

BiP: Master Regulator of the Unfolded Protein Response and Crucial Factor in Flavivirus Biology

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Flaviviruses have an intimate relationship with their host cells, utilizing host proteins during replication. Much of viral genome replication and virion assembly occurs on and within the endoplasmic reticulum (ER[†]). As a cellular protein folding hub, the ER provides an ideal environment for flaviviruses to replicate. Flaviviruses can interact with several ER processes, including the unfolded protein response (UPR), a cellular stress mechanism responsible for managing unfolded protein accumulation and ER stress. The UPR can alter the ER environment in several ways, including increasing ER volume and quantity of available chaperones, both of which can favor viral replication. BiP, a chaperone and master regulator of the UPR, has been demonstrated to play a key role in several flavivirus infections. Here we describe what is known in regard to BiP, its implicated role with flavivirus infection, and what remains to be discovered.

INTRODUCTION

A eukaryotic cell is a tightly controlled biological microcosm. Multiple metabolic and regulatory pathways must function in concert with each other to perform normal cellular processes, maintain homeostasis, and manage a variety of internal and external insults. Many of the cell's functions are dependent upon proteins, and

the proper regulation of protein production is a critical feature of cellular wellbeing. Consequently, cells have evolved systems to cope with aberrant protein synthesis. The unfolded protein response (UPR) is one cellular stress mechanism designed to facilitate proper protein folding and to prevent accumulation of unfolded proteins in the ER lumen. Such accumulation of unfolded proteins often occurs when the regulation of protein synthesis is

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†Abbreviations: BiP, binding immunoglobulin protein; ER, endoplasmic reticulum, GRP78, glucose regulated protein 78; HSP70, heat shock protein 70 kDa; HSPA5, heat shock 70kDa protein 5; UPR, unfolded protein response; ATF6, activating transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, PKR-like ER kinase; S1P, site-1 protease; S2P, site-2 protease; XBP1, x-box binding protein 1; RIDD, regulated IRE1-dependent decay; ERAD, ER associated protein degradation; TRAF2, TNF receptor associated factor 2; TRAF6, TNF receptor associated factor 6; JNK, c-Jun amino-terminal kinase; eIF2 α , eukaryotic initiation factor 2 α ; ISR, integrated stress response; ATF4, activating transcription factor 4; CHOP, CCAAT-enhancer-binding protein homologous protein; GADD34, growth arrest and DNA damage inducible protein 34; ERO1 α , endoplasmic reticulum oxidoreductase 1 α ; PDI, protein disulfide isomerase; GRP94, glucose regulated protein 94; HSC3, heat shock protein cognate 3; MBFV, mosquito-borne flavivirus; TBFV, tick-borne flavivirus; TBEV, tick-borne encephalitis virus; POWV, Powassan virus; DENV, dengue virus; JEV, Japanese encephalitis virus; WNV, West Nile virus; WNV_{KUNJ}, West Nile Virus Kunjin Subtype; E, envelope; NS3, nonstructural protein 3; prM, pre-membrane protein; hpi, hours post infection; EDEM1, ER degradation enhancing alpha-mannosidase-like protein 1; NS4A, nonstructural protein 4A; NS4B, nonstructural protein 4B; MEF, mouse embryonic fibroblast; NS2B, nonstructural protein 2B; VOPBA, viral overlay protein binding assay; LGTV, Langkat virus.

Keywords: BiP, GRP78, unfolded protein response, dengue, Japanese encephalitis, West Nile, Kunjin, tick-borne encephalitis virus

disrupted, either intrinsically, such as by a mutation in a regulatory element, or extrinsically, such as by unrestricted viral protein translation. The UPR functions to mitigate imbalance by increasing capacity for proper protein folding in the ER and decreasing the load of newly synthesized peptides. Binding immunoglobulin protein (BiP) acts as a master regulator for the process, and thus plays a key role in the UPR.

Viruses, as obligate intracellular pathogens, rely heavily on host translation machinery to manufacture proteins. Due to their extrinsic nature, viral protein translation is subject to different restrictions than host proteins, and often results in ER peptide burden stress. Because of the critical role the UPR plays in maintaining protein balance intracellularly, several viruses, including flaviviruses, can co-opt the pathway to modulate the cell environment to better meet their replication needs. In this brief review, we first examine the function of BiP and the UPR, and then describe the current knowledge of UPR-flavivirus interactions.

BIP PROTEIN FUNCTION

BiP, also known as glucose regulated protein 78 (GRP78), is a member of the heat shock protein 70kDa (HSP70) family of proteins (specifically HSPA5). It functions as a chaperone, selectively binding unfolded proteins in the ER lumen by interacting with exposed hydrophobic residues on nascent peptides [1]. Similar to other HSP70 proteins, BiP has two major domains. The N-terminal domain contains an ATP catalytic site and the C-terminal domain contains the substrate-binding site [2]. The two domains communicate by binding and releasing ATP and unfolded peptides respectively to regulate activity. When ATP is bound in the catalytic site, the C-terminus has a low affinity for unfolded proteins. However, in the presence of hydrophobic residues on a nascent peptide, the hydrolysis of ATP to ADP is catalyzed [3], and the affinity for the bound substrate increased, thus allowing time for proper folding. Subsequent exchange of ADP for ATP releases the peptide substrate [4], allowing a new unfolded protein, or the same protein if hydrophobic sites are still exposed, to interact with BiP again. Through the interplay between the domains, BiP is able to temporarily bind and release unfolded proteins until they fold properly or are targeted for degradation [5].

BIP AND THE UNFOLDED PROTEIN RESPONSE

The function of BiP is integral and critical to the UPR. As noted, during times of ER stress induced by an overload of protein, the UPR acts as a corrective pathway, capable of both increasing the ER folding capacity as well

as decreasing the incoming polypeptide load. The UPR provides the cell machinery with situational awareness of the peptide-folding environment via three protein sensors embedded in the ER membrane; activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and PKR-like ER kinase (PERK). BiP transiently binds to the luminal domain of each receptor (Figure 1). If unfolded proteins begin to accumulate in the ER lumen, BiP can detach from the sensors and bind exposed hydrophobic regions on the nascent polypeptides. The decoupling of BiP activates the sensors, by allowing oligomerization and autophosphorylation in the case of IRE1 and PERK [6], and as a yet unknown mechanism for ATF6, which may involve alternate glycosylation [7]. The subsequent downstream pathway of each of the three UPR sensors appears to have an innate preference for a particular type of ER stress. Experiments using different ER stress inducers showed ATF6 responded quickly and robustly to disulfide bonding disruption caused by dithiothreitol, whereas PERK responded quickly to perturbations of Ca^{2+} homeostasis caused by thapsigargin. IRE1 is the most highly conserved of the three sensors and it appears to respond equally well to all types of stress, likely an evolutionary function conserved from its time as the sole sensor of ER stress [8].

Upon dissociating from BiP, each of the three UPR sensors modifies the ER to alleviate stress in its own unique way. ATF6, a basic leucine zipper transcription factor embedded in the ER membrane, is often the first sensor to respond to ER stress. Once ATF6 dissociates from BiP, Golgi localization signals [9] are exposed allowing ATF6 translocation to the Golgi apparatus. In the Golgi apparatus, ATF6 is cleaved by site-1 protease (S1P) and site-2 protease (S2P) [10]. The cytosolic domain of ATF6 is then free to translocate to the nucleus, where it moderates increased expression of several proteins involved in lipid biosynthesis and chaperones, including BiP and x-box binding protein 1 (XBP1) [11]. This increases the volume of the ER and provides more chaperone proteins to aid in folding, thus relieving some of the ER stress.

The other two sensors, IRE1 and PERK, remain as integral ER proteins, but oligomerize and autophosphorylate following BiP disassociation. Some evidence exists to suggest that BiP dissociation does not necessarily activate IRE1, but rather that unfolded proteins activate oligomerization [12]. BiP may play a role instead to modulate IRE1 activity rather than function as a switch [13,14]. IRE1, has two isoforms in mammals, IRE1 α and IRE1 β . IRE1 α is the more common of the two isoforms, and is expressed ubiquitously in mammals. In contrast, IRE1 β is expressed only in the epithelial lining of the lungs and gut where it plays a role in mucin production [15]. Both phosphorylated IRE1 isoforms exhibit en-

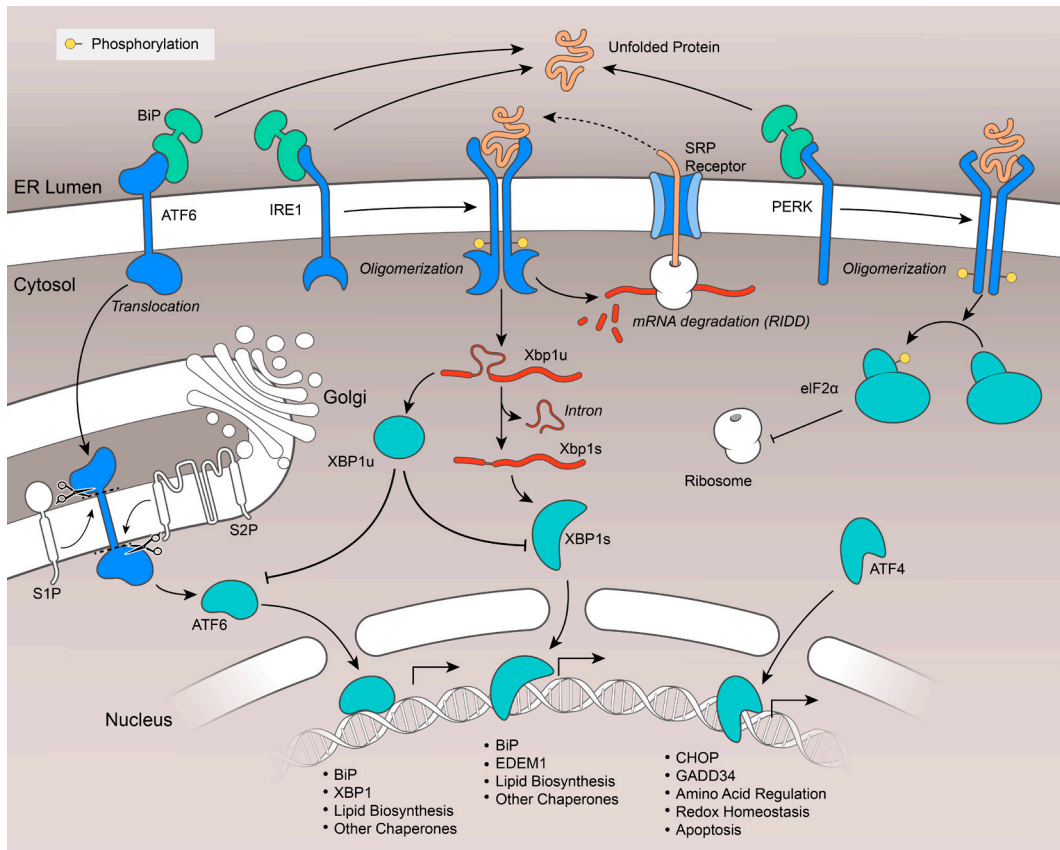


Figure 1. Overview of the unfolded protein response. BiP dissociates from UPR sensors, ATF6, IRE1, and PERK, in the presence of unfolded proteins. ATF6 translocates to the Golgi apparatus where it is subsequently cleaved by S1P and S2P. The cytosolic domain translocates to the nucleus where it promotes transcription of BiP, XBP1, proteins involved with lipid biosynthesis, and other chaperones. IRE1 and PERK both oligomerize and autophosphorylate after dissociating from BiP. IRE1 splices incoming mRNAs, including Xbp1. Spliced Xbp1 is translated (XBP1s) and promotes transcription of BiP, EDEM1, proteins involved in lipid biosynthesis, and other chaperones. The product of unspliced Xbp1 translation (XBP1u) inhibits other components of the UPR, specifically XBP1s and ATF6. Phosphorylated PERK in turn phosphorylates eIF2 α , inhibiting translation. Some proteins, such as ATF4, are unaffected by this block, and are preferentially translated. ATF4 promotes transcription of CHOP, GADD34, and proteins involved with amino acid regulation, redox homeostasis, and apoptosis.

ribonuclease activity, splicing multiple mRNA targets destined for translation in the ER. This process is known as regulated IRE1-dependent decay (RIDD) and reduces the translational burden on the ER. Both IRE1 α and IRE1 β have this nuclease activity. Mammalian IRE1 has been shown to be far more selective than that of *Drosophila melanogaster*, indicating more regulation of RIDD is present in mammals [16]. Although most targets of RIDD are degraded after IRE1 splicing, some of the spliced transcripts have important functions. One such transcript is from the XBP1 gene. While the unspliced Xbp1 transcript (Xbp1u in Figure 1) is translated into a potent inhibitor of the UPR (XBP1u in Figure 1) [17], the spliced form (Xbp1s in Figure 1) is translated into a transcription factor (XBP1s in Figure 1) that promotes expression of several more proteins involved in lipid biosynthesis, ER-associated protein degradation (ERAD),

and also more chaperones, including BiP. The increase in lipid and chaperone protein production has a similar effect to ATF6, increasing both the size of the ER and the density of chaperone proteins within it. Additionally, some of the newly expressed proteins serve to reduce peptide burden in the ER by degrading proteins through ERAD, a process by which unfolded proteins are marked with ubiquitin and targeted for proteasome degradation. IRE1 α may also be linked to innate immune pathways. Phosphorylated IRE1 α interacts with TNF receptor associated factor 2 (TRAF2) and TNF receptor associated factor 6 (TRAF6), linking the UPR to multiple inflammatory pathways including the c-Jun amino-terminal kinases (JNK) [18]. The IRE1 pathway acts as a generalized response to ER stress increasing ER volume and folding capacity while preventing additional peptides from entering the ER.

Table 1. Predicted BiP cellular function and arthropod vector orthologs.

KEGG name(s)	Description	KEGG Ortholog	KEGG Ortholog pathway(s)	Predicted arthropod vector HSPA5/BIP orthologs [◊]
BiP/ HSPA5	Heat shock 70kDa protein 5	K09490	Protein export; protein processing in endoplasmic reticulum; thyroid hormone synthesis; prion diseases	<i>Ixodes scapularis</i> : ISCW017754* <i>Aedes aegypti</i> : AAEL017349** AAEL018061*** <i>Aedes albopictus</i> : AALF021504*** AALF021835*** <i>Culex quinquefasciatus</i> : CPIJ003550**

◊Denoted in VectorBase gene model accession IDs

*Identified as a ortholog from K09490 KEGG Ortholog of KEGG (http://www.kegg.jp/dbget-bin/www_bget?ko:K09490)

**Identified as a ortholog from K09490 KEGG Ortholog of KEGG (http://www.kegg.jp/dbget-bin/www_bget?ko:K09490); also identified as orthologs of ISCW017754 from VectorBase (https://www.vectorbase.org/Ixodes_scapularis/Gene/Comparation_Ortholog?g=ISCW017754;r=DS696337:18885-23790;t=ISCW017754-RA)

***Identified as a ortholog to ISCW017754 from VectorBase (https://www.vectorbase.org/Ixodes_scapularis/Gene/Comparation_Ortholog?g=ISCW017754;r=DS696337:18885-23790;t=ISCW017754-RA)

The third UPR sensor is PERK which also oligomerizes and autophosphorylates its cytoplasmic domain following dissociation from BiP. The oligomerization domain of PERK shares a high degree of homology with IRE1, and may also use BiP as a modulatory element, rather than a switch. However, unlike IRE1, the PERK kinase phosphorylates eukaryotic initiation factor 2 α (eIF2 α), effectively inhibiting translation. Because eIF2 α phosphorylation rapidly reduces the peptide load in the ER, it is a common way to give the cell more time to respond to stimuli. Additional stressors such as amino acid starvation and double-stranded RNA also feed into this pathway; therefore the steps downstream from eIF2 α phosphorylation are commonly referred to as the integrated stress response (ISR) [19]. Although protein synthesis is globally inhibited after eIF2 α phosphorylation, some mRNAs are less susceptible to this inhibition due to upstream open reading frames located in the 5' untranslated region. These unaffected mRNAs allow some key proteins involved in ER stress signaling to be synthesized, thus bypassing the translational block. One of these proteins, crucial for the UPR, is activating transcription factor 4 (ATF4). ATF4 activates transcription of multiple proteins involved with amino acid metabolism, redox homeostasis, and apoptosis, including CCAAT-enhancer-binding protein homologous protein (CHOP), growth arrest and DNA damage inducible protein 34 (GADD34), and to some extent, BiP [20,21]. CHOP activates endoplasmic reticulum oxidoreductase 1 α (ERO1 α), which promotes an oxidizing environment in the ER [22] and leads to eventual apoptosis through caspase 12 [23]. Interestingly, GADD34 de-phosphorylates eIF2 α , thus reversing some of the translation block. The PERK pathway plays multiple roles in the UPR, with responses ranging from global protein synthesis attenuation to apoptosis.

BIP AS A CYTOPROTECTIVE AGENT

BiP has been implicated as a cytoprotective agent in a variety of scenarios, not limited to viral infection. Studies detailing the role of BiP during animal development have deemed it necessary for proper development *in vitro* and *in vivo*. Mouse knockout BiP^{-/-} embryos were able to implant in the uterus, but quickly degenerated and were reabsorbed. Heterozygous BiP embryos are able to survive, although expression of BiP was roughly 50 percent of wild-type mice. Additionally, BiP plays a central role in the survival of tumor cells. In several drug-resistant tumor types, BiP protein is found at significantly elevated levels. Even in tissues with historically low levels of BiP expression such as brain, cancers such as malignant glioma exhibit high levels of BiP expression [24]. Overexpression of BiP in cancer cell lines confers increased resistance to several chemotherapeutic agents. Additionally, suppression of BiP via siRNA knockdown has been shown to reverse this resistance and increase susceptibility [25]. BiP not only protects against chemical agents, but from the natural machinery of the immune system as well. Cancer cells resistant to destruction by T killer cells were found to overexpress BiP. This resistance was also lost following BiP knockdown [26]. BiP has thus been demonstrated to play a cytoprotective role in a variety of stressful cellular conditions involving rapid growth and protein synthesis, including embryo development and tumor biogenesis.

Not only does BiP protect the cell against a myriad of insults, but it along with other components of the UPR machinery are highly conserved across species. The UPR is an ancient cellular stress pathway, conserved from yeast to humans. Yeast has only one sensor, IRE1, which splices a homolog of XBP1, HAC1 [27]. BiP is

Table 2. Amino acid conservation among human BiP and predicted orthologs.

Species	% amino acid coverage* of predicted species ortholog to <i>Homo sapiens</i> ortholog	Number of amino acids making up predicted HSPA5/BIP orthologs
<i>Mus musculus</i> [◊]	98.6%	655
<i>Ixodes scapularis</i> [◊]	83.6%	658
<i>Drosophila melanogaster</i> [◊]	79.5%	656
<i>Aedes aegypti</i> (AAEL017349) [◊]	80.7%	655
<i>Aedes aegypti</i> (AAEL018061) [∞]	78.6%	662
<i>Aedes albopictus</i> (AALF021504) [∞]	76.5%	657
<i>Aedes albopictus</i> (AALF021835) [∞]	81%	656
<i>Culex quinquefasciatus</i> [◊]	79.7%	657

◊Amino acid sequence identified from K09490 KEGG Ortholog of KEGG (http://www.kegg.jp/dbget-bin/www_bget?ko:K09490)

∞Amino acid sequence identified from orthologs of *I. scapularis* ISCW017754 of VectorBase (https://www.vectorbase.org/Ixodes_scapularis/Gene/Compara_Ortholog?g=ISCW017754;r=DS696337:18885-23790;t=ISCW017754-RA)

*% alignment identified from ClustalW = <http://www.genome.jp/tools/clustalw/>

also conserved in yeast through the homolog KAR2 [28] and interacts with HAC1 in a similar manner to mammalian systems [29]. Predicted orthologs of BiP have been identified in several medically-relevant arthropod species, including *Aedes aegypti*, *Aedes albopictus*, *Ixodes scapularis*, and *Culex quinquefasciatus* (Table 1). A BiP homolog, heat shock protein cognate 3 (HSC3) has also been identified in the model fly organism, *D. melanogaster*, a close relative of the *Aedes* and *Culex* mosquito genera. BiP retains a high amino acid conservation (> 76 percent) between mammals and arthropods (Table 2). In *D. melanogaster*, ER stress has been demonstrated to increase HSC3 protein expression in an XBP1-dependent manner [30,31], but still has not been confirmed to interact with the UPR sensors biochemically. Homologs of IRE1 and PERK have both been functionally confirmed in *D. melanogaster*, and a predicted homolog of ATF6 exists, but remains to be tested [32,33]. The arthropod unfolded protein response has many of the predicted orthologs present in the mammalian system, although further research is necessary to confirm the function of these proteins. Due to the conserved nature of these proteins, they may be preferentially targeted by multi-host pathogens like the arthropod-borne flaviviruses.

CELLULAR LOCALIZATION OF BIP

BiP localization in mammalian systems is not exclusively limited to the ER lumen; there are several cases of atypical BiP expression as well. BiP is commonly expressed on the surface of several tumor cell types and proliferating endothelial cells, where it is involved in signal transduction in association with various proteins [34]. A cytosolic splice variant of BiP, GRP78va, was discovered to play a potential role in cytosolic modulation of PERK signaling, resulting in a cytoprotective effect [35].

Additionally, evidence exists to show BiP localization to the mitochondria and nucleus during ER stress, where it may play another role in cell survival. [36,37]. Finally, secreted BiP, along with other ER chaperones, has been detected during ER stress, both in cell culture media and human peripheral circulation, but no function has yet been attributed to it [38]. Although BiP can be found in multiple sites outside of the ER, further research is required to determine the function of many of these variants.

BIP AND FLAVIVIRUS INFECTION

Viral infection can lead to significant ER stress because viral protein production is not subject to the same stringent controls as host protein translation. This often leads to an active UPR during infection. Certain measures taken by the UPR to alleviate ER stress may actually help provide an optimal environment for viral reproduction, including increasing the volume of the ER and the quantity of host chaperone proteins present to assist in protein folding. The UPR has been implicated in the life cycle of several enveloped viruses; however, in this review, focus is on the vector-borne flaviviruses [39,40]. These agents are transmitted by the bite of a mosquito (mosquito-borne flaviviruses; MBFVs) or tick (tick-borne flaviviruses; TBFVs). Although there are fewer cases of infections annually when compared to MBFVs, TBFVs such as tick-borne encephalitis virus (TBEV) and Powassan virus (POWV) are highly neurovirulent agents that cause an estimated 10,000 to 15,000 confirmed encephalitic cases per year worldwide. Flaviviruses, like other viruses, are obligate intracellular pathogens. They hijack the host cell machinery to replicate their genome, to produce and package virions, and to egress from the cell to ultimately infect new ones. Flaviviruses have a single-stranded, positive sense RNA genome roughly 11kb in size, which is direct-

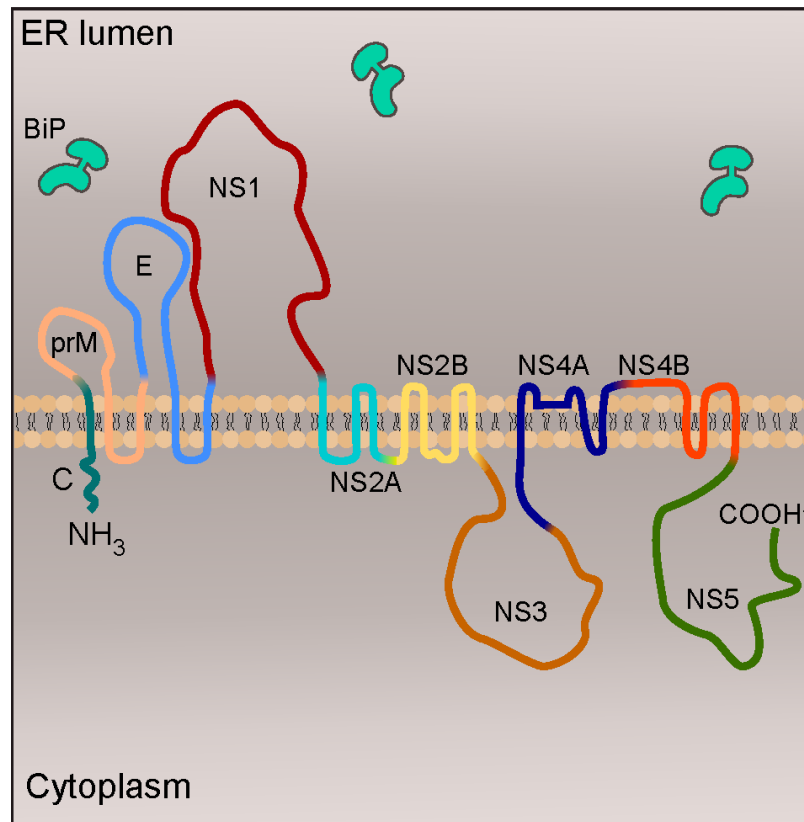


Figure 2. Flavivirus polyprotein synthesis and topology. The flaviviral polyprotein is translated into the ER membrane. It is cleaved at later time points by host and viral proteases to produce the viral proteins, denoted by different colors along the polypeptide chain. PrM (tan), E (light blue), and NS1 (red) are located in the ER lumen, NS3 (light orange) and NS5 (light green) are located in the cytoplasm, and C (dark green), NS2A (teal), NS2B (yellow), NS4A (dark blue), and NS4B (dark orange) remain anchored in the ER membrane. BiP is constitutively expressed throughout the ER lumen.

ly translated to encode a single polyprotein with multiple transmembrane regions (Figure 2). This polyprotein is cleaved into three structural and seven nonstructural proteins using host and viral proteases [41]. RNA replication occurs within spherules on the rough ER whereas virion assembly occurs within the ER lumen [42]. Specifically, the MBFVs dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), and the attenuated WNV, Kunjin virus (WNV_{KUN}) have been shown to interact with the mammalian UPR in some detail.

DENV, JEV, WNV, and WNV_{KUN} infections have been shown to lead to increased BiP protein expression. It remains unclear if this results from specific interaction of viral proteins with factors of the host cell, or if the BiP increase is simply due to the general ER stress caused by an abundance of unfolded viral protein in the ER. Post-translational BiP protein knockdown by Sub_{AB} toxin led to a decrease in release of infectious DENV. Levels of viral genome RNA were unaffected during protein knockdown in these experiments, implying that BiP was involved primarily in viral protein folding and virion assembly, not

genome replication. Interestingly, overexpression of BiP protein prior to DENV infection however, had no effect on infectious virus production [43]. Additionally, BiP protein expression is increased in uninfected bystander cells during DENV infection, suggesting potential stress signaling [43]. While a mechanistic link between BiP and flaviviral proteins has not been observed, yeast two-hybrid assays have shown that DENV envelope (E) protein binds directly to BiP [44]. Similar to DENV, infectious JEV release was reduced 10-fold during siRNA knockdown of BiP transcript, while viral RNA levels remained unchanged [45]. This implies BiP plays a similar role in JEV infection and may play a conserved role in general MBFV infection of mammalian cells. Considering its localization in the ER lumen and its effect on virion assembly, BiP likely interacts with the structural flaviviral proteins that localize within the ER lumen, E and pre-membrane (prM), if at all.

BiP plays a key role in managing the UPR. Therefore, it is imperative to examine the other UPR sensors with which it interacts in order to appreciate the complete

picture in flavivirus infections. ATF6 is one of the first sensors in the UPR to increase BiP protein expression. WNV_{KUN} appears to preferentially activate the ATF6 pathway. A 10-fold increase in XBP1 transcripts, a product of ATF6-mediated transcription, was observed in WNV_{KUN}-infected cells after the viral latent period, 18 hours post infection (hpi). This also correlated with a significant increase in BiP protein expression [46]. Downstream mRNA products of the other pathways, ER degradation enhancing alpha-mannosidase like protein 1 (EDEMI) and ATF4 (IRE1 and PERK, respectively), were only increased 3-fold at peak transcription. Nonstructural protein 4A (NS4A) and nonstructural protein 4B (NS4B) alone each significantly increased XBP1 transcript levels, potentially indicating an interaction between the non-structural WNV_{KUN} proteins and the ATF6 pathway. Additionally, infectious virus release was shown to be lower in ATF6^{-/-} MEFs. This corresponded to an increase in PERK-related factors, suggesting ATF6 may play a role in dampening PERK signaling [47]. In contrast, WNV degrades ATF6 in a proteasome-dependent manner [48]. ATF6 has been shown to activate and translocate to the nucleus during DENV infection [49]; however, infectious DENV release was unchanged in mouse embryonic fibroblast (MEF) ATF6^{-/-} knockouts [50]. ATF6 appears to be preferentially activated during milder infections, such as WNV_{KUN}, but is non-essential for more lethal infections such as DENV and WNV, implying a potential cytoprotective role for the ATF6 pathway during infection.

IRE1, the primordial sensor of the UPR, is conserved in most eukaryotes, including yeasts, plants, and animals, potentially allowing ample time for viruses to co-evolve alongside it. During DENV infection, cleaved ATF6 was not found in the nucleus early in infection [50]; however, XBP1 transcripts spliced by IRE1 were shown to increase [51]. Confocal microscopy also showed the localization of XBP1 in the nucleus 24 to 36 hpi [50]. This suggests that IRE1 is responsible for the increase in BiP protein expression during DENV infection. DENV grown in IRE1^{-/-} knockout MEFs yielded significantly lower infectious virus [50]. The viral nonstructural protein NS2B-3 (fused NS2B and NS3 proteins) was able to increase Xbp1 splicing 6-fold when transfected alone; however, it should be noted that DENV infection increased splicing 140-fold, suggesting multiple viral proteins are involved in enhancing IRE1 splicing activity. JEV also induces Xbp1 splicing, but NS2B-3 does not appear to play a significant role in the process, as demonstrated in a similar transfection experiment [51]. Interestingly, JEV takes advantage of the RIDD activity of IRE1. While the IRE1 endonuclease cleaves most mRNAs encoding proteins destined for the ER, JEV mRNA is unaffected [52]. This allows the JEV genome to be preferentially translated into the ER. Knockdowns of IRE1 significantly reduce

infectious virus release [52]; however, knockdowns of XBP1 have no effect. There is a notable increase in cytopathic effect in XBP1 knockout MEFs, potentially indicating a cytoprotective role of XBP1 downstream targets [51]. WNV_{KUN} also alters IRE1 activity. NS4A and NS4B were shown to enhance Xbp1 splicing, leaving undetectable amounts of unspliced Xbp1 in transfected samples [46]. Like JEV, WNV infection in XBP1^{-/-} MEFs does not cause a significant change in viral release. It remains to be seen if ATF6 has any compensatory effect in the XBP1 knockout MEFs, or if they lack increased BiP expression.

The third pathway of the UPR is mediated via PERK. PERK is responsible for downstream activation of several limiting factors for viral replication, namely translation inhibition and apoptosis. The PERK pathway is considered to have an antiviral effect on most flaviviruses. Knockout studies using PERK^{-/-} [46,50] and CHOP^{-/-} [48] MEFs showed an increase in infectious DENV, WNV, and WNV_{KUN} release. Additional studies in PERK^{-/-} MEFs showed phosphorylation of eIF2 α occurs early in DENV infection, but is rapidly reversed, even when treated with a potent small molecule, ER stress inducer, thapsigargin [50]. However, a recent study showed that PERK may play some proviral role in DENV infection by promoting autophagosome formation and turnover, a process beneficial to infectious virus production [53]. An interesting comparison can be drawn between the highly neurovirulent WNV and the attenuated WNV_{KUN}. WNV induces phosphorylation of eIF2 α and robustly induces CHOP and GADD34 expression through ATF4. Alternatively, WNV_{KUN} shows minimal phosphorylation of eIF2 α and induction of ATF4 during infection. This suggests that PERK signaling may be partially responsible for the deleterious effects observed in particularly neurovirulent flaviviruses.

Recently, atypical variants of BiP have been implicated in flavivirus entry. As noted in a previous section, a small fraction of expressed BiP can localize to the cell surface in certain cell types. BiP was identified as a receptor element for DENV in HepG2 liver cells by the viral overlay protein binding assay (VOPBA) and mass spectrometry fingerprinting [54]. Additionally, in one of the few studies focusing on an arthropod vector, *A. albopictus*, BiP was similarly implicated as a part of the DENV viral entry process in C6/36 cells. It was found to be nonessential for virion binding, but appeared to play some role in entry [55]. With BiP having high amino acid conservation between humans and arthropod vectors (Table 2), it may be possible it is functioning in the same manner in both systems.

Studies examining the ultrastructure of TBFV infection have shown ER enlargement and chaperone recruitment in TBEV and Langkat Virus (LGTV), similar to the MBFVs [56,57]. TBEV infection induced the IRE1 and

Table 3. Summary of predicted proviral or antiviral roles of BiP and UPR pathways on flavivirus infection.

	BiP	ATF6 Pathway	IRE1 Pathway	PERK Pathway
DENV	✓ ¹	- ²	✓ ²	X ²
JEV	✓ ³	?	✓ ⁴	?
WNV	?	?	?	X ⁵
WNV _{KUN}	?	✓ ⁶	?	X ⁷
ZIKV	?	?	?	?
TBEV	?	?	✓ ⁸	?
POWV	?	?	?	?
LGTV	?	?	?	?

✓ denotes protein or pathway plays a proviral role for infectious flavivirus release according to current data

X denotes protein or pathway plays an antiviral role for infectious flavivirus release according to current data

- denotes protein or pathway plays minimal role for infectious flavivirus release according to current data

? denotes at this time, no sufficient functional study of BiP and UPR pathways on infectious flavivirus release has been performed.

¹ [43]

² [50]

³ [45]

⁴ [52]

⁵ [48]

⁶ [47]

⁷ [46]

⁸ [58]

ATF6 pathways as evidenced by increased expression of pathway product transcripts, and inhibition of IRE1 significantly reduced infectious virus release [58]. Proteomic studies of TBEV- and Langat- infected tick cell lines have identified protein evidence for the predicted *Ixodes scapularis* BiP ortholog in infected cells [59,60], but nothing is known regarding specific interactions involving BiP and TBFVs in both mammalian and tick species.

CONCLUSIONS AND OUTLOOK

The UPR clearly plays a significant role in flavivirus infections. A summary detailing the cumulative knock-down data for the above flaviviruses is presented in Table 3. BiP, as the master regulator of the UPR, plays a key part during infection because its expression is increased by all flaviviruses studied so far. Knockdown studies have suggested a pro-viral role of BiP in DENV and JEV infection. The pathways of the UPR which increase protein expression of BiP, ATF6, and IRE1 are preferentially activated during DENV, JEV, and WNV_{KUN} infection. Additionally, the PERK pathway of the UPR, appears to play an antiviral role during flavivirus infection. Despite BiP's role in activating each UPR sensor, each flavivirus appears to preferentially activate different arms of the UPR. This may be due to viral elements interacting with the arms themselves, or perhaps the viruses have different interactions with BiP itself. The primary role of BiP likely involves protein folding and virion assembly, specifically through E and prM, since perturbing BiP tends

to decrease infectious viral titer while having no effect on viral mRNA levels.

Some proteins in the UPR have been identified as potential therapeutic targets, including BiP and all three sensors. Small molecules are available to target these proteins, but much of the research focus utilizing these small molecules has been with protein misfolding diseases and cancer [61,62]. No repurposing studies aimed at using these small molecules for potential therapeutics for flavivirus infection has been attempted. Flavivirus-mammalian host interactions in the UPR have been the subject of considerable study, but examination of the UPR in the arthropod system remains relatively neglected. Studies characterizing the UPR in arthropods have been mostly limited to *Drosophila* and few exist in species more relevant to flavivirus infection. Although studies in mammalian systems are generally more relevant for translation into medical treatments, knowledge concerning the arthropod system may prove invaluable for identifying targets for vector control. By targeting key differences in the viral life cycle between mammals and arthropods, new therapeutic targets specific to arthropods could be developed, providing another option to reduce transmission to humans and ultimately the burden on global health systems.

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