Regulation of gammaherpesvirus lytic replication by endoplasmic reticulum stress-induced transcription factors ATF4 and CHOP

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Abstract

The stress-induced unfolded protein response (UPR) in the endoplasmic reticulum (ER) involves various signaling crosstalks and controls cell fate. B-cell receptor (BCR) signaling, which can trigger UPR, induces gammaherpesvirus lytic replication and serves as a physiological mechanism for gammaherpesvirus reactivation in vivo. However, how the UPR regulates BCR-mediated gammaherpesvirus infection is unknown. Here, we demonstrate that the ER stressors tunicamycin and thapsigargin inhibit BCR-mediated murine gammaherpesvirus 68 (MHV68) lytic replication by inducing expression of the UPR mediator Bip and blocking activation of Akt, ERK, and JNK. Both Bip and the downstream transcription factor ATF4 inhibited BCR-mediated MHV68 lytic gene expression, whereas UPR-induced C/EBP homologous protein (CHOP) was required for and promoted BCR-mediated MHV68 lytic replication by suppressing upstream Bip and ATF4 expression. Bip knockout was sufficient to rescue BCR-mediated MHV68 lytic gene expression in CHOP-knockout cells, and this rescue was blocked by ectopic ATF4 expression. Furthermore, ATF4 directly inhibited promoter activity of the MHV68 lytic switch transactivator RTA. Altogether, we show that ER stress–induced CHOP inhibits Bip and ATF4 expression and that ATF4, in turn, plays a critical role in CHOP-mediated regulation of BCR-controlled MHV68 lytic replication. We conclude that ER stress–mediated UPR and BCR signaling pathways are interconnected and form a complex network to regulate the gammaherpesvirus infection cycle.

Gammaherpesviruses are lymphotropic viruses that are associated with the development of lymphoproliferative diseases and other nonlymphoid cancers, containing two genera, lymphocryptoviruses and rhadinoviruses (1). Human gammaherpesviruses include Epstein-Barr virus (EBV) which belongs to lymphocryptovirus and Kaposi sarcoma–associated herpesvirus (KSHV) which is a member of rhadinoviruses (2-5). Due to the narrow host tropism of human gammaherpesviruses, murine gammaherpesvirus 68 (MHV68) which is isolated from bank voles and yellow-necked field mice provides a valuable mouse model to define gammaherpesviral pathogenesis (6,7). MHV68 infection of laboratory mice mimics EBV infection in human. During chronic infection, MHV68 drives the infected B cells to undergo proliferation and differentiation into memory B cells as latency reservoir and plasma cells as reactivation reservoir (8-10), sharing the common feature of gammaherpesviruses that establish latency in B lymphocytes.

Although various stimuli that activate B cells could induce lytic cycle of gammaherpesvirus from latency, anti-immunoglobulin (anti-Ig) cross-linking by binding to surface Ig mimics the effect of antigen binding to Ig molecules of antigen-specific B cells and subsequently induces B cell receptor (BCR) signaling pathway, which serves as one more physiologically relevant activator of gammaherpesvirus lytic reactivation (11,12). Anti-Ig mediated BCR signaling via activating cellular kinases can trigger viral reactivation of EBV, KSHV or MHV68 latently infected B cells (13-17). The induction of the gammaherpesvirus lytic cycle is initiated by activation of conserved lytic switch gene, which is encoded by highly conserved immediately-early gene BRLF1 in EBV and ORF50 in KSHV and MHV68. It is also commonly referred as RTA, whose expression is sufficient to disrupt EBV, KSHV and MHV68 latency in some cell lines.
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(11,18-22). In the case of EBV, there is an additional immediate-early transcriptional activator, Zta, encoded by the BZLF1 gene that is essential and sufficient to trigger EBV lytic cycle (11,22).

Antigen binding to surface Ig induces aggregation of the BCR and leads to phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) tyrosines by the SRC-family kinases Lyn, Fyn, and Blk. The tyrosine kinase SYK is subsequently recruited to phosphorylated ITAM and forms a signalosome with the SRC-family kinases and other adaptors, activating downstream Akt, Phosphatidylinositol 3-Kinase (PI3K), c-Jun N-terminal kinases (JNKs), MAPK/extracellular signal-regulated kinases (ERKs), NF-κB and other signaling pathways (23). Activation of BCR signaling pathways like PI3K and downstream ERK and p38 is important for BCR-mediated EBV activation (24).

The unfolded protein response (UPR) is an endoplasmic reticulum (ER)-to-nucleus signaling pathway initiated by the protein folding demand overwhelming folding capacity in ER, which is an ER stress response pathway that controls cell fate (25-27). UPR is initiated and mediated by three ER transmembrane stress sensors, protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6) (28,29). In resting state, these sensors associated with binding immunoglobulin protein (Bip). ER stress accumulates unfolded proteins, activates the three ER stress sensors by dissociating Bip and induces PERK, IRE1α, and ATF6-mediated UPR response pathways, leading to the UPR-related gene expression such as ATF4 and C/EBP homologous protein (CHOP) (29). ER stress differentially regulates gammaherpesvirus lytic replication, such as, ER stress inducer thapsigargin (TG), which inhibits the ER Ca\(^{2+}\) ATPase to recover luminal ER calcium stores (30), triggers EBV lytic replication in lymphoblastoid cell lines (LCLs) (31), whereas induction of ER stress by 2-deoxy-d-glucose inhibits KSHV and MHV68 lytic gene expression (32).

It has been demonstrated that BCR signaling is a physiologic UPR trigger and induces an adaptive UPR characterized by upregulation of Bip and CHOP (33). Surface immunoglobulin M-mediated BCR signaling induces a UPR which is dependent on BCR signaling molecule BTK and SYK in chronic lymphocytic leukemia cells, the activation level of UPR correlates with disease progression (34). Since both BCR and UPR signaling could mediate gammaherpesvirus lytic replication, in line with the induction of UPR by BCR signaling, we investigate the role of UPR in BCR-mediated gammaherpesvirus lytic replication. Here, we show that ER stress by TG and tunicamycin (TM) inhibited BCR-mediated MHV68 viral DNA replication and lytic gene expression in MHV68-immortalized SL-1 lymphoma B cells, in concomitant with the inhibition of constitutive Akt, ERK, and JNK activation after prolonged TG or TM treatment, preceded by Bip and CHOP induction. Ectopic CHOP expression promoted BCR-mediated MHV68 lytic gene expression but did not activate the transcription of MHV68 RTA promoter, whereas CHOP knockout abolished BCR-mediated MHV68 lytic replication without influencing BCR signaling, which can be fully rescued by Bip knockout. Importantly, CHOP inhibited Bip and downstream transcription factor ATF4 expression. ATF4 directly inhibited RTA promoter activity, suppressed BCR-mediated MHV68 lytic gene expression and correspondingly contributed to the regulatory role of CHOP in BCR-mediated MHV68 lytic replication.

Results

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Anti-Ig cross-linking not only efficiently induces EBV lytic cycle (15,16), also efficiently triggers MHV68 lytic replication in B cells expressing surface Ig (14,35,36). Furthermore, ER stress inducers, TM and TG, can also trigger EBV lytic activation in Burkitt’s lymphoma cells and LCLs (31,37). Based on the link between BCR-mediated signaling and UPR (33,38), we wonder whether UPR interferes with BCR-mediated gammaherpesvirus lytic replication. To test this possibility, we used TM which blocks N-link protein glycosylation and TG which disrupts ER calcium homeostasis. MHV68–immortalized SL-1 cells were treated with 5μg/ml TM or 5μM TG in the presence or absence of 5μg/ml F(ab’)2 anti-mouse IgG for 24 hr and 48 hr. Immunoblot analyses were performed using specific antibodies against UPR-induced gene Bip, MHV68 vCyclin, ORF59 and lytic antigens as well as loading control GAPDH. As expected, Bip expression was induced at 24 and 48 hr after TM or TG stimulation (Fig. 1A). Consistent with the previous report that BCR signaling induces UPR (33), anti-Ig treatment upregulated Bip expression, regardless of TG or TM treatment (Fig. 1A). However, either TM or TG stimulation completely blocked protein expression of MHV68 vCyclin, ORF59, and lytic antigens mediated by surface Ig cross-linking (Fig. 1A). Likewise, quantitative RT-PCR analyses showed that TM and TG significantly decreased mRNA levels of MHV68 latency-associated gene ORF73, immediate-early gene ORF50, early gene ORF59, and late gene ORF25 induced by surface Ig cross-linking (Fig. 1B). Although ORF73 is a latency-associated gene, its expression can be induced upon MHV68 lytic reactivation (35).

Next, we determined the effects of TM and TG on MHV68 viral DNA replication. SL-1 cells were exposed to TM or TG, together with anti-Ig stimulation for 48 hr. To detect MHV68 viral genome, we performed quantitative PCR with specific primers corresponding to the ORF50 (RTA) coding region. Both TM and TG dramatically reduced MHV68 viral DNA induced by surface Ig cross-linking, whereas DMSO had little effect (Fig. 1C), indicating that TM and TG inhibit BCR-mediated MHV68 viral DNA replication. We then tested whether TM or TG has any effect on the activation of MHV68 lytic switch protein RTA promoter. M12 murine B cells were transfected with RTA luciferase promoter construct in the presence or absence of TM treatment, together with or without anti-Ig stimulation. TM treatment had no direct significant effect on RTA promoter activity regardless of anti-Ig treatment (Fig. 1D). Altogether, these data illustrate that UPR by TM or TG inhibits BCR-mediated MHV68 lytic replication.

Prolonged ER stress inhibits Akt, ERK, and JNK activation

BCR-mediated signaling pathways including PI3K pathway are important for BCR-mediated EBV and KSHV lytic reactivation (16,17,24). As such, we wonder whether TM and TG block MHV68 lytic replication through inhibiting BCR signaling pathways. To test this, we performed a time-course experiment in SL-1 cells treated with TM or TG in the presence or absence of anti-Ig. We examined three main BCR signal transduction pathways by detecting the level of phosphorylated ERK, Akt, and JNK. Immunoblot analyses showed that both TM and TG did not reduce activation of ERK, Akt, and JNK at 0.5 hr post-treatment, instead, increased the magnitude of JNK activation upon anti-Ig treatment (Fig. 2A and 2B), which is in agreement with the previous report that ER stress activates JNK (39). At 3 hr and 6 hr TM or TG post-stimulation, we started to observe significant increase of UPR-induced...
transcription factors ATF4 and CHOP regardless of surface Ig cross-linking and Bip upregulation primarily in the presence of surface Ig cross-linking, induction of Bip, ATF4 and CHOP maintained over the course of the experiment, although the magnitude of induction varied at the later time point (Fig. 2A). ATF4 induction was prior to CHOP induction and showed similar pattern to CHOP, supporting that ATF4 is an upstream transcription factor of CHOP upon ER stress. Concomitantly, Activation of ERK, Akt, and JNK was dramatically blocked by 24 and 48 hr TM or TG post-stimulation (Fig. 2A and 2B). We also detected JNK downstream effector c-Jun activation and observed that both TM and TG did not inhibit c-Jun phosphorylation as compared to control DMSO treatment (Fig. 2A).

To rule out the effect of ER stress-related apoptosis on BCR activation and MHV68 lytic replication, we measured the cell viability for SL-1 cells treated with TM or TG in the presence or absence of anti-mouse IgG for 24 hr and 48 hr. We observed slight increase instead of the decrease of cell viability upon TM or TG treatment as compared to DMSO treatment in the presence of anti-mouse IgG (Fig. 2C), suggesting that apoptosis does not play a role in the attenuation of BCR activation and inhibition of MHV68 lytic replication by TM or TG upon anti-Ig stimulation. Altogether, these data illustrate that TM and TG have no effect on initial activation of BCR signal transduction, but block BCR-mediated constitutive activation of ERK, Akt, and JNK at the later time point after 24 hr prolonged ER stress treatment, which might contribute to the inhibition of BCR-mediated MHV68 lytic replication at 48 hr post anti-Ig stimulation.

To determine the link between inhibition of ERK, Akt, and JNK activation and inhibition of BCR-mediated MHV68 lytic replication, SL-1 cells were treated with Akt inhibitor VIII, ERK inhibitor PD98059, and JNK specific inhibitor SP600125, respectively, together with surface Ig cross-linking. All inhibitors completely blocked MHV68 lytic antigen expression induced by anti-Ig stimulation (Fig. 3A), indicating that ERK, Akt, and JNK activation of BCR signaling is required for BCR-mediated MHV68 lytic gene expression. Next, we examined the cell viability for SL-1 cells treated with inhibitors in the presence of anti-mouse IgG for 48 hr and observed that only 100μM Akt inhibitor reduced cell viability significantly as compared to DMSO (Fig. 3B). Thus cell cytotoxicity induced by inhibitors does not play an important role in MHV68 lytic gene expression since the lower concentration of inhibitor treatment without eliciting cytotoxicity still showed the inhibition of MHV68 lytic gene expression. Therefore, TM and TG possibly block BCR-mediated MHV68 lytic replication through inhibiting constitutive BCR signaling pathways. Given that TM and TG-induced CHOP and Bip expression preceded the inhibition of BCR-mediated ERK, Akt, and JNK activation (Fig. 2), it is also likely that ER stress-induced CHOP, ATF4 or Bip is the main regulator for UPR-mediated inhibition of BCR-mediated MHV68 lytic replication.

**CHOP is required for BCR-mediated MHV68 lytic replication**

We analyzed the possibility of ER stress-induced downstream transcription factor CHOP involving in BCR-mediated MHV68 lytic replication. To delineate the role of CHOP, we used CRISP/Cas9 system with puromycin selection to generate CHOP knockout SL-1 cells and performed limiting dilution culture to select individual clones in which CHOP gene was efficiently knocked out and confirmed by sequencing analyses. We chose the clone C-4 in which the CHOP genomic sequences between 161939-162032 were deleted and CHOP expression was completely knocked out and clone C-2 in which the CHOP genomic sequences between 161990-162062 were
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deleted and CHOP expression was partially knocked out for further analyses. Considering the potential effect of puromycin on cell physiology, SL-1 cells transfected with CRISP/Cas9 vector alone and parallelly selected with puromycin were used as control cells. Surprisingly, CHOP knockout completely inhibited expression of MHV68 vCyclin, ORF59 and lytic antigens induced by surface Ig cross-linking in C-4 cells, whereas MHV68 lytic gene expression was partially blocked in C-2 cells, correlated with a partial knockout of CHOP gene (Fig. 4A). It’s worth noting that Bip expression was increased in CHOP knockout C-4 and C-2 cells regardless of anti-Ig treatment, the increased level of Bip expression corresponded to the knockout level of CHOP (Fig. 4A and 4B), indicating that CHOP inhibits Bip expression.

Next, we examined the effect of CHOP on MHV68 viral DNA replication and temporal gene expression during lytic reactivation. Clones C-4, C-2, and control cells were treated with anti-Ig for 48 hr, followed by quantitative PCR analyses to detect MHV68 viral genome and qRT-PCR analyses to detect MHV68 gene expression. As compared to control cells, MHV68 viral DNA replication was significantly blocked in C-4 cells and partially blocked in C-2 cells in response to anti-Ig treatment (Fig. 4C). Similarly, mRNA expression of MHV68 ORF73, ORF50, ORF59, and ORF25 was also dramatically inhibited in C-4 and C-2 cells (Fig. 4D). Altogether, these data demonstrate that CHOP is required for BCR-mediated MHV68 viral DNA replication and lytic gene expression.

**Ectopic CHOP expression promotes BCR-mediated MHV68 lytic gene expression without activating RTA promoter**

To further define the role of CHOP in BCR-mediated MHV68 lytic replication, C-4 and control cells with were transfected with a CHOP-expressing plasmid with AU1 tag or vector. At 24 hr post-transfection, cells were treated with anti-Ig for 48 hr. CHOP overexpression not only augmented expression of MHV68 ORF59, vCyclin and lytic antigens mediated by surface Ig cross-linking in control cells, also rescued MHV68 lytic antigen expression induced by anti-Ig cross-linking in C-4 cells (Fig. 5A). Both vCyclin and ORF59 expression were also slightly induced by CHOP overexpression in C-4 cells (Fig. 5A). To further confirm this, we transfected C-4 cells with the CHOP-expressing plasmid, followed by anti-Ig treatment and immunofluorescent staining analyses. Notably, CHOP-expressing cells showed the expression of MHV68 lytic antigen (Fig. 5B, left panel). CHOP expression significantly increased the frequency of MHV68 lytic antigen-positive cells (Fig. 5B, right panel), indicating that CHOP expression could rescue MHV68 lytic gene expression in C-4 cells upon anti-Ig treatment.

The essential role of CHOP in BCR-mediated MHV68 lytic replication prompted us to investigate whether CHOP could activate the promoter of MHV68 lytic switch gene RTA directly. To test this possibility, murine M12 B cells were transfected with MHV68 RTA luciferase reporter plasmid, together with a CHOP-expressing plasmid or vector. Instead of activating RTA promoter, ectopic CHOP expression inhibited RTA promoter activity in the absence of anti-Ig stimulation and had no significant effect on RTA promoter in the presence of anti-Ig treatment (Fig. 5C). Thus, we conclude that CHOP is not only required, also promotes BCR-mediated MHV68 lytic replication, independent of CHOP regulation of RTA transcription.

**CHOP is not essential for BCR signaling activation but required for constitutive JNK activation**

Based on the requirement of both CHOP and BCR signal transduction for BCR-mediated
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MHV68 lytic replication, we examined whether CHOP interfered with BCR signaling pathways. To test this, a time-course experiment was performed in CHOP knockout C-4 and control cells treated with anti-Ig. We did not observe any reduced activity for Akt, ERK, JNK, p38 and p65 in C-4 cells as compared to control cells at 15, 30 and 60 min post anti-Ig treatment, instead, Akt and JNK activity was slightly increased in C-4 cells at 15, 30 and 60 min post anti-Ig stimulation (Fig. 6A), suggesting that CHOP is not required for the initiation of BCR signaling activation. However, JNK activity was significantly blocked in C-4 cells at 24 and 48 hr post anti-Ig treatment, whereas ERK activity was only inhibited at 24 hr, but not at 48 hr post anti-Ig treatment in C-4 cells (Fig. 6B), indicating that CHOP is required for constitutive JNK activation, which is required for MHV68 lytic replication. Overall, our data demonstrate that CHOP is not necessary for the initiation of BCR signal transduction but required for constitutive JNK activation, which might due to the indirect effect and ultimately contribute to the role of CHOP in facilitating BCR-mediated MHV68 lytic replication.

Bip inhibits BCR-mediated MHV68 lytic gene expression and mediates CHOP regulation of MHV68 lytic gene expression

As aforementioned, we consistently observed the upregulation of Bip expression in CHOP knockout cells irrespective of anti-Ig stimulation (Fig. 4A, 5A, and 6A), which prompted us to examine whether Bip regulates BCR-mediated MHV68 lytic replication and mediates CHOP function. To test this possibility, SL-1 cells were transfected with a Bip-expressing plasmid with Flag epitope tag or vector, followed by anti-Ig treatment for 48 hr. Ectopic Bip expression significantly inhibited BCR-mediated expression of MHV68 ORF59, vCyclin and lytic antigens (Fig. 7A). It’s worth noting that Bip overexpression led to the decrease of ATF4 and CHOP expression regardless of anti-Ig treatment, which is consistent with the previous report that ectopic Bip expression does not lead to ER stress and is able to attenuate ER stress signal (40). Immunofluorescent staining also showed that MHV68 lytic antigen was only detected in the cells without Bip expression but not in the cells expressing Bip upon anti-Ig stimulation (Fig. 7B, left panel). Bip-expressing cells showed significant percentage reduction of MHV68 lytic antigen positive cells as compared to the cells transfected with vector alone (Fig. 7B, right panel), supporting the role of Bip in inhibiting BCR-mediated MHV68 lytic gene expression. In consistent with this, Bip knockout increased the expression of the MHV68 lytic antigen, ORF59, and vCyclin in SL-1 cells upon anti-Ig stimulation (Fig. 7C).

In line with the role of CHOP in inhibiting Bip expression and the effect of Bip on blocking BCR-mediated MHV68 lytic gene expression, it is likely that CHOP promotes BCR-mediated MHV68 lytic replication through negatively regulating Bip expression. To test this possibility, we generated Bip knockout in CHOP knockout C-4 and control cells by transfecting Bip single guide RNA (sgRNA)-expressing CRISP/Cas9 plasmid or vector alone. The selected clone was confirmed by sequencing analyses and showed the deletion of Bip genomic sequences between 63760-63852 in Bip knockout C-4 cells. The selected individual clones were treated with anti-Ig stimulation for 48 hr and subsequently subjected to immunoblot analyses. Strikingly, Bip knockout substantially rescued MHV68 lytic antigen, vCyclin, and ORF59 expression in CHOP knockout C-4 cells upon anti-Ig stimulation (Fig. 7D), suggesting that Bip is negatively regulated by CHOP and responsible for the role of CHOP in promoting BCR-mediated MHV68 lytic gene expression. In Bip knockout cells, we consistently
observed the reduction of ATF4 expression in the absence of anti-Ig (Fig. 7C and 7D), suggesting that ATF4 is a downstream transcription factor mediated by Bip. However, Bip knockout had no significant effect on CHOP expression when Bip was knocked out in parental SL-1 cells (Fig. 7C), but increased the level of CHOP when Bip was knocked out on the selected control cells (Fig. 7D), indicating that the second hit of nucleofection and puromycin selection might have some effect on cell physiology.

Next, we examined the effect of ectopic Bip expression on Akt, ERK, and JNK activation. Bip overexpression did not inhibit constitutive phosphorylation of Akt, ERK, and JNK (Fig. 7E), which rules out the possibility that Bip upregulation contributes to the inhibition of Akt, ERK, and JNK activity by TM and TG at the later time point as shown in Figure 2. Furthermore, ectopic Bip expression activated rather than inhibited MHV68 RTA promoter transcription irrespective of anti-Ig stimulation (Fig. 7F), suggesting that Bip inhibition of MHV68 lytic gene expression is not through direct regulation of RTA promoter, Bip regulation of CHOP function in BCR-mediated MHV68 lytic gene expression is most likely indirect.

**ATF4 inhibits RTA promoter activity and plays a key role in CHOP regulation of MHV68 lytic replication**

ER stress induces multiple cellular responses mediated by Bip-associated stress sensors IRE1α, ATF6, and PERK. PERK activation initiates the phosphorylation of eIF2α and induces translation of transcription factor ATF4 and subsequent downstream CHOP expression, whereas active IRE1α processes transcription factor XBP1 to produce an active spliced form, XBP1s (28). Given that CHOP inhibited Bip expression and Bip did not play a direct role in CHOP regulation of MHV68 lytic replication, we examined Bip downstream molecules in CHOP knockout C-2 and C-4 cells. Correspondingly, expression of Bip downstream transcription factors ATF4, ATF6, and XBP1 was upregulated in CHOP knockout cells (Fig. 8A), indicating that CHOP also inhibits expression of Bip downstream transcription factors. CHOP has been shown to mediate ER stress-induced apoptosis and downregulate BCL-2 expression (28). We also observed that BCL-2 expression was upregulated in CHOP knockout C-2 and C-4 cells (Fig. 8A). Next, we tested whether BCL-2 upregulation had any effect on MHV68 lytic gene expression, SL-1 cells were transfected with a BCL-2-expressing plasmid with Flag-tag or vector alone. BCL-2 overexpression had no effect on MHV68 lytic antigen expression upon anti-Ig stimulation (Fig. 8B). Considering XBP1s activation of gammaherpesviral lytic replication and ATF6 ER-Golgi translocation upon activation (28,41,42), we postulated that ATF4 could be more likely to play an important role. To test this, M12 cells were firstly co-transfected with an ATF4-expressing plasmid and RTA luciferase promoter construct. Strikingly, ATF4 overexpression significantly blocked MHV68 RTA promoter activation in M12 cells regardless of anti-Ig treatment (Fig. 8C). Next, SL-1 cells were transfected with an ATF4-expressing plasmid with Flag epitope or vector alone, together with or without anti-Ig stimulation. Consistently, ectopic ATF4 expression blocked expression of the MHV68 lytic antigen, ORF59, and vCyclin induced by anti-Ig (Fig. 8D). Furthermore, ectopic ATF4 expression alone slightly induced Bip expression but not CHOP expression in the absence of ER stress (Fig. 8D).

As demonstrated above, Bip knockout rescues MHV68 lytic gene expression in CHOP knockout C-4 cells (Fig. 7D). However, Bip has no direct effect on MHV68 lytic gene
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expression. Thus we wonder whether Bip knockout-derived rescue would result from downstream transcription factor ATF4 which could directly inhibit MHV68 RTA activation and lytic gene expression. To test this, an ATF4-expressing plasmid with Flag epitope tag was expressed in CHOP and Bip double knockout cells in the presence or absence of anti-Ig stimulation. Dramatically, ectopic ATF4 expression blocked MHV68 lytic gene expression rescued by Bip knockout in CHOP knockout cells upon anti-Ig induced viral reactivation (Fig. 8E and 8F), suggesting that CHOP knockout phenotype can be directly restored by ATF4 expression when Bip was knocked out. Altogether, these results illustrate that ATF4 inhibits MHV68 lytic gene expression through directly blocking RTA promoter activity; ATF4 expression is negatively regulated by CHOP and in turn, plays a key role in CHOP requirement of MHV68 lytic gene expression.

Discussion
ER stress buffered by the activation of UPR is involved in the pathogenesis of many human diseases. UPR signaling network regulates various processes like metabolism, immunity and cell differentiation (28,29). BCR signaling and plasma cell differentiation activate UPR (33,43,44). UPR, BCR signaling, and plasma cell differentiation are interconnected and form a complex crosstalk network. BCR signaling mediated by surface Ig cross-linking is a common way to induce gammaherpesvirus lytic cycle in vitro and has been viewed as main physiological stimuli that govern EBV and MHV68 lytic reactivation from latency in vivo as latently infected memory B cells differentiate towards plasma cells (11,12,45), whereas UPR also mediated EBV lytic replication in vitro (31,37). Here we demonstrate that ER stress induced by TM and TG inhibits BCR-mediated MHV68 viral DNA replication and lytic gene expression. UPR mediator Bip and downstream transcription factor ATF4 inhibit BCR-mediated MHV68 lytic gene expression, whereas UPR-induced CHOP is essential and promotes BCR-mediated MHV68 lytic replication by negatively regulating Bip and ATF4 expression. ATF4 can directly inhibit MHV68 RTA promoter activity. These results illustrate the complex crosstalk among UPR, BCR, and gammaherpesvirus infection cycle (Fig. 9).

Consistent with the previous report that BCR signaling is a physiologic UPR trigger (33,34), we also observed upregulation of CHOP and Bip expression upon anti-Ig treatment (Fig. 1A, 2A, and 4A). Furthermore, BCR signaling activation is very rapid and precedes induction of CHOP and Bip by TM or TG (Fig. 2A). We consistently observed higher CHOP expression in SL-1 cells without anti-Ig stimulation as compared to the cells with anti-Ig stimulation at 48 hr post-culture, but not at 24 hr post-culture (Fig. 2 and 6B), the reason for this might due to certain signal derived from prolonged cell proliferation in the absence of anti-Ig, leading to CHOP induction, whereas in the presence of anti-Ig, SL-1 cells undergo reactivation process and proliferation arrest. Furthermore, we consistently observe that the selected knockout clone after puromycin selection was more vulnerable than parental cells upon nucleofection, more susceptible to cell death. We chose D17 nucleofection programs which showed higher survival rate and lower transfection efficiency to perform CHOP overexpression rescue experiment in C-4 cells instead of using T20 program which showed higher transfection efficiency and lower survival rate and was used in parental SL-1 cells.

UPR mediated by ER stressor TM and TG triggers EBV lytic replication in some virus-positive lymphoma cell lines (31,37). However, we showed that TM and TG stimulation alone failed to induce MHV68 lytic gene expression in SL-1 lymphoma cells as they
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do in EBV-positive lymphoma cells. The possible cause might result from MHV68 tight latency that can only be reactivated by surface Ig cross-linking for 48 hr in SL-1 cells (36), whereas EBV latency-lytic cycle switch is more readily rapid and efficient by surface Ig cross-linking and other stimuli (15,17). Additionally, TM and TG block BCR-mediated JNK, Akt, and ERK constitutive activation which is an indirect effect as suggested by our results. Other signaling pathways involved need further investigation.

Bip inhibits BCR-mediated MHV68 lytic gene expression, which is different from the observation that Bip positively regulates tetradecanoylphorbol acetate (TPA)-induced KSHV lytic cycle progression in primary effusion lymphoma cells (46). The discrepancy might be attributed to the distinct signaling pathways regulated by TPA and BCR. As all known KSHV-positive lymphoma B cells naturally do not express surface Ig, we reason that UPR regulation of BCR-mediated lytic replication would possibly be conserved among gammaherpesviruses.

UPR-induced gene CHOP mainly mediates apoptosis and cytokine production (47,48). Here, we demonstrate for the first time that CHOP is required for and promotes BCR-mediated MHV68 lytic replication by inhibiting Bip and downstream ATF4 expression. ER stress-induced UPR initiates IRE1α, PERK, and ATF6 three main signaling pathways by dissociation with Bip, whereas CHOP is mainly induced by PERK-mediated signaling (28). Although we haven’t analyzed the effect of IRE1α and ATF6-mediated UPR signaling pathways on BCR-mediated MHV68 lytic replication, we do not exclude the possibility of both signaling pathways playing a role in BCR-mediated gammaherpesvirus lytic replication even though our data appear not to support this hypothesis, because activation of IRE1α downstream molecules JNK and XBP1s positively regulates MHV68 lytic replication as shown in Figure 3 and demonstrated previously (49). Additionally, ATF6 represents a group of ER stress transducers, translocates to the Golgi under ER stress conditions, and releases its cytosolic domain fragment ATF6f to control other gene expressions like XBP1 (28). It is less likely that ATF6 would inhibit RTA transcription directly. PERK-mediated ER stress signaling branch induces translation of transcription factor ATF4 which subsequently regulates CHOP expression. Previous reports have shown that ATF4 plays a critical role to promote murine cytomegalovirus (MCMV) DNA replication and late gene expression in a multiplicity of infection-dependent manner (50), whereas EBV LMP1 can activate PERK and upregulate ATF4 which in turn transactivates LMP1 promoter (51). Therefore, it is reasonable for us to propose that ATF4 might involve in the regulation of viral gene expression. Our data show that ATF4 inhibits MHV68 RTA transcription and lytic gene expression upon viral reactivation, in contrast to the observation that ATF4 promotes late gene expression during MCMV infection. This discrepancy might result from distinct scenario between viral reactivation and acute infection. Further experiments are needed to investigate the role of ATF4 in gammaherpesvirus de novo infection and reveal whether ATF4 exhibits different function between de novo infection and viral reactivation, or between beta-herpesviruses like CMV and gammaherpesviruses in the future.

In summary, our study reveals a complex and interconnected regulation network between UPR and BCR-mediated gammaherpesvirus lytic reactivation. This network cross-talk might shed light on a complex physiological scenario for the regulation of gammaherpesvirus latency and lytic cycle in vivo. It would be interesting to characterize this network cross-talk in other gammaherpesviruses and MHV68 infection mouse model and will be critical to extend our
understanding of gammaherpesvirus pathogenesis.

**Experimental procedures**

**Cell lines**

MHV68-transformed SL-1 cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin as previously described (36). Virus free murine B cell line M12 was cultured in RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin.

**Plasmids**

The MHV68 Rta-Luc plasmid containing the 410-bp proximal promoter region was a gift from Samuel Speck (52). The Bip-expressing plasmid with C-terminal Flag epitope tag was generated by insertion of PCR-amplified BIP coding fragments into pLVX-tdtomato or pLVX-puro vector (Clontech) with specific primers: forward primer with NotI restriction enzyme site:

ATGCGGCCGCATGATGAAGTTCACTGTGGTGGCGG

reverse primer with BamHI restriction enzyme site and Flag epitope tag:

GCGGATCCCTACTTGTCATCGTCGTCCTTGTAGTCCAACTCA

Two sgRNA sequences targeting CHOP gene were: CACCGGGCACCTATATCTCATCTCATCCCC and AAACGGGGATGAGATATAGGTGCCC.

Two sgRNA sequences targeting Bip gene were: CACCGATGATGAAGTTCACTGTGGT and AAACACCACGTAACCCATCATC.

**Generation of knockout cell lines**

We used the CRISPR-Cas9 genome-editing system to generate CHOP knockout SL-1 cells. 2x10^6 SL-1 cells were transfected with 4µg lentiCRISPRv2-vector, lentiCRISPRv2-CHOP-1 or lentiCRISPRv2-CHOP-2, respectively, using Amaxa Nucleofector II system (Lonza) according to the manufacturer’s instruction. After 48 hr post-nucleofection, cells were selected with puromycin at a concentration of 2µg/ml for two weeks. Cells were subsequently cloned by limiting dilution culture. Individual clones were subjected to immunoblot and sequencing analyses to confirm the depletion of the target protein CHOP. LentiCRISPRv2 vector-nucleofected cells were parallelly selected and used as a control cell line. Bip knockout cells were similarly generated with Bip sgRNA-expressing plasmids. The primers used to sequence genomic CHOP and Bip DNA:

CHOP: 5'-TGCCCTTACCTATCGTGCAA-3' and 5'-CAGTGCAGGGTCACATGCTT-3'.

Bip: 5'-TCGATACTGGCCGAGACAAC-3' and 5'-AGTTGGCAACCCCTAAATCC-3'.

**Western blot, antibodies, and reagents**

Western blot was performed following standard procedure. The antibodies used were as follows: GAPDH (sc-32233), Akt1 (sc-5298)
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and Actin (sc-1616) were from Santa Cruz Biotechnology. phospho-Akt (Ser473) (193H12), phospho-Akt (Thr308) (244F9), PhosphoPlus p44/42 mitogen-activated protein kinase (MAPK) (Erk1/2) antibody kit (9100), PhosphoPlus p38 MAP Kinase antibody kit (9210), PhosphoPlus SAPK/JNK Antibody kit (9250), c-Jun (9165) and p-c-Jun (3270) were from Cell Signaling Technology. Goat F(ab’)2 anti-mouse IgG was obtained from Jackson Immuno Research. Polyclonal MHV68 antiserum to detect lytic antigen was obtained from C57BL/6 mice 6 weeks postinfection (53). vCyclin and ORF59 antibodies were described previously (35). Anti-Flag antibody (A8592), secondary antibodies anti-rabbit IgG (R2004) and anti-mouse IgG (M8642) were from Sigma-Aldrich. AU1 epitope tag antibody was from Novus Biologicals. All Alexa Fluor-conjugated secondary antibodies were from Invitrogen. Tunicamycin and Thapsigargin were from Cell Signaling Technology. Akt1/2 kinase inhibitor (A6730) was from Sigma. ERK inhibitor PD98059 (HY-12028) was from MedChem Express. JNK inhibitor SP600125 was from Invivogen.

Nucleofection and luciferase assay

All SL-1 and M12 cells were transfected by nucleofection. Cells were washed with PBS and resuspended in 100μl transfection buffer from Ingenio® kit (MIR 50118, Mirus) and nucleofected with plasmids using Amaxa Nucleofector II system (Lonza) according to the manufacturer’s instructions. The program T20 was used for SL-1, M12, and other selected cells, whereas the program D17 was used for CHOP knockout C-4 cells in the CHOP overexpression rescue experiments. MHV68 RTA promoter luciferase assay was performed as described previously (54).

Luciferase assays were carried out in triplicate. Luciferase activity was measured using the Dual-Luciferase Reporter Gene Assay kit (Beyotime Biotechnology) according to the manufacturer’s instructions and normalized to Renilla activity.

qRT-PCR and qPCR

Total RNA was extracted using TRIzol reagent (Life Technologies) and reverse transcribed with FastQuant RT kit (TIANGEN) according to the manufacturer’s instruction. Quantitative PCR was performed with SuperReal PreMix Plus (SYBR Green) kit (TIANGEN) on the 7900HT sequence detection system (Life Technologies) using the following primers: ORF50: 5’-GGCCGAGACATTTACTGAC-3’ and 5’-GCTCAACTTCTCTGGAATG-3’; MHV68-ORF73: 5’-GGGAATCTCCTTCAAGGATAG-3’ and 5’-CTCCTCTTCTCTGGAATG-3’; MHV68-ORF59: 5’-GACACCGGGGTGGGAATAAGG-3’ and 5’-GGGGCCCCATCTACCTCTAAAG-3’; MHV68-ORF25: 5’-CAGCGGCTCTTTGAACAA-3’ and 5’-GTAGGCAGATGTTTCTC-3’; GAPDH: 5’-AACGACCCCTTCAATGAC-3’ and 5’-ATGTTAGTGGGGTCTCGCTC-3’. The GAPDH housekeeping gene was used as an internal control for normalization. For viral DNA quantification, genomic DNA was isolated with a Tianamp Genomic DNA kit (DP304-02; TianGen) and used for qPCR. Real-time DNA PCR for the g50 gene was used to measure the MHV68 genome, Relative genome copy numbers were calculated based on normalization with the GAPDH housekeeping gene.

Immunofluorescence

Transfected cells were fixed with 4% paraformaldehyde for 30 min and washed with PBS for three times. After penetration with methanol: acetone (1:1) for 20 min, cells were washed with PBS for three times and subjected to blocking with 5% BSA, followed by incubation with primary antibodies and detection with secondary antibodies (Alexa-488
goat anti-mouse IgG for MHV68 lytic antigen, Alexa-568 donkey anti-rabbit IgG for AU1, Alexa-568 donkey anti-mouse IgG for Flag. Images were acquired by using a confocal microscope (Olympus FV1200). Quantification was calculated by counting three different images.

Cytotoxicity and cell viability assays
Cytotoxic activity and cell viability were determined using a colorimetric Cell Counting Kit-8 (Beyotime, C0037) according to the manufacturer’s instructions. Briefly, The SL-1 cells were seeded in 96-well plates at a density of 5000 cells/well and stimulated with individual stimuli. Cell viability was determined by measuring the absorbance of OD450 using a microplate reader. Individual treatment was assayed in triplicate and at least two independent experiments were performed for each stimulus.

Statistical analyses
Statistical analysis was performed in Prism (Graph Pad Software). The data are reported as means and standard deviations (SD). Differences between groups of research subjects were analyzed for statistical significance with two-tailed Student t-tests. A P value of 0.05 was considered significant.

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Conflict of Interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions
X.C.Z performed the experiment, analyzed the data and drafted the article. S.H.D, Z.S.L, S. L and C.C.Z performed the experiment and analyzed the data. X.Z.L conceived and designed the experiments, drafted and edited the manuscript.

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Figure 1. ER stress inhibits BCR-mediated MHV68 lytic replication. A, MHV68 lytic protein expression after induction of ER stress and anti-Ig stimulation. SL-1 cells were treated with TM (5μg/ml), TG (5μM), or DMSO, together with (+) or without (-) anti-mouse IgG (5μg/ml) for 24 hr and 48 hr. Immunoblot analyses were performed with specific antibodies as indicated. Molecular weight for each blot was marked as indicated. B, mRNA expression of MHV68 viral genes after 48 hr TM or TG treatment. SL-1 cells were cultured in the presence (+) or absence (-) of anti-mouse IgG (5μg/ml). Total RNA isolated from treated cells was subjected to quantitative RT-PCR analyses with specific primers corresponding to MHV68 ORF73, ORF50, ORF59, and ORF25, respectively. Viral gene mRNA was normalized to GAPDH mRNA. Relative fold was calculated by comparison to DMSO treatment without anti-Ig. Each sample was done in triplicate. C, MHV68 viral DNA replication after 48 hr TM or TG treatment. SL-1 cells were cultured in the presence (+) or absence (-) of anti-mouse IgG (5μg/ml). Genomic DNA was isolated from treated cells and subjected to quantitative PCR analyses with primers corresponding to the ORF50 coding region. MHV68 viral DNA level was normalized to GAPDH. Relative fold was calculated by comparison to DMSO treatment without anti-Ig. Each sample was done in triplicate. D, The effect of TM on RTA promoter activity. M12 cells were co-transfected with renilla reporter and RTA luciferase promoter (RTA-p) or vector pGL2, with or without TM (5μg/ml) and anti-mouse IgG (5μg/ml) treatment (+). Luciferase activity was normalized to renilla activity. Histograms represent mean ± s.d. of triplicate samples (two experiments). A p value of < 0.05 was considered significant.

Figure 2. ER stress inhibits constitutive activation of BCR signaling Akt, ERK, and JNK pathways. SL-1 cells were treated with TM (5μg/ml), TG (5μM), or DMSO, together with (+) or without (-) anti-mouse IgG (5μg/ml) for 0.5, 3, 6, 24 and 48 hr. A, Immunoblot analyses were performed using specific antibodies as indicated. Molecular weight of each protein was marked as indicated. The samples for 24 and 48 hr were the same as in Fig.1A, Bip detection at 24 and 48 hr in Fig. 1A was also used in this panel. B, Quantitation of phosphorylated ERK, Akt, and JNK by normalizing to GAPDH for the immunoblot analyses in A. C, Cell viability of SL-1 cells stimulated with DMSO, TM (5μg/ml), or TG (5μM), in the presence (+) or absence of anti-mouse IgG (5μg/ml) for 24 hr and 48 hr. The result for each treatment was normalized to that of the untreated control. Histograms represent mean ± s.d. of triplicate samples (two experiments).

Figure 3. Activation of Akt, ERK, and JNK is required for BCR-mediated MHV68 lytic gene expression. A, SL-1 cells were treated with different concentration of Akt1/2 specific inhibitor (AktI), ERK inhibitor PD98059 (ERKI), or JNK inhibitor SP600125 (JNKI), respectively, together with (+) or without (-) anti-mouse IgG (5μg/ml) for 48 hr. Immunoblot analyses were performed with specific antibodies as indicated. Molecular weight for each blot was marked as indicated. B, Cell viability of SL-1 cells treated with DMSO, Akt1/2 specific inhibitor (AktI), ERK inhibitor PD98059 (ERKI), or JNK inhibitor SP600125 (JNKI) in the presence (+) of anti-mouse IgG (5μg/ml) for 48 hr. The result for each treatment normalized
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to that of the untreated control. Histograms represent mean + s.d. of triplicate samples (two experiments). A p value of < 0.05 was considered significant.

Figure 4. CHOP is required for BCR-mediated MHV68 lytic replication. CHOP knockout individual SL-1 clones C-4 and C-2, as well as control cells (Con), were treated with anti-mouse IgG (5μg/ml) for 48 hr and subjected to further analyses. A, Immunoblot analyses detected by indicated antibodies. Molecular weight for each blot was marked as indicated. B, Quantitation of Bip by normalizing to GAPDH for the immunoblot analyses in A. C, MHV68 viral DNA detected by quantitative PCR with specific primers corresponding to ORF50. Viral DNA level was normalized to GAPDH. Relative fold was calculated by comparison to DMSO treatment without anti-Ig. Each sample was done in triplicate. D, mRNA expression of the MHV68 viral genes detected by quantitative RT-PCR with specific primers against ORF73, ORF50, ORF59, and ORF25. Viral gene mRNA was normalized to GAPDH mRNA. Relative fold was calculated by comparison to DMSO treatment without anti-Ig. Each sample was done in triplicate.

Figure 5. CHOP overexpression promotes BCR-mediated MHV68 lytic gene expression. A, CHOP knockout C-4 cells and control cells (Con) were transfected with vector or CHOP-expressing plasmid with AU1 tag, followed by anti-mouse IgG (5μg/ml) treatment for 48 hr at 24 hr post-transfection. Immunoblot analyses were performed with indicated antibodies. The AU1 antibody was used to detect ectopic CHOP expression. Actin was used as a loading control. Molecular weight was marked as indicated. B, Immunofluorescent imaging of MHV68 lytic gene expression in CHOP-expressing C-4 cells. CHOP knockout C-4 cells were transfected with CHOP-IRES-tdTomato expressing plasmids, followed by anti-mouse Ig treatment for 48 hr and indirect immunofluorescent staining with specific antibodies against MHV68 lytic antigen (green), CHOP-IRES-tdtomato was shown as red. DNA was stained blue with DAPI. Percentage of MHV68 lytic antigen positive cells in CHOP-expressing cells and CHOP-negative cells was quantified as shown in the right panel. C, CHOP regulation of RTA promoter activity. M12 cells were co-transfected with renilla reporter and RTA luciferase promoter (RTA-p) or vector pGL2, together with CHOP-expressing plasmid with AU1 epitope tag or vector, in the presence (+) or absence (-) of anti-mouse IgG (5μg/ml) treatment. CHOP expression was detected by AU1 antibody. Luciferase activity was normalized to renilla activity. Histograms represent mean + s.d. of triplicate samples (two experiments). A p value of < 0.05 was considered significant.

Figure 6. CHOP has no effect on the initiation of B cell signaling but indirectly inhibits constitutive JNK activation. CHOP knockout C-4 cells and control cells (C) were treated with anti-mouse IgG (5μg/ml), followed by immunoblot analyses with indicated antibodies: A, 0, 15, 30, and 60 min; B, 24 and 48 hr. Molecular weight for each blot was marked as indicated.

Figure 7. Bip inhibits BCR-mediated MHV68 lytic gene expression and mediates CHOP function on MHV68 lytic gene expression. A, SL-1 cells were transfected with a
Bip-expressing plasmid with Flag-tag or vector, followed by anti-mouse IgG (5μg/ml) treatment for 48 hr at 24 hr post-transfection. Immunoblot analyses were performed with specific antibodies as indicated. Molecular weight for each blot was marked as indicated. B, Immunofluorescent imaging of MHV68 lytic antigen expression. SL-1 cells were transfected with a Bip-IRES-tdTomato expressing plasmid and treated with anti-mouse IgG for 48 hr, followed by immunofluorescent staining with specific antibodies against MHV68 lytic antigen (green). CHOP-IRES-tdTomato was shown as red. DNA was stained blue with DAPI. Percentage of MHV68 lytic antigen positive cells in Bip-expressing cells was quantified as shown in the right panel. C, Bip knockout SL-1 cells and control cells (Con) were treated with anti-mouse IgG (5μg/ml) for 48 hr and subjected to immunoblot analyses with indicated antibodies. Molecular weight for each blot was marked as indicated. D, CHOP knockout C-4 cells and control cells (Con) were transfected with empty vector or the CRISP/Cas9 plasmid expressing Bip sgRNA, single cell clones were selected and treated with anti-mouse IgG (5μg/ml) for 48 hr. Immunoblot analyses were performed with specific antibodies as indicated. Molecular weight for each blot was marked as indicated. E, Sample from A was detected with specific phosphorylated antibodies as indicated. Molecular weight for each blot was marked as indicated. F, M12 cells were co-transfected with renilla reporter and RTA luciferase promoter (RTA-p) or vector pGL2, together with Bip-expressing plasmid with Flag epitope tag or vector. Bip expression was detected with Flag antibody. Luciferase activity was normalized to renilla activity. Histograms represent mean + s.d. of triplicate samples (two experiments). A p value of < 0.05 was considered significant.

Figure 8. ATF4 expression blocks MHV68 RTA transcription and suppresses MHV68 lytic gene expression rescued by Bip knockout in CHOP knockout cells. A, Control cells (Con) and CHOP knockout clones C-2 and C-4 were stimulated with anti-mouse IgG (5μg/ml) for 48 hr and subjected to immunoblot analyses with indicated antibodies. Molecular weight for each blot was marked as indicated. Quantitation of ATF4 and BCL-2 amount by normalizing to GAPDH was shown, respectively. B, SL-1 cells were transfected with a BCL-2-expressing plasmid with Flag-tag or vector, followed by anti-mouse IgG (5μg/ml) treatment for 48 hr at 24 hr post-transfection. Immunoblot analyses were performed with specific antibodies as indicated. Molecular weight for each blot was marked as indicated. C, ATF4 inhibits RTA promoter activity. M12 cells were co-transfected with renilla reporter and RTA luciferase promoter (RTA-p) or vector pGL2, together with ATF4-expressing plasmid with Flag-tag or vector, in the presence (+) or absence (-) of anti-mouse IgG (5μg/ml) treatment. ATF4 expression was detected with Flag antibody. Luciferase activity was normalized to renilla activity. Histograms represent mean + s.d. of triplicate samples (two experiments). A p value of < 0.05 was considered significant. D, SL-1 cells were transfected with an ATF4-expressing plasmid with Flag-tag or vector, followed by anti-mouse IgG (5μg/ml) treatment for 48 hr at 24 hr post-transfection. Immunoblot analyses were performed with specific antibodies as indicated. Molecular weight for each blot was marked as indicated. E, CHOP and Bip double knockout cells were transfected with vector or an ATF4-expressing plasmid, followed by stimulation with anti-mouse IgG (5μg/ml) for 48 hr and subsequent immunoblot analyses with indicated antibodies. Molecular weight for each blot was marked as indicated. F, Quantitation of MHV68 lytic antigen by normalizing to GAPDH for the
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Immunoblot analyses in E. DKO represents CHOP-/-/Bip-/- cells, V represents vector. + or - represents the presence or absence of anti-Ig.

**Figure 9. Regulation model of BCR-mediated MHV68 lytic replication by ER stress.** ER stress inhibits BCR-mediated MHV68 lytic replication. ER stress mediator Bip and downstream transcription factor ATF4 inhibit BCR-mediated MHV68 lytic gene expression, whereas ER stress-induced gene CHOP is essential and promotes BCR-mediated MHV68 lytic gene expression by negative regulation of Bip and ATF4 expression. ATF4 can directly block MHV68 RTA promoter activity, which ultimately leads to inhibition of MHV68 lytic gene expression and contributes to the essential function of CHOP on BCR-mediated MHV68 lytic replication.
Figure 1

A

24 hr

|       | DMSO | TM | TG |
|-------|------|----|----|
| ORF59 mRNA | -    | +  | +  |
| ORF73 mRNA | +    | +  | +  |
| ORF50 mRNA | -    | +  | +  |
| vCyclin     | -    | +  | +  |
| GAPDH       | -    | +  | +  |

48 hr

|       | DMSO | TM | TG |
|-------|------|----|----|
| ORF59 mRNA | -    | +  | +  |
| ORF73 mRNA | +    | +  | +  |
| ORF50 mRNA | -    | +  | +  |
| vCyclin     | -    | +  | +  |
| GAPDH       | -    | +  | +  |

B

ORF73

|       | DMSO | DMSO+ | TM- | TM+ | TG- | TG+ |
|-------|------|-------|-----|-----|-----|-----|
| ORF73 mRNA | 0.0  | 2.0   | 1.0 | 1.5 | 2.0 | 1.0 |

ORF50

|       | DMSO | DMSO+ | TM- | TM+ | TG- | TG+ |
|-------|------|-------|-----|-----|-----|-----|
| ORF50 mRNA | 0.0  | 1.0   | 1.0 | 1.0 | 1.0 | 1.0 |

ORF59

|       | DMSO | DMSO+ | TM- | TM+ | TG- | TG+ |
|-------|------|-------|-----|-----|-----|-----|
| ORF59 mRNA | 0.0  | 3.0   | 2.0 | 5.0 | 7.0 | 10.0 |

ORF25

|       | DMSO | DMSO+ | TM- | TM+ | TG- | TG+ |
|-------|------|-------|-----|-----|-----|-----|
| ORF25 mRNA | 0.0  | 2.0   | 1.0 | 1.5 | 2.0 | 1.0 |

C

MHV68 Viral DNA (Fold)

|       | DMSO | TM- | TM+ | TG- | TG+ |
|-------|------|-----|-----|-----|-----|
| MHV68 Viral DNA (Fold) | 0.0  | 1.0 | 1.5 | 2.0 | 1.0 |

D

Relative Luciferase Fold

|       | pGL2 | pGL2+TM | RTA-p | RTA-p+TM | RTA-p+TM (+) |
|-------|------|---------|-------|----------|-------------|
| Relative Luciferase Fold | 0.0  | 0.5461  | 0.7769 | 0.3456    | 0.2345     |
|          | 0 hr | 3 hr | 6 hr | 24 hr | 48 hr |
|----------|------|------|------|-------|-------|
| **kDa**  |      |      |      |       |       |
| 42       | +    | +    | +    | +     | +     |
| 44       | -    | -    | -    | -     | -     |
| 42       | -    | -    | -    | -     | -     |
| 60       | -    | -    | -    | -     | -     |
| 60       | -    | -    | -    | -     | -     |
| 54       | -    | -    | -    | -     | -     |
| 54       | -    | -    | -    | -     | -     |
| 48       | -    | -    | -    | -     | -     |
| 43       | -    | -    | -    | -     | -     |
| 43       | -    | -    | -    | -     | -     |
| 27       | -    | -    | -    | -     | -     |
| 55       | -    | -    | -    | -     | -     |
| 70       | -    | -    | -    | -     | -     |
| 35       | -    | -    | -    | -     | -     |

**Figure 2**
Figure 2
Figure 3

A

|        | AktI | ERK1 | JNK1 |
|--------|------|------|------|
| DMSO   | -    | -    | -    |
| 50µM   | +    | -    | +    |
| 100µM  | +    | +    | +    |

|        | Anti-Ig | Anti-Ig | Anti-Ig |
|--------|---------|---------|---------|
| kDa    | -       | +       | -       |

B

Relative cell viability

$\text{p}=0.0216$

$\text{p}=0.0513$

$\text{p}=0.3846$
Figure 4

A. Western blot analysis showing the expression levels of various proteins (Anti-Ig, BH3-only proteins, vCyclin, and BIP) under different conditions (Con, C-4, C-2).

B. Bar graph depicting the fold change in Bip/GAPDH mRNA expression under different conditions.

C. Bar graph showing the fold change in MHV68 Viral DNA expression under different conditions.

D. Bar graphs illustrating the fold change in mRNA expression of ORF73, ORF50, ORF59, and ORF25 under different conditions.
Figure 5

A. Western blot analysis showing expression levels of various proteins under different conditions.

B. Immunofluorescence images of MHV68 lytic antigen expression in cells transfected with different vectors.

C. Relative luciferase fold comparison between control and experimental conditions.

MHV68 CHOP-tdTomato DAPI Merge

Con

C-4

Vector

CHOP

Anti-Ig

MHV68

vCyclin

ORF59

AU1

ATF4

CHOP

BIP

Actin

Relative Luciferase Fold

p=0.0013

p=0.8397

0

20

40

60

80

100

pGL2+Vector

pGL2+Chop

RTA-p+Vector

RTA-p+Chop

0

2

4

6

pGL2+Vector

pGL2+Chop

RTA-p+Vector

RTA-p+Chop

Relative Luciferase Fold

p=0.0013

p=0.8397

0

10

20

30

40

MHV68 lytic antigen+ cells (%)

by guest on July 24, 2018http://www.jbc.org/Downloaded from
Figure 6

A

|          | 0        | 15 min   | 30 min   | 60 min   |
|----------|----------|----------|----------|----------|
| kDa      | C        | C-4      | C        | C-4      |
| Antigen  | -        | -        | +        | +        |
| p-Akt(S473) | p-ERK1/2 | p-JNK1/2 | Akt      |
| p-ERK1/2 | 42       | 44       | 42       | 42       |
| ERK1/2   | 44       | 42       | 44       | 42       |
| p-JNK1/2 | 54       | 46       | 54       | 46       |
| JNK1/2   | 54       | 46       | 54       | 46       |
| p-p38    | 46       | 54       | 46       | 54       |
| p38      | 60       | 60       | 60       | 60       |
| p-p65    | 60       | 60       | 60       | 60       |
| p65      | 65       | 65       | 65       | 65       |
| Bip      | 70       | 70       | 70       | 70       |
| CHOP     | 46       | 46       | 46       | 46       |
| Actin    | 42       | 44       | 42       | 44       |

B

|          | 24 hr    | 48 hr    |
|----------|----------|----------|
| kDa      | C        | C-4      |
| Antigen  | -        | +        |
| p-ERK1/2 | 44       | 42       |
| ERK1/2   | 44       | 42       |
| p-JNK1/2 | 54       | 54       |
| JNK1/2   | 54       | 54       |
| p-Akt(S473) | Akt      |
| Akt      | 46       | 54       |
| p-p65    | 60       | 60       |
| p38      | 60       | 60       |
| Bip      | 70       | 70       |
| CHOP     | 46       | 46       |
| GAPDH    | 35       | 35       |
Figure 7
Figure 7
Figure 8

A

|  | Con | C-2 | C-4 |
|---|-----|-----|-----|
| kDa | - | + | - | + | - | + |
| Anti-Ig |  |  |  |  |  |  |
| Bip |  |  |  |  |  |  |
| CHOP |  |  |  |  |  |  |
| ATF4 |  |  |  |  |  |  |
| p-eIF2α |  |  |  |  |  |  |
| eIF2α |  |  |  |  |  |  |
| ATF6 |  |  |  |  |  |  |
| Xbp-1 |  |  |  |  |  |  |
| BCL-2 |  |  |  |  |  |  |
| GAPDH |  |  |  |  |  |  |

B

|  | Vector | BCL-2 |
|---|--------|--------|
| kDa | - | + | - | + |
| Anti-Ig |  |  |  |  |
| MHV68 |  |  |  |  |
| Flag |  |  |  |  |
| BCL-2 |  |  |  |  |
| GAPDH |  |  |  |  |
C  (-) Anti-Ig  (+) Anti-Ig

D  Vector  ATF4

E  CHOP-/-Bip-/-

Figure 8
Figure 9
Regulation of gammaherpesvirus lytic replication by endoplasmic reticulum stress-induced transcription factors ATF4 and CHOP

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