EC_5S Ubiquitin Complex Is Recruited by KSHV Latent Antigen LANA for Degradation of the *VHL* and *p53* Tumor Suppressors

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Cellular protein degradation pathways can be utilized by viruses to establish an environment that favors their propagation. Here we report that the Kaposi's sarcoma–associated herpesvirus (KSHV)-encoded latency-associated nuclear antigen (LANA) directly functions as a component of the EC_5S ubiquitin complex targeting the tumor suppressors von Hippel-Lindau *(VHL)* and *p53* for degradation. We have characterized a suppressor of cytokine signaling box-like motif within LANA composed of an Elongin B and C box and a Cullin box, which is spatially located at its amino and carboxyl termini. This motif is necessary for LANA interaction with the Cul5–Elongin BC complex, to promote polyubiquitylation of cellular substrates *VHL* and *p53* in vitro via its amino- and carboxyl-terminal binding domain, respectively. In transfected cells as well as KSHV-infected B lymphoma cells, LANA expression stimulates degradation of *VHL* and *p53*. Additionally, specific RNA interference–mediated LANA knockdown stabilized *VHL* and *p53* in primary effusion lymphoma cells. Thus, manipulation of tumor suppressors by LANA potentially provides a favorable environment for progression of KSHV-infected tumor cells.

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Introduction

Kaposi's sarcoma–associated herpesvirus (KSHV) is a DNA tumor virus of the rhadinovirus subfamily associated with rare endothelial and lymphoid cancers which was first discovered in Kaposi's sarcoma (KS) lesions in 1994 [1]. It is also tightly linked to other lymphoproliferative disorders including primary effusion lymphoma (PEL) and multicentric Castleman's disease [2,3]. The rate of KS disease is increased by immunosuppression in both organ transplant patients and AIDS patients [4]. The KSHV-encoded latency-associated nuclear antigen (LANA) is initially identified as a DNA binding, nuclear transcription factor that also contributes to KSHV latent replication by maintaining the viral episome and is also involved in regulation of latency [5–8]. LANA targets downstream viral promoters through both direct DNA binding [9,10] and formation of protein complexes with at least four other cellular DNA binding transcription factors: CREB, CBP, Sp1, and STAT3 [11–15]. LANA also interacts physically with a number of cellular proteins, such as p53, pRB, and GSK3β, resulting in inhibition of *p53*-mediated apoptosis, chromosome instability, derepression of Rb-transcriptional activities, and dysregulation of β-catenin and the Wnt signaling pathway [16–19].

Protein ubiquitylation is important for regulation of a range of cellular processes in eukaryotes, including signal transduction, development, apoptosis, cell cycle progression, endocytosis, and immune response [20–22]. Many pathogens such as HPV (E6 deregulates *p53*) and HIV (Vif targets APOBEC3G) [23,24] have evolved numerous strategies to control protein degradation by mimicking, blocking, or redirecting the activity of the ubiquitin system [25]. In the ubiquitin proteolytic pathways, there are two distinct steps as follows: the covalent attachment of multiple ubiquitin molecules to the protein substrate and the degradation of the ubiquitylated protein by the 26S proteasome complex [22]. The system responsible for ubiquitin attachment consists of several components that act in concert. Using ATP as a substrate, a ubiquitin-activating enzyme E1 (i.e., Uba1) catalyzes the formation of a thioester bond between itself and ubiquitin and then transfers the activated ubiquitin to a ubiquitin-conjugating enzyme E2 (i.e., Ubc5a), which together with a ubiquitin-protein ligase E3 or not specifically transfers ubiquitin to the target protein [21,26,27]. The E3 ligase is generally thought to be a group of multisubunit complexes which are mostly responsible for substrate specific recognition [21]. To date, this group consists of a superfamily of E3 ligase complex including the following: (1) SCF (Skp1-Cul1-F-box)—in this complex, Skp1 links the scaffold protein Cullin

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Abbreviations: aa, amino acids; Elongin BC, Elongin B and C; Cul, Cullin; ECS, Elongin C-Cullin2/5-SOCS-box; HA, hemagglutinin; HIF-1α, hypoxia-inducible factor 1α; GST, glutathione *S*-transferase; IB, immunoblotting; IP, immunoprecipitation; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma–associated herpesvirus; LANA, latency-associated nuclear antigen; PEL, primary effusion lymphoma; SCF, Skp1-Cullin1-F-box; SOCS, suppressors of cytokine signaling; Ub, ubiquitin; *VHL*, von Hippel-Lindau gene; WT, wild-type

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Synopsis

Ubiquitin is a small 8.5-kDa polypeptide with 76 amino acids which is highly conserved in eukaryotes. Cellular proteins destined for degradation are covalently linked to ubiquitin by a process called ubiquitylation. This highly regulated process controls a broad range of fundamental cellular functions, including signal transduction, development, and apoptosis. Many pathogens invade host cells by mimicking, blocking, or redirecting the activity of the cellular ubiquitin system. Understanding the unique biological functions targeted by these pathogens is a key goal in developing strategies for prevention and protection against their invasions. This report describes a unique functional role of the latency-associated nuclear antigen (LANA) encoded by Kaposi's sarcoma–associated herpesvirus, a large DNA virus that persists in primary effusion lymphoma and multicentric Castleman's disease. LANA can modulate hypoxia-inducible factor 1α activities by down-regulation of the critical tumor suppressors von Hippel-Lindau *(VHL)* and *p53* in B lymphoma cells. In this pathway, LANA directly mimics and serves as an adaptor molecule for a specific E3 ubiquitin complex through an unconventional protein motif, to stimulate the ubiquitylation and degradation of both *VHL* and *p53*. This is of fundamental importance because it raises the interesting question as to whether this process is linked to regulation of infection and pathogenesis by tumor viruses associated with human cancers.

(Cul)1 to the substrate-specific adapter referred to as an F-box protein (β-TrCP) [28]; (2) ECS (Elongin C–Cul2/5-SOCS [suppressors of cytokine signaling]-box) complex, in which Elongin C bridges Cul2 or Cul5 to the SOCS-box–containing adaptor protein (SOCS and VHL) [29,30]; (3) BC3B (BTB-Cul3-BTB), in which the Skp1 and Elongin C structurally homologous protein BTB interacts with Cul3 to degrade target substrates (MEL-26) [31–34]; and (4) APC/C (APC2-C) E3 ligase complex, which probably using Cul4 protein as a scaffold [35,36]. Interestingly, in KSHV, the viral K3 and K5 proteins identified as PHD domain–containing E3 ubiquitin ligases enrich this superfamily [37–40].

Hypoxia-inducible factor 1α (HIF-1α) is a subunit of the heterodimer HIF-1 transcription factor that specifically responds to hypoxia and activates a set of genes known to be involved in angiogenesis, cell proliferation, survival, and glucose metabolism [41–43]. In normoxia, HIF-1α is targeted for ubiquitylation and degradation by both the tumor suppressor von Hippel-Lindau *(VHL)* as a component of the EC_2S E3 ubiquitin ligase [29] and *p53*-associated Mdm2 as an SCF E3 ligase [44]. Previous studies have shown that the transcriptional activity of HIF-1α is increased not only by intratumoral hypoxia but also by genetic alterations and loss of function of cellular regulators including *VHL* and *p53* [29,44].

In this report, we investigated the mechanisms by which the KSHV encoded LANA targets the HIF-1α suppressors *VHL* and *p53* for degradation. LANA recruits the ubiquitin-proteasome pathway resulting in a powerful inhibitory effect on *VHL*- and *p53*-mediated HIF-1α degradation. The mechanism that involves binding to and targeting *VHL* and *p53* for degradation is mediated by an unconventional intrinsic ubiquitin E3 ligase activity encoded by the SOCS-box–like motif of LANA. These results imply that this ubiquitin-proteasome pathway regulates normal *VHL* and *p53* turnover and show that their destabilizations are greatly enhanced by

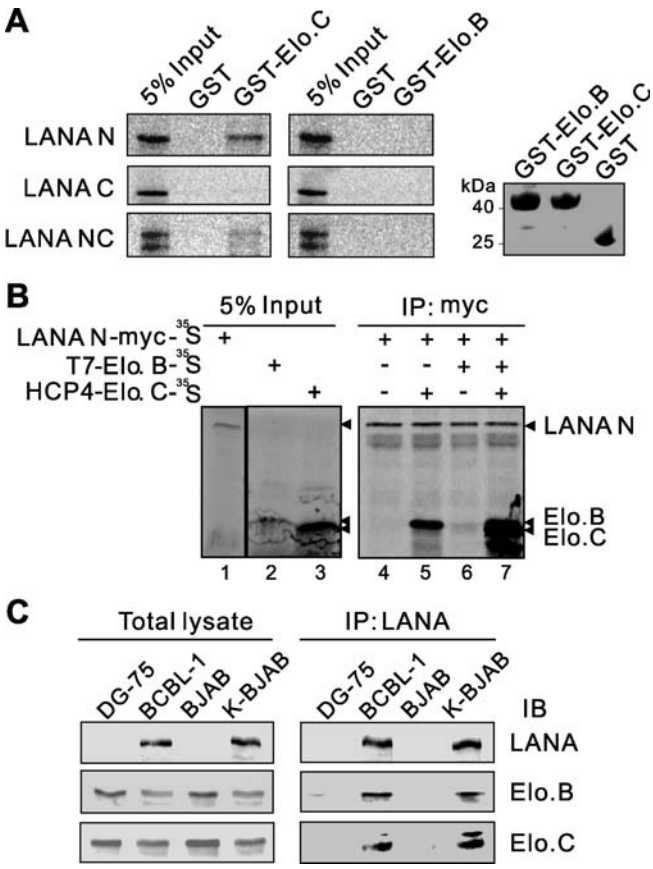

Figure 1. LANA Associates with the Elongin BC Complex

(A) The amino-terminal domain of LANA directly interacts with Elongin C but not Elongin B in vitro. The cDNA encoding N (1–340 aa), C (762-1162 aa), or NC (1–327˜929-1162 aa) truncation of LANA with myc tagged were expressed in a coupled transcription/translation system in the presence of [^{35}S]methionine. Aliquots (20 μl) of the translation products were pulled down by GST, GST–Elongin C, or GST–Elongin B, respectively. The pulldown complex as well as a 1-μl aliquot of the transcription/translation reaction (Input) were fractionated by SDS-PAGE, and detected by a PhosphorImager. The proteins of GST and GST fused with Elongin B or Elongin C were shown by Coomassie staining in the right panel.

(B) Elongin C is critical for LANA binding to Elongin B. In vitro produced and [^{35}S]-labeled proteins LANA N (1–340 aa), Elongin C, and Elongin B were incubated with anti-myc antibodies. The interacting complexes were resolved by SDS-PAGE and examined by a PhosphorImager.

(C) LANA associates with Elongin BC complex in KSHV-infected cells. Cell lysates of BCBL-1, DG-75, BJAB, or BJAB with KSHV infection (K-BJAB) were subjected to IP against LANA or Elongin C (Elo. C) followed by IB against LANA, Elongin C, and Elongin B (Elo. B).

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interaction with LANA. This can contribute to a disruption of the induced antiviral state during the KSHV latent infection.

Results

Identification of LANA as an Elongin BC Interacting Protein

Previously, to identify binding partners for KSHV-encoded latent protein LANA, a yeast two-hybrid screen was performed using LANA as bait, together with a cDNA library from human endothelial cells. One cDNA identified in this screen was found to encode Elongin C. Since Elongin C has been shown to directly bind to BC-box containing proteins which usually interact with Elongin B and C complex (Elongin

BC), the association of LANA with Elongin C in yeast is likely to also involve Elongin B. To confirm the binding results from the yeast two-hybrid screen, we examined in vitro binding assays of Elongin BC to LANA. In in vitro translated [^{35}S]-labeled methionine, different truncated forms (N: 1–340 amino acids [aa], C: 762-1162 aa, and NC: 1–327^929-1162 aa) of LANA with myc tag were incubated with glutathione *S*-transferase (GST)-fused Elongin B (Elo. B) or Elongin C (Elo. C) beads. As shown in Figure 1A, LANA did not bind Elongin B, whereas it clearly bound to Elongin C. Moreover, the amino-terminal domain of LANA predominantly interacted with Elongin C. This was further confirmed by reverse immunoprecipitation (IP) using a myc-tagged LANA N truncated polypeptide (Figure 1B, lanes 5 and 6). In further experiments, association between LANA and the Elongin BC complex was assessed in co-IP experiments. Interestingly, Elongin B efficiently precipitated with the amino-terminal domain of LANA only when incubated along with Elongin C (Figure 1B, compare lanes 6 and 7).

To specifically address the question as to whether or not LANA does associate with the Elongin BC complex in KSHV-infected cells, co-IP assays were done in PEL cells positive for KSHV as well as an isogenic set of BJAB cell lines with and without KSHV infection. The results of this assay nicely showed that LANA associates with Elongin B as well as Elongin C in the PEL cell line BCBL-1 as well as in a KSHV-infected BJAB cell line (Figure 1C). This suggests that LANA does exist in complex with Elongin BC in KSHV-positive cells.

The LANA/Elongin BC Complex Interacted with a Cul5/Rbx1 Module

The Elongin BC complex was shown to be involved in ubiquitylation as a component of the ECS E3 complex. To determine whether the Elongin BC complex could function as an adaptor which links LANA to a Cullin/Rbx1 module, thus reconstituting an E3 ubiquitin ligase complex, IP assays against myc tag were performed on lysates of Saos-2 cells cotransfected with hemagglutinin (HA)-Rbx1 and HA-Cullin (Cul2 or Cul5) in the presence or absence of myc-tagged LANA expression vectors (Figure 2A). The results showed that LANA did not associate with the Cul2/Rbx1module. However, LANA did assemble with the Cul5/Rbx1 module (Figure 2A). Further experiments with or without Elongin BC coexpression demonstrated that LANA–Elongin BC complex did assemble with the Cul5/Rbx1 module to reconstitute a multiprotein complex containing LANA, Elongin BC, Rbx1, and Cul5 (Figure 2B) and the Elongin BC complex can increase the affinity of LANA interaction with Cul5 and Rbx1 module (Figure 2B).

The SOCS-Box–Like Motif Is Essential for LANA Association with Cul5–Elongin BC Complex

A well-known family of Elongin BC–interacting proteins, including *VHL* tumor suppressor and SOCS proteins, has been recently identified as SOCS-box proteins [30]. These proteins directly interact with Elongin BC through their conserved BC-box motif [(A/P/S/T)L-x_3-C-x_3-(A/I/L/V)] and associate with Cullin proteins through the conserved Cul-box motif [ΦxxLPxP] [30]. Moreover, the two Cul boxes, the Cul2 box and the Cul5 box, are specific for distinguishing binding with the different Cullin proteins (Cul2 or Cul5). To determine whether LANA interacts with Elongin BC and the Cullin protein through its SOCS-box motif, we first aligned the sequence of LANA with *VHL* and SOCS (1, 2, and 3) proteins. Our analysis showed that LANA probably also contains an SOCS-box–like motif based on the hydrophobic homology of consensus sequences. However, the BC box and Cul box in this viral SOCS-box motif are atypical as distinguished from the BC box which is usually close to the Cul box found in SOCS-box motifs of cellular proteins. In KSHV LANA these motifs were separately located from each other in the amino-terminal and the carboxyl-terminal domains. Furthermore, in contrast to the *VHL* Cul2 box and SOCS protein Cul5 box, the LANA Cul box was significantly similar to the Cul box of the SOCS protein family. This supports the evidence of LANA associated with Cul5, not Cul2, protein in the IP assay above. Additionally, the ORF73 homolog of LANA encoded by other gammaherpesvirus, including the rhesus monkey rhadinovirus (RRV) or herpesvirus saimiri (HVS A11 and C488), also contains a similar SOCS-box–like motif (Figure 3A).

To confirm that LANA associates with Elongin BC and Cullin 5 protein through its BC-box and Cul box motif, the corresponding site-specific mutation and deletion of the LANA SOCS-box–like motifs were generated. Co-IP assays using different LANA mutants showed that the BC-box motif deletion mutant resulted in a failure of LANA to bind with the Elongin BC complex (Figure 3B). Additionally, LANA

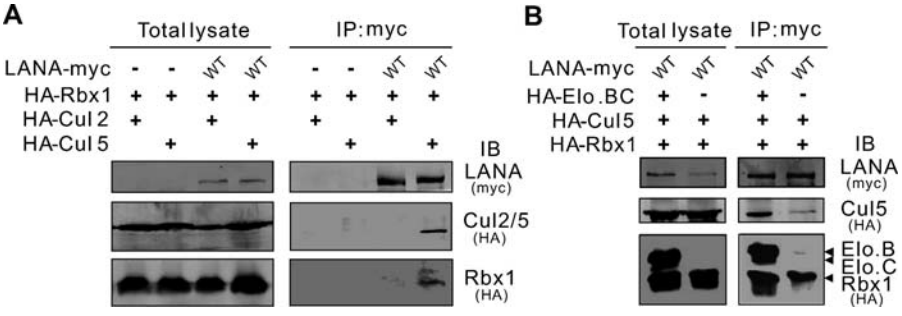

Figure 2. The LANA–Elongin BC Complex Assemble with the Cul5/Rbx1 Module

(A) LANA protein associates with the Cul5/Rbx1 module but not Cul2/Rbx1 module, and (B) Elongin BC complex increases interaction of LANA with the Cul5/Roc1 module. Saos-2 cells were transfected with expression vector encoding the indicated proteins in the figure. The lysates underwent IP using anti-myc antibodies. Crude extracts (left panels) and immune complexes (right panels) were separated by SDS-PAGE and IB with the indicated antibodies.

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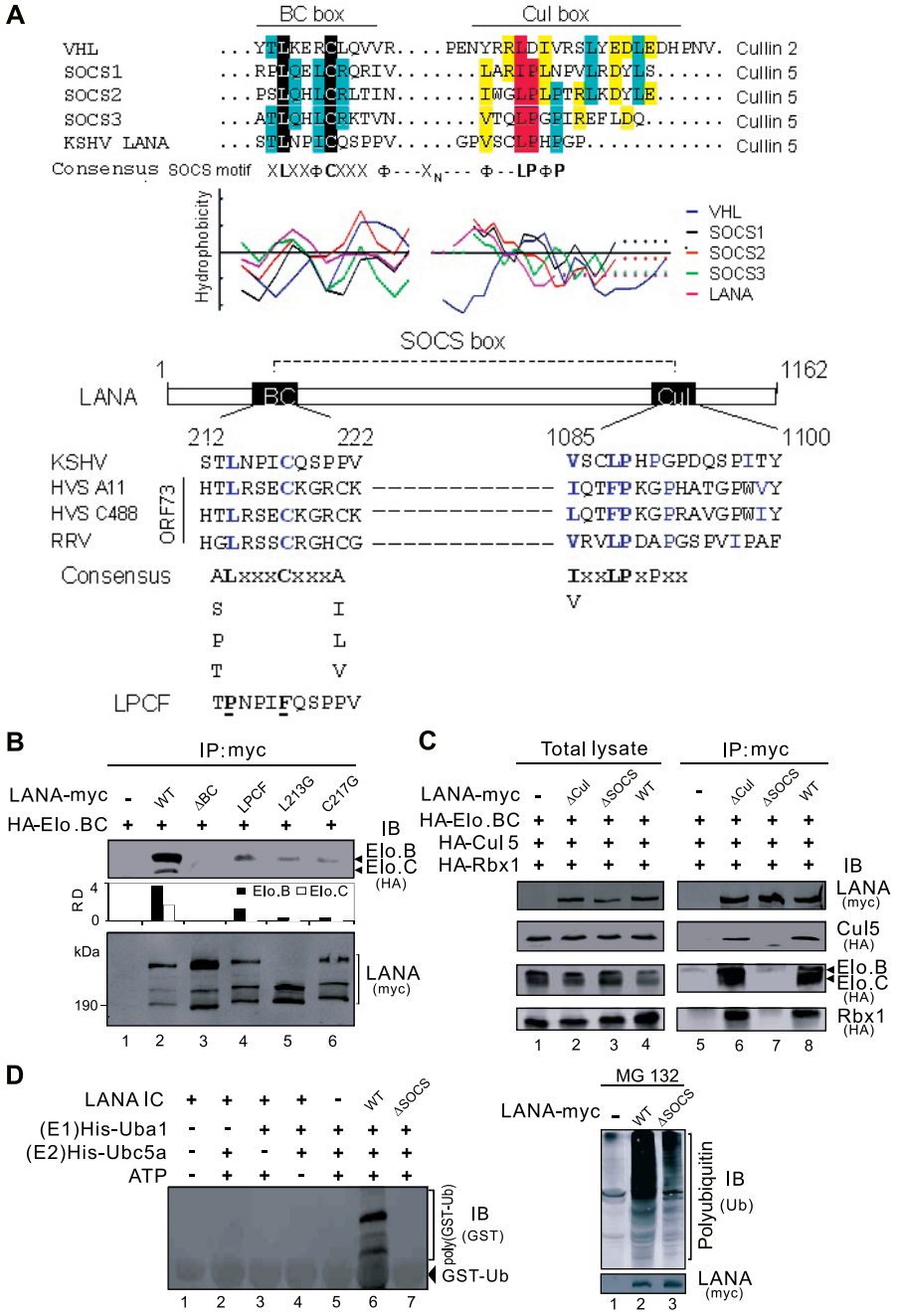

Figure 3. LANA Associates with the Elongin BC Complex and Cul5 through the SOCS-Box–Like Motif

(A) KSHV LANA contains a putative SOCS-box motif which includes a consensus of BC-box and Cul-box motifs. Upper panels show hydrophobic sequence alignment of the BC-box and Cul-box motifs from viral LANA and cellular SOCS-box–containing proteins that bind Elongin BC and Cullin proteins individually. Conserved residues with similarity are shaded in the upper panel. Lower panels show ORF73 proteins from other gammaherpesvirus contain an SOCS-box–like motif which is similar to KSHV LANA. HVS, herpesvirus saimiri; RRV, rhesus monkey rhadinovirus. The site mutations of LANA in leucine (L) and/or cystine (C) are indicated with underlining.

(B) LANA associates with the Elongin BC complex through the BC-box motif. Saos-2 cells were transfected with expression vector encoding the indicated proteins. The WT LANA and its mutant with BC-box (ΔBC) deletion and point mutation (LPCF, L213G or C217G) within BC box were compared. Since LANA lost its normally top bands (approximately 230 kDa) in the L213G mutant, another two lower bands of LANA (between 190 kDa and 230 kDa) were specifically present. The lysates underwent IP and IB assays as described previously. RD, relative density.

(C) LANA associates with Cullin 5 through the Cul-box motif. Saos-2 cells were transfected with expression vector encoding the indicated proteins. The WT LANA with Cul-box (ΔCul) or SOCS-box (ΔSCOS) deletion was compared. The lysates underwent IP and IB assays as described previously. Crude extracts (left panels) and immune complexes (right panels) are shown.

(D) LANA assembles with Elongin BC complex and Cul5/Rbx1 module to reconstitute a multiprotein complex with E3 ubiquitin ligase activity. Left panel shows the LANA–Elongin BC–Cul5–Rbx1 complex had ubiquitin ligase activity. The cell lysates of WT LANA (Figure 3C, lane 4) or SOCS-box–like motif deletion mutant (Figure 3C, lane 3) as control was subjected to anti-myc immunoaffinity purification. The purified LANA immune complex (IC) was incubated with various combinations of Uba1 (E1), Ubc5a (E2), and GST-ubiquitin (GST-Ub) in the absence or presence of ATP, to assess its ability to stimulate ubiquitylation in vitro. The proteins were separated by SDS-PAGE and IB using anti-GST antibodies. Right panel shows SOCS-box–like motif deletion reduced the capacity of LANA to induce polyubiquitylation. Saos-2 cells were transfected with WT LANA or its SOCS mutant. After 24-h transfection, cells were treated with MG132 (0.5 μM) for 2 h. Lysates were subjected to IB against Ub or myc antibodies.

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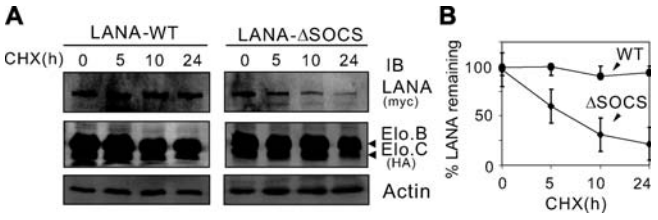

Figure 4. Binding to the Elongin BC Complex Stabilizes LANA

(A) The stabilities of WT LANA and its SOCS motif mutant. Five million Saos-2 cells were transfected with 5 μg of LANA-WT (left panel) or LANA-ΔSOCS (right panel) expression vector in the presence of 5 μg of Elongin B and 5 μg of Elongin C expression vectors, as indicated. Twenty-four hours after transfection, cells were treated with 100 μg/ml cyclohexamide (CHX) for different times (0, 5, 10, and 24 h). Aliquots (40 μg) of each whole-cell extract underwent IB with the indicated antibodies.

(B) The relative levels of WT LANA and its SOCS mutant coexpression with Elongin BC complex. Protein density was quantitated by densitometry of immunoblots using Odyssey Image v1.2 from three separate experiments.

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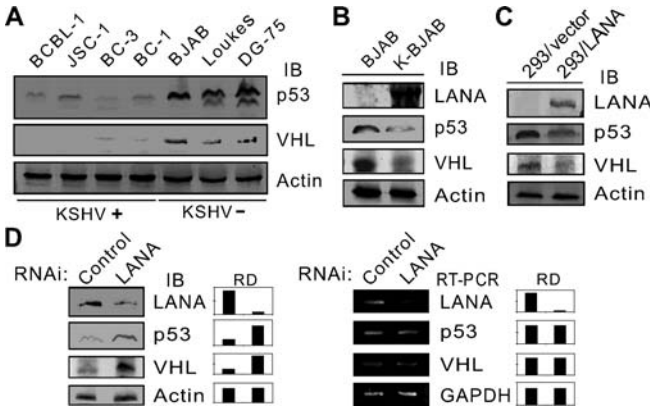

Figure 5. The Tumor Suppressors *p53* and *VHL* Are Inhibited in the KSHV Latently Infected and LANA-Expressing Cells

(A) IB analysis of KSHV-positive cell lines (BC-3, BCBL-1, JSC-1, and BC-1) and KSHV-negative cell lines (BJAB, DG-75, and Louckes) against *p53*, *VHL*, and β-actin. Ten million of each cell line were lysed and coimmunoprecipitated using anti-*VHL* antibodies followed by detection with anti-*VHL*. Cell lysate (5%) was used in IB assays with anti-*p53* and anti–β-actin. The data show that *p53* and *VHL* in KSHV-positive cells are more strongly inhibited than in KSHV-negative cells.

(B) The levels of p53 and VHL protein in B-lymphoma cells with KSHV latent infection. Ten million BJAB- or KSHV-infected BJAB (k-BJAB) cells were lysed and coimmunoprecipitated using anti-*VHL* antibodies followed by detection with anti-*VHL*. Cell lysate (5%) was used in IB assays against LANA, *p53,* and β-actin. The data showed that the tumor suppressor proteins p53 and VHL were inhibited during KSHV infection.

(C) *p53* and *VHL* are inhibited in the LANA stable expressing cells. Ten million 293/LANA or 293/Vector stable cell lysates were analyzed by IB against LANA, *p53,* and *VHL* as described previously. The data showed that LANA decreases tumor suppressor *p53* and *VHL* levels.

(D) *p53* and *VHL* turnover in the LANA knockdown BCBL-1 cells. Cell lysates from stable BCBL-1 cells with LANA or control firefly luciferase knockdown (RNAi) were subjected to IB assays (left panel) against LANA, *p53, VHL,* and β-actin, or total RNA underwent RT-PCR analysis (right panel). RD, relative density.

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mutants with specific site mutations in the BC-box motif also resulted in a decrease in the ability of LANA to bind to the Elongin BC complex when compared with wild-type (WT) LANA (Figure 3B). LANA binding with Cul5/Rbx1 module and Elongin BC complex was completely disrupted when the SOCS-box motif was deleted (Figure 3C). Cul5 is known to interact with Elongin BC complex [24,30,45–47]. Thus, when the LANA Cul-box motif deletion mutant was coexpressed with Elongin BC and tested for binding to the Cul5/Rbx1 module and Elongin BC complex, we observed that the LANA mutants with Cul5 box deletion had same remaining binding activity to the Cul5/Rbx1 module and Elongin BC complex (Figure 3C). To confirm Cul-box motif is necessary for LANA to specifically bind Cul5 protein, further experiments were performed and demonstrated that LANA with the Cul-box deleted lost binding to the Cul5 protein when assayed without the Elongin BC complex coexpression (unpublished data). Taken together, these data suggest that these motifs are critical for LANA interaction with the Elongin BC/Cul5/Rbx1 module and indicate that BC-box motif and Cul-box motif are likely to be in close proximity in terms of the spatial structure of the LANA protein in order to constitute an intact SOCS-box motif.

The LANA–Elongin BC Complex Is Capable of Assembling with Cul5/Rbx1 Module to Reconstitute an E3 Ubiquitin Ligase

To investigate whether the LANA–Elongin BC–Cul5–Rbx1 complex possesses ubiquitin ligase activity, the complex was immunoaffinity purified and assayed for its ability to activate formation of polyubiquitin chains by the E2 ubiquitin–conjugating enzyme Ubc5a in the presence of ATP, the E1 ubiquitin–activating enzyme Uba1, and GST-ubiquitin. As shown in Figure 3D, the LANA–Elongin BC–Cul5–Rbx1 complex stimulated formation of a ladder suggesting polyubiquitylation of the GST-ubiquitin, whereas the LANA SOCS-box mutant did not (Figure 3D, left panel, lanes 6 and 7). As expected, formation of polyubiquitin conjugates depended on the presence of the E1 ubiquitin–activating enzyme, E2 ubiquitin–conjugating enzyme, and ATP (Figure 3D). In addition, in vivo proteasome inhibitor assays further showed that WT LANA was capable of significantly enhancing polyubiquitylation activities when compared to that of the LANA ΔSOCS mutant (Figure 3D, right panel, lanes 2 and 3).

Elongin BC Binding Increases Stability of LANA Expressed in Cells

During our studies, we observed that the levels of WT LANA coexpressed with Elongin BC were consistently increased. Therefore, to determine whether this increase in levels of WT LANA could be attributed at least in part to potential stabilization of LANA by Elongin BC, WT LANA or the SOCS-box–like motif deletion mutant (ΔSOCS) was transiently expressed in Saos-2 cells with or without Elongin BC. Twenty-four hours after transfection, cyclohexamide was added to cultures to block further protein synthesis, and the rate of decay of WT and mutant LANA protein was measured by Western blot analysis. The results showed that the level of WT LANA protein remained constant for at least 24 h after treatment of cyclohexamide to cells cotransfected with LANA along with Elongin BC (Figure 4A, left panel, and 4B). However, in the absence of cotransfected Elongin BC when an SOCS-box deletion mutant of LANA was used, almost 50% of the LANA protein was lost within 5 h. Thus, WT LANA protein exhibits increased stability when coexpressed with Elongin BC (Figure 4A, right panel, and 4B). In contrast to

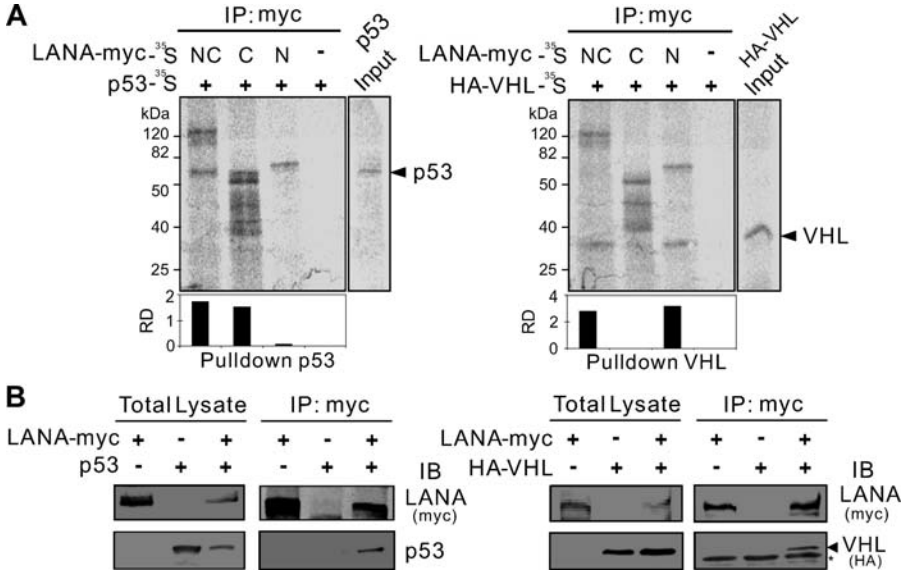

Figure 6. Distinct Domains of LANA Interact with Tumor Suppressors *p53* and *VHL*

(A) The amino-terminal domain of LANA binding *VHL* and carboxyl-terminal domain binding *p53* in vitro. The cDNA encoding N (1–340 aa), C (762-1162 aa), and NC (1–327˚929-1162 aa) LANA with myc tag, HA-VHL, and *p53* were translated in a coupled transcription/translation system in the presence of [^{35}S]methionine. Fifteen-microliter aliquots of the translation products were immunoprecipitated with anti-myc antibodies. Immunoprecipitated proteins (IP) and a 1-μl aliquot of the transcription/translation reaction (Input) were fractionated by SDS-PAGE and detected by autoradiography. RD, relative density.

(B) LANA is associated with *p53* and *VHL* in cells. Saos-2 cells were cotransfected with expression vector encoding the indicated proteins. The lysates underwent IP using anti-myc antibodies. Crude extracts (left panels) and immune complexes (right panels) were separated by SDS-PAGE and IB with antibodies indicated. Asterisk indicates IgG heavy chain.

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WT LANA, the ΔSOCS LANA mutant exhibited similar instability in the presence or absence of cotransfected Elongin BC (unpublished data), suggesting a requirement for the SOCS-box–like motif of LANA for enhanced stability.

Levels of the Tumor Suppressors *p53* and *VHL* Are Significantly Reduced in LANA-Expressing Cells

To determine the effect of LANA on targeted protein degradation, we examined the endogenous levels of some cellular tumor suppressors (such as *p53* and *VHL*) expression in KSHV-positive (BCBL-1, JSC-1, BC-3, and BC-1) and -negative (BJAB, Loucks, and DG75) cell lines. Similar to previous studies which showed that p53 protein expression was decreased in KSHV-positive cells [17], our Western blot analysis of four KSHV-positive PEL cell lines showed that p53 levels were significantly reduced compared to KSHV-negative B cell lines (Figure 5A). Additionally, the levels of the HIF-1α–suppressor protein VHL were also reduced in KSHV-positive cells (Figure 5A). To avoid the inconsistency of genotype among different cell lines, we also compared the levels of p53 and VHL in cell lines with the same genotypic background BJAB and KSHV-infected BJAB. The resulting data further confirmed that levels of the tumor suppressor proteins p53 and VHL did dramatically reduce after the KSHV infection (Figure 5B). To determine that the results for both *p53* and *VHL* inhibition was associated with LANA, stable 293 cell lines expressing LANA were generated. Similar results of tumor suppressor *p53* and *VHL* inhibition by LANA were observed when immunoblotting (IB) assays were performed (Figure 5C). To further confirm the role of LANA on *p53* and *VHL* inhibition, LANA in the KSHV-positive BCBL-1 cell line was knocked down by the introduction of small interference RNA specifically against LANA. Again, analysis using IB assays showed that the tumor suppressors *p53* and *VHL* were greatly rescued on inhibition of LANA expression (Figure 5D, left panel). Furthermore, no obvious change was observed for *p53* and *VHL* mRNA transcripts after LANA transcripts were decreased, supporting the hypothesis that LANA can induce the degradation of p53 and VHL proteins (Figure 5D, right panel).

LANA Mediates the Degradation of *p53* and *VHL* through Ubiquitylation

We wanted to explore whether the potential mechanism for LANA-mediated degradation of the tumor suppressors *p53* and *VHL* was related to LANA recruitment of the EC_5S ubiquitin E3 ligase complex. As *p53* was shown to interact with the carboxyl-terminal domain of LANA [17], we first determined the binding domain of LANA with *VHL* with in vitro [^{35}S]-labeled methionine translated proteins using the p53 protein as a positive control. The results of the in vitro pulldown assays showed that distinct from LANA binding to *p53* through its carboxyl-terminal domain, the region of LANA binding to *VHL* was located in the amino-terminal domain using in vitro IP assays (Figure 6A). Supporting in vivo IP assays further confirmed that full-length LANA was associated with the tumor suppressors *p53* and *VHL* (Figure 6B).

To determine the extent of the involvement of LANA recruitment of the EC_5S E3 ligase for degradation of *p53* and *VHL*, we utilized the specific SOCS-box mutant of LANA which lacks association with the EC_5S complex. The constructs expressing *p53*, HA-ubiquitin (Ub), and WT LANA or its SOCS-deleted mutant were cotransfected into Saos-2 cells.

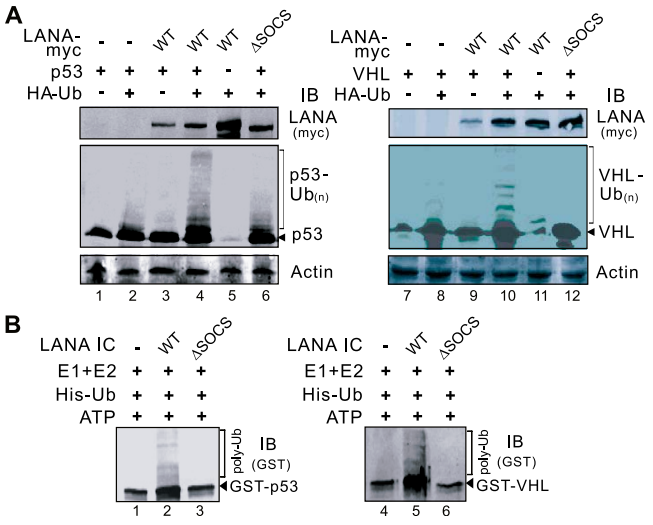

Figure 7. LANA Promotes Polyubiquitylation of Tumor Suppressors *p53* and *VHL*

(A) LANA induces *p53* and *VHL* polyubiquitylation. Saos-2 (left panel) or 786-O (right panel) cells were cotransfected with different combinations of myc-LANA (WT and ΔSOCS), myc-*p53* (or HA-VHL), and HA-Ub as indicated. At 48 h posttransfection, transfected cells were harvested, lysed, and protein normalized. Total protein (40 μg) was subjected to resolve and IB assays against myc (LANA), *p53* (or *VHL*), and β-actin. The data showed LANA can induce *p53* and *VHL* ubiquitylation in vivo.

(B) The LANA–Elongin BC–Cul5–Rbx1 complex induces *p53* and *VHL* polyubiquitylation in vitro. The cell lysates of WT LANA (Figure 3C, lane 4) or SOCS-box–like motif deletion mutant (Figure 3C, lane 3) as control was subjected to anti-myc immunoaffinity purification. The purified LANA immune complex (IC) was incubated with various combinations of Uba1 (E1), Ubc5a (E2), His-Ub, or GST-VHL (or GST-p53) in the presence of ATP in vitro. The proteins were separated by SDS-PAGE and IB against GST antibody. Left panel, GST-p53; right panel, GST-VHL.

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The Western blot data showed that ubiquitylation of *p53* in the presence of LANA was dramatically increased but minimally so when the ΔSOCS mutant of LANA was coexpressed (Figure 7A, left panel, lanes 4 and 6). Similarly, WT LANA also can induce *VHL* polyubiquitylation more efficiently when compared to the ΔSOCS mutant in *VHL*-deficient cell line 786-O (Figure 7A, right panel, lanes 10 and 12). Unexpectedly, the level of LANA could be seen to be decreased when *p53* was coexpressed (Figure 7A, compare lane 5 with lanes 3 and 4) and slightly decreased when *VHL* was coexpressed (Figure 7A, compare lane 11 with lanes 10 and 12). This indicated that although LANA can mediate degradation of the tumor suppressors *p53* and *VHL* through interaction with its amino and carboxyl-terminal domains, the tumor suppressors *p53* and *VHL* are also likely to contribute to regulation of LANA levels, as both are components of two separate E3 ligase complex that may also regulate LANA's activity, and these activities may functionally compete in virally infected cells [29,44].

To detect the specificity of LANA for targeting *p53* and *VHL* for degradation, in vitro, LANA–Elongin BC–Cul5 complex ubiquitylation assays were performed using purified GST-*p53* or GST-*VHL* as substrates. The results showed that only WT LANA could specifically recruit ubiquitin complex to induce GST-*p53* or GST-*VHL* polyubiquitylation, but not its SOCS-deleted mutant or vector control in this in vitro assay (Figure 7B).

Aberrantly Expressed LANA Conquers *p53* or *VHL* Function as Components of E3 Ubiquitin Complexes Leading to Their Degradation

As both *p53* and *VHL* are components of two separate E3 ubiquitin complexes, we wanted to determine whether aberrantly expressed LANA can conquer *p53* and *VHL* functions as components of E3 ubiquitin complex leading to their degradation in KSHV-infected cells. We tested the interaction of the tumor suppressor *p53* or *VHL* with LANA at different expression levels, as well as the effect on one of their downstream effectors proteins, HIF-1α [29,44]. As expression of LANA was increased without exogenous *p53* or *VHL* coexpression, we found that LANA reduced levels of *p53* and *VHL* in a dose-dependent manner (Figure S1A and S1B, right panels). As expected, when *p53* was coexpressed with LANA and HIF-1α with increasing concentrations of *p53,* the levels of LANA as well as of HIF-1α levels were reduced, which was similar to expression of *p53* and HIF-1α alone (Figure S1A, left panels). Similarly, increasing concentrations of *VHL* also inhibit LANA expression in a dose-dependent manner (Figure S1B, left panels). To determine the functional consequences of LANA on HIF-1α transcriptional activities in the context of *p53* and *VHL*, reporter assays were performed using an HIF-1α response element reporter in various cell lines. The reporter assays further confirmed that the transcriptional activity of HIF-1α was strikingly reduced when *p53* and *VHL* were cotransfected, whereas increased levels of LANA coexpressed rescued HIF-1α transcriptional activity most likely through targeting and degrading the HIF-1α suppressors *p53* and *VHL* (Figure S2). Therefore, these results suggested that LANA expression is involved in regulating the degradation of the tumor suppressors *p53* and *VHL* during KSHV-mediated oncogenesis, thus inhibiting their suppressive effects on HIF-1α transcriptional activities.

Discussion

In humans, targeting regulators of angiogenesis to proteasome-mediated degradation in virus-infected cells is getting increasing attention. The KSHV-encoded latent protein LANA was originally identified as a major viral antigen whose expression plays a crucial role in replication and maintenance of the viral episomes in PEL cells [6,48–51]. Here we demonstrated that the KSHV-encoded LANA protein interacts with the Elongin BC complex through the binding of Elongin C to the LANA BC-box motif located within its amino terminus. The LANA–Elongin BC complex was capable of assembling with a Cullin/Rbx1 module to reconstitute a multiprotein complex with ubiquitin ligase activity. Furthermore, the facts that LANA only associated with Cul5, and not with Cul2, and that a mutation in the Cul-box motif of LANA at its carboxyl terminus abolished Cul5 binding in the absence of Elongin BC indicate that LANA can directly interact with Cul5. Interaction of the Elongin BC complex with the BC box markedly increased the stability of LANA protein, suggesting that it may be necessary to protect LANA from degradation in order to assemble the functional E3 ubiquitin ligase complex, as previously proposed for SOCS-box family proteins [52,53]. Thus, our results suggest that LANA can recruit the Elongin BC complex and Cul5/Rbx

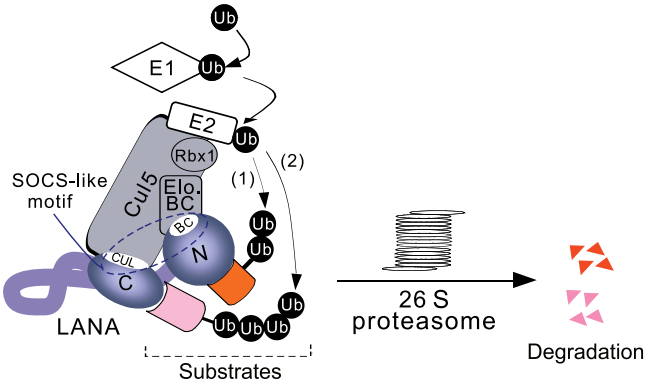

Figure 8. A Model for KSHV LANA Assembles EC_5S Ubiquitin Complex to Target Downstream Substrates for Degradation

LANA is predicted to form a complex with Cul5/Rbx1 that interacts with Elongin BC but not LANA ΔSOCS (Cul box and BC box) mutant. LANA acts as adapter to link substrates which bind at its amino (1) or carboxyl (2) -terminal domain (like *VHL* and *p53*) to EC_5S ubiquitin complex and induces the pathway of ubiquitin E1 activation, E2 conjugation, and substrate polyubiquitylation as well as 26S proteasome–mediated degradation.

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module through its SOCS-box–like motif to function as an EC_5S ubiquitin ligase. Binding of the Elongin BC complex served to stabilize LANA, and binding to Cul5 provided a bridge to recruit Rbx1 and activate the ubiquitin-conjugating process.

Ubiquitin conjugation to protein substrates is strictly regulated by the specificity of the ubiquitin–protein E3 ligase complex. Therefore, identification of key regulators of angiogenesis in human cancers targeted for proteasomal degradation and mediated by KSHV-encoded LANA is highly significant. Previous evidence has shown that HIF-1α is a critical regulator of numerous cellular growth factors and can also function as a regulator of tumor angiogenesis, growth, and metastasis and is commonly overexpressed in a number of human cancers [43,54–56]. Recently, *VHL* and *p53* have been demonstrated to be involved in the regulation of HIF-1α through ubiquitin-mediated degradation [29,44]. In this study, we present data to show that the levels of the HIF-1α–suppressor protein VHL were dramatically decreased in KSHV-positive compared with KSHV-negative cells. Additionally, the tumor suppressor protein p53 was also significantly reduced in KSHV-infected cells as previously reported [17]. The WT LANA, but not mutant deleted for the SOCS-box motif, facilitated *VHL* and *p53* ubiquitylation, suggesting that LANA is involved in the degradation of these HIF-1α–suppressor molecules and can function as an adaptor molecule to the EC_5S ubiquitin ligase complex which contributes to the rescue of HIF-1α. These findings shed light on the unique property of KSHV latently infected KS lesions predominantly seen in the lower extremities in elderly patients in whom oxygen is typically poor [57].

Interestingly, both Cul2 and Cul5 are known to be involved in ECS-type E3 ubiquitin ligase complex. As to why LANA preferentially associates with the Cul5 and not the Cul2 protein, our data suggest that the Cul-box motif as well as additional sequences in the vicinity located in the carboxyl terminus of LANA may be important in this selection process. The Cul5–Elongin BC complex was shown to interact with a number of SOCS-box–containing proteins, such as MUF1, ASB2, and the SOCS family of proteins [45,47,58]. Although the mechanism by which Cul5 functions in these processes remains unclear, some viral proteins recruiting Cul5-containing ECS ubiquitin ligase have been shown to regulate the turnover of molecules involved in cell-cycle control, such as adenovirus E4 orf6 [59] and the HIV vif-1 proteins [46]. Here the interaction of LANA with Cul5 further emphasizes a role for Cul5 in the regulation of virus-mediated dysregulation of cell growth and oncogenesis.

A major difference between the SOCS-box motif of KSHV LANA and the consensus SOCS-box sequence of cellular proteins is the greater distance between the BC box and the Cul box in this motif [30]. Our data strongly suggest that this specific characteristic of the SOCS-box–like motif in ORF73 proteins encoded by other gammaherpesvirus is highly conserved and that this structure allows LANA greater flexibility for interaction with the Elongin C and Cullin proteins to achieve its function. The fact that LANA can bind to *VHL* and *p53* via its amino- and carboxyl-terminal domains, respectively, inducing their degradation, is consistent with this hypothesis (Figure 8).

It is noteworthy to mention that the cellular tumor suppressors *VHL* and *p53* can also inhibit the ability of the viral protein LANA to regulate HIF-1α levels when they are highly expressed, as they can also act as adaptors of EC_2S– and SCF–type E3 ligase complex, respectively. Thus, the utilization of *VHL*-deficient 786-O cells or *p53*-deficient Saos-2 cells was critical for clearly defining the functional role of LANA in the regulation of *VHL* and *p53* [29,44]. This also indicates that the balance between the cellular mechanisms usurped by KSHV and the host cellular defense mechanisms is critical for determination of cell fate. The higher levels of LANA expression in KSHV-infected cells are likely to be crucial to subdue the cellular defenses during KSHV infection (Figure S3), and this is consistent with the fact that LANA is highly expressed in KS and PEL cells [60–62].

This study reinforces the view that regulation of protein degradation is an important mechanism for the control of angiogenesis and oncogenesis during KSHV latent infection.

Materials and Methods

Plasmids, cell lines, and culture. Constructs expressing LANA full length and its truncated LANA1–340 (N), LANA762-1162 (C), and LANA1–327^929-1162 (NC) in the pA3M vector were described previously [63]. Constructs expressing LANA with 212–222 aa (BC box), 1085–1091 aa (Cul box), or 212–222^1085–1091 (SOCS box) aa deletion or site mutation (L213G, C217G, and L213PC217F) in pA3M were prepared by PCR mutagenesis. pA3M/*p53* was constructed by Jason S. Knight. GST-*p53* was as described previously [64]. Plasmids pCEP4/HIF-1α and pCEP4 were provided by Gregg L. Semenza (Johns Hopkins University School of Medicine, Baltimore, Maryland, United States). Plasmid pCMV/HA-VHL was provided by William G. Kaelin (Howard Hughes Medical Institute, Chevy Chase, Maryland, United States). Multimeric hypoxia response element (HRE) in pGL2-Basic vector was a gift from Craig B. Thompson (University of Pennsylvania Medical School, Philadelphia, Pennsylvania, United States). pcDNA3/HA-Rbx1 was provided by Yue Xiong (University of Rochester Lineberger Cancer Center, Rochester, New York, United States). pcDNA3/HA-Ub was provided by George Mosialos (Alexander Fleming Biomedical Sciences Research Center, Vari, Greece). VR1012/HA-Cul2 and VR1012/HA-Cul5 were provided by Xiao-Fang Yu (Johns Hopkins University School of Medicine). T7-Elongin B in pcDNA3.1, His-HPC4-Elongin C in pcDNA3.1, GST-ubiquitin in pGEX-4T-2, myc-Uba1-His in pET-23b, and His-hUbc5a-Flag in pRSETB were provided by Joan W. Conaway (University of Kansas

Medical Center). GST-*VHL* (54–213) in pGEX-4T-3 was provided by Bill Kaelin (Howard Hughes Medical Institute). Constructs HA–Elongin B, HA–Elongin C, GST–Elongin B, and GST–Elongin C were obtained from Elongin B and Elongin C PCR products cloned into pcDNA-3HA and pGEX-2T vectors, respectively.

Renal carcinoma *VHL*-null cell line 786-O was kindly provided by Volker H. Haase (University of Pennsylvania Medical School). Human osteosarcoma *p53*-null cell line Saos-2 was obtained from Jon Aster (Brigham and Women's Hospital, Boston, Massachusetts, United States). BJAB, DG75 and Louckes KSHV-negative type cells and BC-3, BCBL-1, JSC-1 and BC-1 KSHV-positive type cells were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, California, United States) with 7% FBS, 4 μM L-glutamine, penicillin, and streptomycin. The other cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum, penicillin, and streptomycin. BJAB cells latently infected with KSHV were kindly provided by Michael Lagunoff of the University of Washington [65]. Human embryonic kidney (HEK) 293/pA3M-LANA and 293/pA3M stable cell lines were selected by 1 μg/ml Puromycin. Cells were transfected by electroporation with a Bio-Rad (Hercules, California, United States) Gene Pulser in 0.4-cm cuvettes at 220 V and 975 μF.

Antibodies, IB, and IP. The monoclonal antibodies mouse anti-HA (12CA5) and mouse anti-myc (9E10) were purchased from Covance Research Products (Denver, Pennsylvania, United States). Anti-ubiquitin (FL-76) and anti-*p53* (DO-1) were obtained from Santa Cruz Biotechnology (Santa Cruz, California, United States). Mouse anti–HIF-1α (BD Transduction Laboratory, San Jose, California, United States), mouse anti-*VHL* (BD Transduction Laboratory), mouse anti–Elongin B (BD Transduction Laboratory), mouse anti–Elongin C (BioLegend, San Diego, United States), rabbit anti-GST (Amersham Biosciences, Little Chalfont, United Kingdom), rabbit anti-LANA polyclonal antibody (provided by Bala Chandran of Rosalind Franklin University of Medicine and Science, North Chicago, Illinois, United States), and anti–β-actin (Cell Signaling Technology) were also used.

Twenty million whole-cell lysates were extracted in 200 μl of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin). Protein concentration was determined by Bio-Rad protein assay. Protein was separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk in PBS (2 mM KCl, 120 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) containing 0.1% Tween 20, proteins were probed with the corresponding antibodies. For detection of proteins, appropriate infrared-conjugated secondary antibodies (Alexa Fluor 800 or 680) were used with the Li-COR (Lincoln, Nebraska, United States) Odyssey scanner. For IP, after one-step preclearing (rotation 1 h, 4 °C) with protein A/G (50/50) Sepharose fast flow (Amersham Biosciences), antibodies were added to the cell protein extract in a binding buffer adjusted to 20 mM Tris (pH 7.5), 200 mM NaCl, and 0.1% Nonidet P-40. After overnight incubation, immunocomplexes were recovered with protein A/G (50/50) Sepharose for 1-h incubation. After three washes with binding buffer, proteins were eluted in sample buffer and resolved by SDS-PAGE electrophoresis.

RNA interference. siRNAs complementary to the C-terminal (GCTAGGCCACAACACATCT) fragment of LANA as described previously [16] were cloned into the pSIREN vector according to the instruction of manufacture (Clontech, Palo Alto, California, United States) to generate si-LANA construct. pSIREN vector with luciferase target sequence was used as control. Ten million BCBL-1 cells were cotransfected by electroporation with 5 μg of si-LANA (or si-Luc). BCBL-1 knockdown stable cells were selected and maintained in 4 μg/ml puromycin.

Expression and purification of recombinant proteins in *Escherichia coli*. Overnight starter cultures (50 ml) of *E. coli* BL21 (DE3) transformed with pGEX-4T-GST-ubiquitin, pGEX-2T-Elongin B, pGEX-2T Elongin C, pET-23b-myc-Uba1-His, and pRSETB-His-hUbc5a-Flag were incubated into 500 ml of culture medium and grown at 30 °C to an optical density of about 0.6 at 600 nm. After IPTG induction (0.5 mM, 2 h at 30 °C), bacteria were collected and sonicated in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 M DTT, 5% Sarkosyl, and the protease inhibitor cocktail for use with mammalian cell extracts. Uba1, Ubc5, and His-ubiquitin proteins were purified by Ni^{2+}-NTA agarose chromatography (Qiagen, Valencia, California, United States), and GST-ubiquitin, GST–Elongin B, or GST–Elongin C was purified by glutathione sepharose chromatography (Amersham Biosciences) and then dialyzed against 40 mM HEPES-NaOH (pH 7.9), 60 mM potassium acetate, 1 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, and 2 mM DTT.

Pulldown assays. T7 promoter fused cDNA for expressing Elongin B, Elongin C, *p53, VHL,* or LANA derivatives was incubated in the presence of [^{35}S]methionine for 3 h at 30 °C according to the manufacturer's instructions (Promega, Madison, Wisconsin, United States). Translation products of 15 μl of each protein were incubated with 1 μl of anti-myc (9E10) antibody (for GST pulldown, incubated with GST, or GST-tagged recombinant protein) in 500 μl of binding buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 μM $ZnCl_2$, 10% glycerol, freshly supplemented with 0.1 mM DTT and protease inhibitor) overnight at 4 °C. Proteins were recovered with protein A/G (50/50) Sepharose for 1-h incubation. After three washes with washing buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$, 5% glycerol, 0.1% Nonidet P-40, and 0.3 mM DTT with protease inhibitor), proteins were eluted in sample buffer and resolved by SDS-PAGE and then autoradiographed and scanned by a PhosphoImager (Amersham Biosciences).

Ubiquitylation assays. For ubiquitylation assays, cell extracts with WT or mutant myc-tagged LANA expression were immunoprecipitated with anti-myc antibody and protein A/G Sepharose fast flow as described previously [47]. After three washes with binding buffer and two washes with a buffer containing 40 mM HEPES-NaOH (pH 7.9), 60 mM potassium acetate, 1 mM $MgCl_2$, 0.5 mM EDTA, 10% glycerol, and 2 mM DTT, the beads were mixed with 500 ng of Uba1, 1.4 μg of Ubc5a, and 2.5 μg of GST-ubiquitin or His-ubiquitin with 2.5 μg GST-*p53* (GST-*VHL*) in a 15-μl reaction containing 4 mM HEPES-NaOH (pH 7.9), 6 mM potassium acetate, 5 mM $MgCl_2$, 1 mM DTT, and 1.5 mM ATP. Reaction mixtures were incubated for 1 h at 30 °C. Reaction products were fractionated by SDS-PAGE and analyzed by IB with anti-GST antibody.

RT-PCR. RT-PCR was used to compare the mRNA levels of *p53* or *VHL* in BCBL-1 cells with LANA knockdown or without. Total RNA was isolated by using TriZOL reagent (Invitrogen) following the manufacturer's protocol. RT-PCR was performed by using Super-Script II RT kit (Invitrogen) following the manufacturer's instructions. Briefly, RT-PCR was done in a total volume of 20 μl containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM concentration of each deoxynucleotide triphosphate, 2 μM concentration of each primer, 2 μl of randomly primed cDNA, and 0.5 unit of AmpliTaq (Applied Biosystems, Foster City, California, United States. Primers for desired gene (LANA, 5′-ATGTGACTTCGCCAACCGTAG-3′ and 5′-TGCTTCTT CTGCAATCTCCG-3′; *p53,* 5′-CTCTCGGAA-CATCTCGAAGC-3′ and 5′-GCG CACAGAGGAAGAGAATC-3′; *VHL*, 5′-GAAGAGTACGGCCCTGAA-3′ and 5′-GGCAGTGTGA-TATTGGCA-3′; *GAPDH,* 5′-TGCACCACCAACTGCTTAG –3′ and 5′-GATGCAGGGATGATGTTC-3′) were included in some PCRs where indicated. PCR was done on an MJ Research Opticon II thermocycler (Waltham, Massachusetts, United States) by first heating the reaction at 94 °C for 5 min and then cycling the reaction 35 times with conditions set as 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s. Five microliters of the amplification products was analyzed by electrophoresis on 2% agarose gel and visualized after staining with ethidium bromide.

Luciferase reporter assays. Luciferase reporter assays were performed after transient transfection of each construct as a reporter plasmid by following the manufacturer's protocol. Briefly, at 48 h posttransfection, 10 million transfected cells from each sample were harvested and subsequently washed once with PBS (Invitrogen), followed by lysis with 200 μl of reporter lysis buffer (Promega, Madison, Wisconsin, United States). A 40-μg total protein of the lysate was mixed with 100 μl of luciferase assay reagent. Luminescence was measured by the OpticompI Luminometer (MGM Instruments, Hamden, Connecticut, United States) for 10 s. Relative luciferase activity was expressed as fold activation relative to the reporter construct alone. The results shown represent experiments performed in triplicate.

Supporting Information

Figure S1. Aberrant Expression of LANA Conquers *p53* or *VHL* as a Component of E3 Ubiquitin Complex for Degradation

Tumor suppressor *p53* (A) and *VHL* (B) competes with LANA. 786-O and Saos-2 cell lines were individually transfected with different doses (0, 1, 5, 10 μg) of LANA or cotransfected HIF-1α (5 μg) with different doses (0, 1, 5, 10 μg) of *p53* (or *VHL*) in the presence or absence of LANA (2 μg) as indicated. Cell lysates (40 μg) underwent IB assays against myc (*p53* or LANA), HA *(VHL),* and β-actin as described previously. The data show that LANA can inhibit endogenous *p53* or

VHL in a dose-dependent manner, but higher-level exogenous *p53* or *VHL* also can inhibit LANA expression and further suppress HIF-1α. Relative density (RD) of *VHL*, *p53*, and LANA protein was shown in lower panels.

Found at DOI: 10.1371/journal.ppat.0020116.sg001 (92 KB PDF).

Figure S2. LANA Attenuates the Inhibition of *p53* and *VHL* to Regulate the Transcriptional Activities of HIF-1α

(A) BJAB, (B) 786-O, and (C) Saos-2 cells were cotransfected individually as indicated (10 μg of LANA, 5 μg of *VHL*, and 5 μg of *p53*). The multimeric hypoxia response element (HRE) promoter with luciferase reporter was used to detect the transcriptional activity of HIF-1α affected by *VHL* and *p53*. The transfected cell lysates underwent luciferase reporter assay as described previously. The data showed the LANA attenuates the inhibition of *VHL* or *p53* to HIF-1α to increase transcription of HRE-containing promoter. The transcriptional activity of HIF-1α was expressed as the fold activation relative to activity with responding reporter construct alone (control). Means and standard deviations from three independent experiments are shown.

Found at DOI: 10.1371/journal.ppat.0020116.sg002 (19 KB PDF).

Figure S3. Proposed Model for Viral Protein LANA Interacts with Cellular Protein *VHL* and *p53*

During virus invasion, KSHV-encoded LANA is aberrantly expressed and possesses cellular EC_5S ubiquitin complex to induce tumor suppressors *VHL* and *p53* for degradation. In the other hand, the housekeeper genes encoded proteins in host cells together perform regular cellular mechanism, like *VHL* EC2S and *p53* SCF ubiquitin pathway, to defense against infection via inhibiting viral protein expression.

Found at DOI: 10.1371/journal.ppat.0020116.sg003 (14 KB PDF).

Accession Numbers

The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) accession numbers are KSHV (NC__003409), LANA (U52064), Elongin C (BC013809), Elongin B (BC065000), Cullin 2 (NM__003591), Cullin 5 (NM__003478), Rbx 1 (BC001466), *VHL* (NM__198156), *p53* (NM__000546), and HIF-1α (U22431).

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Author contributions. ESR supervised the overall design and execution of all experiments from initiation to submission of the manuscript. QLC designed and executed the experiments. JSK, SCV, and PZ assisted in performing some of the experiments and analysis of the generated data. ESR and QLC reviewed the analysis of all the data. QLC, JSK, SCV, and PZ contributed reagents/materials/analysis tools. ESR and QLC wrote and submitted the manuscript.

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Competing interests. The authors have declared that no competing interests exist.

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