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VIRUS-CELL INTERACTIONS

# The Kaposi's Sarcoma-Associated Herpesvirus ORF34 Protein Interacts and Stabilizes HIF-2$\alpha$ via Binding to the HIF-2$\alpha$ bHLH and PAS Domains

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**ABSTRACT** Hypoxia and hypoxia inducible factors (HIFs) play important roles in the Kaposi's sarcoma-associated herpesvirus (KSHV) life cycle. KSHV is the causative agent of Kaposi's sarcoma (KS) and other AIDS-related malignancies. Kaposi's sarcoma is a highly vascular tumor, which preferentially develops in the lower extremities of the body where blood vessels are often poorly oxygenated. The main cellular responses to hypoxia are mediated mainly by two isoforms of HIF, HIF-1$\alpha$ and HIF-2$\alpha$. HIF-1$\alpha$ and HIF-2$\alpha$ have common as well as distinct functions, although they are similar in structure and function. Previously, we showed that the KSHV ORF34 protein binds HIF-1$\alpha$ and facilitates its degradation through the ubiquitin-proteasome pathway causing negative regulation of HIF-1$\alpha$-dependent genes (Haque and Kousoulas, J Virol 87:2164-2173, 2013, https://www.doi.org/10.1128/JVI.02460-12). Herein, we show that the *ORF34* gene is involved in the regulation of KSHV lytic gene expression, since deletion of *ORF34* resulted in reduced immediate early and early lytic gene expression and blocked late gene expression. Coimmunoprecipitation experiments revealed that the ORF34 protein physically interacted with HIF-2$\alpha$ in transfected as well as in KSHV-infected cells. Utilization of ORF34 truncations revealed that three distinct domains bind HIF-2$\alpha$ and that both bHLH and PAS domains of HIF-2$\alpha$ interacted with ORF34. Unlike HIF-1$\alpha$, dose-dependent coexpression of ORF34 stabilized the HIF-2$\alpha$ protein, ensuring HIF-2$\alpha$-dependent transcriptional activity. The ORF34 protein enhanced HIF-2$\alpha$ ubiquitination at the bHLH and PAS domains. The results show that the KSHV ORF34 protein is involved in the KSHV life cycle by regulating the expression of HIF-1$\alpha$ and HIF-2$\alpha$ proteins.

**IMPORTANCE** Hypoxia inducible factor 1$\alpha$ (HIF-1$\alpha$) and HIF-2$\alpha$ are transcription factors which play important roles in the Kaposi's sarcoma-associated herpesvirus (KSHV) latent and lytic gene replication. Herein, we show that the *ORF34* gene is involved in the regulation of KSHV lytic gene expression, since deletion of *ORF34* resulted in reduced immediate early and early lytic gene expression and blocked late gene expression. In addition, we demonstrate that the KSHV ORF34 protein binds and stabilizes HIF-2$\alpha$, in contrast to its role in binding HIF-1$\alpha$ and causing its degradation via the proteasome pathway. Thus, the KSHV ORF34 protein plays a regulatory role in the KSHV life cycle by regulating HIF-1$\alpha$ and HIF-2$\alpha$ expression.

**KEYWORDS** hypoxia inducible factors (HIFs), Kaposi's sarcoma-associated herpesvirus, ubiquitination

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a gamma-2 herpesvirus related to Epstein-Barr virus (EBV) and herpesvirus saimiri (1, 2). KSHV is the causative agent for Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (3–5). KSHV displays two distinct

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phases in its life cycle: latent and lytic replication (6, 7). During latency, a limited number of viral genes are expressed, which play key roles in the replication and maintenance of the viral genome (8–10). Lytic replication can be induced by treatment of latently infected cells with chemical agents such as 12-0-tetradecanoyl 13-acetate (TPA) or sodium butyrate (6, 11) or low-oxygen condition (hypoxia) (12). Hypoxia provides physiologic stimuli exhibited at least through the induction of hypoxia inducible factors (HIFs), indicating that hypoxia and HIFs play important roles at least in the latent KSHV life cycle (13–21).

HIFs are heterodimeric transcription factors composed of oxygen-labile HIF-$\alpha$ subunits and a constitutively expressed HIF-1$\beta$ subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT) (22–27). Under normal cellular oxygen level (normoxic) conditions, HIF-$\alpha$ proteins are targeted for proteasomal degradation by interaction with the von Hippel-Lindau (pVHL) tumor suppressor protein and subsequent polyubiquitination (28–31). The interaction of pVHL and HIF-$\alpha$ proteins is regulated by the hydroxylation of specific proline residues in the oxygen-dependent degradation domain (ODD) of HIF-$\alpha$ (32–34). In mammalian cells, a family of four HIF prolyl hydroxylases, named prolyl hydroxylase domain (PHD) enzymes (PHD1 to -4), were shown to hydroxylate the oxygen-sensitive alpha subunit (35–38). Under hypoxic conditions, PHD activity is limited, causing increased stability, rapid accumulation, and increased ability to recruit coactivators (39, 40). Stabilized HIF-$\alpha$ proteins translocate to the nucleus and heterodimerize with constitutively expressed HIF-$\beta$ subunits and bind to hypoxia-responsive elements (HREs) within the promoter or enhancer region of hypoxia-responsive target genes, upregulating their expression (41). The core consensus sequence that HIF heterodimers bind to has been identified as 5′-R(A/G)CGTG-3′ (42). Hypoxia induces the accumulation of hypoxia inducible factors (HIF-1$\alpha$ and HIF-2$\alpha$), and most human tumors are positive for both HIF-$\alpha$ subunits (HIF-1$\alpha$ and HIF-2$\alpha$). Recently, both subunits were detected in KS biopsy specimens, supporting a possible role of HIFs in KSHV-induced tumors (16, 43). HIF-1$\alpha$ and HIF-2$\alpha$ have similar structural and functional domains but target both common and unique viral and cellular genes (25, 44–46). HIF-1$\alpha$ is expressed ubiquitously, while HIF-2$\alpha$ has more tissue-specific expression (47, 48). Hypoxia and HIFs can upregulate the main latent *LANA1* gene, as well as specific viral lytic genes, including the major replication and transcription activator (RTA), by binding to specific HRE sequences within the RTA promoter region (13, 15, 19). In addition, LANA1 synergizes with hypoxia in endothelial cells to increase levels of HIFs (16, 19, 49). The viral G protein-coupled receptor (vGPCR) encoded by *ORF74* is a KSHV lytic gene shown to upregulate vascular endothelial growth factor (VEGF) production by stimulating the activity of HIF-1$\alpha$ (18). Thus, KSHV infection increases levels of HIFs that appear to be involved in both lytic and latent life cycle stages. Previously, we have shown that the gene in the KSHV open reading frame (ORF) 34 is regulated by hypoxia acting through a functional hypoxia response element (HRE) (13). Furthermore, we also demonstrated that this HRE could be used to regulate the expression of the downstream genes (*ORF35-37*) under hypoxic conditions (14). Recently, we showed that the ORF34 protein physically interacts and facilitates HIF-1$\alpha$ degradation (50). Herein, we show that the ORF34 protein is involved in the regulation of KSHV lytic gene expression, since deletion of *ORF34* resulted in reduced immediate early and early lytic gene expression and blocked late gene expression. In addition, we show that the ORF34 protein interacts with HIF-2$\alpha$. Specifically, ORF34 promotes HIF-2$\alpha$ stability and transcriptional activity by enhancing HIF-2$\alpha$ ubiquitination. Thus, the KSHV ORF34 protein is involved in the KSHV lytic replication by differentially regulating the expression of HIF-1$\alpha$ and HIF-2$\alpha$ proteins.

## RESULTS

**Deletion of the *ORF34* gene results in decreased lytic gene expression.** To investigate the roles of ORF34 during lytic reactivation of KSHV, we constructed a bacterial artificial chromosome 16 (BAC16) mutant plasmid that fails to express ORF34 by introducing a premature stop codon at the N terminus of the *ORF34* coding region.

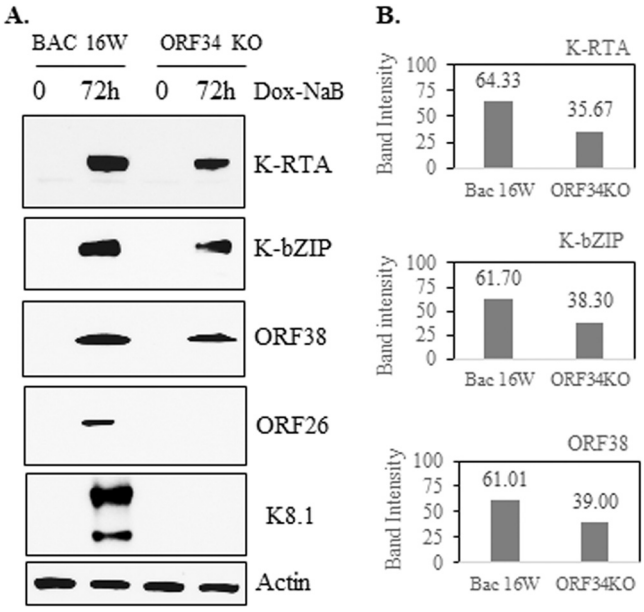


**FIG 1** Effects of the ORF34 knockout on lytic replication of KSHV. (A) iSLK cells harboring BAC16 wild-type or ORF34 KO viruses were induced with doxycycline and sodium butyrate. Cell lysates were harvested at the indicated times postinduction and analyzed by Western blotting with the indicated antibodies to check the expression of these proteins. (B) ImageJ analysis software was used to quantitate and compare the band intensities of proteins expressed in cells harboring BAC16 wild-type and ORF34 knockout virus.

Stable iSLK cells carrying wild-type BAC16 and ORF34 knockout (KO) recombinant viruses were generated. We treated these cells with a combination of doxycycline and sodium butyrate to induce lytic replication of this virus. Whole-cell lysates were prepared at 72 h postinduction, and we checked the KSHV viral protein expression by Western blotting with antibodies against RTA, K-bZIP, ORF26, and K8.1. As shown in Fig. 1, lytic induction of wild-type BAC16 cells resulted in accumulation of viral lytic proteins, including the immediate early protein RTA, the early proteins K-bZIP and ORF38, and the late proteins ORF26 and K8.1. In contrast, cells infected with ORF34 null virus resulted in low expression levels of RTA, K-bZIP, and ORF38 proteins and completely blocked the viral late lytic proteins ORF26 and K8.1. These results indicated that in the absence of ORF34, KSHV can undergo lytic replication normally but produced much lower levels of immediate early protein (RTA) and early proteins K-bZIP and ORF38, and completely inhibited the expression of late proteins (ORF26 and K8.1).

**ORF34 binds to HIF-2α.** We previously showed that KSHV ORF34 protein binds to HIF-1α (50). The HIF-2α protein has a 48% amino acid sequence identity to HIF-1α and possesses similar structure and location of functional domains, but differs in its target gene repertoire (23–25, 51). We tested whether the HIF-2α protein can also interact with the ORF34 protein. Coimmunoprecipitation was utilized to test for ORF34 interaction with HIF-2α *in vivo*. HEK293 cells were cotransfected with the full-length green fluorescent protein (GFP)-tagged ORF34, the 3×Flag wild-type (W), or a mutant form of HIF-2α (mut) containing a specific proline mutation at the ODD domain of HIF-2α (Fig. 2A) that results in escape of pVHL-mediated degradation of HIF-2α while the mutant HIF-2α protein remains functional under normoxic conditions (30, 32, 33). At 30 h posttransfection, cell lysates were first immunoprecipitated with GFP trap beads (Bulldog Bio, Portsmouth, NH) to precipitate GFP fusion protein and then immunoblotted with an anti-Flag monoclonal antibody to detect the presence of HIF-2α bound to GFP34. The anti-GFP trap coimmunoprecipitated flag-tagged HIF-2α from cell lysates cotransfected with wild-type as well as mutant HIF-2α plasmid but not from the cell lysates transfected with the control vector (Fig. 2B). Reciprocally, anti-Flag gel beads coimmunoprecipitated GFP34 only in the presence of either the wild-type or mutant

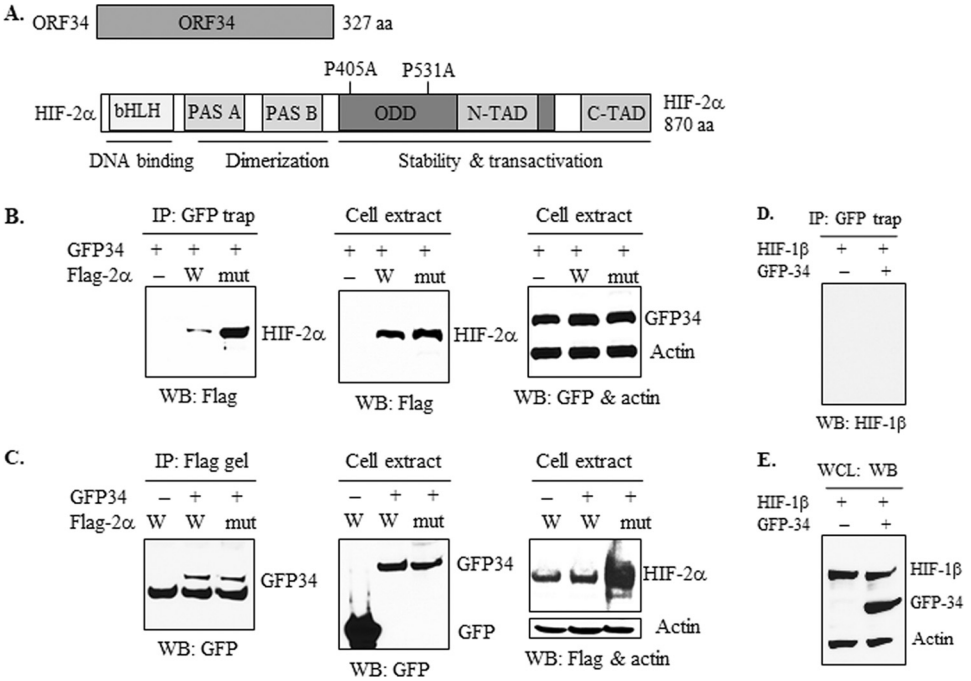


**FIG 2** Interactions of ORF34 and HIF-2$\alpha$ in transfected cells. (A) Schematic diagram of the ORF34 and HIF-2$\alpha$ proteins. HIF-2$\alpha$ encodes an 870-amino-acid protein and has bHLH and PAS domains at the N terminus, which mediate DNA binding and protein-protein interactions, an ODD domain at the central region, which regulates the oxygen-dependent stability of HIF-2$\alpha$ protein through the hydroxylation of two specific proline residues as indicated, and two transactivation domains at the C terminus (N-TAD and C-TAD) that are involved in the *trans*-regulatory functions of HIF-2$\alpha$ protein. (B and C) ORF34 and HIF-2$\alpha$ proteins interact in transient-transfection assays. HEK293 cells were transiently cotransfected with GFP-tagged ORF34 and Flag-tagged wild-type or degradation-resistant HIF-2$\alpha$ as indicated. Thirty hours posttransfection, whole-cell extracts were prepared and proteins were immunoprecipitated (IP) using either anti-GFP trap agarose (B) or anti-Flag gel (C). The immunoprecipitates were analyzed by Western blotting with anti-Flag or anti-GFP antibody. The expression of GFP34 and Flag-HIF-2$\alpha$ in the cell extracts was analyzed by immunoblotting with specific antibodies. (D) ORF34 and HIF-1$\beta$ does not interact in cells. HEK293 cells were transiently cotransfected with HIF-1$\beta$ in the absence or presence of GFP-tagged ORF34, as indicated. Thirty hours posttransfection, whole-cell extracts were prepared and proteins were immunoprecipitated using anti-GFP trap agarose. The immunoprecipitates were analyzed by Western blotting with HIF-1$\beta$ antibody. (E) The expression of each protein in the cell extracts was analyzed by immunoblotting with specific antibodies.

form of HIF-2$\alpha$ protein but not from control cell lysates (Fig. 2C). The presence of GFP34 and Flag-HIF-2$\alpha$ in the cell extracts was detected with specific antibodies, and actin was used to monitor equal loading (Fig. 2B and C). As a negative control, ORF34 did not bind to HIF-1$\beta$, which binds HIF-$\alpha$ proteins (Fig. 2D and E). These results showed that ORF34 interacted with HIF-2$\alpha$ in transfected cells.

**ORF34 protein binds HIF-2$\alpha$ in KSHV-infected cells.** In the absence of available antibody against ORF34, we utilized the BAC16 plasmid to introduce a 2×Myc-tagged epitope tag at the N terminus of the ORF34 gene to facilitate detection in infected cells. We established stable iSLK cells expressing 2×Myc-tagged ORF34 and utilized anti-Myc antibody and anti-Myc trap agarose to detect ORF34 expression in infected cells (Fig. 3B and D). Two experiments were conducted to confirm the interaction of ORF34 and HIF-2$\alpha$ in KSHV-infected cells. First, iSLK cells expressing 2×Myc-tagged ORF34 were transiently transfected with a degradation-resistant Flag-tagged HIF-2$\alpha$ plasmid to stabilize HIF-2$\alpha$ protein or treated with $CoCl_2$ to block degradation of HIF-2$\alpha$. Subsequently, lytic replication was induced with doxycycline and sodium butyrate as described in Materials and Methods. Cell lysates were prepared at specific times postinduction, immunoprecipitated with anti-Myc antibody (GenScript, Piscataway, NJ) to precipitate the Myc-tagged ORF34 protein, and immunoblotted with either anti-Flag antibody (Fig. 3A) or anti-HIF-2 antibody (Fig. 3C) to detect the HIF-2$\alpha$ bound to the ORF34 protein. As expected, the anti-Myc antibody coimmunoprecipitated Flag-HIF-2$\alpha$ or HIF-2$\alpha$ from transfected or $CoCl_2$-treated cells but not from the control cells (Fig. 3A

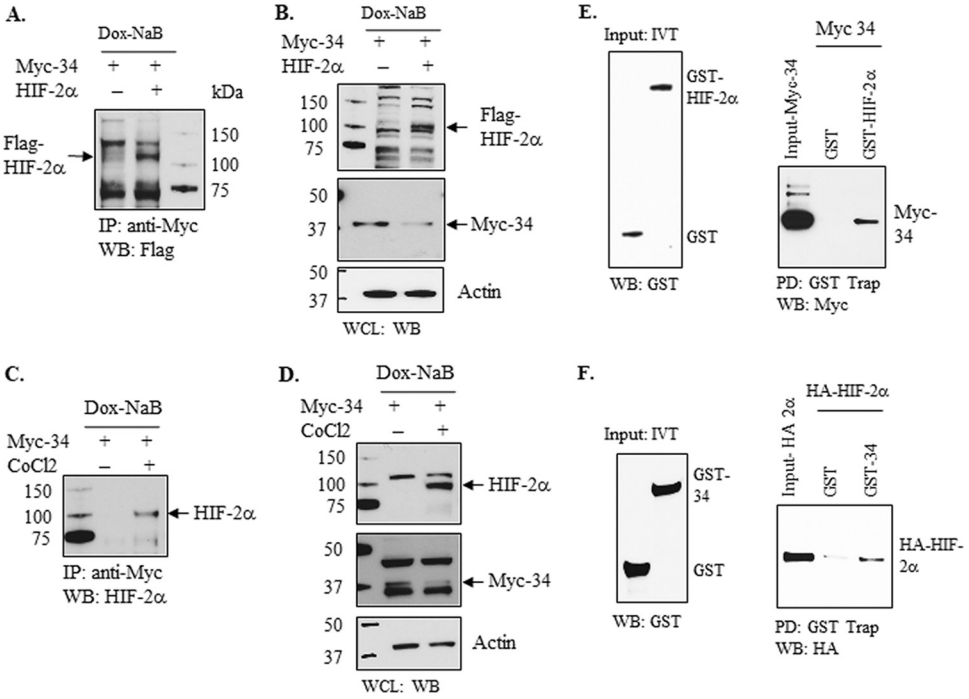


**FIG 3** Interactions of ORF34 and HIF-2α in KSHV-infected cells. (A) iSLK cells expressing Myc-tagged ORF34 were transiently transfected with Flag-tagged degradation-resistant HIF-2α for 17 h. After that, viral lytic replication was induced with doxycycline and sodium butyrate for 65 h. Whole-cell extracts were prepared and proteins were immunoprecipitated (IP) using anti-Myc antibody. The immunoprecipitates were analyzed by Western blotting with anti-Flag antibody. (B) The expression of Flag-HIF-2α in the cell extracts was detected by anti-Flag antibody; Myc trap agarose was used to pulldown Myc-34 protein and was detected by anti-Myc antibody. Specific interaction and detections of each protein are indicated by the arrows. (C) Lytic replication was induced in iSLK cells expressing Myc-tagged ORF34 by doxycycline and sodium butyrate for 60 h. After that, one plate was treated with $CoCl_2$ for 24 h to express HIF-2α protein. Whole-cell extracts were prepared and proteins were immunoprecipitated (IP) using anti-Myc antibody. The immunoprecipitates were analyzed by Western blotting with anti-HIF-2α antibody. (D) The expression of HIF-2α and Myc-34 in the cell extracts was analyzed by immunoblotting with specific antibodies. Specific interaction and detections of each protein are indicated by the arrows. ORF34 and HIF-2α proteins interact in a GST pulldown assay. Equal amounts of *in vitro*-translated GST and GST-HIF-2α (E) or GST-34 (F) protein immobilized to GST trap agarose and incubated either with Myc-tagged ORF34 or HA-tagged HIF-2α and analyzed by Western blotting with anti-Myc or anti-HA antibody. The relative purities and expression levels of *in vitro*-synthesized GST, GST-HIF-2α, and GST-34 proteins used in the binding experiment, as evaluated by Western blotting, are shown at the left side. IVT, *in vitro* translation.

and C). The presence of HIF-2α and Myc-34 in the cell extracts were detected by immunoblotting with specific antibodies (Fig. 3B and D). Since the expression of Myc-34 was low in iSLK cells and the anti-Myc antibody detected nonspecific binding near the Myc-34-specific band, we used anti-Myc trap agarose to pull down Myc-34 protein (Fig. 3B). These results confirm the interaction of ORF34 and HIF-2α in KSHV-infected cells.

Next, to confirm the observed HIF-2α and ORF34 interaction in a cell-free system, both proteins were expressed by *in vitro* transcription-translation, and two-way pulldown experiments were performed as described in Materials and Methods. The Myc-34 protein interacted specifically with glutathione transferase (GST)-HIF-2α but not GST alone (Fig. 3E). In the reverse experiment, hemagglutinin (HA)-HIF-2α specifically interacted with GST-34 but not GST alone (Fig. 3F). The relative expression of GST, GST-HIF-2α, GST-ORF34, Myc-34, and HA-HIF-2α proteins used in these binding assays were ascertained by immunoblotting of 10% of the input samples using specific antibodies (Fig. 3E and F). These results demonstrate that KSHV ORF34 protein interacts with HIF-2α *in vitro* and is a novel binding partner of the HIF-2α protein.

**Delineation of ORF34 and HIF-2α interacting domains.** To delineate interacting domains ORF34 and HIF-2α, several truncated versions of both ORF34 and HIF-2α proteins were generated, and we tested their interaction by coimmunoprecipitation and *in vitro* pulldown assays. First, to locate the interacting domains of ORF34 protein

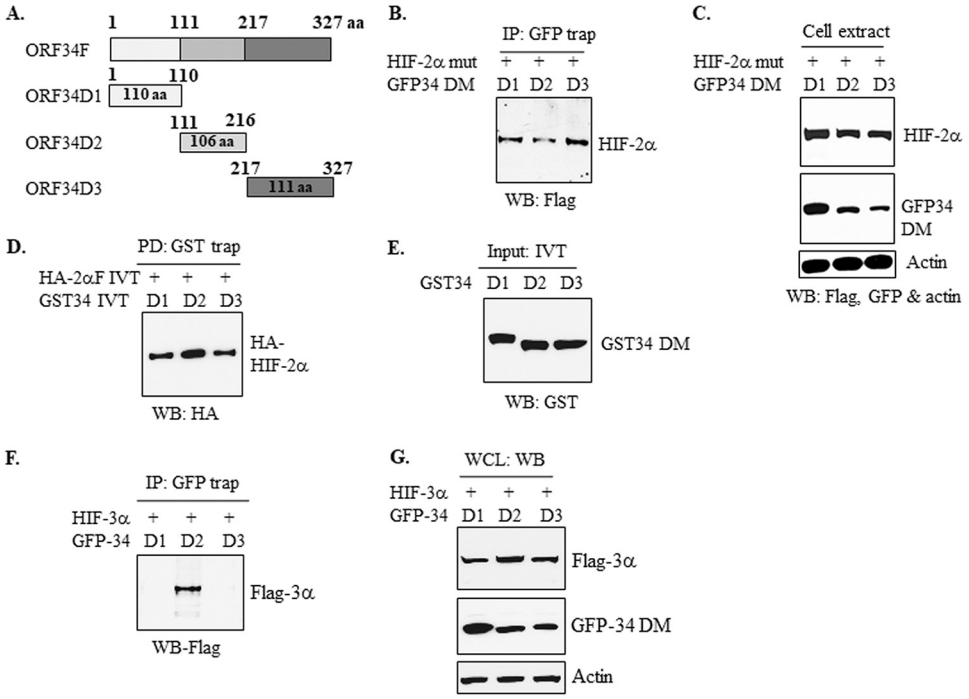


**FIG 4** Mapping of ORF34 interacting domains with HIF-2$\alpha$. (A) Schematic diagram showing the different deletion mutants of ORF34 used in binding assays. (B) Identification of ORF34 binding domains to HIF-2$\alpha$. HEK293 cells were cotransfected with expression plasmids encoding different deletion mutants of GFP-tagged ORF34 and Flag-tagged degradation-resistant HIF-2$\alpha$. Proteins from cell lysates were immunoprecipitated with anti-GFP trap beads, and Western blotting was performed with anti-Flag antibody. (C) Cell extracts were used to detect HIF-2$\alpha$ and deletion mutants of ORF34 proteins using specific antibodies. (D) *In vitro* binding assay showing the regions of ORF34 that bind to HIF-2$\alpha$. *In vitro*-translated deletion mutants of GST34 protein immobilized to GST trap agarose, incubated with *in vitro*-translated HA-tagged HIF-2$\alpha$, and subjected to Western blotting with anti-HA antibody. (E) Expression levels of *in vitro*-synthesized deletion mutants of GST34 proteins used in the binding experiment detected by Western blotting with anti-GST antibody. IVT, *in vitro* translation. (F) Identification of ORF34 binding domains for HIF-3$\alpha$. HEK293 cells were cotransfected with expression plasmids encoding different deletion mutants of GFP-tagged ORF34 and Flag-tagged HIF-3$\alpha$. Thirty hours posttransfection, proteins from whole-cell extracts were immunoprecipitated with anti-GFP trap agarose, and Western blotting was performed with anti-Flag antibody. (G). Whole-cell lysates were used to detect HIF-3$\alpha$ and deletion mutants of ORF34 proteins using specific antibodies.

in HIF-2$\alpha$, the three GFP-tagged truncation mutants of ORF34, including GFP34 D1, GFP34 D2, and GFP34 D3 (Fig. 4A), were coexpressed with full-length 3$\times$Flag-tagged HIF-2$\alpha$ mut plasmid (Fig. 4B) in HEK293 cells. At 30 h posttransfection, cell extracts were immunoprecipitated with GFP trap beads, and the immunoprecipitates were probed with anti-Flag antibody to detect the regions of ORF34 bound to HIF-2$\alpha$. Results showed that the C terminus of ORF34 (GFP34 D3), which encompasses amino acids 217 to 327 of ORF34, bound strongly to HIF-2$\alpha$, whereas GFP34 D1 and D2 bound to a lesser extent to HIF-2$\alpha$ (Fig. 4B).

*In vitro* pulldown experiments using full-length HA-tagged HIF-2$\alpha$ and GST-tagged truncation mutants of ORF34 (GST34 D1, D2, and D3) showed that GST34 D2 bound substantially to HIF-2$\alpha$ compared to GST34 D1 and D3 (Fig. 4D). The expression level of Flag-HIF-2$\alpha$ truncation mutants of GFP34, as well as *in vitro*-synthesized truncation mutants of GST34, was detected with specific antibodies (Fig. 4C and D). To exclude the possibility that ORF34 did not merely bind to any protein, we performed additional coimmunoprecipitation experiments showing that only ORF34D2 interacted with the HIF-3$\alpha$ protein, the other members of the HIF family (Fig. 4F and G). These results showed that all three domains of ORF34 interacted with HIF-2$\alpha$.

Next, to identify the domains of HIF-2$\alpha$ involved in binding to ORF34, three different truncation mutants of HIF-2$\alpha$ were constructed using the GFP-tagged vector (Fig. 5A). These truncation mutants were coexpressed with full-length 3$\times$Flag-tagged ORF34 in HEK293 cells. At 30 h posttransfection, cell extracts were immunoprecipitated with GFP

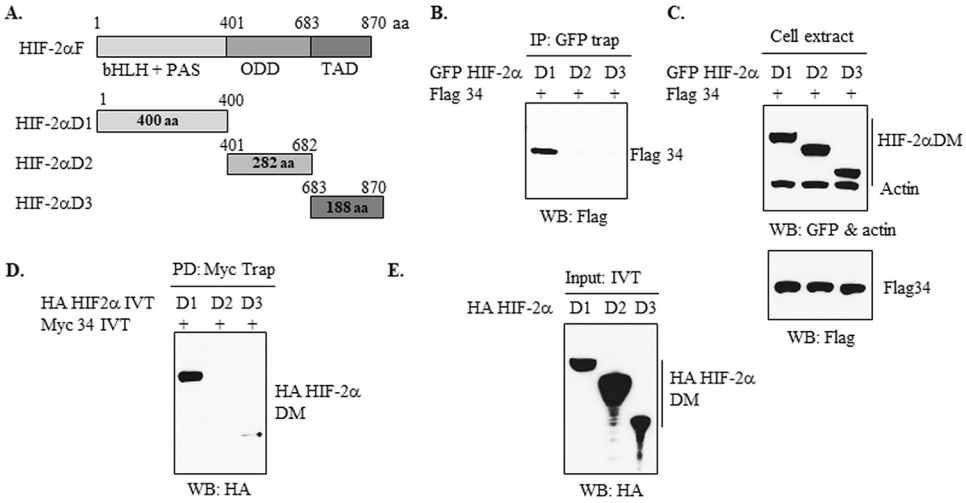


**FIG 5** Mapping of HIF-2α interacting domains with ORF34. (A) Schematic diagram showing the different deletion mutants of HIF-2α used in binding assays. (B) Map of HIF-2α domains that bind ORF34. HEK293 cells were cotransfected with expression plasmids encoding different deletion mutants of GFP-tagged HIF-2α and Flag-tagged ORF34. Proteins from cell lysates were immunoprecipitated with anti-GFP trap beads, and Western blotting was performed with anti-flag antibody. (C) Cell extracts were used to detect ORF34 and deletion mutants of HIF-2α proteins using specific antibodies. (D) *In vitro* binding assay showing the regions of HIF-2α that bind ORF34. *In vitro*-translated Myc-tagged ORF34 protein immobilized to Myc trap agarose was incubated with *in vitro*-translated deletion mutants of HA-tagged HIF-2α and detected by Western blotting with anti-HA antibody. (E) Expression levels of *in vitro*-synthesized deletion mutants of HIF-2α proteins used in the binding experiment detected by Western blotting with anti-HA antibody. IVT, *in vitro* translation.

trap beads (Fig. 5B) followed by immunoprecipitation with anti-Flag antibody to detect the HIF-2α domains bound to ORF34. Results revealed that only the GFP HIF-2α D1, which is harbored by the bHLH and PAS domains of HIF-2α (HIF-2αD1) bound to the ORF34 protein (Fig. 5B). *In vitro* pulldown experiments using the full-length Myc-tagged ORF34 and the three HA-tagged HIF-2α truncation mutants (HA HIF-2α D1, D2, and D3) revealed that in addition to bHLH and PAS domains, the C-terminal TAD also interacted weakly with the ORF34 protein (Fig. 5D). In these experiments, the truncation mutants of GFP-tagged HIF-2α, the Flag-tagged ORF34, and the *in vitro*-synthesized truncation mutants of HA-tagged HIF-2α were detected with specific antibodies (Fig. 5C and E). Taken together, these results showed that the bHLH plus PAS domains and TAD of HIF-2α interacted with ORF34, and all three domains of ORF34 interacted with HIF-2α.

**The ORF34 protein stabilizes HIF-2α expression.** Previously, we showed that the ORF34 protein can cause degradation of the HIF-1α protein as well as inhibit the transcriptional activity of a HIF-1α-dependent reporter gene (50). To examine the effect of ORF34 on HIF-2α protein expression, Flag-HIF-2α was coexpressed with increasing amounts of GFP34 in HEK293 cells (Fig. 6A). After 30 h of transfection, the level of HIF-2α protein was evaluated by immunoblotting with anti-Flag antibody. Compared with the cells expressing Flag-HIF-2α alone, HIF-2α protein levels remained practically unaffected in cells in which GFP34 was coexpressed in a dose-dependent manner (Fig. 6A). Next, to test the effects of ORF34 on the transcriptional activity of HIF-2α, we utilized a HIF-2α-specific reporter plasmid, the prototype human erythropoietin luciferase promoter (EPO), where the expression of luciferase is driven by a 50-bp EPO 3′ enhancer sequence inserted upstream of the SV40 promoter (52). HEK293 cells were cotransfected with a fixed amount of pEpoE-luc HRE reporter plasmid and Flag-tagged HIF-2αw plasmids with increasing concentrations of GFP34. Results revealed that cotransfection of HIF-2α in the absence of ORF34 enhanced the EPO HRE reporter activity approximately 5-fold that of the control vector (Fig. 6B). However, when the HIF-2α plasmid was transfected with increasing amounts of ORF34, the transcriptional activity of this reporter by ORF34 remained unaffected.

**ORF34 is involved in the ubiquitination of HIF-2α.** The key regulator of HIF-α protein is pVHL, which is regulated by oxygen-dependent proteasomal degradation of

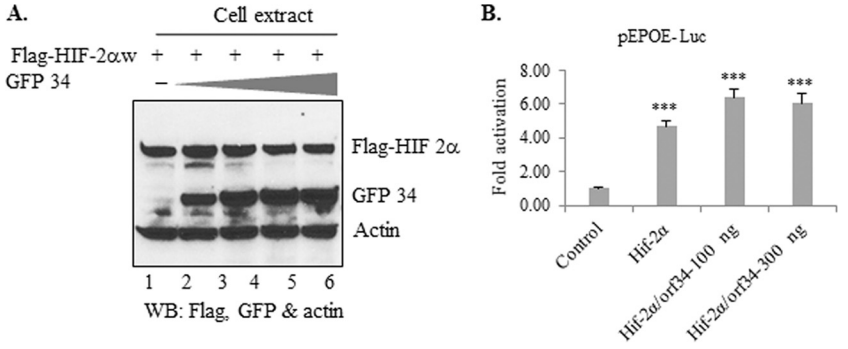


**FIG 6** Effects of ORF34 on HIF-2$\alpha$ protein levels and transcriptional activity. (A) HEK293 cells were cotransfected with a fixed amount of Flag-tagged wild-type HIF-2$\alpha$ plasmid and with increasing amounts of GFP34 plasmid. Whole-cell extracts were prepared at 30 h posttransfection and immunoblotted with antibodies against Flag, GFP, or actin to detect the relative expression levels. (B) HEK293 cells were cotransfected with a fixed amount of pEpoE-luc plasmid along with the wild-type HIF-2$\alpha$-expressing plasmid in the presence of increasing amounts of GFP34 plasmid. After 30 h of normoxic incubation, cells were assayed for luciferase activity and analyzed.

HIF-$\alpha$ in normoxia by binding to specific HIF-$\alpha$ proline residues and promoting ubiquitination (30, 33). Previously, we showed that ORF34 protein decreases the polyubiquitination of HIF-1$\alpha$ protein in an *in vivo* ubiquitination assay (50). To ascertain the potential role of ORF34 protein in HIF-2$\alpha$ ubiquitination, we examined the ubiquitination state of HIF-2$\alpha$ by an *in vivo* ubiquitination assay (Fig. 7A). The HA-ubiquitin (HA-Ub) expression plasmid was coexpressed with the GFP-tagged wild-type HIF-2$\alpha$ in the absence or presence of Flag-tagged ORF34 in HEK293 cells (Fig. 7A). After 30 h of transfection, cell lysates were immunoprecipitated with anti-GFP trap beads, and the ubiquitination status of HIF-2$\alpha$ was detected by immunoblotting with an anti-HA antibody. In the absence of ORF34, a lower level of ubiquitinated HIF-2$\alpha$ was observed. In contrast, the level of ubiquitinated HIF-2$\alpha$ was substantially increased in cells coexpressing the ORF34 protein (Fig. 7A). Immunoblotting of cell extracts with specific antibodies revealed the expression of HIF-2$\alpha$, ORF34, and ubiquitin proteins in cell extracts (Fig. 7B and C). These results demonstrated that ORF34 can enhance the ubiquitination of HIF-2$\alpha$ through direct protein-protein interaction.

**Delineation of HIF-2$\alpha$ domains ubiquitinated by ORF34.** We have shown that the ORF34 protein enhanced the ubiquitination of HIF-2$\alpha$. To map the HIF-2$\alpha$ domains

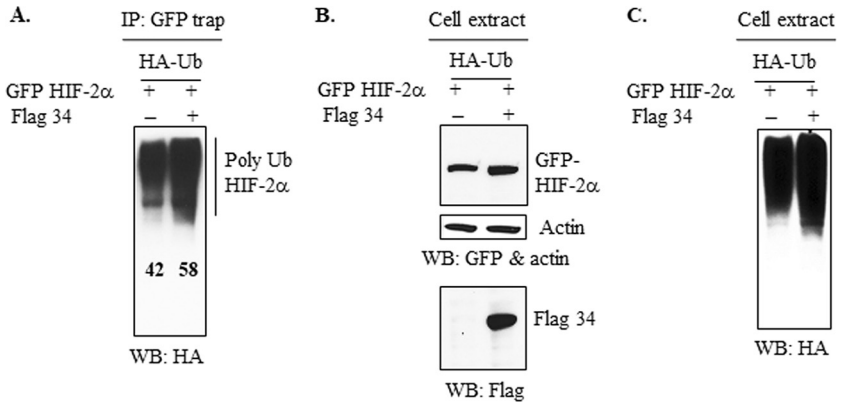


**FIG 7** ORF34 promotes ubiquitination of HIF-2$\alpha$. (A) HEK293 cells were cotransfected with HA-tagged ubiquitin and GFP-tagged HIF-2$\alpha$ plasmids in the presence or absence of Flag-tagged ORF34 plasmid. Thirty hours posttransfection, proteins from cell lysates were immunoprecipitated with anti-GFP trap agarose, and Western blotting was performed with anti-HA antibody. ImageJ analysis was used to quantitate band intensities, and the numbers representing the ubiquitinated area in each lane are shown. (B and C) Expression of HIF-2$\alpha$, ORF34, and ubiquitin proteins in cell extracts was detected using specific antibodies.

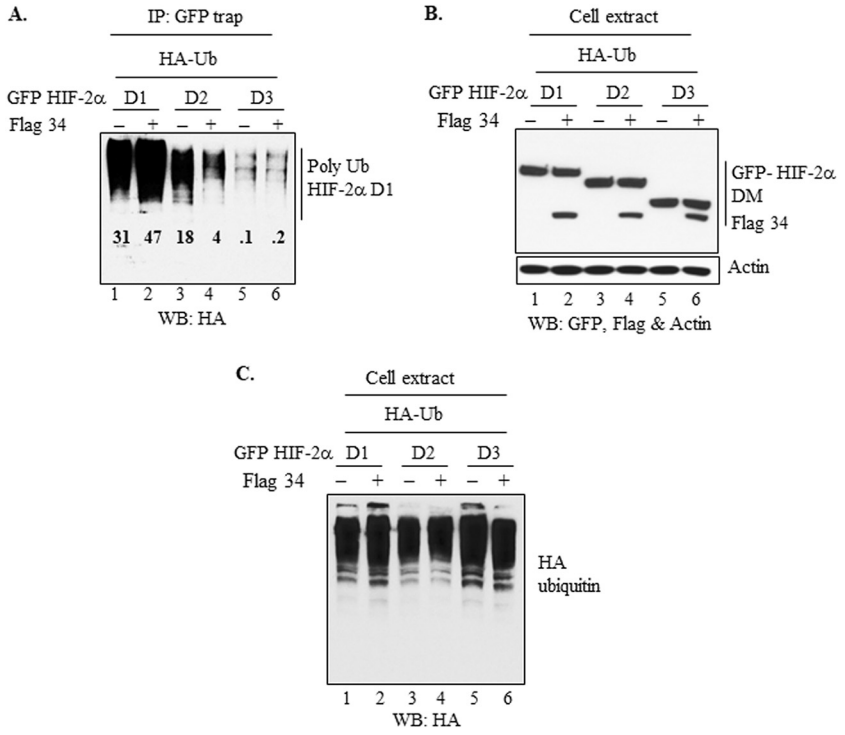


**FIG 8** Mapping of the ubiquitinated HIF-2α domains. (A) HEK293 cells were cotransfected with HA-tagged ubiquitin and GFP-tagged deletion mutants of HIF-2α in the presence or absence of Flag-tagged ORF34. Thirty hours posttransfection, proteins from cell lysates were immunoprecipitated with anti-GFP trap agarose, and Western blotting was performed with anti-HA antibody. ImageJ analysis was used to quantitate band intensities, and the numbers representing the ubiquitinated area in each lane are shown. (B and C) Expression of deletion mutants of HIF-2α, ORF34, and ubiquitin proteins in cell extracts was detected using specific antibodies.

associated with this ubiquitination process, we constructed different truncation mutants of HIF-2α and tested them in an *in vivo* ubiquitination assay (Fig. 8A). HEK293 cells were cotransfected with HA-ubiquitin expression plasmid and different GFP-tagged truncation HIF-2α mutants in the absence or presence of Flag-tagged ORF34 (Fig. 8A). After 30 h of transfection, cell lysates were immunoprecipitated with anti-GFP trap beads followed by immunoblotting using an anti-HA antibody to delineate the regions of HIF-2α involved in the ubiquitination process. In the absence of ORF34, a lower level of ubiquitinated HIF-2α was observed in cells cotransfected with GFP HIF-2α D1. In contrast, the level of ubiquitinated HIF-2α was substantially increased in cells cotransfected in the presence of ORF34 (Fig. 8A). The levels of ubiquitinated HIF-2α practically remained unaffected regardless of whether ORF34 was present or absent in cells cotransfected with plasmids that express GFP HIF-2α D2 or -D3 (Fig. 8A). Expression of different truncation mutants of GFP HIF-2α, ORF34, and ubiquitin proteins in cell extracts were detected using specific antibodies (Fig. 8B and C). These results clearly showed that ubiquitinated sites of HIF-2α are located within the bHLH and PAS domains.

## DISCUSSION

The KSHV ORF34 is activated by hypoxia through a functional HRE element located within the *ORF34* promoter region, which can also activate transcription of the downstream *ORF35-37* genes (14). Transcriptional activity of this promoter is strongly activated by both HIF-1α and HIF-2α proteins (13). Recently, we showed that ORF34 protein binds to and ubiquitinates HIF-1α, targeting it for proteasomal degradation. Moreover, expression of ORF34 lowered HIF-1α protein levels and decreased HIF-1α transcriptional activity (50). Herein, we show that the *ORF34* gene is involved in the regulation of KSHV lytic gene expression, since deletion of *ORF34* resulted in reduced immediate

early and early lytic gene expression and blocked late gene expression. Also, we show that ORF34 also binds HIF-2$\alpha$, and unlike its effect on HIF-1$\alpha$, it causes HIF-2$\alpha$ stabilization and HIF-2$\alpha$-dependent transcription. These results suggest that the ORF34 protein modulates the KSHV virus life cycle via regulation of the HIF-1$\alpha$ and HIF-2$\alpha$ proteins.

Recently, ORF34 has been shown to form a complex with other late genes and activate the transcription of late viral genes and their promoters (53–55). Our initial findings that the ORF34 protein interacted with HIF-1$\alpha$ motivated us to investigate the potential role of the ORF34 protein in the virus life cycle by generating an ORF34-null virus. Investigation of the relative gene expression of immediate early (RTA), early (K-bZIP), ORF38, and late (ORF26), K8.1 genes revealed that both immediate early and early gene expression levels were reduced in comparison to those from the wild-type virus in iSLK infected cells, while ORF26 and K8.1 expression was completely inhibited (Fig. 1). This results strongly suggest that the ORF34 protein plays a regulatory role in lytic gene expression and further suggest that downregulation of ORF34 gene expression may favor KSHV latency.

Herein, we show that the ORF34 protein interacted with HIF-2$\alpha$ in KSHV-infected cells (Fig. 3A and C) and that this interaction requires the HIF-2$\alpha$ N-terminal bHLH plus PAS domains and at least the C-terminal TAD domain (Fig. 5B and D). All three domains of ORF34 representing the amino, central, and carboxyl termini interact with HIF-2$\alpha$ (Fig. 4B and D) in a manner similar to ORF34 interactions with HIF-1$\alpha$ (50). HIF-1$\alpha$ and HIF-2$\alpha$ share 48% overall amino acid sequence similarity, 85% identity in their basic helix-loop-helix domains, and 70% homology between their PAS regions. Therefore, it is not surprising that these homologous domains both bind the ORF34 protein, although the relative strengths of binding may be different due to amino acid differences between HIF-1$\alpha$ and HIF-2$\alpha$ interacting domains.

HIF-2$\alpha$ is relatively stable in normoxia, as detected by immunoprecipitation and Western blot experiments (Fig. 2B and C). In normoxia, the stability and activity of HIF-$\alpha$ proteins are regulated by four HIF-specific prolyl hydroxylases (PHD1 to -4) (33, 35, 36, 38). HIF-2$\alpha$ is hydroxylated more actively than HIF-1$\alpha$ by PHD3, while HIF-1$\alpha$ is hydroxylated at a much lower efficiency than HIF-2$\alpha$ by PHD1 and -2 (56, 57), which may result in the stabilization and activation of HIF-2$\alpha$ at higher oxygen tensions than HIF-1$\alpha$. Hypoxia and at least HIF-1$\alpha$ play important roles in the KSHV life cycle as indicated by the fact that HIF-1$\alpha$ has been shown to physically interact with LANA1, LANA2, and ORF34 (15, 17, 19, 50). Specifically, the KSHV latency-associated nuclear antigen 1 (LANA1) binds and stabilizes HIF-1$\alpha$, causing enhanced RTA expression in PEL cell lines expressing LANA1. Moreover, LANA1 can bind to HIF-1$\alpha$ ODD domain, resulting in HIF-1$\alpha$ stable activity in normoxia (49). The KSHV viral interferon (IFN) regulatory factor 3 (vIRF3), known as latency-associated nuclear antigen 2 (LANA2), binds to the bHLH domain of HIF-1$\alpha$ and enhances the transcriptional activity of the HIF-1$\alpha$ protein (17). Furthermore, expression of vIRF3 led to an increased level of vascular endothelial growth factor (VEGF) expression and facilitated endothelial tube formation (17). The *ORF74*-encoded lytic protein, also known as G-protein coupled receptor (vGPCR), was shown to upregulate the expression of VEGF by stimulating the activity of HIF-1$\alpha$, resulting in the activation of p38 mitogen-activated protein kinase (18) and indicating that there may be regulatory interactions between HIFs and ORF74.

Of particular interest is the fact that ORF34 interacts with both HIF-1$\alpha$ and HIF-2$\alpha$ proteins, but these interactions lead to proteasome-dependent degradation and stabilization, respectively, indicating that ORF34 plays a unique regulatory role in HIF-dependent regulation for the KSHV life cycle. Recently, the hypoxia-associated factor (HAF), which is an E3 ubiquitin ligase, was shown to differentially regulate HIF-1$\alpha$ and HIF-2$\alpha$ proteins. Specifically, HAF has been shown to degrade HIF-1$\alpha$ and target it for proteasomal degradation, while in contrast, HIF-2$\alpha$ levels were stabilized, resulting in increased HIF-2$\alpha$-dependent transcriptional activity (58, 59). Examination of the stability of HIF-2$\alpha$ protein in the presence of ORF34 revealed that unlike HIF-1$\alpha$, the stability of HIF-2$\alpha$ protein was not affected even with increasing concentrations of ORF34 protein. In agreement with the observed HIF-2$\alpha$ stabilization by ORF34, HIF-2$\alpha$-dependent

transcriptional activity was enhanced. Regulation of the HIF-α protein stability is critical in maintaining the function of HIF-α. Like HIF-1α, HIF-2α also undergoes ubiquitin modification by binding to the von Hippel-Lindau (pVHL) protein complex followed by proteasomal degradation, resulting in loss of activity (33, 60–63). The hydroxylation of specific proline residues of HIF-2α resulting in alanine changes (P405A and P531A) is critical to impair ubiquitination, resulting in stable HIF-2α protein levels (27).

We show here that the presence of ORF34 resulted in a higher level of HIF-2α ubiquitination. This result demonstrates that ORF34 can enhance the ubiquitination of HIF-2α through direct protein-protein interaction (Fig. 7A). Furthermore, we showed that the bHLH and PAS domains of HIF-2α are ubiquitinated by ORF34 (Fig. 8A). Additional studies are necessary to identify the specific amino acids associated with this ubiquitinated process and to elucidate the mechanism by which KSHV ORF34 differentially regulates HIF-α protein expression.

The main cellular response to hypoxia is mediated by HIF-1α and HIF-2α. A variety of published reports indicate that HIF-1α and HIF-2α have significantly different roles in regulating the cellular environment in response to hypoxia. For example, HIF-2α but not HIF-1α has been linked to higher levels of neuronal differentiation in glioblastomas and better prognosis, indicating that it may play a tumor-suppressive role (64). In endothelial cells, both HIF-1α and HIF-2α mediate the cellular transcriptional response to hypoxia and regulate multiple processes that are required for angiogenesis for the restoration of perfusion and oxygen supply. Extensive transcriptional analysis in endothelial cells revealed that HIF-1α and HIF-2α regulated 701 and 1,454 genes, respectively. HIF-1α transcription involved primarily metabolic reprogramming, whereas HIF-2α appeared to regulate angiogenic signaling and extracellular matrix remodeling (65). In another study, vGPCR was found to be a novel target of HIF-1α and one of the main viral angiogenic factors regulating the metabolic reprogramming of KSHV (21). Finally, studies in primary effusion lymphoma (PEL) cells, revealed that knockdown of HIF-1α in PEL lines led to reduction in both aerobic and anaerobic glycolysis as well as lipid biogenesis, suggesting that it is necessary for maintaining an optimal metabolic state for PEL growth (20). Interestingly, HIF-1α suppression led to a reduction in the activation of lytic KSHV genes and a decrease in the expression of KSHV latent genes, including those encoding LANA, vCycline, and kaposin, under both hypoxic and normoxic conditions (20).

We show here for the first time that KSHV ORF34 binds and stabilizes HIF-2α, preserving HIF-2α-dependent cellular transcription. In contrast, we have shown that ORF34 binds HIF-1α and causes its proteasome-dependent degradation. This differential action of ORF34 suggests that it plays a critical role in regulating HIF-1α- and HIF-2α-dependent cellular processes required for successful KSHV infection and tumorigenesis. It appears that HIF-1α-dependent transcription promotes a lytic replication state, while HIF-2α may act to dampen HIF-1α effects by competing for ORF34 binding, thus promoting a latent KSHV state. Additional experimentation is needed to ascertain the roles of HIF-1α and HIF-2α in conjunction with ORF34 in the KSHV life cycle.

## MATERIALS AND METHODS

**Cell culture and chemicals.** Human embryonic kidney 293 (HEK293), iSLK, and iSLK BAC16 cells (kindly provided by Jae U. Jung) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics. iSLK cells were generated from uninfected endothelial cells as described previously (66) and maintained in the presence of 1 μg/ml puromycin and 250 μg/ml G418. iSLK cells expressing wild-type or mutant BAC16 were cultured in the presence of 1 μg/ml puromycin, 250 μg/ml G418, and 600 μg/ml of hygromycin B. All cells were maintained at 37°C in a 5% $CO_2$ and 95% air incubator. Sodium butyrate (NaB), doxycycline (Dox), and $CoCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO). Puromycin, hygromycin, and G418 were from Thermo Fisher Scientific, Illinois.

**Plasmid constructs.** Construction of full-length GFP34 and its different truncated mutants was described previously (50). For Flag-tagged expression, the *ORF34* gene was amplified from the GFP34 plasmid with specific primers and cloned into the BamHI-XhoI sites of the 3×Flag-tagged expression vector, pCMV-3Tag6 (Agilent Technologies, Santa Clara, CA). The mammalian expression plasmid encoding the full-length wild-type human HIF-2α hEPAS1-pcDNA3 (25) was used as a PCR template to generate

full-length wild-type and different truncated mutants of HIF-2$\alpha$, which were subsequently cloned into BamHI-XhoI sites of the 3×Flag-tagged expression vector pCMV-3Tag6. A mutant form of the HIF-2$\alpha$ plasmid containing two alanines in place of prolines at positions P405A and P531A, which are known to escape pVHL-mediated degradation, was generated using the Quick Change site-directed mutagenesis kit (Stratagene, San Diego, CA) with specific primers according to the manufacturer's protocol, as described elsewhere (14). To generate a green fluorescent protein (GFP)-tagged HIF-2$\alpha$ protein, full-length and different truncated versions of HIF-2$\alpha$ were cloned into the BglII-SalI sites of the pEGFP-C1 vector (Clontech, Mountain View, CA). The construction of the truncated versions of HIF-2$\alpha$ is shown schematically in Fig. 5A.

The mammalian expression plasmid encoding the full-length human HIF-1$\beta$ (ARNT-pcDNA) has been described previously (29). Myc-Flag-tagged HIF-3$\alpha$ plasmid (MR224296) was purchased from OriGene Technologies, Inc., Rockville, MD.

For the *in vitro* binding assays, the full-length ORF34 and HIF-2$\alpha$ and their different truncated mutants were cloned into an appropriate expression vector containing either GST-tagged (88870), HA-tagged (88861), or Myc-tagged (88863) vectors (Thermo Fisher Scientific, Illinois). The HA-tagged ubiquitin expression plasmid was described previously (50). The wild-type human erythropoietin (EPO) HRE-luciferase reporter (pEpoE-luc) containing a 50-bp EPO 3′ enhancer sequence, was described previously (52).

**Construction of BAC16 Myc-tagged ORF34 and ORF34 knockout recombinant viruses.** KSHV BAC16 containing a recombinant full-length KSHV genome and iSLK cells were kindly provided by Jae U. Jung (67). BAC16 was used to generate 2×Myc-tagged ORF34 and ORF34 knockout viruses via two-step Red-mediated mutagenesis as described previously (68, 69) using 4 nmol ultra-mer primers synthesized by IDT. The Myc-tagged ORF34 has two Myc epitope tags at the N terminus just after the start codons of the *ORF34* gene. ORF34 knockout virus has a premature stop codon at amino acid position 24 of the ORF34 coding region. The following primers were used to generate recombinant viruses.

ORF34-2xMycF: 5′-agg ttg gcg gca agg cgc tcc ctg tga cgg ctg agc agc **atg** GAG CAG AAA CTC ATC TCT GAA GAA GAT CTG GAA CAA AAG TTG ATT TCT GAA GAA GAT CTG *agg atg acg acg ata agt agg g*-3′ (lowercase letters indicate the KSHV genomic sequence, boldface letters indicates *ORF34* start codon, uppercase letters indicate Myc-tag epitope sequence, and the sequence in italics is AphAI specific).

ORF34-2xMycR: 5′-cac ctc cgg gtc acc ctc gga cac gag cga gct caa agc aaa CAG ATC TTC TTC AGA AAT CAA CTT TTG TTC CAG ATC TTC TTC AGA GAT GAG TTT CTG CTC *caa cca att aac caa ttc tga tta g*-3′ (lowercase letters indicate the KSHV genomic sequence, uppercase letters indicate Myc-tag epitope sequence, and the sequence in italics is AphAI specific).

ORF34-24s F: 5′-GAG GGT GAC CCG GAG GTG ACC AGT AGG TAC GTC AAG GGC GTA **T**AA CTT GCC CTG GAC CTT AGC GAG *agg atg acg acg ata agt agg g*-3′ (uppercase letters indicate KSHV genomic sequence, boldface underlined letter indicates changed nucleotide at amino acid position 24 of ORF34 to introduce a stop codon, and the sequence in italics is AphAI specific).

ORF34-24s R: 5′-CTT AAA TTG TCC AGG TGT GTT CTC GCT AAG GTC CAG GGC AAG TT**A** TAC GCC CTT GAC GTA CCT ACT *caa cca att aac caa ttc tga tta g*-3′ (uppercase letters indicate KSHV genomic sequence, boldface underlined letter indicates changed nucleotide at amino acid position 24 of ORF34 to introduce a stop codon, and the sequence in italics is AphAI specific).

The integrity of the engineered insertion or point mutation of the recombinant virus was verified by PCR with specific primers and sequencing for the presence of the engineered mutation.

For transfection and reconstitution of infectious virus, BAC DNAs were isolated from bacteria harboring parental BAC16, Myc-tagged ORF34, and ORF34 knockout viruses by using a NucleoBond Xtra Midi kit (Macherey-Nagel, Germany).

**Generation of iSLK-BAC16 and ORF34 derivatives stable cell lines.** The day before transfection, iSLK cells were seeded in a 12-well plate at 60% to 70% confluence. Then, the cells were cultured in the absence of antibiotics in medium containing 5% fetal bovine serum (FBS) and transfected with 1 $\mu$g of BAC DNA using ViaFect transfection reagent (Promega, Madison, WI) as recommended by the manufacturer. Three days posttransfection, cells were selected in medium containing 2 $\mu$g/ml puromycin, 250 $\mu$g/ml G418, and 600 $\mu$g/ml hygromycin. The culture medium was then refreshed with selection medium every week. After 30 days of selection, hygromycin-resistant colonies were trypsinized and transferred to a 10-cm dish or T-75 flask. To induce lytic viral replication, iSLK cells harboring recombinant KSHV BAC were plated in a 6-well plate or 10-cm dish in the absence of selection marker and the following day induced with 1 $\mu$g/ml doxycycline and 1 mM sodium butyrate for specific times.

**Antibodies and conjugates.** Mouse monoclonal antibodies to Flag (A00187) and anti-cMyc (A00704) were from GenScript (Piscataway, NJ). Rabbit polyclonal antibody against the green fluorescent protein (GFP) (G1544) and mouse monoclonal anti-actin (A5316) were from Sigma (St. Louis, MO). Rabbit polyclonal antibody to GST (sc-459), mouse monoclonal antibody to HA (sc-7392), mouse monoclonal antibody to ARNT1 (sc-55526), mouse monoclonal antibody to KSHV K-bZIP (sc-69797), and mouse monoclonal antibody to KSHV K8.1A/B (sc-65446) were from Santa Cruz Biotechnology (Dallas, TX). Mouse monoclonal antibody to HA (26183) was from Thermo Fisher Scientific, Illinois. HIF-2$\alpha$ rabbit monoclonal antibody (A700-003) was from Bethyl Laboratories, Inc. (Montgomery, TX).

Mouse monoclonal antibody to KSHV ORF26 (2F6B8) was from Novus Biologicals (Centennial, CO). Rabbit polyclonal antibody against KSHV K-RTA was raised in our laboratory. GFP trap, GST trap, and Myc trap agarose were obtained from Bulldog Bio (Portsmouth, NH). Anti-Flag affinity gel was obtained from Bimake (Houston, TX). Western blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (sc-2005) or goat anti-rabbit IgG (sc-2004; Santa Cruz

Biotechnology) secondary antibodies and reacted with Clean-blot IP detection reagent (21230) from Thermo Fisher Scientific, Illinois.

**Transfection and immunoblot analysis.** Subconfluent HEK293 cells in 6-well plates were transfected with 2 $\mu$g each of appropriate plasmids using either the GenJet (SignaGen, Rockville, MD) or ViaFect (Promega, Madison, WI) transfection reagent, as recommended by the manufacturers. An appropriate control plasmid was used to adjust the total amount of DNA as needed. iSLK cells expressing Myc-tagged ORF34 were plated in a 10-cm dish, transfected the following day with 6 $\mu$g of HIF-2$\alpha$ mutant plasmid for 17 h, followed by induction with doxycycline and sodium butyrate for 55 h. Untransfected cells were used as the control. Lytic replication in iSLK cells harboring BAC16 wild-type or ORF34 knockout viruses was induced with doxycycline and sodium butyrate for 72 h. At specific times posttransfection or induction, cells were harvested and lysed in 0.5% IGEPAL lysis buffer (50 mM Tris-HCl [pH 8.00], 150 mM NaCl, 0.5% IGEPAL CA-630, 1 mM EDTA) supplemented with protease inhibitor, incubated on ice for 30 min, and cleared by centrifugation at 14,000 rpm for 10 min. Cleared lysates were mixed with sample buffer, heated at 92°C for 5 min, separated through a 4% to 20% SurePAGE gel (M00656; GenScript, Piscataway, NJ), and transferred to nitrocellulose membrane using the iBlot transfer device. After blocking the nonspecific binding sites in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk, the membranes were probed with the specific primary antibodies and then either with goat anti-mouse or goat anti-rabbit secondary antibody conjugated with HRP (sc-2005 or sc-2004, respectively; Santa Cruz Biotechnology, Dallas, TX), and then reacted with Clean-blot IP detection reagent (for HRP) (21230; Thermo Fisher Scientific, IL). After extensive washing of the membranes with TBS-T, specific immunoreactive protein species were detected either with Luminata Classico or Forte substrate (MilliporeSigma, Burlington, MA) depending on the signal intensity of targeted proteins. For some experiments, band intensities were quantitated using ImageJ software (National Institutes of Health, Bethesda, MD), and the numbers representing the band intensities are shown on specific lanes.

**Coimmunoprecipitation assays.** Transfected or induced cells were collected at specific times posttransfection or induction and used for immunoprecipitation assays. Cell lysates were immunoprecipitated with the addition of either anti-GFP trap agarose (Bulldog Bio, Portsmouth, NH) or anti-Flag gel (Bimake, Houston, TX) according to the manufacturer's instructions. The agarose was washed four times in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and bound proteins were eluted in 30 $\mu$l 2$\times$ sample buffer. For coimmunoprecipitation of ORF34 and HIF-2$\alpha$ in KSHV-infected cells, iSLK cells expressing Myc-tagged ORF34 were induced in 10-cm dishes with doxycycline and sodium butyrate for 55 h, and then one dish was treated with 200 $\mu$M $CoCl_2$ for 20 h. Aliquots of 750 $\mu$g of whole-cell lysates were immunoprecipitated with 5 $\mu$g of anti-Myc antibody overnight at 4°C. Forty microliters of Dynabeads protein G (Thermo Fisher Scientific, Illinois) were added to each tube and incubated at room temperature (RT) for 45 min to precipitate the target protein. Beads were washed four times in TBS-T, and bound proteins were eluted in 40 $\mu$l 2$\times$ sample buffer, separated through a 4% to 20% SurePAGE gel, and transferred to nitrocellulose membranes. After blocking the nonspecific binding sites, the membranes were probed by immunoblotting with specific antibodies, and immunoreactive protein species were detected as described above for immunoblot analysis.

***In vitro* pulldown assays.** For pulldown assays, full-length and different truncated versions of ORF34 and HIF-2$\alpha$ proteins were cloned into the different epitope-tagged *in vitro* translation vectors (PT7CFE1; Thermo Fisher Scientific, Illinois). These epitope-tagged plasmids were *in vitro* synthesized using the one-step human coupled IVT kit (88881; Thermo Fisher Scientific, Illinois), as recommended by the manufacturer. Briefly, 1 $\mu$g of DNA for each of these plasmids was incubated with translation mix at 30°C for 5 to 6 h to synthesize *in vitro* translation products. The pulldown assay was performed as described earlier (69). Briefly, 1 to 2 $\mu$l of appropriate *in vitro*-synthesized translation products were first immobilized to Tag-specific beads (either GST trap or Myc trap) in dilution buffer at room temperature for 30 min. Unbound particles were washed away, and equal amounts of *in vitro*-synthesized binding proteins were added to specific protein immobilized beads and incubated at room temperature for another 20 to 30 min. The beads were washed four times in TBS-T, and bound proteins were eluted in 30 $\mu$l 2$\times$ sample buffer, separated through a 4% to 20% SurePAGE gel, and immunoblotted with specific antibodies.

**Reporter assay.** HEK293 cells were cotransfected at 60% to 70% confluence in a 24-well plate with 150 ng of the pEpoE-luc reporter plasmid along with 150 ng of the flag-tagged HIF-2$\alpha$w plasmid in the absence or presence of increasing amounts of GFP34 expression plasmid. The total amount of DNA was kept constant by using an appropriate control vector. After 30 h under normoxic incubation conditions, cells were lysed using passive lysis buffer. Firefly and *Renilla* luciferase activity was measured with a Victor-2 luminometer (Perkin Elmer, Waltham, MA) using a dual luciferase kit (Promega, Madison, WI) and was normalized to internal control plasmid-obtained values. Luciferase activity was calculated by dividing the activity for each reporter with that of the empty vector and compared to the results obtained with those for HIF-2$\alpha$ alone or HIF-2$\alpha$ and ORF34 together.

**Ubiquitination assay.** HEK293 cells were cotransfected with full-length or deletion mutants of GFP-tagged HIF-2$\alpha$ with or without Flag-tagged ORF34 in the presence of HA-ubiquitin in different combinations as shown in Fig. 7 and 8. After 30 h of transfection, cell lysates were prepared in 0.5% IGEPAL lysis buffer and immunoprecipitated with anti-GFP trap beads. Ubiquitinated HIF-2$\alpha$ was then detected by immunoblotting with an anti-HA antibody.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism 5 software. For comparison of control reporter with HIF-2$\alpha$ along with increasing concentrations of ORF34, data were

analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple-comparison test; a *P* value of less than 0.05 was considered statistically significant.

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