Heinrich Pette Institute
Leibniz Institute for Experimental Virology

Modulation of the Unfolded Protein Response by Kaposi's Sarcoma-associated Herpesvirus

Dissertation

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بسم الله الرحمن الرحیم

((لهم دار السلام عند ربهم و هو ولیهم بما کانو یعملون))

سوره انعام آیه 127

In the name of God

''For them will be a home of peace in the presence of their Lord: He will be their friend, because they practiced''

Quran, Anaam, 127

Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning (knowing).

''Albert Einstein''

از دیروز یاد بگیر، برای امروز زندگی کن، و به فردا امیدوار باش. مهم دست نکشیدن از پرسشگری است.

آلبرت اینشتن

This study was conducted between February 2013 and December 2017 at the Heinrich Pette Institute Leibniz Institute for Experimental Virology under the supervision of Prof. Dr. Wolfram Brune and Prof. Dr. Adam Grundhoff.

Contents

1.1 List of abbreviations

AIDS	acquired immunodeficiency syndrome
ATF6	activating transcription factor 6
bp	base pair(s)
BCL-2	B-cell leukemia/lymphoma 2 (BCL-2) homologs
CMV	cytomegalovirus
DDR	DNA damage response
dsDNA	double stranded DNA
E	early
EBV	Epstein-Barr virus
E-L	early-late
ER	endoplasmic reticulum
FKBP	FK506 (Tacrolimus) binding protein
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GFP	green fluorescent protein
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
h.pi	hours post infection
HSV	herpes simplex virus
IB	immunoblot
IE	immediate early
IRE1	Inositol requiring enzyme 1
IRF3	Interferon regulatory factor 3
IP	immunoprecipitation
K	kaposin
kb	kilo base pairs (1000 bp)
kDa	kilo Dalton
KS	Kaposi’s sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L	late
LANA	Latency-associated nuclear antigen
MCD	Multicentric Castleman disease
MHV68	Murine herpesvirus 68

MOI	multiplicity of infection
NLS	nuclear localization signal
*ori*Lyt	origin of replication (lytic)
ORF	Open reading frame
PCR	polymerase chain reaction
PEL	primary effusion lymphoma
PERK	Protein kinase RNA- like endoplasmic reticulum kinase
PKRDC	DNA-dependent protein kinase
PML	promyelocytic leukemia (associated)
P_{PGK}	promoter of phosphoglycerate kinase 1
Rev	revertant
RTA	replication and transcription activator
RRV	Rhesus rhadino virus
SOX	shutoff and exonuclease
$TCID_{50}$	tissue culture infectious dose 50 %
Tg	Thapsigargin
TIME	telomerase-immortalized microvascular endothelial cells
TR	terminal repeat
Tun	Tunicamycin
UPR	Unfolded protein response
vCyclin	Viral cyclin
vIFN	Viral interferon
vFLIP	viral FLICE inhibitory protein
WB	western blot
wt	wild type
XBP1	X-box-binding protein 1
xCT	component of the xc-amino-acid transporter

Abstract

Kaposi's Sarcoma-Associated Herpesvirus (KSHV) is a member of the large *Herpesviridae* family. As with the other family members, two stages to its life cycle have been detected, namely latency and lytic replication. The main state of KSHV is latent infection with only a small population undergoing lytic replication. Most of the viral proteins are expressed during lytic infection which results in the production of progeny virions. Like other viruses, KSHV modulates several cellular signaling pathways for its own benefit. It is known that endoplasmic reticulum (ER) stress and consequently activation of the unfolded protein response (UPR) triggers KSHV lytic infection, however little is published about how KSHV modulates the UPR. Upon accumulation of unfolded and misfolded proteins in the ER (as it can occur during viral infection), cells trigger a signaling pathway in order to restore ER homeostasis, designated as UPR. The UPR consists of three ER-to-nucleus signaling pathways that regulate synthesis, folding, and degradation of proteins in the ER. One of the three UPR sensors, IRE1, activates the transcription factor XBP1, which induces the expression of chaperones and ER-associated degradation factors, thereby alleviating ER stress. Although ER stress can trigger lytic KSHV replication by reactivating the KSHV replication transcription activator factor (RTA) promoter via XBP1s, the effect of KSHV on the UPR is not clearly understood. The aim of this study was to investigate the influence of a lytically replicating KSHV ($KSVH_{LYT}$) on the UPR via the IRE1 signaling pathway. Here I show that IRE1 protein levels remain largely unchanged at early times but are substantially reduced at late times post infection. Consequently, XBP1s is also decreased during infection. Two cytomegalovirus proteins, M50 and UL50, respectively, have been shown to interact with IRE1 and induce its degradation. However, my experiments show that IRE1 downregulation is not mediated by the ORF67 protein, the homolog of M50/UL50 in KSHV. Instead, IRE1 expression is reduced on the transcriptional level. As KSHV is known to degrade host mRNAs using the viral host shutoff exonuclease, SOX, I constructed a KSHV mutant expressing catalytically inactive SOX. The results indicate that IRE1 is also downregulated in cells infected with the SOX mutant, excluding an effect of the host shutoff in the regulation of IRE1. In contrast, IRE1 levels were not downregulated in cells infected with KSHV mutants containing a deletion of either the latent nuclear antigen (ΔLANA) or the viral cyclin (ΔvCyclin). Moreover, IRE1 levels were reduced in cells transiently transfected with LANA expression plasmids. Based on these results I hypothesize that LANA downregulates IRE1 to curb RTA expression and promote latency.

Zusammenfassung

Das Kaposi-Sarkom-assoziierte Herpesvirus (KSHV oder Humanes Herpesvirus Typ 8, HHV-8) ist ein großes und komplexes DNA-Virus aus der Familie der *Herpesviridae*. Wie andere Vertreter der Herpesviren besitzt auch KSHV zwei Phasen der Replikation: die latente und die lytische Phase. Nach Erstinfektion etabliert dieses Virus sehr schnell eine latente Infektion, das vorherrschende Infektionsstadium bei KSHV. Nur ein geringer Prozentsatz der Viren durchläuft eine lytische Replikation, in der fast alle viralen Proteine exprimiert werden. Dies resultiert in der Bildung von Nachkommenviren. KSHV moduliert während einer lytischen Replikation verschiedene zelluläre Signalwege, um seine eigene Replikation zu begünstigen, darunter auch die Signalwege der *Unfolded Protein Response* (UPR). Die molekularen Mechanismen sind weitgehend unbekannt. Die UPR wird als Gegenmaßnahme der Wirtszelle aktiviert, wenn die ER-Homöostase durch die Akkumulation von ungefalteten und fehlgefalteten Proteinen im Endoplasmatischen Retikulum (ER) gestört ist, wie es bei einer Virusinfektion geschehen kann. Die UPR umfasst drei Signalwege, die von den ER-Stress-Sensoren ATF6, PERK und IRE1 ausgehen. IRE1 ist der evolutionär konservierteste der drei Signalwege. IRE1 aktiviert den Transkriptionsfaktor XBP1s, der die Expression von Chaperonen und ER-assoziierten Proteindegradierungsfaktoren induziert, wodurch ER-Stress reduziert werden kann. Es ist bereits bekannt, das ER-Stress und die Expression von XBP1s zu einer Reaktivierung durch eine erhöhte Expression des Replikations- und Transkriptionsaktivators (RTA) von KSHV führt. Jedoch ist die Modulation der UPR während der lytischen KSHV-Replikation weitgehend unbekannt. Der Einfluss auf den IRE1-XBP1-Signalweg eines lytisch-replizierenden Virus ($KSVH_{LYT}$) sollte während meiner Arbeit im Detail untersucht werden. Ich konnte zeigen, dass die IRE1-Proteinlevels zu frühen Zeiten einer KSHV-Infektion unverändert blieben. Zu späten Zeitenpunkten der Infektion, d.h. nach 48-72 Stunden, war IRE1 jedoch stark reduziert, was auch zu einer Reduktion des XBP1s-Proteins führte. Aus früheren Arbeiten war bekannt, dass Proteine des Cytomegalovirus (M50 bzw. UL50) mit IRE1 interagieren und dessen Abbau induzieren können. Das Homolog von M50/UL50 in KSHV ist das ORF67-Protein. Eine Reduktion von IRE1 durch ORF67 konnte jedoch nicht nachgewiesen werden. Meine Experimente zeigten jedoch, dass KSHV die Expression von IRE1 auf Transkriptionsebene reduziert. Da bekannt war, dass das KSHV mRNAs der Wirtszelle durch das virale Protein SOX (*Virus Host-Shutoff Exonuclease*) abbaut, wurde eine KSHV-Mutante konstruiert, die ein katalytisch-inaktives SOX-Protein exprimiert. Jedoch konnte kein Effekt der KSHV-SOX-Mutante auf IRE1 identifiziert

werden, weswegen ein Effekt des *Host-Shutoff* bei der Regulation von IRE1 ausgeschlossen wurde. Im Gegensatz dazu konnten die IRE1-Proteinlevels wiederhergestellt werden, wenn Zellen mit zwei KSHV-Mutanten infiziert wurden, denen das *latent nuclear antigen* (LANA) oder das virale Cyclin fehlt. Außerdem waren die IRE1-Proteinlevels in Zellen reduziert, die transient mit einem LANA-Expressionsplasmid transfiziert wurden. Aus diesen Befunden schließe ich, dass LANA maßgeblich an der Regulation von IRE1 beteiligt ist. Dieser Effekt könnte dazu dienen, die RTA-Expression einzudämmen und somit die Latenz zu fördern.

2 Introduction

2.1 *Herpesviridae*

The *Herpesviridae* form a large family of DNA viruses which causes disease in humans and animals. There are nine distinct herpesvirus types within this family which in turn belong to three different subfamilies, the *Alpha*, *Beta* and *Gamma-herpesvirinae*. In total there are more than 130 known herpesviruses [1-3].

The subfamily of *Gammaherpesvirinae* includes two genera of γ1 (lymphocryptoviruses) and γ2 (rhadinoviruses) herpesviruses. The genus *lymphocrypticalvirus* includes Epstein Barr virus (EBV) in human, Rhesus lymphocryptovirus in (Rhesus monkeys) and herpesvirus papio in baboons. In the genus *rhadinovirus* there are five members including Herpesvirus saimiri (HVS), Kaposi's sarcoma-associated herpesvirus (HHV8/KSHV), rhesus rhadinovirus, equine herpesvirus 2 and murine herpesvirus 68 (MHV68) [4, 5].

According to phylogenetic analysis of the primate virus genomes, there exists three separate *Rhadinovirus* lineages. Members of the first lineage are T-lymphotropic viruses infecting New World monkeys, including the archetypal rhadinovirus, herpesvirus saimiri (HVS) in squirrel monkeys (Saimirisciureus) and the closely related herpesvirus ateles (HVA) in spider monkeys(Atelesgeoffryi) [6]. Rhadinoviruses have also been identified in Old World primates such as chimpanzees [7, 8], gorillas [9], and gibbons with two lineages [10]. KSHV is classified into the first genogroup, RV1, as an agent in Kaposi's sarcoma (KS). The second group, RV2, contains the rhesus rhadinovirus (RRV). There are currently nine species of *Rhadinovirus* genus associated with severe malignancies in immunocompromised patients [1, 11].

2.1.1 Kaposi's Sarcoma-associated Herpesvirus and diseases

Four diseases are found to be related to KSHV; Kaposi's sarcoma (KS), Primary Effusion Lymphoma (PEL), Multicentric Castleman Disease (MCD), and KSHV inflammatory cytokine syndrome, a syndrome with MCD-like symptoms but without the associated pathology [1, 12-15]. Other KSHV like other gammaherpesviruses are characterized by three important properties: a limited host range; lymphocyte tropism and sequence homology to herpesvirus Saimiri (HVS). [16].

KSHV is closely related to RRV and to MHV68, although the most closely-related human virus to KSHV is Epstein-Barr virus (EBV) from the lymphocryptovirus genus. In general,

rhadinoviruses infect lymphocytes and adherent cell such as fibroblast, epithelial cells, and endothelial cells [17, 18].

2.1.2 KSHV virion structure

Like with other herpesvirus members, the KSHV linear non-segmented double-stranded DNA genome is enclosed by a capsid made up of an icosahedral symmetric genome containing 162 capsomeres and 4 structural proteins. The capsid is enveloped by a phospholipid layer containing glycoproteins, which help the virion attach to and enter the host cell. Between the capsid and the envelope is the tegument, a protein-rich region with the total diameter of the KSHV virion being 150-200 nm [1, 2] (Figure1).

Common genes which have homology to herpesvirus Saimiri (HVS) genes are known as ORF (open reading frame) while the unique specific KSHV genes with no homology to HVS genes are annotated as K and, contain identified codes relating to its location (e.g. K1-K10.1). The KSHV genome encodes up to 100 genes in a single long chromosome, flanked by repetitive non-coding GC-rich terminal repeats (TR) containing 801 base pairs (bp) [1, 19]. KSHV replicates in the nucleus.

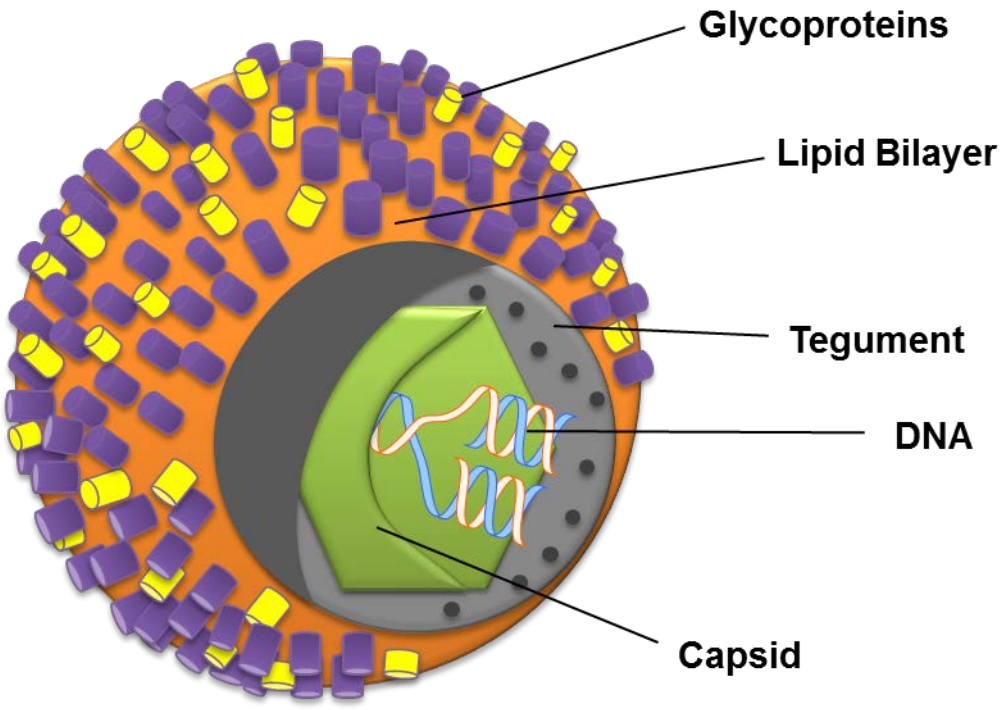


Figure 1: Schematic cross-section through the outer coat membrane and the capsid of Kaposi's Sarcoma-associated herpes virus particles. The linear double-stranded DNA is surrounded by capsid and tegument proteins. The nucleocapsid is covered by a lipid bilayer with glycoproteins known as the envelope.

2.1.3 KSHV attachment and entry

KSHV engages with a number of host cell plasma membrane molecules in order to penetrate the target cells. Some of these molecules are involved in virus entry as binding receptors, which enable the virus to attach to the cell surface [20]. The initial virus–cell interactions of many herpesviruses (e.g. herpes simplex types 1 and 2, human cytomegalovirus, human herpesvirus 7, bovine herpesvirus 4, and KSHV) involve binding to heparan sulfate (HS) as an initial binding receptor [21-23]. KSHV gB is as a key envelope glycoprotein involved together with gpK8.1A in the initiation of entry and binding to the cell surface HS molecules. Other envelope glycoproteins are utilized to either facilitate fusion of the viral envelope with the plasma membrane at normal pH, or entry of the whole virus particle via endocytosis and fusion of the viral envelope with the endosomal membrane at acidic or normal pH [20]. This fusion with the endosomal membrane is mediated by viral glycoproteins which in turn act to to deliver the capsid into the cytoplasm. For example, KSHV glycoproteins gH and gL interact with integrins (α3β1, αVβ3, and αVβ5), xCT (12-transmembrane glutamate/cystine exchange transporter) molecules and ephrin A2 (EphA2 belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family) receptor tyrosine kinase (EphA2R), all of which facilitate entry of the virus in endothelial and fibroblast cells. The KSHV gM and gN glycoproteins as a heterodimeric complex are also important in virus penetration and egress. Depending on the cell type, KSHV utilizes two major endocytosis pathways [24]. KSHV enters the endothelial cells by micropinocytosis whereas in human Burkitt lymphoma B cell line (BJAB) and HEK293 cells it is mediated through the clathrin endocytic pathway [21, 25-28].

The interaction of KSHV glycoproteins with cellular receptors induces several signaling pathways, such as intracellular tyrosine kinases, organization of the actin cytoskeleton, phosphatidyl inositide 3-kinases (PI3-K), and the Ras superfamily (RhoA-GTPase). These activations facilitate intracellular capsid movement, modulate trafficking and deliver the viral genome to the nucleus [29]. KSHV needs to organize the induction of cytoplasmic transcription factors to initiate viral gene expression [24]. Proteins expressed from the viral genome are involved in cell cycle regulation (e.g. vCyclin and LANA), signal transduction (e.g. K1 and K15), inhibition of apoptosis (e.g. K1, vFLIP and vBCL-2), and immune modulation (e.g. viral chemokine receptors, vIRFs, K3 and K5) [1, 30].

2.1.4 Viral life cycle

Replication of the KSHV occurs upon entry and release of the viral DNA into the nucleus [27]. As with other herpesviruses, there are two phases of viral life cycle in KSHV the latent (the most prominent) and the lytic phases [1-3, 11, 31]. The latent KSHV DNA persists not only in vascular endothelial and spindle cells of KS lesions, but also in B cells, and indeed monocytes serve as a major reservoir in invivo infection [27]. In the latent phase, the genome is circularized and tethered to cellular chromosome as an episome. During latency, no functional or infectious particles are produced. Lytic infection is detected in monocytic cells in KS lesions [27]. Although lytic replication can be induced by treatment with phorbolesters or sodium butyrate, the efficiency is very low [31]. It has been demonstrated that a variety of cells are targeted by KSHV in vitro, which does not result in sustained latent infection and immortalization, including HEK293A (epithelial from human kidney embryo), HUVEC (human umbilical vein endothelial cells), TIME (telomerase-immortalized microvascular endothelial cells), BCBL-1 (body-cavity-based lymphoma cell line), human Burkitt lymphoma B cell line (BJAB) and RPE1 (retinal pigment epithelial cells immortalized with human telomerase reverse transcriptase, hTERT) [27, 32]. The most characterized property of the lytic cycle is the replication of the linear viral genome and expression of more than 80 transcripts during the lytic phase [1, 27]. Viral life cycles are important for maintenance of the virus and for pathogenesis of KSHV-associated diseases [11, 27] (Figure2).

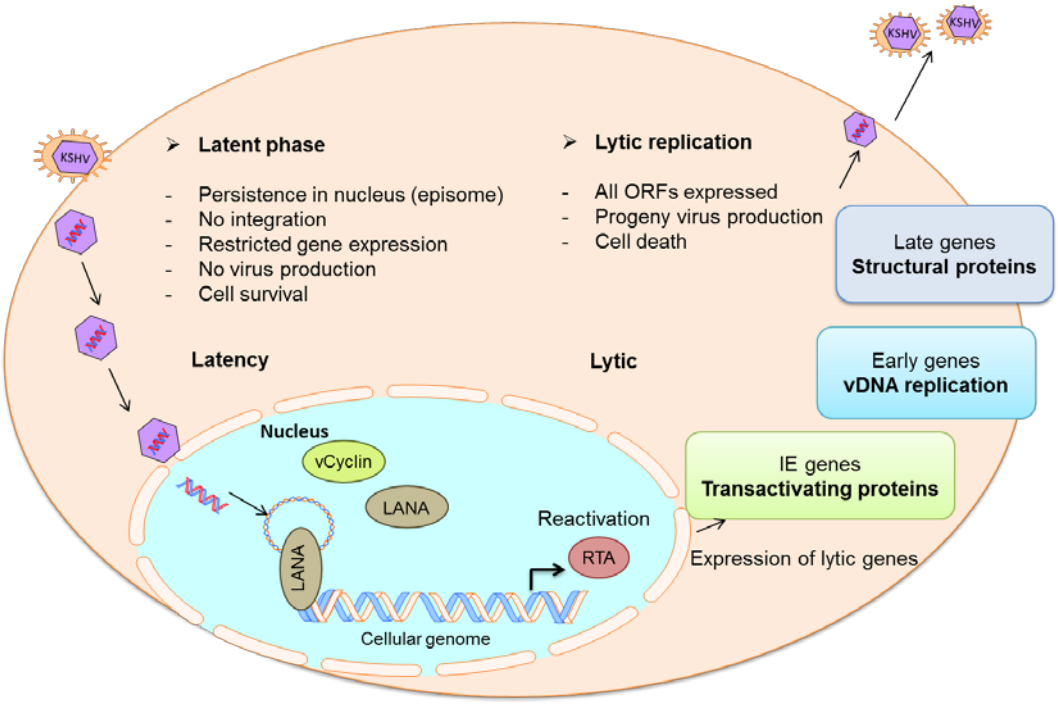


Figure 2: KSHV life cycle. Kaposi's Sarcoma-Associated Herpesvirus (KSHV) has two stages in its life cycle, latency and lytic replication. During latency the viral episome is maintained, few viral genes are expressed and no infectious virus is produced. The switch between phases is controlled by the viral transcription factor, RTA.

The lytic replication results in the production of most proteins such as immediate early, early and late proteins and finally progeny virion production.

2.1.5 KSHV latent proteins

During latency in tumor cells, the viral episome is replicated once per cell cycle. Most KSHV latent genes are expressed in latency, including latency-associated LANA-1 (ORF73), vCyclin D (ORF72), vFLIP (K13), and kaposin (K12) [33]. 12 microRNAs have been discovered in the KSHV genomes which are also expressed during latency. They are upregulated during the lytic cycle [1, 11]. I describe below some latent KSHV genes that have been investigated during this work as genes of interest or for control purposes.

2.1.5.1 ORF73 (LANA)

ORF71, ORF72 and ORF73 are encoded as a tri-cistronic mRNA [33]. The latency-associated nuclear antigen (LANA) is encoded by KSHV- ORF73 and is expressed in all KSHV infected cells. LANA is particularly important in establishing KSHV latency. LANA plays a role in the replication and viral episome maintenance during latent infection. The deletion of LANA from the KSHV genome causes the loss of viral episome and the failure to establish latent infection [34]. In spite of the fact that these genes are latent transcripts, an increased expression of these mRNAs early (ORF71/ORF72) or late (ORF73) was observed after triggering the lytic cycle by RTA expression which has not been seen when induced by Na-butyrate or phorbol esters [35]. The main function of LANA at the early stage of KSHV primary infection is to repress the RTA promoter which results in the inhibition of RTA expression [36].Published data has provided evidence for the presence of an isoform of LANA that may perform alternative functions in KSHV-infected cells. A 3.2kb transcript has been identified in KSHV-positive primary effusion lymphoma (PEL) cells (BCP-1andBC-3) that encodes for a C-terminally truncated form of LANA (LANA-D76). Although this isoform does not bind to the KSHV TR or associate with the full-length LANA, LANA-D76 does contain the domains responsible for interacting with the tumor suppressor protein -p53-, the retinoblastoma protein (Rb) and the nuclear-localized kinase (RING3) [37].While LANA full-length is located in the nucleus, it has also been shown that other cytoplasmic isoforms localize to perinuclear and cytoplasmic sites which themselves may also have cytoplasmic activities [38]. Although a novel function of the cytoplasmic isoforms of LANA during lytic replication has been demonstrated, the function of LANA from its role has been extended during latency to the lytic replication cycle. For instance, to inhibit the innate immune response, the cytoplasmic KSHV LANA isoforms recruit and antagonize cGAS (cellular

DNA sensor proteins) as well as the Rad50-Mre11-CARD9 complex (IFN-βand NF-κB) and promote KSHV lytic reactivation from latency [39].

2.1.5.2 ORF72 (vCyclin)

KSHV encodes for another latent gene designated as vCyclin which is a homolog of the cellular cyclin D. The vCyclin functions as a protein promoting passage through G1 to S phase of the cell cycle. The cellular cyclin D binds and directs cyclin-dependent kinase component (CDKs), which in turn mediates the phosphorylation of tumor suppressor pRb. This phosphorylation leads to the release of the repression of E2F activity which transactivates promoters of genes that are required for DNA replication. The expression of the vCyclin protein however aids in the pathogenesis of Kaposi's sarcoma by promoting cell proliferation [40]. In contrast to the cellular cyclin, the vCyclin is resistant to inhibition by cyclin-dependent kinase inhibitor (CDKIs) [33]. This resistance to the negative regulation of the CDKIs initiate nuclear DNA replication, induces entry into S phase, and finally promotes cell proliferation [33].

2.1.6 KSHV lytic proteins

Upon KSHV lytic reactivation, the first genes to be expressed are the immediate early genes (IE), (e.g. K4.2, K8, ORF45, 48, 50). IE gene expression is silenced during latency [11, 19]. Early genes (E) are activated by IE proteins, and encode a large variety of proteins that have a role in the modulation of the immune response (K1/3/5/15, ORFs 10, 11) and/or in KSHV pathogenesis (K2/4.1/6/9, ORF74), in apoptosis and in DNA synthesis [30]. All proteins necessary for the assembly and egress of the viral particle are defined as late genes [19]. While the innate immune system is capable of decreasing viral replication and inducing the production of cytokines, the adaptive immune response neutralizes virus particles and destroys infected cells. Therefore, KSHV has to modulate this system by hijacking cellular machinery [15, 30]. The production of a wide range of immune modulators during latent and lytic infection is an effective mechanism that is used by KSHV. The numbers of the KSHV lytic proteins investigated in this thesis are mentioned.

2.1.6.1 ORF50 (RTA)

RTA (replication and transcription activator)/ORF50 is a KSHV immediate-early protein and activator for initiating the lytic gene expression cascade [41]. During latency, the RTA promoter associates with histone deacetylases (HDACs), leading to the hypoacetylation of histones. After chemical reactivation of the RTA (such as by butyrate treatment), histones are hyperacetylated and RTA recruits histone acetyltransferases, resulting in RTA expression and

completion of the viral gene expression [19]. It has been demonstrated that, while XBP1 is a necessary transcription factor for terminal differentiation of B cells to plasma cells it can also promote reactivation of the RTA in PEL cells. This means that latency is maintained in KSHV infected B cells until plasma cell differentiation, with XBP1s then inducing the KSHV lytic cycle [42, 43]. Previous studies have shown that RTA also has other functions. For instance, it has an E3 ubiquitin ligase activity, which can mediate proteasomal degradation of cellular proteins such as interferon regulatory factor (IRF7), in order to inhibit interferon responses [44]. Moreover, RTA degrades some RTA repressors, such as hairy/enhancer-of-split related with YRPW motif protein 1(Hey1), LANA and nuclear factor 'kappa-light-chain-enhancer of activated B-cells (NFκB/p65) [44]. It has been also reported that the expression of RTA can be inhibited by LANA by repressing its promoter [36].

2.1.6.2 ORF47/45

The glycoprotein L (gL), uracil DNA glycosylase, and a viral tegument protein are encoded by the ORF47-ORF46-ORF45 gene of KSHV. The tricistronic ORF47-ORF46-ORF45 mRNA are expressed during early stages of viral reactivation. The ORF47/45-A and ORF47/45-B, are spliced gene products consisting a partial region of gL (ORF47), a unique 7-amino-acid motif, and the complete tegument protein ORF45 [45]. Unlike ORF45, both ORF47/45-A and ORF47/45-B contain a signal peptide sequence and are localized at the endoplasmic reticulum (ER). It has been demonstrated that ORF47/45-A and ORF47/45-B have an additional function that mediates the upregulation of GRP78, a master regulator of ER homeostasis [46]. It has been revealed that upregulation of GRP78 is essential for the progression of the KSHV late lytic cycle. It has also been proposed that expression of GRP78 induced by viral proteins at the early lytic stage may protect host cells from severe ER stress and may directly involve the assembly or release of virions [46].

2.1.6.3 ORF67 (p29)

All herpesviruses encode two proteins known as the Nuclear Egress Complex (NEC), which has a key role in facilitating the egress of capsids from the nucleus [47]. For example, in HSV1, the NEC contains two proteins, UL31 and UL34, and in HCMV these components areUL50 and UL53 [48, 49]. The homologs of these proteins in KSHV are p29 encoded by ORF67 and p33 encoded by ORF69. The interaction between p29 and p33 is important for primary egress from the nucleus through the nuclear membrane [47, 48].

2.1.6.4 ORF40/41, PAF, primase-helicase factor

Six KSHV proteins have homology with other herpesvirus core DNA replication proteins and are essential for viral DNA synthesis [50]. ORF6/SSB (single-stranded DNA binding protein), and ORF59/ PPF (polymerase processivity factor) are intrinsic nuclear proteins, whereas ORF9/POL (polymerase) is localized in the cytoplasm. ORF40/41/PAF (primase-associated factor) is a component of the primase-helicase tripartite complex, forming a complex together with ORF56/PRI (primase) and ORF44/HEL (helicase). Efficient nuclear translocation of ORF40/41 requires the presence of all five other replication proteins [50]. ORF40/41 contains a nuclear localization signal and a binding domain for transporting the viral DNA polymerase, ORF9, into the nucleus. Unspliced PAF transcripts are encoded by the PAF gene, which is UL102 in HCMV and UL8 in HSV-1. However, the corresponding loci in Epstein–Barr virus (EBV) and KSHV contain two short ORFs, which in KSHV are spliced to form a continuous ORF. A spliced transcripthas been identified for ORFs 40/41 [51], however, its homologue in MHV68 ORF40, is translated from a unique transcript [52].

2.1.6.5 ORF37 (Sox)

A common strategy of some viruses to decrease and control host gene expression is RNA degradation, which is also called host-shutoff [53, 54]. KSHV ORF37 encodes a shutoff exonuclease (SOX) factor. Degradation of host RNA has also been observed in homologs of SOX in Epstein Barr virus (EBV BGLF5), as well as in MHV68 mSOX [54, 55]. KSHV SOX has two separate important functions namely, DNase and a RNase function [55, 56]. The DNA activity is involved in processing and packaging the viral genome and is conserved across all herpesviruses; however, the RNase host-shutoff activity of SOX, which targets mRNA at the early stage of translation by endonucleotic cleavage, is not conserved. This activity allows the virus to selectively eliminate competing host mRNAs by cosedimenting and cleaving translated mRNAs [54]. A cellular endonuclease, Xrn1, then completes degradation of mRNA cleaved by SOX [56]. Most cellular genes such as Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) and DNA-dependent protein kinase (PKRDC), are directly degraded by SOX. However, some cellular RNAs such as cytokine interleukin 6 (IL-6), apoptosis enhancing nuclease (AEN), and 18s rRNA, are resistant to the host-shutoff induced by SOX [57]. For example, IL-6 protection is conferred by the presence of a protective sequence in the 3′ untranslated region (UTR) [54]. A described SOX mutant (P176S) is defective for the RNA activity, while the DNA function stays intact [56, 58].

2.1.6.6 K8.1-A/B

KSHV viral glycoproteins play important roles in the infectious life cycle as well as promote endothelial cell transformation, angiogenesis, and KS-induced malignancies[59]. K8.1A, K8.1B, K1, K14, and K15 are KSHV genes that do not have homolog in other herpesviruses and are expressed during lytic replication. The glycoprotein K8.1has 228 amino acids and contains a signal sequence, a transmembrane domain, and four glycosylation sites which mediate virus attachment to the cellular receptor, heparan sulfate [60]. While K8.1A is found in the virion envelope and facilitates virion binding to cells, the smaller glycoprotein (K8.1B) activates the alpha/beta interferon (IFN-α/β) signaling pathway [59, 61].

2.2 Cellular stress

The function of the endoplasmic reticulum (ER) is to maintain the correct folding of secretory and transmembrane proteins [62]. Many conditions such as unfolded or misfolded proteins in the lumen of the endoplasmic reticulum induce ER stress [63].To maintain proper ER function and homeostasis, a signaling network known as unfolded protein response (UPR) is used [64]. The UPR involves transcriptional programs inducing expression of genes which enhances the protein folding capacity of the ER and promotes ER-associated protein degradation, leading to a decrease in misfolded/unfolded proteins [64].

PERK, IRE1 and ATF6 are three main branches involved in inducing the UPR. Normally, the N-termini of these transmembrane ER proteins bind to the ER chaperone, binding immunoglobulin protein, BiP (GRP78), which inhibits their aggregation. Upon ER stress, GRP78 dissociates, triggering activation of these signaling proteins [63] (Figure 3).

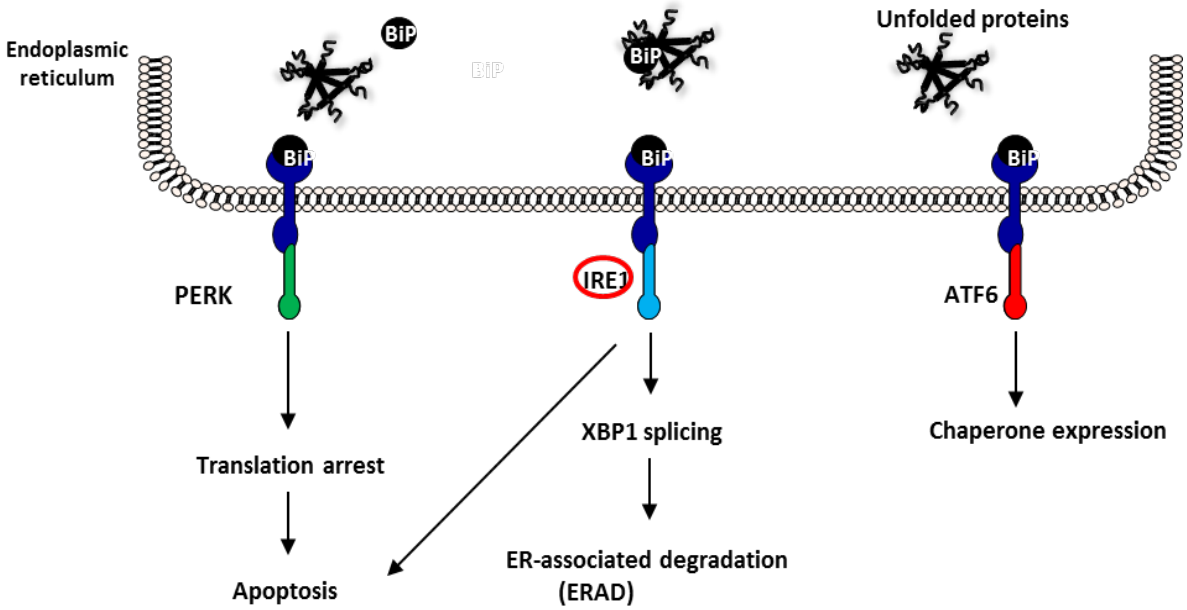


Figure 3: Unfolded Protein Response (UPR). Detection of the accumulation of unfolded proteins is performed by the three ER-stress sensors PERK, IRE1 and ATF6.The activation of PERK results in a translational arrest, which can lead to the induction of apoptosis. IRE1 activation leads to XBP1 splicing and expression of degradation factors that allow for degradation of unfolded proteins, ATF6 activation then induces expression of chaperone genes. This picture is modified from the original by F.Hinte (unpublished data).

2.2.1 PERK

PKR-like ER kinase (PERK) is a protein kinase, which is oligomerized upon activation. Phosphorylation and activation of the kinase domain takes place in the ER membrane, and lead to phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α), resulting in translational attenuation [64]. If ER stress persists, phosphorylation of eIF2α selectively promotes the translation of the activating transcription factor 4 (ATF4), a downstream target of PERK, in turn inducing the expression of the proapoptotic transcription factor C/EBP-homologous protein, CHOP, a protein involved in apoptosis [65].

2.2.2 IRE1

Inositol-requiring enzyme 1 (IRE1) is activated by oligomerization and autophosphorylation in the ER membrane after BiP dissociation. The active form of IRE1 removes 26 nucleotides of XBP1unspliced mRNA by an unconventional splicing which occurs in the cytoplasm [66]. XBP1s the protein product of the spliced XBP1 mRNA is translocated to the nucleus and modulates the transcription of targets such as ER chaperones and the ER-associated degradation (ERAD) components [63, 67-69]. However, when protein misfolding is persistent or prolonged, the IRE1 pathway also triggers cell death, especially apoptosis [64, 68]. This protects the organism from harmful substances produced by damaged cells [67] (Figure 4).PERK and ATF6 increase XBP1s mRNA expression by either increasing IRE1 expression in the PERK signaling pathway or by enhancing the transcription of XBP1 [70].

2.2.3 ATF6

Activating transcription factor 6 (ATF6) is another UPR pathway. The separation of BiP from the N-terminus of ATF6 triggers protein activation by a different mechanism than the PERK and IRE1 signaling pathway. Free ATF6 translocates to the Golgi apparatus, and is cleaved proteolytically by Site-1 protease (S1P) and Site-2 protease (S2P) [71]. The cleaved cytosolic part of ATF6 translocates to the nucleus and activates transcription of the ER chaperones GRP78, GRP94, and calnexin, which then restores the folding of proteins in the ER lumen in the nucleus. ATF6 also collaborates with the IRE1 pathway to increase XBP1 mRNA synthesis [64].

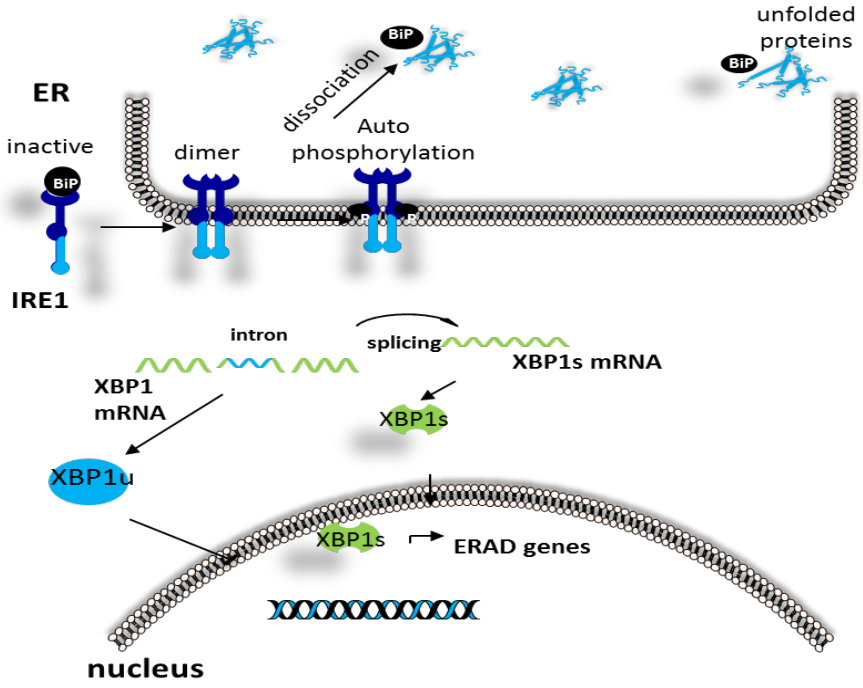


Figure 4: Inositol-requiring enzyme 1 pathway. Upon ER stress e.g. viral infection, the chaperone BiP binds to unfolded proteins, activating ER stress transducers, including Inositol-requiring enzyme 1 (IRE1). The dissociation of BiP from IRE1 allows for its dimerization and phosphorylation. Activated IRE1 catalyzes an unconventional splicing of the XBP1 mRNA in the cytosol, which then expresses a transcription factor regulating ER-associated degradation (ERAD) factors. This figure is taken with permission from F.Hinte (unpublished data).

2.3 Viral infection and UPR signaling

ER stress plays an important role in viral infection in mammalian cells. Activation of all pathways of the UPR causes translation arrest, degradation of misfolded/unfolded proteins, expression of ER molecular chaperones and ultimately decreases protein loads in order to restore proper protein-folding in the ER [67]. In virus-infected cells, the cellular translation machinery is hijacked in order to produce large amounts of viral proteins. This can disturb ER homeostasis and increases ER stress [67, 69]. Numerous viruses manipulate the UPR, as it has an essential role in the establishment of acute, chronic and latent infections. For example, Herpes Simplex Virus 1(HSV1), an alpha-herpesvirus, impacts the UPR during lytic replication. Only ATF6 activation was detected during early infection; however, no upregulation of target chaperone proteins was observed [72]. At the later stages of the virus replication the activity of the eIF2α/ATF4 signaling arm was increased. HSV-1 uses an immediate early protein, ICP0, as a sensor to modulate the cellular stress response. The ICP0 mimics cellular UPR genes and its promoter is responsive to ER stress [73] and activated the UPR enhancers during HSV1 replication. XBP1 has also been identified as a novel target of a

tegument protein, UL41, in HSV1. The mechanism employed for suppression of the IRE1/XBP1 pathway involves reducing the accumulation of XBP1mRNA [74].

In beta-herpesviruses several studies have discovered the ability of the human CMV to interfere with ER stress by manipulation of the UPR for its own benefit [72, 75, 76]. CMV infection causes an increase in BiP levels at the early phase of viral replication [72]. The N-terminal conserved region of M50 in murine cytomegalovirus (MCMV) has been shown to be required for IRE1 binding and degradation. it was also confirmed that UL50 as a M50 homolog in HCMV downregulates the IRE1, but the mechanism has not yet been fully established [76].

Epstein-Barr virus (EBV) encodes latent membrane protein 1(LMP1), which activates PERK to induce phosphorylation of eIF2α leading to the upregulation of the activating transcription factor 4 (ATF4)**.** In addition to PERK, LMP1 activates IRE1 and ATF6 [77].

In murine gammaherpesvirus 68 (MHV68), the M1 protein selectively induces the chaperone-producing pathways (IRE1, ATF6) at a late stage of replication [78]. Moreover, MHV68, inhibits host UPR at the early stage of infection via a helicase-primase ORF40 [52].

The roles played by and the regulation of the UPR during KSHV infection remain poorly understood. Several studies have shown that the UPR plays a role in KSHV infection. The splicing of XBP1mRNA is required for normal plasma cell differentiation and for KSHV reactivation from the latent phase to lytic replication [43, 79, 80]. The mechanism of XBP1s is strongly related to the activation of the RTA promoter [81]. Moreover, XBP1s contributes to the activation of vIL-6 in KSHV-MCD (multicentric Castleman disease) lymph node plasmablasts and in primary effusion lymphoma, PEL [82].

The KSHV tegument protein ORF47-ORF46-ORF45 contains two different mRNA variants, ORF47/45-A and ORF47/45-B, which are spliced from a transcript mRNA and expressed during early stages of viral replication. ORF47/45 plays a key role in controlling BiP expression and ER homeostasis in infected cells by means of upregulation. The upregulation of the GRP78/BiP is essential for the progression of the KSHV lytic cycle, especially at late stages, the activation of GRP78 expression by viral proteins at the early lytic stage leads to the protection of host cells from severe ER stress which can involve the assembly or release of the virions [46]. It has been demonstrated that the KSHV latent genes, LANA and vCyclin suppresses IRE1 transcripts in PEL cells [83]. However, the exact underlying mechanism is yet to be understood.

3 Aim of the work

The function of the endoplasmic reticulum (ER) is to fold and process proteins and translocate them to the Golgi apparatus [84]. Many environmental and genetic factors (as well as viral infections) cause the accumulation of misfolded and unfolded proteins in the ER lumen, leading to the disruption of the ER function, known as ER stress. ER stress leads to the activation of a signaling network, namely the unfolded protein response (UPR), to restore ER homeostasis [63, 84]. Viruses therefore have to interact with the UPR for their own benefit. The influence of the UPR on KSHV replication has already been investigated [67, 69, 74, 83]. The inositol-requiring enzyme 1 (IRE1) signaling pathway is the most conserved branch of the UPR; its activation results in the splicing of the transcription factor, XBP1s [64]. Several studies have shown that XBP1s is required for B cell differentiation into plasma cells, and B cells are the main reservoir cells for KSHV latency [85, 86]. KSHV recruits XBP1s to reactive RTA to switch from latency to the lytic replication cycle. Therefore, the aim of this thesis project was to analyze whether or not KSHV modulates the IRE1 branch of the UPR and to investigate the underlying mechanism(s). I hypothesized that KSHV most likely modulates this pathway during the lytic replication cycle. I therefore used constitutively lytic ($KSHV_{LYT}$) and inducible-lytic ($KSHV_{IND}$) KSHV mutants to determine the effect of the virus on the IRE1 branch of the UPR during latency and lytic replication.

4 Results

4.1 IRE1 signaling pathway is downregulated during KSHV infection

Since it has been shown that herpesviruses such as MCMV, HCMV, EBV and MHV68 manipulate UPR signaling pathways, the aim of my thesis was to investigate whether or not UPR signaling pathways are modulated during KSHV lytic infection. The most conserved branch of the UPR signaling pathways is the IRE1-dependent pathway. Once activated, IRE1 removes an intron from the XBP1 mRNA in the cytoplasm (XBP1s) resulting in a spliced (XBP1s) mRNA. The translated XBP1s protein translocates into the nucleus and regulates the promoter at the ER stress response element (ERSE). To test whether KSHV can modulate IRE1 activity, the splicing of XBP1s at the protein levels was measured as was IRE1 protein. To facilitate the detection of XBP1, mock-infected cells as well as cells infected with KSHV at a MOI of 0.5 TCID50/cell were treated with Thapsigargin (Tg), a UPR inducer, at a concentration of 2 µg for 4 hours before harvesting. The upregulation of XBP1s was observed after Tg-induction (Figure 5A). IRE1 protein level initially remained unchanged, however, at 48hpi, corresponding to late time point, IRE1 was downregulated. The inhibition of IRE1 phosphorylation (pIRE1) was observed upon infection and consequently leads to the subsequent suppression of XBP1 splicing. At early infection stages (from 5hpi) the reduction of XBP1s was observed in infected cells only. Indeed, this early downregulation was not observed in Tg-induced infected cells at all. As expected in response to virus-induced ER stress, the level of BiP also increased during infection, but not in infected cells treated with Thapsigargin (Figure 5B). One possible explanation is the fact that hyperactivation of the UPR leads to a decrease of BiP level in induced samples. K8.1A/B was used as an infection and Actin as a loading control. This initial data suggests the importance of downregulating the IRE1 pathway during KSHV lytic infection.

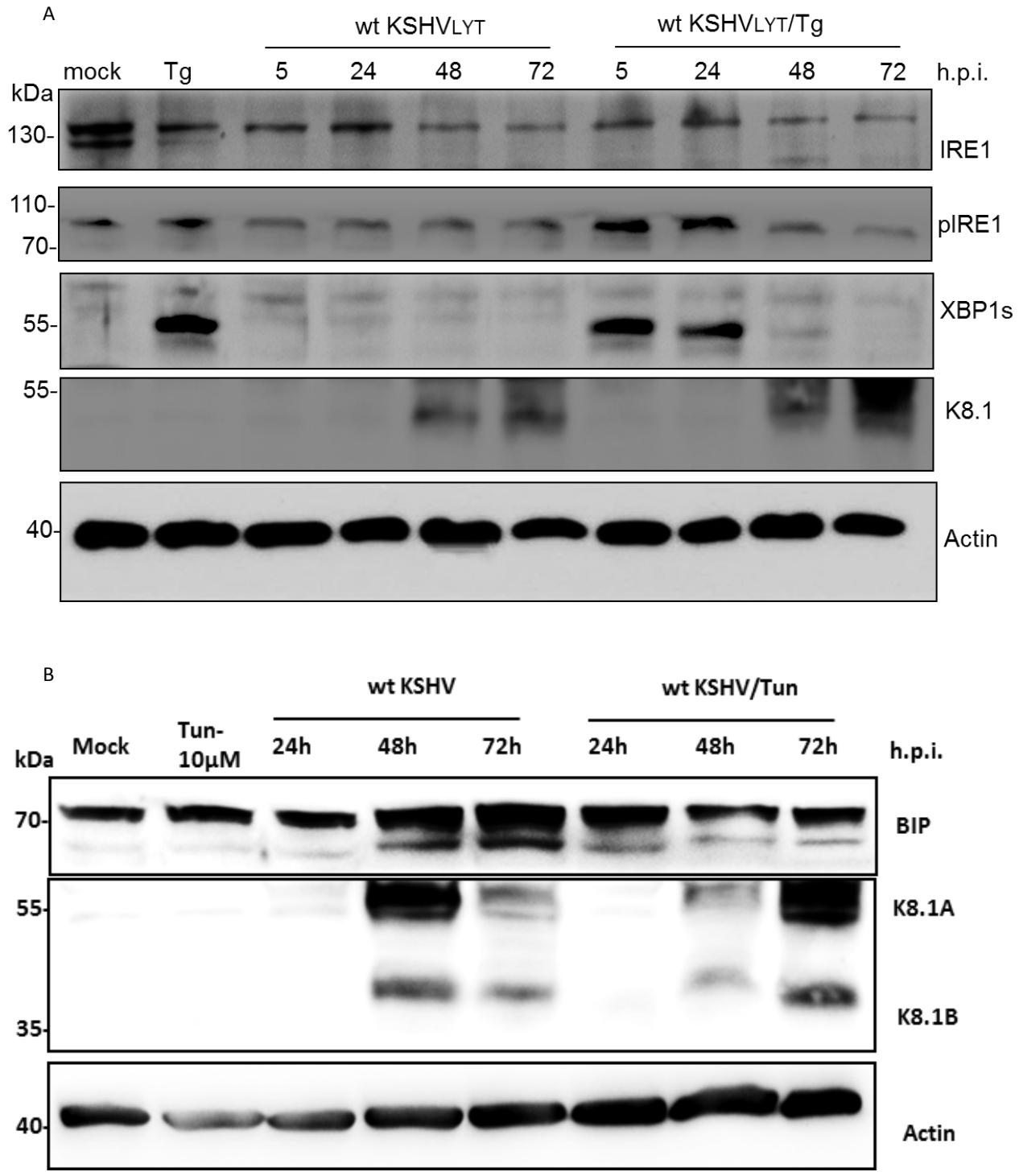


Figure 5: IRE1and XBP1s expression during infection with KSHV. (A) RPE1 epithelial cells were infected with wt $KSHV_{LYT}$ at MOI 0.5 TCID50/cell and treated with 2µM Thapsigargin for 4 hours. Samples were lysed at indicated times by RIPA buffer and were then subjected to western blot to detect endogenous IRE1, pIRE1 and XBP1s. (B) BiP protein level was also determined by western blot. K8.1-A/B was used as infection control and actin as a loading control.

4.2 Overexpressed IRE1 is downregulated during KSHV infection

Next, to test whether or not KSHV is capable of downregulating overexpressed IRE1, RPE1 epithelial cells were transduced with a retroviral vector, encoding HA-labeled IRE1. After selection, the cells were infected with KSHV (MOI 0.5 TCID50/cell). It was observed that even overexpressed IRE1 protein is downregulated in infected cells in comparison to uninfected cells (Figure 6). The overexpressed IRE1 is detected by the HA antibody, while the IRE1 antibody can detect both the endogenous and the overexpressed IRE1. Similar results to those in section 4.1 can be observed, i.e. the downregulation of both overexpressed and endogenous IRE1 in the later stages of KSHV infection (see Figure 5). When IRE1 is overexpressed, its phosphorylated form is also strongly induced, and only a slight inhibition can be observed at 48 and 72hpi. It can be explained by the fact that the overexpression of IRE1 leads to auto-activation of the IRE1 pathway which triggers its autophosphorylation. The level of XBP1s follows the same kinetic as IRE1, with a downregulation at a later time point.

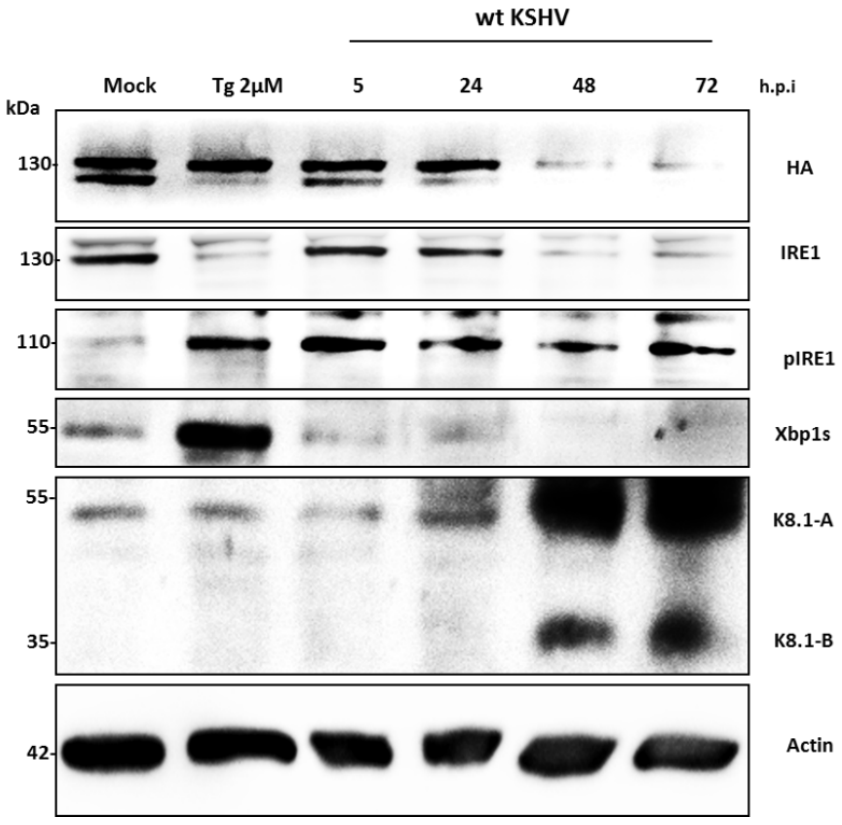
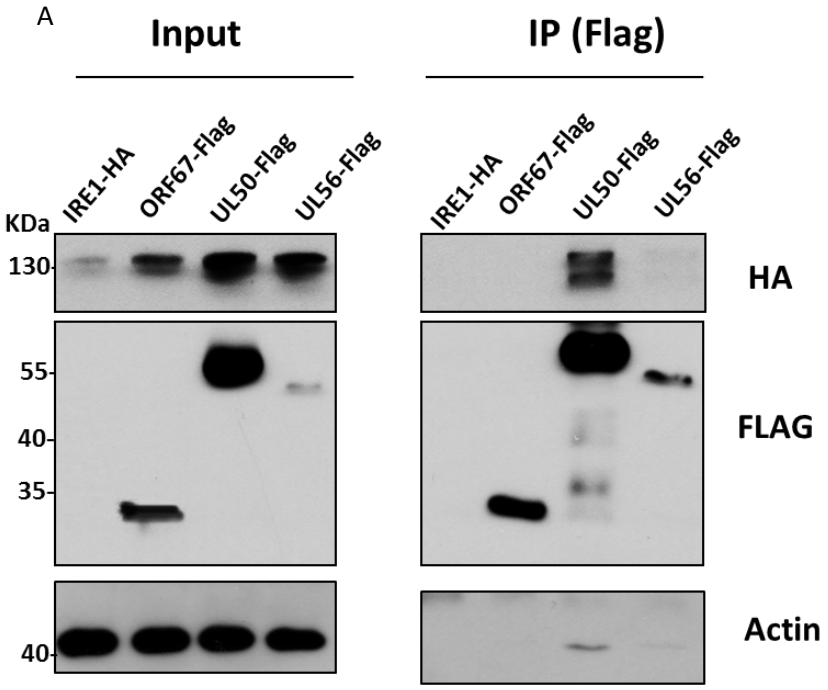
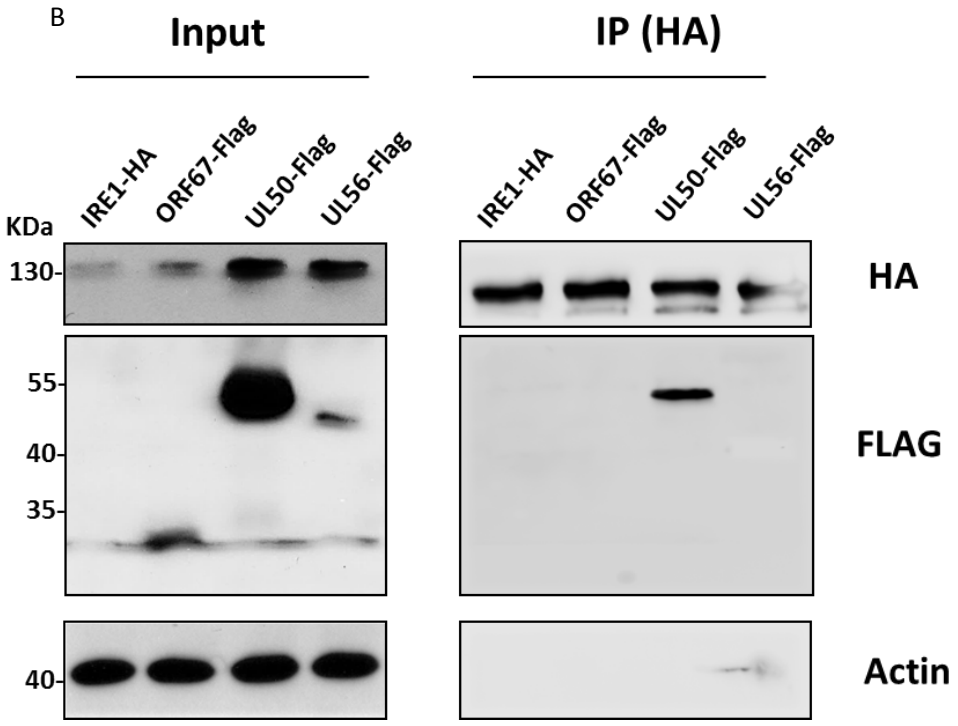


Figure 6: Effect of KSHV infection on overexpression of IRE1. RPE1 epithelial cells were transduced with pMSCV-IRE1-HA and after selection with puromycin were then infected with wt $KSHV_{LYT}$ at MOI 0.5 TCID50/cell. Samples were lysed at indicated times by RIPA buffer and were then subjected to western blot to detect endogenous IRE1, pIRE1 and XBP1s as well as exogenous IRE1 by HA antibody. K8.1-A/B was used as an infection control and actin as a loading control.

4.3 IRE1 is not downregulated by ORF67

It was already shown that HCMV and MCMV downregulate IRE1 through viral proteins, UL50 and M50, respectively, in transfection and infection experiments [69]. ORF67 is a homolog of UL50 and M50 and one of the two nuclear egress complex proteins in KSHV. To test whether ORF67 interacts with IRE1, 293A cells were co-transfected with plasmids (pCDNA3), expressing HA-tagged IRE1 and FLAG-tagged ORF67 in a co-immunoprecipitation experiment; these were compared with FLAG-tagged UL50 as a positive control and FLAG-tagged UL56 as a negative control for interaction with IRE1. Results showed that ORF67 does not interact with IRE1whereas UL50 does (Figure 7A and B). When ORF67 was transfected with increasing amount of protein, the level of IRE1 was stable; however, IRE1 was downregulated with increasing amount of UL50. ORF67 did not downregulate IRE1 (Figure 7C). As ORF67 neither interacted with IRE1 nor downregulated IRE1, it was not further investigated as a modulator of the IRE1 signaling pathway.





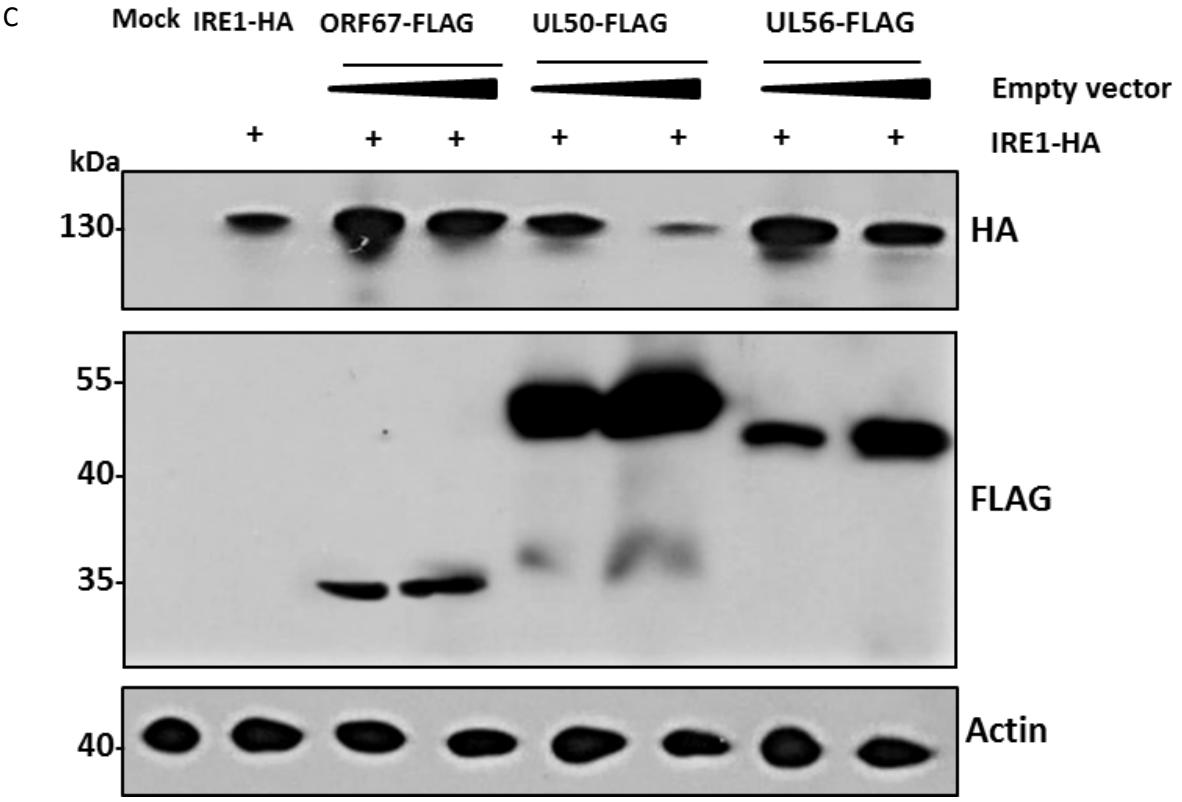
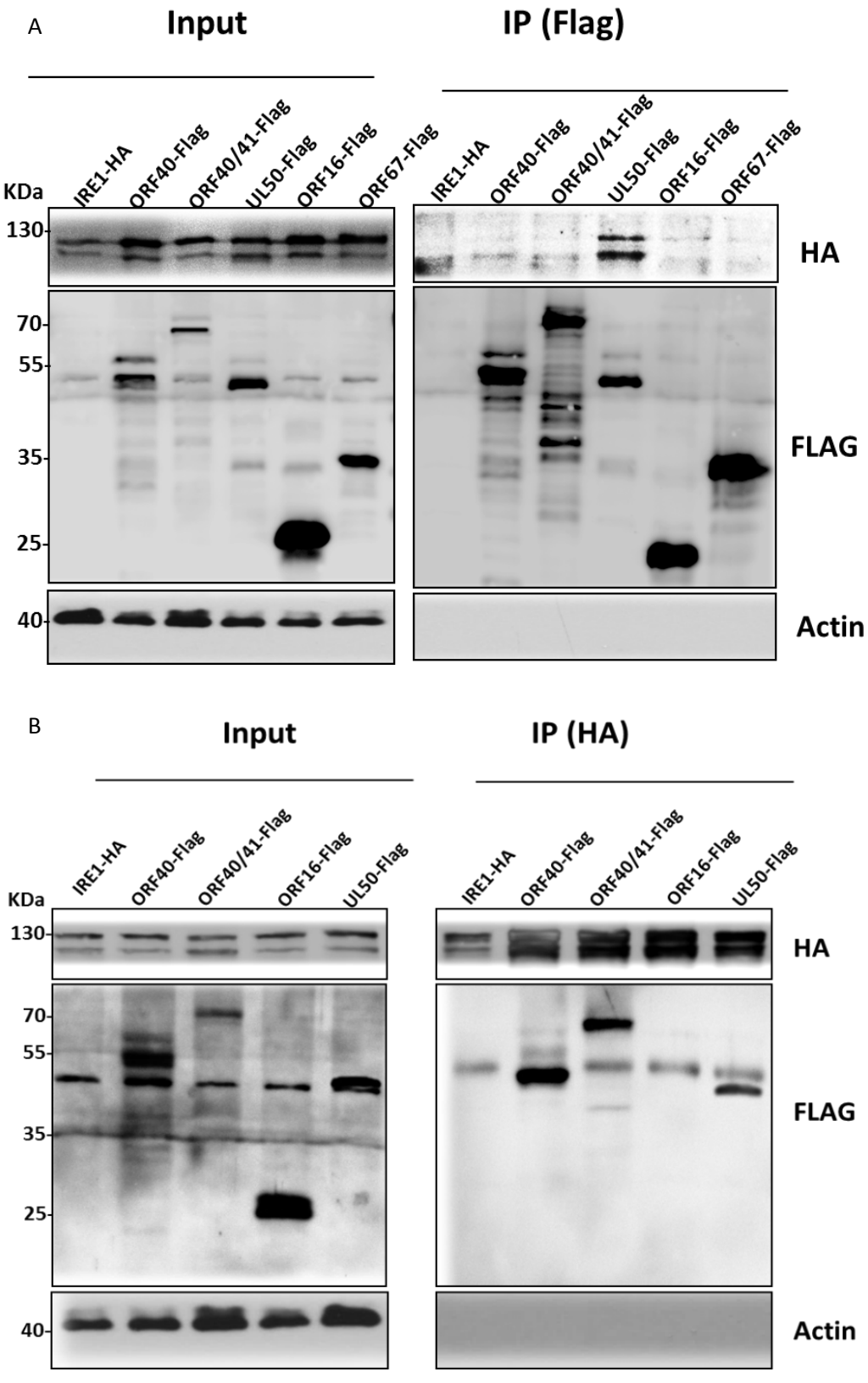


Figure 7: Interaction and effect of ORF67 with and on IRE1 level in co-immunoprecipitation and co-transfection experiment. (A, B) 293A cells were co-transfected with plasmids expressing either ORF67 or UL50 (positive control) or UL56 (negative control) (all flag-tagged) and IRE1-HA. Samples were harvested 48 hours after transfection using RIPA buffer. The lysates were coimmunoprecipitated. Samples were then

subjected to western blot. Proteins were detected with specific antibodies against HA or FLAG. (C) 293A cells were co-transfected with plasmid expressing IRE1-HA and increasing amount of FLAG-tagged ORF67or FLAG-tagged UL50 or FLAG-tagged UL56 as a positive and negative control. Actin was used as loading control**.**

4.4 IRE1 is not downregulated by ORF40/41

The mouse homologue of KSHV, Murine gammaherpesvirus 68 (MHV68), encodes the M1 protein which is involved in IRE1 induction at a later stage of infection [78]. Some data has shown that in early stage of MHV68 infection, a primase –helicase factor (ORF40) might be responsible for the downregulation of IRE1 [52]. Hence, the ORF40 homolog in KSHV, ORF40/41, was tested to see whether or not it has a similar function. A spliced transcript for ORFs 40/41 has been identified [51]. To check the interaction and function of ORF40/41with and on IRE1 modulation, plasmids (pCDNA3) expressing FLAG-tagged ORF40/41, FLAG-tagged ORF40, FLAG-tagged UL50, FLAG-tagged ORF67 and FLAG-tagged ORF16 were co-transfected with HA-tagged IRE1, and were then tested either in a co-immunoprecipitation assay or in a co-transfection dose-dependent manner. These results indicated that IRE1 does not co-precipitate with ORF40/41 (Figure 8A) while ORF40/41 does co-precipitate with IRE1 (Figure 8 B). This may be the result of a weak or indirect interaction. Moreover the downregulation of IRE1 was not observed in co-transfection experiment (Figure 8 C). As ORF40/41 displayed an unspecific or indirect interaction with IRE1 and did not downregulate IRE1, it was not further investigated as a modulator of the IRE1 signaling pathway.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IRE1-HA
ORF40-Flag
ORF40/41-Flag
UL50-Flag
ORF16-Flag
ORF67-Flag
KDa
130
70
55
35
25
40
HA
FLAG
Actin
B
Input
IP (HA)
IRE1-HA
ORF40-Flag
ORF40/41-Flag
ORF16-Flag
UL50-Flag
KDa
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Actin

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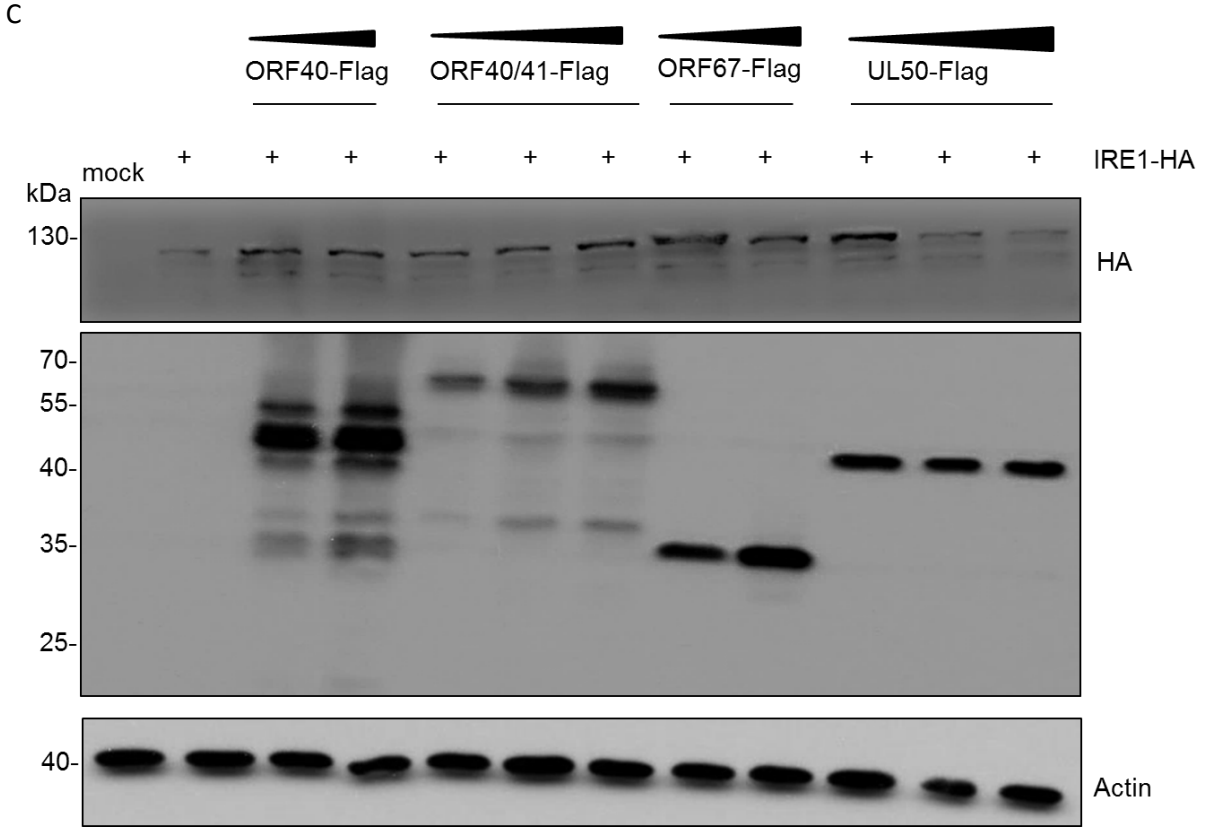


Figure 8: Interaction and effect of ORF40/41 with and on IRE1 expression in co-immunoprecipitation and co- transfection experiment. (A, B) 293A cells were co-transfected with plasmid expressing either FLAG-tagged ORF40/41 or FLAG-tagged ORF67 (negative control) or FLAG-tagged UL50 (positive control) and IRE1-HA. Samples were harvested 48 hours after infection using RIPA buffer. The lysates were co-immunoprecipitated antibody against HA or FLAG and subjected to western blot. The proteins were detected with specific antibodies against HA or FLAG. (C) 293A cells were co-transfected with IRE1-HA and increasing amount of plasmid expressing either FLAG-tagged ORF40 or, FLAG-tagged ORF40/41 or with positive and negative control plasmids expressing either FLAG-tagged UL50 or FLAG-tagged ORF67. Actin was used as loading control.

4.5 KSHV downregulates IRE1 mRNA

As no effect of ORF67 and ORF40/41 proteins on IRE1 modulation was observed, was ascertained whether or not IRE1 protein downregulation is due to transcripts regulation. To that end a quantitative real-time PCR (q-RT PCR) was performed to quantify mRNA levels of IRE1 and XBP1s. Figure 9A shows that IRE1 mRNA is downregulated 48 hours post infection. This was also observed in infected cells stimulated with Tg. During KSHV infection XBP1s mRNA level started to decrease 24 hours post infection however in the presence of Tg, this reduction was observed only at 48hpi and later (Figure 9 B). RPE1 epithelial cells transduced with a retroviral vector encoding HA-labeled IRE1 were used to test whether or not KSHV is capable of downregulating overexpressed IRE1. The results showed that even over expressed IRE1 was reduced by KSHV after 24h, compared to

uninfected cells, suggesting that KSHV not only downregulates endogenous IRE1, but also reduces exogenous IRE1 (Figure 9 C and D). This suggests that regulation of KSHV on IRE1 mRNA does not depend on its promoter because, in transduced cells, IRE1 is under the retroviral promoter control. Additionally, the level of XBP1s decreased in transduced-infected cells only at 72hpi.

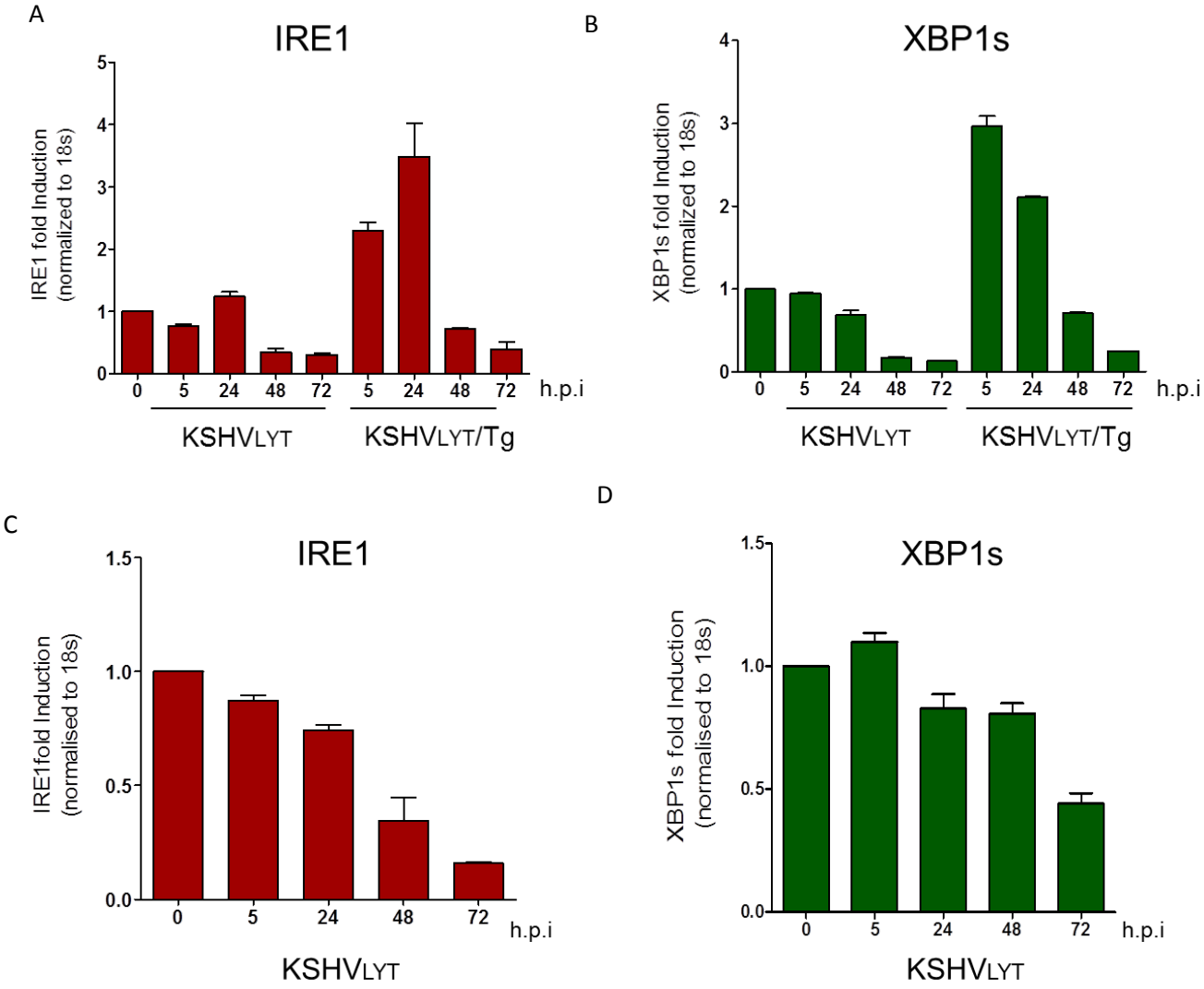


Figure 9: IRE1 mRNA during KSHV infection. (A, B) RPE1Cells were infected with $KSHV_{LYT}$ at MOI 0.5 TCID50/cell and were then treated with Thapsigargin (Tg 2µg/ml) four hours before harvesting at determined time points. RNA samples were then extracted from infected cells using RNA lysis buffer and the RNA was reverse transcribed into complementary DNA (cDNA). The cDNA was further measured by qPCR. (C and D) pMSCV-IRE1-HA transduced RPE1 were infected with $KSHV_{LYT}$ at MOI 0.5 TCID50/cell. Samples were harvested at determined time points using RNA lysis buffer. The RNA was reverse transcribed into complementary DNA (cDNA). The cDNA were subjected to qPCR. All samples were normalized to 18s rRNA. The relative values to the mock value are shown. Means ±SEM of three replicates are shown.

4.6 IRE1 protein level decreases after lytic reactivation

To establish whether IRE1 is downregulated only in lytic replication, the kinetic of IRE1 expression in the cell infected with latent KSHV was investigated. To this end, the IRE1 and XBP1s protein levels were detected in HEK293 transfected with $KSHV_{IND}$ [31]. The lytic reactivation was confirmed by the expression of the immediate early protein RTA, as well as the early lytic protein ORF45 and the late protein K8.1 (Figure 10). No changes could be detected in the protein level of IRE1 during latency, however after reactivation the kinetic of IRE1 similar to the kinetic was observed in samples infected with $KSHV_{LYT}$, with a decrease observed 48h post-induction. The XBP1s protein levels were also decreased 48 hours after lytic reactivation (Figure 10); however XBP1s protein level was increased soon after induction in comparison to the un-induced cells. These data indicated that lytic reactivation leads to downregulation of the IRE1-XBP1s pathway.

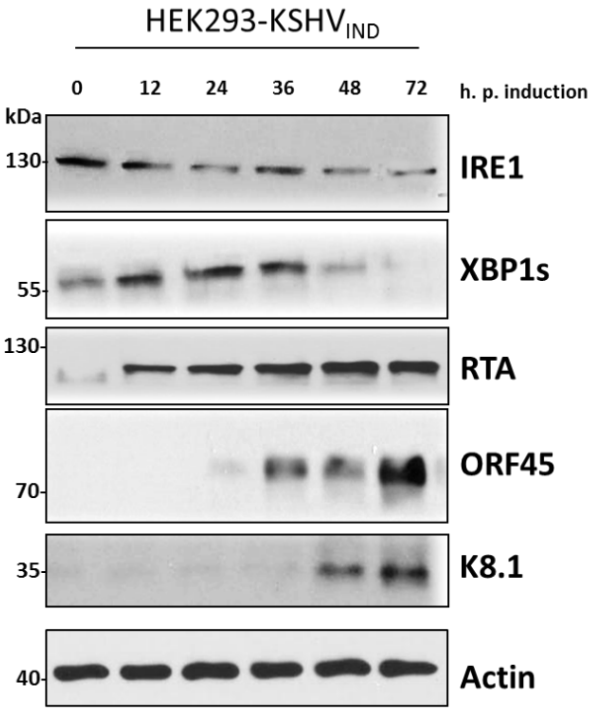


Figure 10: **IRE1 protein level after reactivation of lytic replication in HEK293 cells transfected with $KSHV_{IND}$.** HEK293cells were transfected with $KSHV_{IND}$ and treated with 10μM FK506 (Tacrolimus). The lysate were harvested using RIPA buffer at varying time points and subjected to western blot. The protein levels of endogenous IRE1 and XBP1s were detected using antibodies. The KSHV immediate early (RTA), early (ORF45) and late (K8.1) proteins were tested as control for infection and using actin as a loading control.

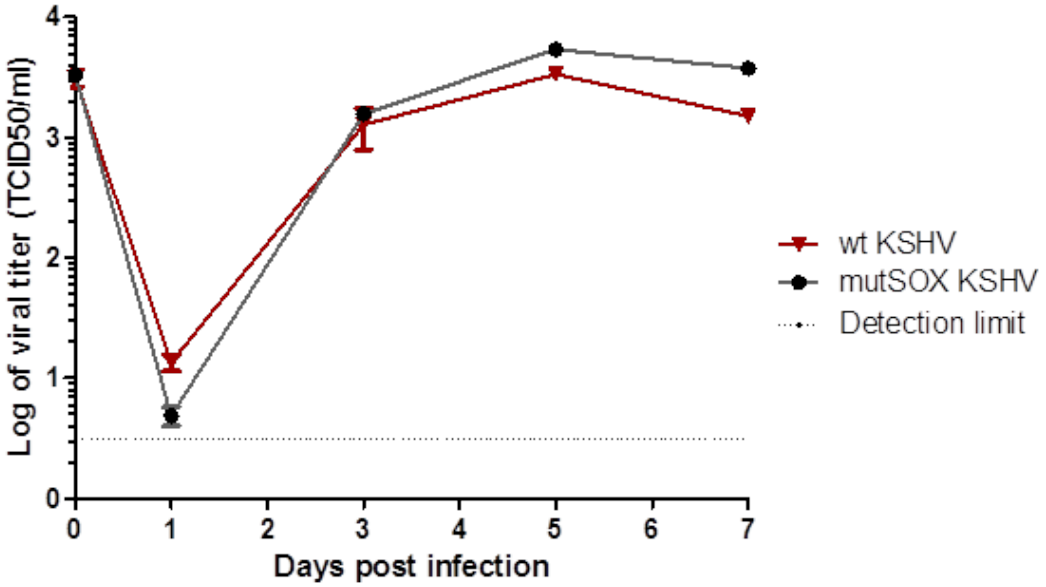
4.7 KSHV host-shutoff protein (SOX) does not affect the modulation of IRE1

Turnover of global mRNA is a very common strategy used to inhibit host gene expression[58]. Like some other herpesviruses, KSHV promotes shutoff of host cell gene

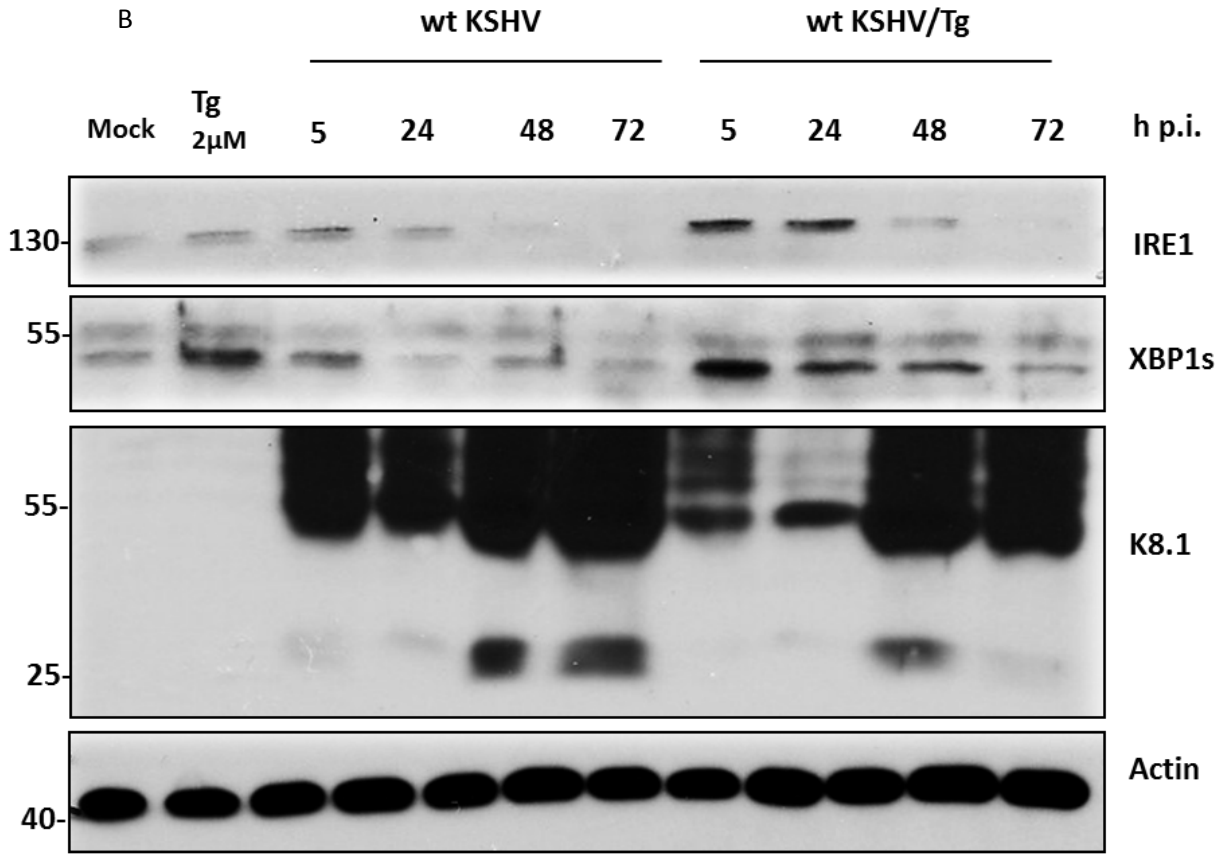
expression during lytic viral replication by dramatically impairing mRNA accumulation. KSHV exonuclease and host-shutoff protein (SOX) is encoded by the ORF37 gene, and downregulation of IRE1 at the transcript level (Figure 11) may be the result of the host-shutoff induced by KSHV SOX. However, not all cellular genes are affected in the same way by SOX. To check this possibility, a mutant was created using ‘’en passant’’ BAC mutagenesis technology. The mutant virus in ORF37 (P176S), called mutSOX KSHV, retains its DNA function but has an abolished shutoff function [87]. Firstly, a comparison was made between the replication of wt KSHV with mutSOX KSHV. The mutSOX KSHV showed a slight enhancement in its replication compared to the wt KSHV, observed on days 5 and 6, whereas the KSHV mutSOX exhibits one log higher titer at the same timepoints (Figure 11 A). This may be the result of the degradation of cellular factors involved in KSHV replication by SOX leading to less efficient replication in wt KSHV. The IRE1 protein levels were detected by western blot. The SOX protein starts to be expressed at 18hpi therefore at 5hpi the protein should not be present. In this state, no IRE1 downregulation was detected in cells infected with both viruses (Figure 11 B and C). No changes were detected in the level of IRE1 even at a later stage (24hpi), when SOX should be expressed. Downregulation of IRE1 occurs only after 48hpi, both in the wt KSHV and the mutSOX KSHV, as well as in cells induced with Tg. However, it seems that the decreased of XBP1s was greater in the cells treated with Tg and infected with mutSOX KSHV in cells infected with wt KSHV (Figure 11 B and C). This suggests that another factor is present in the mutSOX virus that is capable of reducing the level of XBP1s protein. This factor seems to be absent in wt KSHV, where it is probably degraded by the shutoff activity of the wtSOX.

The abolition of mutSOX protein-shutoff function was confirmed by q-PCR analysis on genes sensitive to SOX-induced degradation. As seen in Figure 11F and G, during wt KSHV infection, the level of PKRDC and GAPDH mRNA - as sensitive genes to SOX - was reduced after 24hpi. This is not observed during mutSOX KSHV infection, where the level of this mRNAs remained constant. In contrast, IRE1 and XBP1s mRNAs were downregulated 24hpi or 48hpi, in both viral infections, which confirms the SOX-independence of this reduction (Figure 11 D and E). It should be noted that 18s rRNA was used as a resistance gene to SOX for normalization.

A


Log of viral titer (TCID50/ml)
Days post infection
wt KSHV
mutSOX KSHV
Detection limit

B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wt KSHV/Tg
Mock
Tg 2μM
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72
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72
h p.i.
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XBP1s
55-
K8.1
25-
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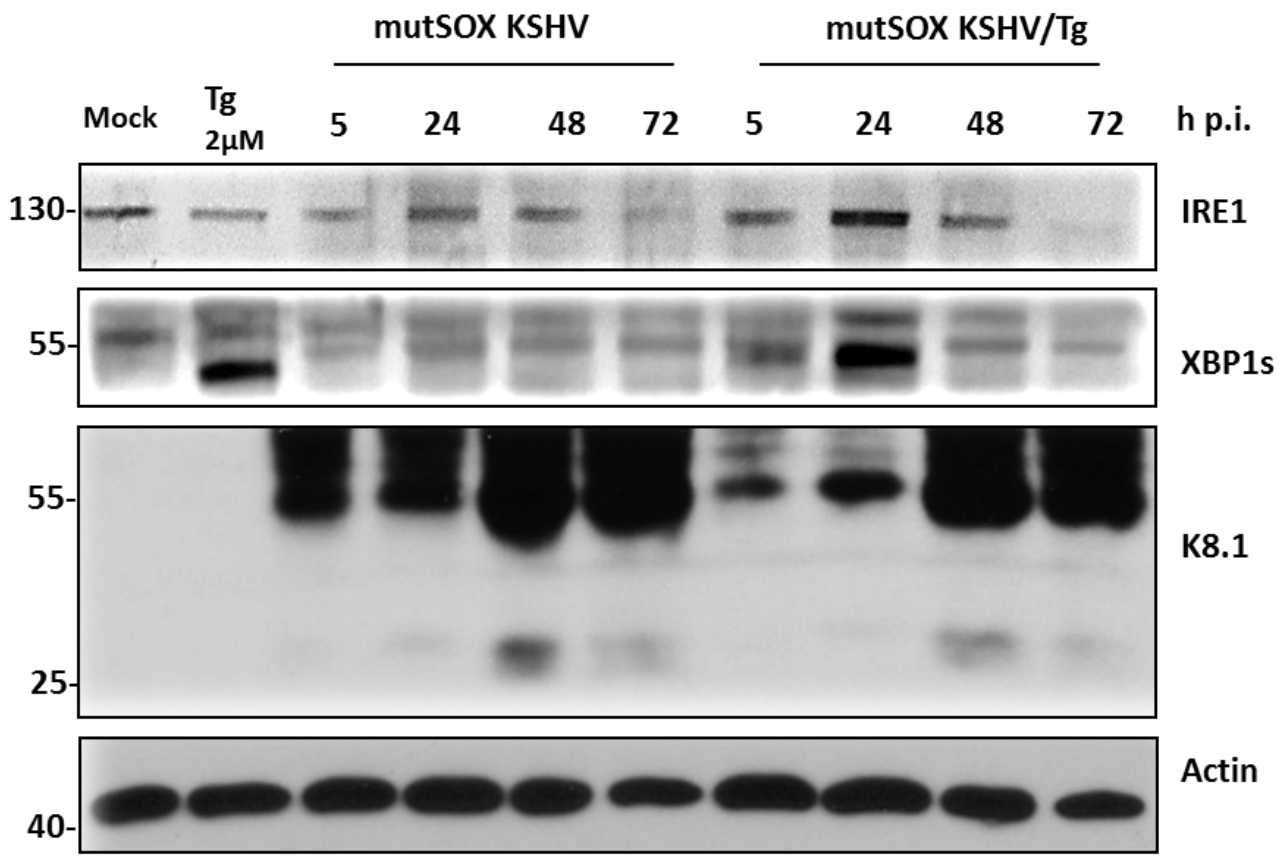
Actin

C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mutSOX KSHV/Tg
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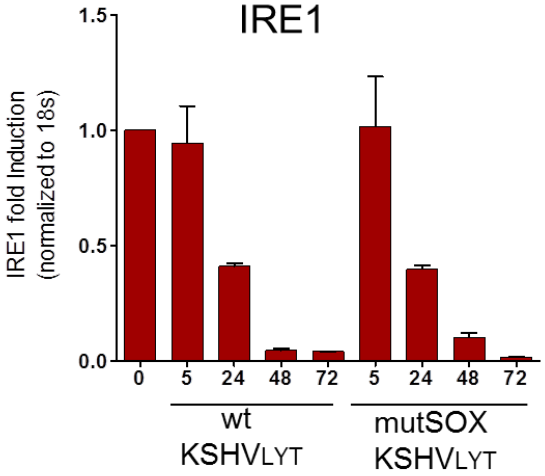
130-
IRE1
55-
XBP1s
55-
25-
K8.1
40-
Actin

D


IRE1
IRE1 fold Induction (normalized to 18s)
1.5
1.0
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72
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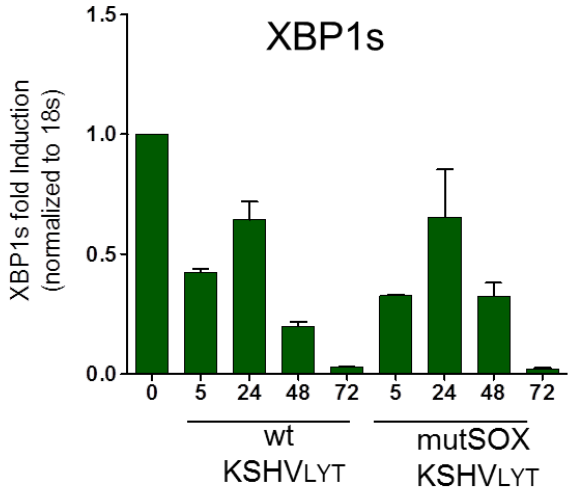
72
wt KSHVLYT
mutSOX KSHVLYT

E


XBP1s
XBP1s fold Induction (normalized to 18s)
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mutSOX KSHVLYT

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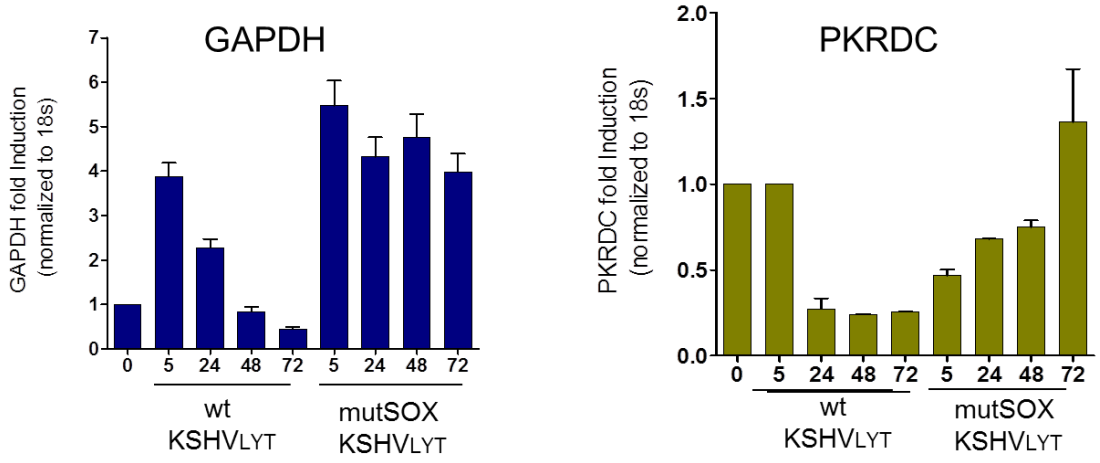


Figure 11: Effect of KSHV host-shutoff protein (SOX) on IRE1 signaling. (A) Multi-step growth curve was carried out in RPE1 epithelial cells. RPE1 cells were infected with either wt or mutSOX KSHV at MOI 0.05 TCID50/cell. The supernatants containing virus were harvested at indicated times and titrated in RPE1 cells. Each datum point represents the mean ± SD from triplicate experiments. DL is the detection limit. (B, C) RPE1 epithelial cells were infected with wt or mutSOX KSHV (MOI 0.5 TCID50/cell) or mock-infected and where indicated treated with 2µMThapsigargin 4 hours before harvesting. The cells were lysed after indicated time with RIPA buffer. Endogenous IRE1 and XBP1s were detected using western blot. The detection of K8.1-A/B served as control for infection and actin as loading control. (D, E,) RPE1 epithelial cells were infected with wt or mutSOX KSHV (MOI 0.5 TCID50/cell) or mock-infected and where indicated stimulated with 2µM Thapsigargin 4 hours before harvesting. RNA was then extracted from infected cells using RNA lysis buffer reverse transcribed into complementary DNA (cDNA). The cDNA was further investigated by qPCR. All samples were normalized to 18s rRNA as a gene insensitive to SOX. The relative values to the mock value are shown. Means ±SEM of three replicates are shown. (F, G) DNA-dependent protein kinase (PRKDC) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA were detected as controls for sensitive genes to SOX degradation.

4.8 IRE1 knockout RPE1 cells enhances KSHV replication

To compare the viral growth of $KSHV_{LYT}$ wild type (wt KSHV) in the presence or absence of IRE1, an IRE1 knock out (KO) RPE1 epithelial cell line was generated using the CRISPR-Cas9 method. As this system required guide RNA (gRNA) to target the IRE1 gene, three individual gRNA were designed and tested to knock out the IRE1 gene from epithelial RPE1 cells. Neither the gRNA1 nor the gRNA2 resulted in a complete knock out of IRE1 (Figure 15A).However, two clones (clone 5-1 and 5-6) from gRNA3 (g3), have demonstrated good reduction of IRE1, even with Tunicamycin stimulation. This stimulation was used to induce the expression of IRE1 and XBP1s, in order to confirm the silencing of IRE1 and the absence of the splicing of XBP1 (Figure 12A, B). To evaluate the effect of the absence of IRE1 on KSHV replication, the cells from the clone g3 -C5-6 were infected with $KSHV_{LYT}$ at MOI

0.05 TCID50/cell. The multistep growth curve showed that KSHV replicate more efficiently in IRE1 KO cells than in parental RPE1 cells. This is particularly visible after 3 days of infection. However, at later time points, it seems that both cell lines produce the same amount of the virus (Figure 12C), suggesting that KSHV may not require IRE1 during the later period of lytic replication. However, this difference may be not significant, and indeed this experiment was repeated only once, and meaning the results need to be confirmed by additional independent experiments.

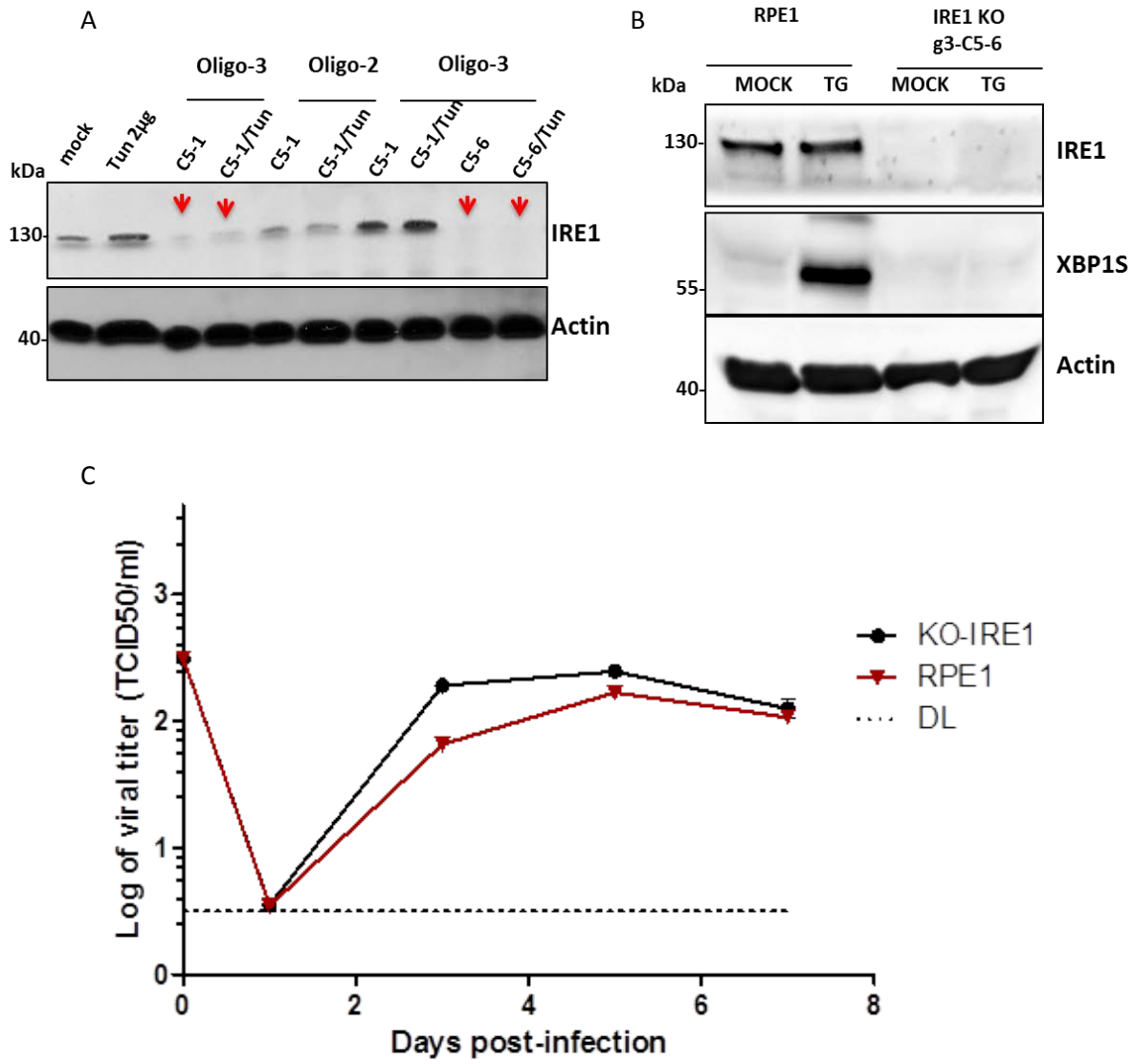
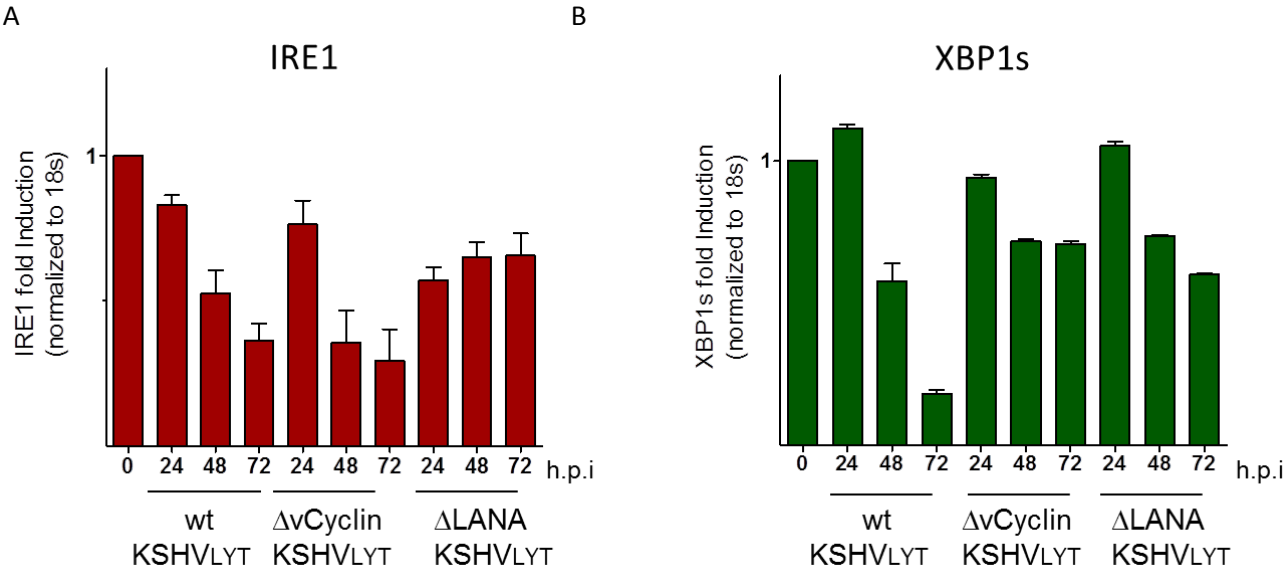


Figure 12: KSHV replication in the presence and absence of IRE1. (A) IRE1-KO RPE1 cells were created by CRISPR-Cas9 and the level of IRE1 protein in cells (stimulated or unstimulated with 10µg/ml Tunicamycin) was investigated by western blot. (B) The level of XBP1s protein was tested in single clone (g3-C5-6) by western blot. (C) RPE1 cells and IRE1-KO RPE1 cells (g3-C5-6) were infected with wt $KSHV_{LYT}$ (MOI 0.05 TCID50/cell). The supernatants containing the virus were harvested at indicated times and titrated in RPE1 cells. Each datum point represents the mean ± SD from triplicate.

4.9 LANA and vCyclin deletion KSHV mutant failed to downregulate IRE1 during infection

During the investigation of potential candidates responsible for the downregulation of IRE1, Shigemi et al[88] published data that indicated that LANA and vCyclin has an inhibitory effect on IRE1 transcription in a co-transfection assay. They also demonstrated that both N- and C-terminal regions of LANA-dCR (dilated central region) are important for the suppression of IRE1. The LANA-dCR is a LANA mutant with a deleted central region between amino acids 329-928, therefore containing only the C- and N- terminal parts [88]. To verify whether or not the same phenotype can be observed during lytic infection, two deletion mutants in KSHV were used which were previously constructed in the lab [31]: ORF72 (ΔvCyclin) and ORF73 (ΔLANA).

In the absence of those genes, the IRE1 RNA level was rescued in ΔLANA KSHV infection; however, the rescue in ΔvCyclin was not as efficient as in ΔLANA KSHV infection suggesting that only LANA is important for inhibiting IRE1 expression (Figure 13A, B). The level of XBP1s mRNA was also restored during ΔLANA and ΔvCyclin infection which suggests that these two viral proteins are involved in the regulation of XBP1s mRNA during infection. At the protein level, the expression of IRE1 and XBP1s is restored in ΔLANA and ΔCyclin KSHV in comparison to the wt KSHV infection (Figure 13C). The level of chaperone BiP was also investigated with no differences observed between the three viruses (Figure 13C). The absence of LANA was confirmed by using a specific antibody (Figure 13C).



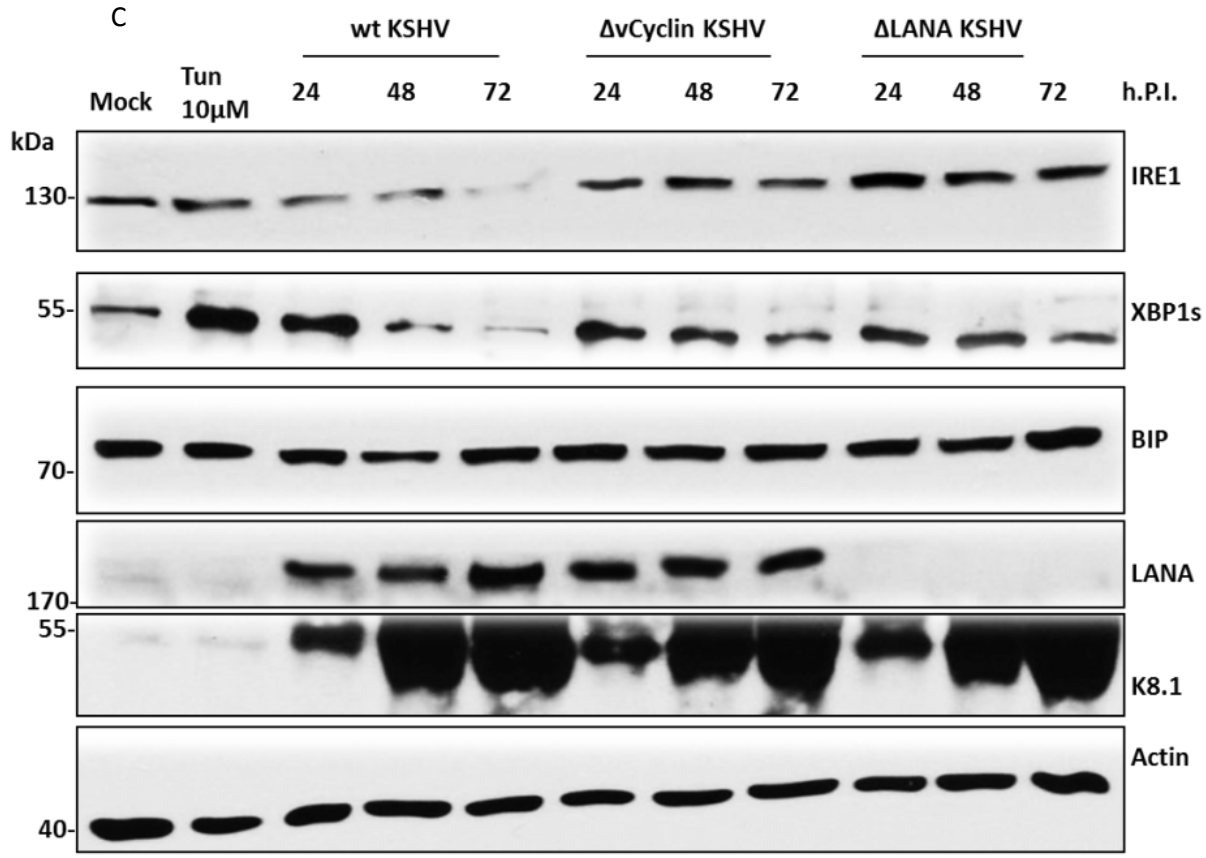


Figure 13: IRE1 and XBP1s mRNA and proteins during infection with KSHV vCyclin and LANA deletion mutants. RPE1 epithelial cells were infected with wt, ΔvCyclin and ΔLANA $KSHV_{LYT}$ mutants at MOI of 0.5 TCID50/cell. Samples were harvested at the indicated time points. (A, B) mRNA level of IRE1 and XBP1s were measured by quantitative-RT-real time PCR. Means ± SEM of triplicates are shown relative to uninfected cells (mock). (C) The RPE1 epithelial cells were infected with wt or ΔvCyclin or ΔLANA $KSHV_{LYT}$. The samples were lysed with RIPA buffer after the indicated time points and subjected to western blot. Indicated cells were then treated with 10µg/ml Tunicamycin. K8.1 and Actin served as infection and loading control respectively.

4.10 The full length and truncated N-termini LANA downregulate IRE1 in transfection assay

As only the ΔLANA KSHV was able to rescue IRE mRNA expression, this protein was studied in more details. Previous studies reported the existence of several isoforms of LANA with the most studied isoform being an N-terminally truncated protein, which localizes to the cytoplasm with a different function than the nuclear full length LANA [39, 89]. After observing IRE1 downregulation during wt KSHV infection but not ΔLANAKSHV infection, the next step was to detect which isoforms of LANA play a role in IRE1 downregulation, the full length, the shorter isoform, or both. As it has already been published that the N and C-terminal part or LANA are required for IRE1 mRNA downregulation in transfection [83] plasmids expressing either the full length (FL-1-1026) or a C- (ΔC-1-910) or N-(ΔN-161-

1026) terminally truncated protein were used to check the function of the known isoforms more specifically. The N-terminal truncation is a cytoplasmic isoform and is based on a deletion of 161 amino acids of LANA (LANAΔN161). This cytoplasmic isoform of LANA contains other functions in innate immune signaling pathways [39, 89]. The results showed that the protein level of IRE1 was reduced by full length LANA and also by LANAΔN161. The same phenotype was not observed with C-terminally truncated LANA, in agreement with what was published at the mRNA level (i.e. the importance of C-terminal for IRE1 downregulation) [88] (Figure 14).

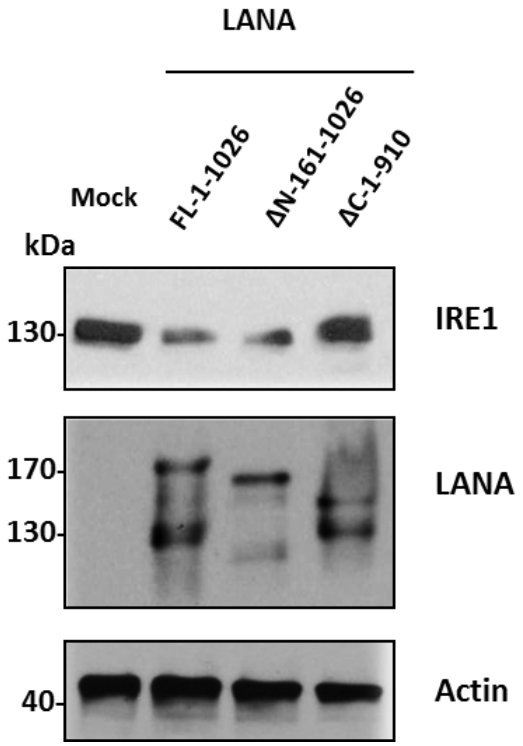


Figure 14: **IRE1 expression in the presence of LANA full length and truncated mutants**. HEK293 cells were transfected with pcDNA3 expressing LANA, either full length or ΔN-161 amino acids orΔC-113 amino acid terminal truncated mutants. Samples were harvested 48 hours after transfection by RIPA lysis buffer. The lysates were subjected to western blot with indicated antibodies. Actin was used as a loading control.

5 Discussion

KSHV is a causative agent of Kaposi's sarcoma (KS), lesions based on endothelial cells, primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD), all primary disorders of B cells. KSHV is classified as a γ2 herpesvirus subfamily and is closely related to Rhesus rhadinovirus (RRV) and murine γ-herpesvirus 68 (MHV68). KSHV displays two distinct life cycles, latency and lytic replication [85, 90, 91]. Whereas only a few genes are expressed during the latent phase, such as LANA, vCyclin and vFLIP, lytic replication allows the virus to express a number of genes which interfere with the host immune response and lead to virus production. Lytic replication is a critical step for KSHV tumorigenesis and can be inhibited or reactivated by various inflammatory cytokines[90]. In endothelial cells, inflammatory cytokines inhibit spontaneous KSHV lytic gene expression. However IFN-γ induces lytic replication and, in contrast, IFN-α inhibits lytic replication[90]. In this thesis, a KSHV constitutively expressing replication and transcription activator (RTA) protein ($KSHV_{LYT}$) and a KSHV inducible ($KSHV_{IND}$) virus, which has already been described was used [31].

Unfolded protein response (UPR) is a cellular homeostatic response to endoplasmic reticulum (ER) stress. IRE1 is one of the most conserved branches of the UPR pathway. Upon ER stress, IRE1 is activated and, by an unconventional splicing, removes an intron of 26 nucleotides from the XBP1 mRNA. The spliced XBP1 (XBP1s) protein translocates into the nucleus to control the expression of the ER associated degradation factors (ERAD) [74].

During viral infection, the accumulation of viral proteins can induce ER stress. The balance between viruses, the UPR and the self-degradative process of autophagy has been addressed in several studies [36, 69]. The UPR is prevalent in viruses establishing latent infections such as herpesviruses. During viral evolution, herpesviruses hijack a number of molecules from their host in order to use them in its life cycle. The UPR is no exception; some herpesviruses share molecular mimicry with the key UPR molecules, such as ICP0 in HSV1 [73]. They also utilize the UPR to establish lytic infection and to break latency [92]. Previous studies have reported that XBP1s reactivates RTA in plasma cell differentiation, a key factor for switching from latency to the lytic cycle [42, 43, 82]. Therefore, a better understanding is required of the entire interaction of KSHV with the host UPR. The aim of this study was to address whether and how KSHV modulates the IRE1 signaling pathway.

5.1 KSHV downregulates IRE1

To determine whether KSHV is able to modulate IRE1 during lytic replication, a KSHV constitutively expressing RTA, already established in the lab was used[31]. While the levels of IRE1 protein were not affected by KSHV shortly after infection, the protein was decreased later after infection. In agreement with the downregulation of IRE1, a lower expression of XBP1s was observed at the early and late time points of infection. This reduction was not observed at early stage in stimulated-infected cells. This can be explained by the fact that at the early stage of infection in infected cells stimulated with Tg, the expression of the lytic genes involved in UPR modulation are not efficient enough to inhibit high activation of Tg-triggered XBP1 splicing.

The level of BiP was stable in the initial phase of infection and then subsequently upregulated. This is consistent with previous data, suggesting that KSHV-ORF47/45 upregulates and selectively induces UPR signaling receptors [46]. It is possible that ORF47/45 is the protein responsible for maintaining the unchanged level of IRE1 by BiP which was observed in the very early stages of infection by the $KSHV_{LYT}$. This early upregulation is important for completion of the lytic replication [46].

It is helpful to know whether the UPR is beneficial for KSHV latency or for lytic replication. The level of IRE1 protein in cells induced with KSHV ($KSHV_{IND}$) was detected before and after the induction of RTA. Even during latency, the level of IRE1 was stable. At the beginning of the lytic reactivation, this level also remained unchanged. This basal level of IRE1 is probably important for maintaining a constant level of XBP1s, which is required for RTA reactivation [42, 43, 86] and for induction of lytic reactivation [88]. The IRE1 protein level was shown to be lower after 24 hours post-reactivation, which was consistent with the results obtained from the constitutively lytic infection experiment.

The first KSHV factor tested to have a possible role in IRE1 downregulation was ORF67. This viral protein is the homolog of M50 and UL50 and is responsible for the downregulation of IRE1 during MCMV and HCMV infection, respectively [69]. However in KSHV, ORF67 did not have an effect on IRE1 modulation.

MHV68-helicase-primase factor, ORF40, may inhibit UPR in the initial phase of infection by binding and stabilizing the interaction between BiP and the transmembrane stress sensors [52]. However, the interaction of IRE1 and ORF40/41 (homolog of MHV68-ORF40) was detectable only in one direction, but not in the other. The observed interaction can only be

either physical or non-specific. This observed interaction did not result in an effect on IRE1 protein level, as described for other herpesvirus protein, such as UL34, which interacts with IRE1 without inhibiting it [69].

Since none of the HCMV, MCMV and MHV68 homologous genes in KSHV is responsible for the reduction of IRE1 at the protein level, the mechanism used by KSHV differs from that used by other member in the herpesvirus family. The downregulation could be the result of transcriptional regulation. QRT-PCR analysis revealed that the mRNA of IRE1 was upregulated at the early time of infection, but at 24 hours after infection, this level dropped dramatically. The transcription of XBP1s was reduced at later stages of infection, which was consistent with previous western blot results. In conclusion, IRE1 mRNA is downregulated by KSHV after 48 hpi but no earlier than 24 hpi, whereas XBP1 splicing is inhibited at an earlier stage (within 24 hours of infection). Therefore, KSHV might make use of an additional viral protein or mechanism to regulate XBP1 splicing.

When the reduction of IRE1 was observed at the mRNA level, the speculation was that this downregulation occur by regulation of the IRE1 promoter. However, the level of IRE1 transcripts was investigated in the overexpression system; both RNA and protein levels were decreased. In the overexpression system, IRE1 is under the control of a retroviral promoter; the observed reduction is probably not due to promoter regulation, unless a KSHV protein can regulate both the IRE1 promoter and the retroviral LTR promoter. Other mechanisms are also possible, such as downregulation at the post-transcriptional level of IRE1. A viral protein may control the IRE1 half-life by manipulating the poly (A) tail, or by removing the 5′CAP from IRE1 mRNA.

5.2 IRE1 downregulation is not due to host-shutoff

Several viruses encode factors that promote host RNA degradation and KSHV is no exception. KSHV expresses exoribonuclease shutoff protein, SOX, encoded by the ORF37 gene [53]. This protein is not packaged into virions but is expressed in infected cells with early kinetic (8-10 hpi). Following SOX expression, cellular and viral mRNA degradation occurs and continues for the remainder of the viral lifecycle [55]. To determine whether SOX plays a role in IRE1 mRNA reduction, a KSHV ORF37 (P176S) mutant (mutSOX) was constructed. This mutation (P176S) causes SOX to fail to degrade host mRNAs, while retaining its DNA activity, which is required for virus replication remains unaffected [58]. No differences between the effects induced by the wt KSHV or by the mutSOX KSHV on the IRE1 transcripts and proteins could be observed. In contrast to UL41, the host-shutoff

homolog in HSV1 [74], KSHV SOX does not degrade IRE1 and XBP1s indicating again that KSHV uses a different mechanism to degrade IRE1 than other member of the herpesviruses family.

5.3 KSHV full length and cytoplasmic isoform of LANA modulate IRE1

It has been demonstrated that the two latent proteins, LANA and vCyclin, suppress IRE1 transcription in transfection experiments [88], but this was not investigated during lytic infection. Using the $KSHV_{LYT}$ virus, the downregulation of IRE1 mRNA and protein levels were confirmed in the absence of LANA; however the IRE1 level did not change in the absence of vCyclin. The XBP1s mRNA level was affected by both LANA and vCyclin during infection. As vCyclin seems to regulate XBP1s but not IRE1, this would imply that the function of vCyclin is different from that of LANA. More investigations are required to clarify this function of vCyclin.

LANA (ORF73) is a KSHV latent gene that localizes to and functions in the nucleus of latently infected cells [39]. In addition, expression of LANA can also be directed by a lytic promoter [35]. A recent study suggests that LANA recruits Krüppel-associated box domain-associated protein 1 (KAP1) to the RTA promoter region of the KSHV genome in order to silence lytic gene expression and facilitate the establishment of KSHV latency [93]. It is reported that KSHV ORF73 encodes several short cytoplasmic isoforms, in addition to the full length protein. One of these shorter isoforms has different functions during lytic replication [39, 89]. The cytoplasmic isoform of LANA, which lacks the NLS-containing N-terminal region, is more abundantly expressed during lytic reactivation, and this may be the result of the alternative in-frame translational start codons or the cleavage of an N-terminal LANA fragment by Caspase 3 [89]. It has been demonstrated that the nuclear LANA antagonizes the cytoplasmic isoform in some functions, such as blocking the interferon response triggered by cGAS, and then promoting lytic reactivation [39], and also interfering with innate sensors of cytoplasmic DNA, which control KSHV replication [89].

To understand the role of the cytoplasmic isoform of LANA (N-terminally truncated) in IRE1 suppression, the IRE1 protein level was evaluated by using overexpression of truncated forms of LANA. The results of this experiment showed that the expression of full-length LANA or the N-terminally truncation mutant can influence the IRE1 protein level, but the C-terminally truncated protein did not impact the IRE1 level. One hypothesis is that either the N-terminus of LANA is not required to downregulate IRE1 or that this effect is a new function of this isoform. This finding should be confirmed in cells infected with $KSHV_{LYT}$ expressing only

the cytoplasmic isoform (N-terminally truncated) of LANA. Using this mutant virus, the level of IRE1 is expected to be reduced during lytic infection. Moreover, LANA was not able to downregulate IRE1 without its C-terminal part, which may suggest that the C-terminal is required for this function. It has been shown that the C-terminal of LANA leads to activation and/or repression of certain cellular and viral promoters, probably through interaction with cellular transcription factors such as CREB, CBP and Sp1 [37]. It would also be interesting to investigate the role of the C-terminal part of LANA in the regulation of the IRE1-XBP1s pathway.

5.4 General conclusion

In KSHV lytic infection BiP is upregulated by ORF47 [46], which was also determined in this thesis. This upregulation can result in the stability of IRE1 at early time of infection that was observed in this study. However, the data in this dissertation showed that KSHV downregulates IRE1 after 24hpi, and that the mechanism used differs from those employed by other members of the herpesvirus family. For instance, ORF67 and ORF40/41 were not able to downregulate IRE1 protein level in contrast to beta herpesviruses such as HCMV and to other gammaherpesviruses such as MCMV [52]. Moreover, data showed that IRE1 is reduced at the mRNA level. However, despite the fact that most of the cellular RNAs are sensitive to host shutoff induced by KSHV-SOX, IRE1 is resistant to SOX-induced degradation. Therefore, KSHV might downregulate IRE1 at both the protein and mRNA levels. It was confirmed that in absence of LANA, IRE1 cannot be downregulated during infection, which is in agreement with published data in transfection experiments which showed that IRE1 transcripts are suppressed by overexpressing of the LANA and vCyclin [88],for which, the C-terminal part of LANA (present in both the full length and the cytoplasmic isoform) seems to be responsible. As these two forms have different functions during infection [39], they may downregulate IRE1 for different purposes. The full length LANA can downregulate IRE1 to restrict RTA expression and promote latency, whereas, the cytoplasmic isoform may downregulate IRE1 and XBP1s protein, to protect the infected cells from severe activation of the UPR via modulation of the IRE1- XBP1s pathway during lytic infection (Figure 17). To understand the exact mechanism used by cytoplasmic isoform (N-terminally truncated) to suppress IRE1, experiments have to be performed in cells infected with mutant $KSHV_{LYT}$ only expressing cytoplasmic isoform of LANA. Additional factors may also be involved in regulating IRE1-XBP1 pathway, such as vCyclin, that seems to directly modulate XBP1.This however, remains to be elucidated in further studies. It may also be useful to investigate the influence of KSHV on the other UPR signaling pathways, PERK and ATF6.

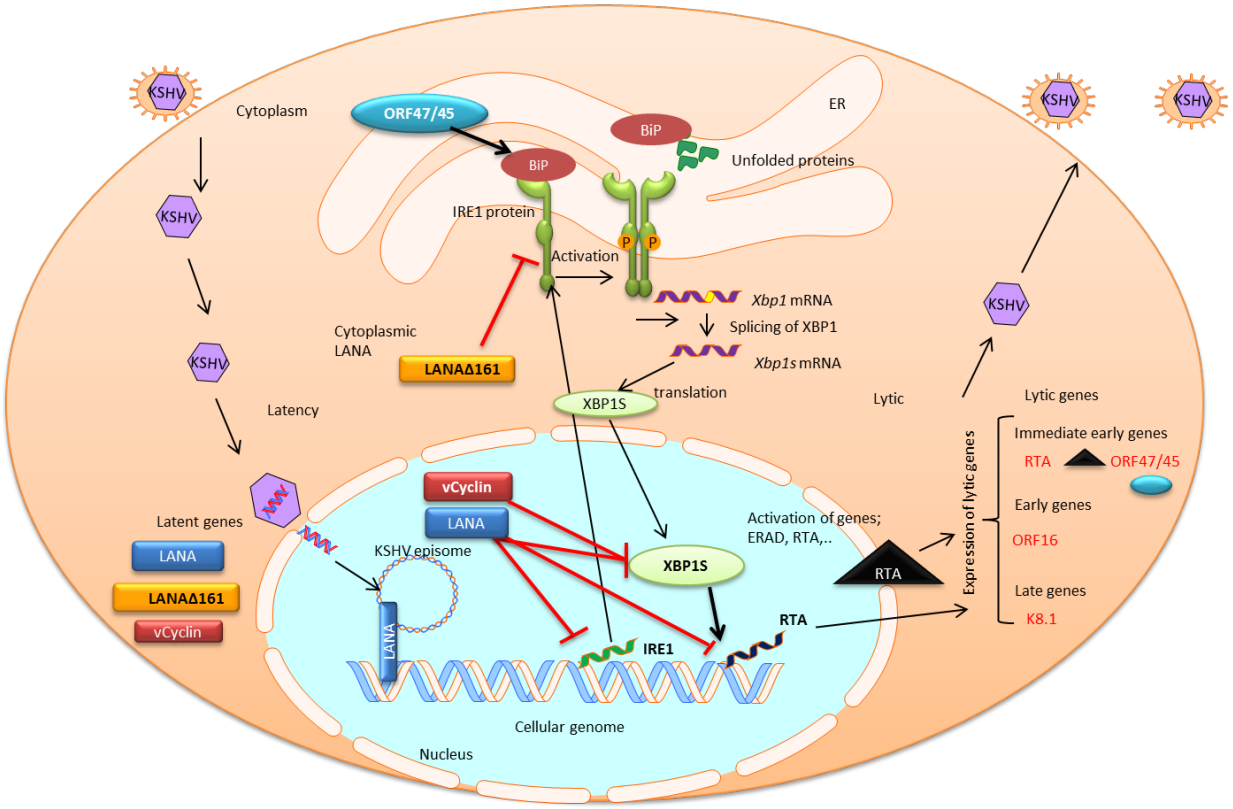


Figure 15: Scheme of IRE1 downregulation during KSHV infection by expression of LANA. Upon KSHV latent infection, the dsDNA linear genome is circularized and binds to the cellular chromatin via LANA [93]. RTA induces the expression of IE, E and L genes lytic genes in temporal cascade manner [32]. LANA is expressed in the nucleus and represses RTA promoter at the early stage of latency resulting in the inhibition of RTA [36].The cytoplasmic isoform (LANAΔ161) expression is extended into the lytic phase [39, 89], activating the unfolded protein response. Upon ER stress the chaperon BiP dissociated from IRE1 and IRE1is dimerized and autophosphorylated. Activation of the IRE1 leads to the unconventional splicing of XBP1 mRNA and producing XBP1s [69]. XBP1s induces RTA reactivation which initiates the switch from latent to the lytic replication [43]. In lytic replication the KSHV early protein, ORF47/45, activates GRP78/BiP expression at the early lytic stage [46]. Both isoforms of LANA are able to influence IRE1 mRNA. vCyclin regulates XBP1s mRNA by reducing IRE1 protein level.

6 Materials

6.1 Cells

Table 1

Cell	Description	Medium	Source/ reference
RPE1	Retinal pigment epithelial cells, human; Immortalized adherent epithelial cells, chromosomally nearly diploid, and ciliated	DMEM 10%FCS 1% P/S	[94]
HEK293A	Human embryonal kidney epithelial cells, subclone of the 293 cell line, selected on flat morphology	DMEM 10%FCS 1% P/S	Invitrogen (R705-07)
HEK293T	Adenovirus transformed human embryonic Kidney cells stably express SV40 large T antigen	DMEM 10%FCS P/S	Invitrogen (R705-07)
Phoenix	Retroviral packaging cell line stably expressing gag, pol and env; based on HEK-293T cells	DMEM 5 % FCS 1% P/S	[95]

6.2 Cell culture medium

Table 2

Cell Culture	Reference
Dulbecco's Modified Eagle Medium (DMEM), high glucose	Sigma-Aldrich
Dulbecco's Phosphate Buffered Saline (PBS) (1x)	Sigma-Aldrich
fetal calf serum (FCS)	PAN Biotech
OptiMEM-I	Thermo Fisher Scientific
penicillin/streptomycin (100x)	Sigma-Aldrich
trypsin-EDTA (1x)	Sigma-Aldrich
DMEM 10 % FCS	DMEM + 10 % (v/v) FCS and 1 % (v/v) penicillin/streptomycin

6.3 Viruses

Table 3

Virus	Description	Source/ reference
Kaposi's Sarcoma associated herpes virus(KSHV/HHV8) lytic expressing gfp	Recombinant Human KSHV-lytic cloned as BAC 16 Construct with expression of the green fluorescent protein (GFP**)**, selection marker in bacteria chloramphenicol resistance	[96]
Kaposi's Sarcoma associated herpes virus(KSHV/ HHV8) lytic expressing gfp with P176S mutSox	Recombinant Human KSHV-lytic cloned as BAC Construct with expression of the green fluorescent protein (GFP), selection marker in bacteria chloramphenicol resistance, mutation in ORF37(sox) gene	Generated KSHV-lytic GFP with point mutation of P176S within ORF37 gene by En-Passant BAC mutagenesis
Kaposi's Sarcoma associated herpes virus(KSHV/HHV8) lytic expressing gfp with deletion in ORF72 gene (v-cyclin D)	Recombinant Human KSHV-lytic cloned as BAC Construct with expression of the green fluorescent protein (GFP), selection marker in bacteria chloramphenicol resistance, point mutation in ORF72 gene	Constructed by Melanie Lampe, (2011)
Kaposi's Sarcoma associated herpes virus (KSHV/HHV8) lytic expressing gfp with deletion in ORF73 (LANA) gene	Recombinant Human KSHV-lytic cloned as BAC Construct with expression of the green fluorescent protein (GFP), selection marker in bacteria chloramphenicol resistance, deletion in ORF73 gene	Constructed by Melanie Lampe, (2011)

6.4 Bacteria and bacterial culture medium

Table 4

Name	Deskription	Growth Temperaturen	Reference
E.coli DH10B	F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara,leu)7697	37 °C	Life technologies

	araD139 galUGalKnupGrpsL λ-		
E.coli GS1783	DH10B l cI857 Δ(cro-bioA)<>araC-PBADI-sceI	30 °C	[97]

6.5 Antibiotics

Table 5

Antibiotic	Concentration	Application	Reference
Ampicillin	100 µg/ml	Selection of bacteria	Roth
Chloramphenicol	15 µg/ml	Selection of bacteria	Roth
Kanamycin	50 µg/ml	Selection of bacteria	Roth
Penicillin	100 U/ml	Selection of bacteria	Sigma-Aldrich
Streptomycin	100 µg/ml	Cellculture supplement	Sigma-Aldrich
Puromycin	5 µg/ml	Selection of transduced cells	Sigma-Aldrich

6.6 Plasmids

Table 6

Plasmid	Description	Source/ reference
pcDNA3	5.4 kb, high copy, eukaryotic expression vector, CMV promoter, neomycin and ampicillin resistance	Invitrogen
pMSCVHyg- IRE1-HA	9.25 kb, low copy, retroviral Vector, LTR Promoter, Hygromycin resistance, expressed IRE1 with C-Terminal HA-Tag	PCR amplification of the hIRE1 gene from RPE1 cell using primer hIRE1 BglII fwd and hIRE1 HA EcoRI rev. Insertion into pcDNA3.1 using BglII and EcoRI restriction sites
pcDNA-LANAΔ161-FLAG	9.6 kb, high copy, based on pcDNA3 eukaryotic expression vector for the KSHV 161 deletion in N-terminal of LANA gene	9.6 kb, high copy, based on pcDNA3 eukaryotic expression vector for the KSHV LANA gene
pcDNA-LANA-FL-	9.54 kb, high copy, based on	9.54 kb, high copy, based on pcDNA3

FLAG	pcDNA3 eukaryotic expression vector for the KSHV full length of LANA gene	eukaryotic expression vector for the KSHV LANA gene
pcDNA-3xFlag- ORF67	6.273kb, high copy, based on pcDNA3 eukaryotic expression vector for the KSHV gene ORF67	PCR amplification of the ORF67 gene from KSHV-lytic BAC using primer ORF67 BglII fwd and ORF67 flag pmscvEcoRI rev. Insertion into pcDNA3.1 using BglII and EcoRI restriction sites
pcDNA-3xFlag- ORF40/41	8.40kb, high copy, based on pcDNA3 eukaryotic expression vector for the KSHV gene ORF40/41	PCR amplification of the ORF40/41 gene from KSHV-lytic BAC using primer ORF40/41 BglII fwd and ORF40/41 flag EcoRI rev. Insertion into pcDNA3.1 using BglII and EcoRI restriction sites.
pcDNA-3xFlag- UL50	55kb, high copy, based on pcDNA3 eukaryotic expression vector for the HCMV gene UL50	[69]
pcDNA-3xFlag- ORF16	5.856kb, high copy, based on pcDNA3 eukaryotic expression vector for the KSHV gene ORF16	[31]
pcDNA-HA- IRE1	8.31kb, high copy, based on pcDNA3 eukaryotic expression vector for the Human gene IRE1α	[69]
RP418	Phenylalanyl - tRNA synthetize subunit beta, Ampicillin resistance, from Addgene	[87, 98]
pCMV8.91	Transgene viral packaging, Ampicillin resistance	[98]
pMDG2	Mammalian expression, Lentiviral ; Envelope , Ampicillin resistance	[98]
pRetroGFP	based on pRetroEBNA, retroviral expression vector to generate retrovirus for GFP expression in eukaryotic cells	T. Shenk, Princeton University, USA
pMSCVpuro	retroviral expression vector to generate retrovirus for transduction	Clontech Laboratories

	of eukaryotic cells, amp^R, $puro^R$	
pEPkan-S	template plasmid for en passant mutagenesis, contains I-Sce-aphA1 cassette, kan^R	[97]
pCMN-ΔC113LAN(1-910)	5.178 bp, high copy, based on pCMN eukaryotic expression vector for the KSHV gene LANA mutant with deletion of 113 aa at C-terminal	The C-terminal mutant is nuclear and has a deletion of113 amino acid (LANAΔCt). This mutant was kindly provided by Dr. Thomas Gunther from Virus Genomics group at Heinrich-Pette-Institut based on pCMN back bone.

6.7 Oligonucleotide

Table 7

Primer Name	Sequence	Function
BamHI-HindIII hIRE1HA	AAGGATCCAAGCTTATGCCGGCCCGGCGGC TCTCGAGTCAAGCGTAGTCTGGGACGTCGT ATGGGTAGAGGGCGTCTGGAGTCACTGGGG G	PCR– amplification of HA tagged hIRE1 for cloning in BamHI/HindII
EcoRI-XhoI ORF67-1xFlag	TATAGAATTCaccATGAGTGTCGTTGGTAAG CG AATTCTCGAGttaCTTGTCGTCGTCATCCTTGT AGTCGCCGGATCCGCTGGGCCTCATCCAAA C	PCR– amplification of flag tagged ORF67 for cloning in EcoRI/XhoI
HindIII-BamHIORF67-3xFlag	TATAAAGCTTaccGGTACCGAGCTCGATGAG TGTCGTTGG AATTGGATCCTTACAGCTGGGCCTCATCCA AAC	PCR– amplification of flag tagged ORF67 for cloning in HindIII/BamHI
KnpI-BamHI ORF40/41-1xFlag	TATAGGTACCACCATGGCAACGAGCGAAGA AACG AATTGCGGCCGCTCAAAATAAAGATAAAAG CC	PCR – amplification of flag tagged ORF40/41 for cloning in KnpI/ BamHI

KnpI-XhoI ORF40/41-3xFlag	TATAGGTACCACCATGGCAACGAGCGAAGAAACG CTCGAGTTACTTGTCGTCATCATCCTTATAG TCCTTATCGTCATCGTCCTTGTAGTCCTTGT CGTCGTCATCCTTGTAGTC	PCR – amplification of flag tagged ORF40/41 for cloning in KnpI/ BamHI
18s rRNA	AACCCGTTGAACCCCATT CCATCCAATCGGTAGTAGCG	q-RT PCR
GAPDH	CCCACTCCTCCACCTTTGACG GTCCACCACCCTGTTGCTGTAG	q- RT PCR
IRE1	TGGGTAAAAAGCAGGACATCTG GTATTCTGTTCGCCCAAGAT	q- RT PCR
XBP1s	CGCAGCAGTGCAGGC TCCTTCTGGGTAGACCTCTGGGAG	q- RT PCR
mutSox(ORE37)-kan (P176S)	CAGCCCATGGCCTATAACGAACAACCACTT TGTCGCGGGCTCGCTTGCCTTTGGGCTGCGG TAGGGATAACAGGGTAATCGATTT GTTTTCACCACCTCCTCGCACCGCAGCCCAA AGGCAAGCGaGCCCGCGACAAAGTGGTTGT GCCAGTGTTACAACCAATTAACC	BAC Mutagenesis
REV.sox (ORF37)-kan	AGCCCATGGCCTATAACGAACAACCACTTT GTCGCGGGCCCGCTTGCCTTTGGGCTGCGG TAGGGATAACAGGGTAATCGATTT GTTTTCACCACCTCCTCGCACCGCAGCCCAA AGGCAAGCGGGCCCGCGACAAAGTGGTTGT GCCAGTGTTACAACCAATTAACC	BAC Mutagenesis
mutSox (ORE37-P176S)	AGTCGTTAGCGAACTGCTCC ACAATTTGTCTCGTCCGGGT	sequencing
Oligo1	GCATGGCGAGGACTCGGCCC	sequencing
Oligo2	GCTGCTGCTGCCCGGCCTCG	sequencing
Oligo3	GTTTGCATGCTGTCAGCAAG	sequencing
Oligo4	AGCGTATACAGGCTGCCATC	sequencing
EF1A	GCAATTGACCCGGTGCCTAG	Sequencing for CRISPR-CAS (Revers

		primer)

6.8 Antibodies

Table 8

6.8.1 Primary antibodies

Primary Antibodies	Produced in	Antigen	Application	Source/ reference
β-Actin (AC-74)	Mouse	β-Actin	WB 1:20000	Sigma-Aldrich Chemie GmbH, München
c-Myc (4A6)	Mouse	Myc	WB 1:2000	Millipore (Upstate)
Flag M2 Sigma-	Mouse	Flag	WB 1:3000	Aldrich Chemie GmbH, München
GRP 78 (E-4)	Mouse	BiP	WB 1:1000	Santa Cruz
HA (16B12)	Mouse	HA	WB 1:1000	Covance Research Products, Berkeley, USA
IRE1 α (14C10)	Rabbit	IRE1 α	WB 1:1000	Cell Signaling Technology, Inc., Danvers, MA, USA
K8.1A/B (clone 4A4)	Mouse	K8.1A/B	WB 1:2000	SantaCruz Biotechnology, Inc., Santa Cruz, CA, USA
LANA(C-AT4C11)	Rabbit	LANA	WB 1:2000	Acris
ORF45	Mouse	ORF45	WB 1:2000	
Phospho-ERN1/IRE1 (Ser724)	Rabbit	pIRE1	WB 1:1000	Novus Biologicals Inc., Littleton, CO, USA
RTA	Mouse	RTA	WB 1:10000	Kindly provided by Thomas Guenther
XBP1 (M-186)	Mouse	XBP1s	WB 1:200	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA

6.8.2 Secondary antibodies

Antigen	Conjugate	Product in	Application	Source/ reference
Mouse Ig	HRP	goat	IB (1:3000)	Dako Cytomation
Mouse Ig	HRP	goat	IB (1:3000)	Jackson ImmunoResearch
Rabbit Ig	HRP	goat	IB (1:3000)	Jackson ImmunoResearch

6.9 Size standards

Gene Ruler™ DNA Ladder Mix (Fermentas)

Page Ruler™ Prestained Protein Ladder (Fermentas)

6.10 Enzyme

FastDigest-Restriction enzyme (10 U/µl) (Fermentas)

T4 DNA Ligase (5 U/µl) (Fermentas)

PRECISOR High-Fidelity DNA Polymerase (2u/µl) (BioCat)

DreamTaq™ Green DNA Polymerase (5u/µl) (Fermentas)

RNAse A (Roth)

6.11 Kits

Table 9

Kit	Source
BCA Protein Assay Kit	Thermo Fisher Scientific
innuPREP DNA mini kit	Analytics Jena
innuPREP RNA mini kit	Analytics Jena
mi-Plasmid Miniprep Kit	Metabion
NucleoBond Gel and PCR Clean-up	Macherey-Nagel
NucleoBondXtra Midi	Macherey-Nagel
Turbo-DNA free kit	Life Technologies

6.12 Consumable

0.45 µm Filter Attachements Minisart® (Sartorius)

Cyro tubes 2 ml (sterile) (Roth)

Deoxyribonucleotides, 10 mM each dATP, dCTP, dGTP, dTTP (fermentas)

ECL Western Blotting Detection Reagents (Amersham Biosciences)

Electroporation cuvettes (Eurogentec)

Phosphatase Inhibitor Cocktail 2 + 3 (Sigma-Aldrich)

Pipette tips (VWR, Neolab)

Power SYBR Green PCR Master Mix (Applied Biosystems)

Protease Inhibitor Cocktail "Complete Mini" (Roche)

QIAshredder (Qiagen)

StrataClean Resin (Agilent Technologies)

Whatman Paper 3mm (Roth)

Cell Culture Scraper (TPP)

Cell culture products (Greiner, Nunc, Sarstedt, TPP)

6.12.1 Devices

ABI 7500 Real-time PCR System (Applied Biosystems)

Agagel Maxi (Biometra)

Agagel Midi-Wide (Biometra)

Axiovert 25 Microscope (Zeiss)

Bacterial Shaking Incubator GFL3033 (Hilab)

Bacterial shaker Certomat® R (B. Braun Biotech International)

Finnpipette® Multistepper (ThermoFisher)

FUSION-SL 4.2 MP (Peqlab)

Gel DocumentationGelDoc XR (Bio-Rad)

Gene PulserXCell (Bio-Rad)

Glass pipettes (5 ml, 10 ml, and 20ml) (Brandt)

Heraeus Multifuge 1S-R (ThermoFisher)

Inverted Microscope Axiovert 200M (Carl Zeiss)

Cooling Table Centrifuge Hereus Fresco 21 (ThermoFisher)

Cooling Centrifuge Sorvall® RC 5C plus (ThermoFisher)

Magnetic stirrer MR2002 (Heidolph)

Mini-Protean 3 Cells (Bio-Rad)

Multiplate Absorbance Reader Spectrafluor Plus (Tecan)

Nanodrop (Peqlab)

PerfectBlueGelsystem Mini S (Peqlab)

PerfectBlue gel system Maxi S (Peqlab)

PhmeterinoLab pH720 (WTW)

Photometer Ultrospec 10 (GE Healtcare)

Pipettboyacu (IBS)

Pipettes 0.2 - 1000µl (Gilson)

Shakers for Blots Wt-17 (Biometra)

Power-PacTM Universal (Bio-Rad)

TC10 ™ Automated Cell Counter (Bio-Wheel)

Thermomixer 5496 (Eppendorf)

Table Centrifuge 5415D (Eppendorf)

Transblot® SD Semi-dry Transfer Cell (Bio-Rad)

Overhead shaker Rotator SB2 (Stuart)

Vortex Mixer 7-2020 (Neolab)

Cell culture Incubator HeraCell 150

Cell Culture Plant Clean Air DLF / Rec 4 KL 2A (ThermoFisher)

6.13 Reagents

Table 10

DNA preparation from bacteria ("Mini" scale)	
S1	50 mM Tris
	10 mM EDTA
	100 µg/ml RNase A
	pH 8.0
S2	200 mM NaOH
	1 % (v/v) SDS
S3	2.8 M Potasium (K) acetate
	pH 5.1
TE	10 mM Tris
	1 mM EDTA
	pH 7.8

Table 11

SDS polyacrylamide gel electrophoresis (SDS-PAGE)		
2x sample loading buffer (SLB)	150 mM Tris	
	2 mM EDTA	
	20 % (v/v) glycerol	
	4 % (v/v) SDS	
	10 % β-mercaptoethanol	
	Bromophenol blue	
	pH 6.8	
10 x Laemmli running buffer	250 mM Tris	Used 1x for running polyacrylamide gels
	1.92 M glycine	
	1 % (w/v) SDS	
10 x TBS-T	100 mM Tris	Used 1x for preparing antibody dilutions and washing nitrocellulose membranes
	1,5 M NaCl	
	1 % (v/v) Tween	
	pH 7.5	
Transfer Buffer	50 mM Tris	

	150 mM NaCl	
	0.04 % (v/v) SDS	
	20 % (v/v) methanol	
Agarose gel electrophoresis		
50x TAE	2 M Tris	used 1x for pouring agarose gels and as running buffer
	50 mM EDTA	
	5.7 % (v/v) acitic acid	
	pH 8.0	
10 x TBE	990 mM Tris	used 0.5x for pouring agarose gels and as running buffer
	40 mM EDTA	
	990 mM borate (boric acid)	
	pH 8.0	

Wasch buffer B (IP):	**Wasch buffer C (IP):**	**Wasch buffer D (IP):**
150 mM NaCl	500 mM NaCl	100 mM Tris pH 8,0
1 mM Tris pH 7,6	1 mM Tris pH 7,6	1 % NP40
2 mM EDTA	2 mM EDTA	
1 % NP40	1 % NP40	

Table 12

Bacteria medium	
Lysogeny broth (LB) medium (Lennox)	Roth
Lysogeny broth (LB) agar	LB medium with 15 g/l agar

Table 133

RIPA (Rhadioimmunoprecipitation assay)-Buffer	
Tris	50 mM
NaCl	150 mM
TritonX100	1 %
SDS	0,1 %
Deoxycholat	1 %
pH 7,2 Setting	
+ Protease inhibitor Tablet(Complete Mini, Roche) and phosphatase-Inhibitor 1Tablet for each/10ml	

Buffer

Table 144

Chemical	
Thapsigargin (stock concentration 10 mM)	Sigma-aldrich dissolved in DMSO
Tunicamycin (1mg)	Sigma-aldrich dissolved in DMSO

7 Methods

7.1 Cell culture

All cell culture work was carried out in class II biosafety cabinets using sterile technique (HeraSafe, Heraeus). Human embryonic kidney (HEK293A), 293T and retinal pigment epithelial (RPE1) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100U/ml of penicillin and 100mg/ml streptomycin (P/S). All cells were cultured in a humidified 5% CO2 atmosphere at 37°C.

7.2 Freezing the cells

For freezing, a 90 % confluent 145 mm dish of cells was trypsinized and neutralized using 10 ml of fetal calf serum (FCS) supplemented medium. Cells were then pelleted using a centrifuge (5810R, Eppendorf) at 320xg for 5 minutes. Supernatant was discarded and pellet was washed in PBS. Next, the pellet was resuspended in cold FCS containing 10 % dimethyl sulphoxide (DMSO, Sigma). Finally, the cells were aliquoted into cryovials and were stored at –80°C overnight, and then transferred to liquid nitrogen.

7.3 Thawing cells

Cells were removed from liquid nitrogen and thawed rapidly at 37°C. The contents of the cryotube were layered onto 10 ml of DMEM medium containing 10 % FCS and P/S. Cells were then pelleted by centrifugation at 320xg for 5 minutes. The cell pellet was washed with 10 ml culture media. The cells were centrifuged for a further 5 min. The pellet was resuspended in a suitable volume of culture medium depending on the cell number and was then counted using a cell counter (TC10 ™ Automated Cell Counter (Bio-Wheel).

7.4 Virus stock

7.4.1 Production of $KSHV_{LYT}$-stock and determination of its titer

In this study a $KSHV_{LYT}$ was used. The constitutive RTA expressing KSHV-lytic has already been constructed in the lab by inserting a constitutively active promoter (promoter of phosphoglycerate kinase 1 (P_{PGK}) in front of ORF50 [31].The gene encoding RTA is depicted in Figure 16.

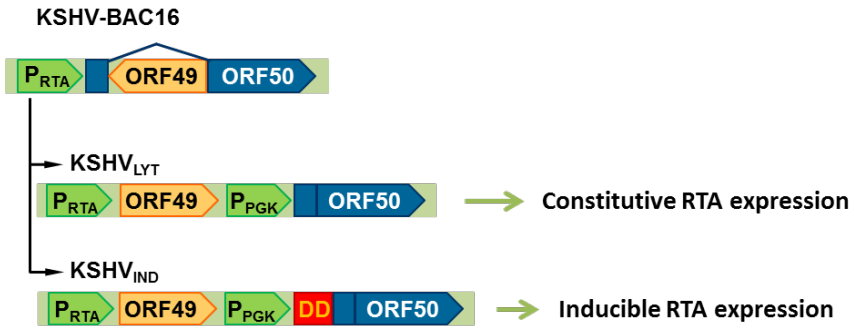


Figure 16: Construction of $KSHV_{LYT}$ via insertion of promoter of phosphoglycerate kinase 1. A promoter, encoded in humans by the PGK1 gene (PGK), was inserted between locus of ORF49 and ORF50 genes [31].

7.4.1.1 Inoculation of the virus stock

$2.5x10^5$ RPE1 cells were grown in 6 well plates a day before and infected at MOI of 0.05 TCID50/cell either wt $KSHV_{LYT}$ or mutant $KSHV_{LYT}$. Infected cells then were distributed in 15cm-dishes (10x, 20ml/dish) and incubated at 37°C until all cells are infected, detectable by foci (about 4 days after inoculation).

7.4.1.2 Harvest of the viruses

The supernatant of the infected cells were pooled into 250ml centrifuge flasks. Centrifugation was done for 15 minutes at 4°C and at, 5000xg (rotor F14-6x250y) after which the supernatant was transferred into a fresh centrifuge flask. These were then again centrifuged for 3 hours at 4°C, 15000xg (rotor F14-6x250y). The first short centrifugation was needed to remove all cell debris from the medium and the second long centrifugation step was required to pellet the virus. The supernatants were then discarded. The pellet were resuspended in 1ml DMEM and stored over night at 4°C. After resuspension pellets were distributed in small aliquots in 1.5 ml microtube and stored at -80°C.

7.4.1.3 Titration of the virus stock

$1.15x10^6$ RPE1 cells were suspended in 65 ml DMEM medium, with 10% FCS and 1% P/S and were then seeded 100µl in 96-well plates (6 plates in total). On the next day, virus dilutions were prepared in 3 rows of glass test-tubes; each row was distributed in 2 plates (one centrifuged and one not.), in order to have three different plate-replicate per condition. First

10µl of virus stock was added to 990 µl medium (1:100, 10^{-2} dilution) in a 1.5ml tube and vortexed. All glass test-tubes were filled with 2.7ml medium. 300µl of diluted virus was added to 3 tubes, vortexed and 300µl was transferred stepwise to the next 3 tubes. This step must be performed 8 times insuccession to prepare a dilution row from 10^{-3} to 10^{-10}. By using a multi-step pipette (with an extra 200µl tip mounted on the reservoir), 100µl of virus dilution was pipetted in the appropriate wells. 3 plates with 3 different dilution rows were centrifuged for 30 minutes at speed of 1065 x g and 37°C. All plates were read 7-10 days after titration.

7.4.1.4 Viral replication kinetics

For replication kinetics of the virus, cells were seeded in 6-well dishes. The cells were infected in triplicates without centrifugal enhancement by seeding 1.5 x 10^5 RPE1 or IRE1-knock out RPE1 or transduced RPE1-IRE1-HA at MOI of 0.05 TCID50/cell. Virus dilution used for initial infection was titrated to determine input titer. At 4 hours after infection cells were washed twice with PBS and 3 ml full DMEM medium with antibiotic and 10% FCS was added. Supernatants were then subsequently harvested at different times post-infection and 3 ml of medium was added to the infected cells. The collected supernatants were subjected to virus titration (see section 7.4.4).

7.4.2 Transfection and transduction

7.4.2.1 Transfection of plasmid DNA

$3x10^5$ HEK293A cells were seeded on 6 well plates. The day after seeding, the cells were transfected using 1-3 µg of plasmid DNA. To this end, plasmid DNA was first diluted in 200 µl DMEM without antibiotic and protein supplements. After 2 minutes of incubation time polyethyleneimine (PEI) was added in a ratio of 1:4 (pcDNA/PEI) and DNA-PEI mixes were incubated for an additional 20 minutes. After the final incubation the DNA-PEI mixes were diluted in 500µl DMEM + 10%FCS and added to the cells. 6 hours after transfection all old media was replaced with fresh DMEM+10% FCS and 1% penicillin/ streptomycin.

7.4.2.2 Transfection of BAC DNA

In order to reconstitute $KSHV_{LYT}$ wild type or mutant SOX (P176S), BAC DNA was transfected in RPE1 cells. 1.5 x 10^5 cells were seeded on 6 well plates. After overnight incubation, the cells were transfected with 3 µg of BAC DNA using the Polyfect Reagent. BAC DNA was diluted in 200 µl DMEM withoutantibiotic and protein supplements. 12 µl of Polyfect was added and DNA-Polyfect mixes were incubated at RT for 20 minutes. After incubation the DNA-Polyfect mixes were diluted in 500 µl DMEM + 10% FCS and added to

the cells very carefully. When green cells were observed, all cells were trypsinized and transferred into a 10 cm plate. These cells were then used for virus expanding.

Another KSHV mutant used in this study is $KSHV_{IND}$ which was constructed on the basis of $KSHV_{LYT}$ by inserting a destabilizing domain with the FKBP12 sequence in front of ORF50. Fusion of DD-FKBP12 to the protein results in degradation of the protein upon translation. The fusion protein is then stabilized by addition of an FKBP12 ligand such as FK-506 [31, 96]. The gene encoding RTA is depicted in Figure 16.

7.4.2.3 Retroviral transduction

Retroviral transduction is a widely-used method for the stable delivery and the expression of genetic material into a eukaryotic cell. The Phoenix cell line was used for production of retroviral vectors. This packaging cell line is based on HEK293T cells that express the gag, pol, and env genes of the Moloney murine leukemia virus.

7.4.2.3.1 Production of retrovirus

7.4.2.3.2 Transfection of Phoenix cells

Phoenix cells were transfected using polyethylenimine (PEI). 3 x 10^6 cells were seeded on 100 mm dishes. After overnight incubation cells were transfected using 8 µg of plasmid DNA (pMSCV or pRetro). In order to do this plasmid DNA was first diluted in 500 µl DMEM without supplements. In parallel 32 µl PEI was also diluted in 500 µl DMEM without supplements. After 10 minutes of incubation both mixes were combined and incubated for an additional 20 minutes at room temperature. The mixture was then added into the cells. The medium of PEI-transfected cells was changed after 12 hours. Supernatant containing virus were collected 48 h and 72 h hours after transfection, sterile filtered using a 0.45 µm filter and stored at -80°C.

7.4.2.4 Retroviral transduction of cells

For transduction 3 x 10^4 RPE1 cells were seeded in 2 wells of a 12-well plate. Old media was replaced the following day with 1.5 ml of retrovirus containing supernatant supplemented with 5µg/µl Polybrene. Cells were then centrifuged at 37 °C and 1000x g for 30 minutes. After 6 hours the virus-containing media was replaced with DMEM + 10 % FCS. The transduction procedure was repeated following an overnight incubation. If cells were transduced with pRetro-derived retrovirus they were used up to 4 weeks post transduction. If cells were transduced with pMSCV derived retrovirus they were selected by using 1 µg/µl

puromycin. This selective pressure was maintained while those cells were in culture; however the Puromycin was removed before infection or transfection experiments.

7.4.3 RT PCR

7.4.3.1 RNA extraction and DNase treatment

Total RNA was extracted from 2.5-5x10^5 RPE1 cells. Cells were lysed in 400µl lysis solution RL (innuPREPRNA Mini Kit, Qiagen, Germany). Washing steps and the elution step were done following the manufacturer's instructions. Finally RNA was eluted with 44µl of RNAse free water. To remove trace DNA contamination from purified RNA the TURBO-DNA-free kit was used. The extracted RNA was incubated with 5µl 10x DNase Buffer and 1µl of DNase for 30 min at 37°C. To inactivate and remove the enzyme DNase reagent was then added and incubated for 5 min at RT. Elutions were then pelleted by subsequent centrifugation at 11000xg for 1.5 min. The RNA-containing supernatant was transferred into a new microtube. RNA was quantified using a ND-1000 spectrophotometer.

7.4.3.2 cDNA synthesis

For cDNA synthesis, 1 µg of RNA was used. The obtained RNA was set with water to a total volume of 11µl. A mixture of 1µl (100 pmol) oligo (dT) or Random oligo, 2µl dNTPs (20 M), 0.5µl (20 U) of RNase inhibitor, 5µl MMLV-RT buffer (10X) and 1µl (200 U) MLV reverse transcriptase was then added. This was followed by incubation for one hour at 42 ° C. In order to inactivate the reverse Transcriptase enzyme, the mixture was heated at 70 ° C for 10 minutes.

7.4.4 Immunoblotting

7.4.4.1 Sample preparation

7.4.4.1.1 Protein extraction

5x10^5 cells were washed with PBS and then lysed directly with 100 µl RIPA buffer. Samples were harvested and incubated on ice for 30 min and centrifuged 10 minutes at full speed at 4°C. Supernatant were collected in fresh 1.5 ml microtubes. The protein concentration was measured by BCA Protein Assays. 6xLaemmli buffer was then added to the samples according to their concentration and heated at 95°C for 10 min. Samples were loaded in a SDS acrylamide gel and run at 80V, and 400mA until marker bands moved to the end of gel.

7.4.4.2 Western Blot

7.4.4.2.1 Blotting, blocking and incubating

After running, the gel was blotted into the nitrocellulose membrane (0.1A, 60 min). The membrane was blocked for 1 hour with 5 % non-fat dry milk or 5% BSA in 1% TBS–Tween, and incubated overnight at 4 °C with primary antibodies against the proteins of interest. Blots were washed 5 times with TBS-T at intervals of 5 min before 1 hr incubation with the appropriate horseradish peroxidase (HRP) conjugated secondary antibodies in 5 % non-fat dry milk in TBS-T at room temperature. All blots were washed 5 times with TBS-T at intervals of 5 minutes each before developing. HRP-conjugated antibody was detected using ECL western blotting detection reagent. Chemiluminescence was measured by autoradiography using Hyperfilm ECL or the Fusion machine FUSION-SL 4.2 MP (Peqlab).

7.4.5 *En passant* BAC mutagenesis

7.4.5.1 Construction of recombinant KSHV P176S mutSox

Because of the large size of the virus genomes (140kb for KSHV), they are unable to be cloned into a standard plasmid. Instead they can be cloned into BACs and propagated in bacteria using a two-step red-mediated recombination method previously reported as “En Passant” mutagenesis. First, the en passant marker cassette was PCR amplified. The resulting PCR product was then cloned into the unique restriction site chosen in the insert, which resulted in an applicable transfer construct. The transfer construct was then amplified by PCR using primers that contain the extensions of 40–50 bases for Red based recombination into the target sequence. With first Red recombination, the linear DNA was introduced into the target sequence. At the end of the procedure, the marker cassette was released from the co-integrate following in vivo cleavage of the I-SceI site and a second Red recombination [97].

7.4.5.2 Preparation of electrocompetent GS1783

E. coli strain GS1783, a derivate of EL250 (16) containing an L-arabinose inducible I-sceI expression cassette, was used. The Red recombination system is heat-inducible at 42°C. Small cultures of GS1783 with the KSHV-BAC were grown overnight, following which they were incubated in 200 ml LB containing 30μg/ml chloramphenicol at 30°C until OD600 reached 0.5–0.6.The culture was immediately transferred to a heat bath set to 42°C, 220 rpm for 30 min. The bacteria culture were then chilled for 20 min in ice bath, and centrifuged for 5 min at 4°C, 5000xg. The supernatant was discarded and the pellets were resuspended in 20 ml 10% ice-cold glycerol, spun down for 10 min at 4°C, 5000xg. The supernatant was discarded and

the bacteria were resuspended with 10% glycerol in a total volume of 2ml.About 130µl of suspension were aliquoted in fresh cooled 1.5ml micotubes and stored at -80°C.

7.4.5.3 PCR product for "En passant" mutagenesis

The PCR consisted of 10 ng template plasmid (pEPkan-S) and 10 pmol of each primer using a standard Taq polymerase protocol. Cycles: 2 min 95°C, 35x (30 s 95°C, 30 s 58°C, 1 min 72°C), 5 min 72°C, ∞ 4°C. The PCR products were digested with 1µlDpnI and 5µl 10x FD buffer and incubated 15min at 37°C to remove template DNA. The PCR product was run on a 0.6% agarose gel and purified with a gel extraction kit (NucleoBond Gel and PCR Clean-up, Macherey-Nagel).

7.4.5.4 Electroporation and first red recombination

200ngof PCR product (or other linear DNA) was added to 50µl of recombination and electrocompetent bacteria. This DNA/bacteria mixture was then transferred to a chilled electroporation cuvette. Samples were immediately electroporated in 2 mm cuvettes with 25 kV/cm (1.5 kV), capacitance of 25µF and 200 Ω resistances. Bacteria were removed from the cuvette with 1 ml LB broth without antibiotics and were shaken for 1h at 30°C. 100µl bacteria suspensions were then plated on a LB agar plate with 30µg/ml chloramphenicol and 30µg/ml kanamycin with the remaining volume plated on another identical plate. Plates were incubated for approximately 24h at 30°C. The positive co-integrates were identified from colonies using colony PCR, and positive clones were sent for sequencing.

7.4.5.5 Resolution of co-integrates (second recombination step)

Bacteria harboring positive co-integrates were inoculated into 2 ml of LB broth with 30 µg/ml chloramphenicol while shaking at 220 rpm for 1–2 h at 32°C until the solution became faintly cloudy. 1 ml of pre-warmed LB broth with 30µg/ml chloramphenicol and 2% L-arabinose was then added to the culture. After 1 h shaking at 30°C at 220 rpm, the culture was transferred to a 42°C water bath and was shaken for another 30 min at 220 rpm. The culture was then transferred to a shaker set at 30°C and shaken for 2–3 h at 220 rpm. OD600 of the culture was then measured. 5–10µl of a 1:1000 (OD600 < 0.5) or a 1:10,000 (OD600 > 0.5) dilution was plated at an LB agar plate with 30µg/ml chloramphenicol and 1% L-arabinose.

The plates were then incubated at 30°C for 1–2 days until bacteria colonies grew**.** To check colonies, some replicated bacteria were picked onto plates containing30µg/ml chloramphenicol and 30µg/ml chloramphenicol plus 30µg/ml kanamycin**.** The positive clones were confirmed via sequencing. BAC was purified from the bacteria using a midi prep kit.

Purified BAC DNA was confirmed by restriction digestion and was then used in transfection of cells.

7.4.6 IRE1 knocks out RPE1 cell

7.4.6.1 CRISPR CAS9

Targeted nucleases are powerful tools for mediating genome alteration with high precision. The RNA-guided Cas9 nuclease is derived from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system. This system can be used to facilitate efficient genome engineering in eukaryotic cells by simply specifying a 20-nt targeting sequence within its guide RNA. The gRNA forward contains 20 nucleotides begining with G (G-(N19)). The reverse primer (N19) is complement to the forward primer without C at the 3′ end as this is a part of the vector. The gRNA has replaced the linker sequence of the vector pSicoR-CRISPR-Cas without changing or inserting any other nucleotides. The vector must not be dephosphorylated because the oligonucleotides are unphosphorylated. Re-ligation of the vector is excluded due to the asymmetry of the BsmBI (Esp3I) restriction site [87].

7.4.6.2 Oligo design for knockout with CRISPR-Cas

- fwd oligo: 5´ ACCgRNA G-(N19) 3´
- 5´BsmBI overhang: rev oligo: 5´ AAACgRNA (N19) 3´

Table 15

Knock out gene	Location		gRNA sequence	Sorce	PAM sequence
Human-exon1-crispr-IRE1-1ko	Chr 17:771-793	-	**ACC**GCATGGCGAGGACTCGGCCC **AAAC**GGGCCGAGTCCTCGCCATG	IRE1 human	TGG
Human-exon1-crispr-IRE1-2ko	Chr 17:858-880	+	**ACC**GCTGCTGCTGCCCGGCCTCG **AAAC**CGAGGCCGGGCAGCAGCAG	IRE1 human	GGG
Human-exon2-crispr-IRE1-3ko	Chr 17	-	**ACC**GTTTGCATGCTGTCAGCAAG **AAAC**CTTGCTGACAGCATGCAAA	IRE1 human	AGG
Human-exon4-crispr-IRE1-4ko	Chr 17	+	**ACC**AGCGTATACAGGCTGCCATC **AAAC**GATGGCAGCCTGTATACGC	IRE1 human	TGG

7.4.6.3 Dissolve and annealing of oligoes (gRNA)

All oligonucleotides should firstly be dissolved and annealed. Therefore, forward and reverse oligonucleotides were diluted in 90µl dH2O and then heated at 95°c for 5 minutes and incubated on ice for 10 minutes.

7.4.6.4 Linearize CRISPR-CAS9-gRNA vector (RP418- puromycin resistance)

The Master Mix was prepared with the following protocol:

Table 16

RP-418 vector	3µg
BSMBI(FD)	1µL
10X FD buffer	3µl
DTT(1mM)	1.5µl
dH2O	30µl
At 37°c for 30-45 min	

1% agarose gel was prepared in 1% TAE buffer and all samples were run at 120V for 60min. DNA was extracted from the gel using an extraction kit (NucleoBond Gel and PCR Clean-up, Macherey-Nagel). For ligation 10µl of the annealed DNA oligos with 100ng of linearized vector, 1µl T4 ligase and 10xT4 ligase and dH2O were mixed in a total volume of 35 µl and incubated at 16°C overnight.

7.4.6.5 Transformation

50 µl of competent bacteria (DH10B) were transformed with 2 µl of ligation product in a 2mm cuvette at 25 kV. The bacteria were then grown in 1ml LB at 37°C for 1 hour in a shaking thermoblock. 100 µl of samples were then plated on agar plates (containing ampicillin) and incubated at 37°C, overnight.

7.4.6.6 Colony preparation

From each ligation plate, 5-10 colonies were picked and inoculated in 5ml LB with Ampicillin and grown at 37°C at 160 rpm overnight. DNA was extracted by using the mini plasmid isolation protocol. To check whether the insertion was successful, the DNA from each clone was amplified by colony PCR using EF1A as a reverse primer and primers specific to the gRNA as a forward primer. All positive clones were subjected to extract DNA using midi preparation kit.

7.4.6.7 293T cell transfection with lentiviral

In 10cm dishes $4x10^6$ 293 cells were seeded. The master mix was prepared follows; a DNA of transgene: viral package (PMSCV8.912): viral envelope (PMDG2) constructs ratio of 4:3:1 (8µg total DNA) was prepared. PEI was then added to the diluted DNA with a ratio of 4:1 and vortexed and incubated at RT for 15 min. The whole mixture was then added to the cells. The supernatant were harvested 24-48 hours after transfection and stored at -80°C.

7.4.6.8 293A / RPE1 cell infection

$1.5x10^6$ cells (either HEK293A or RPE1) were seeded in 6-well plates. After cell reached confluency of 50-75% the next day, old media was removed and 3 ml of prepared lentiviral supernatant with 1:2000 polybrene was added to each well. All plates were centrifuged for 30min at 1065 x g, 37°C. After three days, all cells were transferred to a 10cm dish and treated with an appropriate selection reagent. In this experiment HEK293A and RPE1 were treated with 2-3µg/µl and 10µg/µl of puromycin (stock concentration of 10mg/ml), respectively.

7.4.6.9 Single clone preparation

To select a single cell of a clone, a serial dilution for each positive clone was performed. 2 x 10^4cells were counted and used to make a first dilution of cells in 96 well plates.200µL of the cell suspension were added to well A1. Then using a single channel pipette 100µL was quickly was transferred from the first well to well B1 and mixed gently by pipetting. Using the same tip these 1:2 dilutions were repeated for each well in the first column of the plate. As a final step 100µL from H1 was discarded. With an 8-channel pipette an additional l00µL of medium was then added to each well in column 1 (final volume of cells and medium is 200µL/well). Then using the same pipette l00µL was transferred from the wells in the first column to the next column (Al through H1 and A2 through H2) and was mixed gently by pipetting. This was prepared in a stepwise fashion for each column in the plate using the same tips, creating multiple 1:2 dilutions. 100µL of media was then discarded from each of the wells in the last column (A12 throughH12) so that all wells have 100µL of cell suspension. The final volume of all wells was brought to 200µL by adding 100µL medium to each well. The plate was incubated at 37°C in a humidified CO2 incubator.(Figure 17) [99]. Cells were checked after 4-5 days and all wells with only one single colony were marked. These colonies were then subcultured into larger vessels.

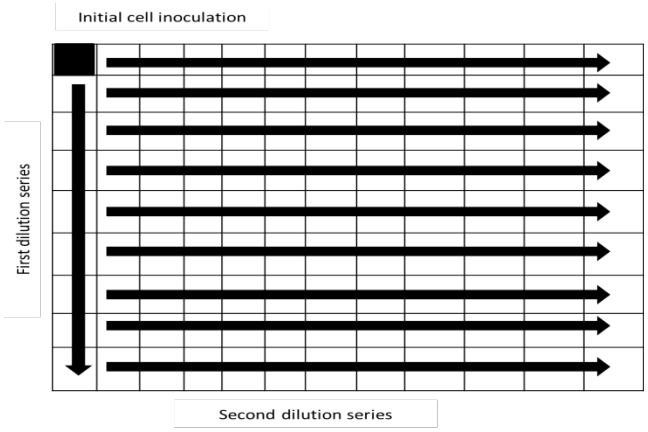


Figure 17: Single Cell Cloning by Serial Dilution (Initial plate setup). Graphical is a representation of preparing a serial dilution in 96 well plates.

7.4.7 Immunoprecipitation

7.4.7.1 Transfection

$4x10^6$ HEK293 cells, maintained in DMEM and 10% FCS plus 1% Penicillin/ Streptomycin, were seeded on 10 cm plates 24h before transfection. Each plate was transfected with 4 µg plasmid expressing ORF67, UL50, UL56 (all flag-tagged), IRE1-HA-tagged in 500µl DMEM (FCS and antibiotic free) and the transfection reagent, PEI (Polyethylenimine), in proportion of 1:4 µg DNA reagent which was mixed gently and incubated at room temperature for 15 minutes. After incubation each transfection reagent/DNA mixture was added to cells in a drop-wise manner and mixed to evenly distribute transfection reagent and DNA complexes. Treated cells were then incubated for 48 hours at 37° C. HEK-293A cells were transfected using Polyfect.

7.4.7.2 Preparation of lysates

The samples were lysed in 1 mL RIPA buffer for 30 min on ice and centrifugated at full speed (15000x g) 4°C (Table Centrifuge 5415D, Eppendorf) after which the cells debris was removed. The lysate was diluted in 2x Laemli buffer at a 1:10 lysate/buffer ration and boiled at 95°C and part of the treated lysate was used as a control with the rest subjected to co-immunoprecipitation.

7.4.7.3 Pre-clearing the lysates

50µl of PGS/ PAS beads were added to the lysate and samples were incubated for 2 hours at 4°C with gentle agitation. The lysate was spun down at full speed (15000x g) at 4°C for 1 min. The beads pellet was discarded and the supernatant was kept for immunoprecipitation.

7.4.7.4 Co-immunoprecipitation

After pelleting the beads, the supernatant was mixed with a target-specific antibody in the ratio of 1: 500. This was followed by incubation at 4°C in an overhead rotary mixer overnight. The next day 50µl of Protein A or G Agarose (PAA, PGA) per sample was added and again agitated for 2 h at 4°C. This was followed by three washing steps with buffer B, two with buffer C and one with buffer D (table 11). The agarose beads were centrifuged after each washing step for 20 sec at 16000x g and the supernatant was discarded. After the last final washing step the buffer was completely removed and the Agarose beads were resuspended in 80µl 4x sample buffer and boiled at 95°C for 5 min. The Agarose was again centrifuged and the supernatant loaded onto a SDS polyacrylamide gel. This was followed by a western blot against the co-precipitated protein.

7.4.8 Plasmids

7.4.8.1 Construction of plasmids (hIRE1, ORF67, ORF40/41)

The ORF67, ORF40/41 and ORF40 coding sequences (C-terminal FLAG tag before the stop codon) were PCR amplified from KSHV BAC DNA using the designed primers. The PCR product and the pCMV mammalian expression vector (pcDNA3) were then digested by restriction enzymes (described in table 6 in material sections). The digested PCR was then inserted into the digested vector. The vector containing PCR fragments were cloned into bacteria (DH10B). All the mutant plasmids were similarly constructed using primer sequences listed in Table 7. The hIRE1 plasmid has already been generated by Sebastian Stahl [69].

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9 Appendix

9.1 Curriculum vitae

9.1.1 Personal Details:

Family Name: Mousavizadeh

Name: Leila

Date of Birth: 1975/09/16

Place of Birth: Zarrinshahr

Nationality: Iranian

Employment Status: PhD student

Relationship Status: Single

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9.1.2 Academic Education:

9.1.2.1.1 PhD student, will graduated in Two months (2013-2017)

Virus-Host Interaction, Heinrich- Pette- Institute, Leibniz Institute for Experimental Virology, Martini strasse. 52, 20251 Hamburg, Germany

Thesis Title: “Modulation of the unfolded protein response by Kaposi’s sarcoma-associated herpesvirus”

Supervisor: Prof. Dr. Wolfram Brune.

9.1.2.1.2 M.Sc. in Medical Virology (2001-2003)

Department of Medical Virology, Medical School, Iran University of Medical Sciences, Tehran, Iran

Third score in MSc. Entrance exam

Thesis Title: “Detection of Cytomegalovirus infection in Transplantation Patients by IF and Comparison with PCR Method”

Supervisor: Prof. Dr. Shamsi-Shahrabadi, Mahmoud.

9.1.2.1.3 B.Sc. in Laboratory Sciences (1997-2001)

Medical School, Shahre-Kord University of Medical Sciences, CharmahaloBakhtiari, Iran and Medical School, Isfahan University of Medical Sciences, Isfahan, Iran.

9.1.2.1.4 Diploma and High school (1990-1993)

Natural sciences, Khadije-Kobra high school, Zarrinshahr, Isfahan, Iran

9.2 Toxicity of chemicals (GHS classification)

substance	GHS symbol	GHS Hazard Phrases	GHS Precaution Phrases& GHS Response Phrases
2-mercaptoethanol		H301 + H331-H310-H315-H317-H318-H373-H410	P261-P280-P301 + P310 + P330-P302 + P352 + P310-P305 + P351 + P338 + P310-P403 + P233
Acetic acid		H226-H314	P280-P305 + P351 + P338-P310
acrylamide		H301-H312 + H332-H315-H317-H319-H340-H350-H361f-H372	P201-P280-P301 + P310-P305 + P351 + P338-P308 + P313
ammoniumbicarbonate		H302	P301 + P312 + P330
ammoniumpersulfate		H272-H302-H315-H317-H319-H334-H335	P220-P261-P280-P305 + P351 + P338-P342 + P311
ampicillin		H315-H317-H319-H334-H335	P261-P280-P305 + P351 + P338-P342 + P311
bis-acrylamide		H302 + H332	
Boric acid		H360FD	P201-P308 + P313
chloramphenicol		H350	P201-P308 + P313
deoxycholate		H302-H335	P301 + P312 + P330
dithiothreitol		H302-H315-H319-H335	P261-P305 + P351 + P338

EDTA		H319	P305 + P351 + P338
ethanol		H225-H319	P210-P280-P305 + P351 + P338-P337 + P313-P403 + P235
Ethidium bromide		H302-H330-H341	P260-P281-P284-P310
FK506		H316-H319	P364-P280-P332+313-P305+351+338-P337+313
Hydrochloric acid		H290-H314-H335	P261-P280-P305 + P351 + P338-P310
hygromycin B		H300 +H310 + H330-H318-H334	P260-P264-P280-P284-P301 + P310-P302 + P350
isopropanol		H225-H319-H336	P210-P261-P305 + P351 + P338
kanamycin		H360	P201-P308 + P313
liquid nitrogen		H281	P202-P271 + P403-P282
methanol		H225-H301 + H311 + H331-H370	P210-P260-P280-P301 + P310-P311
penicillin		H317-H334	P261-P280-P342 + P311
protein A-agarose		H226	
protein G-agarose		H226	
puromycin		H373	

Sodium dodecyl sulfate		H315-H318-H335	P280-P304 + P340 + P312-P305 + P351 + P338 + P310
Sodium hydroxide		H290-H314	P280-P305 + P351 + P338-P310
streptomycin		H302-H361	P281
TEMED		H225-H302-H314-H332	P210-P280-P305 + P351 + P338-P310
Thapsigargin		H315-H319-H334-H335	P264-P280 –P261-P301+310-P330
Triton X-100		H302-H319-H411	P273-P280-P301 + P312 + P330-P337 + P313-P391-P501
Tunicamycin		H300	P264-P301 + P310-P302+352-P332+313-P362-P305+351+338-P337+313-P304+341-P342+311-P309+311-P304+340

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تقدیم به پدر و مادر عزیزم، ممنونم که صبورانه سالها دوری از مرا تحمل کردید تا در موقعیت امروز قرار بگیرم و امیدوارم خداوند عمر طولانی و با عزت نصیبتان کند تا بتوانم ذره ای از محبتتان را پاسخگو باشم.

9.4 Declaration of author

I hereby declare on oath, that I have written the present dissertation by my own and have not used other than the acknowledged resources and aids. The submitted written version corresponds to the version on the electronic storage medium. I hereby declare that I have not previously applied or pursued for a doctorate (Ph.D. studies).

Hamburg, December

Leila Mousavizadeh