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VIRUS-CELL INTERACTIONS

Upregulation of ATF4-LAMP3 Axis by ORF45 Facilitates Lytic Replication of Kaposi's Sarcoma-Associated Herpesvirus

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ABSTRACT Kaposi's sarcoma-associated herpesvirus (KSHV) is a γ-oncogenic herpesvirus, and both lytic and latent infections play important roles in its pathogenesis and tumorigenic properties. Multiple cellular pathways and diverse mediators are hijacked by viral proteins and are used to support KSHV lytic replication. In previous studies, we revealed that KSHV ORF45 promoted KSHV transcription and translation by inducing sustained p90 ribosomal S6 kinase (RSK) activation and the phosphorylation of its substrates c-Fos and eIF4B. However, the cellular mediators required for lytic replication remain largely unknown. Here, we reveal that ORF45 activates eIF2α phosphorylation and ATF4 translation and then upregulates the expression of lysosome-associated membrane protein 3 (LAMP3) in an ATF4-dependent manner during KSHV lytic replication. Consequently, LAMP3 promotes Akt and ERK activation and then facilitates lytic gene expression and virion production. Furthermore, ATF4 enhances lytic replication through LAMP3, and LAMP3 acts in an ATF4-independent manner. Our findings suggest that the ATF4-LAMP3 axis is upregulated by ORF45 through ER stress activation during the KSHV lytic life cycle and, in turn, facilitates optimal lytic replication.

IMPORTANCE The lytic replication of Kaposi's sarcoma-associated herpesvirus (KSHV) reprograms cellular transcription and translation to generate viral proteins and virion particles. Here, we show that the mediator of ER stress ATF4 and the expression of the downstream gene LAMP3 are upregulated by ORF45 during lytic replication. Consequently, increased LAMP3 expression activates Akt and ERK and promotes lytic replication. Although several UPR transcription factors are able to promote KSHV lytic replication, the proviral effect of ATF4 on lytic replication is attenuated by LAMP3 silencing, whereas the effect of LAMP3 does not directly require ATF4 expression, indicating that LAMP3 primarily exerts effects on KSHV lytic replication downstream of ATF4 and ER stress. Taken together, our findings suggest that the ORF45-upregulated ATF4-LAMP3 axis plays an essential role in KSHV lytic replication.

KEYWORDS Kaposi's sarcoma-associated herpesvirus, ATF4, LAMP3, ORF45, lytic replication

As a DNA tumor virus, Kaposi's sarcoma-associated herpesvirus (KSHV) is etiologically associated with three kinds of malignancies in immunosuppressed patients: Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD) (1, 2). The KSHV life cycle has two different phases: latent infection and lytic replication. During latent infection, only specific viral genes are expressed, no virion particles are produced, and viral genomic DNA replicates synchronously with the replication of chromosomal DNA at a low frequency. Lytic replication can be reactivated from latent infection under diverse stimuli, and then all viral genes become

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expressed, viral DNA is autonomously replicated at a high frequency, and virion particles are produced and released. Both latent infection and lytic replication are important for KSHV pathogenesis and disease progression (3, 4). Therefore, lytic replication is an important therapeutic target for the treatment of KSHV-related diseases (5).

A variety of stimuli and cellular pathways have been characterized in both latent and lytic lifecycles, and they play important roles in viral infection and replication (6). RTA is the transcriptional transactivator of lytic replication, its expression is triggered by multiple cellular pathways, including phosphatidylinositol 3-kinase (PI3K)-Akt and extracellular signal-regulated kinase (ERK)-MAPKs, and it initiates lytic gene expression and viral DNA replication (7, 8). Studies have revealed that two lytic viral proteins, ORF24 and ORF45, mediate late gene expression by mimicking the cellular TATA box-binding protein and by activating c-Fos transcriptional activity (9, 10). However, the process and regulation of viral late lytic replication, egress, and release remain incompletely understood. The systemic investigation and understanding of the regulation and mechanisms of late lytic replication would identify more mediators and novel targets for KSHV infection, pathogenesis, and treatment.

Open reading frame 45 (ORF45), a KSHV-encoded, phosphorylated tegument viral protein, is abundantly expressed from the early to late stages of lytic replication (11) and plays several important roles in the KSHV lytic life cycle. It suppresses IRF7-dependent antiviral innate immunity (12), facilitates virion assembly and transport (13, 14), induces sustained ERK-RSK activation, and promotes optimal lytic replication (15, 16). The deletion or F66A point mutation of ORF45 abolishes its interaction with RSK as well as its function in ERK-RSK activation, resulting in great inhibition of KSHV lytic replication and virion production (17, 18). However, the effects and mechanisms of ORF45 on transcription, cellular activity, and behavior remain to be investigated.

During the lytic replication of herpesviruses, abundant viral structural and nonstructural proteins are synthesized and folded in the ER lumen and are then transported to the Golgi for modifications. Excessive, overloaded, or unfolded proteins may disrupt and reprogram the ER function (19), known as ER stress, or the unfolded protein response (UPR), which can cope with the ER damage and restore ER function. Studies have shown that three sensors of the UPR (ATF6, PERK, and IRE1) are activated during KSHV lytic replication and that their downstream mediators play important roles in viral lytic replication (20). In fact, various mediators of ER stress and homeostasis, such as ATF4, XBP1, and GRP78, play essential roles in the different stages of lytic replication (21–23).

Lysosome-associated membrane protein 3 (LAMP3) is a member of the lysosome-associated membrane glycoprotein family, is mainly distributed in lysosomes and late endosomes, and plays important roles in autophagy, apoptosis, viral infection, and antiviral immune responses (24–26). LAMP3 is also called the CD208/dendritic cell-lysosomal-associated membrane protein and is a marker of human dendritic cells because of its high expression in dendritic cells and type II pneumocytes (27, 28). LAMP3 upregulation and dysfunction have been observed in diverse diseases, such as Sjögren syndrome (SS) and several cancers (29, 30), indicating a key role in the progression of these diseases. Importantly, LAMP3 is a downstream target of PERK-eIF2α-ATF4 (31, 32), which plays critical roles in the responses to ER stress.

In the present study, we demonstrate that LAMP3 is upregulated by ORF45 during KSHV lytic replication in an ATF4-dependent manner and that the ATF4-LAMP3 axis plays an essential role in lytic gene expression and virion production, mainly through regulating Akt and ERK activation. This finding reveals that LAMP3 acts as a novel mediator of KSHV lytic replication.

RESULTS

ORF45 enhances eIF2α phosphorylation and ATF4 translation during KSHV lytic replication. Studies have shown that endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) are activated and contribute to the promotion of KSHV lytic replication. The expression of XBP1 activates RTA expression and lytic replication (20, 23), and the expression of Bip/Grp78 is required for the assembly and

release of virions (21). To investigate whether ORF45 regulates ER stress-related gene expression during lytic replication, we performed a RNA-sequencing analysis in iSLK-BAC16 and iSLK-STOP45 cells during late lytic induction. When the differentially expressed genes were filtered and analyzed, we found that ER stress-related gene expression was much lower in iSLK-STOP45 cells than in iSLK-BAC16 cells, including DDX11L2, IGFBP1, WIPI1, KDELR3, STUB1, and ASNS (Fig. 1A), and the pathways associated with the responses to ER stress and the UPR were enriched in iSLK-BAC16 cells, compared with iSLK-STOP45 cells during the lytic life cycle (Fig. 1B), indicating that ORF45 expression is important for the induction of ER stress during lytic replication. The mRNA levels of the ER stress-related transcriptional factors ATF6, ATF4, and XBP1 were slightly affected in iSLK-STOP45 versus iSLK-BAC16 cells; however, the downstream genes of ATF4, such as IGFBP1 (33), WIPI1 (34), and ASNS (35), were greatly decreased in the iSLK-STOP45 cells compared with the iSLK-BAC16 cells. Furthermore, when mRNA was extracted after lytic induction for 72 h and then analyzed via real-time PCR, the levels of eIF2a, ATF4, ATF6, and XBP1 transcription were not affected during lytic replication in iSLK-BAC16 versus iSLK-STOP45 cells (Fig. 1C). In contrast, the mRNA level of IGFBP1, a downstream gene of ATF4, was significantly increased in iSLK-BAC16 cells compared with iSLK-STOP45 cells under lytic induction. These results indicate that ORF45 might not affect the expression of sensors and effectors of ER stress but may promote downstream gene expression, especially ATF4-mediated gene expression. Since ATF4 mRNAs contain two upstream open reading frames (uORFs) that preferentially mediate ATF4 translation via eIF2α phosphorylation under ER stress to act as a key effector of ER stress (36), and since ERK-MAPK signaling can enhance ATF4 translation via translational reprogramming under an asparagine limitation in cancers (37), we hypothesize that ORF45 may enhance ATF4 translation and ATF4-related ER stress during the KSHV lytic cycle. To investigate whether ER stress is induced by ORF45 expression, ATF4-related ER stress signaling was examined in Tet-on inducible ORF45-expressing BJAB cells. The expression level of ATF4 and the phosphorylation of eIF2α were increased in BJAB cells in the presence of ORF45 (Fig. 1D). The levels of ATF4 expression and eIF2α phosphorylation were similarly increased in iSLK-BAC16 cells after lytic induction, though these levels were slightly affected in iSLK-STOP45 cells under lytic replication compared with those during the latent life cycle (Fig. 1D, left). Further, we detected whether their levels were affected during lytic replication in BAC16-ORF45-F66A or revertant ORF45-A66F viruses, which fail in the induction of sustained ERK-RSK activation or reverse this function during lytic replication, respectively (17). The increased eIF2α phosphorylation and ATF4 protein level were abrogated during lytic replication in iSLK-BAC16-ORF45-F66A cells, whereas they were maintained in revertant-harboring cells (Fig. 1D, right), indicating that the ATF4 level was post-transcriptionally increased by ORF45 during lytic replication through sustained ERK-RSK activation. The protein level of ATF6 expression was also detected, and no differences in ATF6 protein levels and cleavage were observed in iSLK-BAC16 cells, compared with iSLK-STOP45 cells under lytic induction (Fig. 1F). In addition, treatment with lytic replication-inducing chemicals (doxycycline [Dox] plus NaB) did not affect ATF4 levels or eIF2α phosphorylation in KSHV-negative SLK cells, excluding the potential effects of Dox+NaB on ER stress during KSHV lytic induction (Fig. 1G). When ORF45 or ORF45 mutants were transfected into HEK293 cells, ORF45 overexpression increased the levels of ATF4 expression and eIF2α phosphorylation (Fig. 1H, Lanes 1 and 2). The single-point F66A mutation or aa19-77-deleted ORF45 truncation, which failed to induce sustained ERK and RSK activation (15, 17), greatly attenuated the induction of ATF4 expression and eIF2α phosphorylation (Fig. 1H, Lanes 3 and 4), indicating that ORF45 alone enhances eIF2α-ATF4 signaling through ERK-RSK activation. To further confirm that ATF4 translation was increased by ORF45 expression, a firefly luciferase-based ATF4 5′-UTR reporter with pTL-TK-mediated Renilla luciferase as an internal control was used to examine ATF4 translation in the absence or presence of ORF45. Both wild-type ORF45 and ORF45 F66A greatly increased the activity of the two kinds of

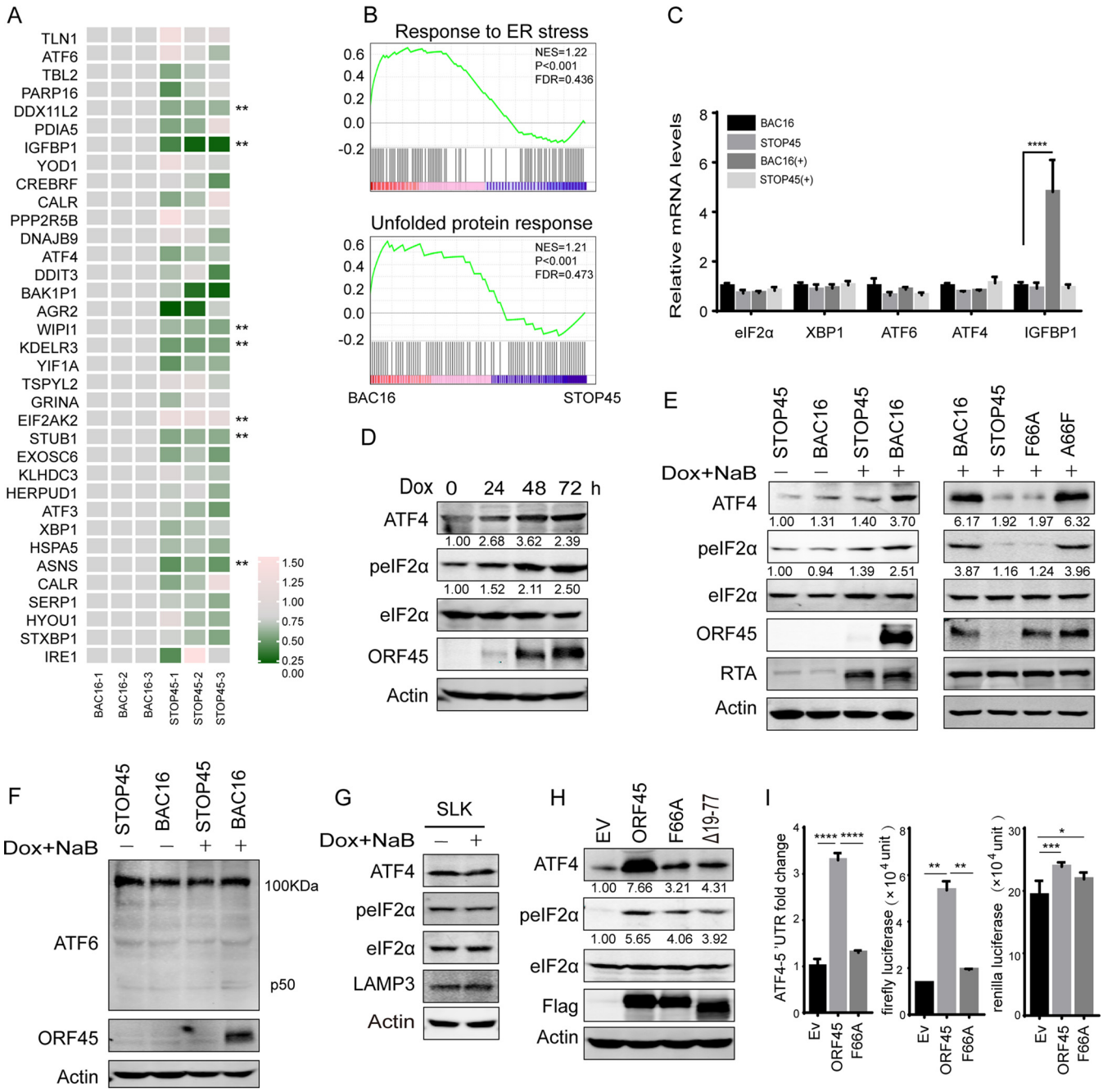

FIG 1 ORF45 activates eIF2α phosphorylation and ATF4 translation during KSHV lytic replication. (A) Heat map of ER stress-related gene expression in iSLK-BAC16 and iSLK-STOP45 cells under lytic induction for 72 h. These differential gene expressions were filtered from the RNA-sequencing analysis, and the relative expression levels in iSLK-BAC16 cells were normalized to those in the iSLK-STOP45 cells. (B) GSEA analyses of the RNA-sequencing data show that both the responses to ER stress and the UPR signaling pathways were significantly enriched. NES, normalized enrichment score; FDR, false discovery rate. (C) iSLK-BAC16 and iSLK-STOP45 cells were either left untreated or treated with 1 μg/mL doxycycline (Dox) plus 1 mM sodium butyrate (NaB) for 72 h. After the cells were collected, the total RNA was extracted and subjected to quantitative RT-PCR analysis to detect ATF4, eIF2α, IGFBP1, ATF6, and XBP1 expression. ****, $P < 0.001$. (D) Stably inducible ORF45-Tet-on BJAB cells were either left untreated or treated with 1 μg/mL doxycycline (Dox) for 48 h, the cells were harvested, and the whole-cell extracts were detected by immunoblots with the indicated antibodies. The densitometry levels of ATF4 and p-eIF2α were normalized to actin and to the total eIF2α, respectively. (E) iSLK-BAC16, -STOP45, -F66A, and-A66F cells were either left untreated or treated with 1 μg/mL Dox plus 1 mM sodium butyrate (NaB) for 72 h. After the cells were collected, the cell extracts were detected by Western blots as indicated for ATF4 expression and eIF2α phosphorylation. (F) iSLK-BAC16 and iSLK-STOP45 cells were either left untreated or incubated with 1 μg/mL Dox plus 1 mM NaB for 72 h. The cell pellets were collected and lysed, and the whole-cell extracts were analyzed by Western blots with the anti-ATF6 antibody. (G) SLK cells were either left untreated or incubated with 1 μg/mL Dox plus 1 mM NaB for 72 h. The cells were collected and the cell extracts were detected by Western blots as indicated. (H) ORF45 wild-type (WT), F66A-mutated, or aa19-77-deleted constructs were transfected into HEK293 cells for 48 h, and then the cell lysates were detected by Western blots as indicated. (I) The firefly luciferase-based reporter of ATF4 5′-UTR activity was cotransfected into HEK293T cells with the empty vector (Ev), ORF45, or ORF45-F66A expressing plasmid for 36 h, with renilla luciferase expressing plasmid as the internal control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$;****, $P < 0.0001$.

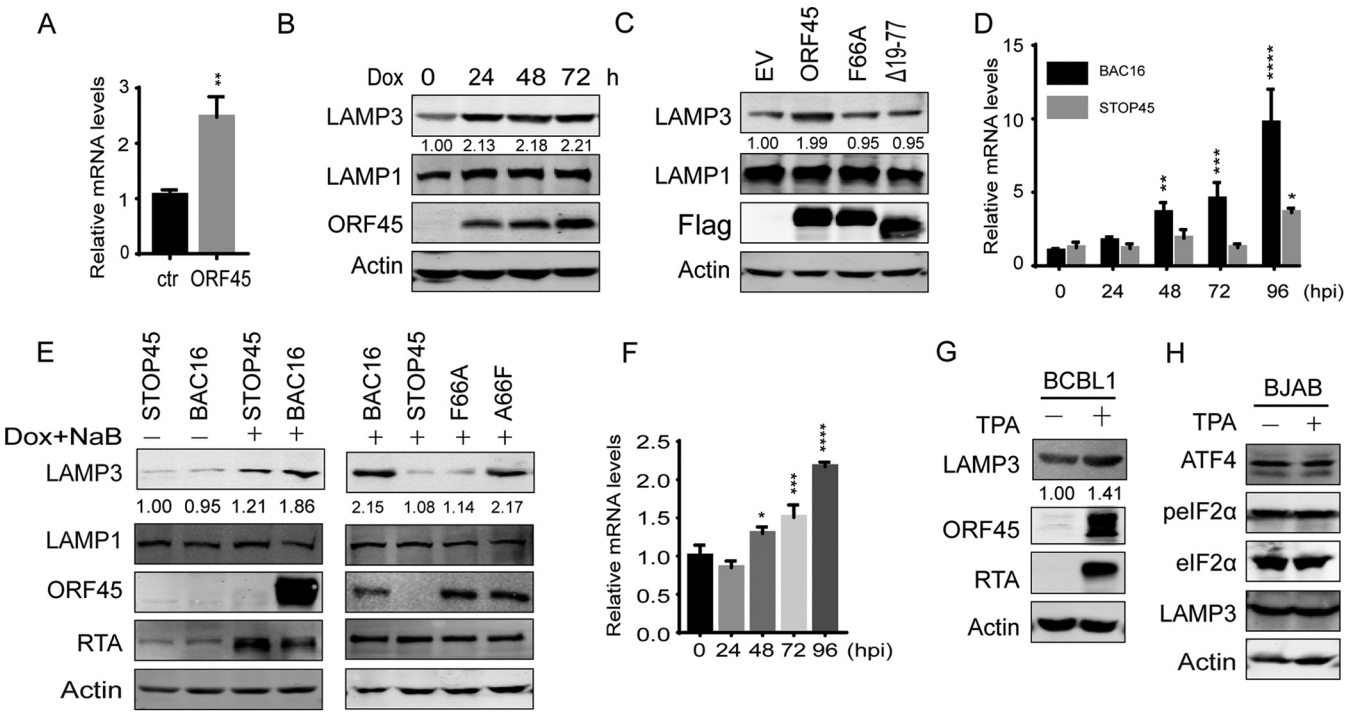

FIG 2 ORF45 increases LAMP3 expression during KSHV lytic replication. (A and B) Stably ORF45-Tet-on BJAB cells were either left untreated or incubated with 1 μg/mL Dox. After 48 h post treatment, total RNA was extracted and subjected to quantitative RT-PCR analysis to detect LAMP3 expression (A). The results are presented as the mean $\pm$ the standard deviation (SD). **, $P < 0.01$. At the different time points after induction, the cells were collected, and the lysates were detected by Western blots with the indicated antibodies (B). (C) ORF45 wild-type (WT), F66A, or aa19-77-deleted mutated constructs were transfected into HEK293 cells for 48 h, and then the cell lysates were immunoblotted with the indicated antibodies. (D) iSLK-BAC16 and iSLK-STOP45 cells were either left untreated or incubated with 1 μg/mL Dox plus 1 mM NaB for different time. The total RNA was extracted and subjected to a quantitative RT-PCR analysis for LAMP3 expression (D). The results are shown as the mean $\pm$ SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (E) iSLK-BAC16, -STOP45, -F66A, and -A66F cells were either left untreated or treated with 1 μg/mL Dox plus 1 mM sodium butyrate (NaB) for 72 h. After the cells were collected, the cell extracts were detected by Western blots as indicated for LAMP3. (F) BCBL1 cells were either left untreated or incubated with 20 ng/mL TPA for a different time. The level of LAMP3 mRNA was analyzed by quantitative RT-PCR, and the results are shown as the mean $\pm$ SD. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. (G) BCBL1 cells were either left untreated or induced by TPA for 48 h. The cells were collected, and the cell extracts were detected by Western blots as indicated. (H) BJAB cells were either left untreated or incubated with 20 ng/mL TPA for 72 h, and then the cell lysates were detected with the indicated antibodies.

luciferase, and much lower activity was induced by the ORF45 F66A mutant than by wild-type ORF45 under normal conditions. The increase in ATF4 5′-UTR-luciferase activities was significantly higher than that of the Renilla luciferase activities in the presence of ORF45 expression, and the relative level of ATF4 5′-UTR-mediated expression was greatly increased by wild-type ORF45 expression, whereas it was slightly elevated by ORF45 F66A expression (Fig. 1I). These data indicate that ORF45 promotes ATF4 translation in both RSK-dependent and RSK-independent manners. These results suggest that ORF45 induces ER stress and promotes ATF4 translation during KSHV lytic replication.

ORF45 increases LAMP3 expression during KSHV lytic replication. Since LAMP3 acts as a direct target gene of PERK/ATF4 during ER stress (32, 38), we further examined whether ORF45 activates LAMP3 gene expression. ORF45 Tet-on inducible BJAB cells were induced with doxycycline, and the expression of ORF45 substantially increased the level of LAMP3 mRNA (Fig. 2A). The increased LAMP3 levels were prolonged for 72 h in the presence of ORF45 expression, while the LAMP1 levels were not affected (Fig. 2B). Notably, when ORF45 or ORF45 mutants were transiently transfected into HEK293 cells, ORF45 increased LAMP3 expression, whereas the single-point F66A mutation or aa19-77-deleted ORF45 mutation almost abolished the increase in LAMP3 expression, and the LAMP1 levels were barely affected (Fig. 2C). These results suggest that ORF45 promotes LAMP3 expression in an RSK-dependent manner.

To further investigate whether the LAMP3 expression was upregulated by ORF45 during lytic replication, iSLK-BAC16 and iSLK-STOP45 cells were induced with 1 μg/mL

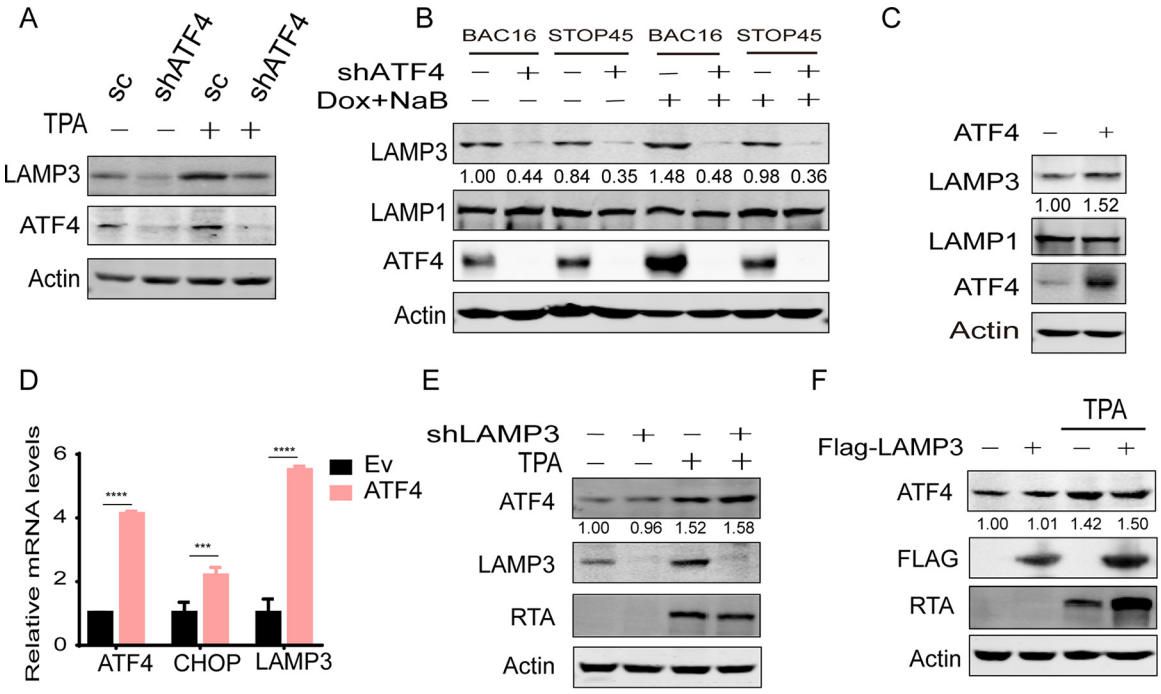

FIG 3 ATF4 is required for the upregulation of LAMP3 expression during KSHV lytic replication. (A) BCBL1 cells were transduced with control scramble shRNA (sc) or shATF4 by lentiviral infection for 24 h. Then, the cells were left untreated or induced by TPA for 48 h. The cells were collected, and the cell extracts were detected by Western blots as indicated. (B) iSLK-BAC16 and iSLK-STOP45 cells were infected with scramble shRNA or shATF4 expressing lentiviruses. 36 h after infection, these cells were either left untreated or treated with Dox plus NaB for 72 h, and then the cell lysates were detected by Western blots with the indicated antibodies. The relative levels of LAMP3 were normalized to actin, based on the density of band grayscale, and they were then calculated to that in the untreated iSLK-BAC16 cells with scramble shRNA. (C and D) BCBL1 cells were infected with ATF4-expressing or empty lentiviruses for 72 h, and then the whole-cell lysates were detected by Western blots as indicated (C). The total RNA was extracted and subjected to quantitative RT-PCR analysis to detect the levels of CHOP, ATF4, and LAMP3 expression (D). ***, $P < 0.001$; ****, $P < 0.0001$. (E and F) BCBL1 cells were infected with scramble shRNA, shLAMP3-expressing lentiviruses (E), or empty or LAMP3-expressing lentiviruses (F). 36 h after infection, the cells were either left untreated or treated with TPA for 72 h, the cells were collected and lysed, and the cell extracts were analyzed by Western blots as indicated.

doxycycline (Dox) and 1 mM sodium butyrate (NaB), and then the LAMP3 expression was examined via real-time PCR and Western blot analysis. Following the induction of lytic replication, the level of LAMP3 mRNA gradually increased in iSLK-BAC16 cells but only slightly increased in iSLK-STOP45 cells, and the LAMP1 mRNA level was barely affected (Fig. 2D). Similarly, the LAMP3 protein level was greatly increased in iSLK-BAC16 and iSLK-ORF45-A66F cells during the lytic life cycle, although the rate of increase was much lower in both iSLK-STOP45 and iSLK-ORF45-F66A cells than in iSLK-BAC16 cells, and LAMP1 exhibited minimal changes (Fig. 2E). These results indicate that KSHV lytic replication promotes LAMP3 expression in both ORF45-dependent and ORF45-independent manners. To further show that the LAMP3 expression was increased during KSHV lytic replication in the primary effusion lymphoma (PEL) cell line, increased LAMP3 mRNA levels and protein accumulation were observed when BCBL1 cells were treated with TPA to induce lytic replication (Fig. 2F and G). As expected, TPA treatment in KSHV-negative cells did not affect ATF4 and LAMP3 expression (Fig. 2H). These results indicate that LAMP3 expression is upregulated during KSHV lytic replication.

KSHV lytic replication promotes LAMP3 expression in an ATF4-dependent manner. To determine the mechanism of the upregulation of LAMP3 expression during lytic replication, the expression of ATF4 was depleted via lentivirus-based shRNA transduction. When wild-type or ATF4-silenced BCBL1 cells were either left untreated or induced by TPA, the LAMP3 level was decreased by ATF4 knockdown (KD) during both latent and lytic infection, and the increase in LAMP3 expression in cells undergoing lytic replication was almost abolished (Fig. 3A). Similarly, the LAMP3 level was greatly

suppressed in ATF4-KD iSLK-BAC16 or iSLK-STOP45 cells during both latent infection and lytic replication, and the increase in lytic iSLK-BAC16 cells was eliminated by ATF4 knockdown (Fig. 3B). In contrast, the depletion of ATF4 barely affected LAMP1 expression during latent or lytic infection. These results suggest that ATF4 expression is essential for the increase in LAMP3 expression during KSHV lytic replication. Furthermore, when ectopic ATF4 overexpression was introduced into BCBL1 cells, LAMP3 expression was increased, whereas the LAMP1 level was minimally affected (Fig. 3C). The mRNA level of LAMP3 was similarly increased by ATF4 overexpression, and the downstream CHOP expression was also significantly increased (Fig. 3D). On the other hand, neither LAMP3 depletion nor overexpression affected ATF4 expression in BCBL1 cells in the presence of latent or lytic replication, even though ATF4 expression was increased by TPA-induced lytic replication (Fig. 3E and F). These results suggest that LAMP3 expression is upregulated in an ATF4-dependent manner during KSHV lytic replication.

ATF4 and LAMP3 facilitate KSHV lytic replication. To investigate the role of LAMP3 in KSHV lytic replication, we performed a RNA-sequencing analysis of wild-type and LAMP3 knockdown iSLK-BAC16 cells under lytic induction to identify LAMP3-regulated viral and cellular transcriptomes. Interestingly, the expression of most KSHV lytic genes was downregulated by LAMP3 shRNA (Fig. 4A). LAMP3 depletion greatly suppressed the expression of lytic genes, including ORF45, ORF64, RTA, and K8, in BCBL1 cells, whereas the expression of the latent genes LANA2 and LANA was not affected (Fig. 4B). To exclude the off-target effect of LAMP3 shRNA, we performed additional rescue experiments in LAMP3 shRNA-transduced cells, and LAMP3 overexpression completely recovered the expression levels of the lytic genes (Fig. 4C). Furthermore, virion production was dramatically inhibited by LAMP3 depletion (Fig. 4D). These results suggest that LAMP3 expression is essential for KSHV lytic replication. Alternatively, LAMP3 overexpression in BCBL1 cells resulted in a robust increase in KSHV lytic gene expression under TPA treatment (Fig. 4E). Similarly, the yield of progeny virions was significantly increased by LAMP3 overexpression under TPA-induced lytic replication (Fig. 4F). Furthermore, we detected the effects of LAMP3 depletion and LAMP3 overexpression on the yield of infectious progeny viral particles from LAMP3-depleted or LAMP3-overexpressing BCBL1 cells. After 100-fold concentration by ultracentrifugation, equal amounts of extracellular virions were used to infect HEK293 cells, the infected cells were stained by immunofluorescence with an anti-LANA antibody, and the expression of LANA was detected via real-time PCR. The number of LANA-positive cells and the level of LANA mRNA were decreased when the HEK293 cells were infected with infectious progeny viral particles from LAMP3-depleted BCBL1 cells, compared with control BCBL1 cells, and similarly, they were increased when the HEK293 cells were infected with viral particles from cells overexpressing LAMP3 (Fig. 4G and H). These results show that LAMP3 promotes the production of infectious KSHV virions. During lytic replication, the mRNA levels of the lytic genes (ORF45, ORF20, and ORF64) were significantly increased by LAMP3 overexpression, and the expression of the latent gene LANA was barely affected (Fig. 4I). In addition, LAMP3 alone slightly induced virion production and lytic gene expression during spontaneous lytic replication in BCBL1 cells without TPA induction (Fig. 4F and I). These results suggest that LAMP3 greatly facilitates both spontaneous and TPA-induced KSHV lytic replication.

To further investigate the function of upstream ATF4 expression during lytic replication, ATF4 expression was silenced in BCBL1 cells. Following TPA induction for 48 h, the expression of the lytic genes ORF45, ORF64, RTA, and K8 was markedly suppressed in ATF4-depleted cells, compared with cells that were transduced with scramble shRNA, whereas the expression of the latent gene LANA was not affected (Fig. 5A). The decreased level of lytic gene expression was recovered when the expression of ATF4 was rescued (Fig. 5B). On the other hand, the expression of these lytic genes was significantly increased by ATF4 overexpression, but LANA was not affected (Fig. 5C). Furthermore, the mRNA levels of KSHV lytic genes were significantly increased in cells in the presence of ATF4 overexpression under both spontaneous and TPA-induced lytic

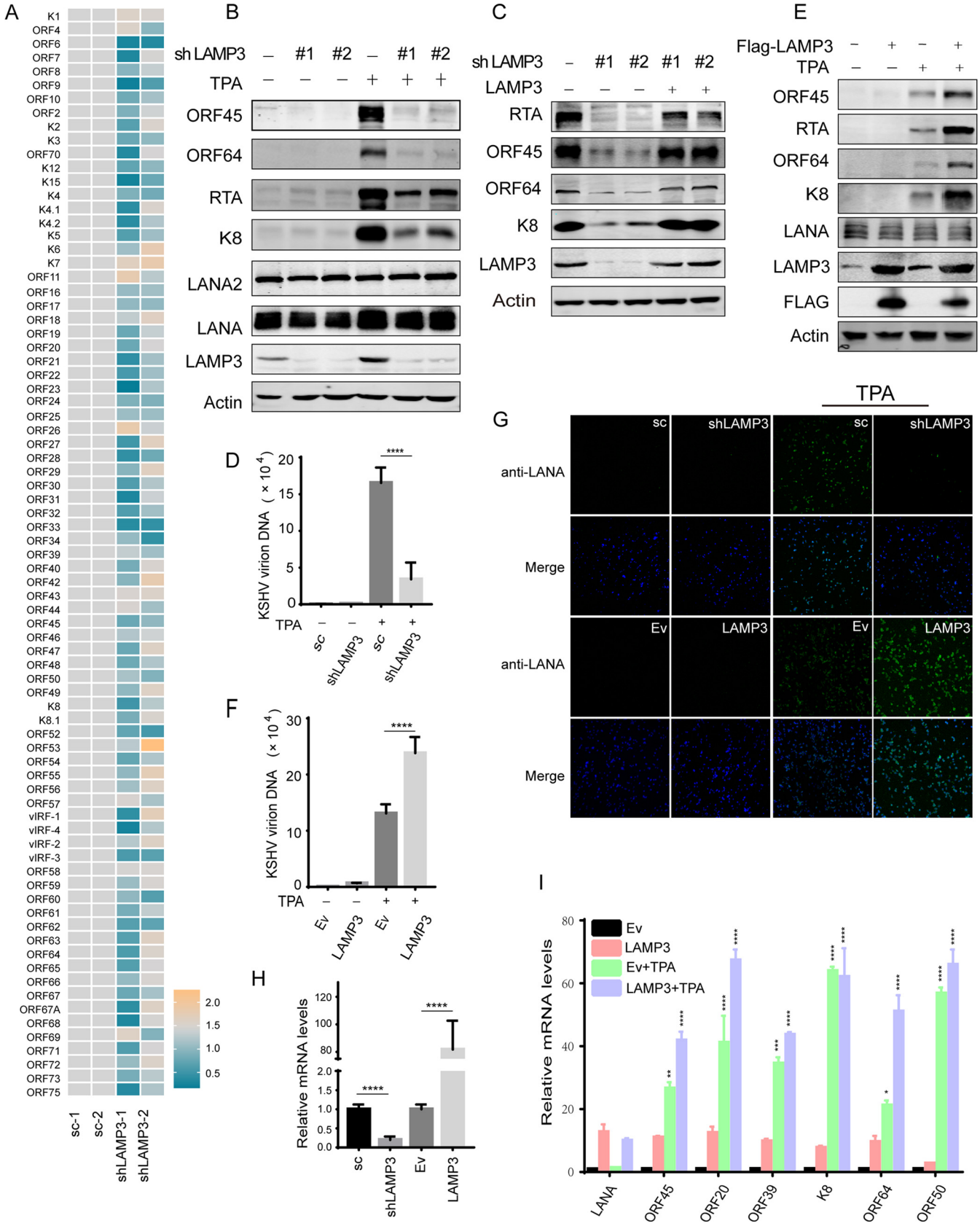

FIG 4 LAMP3 facilitates KSHV lytic replication. (A) Heatmap of the viral gene expression in the control versus LAMP3-knockdown iSLK-BAC16 cells under lytic induction. The viral gene expressions were filtered from the raw data of the RNA-sequencing analysis, and the relative levels of viral gene expression

(Continued on next page)

replication (Fig. 5D). Consequently, extracellular virion production was significantly decreased by ATF4 silencing and was increased by ATF4 overexpression in BCBL1 cells (Fig. 5E and F). Similarly, ATF4 depletion decreased the expression of the immediate early gene ORF45, the delayed early gene ORF64, and the late gene K8.1 in iSLK-BAC16 cells when RTA was expressed under lytic induction, whereas this effect did not occur under latent infection or lytic replication in ORF45-null iSLK-STOP45 cells (Fig. 5G). A similar inhibition of virion production was observed in iSLK-BAC16 cells with ATF4 depletion, and the virion yield was not decreased in iSLK-STOP45 cells (Fig. 5H). Thus, these results suggest that ATF4 promotes both KSHV lytic gene expression and virion production.

The ATF4-LAMP3 axis promotes KSHV lytic replication. Since both LAMP3 and ATF4 are essential for KSHV lytic replication, and since LAMP3 acts as a direct target of ATF4 expression, we wondered which one was the primary mediator of KSHV lytic replication. When LAMP3 was overexpressed in ATF4-knockdown BCBL1 cells, ATF4 knockdown alone or LAMP3 overexpression alone inhibited or promoted KSHV lytic gene expression (ORF45, RTA, and K8), respectively, compared with that in control cells; however, an equivalent increase in KSHV lytic gene expression was observed in ATF4-knockdown cells relative to control cells in the presence of LAMP3 overexpression (Fig. 6A), and there was a minimal effect on the latent gene expression of LANA. Furthermore, virion production was decreased or increased by ATF4 depletion alone or by LAMP3 overexpression alone in BCBL1 cells, respectively, whereas the same level of virion yield was observed in the ATF4 knockdown cells, relative to control cells, in the presence of LAMP3 overexpression (Fig. 6B). These results suggest that LAMP3 is sufficient to promote lytic replication in an ATF4-independent manner.

Next, we investigated whether ATF4-induced lytic replication required LAMP3 expression. When LAMP3 expression was depleted in BCBL1 cells in the absence or presence of ATF4 overexpression, LAMP3 knockdown alone or ATF4 overexpression alone decreased or increased the expression of the lytic genes (ORF45, RTA, and K8), respectively. Interestingly, the levels of these lytic genes were suppressed by LAMP3 silencing, compared with normal LAMP3 expression, in the presence of ATF4 overexpression (Fig. 6C), but there was no obvious effect on latent gene expression (LANA). Furthermore, the virion yield was similarly inhibited by LAMP3 silencing in both control cells and ATF4-overexpressing cells (Fig. 6D). These results indicate that ATF4 promotes lytic gene expression and virion production through increased LAMP3 expression. Thus, we conclude that the ATF4-LAMP3 axis promotes KSHV lytic replication and that LAMP3 acts as a lytic accelerator downstream of ATF4 during the lytic life cycle.

LAMP3 induces Akt and ERK activation during KSHV lytic replication. To further reveal the mechanism and signal transduction of LAMP3 in KSHV lytic replication, RNA-sequencing analysis was performed to identify the differentially expressed genes (DEGs) in normal cells, relative to LAMP3-silenced iSLK-BAC16 cells, during lytic replication. A total of 7,742,298 raw reads, 35,373 cellular transcripts, and 79 viral transcripts were obtained; all of the DEG clusters were then filtered and analyzed by a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. A total of 157 and 171 DEGs were upregulated or downregulated in shLAMP3-transduced cells, compared with control cells, respectively. 11 pathways, including the PI3K-Akt signaling pathway, were significantly enriched by over 10-fold in LAMP3-depleted cells,

FIG 4 Legend (Continued)
in the LAMP3-knockdown cells were normalized to that in the control cells. (B and D) BCBL1 cells were transduced by lentivirus-based scramble or by two different shRNA targeted to LAMP3 for 36 h and then were then either left untreated or treated with TPA for 72 h. The cells were collected and lysed, and then the expression of KSHV genes was analyzed by Western blots as indicated (B). Four days after TPA treatment, the supernatants were collected, and virion DNA was extracted and analyzed via real-time PCR (D). ****, $P < 0.0001$. (C) BCBL1 cells with scramble shRNA or shLAMP3 transduction were infected with empty or LAMP3-expressing lentiviruses as indicated. 36 h after infection, the cells were treated with TPA for 72 h, the cells were collected and lysed, and the cell extracts were analyzed by Western blots as indicated. (E, F, and I) BCBL1 cells were infected with empty or LAMP3-expressing lentiviruses for 36 h. At 72 h after the TPA induction, the whole-cell extracts were detected by Western blots (E), and the total RNA was extracted and subjected to a quantitative RT-PCR analysis (I) to detect viral gene expression. After 4 days of TPA induction, the supernatants were collected, and the virion DNA were extracted and detected via real-time PCR (F). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (G and H) The virions were concentrated and subjected to infection with HEK293 cells for 72 h, the infected cells were stained by inflorescence with the anti-LANA antibody (G), and the total RNA was extracted from the infected cells and subjected to quantitative RT-PCR analysis to detect the levels of LANA (H). ****, $P < 0.0001$.

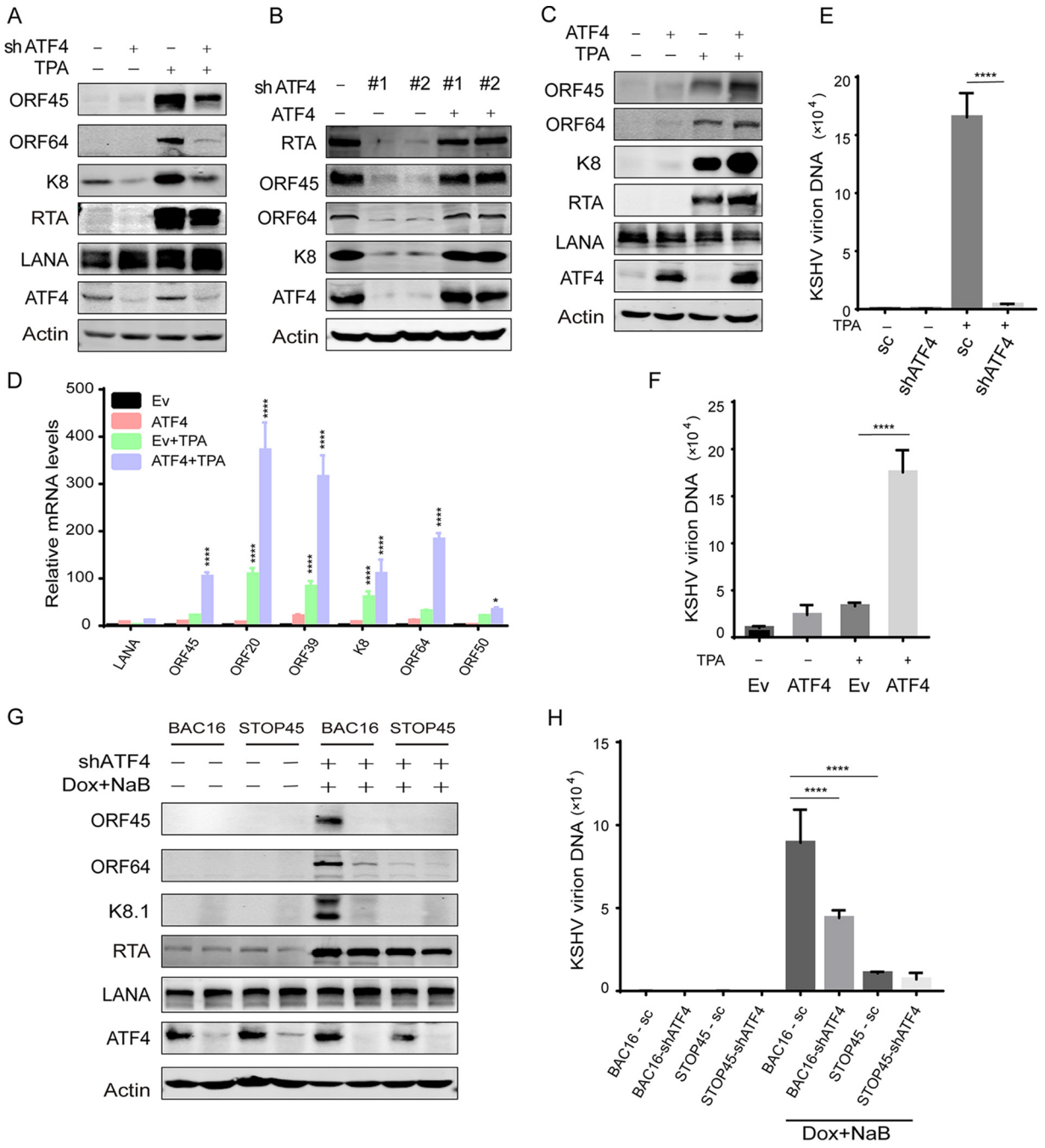

FIG 5 ATF4 facilitates KSHV lytic replication. (A and C) BCBL1 cells were transduced with lentivirus-based scramble shRNA or shATF4 (A) or were infected with control or ATF4-expressing lentiviruses (C) for 24 h, and then the cells were either left untreated or treated with TPA for 72 h. The cells were collected and lysed, and the expression of viral genes was analyzed by Western blots as indicated. (B) ATF4-depleted BCBL1 cells were infected with empty or ATF4-expressing lentiviruses and then analyzed by Western blots as described. (D) BCBL1 cells were infected with control or ATF4-expressing lentiviruses and were then either left untreated or treated as described above for 48 h. The total RNA was extracted and subjected to a quantitative RT-PCR analysis to detect the expression of KSHV genes. *, $P < 0.05$; ****, $P < 0.0001$. (E and F). Scramble shRNA or shATF4-transduced BCBL1 cells (E) and control or ATF4 overexpressing BCBL1 cells (F) were either left untreated or induced by TPA for 4 days. The supernatants were collected, and the KSHV virion DNA was extracted and analyzed via real-time PCR. ****, $P < 0.0001$. (G and H). iSLK-BAC16 and iSLK-STOP45 cells were transduced with scramble shRNA or shATF4 by lentiviruses, and then the cells were either left untreated or treated with Dox plus NaB for lytic induction. After lytic induction for 72 h, the cells were collected, and the cell extracts were analyzed by Western blots as indicated (G). 5 days after induction, the supernatants were collected, and the KSHV virion DNA was extracted and analyzed via real-time PCR (H). ****, $P < 0.0001$.

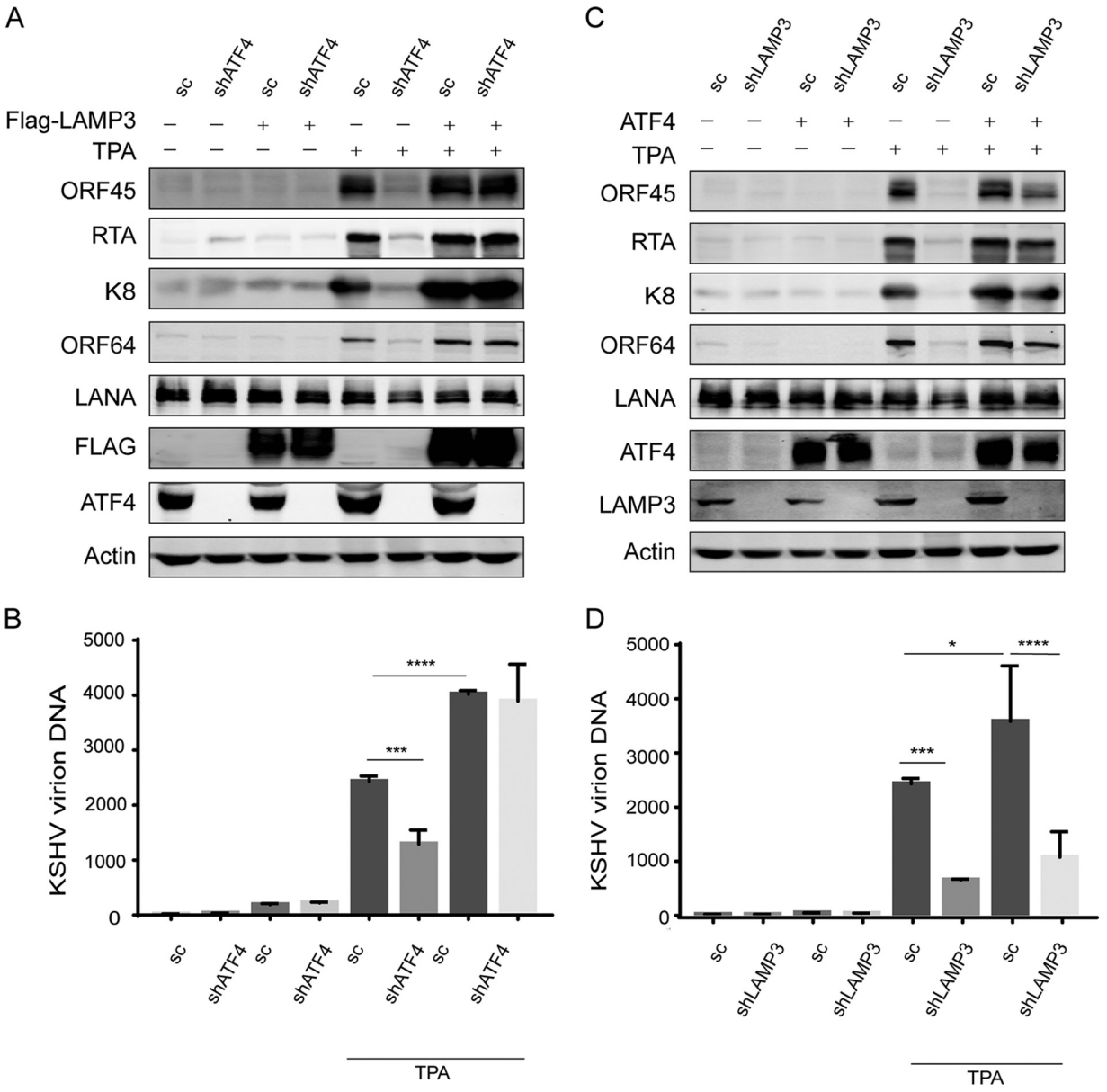

FIG 6 ATF4 silencing does not suppress lytic replication in the presence of LAMP3 overexpression, whereas LAMP3 depletion inhibits KSHV lytic replication in the presence of ATF4 overexpression. (A and B). Scramble shRNA or shATF4-transduced BCBL1 cells were infected with control and LAMP3-expressing lentiviruses and were then either left untreated or treated with TPA for 72 h. Then, the cells were collected and lysed, and the cell extracts were detected with Western blots for the expression of KSHV genes (A). After the cells were either left untreated or treated for 4 days, the supernatants were collected, and the KSHV virion DNA was extracted and analyzed via real-time PCR (B). (C and D) Wild-type or LAMP3-knockdown BCBL1 cells were infected with empty or ATF4-expressing lentiviruses and were then either left untreated or treated with TPA. The expression of KSHV virus genes was analyzed by Western blots (C), and virion DNA were analyzed by real-time PCR (D) as described above. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

compared with control cells (Fig. 7A). Since Akt and ERK activation play central roles in KSHV infection and in lytic replication (39, 40), the phosphorylation of Akt and ERK was examined in LAMP3-overexpressing or LAMP3 shRNA-transduced cells. Akt and ERK phosphorylation was greatly reduced by LAMP3 depletion in HEK293T cells, compared to control cells (Fig. 7B), and the phosphorylation of Akt and ERK was similarly increased by LAMP3 overexpression in HEK293T cells (Fig. 7C). These results confirm that LAMP3 alone promotes Akt and ERK activation. Next, Akt and ERK phosphorylation was examined in KSHV-harboring cells. When BCBL1 cells were transiently infected with shLAMP3-expressing or LAMP3-overexpressing lentiviruses, the phosphorylation

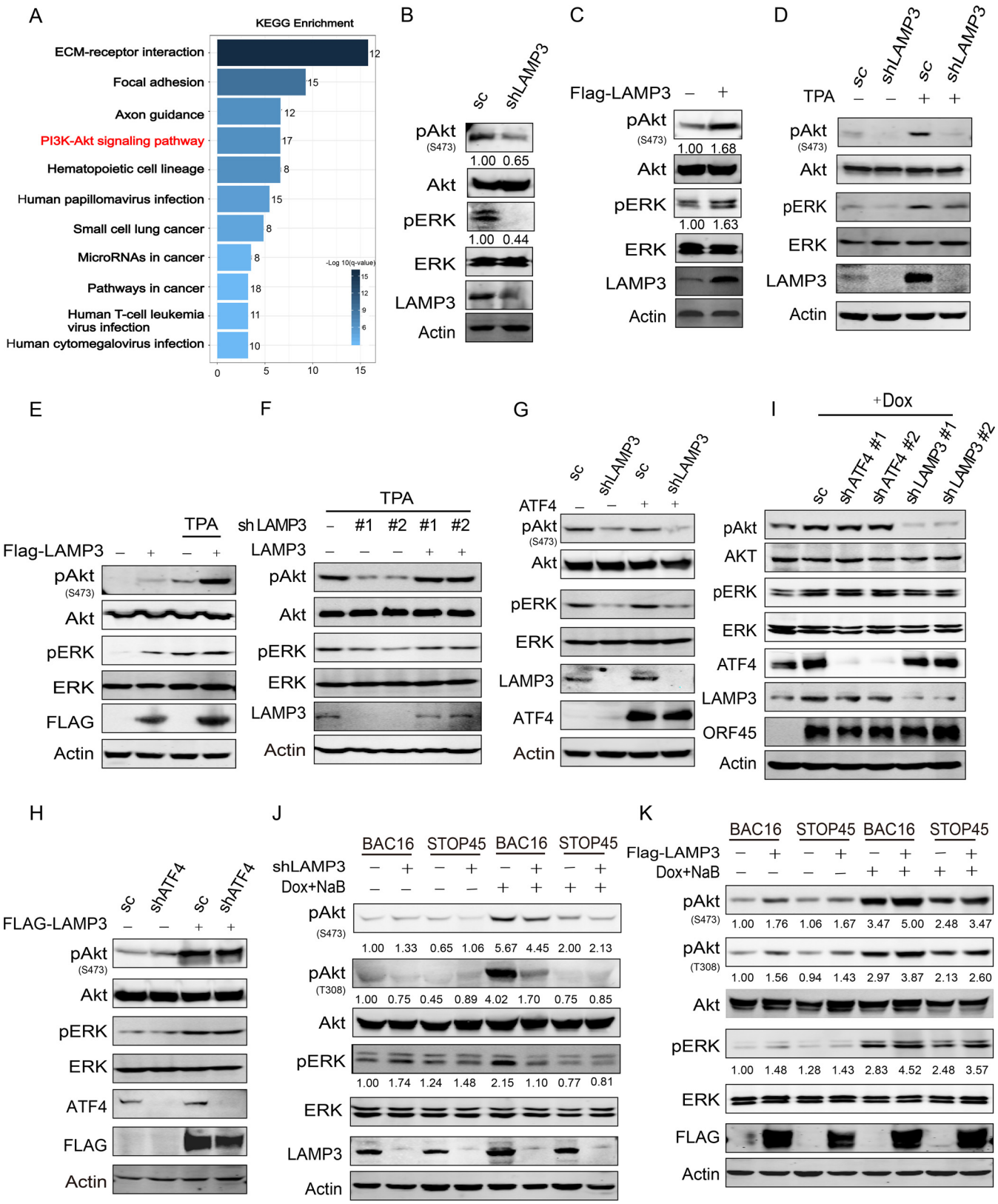

FIG 7 LAMP3 promotes Akt and ERK activation. (A) The differentially expressed genes were filtered from the RNA sequencing analysis in scramble shRNA versus shLAMP3-transduced iSLK-BAC16 cells under lytic replication. The top 11 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the differentially expressed genes are shown ($-\log_{10}$ [*P* values] > 10). (B and C) HEK293T cells were transfected with scramble shRNA or shLAMP3 plasmids (B) or empty control or LAMP3-expressing plasmids (C) for 48 h. The cells were collected and lysed, and the phosphorylation of Akt and ERK in the whole-cell

(Continued on next page)

of Akt and ERK was inhibited by LAMP3 knockdown under both latent infection and TPA-induced lytic replication (Fig. 7D). Similarly, these factors were activated by LAMP3 overexpression under both latent and lytic conditions (Fig. 7E). The decreased ERK and Akt phosphorylation in LAMP3-depleted cells was completely recovered by LAMP3 overexpression (Fig. 7F). Transient ATF4 overexpression minimally affected Akt and ERK activation, and LAMP3 silencing greatly reduced their activation, even in the presence of ATF4 overexpression (Fig. 7G). However, ATF4 silencing did not affect Akt and ERK phosphorylation in the absence or presence of LAMP3 overexpression (Fig. 7H), indicating that LAMP3 overexpression induces Akt and ERK activation in an ATF4-independent manner. To further confirm that ORF45 activates the ATF4-LAMP3-Akt axis, ORF45-Tet-on BJAB cells were infected with shATF4 or shLAMP3-expressing lentiviruses following doxycycline induction, and LAMP3 depletion but not ATF4 depletion decreased Akt activation in the presence of ORF45 overexpression (Fig. 7I), whereas neither greatly affected ERK activation, as ORF45 directly interacted with and induced sustained ERK-RSK activation (16). Since ORF45 induces ERK-RSK and mTOR activation during lytic replication, the phosphorylation of Akt and ERK was further examined in wild-type iSLK-BAC16 and ORF45-null iSLK-STOP45 cells in the absence or presence of LAMP3 expression. Both Akt and ERK phosphorylation were increased during lytic replication and were similarly decreased by LAMP3 knockdown in iSLK-BAC16 cells during lytic replication, and Akt and ERK phosphorylation was slightly affected by LAMP3 knockdown in both iSLK-BAC16 and iSLK-STOP45 cells during latent infection. No difference was observed in iSLK-STOP45 cells during lytic induction, as Akt and ERK phosphorylation were not activated (Fig. 7J). Alternatively, Akt and ERK phosphorylation was increased by LAMP3 overexpression in both iSLK-BAC16 and iSLK-STOP45 cells under latent and lytic conditions (Fig. 7K). These results suggest that LAMP3 promotes Akt and ERK activation during KSHV latent infection and lytic replication, independent of the expression of ORF45 and other viral genes. Given that Akt and ERK activation play important roles in KSHV lytic replication, we conclude that LAMP3 promotes Akt and ERK activation to facilitate KSHV lytic replication.

DISCUSSION

Multiple signaling cascades are activated during KSHV lytic replication to reprogram cellular transcription and translation. Our previous studies have demonstrated that ORF45 induces sustained ERK and RSK activation and promotes viral transcription and translation through c-Fos and eIF4B phosphorylation (9, 15, 39). Here, we further reveal that ORF45 plays an important role in ER stress during lytic replication and promotes eIF2α phosphorylation and ATF4 translation and that, consequently, KSHV lytic replication upregulates LAMP3 expression in an ATF4-dependent manner (Fig. 8). As a result, the ATF4-LAMP3 axis induces Akt and ERK activation and promotes KSHV lytic replication and virion production. Our findings indicate that ORF45 and KSHV lytic replication upregulate the ATF4-LAMP3 axis, which acts as one of the downstream signal cascades of ER stress, to facilitate optimal KSHV lytic replication.

Studies have shown that KSHV lytic replication induces the activation of UPR sensors and downstream mediators of ER stress, such as ATF4, GRP78, and XBP1 (20–22). However, the viral activators of ER stress during lytic replication and the mechanism

FIG 7 Legend (Continued)

extracts was analyzed by Western blots as indicated. (D and E) BCBL1 cells was were infected with scramble shRNA or shLAMP3-expressing lentiviruses (D), or an empty vector or LAMP3-expressing lentiviruses (E) for 36 h, and then the cells were either left untreated or treated with TPA for 72 h, and the cell extracts were detected by Western blot as indicated. (F) Scramble shRNA or shLAMP3 transduced BCBL1 cells were infected with empty or LAMP3-expressing lentiviruses for 36 h. The cells were collected and lysed, and the cell extracts were subjected to Western blotting for ERK and Akt phosphorylation. (G and H) Control or LAMP3-knockdown BCBL1 cells were infected with empty or ATF4-expressing lentiviruses (G), and the control or ATF4-silenced BCBL1 cells were infected with empty or LAMP3-expressing lentiviruses (H) for 48 h. The cells were collected, and the cell extracts were detected as indicated. (I) Stably inducible ORF45-Tet-on BJAB cells were transfected with scramble shRNA, shATF4-expressing lentiviruses, or shLAMP3-expressing lentiviruses for 24 h, and then the cells were either left untreated or incubated with 1 μg/mL doxycycline (Dox) for 48 h, and the cell extracts were analyzed by Western blots as described. (J and K) iSLK-BAC16 or iSLK-STOP45 cells were infected with scramble shRNA or shLAMP3 expressing lentiviruses (J), or the empty vector or LAMP3-expressing lentiviruses (K) for 36 h, and then the cells were either left untreated or induced with Dox plus NaB for 72 h. The cell extracts were prepared and analyzed by Western blots as indicated.

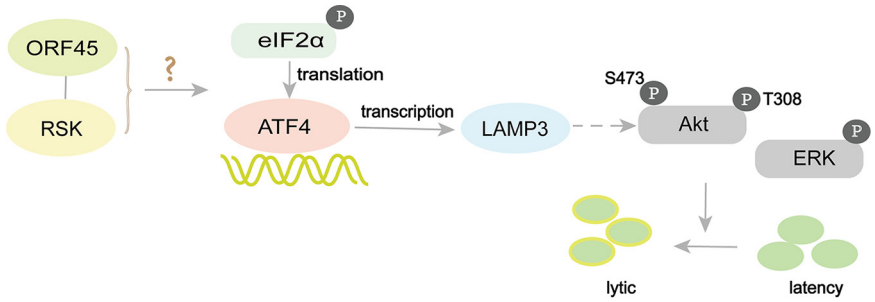

FIG 8 Diagram of the upregulation and function of ATF4-LAMP3 axis during KSHV lytic replication. During late KSHV lytic replication, ORF45 enhances eIF2α phosphorylation and ATF4 translation and subsequently upregulates LAMP3 expression in either an RSK-dependent or RSK-independent manner. As results, LAMP3 induces both ERK and Akt activation and then promotes KSHV reactivation from latency.

remain unknown. Here, we reveal that ORF45 acts as a viral inducer of eIF2α phosphorylation and ATF4 translation and induces the responses to ER stress during KSHV lytic replication. This function depends on RSK signaling, as ORF45 F66A, which is not able to interact with and activate RSK, alleviates this activity. Three potential mechanisms are hypothesized as follows. First, ORF45 induces eIF4B phosphorylation through RSK activation and then promotes the cellular and viral translation of mRNA with a specific 5′-UTR structure; thus, the translation of ER stress-related mediators might be upregulated, and ER stress is activated (39, 41). Second, ORF45 activates the ERK-RSK and mTOR signaling cascades and consequently inhibits autophagy and autophagic clearance (15, 42); therefore, ORF45-induced signaling might cause excessive protein accumulation in the ER or disrupt ER transport and recycling, which also activates ER stress. Finally, studies have shown that ORF45 interacts with Siah1/2 for ubiquitination (43); in turn, Siah1/2 activity can be affected by ORF45 during lytic replication and can finely tune the activation of ER stress and the UPR (44). Of course, we cannot exclude the possibility that ORF45 induces ER stress and ATF4 translation through unknown mechanisms, and further investigation will reveal the function and detailed mechanisms of ORF45 in the induction of ER stress and the UPR.

Both the Akt and ERK pathways play important roles in KSHV primary infection and lytic replication (45, 46), and the inhibition of the activation of either pathway suppresses lytic replication. A RNA-sequencing analysis revealed that LAMP3 silencing in KSHV-harboring cells during lytic replication significantly enriched PI3K-Akt signaling and downregulated lytic replication (Fig. 4A and 7A). LAMP3 expression in KSHV-negative cells promotes Akt and ERK activation, indicating that LAMP3 alone regulates both of the activation pathways but not the viral products. It also remains unclear how LAMP3 activates both pathways, and two hypotheses may explain the mechanism. Studies have revealed that LAMP3 suppresses autophagy, which then results in LC3 accumulation (24). In turn, LC3 binds to ERK and increases ERK activation (47), subsequently activating the ERK-RSK and mTOR-Akt axes. Alternatively, LAMP3 is mainly distributed in endosomes and lysosomes and regulates the trafficking and sorting of the lysosomes and endosomes (48). In turn, signal transduction from cell-surface receptors or integral membrane proteins or the activation of membrane-associated kinases or phospholipase is affected by LAMP3 upregulation, resulting in the activation of the phospholipase C and PI3K-Akt pathways. It would be interesting to further map the functional domains and investigate the partners and signal cascade of LAMP3 in the regulation of PI3K-Akt and ERK-MAPK activation.

Studies have revealed that the different downstream mediators of ER stress affect KSHV lytic replication; for example, XBP1 initiates the reactivation of KSHV from latency in differentiated B cells or under hypoxia (23, 49, 50), Bip/Grp78 play important roles in late lytic replication, including in virion assembly and release (21), and ATF4 plays essential roles in the early stage of lytic replication to regulate lytic gene expression

and proangiogenic properties (22). As a direct downstream target of ATF4, LAMP3 similarly upregulates KSHV early and late gene expression. Importantly, the proviral function of ATF4 in lytic replication requires LAMP3 expression, but the opposite is not true; LAMP3 depletion greatly attenuates lytic replication in the presence of ATF4 overexpression while ATF4 silencing barely affects the LAMP3 promotion of lytic replication, indicating that LAMP3 plays a primary role in KSHV lytic replication downstream of ATF4. Thus, our findings characterize the essential roles of the ATF4-LAMP3 axis in KSHV lytic replication. Certainly, ATF4 has other downstream targets, and these other targets may also play important roles in KSHV lytic replication.

Previous studies have revealed that ORF45 has multiple essential functions during KSHV lytic replication, and a deficiency of ORF45 expression or ORF45-mediated ERK-RSK activation dramatically suppresses KSHV lytic replication (15, 17, 18). Although several downstream substrates of ORF45-RSK signaling, such as c-Fos and eIF4B, are activated to promote viral lytic transcription and translation at the late stage of the lytic life cycle (9, 39), the knowledge of downstream signaling and the mechanisms of ORF45 in the KSHV lytic life cycle largely remain limited. Our present study revealed that ORF45-RSK signaling promotes ATF4 translation and activates the ATF4-LAMP3 axis, one of the downstream signal branches of ER stress, but not the upstream or intact signaling of UPR or ER stress, to facilitate KSHV lytic gene expression and lytic replication. As a downstream signaling pathway, the ATF4-LAMP3 axis plays essential roles in lytic replication in the presence of ORF45 expression, whereas its depletion was not able to enhance the suppression of lytic replication by ORF45 deficiency or by ORF45 F66A mutation, as the ATF4-LAMP3 axis requires ORF45-RSK activation during the lytic life cycle. Thus, our findings demonstrate that the ATF4-LAMP3 axis acts as a downstream cascade of ORF45-RSK signaling to facilitate the optimal KSHV lytic replication.

LAMP3 upregulation has been observed in several kinds of cancers and plays important roles in tumors, including the regulation of autophagy and apoptosis. The regulation of autophagy and apoptosis in KSHV lytically infected cells is important for KSHV lytic replication and KSHV-related tumorigenesis (51–54), and it would be interesting and important to further investigate the role of LAMP3 in KSHV pathogenesis and in the treatment of KSHV-related diseases.

In conclusion, our findings reveal that one of the downstream branches of ER stress, the ATF4-LAMP3 axis, is upregulated by ORF45 during lytic replication, increases Akt and ERK activation in turn, and facilitates KSHV lytic replication and virion production. Our findings show that LAMP3 acts as an important downstream target of ER stress in the KSHV lytic life cycle and is a novel cellular mediator that facilitates KSHV lytic replication.

MATERIALS AND METHODS

Cells, antibodies, and chemicals. KSHV-negative BJAB and KSHV-positive BCBL1 lymphoma cells were maintained in our laboratory and cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). Wild-type BAC16-haboring iSLK-BAC16, ORF45-null iSLK-STOP45, ORF45-F66A, and ORF45-A66F cells were described previously (9, 17, 41) and were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% FBS and antibiotics. The doxycycline (Dox), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and sodium butyrate (NaB) were purchased from Sigma-Aldrich Co. (St. Louis, MO). The anti-LAMP3 and anti-ATF4 antibodies were purchased from Proteintech Group (Rosemont, IL, USA). The anti-LAMP1 antibody was purchased from ABclonal Technology (Wuhan, China). The anti-Akt, p-Akt (Thr308), p-Akt (Ser473), anti-ERK, and anti-pERK antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-K8.1 antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). The anti-RTA, ORF64, K8, and LANA antibodies have been previously described (9, 15).

Plasmids. The shRNA were subcloned into a pLKO.1 vector between the EcoRI and AgeI sites. The sequences of shATF4: 5′-GTTGGTCAGTCCCTCCAACAA-3′ and 5′-GCCAAGCACTTCAAACCTCAT-3′ as well as shLAMP3: 5′-CGTCAGTCAAGACTGGAATTT-3′ and 5′-GTCAGTCAAGACTGGAATTTA-3′. The LAMP3 and ATF4 fragments were amplified via PCR and were subcloned into a pLVX-IRES-ZsGreen vector at the EcoRI and BamHI sites to construct lentivirus-based plasmids for packaging and overexpression.

The induction of KSHV lytic replication and the detection of virion production. To induce KSHV lytic replication, the BCBL1 cells were treated with 20 ng/mL TPA, whereas the iSLK-BAC16 and iSLK-STOP45 cells were treated with 1 μg/mL Dox plus 0.3 mM NaB. After induction for 4 days in BCBL1 cells or for 5 days in iSLK-BAC16 or iSLK-STOP45 cells, the supernatants were collected, and the virion yields

were detected as described previously (55, 56). Briefly, the supernatants were centrifuged twice at 10,000 × *g* for 10 min at room temperature to remove the cell debris and dead cells, and they were then incubated with DNase I at 37°C for 1 h to digest the external DNA. The reaction was stopped by EDTA plus SDS for 10 min, and the supernatants were treated by proteinase K digestion at 55°C for 30 min. The virion DNA was extracted and dissolved in double-distilled water.

Lentivirus packaging and infection. The packaging of lentiviruses for overexpression or for shRNA transduction was performed as described previously (57). In brief, one 10 cm dish of cells was cotransfected with 4 μg of the pLVX lentiviral expression plasmid or pLKO.1 shRNA plasmid as well as 4 μg of the psPAX2 and 4 μg of the pMD.2G packaging plasmids. The supernatants were harvested after 48 h post-transfection to prepare lentiviral stocks. For the lentiviral infection, the lentiviruses were titrated, and the cells were infected in the presence of 4 μg/mL of Polybrene for 6 h, as described previously (58).

KSHV concentration and infection. BCBL1 cells with scramble shRNA or shLAMP3 transfection, empty control, or LAMP3 transfection were induced with TPA treatment for 4 days. The supernatants were harvested and centrifuged twice at 10,000 × *g* for 10 min, and they were then subjected to ultracentrifugation at 100,000 × *g* for 1.5 h, and the pellets were dissolved in a 1/100 volume of PBS. HEK293 cells were infected with KSHV viral stocks in the presence of 4 μg/mL of Polybrene and were centrifuged at 2,000 rpm at room temperature for 1 h.

Real-time PCR. The total RNA was extracted using TRIzol, reverse-transcribed into cDNA, and then analyzed via real-time PCR with SYBR Green and a LightCycler 480 system. The sequences of primer pairs are available upon request.

Western blotting. The cells were collected and lysed in the presence of a protease inhibitor cocktail (Roche) and phosphatase inhibitors. 40 to 60 μg of proteins per lane of the whole-cell extracts were separated by SDS-PAGE and transferred to membranes. The membranes were blocked in 5% dry milk, incubated overnight with primary antibodies at 4°C, and species-matched with IRDye 680 or IRDye 800 secondary antibodies at room temperature for 2 h. The images were visualized and analyzed using a LI-COR Odyssey system.

RNA sequencing analysis and data availability. The iSLK-BAC16 and iSLK-STOP45 cells were either left uninfected or infected with shLAMP3-lentiviruses or empty lentiviruses for 36 h, and then these cells were either left untreated or treated with 1 μg/mL Dox and 0.3 mM NaB for 72 h. The total RNA was extracted with TRIzol, following the manufacturer's procedures, and subjected to a deep RNA-sequencing analysis. The libraries were constructed and sequenced on an Illumina platform, 150 bp paired-end reads were generated, and the data analysis was performed using a Molecule Annotation System 3.0 (Annoroad Gene Technology Co., Beijing, China). The raw data were deposited in the NCBI GEO archive under accession number GSE192724. The data mining, analysis, and enrichment of the differentially expressed genes were performed as described previously (59).

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We declare no competing financial interest.

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