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KSHV LANA AND EBV LMP1 INDUCE THE EXPRESSION OF UCH-L1 FOLLOWING VIRAL TRANSFORMATION

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Abstract

Ubiquitin C-terminal Hydrolase L1 (UCH-L1) has oncogenic properties and is highly expressed during malignancies. We recently documented that Epstein-Barr virus (EBV) infection induces *uch-l1* expression. Here we show that Kaposi's Sarcoma-associated herpesvirus (KSHV) infection induced UCH-L1 expression, via cooperation of KSHV Latency-Associated Nuclear Antigen (LANA) and RBP-Jκ and activation of the *uch-l1* promoter. UCH-L1 expression was also increased in Primary Effusion Lymphoma (PEL) cells co-infected with KSHV and EBV compared with PEL cells infected only with KSHV, suggesting EBV augments the effect of LANA on *uch-l1*. EBV latent membrane protein 1 (LMP1) is one of the few EBV products expressed in PEL cells. Results showed that LMP1 was sufficient to induce *uch-l1* expression, and co-expression of LMP1 and LANA had an additive effect on *uch-l1* expression. These results indicate that viral latency products of both human γ-herpesviruses contribute to *uch-l1* expression, which may contribute to the progression of lymphoid malignancies.

INTRODUCTION

Ubiquitin C-terminal Hydrolase-L1 (UCH-L1) is a cysteine hydrolase that contains the typical active site triad of cysteine, histidine, and aspartic acid and catalyzes hydrolysis of C-terminal esters and amides of ubiquitin (Larsen et al., 1996). In adult humans, UCH-L1 is normally exclusively expressed in the brain and cells of the reproductive system (Kwon et al., 2004; Setsuie and Wada, 2007). Although the physiological function of UCH-L1 in neurons is still unclear, mutations in the *uch-l1* gene have been associated with Parkinson's

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and Alzheimer's diseases (Betarbet et al., 2005). Functional activities, other than acting as an ubiquitin hydrolase, have been proposed for UCH-L1. First, UCH-L1 can dimerize resulting in ubiquitin ligase activity (Liu et al., 2002). Second, in neurons, the stabilization of mono-ubiquitin proteins is not dependent on UCH-L1 deubiquitinating activity (Osaka et al., 2003; Setsuie and Wada, 2007), a finding that points to an ubiquitin-independent function for UCH-L1.

Besides the high levels of expression of UCH-L1 in the brain and reproductive system, *de novo* expression of UCH-L1 has been detected in numerous cancers, such as lung (Hibi et al., 1999; Kim et al., 2008), colorectal (Loeffler-Ragg et al., 2005), bladder (Yang et al., 2006) and breast cancer (Miyoshi et al., 2006), and points to the involvement of this protein in the oncogenic transformation of cells. High levels of UCH-L1 were also observed in transformed cells of lymphoid origin such as Burkitt lymphoma (Ovaa et al., 2004) and multiple myeloma (Otsuki et al., 2004). Recent studies demonstrate that inhibition of the expression of UCH-L1 reduces the tumorigenic phenotype of transformed cells, including virus-transformed B-lymphocytes (Bheda et al., 2009a; Kim et al., 2008; Rolen et al., 2008). UCH-L1 also associates with cytoskeletal components, including microtubules (Bheda et al., 2010; Kabuta et al., 2008) and actin filaments (Basseres et al., 2010), and it physically associates with mitotic spindles (Bheda et al., 2010), which suggests a potential role in the regulation of mitosis. Furthermore, oncogenic transcription factors, such as B-Myb and β-catenin/TCF, up-regulate the expression of the *uch-l1* gene (Bheda et al., 2009b; Long et al., 2003). Together, these findings strongly support the idea of an oncogenic function for UCH-L1, and although the physiological roles of UCH-L1 and the regulation of its expression in normal and transformed cells remain largely unexplored, it has become clear that this multifunctional protein of the ubiquitin system UCH-L1 participates in diverse cellular processes.

Both EBV and KSHV are members of the -herpesvirus subfamily. EBV, the first human tumor virus discovered, causes or is closely associated with both lymphoid and epithelial malignancies, and KSHV is the causative agent of Kaposi's Sarcoma and Primary Effusion Lymphoma (PEL) (Pagano, 2009; Sin et al., 2007). Both viruses produce significant pathology in immunodeficient hosts, most commonly with patients with AIDS (Pagano, 2009; Sin et al., 2007).

During cell transformation by EBV, viral oncoproteins disrupt a variety of host signaling pathways that affect the host ubiquitin system (Pagano, 2009; Shackelford and Pagano, 2005, 2007). The EBV primary oncogene LMP1 inhibits Siah1 ubiquitin ligase and stabilizes the expression of β-catenin (Jang et al., 2005). LMP1 also induces the regulatory ubiquitination of IRF7 (Ning et al., 2008) as well as downregulates the activity of IRF7 via the activation of the ubiquitin-editing enzyme A20 (Ning and Pagano). EBNA1 competes with p53 to interact with HAUSP, the p53 deubiquitinating enzyme, thus indirectly targeting p53 for ubiquitination and degradation (Holowaty and Frappier, 2004; Holowaty et al., 2003). EBNA3C, which possesses intrinsic deubiquitinating activity, inhibits the p53 and Rb pathways by two different mechanisms: deubiquitination of MDM2 and recruitment of SCF4 ligase (Saha et al., 2009; Ying and Xiao, 2006).

The main KSHV protein that directly or indirectly affects the host ubiquitin system is Latency-Associated Nuclear Antigen (LANA), which is expressed in all KSHV latently infected cells and modulates cellular pathways that may contribute to tumorigenesis (Wen et al.). LANA physically associates with p53 and inhibits p53-mediated transcriptional activity and apoptosis (Friborg et al.). LANA also inactivates expression of the tumor suppressor retinoblastoma (Rb) and releases the transactivator E2F, which induces cells to progress through the G1/S cell cycle checkpoint. In addition, LANA interacts with the bromodomain-containing protein RING3/Brd2 and further stimulates cell-cycle progression. Together, p53 regulation and cell-cycle progression are highly regulated by the host ubiquitin system. Finally, LANA rescues β-catenin from phosphorylation-dependent ubiquitination and destruction by interacting with GSK3 (Fujimuro et al., 2007).

LANA functions as a transcriptional modulator of multiple cellular and viral promoters, including its own (Sin et al., 2007). It can both activate as well as repress transcription of multiple viral and cellular genes through a variety of mechanisms. Furthermore, LANA can act as a transcriptional modulator both directly and indirectly. LANA binds DNA, including the KSHV terminal repeat and its own promoter and regulates gene expression. LANA also interacts with p53 and down-regulates its transcriptional activity; however, it can also bind pRb and activate E2F-dependent gene transcription. Additionally, LANA regulates transcription by binding to and inhibiting the histone transferase activity of CREB-binding protein (CBP). Finally, LANA associates with cellular chromatin and remains associated with chromosomes during cell division.

Recently, we have shown that immortalization of peripheral blood mononuclear cells (PBMCs) with EBV activates *uch-l1*. In type III EBV latency, EBV Nuclear Antigen 2 (EBNA2) forms complexes with the transcription factor PU.1, activating the *uch-l1* promoter and inducing UCH-L1 RNA and protein expression (Bheda et al., 2011). In addition, HPV16-mediated transformation induces *uch-l1* expression in normal keratinocytes (Rolen et al., 2009). We therefore hypothesize that in the process of cellular transformation, tumor viruses activate the uch-l1 promoter, thus inducing UCH-L1 protein expression and dysregulation of the host ubiquitin system. We now show, for the first time, that infection of normal human endothelial cells with KSHV results in increased endogenous UCH-L1 expression in these cells and that KSHV LANA along with RBP-Jκ activates the *uch-l1* promoter. In addition, we demonstrate that EBV LMP1 can also activate the *uch-l1* promoter and increase levels of UCH-L1. Finally, we find that in a primary effusion lymphoma (PEL) cell line dually infected with EBV and KSHV endogenous UCH-L1 RNA and protein levels are increased to a greater extent than in PELs infected with KSHV only. These observations demonstrate a mechanism by which KSHV and EBV infections lead to cell transformation and suggest that infection with multiple tumor viruses may have an additive effect on UCH-L1 expression.

MATERIALS AND METHODS

Cells

NIH 3T3 and Cos-7 cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% FBS (Sigma) and penicillin–streptomycin (Sigma).

Primary Effusion Lymphoma cell lines BC-1 (contains both KSHV and EBV genomes) and BC-3 (contains KSHV), were cultured in RPMI 1640 medium (Gibco) plus 10% heat-inactivated FBS, 100 units/ml penicillin–streptomycin, 1% sodium bicarbonate (Sigma) and 0.5% -ME (Sigma). All cell lines were maintained at 37 °C in 5% CO_2 in air.

Plasmids

pcDNA LANA-Flag construct was a gift from Dr. Dirk Dittmer. pECE-RBP-Jκ construct was a gift from Dr. Paul Ling. pGL3-UCH-L1 promoter reporter construct was amplified and cloned as described (Bheda et al., 2009a). pcDNA LMP1 has been previously described (Bentz et al.; Bentz et al., 2011; Ning et al., 2008).

Luciferase Reporter Assays

For luciferase assays, cells were seeded in 6-well plates and transiently transfected with the use of Fugene HD (Roche Diagnostics) with UCH-L1p-Luc promoter plasmid, β-gal constructs, and indicated effector plasmids. The total amount of DNA in all transfections was kept constant with empty vector. Luciferase assays were performed 48 h post-transfection as specified by the manufacturer (Promega). All reporter-assay results are from three independent experiments prepared in triplicate and have been normalized for β-gal activity.

Reverse Transcriptase PCR

NIH 3T3 cells were transiently transfected with a total of 2 μg of DNA with the Fugene HD reagent (Roche Diagnostics). Cells were collected 48 h post-transfection for RT-PCR analysis. Total RNA was extracted with the use of Agilent's Total RNA isolation mini kit per manufacturer's instructions (Agilent Technologies). 500 ng of total RNA were used for RT-PCR reactions using the one step RT-PCR kit (Qiagen) as per manufacturer's instructions at an annealing temperature of 55°C. Samples were analyzed on 1 % agarose gel. Primers used:

UCH-L1: 5'-GGATGGCCACCTCTATGAAC-3', 5'-AGACCTTGGCAGCGTCCT-3'

GAPDH: 5'-AGGTGAAGGTCGGAGTCAACG-3', 5'-AGGGGTCATTGATGGCAACA-3'.

Chromatin Immunoprecipitation

ChIP assays were performed using Active Motif ChIP-IT enzymatic kit (Active Motif) per manufacturer's instructions. KR4 cells were fixed with 37% formaldehyde (1% final concentration) for 10 min at 37°C; the reaction was stopped with cold 0.125 M glycine solution for 5 min at RT. The cells were then washed twice with PBS and collected in 0.5 ml digestion buffer with 1× protease inhibitors. Chromatin was sheared with shearing enzyme for 10 min at 37°C to obtain an average of 200–1000 bp fragments. Sheared chromatin was incubated overnight at 4°C with Protein G magnetic beads, and RBP-Jκ antibody (Santa Cruz- H50 X). Immunoprecipitations were performed per the manufacturer's instructions; cross-linking was reversed by incubating immunoprecipitated complexes with 5 M NaCl and RNase A (final concentration 25 μg /ml) for 2 h at 65°C followed by Proteinase-K (final concentration 50 g/ml) treatment for 2 h at 42°C. PCR reactions were performed with 5 μl

precipitated DNA with primer pairs flanking consensus RBP-Jκ sites in UCH-L1 promoter. PCR conditions: one cycle, 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 30 sec, and 72°C for 2 min, and a final extension at 72°C for 10 min. The primers used in the reaction were:

Site 1 (5' CCTGTTGAATTTGTGCT 3'; 5' CGCCGGTGAGATAATCTG 3')

Site 2/3 (5' GCTCCATACACTCAAGGAAC 3'; 5' GCCAGACGCACTGTGA 3')

Western blotting

Total cell lysates were resolved on 12% SDS-PAGE, transferred to PVDF membrane (GE Healthcare), blocked in 5% milk-Tris-buffered saline solution, and incubated at 4°C overnight with UCH-L1 (1:7500, Invitrogen) and GAPDH (1:5000, Sigma) antibodies followed with horse-radish peroxidase-conjugated secondary antibodies. Proteins were detected with Super Signal West Pico Chemiluminescence Detection Kit (Pierce Biotechnology, Rockford, IL, USA) and exposed to Kodak XAR-5 film.

Immunoprecipitation

KR4 cells were lysed with buffer containing 50 mM Tris-HCl, pH 7.6, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM Na3Ov4, 1 mM NaF and complete protease inhibitor mixture (Roche Diagnostics). Cell lysates were incubated with anti-Flag beads (Sigma) incubated withat 4°C overnight, washed four times with protein lysis buffer, and then eluted from protein anti-Flag beads with 2× Laemmli's buffer.

2D-Gel Electrophoresis

Cells were harvested and washed three times with PBS. The cell pellets were lysed with lysis buffer (10 mM Tris, pH 7.4, and 0.3% SDS) and incubated for 30 minutes on ice. Equivalent micrograms of protein were subjected to 2D gel electrophoresis by the UNC Proteomics Center. The 2D gels were stained with Coomasie blue. Unique spots were identified and sequenced by MALDI TOF/TOF mass spectrometry.

LMP1 Knockdown

BC-1 and BC-3 cells were transiently transfected with siRNA specific for LMP1 (siRNA LMP1 5'-GGAAUUUGCACGGACAGGCUU-3') or with a two-base mutation (siRNA mut 5'-GGAAUGUGCACAGACAGGCUU-3') using Amaxa® Cell Line Nucleofector® Kit V. Nucleofections were performed at 0 and 24 hours, and cells were harvested at 72 hours. RNA was isolated and RT-PCR was performed for GAPDH, UCH-L1, and LMP1 (as described above). pmaxGFP® Vector was used as a transfection control; the transfection efficiencies were approximately 30%.

RESULTS

KSHV infection of endothelial cells induces the expression of UCH-L1

Human Umbilical Vein Endothelial Cells (HUVECs) were infected with a recombinant KSHV virus expressing green fluorescent protein (GFP), and a stable KSHV-HUVEC cell line was generated as described before (Wang et al., 2006). Equivalent amounts of HUVECs

and KSHV-HUVECs were subjected to 2D protein gel electrophoresis analysis, and gels were stained with Coomassie Blue dye. Spots that were differentially expressed in the HUVEC and the KSHV-HUVEC cells were excised from the gel and identified by mass spectrometry (Figure 1A). One protein upregulated in KSHV-HUVECs, but not HUVECs, was the cysteine hydrolase UCH-L1 (Figure 1A), suggesting that KSHV infection induces the expression of UCH-L1.

To determine whether UCH-L1 was upregulated at the transcriptional level, RNA was isolated from these cells, and RT-PCR was performed. KSHV-HUVECs had increased levels of UCH-L1 RNA compared with the uninfected cells (Figure 1B). Similar results were observed when examining UCH-L1 protein levels (Figure 1C); where UCH-L1 levels in infected cells were greater than uninfected cells. Relative UCH-L1 expression was determined for experiments performed in triplicate (Figure 1B and 1C), and results showed a significant ($p < 0.05$) increase in UCH-L1 gene and protein expression in KSHV-infected HUVECs. These results corroborate the 2D proteomic analysis and demonstrate that KSHV infection induces the expression of UCH-L1.

KSHV LANA interacts with UCH-L1 and induces the endogenous expression of UCHL1

To begin to decipher the KSHV-specific protein(s) required for the observed KSHV-induced upregulation of UCH-L1 expression, we focused on LANA, which is expressed in the majority of KSHV-infected cells. First, the ability of KSHV LANA to interact with UCH-L1 was examined in Cos-7 cells, which express intermediate levels of endogenous UCH-L1 (Bheda et al., 2009a). Immunoprecipitations showed that an interaction between overexpressed LANA and endogenous UCH-L1 could be detected (Figure 2A). Additionally, when total cell lysates were probed with UCH-L1-specific antibodies, the results revealed significantly ($p < 0.05$) higher levels of UCH-L1 (two-fold increase) in LANA-expressing cells compared with vector-containing cells (Figure 2A). NIH 3T3 cells, which express very low levels of UCH-L1 (Bheda et al., 2009a), were used to confirm these findings. Results showed that NIH 3T3 cells transfected with Flag-LANA exhibited more than a three-fold increase ($p < 0.001$) in the expression of endogenous UCH-L1 RNA and protein levels (Figure 2B and 2C) when compared with control-expressing cells. In addition, LANA induced UCH-L1 expression in a dose-dependent manner (Figure 2D)

Because UCH-L1 expression appeared to be induced by LANA at the transcriptional level, the ability of LANA to activate the *uch-l1* promoter was tested with the use of a UCH-L1p-LUC reporter construct, which contains a minimal endogenous uch-l1 promoter region (Bheda et al., 2009a). Results showed that LANA expression produced a significant ($p < 0.05$) three-fold increase in activation of the *uch-l1* promoter when compared with control cells (Figure 2D and 2E). Together, these data indicate that KSHV LANA induces UCH-L1 expression at the transcriptional level via its ability to activate the *uch-l1* promoter.

EBV increases levels of UCH-L1 in dually infected Primary Effusion Lymphoma cells

In addition to Kaposi's sarcoma, KSHV is detected in 100% of primary effusion lymphomas (PELs) (Carbone et al., 2000; Carbone and Gloghini, 2005; Fakhari et al., 2006; Sin et al., 2007). PELs, a unique form of non-Hodgkin B-cell lymphomas found only in KSHV-

infected patients with AIDS, are an aggressive, rapidly progressing malignancy that is fatal (Carbone and Gloghini, 2005; Petre et al., 2007). Because LANA is one of the KSHV genes that is expressed in PELs (Fakhari et al., 2006), the effect of KSHV on *uch-l1* expression in two representative PEL cell lines was examined. We reported previously that naïve B cells contain undetectable levels of UCH-L1 RNA and protein (Bheda et al., 2009a; Bheda et al., 2011). However, analysis of two PEL cell lines (BC-1 and BC-3) revealed detectable yet different levels of UCH-L1 RNA and protein (Figure 3A and 3B). BC-1 cells expressed more than four-fold higher ($p < 0.05$) levels of both UCH-L1 RNA and protein compared with BC-3 cells. In addition, endogenous *uch-l1* promoter activity was significantly ($p < 0.05$) greater in BC-1 cells than in BC-3 cells. These findings demonstrate that KSHV-mediated cellular transformation can induce *uch-l1* expression.

One of the major differences between BC-1 and BC-3 cells is that BC-3 cells are only infected with KSHV while BC-1 cells are infected with both KSHV and EBV (Carbone et al., 2000; Carbone and Gloghini, 2005; Fakhari et al., 2006; Sin et al., 2007). While the role of EBV in PEL co-infection has been little explored and remains obscure, there are reports that suggest that EBV and KSHV can regulate each other's viral gene expression (Fan et al., 2005b; Groves et al., 2001a; Krithivas et al., 2000; Xu et al., 2007).

EBV LMP1 induces the expression of UCH-L1

We have implicated EBNA2 in the EBV-mediated induction of UCH-L1 expression (Bheda et al.), however, PEL cells exhibit a restricted expression pattern of EBV proteins and lack detectable expression of EBNA2 as well as EBNA3–6 (Callahan et al., 1999). Instead, dually infected PEL cells express low levels of LMP1(Carbone et al., 2000; Carbone and Gloghini, 2005; Fakhari et al., 2006; Sin et al., 2007), a constitutively active transmembrane receptor that indirectly activates host-cell transcription (Hatzivassiliou and Mosialos, 2002; Lam and Sugden, 2003; Li and Chang, 2003; Zheng et al., 2007). Analysis of the *uch-l1* promoter revealed putative binding sites for NF-κ B, STATs, AP1, c-Jun, SP1, SP3 and AP2- all of which are the major downstream targets of signaling pathways activated by LMP1.

To investigate whether LMP1 can affect endogenous *uch-l1* expression, reporter assays were performed to examine the activation of the endogenous *uch-l1* promoter. Results showed that LMP1 expression correlated with a significant ($p < 0.05$) 2.5-fold increase in the activation of the *uch-l1* promoter when compared with control-expressing cells (Figure 4A). In addition, over five-fold increases ($p < 0.05$) in levels of endogenous UCH-L1 RNA and protein were detected in cells expressing LMP1 compared with control cells (Figure 4B and 4C). LMP1 also induced the expression of UCH-L1 in a dosedependent manner (Figure 4D). These data indicate that EBV LMP1 can induce the UCHL1 endogenous expression in cells by activating its promoter.

KSHV LANA and EBV LMP1 together induce the expression of UCH-L1

Because data showed enhanced activation of the *uch-l1* promoter as well as increased UCH-L1 RNA and protein expression in cells co-infected with KSHV and EBV, we next investigated if LANA and LMP1 could have an additive effect on *uch-l1* expression.

Reporter assays confirmed the previous data demonstrating that expression of LANA or LMP1 resulted in significant ($p < 0.05$) increased activation of the *uch-l1* promoter. Furthermore, when LANA and LMP1 were co-expressed, there was an additive effect on activation of the endogenous promoter (Figure 5A). These findings were confirmed by examining UCH-L1 RNA and protein levels (Figure 5B and 5C). Levels of UCH-L1 were significantly ($p < 0.05$) greater when LANA and LMP1 were co-expressed than when these oncoproteins were expressed alone, which was still significantly ($p < 0.05$) greater than in cells expressing the vector control.

Further confirmation for the additive effects of LMP1 was obtained by knocking down LMP1 expression in PEL cells. BC-3 and BC-1 were transfected with either a LMP1-specific siRNA or a mutant siRNA, in which two bases were changed (Figure 5D). Results showed approximately 40% knockdown in LMP1 RNA levels in BC-1 cells (dually infected with EBV and KSHV), which corresponded with a significant ($p < 0.05$) 35% decrease in relative *uch-l1* levels. No differences in *uch-l1* expression were observed in BC-3 cells, which do not express LMP1. These findings confirm the additive effect of LMP1 on *uch-l1* expression in dually infected PEL cells.

Activation of the *uch-l1* promoter by RBP-Jκ

Finally, the mechanism by which LANA and LMP1 induce the *uch-l1* promoter was explored. Analysis of the UCH-L1 promoter sequence with the use of PATCH software (www.gene-regulation.com) revealed 3 partial putative RBP-Jκ binding sites. LANA interacts with RBP-Jκ, so the ability of LANA to interact with RBP-Jκ to activate the *uch-l1* promoter was tested. Reporter assays revealed that LANA and RBP-Jκ separately activated the *uchl-1* promoter to modest, yet significant ($p < 0.05$), levels. However, co-expression of LANA and RBP-Jκ resulted in significant ($p < 0.05$) additive activation of the endogenous promoter (Figure 6A). These results suggest that that KSHV LANA protein activates the *uch-l1* promoter via its interaction with RBP-Jκ.

The ability of RBP-Jκ to activate the *uch-l1* promoter during EBV infection was confirmed by ChIP assays. Using the EBV-transformed B cell line KR4, which express high levels of LMP1 as well as high levels of endogenous UCH-L1 (Bheda et al., 2009a), RBP-Jκ-specific antibodies were used to pull down RBP-Jκ DNA complexes. Non-immunoprecipitated DNA was used as input DNA, and an isotype-matched IgG antibody served as negative controls. PCR analysis of portions of the *uch-l1* promoter revealed that RBP-Jκ bound to the UCH-L1 promoter through at least 2 of the 3 partial RBP-Jκ binding sites (Figure 6B).

Taken together these data suggest that both KSHV and EBV encode latency-associated proteins (LANA and LMP1) that independently induce the expression of UCH-L1 through the activation of RBP-Jκ. With dual infection, which is detected in more than 60% of PELs, KSHV LANA and EBV LMP1 can have an additive effect on the induction of the expression of UCH-L1, thus potentially enhancing the tumorigenic phenotype in these cells.

Discussion

Our studies are the first to identify a role for KSHV LANA and EBV LMP1 in the activation of UCH-L1 expression. During cell transformation by either virus, the induction of the expression of UCH-L1 is a prominent cellular response (Bheda et al., 2010; Bheda et al., 2009a; Bheda et al., 2011; Bheda et al., 2009b). Because UCH-L1 expression is linked to multiple, observed tumorigenic phenotypes in cells, including increases in cell proliferation, adhesion, migration, and invasion as well as changes in cell morphology and inhibition of apoptosis, (Bheda et al., 2009a; Kim et al., 2008; Rolen et al., 2008), the data presented point to a mechanism by which both viruses can induce the expression of UCH-L1 and contribute to the oncogenicity of these viruses (Figure 7). Furthermore, the two representative PEL cell lines, one infected only with KSHV and one dually infected with KSHV and EBV, revealed that the co-infected cells expressed higher levels of UCH-L1. Indeed, co-expression of KSHV LANA and EBV LMP1 was associated with significant increased activation of the *uch-l1* promoter as well as UCH-L1 RNA and protein and pointed to an additive response by these two very different viral proteins. Knockdown of LMP1 significantly lessened the additive effect of LMP1 in dually infected PEL cells. Together, these data strongly support the hypothesis that that both of these transforming human γ-herpesviruses activate the *uch-l1* promoter, inducing UCH-L1 expression.

We recently documented that EBV EBNA2 activates the *uch-l1* promoter in type III EBV latency (Bheda et al.), resulting in increased UCH-L1 expression (Bheda et al.; Soni et al., 2007). However, EBNA2 cannot be responsible for up-regulation of UCH-L1 in co-infected PELs because the type III latency promoter Cp is not active in EBV-positive PEL cells, and EBNA2 is not expressed (Carbone et al., 2000; Carbone and Gloghini, 2005; Fakhari et al., 2006; Sin et al., 2007). Rather, dually infected PEL cells exhibit a restricted expression pattern of EBV products and may express low levels of LMP1 (Callahan et al., 1999). LMP1 expression was detected in co-infected PEL cells, LMP1 expression alone was capable of inducing the activation of the *uch-l1* promoter and UCH-L1 expression, and knockdown of LMP1 resulted in decreased *uch-l1* expression. These findings identify a second EBV latency protein that induces the expression of UCH-L1 (Figure 7). However, because LMP1 levels are low in dually infected PEL cells (Callahan et al., 1999), and knockdown of LMP1 did not completely abrogate the increase in *uch-l1* expression observed in BC-1 cells compared to BC-3 cells, these findings cannot eliminate the possibility that EBNA1 (Bornkamm, 2009; Kaul et al., 2007) or EBV-encoded non-polyadenylated RNAs (EBER1 and EBER2) contribute to the up-regulation of *uch-l1*. Both EBNA1 and the EBERs have been shown to play roles in malignant transformation, and because we propose that during transformation oncogenic viruses activate the *uch-l1* promoter; it is possible and probable that EBNA1 and the EBERs may also induce UCH-L1 expression either directly or indirectly. Therefore, the higher levels of endogenous UCH-L1 RNA and protein we detected in EBV-positive PELs (Figure 3) and EBV-transformed cells (Bheda et al.) may be the cumulative result of more than one EBV and/or KSHV products.

The role of EBV in PEL co-infection is still unclear. However, there is evidence for interactions between the two viruses: In vitro EBV infection of KSHV-infected PEL enhances the tumorigenicity of the singly infected PEL in SCID mice (Xu et al., 2007);

dually-infected compared with singly KSHV-infected PELs express a unique set of cellular genes (Fan et al., 2005a); KSHV LANA activates the expression of EBV latent membrane protein 1 (LMP1) (Groves et al., 2001b), but reduces the expression of EBV EBNA1 and EBNA2 (Krithivas et al., 2000). Because we show that co-expression of LANA and LMP1 enhanced activation of the *uch-l1* promoter and increased expression of UCH-L1, it is possible that their additive effects on UCH-L1 expression also occur during endogenous infection. The effect of UCH-L1 on the tumorigenic phenotypes of cells has been well documented (Bheda et al., 2009a; Kim et al., 2008; Rolen et al., 2008). We have specifically studied the cellular changes resulting from knockdown of *uch-l1* in EBV-transformed B cells (Bheda et al., 2009a). Our previous results have documented functions for EBV-induced *uch-l1* expression in cell proliferation, adhesion, and migration as well as inhibition of apoptosis (Bheda et al., 2009a). Similar results were observed in different cell lines (independent of viral protein expression) (Bheda et al., 2009a), suggesting a universal role for *uch-l1* in these phenotypic changes regardless of cell origin and method of transformation.

The finding that KSHV LANA can itself induce the expression of EBV LMP1 (Groves et al., 2001b) suggests there is a second mechanism through which UCH-L1 levels are augmented in co-infected cells: LANA activates the expression of LMP1, which in turn activates the *uch-l1* promoter, resulting in greater levels of UCH-L1. Altogether, because UCH-L1 expression is associated with the tumorigenic phenotype of transformed cells (Bheda et al., 2009a; Kim et al., 2008; Rolen et al., 2008), these data suggest that UCHL1 expression may contribute to enhanced tumorigenesis in PEL.

During EBV infection, EBNA2 interacts with PU.1 to activate the *uch-l1* promoter (Bheda et al.). We now document that EBV, as well as KSHV, also induces UCH-L1 expression via RBP-Jκ. Our findings show that RBP-Jκ expression enhanced LANA-induced activation of the *uch-l1* promoter and that endogenous RBP-Jκ binds to endogenous uch-l1 promoter sequences in transformed B-cells. While RBP-Jκ itself can activate the *uch-l1* promoter, indicating that RBP-Jκ binds to the promoter independent of viral protein expression, the strong combined effect of LANA and RBP-Jκ co-expression on the activity of the promoter suggests that LANA interacts with RBP-Jκ and enhances activation of UCH-L1 expression. However, each factor most likely activates the endogenous promoter through independent mechanisms as well. Due to the overlap of the RBP-Jκ sites with other transcription factor binding sites, including NF-κB binding sites, we did not undertake mutational analysis of the partial RBP-Jκ binding sites, but these data do strongly suggest that RBP-Jκ is important in the activation of the *uch-l1* promoter.

In addition to RBP-Jκ, LANA also interacts with SP1, STAT3, c-JUN (Verma et al., 2007), and the *uch-l1* promoter has binding sites for each of these transcription factors. LMP1 also activates multiple signaling pathways resulting in the downstream activation of these factors. Therefore, SP1, STAT3, and c-JUN are likely candidates to contribute to LANA- and LMP1-induced activation of the *uch-l1* promoter and will be the subject of future studies.

UCH-L1 can also up-regulate its own promoter (Bheda et al., 2009b). Previously, UCH-L1 was one of many proteins identified that co-precipitated with LANA, and here we document that LANA does interact with UCH-L1. Therefore, a third mechanism by which LANA induces UCH-L1 expression is through its interaction with UCH-L1, which would result in a positive feed-back loop, further enhancing UCH-L1 expression following KSHV-mediated transformation.

Together, these findings support our hypothesis that in the process of cellular transformation, tumor viruses activate the *uch-l1* promoter, thus inducing UCH-L1 expression. We previously documented that EBV-induced transformation induced UCH-L1 expression via EBNA2 and PU.1 (Bheda et al.) (Figure 7), and now we show KSHV also induces the expression of UCH-L1. Specifically, two viral proteins, LANA and LMP1, which are essential in the viral transformation process, were documented to be sufficient to activate the *uch-l1* promoter via RBP-Jκ, resulting in increased protein expression. Dual expression of LANA and LMP1, both endogenously and exogenously, had an additive effect on UCH-L1 expression (Figure 7). Therefore, in the future, it would be interesting to determine if co-infection with other pairs of viruses, such as HPV and EBV, also enhances UCH-L1 expression, which in light of their different mechanisms may combine to enhance viral pathology.

Finally, we observed that KSHV-infected endothelial cells display upregulated UCH-L1 and that KSHV LANA induces the expression of *uch-l1* in endothelial cells. Thus, UCH-L1 may also play a role in the pathogenesis of Kaposi sarcoma.

Acknowledgments

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- Infection of endothelial cells with KSHV induced UCH-L1 expression.
- KSHV LANA is sufficient for the induction of *uch-l1*.
- Co-infection with KSHV and EBV (observed in some PELs) results in the additive induction of *uch-l1*.
- EBV LMP1 also induced UCH-L1 expression.
- LANA- and LMP1-mediated activation of the *uch-l1* promoter is in part through RBP-Jκ

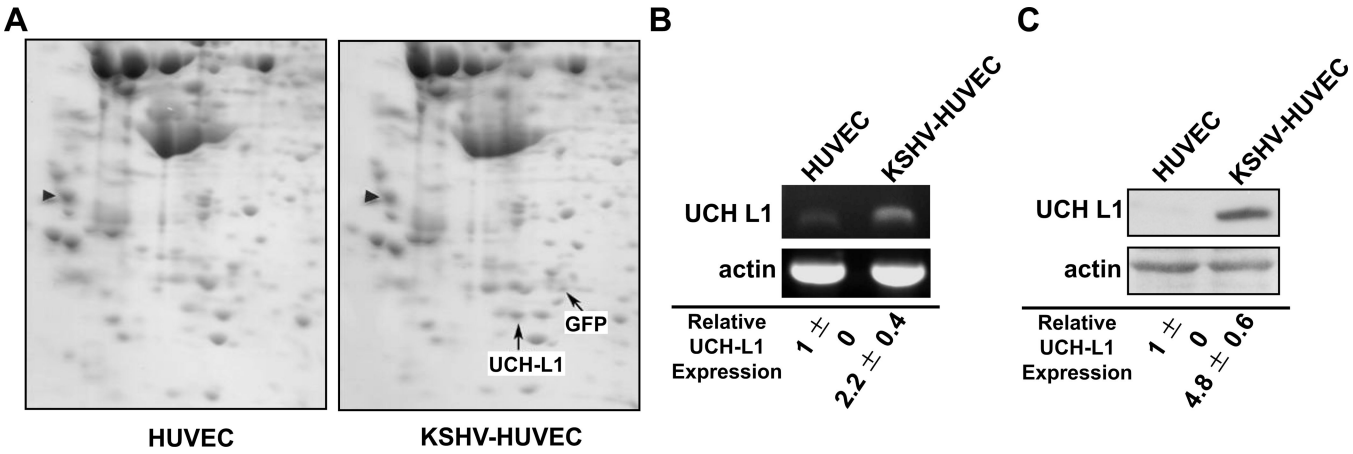

Figure 1. UCH-L1 expression is induced in endothelial cells after infection with KSHV

(**A**) Equivalent amounts of HUVEC and KSHV-HUVEC cell lysates were subjected to 2D gel electrophoresis analysis following which the gels were stained with Coomassie blue. Two of the differentially expressed spots, identified by mass spectrometry, were UCH-L1 and GFP (arrows). (**B**) RNA from HUVEC and KSHV-HUVEC was isolated and subjected to RT-PCR using *uch-l1* and β-*actin* primers. (**C**) Equivalent amounts of KSHVHUVEC and HUVEC cell lysates were subjected to SDS-PAGE and immunoblotted with UCH-L1 or actin antibodies. Relative expression was determined by densitometry. All results are shown as the mean ± standard deviation for experiments performed in triplicate.

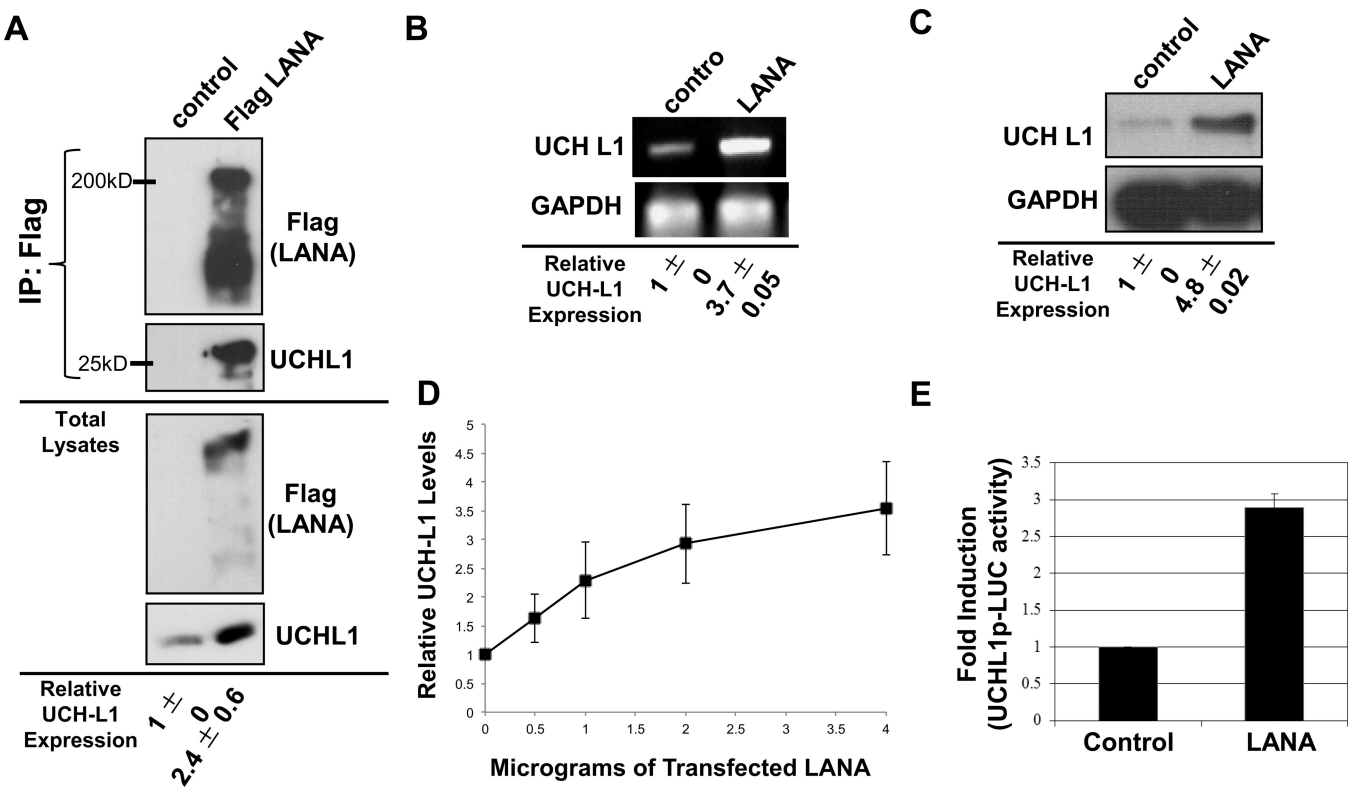

Figure 2. KSHV LANA is associated with endogenous UCH-L1 and induces expression of UCH-L1

(**A**) Cos7 cells were transfected with control or LANA-Flag expression vectors and harvested 48h post-transfection for immunoprecipitation analysis. LANA-Flag was immunoprecipitated with anti-Flag-agarose beads. IPs and cell lysates were resolved on 10–12% SDS-PAGE and probed with UCH-L1 and Flag antibodies. **(B–C)** Total RNA and protein were extracted from cells co-transfected with LANA or control expression constructs. (**B**) RT-PCR analysis was performed using primers specific for *uchl1* and *gapdh*. (**C**) Western blot analyses for UCH-L1 protein levels in lysates from cells transfected with or without LANA were performed with UCH-L1 antibodies. GAPDH was used as loading control. **(D)** Cells were transfected with different amounts of Flag-LANA or vector-control expressing plasmids and Western blot analyses to detect UCH-L1 was performed. Relative expression was determined by densitometry, and results are shown as the mean fold change ± standard deviation for experiments performed in triplicate. (**E**) NIH3T3 cells were co-transfected with control or LANA-Flag expression vectors (350 ng/well) along with UCH-L1p-LUC wildtype reporter plasmid (500 ng/well) and β-gal expression constructs (250 ng/well). Luciferase assays were performed 48 h post-transfection. The data are shown as the mean ± standard deviation for three independent experiments in triplicate and normalized to β-gal activity.

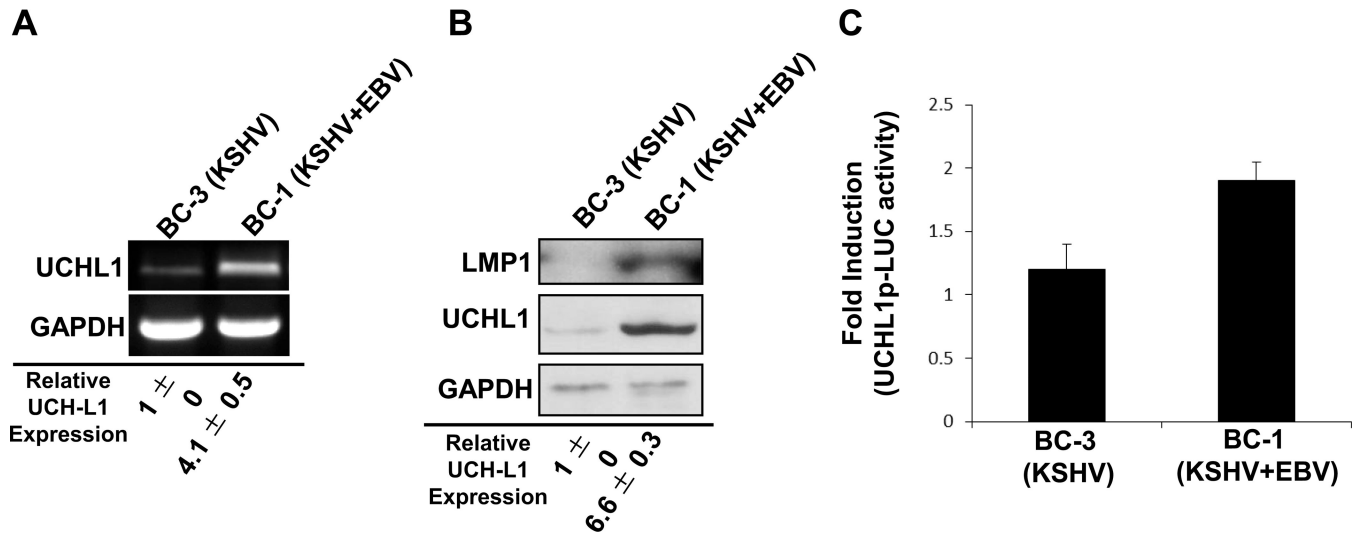

Figure 3. Endogenous UCH-L1 expression is greater in primary effusion lymphoma cells co-infected with EBV

(A–B) Total RNA and protein were extracted from BC-3 and BC-1 cell lines. **(A)** RT-PCR was performed with *uch-l1*- and *gapdh*-specific primers. **(B)** Western blot analyses were performed with UCH-L1 and GAPDH antibodies. Relative expression was determined by densitometry. Results are shown as the mean ± standard deviation for experiments performed in triplicate. **(C)** Endogenous *uch-l1* promoter activity was determined in BC-3 (positive for KSHV only) and BC-1 (co-infected with KSHV and EBV) PEL cells. Cells (2×10^5) were nucleofected with UCH-L1p-LUC reporter and β-gal constructs. Luciferase assays were performed 48 h post-transfection. The experiments were done in triplicate and normalized to β-gal activity.

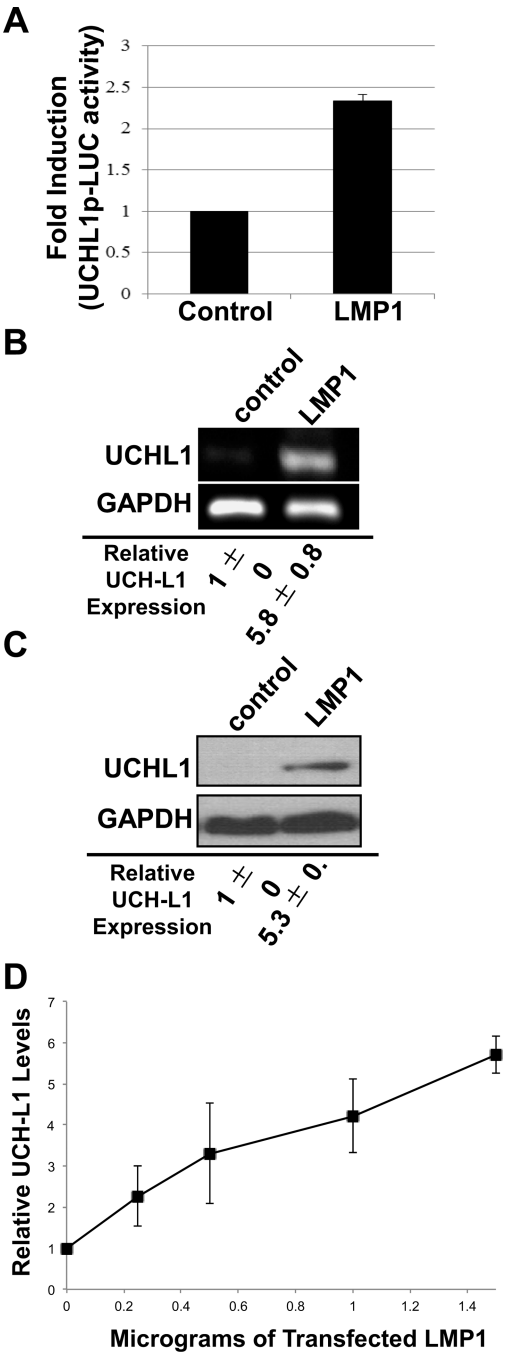

Figure 4. EBV LMP1 induces expression of UCH-L1

(A) NIH3T3 cells were co-transfected with control or LMP1-Flag expression vectors (250 ng/well), along with UCH-L1p-LUC wild type reporter plasmid (500 ng/well) and β-gal expression constructs (250 ng/well). Luciferase assays were performed 48 h post-transfection. The data represent three independent experiments performed in triplicate and normalized to β-gal activity. **(B–C)** Total RNA and protein were extracted from cells co-transfected with LMP1 or control. **(B)** RT-PCR was performed with specific primers for *uch-l1* (*gapdh* was used as a control). **(C)** Western blot analyses for UCH-L1 protein levels

in lysates from cells transfected with or without LMP1 were performed with UCH-L1 antibodies. GAPDH was the loading control. **(D)** Cells were transfected with different amounts of Flag-LANA or vector-control expressing plasmids and Western blot analyses to detect UCH-L1 was performed. Relative expression was determined by densitometry. Results are shown as the mean fold change ± standard deviation for experiments performed in triplicate.

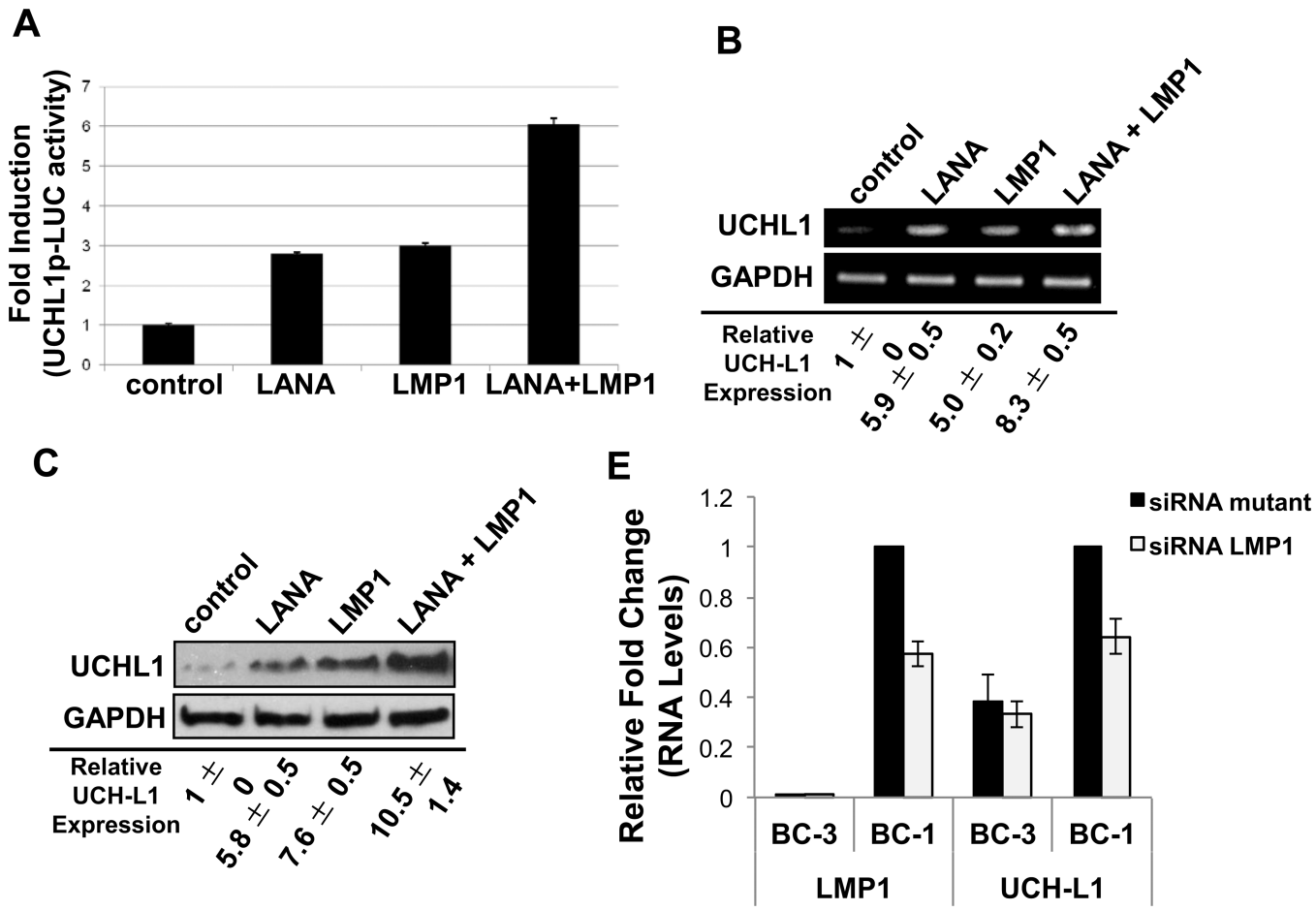

Figure 5. KSHV LANA and EBV LMP1 have additive effects on the expression of UCH-L1

(A) NIH3T3 cells were co-transfected with control, Flag-LANA, and/or pcDNA3-LMP1 expression vectors (250 ng/well), along with UCH-L1p-LUC wild type reporter plasmid (500 ng/well) and β-gal expression constructs (250 ng/well). Luciferase assays were performed 48 h post-transfection. The data represent three independent experiments performed in triplicate and normalized to β-gal activity. **(B–C)** Total RNA and protein were extracted from cells co-transfected with the control, Flag-LANA, and/or pcDNA3-LMP1 expression constructs. **(B)** RT-PCR was performed with specific primers for *uch-l1* (*gapdh* was used as a control). **(C)** Western blot analyses for UCH-L1 protein levels in lysates were performed. GAPDH was the loading control. **(D)** BC-1 and BC-3 cells were transfected with siRNA LMP1. Transfection with a siRNA LMP1 mutant, where two nucleotides were mutated, served as a control. RNA was harvested and RT-PCR was performed with specific primers for *uch-l1* and LMP1 (*gapdh* was used as a control). The fold change in relative *uch-l1* and LMP1 RNA levels (relative to *gapdh*) was determined. Results are shown as the mean ± standard deviation for experiments performed in triplicate.

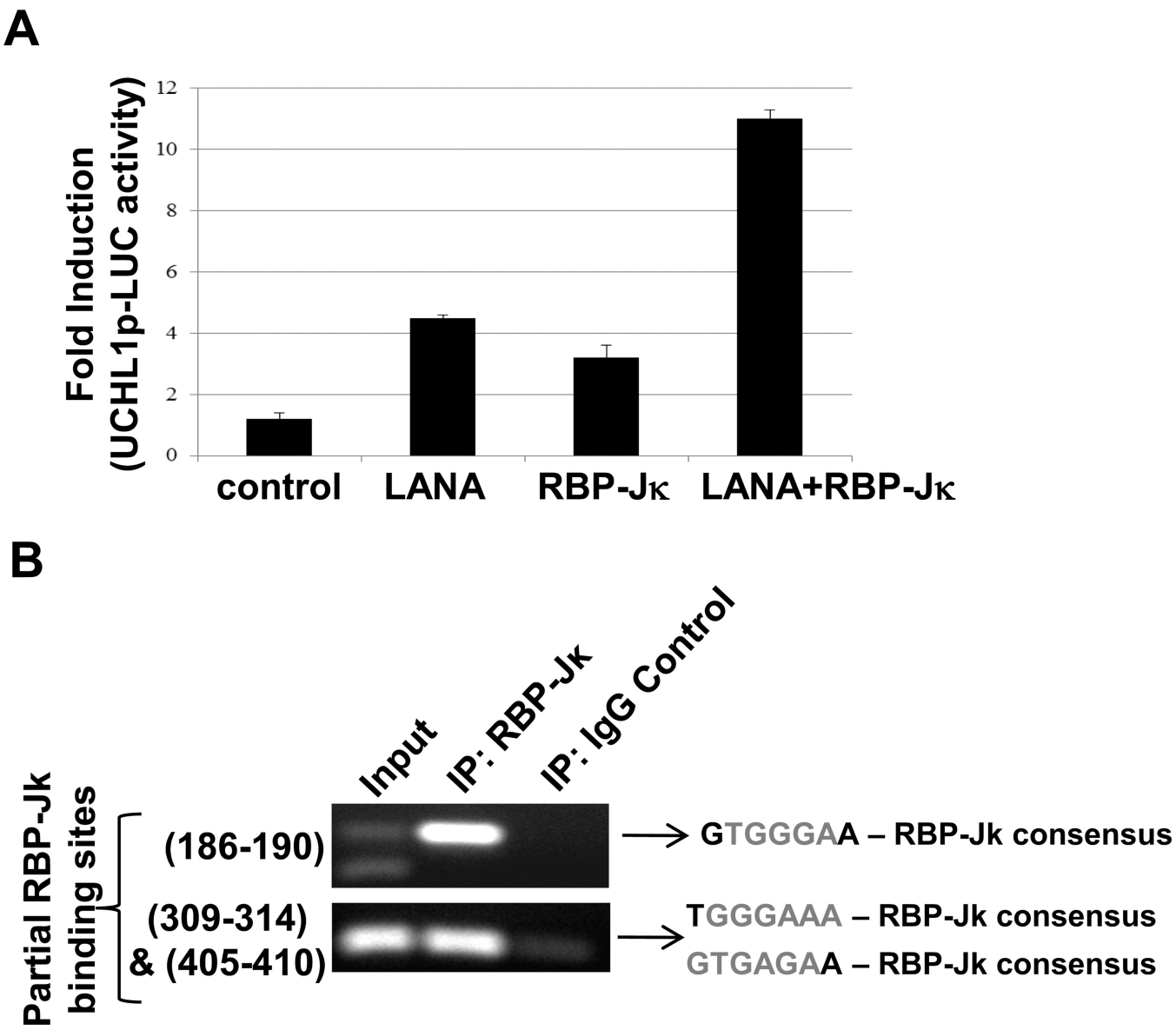

Figure 6. Activation of the *uch-l1* promoter by KSHV-LANA and EBV occurs via RBP-Jκ

(A) NIH3T3 cells were co-transfected with control or RBP-Jκ (100 ng/well) or LANA (350 ng/well) or RBP-Jκ and LANA together, along with UCHL1p-LUC wild type plasmid (500 ng/well) and β-gal expression constructs (250 ng/well). The control DNA was used as filler DNA to maintain the total amount of DNA constant. Luciferase assays were performed 48 h post-transfection. The data represent three independent experiments prepared in triplicate and normalized to β-gal activity. **(B)** ChIP/PCR analyses were performed to determine binding of RBP-Jκ factor to the putative partial binding sites on the UCH-L1 promoter with the use of specific RBP-Jκ antibody in KR4 LCLs. Normal IgG was used as negative control. PCR reactions were performed with primers targeting the partial RBP-Jκ-binding sites (see Materials and Methods), and amplified DNA products were resolved in 2% agarose gels.

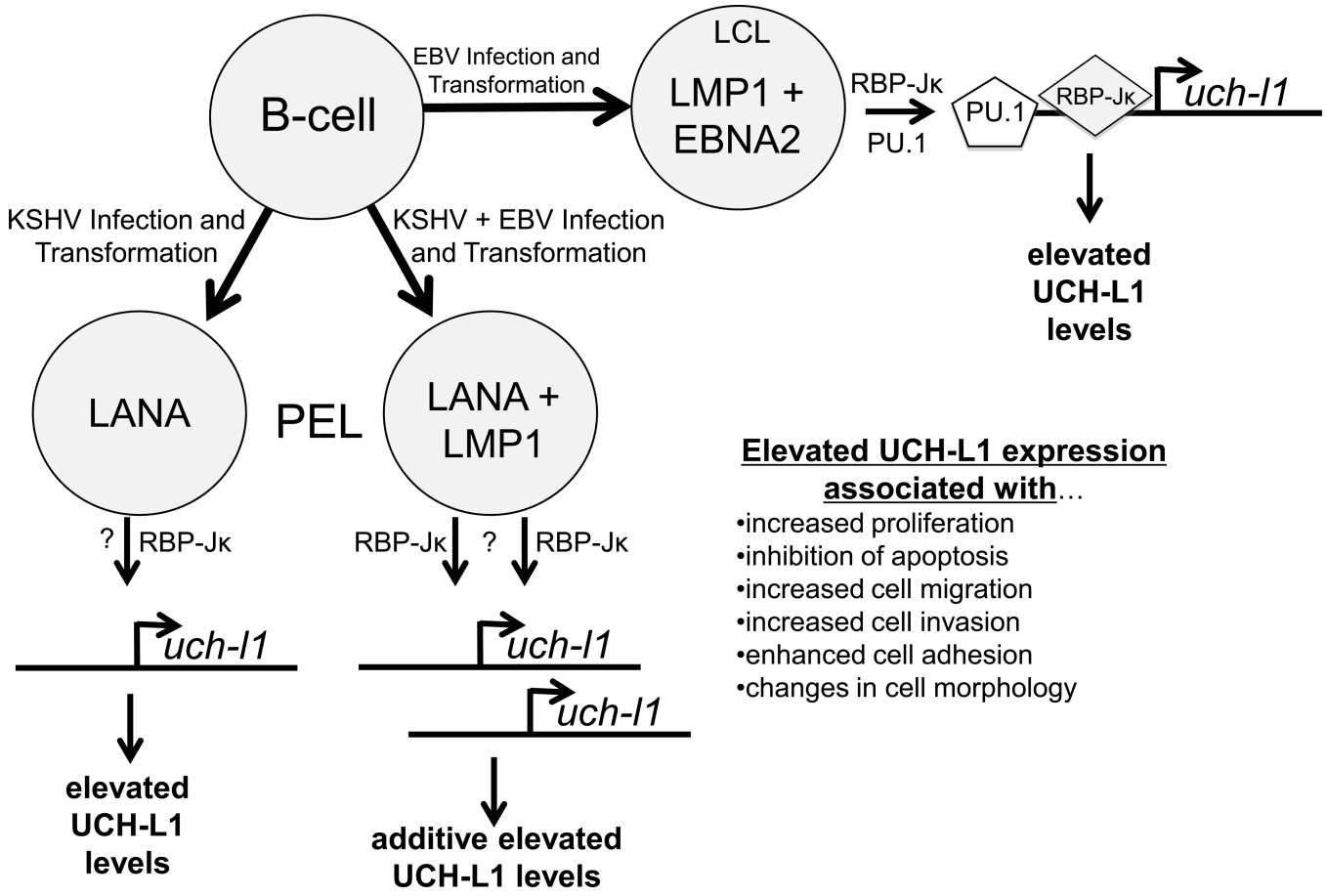

Figure 7. Proposed Model of the Induction of *uch-l1* following transformation of B cells by KSHV and/or EBV

Naïve B cells are infected and transformed by KSHV and/or EBV. EBV-mediated transformation results in EBNA2 and LMP1 expression, resulting in the activation of the *uch-l1* promoter via PU.1 and RBP-Jκ, respectively, and elevated UCH-L1 expression. KSHV-mediated transformation results in LANA expression, resulting in the activation of the *uch-l1* promoter via RBP-Jκ and elevated UCH-L1 expression. Transformation mediated by a dual KSHV/EBV infection results in expression of KSHV LANA and EBV LMP1. Both viral proteins independently activate the *uch-l1* promoter, at least in part through RBP-Jκ, resulting in enhanced activation of the *uch-l1* promoter and an additive elevation of UCH-L1 expression. Known tumorigenic phenotypes associated with elevated UCH-L1 expression include increased cell proliferation, migration, invasion, and adhesion as well as changes in cell morphology and inhibition of apoptosis.