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The ER-Resident Chaperone BiP/GRP78 Is A Pro-viral and Pro-survival Factor In KSHV-Infected Cells

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The ER-Resident Chaperone BiP/GRP78 Is A Pro-viral and Pro-survival Factor In KSHV-

Infected Cells

A Dissertation Submitted In Partial Satisfaction Of The

Requirements For The degree Doctor of Philosophy

in Molecular Cellular and Developmental Biology

By

Guillermo Najarro

Committee In Charge:

Professor Carolina Arias, Co-Chair

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June 2023

The dissertation of Guillermo Najarro is approved.

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1. **Guillermo Najarro**, Kevin Brackett, Catya Faeldonea, Adriana Ramirez Negron, Kevin Osvaldo Moreno, Hunter Woosley, and Carolina Arias. "BiP/GRP78 is a Broad-spectrum Proviral Factor and Promotes Cell Survival and Replication of Cells Infected by the Oncogenic Kaposi's Sarcoma-Associated Herpesvirus" *In Preparation*
2. Kevin Brackett, Ameera Mungale, Mary Lopez-Isidro, Duncan A. Proctor, **Guillermo Najarro**, and Carolina Arias (2021) "CRISPR Interference Efficiently Silences Latent and Lytic Viral Genes in Kaposi's Sarcoma-Associated Herpesvirus-Infected Cells" Viruses, 13: 783

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Dorsten AV, Gutierrez Flores C, Torten G, and Arias C. "The Activity Of
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1. **Najarro G**, Brackett K, Faeldonea C, Ramirez Negron A, Moreno KO,   2022
   Dorsten AV, Gutierrez Flores C, Torten G, and Arias C. "The Activity Of
   The ER-resident Chaperone GRP78/BiP Is Critical For The Lytic
   Reactivation of Kaposi's Sarcoma-Associated Herpesvirus And Survival
   Of Infected Cells" American Society of Virology Conference.
2. **Najarro G**, Torten G, Arias C. "The Endoplasmic Reticulum Chaperone   2019
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Abstract

Productive Kaposi Sarcoma Associated Herpesvirus (KSHV) infection involves translating, folding, and modifying abundant viral proteins in the Endoplasmic Reticulum (ER). The heightened demand for ER function can disrupt ER homeostasis and activate the Unfolded Protein Response (UPR), a collection of stress-signaling pathways in charge of restoring ER homeostasis. Three ER-resident sensors/transducers, the kinase/RNase IRE1, the kinase PERK, and the membrane-bound transcription factor ATF6, govern the UPR. Their activation either promotes adaptation or causes apoptosis if homeostasis is not restored. A master regulator of the UPR and ER function is the HSP70 ER-resident molecular chaperone GRP78/BiP. This abundant chaperone modulates the activation of the UPR sensors and promotes protein folding in the ER lumen to restore homeostasis. Typically, BiP expression is transcriptionally upregulated in response to ER stress by ATF6. However, our results show that lytic reactivation of KSHV in iSLK-219 cells leads to the ATF6-independent accumulation of GRP78/BiP. The upregulation of GRP78/BiP is critical during the lytic cycle as pharmacological inhibition of genetic depletion of GRP78/BiP reduces viral gene expression and viral particle production. Moreover, our results suggest that BiP activity promotes the survival and growth of KSHV-infected B-cells and lymphatic endothelial cells, and that KSHV-infected cells are uniquely sensitive to a BiP inhibitor, HA15. Inhibition of BiP also leads to the reduction in replication of other DNA viruses, including the beta-herpesvirus human cytomegalovirus (HCMV), the alpha-herpesvirus herpes simplex virus 1 (HSV-1), and the poxvirus vaccinia virus (VV), indicating that the pro-viral role for BiP is not exclusive for KSHV. Our results highlight BiP as a promising target for developing broad-spectrum antivirals and potential therapies for treating KSHV-related malignancies.

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List of Abbreviations

KSHV: Kaposi Sarcoma Associated Herpesvirus

ER: Endoplasmic Reticulum

UPR: Unfolded Protein Response

IRE1: Inositol-Requiring Enzyme 1

PERK: Protein Kinase R-like Endoplasmic Reticulum Kinase

ATF6: Activating Transcription Factor 6

GRP78/BiP: Glucose Regulated Protein 78/Binding Immunoglobulin Protein

BiP: Binding Immunoglobulin Protein

GRP94: Glucose-Regulated Protein 94

HCMV: Human cytomegalovirus

HSV-1: Herpes simplex virus 1

VV: Vaccinia virus

SRP: Signal recognition particle

ERAD: ER-associated degradation

PyVs: Polyomaviruses

PDI: Protein disulfide isomerase

VP: Vesicle packets

DMV: Double-membrane vesicle

HCV: Hepatitis C virus

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

ERGIC: ER–Golgi intermediate compartment

HSV-2: Herpes Simplex Virus Type 2

DNJ: 1-Deoxynojirimycin

CAST: Castanospermine

2-DFM: 2-deoxy-2-fluoro-D-mannose

XBP1: X-box-binding protein 1

RIDD: IRE1-dependent decay

TRAF2: Tumor necrosis factor receptor-associated factor 2

MHV: Mouse Hepatitis Virus

JEV: Japanese encephalitis virus

ISR: Integrated stress response

eIF2α: Eukaryotic initiation factor 2 α subunit

Met-tRNAi: Initiating tRNA

GEF: Guanine exchange factor

ATF4: Activating Transcription Factor 4

GADD34: Growth arrest and DNA damage-inducible protein

CHOP: CCAAT-enhancer-binding protein homologous protein

S1P: Site-1 protease

S2P: Site-2 protease

ATF6-N: ATF6-DNA binding domain

ERSE: ER stress response element

SARS-CoV: severe acute respiratory syndrome coronavirus

HSP: Heat Shock Protein

NBD: Nucleotide-binding domain

VSV: Vesicular stomatitis virus

LDL: Low-density lipoproteins

HSP70: Heat Shock Protein 70

SBD: Substrate-binding domain

NEF: Nucleotide exchange factor

MERS-CoV: Middle East respiratory syndrome coronavirus

bCoV-HKU9: Bat coronavirus HKU9

VDAC: Voltage-dependent anion channel

HBV: Hepatitis B virus

KS: Kaposi Sarcoma

PEL: Primary Effusion Lymphoma

MCD: Multicentric Castleman Disease

HS: Heparan sulfate

ICAM-3: Intracellular adhesion molecule-3

DC-SIGN: Dendritic cell-specific intracellular adhesion molecule-3 (ICAM-3) grabbing non-integrin

EphA2: Ephrin receptor A2

LANA: Latent Antigen

RTA: Replication and transcription activator

IE: Immediate early

E: Early

L: Late

ROS: Reactive oxygen species

HDAC: Histone deacetylase

NaB: Sodium butyrate

ValA: Valproic Acid

HUVEC: Human umbilical vein endothelial cell

TG: Thapsigargin

SERCA: Sarco/endoplasmic reticulum Ca^{+2} ATPase

HHV: Human herpesviruses

LEC: Lymphatic endothelial cells

Dox: Doxycycline

IRE1-P: Phosphorylation of IRE1

CRISPRi: CRISPR interference

kDa: kiloDalton

ER-ATF6: ER-bound ATF6

N-ATF6: Nucleus-bound fragment

PFA: Phosphonoformate

IRES: Internal ribosome entry site

RTC: Replication and transcription compartments

NT: Non-targeting

Erdj3/DnaJB11: HSP40

PPBC: primary peripheral B-cell

dsDNA: Double-Stranded DNA

PHF: Primary human fibroblasts

MOI: Multiplicity of infection

SubAB: Subtilase cytotoxin

BEC: Blood endothelial cell

Co-IP: Co-immunoprecipitation

La: La autoantigen

ITAF: IRES trans-acting factor

NSAP-1: NS1-associated protein-1

eIF4G: eukaryotic initiation factor 4 γ

CLIP: Cross-linking and immunoprecipitation

MS: Mass spectrometry

FBS: Fetal bovine serum

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

RT-PCR: Reverse Transcription PCR

qPCR: Quantitative PCR

Chapter 1: Introduction

The Arms Race Between Virus And Host: Prelude To Viral Endoplasmic Reticulum

Modulation

As obligate intracellular parasites, viruses require the host translational and protein folding machinery to produce the viral factors necessary for genome replication and virion assembly [1,2]. These viral factors can rewire the host environment and alter organelle and cellular architecture to promote virion replication, long-term infection, and immune escape. In turn, the host responds to this attack by activating immune responses, halting protein synthesis to prevent the expression of viral factors, and triggering cell death to eliminate infected cells [3]. Increasing our knowledge and understanding of how viruses interact with cellular components, such as the endoplasmic reticulum (ER), will help us gain an advantage in this arms race.

The Importance of the ER During Viral Infections

The ER is a large cellular structure comprised of a network of membranes consisting of the smooth ER and rough ER [4]. The smooth ER encompasses tubular membrane vesicles, and its primary function involves the metabolism of lipids and glycogen. The rough ER consists of flattened sacs with ribosomes on the outer surface. The continuous ER membrane encloses a subcellular space known as the ER lumen. Here, transmembrane and secreted proteins are synthesized and processed to be transported to specific subcellular compartments or outside the cell. The synthesis of proteins in the ER begins with the cotranslational translocation of nascent polypeptides containing an ER signal sequence into the ER. This process is mediated by the signal recognition particle (SRP), which recognizes the ER-signal sequence of the nascent polypeptides and interacts with the Sec61 translocon at

the ER, allowing the polypeptides to enter the ER lumen. Inside the ER lumen, the nascent proteins interact with ER-resident chaperones and host factors that assist in their protein folding and processing. The correctly folded and modified proteins are then transported to their respective destinations. When the folding capacity of the ER is exceeded and unfolded proteins accumulate, or in cases when proteins do not achieve their functional form in the ER, they are retrotranslocated to the proteasome for degradation via the ER-associated degradation (ERAD) pathway.

The function of the ER is critical during viral infection as multiple viruses exploit this organelle for viral entry, genome replication, protein synthesis, and virion assembly [Table 1-1] [5]. All viral infections start when the particles are internalized and transported to the subcellular compartment, where transcription, replication of the viral genome, and virion assembly will occur. One of the best-characterized examples of ER-dependent viral entry is the double-stranded DNA non-enveloped Polyomaviruses (PyVs) [6]. Once the virus recognizes the receptor on the cell surface, it undergoes receptor-mediated endocytosis and traffics to the ER. At the ER, the virus hijacks the functions of ER-resident proteins, including protein disulfide isomerase (PDI)-family members and BiP to disassemble the viral particle and allow entry to the cytosol. Following disassembly, the viral genome goes from the cytosol to the nucleus, allowing genome replication and continuing the infection cycle [7].

Table 1-1. Examples Of ER Functions In The Context Of Viral Infections.

Virus	ER Function
Polyomaviruses (PyVs)	Double-stranded DNA non-enveloped virus that hijacks ER functions for Entry into the cell.
Dengue virus (DENV)	Positive-sensed single-stranded RNA-enveloped virus that hijacks ER functions for synthesizing/modifying viral proteins and generating ER-derived vesicle packets (VPs) for virion replication.
Hepatitis C virus (HCV)	Positive-sensed single-stranded RNA-enveloped virus that hijacks ER functions for synthesizing/modifying viral proteins and generating ER-derived double-membrane vesicles (DMVs) for virion replication.
Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)	Positive-sensed single-stranded RNA-enveloped virus where virion release occurs in the Golgi and the ER–Golgi intermediate compartment (ERGIC).
Herpes Simplex Virus Type 2 (HSV-2)	Double-stranded DNA enveloped virus that requires cellular α-glucosidases I for N-linked glycosylation of viral glycoproteins that are normally glycosylated in the ER.
Human Cytomegalovirus (HCMV)	Double-stranded DNA enveloped virus that requires cellular α-glucosidases II for N-linked glycosylation of viral glycoproteins that are normally glycosylated in the ER.
Kaposi's Sarcoma-Associated Herpesvirus (KSHV)	Double-stranded DNA enveloped virus that requires N-linked glycosylation of viral glycoproteins that are normally glycosylated in the ER.

Table 1-1 Example list of viruses known to modulate or require ER-affiliated functions for viral replication.

The ER is also hijacked as a site for translating viral structural and nonstructural proteins required for genome replication and virion assembly. Viral replication at the ER has been well characterized for members of the Flaviviridae family, composed of several medically relevant enveloped viruses (Zika, Dengue, West Nile Virus) with a positive-sense single-stranded RNA genome [8]. Following entry, the viral genome is translated as a single polyprotein, which is proteolytically processed by viral and cellular proteases at the ER to generate structural and nonstructural proteins. Flaviviruses induce ER invagination via nonstructural transmembrane proteins and host proteins that create ER-membrane-derived replication sites called vesicle packets (VPs) [9]. Similar replication sites derived from the ER membrane called double-membrane vesicles (DMVs) have been reported in cells infected with the flavivirus Hepatitis C virus (HCV), as well as those infected with Coronaviruses and Picornaviruses [10]. The viral genome is synthesized, and immature virions are assembled within these replication sites. It is worth noting that the loss of one of the host proteins,

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atlastin, that assists in forming the replication centers results in decreased viral replication, thus demonstrating a reliance on specific ER components [11].

Once assembled, mature virions are released from the cell, where they can infect other cells. Non-enveloped viruses usually release virions by causing cell lysis [12]. For enveloped viruses, mature virions acquire a double lipid membrane from the host by budding from the plasma membrane or intracellular membranes, including the ER. For Coronaviruses such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), budding starts in the Golgi and the ER–Golgi intermediate compartment (ERGIC), where virions are assembled and matured [13]. Within the ERGIC are the structural proteins, translocated from the ER post-processing. Once developed, the virion is released from the cell via lysosomal trafficking [14].

Some structural proteins, like glycoproteins, are synthesized and processed in the ER for DNA and RNA viruses. Glycoproteins are folded into their correct conformation with assistance from ER-resident chaperones and are modified via glycosylation in the ER and Golgi [15,16]. Without the necessary glycoproteins, viruses cannot infect their host and form virions. Among the viruses that require the host to synthesize and modify their glycoproteins is the Herpesviridae family [17]. N-linked glycosylation of viral proteins is necessary for the replication of Herpes Simplex Virus Type 2 (HSV-2), Human Cytomegalovirus (HCMV), and KSHV [18,19,20]. Inhibition of the cellular enzymes involved in N-linked glycosylation processing, α-glucosidases I and II with the compounds 1-deoxynojirimycin (DNJ) and castanospermine (CAST), or treatment of cells with the mannose analogue 2-deoxy-2-fluoro-D-mannose (2-DFM) results in strong inhibition of HSV, HCMV and KSHV replication

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[18,19,20]. Without the N-linked glycosylation machinery, unprocessed glycoproteins remain in the ER and are promptly retrotranslocated to the cytosol for degradation via ERAD.

The complex mechanisms used by viruses to hijack multiple ER functions and the processing and accumulation of abundant viral proteins in the lumen of this organelle can perturb ER homeostasis, leading to the activation of cellular responses that can be detrimental to viral replication [21]. Viruses have long-evolved mechanisms to evade, disrupt, or usurp these cellular responses to allow infection to continue [Table 1-2].

Table 1-2. Examples Of UPR Functions In The Context Of Viral Infections.

Inositol-Requiring Enzyme 1 (IRE1)	
Virus	**Function**
Mouse Hepatitis Virus (MHV)	Positive-sensed single-stranded RNA-enveloped virus that can cause splicing of XBP1 mRNA but prevents efficient translation of XBP1s transcription factor.
HCMV	Early infection can activate IRE1 but induces its degradation during late infection via the viral protein UL50.
Influenza Virus and SARS-CoV-2	Both are positive-sensed single-stranded RNA-enveloped viruses that require IRE1 activity for efficient viral replication.
Japanese encephalitis virus (JEV)	Positive-sensed single-stranded RNA-enveloped virus that requires IRE1 endonuclease activity with RIDD being suggested as a proviral mechanism.
Protein Kinase R-like ER Kinase (PERK)	
Virus	**Function**
herpes simplex virus 1 (HSV-1)	Counteracts Downstream PERK activation via the viral protein γ34.5 and inhibits PERK activation via glycoprotein B.
HCV	Inhibits PERK activation via viral proteins E1 and E2.
Activating Transcription Factor 6 (ATF6)	
Virus	**Function**
West Nile Virus (WNV)	Positive-sensed single-stranded RNA-enveloped virus that requires ATF6 for viral replication.
Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV)	Positive-sensed single-stranded RNA-enveloped virus that activates ATF6 and upregulates downstream target genes BiP and GRP94 via accessory protein 8ab.
SARS-CoV-2	Virus requires ATF6 for viral replication.

Table 1-2 Example list of viruses known to modulate or require UPR-affiliated functions for viral replication.

Modulation of the Unfolded Protein Response During Viral Infection

A critical aspect of protein synthesis quality control is to ensure that only correctly folded proteins are released from the ER, while misfolded proteins are targeted for degradation. In cases when the demand for protein folding surpasses the biosynthetic

capacity of the ER, the ER lumen can be saturated with unfolded or misfolded proteins, leading to ER stress. To cope with ER stress, cells activate the Unfolded Protein Response (UPR) [Figure 1-1] [22]. The activation of this evolutionarily conserved response relieves ER stress and restores homeostasis by decreasing the protein load in the ER, increasing protein folding capacity, and bolstering protein and mRNA degradation. However, if the cell cannot return to homeostasis, chronic UPR triggers apoptotic pathways to eliminate the affected cell and protect the organism. The UPR is governed by three ER transmembrane sensors activated in the presence of unfolded proteins: Inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6).
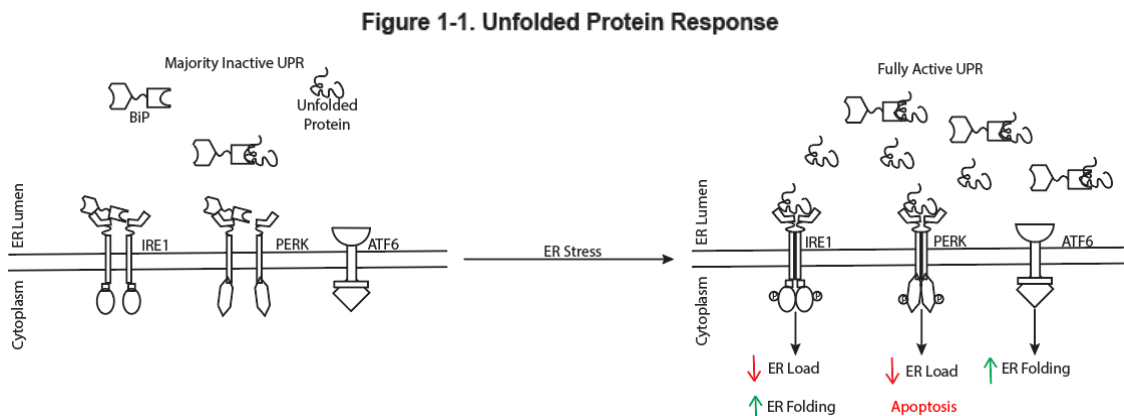
Figure 1-1 Simplified illustration of the Unfolded Protein Response (UPR). Consists of three transmembrane sensors/signal transducers: Inositol-Requiring Enzyme 1 (IRE1), Protein Kinase R-like Endoplasmic Reticulum Kinase (PERK) and Activating Transcription Factor 6 (ATF6). Most of the sensors are inactive until ER homeostasis has been perturbed, activating pathways involved in decreasing ER load, increasing ER folding capacity, or triggering apoptosis if homeostasis cannot be achieved.

Inositol-Requiring Enzyme 1 (IRE1):

Inositol-Requiring Enzyme 1 (IRE1) is a type 1 transmembrane protein that contains a lumenal stress sensing domain and cytoplasmic kinase and endonuclease domains [Figure 1-2] [23]. Under normal conditions, the lumenal domain of IRE1 is bound to the ATPase

binding domain of Binding immunoglobulin protein (BiP) [24]. In the presence of unfolded

proteins, BiP binds them, dissociating from IRE1. The unfolded proteins containing exposed

basic and hydrophobic residues act as activating ligands that can bind to the lumenal domain

of IRE1 [25,26]. This interaction causes the oligomerization of IRE1 and the trans-

autophosphorylation of the cytosolic domains. Active IRE1 oligomerizes, activating the

cytoplasmic RNAse domains, which mediate the non-canonical splicing of the X-box-

binding protein 1 (XBP1) mRNA in the cytosol [27]. The spliced XBP1 (XBP1s) mRNA

encodes for a potent transcription factor that upregulates UPR-related genes to increase the

ERs folding capacity (e.g., chaperones), promote the degradation of proteins (e.g., ERAD)

among other cellular processes [28]. The endonuclease activity of IRE1 also targets mRNAs

at the ER membrane in a process known as IRE1-dependent decay (RIDD), thus helping

reduce protein load further [29]. However, if ER stress persists and ER homeostasis is not

restored, IRE1 can interact with tumor necrosis factor receptor-associated factor 2 (TRAF2),

triggering apoptosis [30].

Figure 1-2. Inositol Requiring Enzyme 1 (IRE1) Signaling Pathway
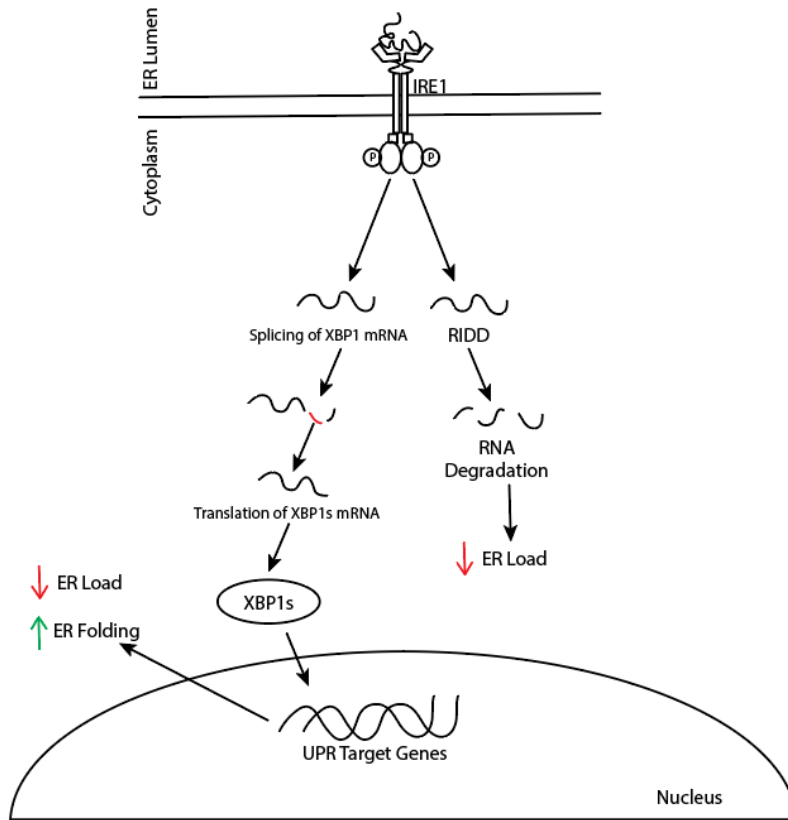
Figure 1-2 Simplified illustration of the IRE1 pathway. Activation leads to unconventional splicing of XBP1, resulting in the translation of the active transcription factor XBP1s. IRE1 activation can also result in RNA degradation via the IRE1-dependent decay (RIDD).

Viruses from multiple families, including the coronaviruses Mouse Hepatitis Virus (MHV) and SARS-CoV-2, the herpesvirus HCMV, the flaviviruses Zika Virus and Japanese encephalitis virus (JEV), and the Influenza virus, among others, modulate IRE1 activity and signaling to promote viral infection and prevent premature cell death. During MHV infection, XBP1 is spliced; however, it is not efficiently translated, and its downstream target genes, like ERAD factors, are not upregulated [31]. In HCMV infection, IRE1 is activated at the early stages of viral replication [32]. Interestingly, at the late stages of infection, the viral protein UL50 binds to IRE1 and causes its degradation by an unknown mechanism

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[33]. During ZIKV infection, IRE1 is activated, and the splicing of XBP1 is required for infection [34].

These few examples show the extent of IRE1 modulation during infection and place this sensor as a potential antiviral target. Indeed, pharmacological inhibition of IRE1 reduces replication of the Influenza virus and SARS-CoV-2; however, the exact mechanism behind the requirement has not been identified [35,36]. A well-characterized example of the requirement of IRE1 comes from studies on JEV [37]. Inhibition of the endonuclease activity of IRE1 resulted in decreased viral protein expression and virion production, but not viral RNA synthesis, suggesting RIDD as a pro-viral mechanism. The requirement for RIDD during JEV infection was confirmed by the knockdown of XBP1, which did not decrease viral replication but exacerbated the cytopathic effects caused by the virus [38]. IRE1 remains an attractive molecule for future studies on the development of potential antivirals.

Protein Kinase R-like Endoplasmic Reticulum Kinase (PERK):

The second branch of the UPR is governed by the protein kinase R-like endoplasmic reticulum kinase (PERK) [Figure 1-3]. PERK is a sensor shared by the UPR and the integrated stress response (ISR) [39]. This homeostatic program responds to various stresses, including hypoxia, glucose deprivation, amino acid deprivation, and viral infection. Like IRE1, PERK is a type 1 transmembrane protein sensor with an ER lumenal domain that senses the accumulation of unfolded proteins. Following recognition of unfolded proteins, PERK is activated by oligomerization which causes autophosphorylation of the C-terminal kinase domain. Active PERK phosphorylates the alpha (α) subunit of the eukaryotic initiation factor 2 at Serine-51 (eIF2α) [40]. eIF2 forms a ternary complex with GTP and the initiating tRNA (Met-tRNAi) and binds the 40S subunit to bring the Met-tRNAi near the start codon.

Upon recognition of the start codon, GTP is hydrolyzed, thus reducing the affinity of eIF2 for Met-tRNAi. The exchange of GDP for GTP in eIF2 is mediated by the guanine exchange factor (GEF) eIF2B, allowing further rounds of translation initiation. The phosphorylation of the α subunit prevents the activity of eIF2B and attenuates global translation. Under these conditions, however, some proteins are preferentially translated [41]. Such is the case of the Activating Transcription Factor 4 (AFT4), which promotes the expression of genes involved in metabolism and the growth arrest and DNA damage-inducible protein (GADD34). The sustained phosphorylation of eIF2α under prolonged ER-stress conditions results in the ATF4-dependent expression of the CCAAT-enhancer-binding protein homologous protein (CHOP), a transcription factor that initiates the apoptotic pathway.

Figure 1-3. Protein Kinase R-Like Kinase (PERK) Signaling Pathway
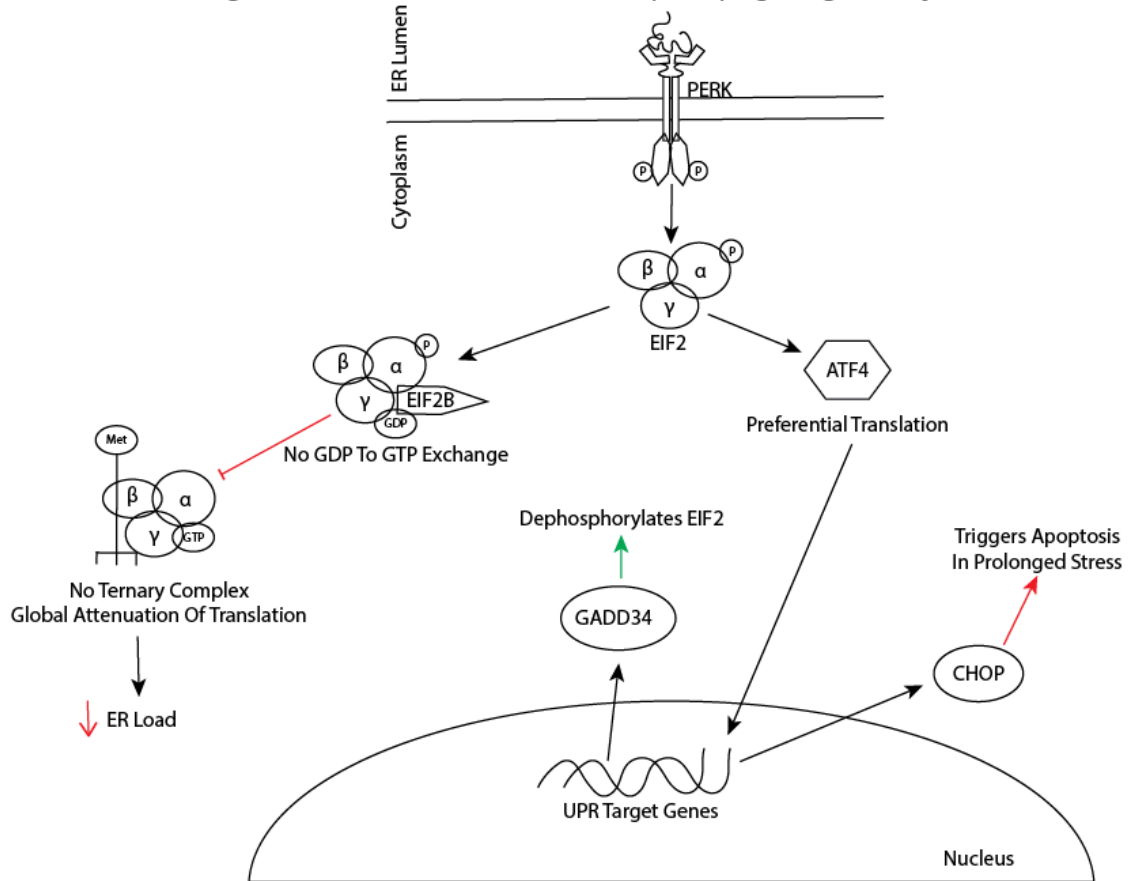
Figure 1-3 Simplified illustration of the PERK pathway. Activation leads to phosphorylation of the alpha subunit of eIF2, which results in global translation attenuation. This event can result in the preferential translation of the transcription factor ATF4, which can lead to the expression of GADD34 to dephosphorylate eIF2. ATF4 can also result in the expression of CHOP, which can trigger apoptosis if the ER stress is prolonged.

The activation of apoptotic pathways due to the persistent activation of PERK during viral infection could result in premature cell death and inhibition of viral replication. To prevent such an outcome, viruses have developed ways to inhibit PERK activation or dephosphorylate eIF2α to drive translation. The herpes simplex virus 1 (HSV-1) protein γ34.5, an ortholog of GADD34, can dephosphorylate eIF2α, thus allowing translation to continue even in conditions when the ISR is activated [42]. HSV1 can also directly inhibit PERK activation by interaction with the viral glycoprotein B, which prevents dimerization

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even under ER-stress conditions [43]. A similar strategy can be seen in HCV infection, where

the E1 and E2 envelope glycoproteins directly interact with PERK [44,45].

Activating Transcription Factor 6 (ATF6):

The third branch of the UPR is governed by the activating transcription factor 6

(ATF6), a type II transmembrane protein with its C-terminus in the ER lumen and an N-

terminal bZIP DNA binding signaling domain facing the cytosol [Figure 1-4] [46]. The

inactive form of ATF6 exists as an ER membrane-tethered precursor held in disulfide-linked

oligomers. Under ER stress conditions, ATF6 is translocated to the Golgi apparatus, where it

is processed by the Site-1 protease (S1P) and Site-2 protease (S2P). The intramembrane

proteolysis liberates the ATF6-DNA binding domain (ATF6-N). ATF6-N translocates to the

nucleus and transactivates the expression of UPR effector genes containing ER stress

response element (ERSE) in the promoters [47]. The primary target genes of ATF6 are ER

chaperones (e.g., BiP and Glucose-Regulated Protein 94 {GRP94}) and UPR mediators (e.g.,

XBP1). The main role of ATF6 is to promote protein folding to restore ER homeostasis.

Figure 1-4. Activating Transcription Factor 6 (ATF6) Signaling Pathway
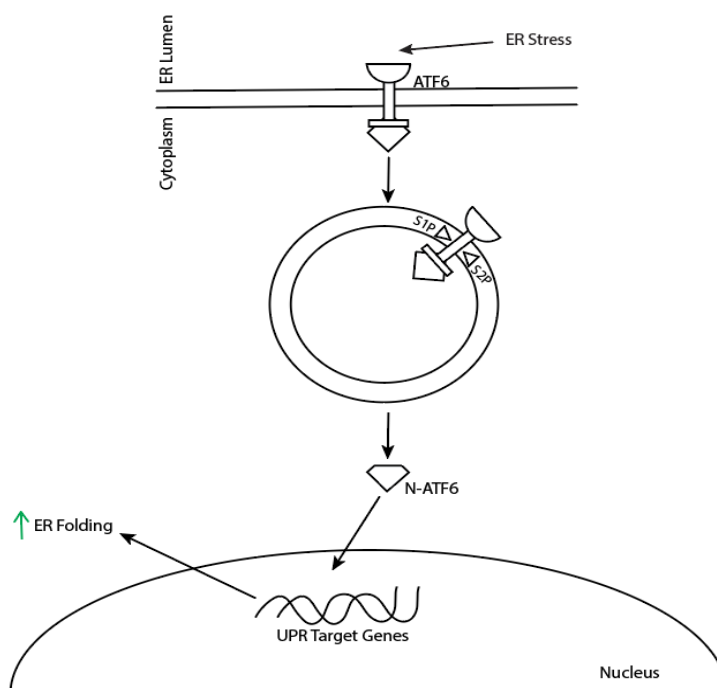
Figure 1-4 Simplified illustration of the ATF6 pathway. Activation leads to the translocation and processing of ATF6, resulting in its active transcription factor form that goes to the nucleus to upregulate genes to increase ER folding capacity.

Like the other branches of the UPR, ATF6 is differentially modulated by viruses during infection. The flaviviruses Dengue, Zika, and West Nile virus activate ATF6 by a mechanism yet to be determined [48,49]. The requirement of flaviviruses for ATF6 activity varies between viruses and cell types infected [50,51]. In Coronavirus infected cells, the mechanism of ATF6 activation has been well described for severe acute respiratory syndrome coronavirus (SARS-CoV) [51]. During SARS-CoV infection, the accessory protein 8ab is located in the ER lumen, where it interacts and activates ATF6, resulting in the upregulation of the ER-resident chaperones BiP and GRP94 [52]. Interestingly, the expression of the SARS-CoV-2 protein ORF8, a homologue of SARS-CoV ORF8ab, results in the activation of ATF6, which is required for efficient virion release [36]. These

observations suggest a conserved mechanism of ATF6 activation during SARS-CoV and SARS-CoV-2 infection.

Understanding how viruses can modulate the ER and UPR drives the ideas for therapeutic intervention against them and further contributes to the expansive knowledge of virology. As mentioned earlier, ATF6 is known for upregulating ER-resident chaperones that contribute to relieving ER stress by either assisting in protein folding, processing, and degradation. Viral infections have also been implicated in utilizing these ER-resident and cytosolic chaperones for their benefit.

ER-Resident Chaperones During Viral Infections

Several viral structural proteins, such as transmembrane and glycoproteins, are translated, folded, and processed within the ER [16]. Most of these viral structural proteins, and on some occasions, nonstructural viral proteins, rely on the ER chaperone machinery. The folding of viral proteins in the ER involves the activity of ER-resident chaperones, including GRP94, Calreticulin, Calnexin, and BiP. There are numerous examples of viral modulation of the activity of ER-resident chaperones. Here I will focus on the viral control of the UPR-regulated molecular chaperones GRP94 and BiP [Table 1-3]. These chaperones belong to the Heat Shock Protein (HSP) family, which plays a critical role in maintaining protein homeostasis (proteostasis) [53]. Genetic and pharmacological targeting of molecular chaperones has interested researchers in unraveling their importance in human diseases and infections.

Table 1-3. Examples Of ER-Resident Chaperones Functions In The Context Of Viral Infections.

Glucose-Regulated Protein 94 (GRP94)	
Virus	**Function**
vesicular stomatitis virus (VSV)	Negative-sense single-stranded enveloped RNA virus that requires GRP94 to chaperone the proper receptors for viral binding and entry.
DENV and Zika Virus (ZIKV)	Both are positive-sensed single-stranded RNA-enveloped virus that requires GRP94 for efficient viral replication.
Binding Immunoglobulin Protein (BiP)	
Virus	**Function**
Middle East Respiratory Syndrome Coronavirus (MERS-CoV), Bat Coronavirus HKU9 (bCoV-HKU9), SARS-CoV-2	All three are positive-sensed single-stranded RNA-enveloped viruses that have been documented to utilize BiP as a coreceptor for cell entry.
DENV and ZIKV	BiP has been documented to be required for viral replication.
Hepatitis B Virus (HBV)	Double-stranded DNA enveloped virus that requires BiP for managing ER stress.

Table 1-3 Example list of viruses that modulate or require molecular chaperones GRPP94 and BiP for viral replication.

Glucose Regulated Protein (GRP94)

GRP94 is an HSP90 molecular chaperone, which contains a nucleotide-binding domain (NBD) for ATP that cycles between a closed (ATP bound) and open (ADP bound) state [54]. It also has a C domain for dimerization and an M domain where the client proteins are thought to bind with calcium ions. Like the other chaperones, GRP94 is known to assist in protein folding and degradation. ATF6 transcriptionally regulates it, and its expression increases during ER stress. GRP94 is required for infection. In GRP94 knockout cells, vesicular stomatitis virus (VSV) does not penetrate the cell due to the lack of low-density lipoproteins (LDL), which act as receptors for the virus [55,56]. LDLs are clients for GRP94, and without the chaperone activity, LDLs would not be folded correctly, thus limiting the receptors necessary for viral entry [57]. In cells infected with the flaviviruses DENV and ZIKV, the pharmacological inhibition and genetic silencing of GRP94 in Huh-7 cells resulted in decreased replication for both viruses [58]. The chaperone activity of GRP94 may be required for viral protein folding. An alternative hypothesis is that the knockdown of GRP94

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results in the accumulation of misfolded proteins that can negatively affect cell health and DENV/ZIKV replication.

Binding Immunoglobulin Protein (BiP)/Glucose Regulated Protein 78 (GRP78)

BiP/GRP78 is the most abundant ER-resident chaperone and has been extensively studied as an essential factor in ER homeostasis regulation. Due to its crucial role, BiP is frequently dysregulated in disease states (cancer, infection, neurodegeneration) [59,60]. It is part of the Heat Shock Protein 70 (HSP70) molecular chaperone subfamily and was initially classified based on its ability to assist in immunoglobulin assembly and increased expression during glucose starvation [61,62,63]. Besides its UPR regulatory functions, BiP can assist in protein importing, folding, complex assembly, and exporting unfolded proteins for degradation via ERAD [64]. Like the other isoforms of HSP70, BiP contains two conserved domains: the nucleotide-binding domain (NBD) and the substrate-binding domain (SBD), where the chaperone activity is regulated by an ATPase cycle [65]. When the NBD is ATP bound, the SBD is in its open conformational state where protein clients can bind with low affinity. When the ATP hydrolyzes to ADP, the lid of the chaperone closes, resulting in a closed conformation with a high affinity for substrate binding, thus allowing the client to fold in its natural conformation and prevent aggregation and misfolding. The ATPase activity for this chaperone is typically regulated by J proteins known to stimulate ATP hydrolysis and nucleotide exchange factors (NEFs) that facilitate the ADP to ATP exchange in the NDB [66,67].

BiP activity has been documented to assist viral entry, replication, and host survival. While predominantly an ER-bound protein, BiP can localize to the cell surface under ER-stress conditions and act as a virus receptor [68]. Indeed, BiP has been identified as a

coreceptor for Middle East respiratory syndrome coronavirus (MERS-CoV) and bat coronavirus HKU9 (bCoV-HKU9). While these viruses use different cellular receptors for entry, BiP mediates the attachment of the virus' spike protein to the cell surface [69]. In silico predictive modeling also identified the spike protein from SARS-CoV-2 as a potential binding partner of BiP, thus hinting at the conserved role of BiP as a Betacoronavirus coreceptor [70]. BiP has also been shown as an essential factor for viral replication downstream of entry events. Genetic knockout and silencing of BiP reduces virion release, protein production, and genome replication in Flaviviruses such as DENV and ZIKV [71,72]. The exact mechanism behind the requirement for BiP remains to be elucidated. However, it is known that BiP can interact with the envelope protein of DENV and the voltage-dependent anion channel (VDAC), an essential factor for infection by this virus [73]. BiP also promotes cell survival in hepatocytes infected with the hepatitis B virus (HBV). During infection, BiP is upregulated and prevents apoptosis by keeping the cell under mild ER stress conditions to establish a chronic infection [74]. BiP silencing in HBV-infected cells increases CHOP expression and cell death when ER-stress homeostasis is pharmacologically disrupted. As a master regulator of the UPR, high levels of BiP promote cell survival by assisting in protein folding or degradation and preventing exacerbated ER stress. It remains unclear if BiP prevents the activation of the UPR sensors during HBV infection. These few examples illustrate the importance of BiP for viral infections and motivate the continued exploration of its role as a pro-viral and pro-survival factor.

Kaposi Sarcoma Associated Herpesvirus (KSHV)

Kaposi Sarcoma Associated Herpesvirus (KSHV) is an oncogenic double-stranded DNA virus member of the gammaherpesvirus subfamily [75]. As with all other herpesviruses, KSHV infection establishes latency, a dormant and persistent infection. The virus reactivates from latency and enters a lytic stage of infection upon certain conditions, such as immunosuppression [76]. The lytic phase of infection is a highly transcriptionally and translationally active state in which all the proteins necessary for viral replication are rapidly and abundantly made. The KSHV genome encodes for over 80 ORFs expressed in a highly regulated and timely pattern [77]. KSHV is the causative agent of malignancies such as Kaposi Sarcoma (KS), Primary Effusion Lymphoma, and Multicentric Castleman Disease (MCD). These diseases, while rare, afflict immunocompromised patients reducing their quality of life or resulting in death. Despite our sophisticated understanding of KSHV biology and pathogenesis, currently, there is no cure for KSHV infections or their related malignancies. Future studies on virus and host interactions will allow us to explore and develop better therapeutics to address this unmet medical need.

Epidemiology of KSHV-Based Malignancies

One of the primary diseases associated with KSHV infection, Kaposi's sarcoma, was initially described in the 1800s by Moritz Kaposi in aging Mediterranean men who developed malignant soft tissue lesions in their lower limbs [78]. The infectious nature of the disease was not appreciated until several decades later, during the HIV epidemic of the late 1980s, when several cases of Kaposi's sarcoma were identified in AIDS patients [79]. Although it was suspected that an infectious agent played a role in the disease, it was not until 1994 that differential PCR analysis confirmed the presence of gammaherpesvirus DNA

sequences in KS lesions compared to unaffected skin [80]. Identifying KSHV led to future studies on understanding its epidemiology, pathogenesis, and molecular virology.

KS manifests as highly vascularized soft tissue lesions [81] [Figure 1-5]. Most tumor cells in KS lesions are of endothelial origin and are latently infected with the virus. The malignancy occurs in four epidemiological forms: Classic, Endemic, Iatrogenic, and Epidemic. Classic KS typically involves the appearance of lesions in the lower extremities in elderly individuals whose immune system has weakened due to advanced age. Endemic KS has been described in Sub-Saharan Africa, where KSHV is highly prevalent and afflicts middle-aged adults and children. Iatrogenic KS presents in KSHV-positive patients undergoing immunosuppressant treatment for an organ transplant. The final form, epidemic KS, is associated with HIV infection and is an AIDS-defining malignancy. KS is still highly prevalent in areas in Africa and is still the second most recorded cancer in individuals with AIDS [82]. Treatments for KS include chemotherapy, radiation therapy, immunotherapy, surgery, and antiviral therapy if the individual is infected with HIV [83]. However, all these treatments do not eradicate KSHV but alleviate the symptoms and slow the progression of the disease.

Figure 1-5. KSHV Associated Diseases
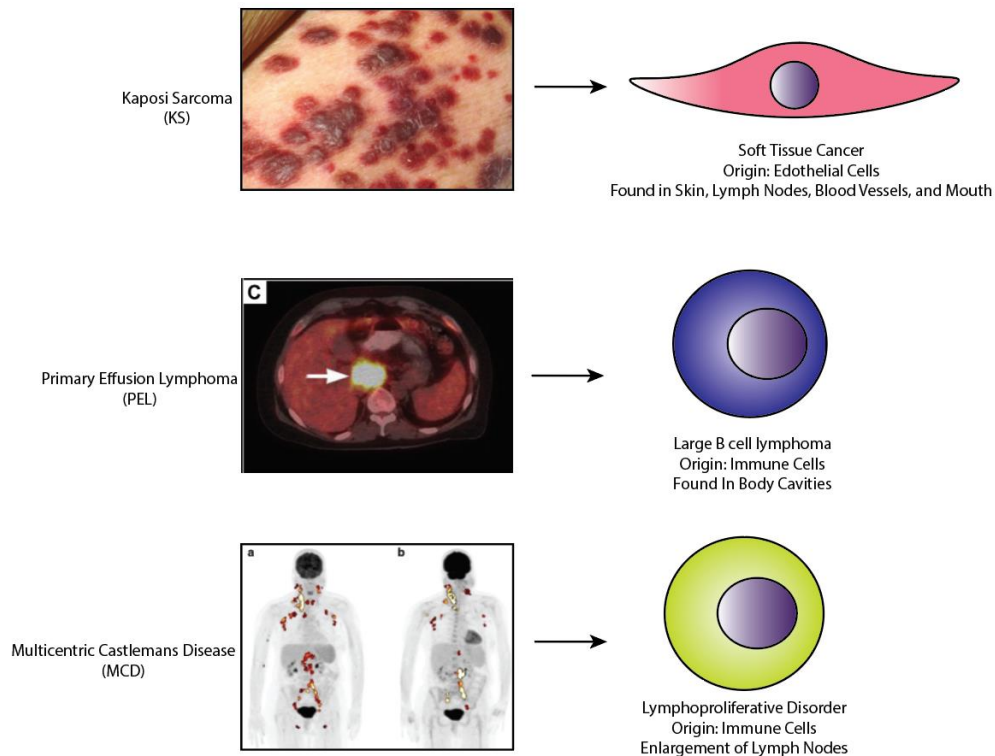
Kaposi Sarcoma (KS)

Soft Tissue Cancer
Origin: Edothelial Cells
Found in Skin, Lymph Nodes, Blood Vessels, and Mouth

Primary Effusion Lymphoma (PEL)

Large B cell lymphoma
Origin: Immune Cells
Found In Body Cavities

Multicentric Castlemans Disease (MCD)

Lymphoproliferative Disorder
Origin: Immune Cells
Enlargement of Lymph Nodes

Figure 1-5 KSHV-associated diseases: Kaposi Sarcoma (KS), Primary Effusion Lymphoma (PEL), and Multicentric Castlemans Disease. Includes images of the diseases, information on what kind of disease, cellular origin, and localization in the human body.

Other KSHV-associated diseases, Primary Effusion Lymphoma (PEL) and Multicentric Castleman's Disease (MCD), were also reported during the HIV epidemic in the 1980s and 1990s [84,85]. PEL is a large B-cell lymphoma described in 1989 in AIDS patients. The presence of KSHV in PEL-derived tumor cells was confirmed in 1995, a year after the discovery of the virus. As the name of the disease indicates, the main symptom of PEL is the development of malignant effusions in body cavities derived from large immunoblastic or plasmablastic cells. Gene expression profiling of PEL cells shows a malignant plasma cell gene expression compared to other B-cell lines [86]. MCD, a lymphoproliferative disease associated with KSHV, is characterized by enlarged lymph

nodes due to the hyperproliferation of immune cells. Like KS, PEL and MCD are endemic in regions with high KSHV prevalence, middle-aged AIDS patients, and organ transplant patients. Treatments for these malignancies include chemotherapy and anti-retroviral therapy in HIV-positive patients. Though remission has been documented after treatment, none of the therapies eradicate the KSHV genome as it resides within the nucleus as a minichromosome, causing a life-long infection.

KSHV Background Of Infection

KSHV has a broad cell tropism, including human endothelial cells, B-cells, epithelial cells, and normal human fibroblasts [87]. The KSHV virion is an enveloped particle with an icosahedral capsid containing the dsDNA linear genome [Figure 1-6]. The viral glycoproteins gB, gH, gL, gM, gN, ORF4, and gpK8.1A are displayed on the surface of the virus and mediate host attachment, viral entry, and virion assembly. The primary KSHV receptor identified to date is the cell surface heparan sulfate (HS). This proteoglycan is ubiquitously expressed and can interact with KSHV glycoproteins (gB, gpK8.1A, ORF4, and gH). After attachment, virus entry requires internalization receptors such as integrins, xCT, Dendritic cell-specific intracellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN), and ephrin receptor A2 (EphA2). Using these receptors, the virion enters the cell via endocytosis, and the capsid is transported to the nucleus periphery via the cytoskeleton. The genome is released in the nucleus, circularized into an episome, and associated with histones [88]. The resulting chromatinized episome binds host nucleosomes in an interaction mediated by the viral Latent Antigen (LANA). During latency, viral gene expression is repressed by epigenetic silencing, and the episome is replicated and segregated during cell division.

Figure 1-6. KSHV Entry And Establishment Of Latent Infection
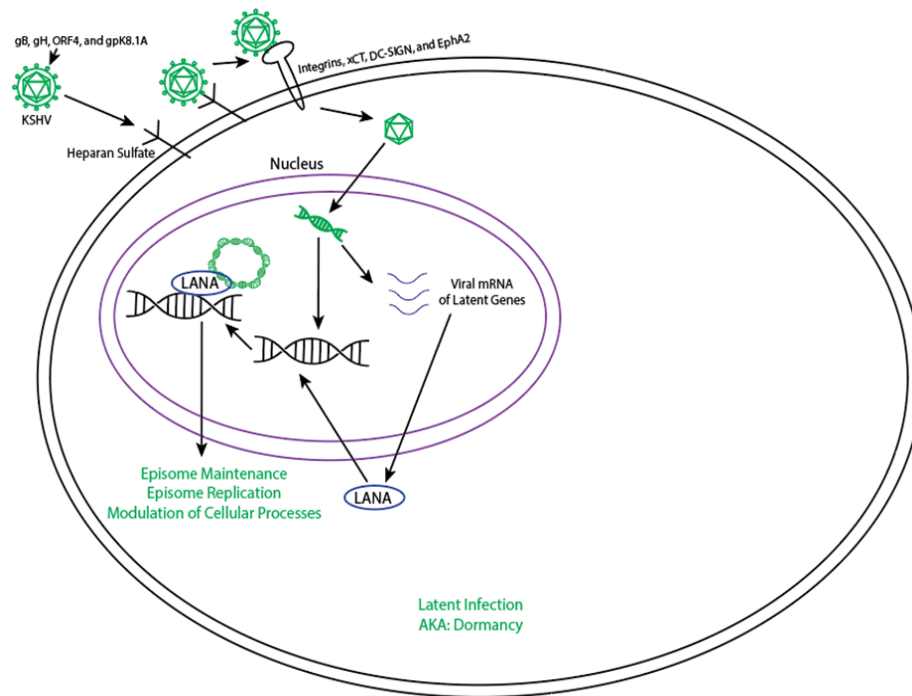
Figure 1-6 Illustration of KSHV entry and establishment of latent infection. KSHV entry involves the virion attaching to the cell via glycoproteins and Heparan sulfate. After attachment, the virion is then internalized by internalization receptors (Integrins, xCT, DC-SIGN, and EphA2). The capsid is then transported to the nucleus periphery, where the genome is released into the nucleus. The genome is then associated with histones with assistance from the latency-associated nuclear antigen (LANA). The virus is then in a dormant state where only a handful of viral genes are expressed.

KSHV latency is characterized by the expression of a handful of genes dedicated to maintaining infection by preventing host apoptosis, inducing cell growth/proliferation, inhibiting immune signaling, and promoting episome preservation/replication [Table 1-4]. The switch from the latent to lytic cycles has been attributed to the expression of the viral transcription factor ORF50 (replication and transcription activator [RTA]), an immediate early (IE) lytic gene that is sufficient and necessary to initiate a temporal cascade of genes resulting in viral replication and virion production [Figure 1-7] [89]. Lytic reactivation in KSHV-infected cells requires epigenetic changes in the viral chromatin allowing expression

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of the lytic genes. Some factors that trigger KSHV lytic reactivation include co-infection with other viruses, cellular stress, and treatment with chemical epigenetic modulators. The expression of the HIV-soluble factor Tat triggers KSHV reactivation and promotes KS progress [90,91,92]. Cellular stress stimuli such as hypoxia and reactive oxygen species (ROS) have been documented to cause the switch from latent to lytic via the accumulation of stress-induced proteins. For example, hypoxic conditions increase hypoxia-inducible factor 1-α expression, a transcription factor that can interact with the RTA promoter in PEL cells and promote its expression leading to lytic reactivation [93]. Treatment of latently infected cells with Histone deacetylase (HDAC) inhibitors like sodium butyrate (NaB) and Valproic Acid (ValA) directly modify the chromatin landscape of the viral genome and triggers entry to the lytic cycle in multiple cell lines [94].

Figure 1-7. KSHV Lytic Cycle Towards Virion Replication And Release
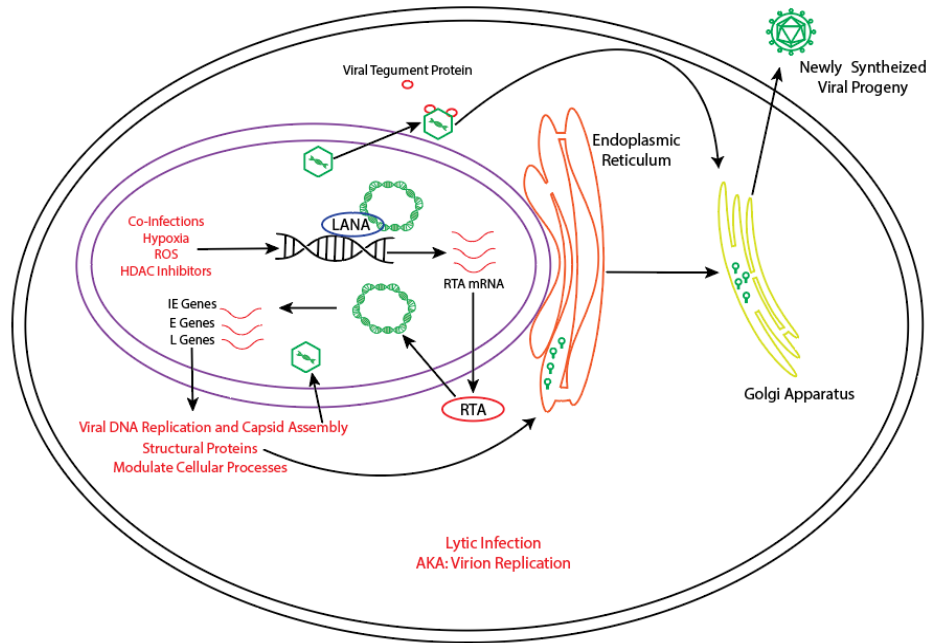
Figure 1-7 Illustration of KSHV lytic infection and virion release. The lytic cycle has been reported to occur after Co-infection with another virus, exposure to a hypoxic environment, exposure to reactive oxygen species (ROS), and exposure to HDAC inhibitors. Lytic cycle induction results in the expression of RTA, the critical transcription factor for lytic gene expression. Lytic genes include the immediate early (IE), Early (E), and late (L). Expression of the lytic genes produces the components necessary for viral DNA replication, capsid assembly, structural proteins, and proteins involved in modulating cellular processes. The immature nucleocapsids acquire the tegument proteins and envelope from the trans-Golgi network. The mature enveloped particles are then released via the fusion of Golgi-derived vesicles and the plasma membrane.

HDAC inhibitors have been a staple in KSHV vitro studies, but their pleiotropic effects have made it challenging to discern compound versus virus-dependent phenotypes. To circumvent the use of HDAC inhibitors, several groups have developed stable cell lines expressing inducible RTA to promote viral transcription and lytic reactivation [95,96]. One such model is the BCBL1-RTA PEL-derived cell line obtained from a KSHV-positive patient. These cells are latently infected and stably express a doxycycline-inducible RTA

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transgene. While valuable for studying virus/host interactions, a limitation of the PEL-derived cell models is spontaneous viral lytic reactivation seen in ~5-10% of cells, which hinders the analysis of latent gene expression. A second restriction of this system is the lack of an uninfected counterpart cell line that can serve as a control in experiments. An alternative model cell line, the iSLK-219 system, was developed to circumvent these limitations. These kidney epithelial carcinoma cells were engineered to express a doxycycline-induced RTA transgene, similar to the one used in BCBL1-RTA cells. The RTA-expressing cell line, deemed iSLK, was infected with a recombinant KSHV virus called the rKSHV219. This virus expresses a constitutive GFP reporter detected during latent infection, and an RTA-inducible RFP reporter expressed only during the lytic cycle. The resulting cell line, iSLK-219, is latently infected with KSHV, has very low levels of spontaneous reactivation (below 1%), and has an uninfected isogenic counterpart (iSLK cells) [95]. These characteristics make iSLK-219 cells a great model for studying KSHV biology and virus/host interactions.

The KSHV lytic cycle is tightly regulated at the transcriptional level and temporally divided according to viral gene expression into three states: immediate early (IE), early (E), and late (L) lytic [89] [Table 1-4]. The lytic cycle typically begins when RTA is expressed, causing the transcriptional upregulation of IE and E lytic genes that modulate host responses, enhance viral transcription, and initiate viral DNA replication. The late (L) lytic genes are expressed after DNA replication occurs and include most structural proteins. Once DNA replication and late lytic gene expression are complete, the viral particle is assembled in the host cell nucleus by incorporating the newly replicated DNA genome into a capsid to form a nucleocapsid which is then released into the cytoplasm. The immature nucleocapsids acquire

the tegument proteins, located in the space between the envelope and the capsid, and the

envelope from the trans-Golgi network. The mature enveloped particles are then released via

the fusion of Golgi-derived vesicles and the plasma membrane. After release, the mature

virions can infect other cells, thus propagating infection.

Table 1-4. Current List Of KSHV Genes.

Gene Name	Function	Time of Expression
ORFK1	Glycoprotein	Latent
ORFK2	Viral Interleukin 6 Homologue (vIL6)	Latent
ORFK12	Kaposin	Latent
ORF71	viral FLICE-inhibitory protein (vFLIP)	Latent
ORF72	Viral Cyclin (vCyclin)	Latent
ORF73	Latency-Associated Nuclear Antigen (LANA)	Latent
ORF11	Predicted dUTPase	Immediate Early
ORF16	Viral Bcl-2 (vBcl-2) Homologue	Immediate Early
ORF45	p90 Ribosomal S6 Kinase (RSK) Activator	Immediate Early
ORF50	Replication And Transcription Activator (RTA)	Immediate Early
ORF57	mRNA export/splicing	Immediate Early
ORF70	Thymidylate Synthase	Immediate Early
ORFK4	viral macrophage inflammatory protein-II (vMIP-II)	Immediate Early
ORFK5	RING-CH E3 Ubiquitin ligase	Immediate Early
ORFK6	viral macrophage inflammatory protein-I (vMIP-I)	Immediate Early
ORFK8	KbZIP	Immediate Early
1.4 kb	Unknown	Immediate Early
PAN	Late gene expression	Immediate Early
ORF2	dihydrofolate reductase	Early ~
ORF4	Complement Binding Protein	Early
ORF6	ssDNA Binding protein	Early
ORFK3	RING-CH E3 Ubiquitin Ligase	Early
ORF17.5	Assembly Protein	Early
ORF18	Late Gene Regulation	Early
ORF34	Scaffold Protein	Early
ORF35	Unknown	Early
ORF36	Serine protein kinase	Early
ORF37	SOX	Early
ORF38	Myristylated Protein	Early
ORF39	Glycoprotein M	Early
ORF46	Uracil Deglycosylase	Early
ORF47	Glycoprotein L	Early
ORF49	Activates JNK/p38	Early ~
ORF58	EBV BMRF2 homologue	Early
ORF59	Processivity factor	Early
ORF60	Ribonucleoprotein Reductase Small Subunit	Early
ORF61	Ribonucleoprotein Reductase Large Subunit	Early
ORFK14	Viral OX2 (vOX2)	Early
ORF74	vGPCR	Early
ORFK3A	Immune modulator	Early
K5/6-AS	Unknown	Early
ORF17	Protease	Early
ORF45.1	Unknown	Early

Gene Name	Function	Time of Expression
ORF8	Glycoprotein B	Late
ORF9	DNA Polymerase	Late
ORF10	Regulator of Interferon Function	Late
ORF21	Thymidine kinase	Late
ORF22	Glycoprotein H	Late
ORF23	Glycoprotein (predicted)	Late
ORF24	Essential for replication	Late
ORF25	Major capsid protein	Late
ORF26	Minor capsid protein	Late
ORF27	Glycoprotein	Late
ORF28	BDLF3 EBV homolog	Late
ORF29	Packaging Protein	Late
ORF30	Late Gene Regulation	Late
ORF31	Nuclear and Cytoplasmic	Late
ORF32	Tegument Protein	Late
ORF33	Tegument protein	Late
ORF40/41	Helicase-Primase	Late
ORF42	Tegument protein	Late
ORF43	Portal Protein (Capsid)	Late
ORF44	Helicase	Late
ORF52	Tegument protein	Late
ORF53	Glycoprotein N	Late
ORF54	dUTPase/Immunomodulator	Late
ORF55	Tegument protein	Late
ORF56	DNA replication	Late
ORFK8.1	Glycoprotein	Late
ORFK9	Viral Interferon Regulatory Factor 1 (vIRF1)	Late
ORFK10	Viral Interferon Regulatory Factor 4 (vIRF4)	Late
ORFK10.5	Viral Interferon Regulatory Factor 3 (vIRF3)	Late
ORFK11	Viral Interferon Regulatory Factor 1 (vIRF2)	Late
ORF62	Capsid	Late
ORF63	NLR homolog	Late~
ORF65	Capsid	Late
ORF66	Late Gene Regulation	Late
ORF67	Nuclear egress complex	Late
ORF67.5	DNA packaging protein	Late
ORF68	DNA Binding Protein	Late
ORF69	BRLF2 Nuclear egress	Late
ORF75	FGARAT	Late
ORF7	Terminase Complex Subunit	Early/Late
ORF48	Virion Protein	Latent/Immediate Early
ORF64	Deubiquitinase	Lytic
ORFK15	LMP1/2 Homologue	Latent/Early
ORFK4.1	Unknown	Unknown

Table 1-4 Current list of KSHV genes recorded to be expressed in different KSHV-infected cell models. The list includes the gene name, function, and expression time denoted as latent, immediate early, early, and late.

Current Knowledge of The UPR and ER Factors In The Context Of KSHV Infection

The ER has been known to be implicated in the replication of KSHV for over a decade. The virus encodes 14 proteins predicted to contain a signal peptide necessary for ER translocation, which suggests they are translated, folded, and modified in the ER [Table 1-5]. For the past decade, research on KSHV and the ER has been focused on understanding UPR modulation in the context of latent and lytic infection, understanding what aspects of the UPR are required for KSHV infection, the effects of ER stress in KSHV-infected cells, and what ER proteins are essential for KSHV infection [Table 1-6].

Currently, only one study by the Fujimuro group compared the basal protein and mRNA levels in latently infected PEL cells and uninfected B cell lymphomas [97]. The PEL cells were found to have lower overall basal mRNA levels even in the presence of ER stress inducers. Transfection experiments in HeLa cells with latent genes LANA and the cellular cyclin homolog called vCyclin identified suppression of the transcription of IRE1, but not the other branches, thus illustrating a specificity in UPR sensor modulation. What combination of viral factors in latent infection modulates the sensors has yet to be fully shown.

Previous de novo infection studies in primary cells have shown what occurs at the downstream signaling of the UPR but not the sensors themselves [98]. Acute KSHV infection in human umbilical vein endothelial cells (HUVECs) resulted in the upregulation of the molecular chaperone BiP, a transcriptional target of ATF6. CHOP expression was also elevated after infection, but whether this is due to PERK activation was not explored further. Similar results were found in primary macrophages where infection increased BiP, ATF4, CHOP, IRE1, and XBP1s protein levels [99]. In both cases, it has been hypothesized that this

manipulation of the downstream signaling of the UPR results in increasing the production and secretion of host factors that are pro-viral and pro-tumorigenic.

As for the lytic cycle, previous work done by the McCormick lab determined that the UPR sensors are activated in the PEL cell line BCBL1-RTA. However, the downstream transcriptional response is suppressed. Specifically, IRE1 was shown to be activated during the lytic cycle, with evidence of XBP1 being spliced at the mRNA level but decreasing as the cycle progressed. At the protein level, XBP1s expression was evident within the first 24 hours but not as dramatic as latent cells acutely treated with the ER toxin Thapsigargin (TG). Thapsigargin inhibits the sarco/endoplasmic reticulum Ca+2 ATPase (SERCA), which prevents Calcium ions from pumping into the ER, causing an influx of cytoplasmic Calcium and depletion of ions in the ER. Interestingly, when the lytically active cells were acutely stressed with TG, XBP1 at the protein and mRNA levels decreased dramatically as the lytic cycle progressed. Similar results were found in the PERK branch where sensor activation was evident, but there was no accumulation of ATF4 even though eIF2α was phosphorylated. Like XBP1, ATF4 increased in the presence of TG but decreased as the lytic cycle progressed, demonstrating a trend of sensor activation followed by silencing the downstream effects of the UPR. ATF6 is no exception, as lytic induction increases the DNA binding domain that translocates to the nucleus but decreases at the protein level in the late stages. Even though the transcription factor fragment is evident, there was no upregulation of known ATF6 targets at the protein and mRNA levels.

The mechanism behind suppressing the transcriptional responses has yet to be discovered. Still, one of the current hypotheses is that the modulation supports viral

replication by preventing the unwanted accumulation of UPR transcription factors from upregulating genes that can be detrimental to the lytic cycle. Evidence that supports this hypothesis is that the overexpression of XBP1s in the KSHV-infected epithelial cell line iSLK-219 resulted in decreased viral replication. No viral factors have been identified as the culprits behind the suppression of the transcriptional response. The only experiments focusing on the subject were on the KSHV lytic-induced host shut-off protein and exonuclease ORF37 (SOX), which can cleave mRNAs resulting in their degradation via the host exonuclease XRN1 [91]. However, overexpression of SOX in HEK293s by the McCormick lab did not display modulation of the downstream effects of the UPR even in the presence of ER toxins, thus leaving this question for future interpretation.

When focusing on the three sensors of the UPR, pharmacological inhibitors and genetic silencing were developed to test what occurs in cellular systems and infections for various diseases. For KSHV, the inhibition and silencing of IRE1 resulted in total downregulation of viral replication and infected cell survival [100,101]. Specifically, the lack of IRE1 reduced viral replication during the lytic cycle in iSLK-219 and BCBL1-RTA cells. Previous work in PEL cells identified that XBP1s could transactivate RTA expression, but whether XBP1s is required for immediate early gene expression has yet to be proven [102]. Inhibition of IRE1 and genetic silencing of the downstream target XBP1 resulted in increased apoptosis in different PEL cell lines. The same could not be said for ATF6 and PERK inhibition, which only slightly affected cell survival. However, inhibition in iSLK-219 cells did reduce viral replication when the lytic cycle was induced. This illustrates an odd scene regarding KSHV and the UPR, where UPR activation can occur during the lytic cycle, but the downstream transcription is suppressed. On the other hand, the lack of one of the sensors

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disrupts KSHV replication, thus hinting that the sensors are essential at some point for KSHV. For the sensors to activate during the lytic cycle indicates the ER is stressed to some capacity but by what can only be hypothesized, with one of them being the viral proteins capable of being translocated to the ER.

ER stress has been an interesting topic regarding KSHV infection. Previous work in the Lieberman lab found that ER stress can result in the cleavage of RAD21. This cohesin complex component can interact with the KSHV genome, specifically in the latency and lytic control regions that form a DNA loop with the transcriptional repressor CTCF [103,104]. The cleavage results in the reactivation of the KSHV virus only in PEL cell lines, as evidenced by the increase of various KSHV lytic genes at the mRNA level. ER, stress was also shown in PEL cells to augment virion production and lytic gene expression [97]. Based on the research, ER stress can potentially induce and increase viral replication, but how this occurs is not thoroughly understood. On the opposite side of the spectrum, ER stress can be detrimental to KSHV replication due to the downstream effects of the UPR preventing efficient translation and causing apoptosis in KSHV-infected cells. Exposure to different ER toxins also decreased cell survival in multiple PEL cell lines, thus showing sensitivity toward ER stress [97].

Besides the UPR, other well-known ER proteins are modulated by KSHV. One of the proteins recorded is the dynamin-related GTPases atlastins which are essential for controlling the structure of the ER membrane [105]. Overexpression of atlastins enhanced the lytic cycle and spontaneous viral reactivation in iSLK-219 cells [106]. When the atlastins were silenced, there was a decrease in KSHV replication and transcription of UPR-related genes CHOP,

XBP1s, and BiP when introduced to ER toxins. The results indicate that the loss of atlastin

inhibits the UPR. How this occurs has yet to be thoroughly explored but demonstrates a

dependency KSHV has on a specific ER factor. Another host protein is the ER-resident

chaperone BiP, which is upregulated in PEL cell lines when induced by the lytic cycle [97].

However, HDAC inhibitors with pleiotropic effects were used to induce the lytic cycle,

which may confound these observations. However, the silencing of the chaperone did disrupt

virion replication, further hinting that BiP is essential for virion replication [107]. It is not

surprising that BiP and molecular chaperones have frequently become therapeutic targets

since their increased expression is required for different diseases and viral infections.

Table 1-5. List of KSHV Genes That Can Potentially Translocate To The ER

Gene Name	Function	Time of Expression	Signal Peptide	Transmembrane domains
K1	Glycoprotein	Latent	1-18	224-247
ORF4	Complement Binding Protein	Early	1-19	533-551
ORF8	Glycoprotein B	Late	1-26	743-762
K2	Viral Interleukin 6 Homologue	Latent	1-22	N/A
K4	v-Macrophage Inflammatory Protein 2	Immediate Early	1-25	N/A
K4.1	v-Macrophage Inflammatory Protein 3	Immediate Early	1-27	N/A
K6	v-Macrophage Inflammatory Protein 1	Immediate Early	1-24	N/A
ORF22	Glycoprotein H	Late	1-21	715-736
ORF39	Glycoprotein M	Early	1-29*	14-25, 79-103, 119-135, 153-171, 211-231, 240-260, 274-293, 307-325
ORF47	Glycoprotein L	Early	1-20	N/A
K8.1	Glycoprotein	Late	1-26	200-220
ORF53	Glycoprotein N	Late	1-23	79-99
K14	Viral OX2	Early	1-24	230-250
K15	LMP1/2 Homologue	Latent/Early	1-25	10-25, 35-50, 69-81, 91-100, 123-139, 150-167, 178-194, 207-223, 240-250, 272-287, 299-308, 329-349

Table 1-5 List of KSHV genes that contain signal peptides and transmembrane domains, which can result in ER translocation.

Table 1-6. Current Knowledge Of The UPR In The Context Of KSHV Infection

ER Function	Cell Type	Description
IRE1	Primary Effusion Lymphoma (PEL)	Latent viral Factors LANA and vcyclin downregulate basal mRNA levels of IRE1. Lytic reactivation activates the sensor and splicing of XBP1s but is downregulating as the lytic cycle progresses. Lack of IRE1 disrupts viral replication and decreases cell survival. Transfection of XBP1s activates the immediate early gene RTA.
IRE1	Macrophages	KSHV infection resulted in increased IRE1 and XBP1s protein expression.
IRE1	Epithelial ISLK-219	Overexpression of XBP1s disrupted late lytic replication. Disruption of IRE1 activity disrupted viral replication.
PERK	Human Umbilical Vein Endothelial Cells (HUVECs)	Infection results in the upregulation of PERK downstream target gene CHOP.
PERK	Macrophages	Infection results in the upregulation of PERK downstream target gene CHOP.
PERK	Primary Effusion Lymphoma (PEL)	Lytic reactivation activates the sensor and eIF2α phosphorylation but there is no accumulation of ATF4 and CHOP expression.
PERK	Epithelial ISLK-219	Disruption of PERK activity disrupted viral replication.
ATF6	Human Umbilical Vein Endothelial Cells (HUVECs)	Infection results in the upregulation of ATF6 downstream target gene BiP.
ATF6	Macrophages	Infection results in the upregulation of ATF6 downstream target gene BiP.
ATF6	Primary Effusion Lymphoma (PEL)	Lytic reactivation activates the sensor, but downstream target genes do not accumulate. Lytic reactivation can also upregulate BiP protein levels but under the influence of chemical inducers. Lack of BiP disrupts viral replication.
ATF6	Epithelial ISLK-219	Disruption of ATF6 activity disrupted viral replication.

Table 1-6 List of current knowledge on the UPR in the context of KSHV infection. The list includes what branch of the UPR, what cells were utilized in the study, and what was discovered.

Prelude To Chapter Two (Herpesviruses As A Whole)

The *Herpesviridae* family is a large group of viruses with over 100 members that can infect reptiles, birds, and terrestrial and marine mammals. Within this family, eight human herpesviruses (HHV 1-8) infect and cause long-life human diseases [17] [Table 1-7]. Herpesviruses are highly prevalent worldwide, and it is considered that most of the population is infected with one or more herpesvirus. The family is divided into three subfamilies alpha, beta, and gamma. Members of these subfamilies have specific cell tropisms and use unique strategies to interact with their host. However, human herpesviruses

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share some mechanisms to control the cellular machinery and depend on similar host factors for infection. The common tactics used by herpesviruses to regulate the UPR and protein folding are of particular interest to us, as these may represent nodes for broad-spectrum intervention against herpesviruses [108].

Here, we explore the modulation of the UPR during KSHV infection in three distinct cellular models, PEL-derived B-cells, epithelial cells (iSLK-219), and primary lymphatic endothelial cells (LEC). In agreement with previous research, we found that KSHV mediates the activation of IRE1 and PERK after lytic induction, but an undetermined viral/host factor suppresses the downstream effects of IRE1 and PERK. Despite the disruption of the UPR signaling, we discovered that the ER HSP70 chaperone BiP is upregulated in the KSHV-infected iSLK-219 epithelial and primary LEC cell models. The upregulation of BiP is post-transcriptional and independent of the UPR (ATF6 and XBP1s).

The higher levels of BiP, even in the face of the strong host shutoff mediated by a viral nuclease, motivated us to test if the activity of this molecular chaperone is essential for KSHV infection. To study this, we treated cells before and during infection with HA15, a thiazole benzenesulfonamide, a cancer cell-killing compound that targets BiP for inhibition [109]. During the lytic cycle, HA15 treatment decreases viral DNA replication, protein expression, and the release of infectious virions in the epithelial cell and B-cell models. We also tested the cytotoxic effects of HA15 on latently infected cells. HA15 treatment causes cytostasis in multiple PEL-derived cell lines and potent cytotoxicity in the KSHV-infected lymphatic endothelial cells (LECs) but not in the uninfected counterpart. These results demonstrate the importance of BiP for KSHV replication and infected cell survival. The

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findings also highlight BiP as a possible target for treating KSHV-related diseases such as KS and PEL.

Table 1-7 Human Herpesviruses Cell Tropism and Associated Diseases

Virus	HHV (1-8)	Cell Type	Associated Disease
Herpes simplex virus 1	1	Stem cells, epithelial cells, neurons, and fibroblasts	Oral herpes, Genital herpes, Conjunctivitis, Meningitis, and Encephalitis
Herpes simplex virus 2	2	epithelial cells, neurons, and fibroblasts	Genital herpes, Meningitis, and Encephalitis
Varicella-zoster virus	3	T-cells, neurons, and epithelial cells	Chicken Pox, Shingles, and Congenital varicella syndrome
Epstein-Barr virus	4	B cells and epithelial cells	Infectious mononucleosis, Hodgkin's lymphoma, Non-Hodgkin lymphoma, Burkitt's lymphoma, and Nasopharyngeal carcinoma
Human cytomegalovirus	5	endothelial cells, macrophages, dendritic cells, and fibroblasts	pneumonitis, encephalitis, bronchiolitis, retinitis, hepatitis and gastroenteritis
Human betaherpesvirus 6A-B	6	T cells and neural cells	Roseola, mononucleosis, colitis, myocarditis, and hepatitis
Human betaherpesvirus 7	7	T cells and epithelial cells	Roseola, Encephalitis, hepatitis, febrile convulsions, and pityriasis rosea
Kaposi's sarcoma-associated herpesvirus	8	endothelial cells, B-cells, epithelial cells, and fibroblasts	Kaposi sarcoma, Primary effusion lymphoma, and Multicentric castleman disease

Table 1-7 List of the current human herpesviruses (1-8), examples of the different cell types they can infect, and the diseases associated with each virus.

Chapter 2: Modulation Of The UPR In Multiple KSHV Infected Cell Models

Introduction

As an obligate intracellular pathogen, KSHV hijacks the cells' translational machinery to synthesize viral proteins and support their post-translation processing and modifications. The function of the ER is especially crucial for infection as KSHV glycoproteins, transmembrane, and secreted proteins need to be synthesized and modified in this organelle before they are trafficked to their final subcellular destinations [Table 1-5]. As discussed in the first chapter, viruses usurp ER molecular chaperones during all stages of infection. Notably, ER-associated factors, including chaperones and UPR sensors/transducers, may vary between cell lines, impacting the ER's folding protein capacity and the cell's tolerance to ER stress. This is particularly relevant for viruses like KSHV, which have a broad cellular tropism.

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In this chapter, we delved into the differences and similarities in the branches of the UPR between three different KSHV-infected models and their uninfected counterparts: epithelial cells (iSLK.219 vs. iSLK-Hygro), B-cells (BCBL1-RTA vs. BJAB), and primary lymphatic endothelial cells (LEC-219 vs. LEC). We initially determined the basal protein levels of the sensors IRE1 and PERK in latent vs. uninfected cells. Then we determined changes in the UPR activation and signaling during the lytic cycle in iSLK-219 and BCBL1-RTA cell lines. We selected these model systems due to the ability to trigger lytic reactivation in a controlled way with the doxycycline (Dox) -inducible RTA-transgene.

Our results confirm modulation of the sensors PERK and IRE1 at the basal protein level during latent infection. As previously reported in PEL-derived cells, we see a modest activation of IRE1 and PERK in iSLK-219 and BCBL1-RTA post-lytic induction. However, signaling downstream of these sensors is suppressed even in conditions of acute ER stress caused by treatment with an ER toxin. To study the third branch of the UPR governed by ATF6, we evaluated the levels of BiP, a transcriptional target of ATF6, as a proxy for the sensor activation. Interestingly, BiP protein levels also varied during latent and lytic infection. Specifically, in the iSLK-219 cells, BiP was upregulated during the lytic cycle. The upregulation of BiP is independent of the UPR (ATF6 and XBP1s), thus suggesting a post-transcriptional mechanism behind the upregulation. These results demonstrate that the UPR sensors and downstream response are modulated during KSHV infection in a cell-type-specific manner.

Results

KSHV Latent Infection Modulates the Basal Protein levels of IRE1 and Disrupts

The Downstream Signaling During The Lytic Cycle

Our knowledge of the regulation of IRE1 during KSHV infection comes mostly from previous studies in PEL-derived cells, which show IRE1 activation during the lytic cycle, suppression of its downstream effects, and its requirement for PEL cell survival [100,101]. KSHV-infected B cells have a gene expression profile similar to plasma cells, which require IRE1 activity for their differentiation and secretion of immunoglobulins [110] [86]. Considering the essential role of IRE1 in B cell biology, we hypothesized that in other cellular targets of KSHV, including endothelial and epithelial cells, IRE1 might be regulated by the virus differently [87].

Our initial studies determined the basal levels of IRE1 in three cellular contexts the BCBL1-RTA (PEL-derived), LEC, and iSLK-219. For this purpose, we collected five hundred thousand cells from uninfected and latently infected cell lines and lysed them for immunoblot analysis of IRE1 **[Figure 2-1 (A), (B), (C)]**. Since infected BCBL1-RTA cells do not have an uninfected counterpart, we used the uninfected large B cell lymphoma BJABs as a control. Latently infected BCBL1-RTA cells displayed higher basal levels than BJABS. We observe the opposite phenotype in the epithelial cell model in which the levels of IRE1 are lower in iSLK-219 compared to the uninfected iSLK-Hygro. This is the first evidence supporting that the viral modulation of IRE1 is cell-type dependent. To further corroborate these results, we infected LECs with the recombinant virus rKSHV219 for 2-3 weeks to achieve stable infection before they were harvested for protein expression analysis [111].
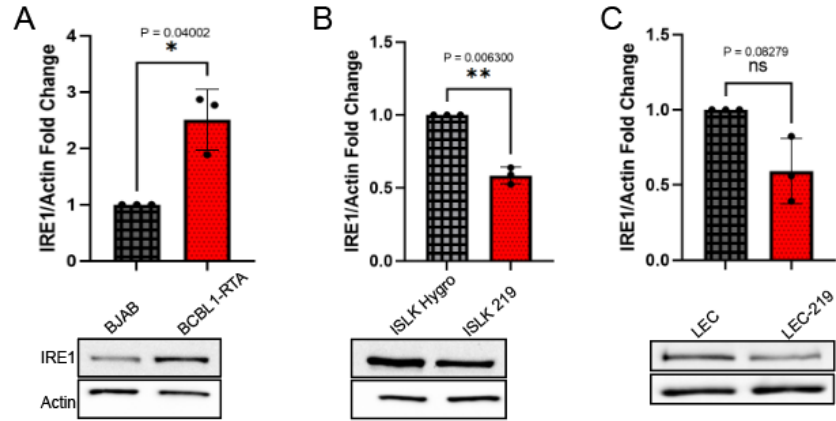
Compared to its uninfected counterpart, IRE1 protein levels were lower in infected LEC-219s resembling the trend found in the iSLK-219. These results demonstrate that latent KSHV infection can modulate the basal protein levels of IRE1 in a cell-type-specific way. We next aimed to determine if the control of IRE1 activity and signaling during the KSHV lytic cycle is conserved or if it differs between cell types.

Research on IRE1 confirmed its activation in the BCBL1-RTA cell line and splicing of the XBP1 mRNA when the lytic cycle was induced [100]. However, XBP1s protein and mRNA levels do not accumulate significantly, or at least not to levels seen during pharmacologically induced ER stress. To test whether the modulation of the IRE1 pathway during the KSHV lytic cycle in B-cells is conserved in the epithelial ISLK-219 cells, we induced lytic reactivation in BCBL1-RTA and iSLK-219 cells in parallel. We then analyzed the levels and activation of IRE1 and the accumulation of the XBP1s protein **[Figure 2-1 (D: Lanes 1-5), (E: Lanes 1-3)]**. The levels of IRE1 do not change throughout the lytic cycle in the ISLK -219 cells but are reduced in BCBL1-RTA cells as early as 24 hours after reactivation. The phosphorylation of IRE1 (IRE1-P), indicative of IRE1 activation, was detected in both KSHV-infected cell lines after lytic induction. Active IRE1 mediates the unconventional splicing of XBP1 mRNA and the translation of XBP1s. Interestingly, we could not see the translation of XBP1s in both cell lines. To determine if this is a transcriptional or translational effect, we evaluated the mRNA levels of this spliced transcript by RT-PCR. The primers we used in the experiment can amplify the unspliced (Upper band) and spliced (Lower band) forms of XBP1 mRNA. By analyzing the levels of unspliced and spliced forms of XBP1 using gel densitometry, we can determine the ratios between these two transcripts **[Figure 2-1 (F: Lanes 1-5), (G: Lanes 1-3)]** [28,112]. Our results show

minimal splicing of XBP1s during the lytic cycle in epithelial and B-cells, corroborate previously published results, and reveal a common trend in IRE1 activation in different KSHV-infected cell lines.

The lack of significant splicing of XBP1 mRNA, even when IRE1 is active during the lytic cycle, suggests a disruption of IRE1 activity by an unknown mechanism. Published work on BCBL-RTA cells found that acute treatment with the ER toxin Thapsigargin could force translation and splicing of XBP1s, but the protein levels decreased during lytic infection [100]. To confirm this in the iSLK-219 model, we pretreated the cells with 100 nM of TG for four hours before collection. At the protein level, TG treatment activates IRE1 robustly, leading to XBP1s expression in both cell lines **[Figure 2-1 (D: Lanes 6-10), (E: Lanes 4-6)]**. However, the protein levels of XBP1s dramatically decreased as the lytic cycle progressed, with minimal expression late in the lytic in the iSLK-219 (48-72 Hours) and the BCBL1-RTA (48 Hours) cells. At the mRNA level, TG treatment was able to increase the mRNA and splicing of XBP1 in the latently infected cells **[Figure 2-1 (F: Lanes 1-5), (G: Lanes 1-3)]**. For the iSLK-219 cells, similar to what was found at the protein level, unspliced and spliced XBP1 appeared to decrease as the lytic cycle progressed. The same scenario occurred in the BCBL1-RTA cells, which showed a dramatic decrease in unspliced and spliced XBP1 mRNA levels after just 24 hours of lytic induction. Our results show that IRE1 is activated, but an unknown mechanism disrupts its downstream signaling during the KSHV lytic cycle independently of the cellular context, even under strong acute ER stress conditions.

2-1

A

P = 0.04002
*

IRE1/Actin Fold Change

BJAB   BCBL1-RTA

IRE1
Actin

B

P = 0.006300
**

IRE1/Actin Fold Change

iSLK Hygro   iSLK 219

C

P = 0.08279
ns

IRE1/Actin Fold Change

LEC   LEC-219

D

iSLK.219 Time Course

TG -   TG +
U   L   24 48 72   U   L   24 48 72

IRE1
IRE1-P
XBP1s
Actin
Lanes   1   2   3   4   5   6   7   8   9   10

E

BCBL1-RTA Time Course

TG -   TG +
L   24   48   L   24   48

IRE1
IRE1-P
XBP1s
Actin
Lanes   1   2   3   4   5   6

L: Latent: 0 Hrs + Dox
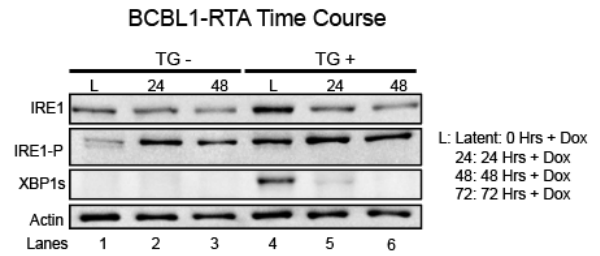24: 24 Hrs + Dox
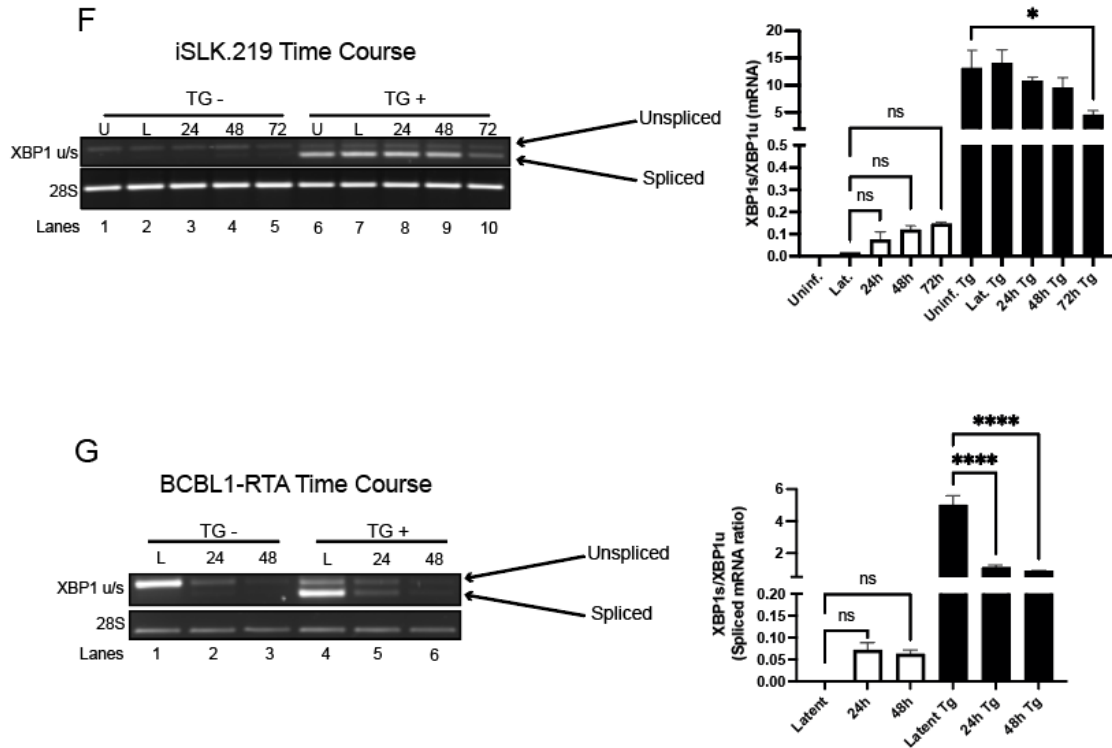48: 48 Hrs + Dox
72: 72 Hrs + Dox

40

Figure 2-1 Modulation of IRE1Pathway In Different KSHV infected cell models. **A-C**. Immunoblots and Densitometry Graphs of IRE1 in KSHV infected cells (ISLK-219, BCBL1-RTA, LEC-219) and their respective uninfected cells (ISLK-Hygro, BJAB, LEC). **D-E**. Immunoblot of the IRE1 pathway (IRE1 and XBP1s) in latent and lytic iSLK-219 and BCBL1-RTA cell lines. Cells were also acutely stressed (4 hrs) with the ER toxin Thapsigargin **F-G**. RT-PCR of spliced/unspliced XBP1 and 28s, along with densitometry of the ratios between spliced and unspliced.

KSHV Infection Modulates The Dynamics of The Sensor PERK and Prevents The Efficient Protein Expression of Downstream Targets ATF4 and CHOP

Motivated by our observations on the modulation of IRE1 during the lytic cycle, we explored the changes in the levels and activity of PERK during latent and lytic KSHV infection. Like IRE1, most of the information on PERK and KSHV infection comes from studies focused on what occurs in PEL cells. In this cellular context, latent infection reduces PERK basal protein and mRNA levels compared to other well-established B-cell lymphomas [97,100]. To determine if the basal levels of PERK are lower in other infected cells, we

collected five hundred thousand cells from uninfected and latently infected cell lines and lysed them for immunoblot analysis of PERK. Contrary to what we found in IRE1, PERK basal protein levels in infected ISLK-219 and BCBL1-RTA cells are lower when compared to their uninfected counterparts ISLK-Hygro and BJAB **[Figure 2-2 (A), (B), (C)]**. Interestingly, in the infected LECs, infection increases basal PERK levels. It is important to note that LEC-219 cells show an abortive lytic phenotype, in which a subset of the population (~20%) express lytic genes without robust viral particle production. The expression of some lytic proteins may impact PERK levels in this cellular context [111]. These results show that PERK basal levels are modulated during latent KSHV infection and appear conserved in two KSHV-infected cell models.

PERK activation has been reported in BCBL1-RTA cells undergoing the lytic cycle [100]. Still, the downstream transcriptional response of ATF4 and CHOP was suppressed by an unknown mechanism with evidence of increased eIF2α phosphorylation. In the case of iSLK-219 cells, it has not been confirmed if PERK and the downstream effects are also affected similarly. We determined the activity of the PERK pathway during the KSHV lytic cycle in iSLK-219 and BCB1-RTA cell lines by immunoblot analyses of the sensor PERK, the kinase target eIF2α, and the transcriptional effectors ATF4, and CHOP **[Figure 2-2 (D: Lanes 1-10), (E: Lanes 1-10)]**. The phosphorylation of PERK can be detected as a shift in the protein migration in SDS-PAGE [113]. We included acute TG treatment as a positive control. As previously documented in BCBL1-RTA cells, we can detect a modest activation of PERK and the consequent phosphorylation of eIF2α in lytic cells. Interestingly, PERK activation was not evident during the KSHV lytic cycle in the iSLK-219 cells, and the total protein levels of this sensor decreased as the lytic infection progressed. Even when PERK is

not active in iSLK-219 cells, we detect high basal levels of eIF2α phosphorylation in these cells during latent infection, suggesting the activity of other ISR eIF2a kinases (PKR, GCN2, or HRI)[39]. At late times during the lytic cycle in BCBL1-RTA and iSLK-219, we see a lower band in total eIF2α immunoblots which could reflect caspase activation and cleavage of this protein [114].

Even when eIF2a is robustly phosphorylated in lytic BCBL1-RTA and iSLK-219 cells, we failed to detect the PERK effectors ATF4 and CHOP expression at the protein level. Acute TG treatment induces the expression of these effectors, indicating that the lytic cells can still respond to ER stress, but ATF4 and CHOP decrease as the lytic cycle progresses. Our results indicate that PERK activation and eIF2α levels and phosphorylation are differentially modulated during the lytic cycle in the BCBL1-RTA and iSLK-219 cells. However, as we saw with the IRE1 pathway, unknown mechanisms muted PERK signaling in both cell lines.
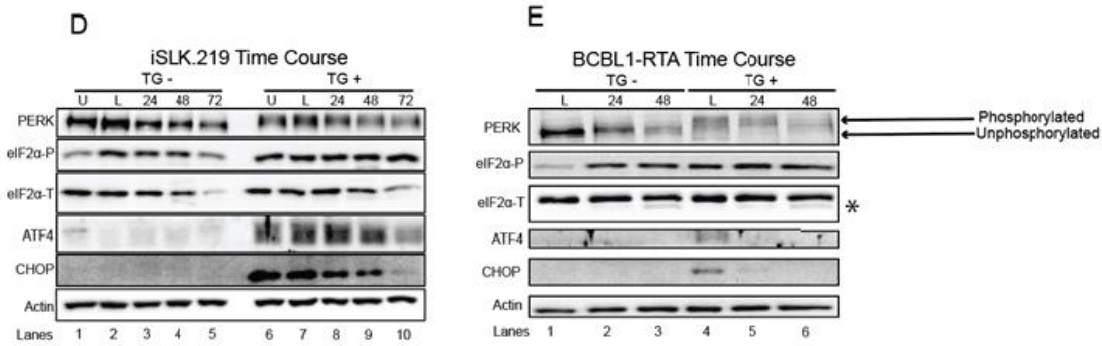

2-2
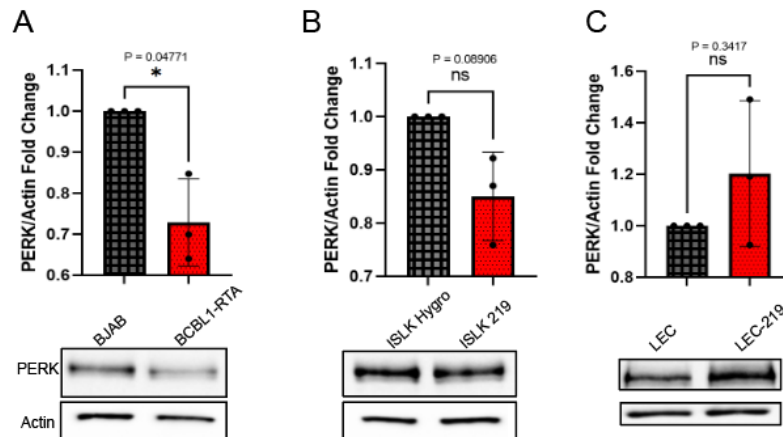
Figure 2-2 Modulation of PERK Pathways In Different KSHV infected cell models. **A-C**. Immunoblots and Densitometry Graphs of PERK in KSHV infected cells (ISLK-219, BCBL1-RTA, LEC-219) and their respective uninfected cells (ISLK-Hygro, BJAB, LEC). **D-E**. Immunoblot of the PERK pathway (PERK, eIF2α-Total, eIF2α-Phosphorylated, ATF4, and CHOP) in latent and lytic iSLK-219 and BCBL1-RTA cell lines.

The Viral Nuclease ORF37 Does Not Contribute To The Muting of The UPR signaling

One of the main questions that remain from our studies is the mechanism behind the disruption of IRE1 and PERK signaling. Previous work by others and our results showed the downregulation of XBP1, ATF4, and CHOP during KSHV lytic infection at the mRNA level [100]. Based on these observations, we hypothesized that the mRNA of the effectors could be a target of the viral nuclease ORF37 (SOX) [115]. This nuclease causes widespread host mRNA degradation in concert with XRN1 to cause host shutoff [116]. To test whether ORF37 has a role in downregulating UPR transcription factors, we silenced ORF37 in iSLK-219 and BCBL1-RTA cells using CRISPR interference (CRISPRi) [117]. We then confirmed the depletion of ORF37 by immunoblot **[Figure 2-3 (A: Lanes 1-10), (B: Lanes 1-10)]**. The knockdown of ORF37 reduced viral gene expression (ORF45 and K8.1) and replication efficiency in both cell lines, as previously characterized by the Glaunsinger group [118] **[Figure 2-3 (C)]**. Interestingly, we do not see the recovery of XBP1s or CHOP protein levels in iSLK-219 or BCBL1-RTA after ORF37 KD, even after acute treatment with TG. The

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results here show that ORF37 is not the major contributor to IRE1 or PERK signaling

disruption or the modulation of the transcription factors XBP1s and CHOP during the lytic

cycle of KSHV.

2-3

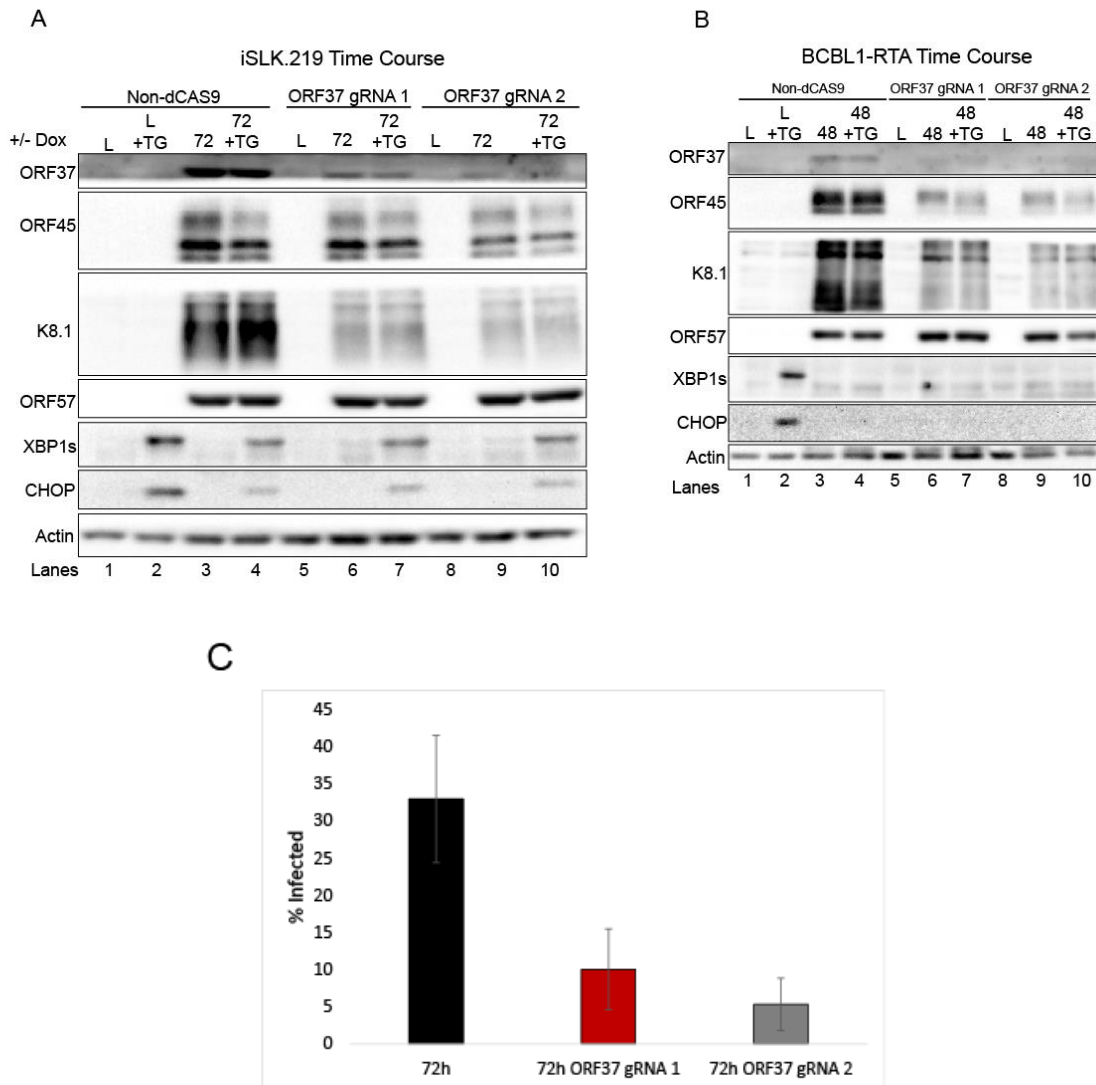**Figure 2-3** ORF37 is not the major contributor towards UPR modulation. **A-B**. Immunoblot of KSHV lytic genes (ORF37,

ORF45, K8.1, ORF57) and UPR transcription factors (XBP1s and CHOP) in latent and lytic in iSLK-219 and BCBL1-RTA

cell lines with and without ORF37 gRNAs. **C**. Infectivity graph of uninfected ISLK-Hygro cells exposed to supernatant

from late lytic ISLK-219 cells with and without ORF37 gRNAs.

BiP Modulation In KSHV Infected Cells Is Cell Type Dependent and Is Post-transcriptionally Regulated In ISLK-219 Cells

The third and the most enigmatic branch of the UPR, governed by ATF6, activates signaling pathways destined to help alleviate ER stress by inducing the expression of genes that increase the protein folding capacity. Previous studies in BCBL1-RTA cells show that ATF6 is active, but the downstream transcriptional response was inhibited by an unknown mechanism [100]. To study ATF6 activation in iSLK-219 cells after lytic induction, we used an antibody that recognizes the full 100 kiloDalton (kDa) ER-bound ATF6 (ER-ATF6). Our results show that ER-ATF6 is detected during latency and steadily declines during the late lytic cycle **[Figure 2-4 (A: Lanes 1-10)]**. Unfortunately, due to high background levels in the ATF6 immunoblot, we cannot conclude that the reduction in the detection of the full-length isoform of ATF6 is due to the sensor being processed into its 55 kDa nucleus-bound fragment (N-ATF6). Even under acute TG treatment, the N-ATF6 was not perceptible, limiting our ability to study ATF6 using this reagent. Also, an unidentifiable background band (Unknown) appeared during the lytic cycle. It is unclear if this band is a cross-reactive viral or host antigen spuriously detected by the ATF6 antibody. Due to these limitations, we evaluated the levels of the molecular chaperone BiP as a proxy of ATF6 activation. BiP has long been established as a transcriptional target of ATF6 [46].

We initially determined the basal levels of BiP by collecting five hundred thousand uninfected and latently infected cells for immunoblot. Basal BiP protein levels in latently infected BCBL1-RTA and iSLK-219 cells were significantly lower than their uninfected counterparts, which may reduce their folding capacity and sensitize cells to ER stress **[Figure**

2-4 (B), (C)]. Interestingly, the basal levels of this chaperone are higher in the KSHV-infected LECs than in the uninfected cells **[Figure 2-4 (D)]**. As mentioned above, it has been previously reported that LECs infected with KSHV can result in a complex gene expression profile that contains lytic genes, which could promote the upregulation of BiP in this cell line [111].

Previous reports indicate the upregulation of BiP in PEL cells during the KSHV lytic cycle [97]. However, in these experiments, the cells were treated with HDAC inhibitors for lytic induction, which can cause pleiotropic effects that make it difficult to discern between what is modulated by the virus and the inhibitors. Thanks to the development of doxycycline-inducible systems for RTA, we can determine what is mainly modulated by the virus. When we explored BiP protein expression during the lytic cycle, the iSLK-219 cells displayed a sustained upregulation of the chaperone at the protein level **[Figure 2-4 (E: Lanes 1-4)]**. Interestingly, we do not see this effect in BCBL1-RTA cells, with evidence of BiP decreasing as the lytic cycle progressed, even in TG treatment conditions. These observations indicate that the upregulation of BiP during KSHV lytic infection is cell-type dependent and seen in epithelial and primary endothelial cells **[Figure 2-4 (F: Lanes 1-6)]**. To determine if the upregulation of BiP is an early event in the lytic cycle, we pretreated iSLK-219 cells with phosphonoformate (PFA), which inhibits viral DNA replication and prevents late lytic gene expression. **[Figure 2-4 (G: Lanes 1-3)]**. The sustained upregulation of BiP, even in the presence of PFA, indicates that KSHV modulation of this chaperone is an early event that occurs within the first 24 hours of lytic induction and is independent of DNA replication.
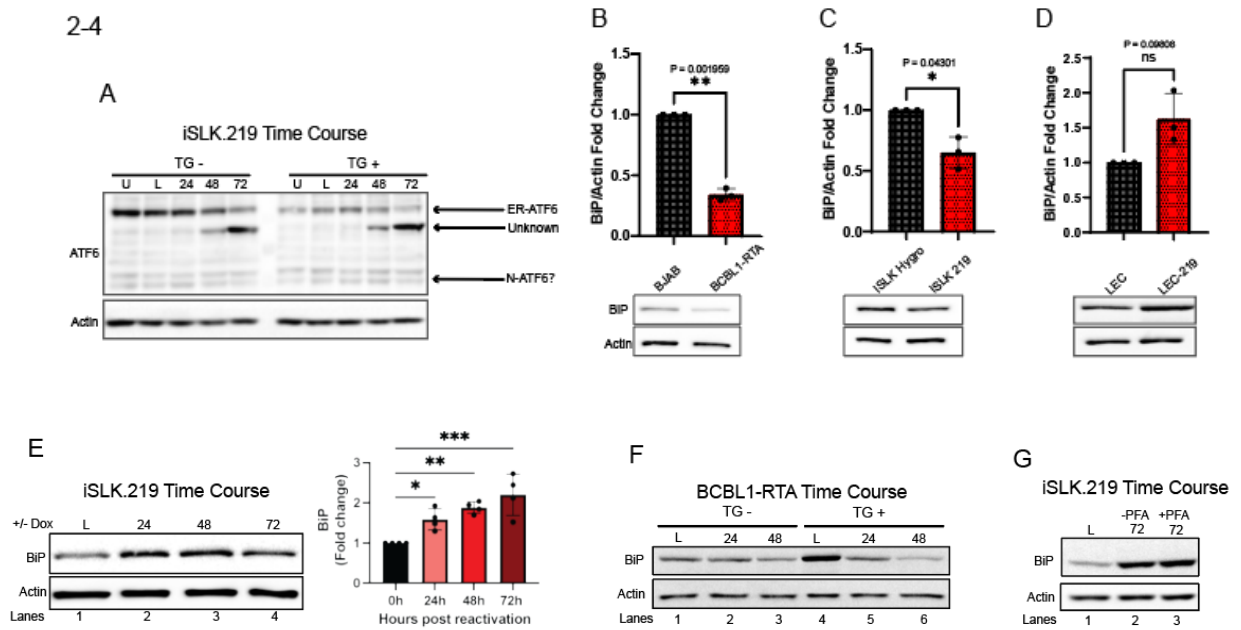
Figure 2-4 Modulation of BiP In Different KSHV infected cell models. **A**. Immunoblot of ATF6 shows difficulties discerning the nuclear fragment and background. **B-D**. Immunoblots and Densitometry Graphs of BiP in KSHV infected cells (ISLK-219, BCBL1-RTA, LEC-219) and their respective uninfected cells (ISLK-Hygro, BJAB, LEC). **E-F**. Immunoblot of BiP in latent and lytic in iSLK-219 and BCBL1-RTA cell lines, along with Densitometry graph for ISLK-219 cells. **G**. Immunoblot of BiP in latent and lytic ISLK-219 cells pretreated with and without the viral DNA polymerase Phosphonoformate (PFA).

We then determined if the higher levels of BiP are due to transcriptional upregulation by measuring BiP mRNA levels by qRT-PCR. Interestingly, the BiP transcript levels did not accumulate significantly in iSLK-219 cells **[Figure 2-5 (A)]**. To study ATF6 involvement in BiP expression, we pretreated iSLK-219 cells with the pharmacological inhibitor Ceapin which prevents ATF6 translocation from the ER to the Golgi by creating a tether between the cytosolic N-terminal domain of ATF6 and the peroxisomal ABCD3 membrane protein [119]. Ceapin treatment did not prevent BiP protein expression from increasing in lytic cells **[Figure 2-5 (B)]**. GRP94, another molecular chaperone known to be a target of ATF6, protein levels do not change in both Ceapin-treated and untreated conditions, thus alluding to

BiP upregulation being independent of ATF6 activity. These results were further

corroborated after CRISPRi-mediated silencing of ATF6 **[Figure 2-5 (C)]**. Another UPR

transcription factor that induces BiP expression is XBP1s [120]. However, silencing of XBP1

did not cause any effect on BiP upregulation **[Figure 2-5 (D)]**. These results indicate that BiP

upregulation is independent of the UPR transcription factors ATF6 and XBP1s in lytically

reactivated ISLK-219s. The exact cause of this upregulation is still unknown, but we know it

is a post-transcriptional mechanism. With BiP being a significant player in different viruses,

it stands to reason that this upregulation could be substantial for viral replication, thus

suggesting that the loss of BiP activity would be detrimental to KSHV infection.

2-5
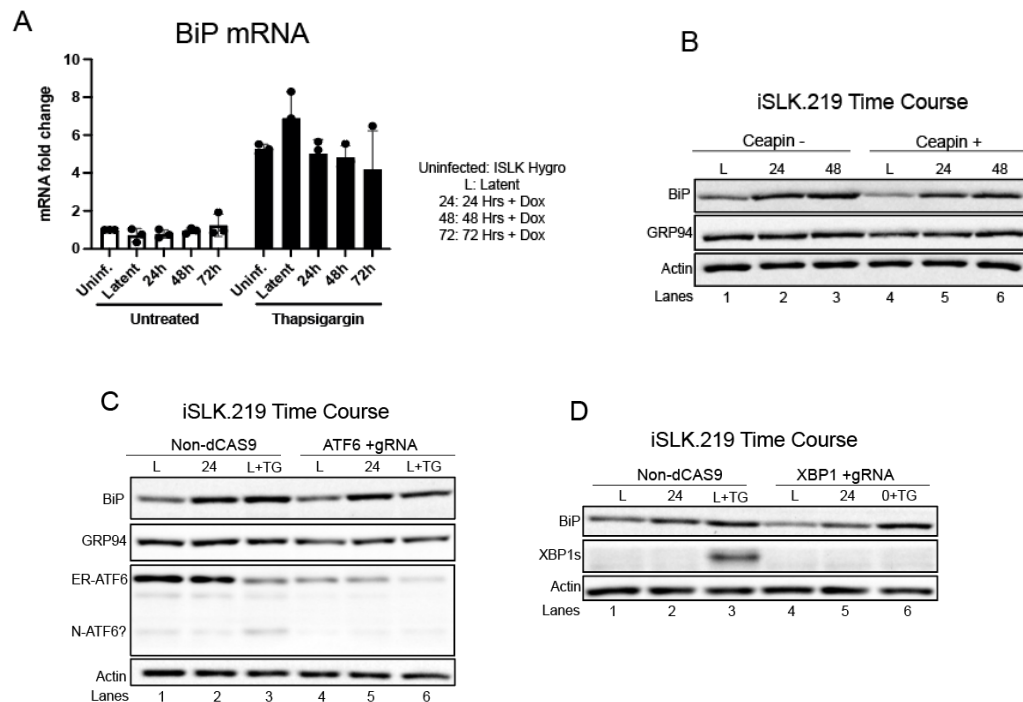
Figure 2-5 BiP upregulation in ISLK-219 lytic cycle is independent of ATF6 and XBP1s. **A**. QPCR analysis of BiP mRNA in iSLK-219 cells during the lytic cycle. **B**. Immunoblot of BiP and GRP94 in iSLK-219 cells pretreated with Ceapin before lytically reactivated. **C**. Immunoblot of ATF6, BiP, and GRP94 in iSLK-219 cells with and without dCAS9 and ATF6 gRNA. **D**. Immunoblot of BiP and XBP1s in ISLK-219 cells with and without dCAS9 and XBP1 gRNA.

Discussion

In this study, we report modulation of the UPR in multiple cell types in the context of KSHV infection. Specifically, we found that the basal protein levels of IRE1, PERK, and BiP in latently infected cells differed from their uninfected counterparts. These findings imply that KSHV infection changes the expression of host factors by an unknown mechanism. For PEL cells, the higher basal levels of IRE1 may have resulted from the virus-driven oncogenic transformation of B-cells. Previous gene expression profile studies had identified PEL cells as having a similar profile to malignant plasma cells that have been shown to rely on IRE1 for cell survival, and it is a critical protein for normal plasma cell differentiation [86,121]. In the future, it would be interesting to compare PEL cells with primary B cells and plasma cells to see if there are any discrepancies in IRE1 basal levels. In the epithelial iSLK-219 cells and the primary LEC-219 cells, the lower basal levels of IRE1 could benefit cell survival. Overexpression of IRE1 in colorectal and lung cancer cells has been found to reduce tumor growth and cause apoptosis [2]. Future studies will help determine if the differences in IRE1 basal levels between KSHV-infected B-cells, epithelial and endothelial cells modulate their response and tolerance to unfolded proteins.

Similarly to IRE1, PERK basal levels are lower in iSLK-219 and BCBL1-RTA cells. The lower levels of this sensor may also promote infected cell survival or replication, as overexpression of PERK has been previously shown to cause cell cycle arrest [3]. In contrast to PEL-derived cells and iSLK-219 cells, PERK is higher in LEC-219. It is possible that the abortive lytic gene expression profile in these cells contributes to the upregulation of PERK by a yet-to-be-characterized mechanism. Future studies in which we knockdown the

expression of PERK in the LEC-219 model system will help address the requirement of this UPR sensor for cell survival.

Our analyses of BiP as a proxy for ATF6 activation show lower basal levels of this chaperone in iSLK-219 and BCBL1-RTA cells. BiP is known to be upregulated in several malignancies and is a prosurvival factor for cancer cells, thus making it unclear what benefits lower levels might have [4]. These events seen in latently infected cells could result from the virus modulating its expression. Future overexpression experiments on KSHV genes known to downregulate host gene expression may prove fruitful in identifying latent BiP modulation. In LEC-219, we detect higher basal levels of BiP, which may have occurred due to the known expression of lytic genes in these cells [6]. However, previous HUVEC studies also identified BiP upregulation post-infection, thus requiring further investigation if this upregulation is specific to endothelial cells [98]. Despite these findings, it is still unclear what the basal levels of ATF6 are in these cell lines and what occurs during the lytic cycle. Future experiments on ATF6 should focus on utilizing antibodies that can visualize the protein efficiently or utilizing tagged ATF6 to investigate.

In addition to seeing differences in the UPR sensor levels in latently infected cells, congruent with previous reports, we show evidence of differential modulation of their levels and activation during the lytic cycle of KSHV in the three model systems studied [100]. One of the most noteworthy effects is the robust activation of IRE1 as the viral cycle progresses. It is unclear what factors trigger IRE1 phosphorylation, but the timing of activation indicates that it occurs during the immediate early or early phase of the lytic cycle. With our current knowledge of KSHV genes that contain signal peptides, future pull-down experiments for IRE1 can be done to identify what factors, such as unfolded viral or host proteins, bind to

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IRE1 to trigger its activation. In contrast to IRE1, the differential activation of PERK

between cell lines may be due to how the branch responds to the lytic cycle. Multiple studies

have indicated that the UPR has different effects depending on the cell type [23,28,124].

Examples can include PERK being required for insulin-producing beta cell differentiation,

but its activities are silenced during plasma cell differentiation [125,126]. Another

speculative explanation is that the iSLK-219 cells developed a tolerance of ER stress after

being infected with KSHV, which is corroborated by the high basal levels of eIF2α

phosphorylation. Previous research on ISR and chronic stress identified cells adjusting to

stress in a PERK-dependent manner with evidence of sustained levels of eIF2α

phosphorylation [12]. However, since we do not see PERK activation and the sensor is

downregulated during the lytic cycle, it can also be speculated that another one of the ISR

kinases may be responsible. Future work on this topic would focus on the dynamics of PERK

activation and ISR activity on both cell lines.

IRE1 and PERK post-activation downstream effects (XBP1s, ATF4, and CHOP)

were found to be downregulated during the KSHV lytic cycle, even in the presence of acute

ER stress caused by TG treatment. Forcing the downregulation of such genes would be

beneficial for the virus, as these UPR factors have the capability to be detrimental to

infection. Examples can be seen in ATF4 and CHOP, which are well-known transcription

factors that are known to upregulate genes to manage stress or reinstate protein translation.

However, prolonged stress can result in CHOP-inducing apoptosis via the death receptor

pathway. To prevent premature apoptosis in infected cells, the lytic cycle of KSHV appears

to have a mechanism to downregulate their expression despite evidence of eIF2α

phosphorylation in both cell lines. Another example can be seen in XBP1s with this

transcription factor upregulating genes involved in ERAD, which may lead to the

degradation of KSHV proteins that are translocated to the ER. This idea can be further

corroborated with previous overexpression of XBP1s disrupting KSHV infection in both

iSLK-219 and BCBL1-RTA cells. Inhibition of IRE1 activity has also been shown to disrupt

lytic replication, thus demonstrating a reliance on the sensor at some point during the lytic

cycle. XBP1s has been reported to transactivate the RTA promoter, trigger the lytic cycle,

and promote the expression of a few lytic genes, thus further hinting at the transcription

factor as a key player for KSHV infection [13,14]. Future experimentation in our XBP1

knockdown cells could answer questions if the virus relies on this transcription factor for

replication, but if there are no changes, then focus on the other functions of IRE1, such as

RIDD, should be considered.

The viral mechanism behind the truncation of the UPR signaling remains unclear.

Given the general downregulation of the effectors of the UPR (ATF4, CHOP, XBP1s), we

hypothesized that this is a general and indiscriminate mechanism, likely mediated by viral

RNA nuclease ORF37. However, genetic silencing of ORF37 did not efficiently recover

CHOP and XBP1s expression. Similarly, previous work by our colleagues in the McCormick

lab at McGill, shows that overexpression of ORF37 in HEK293s does not modulate the UPR

signaling even in the presence of ER toxins, thus confirming that this viral nuclease is not the

major contributor to the truncation of this response [100]. The knockdown of ORF37 also

resulted in the reduction of KSHV replication which could be due to the lack of its host

shutoff properties or the lack of the DNase activity in ORF37, which was recorded to be

essential for viral genome encapsidation [130]. Though we could not determine the viral

factors modulating the UPR, it is worth noting that the downregulation of XBP1s and CHOP

still occurred despite lytic gene expression and virion replication being stunted. Future experiments using CRISPR screens of viral functions could help unveil the KSHV genes involved in muting the UPR signaling.

Lastly, one important observation from our studies is the post-transcriptional upregulation of BiP during the lytic cycle in iSLK-219 cells and KSHV-infected primary LECs. Though we could not show the active translation of BiP, evidence backing this idea is the lack of significant BiP mRNA accumulating during the lytic cycle and upregulation still occurring in ATF6 and XBP1 knockdown conditions. Previous research on HCMV infection identified a similar pattern of BiP upregulation attributed to the internal ribosome entry site (IRES) in the BiP transcript [131]. This RNA element, along with the assistance of IRES interacting proteins, can bypass cap-dependent translation. For HCMV infection, the BiP IRES interactor La Autoantigen contributes to the translation of this chaperone and silencing of La results in decreased BiP translation and viral replication. Future experiments focusing on the BiP IRES and the IRES interacting proteins may prove fruitful in identifying if the RNA element is involved in the upregulation of BiP during KSHV infection. It would also be interesting to explore if any of the KSHV viral proteins that interact with host mRNAs can also interact with the BiP IRES. Since the upregulation occurs within the first 24 hours of the lytic cycle in iSLK-219 cells, it can be speculated that an immediate early or early lytic gene is involved. An example can be seen in the immediate early gene ORF57, where cross-linking and immunoprecipitation studies have identified interactions between ORF57 and host mRNAs in different PEL cells [132]. Whether this viral protein can activate BiP translation in iSLK-219 cells is a subject of future discussion.

Chapter 3: BiP is Required For DNA Virus Infection and KSHV Infected Cell

Survival

Introduction

Researchers have long examined molecular chaperones as therapeutic targets against multiple diseases, such as cancer and viral infections [16]. Like most of the hosts' proteome, viruses require molecular chaperones to assist protein folding and, in some cases, complex viral functions. In the case of the gammaherpesvirus KSHV, known for causing malignancies such as KS and PEL, the molecular chaperones HSP70 and HSP90 are essential for infection [133,134]. As previously mentioned, KSHV has two stages of infection: latent and lytic cycle. The virus can switch to the lytic cycle via the expression of the immediate early gene RTA, which then causes a temporal cascade of viral gene expression [89]. HSP90 studies identified KSHV proteins LANA and K1 as potential clients for the chaperone [135,136]. LANA is a latent protein required to maintain the viral DNA within the infected cell's nucleus, allowing KSHV infection to persist. LANA is localized in the nucleus, and the virus can translocate the cytosolic chaperone to the nucleus to aid in folding this protein. K1 is a viral glycoprotein that transforms cells and prevents apoptosis [136,137]. Inhibition of HSP90 resulted in reduced protein levels for both genes, with evidence of LANA being degraded by the proteasome. It has also been shown that a lack of HSP90 activity results in apoptosis in multiple PEL cell lines, reduced virion replication, immediate early gene expression of RTA, and late lytic gene expression [134].

Another chaperone that plays a critical role in KSHV replication is the cytosolic HSP70s recruited to the nuclear replication and transcription compartments (RTCs), where KSHV transcription, DNA replication, and capsid assembly occur [133]. Pharmacological

inhibition and genetic knockdown of these cytosolic HSP70s in lytic PEL cells reduced RTC formation, implicating that this chaperone is essential for viral replication. Inhibition of stress-induced HSP70 causes apoptosis in PEL cells by preventing the chaperones' ability to stabilize the lysosomal membrane, which releases Cathepsin D, a protease in the lysosome known to be involved in programmed cell death [138]. The requirement for molecular chaperones has been well documented in KSHV infection, but more investigation needs to be done on organelle-specific chaperones.

A handful of the KSHV proteins, including K1, were predicted to have the signal sequence peptide for ER translocation where the ER-resident chaperone BiP resides [Table 1-5]. Previous research suggests that the ER-resident chaperone BiP is modulated during KSHV infection in certain cellular models [97]. Specifically, BiP protein levels were found to upregulate during the lytic cycle in various PEL cell lines. However, these studies used HDAC inhibitors to induce lytic reactivation, which may have numerous and undesired pleiotropic effects. Interestingly, the silencing of BiP resulted in reduced viral replication with evidence of reduced cell viability [107]. Our results on BiP confirmed upregulation at the protein level in the ISLK-219 epithelial cell model when the lytic cycle was induced via a doxycycline-inducible promoter for RTA. However, BCBL1-RTA cells induced in the same manner we could not achieve similar results, thus hinting towards the upregulation of this chaperone being cell-specific. The upregulation of BiP resulted in a more profound question of whether BiP is essential for KSHV infection. To study this pharmacologically, we treated KSHV-infected cells with HA15, a thiazole benzenesulfonamide tested to be a cancer cell-killing compound that targets BiP for inhibition [109]. HA15 treatment decreased viral DNA replication, transcription, protein expression, and the release of infectious virions in different

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KSHV infected cell models. Genetic silencing of BiP via siRNA corroborated the results found in the iSLK-219 cell line. Cytotoxicity was also tested in KSHV-infected cell lines with minor cytotoxicity in the latent infected ISLK-219 and the uninfected LECs but proved toxic to infected LECs and cytostatic towards multiple PEL cell lines. These observations demonstrate the importance of BiP for KSHV infection and how it can be a possible target for therapeutics.

Previous work on RNA viruses corroborated HA15 as a unique tool for testing dependency on the molecular chaperone BiP. Inhibition of BiP in alphaviruses and coronaviruses showed reduced viral replication in various experiments, including mouse models showing reduced viral infections [139,140]. Our HA15 findings in KSHV are the first to be reported in a DNA herpesvirus, which leaves the additional question if the results can be replicated in other extensively studied DNA viruses in the field. HSV-1 is an alpha herpesvirus known to infect different cell types, including epithelial and neural cells [141]. It is mainly known for causing sores and neurological diseases such as encephalitis. HCMV is a beta herpesvirus known to infect multiple cell types, including endothelial cells and macrophages [142]. Diseases affiliated with HCMV include hepatitis in immunocompromised patients and encephalitis in fetuses. Like KSHV, HSV1 and HCMV are lifelong infections and have been documented to upregulate the molecular chaperone BIP, thus making them valid candidates for HA15 experiments [131,143]. To see if HA15 reduces the replication of other DNA viruses, a recombinant vaccinia virus (VV) was also tested. VV is part of the poxvirus family, known for its long history with smallpox [144]. In all three cases, HA15 treatment reduced viral infection in various concentrations, thus

providing further evidence of BiP being a broad-spectrum therapeutic target for viral

infections and HA15 as a unique tool for viral studies.

Results

Inhibition Of BiP Via HA15 Disrupts KSHV Replication In ISLK-219 Cells

BiP is essential for multiple viral infections ranging from viral entry, inhibition of

apoptosis, and folding of viral proteins. The upregulation in the iSLK-219 cells left us

wondering if it is required for the lytic cycle or if it was a bystander effect. We approached

this hypothesis by pretreating cells for 24 hours with the pharmacological inhibitor HA15

before reactivation. The BiP inhibitor has been found to inhibit the chaperone and UPR

regulatory activity of BiP. After reactivation, cells were harvested for immunoblot studies

focusing on immediate early genes KbZIP, ORF57, and ORF45, known to increase

throughout the lytic cycle **[Figure 3-1 (A: Lanes 1-8)]**. The late lytically expressed

glycoprotein K8.1 was detected as a late lytic protein expression marker. Overall, protein

levels of all these genes decreased drastically in HA15-treated iSLK-219 cells, suggesting

BiP inhibition can disrupt the KSHV lytic cycle. The lack of K8.1 expression indicated that

HA15 blocks the lytic cycle. To confirm if HA15 disrupts virion release, we performed

infectivity studies by infecting iSLK-Hygro cells (uninfected) with the viral supernatant from

late lytic iSLK-219s treated with and without HA15 **[Figure 3-1 (B)]**. We measured infection

by detecting the expression of GFP in spinoculated cells, the latent infection marker found in

the rKSHV219 virus. As expected, HA15 treatment reduced infected cells compared to

untreated conditions. The results indicate that the inhibition of BiP via HA15 treatment can

disrupt the efficient protein expression of KSHV genes, reducing virion release and possibly virion replication.

The reduced protein expression suggests either an issue with the translation or transcription, thus requiring an investigation of overall KSHV gene expression. RNA-seq was implemented with a KSHV gene library to generate a heatmap of the fold change to compare gene expression of late lytically reactivated ISLK-219 cells with and without HA15 **[Figure 3-1 (C)]**. When comparing untreated and HA15-treated iSLK-219 cells, there is an evident reduction of mRNA levels of most KSHV genes, including the KSHV genes shown earlier that are known to progress the lytic cycle. Along with those genes, there was also a reduction in the mRNA levels of most KSHV genes known to have signal peptides for ER translocation, including the glycoproteins required for KSHV infection. The results suggest that BiP activity is essential for the KSHV lytic cycle, as evidenced by the decreased protein levels, infectivity, and gene expression. To corroborate our findings, genetic silencing of BiP via siRNA was done in the ISLK-219 cell line before lytic reactivation, and samples were collected for immunoblot and infectivity experiments **[Figure 3-1 (D: Lanes 1-6)(E)]**. Similar to HA15, protein levels of most KSHV genes tested earlier were also lower when BiP was genetically silenced except for the immediate early gene ORF57. We could also see decreased infected cells compared to non-targeting (NT) siRNA. Based on these results, BiP is essential for the lytic cycle in the ISLK-219 cells. However, since this cell line is not attributed to a natural context of infection, this leaves the question: Can similar results be found in a wild-type infected cell line?
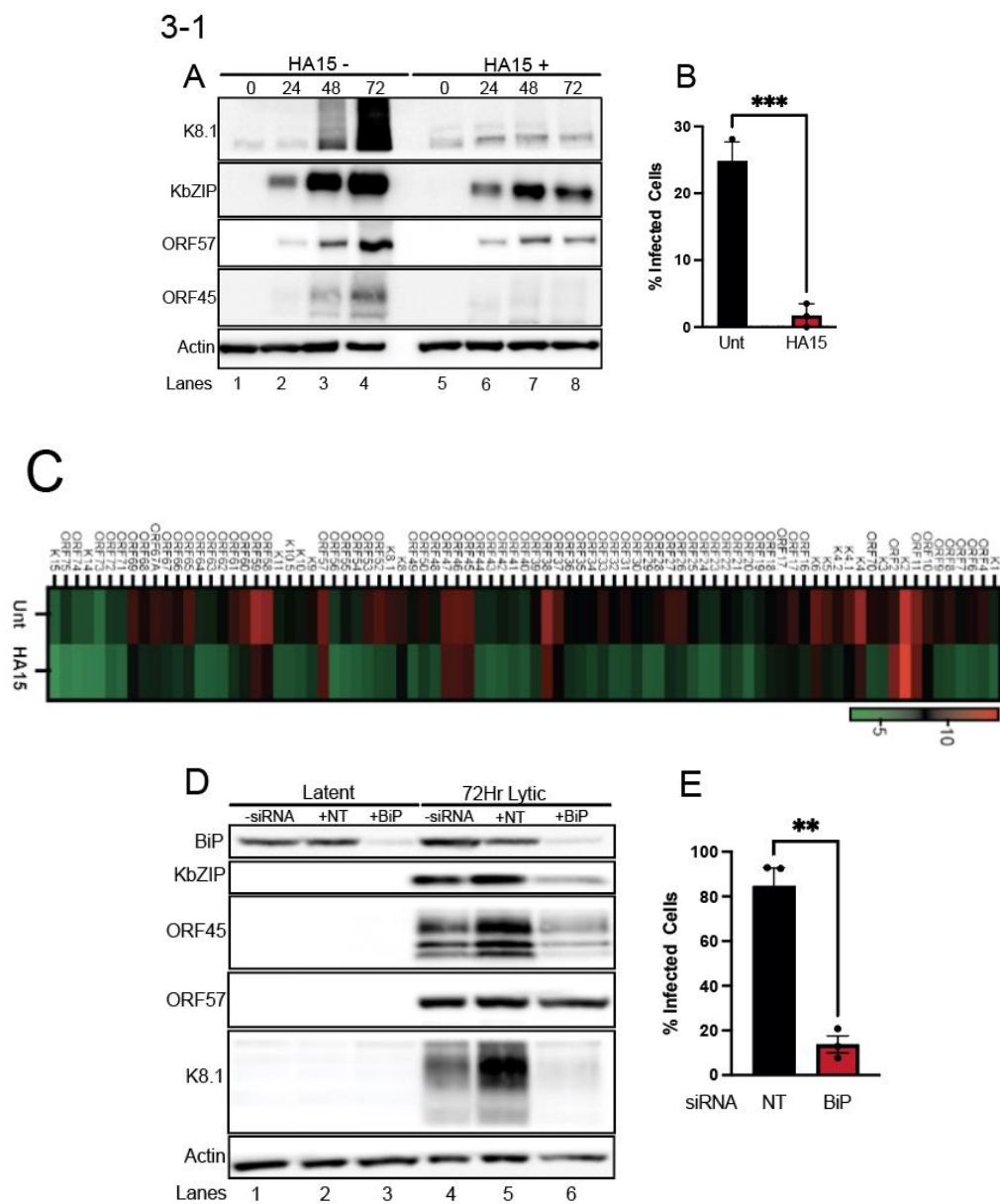
Figure 3-1. Inhibition and silencing of BiP disrupt KSHV replication in ISLK-219 cells. **A.** Immunoblot of KSHV proteins (ORF57, KbZIP, ORF45, and K8.1) and Actin in ISLK219 cells with/without HA15 treatment post lytic reactivation. **B.** Infectivity graph of ISLK-Hygro cells after exposure to supernatant from late lytic ISLK-219 cells that were treated with/without HA15. **C.** Heatmap of RNA-seq analysis on KSHV genes in late lytic ISLK-219 with/without HA15. **D.** Immunoblot of KSHV proteins (ORF57, KbZIP, ORF45, and K8.1), BiP, and Actin in ISLK 219 cells with/without BiP siRNA. **E.** Infectivity graph of ISLK-Hygro cells after exposure to supernatant from late lytic ISLK-219 cells that were treated with Non-targeting (NT) and BiP siRNA.

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Inhibition Of BiP Via HA15 Disrupts KSHV Replication In BCBL1-RTA Cells

The BCBL1-RTA cells were PEL cells obtained from a patient that were naturally infected with KSHV before being immortalized and given an RTA transgene. As previously mentioned, research on genetic silencing of BiP in PEL cells prevented efficient viral replication, laying the groundwork for identifying if pharmalogical inhibition of BiP by HA15 can accomplish similar results. To test if HA15 treatment affects viral protein expression, we treated BCBL1-RTA with this compound and collected cell lysates for immunoblot studies **[Figure 3-1 (F: Lanes 1-6)]**. Like in the ISLK-219 cells, viral protein expression of K8.1, ORF45, and KbZIP was also reduced in HA15-treated BCBL-1-RTA cells. With this information in mind, it can be inferred that the KSHV lytic cycle has also been disrupted in the BCBL1-RTA cells. To confirm this outcome further, we evaluated KSHV DNA replication in these cells. KSHV DNA replication is essential for late lytic gene expression to progress in its final stages. To see if there are any changes in viral DNA replication, DNA from late lytically induced BCBL1-RTA was harvested with the fold change analyzed after real-time PCR experiments **[Figure 3-1 (G)]**. Compared to untreated cells, HA15 treatment reduced viral DNA in late lytic cells, thus further supporting the hypothesis of BiP being required for KSHV replication. Without the efficient chaperone activity of BiP, viral proteins that are predicted to be translocated to the ER and are necessary for KSHV infection may not fold into their correct conformation or be able to be translocated efficiently. With this in mind, we immunoblotted for the viral transmembrane glycoprotein K1 **[Figure 3-1 (H: Lanes 1-6)]**. This glycoprotein has been predicted to have the signaling peptide necessary for ER translocation and can interact with BiP cochaperone HSP40 (Erdj3/DnaJB11), which assists in transferring client proteins to BiP [136]. It has also been

studied to augment the lytic cycle, influence host cell survival, and cause cell transformation [137]. HA15 treatment does appear to reduce K1 protein expression even during the lytic cycle. These results demonstrate that BiP inhibition in BCBL1-RTA cells can disrupt the lytic cycle and reduce the expression of a potential ER-localized viral protein. The reduction of K1 expression led to another query as to what is occurring to the viability of the cells, as it has been proposed that the glycoprotein can enhance infected cell survival.

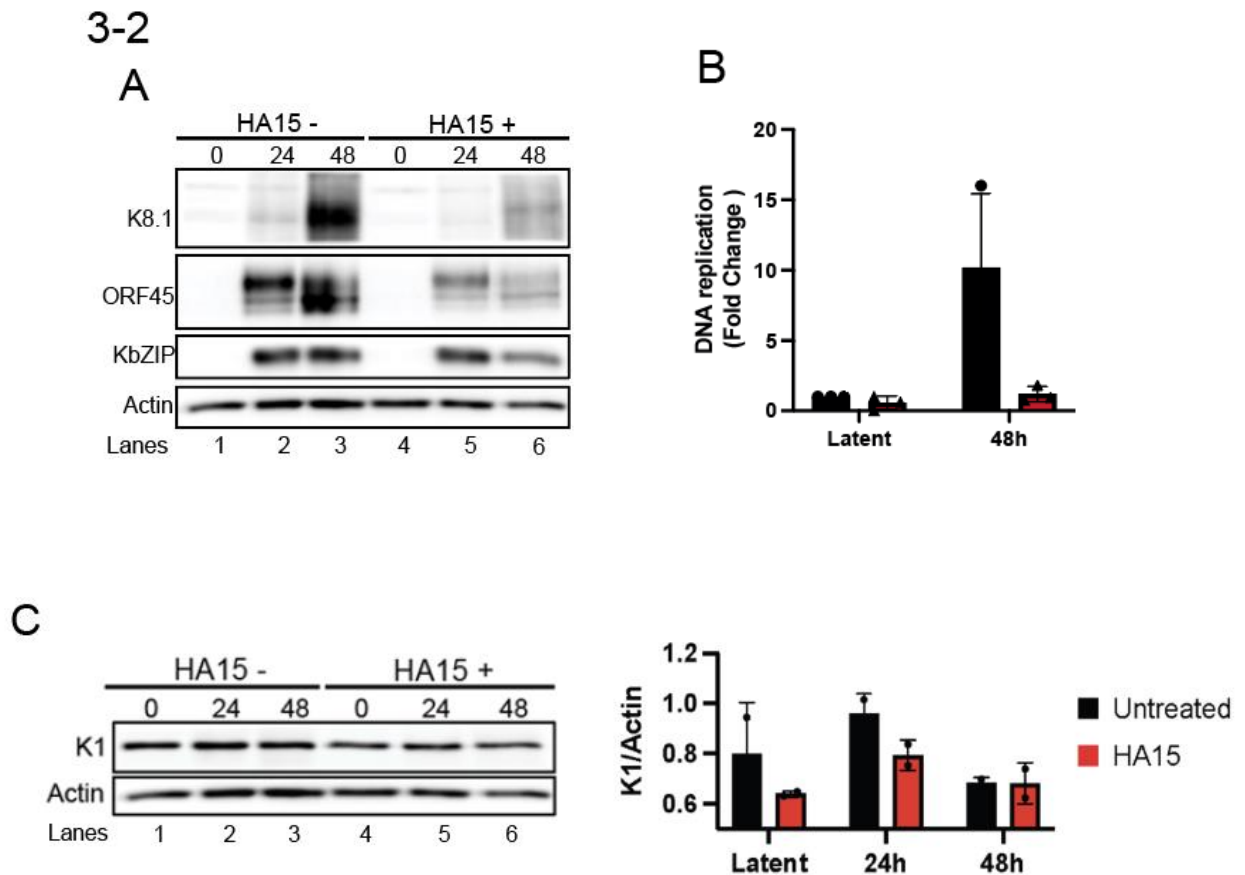**Figure 3-2.** Inhibition of BiP disrupts KSHV replication in BCBL1-RTA cells. **A.** Immunoblot of KSHV proteins (KbZIP, ORF45, and K8.1) and Actin in BCBL1-RTA cells with/without HA15 treatment post lytic reactivation. **B.** BCBL1-RTA viral DNA replication graph with/without HA15 treatment. **C.** Immunoblot of KSHV glycoprotein K1 with/without HA15 treatment in BCBL1-RTA cells with densitometry results.

Cytopathic Effects of HA15 In The Different KSHV Infected Cell Models

HA15 was initially designed to be an anticancer agent against melanoma but is now being tested in multiple malignancies [145,146]. With KSHV being the etiological agent of various malignancies, it can be hypothesized that the compound is also capable of being cytotoxic to KSHV-infected cells. We initially approached this idea by testing HA15 on the different types of available KSHV-infected cell models (ISLK-219, BCBL1-RTA, and LEC-219). To test if HA15 has cytotoxic effects in the adherent ISLK-219 cells, about ten thousand cells were seeded in a 6-well plate and exposed to 10 µM of the inhibitor for 96 hours before being stained with crystal violet, which only stains viable cells that are adhered to the plate **[Figure 3-2 (A)]**. The specific concentration of HA15 was administered because the original publication found this dosage to be the most optimal for decreasing cell viability in melanoma cells. We also tested HA15 on the uninfected ISLK-Hygro to see if the viral infection is the main contributor to cytotoxicity or if the compound has cytotoxic effects since both were derived from the KS malignancy [95]. Treatment with a high dose of TG was used as a positive control to distinguish between the two conditions. Interestingly, there was no noticeable difference between both cell lines when treated with HA15 and TG-treated cells that proved cytotoxic. We confirmed this lack of change by taking confocal microscopy images of ISLK-219 cells **[Figure 3-2 (B)]**. The results suggest that BiP inhibition does not affect these cell lines with the recommended dosage of HA15 and possibly requires a higher dose to see any effects.
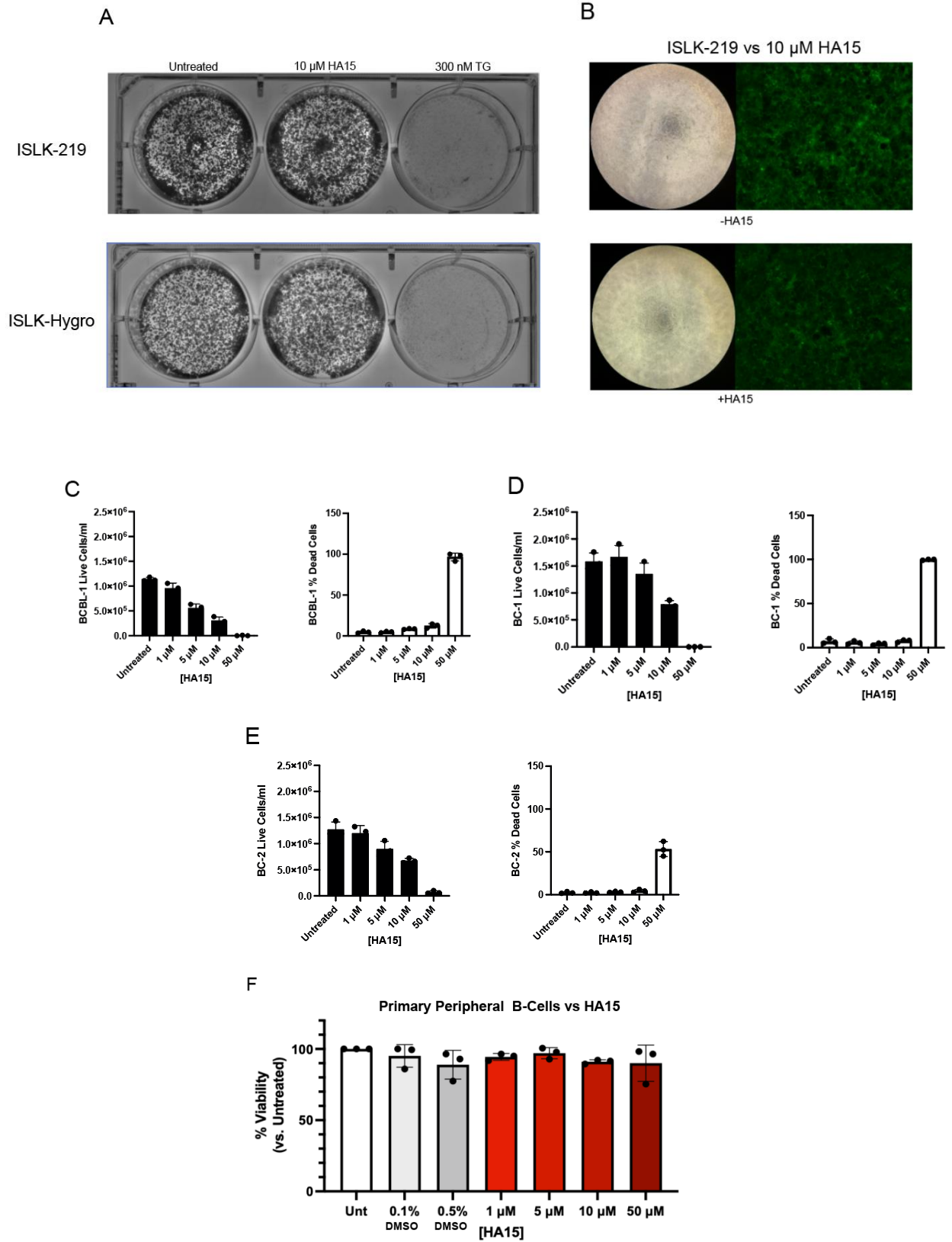
The lack of changes in ISLK-219 cells left us questioning the capabilities HA15 has on cell viability. However, since KSHV does not naturally infect these cells, we wanted to

see if there was an effect on PEL cells derived from KSHV-infected patients. Cell viability was tested via trypan blue staining in various PEL cell lines, including the BCBL1-RTA, BC1, and BC2 cells, after being treated with HA15 for 72 hours **[Figure 3-2 (C-E)]**. Different concentrations of HA15 (0-50 μM) were tested to see if lower or higher impact dosage affects cell viability. Bar graphs were generated on the viable cell number per milliliter and the percentage of cell death. In every PEL cell line tested, cell growth decreased significantly after 72 hrs in a dose-dependent way, with cell death showing no changes until given the highest dose (50 μM). This cytostatic effect appears specific to PEL cells, as it occurs in every PEL cell line tested. HA15 research found no significant changes in cell viability in primary human cells such as melanocytes and fibroblasts, suggesting that the compound only affects cancer cell lines. With our evidence with PEL cells, we can infer that primary B-cells could also be unaffected. However, since B-cells require ER molecular chaperones like BiP to regulate the ER load, we also had to see if HA15 affected their cell viability [110]. Similar to what was done in the PEL cells, primary peripheral B-cells (PPBCs) were treated at various concentrations with a bar graph generated on the percent viability of cells after experimentation with trypan blue staining **[Figure 3-2 (F)]**. Despite the inhibition of BiP, there was no dramatic reduction in cell viability, further demonstrating HA15's capabilities when treated in primary cells. The results indicate that inhibition of BiP via HA15 has a specific cytostatic effect in PEL cell lines and does not significantly impact PPBC viability.

Both KSHV-infected cell models, epithelial and B-cell, clearly show a difference in how they respond in the presence of BiP inhibition. Despite these findings, we kept in mind that each infected cell line tested was immortalized and derived from cancer cell lines. For

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the next cell model tested, LECs, we attempted to simulate a natural infection of KSHV onto primary LECs and then observe what occurs after 72 hours with HA15. LECs have long been a hypothesized natural target of KSHV due to their spindle cell formation post-infection, a hallmark of KS lesions [111]. The LEC-219 cells were obtained after infecting primary LECs with the rKSHV219 virus with the GFP reporter confirming infection. After exposure to the BiP inhibitor, confocal microscopy images were taken of uninfected LECs and infected LEC-219 **[Figure 3-2 (G)]**. Visually, there were no apparent changes in the cell viability of LECs treated with HA15, which shouldn't be surprising since they are primary cells. Interestingly, though there does appear to be a change in the morphology of the cells, it is unclear what is causing such an outcome. As for the LEC-219, GFP expression and the hallmark spindle cell morphology were confirmed. HA15 treatment in these infected cells showed a drastic visual decrease in cell number, with minimal cells being identified compared to untreated cells. We then analyzed cell viability by Cell-Titer-Glo to corroborate what was found visually **[Figure 3-2 (H)]**. As expected, LEC-219 had reduced cell viability post 72 hours of HA15 compared to untreated cells, with uninfected LECs showing no changes. Based on these results, HA15 can affect the cell viability of LECs post-KSHV infection without detrimental effects on uninfected cell viability. The results indicate that BiP inhibition can disrupt KSHV viral replication and affect infected cell survival.
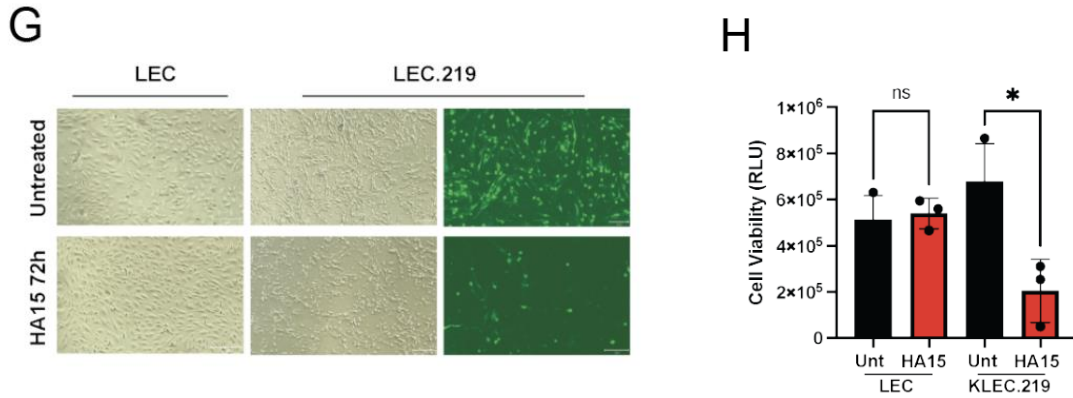
3-3

A

ISLK-219

ISLK-Hygro

B

ISLK-219 vs 10 μM HA15

-HA15

+HA15

C

D

E

F

Primary Peripheral B-Cells vs HA15

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Figure 3-3. HA15 Treatment Results In Different Cytopathic Effects In Different KSHV Infected Cell Models. **A.** Crystal violet stain of latently infected iSLK-219 cells treated with either 10μM HA15 or 300 nM TG for 96 Hrs. **B.** Confocal microscopy images of ISLK-219 cells that were treated with/without HA15. **C.** Trypan Blue viability results in BCBL1-RTA post 72 Hrs HA15 treatment at different concentrations (1μM, 5μM, 10μM, 50μM). **D.** Trypan Blue viability results in BC1 post 72 Hrs HA15 treatment at different concentrations. **E.** Trypan Blue viability results in BC2 post 72 Hrs HA15 treatment at different concentrations. **F.** Trypan Blue viability results in Primary Peripheral B-cells post 72 Hrs HA15 treatment at different concentrations. **G.** Confocal microscopy images of uninfected LECs and infected LEC-219s that were treated with/without HA15. **H.** Cell titer-glo analysis of infected and uninfected LECs that were treated with/without HA15.

HA15 Treatment Results In Decreased Infectivity In Other Well-Studied DNA Viruses

BiP inhibition with HA15 has been shown as a potential antiviral strategy for RNA viruses, including alphaviruses and, more recently, coronaviruses [139,140]. Our results in KSHV infection indicate that this compound is also active against Double-Stranded DNA (dsDNA) viruses. To expand on this observation, we tested the potential of HA15 to inhibit viral replication in primary human fibroblasts (PHF) infected with three other dsDNA viruses; the alphaherpesvirus Herpes Simplex Virus-1 (HSV-1), the betaherpesvirus Human Cytomegalovirus (HCMV) a betaherpesvirus, and the Poxvirus Vaccinia Virus (VV). For this purpose, we infected cells with a low multiplicity of infection (MOI) (HSV-1 MOI 0.001, HCMV at MOI 0.1, and VV at MOI 0.01) in the presence or absence of different concentrations of HA15. We determined the spread of infection at different times post-

infection by measuring the expression of virus-encoded GFP in HSV-1-GFP and HCMV-GFP infected PHF via flow cytometry **[Figure 3-3 (A)]**. The results from the experiment indicate that viral replication was reduced in the standard 10 µM dose, along with a higher dose at 30 µM. As for VV, immunofluorescence using a polyclonal antibody was done to measure expression. Similar to what was found in HSV-1 and HCMV, viral replication also appears to be reduced. Cell survival was also measured to confirm that HA15 capabilities did not affect primary cell viability **[Figure 3-3 (B)]**. As expected and corroborating what was previously published, HA15 treatment on PHF has no effect on cell survival at 30 µM or a long treatment time. The results demonstrate HA15s capabilities in inhibiting viral replication in various DNA viruses, thus making it a novel tool for virology.

3-4
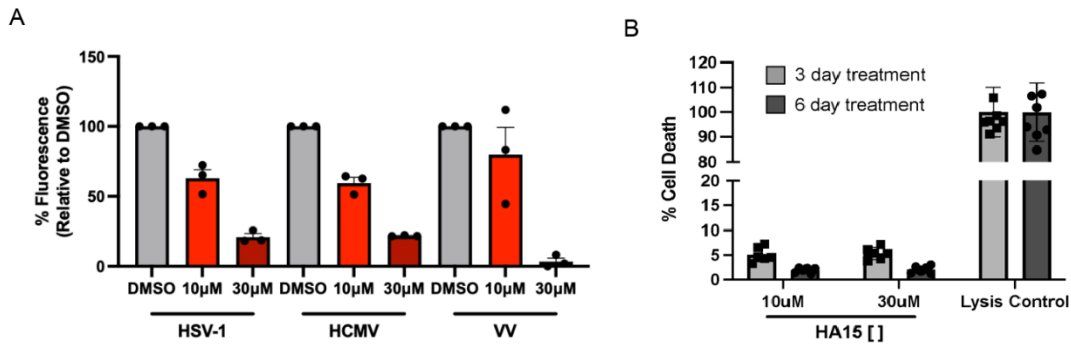
Figure 3-4. HA15 treatment represses viral replication in other well-studied DNA viruses. **A.** Percent fluorescence measured in primary human fibroblasts that have been infected with GFP-tagged HSV-1 and HCMV post-HA15 treatment. Also, percent fluorescence measurement of a polyclonal antibody for VV in primary human fibroblasts that have been infected with VV post-HA15 treatment. (10µM and 30 µM) **B.** CytoTox-Glo viability Assay for primary human fibroblasts.

Discussion

HSP70 inhibitor experiments in KSHV-infected cell lines have shown that constitutively and stress-induced expression of cytosolic HSP70 is essential for viral replication, with minimal work demonstrating if the ER chaperone BiP is explicitly essential

for KSHV replication [133]. Here we answered this question by employing the pharmacological inhibitor HA15. Specifically, the introduction of this compound to ISLK-219 and BCBL1-RTA cells decreased viral gene expression, viral DNA replication, infectivity, and infected cell survival. These results implicate that BiP activity is required for viral infection, bringing about the next major question: what function of the molecular chaperone is required? BiP is a well-studied ER chaperone that assists protein folding and cytosol to ER translocation of nascent polypeptides, targeting misfolded proteins for ERAD and regulating the UPR [66,147]. Each function plays a part in maintaining homeostasis within the ER, thus making BiP a significant player in KSHV-infected cellular health and replication.

The original study for HA15 was able to identify the inhibition of the ATPase activity in BiP, which is critical for its chaperone activity and binding of protein clients [109]. Without the chaperone activity, it can be hypothesized that viral and host proteins that BiP clients cannot fold into their correct conformation, thus requiring them to be degraded via ERAD or autophagy [148]. A similar idea can be considered for BiP, the SEC61 translocon, and SEC63. The current research involving all three proteins identified BiP assisting nascent protein translocation from the SEC61 translocon after the chaperone was recruited by SEC63 [147]. Without the ATPase activity, the nascent proteins would not be able to translocate efficiently, thus requiring them to be degraded by the proteasome. A possible example is the reduction of K1 protein expression in BCBL1-RTA cells after HA15 treatment. Without efficient BiP activity, K1 may not be able to fold into its proper conformation or cannot translocate efficiently into the ER, thus resulting in a reduction in viral replication and cell survival. This idea suggests that K1 may interact with BiP, which requires further exploration

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to identify BiP and potential interactors. The idea also includes identifying if BiP interacts with any other viral proteins identified to have a signal sequence.

Another complication in HA15 treatment is decreasing the pool of available BiP, which may cause sensitivity to the ER that can prematurely activate the UPR. The differences in the UPR in each cell line must be further examined to consider such an idea. We previously showed that IRE1 and PERK sensors of the UPR share differences in basal levels and a common theme of modulation during the lytic cycle. However, what HA15 does to the UPR in each cell model needs to be clarified, which is a question left for future experimentation. An example can be seen in the various PEL cells, which have a cytostatic effect after 72 hours of HA15 treatment. Besides the viral proteins that may be involved in cell growth, PERK has been implicated in cell cycle progression, thus requiring further investigation on what is happening to the UPR post-HA15 treatment [123]. Interestingly, we did not see such an effect on PPBCs. These immune cells were well-studied to have robust UPR for differentiating into antibody-secreting plasma cells [110]. Why HA15 causes such an effect in PEL cells and not PPBCs is a subject of future experimentation since we did not distinguish the genetic profile of both cell lines post-treatment. A similar case can be said for uninfected and infected LECs that show a disparity in how they react to HA15 treatment. Current knowledge of KSHV infection and the UPR in endothelial cells is limited, with HUVECs being the only cell line to show upregulation of the downstream effects, including BiP [98]. We determined BiP upregulation in only infected LECs, but the reason behind this upregulation has yet to be determined. Infected LECs have a complex genetic signature, including lytic genes, and have shown protein expression of viral glycoproteins [111]. The inhibition of BiP via HA15 could have resulted in the activation of the UPR, including the

PERK branch, which can result in apoptosis after long exposure by induction of the apoptotic factor CHOP. These are only a couple of examples of what HA15 treatment could do to the KSHV-infected cell lines, thus opening a wide array of studies to consider involving BiP.

Finally, our work on KSHV and HA15 is the first to focus on a DNA herpesvirus, which left us wondering if the same results can be done in other well-established DNA viruses, such as HSV-1, HCMV, and VV. The results show that HA15 can also decrease their replication, as evidenced by flow cytometry. For HCMV and HSV-1, it comes as no surprise since both herpesviruses have an interesting story to tell with BiP. For HCMV, BiP was upregulated during viral replication, with the molecular chaperone also moving to the nucleus where virion replication occurs [131]. It has been proven that BiP cleavage by the subtilase cytotoxin (SubAB) could disrupt viral replication, and now with HA15, it further corroborates BiP as an essential host factor for HCMV [149]. For HSV-1, BiP was also found to be upregulated but only in specific virus strains [143]. These results in HSV-1 corroborate BiP as an essential host factor for herpesvirus infections. VV is an interesting case since it does not have prior research on BiP modulation, thus making HA15 studies the first to identify the molecular chaperone as crucial for infection. It would be interesting to see in the future what functions of BiP are required for each virus, which can include any of the previous functions mentioned earlier. Further investigation on the genetic expression of each virus should give a clue into what viral mechanism may have also been disrupted after HA15 treatment. With this new knowledge, HA15 demonstrates the capabilities of further exploring therapeutics against specific host factors essential for viral infections. It opens the door to exploring how the compound works in other well-established viruses.

Chapter 4. Conclusions and Future Directions

Modulation of The UPR In Multiple KSHV Infected Cell Models

Research on how viruses modulate ER functions has been extensive, focusing on proviral and antiviral mechanisms. The UPR is an evolutionarily conserved stress response that becomes proactive whenever the ER is under duress, including an accumulation of unfolded and misfolded proteins [23]. Such stressful conditions, though, can occur in different ways, such as the dysregulation of ER functions that assist in protein trafficking, folding, and modifications [21]. Another example is foreign entities like viruses that hijack the cellular translational machinery to synthesize abundant proteins that are translocated to the ER. To alleviate ER stress, the UPR triggers signaling pathways that increase the protein fold capacity and ways involved in decreasing the protein load. However, if stress persists, the UPR will trigger apoptotic pathways. Viruses, such as KSHV, have found ways to disrupt the antiviral aspects of the UPR and upregulate the functions essential for infection.

Future of Cellular Models For KSHV Infection

This section of the thesis explored the differences in the UPR between KSHV-infected cell models (BCBL1-RTA, ISLK-219, and LEC-219) to evaluate if modulation is conserved. The results show that basal protein levels of the UPR sensors, IRE1 and PERK, differ depending on the cell type when latent cells are compared to uninfected cells. ATF6's downstream target gene BiP was visualized as a proxy for ATF6 activity. Like IRE1 and PERK, BiP basal levels also differ, thus demonstrating latent infection-specific modulation of the UPR. These results bring perspective to what lifelong infection can do to cellular processes. Despite these findings, what occurs at the basal transcriptional level in all the cell lines tested is unclear. It has been shown that overexpression of latent genes LANA and

vCyclin suppress IRE1 transcription, but our evidence of higher basal protein levels in BCBL1 compared to BJABs shows a conflict [97]. Identifying what is happening at the transcriptional levels would prove helpful in corroborating the protein levels.

Another set of comparisons that should be made in the future is primary B-cells, primary plasma cells, and PEL cells' UPR basal levels since the malignancy has been documented to have a plasma cell gene profile [86]. This observation would show the difference in basal UPR levels between normal uninfected and malignant infected cells. The upregulation of BiP post-KSHV infection in LECs may have occurred due to a lytic gene being expressed. KSHV infection in LECs has been documented to have an abstract gene profile with the upregulation of lytic genes [111]. To confirm if it is a lytic gene, a comparison should be made between LECs and other endothelial cell models utilized for KSHV studies, such as blood endothelial cells (BECs), which are another hypothesized origin of infection that causes KS. Future experimentation on KSHV modulation of the UPR further brings to light the mechanisms viruses implement to provide a proviral environment.

Future Perspectives On UPR Modulation During KSHV Lytic Infection

For lytic infection, ISLK-219 and BCBL1-RTA reactivation results in activation of the IRE1 sensor, but transcription and translation of the downstream target gene XBP1s do not robustly accumulate. Similar results were also found in PERK and its downstream products ATF4 and CHOP. Still, no sensor activation was found in the ISLK-219 cell line, while BCBL1-RTA cells show a partial activation, thus demonstrating a difference in sensor activity modulation between KSHV-infected cell models. These results demonstrate that there could be a conserved method of activating the sensor and disruption to downstream signaling. It has been a running hypothesis that the KSHV host shut off ORF37 is responsible

for the lack of efficient expression of the UPR genes. With CRISPRi, the silencing of ORF37 confirmed that it is not the major contributor to the lack of UPR gene expression, suggesting additional mechanisms behind the modulation.

Currently, it is unknown what is activating IRE1 during the lytic cycle. Still, since it occurs within the first 24 hours of the KSHV lytic cycle in ISLK-219 cells, it can be inferred that it is immediate early or early lytic proteins that could be potentially binding to the luminal domain. With the predicted KSHV proteins that are capable of ER translocation in mind, co-immunoprecipitation (Co-IP) experiments can be done in extracting IRE1 and potentially the viral protein interacting with it [Table 1-5] [150]. A similar approach can be made in PERK, but in this case, a possible viral protein of interest would be glycoprotein B, which is conserved in all herpesviruses [151]. HSV-1 studies identified this glycoprotein in inactivating PERK [152]. However, since glycoprotein B is a late lytic gene in KSHV infection, another viral glycoprotein may prevent efficient activation of the sensor. The last sensor of the UPR, ATF6, proved challenging to identify what is occurring during lytic reactivation. Experimentally tagged ATF6 may prove helpful in determining what happens to the ER fragment during the lytic cycle [153]. There is still much to be studied on the UPR during the lytic cycle, especially the downstream effects.

The lack of robust XBP1s splicing and protein expression is remarkable since IRE1 is highly active during the lytic cycle. It has also been documented that IRE1 inhibition and genetic silencing disrupt lytic replication, thus demonstrating a reliance on the sensor at some point [99,100]. Further analysis of the lytic cycle XBP1s KD cells may clarify if the virus depends directly on the activity of this transcription factor or other functions of IRE1. To

address this question, we can study KSHV reactivation in cells encoding mutant versions of the nuclease or the kinase domain of IRE1. Another function of IRE1 which remains to be explored in the context of infection is RIDD. To determine if RIDD is important for KSHV reactivation, we can use reported mutants of the RNase domain of IRE1 [154]. The downstream of PERK activation is eIF2α phosphorylation, which was only found to occur in BCBL1-RTA when reactivated. The ISLK-219 cells appear to have high basal levels of eIF2α phosphorylation, which could be caused by another kinase from the ISR [39]. Visualization of the phosphorylated states of all the kinases would provide details into why there is a high phosphorylated eIF2α. Despite the phosphorylated conditions, there is no proof of elevated CHOP and ATF4 expression, thus requiring investigations on viral factors that could be responsible for suppressing the downstream signaling.

The silencing of ORF37 could not recover the expression of XBP1s and CHOP. Previous overexpression studies of ORF37 could also not mitigate their expression of these UPR effectors during ER stress, which leaves future experiments to consider what other viral factors might be involved in downregulating the expression of XBP1s and CHOP [100]. Observations that shed some light on where to start looking is that the knockdown of ORF37 resulted in disruption of late lytic gene expression due to the viral protein being essential for viral DNA processing [130]. With this in mind, it can be inferred that an immediate early or early lytic gene may be involved. To investigate this further, the genetic knockdown of these genes may provide insight, along with studying the literature, if any of the genes can prevent UPR gene expression [155].

Future on identifying the mechanism and factors behind BiP modulation

An interesting observation made from this study is the upregulation of the ER-resident molecular chaperone BiP during the lytic cycle in ISLK-219 cells. BiP has long been known for its chaperone activity, protein translocation, and targeting proteins for degradation. As previously mentioned, BiP was studied as a proxy of ATF6 activity due to the visualization of the sensor being inconclusive. Interestingly, the upregulation of BiP proved cell-specific, as the levels of BiP decreased in BCBL1-RTA cells within 24 hours of lytic reactivation. To investigate the mechanism behind the upregulation of BiP, we silenced the expression of ATF6 and XBP1s, which did not affect the levels of the BiP, indicating that this process is independent of the UPR [120]. This idea was corroborated by the lack of significant increase of BiP mRNA in ISLK-219 cells post lytic induction, thus opening room to interpret the cause behind BiP upregulation.

One hypothesis supported by our data is the post-transcriptional regulation of BiP during the lytic cycle. To test this hypothesis further, we can monitor the translation of the BiP transcript. Briefly, we can isolate polysomes, groups of actively translating ribosomes, from cells and then fractionate them by a sucrose gradient [156]. This technique separates the free ribosomal subunits (60s and 40S) and complete ribosomes (80s) associated with mRNAs via ultracentrifugation. We can isolate RNA from the different polysomal fractions and evaluate by qRT-PCR if BiP mRNAs are actively being translated at 24h-48h post lytic reactivation in iSLK-219 or LEC-219.

BiP upregulation has also been documented in the betaherpesvirus HCMV by increasing its transcription via a viral protein and cap-independent translation via the IRES

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element within the mRNA [131]. BiP translation was modulated by the host factor La autoantigen (La), a known IRES trans-acting factor (ITAF) [157]. Since BiP mRNA levels do not increase during KSHV lytic replication, it can be inferred that the IRES and ITAFs may be the mechanism behind upregulating BiP protein levels. To test this hypothesis, the genetic targeting of BiP IRES-specific trans-acting factors and assessing BiP protein levels along with viral gene expression during the lytic cycle in ISLK-219 cells may provide insight. Besides La, there are only two other ITAFs known to activate BiP IRES-mediated translation, including human NS1-associated protein-1 (NSAP-1) and Caenorhabditis elegans eukaryotic initiation factor 4 γ (eIF4G) homolog IFG-1 [158,159]. However, a caveat behind such testing may prove toxic to the cells as some of these ITAFS are involved in responding to cellular stress.

Another approach to consider is isolating the ITAFS while interacting with the BiP mRNA. Cross-linking and immunoprecipitation (CLIP) is an experimental process that force-links the ITAFs with the mRNA, usually by ultraviolet light exposure [160]. After that, an antibody specific to the ITAFs would isolate the protein with the mRNA attached. The protein would be removed later, and the mRNA would be sequenced for gene identification. Though this technique could show if any ITAFs interact with the BiP mRNA, antibody specificity and primer design must be thoroughly considered to carry out such an experiment.

The Requirement of BiP For DNA Virus Infection and KSHV Infected Cell Survival

Molecular chaperones have long been established as critical factors that assist cellular protein clients in obtaining their three-dimensional functional conformation and transport protein clients to specific cellular compartments [161]. Unfortunately, viruses hijack these critical factors for various stages of infection[162]. A key example explored here is the

molecular chaperone BiP, which is part of the HSP70 family and is known to be located within the ER. Previous research on the relationship between viruses and BiP has found that it can be essential for different stages of viral infection [16]. KSHV studies have previously identified cytosolic HSP70 as necessary for replication. Still, there is minimal knowledge on the impact of BiP on KSHV infection, with genetic silencing in PEL cells being the only report to date [107,133]. With this in mind, we inhibited BiP and saw a dramatic effect on viral replication and cell survival/replication in almost all the cellular models tested. Targeting host factors has been a growing interest in therapeutics against multiple diseases, including cancer and viral infections.

Future Research On The Requirement of BiP During The Lytic Cycle

The upregulation of BiP in lytically active ISLK-219 left us wondering if BiP is required for KSHV infection, which was previously shown in PEL cells post-genetic silencing. The pharmacological inhibitor HA15 was administered to the cells to answer this question [109]. HA15 treatment has been documented to disrupt the ATPase activity of BiP, which is required for protein client binding and release. Typically, ATP-bound BiP is in an open state where cochaperones like HSP40 provide clients to the molecular chaperone causing hydrolysis of the ATP to ADP, causing a conformational change into a closed state [65]. Nucleotide exchange factors (NEFs) may later come to exchange the ADP for ATP, thus opening BiP and releasing the properly folded client, with the cycle continuing for other clients. The loss of this activity disrupts the normal chaperone functions, thus limiting the pool of available BiP within the ER. The results from the experiment show that a lack of efficient BiP activity disrupts the lytic cycle, as shown in reduced viral gene expression and virion replication. The results were further corroborated in the wild-type infected BCBL1-

RTA cells, with viral translation being disrupted and viral DNA replication, which is a hallmark for KSHV replication to progress to late gene expression. The results show that BiP is essential for KSHV, thus leaving room for future experimentation based on this requirement.

What exactly BiP interacts with within the context of KSHV infection is unknown. Predictive analysis of the KSHV genes identified a handful of KSHV proteins capable of translocating into the ER [Table 1-5]. Besides chaperone activity, BiP has also been documented to assist protein translocation via the SEC61 translocon and SEC63 [147]. HA15 treatment in BCBL1-RTA cells confirmed a decrease in K1 expression, which may suggest that the glycoprotein interacts with BiP. The idea can be further corroborated by published work on K1 interacting with the BiP cochaperone HSP40[136]. To test if BiP interacts with any KSHV proteins, whole-cell co-IP experiments that focus on isolating BiP with its bound clients could be informative. This approach includes visualizing K1 and all the KSHV proteins potentially translocated within the ER. The ISLK-219 and BCBL1-RTA cells would be used since both require BiP for efficient viral replication. However, antibody selection for BiP would be critical for the experiment's success. The results can be further corroborated by isolation of the ER followed by mass spectrometry (MS) to evaluate the remodeling of the organelle composition and changes in the client proteins and corroborate the results of the Co-IP. The results obtained from these experiments would further understand how KSHV can utilize BiP for its replication.

Another complication from HA15 is that the depleting pool of BiP that can result in the sensitization of the ER, which could result in premature UPR activation that could be

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detrimental to KSHV replication. Previous work confirmed such an outcome in melanoma cells, with HA15 treatment leading to PERK and IRE1 activation, CHOP expression, and splicing of XBP1 [109]. Premature activation of the UPR may lead to the upregulation of the negative aspects of the stress pathway that can be detrimental to KSHV replication, thus requiring validation. This process would include looking at BCBL1-RTA and ISLK-219 cells during the lytic cycle to confirm UPR activation and its downstream effects. Though everything future-wise can only be speculated, HA15 treatment does prevent the efficient replication of KSHV, thus highlighting BiP as a proviral factor.

Future Research On The Requirement of BiP For KSHV Infected Cell Survival

KSHV is known to be the etiological agent of KS and PEL, which are malignancies with different cellular origins. HA15 is a pharmacological BiP inhibitor initially designed to be an anticancer agent for melanoma cells. The BiP inhibitor was also cytopathic to various other malignancies, such as mesothelioma and lung cancer cells [145,146]. Considering this information, we tested if HA15 has any cytopathic effects in any available KSHV-infected cell lines since many are derived from malignant cells. Our results show that HA15 has a cytostatic effect in various PEL cell lines, while no distinct impact was observed in primary B cells. Interestingly, uninfected and infected ISLK cells did not display any sensitivity to HA15, thus showing specificity in how the compound reacts to different KSHV-infected cells. We also tested HA15 on the long-hypothesized KS-origin cell LECs and found it cytotoxic only for infected cells.

As previously mentioned, HA15 may have led to premature activation of the UPR, which may negatively affect the cells. The PEL cells utilized in this portion of the study had cell division stunted after 72 hours in HA15. Previous research involving the cell cycle and

the UPR identified PERK activation as capable of inducing cell cycle arrest [123]. With this in mind, exploring the protein expression of cell cycle genes and the PERK pathway would confirm that the UPR is the culprit behind the cytostatic effects. Though no effects were seen in the ISLK-219, the amount administered may not have been enough to cause cytopathic effects. This raises speculation as to what dosage would be necessary for ISLK-219 cells as they were derived from malignant cells. Testing different dosages would determine what effects HA15 has on latently infected cell models. After such testing, the next step is to see what would occur in different contexts of KSHV infection.

The current model of studying KSHV infection is immortalizing cells from patients naturally infected with the virus or infecting immortalized cells with a recombinant virus in two-dimensional cell culturing [95,96]. A key issue with two-dimensional cell culturing is the need to mimic a physiological environment more efficiently, which three-dimensional attempts to do, especially regarding how cells form in normal human tissues [163]. Previous research on uninfected and infected LECs created three-dimensional spheroids with characteristics known to occur in endothelial cells. Testing HA15 onto these spheroids would further validate the compounds' capabilities in a three-dimensional format similar to normal cell tissues [164]. Another interesting experiment that can be done is introducing mice with previously published infected endothelial cells that are capable of forming cell colonies and then treating the mice with HA15 to see what can occur [165]. In vivo models with HA15 have been explored before in melanoma xenografted and coronavirus-infected mice with no reported detrimental effects [109,139]. These experiments would broaden further BiP as a therapeutic target for KSHV-associated diseases.

The Future In Utilizing HA15 For Virology.

The results found in KSHV are the first documented case where a DNA virus infection is disrupted by the BiP inhibitor HA15. Previous work on the compound has shown the same in different RNA viruses, thus presenting the idea that HA15 can be an efficient tool for understanding the reliance on BiP in viral infections [139,140]. We therefore tested the compound on other well-established viral infections such as VV, HCMV, and HSV-1. The results from this portion of the thesis confirmed that HA15 disrupts infection of all three viruses when introduced to primary human fibroblasts, which were also shown not to have a drastic effect on cell viability. These findings open opportunities to explore what aspects of BiP function promote viral infection and consider the next steps for HA15 experimentation.

HSV-1 and HCMV are well-known herpesviruses with a broad cell tropism with diseases affiliated with the human body's skin, nervous system, liver, and eyes [141,142]. These organs have different cell types to consider regarding viral infections. The experiments in fibroblasts are just one piece of the puzzle, as these cell types are primarily found in the skin and tendons. To truly understand the effect of HA15 on viruses, different cellular models that focus on the diseases caused by the viruses should be implemented. It is still not been established what HA15 does to other human cells, thus requiring future experimentation on uninfected and infected cells. As for VV, the decrease in infection is the first documented case of the virus to require the ER chaperone BiP. Previous research on VV and the ER identified it as the location of viral DNA synthesis and where membrane proteins are stabilized [166,167]. It is unclear from there what viral proteins would interact with BiP, thus requiring further investigation. These discoveries in HA15 broaden the implementation of the

compound for future virology work and should bring further attention to BiP being a therapeutic target for virus-associated diseases.

Chapter 5: Materials And Methods

Cell Culture

The iSLK-Hygro, iSLK-219, and primary human fibroblasts cells were incubated in DMEM supplemented with penicillin, streptomycin, glutamine, and 10% fetal bovine serum (FBS) at 37°C with a 5% CO_2 atmosphere. They were maintained in 10 µg/ml of puromycin (From InvivoGen Catalog# ant-pr-1) to keep the viral episome within the cells. The TREx BCBL1-RTA, BC1, and BC2 cells were incubated in RPMI supplemented with penicillin, streptomycin, glutamine, and 10% FBS at 37°C with a 5% CO_2 atmosphere. All Primary Effusion Lymphoma (PEL) cells (BCBL1-RTA, BC1, and BC2) were generous gifts from the Jung Lab from the Lerner Research Institute at Cleveland Clinic and the Manzano Lab from the University of Arkansas for Medical Sciences. Lymphatic endothelial cells (LECs) from PromoCell were maintained in EBM-2 media supplemented with the EGM-2 MV bullet at 37°C with a 5% CO2 atmosphere. Infections of LECs with the recombinant KSHV 219 viruses were conducted as described by Henry H Chang and Don Ganem, which included keeping the cells in the presence of 0.25 µg/mL puromycin and allowing a wait time of 14 days post-infection or more before any experimentation. All cells were kept in a HERAcell Vios 160i CO2 incubator from Thermo Scientific.

The lytic cycle in iSLK-219 was activated with the doxycycline (Dox)(From Tocris Catalog# 4090) inducible RTA transgene and the recombinant KSHV-219 virus. Time points for collection included 0 hr (Latent), 24 hr (Early Lytic), 48 hr (Early late lytic), and 72 hr (Late lytic). The same method of reactivation was utilized in BCBL1-RTA cells, where collection timepoints included 0 hr (Latent), 24 hr (Early lytic), and 48 hr (Late lytic). The

concentration of Dox used included 1 µg/mL for the ISLK-219 cells and 2 µg/mL for the BCBL1-RTA cells.

To distinguish if the upregulation of BiP is an early lytic or a late lytic phenomenon, iSLK-219 cells were pretreated for 24 hours with 100 µM phosphonoformate (PFA) (From Sigma Aldrich Catalog# P6801), which is a viral DNA polymerase inhibitor preventing efficient lytic reactivation. Assessment of the UPR during lytic reactivation in the iSLK-219 and BCBL1-RTA cells was done via treatment with 100nM of Thapsigargin (Tg) (From Tocris Catalog# 1138) for 4 hours before each collection time point. This ER stressor blocks the ER calcium pump, thus triggering the UPR. To study if ATF6 is involved in the upregulation of BiP, iSLK-219 cells were pretreated for 24 hours with 6 µM of Ceapin A7 (From Sigma Aldrich Catalog# SML2330) before lytic reactivation with 1 µg/mL. This compound was found to form an interorganic-tether between the cytosolic fragment of ATF6 and the peroxisomal ABCD3 membrane protein. To study the importance of GRP78/BiP for the lytic cycle and cell survival, we pretreated the cells with the GRP78/BiP inhibitor HA15 (From Selleckchem Catalog# S8299) at a concentration of 10 µg/ml for 24 hours before lytic reactivation. This drug can bind to the protein, preventing its chaperone and UPR regulatory activity.

Viral Tittering

Tittering experiments were done by obtaining the supernatant from late lytic (72 Hr) ISLK-219 cells and filtering it into a 5-15 mL conical tube via a 0.45 um syringe filter. About 100,000 uninfected iSLK-Hygro cells were seeded in a 12-well plate and allowed to rest overnight before experimentation. Approximately 1 mL of the supernatant was introduced to the cells and spun at 2000 RPM for two hours in a Thermo Scientific Sorvall

ST 40R centrifuge, then moved to the incubator for another hour before changing the supernatant to a fresh growth medium. After 24 to 48 hours, cells were trypsinized in 500 µL and placed in Eppendorf tubes before counting. About 12 µL of the sample was mixed in 12 µL of Trypan blue solution before putting them into a cell counting chamber slide and then into the Countess II FL. The solution is utilized to distinguish between dead cells and viable cells. Infectivity was analyzed based on GFP expression indicating these cells were infected with the recombinant 219 and those lacking GFP were uninfected. HSV1-US11-GFP (Patton strain) (Mohr lab, NYU School of Medicine) was propagated and titrated in Vero cells. HCMV-TB40/E-GFP (Murphy Lab SUNY) was propagated and titrated in NHDFs. Vaccinia Virus Western Reserve (ATCC) was expanded in HeLa cells and titrated in BSC1 cells.

CRISPRi and siRNA Experiments

All CRISPRi iSLK-219 cell lines were generated by the following protocol, and information in K. Brackett and others' article on utilizing CRISPRi for KSHV latent and lytic genes (gRNA sequences can be found below). All ON-TARGETplus siRNA transfections were done via the protocol supplied by horizondiscovery. 200,000 ISLK-219 cells were seeded in a 6-well plate format and allowed to grow overnight before all transfections. The cells were then incubated with the Dharmafect transfection reagent 1 for 5 minutes in serum-free growth media before mixing with 100nM of the siRNA and leaving it for 20 minutes. The mixture was then added to the desired complete growth medium amount with the cells. Lytic reactivation was induced post-24 hours with 1 µg/mL Dox.

gRNA sequences: ATF6 - 5`GTTAATATCTGGGACGGCGG3`, XBP1 - 5`GCCGCCACGCTGGGAACCTA3`, ORF37 1 - 5`GGCCGGTGCAATCTAGTCCC3`, and ORF37 2 - 5` GGGACCATGACGCTTGACCT`

Cell Viability Experiments

For Crystal violet staining experiments, about 5,000 to 10,000 cells were seeded in a 6-well plate before HA15, and TG was administered the next day. After 72-96 hours, the cells were fixed with a 1:1 mixture of acetone and methanol for ten minutes at room temperature. After that, the cells were exposed to the crystal violet solution consisting of Crystal violet Powder, 20% methanol, and H_2O for another ten minutes. The cells were washed three times with H2O and allowed to dry before images were taken with the c300 Azure Biosystems imager. All viability tests were done so in triplicate before the analysis was done. Photos of cells were taken with an ECHO RVL-100-M microscope before cell fixing with the acetone and methanol mixture.

Trypan viability tests in PEL cell lines BCBL1-RTA, BC1, and BC2 were done in the matter described earlier with an automated cell counter to distinguish dead and viable cells. About 30,000 BCBL1-RTA cells were seeded in a 96-well plate overnight before the addition of the different doses of HA15 (1µM, 5µM, 10µM, and 50µM). After 72 hours, the cells were counted with trypan blue, and analysis was done on the cell number and percentage of dead cells.

CellTiter-Glo Luminescent Cell Viability Assay from Promega was utilized to measure cell viability in infected and uninfected LECs with and without HA15. About 7,500-10,000 cells were seeded onto a 96-well white plate overnight before HA15 was introduced to their respective wells. When the day arrived for luminescence quantification, the cells' old growth media was exchanged with fresh media before adding the CellTiter-Glo reagents at an equal amount of growth media. The plate was left in the dark for 10-15 minutes before

luminescence was read on a Perkin Elmer Victor^{3}V 1420 Multilabel Counter. Pictures of uninfected and infected LEC with/without HA15 were done by seeding 200,000 cells in a 12-well plate and allowing them to acclimate for a couple of days before HA15 treatment. After 72 hours, the images were taken with an ECHO RVL-100-M microscope.

Primary human fibroblast (PHF) cells were plated at a density of 30,000 cells per well in a 96-well plate. The following day, cells were treated with HA15 (DMSO final concentration 0.1%) and incubated at 37 °C. At 24 h or 6 days (corresponding to viral infection period), 50 uL of supernatant was transferred to a new 96-well plate. 50 uL of CytoTox-ONE reagent was added to the plate and incubated at RT for 10 minutes. After 10 minutes, 25 uL of Stopping Reagent was added to the plate, and the plate was incubated at RT for 10 minutes. After incubation, the plate was transferred to the SpectraMax i3x plate reader, and fluorescence was read at 560/590 to determine percent cytotoxicity.

Western Blot Analysis And Antibodies

Cell lysates from each timepoint were prepared by adding 1X laemmli sample buffer to the cells and boiled at 95-100°C for 5 minutes. Samples may have been sonicated to remove excess DNA content and introduced to 2-Mercaptoethanol. Protein separation was done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred onto a nitrocellulose membrane. The membrane was blocked for an hour with 5% nonfat milk and incubated with specific primary antibodies overnight at 4°C. The membrane was then incubated for an hour with an HRP-conjugated secondary antibody in 5% nonfat milk. Imaging and processing were done in a c300 Azure Biosystems imager. Pierce ECL Western Blotting substrate from Thermo Scientific was utilized for enhanced chemiluminescence for HRP detection before images were taken. All primary antibodies

were diluted in 1X TBST with 3-5% BSA; information about their dilutions can be found below. All densitometries for the western blots were done in ImageJ software.

Antibodies for BiP (1:1000), PERK (1:1000), IRE1 (1:1000), GRP94 (1:1000), eIF2α-P (1:1000), eIF2α-T(1:1000), xBP1s(1:1000), GRP94(1:1000), were obtained from Cell Signaling Technology (CST). Antibodies for K8.1 (1:1000) were obtained from bleeds. Antibodies for ORF57 (1:1000), KbZIP (1:1000), and ORF45 (1:1000) were obtained from Santa Cruz Biotechnology. Antibody for Actin (1:20000-1:30000) was obtained from Sigma Aldrich. Antibody for Phosphorylated IRE1 (1:1000) was obtained from Novus Biologicals. Antibody for K1 (1:100) was a generous gift from the Damania Lab at The University of North Carolina at Chapel Hill.

RNA and DNA Extraction

RNA was extracted by RNeasy Mini Kit (Supplied by QIAGEN). DNA was extracted by DNeasy Blood & Tissue Kit (Supplied by QIAGEN) and was exposed to 50ug/mL of RNase A (From VWR Life Science Catalog# E866) to remove any excess RNA. Concentration for DNA and RNA was quantified by the NanoDrop One (Supplied by ThermoFisher).

Reverse Transcription PCR (RT-PCR) and Quantitative PCR (qPCR)

Preparation of cDNA with 0.5-1 µg of sample RNA was done by reverse transcriptase (iScript Reverse Transcription Supermix for RT-qPCR). Samples were subjected to DNase I (New England Biolabs Inc. M0303) to remove any excess genomic DNA that may have been captured during extraction before cDNA was synthesized. RT-PCR in a SimpliAmp Thermal Cycler and electrophoresis of the PCR products for XBP1u/s were done in 3% agarose gels

to distinguish spliced and unspliced XBP1. Densitometry of the ratios between spliced and unspliced was done with ImageJ software.

Quantification of mRNA expression of BiP in iSLK-219 cells and viral DNA synthesis in BCBL1-RTA cells were done by qPCR via PowerUp SYBR Green Master Mix. All qPCR reactions were done in a C1000 Touch Thermal cycler with a CFX96 Real-Time System, and analysis of fold change was done in Excel. For mRNA expression of BiP and 28S, cDNA was generated before 2 µL of samples was loaded in a 96-well qPCR plate for a 20 µL reaction. All samples were normalized to 100ng of DNA for viral DNA synthesis before making the proper mixtures in the PowerUp SYBR Green Master Mix for a 20 µL reaction. Primers for ORF57 were utilized to distinguish an increase of viral DNA from latent and late lytic BCBL1-RTA cells, and primers for Heterochromatin were utilized as a loading control.

Primers used in the following experiments: 28S: F: 5`AAACTCTGGTGGAGGTCCGT3` R: 5`CTTACCAAAAGTGGCCCACTA3`, XBP1u/s: F: 5`GGAGTTAAGACAGCGCTTGG3` R: 5`ACTGGGTCCAAGTTGTCCAG3`, BiP (HSAP5): F: 5`AGTTCCAGCGTCTTTGGTTG3` R: 5`TGCAGCAGGACATCAAGTTC3`, ORF57: F: 5`GGGTGGTTTGATGAGAAGGACA3` R: 5`CGCTACCAAATATGCCACCT, and Heterochromatin: F: 5`TAACTGGTCTTGACTAGGGTTTCAG3` R: 5`ACCACAACAAAAAGCCTTATAGTGG3`

RNA Sequencing and Analysis

Total cellular and viral RNA was isolated from iSLK-219 cells at 72h post reactivation in the presence or absence of 10uM HA15, using the RNAeasy Plus Mini kit

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(QIAGEN 74134) following manufacturers' recommendations, including a DNAse treatment step. RNA sequencing libraries were generated using the NEBNext Ultra II RNA Library Prep Kit (New England BioLabs E7760) and sequenced using a 150bp paired-end protocol on an Illumina Novaseq 6000 instrument. Following demultiplexing, the sequenced reads were analyzed using the CZ ID platform (czid.org). Samples were aligned to the human genome GRcHg38, and all remaining reads were saved as non_host. These files were aligned to the KSHV genome GQ994935.1, and the transcripts were quantified using Salmon (Patro reference science). Heatmaps were generated and annotated in Prism.

Fluorescence Assay

Primary human fibroblast (PHF) cells were plated at a density of 30,000 cells per well in a 96-well plate. The following day, cells were pretreated for two hours with HA15 (DMSO final concentration 0.1%) and incubated at 37 °C. After pretreatment, NHDF cells were either mock-infected or infected with Herpes simplex virus 1 (HSV-1) US11-GFP (Patton strain) at MOI 0.01, Human cytomegalovirus (HCMV) EGFP (TB40/E strain) at MOI 0.1, or Vaccinia virus (VV) (Western Reserve strain) at MOI 0.1 and incubated for 1 h at 37 °C. After 1 h incubation, the supernatant from cells was removed and replaced with fresh DMEM and HA15. Cells were incubated at 37 °C for 24 h (HSV-1 and VV-infected cells) or 6 days (HCMV-infected cells). After respective incubation periods, the supernatant was removed and replaced with PBS. Because VV lacked a fluorescent reporter, infected cells were stained with primary antibody (Vaccinia Virus Polyclonal FITC Antibody, 1:1000) and Hoechst 33342 (1:10,000). The fluorescent signal (GFP/Hoescht) was analyzed using the SpectraMax i3x plate reader. GFP fluorescence was measured at 485/535 and Hoescht fluorescence at 350/461.

References

1. Beachboard DC, Horner SM. Innate immune evasion strategies of DNA and RNA viruses. Current Opinion in Microbiology 32 2016 113–119.
2. Rozman B, Fisher T, Stern-Ginossar N. Translation—A tug of war during viral infection. Mol Cell 2022;
3. Barber GN. Host defense, viruses and apoptosis. 2001.
4. Schwarz DS, Blower MD. The endoplasmic reticulum: Structure, function and response to cellular signaling. Cellular and Molecular Life Sciences 73 2016 79–94.
5. Ravindran MS, Bagchi P, Cunningham CN, Tsai B. Opportunistic intruders: How viruses orchestrate ER functions to infect cells. Nature Reviews Microbiology 14 2016 407–420.
6. Ravindran MS, Bagchi P, Inoue T, Tsai B. A Non-enveloped Virus Hijacks Host Disaggregation Machinery to Translocate across the Endoplasmic Reticulum Membrane. PLoS Pathog 2015; 11.
7. Giannecchini S. Evidence of the mechanism by which polyomaviruses exploit the extracellular vesicle delivery system during infection. Viruses 12 2020.
8. van den Elsen K, Quek JP, Luo D. Molecular insights into the flavivirus replication complex. Viruses 2021; 13.
9. Chatel-Chaix L, Bartenschlager R. Dengue Virus- and Hepatitis C Virus-Induced Replication and Assembly Compartments: the Enemy Inside—Caught in the Web. J Virol 2014; 88: 5907–5911.
10. Roingeard P, Eymieux S, Burlaud-Gaillard J, Hourioux C, Patient R, Blanchard E. The double-membrane vesicle (DMV): a virus-induced organelle dedicated to the replication of SARS-CoV-2 and other positive-sense single-stranded RNA viruses. Cellular and Molecular Life Sciences 79 2022.
11. Rajah MM, Monel B, Schwartz O. The entanglement between flaviviruses and ER-shaping proteins. PLoS Pathog 2020; 16.
12. Welsch S, Müller B, Kräusslich HG. More than one door - Budding of enveloped viruses through cellular membranes. FEBS Letters 581 2007 2089–2097.
13. V'kovski P, Kratzel A, Steiner S, Stalder H, Thiel V. Coronavirus biology and replication: implications for SARS-CoV-2. Nature Reviews Microbiology 19 2021 155–170.
14. Ghosh S, Dellibovi-Ragheb TA, Kerviel A *et al.* β-Coronaviruses Use Lysosomes for Egress Instead of the Biosynthetic Secretory Pathway. Cell 2020; 183: 1520-1535.e14.
15. Yap SSL, Nguyen-Khuong T, Rudd PM, Alonso S. Dengue virus glycosylation: What do we know? Frontiers in Microbiology 8 2017.
16. Kohli E, Causse S, Baverel V *et al.* Endoplasmic Reticulum Chaperones in Viral Infection: Therapeutic Perspectives. 2021.
17. Sehrawat S, Kumar D, Rouse BT. Herpesviruses: Harmonious pathogens but relevant cofactors in other diseases? Frontiers in Cellular and Infection Microbiology 8 2018.
18. Ahmed SP, Nash RJ, Bridges CG *et al.* Antiviral Activity and Metabolism of the Castanospermine Derivative MDL 28,574, in Cells Infected with Herpes Simplex Virus Type 2. Biochem Biophys Res Commun 1995; 208: 267–273.

19. Taylor DL, Fellows LE, Farrar GH *et al.* Loss of cytomegalovirus infectivity after treatment with castanospermine or related plant alkaloids correlates with aberrant glycoprotein synthesis. 1988.
20. Schlesinger M, McDonald C, Ahuja A *et al.* Glucose and mannose analogs inhibit KSHV replication by blocking N-glycosylation and inducing the unfolded protein response. J Med Virol 2023; 95.
21. Lin JH, Walter P, Yen TSB. Endoplasmic reticulum stress in disease pathogenesis. Annual Review of Pathology: Mechanisms of Disease 3 2008 399–425.
22. Walter P, Ron D. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. .
23. Preissler S, Ron D. Early events in the endoplasmic reticulum unfolded protein response. Cold Spring Harb Perspect Biol 2019; 11.
24. Kimata Y, Oikawa D, Shimizu Y, Ishiwata-Kimata Y, Kohno K. A role for BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1. Journal of Cell Biology 2004; 167: 445–456.
25. Gardner BM, Walter P. Unfolded Proteins are Ire1-Activating Ligands that Directly Induce the Unfolded Protein Response. .
26. Kay LE, Elif Karagö G, Acosta-Alvear D *et al.* An unfolded protein-induced conformational switch activates mammalian IRE1. 2017;
27. Park SM, Kang T il, So JS. Roles of XBP1s in transcriptional regulation of target genes. Biomedicines 9 2021.
28. Acosta-Alvear D, Zhou Y, Blais A *et al.* XBP1 Controls Diverse Cell Type- and Condition-Specific Transcriptional Regulatory Networks. Mol Cell 2007; 27: 53–66.
29. Maurel M, Chevet E, Tavernier J, Gerlo S. Getting RIDD of RNA: IRE1 in cell fate regulation. Trends in Biochemical Sciences 39 2014 245–254.
30. Brown M, Strudwick N, Suwara M *et al.* An initial phase of JNK activation inhibits cell death early in the endoplasmic reticulum stress response. J Cell Sci 2016; 129: 2317–2328.
31. Fung TS, Liu DX. Coronavirus infection, ER stress, apoptosis and innate immunity. Frontiers in Microbiology 5 2014.
32. Isler JA, Skalet AH, Alwine JC. Human Cytomegalovirus Infection Activates and Regulates the Unfolded Protein Response. J Virol 2005; 79: 6890–6899.
33. Stahl S, Burkhart JM, Hinte F *et al.* Cytomegalovirus Downregulates IRE1 to Repress the Unfolded Protein Response. PLoS Pathog 2013; 9.
34. Kolpikova EP, Tronco AR, Den Hartigh AB, Jackson KJ, Iwawaki T, Fink SL. IRE1α promotes zika virus infection via XBP1. Viruses 2020; 12.
35. Hassan IH, Zhang MS, Powers LS *et al.* Influenza A viral replication is blocked by inhibition of the inositol-requiring enzyme 1 (IRE1) stress pathway. Journal of Biological Chemistry 2012; 287: 4679–4689.
36. Echavarría-Consuegra L, Cook GM, Busnadiego I *et al.* Manipulation of the unfolded protein response: A pharmacological strategy against coronavirus infection. PLoS Pathog 2021; 17.
37. Bhattacharyya S, Sen U, Vrati S. Regulated IRE1-dependent decay pathway is activated during Japanese encephalitis virusinduced unfolded protein response and benefits viral replication. Journal of General Virology 2014; 95: 71–79.

38. Yu C-Y, Hsu Y-W, Liao C-L, Lin Y-L. Flavivirus Infection Activates the XBP1 Pathway of the Unfolded Protein Response To Cope with Endoplasmic Reticulum Stress. J Virol 2006; 80: 11868–11880.
39. Pakos-Zebrucka K, Koryga I, Mnich K, Ljujic M, Samali A, Gorman AM. The integrated stress response. EMBO Rep 2016; 17: 1374–1395.
40. Sudhakar A, Ramachandran A, Ghosh S, Hasnain SE, Kaufman RJ, Ramaiah KVA. Phosphorylation of serine 51 in initiation factor 2α (eIF2α) promotes complex formation between eIF2α(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B. Biochemistry 2000; 39: 12929–12938.
41. Wek RC. Role of eIF2α kinases in translational control and adaptation to cellular stress. Cold Spring Harb Perspect Biol 2018; 10.
42. He B, Gross M, Roizman B. The 1 34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1 to dephosphorylate the subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase (growth arrest and DNA damage gene 34). 1997.
43. Mulvey M, Arias C, Mohr I. Maintenance of Endoplasmic Reticulum (ER) Homeostasis in Herpes Simplex Virus Type 1-Infected Cells through the Association of a Viral Glycoprotein with PERK, a Cellular ER Stress Sensor. J Virol 2007; 81: 3377–3390.
44. Pavio N, Romano PR, Graczyk TM, Feinstone SM, Taylor DR. Protein Synthesis and Endoplasmic Reticulum Stress Can Be Modulated by the Hepatitis C Virus Envelope Protein E2 through the Eukaryotic Initiation Factor 2α Kinase PERK. J Virol 2003; 77: 3578–3585.
45. Egan PA, Sobkowiak M, Chan S-W. Hepatitis C Virus Envelope Protein E1 Binds PERK and Represses the Unfolded Protein Response. 2013.
46. Hillary RF, Fitzgerald U. A lifetime of stress: ATF6 in development and homeostasis. Journal of Biomedical Science 25 2018.
47. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian Transcription Factor ATF6 Is Synthesized as a Transmembrane Protein and Activated by Proteolysis in Response to Endoplasmic Reticulum Stress. 1999.
48. Ambrose RL, Mackenzie JM. ATF6 Signaling Is Required for Efficient West Nile Virus Replication by Promoting Cell Survival and Inhibition of Innate Immune Responses. J Virol 2013; 87: 2206–2214.
49. Lee YR, Kuo SH, Lin CY *et al.* Dengue virus-induced ER stress is required for autophagy activation, viral replication, and pathogenesis both in vitro and in vivo. Sci Rep 2018; 8.
50. Blázquez AB, Escribano-Romero E, Merino-Ramos T, Saiz JC, Martín-Acebes MA. Stress responses in flavivirus-infected cells: Activation of unfolded protein response and autophagy. Frontiers in Microbiology 5 2014.
51. Xue M, Feng L. The Role of Unfolded Protein Response in Coronavirus Infection and Its Implications for Drug Design. Frontiers in Microbiology 12 2021.
52. Sung SC, Chao CY, Jeng KS, Yang JY, Lai MMC. The 8ab protein of SARS-CoV is a luminal ER membrane-associated protein and induces the activation of ATF6. Virology 2009; 387: 402–413.
53. Hartl FU, Bracher A, Hayer-Hartl M. Molecular chaperones in protein folding and proteostasis. Nature 475 2011 324–332.

54. Marzec M, Eletto D, Argon Y. GRP94: An HSP90-like protein specialized for protein folding and quality control in the endoplasmic reticulum. Biochim Biophys Acta Mol Cell Res 2012; 1823: 774–787.
55. Bloor S, Maelfait J, Krumbach R, Beyaert R, Randow F. Endoplasmic reticulum chaperone gp96 is essential for infection with vesicular stomatitis virus. Proc Natl Acad Sci U S A 2010; 107: 6970–6975.
56. Finkelshtein D, Werman A, Novick D, Barak S, Rubinstein M. LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. Proc Natl Acad Sci U S A 2013; 110: 7306–7311.
57. Weekes MP, Antrobus R, Talbot S *et al.* Proteomic plasma membrane profiling reveals an essential role for gp96 in the cell surface expression of LDLR family members, including the LDL receptor and LRP6. Journal of Proteome Research, 2012, 1475–1484.
58. Rothan HA, Zhong Y, Sanborn MA *et al.* Small molecule grp94 inhibitors block dengue and Zika virus replication. Antiviral Res 2019; 171.
59. Gorbatyuk MS, Gorbatyuk OS. The Molecular Chaperone GRP78/BiP as a Therapeutic Target for Neurodegenerative Disorders: A Mini Review. J Genet Syndr Gene Ther 2013; 04.
60. Lewy TG, Grabowski JM, Bloom ME. BiP: Master Regulator of the Unfolded Protein Response and Crucial Factor in Flavivirus Biology. 2017.
61. Mum-O S, Pelham HRB. An Hsp704ike Protein in the ER: Identity with the 78 kd Glucose-Regulated Protein and lmmunoglobulin Heavy Chain Binding Protein. 1986.
62. Shiu RPC, Pouyssegur J, Pastan I. Glucose depletion accounts for the induction of two transformation-sensitive membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts (glucose starvation/membrane proteins). 1977.
63. Hendershot LM. Immunoglobulin Heavy Chain and Binding Protein Complexes Are Dissociated In Vivo by Light Chain Addition. .
64. Ibrahim IM, Abdelmalek DH, Elfiky AA. GRP78: A cell's response to stress. Life Sciences 226 2019 156–163.
65. Rosenzweig R, Nillegoda NB, Mayer MP, Bukau B. The Hsp70 chaperone network. Nature Reviews Molecular Cell Biology 20 2019 665–680.
66. Pobre KFR, Poet GJ, Hendershot LM. The endoplasmic reticulum (ER) chaperone BiP is a master regulator of ER functions: Getting by with a little help from ERdj friends. Journal of Biological Chemistry 294 2019 2098–2108.
67. Behnke J, Feige MJ, Hendershot LM. BiP and Its Nucleotide Exchange Factors Grp170 and Sil1: Mechanisms of Action and Biological Functions. Journal of Molecular Biology 427 2015 1589–1608.
68. Gonzalez-Gronow M. Cellular Trafficking of Cell Stress Proteins in Health and Disease. 2012.
69. Chu H, Chan CM, Zhang X *et al.* Middle East respiratory syndrome coronavirus and bat coronavirus HKU9 both can utilize GRP78 for attachment onto host cells. Journal of Biological Chemistry 2018; 293: 11709–11726.
70. Ibrahim IM, Abdelmalek DH, Elshahat ME, Elfiky AA. COVID-19 spike-host cell receptor GRP78 binding site prediction. Journal of Infection 2020; 80: 554–562.

71. Limjindaporn T, Wongwiwat W, Noisakran S *et al.* Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production. Biochem Biophys Res Commun 2009; 379: 196–200.
72. Royle J, Ramírez-Santana C, Akpunarlieva S *et al.* Glucose-regulated protein 78 interacts with zika virus envelope protein and contributes to a productive infection. Viruses 2020; 12.
73. Jitobaom K, Tongluan N, Smith DR. Involvement of voltage-dependent anion channel (VDAC) in dengue infection. Sci Rep 2016; 6.
74. Shu W, Guo Z, Li L *et al.* Regulation of Molecular Chaperone GRP78 by Hepatitis B Virus: Control of Viral Replication and Cell Survival. Mol Cell Biol 2020; 40.
75. Cesarman E. Gammaherpesviruses and lymphoproliferative disorders. Annual Review of Pathology: Mechanisms of Disease 2014; 9: 349–372.
76. Aneja KK, Yuan Y. Reactivation and lytic replication of Kaposi's sarcoma-associated herpesvirus: An update. Frontiers in Microbiology 8 2017.
77. Mesri EA, Cesarman E, Boshoff C. Kaposi's sarcoma and its associated herpesvirus. Nature Reviews Cancer 10 2010 707–719.
78. Cesarman E, Damania B, Krown SE, Martin J, Bower M, Whitby D. Kaposi sarcoma. Nat Rev Dis Primers 2019; 5.
79. Curtiss P, Strazzulla LC, Friedman-Kien AE. An Update on Kaposi's Sarcoma: Epidemiology, Pathogenesis and Treatment. Dermatol Ther (Heidelb) 2016; 6: 465–470.
80. Atrick P, Oore SM, Uan Y, Hang C. DETECTION OF HERPESVIRUS-LIKE DNA SEQUENCES IN KAPOSI'S SARCOMA IN PATIENTS WITH AND THOSE WITHOUT HIV INFECTION. 1995.
81. Vincenzi B, D'Onofrio L, Frezza AM *et al.* Classic Kaposi Sarcoma: To treat or not to treat? BMC Res Notes 2015; 8.
82. Gaglia MM. Kaposi's sarcoma-associated herpesvirus at 27. Tumour Virus Research 12 2021.
83. Htet KZ, Waul MA, Leslie KS. Topical treatments for Kaposi sarcoma: A systematic review. Skin Health and Disease 2022; 2.
84. Shimada K, Hayakawa F, Kiyoi H. Biology and management of primary effusion lymphoma. 2018.
85. Dispenzieri A, Fajgenbaum DC. Overview of Castleman disease. 2020.
86. Jenner RG, Maillard K, Cattini N *et al.* Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphoma has a plasma cell gene expression profile. .
87. Veettil MV, Bandyopadhyay C, Dutta D, Chandran B. Interaction of KSHV with host cell surface receptors and cell entry. Viruses 6 2014 4024–4046.
88. Purushothaman P, Dabral P, Gupta N, Sarkar R, Verma SC. KSHV genome replication and maintenance. Frontiers in Microbiology 7 2016.
89. Purushothaman P, Uppal T, Verma SC. Molecular biology of KSHV lytic reactivation. Viruses 7 2015 116–153.
90. Zhou F, Xue M, Qin D *et al.* HIV-1 Tat Promotes Kaposi's Sarcoma-Associated Herpesvirus (KSHV) vIL-6-Induced Angiogenesis and Tumorigenesis by Regulating PI3K/PTEN/AKT/GSK-3β Signaling Pathway. PLoS One 2013; 8.

91. Ensoli B, Barillari G, Zaki Salahuddin S, GaDo RC, WongStaal F. Tat protein of HIV-l stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. 1990.

92. Varthakavi V, Smith RM, Deng H, Sun R, Spearman P. Human immunodeficiency virus type-1 activates lytic cycle replication of Kaposi's sarcoma-associated herpesvirus through induction of KSHV Rta. Virology 2002; 297: 270–280.

93. Davis DA, Rinderknecht AS, Zoeteweij JP *et al.* Hypoxia induces lytic replication of Kaposi sarcoma-associated herpesvirus. 2001.

94. Shin HJ, DeCotiis J, Giron M, Palmeri D, Lukac DM. Histone Deacetylase Classes I and II Regulate Kaposi's Sarcoma-Associated Herpesvirus Reactivation. J Virol 2014; 88: 1281–1292.

95. Myoung J, Ganem D. Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin: Maintenance of tight latency with efficient reactivation upon induction. J Virol Methods 2011; 174: 12–21.

96. Nakamura H, Lu M, Gwack Y, Souvlis J, Zeichner SL, Jung JU. Global Changes in Kaposi's Sarcoma-Associated Virus Gene Expression Patterns following Expression of a Tetracycline-Inducible Rta Transactivator. J Virol 2003; 77: 4205–4220.

97. Shigemi Z, Baba Y, Hara N *et al.* Effects of ER stress on unfolded protein responses, cell survival, and viral replication in primary effusion lymphoma. Biochem Biophys Res Commun 2016; 469: 565–572.

98. Santarelli R, Arteni AMB, Gilardini Montani MS *et al.* KSHV dysregulates bulk macroautophagy, mitophagy and UPR to promote endothelial to mesenchymal transition and CCL2 release, key events in viral-driven sarcomagenesis. Int J Cancer 2020; 147: 3500–3510.

99. Gilardini Montani MS, Falcinelli L, Santarelli R *et al.* KSHV infection skews macrophage polarisation towards M2-like/TAM and activates Ire1 α-XBP1 axis up-regulating pro-tumorigenic cytokine release and PD-L1 expression. Br J Cancer 2020; 123: 298–306.

100. Johnston BP, Pringle ES, McCormick C. KSHV activates unfolded protein response sensors but suppresses downstream transcriptional responses to support lytic replication. PLoS Pathog 2019; 15.

101. Gonnella R, Gilardini Montani MS, Guttieri L, Romeo MA, Santarelli R, Cirone M. Ire1 alpha/xbp1 axis sustains primary effusion lymphoma cell survival by promoting cytokine release and stat3 activation. Biomedicines 2021; 9: 1–11.

102. Yu F, Feng J, Harada JN, Chanda SK, Kenney SC, Sun R. B cell terminal differentiation factor XBP-1 induces reactivation of Kaposi's sarcoma-associated herpesvirus. FEBS Lett 2007; 581: 3485–3488.

103. Li DJ, Verma D, Mosbruger T, Swaminathan S. CTCF and Rad21 Act as Host Cell Restriction Factors for Kaposi's Sarcoma-Associated Herpesvirus (KSHV) Lytic Replication by Modulating Viral Gene Transcription. PLoS Pathog 2014; 10.

104. de Leo A, Chen HS, Hu CCA, Lieberman PM. Deregulation of KSHV latency conformation by ER-stress and caspase-dependent RAD21-cleavage. PLoS Pathog 2017; 13.

105. Chen S, Novick P, Ferro-Novick S. ER structure and function. Current Opinion in Cell Biology 25 2013 428–433.

106. Long WY, Zhao GH, Wu Y. Endoplasmic Reticulum-Shaping Atlastin Proteins Facilitate KSHV Replication. Front Cell Infect Microbiol 2022; 11.

107. Chang P-J, Hung C-H, Wang S-S *et al.* Identification and Characterization of Two Novel Spliced Genes Located in the orf47-orf46-orf45 Gene Locus of Kaposi's Sarcoma-Associated Herpesvirus . J Virol 2014; 88: 10092–10109.

108. Grinde B. Herpesviruses: latency and reactivation – viral strategies and host response. J Oral Microbiol 2013; 5: 22766.

109. Cerezo M, Lehraiki A, Millet A *et al.* Compounds Triggering ER Stress Exert Anti-Melanoma Effects and Overcome BRAF Inhibitor Resistance. Cancer Cell 2016; 29: 805–819.

110. Trezise S, Kong IY, Hawkins ED, Herold MJ, Willis SN, Nutt SL. An arrayed CRISPR screen of primary B cells reveals the essential elements of the antibody secretion pathway. Front Immunol 2023; 14.

111. Chang HH, Ganem D. A unique herpesviral transcriptional program in KSHV-infected lymphatic endothelial cells leads to mTORC1 activation and rapamycin sensitivity. Cell Host Microbe 2013; 13: 429–440.

112. Jin HR, Liao Y, Li X *et al.* Anticancer compound Oplopantriol A kills cancer cells through inducing ER stress and BH3 proteins Bim and Noxa. Cell Death Dis 2014; 5.

113. Durose JB, Tam AB, Niwa M. Intrinsic Capacities of Molecular Sensors of the Unfolded Protein Response to Sense Alternate Forms of Endoplasmic Reticulum Stress □ D. Mol Biol Cell 2006; 17: 3095–3107.

114. Marissen WE, Guo Y, Thomas AAM, Matts RL, Lloyd RE. Identification of caspase 3-mediated cleavage and functional alteration of eukaryotic initiation factor 2α in apoptosis. Journal of Biological Chemistry 2000; 275: 9314–9323.

115. Glaunsinger B, Chavez L, Ganem D. The Exonuclease and Host Shutoff Functions of the SOX Protein of Kaposi's Sarcoma-Associated Herpesvirus Are Genetically Separable. J Virol 2005; 79: 7396–7401.

116. Pardamean CI, Wu TT. Inhibition of Host Gene Expression by KSHV: Sabotaging mRNA Stability and Nuclear Export. Frontiers in Cellular and Infection Microbiology 11 2021.

117. Brackett K, Mungale A, Lopez-Isidro M, Proctor DA, Najarro G, Arias C. Crispr interference efficiently silences latent and lytic viral genes in kaposi's sarcoma-associated herpesvirus-infected cells. Viruses 2021; 13.

118. Hartenian E, Mendez AS, Didychuk AL, Khosla S, Glaunsinger BA. DNA processing by the Kaposi's sarcoma-associated herpesvirus alkaline exonuclease SOX contributes to viral gene expression and infectious virion production. Nucleic Acids Res 2023; 51: 182–197.

119. Torres SE, Gallagher CM, Plate L *et al.* Ceapins block the unfolded protein response sensor ATF6a by inducing a neomorphic inter-organelle tether. 2019;

120. Chen TH, Chiang YH, Hou JN *et al.* XBP1-Mediated BiP/GRP78 Upregulation Copes with Oxidative Stress in Mosquito Cells during Dengue 2 Virus Infection. Biomed Res Int 2017; 2017.

121. White-Gilbertson S, Hua Y, Liu B. The role of endoplasmic reticulum stress in maintaining and targeting multiple myeloma: A double-edged sword of adaptation and apoptosis. Frontiers in Genetics 4 2013.

122. Martinez-Turtos A, Paul R, Grima-Reyes M *et al.* IRE1α overexpression in malignant cells limits tumor progression by inducing an anti-cancer immune response. Oncoimmunology 2022; 11.

123. Brewer JW, Diehl JA. PERK mediates cell-cycle exit during the mammalian unfolded protein response. .

124. Coleman OI, Haller D. ER Stress and the UPR in Shaping Intestinal Tissue Homeostasis and Immunity. Frontiers in Immunology 10 2019.

125. Zhang W, Feng D, Li Y, Iida K, McGrath B, Cavener DR. PERK EIF2AK3 control of pancreatic β cell differentiation and proliferation is required for postnatal glucose homeostasis. Cell Metab 2006; 4: 491–497.

126. Ma Y, Shimizu Y, Mann MJ, Jin Y, Hendershot LM. Plasma cell differentiation initiates a limited ER stress response by specifically suppressing the PERK-dependent branch of the unfolded protein response. Cell Stress Chaperones 2010; 15: 281–293.

127. Guan BJ, van Hoef V, Jobava R *et al.* A Unique ISR Program Determines Cellular Responses to Chronic Stress. Mol Cell 2017; 68: 885-900.e6.

128. Wang V, Davis DA, Deleage C *et al.* Induction of Kaposi's Sarcoma-Associated Herpesvirus-Encoded Thymidine Kinase (ORF21) by X-Box Binding Protein 1. J Virol 2020; 94.

129. Dalton-Griffin L, Wilson SJ, Kellam P. X-Box Binding Protein 1 Contributes to Induction of the Kaposi's Sarcoma-Associated Herpesvirus Lytic Cycle under Hypoxic Conditions. J Virol 2009; 83: 7202–7209.

130. Uppal T, Meyer D, Agrawal A, Verma SC. The DNase Activity of Kaposi's Sarcoma-Associated Herpesvirus SOX Protein Serves an Important Role in Viral Genome Processing during Lytic Replication. 2019;

131. Buchkovich NJ, Yu Y, Pierciey FJ, Alwine JC. Human Cytomegalovirus Induces the Endoplasmic Reticulum Chaperone BiP through Increased Transcription and Activation of Translation by Using the BiP Internal Ribosome Entry Site. J Virol 2010; 84: 11479–11486.

132. Ma Y, Liu P, Majerciak V, Zhu J, Zheng ZM. CLIP-seq to identify KSHV ORF57-binding RNA in host B cells. Curr Protoc Microbiol 2016; 2016: 1E.11.1-1E.11.18.

133. Baquero-Pérez B, Whitehouse A. Hsp70 Isoforms Are Essential for the Formation of Kaposi's Sarcoma-Associated Herpesvirus Replication and Transcription Compartments. PLoS Pathog 2015; 11.

134. Nayar U, Lu P, Goldstein RL *et al.* Targeting the Hsp90-associated viral oncoproteome in gammaherpesvirus-associated malignancies Key Points. 2013;

135. Chen W, Sin SH, Wen KW, Damania B, Dittmer DP. Hsp90 Inhibitors Are Efficacious against Kaposi Sarcoma by Enhancing the Degradation of the Essential Viral Gene LANA, of the Viral Co-Receptor EphA2 as well as Other Client Proteins. PLoS Pathog 2012; 8.

136. Wen KW, Damania B. Hsp90 and Hsp40/Erdj3 are required for the expression and anti-apoptotic function of KSHV K1. Oncogene 2010; 29: 3532–3544.

137. Zhang Z, Chen W, Sanders MK, Brulois KF, Dittmer DP, Damania B. The K1 Protein of Kaposi's Sarcoma-Associated Herpesvirus Augments Viral Lytic Replication. J Virol 2016; 90: 7657–7666.

138. Granato M, Lacconi V, Peddis M *et al.* HSP70 inhibition by 2-phenylethynesulfonamide induces lysosomal cathepsin D release and immunogenic cell death in primary effusion lymphoma. Cell Death Dis 2013; 4.

139. Shin WJ, Ha DP, Machida K, Lee AS. The stress-inducible ER chaperone GRP78/BiP is upregulated during SARS-CoV-2 infection and acts as a pro-viral protein. Nature Communications 13 2022.

140. Barrera MD, Callahan V, Akhrymuk I *et al.* Proteomic Discovery of VEEV E2-Host Partner Interactions Identifies GRP78 Inhibitor HA15 as a Potential Therapeutic for Alphavirus Infections. 2021;

141. Karasneh GA, Shukla D. Herpes simplex virus infects most cell types in vitro: Clues to its success. Virology Journal 8 2011.

142. Shenk T, Stinski MF. Human Cytomegalovirus. .

143. Mao H, Palmer D, Rosenthal KS. Changes in BiP (GRP78) levels upon HSV-1 infection are strain dependent. 2001.

144. Jayswal S, Kakadiya J. A narrative review of pox: smallpox vs monkeypox. Egypt J Intern Med 2022; 34.

145. Xu D, Yang H, Yang Z *et al.* Endoplasmic reticulum stress signaling as a therapeutic target in malignant pleural mesothelioma. Cancers (Basel) 2019; 11.

146. Wu J, Wu Y, Lian X. Targeted inhibition of GRP78 by HA15 promotes apoptosis of lung cancer cells accompanied by ER stress and autophagy. Biol Open 2020; 9.

147. Sun S, Li X, Mariappan M. Signal sequences encode information for protein folding in the endoplasmic reticulum. .

148. Yan MM, Ni JD, Song D, Ding M, Huang J. Interplay between unfolded protein response and autophagy promotes tumor drug resistance (Review). Oncol Lett 2015; 10: 1959–1969.

149. Buchkovich NJ, Maguire TG, Yu Y, Paton AW, Paton JC, Alwine JC. Human Cytomegalovirus Specifically Controls the Levels of the Endoplasmic Reticulum Chaperone BiP/GRP78, Which Is Required for Virion Assembly. J Virol 2008; 82: 31–39.

150. Miernyk JA, Thelen JJ. Biochemical approaches for discovering protein-protein interactions. Plant Journal 2008; 53: 597–609.

151. Fan Q, Longnecker R, Connolly SA. Herpes Simplex Virus Glycoprotein B Mutations Define Structural Sites in Domain I, the Membrane Proximal Region, and the Cytodomain That Regulate Entry. J Virol 2021; 95.

152. Johnston BP, McCormick C. Herpesviruses and the unfolded protein response. Viruses 12 2019.

153. Maiuolo J, Bulotta S, Verderio C, Benfante R, Borgese N. Selective activation of the transcription factor ATF6 mediates endoplasmic reticulum proliferation triggered by a membrane protein. Proc Natl Acad Sci U S A 2011; 108: 7832–7837.

154. Coelho DS, Domingos PM. Physiological roles of regulated Ire1 dependent decay. Front Genet 2014; 5.

155. Arias C, Weisburd B, Stern-Ginossar N *et al.* KSHV 2.0: A Comprehensive Annotation of the Kaposi's Sarcoma-Associated Herpesvirus Genome Using Next-Generation Sequencing Reveals Novel Genomic and Functional Features. PLoS Pathog 2014; 10.

156. Panda A, Martindale J, Gorospe M. Polysome Fractionation to Analyze mRNA Distribution Profiles. Bio Protoc 2017; 7.
157. Godet AC, David F, Hantelys F *et al.* IRES trans-acting factors, key actors of the stress response. International Journal of Molecular Sciences 20 2019.
158. Cho S, Park SM, Kim TD, Kim JH, Kim K-T, Jang SK. BiP Internal Ribosomal Entry Site Activity Is Controlled by Heat-Induced Interaction of NSAP1. Mol Cell Biol 2007; 27: 368–383.
159. Morrison JK, Friday AJ, Henderson MA, Hao E, Keiper BD. Induction of cap-independent BiP (hsp-3) and Bcl-2 (ced-9) translation in response to eIF4G (IFG-1) depletion in C. elegans . Translation 2014; 2: e28935.
160. Hafner M, Katsantoni M, Köster T *et al.* CLIP and complementary methods. Nature Reviews Methods Primers 1 2021.
161. Saibil H. Chaperone machines for protein folding, unfolding and disaggregation. Nature Reviews Molecular Cell Biology 14 2013 630–642.
162. Paladino L, Vitale AM, Bavisotto CC *et al.* The role of molecular chaperones in virus infection and implications for understanding and treating covid-19. Journal of Clinical Medicine 9 2020 1–15.
163. Kapałczyńska M, Kolenda T, Przybyła W *et al.* 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. Archives of Medical Science 2018; 14: 910–919.
164. Cheng F, Pekkonen P, Laurinavicius S *et al.* KSHV-initiated notch activation leads to membrane-type-1 matrix metalloproteinase-dependent lymphatic endothelial-to-mesenchymal transition. Cell Host Microbe 2011; 10: 577–590.
165. Tuohinto K, DiMaio TA, Kiss EA *et al.* KSHV infection of endothelial precursor cells with lymphatic characteristics as a novel model for translational Kaposi's sarcoma studies. PLoS Pathog 2023; 19.
166. Tolonen N, Doglio L, Schleich S, Locker JK. Vaccinia Virus DNA Replication Occurs in Endoplasmic Reticulum-enclosed Cytoplasmic Mini-Nuclei. 2001.
167. Maruri-Avidal L, Weisberg AS, Moss B. Vaccinia Virus L2 Protein Associates with the Endoplasmic Reticulum near the Growing Edge of Crescent Precursors of Immature Virions and Stabilizes a Subset of Viral Membrane Proteins. J Virol 2011; 85: 12431–12441.