting a certain amount of cross-talk between them. Additionally, certain stimuli such as nutrient starvation that have been documented to induce mild ER stress can also induce autophagy. The interplay between the two has mostly been described in some specific disease conditions such as kidney and neurodegenerative diseases. Some autophagy genes have been shown to be activated after activation of ATF4, a PERK-mediated factor during the unfolded protein response. Both IRE1 and ATF6 have also at one time or another been implicated in the modulation of the cellular autophagy process (Cai et al., 2016; Cybulsky, 2017; Hosoi et al., 2017).

More generally though, in conditions where this interplay exists, UPR elicited by ER stress stimulates autophagy and the three UPR sensors may distinctively modulate certain points of the autophagy process. PERK- dependent eIF2α phosphorylation has been shown to contribute in the activation of autophagy in response to the accumulation of unfolded proteins. Activation of IRE1 has also been shown to regulate autophagy when cells were treated with ER stress inducers TM or TG or proteasome inhibitors led to an IRE1-dependent activation of autophagy which relies on the interaction of IRE1 with TRAF2 and JNK activation. These and other distinct ways on how individual arms of UPR converge into the autophagy pathway are comprehensively reviewed by Verfaillie et al., 2009; Senft & Ronai, 2015; Kabir et al., 2018.
1.5 Aim of thesis

My thesis stemmed from previous observations pertaining to the role of UPR in the elicitation of a robust antiviral state during flavivirus infections.

- As a prerequisite, my first task was to analyze the effects of UPR inducers TM and TG on cell growth and viability in our current experimental settings.

- Additional to this, my central objective was rooted into the search for alternative UPR inducers, an attempt to induce UPR in a more specific manner targeting the endoplasmic reticulum, which is the site of replication for many flaviviruses but the center for protein folding and therefore central in ER stress and UPR signaling.

- Upon success, to evaluate this alternative of UPR induction in the context of antiviral signaling during TBEV infection as I also analyzed the same with the inducer Tunicamycin.
2. MATERIALS AND METHODS
2.1 Materials

2.1.1 Cells

Human cells

- U2OS: Human osteosarcoma cell line (ECACC No. 92022711)
- Vero: African green monkey kidney (ECACC No. 84113001)
- HEK 293T: Human embryonic kidney cell line with the SV40 T-antigen

Bacteria cells

- **MAX Efficiency DH10B Competent Cells** (Invitrogen – cat. no. 18297-010).
  Genotype: F- mcrA Δ (mrr-hsdRMS-mcrBC) φ 80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ (ara, leu)7697 galU galK λ -rpsL nupG
- **MAX Efficiency DH5α Competent Cells** (Invitrogen – cat. no. 18258012)
  Genotype: F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ thi1 gyrA96 relA1

2.1.2 Media

Human cells

- Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) Gibco – cat. no. 31885-023 (Life Technologies) supplemented with 10% fetal bovine serum (FBS; REF 10270-106, EU approved, Brazil, Life Technologies), 0.1 U/ml penicillin, and 0.1μg/ml streptomycin at 37°C in 5% CO₂.
- Reduced serum medium (Opti-MEM) was used during transfection. The media and serum were purchased from Gibco – cat. no. 31985-070.
- Cell freezing medium was made by 90% FBS and 10% DMSO (Sigma-Aldrich cat no. 472301-500ML) for long-term storage.
**Materials and Methods**

**Bacteria cells**

- Luria-Bertani (LB) Medium: 10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl per 1 liter medium. Ampicillin or Kanamycin was added at a concentration of 100μg/ml and 50μg/ml respectively. For hardening 1.5% agar-agar was added to the liquid medium.

- SOC Medium: Super Optimal Broth (SOB) medium (20g bactotrypton, 5g bacto-yeast extract, 0.5g NaCl per 1L medium) was enriched with 20mM glucose.

**2.1.3 Antibodies**

**2.1.3.1 Primary Antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4G2</td>
<td>Mouse</td>
<td>1:20000 WB</td>
<td>Kindly provided by Dr. Vivian Huerta Center for Genetic Engineering and Biotechnology (CIGB), Cuba</td>
</tr>
</tbody>
</table>
| FLAG®M2  | Mouse   | • 1: 1000 WB  
          |          | • 1: 5000 FC       | Sigma-Aldrich - cat. no. F1804                                                      |
| SV5      | Mouse   | • 1: 10000 WB 
          |          | • 1: 5000 FC        | Kindly provided by Oscar Burrone; Laboratory of Molecular Immunology (ICGEB)       |
| BiP      | Mouse   | 1: 1000 WB     | • BD Biosciences - cat. no. 610979                                               
|          |         |                | • Kindly provided by Oscar Burrone; Laboratory of Molecular Immunology (ICGEB)   |

**Table 1:** Primary antibodies used in this study (WB= Western Blot, FC=Flow Cytometry)
2.1.3.2 Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse AlexaFluor 488</td>
<td>Donkey</td>
<td>1:500 FC</td>
<td>Molecular probes (Invitrogen cat.no. A32723)</td>
</tr>
<tr>
<td>Anti-mouse/HRP</td>
<td>Rabbit</td>
<td>1:10000 WB</td>
<td>DakoCytomation (cat. no. P0447)</td>
</tr>
<tr>
<td>Anti-rabbit/HRP</td>
<td>Goat</td>
<td>1:10000 WB</td>
<td>DakoCytomation (cat. no. P0449)</td>
</tr>
<tr>
<td>Human β-Actin Peroxidase</td>
<td>Mouse</td>
<td>1:50000 WB</td>
<td>Sigma-Aldrich (cat.no. A3854)</td>
</tr>
</tbody>
</table>

Table 2: Secondary antibodies used in this study (WB= Western Blot, FC=Flow Cytometry)

2.1.4 siRNAs

Pools of small interfering RNAs (siRNAs) against BiP (ON-TARGET plus SMARTpool, 5nmol siRNA cat.no. L-008198-00-0005) and control pool ON-TARGETplus Non-targeting Pool, 5nmol siRNA cat.no. D001810-10-05) were purchased from Dharmacon and used according to manufacturer’s instructions.

2.1.5 Vectors

Plasmid analysis was performed on SerialCloner version 2-6-1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3 BIP (grp78) human</td>
<td>• Encodes Wild type BiP/grp78 • AMPR</td>
<td>• Kindly provided by Dr.Oscar Burrone; Laboratory of Molecular Immunology (ICGEB)</td>
</tr>
<tr>
<td>pcDNA3 BIP (grp78) human G227D</td>
<td>• Encodes a non-ATP binding BiP mutant • AMPR</td>
<td>• Sasset et al., 2015</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Vector/Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3 BIP (grp78) human T47G</td>
<td>Encodes an ATP-conformational change induction BiP mutant</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>pcDNA3-AAT-SV5-BAP</td>
<td>Encodes an SV5-tagged wild type α1AT</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>pcDNA3-AAT-D318-SV5-BAP</td>
<td>Encodes an SV5-tagged null Hong Kong mutant of α1AT</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>pEGFP-C1</td>
<td>Encodes the GFP mutant 1 variant</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt; BD Biosciences Clontech cat. no. 6084-1 Cormack et al., 1996</td>
</tr>
<tr>
<td>pcDNA 3.1(+)</td>
<td>Empty vector</td>
<td>Invitrogen-cat.no. V79020</td>
</tr>
<tr>
<td>pl.18 TBE Hy C 3xFlag</td>
<td>Encodes Flag-tagged TBE Capsid protein</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt; Kindly provided by Dr. Anna K. Överby; MIMS, Umeå Universitet, Sweden Överby et al., 2010 Tarpey and Greenwood, 2001.</td>
</tr>
<tr>
<td>pl.18 TBE Hy prM 3xFlag</td>
<td>Encodes Flag-tagged TBE pre-Membrane protein</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>pl.18 TBE Hy E 3xFlag</td>
<td>Encodes Flag-tagged TBE E protein</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
<tr>
<td>pl.18 TBE Hy NS1 3xFlag</td>
<td>Encodes Flag-tagged TBE non-structural protein 1</td>
<td>AMP&lt;sup&gt;R&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pl.18 TBE Hy NS2A 3xFlag</td>
<td>Encodes Flag-tagged TBE non-structural protein 2A • AMP^R</td>
</tr>
<tr>
<td>pl.18 TBE Hy NS2B 3xFlag</td>
<td>Encodes Flag-tagged TBE non-structural protein 2B • AMP^R</td>
</tr>
<tr>
<td>pl.18 TBE Hy NS3 3xFlag</td>
<td>Encodes Flag-tagged TBE non-structural protein 3 • AMP^R</td>
</tr>
<tr>
<td>pl.18 TBE Hy NS4A 3xFlag</td>
<td>Encodes Flag-tagged TBE non-structural protein 4A • AMP^R</td>
</tr>
<tr>
<td>pl.18 TBE Hy NS4B 3xFlag</td>
<td>Encodes Flag-tagged TBE non-structural protein 4B • AMP^R</td>
</tr>
<tr>
<td>pl.18 TBE Hy NS5 3xFlag</td>
<td>Encodes Flag-tagged TBE non-structural protein 5 • AMP^R</td>
</tr>
<tr>
<td>pGL3 IFN-β Luc</td>
<td>Encodes the Firefly Luciferase gene under control of the IFN-β promoter • AMP^R</td>
</tr>
<tr>
<td>pEF-BOS-FLAG-RIG-IN</td>
<td>Encodes Flag-tagged RIGI (active domain only, not full-length) • AMP^R</td>
</tr>
</tbody>
</table>

Table 3: Plasmids used in this study

#### 2.1.6 Primers

Primers were designed using the NCBI Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), following optimal parameters and selecting only the primers spanning exon–exon junctions to minimize genomic DNA amplification. All primers were synthesized by and purchase from Sigma-Aldrich or Integrated DNA Technologies (IDT).
### Materials and Methods

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Sequence: 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Actin Fw</strong></td>
<td>CAT GTG CAA GCC CGG CTT CG</td>
</tr>
<tr>
<td><strong>β-Actin Rv</strong></td>
<td>GAA GGT GTG GTG CCA GAT TT</td>
</tr>
<tr>
<td><strong>BiP Fw</strong></td>
<td>CCC GAG AAC ACG GTC TTT GA</td>
</tr>
<tr>
<td><strong>BiP Rv</strong></td>
<td>TCA ACC ACC TTG AAC GGC AA</td>
</tr>
<tr>
<td><strong>CHOP Fw</strong></td>
<td>TAA AGA TGA GCG GGT GGC AG</td>
</tr>
<tr>
<td><strong>CHOP Rv</strong></td>
<td>CTG CCA TCT CTG CAG TTG GA</td>
</tr>
<tr>
<td><strong>DNAJC3 Fw</strong></td>
<td>CGT TTG CGT TCA CAA GCA CT</td>
</tr>
<tr>
<td><strong>DNAJC3 Rv</strong></td>
<td>CCC GAA CTT CAC TGA GGG AC</td>
</tr>
<tr>
<td><strong>EDEM1 Fw</strong></td>
<td>AGG ACC AAG GGG GAA AGT CT</td>
</tr>
<tr>
<td><strong>EDEM1 Rv</strong></td>
<td>GTA CAC GAT TGC AGT TGG AGC</td>
</tr>
<tr>
<td><strong>GADD34 Fw</strong></td>
<td>CCC AGA AAC CCC TAC TCA TGA T</td>
</tr>
<tr>
<td><strong>GADD34 Rv</strong></td>
<td>CTC GGA GAA GCG CAC CTT T</td>
</tr>
<tr>
<td><strong>GAPDH Fw</strong></td>
<td>CAT GAG AAG TAT GAC AAC AGC</td>
</tr>
<tr>
<td><strong>GAPDH Rv</strong></td>
<td>AGT CCT TCC ACG ATA CCA AAG</td>
</tr>
<tr>
<td><strong>IFI44 Fw</strong></td>
<td>AGA CGA ATG CTA TGG GCT GC</td>
</tr>
<tr>
<td><strong>IFI44 Rv</strong></td>
<td>CCT CCC TTA GAT TCC CTA TTT GCT C</td>
</tr>
<tr>
<td><strong>IFI44L Fw</strong></td>
<td>TCA AAG CCG GGT CAT GAA TG</td>
</tr>
<tr>
<td><strong>IFI44L Rv</strong></td>
<td>CCT TCA TGG GGT CCA GTT CC</td>
</tr>
<tr>
<td><strong>IFIH1 Fw</strong></td>
<td>GAT TCA GGC ACC ATG GGA AGT</td>
</tr>
<tr>
<td><strong>IFIH1 Rv</strong></td>
<td>AGG CCT GAG CTG GAG TTC TG</td>
</tr>
<tr>
<td><strong>IFIT1 Fw</strong></td>
<td>GAA ATA TGA ATG AAG CCC TGG A</td>
</tr>
<tr>
<td><strong>IFIT1 Rv</strong></td>
<td>GAC CTT GTC TCA CAG AGT TCT CAA</td>
</tr>
<tr>
<td><strong>IFNβ Fw</strong></td>
<td>AGG ACA GGA TGA ACT TTG AC</td>
</tr>
<tr>
<td><strong>IFNβ Rv</strong></td>
<td>TGA TAG ACA TTA GCC AGG AG</td>
</tr>
<tr>
<td><strong>OASL Fw</strong></td>
<td>TAC CAG CAG TAT GTG AAA GCC A</td>
</tr>
<tr>
<td><strong>OASL Rv</strong></td>
<td>GGT GAA GCC TTC GTC CAA CA</td>
</tr>
<tr>
<td><strong>TBEV 5’ NCR Fw</strong></td>
<td>GCG TTT GCT TCG GA</td>
</tr>
<tr>
<td><strong>TBEV 5’ NCR Rv</strong></td>
<td>CTC TTT CGA CAC TCG TCG AGG</td>
</tr>
</tbody>
</table>
Materials and Methods

### Table 4: Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viperin Fw</td>
<td>CCC CAA CCA GCG TCA ACT AT</td>
</tr>
<tr>
<td>Viperin Rv</td>
<td>TTG ATC TTC TCC ATA CCA GCT TCC</td>
</tr>
<tr>
<td>XBP1s Fw</td>
<td>CTG AGT CGG CAG CAG GTG</td>
</tr>
<tr>
<td>XBP1s Rv</td>
<td>GGC TGG TAA GGA ACT GGG TC</td>
</tr>
<tr>
<td>XBP1u Fw</td>
<td>AGC CAA GGG GAA TGA AGT GAG G</td>
</tr>
</tbody>
</table>

#### 2.1.7 Solutions and Buffers

- **1X PBS (1 litre)**
  
  In 800 ml of distilled water, the following were added: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na$_2$HPO$_4$, 0.24 g of KH$_2$PO$_4$. pH adjusted to 7.4 and distilled water was added to bring solution to volume of 1 liter.

- **2X HBS buffer**
  
  42mM HEPES (Sigma-Aldrich cat. no. H4034-100G), 274mM NaCl, 1.5mM Na$_2$HPO$_4$.12H$_2$O, 15mM D-glucose and pH adjusted to 7.

- **5X TBE (1 litre)**
  
  54g Tris base (Invitrogen cat.no. 15504-020), 27.5g Boric acid (Sigma-Aldrich cat.no. 31146), 20ml 0.5M EDTA (Sigma-Aldrich cat.no. E5134-1KG) and distilled water to final volume of 1 litre.

- **3.7% Paraformaldehyde (PFA)**
  
  5ml of 37% PFA: 1.85g of PFA powder, reagent grade crystalline (Sigma-Aldrich cat. no. P6148-500G) dissolved in 5ml of distilled water, 10μl 10M KOH. 37% PFA was diluted in 2x PHEM buffer (18.14g PIPES (ChemCruz cat.no. sc-216099); 6.5g HEPES; 3.8g EGTA; 0.99g MgSO$_4$; pH adjusted to 7 with 10M KOH) to produce 3.7% PFA.

- **6% carboxymethyl cellulose (CMC)**
  
  500 ml; 30g CMC powder (Sigma-Aldrich cat. no. C5013-500G) dissolved in 500ml PBS

- **1% Crystal violet solution**
  
  1L; 10g Crystal violet powder (Sigma- Aldrich cat.no. C6158-50G), 200ml PBS and 800ml Methanol (Sigma-Aldrich cat.no. 32213-2.5L-M)
Materials and Methods

- **SDS electrophoresis buffer (10X)**
  
  30.2g Tris, 188g Glycine (Sigma-Aldrich 33226-1KG), 50ml 10% SDS (Sigma-Aldrich L5750-500G), add water to 1 litre

- **10X Transfer Buffer (1 litre)**
  
  30.3g Tris, 144g Glycine, add water to 1 liter
  
  - **1X Transfer buffer (1 litre):** 100ml 10X Transfer Buffer, 200ml Methanol and 700ml water.

- **10X TBS (1 litre)**
  
  60g Tris, 2g KCl, 80g NaCl, pH adjusted to 8.5 with 37% HCl, add water to 1L
  
  - **1X TBS-T (1 litre):** 100ml 10X TBS, 900 ml water, 1ml Tween-20 (Sigma-Aldrich P2287)

- **100mM PMSF**
  
  17.4 mg of PMSF (Sigma-Aldrich cat.no. P7626) in 1ml of isopropanol (Riedel-de Haën cat.no. 603-117-00-0)

- **EDTA and EGTA solutions**
  
  EDTA (Sigma-Aldrich cat.no. E5134-1KG) and EGTA (Sigma-Aldrich cat.no. E3889-100G) dissolved in H₂O.

- **Nonidet-P40 (NP-40)**
  
  20mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol (Sigma-Aldrich cat.no. 49770-1L), 1% nonidet P-40 (Calbiochem cat.no. 492015) and 2 mM EDTA

### 2.1.8 Size Markers and Dyes

- **PageRuler Plus Prestained Protein Ladder, Thermo Scientific cat.no. 26619**
- **100bp DNA Ladder (5u/μl), Promega cat.no. G2101**
- **1kb DNA ladder (100μg/ml), Promega cat.no. G571A**
  
  - DNA markers supplied with Blue/Orange 6X Loading dye, Promega cat.no. G190A
2.2 Methods

2.2.1 Cell culture

Cells were grown at 37°C, 5% CO₂ in DMEM complete medium. Cell passaging was done by treatment with 0.05% Trypsin – 0.02 % EDTA and seeded at appropriate dilutions. Cell culture was done in aseptic conditions, cells were routinely screened for Mycoplasma contamination and new cell stocks were revived at regular intervals.

2.2.2 Whole genome Transcription analysis

U2OS cells were seeded into 10cm plates, the next day, they were infected with TBEV at a MOI = 1, after one hour of infection with serum free medium, this medium was replaced with complete growth medium alone for the cells with just infection or complete medium plus TM in sterile treatment and infection and treatment with TM. RNA samples were then collected at 0, 10 and 24 hours post infection in EuroGOLD TriFast lysis buffer (EuroClone EMR 507100) and total RNA was extracted. Quality of extracted RNA was checked by spectrophotometric analysis (260/280>1.8) and Agilent bioanalyzer (RNA integrity number, RIN≥8). Our collaborator Danilo Licastro, CBM-Trieste performed further analyses and I report herein the protocol that was followed.

Briefly, cDNA libraries of polyA-containing mRNA molecules were prepared using Illumina TruSeq standard protocol. Libraries were pooled and sequenced on two different Illumina Platforms. The first run was performed on Hiseq2000 4-plex run single reads, 50 bp reads while the second run was performed on HiscanSQ 8-plex run pair-end reads, 2x100bp reads. All data were subjected to quality control using FastQC software.

Briefly, bioconductor packages DESeq2 version 1.4.5. and EdgeR version 3.6.2 in the framework of R software version 3.1.0 were used to perform differential gene expression analysis of RNA-seq data. Both packages are based on the negative binomial distribution (NB) to model the gene reads counts and shrinkage estimator to estimate the per-gene NB dispersion parameters.
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Specifically, rounded gene counts were used as input and the per-gene NB dispersion parameter was estimated using the function DESeq for DESeq2 while, for edgeR the function calcNormFactors with the default parameters was used. To detect outlier data after normalization, R packages Array Quality Metrix were used and before testing differential gene expression all genes with normalized counts below 14 were eliminated to improve testing power while maintaining type I error rates. Estimated p-values for each gene were adjusted using the Benjamini-Hochberg method. Genes with adjusted P<0.05 and absolute Logarithmic base 2-fold change > 1 were selected.

2.2.3 Ingenuity Pathway Analysis

Significantly changed genes (up-regulated, down-regulated, or both) were analyzed by Danilo Licastro using online bioinformatics tool Ingenuity Pathway Analysis (Qiagen). Direct relationships were included with experimentally observed or highly predicted confidence from human species. Canonical pathways, diseases and disorders, and molecular and cellular functions were analyzed.

2.2.4 Plasmid transformation

DH10β or DH5α competent cells were used for transformation of all plasmids in this study. Cells were incubated with the plasmids on ice for 30 min. They were then heat-shocked at 42°C for 40 seconds, left on ice for 2 minutes before addition of SOC medium. Cells were then incubated at 37°C for 1.5 hours before they were plated onto LB agar with the required antibiotic. They were grown overnight at 37°C. On the next day, selected colonies were picked and inoculated into 5 ml of LB medium containing the required antibiotic.

2.2.5 Plasmid DNA extraction

After approximately 12-15 hours, plasmid DNA was extracted using NucleoSpin Plasmid (Macherey-Nagel cat.no.1801/003) for minipreps and/or NucleoBond Xtra Midi (Macherey-Nagel cat. no. 1803/009) for midipreps. Extracted DNA was authenticated by restriction endonuclease digestion and sequencing.
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endonucleases and their specific buffers were purchased from New England Biolabs (NEB).

2.2.6 Plasmid transfection

- **Lipofectamine LTX transfection**

Plasmids were transfected into U2OS cells using Lipofectamine™ LTX Reagent with PLUS™ Reagent (Invitrogen cat.no. 15338-100) according to a standard, optimized protocol. Transfection was done using reduced serum medium (Opti-MEM) and further grown in DMEM supplemented with 10% FBS.

- **Calcium Phosphate transfection**

Transfections for luciferase reporter assay were performed in HEK293T cells using a standard, optimized protocol. Transfection was done in DMEM supplemented with 10% FBS and further incubated at 37°C, 5% CO₂ until sample collection.

2.2.7 siRNA transfection of BiP

U2OS cells were reverse transfected that is cells were seeded onto the transfection mix. siRNAs used were at a concentration of 20nM and transfection was performed using Lipofectamine RNAiMAX transfection Reagent (Invitrogen cat.no. 13778-075) according to the manufacturer’s instructions. Cells were then incubated at 37°C, 5% CO₂ in DMEM complete medium for 48 hours before infection with TBEV.

2.2.8 Flow Cytometry

Efficiency of transfection was checked for all experiments involving plasmid transfection. Cell monolayers were treated with 0.05 % Trypsin – 0.02 % EDTA to prepare single cells suspensions. Cells were then washed twice with PBS. For EGFP control, cells resuspended with 500μl PBS and immediately analyzed by flow cytometry.

Anti-SV5 and FLAG assays involved the fixation of cells with 3.7% paraformaldehyde (PFA) for 15 minutes at room temperature, followed by
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centrifugation at 1000 rpm for 5 minutes followed by two washes, the first with a buffer made up of a 0.1% Tween 20 in PBS solution and the second with 1X PBS, at 1000 rpm for 5 minutes per wash.

Permeabilization was done by 0.05% Saponin-0.1% BSA solution incubated for 15 minutes at room temperature followed by two washes as described above for fixation.

Primary and secondary antibodies (anti-SV5/anti-FLAG or Alexa Fluor 488) were diluted in 0.1% Tween 20 in PBS solution. Anti-SV5 was used at 1:5000, anti-FLAG at 1:50 and Alexa Fluor 488 at 1:500 dilutions. Cells were incubated with primary antibodies for 30 minutes at room temperature followed by two washes. The same conditions were used for secondary incubation but without exposure to light followed by two washes as described previously.

Cells were finally resuspended in 500μl PBS and analyzed on a FACS Calibur machine (Becton Dickinson) and the Cell Quest Pro software.

Appropriate controls were employed every time including untreated cells and those only treated with the secondary antibodies as well as empty vector controls for normalization.

2.2.9 Infection and Tunicamycin treatment of Cells

- Virus stocks

Neudoerfl strain of TBEV was used for all experiments reported herein.

Viral stocks were prepared by infection of Vero cells at multiplicity of infection of 0.1. After observation of cytopathic effects (CPE), the supernatant was collected, clarified by centrifugation, supplemented with 20 % FBS, and stored in aliquots at -80°C. Viral titres were determined by using a plaque-forming assay.

When performing infection experiments, U2OS cells were seeded in a 12 well plate at an appropriate confluence. 24 hours later, cells were infected at multiplicity of infection (MOI=1) by adding 400μl of virus stock properly diluted in serum-free medium.
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After 1 hour incubation at 37°C with 5% CO2, the inoculum was replaced with DMEM supplemented with 4% decomplemented FBS except for the condition of Tunicamycin treatment where at medium change, the medium contained 1μg/ml of Tunicamycin (Sigma-Aldrich cat. no. T7765-1MG) or 0.5mM of Thapsigargin (Sigma-Aldrich cat. no. T9033-1MG). T0 (0 h.p.i) is the time after this first hour of incubation with the virus and subsequent time points are counted from this initial point. Control cells, mock infected and cells untreated with inducer(s) were also used.

2.2.10 Cell Lysis

Depending on the type of analysis to be performed, cells were lysed in one of the following lysis buffers:

- Laemmli Buffer (50 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 0.1% bromophenol blue-Bio-Rad cat.no. 161-0404)
- RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1mM EDTA, 1mM EGTA, 1mM PMSF, 0.5% Sodium deoxycholate (Sigma-Aldrich cat.no. D6750-100G) and Proteinase Inhibitors (Roche - 11836170001) and Phosphatase Inhibitors: Sodium Fluoride (Riedel-deHaën 30105) and Sodium Orthovanadate (Sigma-Aldrich S6508-10G 028K0117)
- 5X Reporter Lysis Buffer (RLB) Promega cat.no. E397A, diluted to 1X for harvest of luciferase samples

2.2.11 Bradford Assay

Quantification of cell lysates was performed by Bradford assay. Briefly, 250μl of 1X Bio-Rad Protein Assay Dye reagent (Bio-Rad Laboratories GmbH cat.no.500-0006) was pipetted into clear 96-well plate according to the number of samples in duplicates. Known concentrations of BSA diluted from a 10mg/ml 100X stock (Promega cat.no. R396D 24122438) ranging from 0.5 to 4μg/μl were then added into respective wells, likely 2μl of cell lysates were added into respective wells. 1X Bio-Rad Protein Assay Dye reagent was used as blank. All samples were
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carefully mixed by pipetting up and down and immediately read at wavelength 595 nm on a Wallac Envision 2104 Multilabel Plate Reader (Perkin Elmer). The known concentrations were used to plot a standard curve that was subsequently used to calculate the concentrations of unknown samples.

2.2.12 SDS PAGE
Whole cell lysates were resolved by SDS-PAGE at appropriate acrylamide percentages. The lysates were boiled at 95°C for 10 minutes, centrifuged for 1 minute at RT at 1000 rpm, and subsequently loaded into the acrylamide gel. Gels were run in SDS electrophoresis buffer, initially at 80V into the stacking gel and later at 140V into the running gel.

2.2.13 Western blot
Nitrocellulose membrane (GE Healthcare – cat.no. 10600015) was used at transfer at 350mA for 1.5 hours. Blocking was done for 1 hour in 5% milk/BSA depending on protein to be checked followed by incubation with appropriate primary antibodies diluted in 5% milk/BSA for 1 hour at room temperature or 4°C overnight (O/N) depending on antibody. Secondary antibodies conjugated with HRP (DakoCytomation – cat.no. P0447/8) were diluted in 5% milk/BSA depending on protein to be checked and blots were incubated for 1 hour at room temperature. Washes were done with TBS 0.5% Tween-20 solution. Blots were developed using Immobilon®Crescendo Western HRP Substrate (Millipore – cat. no. WBLUR0500) according to manufacturer’s instructions.

2.2.14 Luciferase Assay
The luciferase assay to assess the IFNβ promoter activity resulting from co-transfection of NS2B and/or RIGI-N with IFNβ was performed using 250μl of 1X RLB per well of a 12-well plate. Cells were then scraped and harvested followed by freeze and thaw cycles in dry ice and room temperature respectively to facilitate cell lysis. A centrifugation step was then carried out at 12000g for 30 seconds and supernatants were collected and stored at -20°C until analysis. For analysis, 50μl of Luciferase substrate was pipetted into white 96-well-plates.
in duplicate followed by addition of 25μl of respective samples, 1X RLB buffer was used as a blank. Samples were mixed well and measurements of IFNβ-luciferase activity in cell lysates were performed using the Wallac Envision 2104 Multilabel Plate Reader (Perkin Elmer). All measurements were normalized to individual protein concentrations that were quantified with the Bradford assay method as described previously.

2.2.15 RNA extraction, cDNA synthesis and qRT-PCR

Samples for quantitative reverse transcription PCR (qRT-PCR) were lysed and collected with EuroGOLD TriFast buffer. Total cellular RNA was extracted according to the manufacturer’s protocol. RNA samples were resuspended in autoclaved water and treated with DNase I recombinant, RNase Free Kit (Roche cat. no. 04716728001) before being quantified by a Thermo Scientific NanoDrop 2000.

500 ng of total RNA was then reverse transcribed using dNTPs (Promega cat. no. U144B), 150ng/μl random primers (Invitrogen cat.no. P/N 58875), 5X First Strand Buffer, 0.1M DTT and M-MLV Reverse Transcriptase (Invitrogen cat.no. 28025-013) RNase Inhibitor (Promega N2518), in a reaction mix.

Quantification of mRNA was obtained by real-time PCR using the 2X qPCR SyGreen Mix Separate ROX cat.no. PB20.14-05 qPCR kit (PCR BIOSYSTEMS) on a CFX96 Bio-Rad thermocycler. The different primers used are listed in Table 4. Analysis of relative gene expression was performed using the 2−ΔΔCT method of normalization (Livak and Schmittgen, 2001) with β-Actin as the housekeeping gene of choice.

2.2.16 XBP1 splicing RT PCR

cDNA was synthesized from 500ng total RNA samples as described previously. Subsequently, RT PCR was performed using the GoTaq®G2 Flexi DNA Polymerase kit (Promega cat.no. M7805). The amplicon spanning XBP1 splicing site was amplified using the Xbp1u Fw and Xbp1s Rv primers and GAPDH as control (Table 4) under the following conditions: 95 °C for 5 minutes, 95 °C 30 seconds, 60 °C 30 seconds, 72 °C
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30 seconds for 35 cycles and 72°C 2 minutes. PCR products were run on a 2% Agarose gel for 45 minutes at 80V. Gel preparation: UltraPure™ Agarose powder (Invitrogen cat.no. 16500-500) melted in 0.5X TBE buffer and mixed with Ethidium Bromide-250ng/ml. Agarose gels were visualized using the UVIdoc HD2 gel documentation system (UVITEC Cambridge)

2.2.17 Plaque assay
Viral yields quantification was done by plaque assay using Vero cells. Cells were seeded in 24-well plates in on the first day. On the next day when the monolayer was formed, cells were infected in duplicate with a 10-fold serial dilution of TBEV in a total volume of 200μl of serum-free medium. After 1 hour incubation at 37°C, 5% CO₂, the inoculum was removed and a 1ml overlay containing 6% CMC and normal medium (DMEM supplemented with 4 % decomplemented FBS) at a ratio 1:1 was added. The cells were incubated for 5 days at 37°C, 5% CO₂. To develop, the medium/CMC solution was removed, cells were then fixed with 3.7% PFA for 20 minutes at room temperature. Cells were stained with 1% crystal violet solution and incubated for 20 minutes at room temperature. After incubation, the staining solution was removed and cells were washed with water until the plaques were visible enough for counting. Viral yields were determined by counting the average number of plaques formed in the duplicate wells and multiplied by the dilution factor.

2.3 Figures and Statistical Analysis
At least two independent experiments in triplicate repeats were conducted unless indicated otherwise. GraphPad Prism 7 for Mac OS X was used to generate all figures and perform all statistical analyses. Mean values are shown with standard deviation and p-values, measured with an unpaired two-tailed t-test or one-way ANOVA as indicated in figure legends. Significant p-values are denoted by asterisks (p≤0.05 = *; significant, p≤0.01=**; highly significant and p≤0.001 = ***; extremely significant). Where asterisks are missing the differences are not significant.
3. RESULTS
3.1 The UPR primes an earlier and more robust antiviral signaling

Our laboratory has extensively studied the UPR and its interplay with innate immunity. When I started my doctoral degree, I was involved in a team effort to make sense of a series of observations pointing at a role of the UPR in concert with the innate signaling system in priming the host cell in its response to flavivirus infection, boosting its antiviral activity.

The key observation was that in the course of TBEV infection, the UPR pathway is active as early as 8 hours after infection, while the interferon response occurred at later time points (starting from 16 hours). When this pathway is pre-activated by Tunicamycin, a commonly used UPR inducer that inhibits protein N-linked glycosylation, there is an up regulation of IFNβ (Fig. 1B) as well as virus inhibition not just for TBEV (Fig. 11A) but also for several other members of the Flaviviridae family such as WNV, DENV and ZIKV. Similar effects were observed also with another drug Thapsigargin (TG) that inhibits ER Ca\textsuperscript{2+}-dependent ATPase and depletes the ER Ca\textsuperscript{2+} supply (Fig. 11A, C).

The results of this work have been recently published and the paper is appended to this thesis for ease of reference. Although I have been involved in replicating several experiments pertaining to this work, which helped me develop essential skills in molecular biology and virology, I am reporting in this thesis only those where I have been the principal responsible scientist, as well as follow up unpublished experiments.
Figure 11: UPR inhibits TBEV and up regulates IFNβ: TBEV infection (white bars) and TBEV infection and treatment with either TM (grey bars) or TG (black bars) at 24 h.p.i A) TBEV yields in PFU/ml as quantified by plaque assay, B-C) IFNβ mRNA quantification by qRT-PCR on TM or TG treatments. Results presented as normalized to β-Actin and relative fold increase over T0 time point (**p≤0.01, ***p≤0.001 unpaired, student’s t-test) – data obtained from Carletti and Zakaria et al., 2019

3.1.1 UPR inducers Tunicamycin and Tharpsigargin are not cytotoxic but cytostatic

Historically, most UPR studies employ the use of pharmaceutical inducers such as Tunicamycin (TM) and Tharpsigargin (TG); our studies on UPR are no different as can be seen from figure 10. However, majority of the said chemical inducers may act pleiotropically and this may probably have some effects to the cellular activities. This certainly depends on the concentrations and/or cell systems used. To exclude any toxic effect of the UPR-inducing drugs and to ensure the reliability of our data obtained from routine use of the UPR inducers TM and TG, I took the initiative to test these two inducers’ effects on cell growth and/or viability. The protocol implemented to perform this experiment imitated an infection experiment except the infection itself.

Briefly, as indicated in figure 12A, I seeded 50000 U2OS cells per well in a 12-well plate format; the next day, I removed the medium and added serum free medium for 1 hour like I would do in an infection experiment. After 1 hour, I added fresh complete medium to the mock control cells (U2OS) or complete
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medium containing 1µg/ml TM or 0.5µM TG, concentrations that we routinely use for our experiments. After the one hour, I collected the T0 RNA samples and counted cells with Trypan blue. More samples were collected at time points 4, 8, 24 and 32 hours after treatment (subsequently referred to as T4, T8, T24, T32, T = time point). The time point beyond 24 hours was purposefully chosen to cover the infection cycle of flaviviruses that take longer than 24 hours.

When taking the cell counts, I took note of all dead and alive cells at the indicated time points to determine the percentage viability (Fig. 12C) and RNA was used in a qRT-PCR to check the expression of housekeeping genes β-Actin and GAPDH that we commonly use in our qRT-PCR analyses. This was to test whether the TM and TG had any effect on that as well.

As can be observed in figure 12B, the Mock control cells grew exponentially throughout the time course while cells treated with both TM and TG appeared to be somewhat stagnant between 0 and 8 hours but then grew consistently between 8 and 24 hours and even more between 24 and 32 even though at a slightly slower rate than the control cells. The restored growth pattern especially at 24 hours and forward is not unexpected and is quite consistent with the declining exposure of the drugs on cells as the time course progresses.

qRT-PCR of β-Actin and GAPDH revealed that the Cq values of both housekeeping genes ranged between 15 and 17 in all conditions except at 24 hours in the TG treatment where it rose to 19. When compared by a t-test, there was no significant difference between the average Cq values of both genes at all time points (Fig. 12D).

These results indicate that both TM and TG at the concentrations used in our experiments have a rather cytostatic effect on cell growth, since there was no substantial cell death as can be seen in the percentage viability plot (Fig. 12C). This suggests that at the concentrations used, TM and TG have a minimal detrimental effect if any on U2OS cells in the course of our experiments.
Figure 12: TM/TG cytotoxicity assay over the course of 32 hours

A) schematic representation of experimental procedure
B) Average cell counts of mock cells and cells under TM or TG treatment
C) Percentage viability of cells counted in B, D) Average absolute Cq values of β-Actin (black bars) and GAPDH (grey bars) of mock cells and cells treated with TM or TG.
3.1.2 An antiviral signature characterizes the early UPR-driven antiviral response during TBEV infection

During TBEV infection, the kinetics of IFNβ activation are concomitant with IRF3 phosphorylation and its translocation to the nucleus in conditions of infection with a pre-activated UPR. While involving IRF3, this antiviral activity is independent of the canonical interferon signaling and IRF3 has additionally been implicated in other flaviviruses namely, DENV2, WNV and ZIKV. Specific to TBEV, the IRE1 arm of the UPR confers this antiviral activity and other arms may be valid for different viruses. These observations suggest a vital link between UPR and the cellular innate signaling and the two acting in concert to elicit a robust antiviral state against flavivirus infection. (Carletti and Zakaria et al., 2019).

The observation of UPR antiviral signaling being independent of the canonical IFN signaling and dependent on IRF3 instigated a whole genome transcriptome analysis (in collaboration with Dr. Danilo Licastro, CBM-Trieste, Italy) to better characterize this antiviral activity owed to UPR during TBEV infection.

The analysis was carried out on three groups of U2OS cells subjected to different treatments; first group was of cells on sterile Tunicamycin treatment (1μg/ml), second, cells only infected with TBEV and the third group, cells treated with TM and infected with TBEV, MOI = 1. All cells were subjected to their individual treatments for 8 hours, after which RNA samples were collected (Fig. 13A). RNA was extracted and checked for integrity and quality prior to analysis.

Three replicates for each condition were subjected to high-throughput RNA-Sequencing analysis and the data were analyzed using bioinformatics tools by Dr. Licastro as described in the Materials and Methods chapter.

An analysis with Ingenuity Pathway Analysis (IPA) revealed UPR as the most enriched pathway in the condition of sterile TM treatment as expected, and no pathway was particularly enriched in the condition of TBEV infection alone. However, in the condition of both TM treatment and TBEV infection, IPA revealed the following as the most up regulated pathways; “Activation of IRF by
cytosolic PRR”, “Interferon signalling”, “Role of PRRs in recognition of bacterial and viruses” and “Role of RIG-I like receptors in antiviral innate immunity”.

Furthermore, a differential analysis was performed between the group of sterile TM treatment (UPR activation alone) and that of treatment and TBEV infection. This analysis revealed a set of 39 genes that were differentially modulated between the two conditions. Of these genes, 21 representing 53.8% of all genes were ISGs and were up regulated in the condition of both TBEV infection and TM treatment as observed in the heat map. (Fig. 13B – C).
Figure 13: Whole genome transcriptome analysis of U2OS cells under sterile TM treatment, TBEV infection and TBEV infection and TM treatment conditions. A) Schematic diagram for RNA-Seq sample collection. B) Heat map showing differentially modulated genes between conditions of sterile TM treatment (TM) and TBEV infection and treatment with TM (TBEV+TM) performed in U2OS cells (upregulated genes in red, downregulated genes in blue, following the indicated scale). C) Pie chart showing percentages of the 39 differentially modulated genes shown in heat map. Genes with adjusted p<0.05 and absolute logarithmic base two-fold change>1 were selected. Data were analysed with the IPA software as described in Materials and Methods.
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I followed up on the transcriptomic data (Figs. 13B, C) and validated the differential analysis findings by qRT-PCR. I followed the same experimental scheme as shown in figure 14A except that I collected samples at 10 hours and 24 hours after TBEV infection. As opposed to the 8-hour time point used in the transcriptomic analysis (Fig. 13), the 10-hour time point was used to reiterate the time points in a previous differential analysis (not reported herein) that showed UPR as a relevant up regulated response in the window of time between 10 and 24 hours. 10 hours when TBEV replication is already active but no induction of IFNβ, a delay strategy employed by TBEV and at 24 hours when both IFNβ induction and TBEV multiplication are detectable (Carletti and Zakaria et al., 2019). Clearly the events in the transcriptomic analysis (Fig. 13) at 8 hours are still relevant at 10 hours (Fig. 14) and this difference in time points is not at all consequential to the outcomes. From the 21 ISGs identified in the differential analysis (Fig. 13B), I chose the top 4 most up regulated genes namely: OASL, IFI44, IFI44L and IFIH1. Additionally, I tested for the induction of IFIT1 and Viperin, which are known broad antiviral ISGs that have been previously described as having antiviral activity towards several flaviviruses (Wacher et al., 2007; Vonderstein et al., 2017; Lindqvist et al., 2018).

The qRT-PCR data reiterated the differential analysis in that there was a significant induction of the analyzed ISGs in the condition of TBEV infection and treatment with TM (Fig. 14A-F) with OASL, IFIH1, IFIT1 and Viperin showing significant induction at both time points while IFI44 and IFI44L showing significant induction at the 10 hour and 24 hour time points, respectively. These results reinforced the results from the transcriptomic analysis on the fundamental role of ISGs in the UPR-driven antiviral state during TBEV infection.
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Figure 14: Validation of selected ISGs from differential analysis (Fig.13); cells were subjected to conditions of sterile TM treatment (white bars), TBEV infection (grey bars) and TBEV infection and TM treatment (black bars) at 10 and 24 hours post infection. qRT-PCR quantification of A) OASL B) IFIH1 C) IFI44 D) IFI44L E) IFIT1 and F) Viperin. Results presented as normalized to β-Actin and fold change over the T0 time point (*p≤0.05, **p≤0.01, ***p≤0.001 unpaired, student’s t-test).

3.2 Alternative methods of UPR induction

The results shown previously in figure 12 were certainly convincing enough of the minimal effects of TM and TG in our experimental settings. Nevertheless, I sought out to find alternatives that could possibly replace the use of pharmaceutical UPR inducers as employed from figures 11 to 14 and possibly recapitulate the observations in our previous results (Carletti and Zakaria et al., 2019). This was meant to reinforce our previous observations by using a different way of UPR induction but also to attempt that induction in a more organelle-specific manner that is by targeting the ER as opposed to what may be described as widespread/pleiotropic targeting by chemical inducers.
For these reasons, several approaches to induce ER stress and eventually UPR were explored and are reported herein.

3.2.1 UPR induction by depletion of the chaperone protein BiP

GRP78 alias BiP is the main ER chaperone that sequesters the three transmembrane proteins; ATF6, IRE1 and PERK that are principle effectors of UPR and dissociates from them under conditions of ER stress. Taking advantage of this fact, I attempted to deplete BiP and in essence on one hand, making it absent to handle the folding of the incoming protein load but also the elicitation of ER stress due to impairment and/or lack of the sequestration of the UPR effector proteins that is carried out by BiP.

To this end, I reverse-transfected U2OS cells with 20mM of small interfering RNA targeting BiP or the control siRNA following the manufacturer’s protocol. After 48 hours of incubation at 37°C, I infected the cells with TBEV, MOI = 1, and collected supernatants, cell lysates and RNA samples at time points 0, 8 and 24 hours after infection.

The depletion of BiP by siRNA was confirmed by an anti-BiP western blot (Fig. 15A) and in this condition, while not as potent as TM, qRT-PCR analysis showed substantive induction of the spliced form of XBP1 and IFNβ mRNAs at all time points with increasing levels throughout the time course as compared to the siCTRL (Fig. 15B, C), suggestive of an active UPR and innate signaling just as has been observed in our previous results (Carletti and Zakaria et al., 2019).

XBP1s mRNA is considered the classical marker of an active IRE1, the most conserved arm of the UPR (Zhang et al., 2016) and is typically used as the readout for an active UPR.

It is important to note that this induction of XBP1s and IFNβ mRNAs at the T0 time point is purely a consequence of the depletion of BiP and not infection as this time point is only after 1 hour of infection.

However contrary to our previous observations, there was no difference in viral yields between the condition of BiP depletion and siCTRL, in fact, the yields were
similar in both conditions and both more than those observed in the condition of treatment with TM (Fig. 15D). A possible reason could be that as efficient as the knockdown was to induce XBP1s and IFNβ mRNA, this may not be efficient enough to reflect in virus inhibition.

Figure 15: UPR induction by depletion of BiP followed by TBEV infection: siCTRL and TBEV infection (black bars), siBiP and TBEV infection (grey bars), TBEV infection and treatment with TM (white bars), A) Anti-BiP western blot confirming depletion of BiP by siRNA B) qRT-PCR quantification of spliced XBP1 and C) IFNβ mRNAs after BiP depletion and TBEV infection (results presented as normalized to β-Actin and fold change over siCTRL) D) Quantification of TBEV viral yields in PFU/ml as quantified by plaque assay after BiP depletion and TBEV infection (**p≤0.01, ***p≤0.001 unpaired, student’s t-test)

3.2.2 Expression of BiP mutants does not induce UPR

Next I attempted inducing UPR by ectopic expression of two mutants of the chaperone protein BiP; namely G227D and T37G. These mutants are impaired in their ATPase-dependent nuclear binding domains; the ATPase activity is pivotal in the ability of BiP to fold nascent proteins that come into the ER. It was
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therefore, plausible to reason that the mutants’ impairment in protein folding would lead to an increase in the de novo protein load, thereby overloading the ER and in so doing, enable the induction of ER stress and then UPR.

To address this, I seeded 50000 U2OS cells per well of a 12-well plate, the next day I transfected the cells with 500ng of respective plasmids. Transfection was performed using Lipofectamine LTX following a standard, optimized protocol and after approximately 5 hours of incubation at 37°C, the transfection mix was replaced with complete medium and incubated further overnight. The positive control cells were treated with TM for the same amount of time as the transfected cells. After approximately 16 hours, I collected cell extracts and RNA samples. Cell lysates were analyzed for expression by western blot with anti-BiP antibody, while RNA was used for cDNA synthesis and analyzed by qRT-PCR for quantification of the spliced form of XBP1.

Efficiency of transfection was measured by flow cytometry and ranged between 50% and 65% for all constructs. Expression of the BiP proteins was confirmed by an anti-BiP western blot (Fig. 16A) and the difference in expression between ectopic expression and baseline levels as observed in the 3 controls (cells transfected with an empty vector, cells treated with Lipofectamine LTX alone and untreated U2OS cells) is quite obvious.

However, the mutants G227D and T37G were not able to sufficiently induce UPR as observed in the quantitative measurement of the mRNA of the spliced form of XBP1s showing minimal levels such as those seen in the BiP WT (Fig. 16B). This was also true for the induction of IFNβ mRNA (Fig. 16C). The minimal induction of IFNβ in the TM condition is consistent with what is expected in the condition of sterile treatment with TM i.e in the absence of infection (Fig. 14)

One possible reason for this result could be compensation in protein folding by the endogenous BiP in the case of BiP mutants’ expression. A possibly more feasible approach could be to perform a co-transfection of the mutants and wild type BiP with a secretory protein and in this case, the protein folding ability or lack thereof in the case of the mutants would be more comparable.
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3.2.3 UPR induction by expression of Null Hong Kong

The next attempt involved yet again an ectopic expression approach only this time, it was of a verified UPR inducer; null-hong kong (NHK) a truncated mutant of the alpha 1 anti-trypsin (α1AT) that does not fold properly and therefore overloads the ER and induces the UPR (Ordóñez et al., 2013). While α1AT is completely secreted, NHK is considered secretion-incompetent and only a portion of it can be secreted.

To do this, I performed the experiment following the same scheme as in figure 16, this time transfecting SV5-tagged NHK or the control α1AT plasmids using Lipofectamine LTX and like before TM treatment was included as a positive control for UPR induction. Whole cell lysates were collected and tested for protein expression and secretion by anti-SV5 western blot while RNA was used for cDNA synthesis. Successively, these samples were analyzed for their ability to
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induce UPR by quantification of the mRNA of XBP1s by qRT-PCR. An anti-SV5 flow cytometry analysis was also performed to check for transfection efficiency using a standardized protocol and results normalized to an empty vector. The transfection efficiencies ranged between 60% and 64% for all constructs. Expression and secretion of both proteins was confirmed by an anti-SV5 western blot and a difference in size that reflects the difference in amino acids is clearly visible between α1AT and NHK, and only a small portion of NHK was secreted as is expected (Fig. 17A). And as previously documented in the literature, NHK seemed to significantly induce the spliced form of XBP1 (Fig. 17B) but not IFNβ mRNA (Fig. 17C).

While NHK could not induce significant IFNβ levels, its ability to significantly induce XBP1s prompted the next step which was a follow-up with TBEV infection to check whether in the condition of NHK expression and infection, there would be a UPR-driven inhibition of the virus as was observed in the case of infection and treatment with TM.

The same experimental scheme as in figures 17A-C was followed but with an additional step of infection on the following day. Whole cell lysates, supernatants and RNA were then collected at T0, T4, T12 and T24 time points and analyzed by anti-SV5 WB and qRT-PCR.

Upon infection, there was modest induction of IFNβ as a consequence of NHK transfection at T4 and T8, this induction was however not significant. Moreover, an up-regulation in the induction was observed at T24 most likely due to increasing virus multiplication and this was the case in all treatments (Fig. 17D). While this result was not supportive of our hypothetical model, I noticed a trend of modest inhibition of TBEV replication in the condition of expression of NHK from T12 (Fig. 17E), which like the induction of IFNβ mRNA in figure 17D was not significant.

Moreover, I was not able to reproduce this phenotype of the inhibition in TBEV replication in subsequent experiments even with further optimization of the experimental scheme in attempt to improve the efficiency of transfection even more than it was initially.
Figure 17: UPR induction by expression of Null Hong Kong  
A) Anti-SV5 western blot showing expression and secretion of α1AT and NHK  
B) qRT-PCR quantification of spliced XBP1  
C) IFNβ and D) TBEV mRNAs in the conditions of infection with α1AT, NHK transfection or treatment with TM as positive control. Results presented as normalized to β-Actin and fold change over α1AT (A, B) or T0 time point (D, E). C.E=cell extract, sup= supernatant (**p≤0.01, student’s t-test)
Results

3.2.4 A screen of TBE viral proteins identifies some potent UPR inducers

Since previous attempts to induce UPR by inducing protein overload were deemed unsuccessful, I decided to shift my focus to the physiological route of UPR induction following TBE virus infection. From our previous observations and the literature, the process of infection occurs with an unscheduled overload of the viral proteins on the host cell’s machinery and more specifically the ER, thus inducing stress and UPR. In TBEV infection, kinetics of UPR induction range between 8 and 12 hours after infection (Carletti and Zakaria et al., 2019).

By this logic, it was plausible to reason that ectopic expression of TBE viral proteins would also be able to induce the response like it happens during infection. An additional advantage to this approach was the possibility of identifying the viral component(s) that could be responsible for induction of UPR in the course of TBEV infection.

To accomplish this, I began by a screen of all TBE viral proteins to identify candidates for ER stress and UPR induction. All 3 structural and 7 non-structural proteins, a kind gift from Dr. Anna K. Överby; have been individually cloned in a high expression plasmid pL.18 and contain a FLAG tag (Table 3) at her lab in MIMS, Umeå Universitet, Sweden.

To do this, I performed the experiment following the same scheme as in the figures 15 and 16, this time transfecting each construct as well as an empty vector. Additionally, untreated cells (U2OS) and cells treated with TM were included as controls. After approximately 16 hours of transfection, whole cell lysates were collected for anti-FLAG WB and RNA samples for qRT-PCR analysis. Cells treated with TM for the same amount of time were used as a positive control. Assessment for transfection efficiency was performed by an anti-FLAG flow cytometry analysis and normalized to an empty vector. Transfection efficiencies ranged from 50% to 95% for all constructs.

All 10 viral proteins were expressed successfully as confirmed by an anti-FLAG western blot (Fig. 18A) and from the analysis of qRT-PCR data on the quantification of the spliced form of XBP1, 3 out of the 10 viral proteins E, NS1
and NS2B showed a significant induction either comparable or more than the positive control, TM (Fig. 18B). This was also true for the induction of IFNβ for the three proteins in addition to the prM protein (Fig. 18C). These results imply that the ectopic transfection of TBE viral proteins is a viable method of inducing UPR in U2OS cells and some of these proteins can activate the IRE1-XBP1 pathway of UPR and IFNβ mRNAs suggestive of an active UPR, specifically the IRE1 arm and innate signaling respectively.

Figure 18: UPR induction by ectopic expression of TBE viral proteins: A) Anti-FLAG western blot showing expression of 10 TBE viral proteins B) spliced form of XBP1 and C) IFNβ mRNAs quantification by qRT-PCR, results presented as normalized to β-Actin and fold change over untreated U2OS cells – E.V= empty vector, (*p≤0.05, **p≤0.01, ***p≤0.001, one-way ANOVA test).
3.3 UPR induction and Innate signaling by candidate TBE proteins E, NS1 and NS2B

From the above findings, the identified TBEV viral proteins E, NS1 and NS2B were analyzed further in the context of UPR induction and innate signaling and then subjected to TBEV infection. The prM protein was not included in further investigation because while it could significantly induce IFNβ, the same wasn’t true for XBP1s admittedly this could just be a consequence of varying efficiencies between experiments. Nonetheless, the fact that prM is a glycoprotein same as E and NS1 proteins as opposed to NS2B, the effects of UPR induction as a consequence of expression of a glycoprotein at least for the purposes of this study were more than adequately represented by the two. However, this is not to say prM should be completely omitted for investigation in other aspects pertaining to similar interests as those of this project. Although showing an upward trend in the induction of both XBP1s and IFNβ, NS2A was also disregarded from further analyses, as these inductions were not statistically significant (Fig. 18B, C)

As stated previously, E protein and NS1 are glycoproteins and ideally, during their formation require the endoplasmic reticulum where they undergo folding and further modifications in the Golgi before being secreted. This in essence also means their overexpression is therefore quite targeted and indeed more successful in overloading the ER, thereby inducing stress and ultimately resulting into the induction of the unfolded protein response.

Conversely, NS2B, being a transmembrane protein whose most widely studied function has been as the co-factor to NS3 and therefore contributing to protease activity of the NS2B3, poses some intrigue in its ability to induce UPR. This is partly due to its lack of properties for direct UPR induction as compared to the glycoproteins thus favoring an alternative mechanism of UPR induction.
3.3.1 Expression of TBEV E, NS1 and NS2B proteins induces UPR

The three candidate proteins (Fig. 18) identified from the screen of TBE viral proteins were expressed again in subsequent experiments (Fig. 19A) and tested initially for their ability to induce several UPR markers in addition to XBP1s. As observed previously in the quantification of XBP1s by qRT-PCR (Fig. 18B), all three TBE viral proteins showed substantial splicing of XBP1 as compared to the empty vector by XBP1s mRNA quantification by qRT-PCR and by conventional RT-PCR for XBP1 splicing (Fig. 19B). XBP1s RT-PCR is a qualitative method of analyzing XBP1 splicing, the PCR is performed with primers that amplify both spliced and unspliced forms of XBP1. The two forms can then be observed as two bands or a single band on an agarose gel indicating splicing and therefore an active UPR and lack of it, respectively. The existence of the two bands in an event of splicing is due to a 26bp difference between the spliced and unspliced forms of XBP1. This method provides a visual advantage that adequately reiterates and/or complements the qRT-PCR quantification (schematic diagram of amplification by both methods: Fig. 19B, upper panel).

I additionally tested for EDEM1, a downstream marker of IRE1 activation and an ER-resident protein responsible for the disposal of misfolded proteins through the ER-associated degradation pathway (ERAD). EDEM1 was significantly induced by NS1 and NS2B as compared to the control (Fig. 19C), suggesting activation of the ERAD pathway at least by expression of these two TBE proteins.

It is a known fact that an increasing protein load to the ER leads to an up regulation of chaperone and co-chaperone proteins so as to increase the ER’s protein folding capacity and bring back the ER homeostasis. For this reason, I tested for BiP, the major ER chaperone protein and a marker downstream of the UPR arm ATF6. Indeed, BiP was significantly up regulated by the expression of all three viral proteins (Fig. 19D) and so was the co-chaperone DNAJC3, a downstream effector of XBP1s (Fig. 19E). The PERK arm of UPR is responsible for protein translation halting and in cases of extreme ER stress, the pro-apoptotic protein CHOP alias DDIT3 is induced downstream of this pathway. As compared
to the control, the levels of CHOP/DDIT3, a pro-apoptotic protein and GADD34, a target protein for CHOP and a phosphatase that functions as a negative regulator of PERK through the dephosphorylation of eIF2α for protein translation halt recovery were significantly upregulated when all three proteins were expressed (Fig. 19F, G)

These results indicate an activation of not just IRE1-XBP1 pathway of UPR but also other UPR genes that implicate the other two arms of UPR by this approach albeit at varying levels of induction by individual TBE proteins.

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**Results**

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**A**

**B**

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**C**

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**D**

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**E**

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3.3.2 ISG induction in response to expression of TBE viral proteins

The UPR-driven antiviral signaling and maintenance of a robust antiviral state against TBEV is mediated by a subset of ISGs through IRF3 (Carletti and Zakaria et al., 2019). All the candidate proteins showed substantive potential in the induction of UPR (Fig. 19). Additionally, having observed that even 10 hours of sterile TM treatment can induce some amounts of antiviral ISGs (Fig. 14A-F) and the overall transfection time was approximately 16 hours, it was plausible to also check for the ability of the expression of the candidate proteins in inducing some antiviral ISGs. I selected from the strongest induced ISGs that were a result of the differential analysis in figure 13B, that is OASL, IFIH1/MDA5, IFI44, IFI44L (Fig. 14A-D) as well as IFIT1 and Viperin (Fig. 14E, F) broad antiviral ISGs against flaviviruses.

Indeed, all three TBE proteins significantly induced Viperin, IFIT1 and IFI44 as compared to the empty vector control (Fig. 20A-C) while only NS2B was able to significantly induce IFI44L (Fig. 20D). However, the ISGs OASL and IFIH1 were not induced to any significant amounts by any of the viral proteins (Fig. 20E, F)
These results suggest a selective induction of ISGs by TBE E, NS1 and NS2B proteins with NS2B being the strongest of the three proteins in the induction of Viperin, IFIT1, IFI44 and IFI44L ISGs.

**Figure 20:** Expression of E, NS1 and NS2B leads to an induction of antiviral ISGs: qRT-PCR quantification of A) Viperin B) IFIT1 C) IFI44 D) IFI44L E) OASL and F) IFIH1 mRNAs. Results presented as normalized to β-Actin and fold change over E.V=empty vector (*p≤0.05, **p≤0.01, ***p≤0.001 unpaired, student’s t-test)
Results

3.4 Expression of E, NS1 and NS2B in the course of TBEV infection

The UPR-driven antiviral state translates into an up regulation of antiviral ISGs and inhibition of TBEV and other flaviviruses of clinical importance (Fig. 14, Carletti and Zakaria et al., 2019). In line with this, I performed a TBEV infection in a time course experiment in this system of UPR induction by ectopic expression of E, NS1 and NS2B TBE proteins.

3.4.1 Expression of E, NS1 and NS2B protein inhibits TBEV replication

The experimental scheme in this case followed the same procedure as the performed in figures 19 and 20 with an additional step of infection. After an overnight transfection, I infected the cells with TBEV, MOI = 1 and replaced the infection medium with complete medium after 1 hour of incubation. I then collected supernatants, RNA and cell extracts at time points 0, 4, 8 and 24 hours after infection (approximately 16, 20, 24 and 40 hours respectively after transfection).

500ng of RNA was used for cDNA synthesis followed by qRT-PCR quantification of TBEV mRNA and an anti-4G2 western blot for TBEV E protein expression was carried out. Supernatants were used to quantify viral yields by plaque assay as described in the Materials and Methods chapter.

After 8 hours of infection, there was a significant reduction of TBEV viral yields in the conditions of expression of E, NS1 and NS2B proteins as compared to the empty vector as well as untransfected cells. This was also true after 24 hours of infection, notably with increased virus production across all conditions as would be expected at this time point (Fig. 21A).

This phenotype is additionally corroborated with the decreased amounts of TBEV at the protein level as can be observed by an anti-4G2 western blot where in the conditions of ectopic expression of E, NS1 and NS2B at 24 h.p.i, TBEV E protein due to infection is less than that of empty vector and/or TBEV infection alone, whose amounts are quite comparable (Fig. 21B).
3.4.2 Expression of E, NS1 and NS2B proteins induces some antiviral ISGs during TBEV infection

The next set of experiments was prompted from the observations that TBE viral proteins E, NS1 and NS2B can induce both UPR and some antiviral ISGs (Fig. 19, 20), but more importantly was the observation that there was TBEV inhibition in conditions of expression of the three proteins. I wanted to check whether this inhibition was due to a UPR-driven antiviral state that is facilitated by ISGs.

From the RNA samples in the experiment shown in figure 21, I tested for the same ISGs that were previously tested (Fig. 14, 20).
Generally, the expression was not as strong as was observed by transfection alone (Fig. 20) and this was to be expected due to an increased length of time since transfection because until the 24th hour post infection, it is approximately 40 hours of transfection and being a transient transfection, the number of transfectants are most likely dwindling at this point. Additionally, TBEV might have strategies to inhibit the expression of these antiviral genes, a state that is magnified and augmented with the increasing TBEV multiplication at this time point. It is therefore quite possible that at this time, the virus is overpowering the cell machinery in the experimental setting employed.

Despite this fact, there was notable induction of IFIT1, Viperin and IFI44L mRNAs by only NS2B as compared to the empty vector and/or untransfected cells at 4 and 8 hours after infection (Fig. 22A, B, C). Additionally, while transfection alone could not induce OASL (Fig. 20E), at 4 hours of infection, it showed an up-regulation trend by all three proteins but only significantly by NS2B (Fig. 22D). However, at 4 and 8 hours post infection, all proteins could sufficiently induce significant amounts of IFI44 (Fig. 22E) and IFIH1 was not induced by any of the proteins (Fig. 22F) like it was the case with transfection only (Fig. 20F).

Despite a general less expression as compared to transfection alone (Fig. 20), it seems that the ISGs induced by transfection of the proteins is maintained until few hours into infection (Fig. 22) and the effect is enough to inhibit TBEV even until 24 hours post-infection (Fig. 21).
**Results**

B. Viperin mRNA (relative fold increase)

C. IFI44L mRNA (relative fold increase)

D. OASL mRNA (relative fold increase)

E. IFI44 mRNA (relative fold increase)
Results

Figure 22: Expression of E, NS1 and NS2B proteins induces some antiviral ISGs during TBEV infection

A successful innate response relies on the recognition of viral molecular signatures (PAMPs) by specialized cellular receptors (PRRs) that are unique to the said signature. After this recognition event ensues a signaling cascade that encompasses RLR conformational changes and a recruitment of an adaptor protein that is dictated by the initial signal. Adaptor proteins converge at IRFs such as NF-κB and in the case of TBEV, IRF3, the activation of which depends on its phosphorylation by kinases that are deployed in the cascade. The culmination of this complex series of events is the translocation of phosphorylated IRF3 that activates IFNβ molecules and consequently ISGs that are the actual antiviral effectors. However, some ISGs can be induced as a direct consequence of activated IRFs (Fig. 8).

RIG-I recognizes the dsRNA intermediates during TBEV infection. Induction of IFNβ and/or ISGs that characterize the UPR-driven antiviral state (Fig. 11,14) depends on IRF3 activation, which in retrospect depends on the initial PRR-PAMP recognition event and in this case, the RIG-I receptor. For this reason, it was plausible to check whether NS2B being the strongest ISG inducer (Fig. 20, 22), participates in the signaling and/or the mechanism of action of the IFNβ

Figure 22: Expression of E, NS1 and NS2B proteins induces some antiviral ISGs during TBEV infection

A) qRT-PCR quantification of IFIT1 B) Viperin C) IFI44L D) OASL E) IFI44 and F) IFIH1 mRNAs at 4, 8 and 24 time points after TBEV infection. Results presented as normalized to β-Actin and fold change over T0 time point. E.V=empty vector (*p≤0.05, **p≤0.01, ***p≤0.001 unpaired, student’s t-test)
Results

promoter and the resulting effect on the promoter when expressed together with RIGI.

I took advantage of the well-studied IFNβ Luciferase reporter system and the high efficiencies of transfection that can be achieved in HEK293T cells since the readout is solely contingent on what is transfected into the cells and not any cellular responses. Briefly, HEK293T cells were transfected with plasmids encoding NS2B, RIG-I, or both together with a reporter plasmid carrying the firefly luciferase (Fluc) gene under the control of the IFNβ promoter. The same was done with an empty vector in place of NS2B. After an overnight transfection, cell lysates were collected at approximately 16 hours of total transfection time. In every experiment and/or analysis, all samples were analyzed in duplicates and the relative luciferase activity was normalized to total protein concentration in each condition. Total protein concentration was quantified by Bradford assay as described in the Materials and Methods chapter.

As can be observed in figure 23A, all transfected components were successfully expressed as confirmed by an anti-FLAG western blot. The IFNβ promoter activity was significantly up regulated when NS2B was transfected and even more so when only RIG-I was transfected. However, when both NS2B and RIG-I were co-transfected, there was a drastic increase in the IFNβ promoter activity, significantly more than either NS2B or RIG-I transfections alone (Fig. 23B). These results are indicative of an inherent ability of NS2B to potentiate RIG-I activity on the IFNβ promoter and innate signaling. Additionally, it accentuates the UPR-driven activity of NS2B and its role in innate signaling.
Figure 23: Expression of NS2B with RIG-I augments IFNβ promoter activity. A) Anti-FLAG western blot showing expression of RIG-I and NS2B. B) Luciferase assay showing the IFNβ promoter activity after expression of NS2B and RIG-I expression. An empty vector (E.V) was used as a control. (**p≤0.01, ***p≤0.001 unpaired, Student’s t-test).
4. DISCUSSION
Flavivirus infections have devastated mankind at least since the 18th century with the record of the first dengue-like epidemic in Asia, Africa and North America between 1776 and 1780 (Gubler, 1998; 2002). Subsequently, the importance of Flaviviridae has increased well into the 21st century with over 40 discovered pathogens within the family causing a myriad of infections affecting global populations both in morbidity and mortality rates.

The struggle in the control of flaviviral infections is largely owed to the rapid evolving nature of viruses in general and even more so, RNA viruses. Viruses continuously evolve to evade host cell responses. The interplay between viruses and the innate immunity is therefore becoming excessively important, the understanding of which is fundamental in the search for sustainable control of viruses in terms of both vaccines and therapies.

4.1 UPR as a innate immunity signaler

UPR has been extensively studied as a homeostasis process to remediate consequences of ER stress. However, there is a vast wealth of knowledge implicating ER stress and several UPR genes in chronic non-communicable diseases ranging from diabetes to cancer as well as a multitude of autoimmune disorders (Morito and Nagata, 2012; Barrera et al., 2018; Madden et al., 2019, Walczak et al., 2019). Generally, the UPR is quite dynamic with different but systematically integrated actors orchestrating an attempt to recover ER homeostasis. Different ER stress stimuli at varying lengths of exposure activate different UPR sensor(s), in different kinetics and activation patterns. Ultimately, there’s different signaling to the downstream effector UPR genes and put together all these factors define the fate of the cell (Hetz et al., 2015, 2018). This systematically designed system also somewhat applies to viruses in their ability to activate different arms and/or take advantage of one or more arms because the stimuli and other surrounding factors somewhat dictate the ultimate outcome.

In the recent past, the role of ER stress and UPR in infections and of particular interest viral infections is being actively explored. Viruses being dependent on
the host cell machinery disturb the functioning of the cell and particularly the ER. While serving as a remedial process, it is quite possible to imagine how the UPR can also be a danger signal in response to this disruption of cellular processes by viral infections. Indeed, it has been proposed that the UPR can act to potentiate immune system responses and specifically antiviral signaling (Smith, 2018). This is the case in flaviviral infections and extensive studies in our laboratory have proven the central role of UPR in innate immune signaling. Infections with multiple flaviviruses, have shown an induction of UPR much earlier before innate signaling. Without any intervention, innate signaling only kicks in at around 16 hours after infection. However, when pre-activated, the UPR potentiates an earlier and more robust antiviral state that significantly inhibits clinically important flaviviruses (Carletti and Zakaria et al., 2019). This is the basis of the study reported herein and sets the precedent on which this work builds on. My doctoral work has focused on UPR-induced antiviral signaling taking into consideration all our vital findings especially the strong induction of ISGs that plays a significant role of the antiviral signaling (Fig. 14) and indeed the involvement of RLR-sensing in this antiviral signaling that is owed to the UPR.

4.2 Pharmaceutical induction of UPR on cellular viability and growth.

Our findings presenting evidence to these facts have been obtained from experiments performed with chemical inducers of UPR. These are chemicals designed to disrupt the functioning of the endoplasmic reticulum and in doing so interrupting normal activity of the ER, inducing stress and ultimately the UPR. Chemical UPR inducers get this done seamlessly but in some cases their effects may be widespread and pleiotropic. For this reason, there is always some concern on what the drugs do to the general functioning of the cell but also to the antiviral activity that is inherent to some of these drugs such as Tunicamycin, which is a naturally occurring antibiotic active against several viruses, bacteria and fungi (Foufelle and Fromenty, 2016).
Discussion

With that in mind my first task was to rule out any excessive cytotoxic effects of Tunicamycin and Tharpsigargin on cell growth. Ideally, any experimental outcome of a UPR study depends on but not limited to drug concentration, cellular system used, and more importantly the research questions to be answered. For the sake of our studies, the concentrations we use have to be strong enough to induce a notable UPR response without overshadowing the resulting antiviral response in that it has to be possible to distinctly separate the antiviral response that is a result of UPR from any activity that is intrinsic to the drug such as TM. Additionally, ours being infection experiments, the concentrations used should be ones that do not overpower the system; cells need to remain viable enough to support both cellular function and virus infection for the length of the time course.

I was able to show that the concentrations we use for both drugs indeed slow down the cell growth but without significantly impacting viability. Average cell viability ranged between 95% to 100% (Fig. 12C) and growth picked up over time in the course of 32 hours as was shown by cell counts (Fig. 12B). The same was true for the amplification of β-Actin and GAPDH genes (Fig. 12D). While the expression of these genes could be regulated differently in the presence of UPR inducers, the values were not variable compared to my untreated controls all through the time course except for GAPDH at 24 hours after treatment. This was eventually more like the other conditions at 32 hours and this difference was not significant. While this may have just been an odd spike that was probably inconsequential to qRT-PCR data normalization, subsequent data was normalized to β-Actin that was more consistent throughout the time course with the exception of XBP1s RT-PCR that used GAPDH as control (Fig. 19B).

4.3 The pursuit for alternative UPR induction methods

Next came the search for and testing of several alternatives for their ability to induce ER stress and UPR with an aim to eventually minimize or eliminate the use of pharmaceutical inducers that we, like the rest of the UPR field have been accustomed to. Eventually, to check whether this would recapitulate the
induction of antiviral signaling that was a product of UPR induction in the course of flavivirus infections (Carletti and Zakaria et al., 2019).

Simply put, ER stress and UPR induction happens when there is chaperone malfunction, impaired post-translational modification, difficult-to-fold client proteins (Marciniak et al., 2015) among other factors. In the classical model of ER stress and UPR activation, one or more of these factors ultimately leads to a rather direct increase in the intrinsic protein load. The same is true when the ER is provided with an ectopic load of unfolded and/or misfolded proteins. However, it has been suggested severally that activation of the IRE1 arm of the UPR may happen independently of this classical induction as would happen in B-cell development where the response is not due to secretory protein overload but rather a developmental switch as an anticipatory move to be ready for protein folding of the large incoming load (as reviewed by Walter and Ron, 2011). Sundaram et al., 2018 also observed a direct interaction of IRE1α with misfolded secretory proteins and activate preformed IRE1α complexes under conditions of ER stress, stressing on what would be a minor role of BiP in such cases.

The approaches and experiments reported herein have mostly relied on providing the ER with an overload of proteins aiming to imitate the classical model, but not omitting the possibility of an alternative model of UPR induction. What is more relevant however is whether this induction would translate into innate signaling and virus inhibition.

The depletion of BiP by siRNA (Fig. 15) while not as potent as TM was enough to induce significant XBP1s (Fig. 15B) seemingly in line with its function of the sequestration of UPR sensors. The same was true for the induction of IFNβ (Fig. 15C) as was our hypothetical model. However, on the contrary, the viral yields were the same for both the knockdown and control conditions (Fig. 15D). While not following the model to the letter, the increase in virus titres is not completely surprising; knock down of BiP has previously been shown to increase the replication of Hepatitis A virus (HAV), this effect was augmented when BiP was knocked out by CRISPR/Cas 9 in Huh7 cells (Jiang et al., 2017). The same has
been proposed for Hepatitis B virus whose titres increased when BiP was depleted in HepG2 cells (Ma et al., 2009; Zheng et al., 2017). While these studies were not interested in UPR, their findings may partially provide some insight into the observed increase in virus titres (Fig. 15D). Knocking down BiP is probably only enough to induce ER stress and then UPR but this induction is not enough to render the system competent to inhibit TBEV.

On the other hand, the use of BiP mutants could not induce XBP1s (Fig. 16B) or IFNβ (Fig. 16C). One explanation at least for the earlier would be the compensation by an up regulation of intrinsic BiP, other chaperones and co-chaperones in the case of expression of the mutants. This was an observation in a study by Luo et al., 2006 that showed an up regulation of PDI and GRP94 in Grp78+/- mutant mice when compared to their wild-type counterparts in a set-up where BiP activity was considered to be at about 50%. On the other hand, the role of endogenous BiP cannot be underscored either; transient transfection employed in this case was probably not enough to overcome the role of intrinsic BiP in protein folding. Experimentally though, the ER stress or UPR as measured in this experiment relied on XBP1s as the readout, taking into consideration the struggle with superior transfection efficiencies and what may be the role of endogenous BiP, another perhaps indirect UPR readout would probably be more appropriate. An experimental set-up that would allow a co-transfection of BiP and/or its mutants with a secretory protein would enable an assessment of the protein folding ability of the WT versus the mutants by quantifying not only the classical markers but also establish the collective amount of unfolded proteins bound to BiP which would probably be less in the WT than in the case of mutants. Such alternatives to study UPR have been reviewed by Lajoie et al., 2014.

Despite the possibilities and ideas outlined to improve the experimental set-up, I did not pursue this route of targeting BiP any further partly because of the challenge that I continuously experienced in transfection of U2OS cells as explained earlier which would make a co-transfection that much more limiting.
At the same time, I was looking into other approaches that at the time seemed more feasible.

The approach of expressing NHK to induce ER stress and UPR is largely supported by data based on study of diseases involving the serpin family of protease inhibitors and/or interest in ER stress in diseases specifically implicating the ERAD pathway. The overexpression of NHK, a cellular truncated mutant of α1AT has been shown to activate the XBP1-IRE1 pathway and is an appropriate substrate of ERAD, a pathway that serves to dispose off misfolded proteins (Hosokawa et al., 2001; Ordoñez et al., 2013; Jang et al., 2015). EDEM proteins play a major role in the first steps of substrate recognition of NHK and other misfolded proteins and this is crucial for efficient degradation in the ERAD pathway (Jang et al., 2015).

While also not as potent as TM, NHK expression was able to induce considerable XBP1s mRNA as compared to its wild type control (Fig. 17B) and this difference was complemented by the observation of more secreted α1AT than NHK suggesting its accumulation (Fig. 17A). However, there wasn’t significant induction of IFNβ, which has previously characterized the UPR-driven immune response. This however was not reason enough to suspend further experiments considering that XBP1s was significantly induced which fulfilled the minimal requirement but also from previous experiments, TM and TG themselves can induce only minimal IFNβ in the absence of infection (Figs.14, 15C, 16C, 17C).

Upon infection, the lesser TBEV mRNA on expression of NHK as compared to α1AT was not significant and somewhat mirrored the induced IFNβ. In essence it is easy to think that the reduced TBEV mRNA trend observed is what caused the low IFNβ induction observed on expression of NHK (Fig. 17C, D) as opposed to an up regulation of IFNβ as a consequence of UPR that inhibits TBEV as observed in the TM positive control (Fig. 17E) and in previous experiments (Fig. 11B, C). As per our working hypothesis, UPR potentiates innate signalling that results to virus inhibition. This implies that success of the antiviral signalling and virus inhibition depends on and requires an active UPR. It has been previously established that dimers of NHK are much more aggregative than do monomers,
this is because ERAD has to disassemble dimers into monomers which are the actual substrates of EDEM proteins (Hosokawa et al., 2006) suggesting a longer retention of NHK dimers in the ER than its monomers. If the UPR observed in this experimental set-up on exogenous expression of NHK is due to monomer aggregates, it is plausible to speculate that the resulting response may be short-lived and probably not enough to translate into proper IFNβ induction and TBEV inhibition.

4.4 TBE viral proteins: viable UPR inducers

It is common knowledge that multiple viral infections result into the activation of ER stress (He, 2006; Asha and Sharma-Walia, 2018). RNA viruses are no exception; in fact, most of the recorded ER remodeling as a result of virus infections is due to positive-stranded RNA viruses because their replication and assembly relies almost exclusively on the ER. Replication factories for viruses are a product of ER membrane invaginations that eventually provide physical support during replication and viruses additionally use them as protective shields from innate sensors, a strategy adopted by multiple flaviviruses (Romero-Brey and Bartenschlager, 2016, Miorin et al., 2012, 2013). This evidence points to the obvious and vital relationship between flavivirus infection and the ER and a direct link to ER stress during viral infection. A great deal of literature exists providing insights into stress responses and UPR in the course of flavivirus infections, including but not limited to the ways different flaviviruses modulate the response, which of the three arms are activated in response to specific viruses and aspects surrounding apoptosis, autophagy and generally the relationships and/or interplays during infection which many times influence disease pathogenesis (Li et al., 2013; Yu et al., 2013;Blázquez et al., 2014; Perera et al., 2017; Gladwyn-Ng et al., 2018; Tan et al., 2018; Alfano et al., 2019; Zhao et al., 2019). And while there is no doubt on the induction of ER stress by viral infection, all this information opens up an avenue for the inquisition into what could be a specific viral protein(s) responsible for inducing the response. The
Discussion

screen of TBE proteins as a way of inducing ER stress and UPR was therefore an approach presenting an opportunity for both at least in TBEV infection. Indeed, ectopic expression of TBE viral proteins proved to be a feasible and worthwhile approach as it revealed multiple, viable candidates that could strongly induce ER stress and UPR, but also significant innate signaling (Fig. 18-20) same as was already observed in previous experiments that used pharmaceutical inducers of UPR (Fig. 14). While there isn’t much available literature-wise, the search for and identification of viral proteins that induce ER stress is not a completely untapped area (Brunner et al., 2012; Fung et al., 2015; Frabutt et al., 2018; Siddiquey et al., 2018).

Glycoproteins such as E protein and NS1 being secretory proteins with a necessary requirement to go through the ER, provides them with a somewhat expected ability to induce ER stress in cases of ectopic expression, in fact, NS1 glycoproteins of JEV and DENV2 have been shown to increase XBP1 splicing (Yu et al., 2006). Frabutt and colleagues (2018) showed the induction of ER stress by an up regulation of BiP and XBP1s mRNAs after IAV infection. They followed up and showed the specific potency of IAV HA in inducing XBP1 splicing using a luciferase-based reporter assay to measure IRE1-mediated XBP1 activation in transient transfection of HAs of IAV H1 and H5 subtypes as well as Env glycoproteins of HIV-1 of NL43, JRFL, and SF162 strains using NHK as a positive control. This analysis further revealed the specific involvement of the ERAD pathway and highlights on the glycosylation aspect of the proteins regulating ER stress and specifically phosphorylation of IRE1α that defines the host response and pathogenesis (Hrincius et al., 2015; Frabutt and Zheng, 2016).

The relevance of this is that different viral proteins of different viruses may indeed induce ER stress or fail to do so like was the case of some glycoproteins examined by Frabutt et al., 2018 and indeed in my analysis (Fig. 18). But more importantly, it stresses on how ER stress is central in defining infection outcomes and pathogeneses in viral infections which makes it that much more vital in our understanding of viral infections and even more so as we dissect some mechanistic aspects of the processes involved therein.
What seemed like an odd candidate in this screen though is the NS2B protein. NS2B is one of the 4 small, membrane-anchored proteins of flaviviruses and unlike the glycoproteins; it does not require the use of the ER like glycoproteins do in fact, its hydrophobic terminal regions function to anchor the NS2B3 protease to the ER membrane (Clum et al., 1997). Admittedly, ectopic expression of NS2B may indeed cause an overload of proteins to the ER but this could be by a different mechanism as opposed to the glycoproteins, which have the biochemical attributes to suggest that they can directly overload the ER as described previously. While NS2B has not been well described in ER stress and UPR studies, NS2B3, the flavivirus protease complex responsible for several cleavages of the polyprotein has been observed to induce ER stress in DENV (Cheng et al., 2016) and much more than NS2B or NS3 alone (Yu et al., 2006). These two studies are both favoring the possibility of protease activity being involved in ER stress induction as opposed to what I have observed in my tests (Fig.18-19).

This begs the question as to whether NS2B induces the UPR in a mechanism different from the classical one and this aspect is worth looking into especially with the accumulating literature implicating some non-structural proteins in the formation of replication complexes that are the hallmark of ER membrane rearrangements that accompany infection (Leung et al., 2008; Rothan and Kumar, 2019) and even more specific to TBEV, a polyprotein made up of all non-structural proteins except NS5 has been proven to form replication complex-like structures independent of viral replication fortifying the knowledge that NS proteins drive the remodeling that accompanies infection at least in TBEV (Yau et al., 2019) NS2B proteins of DENV and JEV have been suggested to possess membrane permeability and destabilizing properties (Chang et al., 1999; León-Juárez et al., 2016). While membrane remodelling per se has not yet been proven to induce UPR, the above findings may contribute to some of the speculation that NS2B could be one of the non-structural proteins involved in ER membrane rearrangements during infection.
It is important to restate the emphasis of IRE1 pathway in all the analyses carried out in this work. The IRE1 arm is the most conserved UPR transducer in yeast, plants and metazoans (Maurel et al., 2014). Apart from XBP1s being the classical marker of UPR activation as stated previously, the IRE1 is the effector of choice in the UPR-driven antiviral activity in the course of TBEV infection albeit using a mechanism that does not rely on its RNase activity which leads to splicing of XBP1 and IRE1-dependent decay of RNA (Carletti and Zakaria, 2019). And while this is the current hypothesis, it is admissible to probe into the involvement of one or both of the other arms since this is a different way of UPR induction as compared to TM. Indeed, I observed an up regulation of XBP1s by two different methods as well as its downstream effector of the ERAD pathway, EDEM1 by all three proteins (Fig.18, 19B, C). The upregulation of BiP, DNAJC3, CHOP and GADD34 (Fig. 19) by all three proteins may suggest a partial involvement by more than the IRE1 arm but this observation would be more concrete if the phosphorylation of PERK and/or translocation of ATF6 would be analyzed in these conditions in future experiments pertaining to this work.

If indeed there is involvement of another arm of the UPR, this may be a consequence of individual expression of viral proteins as opposed to infection where multiple proteins may act in concert, modulating each other and somehow dampening or accentuating what could be an effect of a single protein as observed in this case and this logic goes for both induction of UPR as well as innate signaling.

4.5 UPR-driven Innate signaling by E, NS1 and NS2B

As discussed previously, the UPR is increasingly becoming important in innate signaling. An important aspect of the UPR-driven antiviral signaling during TBEV infection is the role played by ISGs. (Fig.14, Carletti and Zakaria, 2019). Some work has shown cross talks between certain arms of the UPR with the NF-κB pathway. Phosphorylation of eIF2α has been shown to activate canonical signaling of NF-κB, same as through the catalytic activity of IRE1 on treatment with TM or TG (Schmitz et al., 2018). Innate sensing that is UPR-driven or
modulated has been observed in multiple factors of the response suggesting an element of UPR being a danger signal in viral infection (Smith, 2014).

The induction of ISGs following expression of viral proteins (Fig. 20) is therefore not completely surprising especially after having confirmed that expression of these proteins can induce UPR (Figs. 18, 19). Same as is the case in HCMV where cytokine stimulation can be mediated by interactions between envelope entry proteins and TLR2 (Boehme et al., 2006), the ability of individual viral proteins to induce some innate response and even more when viral proteins are overexpressed is not completely unlikely.

The ISG induction after transfection of E protein, NS1 and NS2B while a significant observation, it was important to see whether this induction is maintained when cells were infected and more importantly if it inhibits the virus as was the case on infection and treatment with TM (Figs. 11, 14). Quite remarkably, the ISGs observed earlier (Fig. 19) are maintained for some time well into early hours of infection (Fig. 21), and visibly suppressing TBEV multiplication as analyzed by viral yield quantification and western blot (Fig. 22). Most notably is IFI44 that is induced by all 3 proteins after transfection until 8 hours after infection. The function of IFI44 is not well studied but has been shown to target HIV-1 LTR promoter activity facilitating viral latency (Power et al., 2015) and has additionally been speculated to be a natural homolog of Ribavirin, a potent known drug against HCV (Hallen et al., 2007). Ribavirin while quite potent in HCV inhibition, when tested in TBEV, it presented weak or no inhibition activity as opposed to other nucleoside analogues in the same studies (Eyer et al., 2015, Lenz et al., 2018). In addition to IFI44, induction of IFIT1 and Viperin (alias RSAD2) was observed during transfection, but this induction was only sustained by NS2B until 8 hours of infection with TBEV. IFIT1 and Viperin are well known broad antivirals against viruses beyond the Flaviviridae family (Helbig and Beard, 2014; Ng and Hiscox, 2018, Richard Lindqvist and Anna K. Överby, 2018) targeting viral protein synthesis and replication steps respectively (Fig. 7, Schoggins, 2019). IFI44L was only induced by NS2B on ectopic expression and this induction lasted until 8 hours of TBEV infection same as IFIT1 and Viperin.
Like IFI44, IFI44L is not a well-studied ISG but was identified in a FACS-based phenotypic screen of ISGs and proposed to have some antiviral activity against HCV (Schoggins et al., 2011; reviewed by Metz et al., 2013). It was also identified in a microarray analysis as one of the highly induced ISGs underlying inhibition of HIV-1 replication by endogenous TRIM56 on treatment with IFNα (Kane et al., 2016).

Quite surprising though is OASL that is not induced with transfection but is significantly induced only at 4 hours after TBEV infection suggesting the possibility of involvement of an infection component in its induction, this induction proves to be a short-lived as it does not last longer than that. OASL belongs to the OAS family of ISGs, which in other mammals targets viral RNA for degradation by an enzyme RNAsel, which is lacking in humans. However, OASL has been suggested to promote antiviral activity by enhancing the sensitivity of RIG-I activation the sensor of dsRNA intermediates of infection (Zhu et al., 2014, 2015) and favoring replication for some DNA viruses (Ghosh et al., 2017; Bussey et al., 2018) suggesting a differential activity in different infections.

Whatever the mechanism of action and/or targets of these ISGs is beyond the current scope of this work but their collective effect seems to translate to TBEV inhibition and quite interestingly, while at 24 hours all the ISGs are not induced to any level of significance, the earlier effects of induction sustain TBEV inhibition until 24 hours after infection (Fig. 22). These results point to a role of ISGs in TBEV inhibition achieved by the UPR-driven activation by E, NS1 and NS2B proteins and pointing to NS2B as the protein with an ability of sustaining longer kinetics of ISG induction. Additionally, it brings forth IFI44 (Fig. 22E) as an important ISG that is induced by all the 3 proteins until 8 hours of infection and probably plays the most central role in TBEV inhibition at least in the conditions of E and NS1 expression (Fig. 21).

4.6 TBEV NS2B, more than just a protease cofactor?

A rather surprising aspect of this what work has been all the findings with NS2B (Fig. 18-23). NS2B has stood out in its potency in inducing UPR and ISG induction
and presents us with an appealing and scarcely studied viral protein that future work may focus on.

Having observed some of the strongest effects on UPR induction and ISG induction in the condition of NS2B expression (Fig. 18-20), the ability of NS2B to increase the IFNβ reporter activity was just another piece to the puzzle that is NS2B (Fig. 23B). This effect was highly significant when NS2B was co-transfected with the sensor RIGI that is the PRR of choice in TBEV infections. The ability of certain viral proteins to initiate the first wave of innate signaling has been suggested in the past (Mogensen and Paludin, 2001) as it would seem is the case with NS2B. And while RIG-I is constitutively expressed, albeit at low levels, its expression is enhanced by activation of IFN-α/β signalling that is also the case when NS2B is transfected (Fig. 23A, B).

However, it would be interesting to understand the mechanism of action of NS2B and its link to the antiviral activity exhibited to a regulatory factor downstream of RLR-sensing such as IRF3.
4.7 Concluding Remarks

In my doctoral study, I embarked on a journey to analyze the effects of current UPR inducers to cell growth in the experimental settings employed for most experiments reported herein and find alternatives if any for ER stress and UPR induction.

The findings of this work have divulged a feasible approach of inducing UPR in U2OS cells using TBE viral proteins and in doing so gave us a way of narrowing down from 10 TBE viral proteins to what may be the culprits of UPR induction during infection. E protein, NS1 and NS2B proteins of TBEV may be the viral components that drive ER stress and UPR induction during TBEV infection. Moreover, they hold promise as tools to investigate the UPR in a more controlled, authentic stance and provide an alternative to the current pharmaceutical inducers.

The induction of UPR by these proteins is accompanied by innate immune signalling and TBEV inhibition, complementing quite well recent findings from our laboratory (Carletti and Zakaria et al., 2019) highlighting a key role played by ISGs in the UPR-induced antiviral state achieved by treatment with TM (Fig. 13), as is observed in the expression of E, NS1 and NS2B viral proteins (Figs. 19, 20) Molecular mechanisms of action of these proteins in their induction of ER stress remains an area to be explored and while the mechanism of E protein and NS1 can be speculated, NS2B is rather more fascinating in this case. This will provide more insights on flavivirus NS2B as a stand-alone protein quite distinctly from how it has been known and studied in literature under the shadow of the NS2B3 protease complex.

NS2B brings forth even more the notion that non-structural proteins may be key players in membrane rearrangements that accompany infection. Whether membrane remodelling is enough to induce UPR independent of infection remains a question to be answered but this question offers one line of speculation as to how NS2B may induce ER stress and UPR. The established ability of NS2B to up regulate the IFNβ promoter activity (Fig. 22) adds to a role it
plays in the collective innate signalling that is observed on transfection of this protein.
Further probing into some of these aspects may be useful in establishing the mechanism(s) of UPR induction and innate signalling achieved on transfection of all proteins studied but even more so for NS2B, having confirmed that it acts in concert with RIG-I, the receptor of choice in TBEV infection. These factors make NS2B subject to more investigation and may add more to the gap of knowledge in the study of membrane rearrangements that is a common feature among flaviviruses.
5. APPENDICES
Appendix 1: Carletti and Zakaria et al., 2019

The innate response to a pathogen is critical in determining the outcome of the infection. However, the interplay of different cellular responses that are activated following viral infection and their contribution to innate antiviral signalling has not been clearly established. This work shows that flaviviruses, including Dengue, Zika, West Nile and Tick-borne encephalitis viruses, activate the unfolded protein response before transcription of interferon regulatory factor 3 induced genes. Infection in conditions of unfolded protein response priming leads to early activation of innate antiviral responses and cell intrinsic inhibition of viral replication, which is interferon regulatory factor 3 dependent. These results demonstrate that the unfolded protein response is not only a physiological reaction of the cell to viral infection, but also synergizes with pattern recognition sensing to mount a potent antiviral response.
Flaviviruses are a family of relevant human pathogens delivered by mosquitoes or ticks. Dengue virus (DENV), Zika virus (ZIKV), West Nile virus (WNV), and tick-borne encephalitis virus (TBEV) are only few examples affecting tropical countries and Europe. The genome of Flaviviruses is a single RNA filament of positive polarity encoding a polyprotein precursor, which is processed into structural and nonstructural proteins. The RNA genome is replicated by the viral RNA-dependent RNA polymerase through a complementary template of negative polarity, which forms transient double-stranded RNA (dsRNA) intermediates. Infection induces important rearrangements of cytoplasmic membranes of the endoplasmic reticulum (ER) with the formation of characteristic replication vesicles containing dsRNA and replicative proteins.

Target cells respond to viral infection by activating innate defense mechanisms. Cytoplasmic pattern recognition receptors (PRRs) recognize viral RNA intermediates as pathogen-associated molecular patterns (PAMPs) to trigger the interferon (IFN)-mediated antiviral response. The innate immune response is generally associated to the activity of IFN leading to the induction of interferon-stimulated genes (ISGs) endowed with antiviral activity. However, at very early time points post infection, cell intrinsic mechanisms of defense play an important role in mediating an antiviral response, while IFN remains essential to protect neighboring uninfected cells and to contain the spread of infection.

Virus infection can also lead to ER stress by unscheduled accumulation of viral proteins or modification of ER membranes. Accumulation of unfolded or misfolded proteins in the ER leads to a stress response by activating the unfolded protein response (UPR) pathway, which restores ER homeostasis. Three transmembrane ER proteins mediate the UPR: the inositol-requiring enzyme 1 (IRE1), the protein kinase RNA-like ER kinase (PERK), and the activating transcription factor 6 (ATF6). Activated IRE1 cleaves the X-box binding protein 1 (XBP1) mRNA in the cytoplasm leading to the spliced form, which is translated into an active transcription factor (XBP1s). Activated PERK phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) causing inhibition of protein synthesis, but also enhanced translation of the activating transcription factor 4 (ATF4). ATF4 in turn promotes transcription of several genes including the CCAAT/enhancer-binding protein-homologous protein (CHOP), a proapoptotic transcription factor, and feedback regulators, which counteract the phosphorylation of eIF2α. Activation of the ATF6 pathway leads to the processing of ATF6 into a cleaved product that translocates to the nucleus to activate UPR-regulated genes including Xbp1.

It has been previously shown that sterile co-stimulation of the UPR with certain PAMPs such as lipopolysaccharide or Polyinosinic:polycytidylic acid greatly potentiates the UPR with certain PAMPs such as lipopolysaccharide or Polyribozyme that translocate to the nucleus to activate UPR-regulated ATF6 pathway leads to the processing of ATF6 into a cleaved protein that translocates to the nucleus containing dsRNA and replicative proteins.

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It has been previously shown that sterile co-stimulation of the UPR with certain PAMPs such as lipopolysaccharide or Polyinosinic:polycytidylic acid greatly potentiates the expression of IFNβ. These observations led to the intriguing hypothesis that UPR and PRR signaling synergize during infection to provide optimal antiviral immunity.

In this work, transcriptome analysis of TBEV infected cells shows upregulation of a number of genes involved in the UPR. Careful temporal analysis demonstrates that the IFN response is a late event preceded by the UPR. Most importantly, preactivation of the UPR during flavivirus infection causes a decrease of viral titers, an earlier induction of IRF3 phosphorylation and translocation, and of IFN and ISGs transcription. IRF3 depletion rescues flavivirus replication induced by UPR priming. Furthermore, depletion of IRE1, but not of ATF6 or PERK, enhance viral replication and rescue specifically TBEV from antiviral priming of the UPR. This IRE1 function is independent of its RNase activity, but dependent on IRF3 and RIG-I. Hence, these data demonstrate that the UPR is a very early cellular response to TBEV infection, which triggers an IRF3/RIG-I dependent cell intrinsic antiviral response through IRE1.

Results

Transcriptome analysis of TBEV infection. In previous studies a consistent delay of the IFN beta (IFNβ) response, following flavivirus infection, was described. Viral replication could be detected as early as 8 h post-infection (h.p.i.), while IFNβ mRNA appeared only after 16 h.p.i. Starting from this observation, in order to investigate the cellular pathways that could be activated in the infected cells before IFNβ induction, an unbiased transcriptome analysis of infected cells was conducted. Newly synthesized TBEV RNA was already high as early as 10 h.p.i., a time point, when IFNβ mRNA could not be detected. As expected, IFNβ mRNA was eventually upregulated at 24 h.p.i. Two these two time points were chosen to conduct the transcriptome analysis of the infected cells. Total RNA was extracted from infected cells in triplicate independent experiments and subjected to high-content sequencing. Differential analysis of the transcriptome of U2OS cells infected with TBEV at 24 versus 10 h.p.i. showed significant upregulation of 437 genes (fold change ≥2) and downregulation of 318 genes (fold change ≤2) with a false discovery rate of <0.05 (DESEQ2 statistical analysis) (Fig. 1A). Ingenuity Pathway Analysis (IPA) indicated the UPR as the most highly significant canonical pathway (−log(p-value) = 7.37) followed by ER stress (−log (p-value) = 3.93). Several genes showed upregulation, including HSPA5 (Heat-Shock 70 kDa Protein 5, BiP), Xbp1, Xbp1s, DDIT3 (CHOP), and the chaperones DnaJ (Hsp40 Homolog, Subfamily C, Member 3 (DNAJC3/P58IPK) and Subfamily B, Member 9 (DNAJB9). To conclude, significant early activation of UPR-related genes was observed during TBEV infection.

UPR is induced before the interferon response following infection. The kinetics of UPR induction was analyzed temporally following TBEV infection. As shown in Fig. 1, TBEV infection of U2OS cells was productive as early as 10 h.p.i. (Fig. 1B) with TBEV RNA being detectable at 8 h.p.i. (Fig. 1C). At variance, IFNβ mRNA appears only after 16 h.p.i. (Fig. 1D). UPR genes such as Xbp1, DNAJC3, and DNAJB9 also appear at late time points, concomitantly with IFNβ mRNA (Fig. 1E and Supplementary Fig. 1A and 1B). However, the spliced form of Xbp1 mRNA, an indicator of IRE1 activation, could be detected as early as 12 h.p.i. (Fig. 1F). Similarly, the PERK-dependent activation of CHOP occurred before the IFNβ response (Fig. 1G). Indeed, PERK phosphorylation could be detected at 8 h.p.i. followed by phosphorylation of the eIF2α (Fig. 1H). The third arm of the UPR response is initiated by nuclear translocation of ATF6. To monitor the ATF6 pathway, GFP-ATF6 was transfected in U2OS cells followed by TBEV infection. As shown in Supplementary Fig. 1C and quantified in Fig. 1I, translocation of GFP-ATF6 into the nucleus of infected cells occurred from 8–12 h.p.i. Consistently, UPR genes that are activated downstream of the ATF6 pathway, such as BiP (Fig. 1I), were also induced following TBEV infection.

Induction of UPR leads to early activation of an innate antiviral response during flavivirus infection. To summarize the above findings, all three arms of the UPR were activated at early time points following TBEV infection, prior to IFNβ induction. Therefore, the UPR could be a prerequisite for a proper antiviral response. To address this hypothesis, U2OS cells were exposed to Tunicamycin (TM), a well-known inducer of the UPR, immediately after TBEV infection. As shown in Fig. 2A, b, viral yields and
viral RNA were markedly reduced following TM treatment. Since TM inhibits N-linked glycosylation and could potentially affect viral infectivity, viral RNA levels were also investigated. To note, at 8 h.p.i., while viral yields were not yet increasing (Fig. 2a), there was a significant inhibition of viral replication in the presence of TM (Fig. 2b), demonstrating that this early antiviral effect is independent of any unspecific activity on the glycosylation of viral proteins. As a control of TM activity, Xbp1s mRNA was also induced at early time points in the presence of TM (Fig. 3). Interestingly, induction of IFNβ mRNA occurred much earlier following preactivation of the UPR. As shown in Fig. 3d, treatment of U2OS cells with TM alone (gray bars) stimulated a weak tenfold increase of IFNβ mRNA only after 24 h of treatment. However, upon both TBEV infection and TM treatment, IFNβ was clearly induced as early as 8 h.p.i. (white bars).

To rule out any unspecific effect of TM, the same approach was repeated using Thapsigargin (TG), which activates UPR by blocking the ER calcium ATPase. As shown in Supplementary Fig. 1D and E, both TM and TG inhibit TBEV, but, while TM showed a partial effect on E protein glycosylation as expected, TG showed a more pronounced effect. However, upon both TBEV infection and TM treatment, IFNβ mRNA was induced at low levels by TG alone after 24 h of treatment, but synergized with TBEV treatment to potentiate the innate response. A similar analysis was conducted on other members of the flavivirus family such as DENV2 (Fig. 2g, h), WNV (Fig. 2i, j), etc.
and ZIKV (Fig. 2k, l). Likewise, induction of the UPR by TM resulted in a decrease of virus yields and an early IFNβ response demonstrating that early activation of the UPR leading to a sustained IFNβ response is a general mechanism within flaviviruses.

The UPR-induced antiviral response is independent of canonical interferon signaling. Since early IFN activation was consistently observed following flavivirus infection in the presence of UPR inducers, it was plausible to hypothesize that the decrease of virus titers could depend on interferon-dependent antiviral signaling. In order to address this, primary embryonic murine fibroblasts, derived from IFN receptor knockout mice (MEF ifnar−/−), were infected in the same experimental conditions. Noteworthy, as shown in Fig. 3, wild-type MEF reproduced the same phenotype observed as in U2OS cells, reinforcing the previous observations also in a nontransformed primary murine cell model. MEF ifnar−/− showed an antiviral response and an early induction of IFNβ in the presence of TM, which was kinetically similar to that observed in wild-type MEFs (Fig. 3a, b). This observation points to an IFN-signaling independent antiviral activity.

A subset of ISGs, with antiviral activity, that are known to be induced directly by the transcription factor IRF3, have been previously described17, 18. IRF3 phosphorylation, measured following infection, occurred at earlier time points and to a greater magnitude in TM-treated cells as compared with control (Fig. 3c). Consistently, nuclear translocation of IRF3 was significantly increased at 8 h.p.i. compared with infection alone in the same experimental conditions (Fig. 3d, e). Transcriptional induction of IRF3-dependent ISGs, such as IFIT1 or Viperin, showed early activation kinetics in the presence of TM, comparable with what was observed for IFNβ (Fig. 3f, g).

Fig. 2 Modulation of the interferon response to flavivirus infection by the UPR. a) Tunicamycin treatment inhibited TBEV yields. U2OS cells were either infected with TBEV at moi = 1 (blue bars) or treated with 1 μg/ml Tunicamycin (TM) immediately after infection (black bars). At the indicated time points, supernatants from infected cells were used to infect Vero cells to measure virus yields (PFU/ml). b–d) Tunicamycin treatment inhibited TBEV RNA, increased Xbp1s and led to early IFNβ induction. U2OS cells were infected as in a. Total RNA was extracted at the indicated time points and used as template for qPCR using primers specific for TBEV 5′-NCR (b), Xbp1s (c) or IFNβ (d). β-actin was used for normalization and data plotted as fold change from time 0. Values of IFNβ mRNA from mock-infected cells treated with Tunicamycin alone were also indicated (d, azure bars). e) Tunicamycin treatment inhibited TBEV yield and led to early IFNβ induction. U2OS cells were infected with TBEV at moi = 1 (blue bars) or treated with 0.5 μM Tunicamycin (TG) immediately after infection (black bars) and treated as above (b, d). f i) Tunicamycin treatment inhibited DENV2, WNv, and ZIKV yields and led to early IFNβ induction. U2OS cells were either infected (blue bars) or treated with Tunicamycin (TM) immediately after infection (black bars). At the indicated time points, supernatants from infected cells were either used to infect Vero cells to measure virus yields (DENV2, f, WNv, g, ZIKV, h) or total RNA was extracted and used as template for real-time qPCR to quantify IFNβ mRNA (DENV2, h, WNv, j, ZIKV, k). Statistics as already described in the legend of Fig. 1. Source data are provided as a Source Data file.
In order to better characterize this phenomenon a transcriptome analysis was conducted on: (i) cells infected with TBEV at 8 h.p.i.; (ii) cells treated with TM for 8 h and (iii) cells both infected and treated with TM at the same time point. IPA indicated the UPR as the most highly enriched canonical pathway ($p$-value = 7.97$^{-12}$) induced by sterile TM treatment, while for TBEV infected cells alone at 8 h.p.i. no enriched pathways were indicated, which is in line with the observation that UPR is activated from 10 h.p.i. (Fig. 1). IPA indicated ‘activation of IRF by cytosolic PRRs’ as the most highly enriched canonical pathway ($p$-value = 3.04$^{-65}$) for the dataset of cells infected with TBEV and treated with TM. Other top-ranked pathways were ‘interferon signaling’, ‘role of PRRs in recognition of bacteria and viruses’, and ‘role of RIG-1 like receptors in antiviral innate immunity’ strongly supporting the hypothesis. Differential analysis of the two groups, i.e., treated with TM alone and infected +TM, indicated that the 39 genes that were differentially modulated in the two conditions partition in two functional clusters that defined each condition as clearly visible in the heatmap (Fig. 3h). The cluster of genes differentially regulated during infection in conditions of UPR induction showed a high prevalence of ISGs. In particular ISG15, IFIH1, IFI44L, SAMD9, SAMD9L, ZC3HAV1, PARP10, DDX58, and OAS1 have been already described as antiviral effectors$^{19–23}$. A subset of these ISGs that include OAS1L, IFIH1, IFI44, and IFI44L have been validated by RT-qPCR.
The UPR-dependent anti-TBEV response is IRE1 and IRF3/RIG-1 dependent. The UPR activates three signaling pathways mediated by the ER transmembrane proteins ATF6, IRE1, and PERK. Each of them was targeted by RNAi to investigate their involvement in antiviral signaling following TBEV infection. U2OS cells were treated with siRNA (IRE1 and ATF6) or with shRNA (PERK) to deplete cells of the respective proteins. As shown in Fig. 4a–d, depletion of PERK or ATF6 did not result in significant changes of viral yields. Depletion of PERK was also obtained by siRNA transfection with similar results (Supplementary Fig. 3A and 3B). Conversely, depletion of IRE1 resulted in a higher TBEV titer (Fig. 4e, f) and enhanced TBEV RNA levels (Fig. 4g), but not IFNβ mRNA (Fig. 4h). Therefore, the IRE1 pathway appears principally responsible for UPR-mediated antiviral signaling for TBEV. Indeed, IRE1 depletion partially rescued replication of TBEV following TM treatment (Fig. 4i, j). From previous studies it was discovered that the IFN response against TBEV infection depends on the activity of the PRR RIG-1 leading to IRF3 induction15. In order to understand if the antiviral activity triggered through IRE1 was dependent on IRF3, U2OS cells were treated with a siRNA against IRF3 and then infected and subjected to TM treatment. As shown in Fig. 4k, l, IRF3 depletion almost completely rescued viral replication in TM-treated cells. U2OS cells were treated as above (i, j) in conditions of IRF3 depletion. Immunoblot for IRF3 and TBEV RNA quantification was performed at 24 h.p.i. m, n RIG-I depletion partially rescued TBEV replication in TM-treated cells. U2OS cells were treated as above (i, j) in conditions of RIG-I depletion. Immunoblot for RIG-I and TBEV RNA quantification was performed at 24 h.p.i. Statistics as already described in the legend to Fig. 1. Source data are provided as a Source Data file.
IRE1, which leads to Xbp1 splicing and IRE1-dependent decay of RNA (RIDD) activity. Xbp1 transcription factor has been shown to directly activate IFN, while the RIDD activity has been proposed to generate substrates for RIG-I signaling31,32. However, pretreatment of TBEV infected cells with 4µg/ml, a specific inhibitor of IRE1 RNAse activity capable of inhibiting Xbp1 splicing (Supplementary Fig. 3C), didn’t show any modulation of virus infectivity (Supplementary Fig. 3D). These data indicate that IRE1-mediated pathways other than Xbp1 splicing and RIDD activity need to be considered to explain the UPR-dependent antiviral signaling induced by TBEV infection.

Other flaviviruses, such as WNV, DENV2, and ZIKV, which have been shown previously to be inhibited by early induction of the UPR with TM (Fig. 2g–i), also respond in terms of rescued viral replication in conditions of IRF3 depletion (Supplementary Fig. 4A–F). These experiments reinforce the notion that there is a causal link between virus-induced ER stress and innate immune sensing and that this feature is shared among different flaviviruses. However, WNV replication inhibited by TM could not be rescued in IRE1 depleted cells (Supplementary Fig. 4G and 4H). This observation indicates that while TBEV appears to depend principally on the IRE1 pathway, other flaviviruses may require the contribution of ATF6 or PERK.

Discussion
In this work, virus-induced UPR is shown to play a pivotal role in the cell intrinsic innate antiviral response to flaviviruses. UPR and innate sensing have been shown previously to synergize following sterile stimulation10–13. However, evidence of this mechanism in infected cells is lacking. The experimental evidence presented here in the context of flavivirus infection points to a direct role of the UPR to trigger a suboptimal activation of the IRF3 pathway, which synergizes with PRR signaling to mount a potent antiviral defense.

This work stems from previous observations that identified a delayed IFN response following flavivirus infection14,19. Transcriptome analysis of TBEV infected cells identified the UPR and ER stress pathways as early responses of the cell to infection prior to the IFN response. Similar findings were recently observed for cells of neuronal origin infected with TBEV25. Hence, a temporal analysis of UPR activation was performed to better understand the cellular events that follow infection (Fig. 4). IRE1 activity was initially assessed in terms of rescued viral replication also for WNV, DENV2, and ZIKV, indicating a general mechanism of UPR priming of antiviral innate immunity. However, IRE1 dependence could be demonstrated for TBEV, but not for WNV (Supplementary Fig. 4G and 4H). This observation is in line with the differences in UPR response among flaviviruses as mentioned above and will require further analysis.

PRR signaling is believed to depend on the masking of specific viral PAMPs during infection, particularly RNA replication intermediates for flaviviruses15. Disruption of these components that allow PRR access to agonist RNA is therefore required for full activation of the IFN response. It is possible to conceive that the UPR also triggers the modification of membranes or membrane-associated protein complexes that eventually mask the viral RNAs allowing PRR function. Recent loss-of-function screens identified novel ER-associated factors required for flavivirus replication, which could provide hints on the cellular factors involved in this process4,35.

To conclude, several lines of evidence point to a direct relationship between the UPR and PRR-mediated activation of cell intrinsic innate antiviral signaling. Independent pathways cooperate to respond to viral infection and to overcome viral subversion strategies in the incessant battle between flaviviruses and the host cell.
Materials and methods

Cells and viruses. TBEV represents a good model of the Flavivirus genus because it is easily manipulated leading to robust infection in vitro. Furthermore, U2OS cells have been previously used to maintain an intact PRR-IFN signaling pathway with respect to TBEV infection 13. Vero clone E6 (ATCC C1008) and human osteosarcoma U2OS cells (ATCC HTB-96) were grown under standard conditions in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Primary murine embryonic fibroblasts (MEF; 5-fluorodeoxyuridine) from mice lacking the α-chain of the IFN-α/β receptor on a B6 background were kindly provided by U. Kallinikos (Tübingen, Germany). Pregnant female mice at 13–14 days of gestation were sacrificed and uteri were removed with the help of forceps and washed with sterile PBS to remove blood. Embryos were carefully separated from the uterus of pregnant mice at 13–14 days of gestation and their head and liver were removed. Each embryo was minced, treated with trypsin EDTA and plated in growth medium. Low-passage MEFs were used for the experiments. Wild-type MEF were similarly obtained from isogenic B6 animals. Animal care and treatments were conducted in conformity with institutional guidelines in compliance with national and international laws and policies.

Working stocks of TBEV (strain NeuDeero), West Nile virus (strain NY99), Dengue virus type 2 (strain New Guinea), and Zika virus (strain Uganda #976) were routinely propagated and titrated on Vero E6 cells. UPR inducers Tunicamycin or Thapsigargin (Sigma-Alrldich) were added to cells 1 h after infection (h.p.i.). IRE1 Inhibitor 4 was diluted and kept at −80 °C until use. U2OS cells were transduced in presence of polybrene (hexadimethrine bromide, Sigma-Alrldich) according to the manufacturer’s instructions. Transduced cells were selected using 2 μg/ml puromycin.

LV Production and shRNA delivery. Lentiviral silencing vectors were derived from pLKO.1 TRC (Addgene). The control short hairpin RNA (shCTRL) was the pLKO.1 scramble sequence from Addgene. For PERK targeting, a specific sequence was designed and cloned into pLKO.1 TRC (shPERK) using the oligonucleotides 5′-CCGGGACACGCTTAACTGTTTAGATTTCCGTTCC-3′ and 5′-AACTTTGAAGAGAACGATCTTCAAGGTATTTCCGTGTTCC-3′. Packaging in HEK 293T cells was performed according to standard procedures using the packaging plasmid psPAX2 and PMD2G (Addgene). Cell supernatants were filtered and kept at −80 °C until use. U2OS cells were transduced in presence of polybrene (hexadimethrine bromide, Sigma-Alrldich) according to the manufacturer’s instructions.

RNA interference. Pools of small interfering RNAs (siRNAs) were obtained from Dharmacon. ON TARGET plus NonTargeting Pool was used as a control in all experiments. ON TARGET plus SMARTpool siRNA was used for the depletion of ATTP1, PERK, IRE1, and RIG-1. U2OS cells were transfected with siRNAs at a concentration of 100 nM, using Lipofectamine RNAiMAX transfection Regent (Invitrogen) according to the manufacturer’s instructions.

Western-blotting and immunofluorescence. For immunoblotting, whole-cell lysates were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. The blots were blocked in 4% nonfat milk in Tris-buffered saline (TBS) plus 0.1% Tween 20 (TBST), followed by incubation with the primary antibody diluted in the same solution at 4 °C overnight. After washing three times with TBST, secondary antibodies were then added to the blots. HRP-conjugated secondary antibodies were incubated for 1 h at room temperature. The blots were developed using a chemiluminescent HRP substrate (Millipore). For immunofluorescence (IF), cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, incubated for 5 min with 100 mM glycine, and permeabilized with 0.1% Triton X-100 for 5 min. Subsequently, the cells were incubated at 37 °C for 30 min with PBS, 1% bovine serum albumin, and 0.1% Tween 20 before incubation with antibodies. The cover slips were rinsed three times with PBS, 0.1% Tween 20 (washing solution) and incubated for 1 h with secondary antibodies. The cover slips were finally washed three times with washing solution and mounted on slides using Vectashield mounting medium (Vector Laboratories). Fluorescence images of fixed cells were captured on a Zeiss LSM510 Meta confocal microscope with a 63 × numerical-aperture 1.4 Plan-Apochromat oil objective. Further details can be found in previous publications 17,37. The following antibodies were used in this study: a rabbit polyclonal against the TBEV E protein produced in our laboratory following immunization with the inactivated virions (1:100 IF; 1:1000 WB), a rabbit polyclonal against TBEV prM kindly provided by Franz Heinz, Vienna (1:1000; WB, cat.no. sc-33826); a rabbit polyclonal against human eIF2α from SCBT (1:1000 WB, cat.no. sc-7680); a rabbit polyclonal against phosphorylated (Ser51) human eIF2α from Cell Signaling (1:500 WB, cat.no. 9271); a rabbit polyclonal against human phosphoS6K from SCBT (1:500 WB, cat.no. sc-15370); a rabbit polyclonal against human PERK from Cell Signaling (1:1000 WB, cat.no. 21921); a rabbit polyclonal against phosphorylated (Thr94) human PERK from SCBT (1:200 WB, cat.no. sc-35277); a mouse monoclonal against human PRK from SCBT (1:200 WB, cat.no. sc-13042); a rabbit monoclonal against human β-catenin from Transduction Lab (1:2000 WB, cat.no. 610153); a mouse monoclonal against human β-actin conjugated with peroxidase from Sigma-Aldrich (1:50000 WB, A3854); a rabbit monoclonal against IRE1 from Cell Signaling (1:1000 WB, cat.no. 34032); a mouse monoclonal against dsDNA from English & Scientific Consulting (1:100 IF, cat.no. I2-1101) a rabbit monoclonal against phospho-IRE3 from Cell Signaling (1:500 WB, cat.no. 4947); a mouse monoclonal against ATF4 from Abcam (1:500 WB, cat.no. ab122897); a rabbit monoclonal against IRE1 from Cell Signaling (1:1000 WB, cat.no. 3294); a mouse monoclonal against RIG-I from Adipogen (WB 1:500, cat.no. AG-20B-0009). Secondary antibodies conjugated with Alexa648(488)/594 were from Life Technologies (1:500 IF, cat.no. anti-mouse 1:5042120 and anti-rabbit 488 A-21206) and peroxidase conjugates from Dako (1:5000 WB, cat.no. anti-rabbit P0448 and anti-mouse P0447).

Real-time quantitative reverse transcription PCR. For real-time quantitative reverse transcription PCR (qPCR) total cellular RNA was extracted with the UPzol (Biotegratech) and total RNA was extracted at time 0, 10, and 24 h post-infection (h.p.i.). For the second transcription analysis (Fig. 3) U2OS cells were infected with TBEV at a moi of 1 PFU/cell and after 1 h media was replaced with normal growth medium or DMEM plus TM. Cells were lysed with UPzol and total RNA was extracted from each condition at 0 and 8 h.p.i. Quality of extracted RNA was checked by gel electrophoresis (ribosomal 18S and 28S), spectrophotometric analysis (260/280 > 1.8), and Agilent bioanalyzer (RNA integrity number, RIN ≥ 8). All cDNA libraries of poly-A-containing mRNA molecules were prepared using Illumina TrueSeq standard protocol. Libraries were pooled and sequenced on two different Illumina Platforms. The first run was performed on Hissequ2000 4 plex run single reads, 50 bp reads, while the second run was performed on Hisconda8 8 plex run pair-end reads, 2×100 bp reads. All data were subjected to quality control using FastQC software. Single reads were mapped against the human genome RNA-reference from NCBI using CLCbio software, while pair-end reads were mapped against Homo sapiens GRC38 37.77 reference from UCSC using STAR software 38. Bioconductor packages DESeq2 version 1.18.18 and BHWE version 1.6.0 in the framework of the R software version 3.4.3 was used to perform differential gene expression analysis of infected cells with TBEV at 24versus 10 h.p.i and cells infected with TBEV and treated with TM at 8 h.p.i versus untreated cells treated with TM at the same time point. The package is based on the negative binomial distribution (NB) to model the gene reads counts and shrinkage estimator to estimate the per-gene NB dispersion parameters. Specifically, rounded gene counts were used as input and the per-gene NB dispersion parameter was estimated using the function DESeq for DESEQ. The RNA-seq workflow recommendations 39 were used to detect outlier data after normalization and to improve testing power, while maintaining type I error rates. Independent Hypothesis Weighting was used as multiple testing procedure. Estimated p-values for each gene were adjusted using the Benjamini-Hochberg method 40. Genes with adjusted p < 0.05 and absolute logarithmic base twofold change >1 were selected. Data were finally analysed with the IPA software.

Statistics. Typically three independent experiments in triplicate repeats were conducted for each condition examined. Average values are shown with standard deviation and p values are reported with a paired two-tailed t-test. Only significant p-values are indicated by the asterisks above the graphs (** p < 0.01 highly significant; *p < 0.05 significant). Where asterisks are missing the differences are calculated as nonsignificant (n.s.).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data underlying Figs. 1 B–D, 2 A–L, 3 A–C and E–H, 4 A–N, Supplementary Figs. 1A, B and D–F, 2 A–L, 3 A–H, and 4 A–H are provided as Source Data files. All other data are available from the corresponding author upon reasonable requests. RNAseq data have been deposited with links to BioProject accession number PRJNA474353 in the NCBI BioProject database [https://www.ncbi.nlm.nih.gov/bioproject/]. Sequence Read Archive (SRA) submission: SUB4411543—1st of June 2018, SRA accession: SRP149625.

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Author contributions

T.C. and M.K.Z. performed experiments, analyzed the data, generated hypothesis, and contributed to the writing of the paper; V.F. contributed to virology experiments; L.R. contributed to the AT6 data; Y.K. contributed to qPCR, virology, and protein analysis; D.L. performed the RNAseq and the analysis of data; A.M. conceived the work, analyzed the data, and wrote the paper.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-11663-2.

Competing interests: The authors declare no competing interests.

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Appendix 2: Interferon-Stimulated Genes identified in the differential analysis from the Whole genome transcriptome analysis (Fig.13)

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<th>ISG Code</th>
<th>Description</th>
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