1 **KSHV activates unfolded protein response sensors but suppresses downstream**
2 **transcriptional responses to support lytic replication**
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Abstract

Herpesviruses usurp host cell protein synthesis machinery to convert viral mRNAs into proteins, and the endoplasmic reticulum (ER) to ensure proper folding, post-translational modification and trafficking of secreted viral proteins. Overloading ER folding capacity activates the unfolded protein response (UPR), whereby displacement of the ER chaperone BiP activates UPR sensor proteins ATF6, PERK and IRE1 to initiate transcriptional responses to increase catabolic processes and ER folding capacity, while suppressing bulk protein synthesis. Kaposi's sarcoma-associated herpesvirus (KSHV) can be reactivated from latency by chemical induction of ER stress, whereby the IRE1 endoribonuclease cleaves XBP1 mRNA, resulting in a ribosomal frameshift that yields the XBP1s transcription factor that transactivates the promoter of K-RTA, the viral lytic switch protein. By incorporating XBP1s responsive elements in the K-RTA promoter KSHV appears to have evolved a mechanism to respond to ER stress. Here, we report that following reactivation from latency, KSHV lytic replication causes activation of ATF6, PERK and IRE1 UPR sensor proteins. UPR sensor activation is required for efficient KSHV lytic replication; genetic or pharmacologic inhibition of each UPR sensor diminishes virion production. Despite strong UPR sensor activation during KSHV lytic replication, downstream UPR transcriptional responses were restricted; 1) ATF6 was cleaved to release the ATF6(N) transcription factor but known ATF6(N)-responsive genes were not transcribed; 2) PERK phosphorylated eIF2α but ATF4 did not accumulate as expected; 3) IRE1 caused XBP1 mRNA splicing, but XBP1s protein failed to accumulate and XBP1s-responsive genes were not transcribed. Remarkably, complementation of XBP1s deficiency during KSHV lytic replication by ectopic expression inhibited the production of infectious virions in a dose-dependent manner. Therefore, while XBP1s plays an important role in reactivation from latency, it inhibits later steps in lytic replication, which the virus overcomes by preventing its synthesis. Taken together, these findings suggest that KSHV hijacks UPR sensors to promote efficient viral replication while sustaining ER stress.

Author summary

Human herpesvirus-8 is the most recently discovered human herpesvirus, and it is the infectious cause of Kaposi's sarcoma, which is why it's also known as Kaposi's sarcoma-associated herpesvirus (KSHV). Like all herpesviruses, KSHV replicates in the cell nucleus and uses host cell machinery to convert viral genes into proteins. Some of these proteins are synthesized, folded and modified in the endoplasmic reticulum (ER) and traverse the cellular secretory apparatus. Because the virus heavily utilizes the ER to make and process proteins, there is potential to overwhelm the system, which could impede viral replication and in extreme cases, kill the cell. Normally, when demands on the protein folding machinery are exceeded then misfolded proteins accumulate and activate the unfolded protein response (UPR). The UPR resolves ER stress by putting the brakes on synthesis of many proteins, while signaling to the nucleus to turn on a program that aims to correct this imbalance. Previous work has shown that KSHV is 'wired' to sense ER stress, which it uses to reactivate from a largely inactive state known as latency, in order to make more viruses. Specifically, a UPR sensor protein called IRE1 senses the accumulation of unfolded proteins in the ER and rededicates a gene called XBP1 to the production of a transcription factor called XBP1s through an unconventional cytoplasmic mRNA splicing event. XBP1s travels to the cell nucleus and stimulates the production of a collection of proteins that mitigate ER stress. In latently infected cells, XBP1s also binds to the KSHV genome and causes the production of K-RTA, a viral transcription factor that initiates the switch from latency to productive lytic replication. This achieves stress-induced initiation of KSHV replication, but nothing is known about how ER stress and the UPR affect progress through the KSHV replication cycle. Here we show that as KSHV replication progresses, all three known UPR sensor proteins, IRE1, ATF6 and PERK, are activated, which is required for efficient viral replication. Normally, activation of each of these three sensor proteins communicates a unique signal to the cell nucleus to stimulate the production of ER stress mitigating proteins, but in KSHV lytic replication all downstream communication is stymied. The failure to resolve ER stress would normally be expected to put the virus at a disadvantage, but we demonstrate that reversal of this scenario is worse; when we add extra XBP1s to the system to artificially stimulate the production of UPR responsive genes, virus replication is blocked at a late stage and no progeny viruses are released from infected cells. Taken together, these observations suggest that KSHV requires UPR sensor protein activation to replicate but has dramatically altered the outcome to prevent the synthesis of new UPR proteins and sustain stress in the ER compartment.

Introduction

Proteins destined to be secreted are synthesized in the endoplasmic reticulum (ER), where they are folded by chaperone proteins and modified by glycosyltransferases and protein disulfide isomerases. Demands on the cellular protein folding machinery that exceed ER folding capacity trigger ER stress, which is the accumulation of ER-localized misfolded proteins [1]. This accrual of misfolded proteins activates the unfolded protein response (UPR) to mitigate the stress [2–4]. The UPR attempts to resolve ER stress by transiently attenuating translation, increasing the folding machinery, increasing lipid biogenesis to expand ER surface area, and degrading misfolded proteins in a process called ER-associated degradation (ERAD). If proteostasis is not re-established the UPR switches from an adaptive to an apoptotic response.

The UPR is coordinated by three transmembrane sensor proteins that sample the ER lumen; activated transcription factor-6 (ATF6), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1 (IRE1). These sensor proteins are maintained in an inactive state by association of their lumenal domains with the ER chaperone BiP [5]. In response to ER stress, BiP is released to participate in re-folding reactions, allowing UPR sensor activation [6]. Together these three UPR sensors coordinate complementary aspects of an integrated transcription and translation program that mitigates ER stress.

ATF6 is a type II transmembrane protein and upon sensing unfolded proteins in the ER lumen, traffics to the Golgi apparatus, where it is cleaved by resident proteases site-1 protease (S1P) and site-2 protease (S2P) [7,8]. These cleavage events release the ATF6(N) fragment, which translocates to the nucleus to transactivate genes encoding chaperones, foldases and lipogenesis factors.

PERK activation by ER stress causes dimerization and autophosphorylation [9]. Active PERK phosphorylates serine 51 of eIF2α, which increases eIF2α affinity for its guanine exchange factor eIF2B [10,11]. This binding depletes the small pool of eIF2B, thereby inhibiting replenishment of the eIF2-GTP-Met-tRNA$^{\mathrm{Meti}}$ ternary complex required for translation initiation [12]. Bulk cap-dependent translation is attenuated, while a subset of uORF-containing mRNAs encoding stress response proteins are preferentially translated [13]. Activating transcription factor 4 (ATF4) is chief among these stress response proteins [14]; it translocates to the nucleus and drives the synthesis of genes products that mitigate ER stress by increasing a catabolic process known as autophagy and the antioxidant response [15,16]. ATF4 upregulates protein-phosphatase 1 α (PP1α) cofactor GADD34 (PPP1R15A) to resume translation by dephosphorylating eIF2α [17]. This direct control of protein synthesis by stress is known as the integrated stress response (ISR) [18,19]. During chronic or severe ER stress, ATF4 also upregulates the apoptotic effector CHOP [20–22].

IRE1 is a kinase and endoribonuclease ER transmembrane protein. ER stress triggers IRE1 dimerization, autophosphorylation, and activation of the cytosolic RNase domain that recognizes a conserved nucleotide sequence in two stem loops in the mRNA of *Xbp1* and excises a 26-nucleotide intron [23–25]. The tRNA ligase RTCB re-ligates cleaved *Xbp1* mRNA, generating a ribosomal frameshift that yields the active transcription factor XBP1s [26,27]. XBP1s translocates to the nucleus and drives production of chaperones, lipid synthesis proteins and proteins involved in ERAD [28]. The combined action of these gene products simultaneously increases ER folding capacity and decreases the load via ERAD. In a process called regulated IRE1-dependent decay (RIDD), IRE1 can cleave ER-targeted mRNAs with a stem loop that resembles that of XBP1, triggering mRNA degradation, which like PERK, is thought to attenuate translation [29,30].

137   UPR signalling is involved in many normal physiological processes and dysregulation can
138 contribute to the development and progression of human disease. The UPR is important for
139 differentiation of professional secretory cells like pancreatic beta cells and antibody-secreting
140 plasma B cells [31–33], and it is mechanistically linked to diseases such as Crohn's disease and
141 type 2 diabetes [34,35]. Several viruses have been shown to modulate UPR signalling during
142 infection [36].
143   KSHV is a gammaherpesvirus that causes Kaposi's sarcoma (KS), primary effusion lymphoma
144 (PEL) and multicentric Castleman's disease (MCD) [37–39]. Like all herpesviruses, KSHV can
145 establish a quiescent form of infection known as latency in which viral gene expression is
146 severely restricted and the genome is maintained as a nuclear episome. The virus can latently
147 infect a variety of cell types, but it is thought that life-long infection of human hosts is primarily
148 enabled by latent infection of immature B lymphocytes [40], followed by viral reprogramming
149 into an intermediate cell type that resembles a plasma cell precursor [41–43].
150   An essential feature of latency is reversibility, which is required for viral replication and
151 production of viral progeny. The true physiologic cues for KSHV lytic reactivation are not
152 known, but *in vitro* studies have implicated ER stress-activated signalling pathways.
153 Reactivation from latency requires the immediate-early lytic switch protein replication and
154 transcriptional activator (RTA), a transcription factor that initiates a temporal cascade of gene
155 expression [44,45]. RTA recruits host cell co-factors to transactivate viral early gene promoters
156 required for genome replication [46]. KSHV has usurped the IRE1/XBP1s pathway to regulate
157 latent/lytic switch. XBP1s binds to canonical response elements in the RTA promoter and drives
158 synthesis of the RTA lytic switch protein required for lytic reactivation [47–49]. Functional
159 XBP1s binding motifs have also been found in the promoter for v-IL6 [50], the KSHV homolog
160 of human IL6, which plays key roles in PEL and MCD cancers [51–53]. Thus, UPR activation
161 causes reactivation from latency and initiation of lytic gene expression. Interestingly, because the
162 XBP1 transcription factor is required for normal B cell differentiation into mature plasma cells
163 [32,33], it is likely that the viral acquisition of XBP1s target sequences hardwires KSHV
164 reactivation from latency to terminal B cell differentiation [47], although the physiological
165 consequences of this link are not known. In sharp contrast to these mechanistic linkages, nothing
166 is known about how ATF6 and PERK impact latent KSHV infection or reactivation from
167 latency.
168   KSHV replicates in the nucleus and usurps host cell machinery to transcribe, post-
169 transcriptionally modify, export and translate viral mRNAs. KSHV encodes structural and non-
170 structural proteins that are folded in the ER and traverse the secretory apparatus. UPR activation
171 during herpesvirus lytic replication has been reported, and there is evidence for UPR sensor
172 engagement by specific gene products, rather than simply by exceeding ER folding capacity [54–
173 56]. HSV-1 gB glycoprotein can bind PERK to promote virus protein production [57]. Nothing is
174 known about how KSHV lytic gene products affect UPR.
175   To better understand how KSHV usurps the UPR, we investigated UPR activation following
176 reactivation from latency in B cell- and epithelial cell-based models. We report that all three
177 proximal UPR sensors are activated following reactivation from latency; ATF6 is cleaved, PERK
178 and eIF2α are phosphorylated, and IRE1 is phosphorylated and *Xbp1* mRNA is spliced.
179 Furthermore, we determined that UPR sensor activation is pro-viral; pharmacologic or genetic
180 inhibition of each UPR sensor diminished virion yield from infected cells. Surprisingly, viral
181 proteins accumulated despite sustained phosphorylation of eIF2α throughout the lytic cycle,
182 suggesting that viral messenger ribonucleoproteins (mRNPs) may have unique properties that

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183 ensure priority access to translation machinery during stress. Remarkably, activation of proximal
184 UPR sensors during lytic replication failed to elicit any of the expected downstream effects:
185 ATF6(N) and XBP1-targets genes were not upregulated and ATF4 was not translated. This
186 suggests that the virus requires proximal activation of UPR sensors, but prevents the UPR from
187 mitigating stress and restoring ER homeostasis. Indeed, despite clear evidence of IRE1 activation
188 and XBP1 mRNA splicing, XBP1s protein failed to accumulate during KSHV lytic replication.
189 Remarkably, complementation of XBP1s deficiency during KSHV lytic replication by ectopic
190 expression inhibited the production of infectious virions. Therefore, while XBP1s plays an
191 important role in reactivation from latency, it inhibits later steps in lytic replication, which the
192 virus overcomes by tempering its synthesis. Taken together, these findings suggest that KSHV
193 hijacks UPR sensors to promote efficient viral replication instead of resolving ER stress.
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Results

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227 **KSHV lytic replication activates IRE1 and PERK but downstream UPR transcription**
228 **factors XBP1s and ATF4 fail to accumulate**
229 ER stress can induce KSHV lytic replication via IRE1 activation and downstream XBP1s
230 transactivation of the latent/lytic switch protein RTA [47,48] (Supplemental Fig. 1A and B).
231 However, it is not known whether the burden of synthesizing secreted KSHV proteins causes ER
232 stress over the course of the lytic replication cycle. To test UPR activation we used the TREx
233 BCBL1-RTA cell line that expresses RTA from a doxycycline (dox)-regulated promoter to
234 reactivate KSHV from latency [58]. We treated cells with dox for 0, 24, and 48 hours (h) and
235 immunoblotted for UPR proteins to determine their activation state and KSHV proteins to
236 monitor the progression of the lytic cycle from early (ORF45) to late (ORF65) stages (Fig. 1A).
237 As a positive control to ensure that UPR sensors were intact in our system, we also treated latent
238 and lytic cells with the SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) inhibitor
239 thapsigargin (Tg) to pharmacologically induce ER stress [59]. After 2 h of Tg treatment of
240 latently-infected cells, IRE1α was phosphorylated as determined by a migration shift in a total
241 protein blot, and a semiquantitative *Xbp1* RT-PCR splicing assay revealed that the majority of
242 *Xbp1* mRNA was spliced, causing a ribosomal frameshift and synthesis of XBP1s protein. Tg
243 activated the ISR as determined by phosphorylation of PERK (also determined by a migration
244 shift in total protein) and its downstream target eIF2α-Ser51, which resulted in translation of the
245 uORF-containing mRNA ATF4. Lysates collected at 24 and 48 h post dox addition displayed
246 strong accumulation of ORF45 and ORF65, respectively, and activation of both IRE1α and
247 PERK pathways. However, PERK activation and eIF2α phosphorylation did not result in full
248 ISR activation in lytic cells because there was minimal induction of ATF4. ATF4 also failed to
249 accumulate in Tg-treated lytic cells, suggesting that lytic replication prevents downstream
250 execution of the ISR when eIF2α is phosphorylated. Tg-treated lytic cells also displayed reduced
251 total IRE1α and XBP1s levels, even though *Xbp1* mRNA splicing was similar to Tg-treated
252 latent cells. These data demonstrate that KSHV lytic replication activates IRE1α and PERK UPR
253 sensor proteins but prevents the accumulation of XBP1s and ATF4 transcription factors required
254 for downstream transcriptional responses.

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256 **KSHV lytic replication activates ATF6 but downstream UPR transcriptional responses are**
257 **inhibited**
258 The third UPR sensor ATF6 is cleaved in the Golgi during ER stress to release its active N-
259 terminal fragment ATF6(N) that translocates to the nucleus and transactivates a variety of UPR
260 genes [8]. We observed elevated total endogenous ATF6 levels at 24 h post dox addition, which
261 diminished over the following 24 h (Fig. 1A). However, the ATF6 antibody that we employed in
262 this study could not detect endogenous ATF6(N) in any samples, including Tg-treated positive
263 controls. Previous reports show that the N-terminal fragment is susceptible to degradation [8,60].
264 Interestingly, we detected the emergence of an additional high MW species that reacted with the
265 ATF6 antiserum in lysates from dox-treated cells (Fig. 1A, marked with a *), which to our
266 knowledge has not been reported in the literature before.
267　　　To further investigate ATF6 cleavage in this system, we transduced TREx BCBL1-RTA cells
268 with a lentiviral vector encoding HA-epitope-tagged full-length ATF6 (HA-ATF6αFL) [61].

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269   Transduced cells treated with Tg revealed the detection of the N-terminal fragment (Fig. 1B, lane
270   2). Consistent with endogenous ATF6 in lytic cells (Fig. 1A), we observed the accumulation of
271   full-length HA-ATF6 at 24 hours post-dox followed by return to basal levels by 48 hours post-
272   dox. This ectopic expression system allowed us to monitor cleavage of the ~100 kDa HA-
273   ATF6αFL precursor into the ~60 kDa HA-ATF6α(N) fragment during lytic replication (Fig. 1B,
274   lanes 3-6). Indeed, by 24 hours post-dox the levels of HA-ATF6α(N) were higher than latently
275   infected cells treated with Tg. Interestingly, ATF6-target genes, GRP78 and HERPUD1 [62–64],
276   were not transactivated during lytic replication, even in cells treated with Tg (Fig. 1C). These
277   data show that ATF6 is activated during lytic replication but the N-terminal transcription factor
278   is unable to transactivate canonical target genes.
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280   PERK-dependent eIF2α phosphorylation during the KSHV lytic cycle
281   Because eIF2α can be phosphorylated on serine 51 by four possible eIF2α kinases activated by
282   different stresses (ER stress activates PERK, dsRNA activates PKR, nutrient stress activates
283   GCN2, oxidative stress activates HRI [9,65–67]), we investigated the specific contribution of
284   PERK to eIF2α phosphorylation during KSHV lytic replication. TREx BCBL1-RTA cells were
285   treated with PERK inhibitor GSK2606414 (PERKi) [68,69] concurrent with the addition of dox.
286   After 24 h and 48 h of treatment PERKi caused a reduction in PERK and eIF2α phosphorylation
287   compared to cells treated with dox alone (Fig. 2). Importantly, PERKi had no appreciable effect
288   on IRE1α activation, indicating that ER stress is still manifested during KSHV lytic replication
289   when PERK is inhibited, and PERK inhibition does not heighten this response. Interestingly, the
290   level of eIF2α phosphorylation following PERKi treatment did not return to baseline levels
291   observed in latently-infected TREx BCBL1-RTA cells, suggesting that other eIF2α kinases
292   besides PERK may be activated during lytic replication. Furthermore, treating lytic cells with
293   PERKi revealed that even though ATF4 fails to accumulate during lytic replication with or
294   without Tg treatment, PERK-mediated phosphorylation of eIF2α may still inhibit bulk protein
295   synthesis, as evidenced by the increased levels of the early viral gene product ORF45 (Fig. 2A,
296   compare lanes 9 vs 10 and lanes 11 vs 12).

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298   Failure of XBP1s and ATF4 to accumulate is not specific to the type or duration of ER
299   stress
300   We previously observed UPR sensor activation but failure to accumulate active UPR
301   transcription factors XBP1s, ATF4 and ATF6(N) following a brief 2 h pulse of Tg (Figs. 1A, B).
302   We confirmed these observations by treating latent and lytic TREx BCBL1-RTA cells with Tg or
303   tunicamycin (Tm), which induces ER stress by blocking protein N-linked glycosylation in the
304   ER [70,71], over a range of incubation times (1h, 4h, 8h). Maximal *Xbp1* mRNA splicing was
305   observed after latent cells were Tg-treated for 1 h or Tm-treated for 4 h (Fig. 3); these slower
306   kinetics are consistent with known properties of Tm, which relies on new protein synthesis to
307   trigger ER stress [72]. As expected, in latently infected cells, *Xbp1* splicing and XBP1s and
308   ATF4 protein accumulation were observed throughout the 8 h course of Tg or Tm treatment. By
309   contrast, during KSHV lytic replication, regardless of duration of Tg or Tm treatment,
310   comparable levels of spliced *Xbp1* mRNA and phospho-eIF2α were detected but XBP1s and
311   ATF4 proteins failed to accumulate. Consistent with previous results, there were slightly higher
312   levels of unspliced *Xbp1* mRNA observed during the lytic cycle that correlated with reduced
313   total IRE1α protein levels, but this was only observed in Tg-treated cells; Tm treatment

314   converted the bulk of the *Xbp1* mRNA pool to the spliced form by 4 h post-treatment, even
315   though IRE1α accumulation was still blocked. Consistent with our previous observations total
316   ATF6 levels increased during the lytic cycle, and protein species with different electrophoretic
317   mobilities could be detected by the ATF6 antiserum. Extended Tg or Tm treatments caused
318   accumulation of the ATF6(N) transcription factor exclusively in the lytic cells. Taken together,
319   these observations confirm that XBP1s and ATF4 transcription factors fail to accumulate in the
320   KSHV lytic cycle despite robust activation of UPR sensors after chemically-induced ER stress.
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322   XBP1s target genes are not transactivated by XBP1s during lytic replication
323   To better understand the kinetics of IRE1 activity during KSHV lytic replication we reactivated
324   TREx BCBL1-RTA cells from latency using dox and monitored IRE1 activity over a 24 h time
325   course. Spliced XBP1 mRNA and XBP1s protein were detected by 6 h post-dox addition,
326   concomitant with a modest increase in IRE1 phosphorylation as determined by retarded IRE1
327   mobility on an immunoblot probed with an anti-IRE1α antibody (Fig. 4A). This induction of
328   *Xbp1* splicing corresponds with the increase in the early viral protein ORF45. By 18 hours post
329   dox addition, which coincides with the beginning of viral genome replication in this model [58],
330   IRE1 phosphorylation, *Xbp1* splicing and XBP1s accumulation had peaked, with almost 40% of
331   *Xbp1* mRNA in the spliced isoform; however, these phenotypes were surpassed by the Tg
332   positive control (Fig. 4A and B). We wanted to test if the low levels of XBP1s that we observe
333   during lytic replication would be sufficient to induce synthesis of XBP1s target genes. We
334   harvested RNA from latent and lytic (24 h post dox addition) TREx BCBL1-RTA cells treated
335   with either Tg or Tm or left untreated and performed semiquantitative RT-PCR analysis. As
336   previously observed, *Xbp1* mRNA was efficiently spliced following Tg or Tm treatment, both in
337   latent and lytic samples, while moderate splicing was observed in the mock-treated lytic samples
338   (Fig. 4C). RT-qPCR was performed to measure relative levels of canonical XBP1s target genes
339   EDEM1 and ERdj4 [73] (Fig. 4D). Indeed, Tg and Tm treatments caused dramatic accumulation
340   of EDEM1 and ERdj4 transcripts in latently infected cells, whereas these transcripts remained at
341   low levels in lytic cells treated with Tg, Tm or mock-treated. Thus, low expression of XBP1s
342   during lytic replication did not induce synthesis of canonical XBP1s target gene products
343   involved in ER stress mitigation.
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345   UPR sensor activation supports efficient KSHV replication
346   We next used a combination of genetic and pharmacologic approaches to inhibit each of the UPR
347   sensor proteins and measure their impact on KSHV lytic replication. To investigate the role of
348   ATF6, we silenced ATF6α expression in TRex BCBL-RTA cells with shRNAs (Fig. 5A) and
349   collected cell supernatants from ATF6-silenced or control cells at 48 h post-dox addition. Cell
350   supernatants were processed to measure relative levels of released capsid-protected viral
351   genomes by qPCR. ATF6 knockdown reduced virus titre by ~50% compared to cells transduced
352   with non-targeting shRNA (Fig. 5B). To determine whether activation of PERK or downstream
353   engagement of the ISR are important for viral replication TREx BCBL1-RTA cells were treated
354   with the selective PERK inhibitor GSK2606414 (PERKi) or the ISR inhibitor ISRIB, a small
355   molecule that blocks phospho-eIF2α-mediated inhibition of translation by maintaining active
356   eIF2B [74,75]. PERKi and ISRIB each inhibited viral particle release by ~50% (Fig. 5C).
357   To determine if IRE1 RNase activity is required for efficient viral replication TREx BCBL1-
358   RTA cells were treated with the IRE1 inhibitor 4μ8c [76], which inhibited virus release in a
359   dose-dependent manner (Fig. 6A). We corroborated these findings in the dox-inducible

360 iSLK.219 cell model that produces KSHV virions bearing GFP transgenes [77]; cells were
361 treated with 4μ8c at the time of dox addition and cell supernatants were harvested 96 hours later,
362 serially diluted, and titered on naive monolayers of 293A cells by flow cytometry. As in the
363 TREx BCBL1-RTA cell model, higher doses of 4μ8c inhibited virion production from iSLK.219
364 cells, with statistically significant inhibition achieved at the 25 μM dose (Fig. 6B). To confirm
365 these observations, we inhibited IRE1α expression in iSLK.219 cells via RNA silencing. Cells
366 transduced with IRE1α-targeting shRNAs or non-targeting controls were treated with dox for 96
367 hours, and cell supernatants were once again collected to titer GFP-expressing KSHV virions by
368 flow cytometry. iSLK.219 cells bearing IRE1α shRNAs inhibited release of infectious virions by
369 more than two-fold (Fig. 6C, 6D). Taken together, these data suggest that all three sensors of the
370 UPR are important for robust virus replication.
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372 **Ectopic XBP1s expression inhibits release of infectious KSHV virions in a dose-dependent**
373 **manner**
374 Our studies to this point suggested that efficient lytic replication depends on activation of all
375 three UPR sensor proteins, but the cell fails to produce UPR transcription factors and
376 downstream transcriptional responses required to mitigate ER stress. This suggests that KSHV
377 may re-dedicate the UPR sensors for a new purpose other than to resolve ER stress, and that
378 sustained ER stress may have little impact on viral replication. We also found it puzzling that
379 IRE1 RNase activity was required for efficient lytic replication but XBP1s could not
380 transactivate XBP1s-target genes, including RTA. Furthermore, XBP1s protein levels were
381 reduced following treatment with chemical inducers of ER stress during the lytic cycle despite
382 clear IRE1 activation and efficient *Xbp1* mRNA splicing. For these reasons, we hypothesized
383 that XBP1s accumulation may negatively impact KSHV replication. To complement the XBP1s
384 deficiency in the KSHV lytic cycle we overexpressed XBP1s using dox-inducible lentiviral
385 vectors. We engineered myc-tagged XBP1s to be weakly expressed under the control of a single
386 tet operator (TetO) element, or strongly expressed under the control of seven tandem TetO
387 elements. We transduced iSLK.219 cells with these constructs or an empty vector control, and
388 selected stable cells with blasticidin antibiotic. With the addition of dox, XBP1s and RTA were
389 concurrently expressed from dox-inducible promoters. To demonstrate that the ectopic XBP1s
390 was functional we measured mRNA levels of the XBP1s-target gene *ERdj4* and observed that it
391 is upregulated in cells expressing XBP1s from the 7xTetO compared to empty vector control and
392 peaks at 24 hours post dox addition (Fig.7A). These cells also displayed higher levels of mRNA
393 and protein for RTA and the RTA target gene ORF45 by 24 hours post-dox treatment; and by 48
394 hours, mRNA encoding the late viral protein K8.1 was markedly increased compared to controls
395 (Fig. 7A and B), suggesting accelerated viral genome replication. Indeed, intracellular levels of
396 viral genomes at 96 h post-dox were two-fold higher in XBP1s-overexpressing cells compared to
397 empty vector (Fig. 7C). We harvested cell supernatants at 48, 72, and 96 hours post dox and
398 measured virion titer as previously described. Surprisingly, despite accelerated viral gene
399 expression and genome replication in XBP1s-overexpressing cells, there was a dramatic, dose-
400 dependent reduction in virion production by 72 and 96 hours post-dox compared to empty vector
401 control (Fig. 7D). There was also a corresponding 20-fold decrease in release of viral particles by
402 XBP1s-overexpressing cells compared to controls, as measured by qPCR for capsid-protected
403 viral genomic DNA (Fig. 7E). Thus, while ectopic XBP1s expression promotes KSHV lytic gene
404 expression and genome replication, it prevents efficient release of infectious progeny.

405   Since XBP1s transactivates the *RTA* promoter, we hypothesized that this defect in virion
406   production could be a negative consequence of *RTA* hyper-activation. We observed a decrease in
407   the accumulation of capsid proteins ORF26 and ORF65 (Fig. 7B), which we speculate could
408   result from RTA hyper-activation and negatively impact late stages of replication [78]. However,
409   experiments with the weaker 1xTetO-XBP1s construct revealed a 2-fold decrease in virion
410   release at 96 hours post dox (Fig. 7D) without affecting RTA, ORF26 and ORF65 protein
411   accumulation (Fig. 7B). To confirm that the diminished production of infectious virions from
412   XBP1s-overexpressing cells is not due to enhanced RTA expression, we also overexpressed
413   RTA in parallel from a 7xTetO in iSLK.219 cells, such that dox addition causes RTA expression
414   by two dox-responsive promoters. KSHV from iSLK.219 cells also express monomeric red
415   fluorescent protein (mRFP) from the viral lytic PAN promoter and can be used to monitor virus
416   reactivation [79]. 48 hours post-dox addition, the levels of mRFP were similar between XBP1s
417   and RTA-expressing cells and noticeably greater than that of the empty vector control (Fig. 7F).
418   We harvested virus-containing supernatants at 24, 48, 72, and 96 hours post-dox and measured
419   virion release by flow cytometry following infection of a naïve 293A monolayer (Fig. 7G). At
420   24h, when virion production is negligible in empty vector- and XBP1s-expressing cells, there is a
421   significantly higher level of virions produced by 7xTetO RTA-expressing cells which continues
422   up until 72 hours. At 96 hours post-dox, virion production from the 7xTetO RTA-expressing
423   cells hits a plateau. Here again, in agreement with our previous observations, virion production
424   from 7xTetO XBP1s-expressing cells is comparable to empty vector control after 48 hours but
425   by 96 hours post-dox virion production is ~20-fold lower. These data demonstrate that the
426   dramatic reduction in virions produced by XBP1-overexpressing cells is not due to hyper-
427   activation of RTA.
428   Our findings indicate that XBP1s is both pro-lytic (by transactivating RTA and promoting
429   reactivation from latency) and anti-viral (by inhibiting a late stage in the lytic replication cycle).
430   These confounding observations prompted us to take a step back and corroborate these
431   observations in a different system, wherein XBP1s is expressed from a constitutive CMV
432   promoter in the absence of doxycycline-induced RTA. To achieve this, we transduced iSLK.219
433   cells with increasing concentrations of lentiviral vectors expressing either myc-tagged XBP1s or
434   FLAG-tagged RTA. To ensure that there were would be an equivalent level of reactivation
435   between cells constitutively expressing RTA or XBP1s, we chose virus dilutions that resulted in
436   a similar range of mRFP levels (indicating lytic cycle initiation) as monitored by fluorescence
437   microscopy (Fig. 8A). Six days after cell transduction, virus-containing supernatants were
438   harvested, and infectious virions were enumerated by infecting naive 293A cells and detecting
439   GFP positive cells by flow cytometry; as a positive control, we harvested virus from
440   untransduced iSLK.219 cells treated with doxycycline for 72h (Fig. 8B). Interestingly, although
441   there is a dose-dependent increase in virion release from RTA-transduced iSLK.219 cells,
442   increasing levels of XBP1s had no impact on virion production and the highest yield of virions
443   was almost 100-fold lower than the corresponding highest yield from RTA-transduced cells or
444   dox-treated iSLK.219 cells. Interestingly, although in RTA-transduced cells, RTA and ORF45
445   protein levels correspond to the increasing levels of mRFP this does not occur in XBP1-
446   expressing cells; increasing XBP1s expression had a nominal impact on RTA and ORF45 protein
447   accumulation (Fig. 8C). In XBP1s-transduced cells there was a striking reduction in ORF65
448   accumulation, and ORF45 displayed a mobility shift compared to RTA-transduced cells. Taken
449   together, our observations from multiple systems suggest that XBP1s negatively impacts late
450   stages of KSHV replication resulting in diminished virion yield.

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Discussion

451 XBP1s-mediated transactivation of the RTA promoter causes reactivation from latency, but little
453 is known about how ER stress and the UPR affect the lytic replication cycle that follows. Here
454 we report that activation of all three UPR sensors (PERK, IRE1, ATF6) is required for efficient
455 KSHV lytic replication, because genetic or pharmacologic inhibition of each UPR sensor
456 diminishes virion production. Despite strong UPR sensor activation during KSHV lytic
457 replication, downstream UPR transcriptional responses were restricted. Paradoxically we found
458 that although XBP1s can trigger reactivation by upregulating RTA, forced expression of XBP1s
459 blocks virion production, despite accelerating lytic gene expression and genome replication.
460 These findings suggest that XBP1s has antiviral properties that KSHV must circumvent during
461 the lytic cycle to ensure efficient viral replication. This provides a tidy rationale for viral
462 blockade of XBP1s production during the lytic cycle, but significantly more work will be
463 required to elucidate precise mechanisms of viral activation of UPR sensors and inhibition of
464 downstream UPR transcriptional responses.
465 All three UPR sensors are activated concurrently during the lytic cycle, which suggests that
466 they may be responding to canonical protein misfolding events in the ER. Such misfolding could
467 be the result of viral glycoprotein biogenesis, but we think this scenario is unlikely because UPR
468 sensor activation in the BCBL-1 and iSLK.219 cell models precedes viral genome replication
469 and bulk synthesis of most structural viral glycoproteins. We favour an alternative hypothesis
470 that lytic replication disrupts ER chaperone function in some way, perhaps through the direct
471 action of viral proteins or indirect activation of signal transduction pathways. It seems
472 counterintuitive that an enveloped virus would actively trigger ER stress, but we speculate that
473 UPR activation in the earliest stages of lytic replication could help prime the ER for the
474 impending increase in viral glycoprotein synthesis. A more comprehensive accounting of
475 changes in gene expression during early lytic replication is required to determine whether there is
476 indeed an activation of an adaptive UPR to prepare the cell for late gene expression. There are
477 multiple examples of viruses that trigger Ca^{2+} release from ER stores, which may impede protein
478 folding and activate an ER stress response [80]. Indeed, KSHV encodes a viral G protein-
479 coupled receptor (vGPCR) that activates stress signalling and increases cytosolic calcium at least
480 in part by inhibiting the SERCA pump [81]. Thus, vGPCR may act similarly to Tg, inducing
481 broad activation of all three UPR sensors by depleting ER calcium stores essential for protein
482 folding.
483 Why would a virus cause the induction of ER stress but block the downstream UPR signalling
484 and transcription events that mitigate stress? UPR signalling is etiologically-linked to
485 inflammatory diseases including Crohn's Disease and type 2 diabetes [34,82,83]. Furthermore,
486 maximal IFN production following Toll-like receptor (TLR) engagement has been shown to
487 require UPR activation and XBP1s activity [84]. Thus, KSHV suppression of UPR transcription
488 may dampen antiviral inflammatory responses. Furthermore, because UPR transcription factors
489 transactivate genes involved in catabolism, we speculate that blockade of their activity may
490 allow newly-synthesized viral gene products to evade degradation. For example, ATF6 and
491 XBP1s transactivate genes involved in ERAD [28,62], so suppression of ATF6/XBP1s
492 transcription could prevent ERAD-mediated degradation of newly-synthesized viral
493 glycoproteins. Similarly, because ATF4 and its downstream target CHOP transactivate
494 autophagy [16] and autophagy restricts herpesvirus replication [85,86], suppression of ATF4 and
495 CHOP accumulation may allow KSHV to evade negative catabolic effects of autophagy. Viral
496 suppression of CHOP synthesis may also prevent the pro-apoptotic effects of CHOP [87],

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497   thereby extending the survival of cells enduring the later stages of lytic replication. However, the
498   consequences of allowing unchecked accumulation of misfolded proteins are unclear. Autophagy
499   has been shown to compensate and assist with bulk protein degradation when ERAD is defective
500   or inadequate for degrading misfolded proteins like aggregation-prone membrane proteins
501   [88,89]. Since ERAD genes are not upregulated during lytic replication, it is possible that
502   autophagy is required to clear misfolded proteins. However, there are multiple KSHV proteins
503   that have been shown to directly inhibit autophagy including vFLIP, K-survivin, and vBCL2
504   [90–92], as well as multiple KSHV proteins that stimulate the pro-growth PI3K-Akt-mTOR
505   signalling pathway [93,94]. Simultaneous inhibition of ERAD and autophagy likely would cause
506   the accumulation of the misfolded proteins, including aggregated membrane proteins, in KSHV
507   infected cells. Mechanisms that KSHV employs (if any) to circumvent a possible accrual of
508   misfolded proteins remain unknown.
509   How might KSHV inhibit ATF4 protein accumulation? ATF4 synthesis relies on eIF2α
510   phosphorylation, which binds and inhibits the eIF4B guanine nucleotide exchange factor and
511   depletes the available pool eIF2α-tRNA$_{Met}^i$-GTP ternary complexes; this causes bypass of AUG
512   start sites that normally initiate uORF translation and favours initiation at the *bona fide*
513   downstream ATF4 AUG start codon [14]. Host shutoff during lytic replication decreases the pool
514   of translation-ready mRNAs [95], which may necessitate increased phosphorylation of eIF2α to
515   suppress eIF4B and allow uORF skipping for ATF4 synthesis. ATF4 accumulation is also
516   controlled by post-transcriptional modifications; when the ISR is activated, host
517   methyltransferases add N^6-methyladenosine (m6A) to the ATF4 mRNA, which further enhances
518   ATF4 ORF translation [96]. The m6A machinery is also required for efficient KSHV lytic
519   replication [97]. Intriguingly, these studies show that knockdown of the m6A methyltransferase
520   METTL3 increases ATF4 synthesis and decreases virion production. Thus, it is possible that
521   KSHV hijacks the m6A machinery to prevent translation of ISR mRNAs while promoting
522   efficient translation of KSHV gene products.
523   Despite efficient XBP1 mRNA splicing, XBP1s protein failed to accumulate in lytic cells in
524   iSLK.219 and BCBL-1 cell models, even after extended Tg or Tm treatments. We don't yet
525   understand the underlying mechanism of viral blockade of XBP1s accumulation, but the
526   interconnected nature of the different arms of the UPR will inform our ongoing studies. For
527   example, we have observed diminished levels of total IRE1 protein at later stages of lytic
528   replication. ATF4 and ATF6(N) have both been shown to upregulate IRE1 [24,98]. Thus, during
529   KSHV lytic replication, failure to accumulate the UPR transcription factors may diminish IRE1
530   protein levels required to support XBP1s synthesis. We are actively investigating these UPR
531   regulatory mechanisms in our KSHV infection models.
532   Many viruses modulate the UPR, including other herpesviruses. Herpes simplex virus type 1
533   (HSV-1) has been shown to dysregulate IRE1 activity [99]; HSV-1 infected cells displayed
534   increased IRE1 protein levels but diminished IRE1 RNase activity, and treatment with the IRE1
535   inhibitor STF-083010 [100] diminished viral replication [101]. Moreover, HSV-1 glycoprotein B
536   (gB) binds and inhibits PERK to support robust viral protein synthesis [57]. Interestingly, ectopic
537   expression of FLAG-tagged XBP1s inhibits HSV-1 replication [101], consistent with our
538   findings in KSHV infection models. By contrast, human cytomegalovirus (HCMV) lytic
539   replication in fibroblast cells selectively activates PERK and IRE1, but not ATF6 [55].
540   Consistent with our model, IRE1 activation causes normal *Xbp1* mRNA splicing but fails to
541   upregulate the XBP1s target gene EDEM (XBP1s protein levels were not evaluated in this
542   study). However, PERK causes ISR activation and normal ATF4 accumulation during HCMV

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543 lytic replication, in sharp contrast to the situation in KSHV infection [55]. A recent study linked
544 HCMV PERK and IRE1 activation to the UL148 glycoprotein [56]. In cells infected with a
545 UL148-deficient HCMV, XBP1s protein levels were diminished compared to wildtype but levels
546 of the XBP1s-target gene EDEM1 are unaffected, suggesting that XBP1s activity may also be
547 inhibited in CMV infected cells. Another study showed that HCMV UL38 induces eIF2α
548 phosphorylation and ATF4 synthesis and promotes cell survival and virus replication during
549 drug-induced ER stress by repressing JNK phosphorylation [102]. HCMV UL50 protein and the
550 murine cytomegalovirus (MCMV) ortholog M50 bind IRE1 and promote its degradation, thereby
551 inhibiting *Xbp1* mRNA splicing [103]. The accumulating evidence indicates that herpesviruses
552 have evolved distinct mechanisms to regulate the UPR to promote viral replication. We suspect
553 that many more viral regulators of the UPR will be discovered in the coming years. Such studies
554 will benefit from the development of appropriate platforms to screen hundreds of viral ORFs for
555 UPR and ISR-modulating activity.
556   IRE1 is modulated by diverse viruses. Hepatitis C virus (HCV) replication triggers chronic
557 ER stress and sustained activation of all three branches of the UPR [104], but other studies
558 showed that HCV suppresses the IRE1/XBP1 pathway and virus replication is enhanced in IRE1
559 KO MEFs [105]. Influenza A virus (IAV) specifically activates IRE1 with little or no activation
560 of PERK or ATF4 and chemical inhibition of IRE1 diminishes virion production [106]. Why
561 does this array of unrelated viruses target IRE1? Beyond the aforementioned XBP1s
562 transcription program, IRE1 can activate the JNK signalling pathway [107], which plays
563 important roles in viral replication, cellular stress responses, and cell fate [108,109]. For HSV-1,
564 IRE1 activation could activate JNK, which has been shown to support viral replication in
565 multiple cell types [101]. JNK has also been shown to play important roles in KSHV infection;
566 *de novo* infection triggers JNK activation, and ER stress-induced JNK activation can reactivate
567 KSHV from latency [110,111]. Later stages of KSHV lytic replication also feature JNK
568 activation, and ectopic expression experiments have shown that KSHV lytic proteins K15 and
569 vGPCR can induce JNK phosphorylation [112,113]. It will be interesting to determine whether
570 IRE1 is required for JNK activation during KSHV infection.
571   IRE1 can also cleave a subset of ER-targeted mRNAs for degradation in a process called
572 RIDD [30]. It is possible that certain viruses may hijack RIDD as a form of host shutoff to
573 ensure preferential translation of secreted viral proteins. Conversely, if viral mRNAs contain
574 XBP1-like cleavage sites, then the virus would likely want to suppress IRE1 RNase activity
575 [114]. The molecular events that direct IRE1 toward RIDD or *Xbp1* mRNA splicing are not well
576 understood but one study suggests that the higher order multimerization of IRE1 can dictate the
577 response, whereby oligomeric IRE1 prefers *Xbp1* as a substrate, whereas dimeric IRE1
578 preferentially cleaves mRNAs through RIDD [115]. Since the levels of IRE1 are impacted by
579 many of these viruses, including CMV, HSV-1, and KSHV, it will be interesting to determine if
580 IRE1 specificity of mRNAs is impacted during infection. A search for XBP1-like stem loops in
581 herpesviruses mRNAs could help inform if RIDD is antiviral.
582   XBP1s is required for the differentiation of B cells into antibody-secreting plasma cells [33].
583 KSHV co-opts the B lymphocyte differentiation program when it infects immature B cells and
584 drives them to differentiate into plasmablast cells [43], which serves as the long-term latent
585 reservoir in the body. It would be interesting to test if the KSHV modulation of the IRE1/XBP1s
586 pathway that we report is required to drive this unique differentiation program. Consistent with
587 our findings and unlike some XBP1s-positive plasmablastic lymphomas, patient-derived PEL
588 cells can lack XBP1s expression [116]. Suppression of XBP1s accumulation may allow KSHV

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589   to prevent reactivation from latency and preserve this viral reservoir. If this hypothesis is correct,
590   then ectopic expression of XBP1s should kill KSHV-positive PEL cells, while interfering with
591   viral replication. It is also possible that the lack of XBP1s-positive PEL cells is because XBP1s-
592   mediated plasma cell differentiation should also trigger reactivation and ultimately cell death.
593   The XBP1s transcriptional program during plasma cell development is likely to have distinct
594   players and analysing some of these downstream effectors for possible KSHV inhibition may
595   help identify why XBP1s potently inhibits virus production in our model.

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Materials and Methods

Cell culture and chemicals

293A (ThermoFisher), HEK293T (ATCC), Hela Tet-Off (Clontech), and iSLK.219 cells (a kind gift from Don Ganem) were cultured in DMEM (ThermoFisher) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 Units/mL penicillin, 100 μg/mL streptomycin, and 2mM L-Glutamine. iSLK.219 cells were also passaged in the presence of 10 μg/mL of puromycin (ThermoFisher) to maintain the rKSHV.219 episomal DNA. TREx BCBL1-RTA cells (a kind gift from Jae Jung) were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 500 μM β-mercaptoethanol and the same concentrations of penicillin, streptomycin, and L-Glutamine as the adherent cell lines. All cells were maintained at 37˚C with 5% CO_2.

To induce lytic replication via expression of the RTA transgene in TREx BCBL1-RTA and iSLK.219 cells, 1 μg/mL of doxycycline (dox; Sigma) was added to the cells. iSLK.219 cells were seeded at density of $2x10^5$ cells/well of a 6-well plate one day prior to lytic induction and TREx BCBL1-RTA cells were seeded at a concentration of $2.5x10^5$ cells/mL immediately prior to lytic induction.

4μ8c (Axon Medchem and Sigma), PERKi (GSK2606414; Tocris), ISRIB (Sigma), Thapsigargin (Sigma), and Tunicamycin (Sigma) were dissolved in DMSO (Sigma) to stock concentrations and diluted to the indicated concentrations in cell media.

Plasmids

To generate the plasmids pLJM1 B* Puro and pLJM1 B* BSD, the genes encoding puromycin N-acetyltransferase and blasticidin S deaminase were amplified from pBMN-IRES-Puro and pBMN-IRES-Blast, respectively (previously generated in the lab by switching GFP with PuroR or BlastR from pBMN-I-GFP that was generated by the Nolan Lab [Stanford]), with forward and reverse primers containing BglII and KpnI RE sites. The antibiotic selection cassettes were cut-and-pasted into pLJM1.D (previously generated in the lab from modifying the MCS of pLJM1 plasmid that was generated by the Sabatini Lab [MIT]), deleting the BamHI RE site. A new MCS was generated by annealing overlapping oligos containing NheI and MfeI forward and reverse RE sites and inserted into the plasmid digested with NheI and EcoRI to replace the existing MCS with unique RE sites in the following order: NheI, AgeI, BamHI, EcoRI, PstI, XbaI, MluI, SalI, EcoRV. 1x TO (tet operator) and 7x TO promoters were generated by annealing overlapping oligos containing one or seven copies of the tetracycline operator (5'-TCCCTATCAGTGATAGAGA-3') and a minimal CMV promoter and using PCR extension to amplify a blunt oligo containing NdeI and NheI RE sites. The amplicon was digested with NdeI and NheI and pasted into the pLJM1 B* BSD replacing the CMV promoter.

To generate pLJM1 B* Puro HA-ATF6, HA-ATF6 was PCR amplified from pCGN-ATF6 (a gift from Ron Prywes, Addgene plasmid # 11974) and cut and pasted into pLJM1 B* Puro with NheI and AgeI. pCMV2B-XBP1s was generated by PCR amplifying XBP1 from total RNA isolated from TREx BCBL1-RTA cells treated with Tg for 4h. After PCR, DNA was digested with PstI (cleaves unspliced XBP1 DNA only) to enrich for the spliced isoform of XBP1 and cut and pasted into pCMV2B with BamHI and XhoI and in-frame of the N-terminal FLAG-tag. pLJM1 BSD CMV, 1x TO, and 7x TO myc-XBP1s were generated by and including the myc-tag nucleotide sequence in the 5' primer and amplifying XBP1s from pCMV2B-XBP1s. Cloned myc-XBP1s was subsequently cut and pasted into their corresponding lentiviral plasmids with

678   BamHI and SalI. FLAG-RTA was cut from pcDNA3-FLAG-RTA (previously generated in the
679   lab) with RE sites EcoRI and XhoI and pasted into pLJM1 B* BSD CMV and 7x TO with RE
680   sites EcoRI and SalI (via compatible sticky ends between XhoI and SalI).
681
682   **Lentiviral vectors**
683   HEK293T cells were seeded on 10cm plates for 60-70% cell density the following day. Cells
684   were transfected with polyethylenimine (PEI; Sigma) and the following plasmids for lentiviral
685   generation: pLJM1 transfer plasmid, pMD2.G, and psPAX2. pMD2.G and psPAX2 are gifts
686   from Didier Trono (Addgene plasmids # 12259 and # 12260). 48 hours post transfection,
687   lentivirus containing supernatants were passed through a 0.45μM filter and frozen at -80˚C.
688   For transducing iSLK.219, iSLK.219 cells were seeded at density of $5x10^4$ cells/well of a 6-well
689   plate and the following day were resuspended in media containing 4 μg/mL polybrene (Sigma).
690   Lentivirus was serially diluted dropwise onto cells and incubated at 37˚C for 24 hours. Following
691   infection, the media was refreshed containing 10 μg/mL blasticidin. For transducing TREx
692   BCBL1-RTA cells, cells were seeded at a concentration of $2.5x10^5$ cells/mL in media containing
693   the same concentration of polybrene and lentivirus was serially diluted onto cells. 24 hours post
694   infection, the media was replaced with media containing 1 μg/mL puromycin. The first lentivirus
695   dilution that resulted in minimal cell death following antibiotic selection was used for subsequent
696   experiments.
697
698   **Luciferase Assays**
699   4x pORF50XRE-FLuc plasmid was generated by annealing oligos that have the XBP1
700   response element sequence 5'-ATGACACGTCCC-3' found in ORF50 promoter (Dalton-Giffin
701   *et al.* 2009 J.Virol) and is repeated 4 times flanked by 10bp repeats. The oligo also contains KpnI
702   and XhoI RE sites and was inserted into the firefly luciferase plasmid
703   pGL4.26[*luc2*/minP/Hygro] (Promega).
704   Hela tet-off cells were seeded at a density of $3.2x10^4$ cells/well of a 12-well plate and
705   transfected the following day with FuGENE HD transfection reagent and the following plasmids
706   per well: either 400 ng pCR3.1 Vector control or pCMV2B-XBP1s, 80ng 4x pORF50XRE-
707   FLuc, and 20ng of the *Renilla* luciferase plasmid pRL-TK for normalization. 24 hours post
708   transfection, cells transfected with pCR3.1 were untreated or treated with 150 nM Tg for 16h.
709   Following treatment, firefly and *Renilla* luciferase activity was detected by Glomax 20/20
710   Luminometer (Promega) after incubating with corresponding substrates from the Dual-
711   Luciferase Reporter System (Promega) according to the manufacturer's instructions. Firefly
712   luciferase relative lights units were normalized to *Renilla* luciferase and expressed as fold change
713   in signal relative to untreated vector control.
714
715   **shRNA lentivirus cloning and knockdown**
716   The pLKO.1 -TRC control plasmid was a gift from David Root (Addgene plasmid # 10879)
717   and the RNAi Consortium was used to design the following oligos for generating shRNA
718   lentiviral vectors against ATF6α and IRE1α with the targeting sequences underlined (Fwd/Rev):
719   shAFT6α (TRCN0000416318): 5'-
720   CCGGACAGAGTCTCTCAGGTTAAATCTCGAGATTTAACCTGAGAGACTCTGTTTTTT
721   G/5'-
722   AATTCAAAAACAGAGTCTCTCAGGTTAAATCTCGAGATTTAACCTGAGAGACTCTG
723   T-3'

724 shIRE1α-1 (TRCN0000356305): 5'-
725 CCGG<u>TCAACGCTGGATGGAAGTTTG</u>CTCGAGCAAACTTCCATCCAGCGTTGATTTTT
726 G -3'/5'-
727 AATTCAAAAATCAACGCTGGATGGAAGTTTGCTCGAG<u>CAAACTTCCATCCAGCGTTG</u>
728 <u>A</u>-3'
729 shIRE1α-2 (TRCN0000235529): 5'-
730 CCGG<u>AGAGGAGGGAATCGTACATTT</u>CTCGAGAAATGTACGATTCCCTCCTCTTTTTT
731 G-3'/5'-
732 AATTCAAAAAGAGGAGGGAATCGTACATTTCTCGAG<u>AAATGTACGATTCCCTCCTC</u>
733 <u>T</u>-3'
734 The cloning strategy on Addgene (http://www.addgene.org/tools/protocols/plko/) was used to
735 clone the oligos into either pLKO.1-Puro or pLKO.1-Blast and lentivirus generation was
736 completed as described previously.
737
738 **Fluorescent imaging**
739 For Fig.7, iSLK.219 cells were seeded at density of $2x10^5$ cells/well of a 6-well plate and and 48
740 hours post addition of doxycycline, brightfield images and fluorescent images were captured
741 with Olympus CKX41 microscope fitted with a QImaging QICAM Fast 1394 digital camera and
742 Lumencor Mira light engine and using the 10x objective. For Fig. 8, iSLK.219 cells were seeded
743 at density of $5x10^4$ cells/well of a 6-well plate and transduced with the indicated lentiviral
744 vectors. Fluorescent images were captured with an EVOS FL Cell Imaging System with 10x
745 objective (ThermoFisher) following 5 days post transduction or 48 hours post dox.
746
747 **Immunoblotting**
748 Cells were washed once with ice-cold PBS and lysed in 2x Laemmli Buffer (120mM Tris-HCl
749 pH 6.8, 20% glycerol, 4% SDS). Lysates were passed through a 21-gauge needle 5-7 times to
750 minimize viscosity and protein concentration was quantified with DC protein assay (Bio-Rad).
751 DTT and bromophenol blue were added to samples, boiled, and 10-20 μg of protein per sample
752 were loaded on 6-12% polyacrylamide gels and resolved by SDS-PAGE. Protein was transferred
753 to PVDF membranes using the Trans-Blot Turbo Transfer System (Bio-Rad) and blocked in 5%
754 skim milk diluted in Tris-buffered-saline (TBS) supplemented with 0.1% Tween 20 (Fisher Bio)
755 (TBS-T) for 1 hour at room temperature followed by overnight incubation at 4˚C with primary
756 antibody diluted in 5% bovine serum albumin in TBS-T. Following washing of primary
757 antibody, membranes were incubated with IgG HRP-linked antibodies followed by exposure to
758 ECL-2 western blotting substrate (Thermo Scientific, Pierce) and imaged on a Carestream Image
759 Station 400mm Pro (Carestream) using the chemifluorescence setting. The following antibodies
760 were used: PERK (Cell Signaling; #5683); IRE1α (Cell Signaling; #3294); ATF6α (Abcam;
761 ab122897); XBP1 (Cell Signaling; #12782); Phospho-eIF2α (Ser51; Abcam; ab32157); Total
762 eIF2α (Cell Signaling; #5324); ATF4 (Santa Cruz; sc-200); Myc-tag (Cell Signaling; #2278);
763 FLAG-tag (DYKDDDDK tag; Cell Signaling; #8146); ORF45 (Thermo-Fisher; MA5-14769);
764 ORF65 (a kind gift from Shou-Jiang Gao); RTA (a kind gift from David Lukac); β-actin HRP
765 conjugate (Cell Signaling; #5125); anti-rabbit IgG HRP-linked (Cell Signaling; #7074); anti-
766 mouse IgG HRP-linked (Cell Signaling; #7076).
767
768 **Xbp1 RT-PCR splicing assay**

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769 RNA was isolated from TREx BCBL1-RTA cells with the RNeasy Plus Kit (Qiagen) and 500 ng
770 total RNA was reverse transcribed with qScript cDNA SuperMix (Quanta) according to
771 manufacturers' protocols. Based on the Ron Lab protocol
772 (http://ron.cimr.cam.ac.uk/protocols/XBP-1.splicing.06.03.15.pdf) and minor modifications, a
773 473 bp PCR product overlapping the IRE1 splice site was amplified with XBP1 Fwd primer (5'-
774 AAACAGAGTAGCAGCTCAGACTGC-3') and XBP1 Rev primer (5'-
775 TCCTTCTGGGTAGACCTCTGGGAG-3'). The amplified PCR product was digested overnight
776 with 40 units of High Fidelity PstI (New England Biolabs) to cleave unspliced XBP1 cDNA. The
777 PCR products were resolved on a 2.5% agarose gel made with 1x TAE (Tris-acetate-EDTA)
778 buffer and stained with SYBR Safe (ThermoFisher) and visualized on a ChemiDoc Imaging
779 Station (Bio-Rad). Percent *Xbp1* mRNA splicing was calculated by densitometry analysis with
780 Image Lab software ver. 6.0.0 (Bio-Rad) and calculated using the following formula:

781
$$\% \textbf{ xbp1 mRNA splicing} = \frac{\mathbf{0.5 * xbp1hybrid + xbp1s}}{\mathbf{xbp1hybrid + xbp1s + xbp1u1 + xbp1u2}} * \mathbf{100}$$

782

783 **Quantitative Reverse-Transcription PCR (RT-qPCR)**
784 RNA was isolated from TREx BCBL1-RTA cells with the RNeasy Plus Kit (Qiagen) and 500 ng
785 total RNA was reverse transcribed with qScript cDNA SuperMix (Quanta) according to
786 manufacturers' protocols. A CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and
787 GoTaq qPCR MasterMix (Promega) was used to perform Real-Time PCR. Changes in mRNA
788 levels were calculated by the $\Delta\Delta$Cq method and normalized using 18S rRNA as a reference gene.
789 The following primer sets were used in the study:
790 *GRP78* F: 5'-GCCTGTATTTCTAGACCTGCC-3'; R: 5'-TTCATCTTGCCAGCCAGTTG-3'
791 *HERPUD1* F: 5'-AACGGCATGTTTTGCATCTG-3'; R: 5'-GGGGAAGAAAGGTTCCGAAG-
792 3'
793 *EDEM1* F: 5'-TTGACAAAGATTCCACCGTCC-3'; R: 5'-TGTGAGCAGAAAGGAGGCTTC-
794 3'
795 *ERdj4* F: 5'-CGCCAAATCAAGAAGGCCT-3'; R: 5'-CAGCATCCGGGCTCTTATTTT-3'
796 *ORF26* F: 5'-CAGTTGAGCGTCCCAGATGA-3'; R: 5'-GGAATACCAACAGGAGGCCG-3'
797 *ORF50* F: 5'-GATTACTGCGACAACGGTGC-3'; R: 5'-TCTGCGACAAAACATGCAGC
798 *K8.1* F: 5'-AGATACGTCTGCCTCTGGGT-3'; R: 5'-AAAGTCACGTGGGAGGTCAC-3'
799 *ORF45* F: 5'-TGATGAAATCGAGTGGGCGG-3'; R: 5'-CTTAAGCCGCAAAGCAGTGG-3'
800 *18S* F: 5'-TTCGAACGTCTGCCCTATCAA-3' R: 5'-GATGTGGTAGCCGTTTCTCAGG-3'
801

802 **Viral genome amplification**
803 DNA was harvested from iSLK.219 cells with DNeasy Blood & Tissue Kit (Qiagen) according
804 to manufacturer's protocol. RT-PCR was carried out as described previously with primers
805 specific to KSHV ORF26 (as listed previously) and β-actin (F: 5'-
806 CTTCCAGCAGATGTGGATCA
807 -3'; R: 5'-AAAGCCATGCCAATCTCATC-3'). Changes in KSHV genome copy number was
808 calculated by the $\Delta\Delta$Cq method and normalized to β-actin.
809

810 **Titering DNAse-protected viral genomes**
811 Virus containing cell supernatants were processed by first pelleting floating cells and debris and
812 then 180 μL cleared supernatant were treated with 20 μL of 3 mg/mL DNase I (Sigma) for 30
813 minutes at 37°C. Viral genomic DNA was then purified from the supernatant with DNeasy Blood

19

814 & Tissue Kit (Qiagen) according to the manufacturer's protocol with the following
815 modifications: 10 μg of salmon sperm DNA (Invitrogen) and 1 ng of luciferase plasmid
816 pGL4.26[*luc2*/minP/Hygro] (Promega) were added to the lysis buffer. RT-PCR was performed
817 as described previously with primers specific to KSHV ORF26 (as listed previously) and *luc2* (F:
818 5'-TTCGGCAACCAGATCATCCC-3'; R: 5'-TGCGCAAGAATAGCTCCTCC-3'). Changes in
819 virus titre was calculated by the ΔΔCq method and normalized to *luc2*
820

821 **rKSHV.219 infection and titering**
822 Virus-containing supernatant was harvested from iSLK.219 cells at the indicated times by
823 pelleting cellular debris at 3300 x g for 5 minutes and then stored at -80˚C until ready to titer the
824 virus. 1×10^5 293A cells/well were seeded in 12-well plates to obtain a confluent monolayer two
825 days later. The thawed viral inoculum was briefly vortexed and centrifuged again at 3300 x g for
826 5 minutes. Two-fold serial dilutions of viral supernatants were applied to the monolayer
827 containing 4 μg/ml polybrene and 25 mM HEPES (Gibco) and centrifuged at 800 x g for 2 hours
828 at 30˚C. The total cell count per well was also determined from an uninfected well. Fresh media
829 was applied immediately after spinoculation. 20-24 hours post infection, two dilutions that
830 resulted in less than 30% GFP-positive cells (the linear range for infection [data not shown])
831 were trypsinized, washed once with PBS and fixed with 1% paraformaldehyde in PBS. GFP-
832 positivity was measured on either FACSCalibur or FACSCanto cytometers (BD) by gating on
833 FSC/SSC and counting 10000-15000 "live" events. Gating and % GFP positive events was
834 determined with FCS Express 6 Flow Cytometry Software (ver.6.0; De Novo). Virus titre was
835 calculated as IU/mL with the following formula:
836

837 **Virus titre (IU/mL) = % GFP positive events ∗ dilution factor ∗ cell count ∗ 100**
838

839 The two dilution series were averaged for the final titre value.
840

841 **Statistical analysis**
842 Prism7 (GraphPad) was used for generating graphs and performing statistical analysis. Unpaired
843 Student's t-tests were used to determine significance between two groups. One-way or two-way
844 ANOVA was used to compare multiple samples or between grouped samples respectively, and
845 an appropriate post-hoc test was done to determine differences between groups. p-values <0.05
846 were considered significant and denoted as the following: <0.05 (*), <0.01 (**), <0.001 (***),
847 <0.0001 (****)
848

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Figure Legends

Fig. 1: KSHV lytic replication activates IRE1, PERK and ATF6 but downstream UPR transcription factors are inhibited. (A) TREx BCBL1-RTA cells were treated with 1μg/ml doxycycline (dox) for 0, 24 and 48 hours to induce lytic replication followed by 75nM thapsigargin (Tg) for 2 hours prior to harvesting for protein and total RNA. Whole cell lysates were analyzed by immunoblots for UPR markers (IRE1α, XBP1s, PERK, phospho- and total eIF2α, ATF4, and full length ATF6α. Migration shift in PERK and IRE1α immunoblots correspond to phosphorylation. KSHV proteins ORF45 and ORF65 were probed for to indicate induction of early lytic and late lytic, respectively. β-actin was used as a loading control. The asterisk (*) corresponds to an unknown protein species that cross-reacted with ATF6 anti-sera. (Bottom) XBP1 mRNA was amplified by RT-PCR, digested with PstI (cleaves unspliced XBP1 isoform only), and separated on SYBR Safe-stained agarose gel. The asterisk (*) corresponds to xbp1u-xbp1s hybrid cDNA. Representative immunoblot and agarose gel of two independent experiments are shown (B) TREx BCBL1-RTA cells were transduced with lentiviral expression vector encoding HA-ATF6α and selected for with 1μg/ml puromycin. Following selection, cells were treated with 1μg/ml doxycycline for 0, 24 and 48 hours and treated with 75nM thapsigargin for 4 hours prior to harvest. Whole cell lysates were analyzed by immunoblots for HA epitope tag, PERK, ORF45 and β-actin (loading control). Immunoblot shown is representative of two independent experiments. (C) As in (B), HA-ATF6a-transduced TREx BCBL1-RTA were treated with doxycycline for 0 and 24 hours and then treated with 75nM thapsigargin for 4 hours prior to total RNA isolation. mRNA levels of ATF6α target genes GRP78 and HERPUD1 were measured by qPCR. Changes in mRNA levels were calculated by the $\Delta\Delta Cq$ method and normalized using 18S rRNA as a reference gene. An average of 3 independent experiments are graphed and error bars denote SEM. Two-way ANOVA and a post-hoc multiple comparisons test were done to determine statistical significance (****, p value < 0.0001).

Fig. 2: PERK-dependent eIF2α phosphorylation during the KSHV lytic cycle
TREx BCBL1-RTA cells were treated with 1μg/ml doxycycline -/+ 500 nM of the PERK inhibitor GSK2606414 (PERKi) for 0, 24 and 48 hours and treated with or without 75 nM thapsigargin for 2 hours prior to harvest. Whole cell lysates were analyzed by immunoblots for PERK, phospho and total eIF2α, ATF4, IRE1α, and ORF45. Migration shift in PERK and IRE1α immunoblots correspond to phosphorylation. β-actin was used as a loading control. Immunoblots shown are representative of two independent experiments.

Fig. 3: The type or duration of ER stress does not account for the lack of ATF4 and XBP1s
TREx BCBL1-RTA cells were pretreated with doxycycline for 0 or 24 hours and treated with 75nM Tg or 5μg/ml tunicamycin (Tm) for 1, 4, or 8 hours prior to harvest for either protein or RNA. Whole cell lysates were analyzed by immunoblots for UPR markers (IRE1α, XBP1s, PERK, phospho- and total eIF2α, ATF4, and ATF6α). Migration shift in PERK and IRE1α immunoblots correspond to phosphorylation. KSHV protein ORF45 was probed to show lytic replication and β-actin was used as a loading control. (Bottom) XBP1 RT-PCR splicing assay was performed as previously indicated. (*) corresponds to xbp1u-xbp1s hybrid cDNA. Immunoblots and agarose gels are representative of two independent experiments.

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Fig. 4: Low levels of XBP1s induced in lytic are insufficient to upregulate XBP1s-target genes.

(A) TREx BCBL1-RTA cells were treated with 1µg/ml doxycycline (dox) for the indicated times or treated with 75nM Tg for 4 hours (as a positive control) and harvested for either protein or RNA. Whole cell lysates were analyzed by immunoblots for IRE1α, XBP1s, ORF45 and β-actin (loading control). (Bottom) XBP1 RT-PCR splicing assay was performed as previously indicated. (*) corresponds to xbp1u-xbp1s hybrid cDNA. A representative immunoblot and agarose gel for three independent experiments are shown. (B) Densitometry analysis from semi-quantitative XBP1 RT-PCR splicing assay in (A) was used to calculate the percentage of XBP1 in the spliced isoform. The mean of three independent experiments are graphed and error bars represent the standard deviation of the mean. (C) TREx BCBL1-RTA cells were treated with 1µg/ml doxycycline (dox) for 0 or 24 hours and treated with 75nM Tg or 5µg/ml Tm for 4 hours prior to RNA isolation. XBP1 mRNA splicing was determined by semi-quantitative RT-PCR splicing assay as previously described. The gel shown is representative of two independent experiments. (D) Total RNA samples from (C) were used to measure the mRNA levels of XBP1 target genes EDEM1 and ERdj4 by qPCR. Changes in mRNA levels were calculated by the $\Delta\Delta Cq$ method and normalized using 18S rRNA as a reference gene. An average of 4 independent experiments are graphed and error bars denote SEM. Two-way ANOVA and a post-hoc multiple comparisons test were done to determine statistical significance (****, p value < 0.0001).

Fig. 5: ATF6α and PERK support robust KSHV replication.

(A) TREx BCBL1-RTA cells were transduced with lentivirus expressing either non-targeting shRNA (NS) or shRNA against ATF6 and selected for with 1µg/ml puromycin. Following selection, cells were treated with 5ug/ml Tm for 6h and whole cell lysates were analyzed by immunoblots for ATF6 and BiP (Grp78), a transcriptional target of ATF6. (B) As in (A), cells were treated with 1µg/ml doxycycline for 48 hours and virus-protected genomic DNA from cell supernatants were column purified for determining virus titre by qPCR using primers against ORF26. Firefly luciferase plasmid DNA was added during DNA purification for normalization using the ΔΔCq method. (B) TREx BCBL1-RTA cells were treated with 1µg/ml doxycycline for 48 hours with or without 500 nM PERKi GSK2606414 or 250 nM ISRIB, and virus titre was measured by qPCR as in (B). Data are represented as 3 (B) or 4 (C) independent experiments and error bars denote SEM. One-way ANOVA and a post-hoc multiple comparisons test were done to determine statistical significance. (*, p value <0.05; **, p value <0.01).

Fig. 6: IRE1 is required for efficient KSHV replication

(A) TREx BCBL1-RTA cells were treated with 1µg/ml doxycycline for 48 hours with or without increasing concentrations of IRE1 inhibitor 4µ8c and virus-protected genomic DNA from cell supernatants was measured by qPCR to determine changes in virus titre using the ΔΔCq method by normalizing to *luc2* DNA levels. The mean of four independent experiments are graphed -/+ SEM. (B) iSLK.219 cells were treated with 1µg/ml doxycycline with or without 10 or 25 µM 4µ8c for 96 hours and virus-containing supernatants were serially diluted and spinfected onto a monolayer of 293A cells. GFP-positive cells (infected) were quantified by flow cytometry the following day and virus titre (IU/ml) was calculated as described in the Materials & Methods. The mean of four independent experiments are graphed -/+ SEM. (C) iSLK.219 cells were transduced with two different pLKO.1-blast shRNA lentiviruses targeting IRE1α or an non-targeting control and selected for with blasticidin. Following selection, cells were treated with

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1391   150nM Tg for 4 hours and harvested for immunoblot analysis to confirm IRE1α knockdown. The
1392   immunoblot shown is representative of two independent experiments performed. (D) Lentivirus
1393   transduced iSLK.219 cells from (C), were treated with doxycycline for 96h and virus titre was
1394   determined by flow cytometry as previously described. The data are represented as the change in
1395   virus titre relative to the non-targeting shRNA control sample and the mean of three independent
1396   experiments are graphed -/+ SEM. One-way ANOVA and multiple comparisons test were done
1397   to determine statistical significance in (A), (B), and (D). (*ns*, not statistically significant; *, *p*
1398   value < 0.05; **, *p* value < 0.01; ***, *p* value < 0.001)

1399

1400   **Fig. 7: XBP1s overexpression inhibits KSHV replication at a late stage**
1401   (A) iSLK.219 cells were transduced with lentiviral doxycycline-inducible expression vectors
1402   encoding myc-XBP1s whose expression is driven from 7x tandem tet operator (TetO) for robust
1403   gene expression. Following blasticidin expression, lytic replication was induced with
1404   doxycycline for 0, 24, 48, 72, and 96 hours or 96 hours with 500 nM PAA (to inhibit genome
1405   replication) and and harvested for total RNA. mRNA levels of XBP1s-target gene ERdj4 and
1406   viral genes RTA (immediate early gene [IE]), ORF45 (early gene [E]), and K8.1 (late gene [L])
1407   were measured by qPCR using 18S rRNA as a reference gene for normalization. An average of 3
1408   independent experiments are graphed and error bars denote SEM. (B) iSLK.219 cells were
1409   transduced with lentiviral doxycycline-inducible expression vectors encoding myc-XBP1s whose
1410   expression is driven from either 1x TetO (weak expression) or 7x TetO (strong expression) and
1411   treated with doxycycline for the indicated times and harvested for total cell lysates. Immunoblots
1412   were done for myc-epitope tag (XBP1s), and the viral proteins RTA (IE), ORF45 (E), ORF65
1413   (L), and ORF26 (L). β-actin was used as a loading control. The presented immunoblots are
1414   representative of 2 independent experiments. (C) 7xTetO-myc-XBP1s and vector transduced
1415   iSLK.219 cells were treated with doxycycline for 96 hours and intracellular DNA purified. qPCR
1416   against ORF26 DNA was done to measure the relative change in viral genome replication using
1417   the ΔΔ*Cq* method and normalized to β-actin DNA. Values are the mean of 3 independent
1418   experiments -/+ SEM. (D) Virus-containing supernatants from (C) were serially diluted and
1419   spinfected onto a monolayer of 293A cells. GFP-positive cells were quantified by flow
1420   cytometry the following day and used to calculate virus titre (IU/ml). The values are the mean
1421   virus titre of 4 independent experiments -/+ SEM. (E) 7xTetO-myc-XBP1s and vector
1422   transduced iSLK.219 cells were treated with doxycycline for 96 hours and DNase-protected
1423   genomic DNA from supernatants were column purified. Firefly luciferase plasmid DNA was
1424   added during DNA purification to allow for normalization. The relative change in virus titre
1425   (infectious and non-infectious) was quantified by qPCR using ORF26 primers and normalized to
1426   luciferase DNA using the ΔΔ*Cq* method. Values are the mean of 4 independent experiments -/+
1427   SEM. (F and G) iSLK.219 cells were transduced with lentiviral expression vectors encoding
1428   either doxycycline-inducible 7xTetO-myc-XBP1s or 7xTetO-FLAG-RTA. Following blasticidin
1429   selection, lytic replication was induced with doxycycline for (F) 48 hours and fluorescence
1430   microscopy was used to image RFP-positive cells (cells undergoing lytic replication) or for (G)
1431   24, 48, 72, and 96 hours and virus supernatants were harvested to measure titre by flow
1432   cytometry. Values are an average of 3 independent experiments -/+ SEM.

1433

1434   **Fig. 8: XBP1s overexpression inhibits KSHV replication at a late stage**
1435   (A,B, and C) iSLK.219 cells were transduced with increasing concentrations of CMV-driven
1436   lentiviral expression vectors encoding myc-XBP1s (LV dilutions: 1:400, 1:200, 1:100, 1:50,
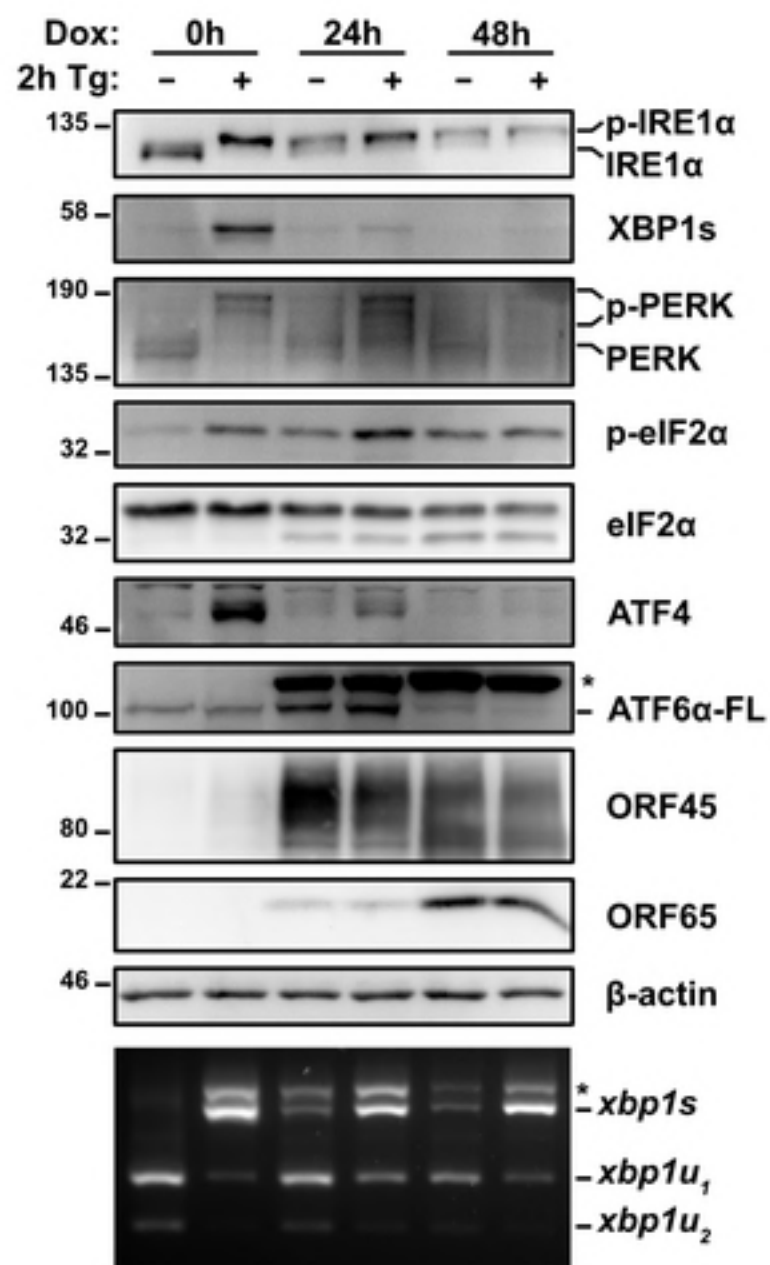
34

1437   1:25) or FLAG-RTA (LV dilutions: 1:32, 1:16, 1:8, 1:4, 1:2) for 6 days or treated with
1438   doxycycline for 72 hours (as a positive control) and (A) a subset of the lentiviral dilutions, cells
1439   were imaged for RFP-positive cells by fluorescence microscopy; (B) virus-containing
1440   supernatants were harvested for measuring virus titre by flow cytometry; and (C) cell lysates
1441   were analyzed by immunoblots for myc-epitope tag (XBP1s), and the viral proteins RTA,
1442   ORF45, and ORF65.). β-actin was used as a loading control.
1443
1444   **Supplemental Figure. 1: XBP1s transactivates RTA to induce lytic reactivation**
1445   (A) Wildtype BCBL1 cells were treated with either 1mM sodium butyrate (NaB) and 20 ng/mL
1446   of the phorbol ester TPA (12-O-Tetradecanoylphorbol-13-acetate) or 150 nM Tg for 6, 12, and
1447   24 hours and RTA mRNA levels were measured by qPCR. Changes in mRNA levels were
1448   calculated by the $\Delta\Delta Cq$ method and normalized using β-actin. An average of 3 independent
1449   experiments are graphed and error bars denote SEM. (B) Hela Tet-Off cells were transfected
1450   with pCMV2B-XBP1s or pCR3.1 Vector control and a firefly luciferase expression construct
1451   that is driven by 4 tandem XBP1 target sequences that are derived from the promoter of ORF50.
1452   A renilla luciferase plasmid was added for normalization and 24 hours later the vector control
1453   samples were treated with 500 nM Tg for 16h. Approximately 48 hours post transfection, cells
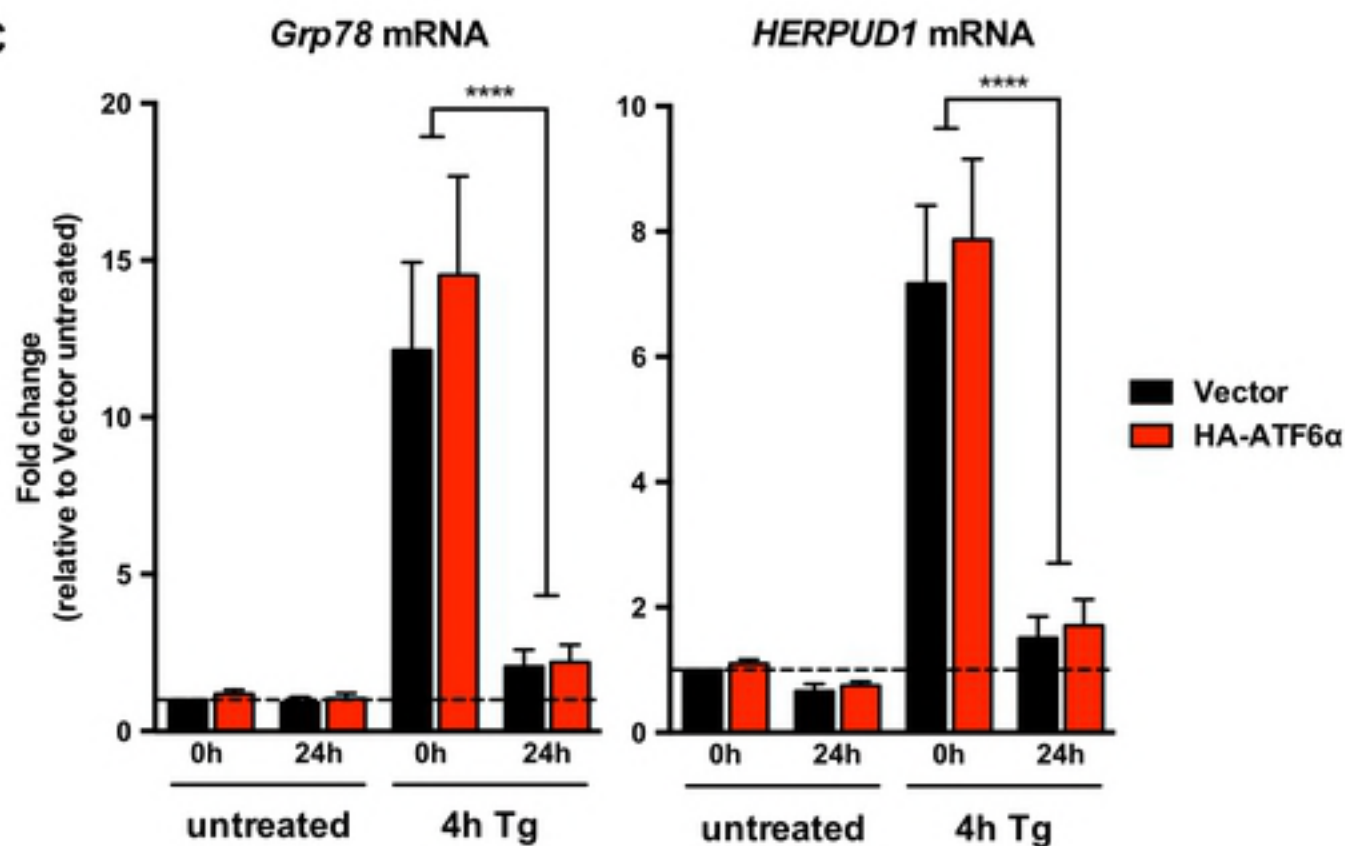1454   were lysed and a dual-luciferase assay was conducted to measure XBP1 activity.
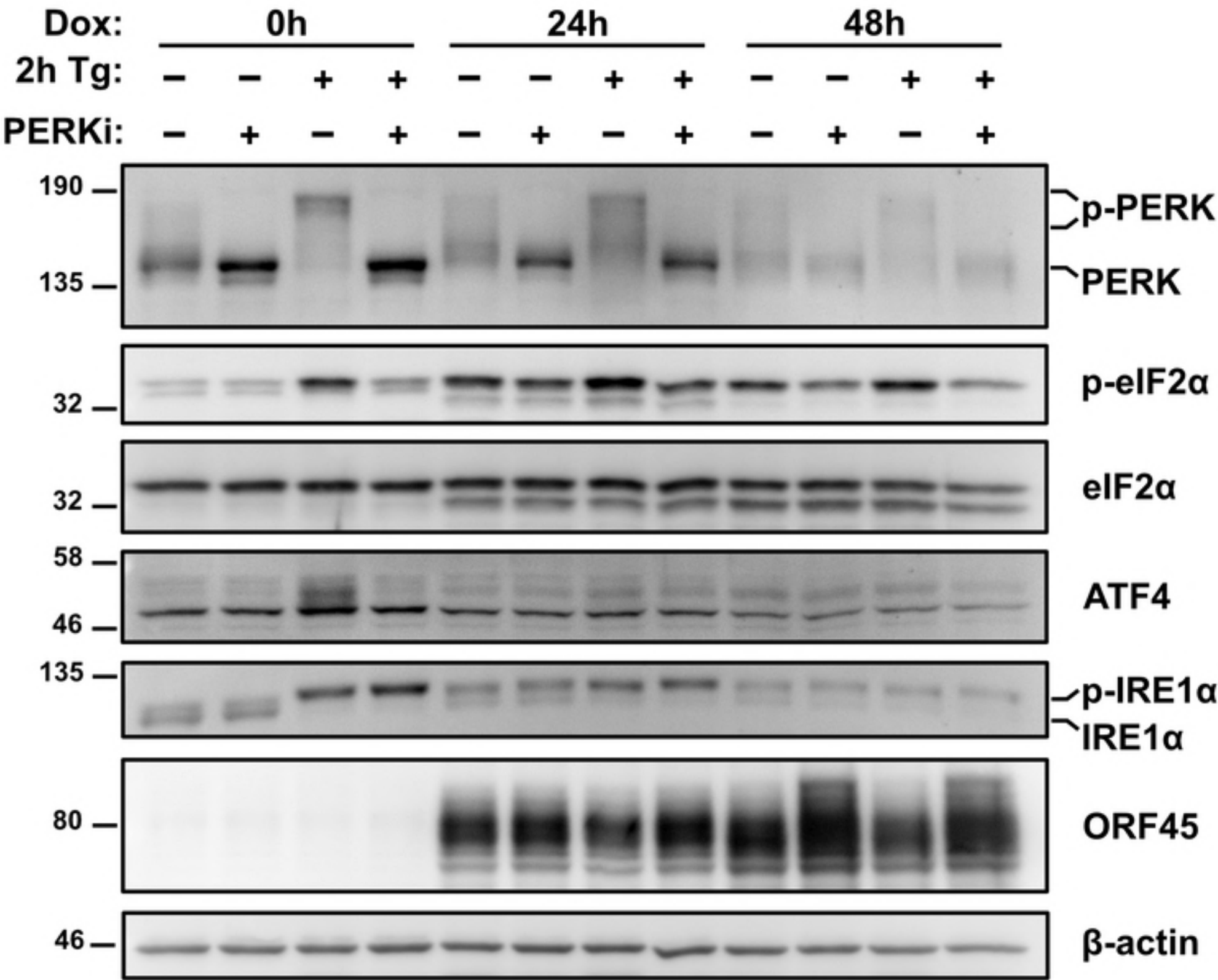1455

Figure 1

A

Dox:	0h	24h	48h
2h Tg:	− +	− +	− +

- p-IRE1α
- IRE1α
- XBP1s
- p-PERK
- PERK
- p-eIF2α
- eIF2α
- ATF4
- * ATF6α-FL
- ORF45
- ORF65
- β-actin
- * xbp1s
- xbp1u₁
- xbp1u₂

135 —
58 —
190 —
135 —
32 —
32 —
46 —
100 —
80 —
22 —
46 —

B

HA-ATF6α

Dox:	0h	24h	48h
4h Tg:	− +	− +	− +

- HA-ATF6α-FL
- HA-ATF6α-N
- p-PERK
- PERK
- ORF45
- β-actin

100 —
80 —
58 —
190 —
135 —
80 —
46 —

C

Grp78 mRNA

HERPUD1 mRNA

Fold change (relative to Vector untreated)

****

untreated   4h Tg

0h 24h   0h 24h

■ Vector
■ HA-ATF6α
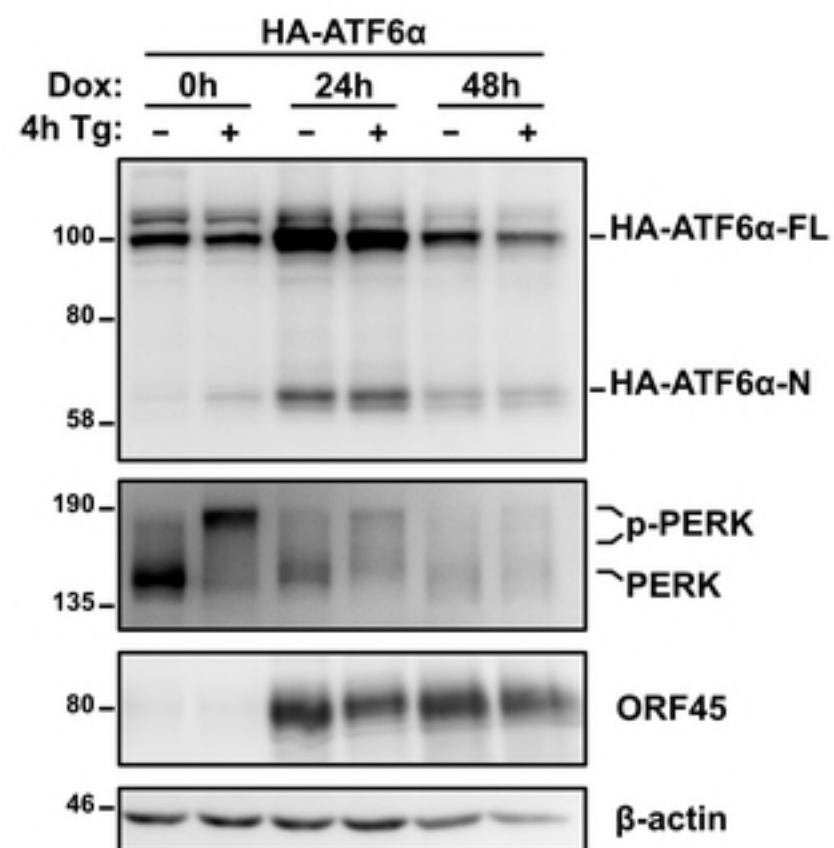
Figure 1

Figure 2

Figure 2
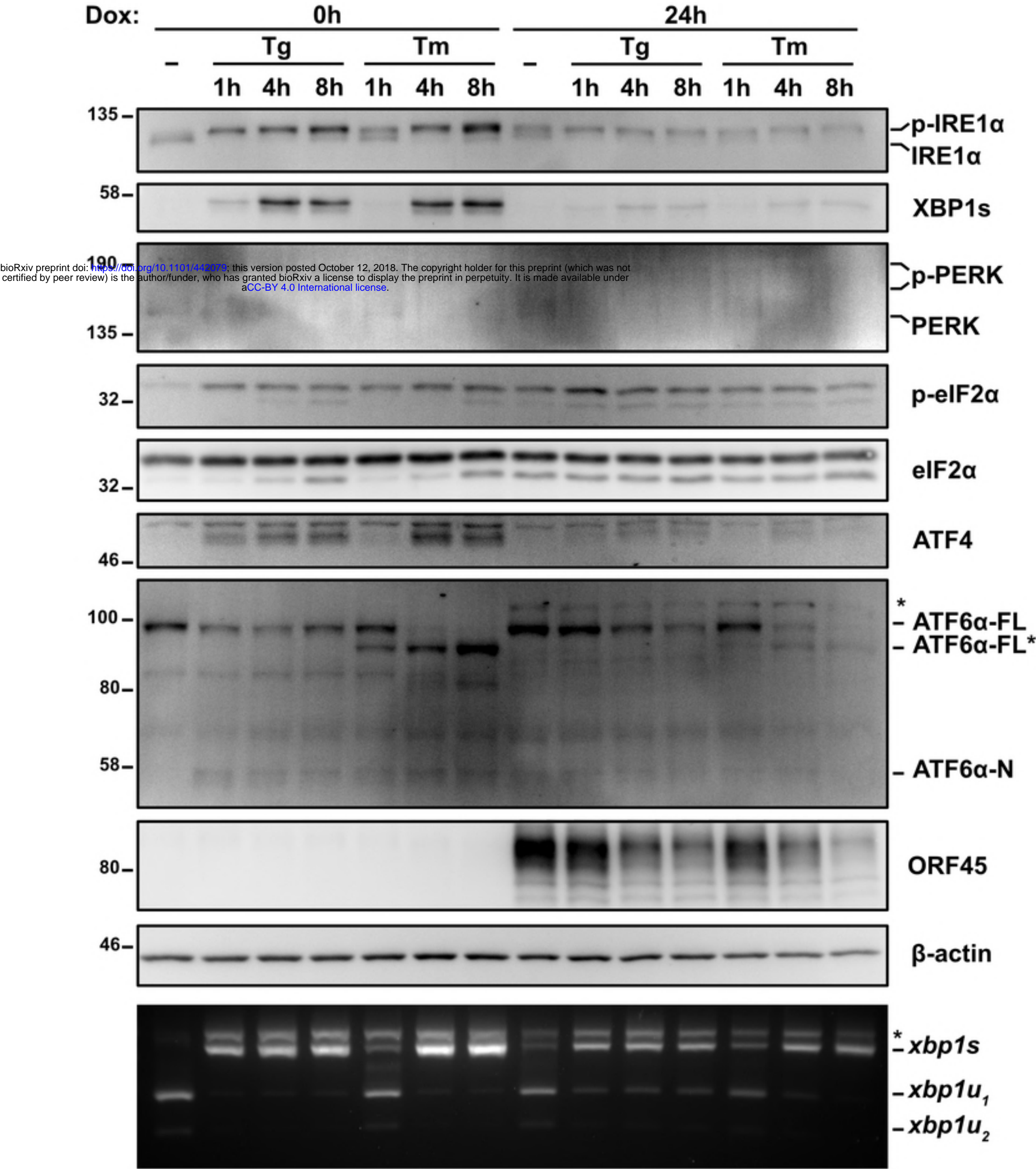
Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

Figure 6

A

B

C

D

Figure 6

Figure 7

A

B

C

D

E

F

G

Figure 7

Figure 8

A

B

C

Figure 8