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RESEARCH ON KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS: PAST, PRESENT, AND FUTURE

Hosted by
Keiji Ueda

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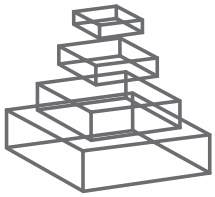
MICROBIOLOGY



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RESEARCH ON KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS: PAST, PRESENT, AND FUTURE

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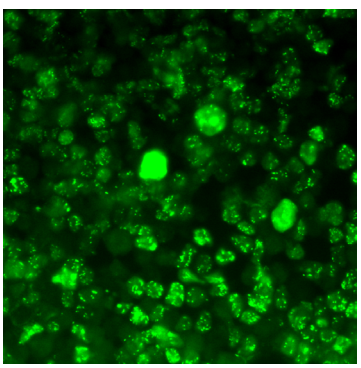

It has been 16 years since Kaposi's sarcoma-associated herpesvirus (KSHV) was found from Kaposi's sarcoma. Very extensive studies on KSHV have been performed and we now know well that KSHV is actually the very etiologic agent to cause Kaposi's sarcoma, primary effusion lymphoma and multicentricCastleman's disease and this virus is an oncogenic DNA virus in such sense. Though a lot of reports have been published, there are lots of enigmas on its epidemiology, entry, lytic replication/induction, viral particle assembly/egress, latency, oncogenesis and so on. At this time point, it is better for us to review what we learned from the studies of this virus and consider what we have to clarify about this virus nature for future by comparing the virus with the other virus research.

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EDITORIAL
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For the future studies of Kaposi's sarcoma-associated herpesvirus

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It is 18 years since Kaposi's sarcoma-associated virus (KSHV), also called human herpesvirus 8 (HHV-8), was found from Kaposis' sarcoma (KS) by Chang et al. (1994). More than 8, 000 reports have been published so far and we have learned many things from this virus. I would like to say it is about time to look back previous studies and to think what to study next on the virus, and planed a topic to think what to study next on the virus for future.

Herpesviruses have relatively big genomes and encode a 100 genes or so. Thus, the virion assembly/structure, gene expression regulation and attachment/entry are complicated and have known only an iceberg of them. Studying the details how the viruses run their life cycles and cause diseases in their processes will lead to exploring new therapeutic drugs/methods.

A viral life cycle starts from attachment on the susceptible cells and then, entry into the cells, followed by the viral gene expression, the genome replication, the particle assembly and finally the daughter viruses egress out of the cells. This process is skillfully built and all the viral genes are required for the process, though there are essential genes and non-essential ones. Viral pathogenesis could be established during this process by the interaction between viruses and host cells, and individual host systems such as immune system. In this topic, although I would like to cover all the processes, thankfully, 15 specialists in each field have contributed for this topic.

Polizzotto et al. (2012) described clinical manifestations of KSHV-associated diseases. So far, there were few reports on clinical manifestations of primary KHSV infection. In this term, KSHV inflammatory cytokine syndrome (KICS) is a new concept and we might have been looked over an important disease sign on KHSV infection. We will have to be more careful about what happens in primary KSHV infection than before.

Fukumoto et al. (2011) describe KSHV infection from a pathologist's points of view. Pathologic study is very important to know what happens in the lesions. Currently, we are able to know what is going on only in the KSHV associated lesions such as Kaposi's sarcoma, multicentric Castleman's disease and primary effusion lymphomas (PEL) of human samples suffered from KSHV infection, but once an infection model is established, chronological pathologic studies will provide a lot of information on how KSHV-associated diseases are formed.

Chakraborty et al. (2012) review the entry mechanism of KSHV into cells. In general, herpesviruses can infect various kinds of cells *in vitro* including non-human cell lines, but the infectivity to B lymphocyte originated cells is very inefficient. Their report will give us a hint why such phenomenon happens.

An immediate early gene, *RTA* (reactivation and transcription activator) is very important for the viral lytic replication induction and shows multifunctions. We still have not understood how the factor functions. Guito and Lukac (2012) and Tsai et al. (2012) review or report mechanistic regulation of this strong transactivator, respectively.

Jackson et al. (2012) describe ORF57, which is also an interesting and multifunctional protein. This is involved in post-translational processes of the viral gene expression as sumoylation and ubiquitination described by Campbell and Izumiya (2012) and Ashizawa et al. (2012) respectively. We had believed that K-bZIP, a homolog of Epstein-Barr virus Zta was a transactivator and origin recognition factor in the lytic replication. K-bZIP, however, has other important roles for KSHV lytic replication. In latency, metabolism of LANA (latency-associated nuclear antigen) could be critical for KSHV-induced tumor formation and/or its phenotype.

Viral particle assembly is virologically an exciting and interesting field. There have been few reports on this, Sathish et al. (2012) try to search this issue.

The detail replication mechanism of KSHV in both lytic and latent phase has been still unclear. In latency, the virus is supposed to utilize host replication machinery including pre-replication complexes (pre-RC) for the viral replication initiation in the presence of LANA. The viral factor, LANA, is an essential factor, but its necessity has not been elucidated well. LANA binds with LANA-biding sites (LBS) and recruits origin recognition complexes (ORCs) on the viral replication origin (ori-P), which cannot account for necessity of the GC-rich element followed by LBS. Ohsaki and Ueda (2012) will give us a hint about this question.

Viral immune evasion system is very tactic to maintain its latency in case of herpesviruses. The maintenance of latency is then critical for the virus to wait for reactivation to produce daughter viruses, whose transition may a step for the viral oncogenic process. Lee et al. (2012) summarize KSHV immune evasion strategy and make a comment on the future landscape.

Kaposi's sarcoma-associated virus mediated tumorigenesis including PEL and KS has been still unclear, though there are many reports on individual viral putative oncogenes. KSHV has not been reported to infect and immortalize and/or transform endothelial cells or peripheral blood mononuclear cells *in vitro*. And thus, we have not known how the viral genes with oncogenic potentials such as *vFLIP*, *vCYC*, *vGPCR* and so on in addition to *LANA* cooperate in the viral oncogenic process. DiMaio and Lagunoff (2012) address on this issue and look forward for this field.

MicroRNA is one of the hottest research fields even in KSHV. This kind of small RNA molecule seems to have profound effects on cellular processes and then viral activities but their details have not been elucidated totally. KSHV lytic and latent phases are regulated by viral but also cellular microRNAs. Two specialists; Liang et al. (2011) and Gottwein (2012) reveals the microRNA world of KSHV.

And finally, we have to think about treatment of KSHV-associated tumors such as KS, PEL and a lympho-proliferative disease, multicentric Castleman's disease. It should be very hard to treat these tumors in the immunodeficient setting. It will be desirable if KSHV specific strategy is designed, since these tumors are very tightly linked with KSHV infection. Dittmer et al. (2012) contribute for this theme and discuss about it.

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I would like to thank all contributors, and hope that this topic will be useful for the future study of KSHV.

REFERENCES

Ashizawa, A., Higashi, C., Masuda, K., Ohga, R., Taira, T., and Fujimuro, M. (2012). The ubiquitin system and Kaposi's sarcoma-associated herpesvirus. *Front. Microbiol.* 3:66. doi: 10.3389/fmicb.2012.00066

Campbell, M., and Izumiya, Y. (2012). Post-translational modifications of Kaposi's sarcoma-associated herpesvirus regulatory proteins–SUMO and KSHV. *Front. Microbiol.* 3:31. doi: 10.3389/fmicb.2012.00031

Chakraborty, S., Veettil, M. V., and Chandran, B. (2012). Kaposi's sarcoma associated herpesvirus entry into target cells. *Front. Microbiol.* 3:6. doi: 10.3389/fmicb.2012.00006

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266, 1865–1869.

DiMaio, T. A., and Lagunoff, M. (2012). KSHV induction of angiogenic and lymphangiogenic phenotypes. *Front. Microbiol.* 3:102. doi: 10.3389/fmicb.2012.00102

Dittmer, D. P., Richards, K. L., and Damania, B. (2012). Treatment of kaposi sarcoma-associated herpesvirus-associated cancers. *Front. Microbiol.* 3:141. doi: 10.3389/fmicb.2012.00141

Fukumoto, H., Kanno, T., Hasegawa, H., and Katano, H. (2011). Pathology of Kaposi's sarcoma-associated herpesvirus infection. *Front. Microbiol.* 2:175. doi: 10.3389/fmicb.2011.00175

Gottwein, E. (2012). Kaposi's sarcoma-associated herpesvirus microRNAs. *Front. Microbiol.* 3:165. doi: 10.3389/fmicb.2012.00165

Guito, J., and Lukac, D. M. (2012). KSHV Rta promoter specification and viral reactivation. *Front. Microbiol.* 3:30. doi: 10.3389/fmicb.2012.00030

Jackson, B. R., Noerenberg, M., and Whitehouse, A. (2012). The Kaposi's sarcoma-associated herpesvirus ORF57 protein and its multiple roles in mRNA biogenesis. *Front. Microbiol.* 3:59. doi: 10.3389/fmicb.2012.00059

Lee, H. R., Brulois, K., Wong, L., and Jung, J. U. (2012). Modulation of immune system by Kaposi's sarcoma-associated herpesvirus: lessons from viral evasion strategies. *Front. Microbiol.* 3:44. doi: 10.3389/fmicb.2012.00044

Liang, D., Lin, X., and Lan, K. (2011). Looking at Kaposi's sarcoma-associated herpesvirus-host interactions from a microRNA viewpoint. *Front. Microbiol.* 2:271. doi: 10.3389/fmicb.2011.00271

Ohsaki, E., and Ueda, K. (2012). Kaposi's sarcoma-associated herpesvirus genome replication, partitioning, and maintenance in latency. *Front. Microbiol.* 3:7. doi: 10.3389/fmicb.2012.00007

Polizzotto, M. N., Uldrick, T. S., Hu, D., and Yarchoan, R. (2012). Clinical manifestations of Kaposi sarcoma herpesvirus lytic activation: multicentric castleman disease (KSHV-MCD) and the KSHV inflammatory cytokine syndrome. *Front. Microbiol.* 3:73. doi: 10.3389/fmicb.2012.00073

Sathish, N., Wang, X., and Yuan, Y. (2012). Tegument proteins of Kaposi's sarcoma-associated herpesvirus and related gamma-herpesviruses. *Front. Microbiol.* 3:98. doi: 10.3389/fmicb.2012.00098

Tsai, W. H., Wang, P. W., Lin, S. Y., Wu, I. L., Ko, Y. C., Chen, Y. L., Li, M., and Lin, S. F. (2012). Ser-634 and Ser-636 of Kaposi's sarcoma-associated herpesvirus RTA are involved in transactivation and are potential Cdk9 phosphorylation sites. *Front. Microbiol.* 3:60. doi: 10.3389/fmicb.2012.00060

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Clinical manifestations of Kaposi sarcoma herpesvirus lytic activation: multicentric Castleman disease (KSHV–MCD) and the KSHV inflammatory cytokine syndrome

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Soon after the discovery of Kaposi sarcoma (KS)-associated herpesvirus (KSHV), it was appreciated that this virus was associated with most cases of multicentric Castleman disease (MCD) arising in patients infected with human immunodeficiency virus. It has subsequently been recognized that KSHV–MCD is a distinct entity from other forms of MCD. Like MCD that is unrelated to KSHV, the clinical presentation of KSHV–MCD is dominated by systemic inflammatory symptoms including fevers, cachexia, and laboratory abnormalities including cytopenias, hypoalbuminemia, hyponatremia, and elevated C-reactive protein. Pathologically KSHV–MCD is characterized by polyclonal, IgM-lambda restricted plasmacytoid cells in the intrafollicular areas of affected lymph nodes. A portion of these cells are infected with KSHV and a sizable subset of these cells express KSHV lytic genes including a viral homolog of interleukin-6 (vIL-6). Patients with KSHV–MCD generally have elevated KSHV viral loads in their peripheral blood. Production of vIL-6 and induction of human (h) IL-6 both contribute to symptoms, perhaps in combination with overproduction of IL-10 and other cytokines. Until recently, the prognosis of patients with KSHV–MCD was poor. Recent therapeutic advances targeting KSHV-infected B cells with the anti-CD20 monoclonal antibody rituximab and utilizing KSHV enzymes to target KSHV-infected cells have substantially improved patient outcomes. Recently another KSHV-associated condition, the KSHV inflammatory cytokine syndrome (KICS) has been described. Its clinical manifestations resemble those of KSHV–MCD but lymphadenopathy is not prominent and the pathologic nodal changes of KSHV–MCD are absent. Patients with KICS exhibit elevated KSHV viral loads and elevation of vIL-6, homolog of human interleukin-6 and IL-10 comparable to those seen in KSHV–MCD; the cellular origin of these is a matter of investigation. KICS may contribute to the inflammatory symptoms seen in some patients with severe KS or primary effusion lymphoma. Additional research is needed to better define the clinical spectrum of KICS and its relationship to KSHV–MCD. In additional, research is needed to better understand the pathogenesis and epidemiology of both KICS and KSHV–MCD, as well as the optimal therapy for both of these disorders.

Keywords: Kaposi sarcoma-associated herpesvirus, human herpesvirus 8, multicentric Castleman disease, KSHV inflammatory cytokine syndrome, interleukin-6, human immunodeficiency virus

KAPOSI SARCOMA-ASSOCIATED HERPESVIRUS AND ASSOCIATED DISEASES

Kaposi sarcoma (KS)-associated herpesvirus (KSHV) is a gamma herpesvirus that is established as the causative agent of a several human illnesses, including KS, primary effusion lymphoma (PEL), and, in the setting of human immunodeficiency virus (HIV) infection, almost all cases of multicentric Castleman disease (MCD; Chang et al., 1994; Cesarman et al., 1995; Soulier et al., 1995). In KS, the predominant cell type is the spindle cell, which expresses endothelial markers, while the cell of origin in PEL and KSHV–MCD is of B-cell lineage. KS and KSHV–MCD are polyclonal in nature, while PEL is considered to be monoclonal. Like other herpesviruses, KSHV infection can exhibits both latent and lytic phases, distinguished by their viral gene expression patterns (Moore and Chang, 1998; Staskus et al., 1999; Sun et al., 1999). KSHV can establish latency in various cell types including endothelial cells and B-lymphocytes (Monini et al., 1999; Wu et al., 2006; Della Bella et al., 2008). Gene expression in latent phase is extremely limited (Jenner et al., 2001), with expressed genes primarily being directed to enhance cell survival, inhibit apoptosis, and evade the host immune response (Moore and Chang, 1998; Wen and Damania, 2010). Following activation of the lytic switch gene, RTA, encoded by open reading frame (ORF) 50, the virus enters the lytic phase, wherein the full complement of viral encoded genes is expressed and the host cellular machinery is redirected to the manufacture and assembly of progeny virions. The triggers of lytic activation *in vivo* remain unclear. It is speculated that intermittent lytic cycles may play a

role in maintaining viral persistence in the host (Gregory et al., 2009).

The KSHV genome is notable for its molecular piracy of cellular genes (Moore et al., 1996; Ganem, 2010). These appear to promote viral survival, and there is evidence that they contribute to the pathogenesis of KSHV-associated diseases through interactions with the human cellular machinery and immune system. These gene mimics include viral homologs of interferon regulatory factors (IRF), ORF K9/vIRF-1, and ORF K11.5/vIRF-2; a viral homolog of human interleukin-6 (hIL-6) called viral IL-6 (vIL-6; encoded by ORF K2); and a constitutively active G-protein coupled receptor (GPCR) with pro-angiogenic and immunomodulatory functions encoded by ORF74 (Aoki et al., 2000; Jenner et al., 2001; Yarchoan, 2006). While vIL-6 is commonly referred to as a lytic gene, in some circumstances it may be expressed in the absence of full lytic activation (Chatterjee et al., 2002). KSHV also encodes at least 12 small non-coding microRNAs (miRNAs), whose functions are still to be comprehensively determined (Ganem and Ziegelbauer, 2008; Umbach and Cullen, 2010). There is evidence that these also interact with host proteins in ways that favor viral survival and may also induce expression of other host inflammatory proteins including IL-10 (Abend et al., 2010; Lei et al., 2010; Lu et al., 2010; Qin et al., 2010).

Viral IL-6 exhibits a number of important differences from its human counterpart. Their sequence homology is approximately 25% (Moore et al., 1996; Neipel et al., 1997). vIL-6 has a signaling potency 100- to 1000-fold lower that that seen with hIL-6 in *in vitro* models (Aoki et al., 2000). However, unlike hIL-6, vIL-6 is able to signal by forming a tetrameric complex with the IL-6 receptor superfamily gp130 component, which is widely expressed in human tissues, without requiring binding to the classical gp80 IL-6 receptor (Heinrich et al., 1998; Boulanger et al., 2004; Meads and Medveczky, 2004; Hu and Nicholas, 2006). As a result, vIL-6 may be able to initiate signaling in a wider variety of cell types than does hIL-6 (Osborne et al., 1999; Meads and Medveczky, 2004). In addition, there is *in vitro* evidence of vIL-6 autocrine action in lytically infected cells, though the function and significance of this activity is as yet uncertain (Chen et al., 2009a,b).

In the absence of HIV infection or other causes of immunodeficiency, most infection with KSHV is clinically silent, even when intermittent lytic activation cycles occur. However, as T cell immunity declines, KSHV-infected patients become increasingly likely to develop KSHV-associated tumors, particularly KS. It is not unusual for patients to manifest more than one KSHV-associated tumor. Each of these tumors has also been reported in other immunosuppressed and older individuals (Teruya-Feldstein et al., 1998). In KS tissue, KSHV is found predominantly in latent phase as assessed by immunohistochemical and genetic analysis of tumor tissue, while there is limited expression of KSHV viral genes, especially vIL-6, in PEL cells (Teruya-Feldstein et al., 1998; Jones et al., 1999; Staskus et al., 1999; Parravicini et al., 2000). By contrast, and unusually among herpesvirus-associated tumors, KSHV–MCD is associated with lytically active KSHV in a substantial proportion of KSHV-infected cells (Oksenhendler et al., 1996; Staskus et al., 1999; Parravicini et al., 2000). This lytic activation, and particularly the production of vIL-6, is thought to underlie its pathogenesis (Oksenhendler et al., 2000; Aoki et al., 2001a; Polizzotto et al., 2011).

MULTICENTRIC CASTLEMAN DISEASE

There are several forms of Castleman disease, which historically have been distinguished by clinical findings (based on whether involved nodes are singular or multiple), pathologic findings, and more recently etiology (idiopathic or KSHV-associated; Cronin and Warnke, 2009). The idiopathic forms, as originally described, include a hyaline vascular form (usually unicentric) and a plasmacytoid form (usually multicentric). The KSHV-associated form is characteristically multicentric, with distinct pathologic features mirroring its unique etiology, as described below (Oksenhendler et al., 1996). Nearly all Castleman disease arising in the setting of HIV infection is KSHV-associated MCD (KSHV–MCD), while KSHV–MCD also rarely occurs in non-HIV infected individuals, particularly the immunocompromised. KSHV–MCD is considered to be rare, although its incidence is not well defined. By contrast to KS, there is evidence that MCD has become more common since the advent of combination anti-retroviral therapy (cART; Powles et al., 2009).

KSHV-MCD is characterized by intermittent flares of inflammatory symptoms, including fevers, fatigue, and cachexia, and edema, together with lymphadenopathy and/or splenomegaly (Oksenhendler et al., 1996; Stebbing et al., 2008). Gastrointestinal and respiratory symptoms are common. Flares are often severe and can be fatal. Common laboratory abnormalities include anemia, thrombocytopenia, hypoalbuminemia, hyponatremia, and elevated inflammatory markers such as C-reactive protein (CRP; Oksenhendler et al., 2000; Oksenhendler, 2009). The clinical course waxes and wanes, but until recently, has generally been fatal within 2 years of diagnosis, with patients succumbing to the severe inflammatory syndrome, concurrent infections, or progressing to lymphoma (Oksenhendler et al., 2000; Stebbing et al., 2008). The differential diagnosis of fever and adenopathy, even with other laboratory abnormalities, in the HIV infected individuals is broad. As a result, KSHV–MCD may be difficult to diagnose and is often missed. Also, while there are few reports of KSHV–MCD in Africa, there is recent evidence that it may be substantially underdiagnosed in this region (Ayers et al., 2011).

The diagnosis of KSHV–MCD is made pathologically by a lymph node biopsy demonstrating hypocellular germinal centers with a vascularized core and by a proliferation of polyclonal, IgM λ-restricted plasmacytoid cells in the interfollicular area (Oksenhendler et al., 1996; Du et al., 2001; Chadburn et al., 2008). A proportion of these cells are KSHV-infected, as demonstrated by expression of the KSHV latency associated nuclear antigen (LANA-1), and of these a subset express KSHV lytic genes, especially vIL-6 (Du et al., 2001; **Figure 1**). Areas of KS spindle cell proliferation in affected nodes are also not uncommon (Naresh et al., 2008). However, the majority of the cellular burden within affected nodes is comprised of uninfected plasmacytoid B-lymphocytes (Chadburn et al., 2008; Naresh et al., 2009).

As noted, the symptom complex of KSHV–MCD is associated with inflammatory dysregulation attributable to KSHV lytic activation. Both production of vIL-6 and induction of host production of hIL-6 have separately been postulated to contribute

to symptoms, perhaps in combination with enhanced production of human interleukin-10 (IL-10) and other human cytokines (Oksenhendler et al., 2000; Aoki et al., 2001a,b; Bower et al., 2009). Viral and human IL-6 over-expression in mouse models can each produce phenotypes resembling that of KSHV–MCD (Brandt et al., 1990; Aoki et al., 2001b). Symptomatic episodes ("flares") are also associated with high KSHV viral loads (Oksenhendler et al., 2000; Polizzotto et al., 2011). Histopathologic evaluation of nodes involved by KSHV–MCD suggests that the cellular origins of vIL-6 and hIL-6 are largely distinct: involved nodes show vIL-6 production within the periphery of affected germinal center, in the lytic subset of the relatively small number of KSHV-infected cells. By contrast, the larger number of KSHV-uninfected cells in the core of affected nodes can produce hIL-6 but not vIL-6 (Chadburn et al., 2008).

KSHV INFLAMMATORY CYTOKINE SYNDROME

In addition to these well-recognized KSHV-associated diseases, our group has recently described what appears to be a novel inflammatory symptom complex associated with KSHV infection (Uldrick et al., 2010a). In six patients with HIV and KSHV co-infection, but without any pathological evidence of MCD, we observed an inflammatory syndrome resembling that seen in MCD. These patients presented with abnormalities including fevers, sweats, fatigue, wasting, cytopenias, hypoalbuminemia, and hyponatremia, associated in some cases with lymphadenopathy or effusions (**Table 1**). Notably, four of the six patients also had severe KS. Each patient underwent a least one tissue biopsy to rule out KSHV–MCD as a cause of symptoms. None had pathologic evidence of KSHV–MCD (or KSHV-associated lymphoma), although abnormal KSHV-infected mononuclear cells were sometimes detected in biopsy specimens. None developed KSHV–MCD on follow-up, and one patient who died had no evidence of KSHV–MCD or lymphoma at autopsy (Uldrick et al., 2010a).

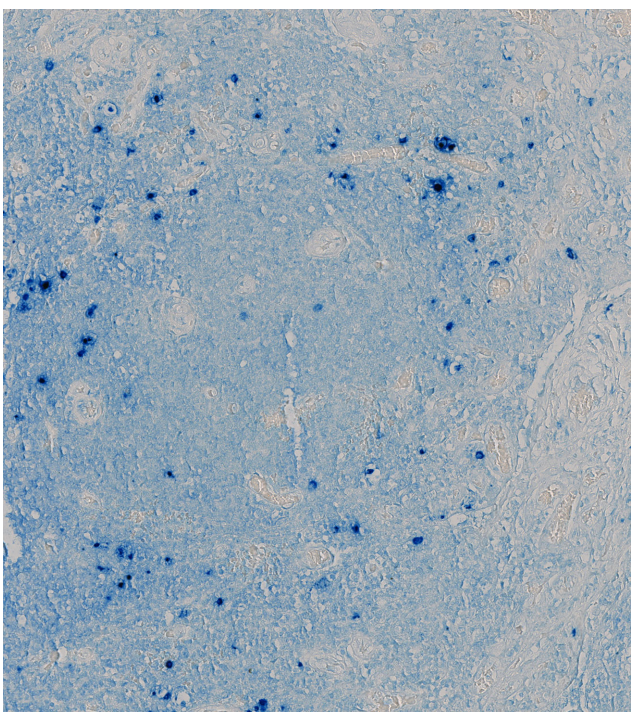

FIGURE 1 | Lymph Node from a Patient with KSHV–MCD Stained for vIL-6. Germinal center of a lymph node from a patient with KSHV–MCD, stained with a monoclonal antibody to vIL-6 and an alkaline phosphatase substrate. Cells expressing vIL-6 stain in blue; these cells are located primarily in the mantle region around the germinal center. The tissue was studied under a protocol approved by the Institutional Review Board of the National Cancer Institute, and the patient gave written informed consent.

We hypothesized that while these symptoms did not appear to be caused by KSHV–MCD, they might nonetheless similarly result from KSHV lytic activation and consequent cytokine production. On investigation, this cohort indeed exhibited KSHV viral burdens and cytokines profiles similar those seen with KSHV lytic activity in MCD flares, including substantial elevation of vIL-6 and hIL-6 (**Figure 2**; Uldrick et al., 2010a). Patients also exhibited markedly elevated KSHV viral loads in peripheral blood mononuclear cells. While the number of patients is small, the large differences in the immunologic and virologic milieu in these patients compared to KS patients without inflammatory symptoms was statistically significant (**Figure 2**). Our findings in this small cohort provide evidence that patients with lytically active KSHV may manifest systemic clinical symptoms even in the absence of the pathological changes diagnostic of MCD. These features are distinguishable from the chronic immune activation of HIV infection by the presence both of markedly elevated KSHV VLs in peripheral blood and by the detection of vIL-6 in KSHV inflammatory cytokine syndrome (KICS). As seen in **Figure 2**, neither of these features are evident in patients with KSHV and HIV co-infection who have KS but do not have systemic inflammatory symptoms. While in the original series all patients were HIV infected, we have subsequently observed this syndrome in patients without HIV.

Based on this original cohort, we have proposed naming this putative clinical syndrome KICS. The assessment of this small retrospective cohort of patients suggest that clinical manifestations of KICS are protean, and none are specific to the syndrome. Rather, they likely reflect common endpoints of proinflammatory

Table 1 | Key clinical and laboratory findings in patients with KICS and KSHV–MICD.

Parameter	KICS	KSHV–MCD
Number of patients	6	8
CD4* (cells/μL; median, range)	255 (28–492)	189 (21–364)
HIV VL* (copies/mL; median, range)	4650 (< 50–110,000)	<50 (<50–664)
Sodium (mEq/L; median, range)	132 (127–139)	130 (127–136)
Albumin (g/dL; median, range)	2.6 (2.0–3.9)	2.7 (1.7–3.9)
Hemoglobin (g/dL; median, range)	8.7 (7.0–14.8)	10.2 (7.4–12.7)
Leukocytes (10^3 cells/μL; median, range)	4.5 (2.3–6.4)	5.7 (2.7–10.1)
Platelets (10^3 cells/μL; median, range)	158 (62–231)	126 (11–262)

**From Uldrick et al., 2010a, used with permission. All patients in both cohorts were HIV-coinfected.*

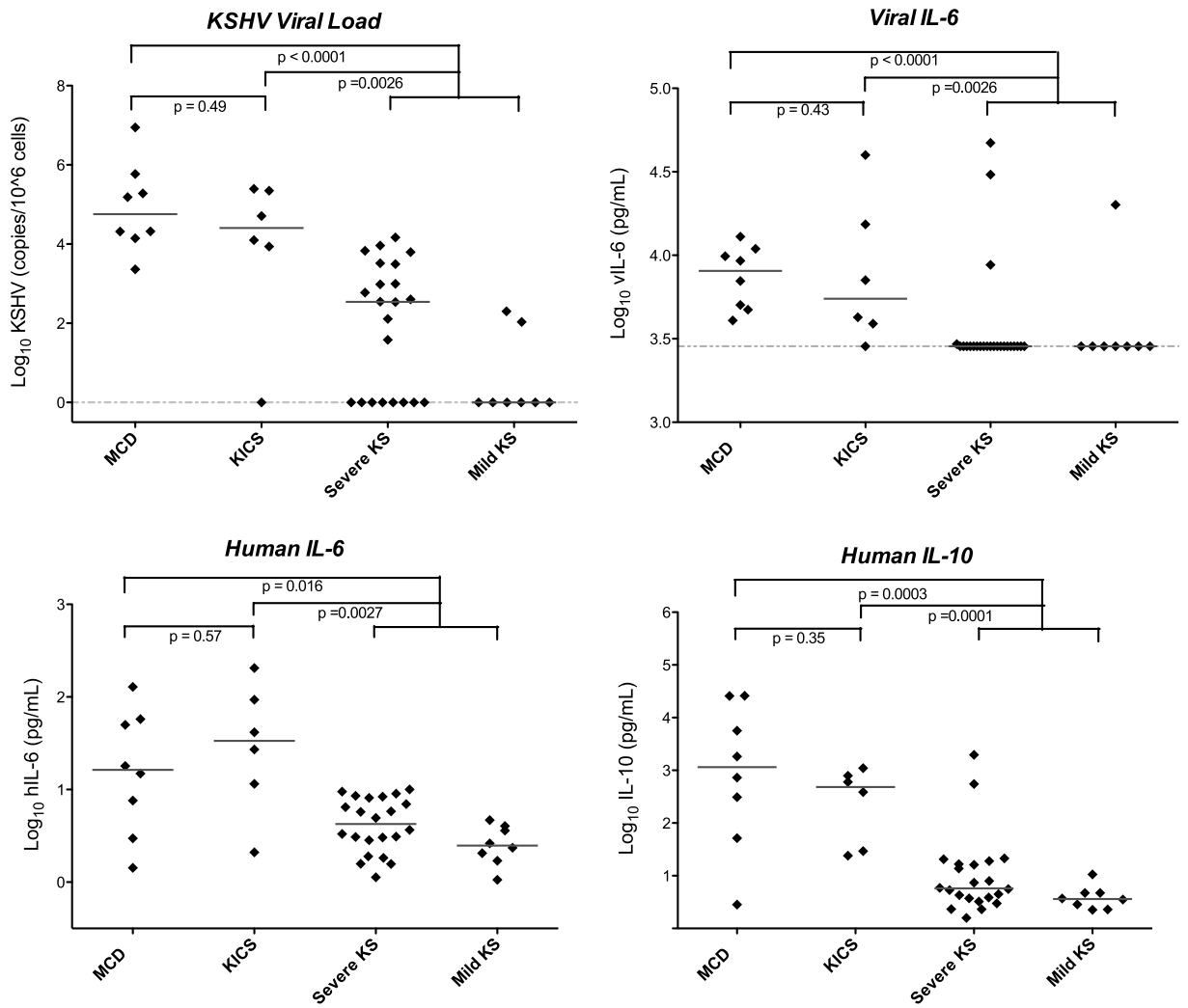

FIGURE 2 | Viral and cytokine elevations in patients with KICS, KSHV–MCD, and KS. Statistically significant differences in KSHV viral load, viral, and human cytokines were seen in KICS patients compared with controls with KS alone, at levels comparable to those seen in KSHV–MCD. Horizontal lines indicate median value, and the dashed line indicates the lower limit of detection. Values are log transformed; viral and human IL-6 and IL-10 are picograms per milliliter; KSHV VL is copies/10^6 cells. Note that several of the patients with severe KS and elevated vIL-6 had laboratory abnormalities suggestive of KICS, but did not meet the operative case definition for this series. Modified from Uldrick et al. (2010a), with permission.

cytokine deregulation in the setting of KSHV lytic replication and pathologic expression of KSHV encoded genes such as vIL-6. These symptoms may also be observed in other inflammatory states, and at times can be confused clinically with sepsis. KICS is therefore a diagnosis of exclusion, requiring pathologic exclusion of MCD; careful evaluation to exclude alternate explanations for the clinical manifestations, such as serious intercurrent infection; demonstration of systemic inflammation; and demonstration of KSHV viral activity. Based on the initial case series, we have proposed a working case definition for KICS (**Table 2**), incorporating clinical, laboratory, and radiographic manifestations together with evidence of KSHV activation and systemic inflammation, and a requirement that KSHV–MCD be excluded on pathological examination. A clinical protocol to study this syndrome prospectively is underway at the National Cancer Institute, National Institutes of Health, based on this working definition (NCT01419561).

The precise relationship of KICS to KSHV–MCD is unclear, and it is possible that KICS is a heterogeneous condition. Pathological evaluation and perhaps functional imaging with 18-[F]-fluorodeoxyglucose positron emission tomography (FDG-PET) may shed additional light on this question, and perhaps assist in identifying lymph nodes to biopsy that may reveal KSHV–MCD. We have preliminary evidence to show that in KSHV–MCD, pathologically involved nodes are detectable by FDG-PET, and that PET findings are distinct from those of suppressed HIV or intercurrent lymphoma (Polizzotto et al., 2010). Comparison of PET findings in KICS and KSHV–MCD may also provide a non-invasive means to assess disease activity and shed light on syndrome pathogenesis, including the possible cellular sites of disease activity. It is

Table 2 | Proposed working case definition of KSHV inflammatory cytokine syndrome.

1. CLINICAL MANIFESTATIONS*	
a. Symptoms	**b. Laboratory abnormalities**
Fever	Anemia
Fatigue	Thrombocytopenia
Edema	Hypoalbuminemia
Cachexia	Hyponatremia
Respiratory symptoms (including cough, dyspnea, airway hyperreactivity)	**c. Radiographic abnormalities**
Gastrointestinal disturbance (including nausea, anorexia, abdominal discomfort, altered bowel habit)	Lymphadenopathy
Arthralgia and myalgia	Splenomegaly
Altered mental state	Hepatomegaly
Neuropathy with or without pain	Body cavity effusions
2. EVIDENCE OF SYSTEMIC INFLAMMATION	
Elevated C-reactive protein ($\geq$3 g/dL)	
3. EVIDENCE OF KSHV LYTIC ACTIVITY	
Elevated KSHV viral load in peripheral blood mononuclear cells ($\geq$100 copies/10^6 cells)	
4. NO EVIDENCE OF KSHV-ASSOCIATED MULTICENTRIC CASTLEMAN DISEASE	
Exclusion of MCD requires pathologic assessment lymph node, bone marrow, or spleen	

The working case definition of KICS requires the presence of at least two clinical manifestations drawn from at least two categories (1a, b, and c), together with each of the criteria in 2, 3, and 4. Clinical manifestations for the working definition are drawn from the initial case series and from findings commonly seen in KSHV–MCD.

also possible that some patients with KICS may represent a prodrome to KSHV–MCD or an unusual or limited presentation of KSHV–MCD, and may eventually evolve into frank KSHV–MCD. Alternatively, certain patients may have another cellular source apart from lymphocytes responsible for the excess vIL-6 cytokine production, perhaps extensive KS spindle cells or KSHV-infected monocytes or macrophages. Notably, while several patients with KICS in the original cohort had severe KS, several other patients in this cohort with severe KS had less severe laboratory abnormalities suggestive of KICS in conjunction with detectable vIL-6 levels, although these abnormalities were not sufficiently severe for the patients to be identified as KICS based on the clinical criteria used for the initial case series. Similarly, PEL patients can exhibit severe inflammatory symptoms with disease activity, and may exhibit elevated KSHV viral loads (Uldrick et al., 2011b). Taken together, these observations raise the possibility that KSHV lytic activation and associated inflammatory clinical manifestations may be more common than appreciated in patients with other KSHV-associated diseases. It is possible that significant clinical overlap exists between KICS, KS, and PEL.

Among patients with KSHV and HIV co-infection, KICS may be an important cause of morbidity and mortality. KICS was associated with 50% mortality in our small series (which likely captured relatively severe cases). We also hypothesize that KS may be relatively refractory to therapy in patients with KICS because the cytokine excess can help drive KS spindle cell proliferation. In a small number of patients our group has seen with KICS and KS that was difficult to treat, strategies to reduce KSHV burden and intercurrent inflammation (including rituximab with cytotoxic chemotherapy) appear to have led to improvements in KS. One can thus hypothesize that KICS may in some cases be associated with the worsening of KS that can be observed after starting cART [KS immune reconstitution syndrome (IRIS)], although KICS has not been formally studied in this setting. Notably, some investigators report elevated hIL-6 levels in IRIS patients without KS, though KSHV and viral cytokines have not, to our knowledge, been assessed in relation to systemic symptoms (Stone et al., 2001).

RATIONAL THERAPEUTIC APPROACHES IN KSHV–MCD AND KICS

There is no standard therapy for KSHV–MCD, and most articles describing therapy have consisted of case reports or very small series. Some of the approaches that have shown activity include interferon-alpha, surgical resection of the spleen, cytotoxic chemotherapy (either single agents or combinations), ganciclovir, glucocorticoids, and rituximab (Boulanger et al., 2004; Waterston and Bower, 2004; Dispenzieri and Gertz, 2005; Neuville et al., 2005; Bower et al., 2007; Mylona et al., 2008; Bower, 2010). Our group has explored selective targeting of KSHV–MCD plasmablasts based on their expression of KSHV lytic genes that phosphorylate certain drugs (Davis et al., 2007). One KSHV lytic gene, ORF36, encodes a phosphotransferase that activates ganciclovir to a cytotoxic triphosphate moiety, and another, ORF21, encodes a thymidine kinase that phosphorylates zidovudine (AZT), again to a cytotoxic moiety (Cannon et al., 1999; Gustafson et al., 2000). Members of our group have shown that PEL cells in which KSHV was lytically activated produced increased amounts of triphosphate moieties of AZT and ganciclovir and that AZT and ganciclovir had synergistic toxicity, at doses attainable in patients, against these PEL lines with activated KSHV (Davis et al., 2007). No comparable cytotoxicity was seen in non-KSHV-infected Burkitt lymphoma cell lines. Based on these *in vitro* results, we explored the combination of high dose zidovudine and valganciclovir, an oral pro-drug of ganciclovir, in 14 patients with HIV infection and KSHV–MCD (Uldrick et al., 2011a). This regimen delivered symptomatic control and normalization of correlates of lytic KSHV activity (including KSHV viral load, vIL-6, and hIL-6) in a high proportion of MCD patients. Responses were evaluated based on

newly established criteria (NCI–HAMB criteria) for improvement in clinical symptoms, laboratory parameters (including hemoglobin, platelets, CRP, albumin, and sodium), and radiographic parameters (Uldrick et al., 2011a). Overall, 12 patients (86%) attained major clinical responses by these criteria, with seven patients (50%) attaining clinical complete responses, three (21%) attaining symptom free disease, and two (14%) attaining partial clinical response. In addition, seven patients (50%) attained major biochemical responses, with three (21%) attaining a complete response, four (29%) attaining a partial response. Finally, five patients (36%) attained a major radiographic responses including four (29%) with complete radiographic responses. Relapses were relatively common with this regimen, and the progression-free survival was 6 months. However, patients overall did well, and with 43 months of median follow-up, the overall survival was 86%.

Several recent studies have reported that KSHV–MCD can respond to therapy with the anti-CD20 monoclonal antibody rituximab (Corbellino et al., 2001; Marcelin et al., 2003; Ocio et al., 2005; Bower et al., 2007). It is postulated that in this setting, it acts by targeting the B-lymphocyte KSHV reservoirs, though it should be noted that the level of expression (weak or absent) of CD20 on lytic KSHV-infected B-lymphocytes themselves is contentious. Alternatively, it may target IL-6 production by other B cells that are stimulated by the KSHV-infected plasmablasts. However, different investigators have reported variable results with this agent, and two studies noted that its use was associated with a worsening of KS (Neuville et al., 2005; Bower et al., 2007). Because of this concern, and because many patients with MCD either have KS or are at risk of KS, we have explored the combination of rituximab and liposomal doxorubicin (Doxil) in KSHV–MCD (R/Dox). Liposomal doxorubicin has a high affinity for the pathogenic spindle cell in KS lesions, and, when available, is currently the standard of care for first line therapy of KS (Northfelt et al., 1998; Cianfrocca et al., 2010). It was also possible that it could help target the KSHV-infected MCD plasmablasts. In an ongoing study, we have treated 12 patients with this R/Dox regimen (Uldrick et al., 2010b). R/Dox was highly effective even in heavily pretreated patients. All achieved a clinical CR by NCI–HAMB criteria (after median two cycles therapy, range 1–5); nine (75) achieved a biochemical CR, one (8%) biochemical PR, and two (17%) biochemical SD; while best radiographic response (nodes and spleen) was CR in six (50%) and PR in six (50%). Toxicity was not marked, comprising predominantly infusion reactions and neutropenia.

Until recently, the expected survival of most patients with KSHV–MCD was 2 years or less (Oksenhendler et al., 2000). With the recent advances in therapy, along with the use of highly active anti-retroviral therapy (HAART) for those patients with HIV-associated KSHV–MCD, the overall prognosis of patients with KSHV–MCD has improved markedly, and a number of patients can now attain long remissions. Nonetheless, patients with flares of KSHV–MCD can rapidly become critically ill, with a clinical presentation similar to patients with sepsis, and may require careful monitoring, sometimes in an intensive care unit, during the initial treatment. Our experience is that some patients may benefit from short-term glucocorticoids during initial KSHV–MCD treatment, although patients should be probably weaned as soon as possible as glucocorticoids may mask intercurrent infections and can exacerbate KS (Gill et al., 1989). It should be noted, however, that there is no standard therapy for KSHV–MCD and it may be worthwhile to consider a variety of approaches, depending on disease severity and other parameters. Our group has been exploring the use of consolidation therapy after patients achieve remissions with several months of either interferon-alpha or high dose zidovudine with valganciclovir. Additional studies will be needed to explore new therapies, to establish the best therapy for KSHV–MCD, and perhaps through a combination of treatments, to establish the best way to optimize therapy for different patients with the goal of durable long-term remission.

Because of the small number of patients with KSHV–MCD, there is little commercial interest in developing drugs specifically for this disease, and it is unlikely that a therapeutic antibody to vIL-6 will be developed in the near future. However, an antibody to the IL-6 receptor is in clinical use for rheumatoid arthritis, while a different antibody against IL-6 is being evaluated in the setting of MCD not associated with KSHV. Because hIL-6 is markedly elevated and believed to contribute to disease pathogenesis in KSHV–MCD, antibodies targeting IL-6 signaling may have activity, and we are currently conducting a clinical study of tocilizumab, an anti-IL-6 receptor antibody in patients with symptomatic KSHV–MCD (NCT01441063).

Kaposi sarcoma-associated herpesvirus inflammatory cytokine syndrome is a newly described syndrome, and there is little clinical experience to guide therapy. In those cases where KICS accompanies KS or PEL, it is reasonable to hypothesize that treatment of the underlying tumor may help reduce the KSHV-associated cytokines. However, it should be noted that in the original series describing this syndrome KS was often quite difficult to treat, perhaps because of stimulation by the IL-6 or other cytokines. It may be worthwhile to consider treatment approaches to KICS similar to those used for KSHV–MCD. These may include, for example, antiviral drugs such as ganciclovir with activity against KSHV, the use of high doses of zidovudine or ganciclovir that can be activated by KSHV to toxic moieties as described above, the use of rituximab to kill B cells that may harbor KSHV or be producing cytokines, or the use of liposomal doxorubicin to kill KS spindle cells. In the original series of six patients of KICS, one patient received high dose zidovudine and valganciclovir, one patient received valganciclovir alone, and two received liposomal doxorubicin alone (Uldrick et al., 2010a). The patient who received zidovudine/valganciclovir demonstrated an initial improvement in laboratory parameters, though his clinical course was later complicated significantly by intercurrent illness, while one patient who received valganciclovir monotherapy had a transient clinical response. The two patients who received liposomal doxorubicin (both with KS requiring therapy) also had transient clinical responses.

SUMMARY AND FUTURE CONSIDERATIONS

Soon after the discovery of KSHV in 1994, it became apparent that KSHV-associated PEL and KSHV–MCD were separate disease entities. Since that time, we have learned much about the

pathogenesis of these diseases, and for KSHV–MCD in particular, therapeutic advances have led to substantial improvement in survival. At the same time, many questions remain concerning KSHV–MCD and its relationship to other KSHV-induced diseases. More recently, KICS has been described as a new syndrome caused by KSHV; but there is much to be learned about this entity. What cells are primarily responsible for the cytokine overproduction in KICS, and what pathophysiologic mechanisms are responsible for its overproduction? What is the spectrum of disease, and what is its incidence and prevalence in different populations? How is it best managed? It is not unreasonable to hope that answers to many of these questions will be forthcoming in the next decade and that this research will help lead to improved tools to diagnose and treat these entities.

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NOTE

REFERENCES

Abend, J. R., Uldrick, T., and Ziegelbauer, J. M. (2010). Regulation of tumor necrosis factor-like weak inducer of apoptosis receptor protein (TWEAKR) expression by Kaposi's sarcoma-associated herpesvirus microRNA prevents TWEAK-induced apoptosis and inflammatory cytokine expression. *J. Virol.* 84, 12139–12151.

Aoki, Y., Jones, K. D., and Tosato, G. (2000). Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. *J. Hematother. Stem Cell Res.* 9, 137–145.

Aoki, Y., Tosato, G., Fonville, T. W., and Pittaluga, S. (2001a). Serum viral interleukin-6 in AIDS-related multicentric Castleman disease. *Blood* 97, 2526.

Aoki, Y., Yarchoan, R., Wyvill, K., Okamoto, S., Little, R. F., and Tosato, G. (2001b). Detection of viral interleukin-6 in Kaposi sarcoma-associated herpesvirus-linked disorders. *Blood* 97, 2173.

Ayers, L. W., Lukande, R., NDung'u, J. R., Chumba, D. K., and Turnwine, L. K. (2011). "Multicentric Castleman's disease (MCD) and other related KSHV lymphoproliferations in East Africa," in *International Conference on Malignancies in AIDS and Other Acquired Immunodeficiencies*, Bethesda, MD.

Boulanger, M. J., Chow, D., Brevnova, E., Martick, M., Sandford, G., Nicholas, J., and Garcia, K. C. (2004). Molecular mechanisms for viral mimicry of a human cytokine: activation of gp130 by HHV-8 interleukin-6. *J. Mol. Biol.* 335, 641–654.

Bower, M. (2010). How I treat HIV-associated multicentric Castleman disease. *Blood* 116, 4415–4421.

Bower, M., Powles, T., Williams, S., Davis, T. N., Atkins, M., Montoto, S., Orkin, C., Webb, A., Fisher, M., Nelson, M., Gazzard, B., Stebbing, J., and Kelleher, P. (2007). Brief communication: rituximab in HIV-associated multicentric Castleman disease. *Ann. Intern. Med.* 147, 836–839.

Bower, M., Veraitch, O., Szydlo, R., Charles, P., Kelleher, P., Gazzard, B., Nelson, M., and Stebbing, J. (2009). Cytokine changes during rituximab therapy in HIV-associated multicentric Castleman disease. *Blood* 113, 4521–4524.

Brandt, S. J., Bodine, D. M., Dunbar, C. E., and Nienhuis, A. W. (1990). Dysregulated interleukin 6 expression produces a syndrome resembling Castleman's disease in mice. *J. Clin. Invest.* 86, 592–599.

Cannon, J. S., Hamzeh, F., Moore, S., Nicholas, J., and Ambinder, R. F. (1999). Human herpesvirus 8-encoded thymidine kinase and phosphotransferase homologues confer sensitivity to ganciclovir. *J. Virol.* 73, 4786–4793.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Chadburn, A., Hyjek, E. M., Tam, W., Liu, Y., Rengifo, T., Cesarman, E., and Knowles, D. M. (2008). Immunophenotypic analysis of the Kaposi sarcoma herpesvirus (KSHV; HHV-8)-infected B cells in HIV+ multicentric Castleman disease (MCD). *Histopathology* 53, 513–524.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266, 1865–1869.

Chatterjee, M., Osborne, J., Bestetti, G., Chang, Y., and Moore, P. S. (2002). Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* 298, 1432–1435.

Chen, D., Choi, Y. B., Sandford, G., and Nicholas, J. (2009a). Determinants of secretion and intracellular localization of human herpesvirus 8 interleukin-6. *J. Virol.* 83, 6874–6882.

Chen, D., Sandford, G., and Nicholas, J. (2009b). Intracellular signaling mechanisms and activities of human herpesvirus 8 interleukin-6. *J. Virol.* 83, 722–733.

Cianfrocca, M., Lee, S., Von Roenn, J., Tulpule, A., Dezube, B. J., Aboulafia, D. M., Ambinder, R. F., Lee, J. Y., Krown, S. E., and Sparano, J. A. (2010). Randomized trial of paclitaxel versus pegylated liposomal doxorubicin for advanced human immunodeficiency virus-associated Kaposi sarcoma: evidence of symptom palliation from chemotherapy. *Cancer* 116, 3969–3977.

Corbellino, M., Bestetti, G., Scalamogna, C., Calattini, S., Galazzi, M., Meroni, L., Manganaro, D., Fasan, M., Moroni, M., and Galli, M. (2001). Long-term remission of Kaposi sarcoma-associated herpesvirus-related multicentric Castleman disease with anti-CD20 monoclonal antibody therapy. *Blood* 98, 3473.

Cronin, D. M., and Warnke, R. A. (2009). Castleman disease: an update on classification and the spectrum of associated lesions. *Adv. Anat. Pathol.* 16, 236–246.

Davis, D. A., Singer, K. E., Reynolds, I. P., Haque, M., and Yarchoan, R. (2007). Hypoxia enhances the phosphorylation and cytotoxicity of ganciclovir and zidovudine in Kaposi's sarcoma-associated herpesvirus infected cells. *Cancer Res.* 67, 7003–7010.

Della Bella, S., Taddeo, A., Calabrò, M. L., Brambilla, L., Bellinvia, M., Bergamo, E., Clerici, M., and Villa, M. L. (2008). Peripheral blood endothelial progenitors as potential reservoirs of Kaposi's sarcoma-associated herpesvirus. *PLoS ONE* 3, e1520. doi:10.1371/journal.pone.0001520

Dispenzieri, A., and Gertz, M. A. (2005). Treatment of Castleman's disease. *Curr. Treat. Options Oncol.* 6, 255–266.

Du, M. Q., Liu, H., Diss, T. C., Ye, H., Hamoudi, R. A., Dupin, N., Meignin, V., Oksenhendler, E., Boshoff, C., and Isaacson, P. G. (2001). Kaposi sarcoma-associated herpesvirus infects monotypic (IgM lambda) but polyclonal naive B cells in Castleman disease and associated lymphoproliferative disorders. *Blood* 97, 2130–2136.

Ganem, D. (2010). KSHV and the pathogenesis of Kaposi sarcoma: listening to human biology and medicine. *J. Clin. Invest.* 120, 939–949.

Ganem, D., and Ziegelbauer, J. (2008). MicroRNAs of Kaposi's sarcoma-associated herpes virus. *Semin. Cancer Biol.* 18, 437–440.

Gill, P. S., Loureiro, C., Bernstein-Singer, M., Rarick, M. U., Sattler, F., and Levine, A. M. (1989). Clinical effect of glucocorticoids on Kaposi sarcoma related to the acquired immunodeficiency syndrome (AIDS). *Ann. Intern. Med.* 110, 937–940.

Gregory, S. M., West, J. A., Dillon, P. J., Hilscher, C., Dittmer, D. P., and Damania, B. (2009). Toll-like receptor signaling controls reactivation of KSHV from latency. *Proc. Natl. Acad. Sci. U.S.A.* 106, 11725–11730.

Gustafson, E. A., Schinazi, R. F., and Fingeroth, J. D. (2000). Human herpesvirus 8 open reading frame 21 is a thymidine and thymidylate kinase of narrow substrate specificity that efficiently phosphorylates zidovudine but not ganciclovir. *J. Virol.* 74, 684.

Heinrich, P. C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. (1998). Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem. J.* 334, 297–314.

Hu, F., and Nicholas, J. (2006). Signal transduction by human herpesvirus 8 viral interleukin-6 (vIL-6) is modulated by the nonsignaling gp80 subunit of the IL-6 receptor complex and is distinct from signaling induced by human IL-6. *J. Virol.* 80, 10874–10878.

Jenner, R. G., Albà, M. M., Boshoff, C., and Kellam, P. (2001). Kaposi's sarcoma-associated herpesvirus latent and lytic gene expression as revealed by DNA arrays. *J. Virol.* 75, 891–902.

Jones, K. D., Aoki, Y., Chang, Y., Moore, P. S., Yarchoan, R., and Tosato, G. (1999). Involvement of interleukin-10 (IL-10) and viral IL-6 in the spontaneous growth of Kaposi's sarcoma herpesvirus-associated infected primary effusion lymphoma cells. *Blood* 94, 2871–2879.

Lei, X., Bai, Z., Ye, F., Xie, J., Kim, C. G., Huang, Y., and Gao, S. J. (2010). Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. *Nat. Cell Biol.* 12, 193–199.

Lu, C. C., Li, Z., Chu, C. Y., Feng, J., Feng, J., Sun, R., and Rana, T. M. (2010). MicroRNAs encoded by Kaposi's sarcoma-associated herpesvirus regulate viral life cycle. *EMBO Rep.* 11, 784–790.

Marcelin, A. G., Aaron, L., Mateus, C., Gyan, E., Gorin, I., Viard, J. P., Calvez, V., and Dupin, N. (2003). Rituximab therapy for HIV-associated Castleman disease. *Blood* 102, 2786–2788.

Meads, M. B., and Medveczky, P. G. (2004). Kaposi's sarcoma-associated herpesvirus-encoded viral interleukin-6 is secreted and modified differently than human interleukin-6: evidence for a unique autocrine signaling mechanism. *J. Biol. Chem.* 279, 51793–51803.

Monini, P., Colombini, S., Sturzl, M., Goletti, D., Cafaro, A., Sgadari, C., Butto, S., Franco, M., Leone, P., and Fais, S. (1999). Reactivation and persistence of human herpesvirus-8 infection in B cells and monocytes by Th-1 cytokines increased in Kaposi's sarcoma. *Blood* 93, 4044.

Moore, P. S., Boshoff, C., Weiss, R. A., and Chang, Y. (1996). Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* 274, 1739–1744.

Moore, P. S., and Chang, Y. (1998). Kaposi's sarcoma-associated herpesvirus-encoded oncogenes and oncogenesis. *J. Natl. Cancer Inst. Monogr.* 23, 65–71.

Mylona, E., Baraboutis, I. G., Georgiou, O., Rondogianni, D., Lekakis, L. J., Papastamopoulos, V., Apostolidis, I., and Skoutelis, A. T. (2008). Solid variant of primary effusion lymphoma in successfully treated HIV infection: a case report. *Int. J. STD AIDS* 19, 570–572.

Naresh, K. N., Rice, A. J., and Bower, M. (2008). Lymph nodes involved by multicentric Castleman disease among HIV-positive individuals are often involved by Kaposi sarcoma. *Am. J. Surg. Pathol.* 32, 1006–1012.

Naresh, K. N., Trivedi, P., Horncastle, D., and Bower, M. (2009). CD20 expression in the HHV-8-infected lymphoid cells in multicentric Castleman disease. *Histopathology* 55, 358–359.

Neipel, F., Albrecht, J. C., Ensser, A., Huang, Y. Q., Li, J. J., Friedman-Kien, A. E., and Fleckenstein, B. (1997). Human herpesvirus 8 encodes a homolog of interleukin-6. *J. Virol.* 71, 839.

Neuville, S., Agbalika, F., Rabian, C., Briere, J., and Molina, J. M. (2005). Failure of rituximab in human immunodeficiency virus-associated multicentric Castleman disease. *Am. J. Hematol.* 79, 337–339.

Northfelt, D. W., Dezube, B. J., Thommes, J. A., Miller, B. J., Fischl, M. A., Friedman-Kien, A., Kaplan, L. D., Du Mond, C., Mamelok, R. D., and Henry, D. H. (1998). Pegylated-liposomal doxorubicin versus doxorubicin, bleomycin, and vincristine in the treatment of AIDS-related Kaposi's sarcoma: results of a randomized phase III clinical trial. *J. Clin. Oncol.* 16, 2445–2451.

Ocio, E. M., Sanchez-Guijo, F. M., Diez-Campelo, M., Castilla, C., Blanco, O. J., Caballero, D., and San Miguel, J. F. (2005). Efficacy of rituximab in an aggressive form of multicentric Castleman disease associated with immune phenomena. *Am. J. Hematol.* 78, 302–305.

Oksenhendler, E. (2009). HIV-associated multicentric Castleman disease. *Curr. Opin. HIV AIDS* 4, 16.

Oksenhendler, E., Carcelain, G., Aoki, Y., Boulanger, E., Maillard, A., Clauvel, J. P., and Agbalika, F. (2000). High levels of human herpesvirus 8 viral load, human interleukin-6, interleukin-10, and C reactive protein correlate with exacerbation of multicentric Castleman disease in HIV-infected patients. *Blood* 96, 2069–2073.

Oksenhendler, E., Duarte, M., Soulier, J., Cacoub, P., Welker, Y., Cadranel, J., Cazals-Hatem, D., Autran, B., Clauvel, J. P., and Raphael, M. (1996). Multicentric Castleman's disease in HIV infection: a clinical and pathological study of 20 patients. *AIDS* 10, 61.

Osborne, J., Moore, P. S., and Chang, Y. (1999). KSHV-encoded viral IL-6 activates multiple human IL-6 signaling pathways. *Hum. Immunol.* 60, 921–927.

Parravicini, C., Chandran, B., Corbellino, M., Berti, E., Paulli, M., Moore, P. S., and Chang, Y. (2000). Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. *Am. J. Pathol.* 156, 743–749.

Polizzotto, M. N., Millo, C., Uldrick, T. S, Aleman, K., Whatley, M., Wyvill, K. M., O'Mahony, D., Marshall, V., Whitby, D., Steinberg, S. M., Little, R. F., and Yarchoan, R. (2010). "[18F]-fluoro-D-deoxyglucose-positron emission tomography findings in Kaposi sarcoma-associated herpesvirus associated multicentric Castleman disease in people with HIV: correlation with clinical, inflammatory and virologic parameters," in *Conference on Retroviruses and Opportunistic Infections*, Boston, MA.

Polizzotto, M. N., Uldrick, T. S., Wang, V., Aleman, K., Wyvill, K. M., Marshall, V., Pittaluga, S., O'Mahony, D., Whitby, D., Tosato, G., Steinberg, S. M., Little, R. F., and Yarchoan, R. (2011). "Distinct human and viral interleukin-6 profiles and other viral and immunologic abnormalities in KSHV-associated multicentric Castleman disease: relationship with disease activity and individual disease manifestations," in *American Society of Hematology Annual Meeting*, San Diego, CA, A1573.

Powles, T., Stebbing, J., Bazeos, A., Hatzimichael, E., Mandalia, S., Nelson, M., Gazzard, B., and Bower, M. (2009). The role of immune suppression and HHV-8 in the increasing incidence of HIV-associated multicentric Castleman's disease. *Ann. Oncol.* 20, 775–779.

Qin, Z., Kearney, P., Plaisance, K., and Parsons, C. H. (2010). Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded microRNA specifically induce IL-6 and IL-10 secretion by macrophages and monocytes. *J. Leukoc. Biol.* 87, 25–34.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and Sigaux, F. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86, 1276–1280.

Staskus, K. A., Sun, R., Miller, G., Racz, P., Jaslowski, A., Metroka, C., Brett-Smith, H., and Haase, A. T. (1999). Cellular tropism and viral interleukin-6 expression distinguish human herpesvirus 8 involvement in Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. *J. Virol.* 73, 4181–4187.

Stebbing, J., Pantanowitz, L., Dayyani, F., Sullivan, R. J., Bower, M., and Dezube, B. J. (2008). HIV-associated multicentric Castleman's disease. *Am. J. Hematol.* 83, 498–503.

Stone, S. F., Price, P., Brochier, J., and French, M. A. (2001). Plasma bioavailable interleukin-6 is elevated in human immunodeficiency virus-infected patients who experience herpesvirus-associated immune restoration disease after start of highly active antiretroviral therapy. *J. Infect. Dis.* 184, 1073–1077.

Sun, R., Lin, S. F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., and Miller, G. (1999). Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. *J. Virol.* 73, 2232.

Teruya-Feldstein, J., Zauber, P., Setsuda, J. E., Berman, E. L., Sorbara, L., Raffeld, M., Tosato, G., and Jaffe, E. S. (1998). Expression of human herpesvirus-8 oncogene and cytokine homologues in an HIV-seronegative patient with multicentric Castleman's disease and primary effusion lymphoma. *Lab. Invest.* 78, 1637–1642.

Uldrick, T. S., Polizzotto, M. N., Aleman, K., O'Mahony, D., Wyvill, K. M., Wang, V., Marshall, V., Pittaluga, S., Steinberg, S. M., Tosato, G., Whitby, D., Little, R. F., and Yarchoan, R. (2011a). High-dose zidovudine plus valganciclovir for Kaposi sarcoma herpesvirus-associated multicentric Castleman disease: a pilot study of virus-activated cytotoxic therapy. *Blood* 117, 6977–6986.

Uldrick, T. S., Polizzotto, M. N., Wyvill, K. M., Aleman, K., Marshall, V., Little, R. F., Filie, A., Raffeld, M., Steinberg, S. M., Pittaluga, S., Whitby, D., and Yarchoan, R. (2011b). "Patients with Kaposi sarcoma herpesvirus

(KSHV)-associated aggressive lymphomas have markedly elevated interleukin-10, abnormalities in other inflammatory cytokines, and high circulating KSHV viral load," in *International Conference on Malignancies in AIDS and Other Acquired Immunodeficiencies*, Bethesda, MD.

Uldrick, T. S., Wang, V., O'Mahony, D., Aleman, K., Wyvill, K. M., Marshall, V., Steinberg, S. M., Pittaluga, S., Maric, I., Whitby, D., Tosato, G., Little, R. F., and Yarchoan, R. (2010a). An interleukin-6-related systemic inflammatory syndrome in patients co-infected with Kaposi sarcoma-associated herpesvirus and HIV but without multicentric Castleman disease. *Clin. Infect. Dis.* 51, 350–358.

Uldrick, T. S., Polizzotto, M. N., O'Mahony, D., Aleman, K., Wyvill, K. M., Steinberg, S. M., Pittaluga, S., Marshall, V., Whitby, D., Tosato, G., Little, R. F., and Yarchoan, R. (2010b). Rituximab combined with liposomal doxorubicin (R-Dox) in HIV-infected patients with severe Kaposi sarcoma-associated herpes virus (KSHV) associated multicentric Castleman disease (MCD). *Infect. Agents Cancer* 5(Suppl. 1), A72.

Umbach, J. L., and Cullen, B. R. (2010). In-depth analysis of Kaposi's sarcoma-associated herpesvirus microRNA expression provides insights into the mammalian microRNA-processing machinery. *J. Virol.* 84, 695–703.

Waterston, A., and Bower, M. (2004). Fifty years of multicentric Castleman's disease. *Acta Oncol.* 43, 698–704.

Wen, K. W., and Damania, B. (2010). Kaposi sarcoma-associated herpesvirus (KSHV): molecular biology and oncogenesis. *Cancer Lett.* 289, 140–150.

Wu, W., Vieira, J., Fiore, N., Banerjee, P., Sieburg, M., Rochford, R., Harrington, W., and Feuer, G. (2006). KSHV/HHV-8 infection of human hematopoietic progenitor (CD34+) cells: persistence of infection during hematopoiesis in vitro and in vivo. *Blood* 108, 141–151.

Yarchoan, R. (2006). Key role for a viral lytic gene in Kaposi's sarcoma. *N. Engl. J. Med.* 355, 1383–1385.

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Pathology of Kaposi's sarcoma-associated herpesvirus infection

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Kaposi's sarcoma-associated herpesvirus (KSHV; human herpesvirus 8) is a human herpesvirus, classified as a gamma-herpesvirus. KSHV is detected in Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and some cases of multicentric Castleman's disease (MCD). Similar to other herpes viruses, there are two phases of infection, latent and lytic. In KSHV-associated malignancies such as KS and PEL, KSHV latently infects almost all tumor cells. Quantitative PCR analysis revealed that each tumor cell contains one copy of KSHV in KS lesions. The oncogenesis by KSHV has remained unclear. Latency-associated nuclear antigen (LANA)-1 plays an important role in the pathogenesis of KSHV-associated malignancies through inhibition of apoptosis and maintenance of latency. Because all KSHV-infected cells express LANA-1, LANA-1 immunohistochemistry is a useful tool for diagnosis of KSHV infection. KSHV encodes some homologs of cellular proteins including cell-cycle regulators, cytokines, and chemokines, such as cyclin D, G-protein-coupled protein, interleukin-6, and macrophage inflammatory protein-1 and -2. These viral proteins mimic or disrupt host cytokine signals, resulting in microenvironments amenable to tumor growth. Lytic infection is frequently seen in MCD tissues, suggesting a different pathogenesis from KS and lymphoma.

Keywords: Kaposi's sarcoma-associated herpesvirus, HHV-8, latency-associated nuclear antigen, LANA-1, primary effusion lymphoma

INTRODUCTION

The 1994 discovery of Kaposi's sarcoma-associated herpesvirus (KSHV, human herpesvirus 8, HHV-8) in Kaposi's sarcoma (KS) tissues had a huge impact, not only in the field of virology, but also on bioscience generally (Chang et al., 1994; Ganem, 2005). Before the discovery of KSHV, almost all viruses had been identified using conventional virus isolation methods with cell cultures. DNA fragments of KSHV were identified in KS tissues by representational difference analysis, which is a subtraction PCR-based method to purify restriction-endonuclease-digested fragments present in one population of DNA fragments but not in others (Chang et al., 1994). Thus, KSHV is the first virus whose fragments were identified directly by the PCR method before any cell culture methods. In 1996, KSHV-infected cell lines were established, based on the fragments' DNA sequences (Renne et al., 1996b). Herpesvirus-like particles of this virus were found in lymphoma cells by electron microscopic analysis. Finally, KSHV's full DNA sequence was determined (Russo et al., 1996). Over the 15-years since the discovery of KSHV, it has been established as a tumor virus (Ganem, 2005). Some KSHV-encoded genes are homologous to oncogenes or cell-cycle-associated genes (Russo et al., 1996); some are transformational genes, able to transform human cells (Gao et al., 1997; Bais et al., 1998; Lee et al., 1998; Muralidhar et al., 1998). However, expression of KSHV-encoded genes is severely restricted; only a few viral genes are expressed in KSHV-infected cells. The KSHV-encoded latency-associated nuclear antigen 1 (LANA-1) is the only protein whose expression is stably detected by immunohistochemistry in KSHV-infected cells (Dupin et al., 1999; Katano et al., 2000b). LANA-1 is a multifunctional protein, but has no full transforming activity. In comparison, Epstein–Barr virus (EBV) encodes a full oncogenic protein, latent membrane protein-1 (LMP1), which is expressed in a subset of EBV-latently infected cells (Cohen, 2000). Thus, KSHV oncogenesis is not simple. Many KSHV-encoded non-transforming proteins apparently collaborate to establish and maintain oncogenesis in KSHV-infected cells. In this review, the pathological aspects of KSHV infection and KSHV-associated diseases are summarized.

VIRUS AND ITS GENE EXPRESSION

Usually, viral particles are not observed in KS samples by electron microscope because of the small number of KSHV copies. However, they can be seen in primary effusion lymphoma (PEL) cell lines stimulated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). A complete viral particle of KSHV, consisting of a capsid and an envelope (Renne et al., 1996b; Said et al., 1996, 1997; Orenstein et al., 1997; Ohtsuki et al., 1999), is 150–200 nm in diameter, which is similar to other human herpes viruses and indistinguishable from other herpes viruses. The unenveloped capsid is produced in the host nucleus and is 100 nm in diameter. It contains a central DNA core, which appears to have a high electron density. The envelope is derived from the inner nuclear membrane, as viral particles bud into the cytoplasm from the nucleus. The tegument protein fills the space between the nucleocapsid and envelope. This feature

of viral particles is apparently quite similar among herpes viruses, but related structures forming in infected cells seem to depend on the type of virus.

The KSHV genome consists of linear, double-stranded DNA of about 170 kbp (Renne et al., 1996a; Russo et al., 1996). The KSHV genome consists of a long unique region (LUR) and a terminal repeat (TR) at both termini, which resembles the herpes virus saimiri structure (Russo et al., 1996). The TRs consist of 801-bp direct repeat units having 84.5% GC content. The number of repeats in TRs may vary. The LUR is 140.5 kbp and has 53.5% GC content. KSHV encodes more than 80 viral proteins on LUR. KSHV also encodes 17 microRNAs (miRNAs), which are derived by processing from 12 pre-miRNAs (Cai et al., 2005). Kinetics of KSHV-encoded genes were mainly investigated in KSHV-infected PEL cell lines stimulated with phorbol ester such as TPA (Sun et al., 1999). Like other herpesviruses, viral genes were categorized into lytic and latent genes, and also into immediate-early (IE), early (E), and late (L) genes based on their expressions. The function of each KSHV-encoded gene was summarized in the **Table 1**. Open reading frame 50 (*ORF50*) is an IE gene that is a homolog of *Rta*, a transcriptional activator encoded by EBV (Lukac et al., 1999; Seaman et al., 1999; Sun et al., 1999; Zhu et al., 1999). Transcription of *ORF50* results in its expression within 4 h after stimulation by TPA. This expression could not be blocked by phosphonoacetic acid (a herpesvirus-DNA polymerase inhibitor) nor cycloheximide (a protein synthesis inhibitor). Transfection of *ORF50* to KSHV-infected cells resulted in the activation of lytic gene expression (Lukac et al., 1999). Thus, ORF50 protein is a lytic switch protein. Expression of ORF50 protein is required for expression of many KSHV-encoded lytic genes such as *K3*, and *K5* (homologs of the IE gene of *BHV-4*), viral interleukin-6 (*vIL-6*), viral macrophage inflammatory proteins (*vMIP*s), polyadenylated nuclear RNA (*PAN*), *vBcl-2*, *K12*, viral G-protein-coupled receptor (*vGPCR*), viral dihydrofolate reductase (*vDHFR*), DNA replication factors, and thymidylate synthase (Sarid et al., 1998). ORF50 protein also induces expression of K8 (K-bZIP, a positional homolog of EBV BZLF1) protein, an early protein. K8 protein plays a role as transactivation repressor for ORF50 protein, leading to a negative autoregulation system during lytic infection (Liao et al., 2003). Late genes, including tegument proteins, and virion-associated protein are then expressed (**Table 1**).

Latent infection is predominant in KSHV infection. KSHV codes a latency-associated gene cluster including *ORF73* (*LANA-1*, *LNA*, or *LNA-1*), v-cyclin (*ORF72*), viral FLICE-inhibitory protein (*K13*, *v-FLIP*), Kaposin (*K12*), and viral-encoded miRNAs. LANA-1 is always detected as a dot-like staining pattern in KSHV-infected cells by immunohistochemistry. KSHV-encoded 17 miRNAs, which are derived by processing from 12 pre-miRNAs, are expressed during viral latency (Cai et al., 2005; Samols et al., 2005).

Table 1 | Kaposi's sarcoma-associated herpesvirus genes and their functions.

Gene	Phase	Functions
LANA-1	Latent	Always express in KSHV-infected cells Maintain and replicate viral genome during mitotic division by holding KSHV episome at chromosome Bind to p53 and inhibit p53-dependent apoptosis Bind to Rb and inhibit Rb-E2F pathway Bind to GSK-3β, and induce accumulation of β-catenin
LANA-2	Latent	Expressed in only PEL cells, not in KS cells Homolog of IRF Inhibit p53-dependent apoptosis
Kaposin	Latent	Kaposin A: transformation activity? Kaposin B, C: associate with cytokine expression as adaptor protein of MAP kinase-associated protein kinase 2 (MK2)
v-cyclin	Latent	Homolog of cyclin D1 Inhibit P27Kip1, and induce cell-cycle to S-phase
v-FLIP	Latent	Anti-apoptosis
ORF50 (RTA)	Lytic (IE)	Lytic switch protein Transactivator for K8
K1	Lytic	Transformation activity
K8	Lytic (early)	Transcriptional repressor for RTA
K3, K5	Lytic (IE/early)	Down-regulation of MHC class I expression
vIL-6	Lytic (early)	Induce VEGF expression Induce constitutional activation of Stat3 Disrupt anti-viral function by IFN-α
vIRF-1	Lytic (early)	Disrupt IFN signal Transformation activity?
vMIPs	Lytic (early)	Bind to chemokine receptors and induce angiogenesis
vBcl-2	Lytic (early)	Inhibit apoptosis
vGPCR	Lytic (early)	Transformation activity Bind to IL-8 Induce VEGF expression
K15	Lytic	Bind to TRAF family, and induction of NF-κB activation

KSHV ONCOGENESIS

The first evidence of transformation activity by KSHV came from a report describing that human umbilical vein endothelial cells (HUVEC) were transformed and immortalized by KSHV infection *in vitro* (Flore et al., 1998). However, such KSHV-infected HUVEC did not express any KSHV gene, and the immortalization by KSHV infection was not confirmed by any other groups (Gao et al., 2003; Tang et al., 2003). KSHV efficiently infects primary cultures of human endothelial cells *in vitro* (Sakurada et al., 2001; Gao et al., 2003). KSHV-infected cells express LANA-1 within several hours after infection. One week after infection, a large portion of culture cells will be infected by KSHV and expressing LANA-1. Interestingly, expression of any lytic proteins encoded by KSHV is not observed at that time. Latent infection is dominant in KSHV-infected cells *in vivo* and *in vitro*. Although some KSHV-encoded proteins such as K1 and vGPCR

are shown to have a transformation activity on mammalian cells, these transforming proteins are not usually expressed in KSHV-infected cells (Bais et al., 1998; Lee et al., 1998; Montaner et al., 2003). However, LANA-1, a major KSHV-encoded latency protein, is always expressed in KSHV-infected cells both *in vivo* and *in vitro* (Dupin et al., 1999; Katano et al., 1999b; Kellam et al., 1999). Moreover, latency is maintained during the presence of KSHV in the cells. Thus, LANA-1 clearly plays an important role in the pathogenesis of KSHV infection, and has been shown to be a multifunctional protein. Probably the most important role of LANA-1 is to establish and maintain the latency in KSHV-infected cells by tethering KSHV DNA to host chromosomes (Ballestas et al., 1999). LANA-1 binds directly to TR sequences of the KSHV genome, and recruits it to the host chromosome (**Figure 1E**). The DNA of KSHV is replicated during host cell divisions using host DNA replicative machinery (Sakakibara et al., 2004). Thus, daughter cells inherit KSHV genome without any virus particle. LANA-1 is also associated with signal transduction in KSHV-infected cells. LANA-1 binds directly to p53, a major tumor repressor and anti-apoptotic factor (Friborg et al., 1999). Viral infection usually induces p53 expression and p53-dependent apoptosis as self-defense system. Direct interaction with p53 by LANA-1 results in inhibition of p53-dependent apoptosis in KSHV-infected cells. Moreover, LANA-1 stabilizes β-catenin by binding to the negative regulator GSK-3β, promoting cell-cycle induction by nuclear accumulation of GSK-3β (Fujimuro et al., 2003). Thus, LANA-1 plays a central role in the pathogenesis of KSHV infection, but LANA-1 itself does not have any full transformation activity. Many other factors besides LANA-1 are required to establish KSHV oncogenesis.

Another important factor in KSHV oncogenesis is that KSHV encodes many homologs of human genes. The viral genes of human gene homologs cooperate to establish suitable growth conditions for KSHV-infected cells. Among them, vIL-6 is the most important factor for KSHV pathogenesis. vIL-6 induces angiogenesis by vascular endothelial cell growth factor (VEGF) expression (Aoki et al., 1999), and stimulates the constitutive Jak-Stat pathway through the Stat3 signal, resulting in cell growth (Aoki et al., 2003). In addition, vIL-6 represses the anti-viral function of interferon by binding to a subunit of human IL-6 receptor and suppressing p21 expression (Chatterjee et al., 2002). KSHV-encoded vMIP-1, vMIP-2, vBcl-2, vIRF-1, v-cyclin D, and v-FLIP mimic their human homologs, and work sometimes as inhibitors and sometimes as mimics, resulting in growth of KSHV-infected cells. Because almost all these mimics are lytic proteins, their expression is not usually observed. However, some cytokines may induce their expression independently to lytic and latent infection as necessary. Thus, KSHV oncogenesis is established by cooperation of many viral proteins such as LANA-1 and by the mimic, rather than the primary functions of oncogenetic transformation genes encoded by the virus.

Recently, miRNA has been shown to affect tumor biology. Several KSHV miRNAs were shown to modulate host gene expression, suggesting some roles for miRNA in the pathogenesis of KSHV-induced malignancies. Thrombospondin 1, a potent inhibitor of angiogenesis that is reportedly downregulated in KS lesions, is targeted by multiple miRNAs (Samols et al., 2007). The target of miR-K5 is Bcl2-associated factor BCLAF1, which promote apoptosis (Lei et al., 2010). MiR-K1 targets IκBα, an inhibitor of NF-κB. NF-κB inhibits the activation of lytic viral promoters. By activating NF-κB, miR-K1 suppresses viral lytic replication, maintaining latent infection (Ziegelbauer et al., 2009). So far, miRNAs' roles in viral infection and replication remain unclear.

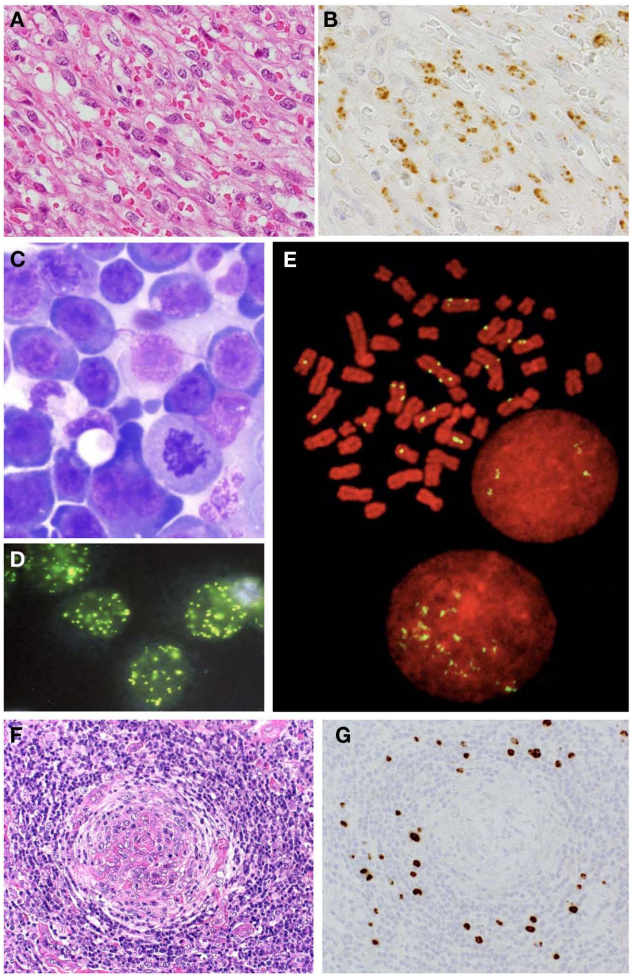

FIGURE 1 | Histological analysis on KSHV-associated diseases. (A) Nodular stage of KS; HE staining. **(B)** LANA-1 immunohistochemistry of KS. **(C)** Giemsa staining of PEL. **(D)** LANA-1 immunofluorescence staining in mitosis of PEL cells. **(E)** LANA-1 immunostaining of mitosis of PEL cell line, TY-1. Yellow signals indicate LANA-1. Red is counter staining of chromosome. **(F)** HE staining of MCD. **(G)** LANA-1 immunohistochemistry of MCD.

EPIDEMIOLOGY

Serological studies have revealed that KSHV-infected individuals are found all over the world. Serum antibody to KSHV is detected with ELISA using lysate of KSHV viral particles or recombinant viral proteins as antigens, or immunofluorescence assay using KSHV-infected cells. The seroprevalence of KSHV infection differs among regions/countries. Among the general population, KSHV seropositivity is less than 10% in northern Europe, America, and Asia, 10–30% in the Mediterranean region, and more than

Table 2 | Kaposi's sarcoma-associated herpesvirus genotypes.

Genotype	Patients, infected persons
A	AIDS–KS patients in the US, Europe, Eurasia
B	KS patients of African heritage
C	Classic KS, iatrogenic, and AIDS–KS in Eurasia, US AIDS–KS, Taiwan, Korea, China, Middle East
D	KS patients of Pacific island
E	South American (partial), Brazil Amerindian, Guinea Amerindian

50% in most of sub-Saharan Africa (Davis et al., 1997; Kedes et al., 1997; Chatlynne et al., 1998; Mayama et al., 1998; Rabkin et al., 1998; Katano et al., 2000a). The homosexual population exhibits higher positivity (8–25%) than the general population (Grulich et al., 2005; Casper et al., 2006; Engels et al., 2007). Although the transmission modes of KSHV have not yet been clarified, transmission though saliva is likely (Pauk et al., 2000), because high KSHV copy numbers are detected in saliva of seropositives. Horizontal transmission through the saliva transmission is suggested among children in endemic countries, while sexual transmission may be predominant among homosexual men in non-endemic countries. Organ transplantation can transmit KSHV (Regamey et al., 1998). Transmission of KSHV through blood transfusion is controversial. While KSHV seroconversion was found in US transfusion recipients (Hladik et al., 2006), later studies found no significant association of KSHV infection between transfusion groups and non-transfusion groups (Cannon et al., 2009).

Genotypes of KSHV are categorized based on sequences of the hypervariable regions in its *K1* gene (Meng et al., 1999; Zong et al., 1999; Biggar et al., 2000; Kazanji et al., 2005; Hayward and Zong, 2007; Kanno et al., 2010). The KSHV *K1* genes are classified into at five groups: A, B, C, D, and E (**Table 2**). Geographical differences in KSHV genotypes may reflect the history of migration of human populations (Zong et al., 1999). Subtypes A and C were detected in Japan and subtype A was seen more frequently in AIDS-associated cases than non-AIDS patients (Kanno et al., 2010). There is no correlation between genotype and KSHV-related disease, including KS, PEL, and multicentric Castleman's disease (MCD).

KSHV-RELATED DISEASES

Fragments of the KSHV genome have been detected in DNA samples extracted from various diseases by PCR. However, the only diseases whose associations with KSHV infection are widely accepted among researchers in this field are KS, PEL, and MCD (**Table 3**). KSHV is distributed all over the world, and there are many individuals with KSHV infections. Therefore, a low KSHV titer, as detected by PCR, does not mean that a disease is associated with KSHV infection. Because KSHV LANA-1 is always expressed in KSHV-infected cells, LANA-1 immunohistochemistry is a powerful and confirmative tool to detect KSHV-infected cells in pathological samples, and the association with KSHV infection in diseases should be examined by LANA-1 immunohistochemistry on tissue samples.

Table 3 | Kaposi's sarcoma-associated herpesvirus and diseases.

Usually detected (Confirmed association in all cases)	Kaposi's sarcoma (all subtypes), primary effusion lymphoma
Partially detected (Confirmed association only in $KSHV^+$ cases)	Multicentric Castleman's disease including POEMS (polyneuropathy, organomegaly, endocrinopathy, M protein, skin changes) syndrome, febrile maculopapular skin rash, hemophagocytic syndrome
Detected in reports, but no association with KSHV infection	Multiple myeloma, primary pulmonary hypertension, Bowen disease, squamous cell carcinoma, Paget disease, actinic keratosis etc.

PRIMARY KSHV INFECTION

A mass study of immunocompetent children in Egypt, where KSHV infection is common, suggested that a febrile maculopapular skin rash was associated with primary KSHV infection (Andreoni et al., 2002). Seroconversion for KSHV was confirmed in those patients and transmission through saliva was implied by DNA sequences in saliva. A study of homosexual men without HIV infection suggested that diarrhea, fatigue, localized skin rash, and lymphadenopathy were also symptoms of primary KSHV infection (Wang et al., 2001). Moreover, active KSHV infection may be associated with non-malignant illnesses such as fever, cutaneous rash, and hepatitis after peripheral blood stem cell/bone marrow transplantation (Luppi et al., 2000).

KAPOSI'S SARCOMA

Kaposi's sarcoma is most important and common of KSHV-associated diseases. Four clinical subtypes have been recognized: classic, AIDS-associated, post-transplantational (iatrogenic or immunodeficient), and African (endemic) subtypes (Antman and Chang, 2000). These four subtypes of KS are histologically indistinguishable. In the AIDS–KS subtype, KS occurs only in homosexual men. KS occurs in the skin, oral cavity, gastrointestinal tract, lung, liver, lymph node, etc. Skin lesions of KS are most common; they are clinically classified as patchy, plaque, and nodular stages. In the patchy stage, small red flat lesions are observed on the skin. Histologically, dilated, abnormally shaped blood vessels with extravasated red blood cells and edema are found in KS lesions. In the plaque stage, patchy lesions fuse together to form plaque lesions. Proliferation of the spindle-shaped cells is seen around vessels in the plaque stage. In the final nodular stage, brown nodular, and elevated lesions are observed. Histologically, proliferation of spindle cells with slit-like vascular spaces is found (**Figure 1A**). Multiple KS lesions in the extremities or face are often complicated with lymphedema. Pulmonary lesions may lead to fatal respiratory compromise.

Kaposi's sarcoma should be diagnosed with histology and immunohistochemistry. Immunohistochemical staining with anti-LANA-1 antibody shows that the viral protein is expressed in KS cells, irrespective of clinical type or disease stage (Dupin et al., 1999; Katano et al., 1999b). Expression of LANA-1 can be seen in nuclei of KS spindle cells with a speckled pattern (**Figure 1B**). The

lymphatic marker, podoplanin (D2-40), is also expressed in KS cells (Weninger et al., 1999). In addition to histological investigation, PCR analysis is useful for the KS diagnosis. Because each KS cell contains about one copy of the KSHV genome, KSHV DNA fragments are consistently detected by PCR, even in formalin-fixed paraffin-embedded KS tissues (Asahi-Ozaki et al., 2006). PCR sometimes, but not always, detects KSHV DNA in the sera of KS patients. Serum antibody to KSHV is usually positive in KS patients.

Highly active anti-retroviral therapy (HAART) is effective on KS. Incidence of KS in HIV-infected persons has dramatically decreased in the HAART era. Regression of KS is often observed in patients administrated with HAART. In patients with low CD4 counts, KS progresses earlier than in patients with high CD4 counts. These data suggest that KS progression depends on the host's immune status (Bower et al., 2009). Recently, patients with KS were administrated with HAART. Patients with aggressive KS received a combination therapy of HAART and chemotherapy of pegylated liposomal doxorubicin (Martin-Carbonero et al., 2008). Irradiation or surgical resection is also performed for the case of small skin lesion in addition to HAART. There is no effective anti-KSHV therapy for KS. Although vaccine is the most effective method to prevent viral diseases, no vaccine against KSHV is commercially available at present.

The pathological roles of KSHV in KS have been intensely investigated for a long time. The origin of KS cells is thought to be endothelial cells. However, cellular protein expression in KS cells is very different from those of endothelial cells. Infection by KSHV induces a dynamic alteration of gene expression in endothelial cells (Hong et al., 2004; Wang et al., 2004). Analysis via DNA array revealed that endothelial cells reduce expression of blood vascular genes and induce markers of lymphatic endothelial cells after KSHV infection *in vitro*. Thus, KSHV can affect the expression level of cellular proteins in endothelial cells. LANA-1 is expressed in the nucleus by almost all KS spindle shaped cells (**Figure 1B**), whereas the expression of lytic proteins is limited in KS lesions. Therefore, it is likely that latent infection by KSHV is important for the pathogenesis of KS. As described above, LANA-1 plays a central role in the establishment and maintenance of latency. In addition to LANA-1, cytokines are important for KS pathogenesis. Some cytokines have been detected in the sera of KS patients at high levels. It has been demonstrated that bFGF, IL-6, oncostatin M (OSM), and tumor necrosis factor (TNF)-alpha are required for growth of KS cells *in vitro* (Liu et al., 1997; Faris et al., 1998; Murakami-Mori et al., 1998). IL-6 is known to be an important growth factor of KS cells especially *in vitro*. KSHV-encoded vIL-6 interacts with the receptor of human IL-6, mimics its function partially, and contributes to immune escape mechanism by KS cells as described above. KSHV-infected cells have several immune escape mechanisms besides that of vIL-6. K5, a lytic protein of KSHV, down-regulates MHC class I and co-activation molecules, enabling productively infected cells to escape both cytotoxic T cell and NK cell responses (Ishido et al., 2000). In addition, latently infected cells are also resistant to cytotoxic T cell responses owing to reduced levels of MHC class I molecules, impaired antigen processing, and expression of the anti-apoptotic KSHV ORF-K13/viral FLICE-inhibitory protein (v-FLIP; Thome et al., 1997).

PRIMARY EFFUSION LYMPHOMA

Primary effusion lymphoma is a rare disease occurring mainly in immunosuppressed patients, in particular HIV-infected homosexual males (Cesarman et al., 1995; Nador et al., 1996). PEL appears as lymphomatous effusions occurring in the pleural, abdominal, or pericardial effusion in the absence of a contiguous tumor mass. Some patients with PEL secondarily develop solid tumors in adjacent structures such as the pleura; these solid tumors have been termed extracavity PEL (Chadburn et al., 2004). About half of PEL patients have KS. These tumors always carry KSHV and are commonly co-infected by EBV. Histologically, the tumor cells exhibit various appearances, from large immunoblastic or plasmablastic cells to cells with more anaplastic morphology (**Figure 1C**). Nuclei vary from large and round to more irregular in shape, with prominent nucleoli. The cytoplasm can be abundant and is deeply basophilic with vacuoles in occasional cells. Binucleated or multinucleated cells resembling Reed–Sternberg cells can be seen. Mitotic figures are typically numerous. PEL cells are derived from post-germinal center B-cells (Jenner et al., 2003). Their immunophenotypes are undetermined, i.e., CD45 (+), CD138 (+), B-cell markers (−), T cell markers (−); however, their immunoglobulin genes are clonally rearranged and hypermutated. PEL cells contain high copy numbers (about 50 copies/cells) of KSHV DNA (Cesarman et al., 1995; Asahi-Ozaki et al., 2006). PEL cells are sometimes co-infected with EBV, while others are infected only with KSHV. However, expressions of LMPs and EBNAs are suppressed in PEL cells. Several KSHV-infected cell lines have been established from PEL cells (Carbone et al., 2010). A $KSHV^+/EBV^-$ cell line, TY-1, was even established from EBV^+ and $KSHV^+$ PEL cases, suggesting that KSHV plays an essential role in the pathogenesis of PEL (Katano et al., 1999a). Infection by KSHV is predominantly latent in PEL cells, which has made PEL cell lines the most widely studied models for KSHV latency. PEL cells express latent genes coded in the latent cluster in KSHV genome (**Figure 1D**). However, it is not easy to detect latent viral protein expressions other than LANA-1. The expression pattern of KSHV-encoded proteins is almost the same as KS, except that PEL cell express LANA-2 protein (Rivas et al., 2001). Most PEL lines display a very small subpopulation of cells that stain for markers of lytic reactivation such as ORF50, ORF59, and K8.1 (Katano et al., 2000b). Although KSHV-encoded vIL-6 is thought as a lytic protein, vIL-6 is detected more frequently in PEL cells than other lytic proteins. It has been demonstrated that vIL-6 is a multifunctional protein; vIL-6 can bind to IL-6 receptor gp130 in the absence of another subunit of IL-6 receptor, gp80, suggesting vIL-6 can induce cytokine signals in a broader range of cell types (Chatterjee et al., 2002). The signal from gp130 often secretes human IL-6 itself, raising the possibility of an autocrine loop. vIL-6 also induces VEGF expression, resulting in an indirect proliferation effect on PEL cells (Aoki et al., 1999).

MULTICENTRIC CASTLEMAN'S DISEASE

Multicentric Castleman's disease is characterized by plasmacytic lymphoadenopathy with polyclonal hyperimmunoglobulinemia

and high levels of IL-6 in the serum. Histologically, follicular hyperplasia with proliferation of plasma cells and hyaline vascular alterations are observed in the lymph nodes (**Figure 1F**). Two distinct histopathologic subtypes have been reported; the hyaline vascular type (HV type) and the plasma cell type (PC-type). The HV type is characterized by enlarged lymphoid follicles, hyalinized germinal centers within an expanded mantle zone, and a highly vascularized interfollicular area. In contrast, in the PC-type, remarkable infiltration of plasma cells is observed in the interfollicular area. Among these mantle zone cells, there are variable numbers of the larger cells, which are approximately twice the size of mantle zone lymphocytes. These cells are characterized by a moderate amount of amphophilic cytoplasm and a large vesicular nucleus containing one or sometimes two prominent nucleoli. These cells have been called plasmablasts, although they frequently have immunoblastic features (Dupin et al., 2000). The plasmablasts are also found in the interfollicular area of PC-type MCD frequently. In some, but not all cases of MCD, KSHV is detected (Soulier et al., 1995). Using PCR, KSHV is frequently detected in tissues obtained from patients with MCD associated with HIV infection, but is very rare in MCD cases without HIV infection (Suda et al., 2001). KSHV was also detected with high frequency in MCD complicated with polyneuropathy, organomegaly, endocrinopathy, M protein, and skin changes (POEMS) syndrome (Belec et al., 1999). Immunohistochemistry for LANA-1 revealed that KSHV-infected cells are localized in the mantle zone of lymphoid follicles (**Figure 1G**). Besides LANA-1, other KSHV-encoded lytic proteins such as vIL-6, K8, and K8.1 are also detected in these cells, suggesting KSHV$^+$ MCD is associated with KSHV-lytic infection (Dupin et al., 1999; Katano et al., 2000b). The KSHV-encoded vIL-6 plays a role in the proliferation of plasma cells, and is also detected in patients' sera at high levels, suggesting high levels of vIL-6 are associated with MCD pathogenesis (Parravicini et al., 1997). High levels of KSHV DNA are also detected in the serum, which can be a marker of progressive MCD.

LARGE B-CELL LYMPHOMA ARISING IN KSHV-ASSOCIATED MCD

Large B-cell lymphoma arising in KSHV-associated MCD is characterized by a monoclonal proliferation of KSHV-infected lymphoid cells resembling plasmablasts expressing IgM, arising in the setting of MCD (Dupin et al., 2000; Oksenhendler et al., 2002). The small confluent sheets of LANA-1$^+$ plasmablasts are seen in the interfollicular zone of KSHV-associated MCD. This type of lymphoma occurs in the lymph node or spleen with generalized lymphadenitis and/or massive splenomegaly. Plasmablasts show stippled nuclear staining for LANA-1 and cytoplasmic staining for vIL-6, and strongly express cIgM with λ light-chain restriction.

CONCLUSION

Since the discovery of KSHV, 16 years have passed. During the period, some useful diagnostic tools have been developed for pathological examination. Anti-LANA-1 antibody is the most powerful tool for diagnosis of pathological samples of KSHV infection. LANA-1 expression is specific to KSHV infection, because all KSHV-infected cells express LANA-1. Real-time PCR is also a powerful tool for diagnosis. Thus, it is not difficult to diagnose KSHV infection in pathological samples. On the other hand, the pathogenesis, and especially the oncogenesis, of KSHV remain unknown. Although many KSHV-encoded proteins have been characterized and their *in vitro* functions revealed, it is still not clear if KSHV can fully transform or immortalize endothelial cells. It has been shown that LANA-1 plays a central role in KSHV pathogenesis. However, LANA-1 is not enough for KSHV oncogenesis. KSHV-encoded non-transforming proteins may collaborate to establish and maintain appropriate environment for KSHV-infected cells. Further studies should reveal the mechanism of the collaboration by KSHV-encoded proteins.

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REFERENCES

Andreoni, M., Sarmati, L., Nicastri, E., El Sawaf, G., El Zalabani, M., Uccella, I., Bugarini, R., Parisi, S. G., and Rezza, G. (2002). Primary human herpesvirus 8 infection in immunocompetent children. *JAMA* 287, 1295–1300.

Antman, K., and Chang, Y. (2000). Kaposi's sarcoma. *N. Engl. J. Med.* 342, 1027–1038.

Aoki, Y., Feldman, G. M., and Tosato, G. (2003). Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. *Blood* 101, 1535–1542.

Aoki, Y., Jaffe, E. S., Chang, Y., Jones, K., Teruya-Feldstein, J., Moore, P. S., and Tosato, G. (1999). Angiogenesis and hematopoiesis induced by Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. *Blood* 93, 4034–4043.

Asahi-Ozaki, Y., Sato, Y., Kanno, T., Sata, T., and Katano, H. (2006). Quantitative analysis of Kaposi sarcoma-associated herpesvirus (KSHV) in KSHV-associated diseases. *J. Infect. Dis.* 193, 773–782.

Bais, C., Santomasso, B., Coso, O., Arvanitakis, L., Raaka, E. G., Gutkind, J. S., Asch, A. S., Cesarman, E., Gershengorn, M. C., and Mesri, E. A. (1998). G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. *Nature* 391, 86–89.

Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* 284, 641–644.

Belec, L., Mohamed, A. S., Authier, F. J., Hallouin, M. C., Soe, A. M., Cotigny, S., Gaulard, P., and Gherardi, R. K. (1999). Human herpesvirus 8 infection in patients with POEMS syndrome-associated multicentric Castleman's disease. *Blood* 93, 3643–3653.

Biggar, R. J., Whitby, D., Marshall, V., Linhares, A. C., and Black, F. (2000). Human herpesvirus 8 in Brazilian Amerindians: a hyperendemic population with a new subtype. *J. Infect. Dis.* 181, 1562–1568.

Bower, M., Weir, J., Francis, N., Newsom-Davis, T., Powles, S., Crook, T., Boffito, M., Gazzard, B., and Nelson, M. (2009). The effect of HAART in 254 consecutive patients with AIDS-related Kaposi's sarcoma. *AIDS* 23, 1701–1706.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B., and Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5570–5575.

Cannon, M. J., Operskalski, E. A., Mosley, J. W., Radford, K., and

Dollard, S. C. (2009). Lack of evidence for human herpesvirus-8 transmission via blood transfusion in a historical US cohort. *J. Infect. Dis.* 199, 1592–1598.

Carbone, A., Cesarman, E., Gloghini, A., and Drexler, H. G. (2010). Understanding pathogenetic aspects and clinical presentation of primary effusion lymphoma through its derived cell lines. *AIDS* 24, 479–490.

Casper, C., Carrell, D., Miller, K. G., Judson, F. D., Meier, A. S., Pauk, J. S., Morrow, R. A., Corey, L., Wald, A., and Celum, C. (2006). HIV serodiscordant sex partners and the prevalence of human herpesvirus 8 infection among HIV negative men who have sex with men: baseline data from the EXPLORE study. *Sex. Transm. Infect.* 82, 229–235.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Chadburn, A., Hyjek, E., Mathew, S., Cesarman, E., Said, J., and Knowles, D. M. (2004). KSHV-positive solid lymphomas represent an extracavitary variant of primary effusion lymphoma. *Am. J. Surg. Pathol.* 28, 1401–1416.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266, 1865–1869.

Chatlynne, L. G., Lapps, W., Handy, M., Huang, Y. Q., Masood, R., Hamilton, A. S., Said, J. W., Koeffler, H. P., Kaplan, M. H., Friedman-Kien, A., Gill, P. S., Whitman, J. E., and Ablashi, D. V. (1998). Detection and titration of human herpesvirus-8-specific antibodies in sera from blood donors, acquired immunodeficiency syndrome patients, and Kaposi's sarcoma patients using a whole virus enzyme-linked immunosorbent assay. *Blood* 92, 53–58.

Chatterjee, M., Osborne, J., Bestetti, G., Chang, Y., and Moore, P. S. (2002). Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* 298, 1432–1435.

Cohen, J. I. (2000). Epstein-Barr virus infection. *N. Engl. J. Med.* 343, 481–492.

Davis, D. A., Humphrey, R. W., Newcomb, F. M., O'Brien, T. R., Goedert, J. J., Straus, S. E., and Yarchoan, R. (1997). Detection of serum antibodies to a Kaposi's sarcoma-associated herpesvirus-specific peptide. *J. Infect. Dis.* 175, 1071–1079.

Dupin, N., Diss, T. L., Kellam, P., Tulliez, M., Du, M. Q., Sicard, D., Weiss, R. A., Isaacson, P. G., and Boshoff, C. (2000). HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. *Blood* 95, 1406–1412.

Dupin, N., Fisher, C., Kellam, P., Ariad, S., Tulliez, M., Franck, N., Van Marck, E., Salmon, D., Gorin, I., Escande, J. P., Weiss, R. A., Alitalo, K., and Boshoff, C. (1999). Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4546–4551.

Engels, E. A., Atkinson, J. O., Graubard, B. I., Mcquillan, G. M., Gamache, C., Mbisa, G., Cohn, S., Whitby, D., and Goedert, J. J. (2007). Risk factors for human herpesvirus 8 infection among adults in the United States and evidence for sexual transmission. *J. Infect. Dis.* 196, 199–207.

Faris, M., Ensoli, B., Kokot, N., and Nel, A. E. (1998). Inflammatory cytokines induce the expression of basic fibroblast growth factor (bFGF) isoforms required for the growth of Kaposi's sarcoma and endothelial cells through the activation of AP-1 response elements in the bFGF promoter. *AIDS* 12, 19–27.

Flore, O., Rafii, S., Ely, S., O'Leary, J. J., Hyjek, E. M., and Cesarman, E. (1998). Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. *Nature* 394, 588–592.

Friborg, J. Jr., Kong, W., Hottiger, M. O., and Nabel, G. J. (1999). p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* 402, 889–894.

Fujimuro, M., Wu, F. Y., Aprhys, C., Kajumbula, H., Young, D. B., Hayward, G. S., and Hayward, S. D. (2003). A novel viral mechanism for dysregulation of beta-catenin in Kaposi's sarcoma-associated herpesvirus latency. *Nat. Med.* 9, 300–306.

Ganem, D. (2005). *Kaposi's Sarcoma-Associated Herpesvirus*. Philadelphia: Lippincott Williams & Wilkins.

Gao, S. J., Boshoff, C., Jayachandra, S., Weiss, R. A., Chang, Y., and Moore, P. S. (1997). KSHV ORF K9 (vIRF) is an oncogene which inhibits the interferon signaling pathway. *Oncogene* 15, 1979–1985.

Gao, S. J., Deng, J. H., and Zhou, F. C. (2003). Productive lytic replication of a recombinant Kaposi's sarcoma-associated herpesvirus in efficient primary infection of primary human endothelial cells. *J. Virol.* 77, 9738–9749.

Grulich, A. E., Cunningham, P., Munier, M. L., Prestage, G., Amin, J., Ringland, C., Whitby, D., Kippax, S., Kaldor, J. M., and Rawlinson, W. (2005). Sexual behaviour and human herpesvirus 8 infection in homosexual men in Australia. *Sex. Health* 2, 13–18.

Hayward, G. S., and Zong, J. C. (2007). Modern evolutionary history of the human KSHV genome. *Curr. Top. Microbiol. Immunol.* 312, 1–42.

Hladik, W., Dollard, S. C., Mermin, J., Fowlkes, A. L., Downing, R., Amin, M. M., Banage, F., Nzaro, E., Kataaha, P., Dondero, T. J., Pellett, P. E., and Lackritz, E. M. (2006). Transmission of human herpesvirus 8 by blood transfusion. *N. Engl. J. Med.* 355, 1331–1338.

Hong, Y. K., Foreman, K., Shin, J. W., Hirakawa, S., Curry, C. L., Sage, D. R., Libermann, T., Dezube, B. J., Fingeroth, J. D., and Detmar, M. (2004). Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. *Nat. Genet.* 36, 683–685.

Ishido, S., Wang, C., Lee, B. S., Cohen, G. B., and Jung, J. U. (2000). Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *J. Virol.* 74, 5300–5309.

Jenner, R. G., Maillard, K., Cattini, N., Weiss, R. A., Boshoff, C., Wooster, R., and Kellam, P. (2003). Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphoma has a plasma cell gene expression profile. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10399–10404.

Kanno, T., Sato, Y., Nakamura, T., Sakamoto, K., Sata, T., and Katano, H. (2010). Genotypic and clinicopathological characterization of Kaposi's sarcoma-associated herpesvirus infection in Japan. *J. Med. Virol.* 82, 400–406.

Katano, H., Hoshino, Y., Morishita, Y., Nakamura, T., Satoh, H., Iwamoto, A., Herndier, B., and Mori, S. (1999a). Establishing and characterizing a CD30-positive cell line harboring HHV-8 from a primary effusion lymphoma. *J. Med. Virol.* 58, 394–401.

Katano, H., Sato, Y., Kurata, T., Mori, S., and Sata, T. (1999b). High expression of HHV-8-encoded ORF73 protein in spindle-shaped cells of Kaposi's sarcoma. *Am. J. Pathol.* 155, 47–52.

Katano, H., Iwasaki, T., Baba, N., Terai, M., Mori, S., Iwamoto, A., Kurata, T., and Sata, T. (2000a). Identification of antigenic proteins encoded by human herpesvirus 8 and seroprevalence in the general population and among patients with and without Kaposi's sarcoma. *J. Virol.* 74, 3478–3485.

Katano, H., Sato, Y., Kurata, T., Mori, S., and Sata, T. (2000b). Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. *Virology* 269, 335–344.

Kazanji, M., Dussart, P., Duprez, R., Tortevoye, P., Pouliquen, J. F., Vandekerkhove, J., Couppie, P., Morvan, J., Talarmin, A., and Gessain, A. (2005). Serological and molecular evidence that human herpesvirus 8 is endemic among Amerindians in French Guiana. *J. Infect. Dis.* 192, 1525–1529.

Kedes, D. H., Ganem, D., Ameli, N., Bacchetti, P., and Greenblatt, R. (1997). The prevalence of serum antibody to human herpesvirus 8 (Kaposi sarcoma-associated herpesvirus) among HIV-seropositive and high-risk HIV-seronegative women. *JAMA* 277, 478–481.

Kellam, P., Bourboulia, D., Dupin, N., Shotton, C., Fisher, C., Talbot, S., Boshoff, C., and Weiss, R. A. (1999). Characterization of monoclonal antibodies raised against the latent nuclear antigen of human herpesvirus 8. *J. Virol.* 73, 5149–5155.

Lee, H., Veazey, R., Williams, K., Li, M., Guo, J., Neipel, F., Fleckenstein, B., Lackner, A., Desrosiers, R. C., and Jung, J. U. (1998). Deregulation of cell growth by the K1 gene of Kaposi's sarcoma-associated herpesvirus. *Nat. Med.* 4, 435–440.

Lei, X., Bai, Z., Ye, F., Xie, J., Kim, C. G., Huang, Y., and Gao, S. J. (2010). Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. *Nat. Cell Biol.* 12, 193–199.

Liao, W., Tang, Y., Lin, S. F., Kung, H. J., and Giam, C. Z. (2003). K-bZIP of Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8) binds KSHV/HHV-8 Rta and represses Rta-mediated transactivation. *J. Virol.* 77, 3809–3815.

Liu, Z. Y., Ganju, R. K., Wang, J. F., Ona, M. A., Hatch, W. C., Zheng, T., Avraham, S., Gill, P., and Groopman, J. E. (1997). Cytokine signaling through the novel tyrosine kinase RAFTK in Kaposi's sarcoma cells. *J. Clin. Invest.* 99, 1798–1804.

Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999). Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. *J. Virol.* 73, 9348–9361.

Luppi, M., Barozzi, P., Schulz, T. F., Trovato, R., Donelli, A., Narni, F., Sheldon, J., Marasca, R., and Torelli, G. (2000). Nonmalignant disease associated with human herpesvirus 8 reactivation in patients who have undergone autologous peripheral blood stem cell transplantation. *Blood* 96, 2355–2357.

Martin-Carbonero, L., Palacios, R., Valencia, E., Saballs, P., Sirera, G., Santos, I., Baldobi, F., Alegre, M., Goyenechea, A., Pedreira, J., Gonzalez Del Castillo, J., Martinez-Lacasa, J., Ocampo, A., Alsina, M., Santos, J., Podzamczer, D., and Gonzalez-Lahoz, J. (2008). Long-term prognosis of HIV-infected patients with Kaposi sarcoma treated with pegylated liposomal doxorubicin. *Clin. Infect. Dis.* 47, 410–417.

Mayama, S., Cuevas, L. E., Sheldon, J., Omar, O. H., Smith, D. H., Okong, P., Silvel, B., Hart, C. A., and Schulz, T. F. (1998). Prevalence and transmission of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in Ugandan children and adolescents. *Int. J. Cancer* 77, 817–820.

Meng, Y. X., Spira, T. J., Bhat, G. J., Birch, C. J., Druce, J. D., Edlin, B. R., Edwards, R., Gunthel, C., Newton, R., Stamey, F. R., Wood, C., and Pellett, P. E. (1999). Individuals from North America, Australasia, and Africa are infected with four different genotypes of human herpesvirus 8. *Virology* 261, 106–119.

Montaner, S., Sodhi, A., Molinolo, A., Bugge, T. H., Sawai, E. T., He, Y., Li, Y., Ray, P. E., and Gutkind, J. S. (2003). Endothelial infection with KSHV genes in vivo reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes. *Cancer Cell* 3, 23–36.

Murakami-Mori, K., Mori, S., and Nakamura, S. (1998). Endogenous basic fibroblast growth factor is essential for cyclin E-CDK2 activity in multiple external cytokine-induced proliferation of AIDS-associated Kaposi's sarcoma cells: dual control of AIDS-associated Kaposi's sarcoma cell growth and cyclin E-CDK2 activity by endogenous and external signals. *J. Immunol.* 161, 1694–1704.

Muralidhar, S., Pumfery, A. M., Hassani, M., Sadaie, M. R., Kishishita, M., Brady, J. N., Doniger, J., Medveczky, P., and Rosenthal, L. J. (1998). Identification of kaposin (open reading frame K12) as a human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) transforming gene. *J. Virol.* 72, 4980–4988.

Nador, R. G., Cesarman, E., Chadburn, A., Dawson, D. B., Ansari, M. Q., Sald, J., and Knowles, D. M. (1996). Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. *Blood* 88, 645–656.

Ohtsuki, Y., Iwata, J., Furihata, M., Takeuchi, T., Sonobe, H., and Miyoshi, I. (1999). Ultrastructure of Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus-8 (HHV-8) in a primary effusion lymphoma cell line treated with tetradecanoyl phorbol acetate (TPA). *Med. Electron Microsc.* 32, 94–99.

Oksenhendler, E., Boulanger, E., Galicier, L., Du, M. Q., Dupin, N., Diss, T. C., Hamoudi, R., Daniel, M. T., Agbalika, F., Boshoff, C., Clauvel, J. P., Isaacson, P. G., and Meignin, V. (2002). High incidence of Kaposi sarcoma-associated herpesvirus-related non-Hodgkin lymphoma in patients with HIV infection and multicentric Castleman disease. *Blood* 99, 2331–2336.

Orenstein, J. M., Alkan, S., Blauvelt, A., Jeang, K. T., Weinstein, M. D., Ganem, D., and Herndier, B. (1997). Visualization of human herpesvirus type 8 in Kaposi's sarcoma by light and transmission electron microscopy. *AIDS* 11, F35–F45.

Parravicini, C., Corbellino, M., Paulli, M., Magrini, U., Lazzarino, M., Moore, P. S., and Chang, Y. (1997). Expression of a virus-derived cytokine, KSHV vIL-6, in HIV-seronegative Castleman's disease. *Am. J. Pathol.* 151, 1517–1522.

Pauk, J., Huang, M. L., Brodie, S. J., Wald, A., Koelle, D. M., Schacker, T., Celum, C., Selke, S., and Corey, L. (2000). Mucosal shedding of human herpesvirus 8 in men. *N. Engl. J. Med.* 343, 1369–1377.

Rabkin, C. S., Schulz, T. F., Whitby, D., Lennette, E. T., Magpantay, L. I., Chatlynne, L., and Biggar, R. J. (1998). Interassay correlation of human herpesvirus 8 serologic tests. HHV-8 Interlaboratory Collaborative Group. *J. Infect. Dis.* 178, 304–309.

Regamey, N., Tamm, M., Wernli, M., Witschi, A., Thiel, G., Cathomas, G., and Erb, P. (1998). Transmission of human herpesvirus 8 infection from renal-transplant donors to recipients. *N. Engl. J. Med.* 339, 1358–1363.

Renne, R., Lagunoff, M., Zhong, W., and Ganem, D. (1996a). The size and conformation of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) DNA in infected cells and virions. *J. Virol.* 70, 8151–8154.

Renne, R., Zhong, W., Herndier, B., Mcgrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996b). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat. Med.* 2, 342–346.

Rivas, C., Thlick, A. E., Parravicini, C., Moore, P. S., and Chang, Y. (2001). Kaposi's sarcoma-associated herpesvirus LANA2 is a B-cell-specific latent viral protein that inhibits p53. *J. Virol.* 75, 429–438.

Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc. Natl. Acad. Sci. U.S.A.* 93, 14862–14867.

Said, J. W., Chien, K., Tasaka, T., and Koeffler, H. P. (1997). Ultrastructural characterization of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) in Kaposi's sarcoma lesions: electron microscopy permits distinction from cytomegalovirus (CMV). *J. Pathol.* 182, 273–281.

Said, W., Chien, K., Takeuchi, S., Tasaka, T., Asou, H., Cho, S. K., De Vos, S., Cesarman, E., Knowles, D. M., and Koeffler, H. P. (1996). Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8) in primary effusion lymphoma: ultrastructural demonstration of herpesvirus in lymphoma cells. *Blood* 87, 4937–4943.

Sakakibara, S., Ueda, K., Nishimura, K., Do, E., Ohsaki, E., Okuno, T., and Yamanishi, K. (2004). Accumulation of heterochromatin components on the terminal repeat sequence of Kaposi's sarcoma-associated herpesvirus mediated by the latency-associated nuclear antigen. *J. Virol.* 78, 7299–7310.

Sakurada, S., Katano, H., Sata, T., Ohkuni, H., Watanabe, T., and Mori, S. (2001). Effective human herpesvirus 8 infection of human umbilical vein endothelial cells by cell-mediated transmission. *J. Virol.* 75, 7717–7722.

Samols, M. A., Hu, J., Skalsky, R. L., and Renne, R. (2005). Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79, 9301–9305.

Samols, M. A., Skalsky, R. L., Maldonado, A. M., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Identification of cellular genes targeted by KSHV-encoded microRNAs. *PLoS Pathog.* 3, e65. doi: 10.1371/journal.ppat.0030065

Sarid, R., Flore, O., Bohenzky, R. A., Chang, Y., and Moore, P. S. (1998). Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). *J. Virol.* 72, 1005–1012.

Seaman, W. T., Ye, D., Wang, R. X., Hale, E. E., Weisse, M., and Quinlivan, E. B. (1999). Gene expression from the ORF50/K8 region of Kaposi's sarcoma-associated herpesvirus. *Virology* 263, 436–449.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., D'Agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and Sigaux, F. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86, 1276–1280.

Suda, T., Katano, H., Delsol, G., Kakiuchi, C., Nakamura, T., Shiota, M., Sata, T., Higashihara, M., and Mori, S. (2001). HHV-8 infection status of AIDS-unrelated and AIDS-associated multicentric Castleman's disease. *Pathol. Int.* 51, 671–679.

Sun, R., Lin, S. F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., and Miller, G. (1999). Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. *J. Virol.* 73, 2232–2242.

Tang, J., Gordon, G. M., Muller, M. G., Dahiya, M., and Foreman, K. E. (2003). Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen induces expression of the helix-loop-helix protein Id-1 in human endothelial cells. *J. Virol.* 77, 5975–5984.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J. L., Schroter, M., Scaffidi, C., Krammer, P. H., Peter, M. E., and Tschopp, J. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 386, 517–521.

Wang, H. W., Trotter, M. W., Lagos, D., Bourboulia, D., Henderson, S., Makinen, T., Elliman, S., Flanagan, A. M., Alitalo, K., and Boshoff, C. (2004). Kaposi sarcoma herpesvirus-induced cellular reprogramming contributes to the lymphatic endothelial gene

expression in Kaposi sarcoma. *Nat. Genet.* 36, 687–693.

Wang, Q. J., Jenkins, F. J., Jacobson, L. P., Kingsley, L. A., Day, R. D., Zhang, Z. W., Meng, Y. X., Pellett, P. E., Kousoulas, K. G., Baghian, A., and Rinaldo, C. R. Jr. (2001). Primary human herpesvirus 8 infection generates a broadly specific CD8(+) T-cell response to viral lytic cycle proteins. *Blood* 97, 2366–2373.

Weninger, W., Partanen, T. A., Breiteneder-Geleff, S., Mayer, C., Kowalski, H., Mildner, M., Pammer, J., Sturzl, M., Kerjaschki, D., Alitalo, K., and Tschachler, E. (1999). Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells. *Lab. Invest.* 79, 243–251.

Zhu, F. X., Cusano, T., and Yuan, Y. (1999). Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 73, 5556–5567.

Ziegelbauer, J. M., Sullivan, C. S., and Ganem, D. (2009). Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. *Nat. Genet.* 41, 130–134.

Zong, J. C., Ciufo, D. M., Alcendor, D. J., Wan, X., Nicholas, J., Browning, P. J., Rady, P. L., Tyring, S. K., Orenstein, J. M., Rabkin, C. S., Su, I. J., Powell, K. F., Croxson, M., Foreman, K. E., Nickoloff, B. J., Alkan, S., and Hayward, G. S. (1999). High-level variability in the ORF-K1 membrane protein gene at the left end of the Kaposi's sarcoma-associated herpesvirus genome defines four major virus subtypes and multiple variants or clades in different human populations. *J. Virol.* 73, 4156–4170.

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Kaposi's sarcoma associated herpesvirus entry into target cells

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Herpesvirus infection of target cells is a complex process involving multiple host cell surface molecules (receptors) and multiple viral envelope glycoproteins. Kaposi's sarcoma associated herpesvirus (KSHV or HHV-8) infects a variety of *in vivo* target cells such as endothelial cells, B cells, monocytes, epithelial cells, and keratinocytes. KSHV also infects a diversity of *in vitro* target cells and establishes *in vitro* latency in many of these cell types. KSHV interactions with the host cell surface molecules and its mode of entry in the various target cells are critical for the understanding of KSHV pathogenesis. KSHV is the first herpesvirus shown to interact with adherent target cell integrins and this interaction initiates the host cell pre-existing signal pathways that are utilized for successful infection. This chapter discusses the various aspects of the early stage of KSHV infection of target cells, receptors used and issues that need to be clarified, and future directions. The various signaling events triggered by KSHV infection and the potential role of signaling events in the different stages of infection are summarized providing the framework and starting point for further detailed studies essential to fully comprehend the pathogenesis of KSHV.

Keywords: KSHV, entry, tropism, integrins, signaling, endocytosis, receptors

INTRODUCTION

Viruses are obligatory parasites that critically rely on their ability to transmit their genome from infected to uninfected host organisms. Being an inert particle, viruses have successfully evolved to exploit the behavior and physiology of their host. Viral infection induces the activation of various endogenous responses that enable it to permeate through cell membranes and other barriers to reach the cytoplasm or nucleus.

Herpesviruses have a large double stranded DNA genome enclosed in the viral protein shell (capsid) surrounded by a tegument layer which is enclosed in a lipid envelope with at least eight distinct viral envelope glycoproteins. A characteristic property of these viruses is that after primary infection they establish lifelong latent infection in the infected host with periodic reactivation and re-infection. KSHV or human herpesvirus-8 (HHV-8) is a member of the γ2-lymphotropic-oncogenic herpesviruses. KSHV is etiologically associated with Kaposi's sarcoma (KS) and with at least two lymphoproliferative malignancies, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD). It is the newest member of the human herpesvirus family and is closely related to γ-1 Epstein–Barr virus (EBV), γ-2 herpesvirus saimiri (HVS) and Rhesus monkey rhadinovirus (RRV; Ganem, 2007a,b). KSHV has a double stranded DNA genome of about ∼160-kb encoding more than 90 ORFs designated 4–75 by their homology to HVS ORFs. The genome contains gene blocks conserved with other herpesviruses as well as divergent regions encoding more than 20 KSHV unique genes (K genes). KSHV encodes several proteins that are homologs of host proteins with immunomodulatory, anti-apoptotic, signal induction, transcriptional regulation, and other functions (Cesarman et al., 1996; Neipel et al., 1997).

The first step of any viral infection is governed by binding and entry into target cells. Therefore, to control KSHV infection, a detailed understanding of how KSHV infects its target cells utilizing the varied set of cellular receptors, envelope glycoproteins, signaling, and modes of entry is essential. Recent advances indicate that KSHV interacts with multiple host cell surface receptors of adherent target cells and these interactions induce a network of rapid intracellular signaling pathways, which facilitate the various steps of successful infection. Here, we review the important steps involved in KSHV entry into target cells utilizing viral envelope–cellular receptor interactions, and signal cascades inducing dynamic cell membrane changes leading to a productive latent infection.

KSHV TROPISM

To better understand the underlying multistep complex entry mechanism(s) initiated by KSHV, one must appreciate the broad variety of cell types infected both *in vivo* and *in vitro*.

In vivo KSHV has a broad tropism as suggested by the detection of its genome and transcripts in a variety of *in vivo* cell types such as CD19+ peripheral blood B cells, endothelial cells, monocytes, keratinocytes, and epithelial cells (Ganem, 2007b). Latent KSHV DNA is present in vascular endothelial and spindle cells of KS lesions, associated with expression of latency-associated ORF73 (LANA-1), ORF 72 (v-cyclin D), K13 (v-FLIP), and K12 (Kaposin) genes and microRNAs (Boshoff et al., 1995; Dupin et al., 1999; Ganem, 2007b). Lytic infection is also detected in <1% of infiltrating inflammatory monocytic cells of KS lesions (Dourmishev

et al., 2003; Ganem, 2007b). Available evidences suggest that B cells and monocytes are the major reservoir of *in vivo* latent infection. Cell lines with B cell characteristics, such as BC-1, BC-3, BCBL-1, HBL-6, and JSC have been established from PEL tumors (Dourmishev et al., 2003; Ganem, 2007b). In PEL cells, in addition to the above set of latent genes, K10.5 (LANA-2) gene is also expressed (Parravicini et al., 2000; Ganem, 2007b). About 1–3% of PEL cells spontaneously enter lytic cycle and virus induced from these cells by chemicals serve as the source of virus. Multiple genome copies of both KSHV and EBV exist in latent form in BC-1, HBL-6, and JSC cells while BCBL-1 and BC-3 cells carry only the KSHV genome (Ganem, 2007b). An endothelial cell line carrying KSHV has not been established from KS lesions since KS cells grow poorly in cell culture and viral DNA is lost within a few passages (Ganem, 2007b).

Kaposi's sarcoma associated herpesvirus has been shown *in vitro* to infect several types of human cells such as B, endothelial, epithelial, fibroblast cells, CD34+ stem cell precursors of dendritic cells (DCs), and monocytes (Ganem, 2007b). KSHV also infects owl monkey kidney cells, baby hamster kidney (BHK-21) cells, Chinese hamster ovary (CHO) cells, and mouse fibroblasts cells (Parravicini et al., 2000; Akula et al., 2001a,b, 2002; Birkmann et al., 2001; Bechtel et al., 2003; Inoue et al., 2003; Garrigues et al., 2008; Jarousse et al., 2008).

Infection of primary B cells by KSHV does not result in immortalization and a lytic KSHV replication is seen in activated B cells. Another characteristic feature of *in vitro* infection of human microvascular dermal endothelial cells (HMVEC-d), human umbilical vein endothelial cells (HUVEC), human foreskin fibroblasts (HFF), human endothelial cells immortalized by telomerase (TIME), and human endothelial cells (HEK-293), monkey kidney cells (VERO, CV-1), and mouse fibroblasts (Bechtel et al., 2003) by KSHV is the expression of latency-associated genes and the absence of productive lytic replication and thus providing a reasonable model for studying *in vitro* latency. However, latent infection of KSHV *in vitro* is not persistent and leads to the loss of viral genome over time (Grundhoff and Ganem, 2004).

Analysis of *in vitro* KSHV interaction with adherent target cells and quantitation of infection has been hampered by the absence of a lytic replication cycle and hence a plaque assay. Since *in vitro* KSHV infection results in the expression of latency-associated genes, various methods have been devised to assess the different phase(s) of KSHV infection (Parravicini et al., 2000; **Table 1**).

KSHV BINDING AND ENTRY INTO TARGET CELLS

Kaposi's sarcoma associated herpesvirus uses multiple envelope glycoproteins to complete the binding and entry processes. KSHV binding to the target cells and identity of the receptors involved in binding and entry were elucidated by using labeled virus binding to the target cells at 4°C as well as other methods (**Table 1**). These studies have demonstrated that KSHV binds and enters a variety of target cells which include human (293, HFF, HeLa, HMVEC-d, HUVEC, TIME, BCBL-1, BJAB, Raji), monkey (Vero, CV-1), hamster (BHK-21, CHO), and mouse (Du17) cells. This is demonstrated by the detection of viral DNA, limited viral gene expression, and GFP expression (**Table 1**). Real-time DNA PCR of internalized KSHV DNA demonstrates a rapid internalization of viral DNA in the infected endothelial and HFF cells (Krishnan et al., 2004).

KSHV ENVELOPE GLYCOPROTEINS-MEDIATORS OF BINDING AND ENTRY

Kaposi's sarcoma associated herpesvirus envelope glycoproteins play critical roles in mediating virus attachment, entry, assembly, and egress of virus. Like other herpesviruses, KSHV encodes five conserved glycoproteins gB (ORF 8), gH (ORF 22), gL (ORF 47), gM (ORF 39), and gN (ORF 53; Cesarman et al., 1996; Neipel et al., 1997; Ganem, 2007a). In addition to these glycoproteins, KSHV also encodes the unique lytic cycle associated glycoproteins ORF 4, gpK8.1A, gpK8.1B, K1, K14, and K15 (Cesarman et al., 1996; Neipel et al., 1997; Ganem, 2007a). Among these, ORF 4, gB, gH/gL, gM/gN, and gpK8.1A are associated with KSHV envelopes (Baghian et al., 2000; Parravicini et al., 2000; Akula et al., 2001a,b; Birkmann et al., 2001; Wang et al., 2001; Naranatt et al., 2002; Koyano et al., 2003).

Kaposi's sarcoma associated herpesvirus gB is a major envelope glycoprotein. It is synthesized as a 110-kDa precursor protein which undergoes cleavage and processing to yield envelope associated disulfide linked 75 and 54-kDa polypeptides with high mannose and complex sugars (Baghian et al., 2000; Akula et al., 2001a; Wang et al., 2003). KSHV-gB mediates viral binding and

Table 1 | Methods employed to study the various stages of *in vitro* KSHV infection.

Stage of infection	Detection methods
1. Binding	[H3] Thymidine labeled virus and FITC labeled (FACS) binding assay; viral DNA (ORF73 gene) DNA quantitation by real-time DNA PCR; electron and confocal microscopy.
2. Signal induction	Quantitation of signal molecules induction by Western blots and ELISA; use of chemical inhibitors or dominant-negative signal molecules.
3. Viral DNA internalization (entry)	Real-time DNA PCR for KSHV ORF73 gene after removal of unbound/partially bound virus by trypsin–EDTA; electron and confocal microscopy.
4. Cytoplasmic trafficking of KSHV	Confocal microscopy by colocalizing virus with microtubules and endosomal vesicles; physiological ligand uptake assays.
5. Nuclear delivery of KSHV DNA	Real-time DNA PCR for ORF73 gene in the isolated nuclei of infected cells.
6. Viral gene expression, host gene manipulation	Real-time RNA PCR for KSHV and host gene expression; confocal microscopy and FACS for KSHV ORF73 gene and GFP expression.

entry by interacting with cell surface heparan sulfate and integrins α3β1, αVβ3, and αVβ5 (Akula et al., 2001a,b, 2002; Veettil et al., 2008). KSHV-gB interaction with target cells modulates the host cell signaling pathways by inducing integrin associated focal adhesion kinases (FAK), Src, PI3-K, and Rho-GTPase activities (Sharma-Walia et al., 2004). Studies have demonstrated that besides playing a role in KSHV binding and entry of target cells, gB is critical for virus maturation and egress (Krishnan et al., 2005).

Distinct from other herpesviruses, the KSHV gpK8.1 gene encodes two alternatively spliced messages yielding glycoprotein gpK8.1A and gpK8.1B. Both gpK8.1A and gpK8.1B contain N- and O-linked sugars, and gpK8.1A is the predominant form detected within infected cells and in the virion envelopes (Neipel et al., 1997; Zhu et al., 1999a,b). Like gB, gpK8.1A also possesses the heparan sulfate binding motif and interacts with cell surface heparan sulfate molecules (Wang et al., 2001). Similar to herpes simplex type 1 (HSV-1) gB glycoprotein, recent studies have reported that KSHV gB and gpK8.1A are enriched in membrane microdomains, lipid rafts (LRs), during early infection in endothelial cells (Bender et al., 2003; Chakraborty et al., 2011).

Like in other α, β, and γ-herpesviruses, KSHV gH and gL form a non-covalently linked complex consisting of 120-kDa gH and 42-kDa gL proteins. KSHV gL is required for processing and intracellular transport of gH and this complex is critical for KSHV entry (Naranatt et al., 2002). Recent studies show that KSHV gH, as well as complement binding KSHV ORF4, also interacts with cell surface heparan sulfate (Mark et al., 2006; Hahn et al., 2009). Studies have demonstrated that anti-gH and anti-gL antibodies inhibit KSHV entry without affecting binding to the target cells (Naranatt et al., 2002).

Kaposi's sarcoma associated herpesvirus gM and gN also form a glycosylated heterodimeric complex and are involved in virus penetration and egress. gN has been shown to be essential for proper post translational modification and transport of gM to the cell surface (Koyano et al., 2003). KSHV gM and gN forms a heterodimeric complex and were shown to inhibit cell fusion in an *in vitro* cell fusion assay (Koyano et al., 2003).

CELLULAR RECEPTORS RECOGNIZED BY KSHV

Most cell surface molecules that herpesviruses bind fall into two main categories depending on the functional consequences of the interaction. The major groups include the attachment or binding factors and entry receptors. Attachment factors promote binding and concentration of viruses on target cells but may not be very specific. Often, these involve charge interactions involving heparan sulfate or other carbohydrate moiety bearing molecules.

The other group comprises entry receptors, including a broad variety of cell surface molecules capable of either viral fusion to cell membranes or initiating signaling to promote endocytosis. Entry receptors are highly specific and vary by cell types. Often, these molecules are internalized along with the virus, hence are actively utilized by viruses.

HEPARAN SULFATE AS KSHV ATTACHMENT FACTOR IN TARGET CELLS

Like many herpesviruses, KSHV utilizes ubiquitous heparan sulfate (HS) molecule for binding to most of its target cells. HS bears carbohydrate residues that facilitate concentration of virus particles on target cells owing to charge interactions. KSHV infection can be inhibited by soluble heparin but not by chondroitin sulfates A and C (Akula et al., 2001a). Pre-treatment of KSHV with soluble heparin prevents virus binding and subsequent signal induction. This is a specificity control for KSHV-induced phenomena. Several B cell lines and primary B cells lack the Ext1 enzyme, which promotes glycosylation in HS biosynthesis. Due to this, B cells have low HS expression, and thereby refractory to KSHV infection. However, expression of HS in BJAB (EBV negative cell line) results in greater susceptibility to KSHV infection (Jarousse et al., 2008). These studies suggest that KSHV infection of BJAB cells depends on the level of expression of HS; BJAB cells expressing HS were readily infected while cells lacking HS were not infected (Kabir-Salmani et al., 2008).

Kaposi's sarcoma associated herpesvirus envelope glycoproteins gB, gpK8.1A, ORF4, and gH bind to cell surface HS molecules, thereby emphasizing the importance of cell surface HS for KSHV attachment to target cells (Birkmann et al., 2001; Akula et al., 2002; Wang et al., 2003; Hahn et al., 2009). Incubation of KSHV with soluble heparin and enzymatic removal of cell surface HS by heparinase I and III inhibits KSHV infectivity. Binding of the soluble forms of gB and gpK8.1A to target cells is saturable and can be blocked by soluble heparin (Birkmann et al., 2001; Wang et al., 2001, 2003).

Many proteins possess more than one of the two heparin binding domains (HBD), containing the XBBXBX and XBBBXXBX sequences, where B is a positively charged basic amino acid (lysine, arginine, or histidine) flanked by an additional positively charged residue separated by hydrophobic amino acids "X." The extracellular domain of KSHV gB possesses 108HIFKVRRYRK117, which is a BXXBXBBXBB type HBD, and is conserved throughout the γ2 herpesviruses. KSHV gpK8.1A also possesses two possible atypical heparin-binding motifs, ^{150}SRTTRIRV^{157} (XBXXBXBX) and ^{182}TRGRDAHY^{189} (XBXBXXBX) whereas KSHV gH lacks the typical HBD.

Several lines of evidence indicate that KSHV-gB and gpK8.1A bind to cell surface HS molecules (Akula et al., 2001b; Birkmann et al., 2001; Wang et al., 2003). Binding of soluble forms of the proteins made in baculovirus is saturable and can be blocked by soluble heparin (Wang et al., 2001, 2003). Full length gB and gpK8.1A in the virion envelope specifically bind heparin–agarose, and can be eluted by high concentrations of soluble heparin, but not by chondroitin sulfates (Wang et al., 2001; Akula et al., 2002). KSHV-gpK8.1A binds to heparin with an affinity comparable to that of glycoproteins B and C of herpes simplex virus and gpK8.1A binds more strongly than gB (Wang et al., 2003).

DC-SIGN AS KSHV ENTRY RECEPTORS

Dendritic cell specific intercellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN; CD209) is a C-type lectin present on the dermal DC surface. It has been shown to be used by many viruses as a receptor including human immunodeficiency virus (HIV) and more recently by Bunyaviruses (Geijtenbeek et al., 2003; Lozach et al., 2011). Similarly, KSHV also appears to utilize DC-SIGN during infection of human myeloid DCs, macrophages,

and activated B cells (Rappocciolo et al., 2006, 2008). DC-SIGN blocking monoclonal antibodies and mannan efficiently inhibited KSHV binding and infection. However, the pre-treatment of cells with anti-DC-SIGN antibodies did not completely block KSHV binding and infection possibly due to binding to HS and/or other receptors. KSHV envelope glycoprotein(s) interacting with DC-SIGN is not known yet and KSHV gB with its high mannose sugar is a potential candidate. Recent studies have shown that activated B cells are infected more efficiently due to increased expression of DC-SIGN. However, whether HS and other KSHV receptors are also expressed at higher levels possibly contributing to the increased infectivity in activated B cells need to be studied further.

Recent studies have also elucidated that KSHV effectively binds and enters THP-1, a human acute monocytic leukemic cell line, using heparan sulfate and integrins (Kerur et al., 2010). Blocking DC-SIGN in these cells did not affect KSHV binding; however KSHV entry was reduced, suggesting that KSHV utilized DC-SIGN as part of the entry receptor(s) in addition to the previously identified integrins (α3β1, αVβ3, and αVβ5) in THP-1 cells (Kerur et al., 2010).

KSHV IS THE FIRST HERPESVIRUS SHOWN TO UTILIZE INTEGRINS AS ENTRY RECEPTORS IN ADHERENT CELLS

Several viruses utilize multiple integrins for target cell infection and engaging integrin receptors leading to induction of potent signaling responses critical for virus infectivity. KSHV-gB possesses an integrin-binding RGD (Arg-Gly-Asp) motif at amino acids 27–29. The RGD motif is the minimal peptide sequence of many integrin ligands known to interact with subsets of cellular integrins. Several studies have demonstrated that α3β1, αVβ3, and αVβ5 integrins play roles in KSHV infection. Using RGD peptides, antibodies against RGD-gB (RGDTFQTSSSPTPPGSSS), and the extracellular matrix (ECM) protein fibronectin studies have shown the role of integrins in KSHV infection of HMVEC-d and HFF cells (Akula et al., 2002). In HMVEC-d and HFF cells, KSHV interacts with integrin α3β1 as demonstrated by a 30–50% reduction in infection by pre-treating cells with function blocking anti-α3 and β1 antibodies and by mixing virus with soluble α3β1 integrin before infection, as well as the immunoprecipitation of virus-α3 and β1 complexes by anti-KSHV-gB antibodies (Akula et al., 2002). Though expression of α3 integrin in CHO cells increases infectivity, the levels of infection do not reach that observed in the HMVEC-d and HFF cells strongly suggesting that KSHV uses multiple receptors (Akula et al., 2002; Naranatt et al., 2003; Krishnan et al., 2004).

Virus binding and DNA internalization studies suggest that integrins αVβ3 and αVβ5 also play roles in KSHV entry (Veettil et al., 2008). Variable levels of inhibition of virus entry into adherent HMVEC-d, 293 and Vero cells, and HFF was observed by pre-incubating virus with soluble αVβ3, αVβ5, and α3β1 integrins, and cumulative inhibition was observed with a combination of integrins. Confocal microscopy studies confirmed the association of KSHV with α3β1 integrins (Veettil et al., 2008; Chakraborty et al., 2011). A study also revealed the roles of integrins (α3β1, αVβ3, and αVβ5) in the entry of KSHV in THP-1 and primary monocyte cells (Kerur et al., 2010).

DISCREPANCIES REGARDING THE ROLE OF INTEGRINS IN KSHV BIOLOGY

Two studies did not detect a role for α3β1 in KSHV infection. However, methodological differences could explain the discrepancies. For example, Inoue et al. (2003) reported the inability of soluble α3β1 integrin and RGD peptides to block KSHV infectivity in the 293-T cell line. However, the validity of this observation is questionable since in this study, cells were pre-treated with RGD peptide and infected with KSHV by using centrifugation and polybrene. Both centrifugation and polybrene are known to enhance virus infection without the need for the virus to interact with specific receptors. It is well known that polybrene forming a complex with viral envelope is used for gene delivery into various target cells bypassing the need of specific receptors. Moreover, Inoue et al. (2003) studies also pre-incubated the cells with integrins, washed, and infected with KSHV. This is not a correct design of experiment since to demonstrate the role of integrin in any viral infection cells need to be incubated first with anti-integrins antibodies, but not soluble integrins, prior to viral infections. Similarly, virus needs to be pre-incubated with soluble integrins before addition to cells to demonstrate the role of respective integrin in viral infection.

Another study utilized a 15-mer-AHSRGDTFQTSSGCG peptide of KSHV-gB and demonstrated that this peptide mediated the human fibrosarcoma HT1080 cell adhesion which was blocked by αVβ3 and αVβ5 antibodies while peptide bound beads detected only αVβ3 integrin (Garrigues et al., 2008). The GCG amino acids in the peptide used in this study are not present in the KSHV-gB sequence and may potentially give rise to dimers and multimers due to the cystine residue (Garrigues et al., 2008). Though HT1080 cell infection was inhibited by anti-αVβ3 antibodies, the ability of anti-α3β1 and αVβ5 antibodies to block infection was not examined (Garrigues et al., 2008). Moreover, co-immunoprecipitation and colocalization studies to demonstrate the direct association of KSHV with integrins were not done in these studies. Hence, to disproof that KSHV infection depends upon α3β1, studies should be carried out with the same target cells that have been used to show the role of α3β1 integrin in KSHV target cell infection. Moreover, the differential ability of integrins to block infection remains questionable in HT1080 cells since another study could not infect these cells (Veettil et al., 2008). Mouse keratinocytes lacking α3β1 were infectable with KSHV and expression of human α3 resulted in only 55% of infection in these cells. Even though the level of αVβ3 in these cells and the ability of anti-αVβ3 to block KSHV infection were not tested, it was concluded that α3β1 expression must have a dominant-negative effect on αVβ3 integrin. Later studies clearly demonstrating the association of KSHV with α3β1 integrin and LR-KSHV and α3β1 integrins (Veettil et al., 2008; Chakraborty et al., 2011), it is clear the conclusions from the above two studies is not correct and should not be considered further.

The discrepancies in the usage of different integrins were expected since it is common for different cells to express different combinations of integrins and a specific integrin could be one of the receptors in some but not in all target cells of KSHV. Since herpesvirus–cell receptor interactions are temporarily coordinated events mediated by interactions of viral glycoproteins with one receptor leading to conformational changes in the viral glycoproteins allowing interaction with the next receptor(s),

detection of α3 and β1 in HFF or HMVEC-d cells incubated with virus or purified gB (Akula et al., 2002) could be representing the event occurring during virus–host cell interactions under physiological conditions. Further studies are required to determine the role of additional receptor(s) in different target cells of KSHV.

Later studies demonstrated that αVβ3 and αVβ5 integrins also play roles in KSHV entry of adherent target cells (Veettil et al., 2008; Chakraborty et al., 2011; **Table 2**; **Figure 1**). Studies have also demonstrated the neutralization of KSHV infection in HMVEC-d and HFF cells upon blocking with anti-αVβ3 and αVβ5 antibodies. Supporting these findings, KSHV entry was inhibited by soluble α3β1, αVβ3, and αVβ5 integrins in HMVEC-d, HFF, 293, and Vero cells (Veettil et al., 2008). Though a cumulative inhibition was observed with combinations of integrins, complete block in infection was not achieved which may be due to KSHV's first interaction with HS. The role of integrin in KSHV infection of HUVEC cells, B cells, monocytes, keratinocytes, and other cells has not been studied (**Table 2**).

ROLE OF xCT AS A KSHV ENTRY RECEPTOR

Kaleeba and Berger (2006) identified the 12-transmembrane glutamate/cysteine exchange transporter protein xCT as a fusion-entry receptor in adherent cells. Ectopic expression of xCT rendered the non-susceptible adherent target cells to become susceptible to KSHV infection (Kaleeba and Berger, 2006). xCT is part of the cell surface 125-kDa disulfide linked heterodimeric membrane glycoprotein CD98 (4F2 antigen) complex containing a common glycosylated heavy chain (80-kDa) and a group of 45 kDa light chains. The xCT molecule is one of the light chains (Fenczik et al., 2001; Feral et al., 2007; Kabir-Salmani et al., 2008; Veettil et al., 2008). CD98 was initially identified as a molecule associated with integrin α3 and plays multiple roles including amino acid transport, cell adhesion, fusion, proliferation, and integrin activation. It is interesting to note that CD98 and integrin α3 were identified as fusion regulation protein 1 (FRP-1) and FRP-2, respectively, as this interaction was shown to play crucial roles in cell–cell fusion and virus-induced cell fusion (Fenczik et al., 2001; Feral et al., 2007; Kabir-Salmani et al., 2008; Veettil et al., 2008). Studies show that xCT is a component of a multimolecular signaling complex formed during KSHV macropinocytosis in HMVEC-d cells (Veettil et al., 2008; Chakraborty et al., 2011). This suggests that xCT plays a role within a multimolecular complex that may help to regulate the signaling pathways associated with the endocytic pathway of the virus. It is possible that the xCT present in the multimolecular complex may initiate a distinct cellular signaling pathway to control the various events associated with KSHV infection. Since a direct interaction of KSHV envelope glycoproteins with xCT has not been shown as yet, the exact role of xCT in the multimolecular complex and its role in KSHV infection need to be investigated further.

MULTIMOLECULAR INTEGRIN COMPLEX FORMATION DURING EARLY KSHV INFECTION

CD98 also mediates membrane clustering, β1 integrin-mediated signaling events, and stimulation of α3β1-dependent adhesion of cells and signal transduction cascade of αVβ3 integrin (Fenczik et al., 2001; Feral et al., 2007; Kabir-Salmani et al., 2008; Veettil et al., 2008). Co-immunoprecipitation and immunofluorescence studies in KSHV infected HMVEC-d cells have shown a time-dependent interaction of CD98/xCT with integrins α3β1, αVβ5, and αVβ3 (Veettil et al., 2008). Three different time-dependent temporal patterns of association and dissociation of KSHV interactions with cell surface molecules were observed. Integrin αVβ5 interaction with CD98/xCT predominantly occurred by 1 min post-infection (p.i.) and dissociated at 10 min p.i., whereas α3β1–CD98/xCT interaction was maximal at 10 min p.i. and dissociated at 30 min p.i., and αVβ3–CD98/xCT interaction was maximal at 10 min p.i. and remained at the observed 30 min p.i. Confocal microscopy studies confirmed the association of CD98/xCT with α3β1 and KSHV. Studies also showed that α3β1–CD98/xCT interactions could be inhibited by pre-incubating KSHV with soluble heparin and α3β1 strongly suggesting that KSHV's first contact occurs with HS, and integrins are essential elements in subsequent CD98–xCT interactions (Veettil et al., 2008). These studies demonstrated temporal interactions of KSHV with a family of functionally related proteins such as HS, integrins, and CD98–xCT molecules in endothelial, epithelial, and fibroblast cells (Wang et al., 2001, 2003; Akula et al., 2002; Veettil et al., 2008).

BIOLOGICAL RELEVANCE OF UTILIZING MULTIPLE ENTRY RECEPTORS BY KSHV

It is fascinating to note that KSHV uses multiple molecules to enter target cells. The presence of multiple receptors is evolutionarily advantageous for KSHV and one of the major reasons for the broad tropism of the virus. Although virus–receptor interactions are highly specific, the affinity of interactions are low (Marsh

Table 2 | Binding and entry receptors and entry pathways of KSHV in various *in vitro* human target cells.

Target cells	Receptors recognized	Entry pathway
Human foreskin fibroblast cells (HFF)	Heparan sulfate (binding), α3β1, αVβ3, αVβ5, xCT/CD98	Clathrin-mediated endocytosis
Human microvascular dermal endothelial cells (HMVEC-d)	Heparan sulfate (binding), α3β1, αVβ3, αVβ5, xCT/CD98	Macropinocytosis
Human umbilical vein endothelial cells (HUVEC)	Heparan sulfate (binding)	Macropinocytosis mediated endocytosis
Human embryonic kidney epithelial cells (HEK-293 with adenovirus 5 DNA nts 1–4344 integrated into chromosome 19 (19q13.2)	Heparan sulfate (binding)	Endocytosis
Monocytes	HS, DC-SIGN	Endocytosis
B cells	HS, DC-SIGN	Endocytosis

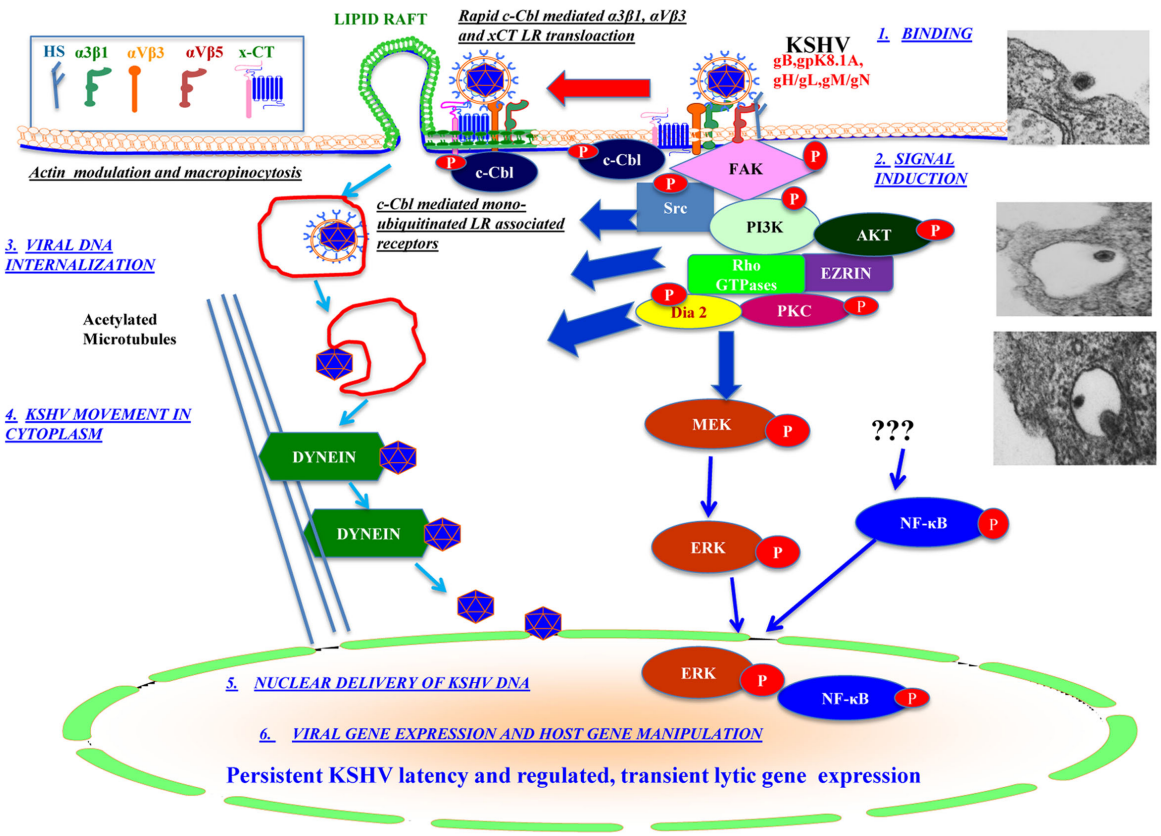

FIGURE 1 | Schematic model depicting the different overlapping phases of KSHV entry and infection in endothelial (HMVEC-d) target cell. KSHV infection is initiated by binding to the cell surface proteoglycan heparan sulfate [HS; Stage 1: binding; inset electron microscopic, (EM) picture], followed by subsequent temporal associations with integrins (α3β1, αVβ3, αVβ5) and xCT molecules in the non-lipid raft (NLR) parts of the membranes. KSHV's interactions with integrins activate FAK at tyrosine 397, which creates a binding site for the SH2 domain containing Src family kinases, subsequently leading to the activation of PI3-K and Rho-GTPases (Stage 2: signal induction). These rapid overlapping host cell signal induction play roles in actin modulation, formation of endocytic vesicles, and virus entry and trafficking through the cytosol leading into a productive infection (Blue arrows). KSHV infection induces the phosphorylation of c-Cbl and the phosphorylated c-Cbl forms a complex with p85-PI3-K, leading into the interaction of c-Cbl with downstream molecules. c-Cbl mediates a rapid selective translocations of KSHV into the lipid rafts (LRs) along with the α3β1, αVβ3, and xCT receptors. KSHV-associated αVβ5 remains in the NLR parts of the membranes. Activated c-Cbl localizes with LRs, associates with myosin IIA and actin, and is rapidly recruited to membrane blebs. This also leads into c-Cbl mediated ubiquitination of actin and myosin. c-Cbl mediated monoubiquitination of translocated receptors is followed by productive macropinocytic entry. Myosin IIA interactions with actin may be providing the ATP-dependent force to generate actomyosin contraction, bleb retraction to form macropinosomes along with KSHV (Stage 3: internalization; inset EM picture). NLR associated KSHV bound αVβ5 and other receptors are polyubiquitinated and directed to a clathrin-dependant lysosomal non-productive pathway. RhoA activates Dia-2 aiding in formation and movement of endosomes through the cytoplasm. KSHV capsid is released from the endocytic vesicles by fusion of viral envelope with endosomal vesicles (Stage 3: internalization; inset EM picture). Released capsids are transported toward the nucleus utilizing a Rho-GTPase dependant pathway that involves acetylation of microtubules (MT; Stage 4: movement in cytoplasm). KSHV capsid disassembly at or near the nuclear pore results in the delivery of KSHV DNA into the infected cell nucleus (Stage 5: nuclear delivery) followed by viral and host gene expression (Stage 6) initiated by KSHV binding and entry induced ERK and NF-κB pathways. All these events demonstrate that KSHV has evolved to utilize its interactions with cellular receptors to manipulate host cell signaling and to induce an environment that is conducive for a productive infection.

and Helenius, 2006). Hence, KSHV binding to multiple receptors (mainly integrins) possibly increases binding avidity that leads to receptor clustering, a key step to activate signaling pathways. It is widely known that integrins are a signaling hub for a variety of processes including adhesion, motility, and endocytosis.

Deciphering the interactions between entry receptors and signaling leading to active endocytosis of viruses has been an active area of research for the past decade. However, in KSHV biology, recent advancements have revealed that receptor recruitment by KSHV is tightly succeeded by an active modulation of host cell membranes involving actin–myosin and other cellular signaling (Raghu et al., 2009; Valiya Veettil et al., 2010; Chakraborty et al., 2011). Detailed understanding of how KSHV induced such changes in the cell membrane very early during infection comes from its ability to manipulate a variety of host cell molecules to regulate signaling. The usage of multiple receptors possibly adds greater gradient to the succeeding signaling events required for efficient entry into target cells.

KSHV-INDUCED SIGNALING DURING EARLY INFECTION

Many viruses, including KSHV make use of host cell signaling to enter target cells and establish productive infection. Binding and interaction of KSHV glycoproteins with integrins and other cellular receptors initiates intracellular signaling cascades to induce internalization. This internalization appears to be very rapid, reaching a peak by 60-min p.i. (Krishnan et al., 2004) and therefore signaling induced by the virus is very active within this time frame. Integrins are closely associated with FAK and Src kinases and is known to regulate them through a variety of other pathways. Since KSHV utilized a variety of integrins as its cellular receptor, it is evident that the virus has evolved ways to induce the integrin associated signaling.

Kaposi's sarcoma associated herpesvirus induces the phosphorylation of FAK and the subsequent phosphorylation of a variety of focal adhesion associated signal molecules such as Src, PI3-K, Rho-GTPases (RhoA, Rac, and Cdc42), and diaphanous-2, as well as several other downstream effector molecules, producing actin rearrangements that eventually lead to the internalization of KSHV. Induction of these signaling pathways is critical for active internalization of KSHV in target cells. The fact that a low multiplicity of infection is able to induce a sequential cascade of signaling events is a unique feature of KSHV infection. Several studies have elucidated that a low dose of KSHV (5–10 DNA copies/cell) is able to induce signaling events critical for entry, expression of viral genes, and latent infection (Sharma-Walia et al., 2005; Veettil et al., 2008; Valiya Veettil et al., 2010; Chakraborty et al., 2011). KSHV induces macropinocytic blebs as early as 1 min p.i., engage clustering of integrins, activate adaptors like c-Cbl, mediate receptor translocations, and induce ERK1/2 for establishing latent infection (Sharma-Walia et al., 2005; Veettil et al., 2008; Valiya Veettil et al., 2010; Chakraborty et al., 2011).

INTEGRIN ASSOCIATED SIGNALING: FOCAL ADHESION KINASES AND THEIR RELEVANCE IN KSHV ENTRY

Focal adhesions are multifunctional organelles that mediate cell–ECM adhesion, force transmission, cytoskeletal regulation, and signaling. Focal adhesions consist of a complex network of trans-plasma-membrane integrins and cytoplasmic linking of the ECM to the actin cytoskeleton (Riveline et al., 2001). FAK, a major component of focal adhesions, is a multidomain non-receptor tyrosine kinase involved in signaling downstream of integrins. Ligand interaction with integrins activates FAK by autophosphorylation at tyrosine 397, a key step initiating integrin outside-in signaling (Calderwood et al., 2000; Giancotti, 2000). Activated FAK associates with a lot of other signal molecules to regulate a broad range of functions including cell growth, endocytosis, and apoptosis (Giancotti, 2000, 2003).

Kaposi's sarcoma associated herpesvirus infection induced tyrosine phosphorylation of FAK within minutes of infection in HMVEC-d, HFF, 293, and FAK +/+ mouse Du17 fibroblasts (Akula et al., 2002; Naranatt et al., 2003; Wang et al., 2003; Sharma-Walia et al., 2004, 2005; Veettil et al., 2006; Raghu et al., 2007). Soluble glycoprotein gB also induced FAK autophosphorylation (Wang et al., 2003; Sharma-Walia et al., 2004) and the phosphorylated FAK colocalizes with Src, RhoA, and cytoskeletal proteins like vinculin and paxillin in the infected cells (Akula et al., 2002; Wang et al., 2003; Sharma-Walia et al., 2004; Veettil et al., 2006). Additionally, virus or gB pre-incubated with soluble α3β1 integrin or a soluble form of gB in which the RGD sequence had been mutated inhibited the activation of FAK (Akula et al., 2002; Wang et al., 2003; Sharma-Walia et al., 2004). Since FAK activation is a hallmark of integrin-mediated signaling, FAK knockout cells or cells transfected with integrin have been used to study the role of FAK in integrin-mediated signaling. KSHV infection studies with FAK negative (Du3) and FAK positive (Du17) mouse fibroblasts and CHO cells transfected with human α3 integrin demonstrated a significant role of FAK in KSHV infection (Naranatt et al., 2003; Krishnan et al., 2006). Heparin inhibited the binding of KSHV in both DU3 and DU17 cell types. FAK negative Du3 cells showed an approximately 70% reduction in KSHV DNA internalization and over-expression of FAK (Du17) increased viral DNA internalization, thus suggesting that FAK plays a significant role in signaling and KSHV entry. In addition to this, over-expression of FAK-related non-kinase (FRNK), a FAK dominant-negative inhibitor, significantly decreased KSHV entry in DU17 cells. Decreased viral entry, nuclear delivery, and viral gene expression in Du3 cells suggest that another protein may be able to compensate for FAK's function early during infection. KSHV infection in Du3 cells induced the phosphorylation of the FAK-related proline-rich tyrosine kinase (Pyk2) molecule, which demonstrated that the Pyk2 molecule compensate for the loss of some of the functions of FAK in FAK negative cells during KSHV infection. Moreover, inhibition of Pyk2 by an autophosphorylation mutant of Pyk2 also significantly reduced viral entry in DU3 cells (Krishnan et al., 2006). Since activation of FAK plays a central role in integrin-mediated signaling, rearrangement of actin and endocytosis, KSHV must have evolved to take advantage of these signaling pathways both to promote entry and the subsequent steps of infection (**Figure 1**).

ROLE OF Src AND PI3-KINASES IN KSHV ENTRY AND INFECTION

The autophosphorylation site of FAK (Tyr397) creates a binding site for the SH2 domain of Src kinases and the p85 subunit of PI3-K. KSHV infection induced a strong phosphorylation of Src within minutes of infection, and the phosphorylated Src colocalized with FAK (Veettil et al., 2006). KSHV-gB also induced the FAK dependent Src phosphorylation in adherent target cells (Sharma-Walia et al., 2004) and the activated Src kinases then phosphorylate a number of FA components. Furthermore, Src is required for the activation of PI3-K and other downstream targets such as Rho-GTPases. The critical role of Src in the KSHV entry process has been validated by multiple lines of evidences such as the failure of KSHV to enter Src negative mouse fibroblast cells (unpublished observation), increase in Src activity by LR disruption resulting in enhanced virus entry (Raghu et al., 2007). Another observation showed that RhoA-GTPase facilitated KSHV entry into adherent target cells in a Src-dependent manner. This study suggests that KSHV-induced Src is involved in RhoA activation, which in turn results in positive feedback activation of Src to increase viral entry.

PI3-Kinases (PI3-K) are heterodimeric proteins, consisting of a p85 regulatory subunit and a catalytic p110 subunit while phosphorylation of specific tyrosine residues on the p85 subunit is an indication of PI3-K activation. PI3-kinases play crucial roles in several signaling pathways and regulate multiple functions such as Rho-GTPase activation, apoptosis, survival, and migration (Giancotti, 2000; Sastry and Burridge, 2000). KSHV induces PI3-K within 5 min p.i. which decreased after 15 min and this response can be inhibited either by pre-incubating virus with integrin or by the PI3-K inhibitors wortmannin and LY294002 (Naranatt et al., 2003). LY294002 and wortmannin did not affect KSHV-gB induced Src phosphorylation whereas the Src kinase inhibitor SU6656 completely blocked KSHV-gB induced p85-PI3-K phosphorylation suggesting that PI3-K is downstream of Src (Sharma-Walia et al., 2004). In FAK positive Du17 cells, KSHV-gB induced PI3-K p85 phosphorylation whereas in FAK-null Du3 cells there was no significant induction of PI3-K p85 phosphorylation. This study suggests that FAK is an essential molecule for the induction of PI3-K during KSHV infection. Treatment of cells with PI3-K inhibitors wortmannin and LY294002 reduced PI3-K activation and viral entry in a concentration dependent manner suggesting the role of PI3-K activation in the entry of KSHV in target cells. Induction of PI3-K eventually leads to the induction of Rho-GTPases and their effectors (Sharma-Walia et al., 2004; Veettil et al., 2006) which in turn regulates the remodeling of actin, endosome formation, and the movement of endocytic vesicles. Therefore, these studies using chemical inhibitors, dominant-negative proteins, or cells lacking these molecules have demonstrated that FAK, Src, and PI3-K activation were necessary for KSHV entry.

ACTIVATION OF RhoA AND ACTIN DYNAMICS DURING KSHV INFECTION

RhoA, Rac, and Cdc42-Rho-GTPases are master regulators of a diverse set of signaling pathways, including cytoskeleton rearrangement and morphological changes (Giancotti, 2000; Hall and Nobes, 2000; Ishizaki et al., 2001; Palazzo et al., 2001). Immediately following infection, KSHV induces PI3-K Rho-GTPase-dependent cytoskeletal rearrangements and the formation of structures such as filopodia (Cdc42), lamellipodia (Rac), and stress fibers (RhoA) in the target cells (Naranatt et al., 2003; Veettil et al., 2006; Greene and Gao, 2009; Raghu et al., 2009). Soluble gB induced the FAK-Src-PI3-K Rho-GTPase signaling pathway and extensive cytoskeletal rearrangement in target cells (Sharma-Walia et al., 2004). KSHV-induced RhoA colocalized with Src in the infected cells (Sharma-Walia et al., 2004). Ezrin, an actin cross linking protein with the plasma membrane, was also induced by KSHV via Rho-GTPases, thereby modulating membrane changes (Sharma-Walia et al., 2004). Treatment of target cells with a potent RhoA inhibitor, *Clostridium difficile* toxin B (CdTxB), or transfecting dominant-negative constructs of RhoA resulted in significant inhibition of KSHV entry by modulation of Src activity (Veettil et al., 2006).

Several studies have demonstrated that RhoA-GTPases mediate rearrangement of cytoskeleton through the activation of its downstream effector molecules, formin family diaphanous 1 and 2 (Hall and Nobes, 2000; Ishizaki et al., 2001; Palazzo et al., 2001). KSHV infection induced diaphenous-2 without any significant activation of Rac-1 and Cdc42-dependent PAK1/2 and stathmin molecules. Dia-2 co-immunoprecipitated and colocalized with activated Src in the infected cells which were inhibited by Src inhibitors (Veettil et al., 2006). Together with the reduced virus entry in RhoA dominant-negative cells, these results suggest that activated RhoA-dependent Dia-2 probably functions as a link between RhoA and Src in KSHV infected cells, mediates the sustained Src activation, and that KSHV-induced Src and RhoA play roles in facilitating entry and nuclear delivery of viral DNA.

KSHV-INDUCED ADAPTOR MOLECULE c-Cbl AND ASSOCIATED MEMBRANE DYNAMICS

The Cbl family of adaptor proteins plays important roles in signal transduction as negative regulators by mediating the ubiquitination and down-regulation of proteins while it acting as a positive regulator through their scaffold function in assembling signaling complexes (Thien and Langdon, 2001; Schmidt and Dikic, 2005). Recent evidences have elucidated that KSHV-induced c-Cbl tyrosine phosphorylation is required for membrane ruffling known as blebs (Valiya Veettil et al., 2010). Blebs are protrusions from the plasma membrane that have often been characterized as a preceding step in macropinocytosis and are known to be induced by many viruses including vaccinia virus and KSHV early during infection (Mercer and Helenius, 2008; Raghu et al., 2009; Valiya Veettil et al., 2010). The role of c-Cbl in the entry and signaling of any viral infection has not been reported. Hence it opens up new directions of studies on the broad cellular requirements required by viruses for infecting target cells.

Kaposi's sarcoma associated herpesvirus is known to induce a variety of endothelial cell membrane alterations including filopodia and bleb formation (Raghu et al., 2009; Valiya Veettil et al., 2010). These studies elegantly deciphered the mechanism behind KSHV-induced membrane bleb formation. KSHV infection increased c-Cbl interaction with PI3-K in a time dependant manner. In KSHV infected cells, activated c-Cbl is recruited to the macropinocytic blebs and associated with its novel interacting partner, myosin IIA inside the membrane blebs very early by 5 min p.i. Studies using shRNA against c-Cbl not only reduced the macropinocytic blebs induced by KSHV, but also significantly reduced viral entry by >70% and hence KSHV gene expression by 90% (Valiya Veettil et al., 2010). This study demonstrates that recruitment of c-Cbl–myosin in macropinocytic blebs very early during KSHV infection is essential for its entry via macropinocytosis in endothelial cells. Although many viruses utilize macropinocytosis to enter target cells, the mechanisms behind it were unclear (Mercer and Helenius, 2008); however, the role c-Cbl and myosin IIA in macropinocytic modulation of KSHV potentiate new avenues of interest in the phenomena of membrane dynamicity induced by viruses.

ROLE OF LIPID RAFTS: A SIGNALING PLATFORM UTILIZED BY KSHV IN ENDOTHELIAL CELLS

Lipid rafts, the detergent resistant microdomains in the exoplasmic leaflet of plasma membranes, are made up of cholesterol and sphingolipids (sphingomyelin and glyco-sphingolipids) and play roles in clustering cell surface receptors and signal molecules (Simmons, 2001). Lipid microdomains favor specific

protein–protein interactions including ligand receptor interactions activating signal cascades. KSHV binding was not affected upon disruption of LRs by methyl beta cyclodextrin or nystatin, but pre-treatment of HMVEC-d with these LR disrupting drugs significantly reduced the expression of ORF73 and ORF50 (Raghu et al., 2007). Although internalization of viral DNA was increased, the association of internalized viral capsids with microtubules was reduced upon LR disruption, microtubules disorganized, and nuclei associated viral DNA decreased (Raghu et al., 2007).

Mechanistic studies on the role of LRs in KSHV infection revealed more intricate details with relation to entry receptors and signal molecules. A recent study reported that very early during infection (1 min p.i.), c-Cbl induced the selective translocation of KSHV into LRs along with α3β1, αVβ3, and xCT receptors, but not αVβ5 (Chakraborty et al., 2011). Evidence also suggests that activated c-Cbl localized to LRs at the junctional base of macropinocytic blebs, thereby aiding macropinocytosis (Chakraborty et al., 2011). Such partitioning of entry receptors in LRs by specific signaling adaptors was not reported before and evokes new insights to the molecular mechanisms of viral–host cell interactions, often favoring viral internalization.

The role of LRs is complex and affects KSHV-induced signaling. For instance Raghu et al. (2007) reported that LR disruption affects signal pathways induced by KSHV such that phospho-Src levels had increased without affecting FAK or ERK1/2. However, KSHV-induced PI3-K, Rho-GTPases, and NF-κB activation were significantly reduced. The fact that p-Src increased with LR disruption is indicative of a strong regulation of Src by LRs. LR disruption also affects PI3-K and RhoA with subsequent reduction in KSHV-induced RhoA mediated acetylation and aggregation of MTs (Raghu et al., 2007).

Cellular signaling can be generated in many ways. Viruses most often activate cellular signaling directly by using receptors or induce signaling by clustering specific cell-surface proteins or lipids (Marsh and Helenius, 2006). KSHV serves an excellent model system in this regard as it utilizes both pathways; firstly by activating associated integrin signaling and secondly by clustering activated integrins and signal molecules in LRs, generating significant amplification of the signaling response; however, the molecular partners behind such signaling amplification are yet to be studied.

RECEPTOR UBIQUITINATION: A CRITICAL STEP REQUIRED FOR PRODUCTIVE KSHV INTERNALIZATION

Ubiquitination of receptors has been recognized as an internalization signal based on the nature and type of ubiquitin modifications (Levkowitz et al., 1998; Dupin et al., 1999). Studies have revealed an important role of c-Cbl, an E3-ubiquitin ligase, in differential ubiquitination of KSHV integrin receptors (Chakraborty et al., 2011). Essentially, LR translocated integrins (α3β1 and αVβ3) were monoubiquitinated leading to productive macropinocytic entry, whereas non-LR associated αVβ5 was polyubiquitinated leading to clathrin mediated entry that was targeted to lysosomes, the non-infectious pathway (Chakraborty et al., 2011). This elucidates the complexities of viral endocytic mechanisms and the ability of KSHV to utilize E3-ubiquitin ligases to regulate and sort out productive pathways.

DIVERSE INTERNALIZATION PATHWAYS OF KSHV IN TARGET CELLS

Current evidences show that KSHV enters human B cells (Rappocciolo et al., 2008), fibroblast (Akula et al., 2003), epithelial (Inoue et al., 2003; Liao et al., 2003), and endothelial cells (Raghu et al., 2009) by endocytosis. KSHV was detected by electron microscopy in large endocytic vesicles within 5 min of HMVEC-d and HFF cell infection while fusion of virion envelope with the endocytic vesicles was also observed (Akula et al., 2001a, 2003). Viral capsids were detected in the vicinity of the nuclear membrane by 15 min p.i. and anti-KSHV-gB and gpK8.1A antibodies colocalized with virus-containing endocytic vesicles (Akula et al., 2001a; Greene and Gao, 2009; Raghu et al., 2009; **Table 2**).

The roles of different endocytic pathways have been studied using specific inhibitors of each type. Clathrin-mediated endocytosis is the predominant pathway of entry in HFF cells. Chlorpromazine, an inhibitor of clathrin-mediated endocytosis, significantly inhibited entry in HFF cells, whereas nystatin, an inhibitor of caveolae and cholera toxin B, a LR inhibiting agent did not have any effect on entry. A significant inhibition of gene expression was also observed after blocking endosomal acidification by NH_4Cl and bafilomycin A in HFF cells (Akula et al., 2003; Raghu et al., 2009) suggesting that post internalization endosomal acidification was required for KSHV trafficking. In addition to this, electron microscopy revealed KSHV virions in large endocytic vesicles within 5-min of HFF cell infection and fusion of virion envelope with endocytic vesicles was also observed (Akula et al., 2003; Raghu et al., 2009). Evidence for clathrin-mediated endocytosis has also been observed in BJAB and 293 cells (Akula et al., 2001a; Inoue et al., 2003). KSHV enters THP-1 cells and primary monocytes by clathrin and caveolin dependant endocytosis, which required endosomal acidification (Kerur et al., 2010).

In HMVEC-d and HUVEC cells, entry and gene expression of KSHV were significantly blocked by macropinocytosis inhibitors EIPA and rottlerin. Macropinocytosis of KSHV is an actin dependant endocytic pathway and it was also inhibited by cytochalasin D (Raghu et al., 2009). Cytochalasin D inhibited actin polymerization and formation of lamellipodial extensions significantly inhibited the entry and expression of KSHV (Naranatt et al., 2003; Greene and Gao, 2009; Raghu et al., 2009). The LR inhibiting agents reduced viral gene expression in HMVEC-d cells but not in HUVEC or HFF cells, indicating the role of LRs in KSHV infection (Greene and Gao, 2009; Raghu et al., 2009). Colocalization studies using clathrin-mediated endocytosis marker, transferrin and macropinocytosis marker, dextran showed significant association of KSHV with dextran but not with transferrin or the caveolar marker caveolin. The dynamin inhibitor, dynasore, did not affect viral entry into endothelial cells while inhibiting entry into HFF cells (Raghu et al., 2009). The small GTPase Rab34, a key regulator of macropinocytosis, associated with KSHV and Rab34-siRNA considerably decreased KSHV gene expression (Raghu et al., 2009). These studies suggested that KSHV utilizes the actin polymerization-dependent, dynamin-independent macropinocytic pathway involving a Rab34 GTPase-dependent late endosome and low-pH environment for its infectious entry into HMVEC-d and HUVEC cells (**Table 2**).

Recent studies have confirmed the role of LRs in aiding the macropinocytosis of KSHV bound receptors in endothelial cells, whereas non-LR bound receptors were targeted toward a clathrin mediated non-infectious lysosomal pathways (Chakraborty et al., 2011). This study suggests that both macropinocytosis and clathrin-mediated endocytosis are occurring in endothelial cells, however the former being productive while the latter is degradative. KSHV internalized by clathrin-mediated endocytosis clearly associated with lysosomal compartments (Chakraborty et al., 2011). Another report suggested that clathrin-mediated endocytosis is the predominant pathway of entry in endothelial cells. This discrepancy could be due to optimal concentration of the inhibitor used and the method of quantification used to analyze the entry of KSHV (Greene and Gao, 2009). The detection of KSHV by ORF65 (KSHV capsid protein) in perinuclear regions of HUVECs does not necessarily correlate with a productive endocytosis pathway and hence needs further clarification (**Table 2**).

Some evidences have also revealed the role of fusion of KSHV envelope glycoproteins with target cell membranes (Akula et al., 2001a; Wang et al., 2001, 2003; Naranatt et al., 2002). The minimal fusion machinery of KSHV probably comprises of gB, gH, and gL since anti-gB, gH, gL, and gpK8.1A antibodies neutralize KSHV infection without affecting virus binding to the target cells (Akula et al., 2001a; Wang et al., 2001, 2003; Naranatt et al., 2002). However the mechanism of neutralization is not known. Multiple mechanisms could be possible depending upon the glycoprotein and the targeted region of the glycoproteins. For example, neutralization could be due to: (a) interference in the interaction of KSHV glycoproteins with integrins and other receptors; (b) interference at the activation of a subset of signal molecules that are essential for the c-Cbl mediated receptor translocation, ubiquitination, bleb formation, and macropinocytosis; and (c) interference of fusion of viral envelope with the endosomal membrane. Further studies need to be carried out to precisely define the mechanism behind the neutralization by these antibodies. Nevertheless, these studies suggested that these glycoproteins play critical roles in the entry process after attachment has occurred, possibly as a result of interaction with additional cell surface molecules.

POST ENTRY STEPS: KSHV TRAFFICKING AND PRODUCTIVE INFECTION OF KSHV

Like most herpesviruses, KSHV replicates in the nucleus of infected cells. Therefore, to reach the nucleus KSHV capsids traffic through the crowded cytosol before releasing viral DNA into the nucleus (Lyman and Enquist, 2009). Delivery of KSHV DNA into the infected cell nucleus reached a peak by 90 min p.i. suggesting the rapidness of the host cellular trafficking utilized by the virus (Naranatt et al., 2003, 2005; Krishnan et al., 2004; Veettil et al., 2006; Raghu et al., 2009).

Productive infection comprised of KSHV internalized by macropinocytosis in endothelial cells or by clathrin-mediated endocytosis in fibroblast cells utilize the extensive microtubule (MT) network to traffic through the cytosol, tightly regulated by Rho-GTPases. Microtubule bundles colocalized with KSHV capsids and this colocalization was abolished by the microtubule destabilizing agent, nocodazole, and PI3-K inhibitor affecting the Rho-GTPases (Naranatt et al., 2003, 2004). Depolymerization of microtubules did not affect KSHV binding and internalization, but nuclear delivery of viral DNA and infection in HFF, HMVEC-d, and HUVEC cells was inhibited (Naranatt et al., 2005; Veettil et al., 2006; Raghu et al., 2009). Inhibition of Rho-GTPase activities by CdTxB abolished microtubular acetylation and subsequently the delivery of viral DNA to the nucleus. Conversely, activation of Rho-GTPases by *Escherichia coli* cytotoxic necrotizing factor significantly increased the intracellular trafficking and delivery of viral capsids to the nucleus. Similarly, nuclear delivery of viral DNA was increased in cells expressing a constitutively active RhoA mutant and decreased in cells expressing a dominant-negative mutant of RhoA (Naranatt et al., 2005). Taken together, these studies indicate that KSHV induces Rho-GTPases, modulates stabilization of microtubules and promotes the rapid trafficking of viral capsids toward the nucleus (**Table 3**).

Kaposi's sarcoma associated herpesvirus utilizes ATP dependant directional transport along microtubules governed by motor proteins. Dynein motor proteins responsible for minus-end transport from the periphery to the cell center actively carry KSHV to the vicinity of the nuclear membrane. Inhibition of dynein activity by sodium orthovanadate significantly reduced the infection and delivery of KSHV DNA into the nucleus (Naranatt et al., 2005). These studies demonstrate that KSHV movement across the cytoplasm to reach the nucleus is a series of well-orchestrated phenomenon probably involving viral proteins and host proteins. Further studies are essential to define this process and the mediating viral and host proteins.

Cross talk between ECM and integrins activates FAK, which initiates a cascade of intracellular signals that eventually activate the mitogen activated protein kinase (MAPK) pathways. As early as 5 min p.i., KSHV activates MEK (MAPK/ERK kinase) and extracellular-signal-regulated kinase (ERK; Naranatt et al., 2005). Soluble KSHV gpK8.1A, but not gB, induced MEK-mediated ERK1/2 phosphorylation as early as 5 min p.i., and ERK1/2 phosphorylation facilitated the establishment of KSHV infection in HFF and HMVEC-d cells (Sharma-Walia et al., 2005). PI3-K and protein kinase C-ζ (PKC-zeta) are recruited as upstream mediators of the KSHV ERK pathway and inhibitors specific for PI3-K, PKC-ζ, MEK, and ERK significantly reduce virus infectivity without affecting virus binding to the target cells (**Table 3**).

Table 3 | Host molecules and their roles in KSHV entry/infection in HMVEC-d and HFF cells.

Molecules	Function in KSHV biology
Heparan sulfate (HS)	Binding receptor
Integrins (α3β1, αVβ3, αVβ5)	Entry receptors
xCT/CD98	Entry (fusion) receptor
FAK	Initiate endocytosis
Src	Initiate endocytosis
PI3-K, RhoA, Dia-2, Ezrin	Entry, actin modulation, endocytosis, and nuclear trafficking
c-Cbl	KSHV-receptor translocations into LR and adaptor for macropinocytosis in HMVEC-d cells
ERK and NF-κB	Viral and host gene expression

Rapid activation of NF-κB as early as 5–15 min p.i. led to the translocation of p65-NF-κB into the nucleus (Sadagopan et al., 2007). KSHV incubated with heparin significantly reduced NF-κB activation. During the observed 72 h periods of *in vitro* KSHV latency, a sustained moderate level of NF-κB induction was observed, and inhibition of IκB phosphorylation by Bay11-7082 drastically reduced this activation (Sadagopan et al., 2007). In contrast, high levels of ERK1/2 activation during the earlier time points and a moderate level of activation at later time points were observed. The p38-MAP kinase was activated only during the later time points, and AKT was activated at lower levels in a cyclic manner. Studies with UV-KSHV suggested a role for virus entry stages in NF-κB induction and requirement of KSHV viral gene expression for sustained induction. Though inhibition of NF-κB did not have any effect on KSHV entry into cells, expression of viral latent ORF 73 and lytic ORF 50 genes was significantly reduced. Several transcription factors were activated during KSHV infection, and inhibition of NF-κB significantly affected the activation of Jun D, Jun B, phospho-c-Jun, cFos, and FosB factors. These results suggested that during *in vitro* infection, KSHV induces sustained levels of NF-κB to regulate viral genes thus possibly regulating the establishment of latent infection.

Productive KSHV primary infection in adherent cells involves an initial lytic phase (2 h p.i.) succeeded by a latent phase (24 h p.i.) with a decline of lytic phase (Krishnan et al., 2004). A report by Yoo et al. (2005) suggests that KSHV infection is permissive in HUVECs at early time points with an initial production of infectious virus particles (lytic cycle), while the surviving cells later enter a latent phase with spontaneous lytic replication. However, the percentage of cells that were infected, cells that entered into lytic cycle and cells that entered latency were not determined in these studies. Hence, these studies need to be reexamined.

PERSPECTIVES AND FUTURE DIRECTIONS

Kaposi's sarcoma associated herpesvirus exemplifies an excellent model for viruses requiring multiple cellular molecules to enter target cells. Although, integrins and associated signaling, adaptor molecules and a host of pre-existing signaling molecules have been identified to play a crucial role in KSHV entry and infection, the nature and mechanism by which the virus is able to successfully utilize the sequential series of host signaling still remains a mystery. Moreover, there is a difference in the entry pathways of KSHV in infectable cell types, making this interesting story more complicated. What is clear from studies so far is that KSHV overcomes cellular barriers making conditions conducive to infection by utilizing more than one endocytic mechanism. However, further studies need to be performed to ensure whether differential activation of signal responses by KSHV coupled to different endosomal vesicles accounts for the varied modes of entry. Moreover, whether KSHV utilizes more than one E3-ubiquitin ligase and their modulation by viral induced cellular targets remains to be explored. Another interesting future study involves the recognition of KSHV in the endosomal vesicles that direct productive trafficking of virus away from the lysosomal compartments. Cellular signaling targets of KSHV are broad and hence critical molecules regulating such multiple pathways need to be targeted for therapeutics. These studies will shed valuable insights into the molecular mechanisms of cytosolic entry of KSHV.

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REFERENCES

Akula, S. M., Naranatt, P. P., Walia, N. S., Wang, F. Z., Fegley, B., and Chandran, B. (2003). Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) infection of human fibroblast cells occurs through endocytosis. *J. Virol.* 77, 7978–7990.

Akula, S. M., Pramod, N. P., Wang, F. Z., and Chandran, B. (2001a). Human herpesvirus 8 envelope-associated glycoprotein B interacts with heparan sulfate-like moieties. *Virology* 284, 235–249.

Akula, S. M., Wang, F. Z., Vieira, J., and Chandran, B. (2001b). Human herpesvirus 8 interaction with target cells involves heparan sulfate. *Virology* 282, 245–255.

Akula, S. M., Pramod, N. P., Wang, F. Z., and Chandran, B. (2002). Integrin alpha3beta1 (CD 49c/29) is a cellular receptor for Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) entry into the target cells. *Cell* 108, 407–419.

Baghian, A., Luftig, M., Black, J. B., Meng, Y. X., Pau, C. P., Voss, T., Pellett, P. E., and Kousoulas, K. G. (2000). Glycoprotein B of human herpesvirus 8 is a component of the virion in a cleaved form composed of amino- and carboxyl-terminal fragments. *Virology* 269, 18–25.

Bechtel, J. T., Liang, Y., Hvidding, J., and Ganem, D. (2003). Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. *J. Virol.* 77, 6474–6481.

Bender, F. C., Whitbeck, J. C., Ponce de Leon, M., Lou, H., Eisenberg, R. J., and Cohen, G. H. (2003). Specific association of glycoprotein B with lipid rafts during herpes simplex virus entry. *J. Virol.* 77, 9542–9552.

Birkmann, A., Mahr, K., Ensser, A., Yaguboglu, S., Titgemeyer, F., Fleckenstein, B., and Neipel, F. (2001). Cell surface heparan sulfate is a receptor for human herpesvirus 8 and interacts with envelope glycoprotein K8.1. *J. Virol.* 75, 11583–11593.

Boshoff, C., Schulz, T. F., Kennedy, M. M., Graham, A. K., Fisher, C., Thomas, A., McGee, J. O., Weiss, R. A., and O'Leary, J. J. (1995). Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. *Nat. Med.* 1, 1274–1278.

Calderwood, D. A., Shattil, S. J., and Ginsberg, M. H. (2000). Integrins and actin filaments: reciprocal regulation of adhesion and signaling. *J. Biol. Chem.* 275, 22607–22610.

Cesarman, E., Nador, R. G., Bai, F., Bohenzky, R. A., Russo, J. J., Moore, P. S., Chang, Y., and Knowles, D. M. (1996). Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. *J. Virol.* 70, 8218–8223.

Chakraborty, S., Valiyaveettil, M., Sadagopan, S., Paudel, N., and Chandran, B. (2011). c-Cbl-mediated selective virus-receptor translocations into lipid rafts regulate productive Kaposi's sarcoma-associated herpesvirus infection in endothelial cells. *J. Virol.* 85, 12410–12430.

Dourmishev, L. A., Dourmishev, A. L., Palmeri, D., Schwartz, R. A., and Lukac, D. M. (2003). Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. *Microbiol. Mol. Biol. Rev.* 67, 175–212.

Dupin, N., Fisher, C., Kellam, P., Ariad, S., Tulliez, M., Franck, N., van Marck, E., Salmon, D., Gorin, I., Escande, J. P., Weiss, R. A., Alitalo, K., and Boshoff, C. (1999). Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4546–4551.

Fenczik, C. A., Zent, R., Dellos, M., Calderwood, D. A., Satriano, J., Kelly, C., and Ginsberg, M. H. (2001). Distinct domains of CD98hc regulate integrins and amino acid transport. *J. Biol. Chem.* 276, 8746–8752.

Feral, C. C., Zijlstra, A., Tkachenko, E., Prager, G., Gardel, M. L., Slepak, M., and Ginsberg, M. H. (2007). CD98hc (SLC3A2) participates in fibronectin matrix assembly by mediating integrin signaling. *J. Cell Biol.* 178, 701–711.

Ganem, D. (2007a). "Kaposi's sarcoma associated herpesvirus," in *Fields Virology*, 5th Edn, Vol. 2, eds D. M. Knipe and P. M. Howley (Philadelphia, PA: Lippincott Williams & Wilkins), 2875–2888.

Ganem, D. (2007b). *KSHV-Induced Oncogenesis. Human Herpesviruses Biology, Therapy, and Immunoprophylaxis*, Chap. 56. Cambridge: Cambridge University Press, 1007–1028.

Garrigues, H. J., Rubinchikova, Y. E., Dipersio, C. M., and Rose, T. M. (2008). Integrin alphaVbeta3 Binds to the RGD motif of glycoprotein B of Kaposi's sarcoma-associated herpesvirus and functions as an RGD-dependent entry receptor. *J. Virol.* 82, 1570–1580.

Geijtenbeek, T. B., Van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M., Appelmelk, B., and Van Kooyk, Y. (2003). Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 197, 7–17.

Giancotti, F. G. (2000). Complexity and specificity of integrin signalling. *Nat. Cell Biol.* 2, E13–E14.

Giancotti, F. G. (2003). A structural view of integrin activation and signaling. *Dev. Cell* 4, 149–151.

Greene, W., and Gao, S. J. (2009). Actin dynamics regulate multiple endosomal steps during Kaposi's sarcoma-associated herpesvirus entry and trafficking in endothelial cells. *PLoS Pathog.* 5, e1000512. doi:10.1371/journal.ppat.1000512

Grundhoff, A., and Ganem, D. (2004). Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. *J. Clin. Invest.* 113, 124–136.

Hahn, A., Birkmann, A., Wies, E., Dorer, D., Mahr, K., Sturzl, M., Titgemeyer, F., and Neipel, F. (2009). Kaposi's sarcoma-associated herpesvirus gH/gL: glycoprotein export and interaction with cellular receptors. *J. Virol.* 83, 396–407.

Hall, A., and Nobes, C. D. (2000). Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 355, 965–970.

Inoue, N., Winter, J., Lal, R. B., Offermann, M. K., and Koyano, S. (2003). Characterization of entry mechanisms of human herpesvirus 8 by using an Rta-dependent reporter cell line. *J. Virol.* 77, 8147–8152.

Ishizaki, T., Morishima, Y., Okamoto, M., Furuyashiki, T., Kato, T., and Narumiya, S. (2001). Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1. *Nat. Cell Biol.* 3, 8–14.

Jarousse, N., Chandran, B., and Coscoy, L. (2008). Lack of heparan sulfate expression in B-cell lines: implications for Kaposi's sarcoma-associated herpesvirus and murine gammaherpesvirus 68 infections. *J. Virol.* 82, 12591–12597.

Kabir-Salmani, M., Fukuda, M. N., Kanai-Azuma, M., Ahmed, N., Shiokawa, S., Akimoto, Y., Sakai, K., Nagamori, S., Kanai, Y., Sugihara, K., and Iwashita, M. (2008). The membrane-spanning domain of CD98 heavy chain promotes alpha(v)beta3 integrin signals in human extravillous trophoblasts. *Mol. Endocrinol.* 22, 707–715.

Kaleeba, J. A., and Berger, E. A. (2006). Broad target cell selectivity of Kaposi's sarcoma-associated herpesvirus glycoprotein-mediated cell fusion and virion entry. *Virology* 354, 7–14.

Kerur, N., Veettil, M. V., Sharma-Walia, N., Sadagopan, S., Bottero, V., Paul, A. G., and Chandran, B. (2010). Characterization of entry and infection of monocytic THP-1 cells by Kaposi's sarcoma associated herpesvirus (KSHV): role of heparan sulfate, DC-SIGN, integrins and signaling. *Virology* 406, 103–116.

Koyano, S., Mar, E. C., Stamey, F. R., and Inoue, N. (2003). Glycoproteins M and N of human herpesvirus 8 form a complex and inhibit cell fusion. *J. Gen. Virol.* 84, 1485–1491.

Krishnan, H. H., Naranatt, P. P., Smith, M. S., Zeng, L., Bloomer, C., and Chandran, B. (2004). Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. *J. Virol.* 78, 3601–3620.

Krishnan, H. H., Sharma-Walia, N., Streblow, D. N., Naranatt, P. P., and Chandran, B. (2006). Focal adhesion kinase is critical for entry of Kaposi's sarcoma-associated herpesvirus into target cells. *J. Virol.* 80, 1167–1180.

Krishnan, H. H., Sharma-Walia, N., Zeng, L., Gao, S. J., and Chandran, B. (2005). Envelope glycoprotein gB of Kaposi's sarcoma-associated herpesvirus is essential for egress from infected cells. *J. Virol.* 79, 10952–10967.

Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998). c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* 12, 3663–3674.

Liao, W., Tang, Y., Kuo, Y. L., Liu, B. Y., Xu, C. J., and Giam, C. Z. (2003). Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 transcriptional activator Rta is an oligomeric DNA-binding protein that interacts with tandem arrays of phased A/T-trinucleotide motifs. *J. Virol.* 77, 9399–9411.

Lozach, P. Y., Kuhbacher, A., Meier, R., Mancini, R., Bitto, D., Bouloy, M., and Helenius, A. (2011). DC-SIGN as a receptor for phleboviruses. *Cell Host Microbe* 10, 75–88.

Lyman, M. G., and Enquist, L. W. (2009). Herpesvirus interactions with the host cytoskeleton. *J. Virol.* 83, 2058–2066.

Mark, L., Lee, W. H., Spiller, O. B., Villoutreix, B. O., and Blom, A. M. (2006). The Kaposi's sarcoma-associated herpesvirus complement control protein (KCP) binds to heparin and cell surfaces via positively charged amino acids in CCP1-2. *Mol. Immunol.* 43, 1665–1675.

Marsh, M., and Helenius, A. (2006). Virus entry: open sesame. *Cell* 124, 729–740.

Mercer, J., and Helenius, A. (2008). Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science* 320, 531–535.

Naranatt, P. P., Akula, S. M., and Chandran, B. (2002). Characterization of gamma2-human herpesvirus-8 glycoproteins gH and gL. *Arch. Virol.* 147, 1349–1370.

Naranatt, P. P., Akula, S. M., Zien, C. A., Krishnan, H. H., and Chandran, B. (2003). Kaposi's sarcoma-associated herpesvirus induces the phosphatidylinositol 3-kinase-PKC-zeta-MEK-ERK signaling pathway in target cells early during infection: implications for infectivity. *J. Virol.* 77, 1524–1539.

Naranatt, P. P., Krishnan, H. H., Smith, M. S., and Chandran, B. (2005). Kaposi's sarcoma-associated herpesvirus modulates microtubule dynamics via RhoA-GTP-diaphanous 2 signaling and utilizes the dynein motors to deliver its DNA to the nucleus. *J. Virol.* 79, 1191–1206.

Naranatt, P. P., Krishnan, H. H., Svojanovsky, S. R., Bloomer, C., Mathur, S., and Chandran, B. (2004). Host gene induction and transcriptional reprogramming in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8)-infected endothelial, fibroblast, and B cells: insights into modulation events early during infection. *Cancer Res.* 64, 72–84.

Neipel, F., Albrecht, J. C., and Fleckenstein, B. (1997). Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? *J. Virol.* 71, 4187–4192.

Palazzo, A. F., Cook, T. A., Alberts, A. S., and Gundersen, G. G. (2001). mDia mediates Rho-regulated formation and orientation of stable microtubules. *Nat. Cell Biol.* 3, 723–729.

Parravicini, C., Chandran, B., Corbellino, M., Berti, E., Paulli, M., Moore, P. S., and Chang, Y. (2000). Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. *Am. J. Pathol.* 156, 743–749.

Raghu, H., Sharma-Walia, N., Veettil, M. V., Sadagopan, S., Caballero, A., Sivakumar, R., Varga, L., Bottero, V., and Chandran, B. (2007). Lipid rafts of primary endothelial cells are essential for Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8-induced phosphatidylinositol 3-kinase and RhoA-GTPases critical for microtubule dynamics and nuclear delivery of viral DNA but dispensable for binding and entry. *J. Virol.* 81, 7941–7959.

Raghu, H., Sharma-Walia, N., Veettil, M. V., Sadagopan, S., and Chandran, B. (2009). Kaposi's sarcoma-associated herpesvirus utilizes an actin polymerization-dependent macropinocytic pathway to enter human dermal microvascular endothelial and human umbilical vein endothelial cells. *J. Virol.* 83, 4895–4911.

Rappocciolo, G., Hensler, H. R., Jais, M., Reinhart, T. A., Pegu, A., Jenkins, F. J., and Rinaldo, C. R. (2008). Human herpesvirus 8 infects and replicates in primary cultures of activated B lymphocytes through DC-SIGN. *J. Virol.* 82, 4793–4806.

Rappocciolo, G., Jenkins, F. J., Hensler, H. R., Piazza, P., Jais, M., Borowski, L., Watkins, S. C., and Rinaldo, C. R. Jr. (2006). DC-SIGN is a receptor for human herpesvirus 8 on dendritic cells and macrophages. *J. Immunol.* 176, 1741–1749.

Riveline, D., Zamir, E., Balaban, N. Q., Schwarz, U. S., Ishizaki, T., Narumiya, S., Kam, Z., Geiger, B., and Bershadsky, A. D. (2001). Focal contacts as mechanosensors: externally applied local mechanical force

induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. *J. Cell Biol.* 153, 1175–1186.

Sadagopan, S., Sharma-Walia, N., Veettil, M. V., Raghu, H., Sivakumar, R., Bottero, V., and Chandran, B. (2007). Kaposi's sarcoma-associated herpesvirus induces sustained NF-kappaB activation during de novo infection of primary human dermal microvascular endothelial cells that is essential for viral gene expression. *J. Virol.* 81, 3949–3968.

Sastry, S. K., and Burridge, K. (2000). Focal adhesions: a nexus for intracellular signaling and cytoskeletal dynamics. *Exp. Cell Res.* 261, 25–36.

Schmidt, M. H., and Dikic, I. (2005). The Cbl interactome and its functions. *Nat. Rev. Mol. Cell Biol.* 6, 907–918.

Sharma-Walia, N., Krishnan, H. H., Naranatt, P. P., Zeng, L., Smith, M. S., and Chandran, B. (2005). ERK1/2 and MEK1/2 induced by Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) early during infection of target cells are essential for expression of viral genes and for establishment of infection. *J. Virol.* 79, 10308–10329.

Sharma-Walia, N., Naranatt, P. P., Krishnan, H. H., Zeng, L., and Chandran, B. (2004). Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 envelope glycoprotein gB induces the integrin-dependent focal adhesion kinase-Src-phosphatidylinositol 3-kinase-rho GTPase signal pathways and cytoskeletal rearrangements. *J. Virol.* 78, 4207–4223.

Simmons, G. (2001). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 2, 216.

Thien, C. B., and Langdon, W. Y. (2001). Cbl: many adaptations to regulate protein tyrosine kinases. *Nat. Rev. Mol. Cell Biol.* 2, 294–307.

Valiya Veettil, M., Sadagopan, S., Kerur, N., Chakraborty, S., and Chandran, B. (2010). Interaction of c-Cbl with myosin IIA regulates Bleb associated macropinocytosis of Kaposi's sarcoma-associated herpesvirus. *PLoS Pathog.* 6, e1001238. doi:10.1371/journal.ppat.1001238

Veettil, M. V., Sadagopan, S., Sharma-Walia, N., Wang, F. Z., Raghu, H., Varga, L., and Chandran, B. (2008). Kaposi's sarcoma-associated herpesvirus forms a multimolecular complex of integrins (alphaVbeta5, alphaVbeta3, and alpha3beta1) and CD98-xCT during infection of human dermal microvascular endothelial cells, and CD98-xCT is essential for the postentry stage of infection. *J. Virol.* 82, 12126–12144.

Veettil, M. V., Sharma-Walia, N., Sadagopan, S., Raghu, H., Sivakumar, R., Naranatt, P. P., and Chandran, B. (2006). RhoA-GTPase facilitates entry of Kaposi's sarcoma-associated herpesvirus into adherent target cells in a Src-dependent manner. *J. Virol.* 80, 11432–11446.

Wang, F. Z., Akula, S. M., Pramod, N. P., Zeng, L., and Chandran, B. (2001). Human herpesvirus 8 envelope glycoprotein K8.1A interaction with the target cells involves heparan sulfate. *J. Virol.* 75, 7517–7527.

Wang, F. Z., Akula, S. M., Sharma-Walia, N., Zeng, L., and Chandran, B. (2003). Human herpesvirus 8 envelope glycoprotein B mediates cell adhesion via its RGD sequence. *J. Virol.* 77, 3131–3147.

Yoo, S. M., Zhou, F. C., Ye, F. C., Pan, H. Y., and Gao, S. J. (2005). Early and sustained expression of latent and host modulating genes in coordinated transcriptional program of KSHV productive primary infection of human primary endothelial cells. *Virology* 343, 47–64.

Zhu, L., Puri, V., and Chandran, B. (1999a). Characterization of human herpesvirus-8 K8.1A/B glycoproteins by monoclonal antibodies. *Virology* 262, 237–249.

Zhu, L., Wang, R., Sweat, A., Goldstein, E., Horvat, R., and Chandran, B. (1999b). Comparison of human sera reactivities in immunoblots with recombinant human herpesvirus (HHV)-8 proteins associated with the latent (ORF73) and lytic (ORFs 65, K8.1A, and K8.1B) replicative cycles and in immunofluorescence assays with HHV-8-infected BCBL-1 cells. *Virology* 256, 381–392.

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KSHV Rta promoter specification and viral reactivation

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Viruses are obligate intracellular pathogens whose biological success depends upon replication and packaging of viral genomes, and transmission of progeny viruses to new hosts. The biological success of herpesviruses is enhanced by their ability to reproduce their genomes without producing progeny viruses or killing the host cells, a process called latency. Latency permits a herpesvirus to remain undetected in its animal host for decades while maintaining the potential to reactivate, or switch, to a productive life cycle when host conditions are conducive to generating viral progeny. Direct interactions between many host and viral molecules are implicated in controlling herpesviral reactivation, suggesting complex biological networks that control the decision. One viral protein that is necessary and sufficient to switch latent Kaposi's sarcoma-associated herpesvirus (KSHV) into the lytic infection cycle is called K-Rta. K-Rta is a transcriptional activator that specifies promoters by binding DNA directly and interacting with cellular proteins. Among these cellular proteins, binding of K-Rta to RBP-Jk is essential for viral reactivation. In contrast to the canonical model for Notch signaling, RBP-Jk is not uniformly and constitutively bound to the latent KSHV genome, but rather is recruited to DNA by interactions with K-Rta. Stimulation of RBP-Jk DNA binding requires high affinity binding of Rta to repetitive and palindromic "CANT DNA repeats" in promoters, and formation of ternary complexes with RBP-Jk. However, while K-Rta expression is necessary for initiating KSHV reactivation, K-Rta's role as the switch is inefficient. Many factors modulate K-Rta's function, suggesting that KSHV reactivation can be significantly regulated post-Rta expression and challenging the notion that herpesviral reactivation is bistable. This review analyzes rapidly evolving research on KSHV K-Rta to consider the role of K-Rta promoter specification in regulating the progression of KSHV reactivation.

Keywords: KSHV, herpesvirus, reactivation, DNA/protein interactions, DNA binding, RBP-Jk, Rta

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV) is one of five DNA tumor viruses implicated in the etiology of human cancers. The study of small DNA tumor viruses has established a paradigm for viral cancers, which result from non-productive, dead-end infections. For example, in human papillomavirus (HPV) infection, extrachromosomal genomes replicate to produce mature progeny viruses (a productive infection), leading to the formation of benign warts. However, if HPV integrates into the host genome, it chronically expresses its oncogenic early proteins, fails to replicate (a non-productive infection), and elicits cervical cancer (Moody and Laimins, 2010). This binary relationship between alternative outcomes of infection and cell transformation in human infections has received further support as a fundamental oncogenesis mechanism from studies of the recently discovered Merkel cell polyomavirus (Chang and Moore, 2011).

A basic difference between the small and large DNA tumor viruses is the relationship between productive and non-productive infections. While the small DNA tumor viruses transform human cells due to non-productive, inescapably dead-end infections, large DNA tumor viruses establish non-productive infections that are reversible. These large DNA tumor viruses, KSHV and Epstein–Barr virus (EBV), belong to the family Herpesviridae, and both replicate with latent/reactivating (i.e., non-productive/productive) patterns of infection. In natural infections of humans, both KSHV and EBV establish latency in B cells and are associated with B lymphoid hyperproliferations and cancers (Ganem, 2007; Rickinson and Kieff, 2007). Reactivation of KSHV from latency leads to increased risk of developing Kaposi's sarcoma (KS) and primary effusion lymphoma (PEL). KS tumors develop following KSHV reactivation; in the KS tumors, the virus is found in endothelial cells.

Dramatic advances in understanding the relationship between EBV infection and mechanisms of pathogenesis have been facilitated by the ease with which EBV infects primary B cells *de novo*. In that model, genetic studies established a strict interdependence between EBV latency and B cell transformation, and led to definition of the minimal EBV genome sufficient for that effect (Kieff and Rickinson, 2007). Similar progress in understanding KSHV biology has been hampered by the difficulty in establishing robust models of *de novo* infection that support long-lived viral persistence and transformation. Nonetheless, the models that have been most revealing suggest a remarkable contribution of productive cycle genes to cellular survival and growth control. Most of these

viral genes are homologous to human genes (Russo et al., 1996). Mechanisms that control expression of the productive cycle genes and progression of the viral reactivation program are thus keys to understanding cellular growth control during KSHV infection.

PEL CELL MODEL OF KSHV LATENCY AND REACTIVATION

Although long-term latency in tissue culture is not established following *de novo* KSHV infection (Renne et al., 1998; Grundhoff and Ganem, 2004), latently infected B cells can be explanted and cultured from human afflicted with PEL (Cesarman et al., 1995; Moore et al., 1996; Renne et al., 1996; Boshoff et al., 1998; Drexler et al., 1998). These PEL cell lines maintain KSHV latency for multiple passages, supporting very limited viral gene expression and little virus production (Renne et al., 1996). Treatment of these cells with the protein kinase C-activator 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) induces a dramatic increase in the number of viral genes transcribed, and release of viral progeny (Renne et al., 1996). Since most of the TPA-induced transcripts encode homologs of productive cycle genes from other herpesviruses (Sarid et al., 1998), these data established PEL cells as authentic models for KSHV latency and reactivation.

Samples derived from KS tissue show transcription patterns similar to un-treated PEL cells (Zhong et al., 1996; Sun et al., 1999) most infected endothelial cells express latent stage transcripts, but 1–10% of cells express productive cycle transcripts (Staskus et al., 1997, 1999; Sun et al., 1999). Most of the viral homologs of cellular genes are expressed with a productive cycle transcription pattern (reviewed in Mesri et al., 2010).

The development of KSHV microarrays permitted initial kinetic classification of the viral transcriptome. Less than 10 KSHV genes are expressed during latency in PEL cells, but most viral genes are induced following reactivation. With a few exceptions, the viral mRNAs that encode homologs of cellular proteins are expressed with kinetics similar to that of the viral transcripts for DNA replication proteins (Jenner et al., 2001; Paulose-Murphy et al., 2001). Most of the viral transcripts that encode structural proteins are expressed following the transcripts for the replication proteins (Jenner et al., 2001; Paulose-Murphy et al., 2001).

Formal definition of stage-specific transcription was established using microarrays and approaches validated for other herpesviruses. Four of the latent transcripts are expressed constitutively in both latency and reactivation, while all other transcripts are induced during reactivation (Fakhari and Dittmer, 2002). Eighteen miRNAs are also expressed during KSHV latency, and further induced during reactivation (Cai et al., 2005; Samols et al., 2005). Nine transcripts were defined as immediate early (IE) based on their expression in the absence of *de novo* protein synthesis (Zhu et al., 1999; Rimessi et al., 2001). Twenty-seven transcripts were defined as late (L) based on their sensitivity to treatment with a viral replication inhibitor (Lu et al., 2004). By default classification, 42 transcripts were expressed with delayed early (DE) kinetics, filling the temporal class that follows IE expression but precedes viral DNA replication. As in the other herpesviruses, the existence of distinct classes of transcription in KSHV implies the existence of mechanisms that regulate gene-specific transactivation.

Recent studies used tiling microarrays and limiting dilution quantitative-reverse-transcription (qRT)-PCR to further define stage-specific transcription. These studies showed that a few transcripts commonly classified as lytic are latently expressed in selected cells or conditions (Chandriani and Ganem, 2010). Following reactivation, nearly the entire KSHV genome, including both DNA strands, is transcribed to high levels (Chandriani et al., 2010; Xu and Ganem, 2010). These analyses identified a complex set of previously un-appreciated and abundant productive cycle transcripts. Many of these transcripts are very large, and are expressed in the anti-sense orientation to coding transcripts and miRNAs.

Rta IS THE KSHV LYTIC SWITCH PROTEIN

Rta IS SUFFICIENT TO REACTIVATE KSHV FROM LATENCY

A series of studies established that a single KSHV gene called Rta encoded the only viral protein both necessary and sufficient to reactivate the virus from latency.

Publication of the complete KSHV genome sequence and the demonstration of its extensive homology and collinearity with EBV (Russo et al., 1996) permitted identification of candidate KSHV lytic switch proteins. KSHV encodes two homologs of the EBV transactivating switches Rta ("E-Rta") and Zta in open reading frames (ORFs) 50 and K8, respectively (Sun et al., 1998). In KSHV, these proteins are called K-Rta and K-bZIP. Transient transfection of vectors that expressed K-Rta in BC-1 PEL cells induced the lytic cycle transcripts PAN (also known as nut-1), K-bZIP, small viral capsid antigen (sVCA), viral interleukin (vIL)-6, and viral macrophage inflammatory protein (vMIP)-II (Sun et al., 1998). Conversely, transfection of a K-bZIP expression vector failed to induce PAN and vIL-6, demonstrating that K-bZIP is not a functional equivalent of EBV Zta (Sun et al., 1998). The amount of PAN expressed was dependent on the input dose of the K-Rta expression vector. vIL-6 and PAN were induced by K-Rta with DE kinetics, similar to induction by reactivating chemicals (Sun et al., 1998). As sVCA expression required viral genome replication (i.e., sVCA is a true late gene), its induction by Rta suggested that Rta could drive the lytic gene expression cascade to completion. Interestingly, sVCA was induced in only 20% of the Rta vector-transfected cells.

Transcription of K-Rta was induced by reactivating chemicals prior to detection of the vIL-6 and K-bZIP transcripts, proving that K-Rta was upstream of those genes in the reactivation cascade (Sun et al., 1998). Truncation at amino acid (aa) 134 eliminated K-Rta's ability to induce lytic cycle gene expression.

Candidate lytic switch proteins were also selected based on early expression during KSHV reactivation in BCBL-1 PEL cells and by homology to EBV transactivators (Lukac et al., 1998). KSHV ORFs 50 (K-Rta), 57, K1, K3, and K5 were transcribed within 6 h following TPA treatment of BCBL-1 cells, prior to expression of suspected DE genes. Moreover, ORF 57 encoded a homolog of the EBV transactivator Mta. Transfection of a vector expressing K-Rta, but not vectors expressing ORF 57, K1, K3, or K5, induced expression of the ORF 59 and K8.1 proteins from the latent viral genome in BCBL-1 cells (Lukac et al., 1998). Induction of these productive cycle proteins by ectopic K-Rta was quantitatively similar to reactivation stimulated by TPA. Induction of the late protein K8.1 by K-Rta was sensitive to a viral replication inhibitor, agreeing with the conclusion that Rta expression was

sufficient to reactivate the complete lytic cycle of KSHV. Remarkably, K8.1 expression occurred in only 6.9% of Rta expressing cells (Lukac et al., 1998).

Despite the identification of K-Rta as the KSHV lytic switch protein, two characteristics of PEL cell lines have hampered molecular studies of KSHV reactivation: PEL cells are poorly transfectable, and undergo low-level spontaneous reactivation under normal culture conditions. Isolation of the HH-B2 PEL cell line overcame these obstacles by permitting demonstration that ectopic expression of K-Rta induced release of encapsidated viral DNA to the culture supernatant (Gradoville et al., 2000). These data formally proved that K-Rta could activate the entire KSHV productive reactivation cycle (Gradoville et al., 2000). More recent studies showed that K-Rta is also sufficient to reactivate the complete productive cycle of KSHV in infected endothelial, CV-1, human fibroblastic, and 293 cells (Bechtel et al., 2003; An et al., 2006).

Rta IS A PHOSPHORYLATED, TRANSCRIPTIONAL TRANSACTIVATOR

Analyses of ORF 50's RNA and protein products enhanced understanding of K-Rta's role in viral reactivation. K-Rta's aa sequence suggested that it was a transcription factor, since it contains an N-terminal basic domain and a C-terminal acidic domain conserved with E-Rta (**Figure 1**; Lukac et al., 1998). In E-Rta, the domains mediate DNA binding and dimerization, and transactivation, respectively. K-Rta also contained two putative nuclear localization sequences (NLSs; **Figure 1**), and a K-Rta-specific polyclonal antiserum confirmed that K-Rta was localized to the nuclei of TPA-treated BCBL-1 cells and transfected CV-1 cells (Lukac et al., 1998). Co-transfection of uninfected CV-1 cells with the K-Rta expression vector and reporter plasmids demonstrated that Rta transactivated the promoters for PAN, thymidine kinase (TK), kaposin, single stranded DNA binding protein (DBP), and DNA polymerase, but not the assembly protein (AP; Lukac et al., 1998). K-Rta transactivated the PAN, TK, and DBP promoters in

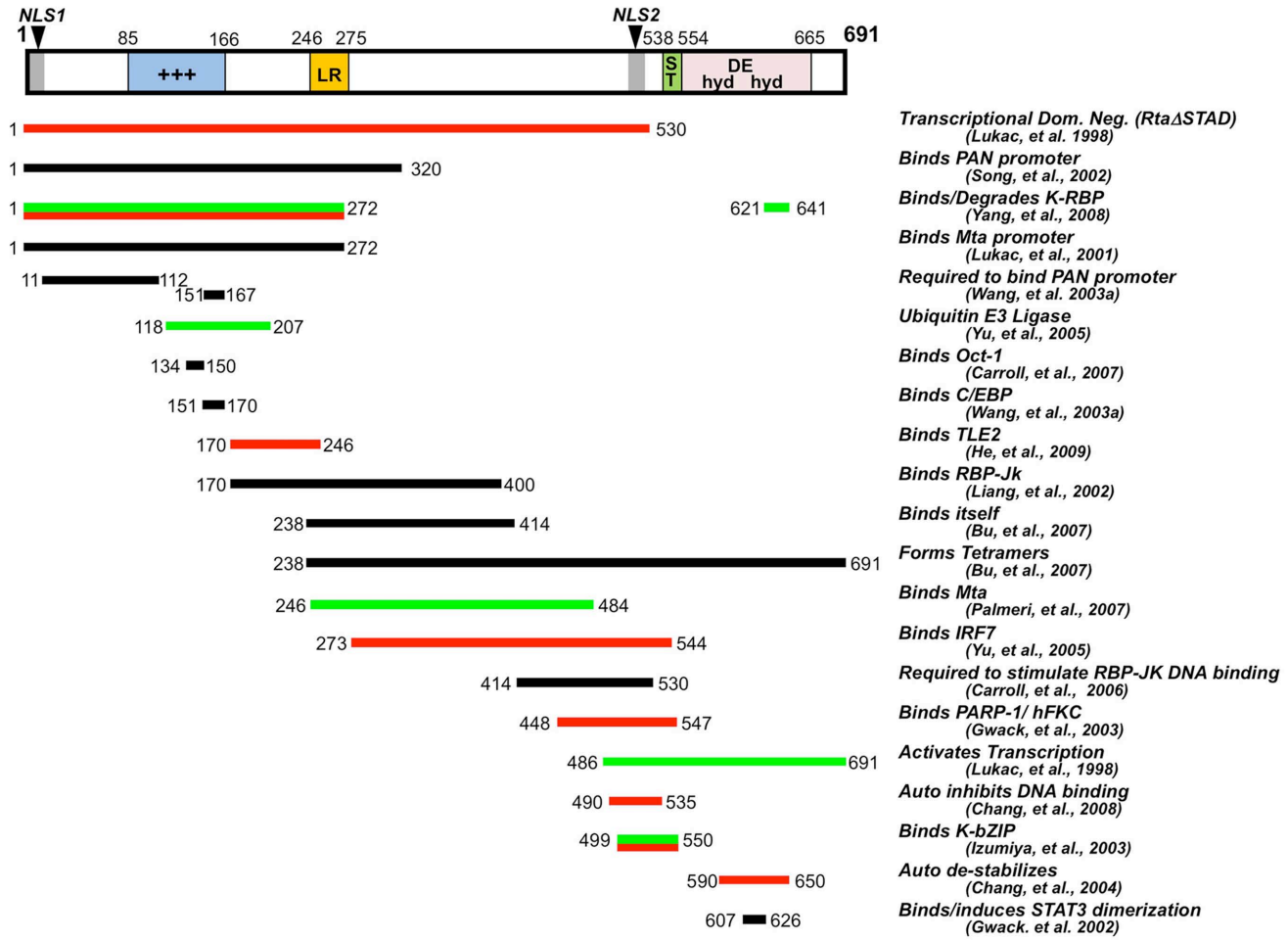

FIGURE 1 | K-Rta structure/function map. A linear representation of the amino acid content and predicted structural motifs of the ORF 50/Rta protein. Numbers refer to amino acid (AA) positions. Positions of each functional domain are shown by the colored bars, with amino acid boundaries indicated by numbers, corresponding to the function shown in the column on the right. Color codes for bars are: black, core functional domain; red, inhibitor of K-Rta; green, stimulator of K-Rta. Citations are listed in parentheses, and described in the text. +++, basic amino acid rich; LR, leucine heptapeptide repeat; ST, serine/threonine-rich; hyd/DE/hyd, repeats of hydrophobic and acidic amino acids, comprising K-Rta's transcriptional activation domain; NLS, nuclear localization sequence; Dom. Neg., dominant negative.

both B cells and endothelial cells (Lukac et al., 1999). These data established that Rta was a direct transactivator of specific viral promoters in the absence of any other viral genes (Lukac et al., 1999).

Northern blotting more clearly distinguished the relative kinetics of K-Rta transcription and that of other early proteins. Abundant Rta expression was detectable within 1 h post-TPA addition to PEL cells, and preceded detection of transcripts for Mta, K-bZIP, K3, K5, and DBP (Lukac et al., 1999). The ORF 50 locus expresses multiple, divergent transcripts, but only a single, 3,402 nt transcript encodes the sense K-Rta sequence (Lukac et al., 1999). This major transcript initiates upstream of ORF 49, is spliced to an acceptor site upstream of ORF 50, and then continues un-spliced through ORF 50. Downstream of ORF 50, the transcript is spliced three additional times, and encodes K-bZIP and K8.1. The major protein expressed from ORF 50 during reactivation is approximately 110 kDa, about 36 kDa greater than the predicted molecular mass of 73.7 kDa (Lukac et al., 1999). The 110-kDa protein is also detected in Cos cells transfected with an ORF 50 expression vector. A second, minor protein of 90 kDa is also detected by the K-Rta antiserum in TPA-induced PEL cell extracts, and migrates identically to the protein produced by programming rabbit reticulocyte lysates (RRL) with the ORF 50 cDNA (Lukac et al., 1999). Phosphatase treatment of the 110-kDa K-Rta protein decreased its abundance and increased accumulation of the 90-kDa species, suggesting that 20 kDa of K-Rta's apparent MW is attributable to phosphorylation (Lukac et al., 1999). Thus, K-Rta has the potential to be regulated by post-translational modification.

Rta IS NECESSARY FOR KSHV REACTIVATION

Ectopic expression of K-Rta in PEL cells had established that K-Rta was sufficient to induce the full lytic cycle of KSHV gene expression. Structure-function studies of K-Rta led to experiments that proved that K-Rta was also necessary for KSHV reactivation. K-Rta's transcriptional activation domain was identified by fusing full-length and truncated mutants of K-Rta to the DNA binding domain of the yeast protein Gal4. Among these truncations, only K-Rta aa 486–691 were sufficient to activate transcription of a reporter gene containing binding sites specific for Gal4, in B and endothelial cells (Lukac et al., 1999). These data indicated that K-Rta's activation domain is contained within its C-terminal 206 aa (**Figure 1**; Lukac et al., 1998; Seaman et al., 1999; Wang et al., 2001a). This sequence is homologous to the activation domains of many cellular and viral transcription factors (Lukac et al., 1999). Deletion of the C-terminal 162 aa from wild type, cognate K-Rta generated a mutant called RtaΔSTAD (also known as ORF50ΔSTAD; **Figure 1**), that was unable to transactivate KSHV promoters or reactivate the virus from latency in PEL cells (Lukac et al., 1999). These data proved that transcriptional activation by K-Rta was required for KSHV reactivation. Furthermore, this RtaΔSTAD bound to WT K-Rta and specifically inhibited K-Rta-mediated transactivation. Proving that K-Rta was necessary for KSHV reactivation, RtaΔSTAD blocked viral reactivation that was either spontaneous or induced by TPA or sodium butyrate in PEL cells (Lukac et al., 1999).

Formal genetic proof that K-Rta is required for KSHV reactivation came from studies of a bacmid clone of KSHV in which ORF 50 was deleted (called BAC36Δ50). Viral infection was established by transfecting 293 cells with BAC36Δ50 DNA and selecting with hygromycin. TPA failed to reactivate BAC36Δ50, as measured by DNA replication, production of infectious virus in cell supernatants, or expression of K-bZIP, ORF 40/41, K8.1, ORF 57, or ORF 59 (Xu et al., 2005). Expression of K-Rta in trans rescued reactivation of BAC36Δ50, implying the deletion of ORF 50 was the only mutation that contributed to the reactivation defect. In addition, a bacmid clone constitutively expressing Rta (called "KSHV-lyt") entered the productive replication cycle by default upon infecting epithelial, endothelial, and Vero cells (Budt et al., 2011).

Rta FUNCTIONS AS A TETRAMER

The ability of the Rta mutant RtaΔSTAD to inhibit KSHV reactivation suggested that Rta must form multimers to reactivate KSHV from latency. Blue Native Polyacrylamide electrophoresis (BN-PAGE) and size exclusion chromatography showed that full-length Rta formed tetramers and decamers in solution (Bu et al., 2007). Deletion of Rta's N-terminal leucine heptapeptide repeat (RtaΔLR; aa 244–275; **Figure 1**) inhibited it's transactivation of the K-bZIP and PAN promoters without affecting its nuclear localization. The ΔLR mutation also did not inhibit DNA binding of K-Rta, but altered the pattern of K-Rta/DNA complexes observed in EMSA. In the context of the dominant negative mutant RtaΔSTAD, deletion of the LR (RtaΔSTADΔLR) inhibited its ability to interact with full-length K-Rta and to block K-Rta-mediated transactivation and reactivation. However, the LR alone was not sufficient for K-Rta self-interaction, but required the entire central portion of K-Rta (aa 245–414; **Figure 1**; Bu et al., 2007).

Despite encoding four leucines spaced at 7 aa intervals, K-Rta's LR is not predicted to form leucine-zipper coiled-coils because it also contains five proline residues (Bu et al., 2007). In fact, the proline content, but not the leucine content, of the LR is critical for determining the oligomeric state of Rta. Mutation of three leucines to prolines in the center of the LR generated a K-Rta mutant (Rta-L3P) that exclusively formed tetramers and retained the ability to transactivate and reactivate the virus (Bu et al., 2007). Mutants of Rta that were unable to form tetramers but retained the ability to form higher-order multimers were reduced in function or were non-functional (Bu et al., 2007). Monomers were never detected with WT or mutant K-Rta. In the context of RtaΔSTAD, the L3P mutant (RtaΔSTAD-L3P) retained the ability to bind and dominant-negatively inhibit WT K-Rta (Bu et al., 2007). As differences in Rta multimerization had dramatic effects on its function, the data suggested that Rta's multimerization state is a prime candidate for regulating KSHV reactivation. The data also suggest that many phenotypes attributed to K-Rta LR mutations could affect both interactions with heterologous proteins and K-Rta homo-oligomerization.

Rta SPECIFIES PROMOTERS FOR TRANSACTIVATION BY BINDING DNA DIRECTLY AND INTERACTING WITH CELLULAR DNA BINDING PROTEINS

The molecular interactions between host and virus that determine transactivation specificity are critical for understanding DNA virus persistence and replication. Identification of K-Rta as KSHV's lytic switch (Lukac et al., 1998; Sun et al., 1998), and demonstration of

K-Rta's promoter selectivity (Lukac et al., 1998), led immediately to the question of how Rta specified promoters for transactivation. Intense investigations of Rta specificity have focused on the PAN and Mta promoters as models. Experimental approaches have followed the classic strategy of comparing K-Rta DNA binding specificity with K-Rta transactivation specificity. Studies of PAN have revealed a largely concordant relationship between K-Rta DNA binding and transactivation. Studies of Mta have revealed a more complex relationship between K-Rta DNA binding and interactions with cellular DBPs. Taken together, the PAN and Mta models support the existence of two apparently distinct mechanisms for K-Rta promoter specification. These mechanisms are distinguished by K-Rta's relative dependence on the cellular protein RBP-Jk, and the relative lengths of K-Rta's binding sites. K-Rta promoter specification is further influenced by cell type. Controversies regarding K-Rta promoter specification might be attributable to variations in experimental systems, and are discussed in the Section "Conclusion and Perspectives."

RBP-Jk INDEPENDENT PROMOTERS

PAN is a non-coding, poly-adenylated nuclear RNA that is the most abundant transcript expressed by reactivating KSHV (Sun et al., 1996; Zhong et al., 1996); recent data shows PAN RNA is essential for viral late gene expression (Borah et al., 2011). The PAN locus from −2974 to +1136 was cloned into a pBluescript vector and transfected into COS-1 and 293T cells (Song et al., 2001). The PAN RNA was undetectable by Northern blotting unless a K-Rta expression vector was co-transfected (Song et al., 2001). Deletion of the promoter to −69 had little effect on K-Rta transactivation, while further deletion to −38 eliminated transactivation (Song et al., 2001). Deletion of the element in the cognate PAN promoter eliminated K-Rta transactivation (Kirshner et al., 2000; Song et al., 2001), and the sequence from −69 to −38 conferred orientation independent Rta responsiveness on a heterologous promoter (Song et al., 2001).

Recombinant, FLAG-tagged, full-length K-Rta, expressed and purified from *E. coli*, bound to PAN promoter DNA −70/−42 in EMSA, providing a direct correspondence between DNA binding and transactivation (Song et al., 2001). Truncated K-Rta (aa 1–320; **Figure 1**), expressed and purified from bacteria, bound to the PAN −78/−37 element with high affinity [a dissociation constant (K_D) of 7.9–8.6 nM; Song et al., 2002]. The minimal DNA sequence for detectable K-Rta binding was −74/−45, and methylation between positions −67/−52 interfered with DNA binding (**Figure 2**; Song et al., 2002). The extra 14 bp flanking the methylation sensitive sequences were A/T-rich. K-Rta made in RRL, requires aa 11–112 and 151–167 to bind the PAN promoter (**Figure 1**; Wang et al., 2003a).

Analyses of an extensive set of mutations across positions −75/−38 of the PAN promoter showed a general correspondence between K-Rta binding and transactivation, with some discrepancies (i.e., incomplete concordance between a mutation's effect on binding with its effect on transactivation; Song et al., 2002). Due to Rta's high binding affinity to the relatively short segment of the PAN promoter, it was concluded that Rta binding to the promoter is necessary and sufficient to activate transcription of PAN (Song et al., 2002).

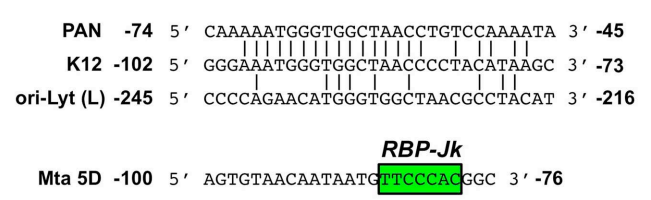

FIGURE 2 | Comparison of RBP-Jk independent and dependent K-Rta responsive elements. Vertical lines indicate homologous bases. Numbers indicate positions relative to transcriptional start sites. The green box indicates position of RBP-Jk binding site.

Comparison of the K-Rta responsive element from PAN to other KSHV promoters revealed a 26-bp element from the kaposin (K12) promoter that contained 20 bp homologous to PAN (**Figure 2**; Song et al., 2002; Chang et al., 2005c). Recombinant K-Rta, cloned as a fusion of aa 1–490 to the VP16 activation domain and expressed in cultured cells, bound to the PAN and kaposin promoters (Chang et al., 2002). The same fusion protein was competent to activate transcription of PAN and kaposin from the latent viral genome in PEL cells (Chang et al., 2002). Mutations in the kaposin element also demonstrated a strict correlation between DNA binding by the K-Rta1-490/VP16 and transactivation (Chang et al., 2002). Further C-terminal truncation of K-Rta to aa 390 was sufficient to bind and transactivate the PAN and kaposin promoters (Chang and Miller, 2004).

The K-Rta binding site from kaposin is also homologous to K-Rta binding sites in the lytic origins of replication (ori-Lyt; **Figure 2**). K-Rta activates transcription from those sites to participate in lytic replication (AuCoin et al., 2004; Wang et al., 2004b).

RBP-Jk-DEPENDENT PROMOTERS

In transient transfections of CV-1 cells with a series of KSHV promoters, K-Rta most robustly transactivated those from the ORF 57 and K-bZIP genes (Lukac et al., 1998). Deletion analysis of the ORF 57 promoter revealed that the element critical for Rta transactivation was located between bps −106 and −54 relative to Mta's transcriptional start site (Lukac et al., 2001). While not required for K-Rta transactivation, the promoter sequence between −218 and −106 also contributed positively to transactivation (Lukac et al., 2001). Fusion of the −106/−54 element to a heterologous TATA box conferred K-Rta responsiveness in an orientation independent manner (Lukac et al., 2001).

Initial studies of K-Rta's interaction with the Mta promoter utilized recombinant K-Rta protein that was partially purified from Sf9 cells that were infected with a K-Rta expressing baculovirus. This protein preparation formed three K-Rta-containing complexes with short, overlapping DNAs that spanned the −106/−54 element (Lukac et al., 2001). The DNAs were cloned as dimers in a heterologous reporter vector, and tested for transactivation by K-Rta in CV-1 cells. K-Rta transactivation corresponded with formation of two of the three K-Rta-containing protein/DNA complexes (Lukac et al., 2001). A 26-bp sequence, called Mta 5D (−100/−76; **Figure 2**), was optimal for K-Rta complex DNA

binding and transactivation (Lukac et al., 2001). Mta 5D contains a 12-bp, partially palindromic sequence that is conserved with the TATA proximal promoter of the K-bZIP gene. Mutations that inhibited DNA binding of the two K-Rta-containing complexes also eliminated K-Rta transactivation of the Mta and K-bZIP promoters (Lukac et al., 2001).

Site-directed mutagenesis of the Mta 5D element revealed nucleotide-specific variability in K-Rta transactivation and DNA binding (Lukac et al., 2001). Mutations in the upstream side of the 5D palindrome reduced K-Rta transactivation 50–80% and reduced, but did not eliminate, DNA binding of the K-Rta-containing complexes. Mutations in the downstream side of the palindrome eliminated K-Rta transactivation and DNA binding. Later studies suggested that the downstream side of the 5D element was a binding site for K-Rta complexed with the cellular protein Recombination Signal Binding Protein (RBP)-Jk (**Figure 2**), which co-purified from Sf9 cells (see next paragraph). Truncations of K-Rta, expressed and fully purified from *E. coli*, demonstrated that K-Rta bound directly to the 5D element, and that K-Rta's DNA binding domain is encoded within aa 1–272 (Lukac et al., 2001).

A two-hybrid screen identified RBP-Jk as a binding partner for K-Rta, and provided a crucial insight into how K-Rta's specifies a sub-set of promoters for transactivation (Liang et al., 2002). RBP-Jk is a nuclear, DBP that specifies Notch-responsive promoters in the canonical model for the Notch signal transduction pathway (Tanigaki and Honjo, 2010). RBP-Jk is essential for KSHV reactivation (Liang and Ganem, 2003). K-Rta and RBP-Jk co-immunoprecipitated from reactivating PEL cells and from cells co-transfected with expression vectors for both proteins (Liang et al., 2002). K-Rta and RBP-Jk also interacted in GST pull-down assays, which identified two independent K-Rta-interacting domains in RBP-Jk, aa 1–180 and 179–360 (Liang et al., 2002). Similar experiments identified the smallest fragment of K-Rta that interacted with RBP-Jk as aa 170–400 (**Figure 1**).

The significance of RBP-Jk in K-Rta promoter specification was shown by transient transfection-reporter assays. K-Rta transactivated artificial promoters containing multimerized WT, but not mutant, RBP-Jk binding sites, in an orientation independent manner (Liang et al., 2002). K-Rta failed to transactivate these promoters in transfected mouse embryonic fibroblasts (MEFs) that were genetically RBP-Jk null (OT-11 cells), unless RBP-Jk was ectopically co-expressed (Liang et al., 2002).

Similar to the right side of the Mta promoter 5D element (**Figure 2**), the KSHV DBP promoter also contains a consensus RBP-Jk site (Liang et al., 2002). However, the PAN promoter does not contain an obvious RBP-Jk site. Rta transactivation of the Mta and DBP promoters, but not the PAN promoter, was eliminated in OT-11 cells, but rescued by ectopic RBP-Jk expression. Mutation of the RBP-Jk sites eliminated K-Rta transactivation of the Mta and DBP promoters in cells genetically intact for RBP-Jk (Liang et al., 2002). K-Rta and RBP-Jk formed a complex with an RBP-Jk site in EMSA assays. Together with studies of the PAN promoter, these data suggested that K-Rta responsive promoters fall into two classes, differentiated by their dependence on RBP-Jk for specification (Liang et al., 2002).

Rta STIMULATES DNA BINDING OF RBP-Jk

While it was firmly established that K-Rta transactivation of the Mta promoter is RBP-Jk-dependent, mutations upstream of the RBP-Jk site in the Mta 5D promoter also reduced K-Rta transactivation, suggesting additional complexity in the K-Rta/RBP-Jk/DNA model for promoter specification (Lukac et al., 2001). Two complementary approaches revealed that the unexplained mutations affected Rta DNA binding (Carroll et al., 2006): (1) analyses of constitutive activators of the Notch pathway, and (2) comparison of DNA binding specificity of fully pure K-Rta and RBP-Jk.

The canonical model for Notch signal transduction holds that RBP-Jk is constitutively bound to DNA as a transcriptional repressor, but provides a nucleation site for activated Notch1 (NICD1) to activate transcription (Tanigaki and Honjo, 2010). The RBP-Jk site in the Mta promoter (**Figure 2**) is a perfect match to the consensus sequence (Liang et al., 2002). Therefore, it was surprising that both NICD1 and transcriptionally active RBP-Jk (WT RBP-Jk fused to the HSV VP16 transcriptional activation domain, called "RBP-Jk/VP16") failed to transactivate the native Mta promoter, nor the dimerized Mta 5D element (Carroll et al., 2006). As a positive control, both proteins activated the dimerized RBP-Jk element from the EBV Latency C promoter (Cp). These data suggested that RBP-Jk did not constitutively bind to the Mta promoter *in vivo*, an exception to the canonical model for Notch regulation.

Remarkably, co-expression of the K-Rta transcriptional mutant RtaΔSTAD, which contained K-Rta's domains for DNA binding, RBP-Jk interaction, and oligomerization (**Figure 1**), dramatically rescued the ability of RBP-Jk/VP16 to activate transcription of the native Mta promoter and the dimerized Mta 5D element (Carroll et al., 2006). These data suggested that RtaΔSTAD stimulated DNA binding of RBP-Jk/VP16 *in vivo*. Proving that RtaΔSTAD could similarly stimulate DNA binding of endogenous, WT RBP-Jk to DNA, RtaΔSTAD also rescued the ability of NICD1 to activate the native Mta promoter (Carroll et al., 2006; Palmeri et al., 2011). This effect of RtaΔSTAD was promoter specific: although the viral TK promoter also contains a consensus RBP-Jk site, and RBP-Jk/VP16 also failed to transactivate TK, RtaΔSTAD did not rescue RBP-Jk/VP16 transactivation of TK (Carroll et al., 2006). RtaΔSTAD also failed to stimulate transactivation of a Gal4 reporter by Gal4/VP16, proving that the effect of RtaΔSTAD on RBP-Jk/VP16 requires the RBP-Jk portion of the VP16 fusion molecule (Palmeri et al., 2011).

Clarification of the relationship between DNA binding specificity of RBP-Jk and K-Rta to the Mta 5D element came from studies of truncated K-Rta (aa 1–272; **Figure 1**) and RBP-Jk expressed and purified from *E. coli* (Carroll et al., 2006). As expected, RBP-Jk bound specifically to its consensus element, while Rta bound specifically to the A/T-rich sequences upstream of the RBP-Jk site in the 5D element. Mutational analyses of 5D showed (1) a strict correlation between RBP-Jk DNA binding and K-Rta transactivation, and (2) reduced, transactivation of 5D mutants that bound to RBP-Jk but not Rta. These data confirmed that RBP-Jk DNA binding was essential for K-Rta activation of the 5D element, and that DNA binding of Rta independently of RBP-Jk was not sufficient to activate the dimerized 5D element (Carroll et al., 2006).

Combinatorial DNA binding of K-Rta and RBP-Jk revealed that ternary Rta/RBP-Jk/DNA complexes formed only on WT and mutant 5D elements to which both proteins bound independently (Carroll et al., 2006). Furthermore, RtaΔSTAD stimulation of RBP-Jk/VP16 binding also required intact binding sites for both proteins, and corresponded to maximal transactivation of these dimerized elements by WT K-Rta. C-terminal truncation of RtaΔSTAD to aa 414 generated a mutant of Rta that retained the ability to bind to the 5D element, but failed to form a ternary complex with RBP-Jk and DNA. This Rta mutant also failed to stimulate transactivation of 5D dimers by RBP-Jk/VP16 (**Figure 2**; Carroll et al., 2006). These data established that RtaΔSTAD stimulation of RBP-Jk/VP16 DNA binding required DNA/protein and protein/protein interactions of both proteins.

Proving that cognate, full-length K-Rta could also stimulate DNA binding of RBP-Jk, chromatin immunoprecipitations (ChIPs) showed that RBP-Jk is virtually undetectable on the Mta, K-bZIP, and K14 promoters during KSHV latency, but is enriched on these promoters during reactivation in a K-Rta-dependent fashion (Carroll et al., 2006). Rta also selectively stimulated RBP-Jk binding to the cellular IL-6 and HES-1, but not CD23, promoters (Carroll et al., 2006). The inability of the RBP-Jk-dependent transactivators NICD1 and Esptein–Barr virus (EBV) nuclear antigen (EBNA)-2 to reactivate KSHV from latency when expressed ectopically in PEL cells (Liang and Ganem, 2003; Chang et al., 2005a; Carroll et al., 2006) confirmed that RBP-Jk is not bound to essential KSHV genes during latency in the absence of K-Rta.

COMPARISON OF RBP-Jk-DEPENDENT AND INDEPENDENT PROMOTERS

K-Rta promoter binding and transactivation were compared for the PAN, kaposin, ORF 57, and vIL-6 genes. Nuclear run-on assays from KS-1 PEL cells that were treated with sodium butyrate demonstrated a hierarchy of transcription initiation rates for the genes, in the order PAN > kaposin > ORF57 > vIL-6 (Song et al., 2003). The hierarchy was similar to the magnitude of transactivation of the promoters of these genes in transient reporter assays in 293T, BJAB, and PEL cells. The hierarchy was also similar in comparative DNA binding affinities of K-Rta to short promoter elements from these genes. In particular, K-Rta's DNA binding affinity for PAN was at least 10-fold greater than for a 30-bp Mta element (similar to Mta 5D; **Figure 2**; Song et al., 2003). Notably, the differences in DNA binding affinities were greater than the differences in transactivation.

The Mta 5D element contained significant homology to the promoter of the KSHV macrophage inflammatory protein (vMIP)-I gene (Chang et al., 2005c). The homologous portion of the vMIP-I promoter conferred K-Rta responsiveness on a heterologous promoter. Rta aa 1–490/VP16, expressed in cultured mammalian cells, did not bind to 39 bp K-Rta responsive elements from the Mta and vMIP-I promoters, although it bound to the PAN and kaposin elements (Chang et al., 2005c). A series of proteins from cellular extracts, including RBP-Jk, bound to the vMIP-I and Mta elements (Chang et al., 2005c). The K-Rta point mutation R160A abolished DNA binding of K-Rta aa 1–490/VP16, reduced transactivation of the short Mta and vMIP-I reporters, and eliminated transactivation of the short PAN and kaposin reporters (Chang et al., 2005c). A series of additional mutations in K-Rta's DNA binding domain had inconsistent effects on DNA binding of Rta aa 1–490/VP16 and transactivation of the short Mta and vMIP-I reporters. Two of the Rta DNA binding mutants, R160A and R161A, showed reduced and delayed transactivation of the viral transcripts from the viral genome in PEL cells (Chang et al., 2005c). The transcription was probably dependent on WT Rta induced from the endogenous virus (see Conclusion and Perspectives). The authors concluded that DNA binding was essential for K-Rta to transactivate the PAN and kaposin elements, but not the Mta and vMIP-I elements (Chang et al., 2005c).

K-Rta BINDS WITH HIGH AFFINITY TO CANT REPEATS IN THE Mta PROMOTER TO STIMULATE DNA BINDING OF RBP-Jk

A puzzling observation was that K-Rta-stimulated RBP-Jk DNA binding to the Mta promoter (Carroll et al., 2006) yet bound with relatively low affinity to the Mta 5D DNA element (Song et al., 2003). Studies of the K-bZIP promoter provided a hint to resolve this conundrum. In the K-bZIP promoter, an extended repeat of phased A/T trinucleotides were bound by K-Rta with high affinity, but a single unit of that repeat bound to K-Rta with only low affinity (**Figure 3**; Liao et al., 2003a). The Mta promoter contains a similar A/T repeat (Liao et al., 2003a), and the 5D element contains only a single A/T unit (**Figure 4A**, bold sequence; Palmeri et al., 2011).

To determine the qualitative interactions of K-Rta with the Mta promoter, the DNAse I footprint of recombinant RtaΔSTAD was determined (Palmeri et al., 2011). WT RtaΔSTAD, but not a DNA binding mutant, footprinted four distinct stretches of the promoter (**Figure 4A**, underlined sequence). The foot print extended over 64 bp flanking the RBP-Jk binding site, within the −136/−62 portion of the promoter. The longest contiguous portion of the footprint protected the previously identified 5D element (**Figure 4A**). Alignment of the foot printed regions revealed a 14-nt consensus sequence, ANTGTAACANT$^{A}/_{T}{}^{A}/_{T}$T, in which N = any base (**Figure 3**; Palmeri et al., 2011). The consensus was repeated seven times in the foot printed region, and two pairs of two of the repeats formed palindromes (**Figure 4A**; Palmeri et al., 2011). Each unit contained the core sequence "CANT," so was termed the "CANT repeat." (Palmeri et al., 2011). Using saturating K-Rta concentrations, the footprint extended farther downstream to −21 to cover additional CANT repeats (Palmeri et al., 2011); this downstream region has been identified as an additional Rta responsive element (Wen et al., 2009).

Liao, et al., 2003a	$[A/T]_3$-N_7-$[A/T]_3$-N_7-$[A/T]_3$ $[A/T]_3$-N_{17}-$[A/T]_3$
Chen, et al., 2009	TTCCAGGAT N_{0-16} TTCCTGGGA
Ziegelbauer, et al., 2006	CCCACTTT
Palmeri, et al., 2011	ANTGTAACANT$^{A}/_{T}{}^{A}/_{T}$T

FIGURE 3 | Comparison of consensus K-Rta binding sites. The indicated K-Rta consensus sequences were derived from the listed studies. "N" means any nucleotide.

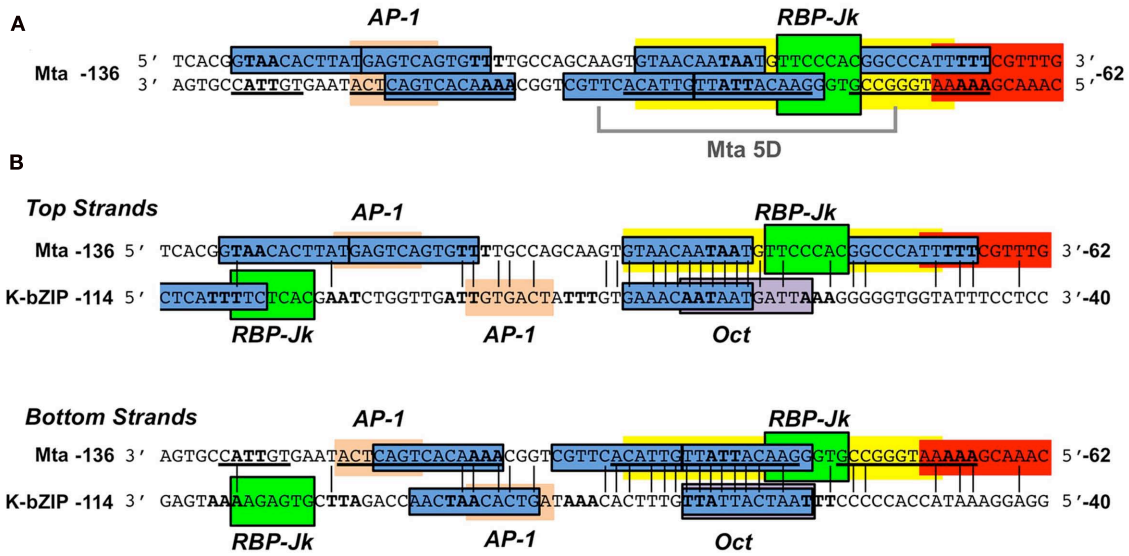

FIGURE 4 | Comparison of Mta and K-ZIP promoters. (A). Schematic of the −136/−62 Mta promoter. Sequences of the top and bottom strands of the indicated portion of the Mta promoter. Numbers indicate positions relative to Mta transcriptional start site. Bold letters indicate four units of the A/T_3 trinucleotide repeat. Underlines indicate Rta's footprint from the bottom strand. Blue boxes indicate regions of highest conservation, nt 4–14, for each unit of the CANT repeat. The green box indicates the RBP-Jk binding site. The peach box indicates the AP-1 binding site. The yellow box indicates the K-RBP binding site. The red box indicates the IRF-7 binding site. The position of the short, Mta 5D element is indicated by the brackets. **(B)** Alignment of the top and bottom strands of the Mta and K-bZIP promoters. Numbering and box designations are as listed in the legend for **Figure 3**, above. The purple box indicates the Oct-1 binding site.

RtaΔSTAD's affinity for Mta −136/−62 was 5.7 nM (Palmeri et al., 2011), similar to K-Rta's affinity measured for its shorter PAN promoter binding site (Song et al., 2003). RtaΔSTAD's affinity for –136/–62 was approximately 35-fold greater than for the 5D element alone (Palmeri et al., 2011), similar to the affinity measured for a short Mta element in (Song et al., 2003). RBP-Jk enhanced Rta binding affinity fivefold to Mta −136/−62, and ternary Rta/RBP-Jk/DNA complex formation on −136/−62 was more robust than on the short 5D DNA (Palmeri et al., 2011). Therefore, classification of the Mta promoter as a high or low affinity K-Rta binding site depended on the length of DNA used as probe (Palmeri et al., 2011).

To test the functional significance of these data, a series of promoters were generated by artificially combining CANT repeats and RBP-Jk sites, or mutating the cognate Mta promoter to alter the number and architecture of CANT repeats (Palmeri et al., 2011). Each was tested for transactivation by the combination of RtaΔSTAD and RBP-Jk/VP16. The data confirmed that K-Rta's stimulation of RBP-Jk DNA binding required an intact RBP-Jk binding site. However, K-Rta's stimulation of RBP-Jk DNA binding was proportional to the number and position of CANT elements for K-Rta, but not RBP-Jk sites. Furthermore, the number of CANT repeats, their presence in palindromes, and their positions relative to the RBP-Jk binding site determined the optimal target for Rta stimulation of RBP-Jk DNA binding. Both CA dinucleotides and A/T-richness of the promoter were critical for the Rta/RBP-Jk/DNA interaction (Palmeri et al., 2011).

The minimum promoter structure required for robust Rta/RBP-Jk transactivation contained a single RBP-Jk site straddled by two pairs of palindromic CANT repeats (Palmeri et al., 2011). DNA binding and tetramerization mutants of K-Rta failed to stimulate RBP-Jk DNA binding (Palmeri et al., 2011). Based on these genetic studies of K-Rta, RBP-Jk, and the Mta promoter, a model was proposed in which K-Rta tetramers make high affinity interactions with repetitive DNA elements in KSHV promoters (**Figure 5**; Palmeri et al., 2011). Tetramerization of Rta allows it to straddle RBP-Jk and contact palindromic pairs of CANT repeats flanking RBP-Jk in the Mta promoter. Two regions of RBP-Jk independently bind to K-Rta in solution (Liang et al., 2002; Carroll et al., 2006) permitting Rta bound to DNA on both sides of RBP-Jk to contact RBP-Jk simultaneously. A second K-Rta tetramer might bind to the upstream CANT repeats, with or without an unidentified cellular protein, to further stabilize K-Rta DNA binding and recruitment of RBP-Jk to DNA (**Figure 5**). Since the upstream CANT repeats partially overlap a functional binding site for the cellular protein AP-1, and AP-1 enhances K-Rta transactivation (Wang et al., 2004a), AP-1 might contribute to formation of the Rta/RBP-Jk/DNA complex.

Chromatin immunoprecipitations showed that RBP-Jk DNA binding was broadly, but selectively, stimulated across the entire KSHV genome during reactivation (Palmeri et al., 2011). Bioinformatics showed that CANT and A/T repeats were located in proximity to many RBP-Jk sites throughout the KSHV genome. This study thus integrated high affinity Rta DNA binding with the requirement for RBP-Jk in Rta transactivation, to define the promoter requirements for formation of transcriptionally productive Rta/RBP-Jk/DNA complexes (Palmeri et al., 2011).

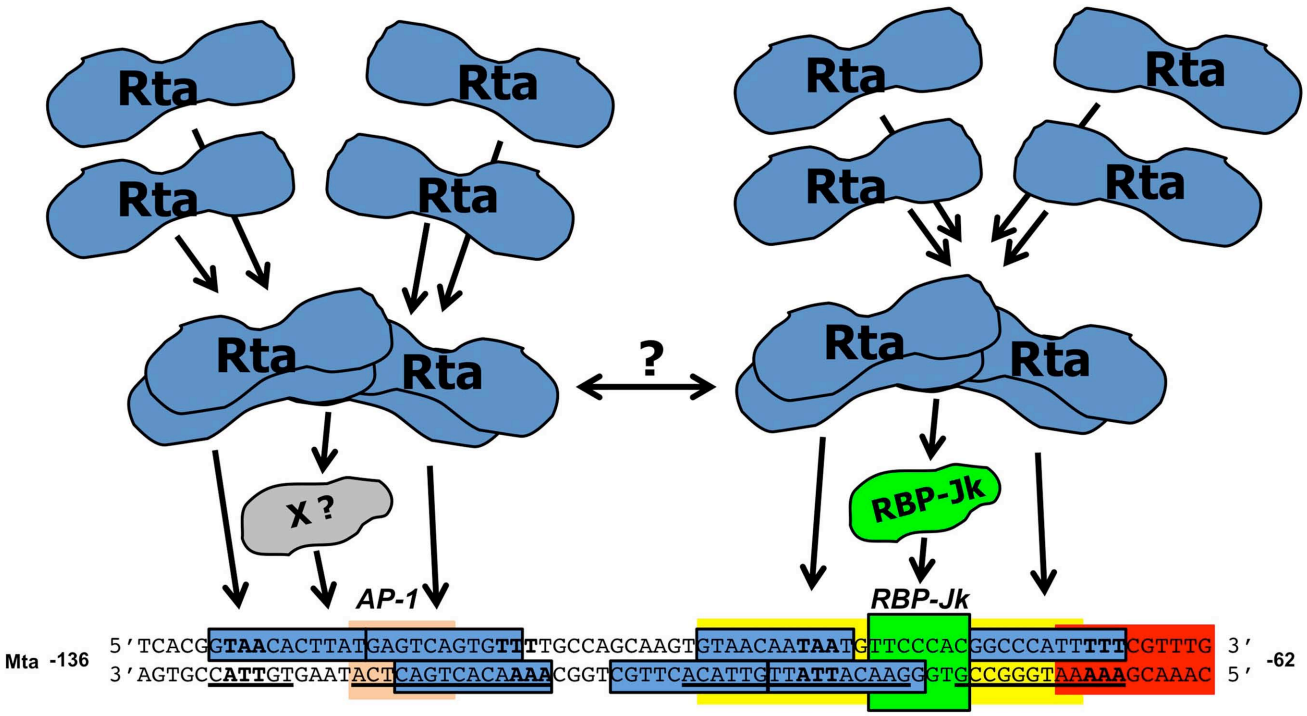

FIGURE 5 | Model for stimulation of DNA binding of RBP-Jk by Rta. The sequence and description of the Mta promoter are as described in the legend for **Figure 4A**. Tetramerization of Rta allows it to straddle RBP-Jk and contact the palindromic CANT repeat units on both sides of RBP-Jk. A second K-Rta tetramer may bind to an unidentified protein ("X") and contact the CANT repeats in the upstream side of the promoter. The two Rta tetramers might contact each other to stabilize the holo-protein/DNA complex.

The PAN and Mta promoter models for Rta DNA binding therefore suggest dramatic differences in mechanisms for forming productive Rta/DNA transcription complexes. The PAN promoter contains a short, high affinity K-Rta binding site from which Rta activates transcription independently of RBP-Jk (Liang et al., 2002; Song et al., 2003). The Mta promoter contains multiple Rta CANT binding sites, each of which is an independent, low affinity binding site, but which act in concert as a high avidity, extended element that bind K-Rta multimers simultaneously, and together with RBP-Jk (**Figure 5**; Palmeri et al., 2011).

RBP-Jk AND OCTAMER-1 PARTICIPATE IN K-Rta SPECIFICATION OF THE K-bZIP PROMOTER

Although CANT repeats are found throughout the KSHV genome (Palmeri et al., 2011), their significance for Rta transactivation has only been formally investigated for the Mta and K-bZIP promoters (see Mta, above). The core sequences of two of the Mta CANT repeats are homologous to the TATA proximal K-bZIP promoter (**Figure 4B**; Lukac et al., 2001). These K-bZIP repeats also bound to recombinant K-Rta purified from *E. coli*. Mutation of the repeats in the full-length K-bZIP promoter reduced K-Rta-mediated transactivation by greater than 80% (Lukac et al., 2001; Carroll et al., 2007).

In the K-bZIP promoter, the CANT repeats overlap an RBP-Jk binding site and a sequence that is well conserved with binding sites for the cellular Octamer (Oct) transcription factors that are found in the immediate early promoters of herpes simplex virus type 1 (HSV-1; **Figure 4B**; Carroll et al., 2007). At least seven protein complexes from infected PEL cells bound to a 26-bp DNA from the K-bZIP CANT repeats (Carroll et al., 2007). Mutation of the K-bZIP sequence eliminated binding of one cellular complex. That complex was super shifted by an Oct-1 specific antibody and was increased following TPA addition to PEL cells. Together, these data suggested that Oct-1 binds to the K-bZIP promoter in a manner that corresponds to viral reactivation and Rta transactivation (Carroll et al., 2007).

Recombinant K-Rta and Oct-1 bound to the WT but not mutant promoter, and Oct-1 and Rta were specifically ChIP'd on the K-bZIP promoter in reactivating cells (Carroll et al., 2007). Their binding sites overlap (**Figure 4B**). Recombinant Oct-1 enhanced Rta DNA binding to the promoter, and co-expression of Oct-1 enhanced Rta-mediated transactivation of the wild type but not the mutant K-bZIP promoter.

Octamer-1 and WT Rta proteins bound to each other directly *in vitro* (Carroll et al., 2007). aa 134–150 of Rta, within its DNA binding domain, contains a sub-domain homologous to the Oct-interacting domains (OIDs) of the Yeast protein MATα2 and the HSV-1 virion protein 16 (**Figure 1**). A series of single point mutations of Rta's OID had individual, negative effects on either DNA or Oct-1 binding, but none completely eliminated transactivation of K-bZIP and viral reactivation (Carroll et al., 2007). However, combining 3 of these point mutations, or insertion of a 4 aa peptide, ablated Rta DNA and Oct-1 binding, transactivation of the K-bZIP promoter and viral reactivation. None of the

mutations inhibited Rta's interaction with RBP-Jk (Carroll et al., 2007).

A second Oct-1 binding site was identified upstream of the CANT repeats (Carroll et al., 2007). In transfections of B cells, mutation of both Oct sites ablated Rta-mediated transactivation (Carroll et al., 2007). However, in transfections of WT and mutant MEFs, Oct-1 deletion only reduced Rta transactivation of K-bZIP by 50%, while RBP-Jk deletion eliminated transactivation. These data suggested that the Rta/Oct-1 interaction is essential for optimal KSHV reactivation, and the requirement for Oct-1 in Rta-mediated transactivation was stricter in B cells than MEFs (Carroll et al., 2007). Oct-1 is also involved in K-Rta transactivation of the K-Rta and K9 promoters (Sakakibara et al., 2001; Ueda et al., 2002; Harrison and Whitehouse, 2008).

K-Rta DNA BINDING SCREENS

Two genome-wide screens were performed using distinct strategies to identify K-Rta DNA binding sequences. In one strategy, FLAG-tagged K-Rta aa 1–390 was stably expressed in PEL cells, and chromatin was ChIP'd with anti-FLAG antibody 6 h after adding TPA to the cells (Chen et al., 2009). ChIP'd sequences were detected by a KSHV genomic tiling array. Nineteen viral loci scored above background, and were compared to previously published Rta binding sites to derive a consensus. The consensus contained two imperfect inverted tandem repeats with the sequence TTCCAGGAT$(N)_{0–16}$TTCCTGGGA (**Figure 3**). The study identified a new Rta responsive element within the first intron of the K15 P gene.

In the other strategy, RtaΔSTAD generated in *E. coli* was incubated with sheared KSHV genomic DNA, and bound DNA was enriched by serial repetition of PCR amplification and incubation with RtaΔSTAD (Ziegelbauer et al., 2006). Bound sequences were detected using a KSHV genomic microarray. Eighteen viral loci scored above background. The highest scoring sequence was in the gB promoter, which the authors confirmed was strongly activated by K-Rta. The authors aligned gB with other known transcriptional targets of Rta and identified a consensus with the sequence $C^C/_TC^A/_C{}^C/_TTTT$ (**Figure 3**). Notably, the core of this consensus is a CANT element (**Figure 3**).

OTHER MECHANISMS FOR K-Rta PROMOTER SPECIFICATION

WT K-Rta, but not a DNA binding mutant, induces the expression of IFN-stimulated genes (Zhang et al., 2005). K-Rta's binding sites are similar to interferon stimulated response elements (ISREs), and K-Rta activates the K14-ORF 74 promoter through an ISRE-like element. K-Rta's DNA binding domain is partly homologous to that of cellular interferon regulatory factor (IRF) proteins, and mutations in conserved aas inhibit K-Rta DNA binding (Zhang et al., 2005). K-Rta also binds to DNAs containing sites matching the A/T trinucleotide consensus (Liao et al., 2003a; **Figure 3**) to activate transcription of the KSHV K1 promoter (Bowser et al., 2006).

K-Rta interacts with the cellular protein C/EBPα to regulate transactivation of various KSHV promoters (Wang et al., 2003a,b). In transient transfections, C/EBPα and Rta cooperate to activate transcription of the K-bZIP, Mta, Rta, and PAN promoters. All of these promoters contain at least one C/EBPα binding site required for contributing to Rta-mediated transactivation. However, since the consensus binding site for C/EBPα (the CAAT box Osada et al., 1996) matches the core of the K-Rta binding CANT repeats (Palmeri et al., 2011; **Figure 3**), CAAT box mutations also have the potential to disrupt binding of K-Rta. K-Rta binds directly to C/EBPα *in vitro* and *in vivo*, and deletion of aa 151–167 of K-Rta inhibits (1) the interaction of K-Rta with C/EBPα or PAN DNA (**Figures 1**), (2) transactivation of the PAN promoter by Rta alone, and (3) cooperative transactivation of the K-bZIP or PAN promoters by Rta with C/EBPα (Wang et al., 2003a,b) ChIPs demonstrated that C/EBPα and Rta bound to the Rta, PAN, and Mta promoters in increasing amounts at 8 and 24 h after TPA addition to PEL cells, but not in un-treated cells (Wang et al., 2003b). Immunodepletion of C/EBPα prior to Rta ChIP suggested that it participated in binding of Rta to the K-bZIP promoter. K-Rta may also bind to the ori-Lyts through C/EBPα sites (Wang et al., 2006; Kato-Noah et al., 2007).

K-Rta also cooperates with AP-1 constituents cJun and cFos to activate transcription (Wang et al., 2004a). Rta binds to cJun and cFos *in vivo* and *in vitro*, and the three proteins cooperatively transactivate the K-bZIP, Mta, and Rta promoters in uninfected cells. cJun ChIPs those promoters in TPA-treated, but not un-treated, BCBL-1 cells.

K-Rta also transactivates STAT-driven reporter genes by stimulating STAT3 dimerization and nuclear localization (Gwack et al., 2002; **Figure 1**).

K-Rta HAS THE POTENTIAL TO ACTIVATE KSHV GENE EXPRESSION BROADLY DURING INFECTION

Transcriptomic analyses indicated that expressing Rta ectopically in PEL cells induced a coordinated cascade of KSHV gene expression (Nakamura et al., 2003; Damania et al., 2004). Transient expression of Rta induced all ORFs except the latency genes (Damania et al., 2004), while uniform expression of K-Rta under the control of doxycycline (dox) induced every viral ORF within 48 h after dox addition (Nakamura et al., 2003). In the latter study, the productive transcriptional cascade induced by dox was more rapid and robust than that induced by TPA treatment, suggesting TPA is a relatively inefficient reactivation signal. Most of the viral homologs of cell growth regulatory genes were expressed as DE transcripts, and infectious viral progeny were produced.

K-Rta activates many KSHV promoters in an RBP-Jk-dependent manner (Liang et al., 2002; Liang and Ganem, 2004; Chang et al., 2005b, 2010; Lan et al., 2005a; Carroll et al., 2006, 2007; Wang and Yuan, 2006; Ziegelbauer et al., 2006; Liu et al., 2008; Persson and Wilson, 2010; Wang et al., 2010). At least 260 putative binding sites for K-Rta's cellular binding partner RBP-Jk were identified *in silico* in the KSHV genome (Persson and Wilson, 2010 and Gonzalez-Lopez and Lukac, unpublished). Three genomic regions with clustered sites were identified as new RBP-Jk-dependent transactivation targets of K-Rta (Persson and Wilson, 2010). Among the putative RBP-Jk sites, the RBP-Jk protein demonstrated a binding preference, and K-Rta demonstrated a transactivation preference. A signature was derived for 17 confirmed RBP-Jk binding sites, which added a cytosine residue at position upstream of the canonical GTGGGAA core sequence (Persson and Wilson, 2010). Further work is needed to determine which of the putative

RBP-Jk sites are co-regulated by K-Rta DNA binding. The observation that the RBP-Jk sites are scattered widely across the unique portion of the genome suggests that K-Rta has the potential to activate transcription of every KSHV ORF during reactivation.

A comprehensive strategy to identify all of K-Rta's transcriptional targets compared 83 putative KSHV promoters for K-Rta transactivation in uninfected 293 cells, with ChIP of dox-inducible Rta from infected PEL cells (Ellison et al., 2009). Rta transactivated 34 of the promoters greater than 10-fold, including many previously identified targets of K-Rta. At 12 h following dox induction, K-Rta was significantly ChIP'd on 22 promoters (Ellison et al., 2009). Fourteen of the promoters corresponded to those identified as targets in 293 cells. Although there was little correspondence between magnitude of transactivation and magnitude of ChIP enrichment, there were cell-specific differences in specificity and magnitude of transactivation (Ellison et al., 2009). A subset of promoters were tested at later time points following K-Rta induction, which indicated that K-Rta associated with increasingly greater numbers of promoters as the infection progressed (Ellison et al., 2009). These data suggest that K-Rta has the potential to activate at least half of putative KSHV promoters during reactivation.

DIRECT K-Rta TRANSACTIVATION IS LIMITED IN INFECTED CELLS

It is well established that K-Rta is the only viral protein necessary and sufficient to initiate the complete KSHV productive cycle. The foundation of this process is the ordered expression of viral genes, with transcriptional initiation of specific genes one of the earliest regulated steps. It was clear that the gene-specific transcriptional cascade could be classified kinetically from the earliest detection of Rta to the replication-dependent expression of the true late genes. It was also clear that Rta had the potential to activate transcription of many, if not most, of the viral genes. However, it was not clear which viral genes Rta directly transactivated during reactivation in infected cells, and which depended on prior expression of Rta's targets.

To answer this question, a modified allele of Rta was engineered that was fully functional, but could be conditionally activated in the presence of protein synthesis inhibitors to eliminate any other protein expression (Bu et al., 2008). The strategy relied upon manipulation of Rta's nuclear/cytoplasmic localization by fusion to the hormone-binding domain of the murine estrogen receptor gene (ER), an approach used successfully for many other transcription factors. In this approach, nuclear transcription factors are constitutively localized to the cytoplasm by fusion to the ER, but re-localize to the nucleus in the presence of 4-hydroxytamoxifen (OHT).

However, fusion of the WT K-Rta protein to ER failed to localize the protein to the cytoplasm, suggesting that Rta's cognate NLS was dominant (Bu et al., 2008). Sequence analysis identified two putative nuclear localization signals in K-Rta, between aa 6–12 (NLS1) and 516–530 (NLS2; **Figure 1**). NLS1 regulated nuclear localization in one publication (Chen et al., 2000), but deletion of NLS1 had no effect on Rta-mediated function in CV-1, BL-41, and PEL cells (Bu et al., 2008). Instead, deletion of aa 516–530 (NLS2) eliminated both K-Rta-mediated transactivation and reactivation (Bu et al., 2008). Deletion of NLS2 re-localized full-length K-Rta to the cytoplasm, and fusion of NLS2 to GFP re-localized GFP to the nucleus. NLS2 was thus a *bona fide* nuclear localization signal for K-Rta (Bu et al., 2008; **Figure 1**). WT K-Rta and each NLS mutant were tested as independent fusions to ER to determine sub-cellular localization and transactivation of Mta and PAN reporter plasmids. In this experiment, K-Rta with both putative NLSs deleted (RtaΔNLS1,2-ER) was fully regulatable by addition or subtraction of OHT to growth media of transfected cells (Bu et al., 2008).

The effect of the regulatable K-Rta allele on KSHV was tested using Vero cells infected with the recombinant virus rKSHV.219. This virus constitutively expresses GFP in all infected cells, and conditionally expresses RFP from a second copy of the PAN promoter as a reporter for viral reactivation (Vieira and O'Hearn, 2004). The RtaΔNLS1,2-ER fusion protein demonstrated OHT-dependent stimulation of the complete viral reactivation cycle, as determined by expression of viral RNAs and proteins, RFP induction, and production of infectious virus in the cell supernatant (Bu et al., 2008).

To determine which viral genes were transactivated by Rta in the absence of any other protein expression, RtaΔNLS1,2-ER was expressed in Vero-rKSHV.219 cells for 3 h prior to addition of the protein synthesis inhibitor hygromycin to the cells (Bu et al., 2008). Shut-off of protein synthesis was confirmed by lack of endogenous RFP and tubulin expression, and lack of luciferase and beta-galactosidase expression from ectopic reporter plasmids. Fifteen hours following hygromycin addition, OHT was added to half of the cultures. Total RNA was harvested from cells, and analyzed using a printed oligonucleotide microarray containing detectors for the entire KSHV transcriptome. Only eight genes were significantly induced by nuclear re-localized K-Rta in the absence of *de novo* protein synthesis: PAN, Mta, ORF 56, vIL-6, ORF 37, K14, K9, and ORF 52 (Bu et al., 2008).

This small number of direct K-Rta targets in infected cells is remarkable considering that the KSHV genome contains at least 260 putative RBP-Jk binding sites, and Rta activates or binds to approximately half of the KSHV promoters in transiently transfected, uninfected cells. These data suggest that additional viral and cellular protein expression is required for the lytic cycle to progress beyond these eight direct transcriptional targets of Rta. The data also support a model in which KSHV reactivation is controlled not only by induction of K-Rta transcription, but also by regulation of K-Rta protein function in a manner that requires on-going translation. In this scenario, K-Rta expression is not sufficient to ensure completion of the entire lytic cascade, but K-Rta's function is regulated to direct the progression of reactivation.

REGULATORS OF K-Rta FUNCTION

MTA COOPERATES WITH Rta AS A COMMITMENT FACTOR FOR REACTIVATION

KSHV Mta was identified as one of eight direct transcriptional targets of K-Rta during KSHV reactivation (Bu et al., 2008). Mta's promoter has served as a model for understanding K-Rta's specification of transactivation (see "RBP-Jk-dependent promoters," above; **Figure 4A**). Genetic studies demonstrated that Mta is required for productive viral reactivation, especially for optimal expression of DNA replication factors (Han and Swaminathan, 2006; Majerciak et al., 2007). Mta was unable to reactivate KSHV

from latency when ectopically expressed in PEL cells, but synergized with K-Rta to stimulate reactivation (Lukac et al., 1998; Palmeri et al., 2007). Synergy with K-Rta was detected by expression of the PAN RNA from the endogenous viral genome in PEL cells, and release of mature virus to the cell medium (Palmeri et al., 2007).

Mta is encoded by KSHV ORF 57, and is homologous to regulators of gene expression in other herpesviruses. In KSHV, ORF 57 is one of the first genes transcribed during reactivation, following transcription of K-Rta and other IE genes (Lukac et al., 1999). The Mta protein localizes to the nuclei of cells transfected with an ORF 57 expression vector (Kirshner et al., 2000; Majerciak et al., 2006b). The major monocistronic transcript that encodes ORF 57 is 1.4 kb and contains a single, small 5′ intron (Kirshner et al., 2000). Mta is expressed as a 49-kDa protein within 1 h of TPA treatment of PEL cells, following K-Rta protein expression (Palmeri et al., 2007). Up until 24 h post-TPA, total K-Rta and Mta proteins increased in abundance, and the percentages of cells expressing either Mta or Rta also increased (Palmeri et al., 2007). From 24 to 72 h post-TPA treatment, the percentage of K-Rta expressing cells continued to increase linearly to 20%, while the percentage of Mta-expressing cells plateaued at 10–12%. Mta expression thus marked a population of cells that diverged from K-Rta positive cells as reactivation progressed (Palmeri et al., 2007).

To determine the relationship between K-Rta and Mta expression, and complete viral reactivation, the percentages of cells co-expressing either K-Rta or Mta, together with the true late protein K8.1, were measured (Palmeri et al., 2007). At all times post-TPA addition, the proportion of Mta positive cells that also expressed K8.1 exceeded that of K-Rta positive cells that expressed K8.1. Beginning at 24 h post-TPA, the proportion of Mta positive cells that also expressed K8.1 increased and continued for the duration of the experiment, while the percent of K-Rta positive cells that expressed K8.1 decreased during that time. At 72 h post-TPA, the latest time point tested, 80% of Mta positive cells also expressed K8.1, while only 20% of K-Rta positive cells expressed K8.1 These data showed that although K-Rta was necessary and sufficient to initiate KSHV reactivation, expression of Mta predicted successful progression of the productive cycle to the late stage (Palmeri et al., 2007). The expression of Mta therefore seemed to be required to commit a reactivating virus to the entire productive cascade.

KSHV Mta has multiple, pleiotropic effects on gene expression. Mta increased the steady-state abundances of the transcripts for ORF 59/58 and PAN, but not ORF 74/vGPCR or K5, when the transcripts were expressed from the CMV promoter in CV-1 cells (Kirshner et al., 2000). However, Mta showed inconsistent and <2-fold effects on firefly luciferase expressed under control of the kaposin, DNA polymerase, PAN, and TK promoters, in CV-1 cells (Kirshner et al., 2000). These data suggested a post-transcriptional role for Mta in transactivating gene expression.

Despite Mta's inability to independently transactivate KSHV promoters, Mta dramatically synergized with K-Rta in a promoter specific fashion (Kirshner et al., 2000; Palmeri et al., 2007). These effects of expressing K-Rta and Mta alone and together were similar to their effects on KSHV reactivation. Mta strongly synergized with K-Rta transactivation of the PAN promoter, and cooperated to a lesser amount for the ori-Lyt (L), Kaposin, ORF 57, and TK promoters (Kirshner et al., 2000; Palmeri et al., 2007). The effect on the PAN promoter was 80- to 243-fold over K-Rta transactivation alone. However, K-Rta/Mta did not synergize on the K-bZIP and ORF 50 promoters (Kirshner et al., 2000; Palmeri et al., 2007). K-Rta/Mta synergy required K-Rta transactivation, since synergy was eliminated by (1) mutation of the K-Rta binding site in the PAN promoter, and (2) co-expression of the K-Rta dominant negative mutant RtaΔSTAD (Kirshner et al., 2000). K-Rta/Mta synergy was reduced but not eliminated when an intron was added to the luciferase transcript in the PAN reporter plasmid (Kirshner et al., 2000). Mta did not increase the amount of K-Rta mRNA and protein levels in co-transfected cells (Kirshner et al., 2000). These data suggested that Mta has a K-Rta-dependent, promoter specific effect on transactivation in CV-1 cells, and the promoter specific effect of Mta is further influenced by the structure of the resulting transcript.

Mta binds directly to K-Rta, and requires two regions of Rta: the LR and aa 275–484 (Palmeri et al., 2007; **Figure 1**). K-Rta DNA binding was necessary for synergy with Mta, but it was not sufficient (Palmeri et al., 2007). Although K-Rta strongly transactivated the PAN promoter when truncated immediately upstream of the K-Rta binding site, or when the K-Rta binding site was cloned upstream of a heterologous TATA box, K-Rta/Mta synergy was severely reduced (Palmeri et al., 2007). Therefore, the K-Rta binding site, plus additional elements, in the PAN promoter were necessary for robust K-Rta/Mta synergy.

Further investigation of K-Rta/Mta synergy revealed a cell-specific, Rta-independent transcriptional activation function for Mta (Palmeri et al., 2007). Mta strongly activated the PAN promoter reporter in 293 cells and in Akata-31 cells, a sub-clone of Akata cells that were cured of EBV infection. Mta transactivation was promoter specific in 293 cells, and the WT and mutant promoters that were activated were those on which Mta synergized with Rta in CV-1 cells (Palmeri et al., 2007). Since Mta and K-Rta did not synergize in 293 cells, and Mta transactivation was independent of basal promoter activity, the data suggested that 293 cells endogenously mimic a K-Rta function required for Mta synergy in CV-1 cells.

Multiple experiments support a direct transcriptional role for Mta in transactivation. Mta transactivated the PAN promoter in nuclear run-on transcription assays, demonstrating that it is a *bona fide* transcriptional transactivator (Palmeri et al., 2007). Mta also activated the ORF 50 promoter in cooperation with Rta (Malik et al., 2004a). Mta and K-Rta ChIP'd the K-Rta and PAN promoters in KSHV infected cells (Malik et al., 2004a; Palmeri et al., 2007).

However, two observations supported an additional post-transcriptional role for Mta in synergy with K-Rta. The magnitude of Mta transactivation in the nuclear run-on experiments was considerably lower than in whole cell reporter assays, and Mta transactivation in the presence of actinomycin D suggested that Mta also stabilized reporter messages (Palmeri et al., 2007). Indeed, the Mta ORF includes protein domains with putative transcriptional and post-transcriptional functions, and Mta binds to proteins that regulate gene expression at various points (Malik et al., 2004a,b;

Nishimura et al., 2004; Majerciak et al., 2006b, 2008, 2011; Boyne et al., 2010a; Jackson et al., 2011).

In fact, an expanding literature demonstrates multiple post-transcriptional roles for Mta. Mta enhances the accumulation of both viral (Kirshner et al., 2000; Han and Swaminathan, 2006; Majerciak et al., 2006a,c, 2008) and cellular (Gupta et al., 2000) RNAs in a gene-specific manner. Mta binds directly to RNAs in a sequence specific fashion to stabilize them or to promote escape from RNA silencing (Majerciak et al., 2006b; Nekorchuk et al., 2007; Sahin et al., 2010; Kang et al., 2011b; Massimelli et al., 2011; Sei and Conrad, 2011), enhances RNA splicing (Majerciak et al., 2008), shuttles between nuclei in heterokaryon assays (Bello et al., 1999), and promotes the nuclear export of un-spliced RNAs (Malik et al., 2004b; Boyne et al., 2008; Jackson et al., 2011; Majerciak et al., 2011; Taylor et al., 2011). Mta also stimulates mRNA translation (Nishimura et al., 2004; Boyne et al., 2010a). Details of Mta's post-transcriptional functions can be found in a number of excellent literature reviews (Swaminathan, 2005; Conrad, 2009; Majerciak and Zheng, 2009; Boyne et al., 2010b).

A model that is supported by all of the publications is that Mta is present at viral promoters prior to or during transcriptional initiation, the earliest step in biogenesis of an RNA. Mta might subsequently associate with the nascent RNA transcript in the nucleus, then remain associated with the transcript to regulate it post-transcriptionally. In this model, sequence specific transactivation by Mta could be governed by direct interaction with DNA or RNA, or indirectly by interaction with K-Rta.

K-Rta AUTOREGULATES ITS FUNCTION

K-Rta encodes two independent regions that auto-regulate its DNA binding and abundance (Chang and Miller, 2004; Chang et al., 2008). K-Rta aa 520–535 inhibits DNA binding to the PAN promoter in cis, and aa 590–650 destabilizes K-Rta in concert with a domain that overlaps aa 520–535 (**Figure 1**). K-Rta mutants in aa 520–535 are better DNA binders, but worse transactivators, of PAN, Mta, and vMIP-I. Fusion of the destabilizing element to glutathione-*S*-transferase (GST) destabilizes that protein, also. Since K-Rta's NLS2 is located at aa 516–530 (**Figure 1**), these functions of K-Rta might be co-regulated with proper sub-cellular localization. Further work is required to determine whether aa 590–650 are involved in K-Rta auto-ubiquitylation.

NEGATIVE REGULATORS OF Rta FUNCTION

Post-translational modifications and heterologous proteins inhibit K-Rta. The cellular protein IRF-7 competes with K-Rta for binding the Mta promoter to inhibit Rta-mediated transactivation (Wang et al., 2005). The IRF-7 binding site overlaps a CANT repeat downstream of the RBP-Jk site in the Mta promoter (**Figure 4A**).

The cellular NF-kB (RelA) protein inhibits K-Rta transactivation in 293 cells and reactivation in PELs, promoter binding by K-Rta and RBP-Jk, and K-Rta binding to RBP-Jk (Izumiya et al., 2009). RelA repression was promoter specific; it did not affect K-Rta transactivation of the Ori-RNA or Kaposin promoters. RelA bound directly to RBP-Jk. Multiple NF-kB family proteins inhibited Rta transactivation in 293 cells (Brown et al., 2003; Izumiya et al., 2009). The NF-kB inhibitor Bay11–7082 induced productive cycle gene expression in latently infected PEL cells, suggesting that high NF-kB levels help maintain latency (Brown et al., 2003). RelA also inhibited K-Rta transactivation of the human IL-6 promoter (Roan et al., 2002).

The cellular protein Trasducin-like enhancer of split (TLE) 2 binds directly to K-Rta aa 170–246 (**Figure 1**), and competes with K-Rta binding to RBP-Jk (He et al., 2010). TLE2 down-regulates K-Rta transactivation of the K-Rta, PAN, K-bZIP, Mta, and ORF 59 promoters, and reduces K-Rta-mediated reactivation. Knockdown of TLE2 enhances KSHV reactivation. K-Rta/TLE2 complexes bind to the PAN, K-bZIP, and ORF 59 promoters, and TLE2 antibody ChIPs the PAN and Mta promoters during reactivation.

K-Rta's promoter association and stimulation of reactivation in PEL cells can be decreased by poly-ADP-ribosylation and phosphorylation of its serine-threonine (ST)-rich domain, mediated by binding to the cellular proteins poly(ADP-ribose) polymerase-(PARP)-1 and human kinase from chicken (KFC; Gwack et al., 2003; **Figure 1**).

DUAL POSITIVE/NEGATIVE REGULATORS OF K-Rta FUNCTION

K-Rta makes complex interactions with the KSHV IE protein K-bZIP that are both positive and negative. In the screen of 83 putative KSHV promoters in 293 cells, K-bZIP activated 21 alone, synergized with Rta on 19, and repressed Rta transactivation of 3 (Ellison et al., 2009). K-bZIP's ability to repress K-Rta-mediated activation required a direct protein-protein interaction (**Figure 1**), and was not relieved by overexpressing transcriptional coactivators (Liao et al., 2003b). Three K-Rta deletions inhibited binding to K-bZIP: aa 1–109, 110–295, and 293–503.

The cellular protein KSHV Rta binding protein (K-RBP) binds K-Rta aa 1-272, and contains a Kruppel-associated box (KRAB) and 12 adjacent zinc finger motifs (Wang et al., 2001b; **Figure 1**). When expressed at low levels, K-RBP synergizes with K-Rta transactivation of the Mta, K-bZIP, vIL-6, K-Rta, and vMIP-1 promoters, but at high levels, represses K-Rta transactivation of K-bZIP, Mta, and PAN promoters (Wang et al., 2001b; Yang and Wood, 2007). K-RBP binds to DNAs that contain a GC-rich core, including a 40-bp region of the Mta promoter that contains the RBP-Jk site and overlaps CANT repeats. K-RBP competes with K-Rta to bind the Mta promoter, and represses KSHV lytic reactivation (Yang and Wood, 2007; Yang et al., 2009).

Rta AND CHROMATIN

Latency and reactivation of KSHV is associated with dynamic chromatin remodeling (Gunther and Grundhoff, 2010; Toth et al., 2010; Chang et al., 2011; Kang et al., 2011a). Early evidence for the dependence of reactivation on relief of repressive chromatin came from the observation that histone deacetylase inhibitors (HDACi) reactivate KSHV from latency (Sun et al., 1996). Dominant negative RtaΔSTAD inhibited reactivation stimulated by the HDACi sodium butyrate, suggesting that repressive chromatin controls expression of K-Rta from the latent genome (Lukac et al., 1999).

Similar to many DBPs, chromatin structure also plays an integral role in K-Rta's accessibility to DNA. ChIPs specific for histone H3-K9 acetylation (H3acK9), a marker for active chromatin, showed that at 12 h post-K-Rta induction, H3acK9 was ChIP'd

on 48 promoters (Ellison et al., 2009). There was a correlation between the increase in active chromatin and progression of reactivation, and H3acK9 was ChIP'd before, or concurrent with, ChIP of K-Rta. The data suggested that chromatin accessibility regulated K-Rta's access to its transcriptional targets.

The cellular protein Kruppel-associated box domain-associated protein-1 (KAP-1) regulates formation of heterochromatin, and was found associated with most KSHV productive cycle promoters during latency (Chang et al., 2009). During reactivation, KAP-1 dissociated from promoters, concomitant with K-Rta DNA binding. KAP-1 knockdown induced reactivation (Chang et al., 2009).

The cellular protein high mobility group protein (HMG) B1 binds and bends DNA, and enhances K-Rta DNA binding. The effect of HMGB1 is greater for low affinity K-Rta binding sites (Song et al., 2004). Ectopic expression of HMGB1 enhanced K-Rta transactivation of the PAN, kaposin, vIL-6, Mta, and K-Rta promoters (Song et al., 2004; Harrison and Whitehouse, 2008).

Rta COUNTERACTS ITS NEGATIVE REGULATORS

K-Rta encodes a ubiquitin E3 ligase activity that regulates KSHV reactivation by targeting transcriptional repressors of K-Rta transactivation (Yu et al., 2005; Yang et al., 2008). K-Rta contains a Cys/His-Rich domain reminiscent of catalytic regions of E3 ligases (**Figure 1**). K-Rta targets the cellular protein IRF-7 for poly-ubiquitylation and degradation in a proteasome and Cys/His-dependent manner (Yu et al., 2005). IRF-7 binds directly to K-Rta aa 273–544 (Yu et al., 2005; **Figure 1**).

K-Rta similarly targets the cellular protein K-RBP for degradation (Yang et al., 2008), but also requires K-Rta aa 621–641 for this effect (**Figure 1**). Poly-ubiquitylation by K-Rta corresponds with its ability to counteract transcriptional repression mediated by K-RBP. K-Rta expression is also associated with reduction of K-bZIP, LANA, and NF-kB p65 protein levels in 293 cells. Proteasome inhibitors reduced Rta-stimulated reactivation of KSHV in PEL cells. While Rta also autoregulates its own degradation by self-ubiquitylation (Yu et al., 2005), the biological significance has not been directly tested.

CONCLUSION AND PERSPECTIVES

SUMMARY

Small DNA tumor viruses transform cells in non-productive, dead-end infections. The large DNA tumor virus KSHV establishes latent, or non-productive infections, which are reversible in a process called reactivation. In reactivation, KSHV genes are expressed in kinetically distinct cascades, leading to viral DNA replication and release of infectious virus. A single KSHV protein, K-Rta, is necessary and sufficient to initiate the productive cascade of gene expression and the complete viral productive cycle. K-Rta is a nuclear, gene-specific transcriptional transactivator. K-Rta specifies promoters for transactivation by binding DNA directly and interacting with cellular DBPs.

Reactivation of KSHV from latency requires the cellular protein RBP-Jk to specify many of K-Rta's transcriptional targets. Thus, K-Rta transactivation of KSHV promoters is either RBP-Jk independent or RBP-Jk-dependent. K-Rta transactivates RBP-Jk independent promoters by binding to a short, conserved, high affinity K-Rta binding site (**Figure 2**). K-Rta transactivates the RBP-Jk-dependent promoter, Mta, by binding to an extended element that contains multiple copies of a repetitive CANT DNA sequence, and stimulating RBP-Jk DNA binding (**Figure 4A**). CANT repeats are found in proximity to many RBP-Jk sites across the KSHV genome, and K-Rta stimulates RBP-Jk DNA binding broadly, but specifically, to the KSHV genome. A putative, working model based on genetic studies proposes that K-Rta stimulates RBP-Jk DNA binding by contacting CANT DNA repeats flanking an RBP-Jk site; biophysical investigations of this model are on-going. The inability of NICD1 and EBNA-2 to reactivate the complete KSHV productive cycle supports the existence of non-canonical regulation of RBP-Jk in KSHV infected cells.

Although K-Rta transactivates many of the KSHV promoters in transient transfections, and the KSHV genome contains at least 260 putative RBP-Jk binding sites, K-Rta only transactivates eight genes in the absence of *de novo* protein synthesis during reactivation. These data suggest that continued progression of reactivation is regulated after K-Rta expression.

Indeed, K-Rta appears to be a relatively inefficient reactivating switch, and can be regulated positively and negatively by many viral and cellular factors. One positively acting, K-Rta regulating factor is the essential KSHV protein Mta, which was identified as one of K-Rta's direct transcriptional targets. Mta cannot reactivate KSHV alone, but synergizes with K-Rta to induce complete reactivation. Single cell expression of Mta suggests that it is a commitment factor that commits a K-Rta expressing cell to complete reactivation. Mta has multiple, pleiotropic effects on gene expression. It is a cell and promoter specific transcriptional transactivator, and a transcript-specific post-transcriptional transactivator. Mta synergizes with K-Rta in a promoter specific manner, then might continue to transactivate post-transcriptionally by maintaining association with nascent RNAs. K-Rta also autoregulates its stability and DNA binding.

CONTROVERSIES IN Rta PROMOTER SPECIFICATION

Promoter specification by K-Rta has been controversial. Disagreements in published studies might be explained by technical variations in the strategies used to evaluate K-Rta DNA binding. Three examples of important technical differences might explain the divergent views. These disagreements raise some interesting biological questions about K-Rta.

In the first example, many reports agree that K-Rta binds to short segments of the Mta promoter directly (Duan et al., 2001; Lukac et al., 2001; Song et al., 2003; Carroll et al., 2006; Wen et al., 2009; Palmeri et al., 2011), yet other reports propose that K-Rta binds only indirectly to the Mta promoter (and others), by "piggybacking" onto DNA-bound RBP-Jk (Zhang et al., 1998; Sakakibara et al., 2001; Wang et al., 2003a,b, 2004a; Liang and Ganem, 2004; Chang et al., 2005c; Wang and Yuan, 2006). A recent publication demonstrated that K-Rta binds to a longer region of the Mta promoter then previously recognized. High affinity DNA binding (i.e., nM) of K-Rta requires seven copies of a CANT DNA element repeated over 62 bp of the Mta promoter. Conversely, K-Rta binds DNAs containing three or fewer CANT repeats with 35-fold lower affinity. The earlier studies that concluded that K-Rta binds the Mta promoter

indirectly all used similar short DNAs that contained three or fewer CANT repeats as K-Rta targets (Duan et al., 2001; Lukac et al., 2001; Song et al., 2003; Carroll et al., 2006; Wen et al., 2009). Thus, the Mta promoter is not an indirect target of K-Rta, but rather, a high affinity RBP-Jk-dependent target. Biologically, the data suggest that K-Rta's affinity will vary significantly for natural promoters that contain varying numbers of CANT repeats.

In the second example, the choice of K-Rta expression system has led to disagreements regarding K-Rta's promoter specificity. *In vitro*, K-Rta only binds to Mta promoter DNAs when made in bacteria (Duan et al., 2001; Lukac et al., 2001; Song et al., 2003; Carroll et al., 2006; Wen et al., 2009; Palmeri et al., 2011), but never when made in mammalian systems (Wang et al., 2003a,b; Chang et al., 2005c). These data suggest that mammalian modifications, or co-purifying proteins, inhibit DNA binding of K-Rta to the Mta promoter. Interestingly, even though K-Rta aa 1–272 made in bacteria is sufficient to bind to the Mta promoter, its specificity seems to be more stringent than FL K-Rta made in bacteria (Carroll et al., 2006).

In the third example, different consensus sequences for K-Rta DNA binding have been derived from KSHV genome-wide screens. In one study, ChIP-on-ChIP of K-Rta aa 1–390 expressed in BCBL-1 cells was performed 6 h after TPA induction, and generated a 40-bp consensus binding sequence that was sometimes found in tandem repeats (Chen et al., 2009). In another study, *in vitro* binding of K-Rta aa 1–530 to 200–300 nt fragments of the KSHV genome generated an 8-bp consensus binding sequence (Ziegelbauer et al., 2006). Besides deriving different K-Rta binding sites, the studies shared only two significant hits. The use of different sources and lengths of K-Rta protein, and an *in vivo* or *in vitro* approach, likely led to the different conclusions of the two studies. A third study screened 83 putative KSHV promoters using ChIP of FL K-Rta expressed in BCBL-1 cells 12 h after inducing reactivation, and identified 22 significant promoter hits (Ellison et al., 2009). Only eight of these promoters were also identified in the ChIP-on-ChIP screen (Chen et al., 2009). However, this study demonstrated that the choice of time point following reactivation could dramatically influence the number of K-Rta targets identified (Ellison et al., 2009). Therefore, it is likely that different conclusions between the two ChIP approaches could be explained by time after stimulation of reactivation, the use of different lengths of K-Rta protein, and different antibodies.

Clearly, there is much to be learned about K-Rta promoter specification and how it is regulated. In fact, the potential exists for disparity in one additional aspect of K-Rta DNA binding: the interpretation of phenotypes of K-Rta N-terminal mutants. The minimal K-Rta DNA binding domain maps to aa 1–272 (Lukac et al., 2001; Carroll et al., 2006), and overlaps with nine domains attributed to other functions of K-Rta (**Figure 1**): homo-oligomerization (Bu et al., 2007), RBP-Jk binding (Liang et al., 2002), Oct-1 binding (Carroll et al., 2007), E3 Ubiquitin ligation (Yu et al., 2005; Yang et al., 2008), inhibiting p53 (Gwack et al., 2001), K-RBP binding and degradation (Wang et al., 2001b; Yang et al., 2008), C/EBP binding (Wang et al., 2003a), and TLE2 binding (He et al., 2010). In fact, two publications have described single N-terminal point mutants that affect both DNA binding and interactions with either Oct-1 or C/EBPα (Wang et al., 2003a; Carroll et al., 2007). Many of these other N-terminal K-Rta functions have the potential to influence K-Rta promoter specification. A full understanding of K-Rta promoter selectivity requires more refinement of the K-Rta structure/function map to establish whether these functions are co-regulated or separable.

K-Rta PROMOTER SPECIFICATION AND PROGRESSION OF VIRAL REACTIVATION

The conclusion that K-Rta is an inefficient switch protein is based on results of single cell assays. K-Rta expressing PEL cells appear to have two fates that can be distinguished by Mta expression (Palmeri et al., 2007). In K-Rta expressing cells that co-express Mta, viral reactivation proceeds down the entire lytic cascade to release mature virus (see top of **Figure 6**). K-Rta activates Mta transcription, then Mta protein cooperates with K-Rta to transactivate additional DE genes, driving progression of reactivation. However, most K-Rta expressing cells do not co-express Mta, and are observed during KSHV reactivation up to 72 h post-TPA addition or post-Rta vector transfection (Palmeri et al., 2007; see bottom of **Figure 6**). Finally, a third fate of K-Rta expressing cells is suggested by the observation that both K-Rta and Mta are gene-specific transactivators: K-Rta expression accompanied by a class of DE genes that are transactivated by K-Rta in the absence of Mta expression (see middle of **Figure 6**).

The other positive and negative regulators of K-Rta function can be considered using the single cell paradigm illustrated in **Figure 6**. Factors that stimulate K-Rta transactivation would drive reactivation toward the productive pathway (upwards), while factors that inhibit or temper K-Rta function would drive reactivation toward the non-productive pathways (downwards).

AUTOREGULATION OF K-Rta EXPRESSION

Numerous positive and negative regulatory events control K-Rta expression, and the K-Rta dosage clearly is proportional to the magnitude of reactivation (Sun et al., 1998). The key regulatory event in the latent to lytic switch is initiation of K-Rta transcription, which is not covered in detail here due to limitations of the publication format. However, following its expression, K-Rta auto-transactivates ORF 50 transcription in collaboration with Mta, Oct-1, AP-1, C/EBPα, K-RBP, HMGB1, and XBP-1s (Deng et al., 2000; Sakakibara et al., 2001; Wang et al., 2001b, 2003a,b, 2004a; Malik et al., 2004a; Wilson et al., 2007; Harrison and Whitehouse, 2008; Di Bartolo et al., 2009). K-Rta auto-transactivation is inhibited by both cellular and viral factors, including TLE2, NF-kB, Oct-2, Hey1, and LANA. (Brown et al., 2003; Lan et al., 2004, 2005a; Yada et al., 2006; Di Bartolo et al., 2009; He et al., 2010). Oct-2 inhibits K-Rta activation of the K-Rta promoter by preventing DNA binding of Oct-1 and K-Rta to the K-Rta promoter (Di Bartolo et al., 2009). LANA binds to both K-Rta and RBP-Jk, and competes with Rta for binding to RBP-Jk (Lan et al., 2004, 2005a), resulting in reduction of productive replication. Interestingly, K-Rta activates Hey1 and LANA, the latter in an RBP-Jk-dependent fashion (Lan et al., 2005b; Yada et al., 2006), to establish negative-feedback loops on its expression. Therefore, expression

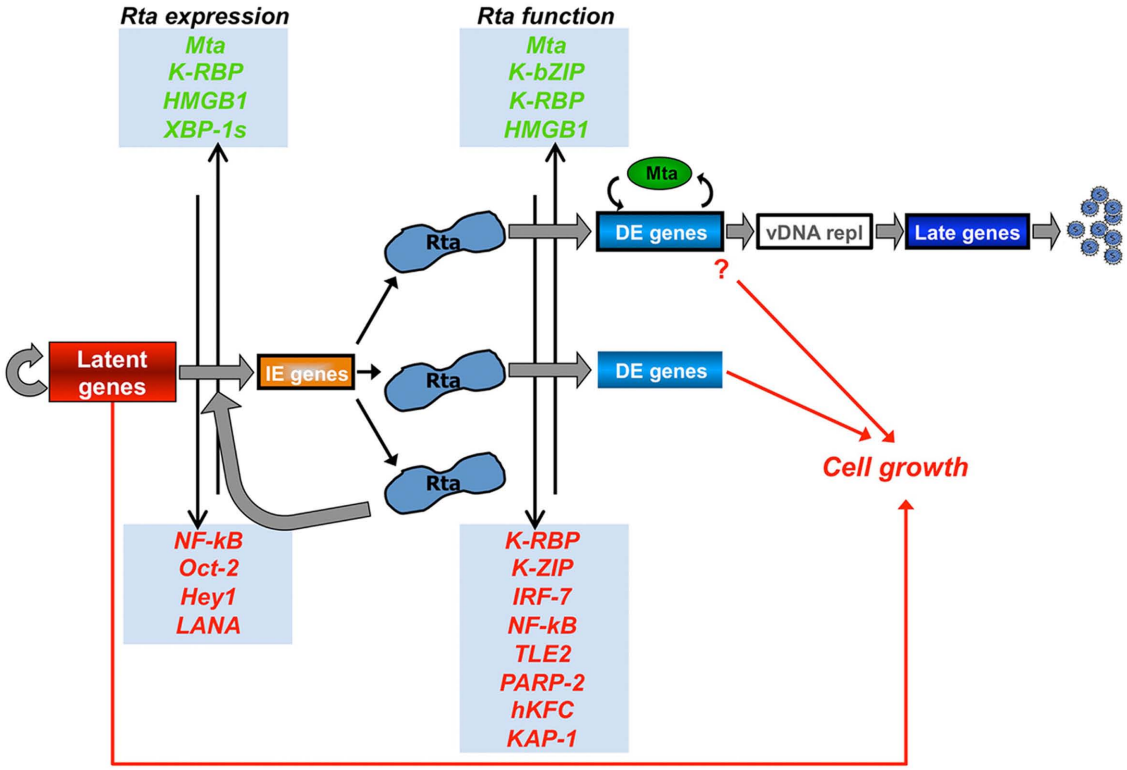

FIGURE 6 | Single cell model for progression of KSHV reactivation and its putative influence on cellular growth. As described in text, Rta expressing PEL cells are depicted as having three fates: complete reactivation (top), expression of a sub-set of DE genes without complete reactivation (middle), and completely abortive reactivation, in which K-Rta might feedback to activate itself (bottom). Modulators of K-Rta function that were discussed in the text are listed in the light blue boxes. Negative regulators (red text) might favor the non-productive reactivation fates, while positive regulators (green text) might favor the productive reactivation fate. DE oncogenes expressed in the top or middle fates, might cooperate with latent proteins to stimulate cell growth (red lines and text). IE, immediate early; DE, delayed early; vDNA repl, viral DNA replication.

levels of K-Rta itself and of K-Rta's regulators of expression and function, work together to drive reactivation toward, or steer reactivation away from, the productive pathway (**Figure 6**). Any of K-Rta's regulatory factors that influence K-Rta's promoter specification could favor a specific path as illustrated, or a hypothetical "sub-path."

PROGRESSION OF VIRAL REACTIVATION AND KSHV DISEASE

Both latent and productive cycle genes are sufficient to transform cells when expressed alone, and animal models of KS demonstrate that productive cycle genes dramatically potentiate transformation driven by latent genes (reviewed in Martin and Gutkind, 2008; Mesri et al., 2010). This combinatorial effect would be consistent with the top and middle pathways of **Figure 6**. Most of KSHV's oncogenes are expressed in the DE class, suggesting that regulation of K-Rta's function can influence cell growth. Indeed, tissue culture models of KSHV infection support a role for K-Rta in contributing to growth of infected cells. In BC-1 PEL cells co-infected by KSHV and EBV, KSHV reactivation induced expression of the EBV oncogene latent membrane protein (LMP)-1 (Spadavecchia et al., 2010). RtaΔSTAD, but not the non-functional mutant RtaΔSTADΔLR, reduced growth of BC-1 cells within 24 h after transfection. As a control, RtaΔSTAD had no effect on growth of uninfected BL-41 cells, proving that its growth suppressing effect required cells to be infected by EBV and KSHV.

Since BC-1 cells were not uniformly transfected by the RtaΔSTAD expression vector, the data suggested that the BC-1 cell population spontaneously expressed endogenous Rta in a non-synchronous and unsustained manner, which contributed to growth of BC-1 cells. In turn, LMP-1 is known to inhibit K-Rta expression and reactivation (Xu et al., 2007). Together, the data suggested a model in which spontaneously expressed Rta activated LMP-1, and LMP-1 provided a negative-feedback loop to promote a cellular environment in which Rta indirectly contributes to PEL growth without productively reactivating KSHV and lysing the infected cell.

In this system, K-Rta activated LMP-1 in an RBP-Jk-dependent manner, as if it was a KSHV DE gene (Spadavecchia et al., 2010). Although the role of KSHV oncogenes in RtaΔSTAD's growth effect in BC-1 cells has not been examined, it is reasonable to predict a similar contribution of RtaΔSTAD to the growth of singly infected, KSHV+ PEL cells. PEL cells characteristically display spontaneous reactivation in a small percentage of cells. Non-uniform expression of RtaΔSTAD in PEL cells inhibits spontaneous KSHV reactivation (Lukac et al., 1999). K-Rta transactivates many of the KSHV productive cycle, cellular survival and growth

genes. In fact, three of these genes were identified as direct transcriptional targets of K-Rta: vIL-6, vIRF1, and K14 (Bu et al., 2008). K14 is co-transcribed with ORF 74, the viral G-protein coupled receptor (vGPCR; Kirshner et al., 1999). vGPCR negatively regulates Rta expression (Cannon et al., 2006) in a negative-feedback loop, similar to the effect of LMP-1 in co-infected cells.

The plethora of regulators of K-Rta function thus have the potential to influence cellular growth by modulating the progression of reactivation (**Figure 6**). In the examples of LMP-1 and vGPCR, the oncogenes downstream of K-Rta are themselves the negative regulators of K-Rta expression (i.e., the center pathway in **Figure 6**). However, the models proposed in **Figure 6** do not require the K-Rta regulators to be the actual oncogenes. Furthermore, expression of productive cycle oncogenes in the absence of K-Rta could also satisfy a model for unproductive reactivation (Chatterjee et al., 2002; Chang et al., 2005a). The applicability of these models to KSHV infection of endothelial cells is tempting but untested.

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REFERENCES

An, F. Q., Folarin, H. M., Compitello, N., Roth, J., Gerson, S. L., Mccrae, K. R., Fakhari, F. D., Dittmer, D. P., and Renne, R. (2006). Long-term-infected telomerase-immortalized endothelial cells: a model for Kaposi's sarcoma-associated herpesvirus latency in vitro and in vivo. *J. Virol.* 80, 4833–4846.

AuCoin, D. P., Colletti, K. S., Cei, S. A., Papouskova, I., Tarrant, M., and Pari, G. S. (2004). Amplification of the Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 lytic origin of DNA replication is dependent upon a cis-acting AT-rich region and an ORF50 response element and the trans-acting factors ORF50 (K-Rta) and K8 (K-bZIP). *Virology* 318, 542–555.

Bechtel, J. T., Liang, Y., Hvidding, J., and Ganem, D. (2003). Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. *J. Virol.* 77, 6474–6481.

Bello, L., Davison, A., Glenn, M., Whitehouse, A., Rethmeier, N., Schulz, T., and Clements, J. (1999). The human herpesvirus-8 ORF57 gene and its properties. *J. Gen. Virol.* 80, 3207–3215.

Borah, S., Darricarrere, N., Darnell, A., Myoung, J., and Steitz, J. A. (2011). A viral nuclear noncoding RNA binds re-localized poly(A) binding protein and is required for late KSHV gene expression. *PLoS Pathog.* 7, e1002300. doi:10.1371/journal.ppat.1002300

Boshoff, C., Gao, S.-J., Healy, L., Matthews, T., Thomas, A., Coignet, L., Warnke, R., Strauchen, J., Matutes, E., Kamel, O., Moore, P., Weiss, R., and Chang, Y. (1998). Establishing a KSHV+ cell line (BCP-1) from peripheral blood and characterizing its growth in Nod/SCID mice. *Blood* 91, 1671–1679.

Bowser, B. S., Morris, S., Song, M. J., Sun, R., and Damania, B. (2006). Characterization of Kaposi's sarcoma-associated herpesvirus (KSHV) K1 promoter activation by Rta. *Virology* 348, 309–327.

Boyne, J. R., Colgan, K. J., and Whitehouse, A. (2008). Recruitment of the complete hTREX complex is required for Kaposi's sarcoma-associated herpesvirus intron less mRNA nuclear export and virus replication. *PLoS Pathog.* 4, e1000194. doi:10.1371/journal.ppat.1000194

Boyne, J. R., Jackson, B. R., Taylor, A., Macnab, S. A., and Whitehouse, A. (2010a). Kaposi's sarcoma-associated herpesvirus ORF57 protein interacts with PYM to enhance translation of viral intronless mRNAs. *EMBO J.* 29, 1851–1864.

Boyne, J. R., Jackson, B. R., and Whitehouse, A. (2010b). ORF57: master regulator of KSHV mRNA biogenesis. *Cell Cycle* 9, 2702–2703.

Brown, H. J., Song, M. J., Deng, H., Wu, T. T., Cheng, G., and Sun, R. (2003). NF-kappaB inhibits gammaherpesvirus lytic replication. *J. Virol.* 77, 8532–8540.

Bu, W., Carroll, K. D., Palmeri, D., and Lukac, D. M. (2007). The Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8 ORF50/Rta lytic switch protein functions as a tetramer. *J. Virol.* 81, 5788–5806.

Bu, W., Palmeri, D., Krishnan, R., Marin, R., Aris, V. M., Soteropoulous, P., and Lukac, D. M. (2008). Identification of direct transcriptional targets of the KSHV Rta lytic switch protein by conditional nuclear localization. *J. Virol.* 82, 10709–10723.

Budt, M., Hristozova, T., Hille, G., Berger, K., and Brune, W. (2011). Construction of a lytically replicating Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 85, 10415–10420.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B., and Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5570–5575.

Cannon, M., Cesarman, E., and Boshoff, C. (2006). KSHV G protein-coupled receptor inhibits lytic gene transcription in primary-effusion lymphoma cells via p21-mediated inhibition of Cdk2. *Blood* 107, 277–284.

Carroll, K. D., Bu, W., Palmeri, D., Spadavecchia, S., Lynch, S. J., Marras, S. A., Tyagi, S., and Lukac, D. M. (2006). Kaposi's sarcoma-associated herpesvirus lytic switch protein stimulates DNA binding of RBP-Jk/CSL to activate the Notch pathway. *J. Virol.* 80, 9697–9709.

Carroll, K. D., Khadim, F., Spadavecchia, S., Palmeri, D., and Lukac, D. M. (2007). Direct interactions of KSHV/HHV-8 ORF50/Rta protein with the cellular protein Octamer-1 and DNA are critical for specifying transactivation of a delayed-early promoter and stimulating viral reactivation. *J. Virol.* 81, 8451–8467.

Cesarman, E., Moore, P. S., Rao, P. H., Inghirami, G., Knowles, D. M., and Chang, Y. (1995). In vitro establishment and characterization of two acquired immunodeficiency syndrome-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. *Blood* 86, 2708–2714.

Chandriani, S., and Ganem, D. (2010). Array-based transcript profiling and limiting-dilution reverse transcription-PCR analysis identify additional latent genes in Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 84, 5565–5573.

Chandriani, S., Xu, Y., and Ganem, D. (2010). The lytic transcriptome of Kaposi's sarcoma-associated herpesvirus reveals extensive transcription of noncoding regions, including regions antisense to important genes. *J. Virol.* 84, 7934–7942.

Chang, H., Dittmer, D. P., Chul, S. Y., Hong, Y., and Jung, J. U. (2005a). Role of Notch signal transduction in Kaposi's sarcoma-associated herpesvirus gene expression. *J. Virol.* 79, 14371–14382.

Chang, H., Gwack, Y., Kingston, D., Souvlis, J., Liang, X., Means, R. E., Cesarman, E., Hutt-Fletcher, L., and Jung, J. U. (2005b). Activation of CD21 and CD23 gene expression by Kaposi's sarcoma-associated herpesvirus RTA. *J. Virol.* 79, 4651–4663.

Chang, P. J., Shedd, D., and Miller, G. (2005c). Two subclasses of Kaposi's sarcoma-associated herpesvirus lytic cycle promoters distinguished by open reading frame 50 mutant proteins that are deficient in binding to DNA. *J. Virol.* 79, 8750–8763.

Chang, P. C., Fitzgerald, L. D., Hsia, D. A., Izumiya, Y., Wu, C. Y., Hsieh, W. P., Lin, S. F., Campbell, M., Lam, K. S., Luciw, P. A., Tepper, C. G., and Kung, H. J. (2011). Histone demethylase JMJD2A regulates Kaposi's sarcoma-associated herpesvirus replication and is targeted by a viral transcriptional factor. *J. Virol.* 85, 3283–3293.

Chang, P. C., Fitzgerald, L. D., Van Geelen, A., Izumiya, Y., Ellison, T. J., Wang, D. H., Ann, D. K., Luciw, P. A., and Kung, H. J. (2009). Kruppel-associated box domain-associated protein-1 as a latency regulator for Kaposi's sarcoma-associated herpesvirus and its modulation by the viral protein kinase. *Cancer Res.* 69, 5681–5689.

Chang, P. J., Boonsiri, J., Wang, S. S., Chen, L. Y., and Miller, G. (2010). Binding of RBP-Jkappa (CSL) protein to the promoter of the Kaposi's sarcoma-associated herpesvirus ORF47 (gL) gene is a critical but not sufficient determinant of transactivation by ORF50 protein. *Virology* 398, 38–48.

Chang, P. J., and Miller, G. (2004). Autoregulation of DNA binding and protein stability of Kaposi's sarcoma-associated herpesvirus ORF50 protein. *J. Virol.* 78, 10657–10673.

Chang, P. J., Shedd, D., Gradoville, L., Cho, M. S., Chen, L. W., Chang, J., and Miller, G. (2002). Open reading frame 50 protein of Kaposi's sarcoma-associated herpesvirus directly activates the viral PAN and K12 genes by binding to related response elements. *J. Virol.* 76, 3168–3178.

Chang, P. J., Shedd, D., and Miller, G. (2008). A mobile functional region of Kaposi's sarcoma-associated herpesvirus ORF50 protein independently regulates DNA binding and protein abundance. *J. Virol.* 82, 9700–9716.

Chang, Y., and Moore, P. S. (2011). Merkel cell carcinoma: a virus-induced human cancer. *Annu. Rev. Pathol.* PMID: 21942528. [Epub ahead of print].

Chatterjee, M., Osborne, J., Bestetti, G., Chang, Y., and Moore, P. S. (2002). Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* 298, 1432–1435.

Chen, J., Ueka, K., Sakakibara, S., Okuno, T., and Yamanishi, K. (2000). Transcriptional regulation of the Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor gene. *J. Virol.* 74, 8623–8634.

Chen, J., Ye, F., Xie, J., Kuhne, K., and Gao, S. J. (2009). Genome-wide identification of binding sites for Kaposi's sarcoma-associated herpesvirus lytic switch protein, RTA. *Virology* 386, 290–302.

Conrad, N. K. (2009). Posttranscriptional gene regulation in Kaposi's sarcoma-associated herpesvirus. *Adv. Appl. Microbiol.* 68, 241–261.

Damania, B., Jeong, J. H., Bowser, B. S., Dewire, S. M., Staudt, M. R., and Dittmer, D. P. (2004). Comparison of the Rta/Orf50 transactivator proteins of gamma-2-herpesviruses. *J. Virol.* 78, 5491–5499.

Deng, H., Young, A., and Sun, R. (2000). Auto-activation of the rta gene of human herpesvirus-8/Kaposi's sarcoma-associated herpesvirus. *J. Gen. Virol.* 81, 3043–3048.

Di Bartolo, D. L., Hyjek, E., Keller, S., Guasparri, I., Deng, H., Sun, R., Chadburn, A., Knowles, D. M., and Cesarman, E. (2009). Role of defective Oct-2 and OCA-B expression in immunoglobulin production and Kaposi's sarcoma-associated herpesvirus lytic reactivation in primary effusion lymphoma. *J. Virol.* 83, 4308–4315.

Drexler, H., Uphoff, C., Gaidano, G., and Carbone, A. (1998). Lymphoma cell lines: in vitro models for the study of HHV-8+ primary effusion lymphomas (body cavity-based lymhomas). *Leukemia* 12, 1507–1517.

Duan, W., Wang, S., Liu, S., and Wood, C. (2001). Characterization of Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8 ORF57 promoter. *Arch. Virol.* 146, 403–413.

Ellison, T. J., Izumiya, Y., Izumiya, C., Luciw, P. A., and Kung, H. J. (2009). A comprehensive analysis of recruitment and transactivation potential of K-Rta and K-bZIP during reactivation of Kaposi's sarcoma-associated herpesvirus. *Virology* 387, 76–88.

Fakhari, F. D., and Dittmer, D. P. (2002). Charting latency transcripts in Kaposi's sarcoma-associated herpesvirus by whole-genome real-time quantitative PCR. *J. Virol.* 76, 6213–6223.

Ganem, D. (2007). "Kaposi's sarcoma-associated herpesvirus," in *Fields Virology*, eds D. M. Knipe and P. M. Howley (Philadelphia: Lippincott Williams and Wilkins), 2848–2888.

Gradoville, L., Gerlach, J., Grogan, E., Shedd, D., Nikiforow, S., Metroka, C., and Miller, G. (2000). Kaposi's sarcoma-associated herpesvirus open reading frame 50/Rta protein activates the entire lytic cycle in the HH-B2 primary effusion lymphoma cell line. *J. Virol.* 74, 6207–6212.

Grundhoff, A., and Ganem, D. (2004). Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. *J. Clin. Invest.* 113, 124–136.

Gunther, T., and Grundhoff, A. (2010). The epigenetic landscape of latent Kaposi sarcoma-associated herpesvirus genomes. *PLoS Pathog.* 6, e1000935. doi:10.1371/journal.ppat.1000935

Gupta, A. K., Ruvolo, V., Patterson, C., and Swaminathan, S. (2000). The human herpesvirus 8 homolog of Epstein-Barr virus SM protein (KS-SM) is a posttranscriptional activator of gene expression. *J. Virol.* 74, 1038–1044.

Gwack, Y., Hwang, S., Byun, H., Lim, C., Kim, J. W., Choi, E. J., and Choe, J. (2001). Kaposi's sarcoma-associated herpesvirus open reading frame 50 represses p53-induced transcriptional activity and apoptosis. *J. Virol.* 75, 6245–6248.

Gwack, Y., Hwang, S., Lim, C., Won, Y. S., Lee, C. H., and Choe, J. (2002). Kaposi's sarcoma-associated herpesvirus open reading frame 50 stimulates the transcriptional activity of STAT3. *J. Biol. Chem.* 277, 6438–6442.

Gwack, Y., Nakamura, H., Lee, S. H., Souvlis, J., Yustein, J. T., Gygi, S., Kung, H. J., and Jung, J. U. (2003). Poly(ADP-ribose) polymerase 1 and Ste20-like kinase hKFC act as transcriptional repressors for gamma-2 herpesvirus lytic replication. *Mol. Cell. Biol.* 23, 8282–8294.

Han, Z., and Swaminathan, S. (2006). Kaposi's sarcoma-associated herpesvirus lytic gene ORF57 is essential for infectious virion production. *J. Virol.* 80, 5251–5260.

Harrison, S. M., and Whitehouse, A. (2008). Kaposi's sarcoma-associated herpesvirus (KSHV) Rta and cellular HMGB1 proteins synergistically transactivate the KSHV ORF50 promoter. *FEBS Lett.* 582, 3080–3084.

He, Z., Liu, Y., Liang, D., Wang, Z., Robertson, E. S., and Lan, K. (2010). Cellular corepressor TLE2 inhibits replication-and-transcription-activator-mediated transactivation and lytic reactivation of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 84, 2047–2062.

Izumiya, Y., Izumiya, C., Hsia, D., Ellison, T. J., Luciw, P. A., and Kung, H. J. (2009). NF-kappaB serves as a cellular sensor of Kaposi's sarcoma-associated herpesvirus latency and negatively regulates K-Rta by antagonizing the RBP-Jkappa coactivator. *J. Virol.* 83, 4435–4446.

Jackson, B. R., Boyne, J. R., Noerenberg, M., Taylor, A., Hautbergue, G. M., Walsh, M. J., Wheat, R., Blackbourn, D. J., Wilson, S. A., and Whitehouse, A. (2011). An interaction between KSHV ORF57 and UIF provides mRNA-adaptor redundancy in herpesvirus intronless mRNA export. *PLoS Pathog.* 7, e1002138. doi:10.1371/journal.ppat.1002138

Jenner, R., Alba, M., Boshoff, C., and Kellam, P. (2001). Kaposi's sarcoma-associated herpesvirus latent and lytic gene expression as revealed by DNA arrays. *J. Virol.* 75, 891–902.

Kang, H., Wiedmer, A., Yuan, Y., Robertson, E., and Lieberman, P. M. (2011a). Coordination of KSHV latent and lytic gene control by CTCF-cohesin mediated chromosome conformation. *PLoS Pathog.* 7, e1002140. doi:10.1371/journal.ppat.1002140

Kang, J. G., Pripuzova, N., Majerciak, V., Kruhlak, M., Le, S. Y., and Zheng, Z. M. (2011b). Kaposi's sarcoma-associated herpesvirus ORF57 promotes escape of viral and human interleukin-6 from microRNA-mediated suppression. *J. Virol.* 85, 2620–2630.

Kato-Noah, T., Xu, Y., Rossetto, C. C., Colletti, K., Papouskova, I., and Pari, G. S. (2007). Overexpression of the kaposi's sarcoma-associated herpesvirus transactivator K-Rta can complement a K-bZIP deletion BACmid and yields an enhanced growth phenotype. *J. Virol.* 81, 13519–13532.

Kieff, E., and Rickinson, A. (2007). "Epstein-barr virus and its replication," in *Fields Virology*, 5th Edn. eds D. M. Knipe and P. M. Howley (Philadelphia, PA: Lippincott Williams and Wilkins), 2603–2654.

Kirshner, J. R., Lukac, D. M., Chang, J., and Ganem, D. (2000). Kaposi's sarcoma-associated herpesvirus open reading frame 57 encodes a posttranscriptional regulator with multiple distinct activities. *J. Virol.* 74, 3586–3597.

Kirshner, J. R., Staskus, K., Haase, A., Lagunoff, M., and Ganem, D. (1999). Expression of the open reading frame 74 (G-protein-coupled receptor) gene of Kaposi's sarcoma (KS)-associated herpesvirus; implications for KS pathogenesis. *J. Virol.* 73, 6006–6014.

Lan, K., Kuppers, D. A., and Robertson, E. S. (2005a). Kaposi's sarcoma-associated herpesvirus reactivation is regulated by interaction of latency-associated nuclear antigen with recombination signal sequence-binding protein Jkappa, the major downstream effector of the Notch signaling pathway. *J. Virol.* 79, 3468–3478.

Lan, K., Kuppers, D. A., Verma, S. C., Sharma, N., Murakami, M., and Robertson, E. S. (2005b). Induction of Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen by the lytic transactivator RTA: a novel mechanism for establishment of latency. *J. Virol.* 79, 7453–7465.

Lan, K., Kuppers, D. A., Verma, S. C., and Robertson, E. S. (2004). Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen inhibits lytic replication by targeting Rta: a potential mechanism for virus-mediated control of latency. *J. Virol.* 78, 6585–6594.

Liang, Y., Chang, J., Lynch, S., Lukac, D. M., and Ganem, D. (2002). The lytic switch protein of KSHV activates gene expression via functional interaction with RBP-Jk, the target of the Notch signaling pathway. *Genes Dev.* 16, 1977–1989.

Liang, Y., and Ganem, D. (2003). Lytic but not latent infection by Kaposi's sarcoma-associated herpesvirus requires host CSL protein,

the mediator of Notch signaling. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8490–8495.

Liang, Y., and Ganem, D. (2004). RBP-J (CSL) is essential for activation of the K14/vGPCR promoter of Kaposi's sarcoma-associated herpesvirus by the lytic switch protein RTA. *J. Virol.* 78, 6818–6826.

Liao, W., Tang, Y., Kuo, Y. L., Liu, B. Y., Xu, C. J., and Giam, C. Z. (2003a). Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 transcriptional activator Rta is an oligomeric DNA-binding protein that interacts with tandem arrays of phased A/T-trinucleotide motifs. *J. Virol.* 77, 9399–9411.

Liao, W., Tang, Y., Lin, S. F., Kung, H. J., and Giam, C. Z. (2003b). K-bZIP of Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8) binds KSHV/HHV-8 Rta and represses Rta-mediated transactivation. *J. Virol.* 77, 3809–3815.

Liu, Y., Cao, Y., Liang, D., Gao, Y., Xia, T., Robertson, E. S., and Lan, K. (2008). Kaposi's sarcoma-associated herpesvirus RTA activates the processivity factor ORF59 through interaction with RBP-Jkappa and a cis-acting RTA responsive element. *Virology* 380, 264–275.

Lu, M., Suen, J., Frias, C., Pfeiffer, R., Tsai, M. H., Chuang, E., and Zeichner, S. L. (2004). Dissection of the Kaposi's sarcoma-associated herpesvirus gene expression program by using the viral DNA replication inhibitor cidofovir. *J. Virol.* 78, 13637–13652.

Lukac, D., Garibyan, L., Kirshner, J., Palmeri, D., and Ganem, D. (2001). DNA binding by the Kaposi's sarcoma-associated herpesvirus lytic switch protein is necessary for transcriptional activation of two viral delayed early promoters. *J. Virol.* 75, 6786–6799.

Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999). Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. *J. Virol.* 73, 9348–9361.

Lukac, D. M., Renne, R., Kirshner, J. R., and Ganem, D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. *Virology* 252, 304–312.

Majerciak, V., Pripuzova, N., Mccoy, J. P., Gao, S. J., and Zheng, Z. M. (2006a). Targeted disruption of KSHV ORF57 in the viral genome is detrimental for the expression of ORF59, K8{alpha}, and K8.1 and the production of infectious virus. *J. Virol.* 81, 1062–1071.

Majerciak, V., Yamanegi, K., Nie, S. H., and Zheng, Z. M. (2006b). Structural and functional analyses of Kaposi sarcoma-associated herpesvirus ORF57 nuclear localization signals in living cells. *J. Biol. Chem.* 281, 28365–28378.

Majerciak, V., Yamanegi, K., and Zheng, Z. M. (2006c). Gene structure and expression of Kaposi's sarcoma-associated herpesvirus ORF56, ORF57, ORF58, and ORF59. *J. Virol.* 80, 11968–11981.

Majerciak, V., Pripuzova, N., Mccoy, J. P., Gao, S. J., and Zheng, Z. M. (2007). Targeted disruption of Kaposi's sarcoma-associated herpesvirus ORF57 in the viral genome is detrimental for the expression of ORF59, K8alpha, and K8.1 and the production of infectious virus. *J. Virol.* 81, 1062–1071.

Majerciak, V., Uranishi, H., Kruhlak, M., Pilkington, G. R., Massimelli, M. J., Bear, J., Pavlakis, G. N., Felber, B. K., and Zheng, Z. M. (2011). Kaposi's sarcoma-associated herpesvirus ORF57 interacts with cellular RNA export cofactors RBM15 and OTT3 to promote expression of viral ORF59. *J. Virol.* 85, 1528–1540.

Majerciak, V., Yamanegi, K., Allemand, E., Kruhlak, M., Krainer, A. R., and Zheng, Z. M. (2008). Kaposi's sarcoma-associated herpesvirus ORF57 functions as a viral splicing factor and promotes expression of intron-containing viral lytic genes in spliceosome-mediated RNA splicing. *J. Virol.* 82, 2792–2801.

Majerciak, V., and Zheng, Z. M. (2009). Kaposi's sarcoma-associated herpesvirus ORF57 in viral RNA processing. *Front. Biosci.* 14, 1516–1528.

Malik, P., Blackbourn, D. J., Cheng, M. F., Hayward, G. S., and Clements, J. B. (2004a). Functional co-operation between the Kaposi's sarcoma-associated herpesvirus ORF57 and ORF50 regulatory proteins. *J. Gen. Virol.* 85, 2155–2166.

Malik, P., Blackbourn, D. J., and Clements, J. B. (2004b). The evolutionarily conserved Kaposi's sarcoma-associated herpesvirus ORF57 protein interacts with REF protein and acts as an RNA export factor. *J. Biol. Chem.* 279, 33001–33011.

Martin, D., and Gutkind, J. S. (2008). Human tumor-associated viruses and new insights into the molecular mechanisms of cancer. *Oncogene* 27(Suppl. 2), S31–S42.

Massimelli, M. J., Kang, J. G., Majerciak, V., Le, S. Y., Liewehr, D. J., Steinberg, S. M., and Zheng, Z. M. (2011). Stability of a long noncoding viral RNA depends on a 9-nt core element at the RNA 5′ end to interact with viral ORF57 and cellular PABPC1. *Int. J. Biol. Sci.* 7, 1145–1160.

Mesri, E. A., Cesarman, E., and Boshoff, C. (2010). Kaposi's sarcoma and its associated herpesvirus. *Nat. Rev. Cancer* 10, 707–719.

Moody, C. A., and Laimins, L. A. (2010). Human papillomavirus oncoproteins: pathways to transformation. *Nat. Rev. Cancer* 10, 550–560.

Moore, P. S., Gao, S. J., Dominguez, G., Cesarman, E., Lungu, O., Knowles, D. M., Garber, R., Pellett, P. E., Mcgeoch, D. J., and Chang, Y. (1996). Primary characterization of a herpesvirus agent associated with Kaposi's sarcomae. *J. Virol.* 70, 549–558.

Nakamura, H., Lu, M., Gwack, Y., Souvlis, J., Zeichner, S. L., and Jung, J. U. (2003). Global changes in Kaposi's sarcoma-associated virus gene expression patterns following expression of a tetracycline-inducible Rta transactivator. *J. Virol.* 77, 4205–4220.

Nekorchuk, M., Han, Z., Hsieh, T. T., and Swaminathan, S. (2007). Kaposi's sarcoma-associated herpesvirus ORF57 protein enhances mRNA accumulation independently of effects on nuclear RNA export. *J. Virol.* 81, 9990–9998.

Nishimura, K., Ueda, K., Guwanan, E., Sakakibara, S., Do, E., Osaki, E., Yada, K., Okuno, T., and Yamanishi, K. (2004). A posttranscriptional regulator of Kaposi's sarcoma-associated herpesvirus interacts with RNA-binding protein PCBP1 and controls gene expression through the IRES. *Virology* 325, 364–378.

Osada, S., Yamamoto, H., Nishihara, T., and Imagawa, M. (1996). DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. *J. Biol. Chem.* 271, 3891–3896.

Palmeri, D., Carroll, K. D., Gonzalez-Lopez, O., and Lukac, D. M. (2011). Kaposi's sarcoma-associated herpesvirus Rta tetramers make high-affinity interactions with repetitive DNA elements in the Mta promoter to stimulate DNA binding of RBP-Jk/CSL. *J. Virol.* 85, 11901–11915.

Palmeri, D., Spadavecchia, S., Carroll, K., and Lukac, D. M. (2007). Promoter and cell-specific transcriptional activation by the Kaposi's sarcoma-associated herpesvirus ORF57/Mta protein. *J. Virol.* 81, 13299–13314.

Paulose-Murphy, M., Ha, N.-K., Xiang, C., Chen, Y., Gillim, L., Yarchoan, R., Meltzer, P., Bittner, M., Trent, J., and Zeichner, S. (2001). Transcription program of Human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus). *J. Virol.* 75, 4843–4853.

Persson, L. M., and Wilson, A. C. (2010). Wide-scale use of Notch signaling factor CSL/RBP-Jkappa in RTA-mediated activation of Kaposi's sarcoma-associated herpesvirus lytic genes. *J. Virol.* 84, 1334–1347.

Renne, R., Blackbourn, D., Whitby, D., Levy, J., and Ganem, D. (1998). Limited transmission of Kaposi's sarcoma-associated herpesvirus in cultured cells. *J. Virol.* 72, 5182–5188.

Renne, R., Zhong, W., Herndier, B., Mcgrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat. Med.* 2, 342–346.

Rickinson, A., and Kieff, E. (2007). "Epstein-barr virus," in *Fields Virology*, eds D. M. Knipe and P. M. Howley (Philadelphia, PA: Lippincott Williams and Wilkins), 2656–2700.

Rimessi, P., Bonaccorsi, A., Sturzl, M., Fabris, M., Brocca-Cofano, E., Caputo, A., Melucci-Vigo, G., Falchi, M., Cafaro, A., Cassai, E., Ensoli, B., and Monini, P. (2001). Transcription pattern of human herpesvirus 8 open reading frame K3 in primary effusion lymphoma and Kaposi's sarcoma. *J. Virol.* 75, 7161–7174.

Roan, F., Inoue, N., and Offermann, M. K. (2002). Activation of cellular and heterologous promoters by the human herpesvirus 8 replication and transcription activator. *Virology* 301, 293–304.

Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc. Natl. Acad. Sci. U.S.A.* 93, 14862–14867.

Sahin, B. B., Patel, D., and Conrad, N. K. (2010). Kaposi's sarcoma-associated herpesvirus ORF57 protein binds and protects a nuclear noncoding RNA from cellular RNA decay pathways. *PLoS Pathog.* 6, e1000799. doi:10.1371/journal.ppat.1000799

Sakakibara, S., Ueda, K., Chen, J., Okuno, T., and Yamanishi, K. (2001). Octamer-binding sequence is a key element for the

autoregulation of Kaposi's sarcoma-associated herpesvirus ORF50/Lyta gene expression. *J. Virol.* 75, 6894–6800.

Samols, M. A., Hu, J., Skalsky, R. L., and Renne, R. (2005). Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79, 9301–9305.

Sarid, R., Flore, O., Bohenzky, R. A., Chang, Y., and Moore, P. S. (1998). Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). *J. Virol.* 72, 1005–1012.

Seaman, W., Ye, D., Wang, R., Hale, E., Weisse, M., and Quinlivan, E. (1999). Gene expression from the ORF50/K8 region of Kaposi's sarcoma-associated herpesvirus. *Virology* 263, 436–449.

Sei, E., and Conrad, N. K. (2011). Delineation of a core RNA element required for Kaposi's sarcoma-associated herpesvirus ORF57 binding and activity. *Virology* 419, 107–116.

Song, M., Brown, H., Wu, T.-T., and Sun, R. (2001). Transcription activation of polyadenylated nuclear RNA by Rta in human herpesvirus 8/Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 75, 3129–3140.

Song, M. J., Deng, H., and Sun, R. (2003). Comparative study of regulation of RTA-responsive genes in Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8. *J. Virol.* 77, 9451–9462.

Song, M. J., Hwang, S., Wong, W., Round, J., Martinez-Guzman, D., Turpaz, Y., Liang, J., Wong, B., Johnson, R. C., Carey, M., and Sun, R. (2004). The DNA architectural protein HMGB1 facilitates RTA-mediated viral gene expression in gamma-2 herpesviruses. *J. Virol.* 78, 12940–12950.

Song, M. J., Li, X., Brown, H. J., and Sun, R. (2002). Characterization of interactions between RTA and the promoter of polyadenylated nuclear RNA in Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8. *J. Virol.* 76, 5000–5013.

Spadavecchia, S., Gonzalez-Lopez, O., Carroll, K. D., Palmeri, D, and Lukac, D. M. (2010). Convergence of Kaposi's sarcoma-associated herpesvirus reactivation with Epstein-Barr virus latency and cellular growth mediated by the notch signaling pathway in coinfected cells. *J. Virol.* 84, 10488–10500.

Staskus, K. A., Sun, R., Miller, G., Racz, P., Jaslowski, A., Metroka, C., Brett-Smith, H., and Haase, A. T. (1999). Cellular tropism and viral interleukin-6 expression distinguish human herpesvirus 8 involvement in Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. *J. Virol.* 73, 4181–4187.

Staskus, K. A., Zhong, W., Gebhard, K., Herndier, B., Wang, H., Renne, R., Beneke, J., Pudney, J., Anderson, D. J., Ganem, D., and Haase, A. T. (1997). Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. *J. Virol.* 71, 715–719.

Sun, R., Lin, S., Gradoville, L., and Miller, G. (1996). Polyadenylated nuclear RNA encoded by Kaposi's sarcoma-associated herpesvirus. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11883–11888.

Sun, R., Lin, S. F., Gradoville, L., Yuan, Y., Zhu, F., and Miller, G. (1998). A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10866–10871.

Sun, R., Lin, S.-F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., and Miller, G. (1999). Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. *J. Virol.* 73, 2232–2242.

Swaminathan, S. (2005). Post-transcriptional gene regulation by gamma herpesviruses. *J. Cell. Biochem.* 95, 698–711.

Tanigaki, K., and Honjo, T. (2010). Two opposing roles of RBP-J in Notch signaling. *Curr. Top. Dev. Biol.* 92, 231–252.

Taylor, A., Jackson, B. R., Noerenberg, M., Hughes, D. J., Boyne, J. R., Verow, M., Harris, M., and Whitehouse, A. (2011). Mutation of a C-terminal motif affects Kaposi's sarcoma-associated herpesvirus ORF57 RNA binding, nuclear trafficking, and multimerization. *J. Virol.* 85, 7881–7891.

Toth, Z., Maglinte, D. T., Lee, S. H., Lee, H. R., Wong, L. Y., Brulois, K. F., Lee, S., Buckley, J. D., Laird, P. W., Marquez, V. E., and Jung, J. U. (2010). Epigenetic analysis of KSHV latent and lytic genomes. *PLoS Pathog.* 6, e1001013. doi:10.1371/journal.ppat.1001013

Ueda, K., Ishikawa, K., Nishimura, K., Sakakibara, S., Do, E., and Yamanishi, K. (2002). Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) replication and transcription factor activates the K9 (vIRF) gene through two distinct cis elements by a non-DNA-binding mechanism. *J. Virol.* 76, 12044–12054.

Vieira, J., and O'Hearn, P. M. (2004). Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. *Virology* 325, 225–240.

Wang, J., Zhang, J., Zhang, L., Harrington, W. Jr., West, J. T., and Wood, C. (2005). Modulation of human herpesvirus 8/Kaposi's sarcoma-associated herpesvirus replication and transcription activator transactivation by interferon regulatory factor 7. *J. Virol.* 79, 2420–2431.

Wang, S., Liu, S., Wu, M., Geng, Y., and Wood, C. (2001a). Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8 ORF50 gene product contains a potent C-terminal activation domain which activates gene expression via a specific target sequence. *Arch. Virol.* 146, 1415–1426.

Wang, S., Liu, S., Wu, M. H., Geng, Y., and Wood, C. (2001b). Identification of a cellular protein that interacts and synergizes with the RTA (ORF50) protein of Kaposi's sarcoma-associated herpesvirus in transcriptional activation. *J. Virol.* 75, 11961–11973.

Wang, S. E., Wu, F. Y., Chen, H., Shamay, M., Zheng, Q., and Hayward, G. S. (2004a). Early activation of the Kaposi's sarcoma-associated herpesvirus RTA, RAP, and MTA promoters by the tetradecanoyl phorbol acetate-induced AP1 pathway. *J. Virol.* 78, 4248–4267.

Wang, Y., Li, H., Chan, M. Y., Zhu, F. X., Lukac, D. M., and Yuan, Y. (2004b). Kaposi's sarcoma-associated herpesvirus ori-Lyt-dependent DNA replication: cis-acting requirements for replication and ori-Lyt-associated RNA transcription. *J. Virol.* 78, 8615–8629.

Wang, S. E., Wu, F. Y., Fujimuro, M., Zong, J., Hayward, S. D., and Hayward, G. S. (2003a). Role of CCAAT/enhancer-binding protein alpha (C/EBPalpha) in activation of the Kaposi's sarcoma-associated herpesvirus (KSHV) lytic-cycle replication-associated protein (RAP) promoter in cooperation with the KSHV replication and transcription activator (RTA) and RAP. *J. Virol.* 77, 600–623.

Wang, S. E., Wu, F. Y., Yu, Y., and Hayward, G. S. (2003b). CCAAT/enhancer-binding protein-alpha is induced during the early stages of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic cycle reactivation and together with the KSHV replication and transcription activator (RTA) cooperatively stimulates the viral RTA, MTA, and PAN promoters. *J. Virol.* 77, 9590–9612.

Wang, S. S., Chen, L. W., Chen, L. Y., Tsai, H. H., Shih, Y. C., Yang, C. T., and Chang, P. J. (2010). Transcriptional regulation of the ORF61 and ORF60 genes of Kaposi's sarcoma-associated herpesvirus. *Virology* 397, 311–321.

Wang, Y., Tang, Q., Maul, G. G., and Yuan, Y. (2006). Kaposi's sarcoma-associated herpesvirus ori-Lyt-dependent DNA replication: dual role of replication and transcription activator. *J. Virol.* 80, 12171–12186.

Wang, Y., and Yuan, Y. (2006). Essential role of RBP-Jkappa in activation of the K8 delayed-early promoter of Kaposi's sarcoma-associated herpesvirus by ORF50/RTA. *Virology* 359, 19–27.

Wen, H. J., Minhas, V., and Wood, C. (2009). Identification and characterization of a new Kaposi's sarcoma-associated herpesvirus replication and transcription activator (RTA)-responsive element involved in RTA-mediated transactivation. *J. Gen. Virol.* 90, 944–953.

Wilson, S. J., Tsao, E. H., Webb, B. L., Ye, H., Dalton-Griffin, L., Tsantoulas, C., Gale, C. V., Du, M. Q., Whitehouse, A., and Kellam, P. (2007). X box binding protein XBP-1s transactivates the Kaposi's sarcoma-associated herpesvirus (KSHV) ORF50 promoter, linking plasma cell differentiation to KSHV reactivation from latency. *J. Virol.* 81, 13578–13586.

Xu, D., Coleman, T., Zhang, J., Fagot, A., Kotalik, C., Zhao, L., Trivedi, P., Jones, C., and Zhang, L. (2007). Epstein-barr virus inhibits Kaposi's sarcoma-associated herpesvirus lytic replication in primary effusion lymphomas. *J. Virol.* 81, 6068–6078.

Xu, Y., Aucoin, D. P., Huete, A. R., Cei, S. A., Hanson, L. J., and Pari, G. S. (2005). A Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 ORF50 deletion mutant is defective for reactivation of latent virus and DNA replication. *J. Virol.* 79, 3479–3487.

Xu, Y., and Ganem, D. (2010). Making sense of antisense: seemingly noncoding RNAs antisense to the master regulator of Kaposi's sarcoma-associated herpesvirus lytic replication do not regulate that transcript but serve as mRNAs

encoding small peptides. *J. Virol.* 84, 5465–5475.

Yada, K., Do, E., Sakakibara, S., Ohsaki, E., Ito, E., Watanabe, S., and Ueda, K. (2006). KSHV RTA induces a transcriptional repressor, HEY1 that represses rta promoter. *Biochem. Biophys. Res. Commun.* 345, 410–418.

Yang, Z., Wen, H. J., Minhas, V., and Wood, C. (2009). The zinc finger DNA-binding domain of K-RBP plays an important role in regulating Kaposi's sarcoma-associated herpesvirus RTA-mediated gene expression. *Virology* 391, 221–231.

Yang, Z., and Wood, C. (2007). The transcriptional repressor K-RBP modulates RTA-mediated transactivation and lytic replication of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 81, 6294–6306.

Yang, Z., Yan, Z., and Wood, C. (2008). Kaposi's sarcoma-associated herpesvirus transactivator RTA promotes degradation of the repressors to regulate viral lytic replication. *J. Virol.* 82, 3590–3603.

Yu, Y., Wang, S. E., and Hayward, G. S. (2005). The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteosome-mediated degradation. *Immunity* 22, 59–70.

Zhang, J., Wang, J., Wood, C., Xu, D., and Zhang, L. (2005). Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 replication and transcription activator regulates viral and cellular genes via interferon-stimulated response elements. *J. Virol.* 79, 5640–5652.

Zhang, L., Chiu, J., and Lin, J. C. (1998). Activation of human herpesvirus 8 (HHV-8) thymidine kinase (TK) TATAA-less promoter by HHV-8 ORF50 gene product is SP1 dependent. *DNA Cell Biol.* 17, 735–742.

Zhong, W., Wang, H., Herndier, B., and Ganem, D. (1996). Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. *Proc. Natl. Acad. Sci. U.S.A.* 93, 6641–6646.

Zhu, F., Cusano, T., and Yuan, Y. (1999). Identification of the immediate-early transcripts of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 73, 5556–5567.

Ziegelbauer, J., Grundhoff, A., and Ganem, D. (2006). Exploring the DNA binding interactions of the Kaposi's sarcoma-associated herpesvirus lytic switch protein by selective amplification of bound sequences in vitro. *J. Virol.* 80, 2958–2967.

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Ser-634 and Ser-636 of Kaposi's sarcoma-associated herpesvirus RTA are involved in transactivation and are potential CDK9 phosphorylation sites

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The replication and transcription activator (RTA) of Kaposi's sarcoma-associated herpesvirus (KSHV), K-RTA, is a lytic switch protein that moderates the reactivation process of KSHV latency. By mass spectrometric analysis of affinity purified K-RTA, we showed that Thr-513 or Thr-514 was the primary *in vivo* phosphorylation site. Thr-513 and Thr-514 are proximal to the nuclear localization signal (^{527}KKRK^{530}) and were previously hypothesized to be target sites of Ser/Thr kinase hKFC. However, substitutions of Thr with Ala at 513 and 514 had no effect on K-RTA subcellular localization or transactivation activity. By contrast, replacement of Ser with Ala at Ser-634 and Ser-636 located in a Ser/Pro-rich region of K-RTA, designated as S634A/S636A, produced a polypeptide with ∼10 kDa shorter in molecular weight and reduced transactivation in a luciferase reporter assay relative to the wild type. In contrast to prediction, the decrease in molecular weight was not due to lack of phosphorylation because the overall Ser and Thr phosphorylation state in K-RTA and S634A/S636A were similar, excluding that Ser-634 or Ser-636 motif served as docking sites for consecutive phosphorylation. Interestingly, S634A/S636A lost ∼30% immuno-reactivity to MPM2, an antibody specific to pSer/pThr-Pro motif, indicating that ^{634}SPSP^{637} motif was *in vivo* phosphorylated. By *in vitro* kinase assay, we showed that K-RTA is a substrate of CDK9, a Pro-directed Ser/Thr kinase central to transcriptional regulation. Importantly, the capability of K-RTA in associating with endogenous CDK9 was reduced in S634A/S636A, which suggested that Ser-634 and Ser-636 may be involved in CDK9 recruitment. In agreement, S634A/S636A mutant exhibited ∼25% reduction in KSHV lytic cycle reactivation relative to that by the wild type K-RTA. Taken together, our data propose that Ser-634 and Ser-636 of K-RTA are phosphorylated by host transcriptional kinase CDK9 and such a process contributes to a full transcriptional potency of K-RTA.

Keywords: Kaposi's sarcoma-associated herpesvirus, replication and transcription activator, phosphorylation, negative elongation factor B, CDK9

INTRODUCTION

In a cell latently infected with Kaposi's sarcoma-associated herpesvirus (KSHV), the switch to viral lytic replication can be achieved via signals transmitted from interferon-γ, phorbol ester, HDAC inhibitors, or with the ectopic expression of KSHV replication and transcription activator, referred to as K-RTA (also known as ORF50 and Lyta, reviewed in Ganem, 2007). Consecutive expression of KSHV lytic genes induced by either chemicals or K-RTA has been previously demonstrated (Sarid et al., 1998; Lukac et al., 1999; Sun et al., 1999; Gradoville et al., 2000; Fakhari and Dittmer, 2002; Nakamura et al., 2003; Yoo et al., 2005). In addition, the combination of sodium butyrate plus ectopic K-RTA yielded synergistic effects in an HEK293 cell background (Vieira and O'Hearn, 2004). K-RTA interacts with various cellular molecules involved in the transcription process. These include transcription factors (POU2F1/Oct-1, Sakakibara et al., 2001; STAT3, Gwack et al., 2002; RBPJ/CSL, Liang and Ganem, 2003; C/EBPα, Wang et al., 2003; ZNF426/K-RBP, Yang and Wood, 2007) and chromatin modifiers/remodelers (CBP, HDAC1, MED12/TRAP230, SMARCA4; Gwack et al., 2001, 2003a). Depending on the cellular context and promoters, association of these host factors results in either activation or repression of K-RTA transactivation activity. It bears noting that K-RTA-mediated gene regulation is not limited to the KSHV genome; a number of cellular genes are also modulated by K-RTA, as evidenced by genome-wide transcriptome analysis (Chang et al., 2005; Brown et al., 2010). Either on viral or host genomes, K-RTA requires host RNA polymerase II (RNA Pol II) for mRNAs synthesis since no RNA polymerase homolog was found in the KSHV genome.

The process of RNA Pol II-directed transcription can be divided into four distinct phases: (1) pre-initiation complex formation, (2) promoter clearance (escape), (3) RNA Pol II stalling at a

promoter-proximal pausing site, and (4) productive elongation (Peterlin and Price, 2006; Saunders et al., 2006). The advancement of the transcription machinery from one stage to the next is tightly regulated by protein phosphorylation. Specifically, promoter clearance depends on CDK7-mediated Ser-5 phosphorylation of the tandem heptapeptide repeats (YSPTSPS) located in the carboxyl terminal domain (CTD) of RNA Pol II. Next, Ser-5 phosphorylated CTD undergoes a conformational change that unmasks the Ser-2 in the heptapeptide repeats (YSPTSPS), which serves to recruit positive transcription elongation factor b (P-TEFb; Lolli, 2009). P-TEFb not only phosphorylates Ser-2 but also phosphorylates negative elongation factor complex (NELF and DSIF) by which RNA Pol II is released from the pausing site and enters the productive elongation stage. Accumulating evidence demonstrate that RNA Pol II pausing is enriched in the 5′ region of genes involved in central regulatory processes, including developmental switches that are required for embryogenesis and rapidly inducible molecules involved in cellular responses to stimuli (Zeitlinger et al., 2007; Gilchrist et al., 2008). Recently, RNA Pol II pausing was also documented in controlling the expressions of viral genes in the KSHV and Epstein–Barr virus (EBV) genomes (Kang and Lieberman, 2011; Palermo et al., 2011).

Because expression of the viral transcriptome is usually RNA Pol II dependent, hijacking P-TEFb activity by viral molecules could be advantageous for viral transcription. The first example was in human immunodeficiency virus type I (HIV-I). P-TEFb is composed of a catalytic subunit (CDK9) and a transcription regulatory cyclin (cyclin T or K). By modifying the substrate specificity of CDK9, HIV-1 Tat increased noticeably the efficiency of the elongation step during HIV-1 transcription (Garber et al., 2000; Zhou et al., 2000). By contrast, CDK9 dominant negative mutants severely impaired HIV-1 replication in cultured human cells (Foskett et al., 2001; Fujinaga et al., 2002). Furthermore, CDK9 was recently shown to complex with ICP22 of herpesvirus type 1 (Durand and Roizman, 2008). The CDK9-bound ICP22 was required for optimal expression of certain viral late genes transcribed by RNA Pol II (Durand and Roizman, 2008). In the case of human cytomegalovirus, recruitment of cyclin T1 and CDK9 by IE2 86 kDa protein was critical to establishing an active immediate-early transcriptome in the first 8 h after viral infection (Kapasi and Spector, 2008; Kapasi et al., 2009). Finally, viral latent promoters activated by nuclear protein of EBV EBNA2 are enriched with the CDK9-mediated, elongated form of RNA Pol II (Bark-Jones et al., 2006).

In addition to moderating components of the RNA Pol II apparatus, phosphorylation of viral factors also contributes to the expression of the viral lytic transcriptome. Phosphorylation of Ser-186 located in the DNA binding domain of EBV BZLF1/ZEBRA is required for the disruption of EBV latency (Francis et al., 1997). Such a phosphorylation event is essential to bind and then activate with high preference methylated EBV immediate-early promoter (Bhende et al., 2005). In addition, phosphorylation of Ser-167 and Ser-173 leads BZLF1/ZEBRA to function as a repressor in expression of one late gene (El-Guindy and Miller, 2004). In human cytomegalovirus, the phosphorylation status of a Ser-rich domain encompassing amino acids 258–275 of immediate-early protein IE2 orchestrates the temporal expression of distinctive viral genes (Barrasa et al., 2005). Furthermore, the immediate-early protein ICP0 of human herpesvirus type 1 contains eleven potential phosphorylation sites clustered into three regions adjacent to domains required for transactivation. Mutation of the first phosphorylation region in ICP0 altered its E3 ligase activity that in turn led to virus replication defect (Boutell et al., 2008).

As every regulator needs to be regulated, in the present study we focus on the role of phosphorylation in the latent-lytic switch activity of K-RTA. Previous studies by Lukac et al. (1999) established that K-RTA is a highly phosphorylated protein evidenced by the alleviation of ∼20 kDa molecular mass on SDS-PAGE by calf intestinal phosphatase treatment. To extend this finding further, we performed computational analysis of K-RTA amino acid sequence using NetPhosK (Blom et al., 2004) and GPS 2.0 (Xue et al., 2008) for potential phosphorylation sites, and, Blast search in various protein databases for homologous protein motifs. We found: (1) K-RTA is a Ser- and Thr-rich protein (122 of 691, 17.7%) with the most abundant region located at amino acids 500–550 (33%). (2) In addition to multiple PKC and casein kinase II consensus sites, K-RTA harbors seven potential CDK phosphorylation motifs (T449, T540, T628, S634, S636, S644, S650). (3) In the *Human Proteinpedia* database, the sequences between amino acids 633–652 share high homology with an *in vivo* phosphorylated tryptic peptide derived from negative elongation factor B (NELF-B; Beausoleil et al., 2004; Olsen et al., 2006). In addition, PredictNLS (Cokol et al., 2000) identified one nuclear localization signal (NLS) in K-RTA, which is equivalent to the NLS-2 designated by Lukac and colleagues (Lukac et al., 1998, 1999; Bu et al., 2008). Among these predictions, we showed that Ser-634 and Ser-636 located in the NELF-B homologous region are involved in CDK9 recruitment and phosphorylation. Substitutions of Ser with Ala at Ser-634 and Ser-636 impaired K-RTA transactivation activity and altered its electrophoretic mobility on SDS-PAGE. In addition, CDK9 inhibitors suppressed the expressions of various K-RTA target genes and KSHV viral production in a HEK293/rKSHV.219 cell model. Together, these results support an emerging notion that some regions of the latent KSHV genome are consistently associated with paused RNA Pol II whose activity can be acutely induced by positive elongation factors such as CDK9 in response to various stimuli.

MATERIALS AND METHODS

PLASMIDS

pLenti4-FLAG-CPO is a gift from Dr. Dan Robinson at MCTP, University of Michigan (Ann Arbor, MI, USA). pLenti4-FLAG-CPO is a modified vector derived from pLenti4/TO/V5-DEST (Invitrogen). Briefly, the original *att*R1 site to V5 epitope region (nt 2405–4203) in pLenti4/TO/V5-DEST was replaced with an in-frame DNA fragment encoding Kozak sequence, ATG, FLAG tag, and a rare cutter CPO I site (5′CGGTCCG). Accordingly, pLenti4-FLAG-K-RTA was constructed by cloning the coding sequences of K-RTA (GeneID: 4961526) into the CPO I site of pLenti4-FLAG-CPO. The expression plasmid pLenti4-FLAG-NLSm was generated by replacing ^{527}KKRK^{530} motif of K-RTA with AAAA in pLenti4-FLAG-K-RTA by QuikChange® site-directed mutagenesis kit (Stratagene). Similarly, expression plasmids for the other NLS and phosphorylation mutants, including $NLSm_1$, $NLSm_2$,

T513A, T514A, T513A/T514A, S634A, S634T, S634D, S636A, S636T, S636D, S644A, S652A, and S634A/S636A were generated by using pLenti4-FLAG-K-RTA as template in the QuikChange® site-directed mutagenesis reactions. In luciferase reporter assays, the upstream sequences of PAN (nt 28159–28660 of U75698) and ORF57 (nt 81556–82005) were cloned into *Sac*I–*Xho*I sites of pGL3-Basic (Promega), yielding pGL3-Basic-PANp, and pGL3-Basic-ORF57p, respectively. Expression plasmids for CDK9, dominant negative CDK9 (D167N), and cyclin T have been described previously (Chang and Li, 2008).

ESTABLISHMENT OF 293TetKR AND 293TetNLSm CONDITIONAL EXPRESSION CELL LINES

The Tetracycline-Regulated Expression HEK293 (TREx™, Invitrogen) cell line were infected with lentivirions harboring pLenti4-FLAG-K-RTA and pLenti4-FLAG-NLSm by using ViraPower™system (Invitrogen). Forty-eight hour after infection, the cells were selected with 400 μg/ml zeocin (Invitrogen) for 2 weeks. Zeocin-resistance clones were subjected to doxycycline-inducibility test for the expression of desired genes by Western blot analysis using M2 anti-FLAG antibody (Sigma-Aldrich). For each conditional expression line, multiple positive clones with similar growth rate and expression level were pooled, collectively designated as 293TetKR and 293TetNLSm, respectively.

CELLS AND CELL CULTURE

Conditional expression cell lines 293TetKR and 293TetNLSm were maintained in DMEM supplemented with 10% Tet System Approved FBS (Clontech Laboratories), 5 μg/ml blasticidin-S-HCl (Invitrogen), and 200 μg/ml zeocin. HEK293 was maintained in DMEM containing 10% FBS. 293/rKSHV.219 was maintained in DMEM supplemented with 10% FBS and 660 ng/ml puromycin (Becton Dickinson). All cells were grown in a humidified 37˚C incubator with 5% CO_2.

TRANSFECTION AND LUCIFERASE REPORTER ASSAY

Transfection was performed in 24-well plates. HEK293 cells (2.4×10^5) were seeded into each well to reach a 90% confluence the next day before transfection. Transfection was carried out using Lipofectamine™2000 (Invitrogen) conveying appropriate plasmids according to the manufacturer's instruction. Twenty-four hour after transfection, cells were harvested for luciferase activity assay. Firefly and renilla luciferase activities were measured by using Dual-Glo Lucifearse Assay Kit (Promega). Transfection efficiency was normalized with a cotransfected renilla luciferase reporter (phRL-TK, Promega).

PROTEIN PURIFICATION AND MASS SPECTROMETRIC ANALYSIS

Immuno-purification of FLAG-tagged proteins from 293Tet-inducible cell lines was carried out according to two previously described procedures (Barlev et al., 2003; Trester-Zedlitz et al., 2005) with minor modifications. Specifically, 10^8 cells with 80% confluence were treated with 50 ng/ml doxycycline for 10 h before cell harvest. Combined cell pellet (∼1 g) was solubilized in 10 ml of lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1× protease inhibitors, 0.2 mM sodium orthovanadate) at 4˚C for 40 min with gentle shaking. Protein extracts were clarified by centrifugation at 12,000 × *g*, 4˚C for 15 min. Collected protein supernatant was further filtrated through a 0.45 micron filter. The filtrated protein supernatant was applied onto 400 μl anti-FLAG M2 affinity resin (A2220, Sigma-Aldrich) equilibrated with lysis buffer. The column eluate was re-loaded onto the column for two more cycles. The column was alternatively washed with one column volume of TBS (50 mM Tris–HCl, pH 7.4) followed by one column volume of high salt (500 mM NaCl)–TBS for three times. The column was washed with additional two column volumes of TBS buffer prior to elution. The FLAG-tagged proteins were eluted from the column by TBS buffer supplemented with 100 μg/ml FLAG peptide (F3290, Sigma-Aldrich) and 20% glycerol. For each purification, four eluted fractions were collected and samples were immediately frozen in −70˚C for further studies. Approximately 500 ng affinity purified K-RTA were resolved in a SDS-PAGE gel, stained with SYPRO® Ruby (Invitrogen) followed by in-gel digestion. Phosphopeptides were enriched by C18-functionalized Fe_3O_4 nanoparticles as described in Hsiao et al. (2007) followed by LC/MS/MS analysis as described previously (Lin et al., 2008).

WESTERN BLOT ANALYSIS AND CO-IMMUNOPRECIPITATION

Cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 1× protease inhibitors, 1× phosphatase inhibitors). The protein concentration was measured spectrophotometrically at 562 nm using BCA protein assay reagent (Pierce, Rockford, IL, USA). For each co-immunoprecipitation assay, the cells were lysed in cold EBC lysis buffer (50 mM Tris–HCl, pH 7.4, 0.5% NP-40, 120 mM NaCl, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 1× protease inhibitors, 1× phosphatase inhibitors). The protein extracts were incubated with M2 anti-FLAG affinity resin or anti-CDK9 bounded Dynabeads-protein G (Invitrogen) with continuous rocking at 4˚C overnight. After centrifugation or magnetic separation, the resin, or beads were washed thoroughly with TBS (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) three times followed by elution of immunocomplex in 1× sample buffer. Cell lysates or the eluted immunocomplex were subjected to SDS-PAGE separation and analyzed by Western blotting as described elsewhere (Chen et al., 2011). For IP-kinase assay (**Figures 5A,B**), the eluted immunocomplex were washed three times with TBS and once with kinase buffer (25 mM Tris–HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM MgCl2, 10 μM ATP). The immunocomplex were resuspended in 29 μl kinase buffer containing 1 μCi [γ-^{33}P] ATP and incubated at 30˚C for 30 min. After eluting proteins from resin, the proteins were resolved by SDS-polyacrylamide gel electrophoresis. The gel was dried, exposed to an X-ray film and developed by autoradiography.

CALF INTESTINAL ALKALINE PHOSPHATASE TREATMENT

Cells were lysed in EBC lysis buffer without phosphatase inhibitors or sodium orthovanadate. For each sample 50 μg of cell extract was incubated with 60 U calf alkaline phosphatase in buffer 3 (New England Biolabs) at 37˚C for 45 min. Extracts were separated by SDS-PAGE followed by Western blot analysis.

IMMUNOFLUORESCENCE ASSAY

In **Figure 1B**, 1 day before transfection, cells at a density of 10^5/0.4 ml/well were seeded on eight-well chamber slide (Nunc) coated with poly-D-Lysine (Sigma-Aldrich). The cells were transfected with indicated plasmids by using Lipofectamine™ 2000 (Invitrogen) and incubated for 24 h. In **Figure 2A**, cells were pretreated with doxycycline for 24 h before assay. Cells were fixed with acetone/methanol (v/v = 1:1) at −20°C for 20 min and permeablized with 0.4% Triton X-100 at RT for 5 min. The slides were blocked for 30 min in blocking buffer (PBS containing 1% FBS) and incubated with anti-FLAG antibody at RT for 2 h. The slides were washed with PBS three times for 5 min each. The slides were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG for 2 h and counterstained with DAPI (Sigma-Aldrich) for 5 min at RT. The slides were observed under a confocal fluorescence microscope.

SUBCELLULAR FRACTIONATION

Subcellular fractionation was conducted according to a previously described procedure (Wang et al., 2005). Briefly, cells were incubated with hypotonic buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M Sucrose, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 1× protease inhibitors) on ice for 10 min. Cells were centrifuged at 1300 × *g* for 4 min at 4°C. The resulting pellet was the nuclear fraction. The supernatant was further centrifuged at 20,000 × *g* for 15 min at 4°C to remove debris. The resulting supernatant was the cytosolic fraction.

FLOW CYTOMETRIC ANALYSIS

After transfection, the cells were harvested by trypsinization and resuspended in 500 µl PBS. A total of 5,000 cells were acquired by using a flow cytometer (FACSCalibur, Becton Dickinson) and analyzed using the WinMDI v2.8 software. GFP and RFP signals were detected at 488 and 540 nm, respectively.

TITRATION OF KSHV VIRAL PARTICLES

Filtrated (0.45 µm) viral supernatant (160 µl) was incubated with 2 U DNase I (Invitrogen) at 37°C for 30 min followed by extraction of encapsidated KSHV DNA using QIAamp MinElute virus spin kit (QIAGEN). Each comparative quantitative PCR reaction was composed of 2 µl diluted viral DNA, 5 µl Power SYBR Green Master Mix (Applied Biosystems), and 3 µl primer mix (0.66 µM). The primers used for detecting KSHV genome are ORF9-forward (5′-CCAACATCATCCAATGCCTC-3′) and ORF9-reverse (5′-GGGAAAAGTCACGGGAATG-3′). Known copy numbers of serially diluted cosmid GB11 DNA encompassing KSHV genome nt 1–35,022 (U75698) were used as standards in titrating KSHV viral particles. The reaction was conducted and detected by StepOnePlusTM Real-Time PCR system (Applied Biosystems).

QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Total RNA was extracted from cells by using RNeasy kit (Qiagen). Reverse transcription of 2.5 µg RNA was performed in a 10 µl SuperScript™III reaction mixtures (Invitrogen) according to the manufacturer's instructions. One percent of the resulting cDNAs were used for each real-time PCR reaction composed of 2 µl diluted cDNA, 5 µl Power SYBR Green Master Mix (Applied Biosystems), and 3 µl primer mix (0.66 µM). The primers used

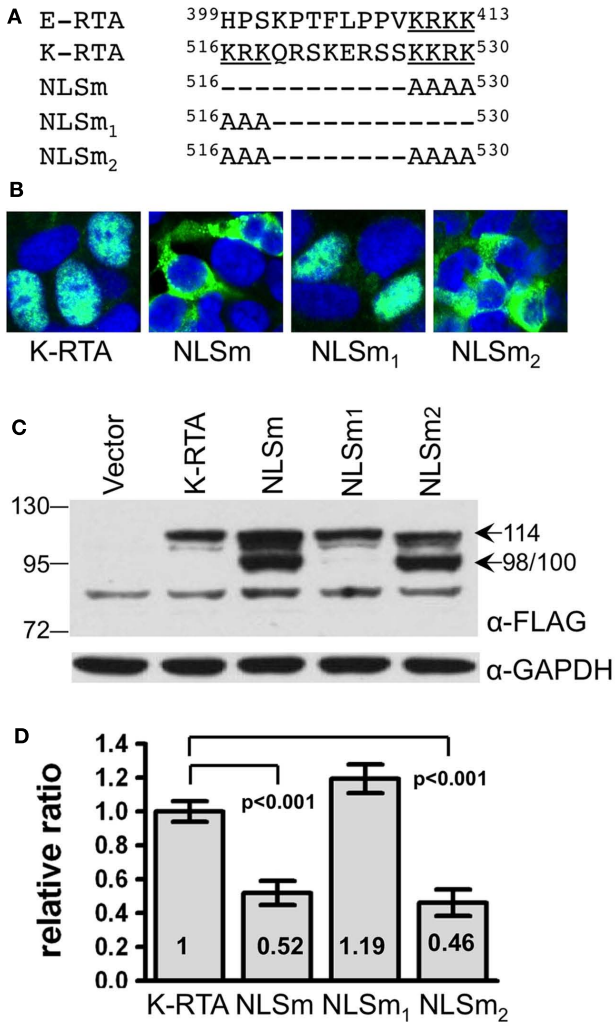

FIGURE 1 | Characterization of a prototypic bipartite nuclear localization signal (NLS) of K-RTA locating at amino acids 516–530. (A) Alignment of NLS from EBV Rta (E-RTA), K-RTA, NLSm, $NLSm_1$, and $NLSm_2$. The KRKK motif required for E-RTA nuclear localization (Hsu et al., 2005) and the two clusters of basic amino acids located in the bipartite NLS of K-RTA are underlined. **(B)** Subcellular localization of K-RTA and the three NLS mutants in HEK293 cells revealed by an immunofluorescence assay using M2 FLAG antibody followed by confocal microscopy. Only mutations locating at the second basic motif affect the nuclear targeting (NLSm and $NLSm_2$). **(C)** Western blot analysis of HEK293 cells transiently transfected with K-RTA or each of three NLS mutants. An additional 98/100 doublet was detected only in the extract of variants harboring the second basic motif mutation (NLSm and $NLSm_2$). GAPDH served as a loading control. **(D)** Luciferase reporter assay of KSHV ORF57 promoter responding to K-RTA and NLS mutants transiently expressed in HEK293 cells. Each firefly luciferase value was normalized to an internal control derived from cotransfected plasmid phRL-TK. The value in K-RTA was set to 1. Data are presented as means ± SD from triplicate transfections in an experiment. Statistical evaluations were performed by using Student's *t*-test. Three independent experiments were performed; a representative result is shown.

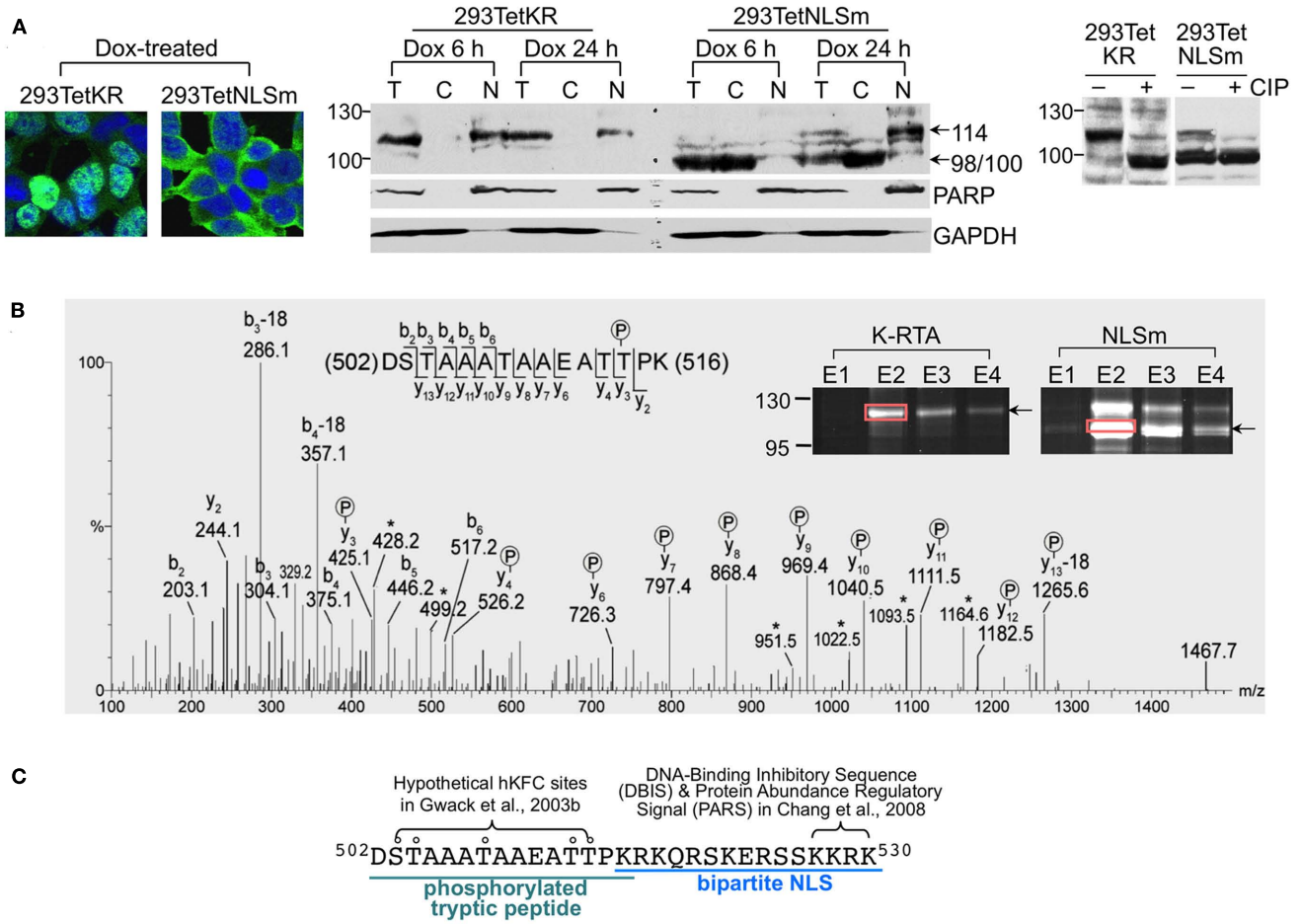

FIGURE 2 | Thr-513 and Thr-514 of K-RTA is the primary *in vivo* phosphorylation motif identified by mass spectrometry. (A) (Left) distinct subcellular localization of K-RTA and NLSm was revealed in doxycycline (Dox, 50 ng/ml)-treated 293TetKR and 293TetNLSm cells by an immunofluorescence assay using M2 FLAG antibody. (Middle) subcellular fractionation of protein lysates prepared from 6 to 24 h Dox-treated 293TetKR and 293TetNLSm cells. T, total lysate; N, nuclear fraction; C, cytosolic fraction. PARP and GAPDH served as the nuclear and cytosolic indicators, respectively. (Right) protein lysate of the 293TetKR and 293TetNLSm was preincubated with (+) or without (–) calf intestinal alkaline phosphatase (CIP) at 37°C for 45 min followed by Western blot analysis. **(B)** MS/MS spectrum on $[M + 2H]^{2+}$ (*m/z* 743.31) ion for the one phosphorylation-modified peptide DSTAAATAAEATTPK from K-RTA (rectangle). The product ion y_3 that carries a phosphate indicated that Thr-514 was phosphorylated but its low intensity among other background/unassigned peaks does not preclude the alternative Thr-513 site. A single phosphorylation on either site was unambiguously supported by additional phosphorylated y ions. Product ions marked with starts resulted from elimination of H_2O. Of note, the same spectrum was observed for 100 kDa NLSm (not shown). **(C)** Summary of various features identified in amino acids 502–530.

in the present study are listed in **Table A1** in Appendix. The reaction was conducted and detected by StepOnePlus™Real-Time PCR system (Applied Biosystems).

ANTIBODIES

Monoclonal and polyclonal anti-K-RTA antibodies were kindly provided by Drs. Keiji Ueda (Osaka University Graduate School of Medicine, Japan) and Yoshihiro Izumiya (University of California at Davis, Sacramento, CA, USA), respectively. Other antibodies used in this study are M2 FLAG (F1804, Sigma), β-actin (A5441, Sigma), mouse monoclonal CDK9 (sc-13130, Santa Cruz), PARP (sc-8007, Santa Cruz), rabbit monoclonal CDK9 (#2316, Cell Signaling), p44/42 MAPK (#9107, Cell Signaling), phospho-p44/42 MAPK (#4376, Cell Signaling), MPM2 (#05-368, Upstate), α-tubulin (05-829, Millipore), phospho-serine (AB1603, Millipore), phospho-threonine (AB1607, Millipore), GAPDH (#54593, AnaSpec, Inc.).

RESULTS

THE KKRK MOTIF OF THE BIPARTITE NUCLEAR LOCALIZATION SIGNAL CONTRIBUTES TO NUCLEAR TARGETING AND TRANSACTIVATION ACTIVITY OF K-RTA

Computer-assisted identification of the NLS sequence in K-RTA was achieved using PredictNLS, a program serving to identify *in silico* potential NLS present in a given sequence (Cokol et al., 2000). Accordingly, a prototypic bipartite NLS located at amino acids 516–530 (KRKQRSKERSSKKRK) was identified with 97.92% probability (**Figure 1A**). This subclass of bipartite NLS can be found in 193 nuclear proteins collected in the PredictNLS database as of September 2011. In agreement, functional domains

encompassing amino acids 516–530 of K-RTA were recently shown to be a *bona fide* NLS (Bu et al., 2008) or to possess inhibitory effects on DNA binding (Chang et al., 2008). Noteworthy, Chen et al. (2000) showed that amino acids 6–12 of K-RTA, designated as NLS-1 in Lukac's et al. (1998) publication, was demonstrated to be a functional NLS when fused to β-Gal protein, and, is required for efficient nucleus-targeting since product of genomic ORF50 devoid of NLS-1 tends to localize to both the nucleus and the cytoplasm. This finding suggests that NLS-1 can serve as a cryptic NLS and may participate in correct folding of K-RTA that is required for the exposure of the C-terminal bipartite NLS.

Alignment of amino acids 516–530 with the NLS derived from EBV Rta (Hsu et al., 2005), a functionally homologous protein to K-RTA, revealed little similarity except for the last four basic residues (**Figure 1A**). To characterize further which of the two basic motifs is responsible for nuclear localization, the Lys and Arg located at amino acids 516–518 and 527–530, respectively, were mutated to Ala (**Figure 1A**). Subcellular localization of K-RTA and NLS mutants was inspected by an immunofluorescence assay. As shown in **Figure 1B**, NLSm and $NLSm_2$ were clearly retained in the cytoplasm, indicating that the mutation of the second basic motif composed of KKRK is the determinant of nuclear targeting. By contrast, modification of the first basic motif from KRK to AAA did not seem to interfere with the nuclear targeting of K-RTA, as represented by $NLSm_1$. In addition, compared with the wild type that was estimated to be 114 kDa in size, mutation of the second basic motif consistently yielded more abundant but shorter 98/100 kDa doublet bands in Western blot analysis (**Figure 1C**). To assess whether mutations affect the transactivation capability of K-RTA, a luciferase reporter assay using ORF57 promoter sequences was performed. Again, mutation in the second (NLSm and $NLSm_2$), but not in the first ($NLSm_1$) basic motif showed a dramatic decrease (50–60%) in transactivation activity (**Figure 1D**). A similar result was obtained in a PAN promoter-driven luciferase reporter assay (data not shown). Collectively, these results revealed that the second basic motif KKRK of the bipartite NLS is responsible for the targeting of K-RTA to the nucleus and the full potency of transactivation, and is involved in regulating protein abundance. Because NLSm requires the least changes in altering subcellular localization, it was used as the cytoplasmic version of K-RTA in the following studies.

K-RTA IS PHOSPHORYLATED IN BOTH CYTOPLASM AND NUCLEUS

The most probable explanation for the 14 kDa difference between K-RTA and NLSm was that it is caused by different extents of phosphorylation taking place in different compartments of the cell. To corroborate this hypothesis, cell lines conditionally expressing K-RTA and NLSm were established by using the Virapower system (Invitrogen). Briefly, TREx-293^{TM} cells were stably transduced with a lentiviral-based plasmid expressing FLAG-tagged K-RTA or NLSm under the control of a doxycycline (Dox)-inducible promoter, CMVTetO2™. The resulting cell lines were designated as 293TetKR and 293TetNLSm, respectively. As expected, upon Dox treatment, K-RTA was found predominantly in the nucleus of 293TetKR cells using an immunofluorescence assay, while NLSm was largely confined to the cytoplasm of 293TetNLSm cells (**Figure 2A**, left). The protein extracts of Dox-treated 293TetKR and 293TetNLSm cells were subjected to subcellular fractionation followed by Western blot analysis. Clearly, the 114 kDa polypeptide and the 98/100 kDa protein bands existed in distinct fractions: the 114 kDa was exclusively in the nucleus and the 98/100 kDa doublet in the cytoplasm (**Figure 2A**, middle). Further, when protein extract was pre-treated with calf intestinal alkaline phosphatase (CIP), which should remove most of the phosphate groups attached to K-RTA and NLSm, both molecules were converted to a single 98 kDa species (**Figure 2A**, right). These results indicated that the non-phosphorylated forms of K-RTA and NLSm were similar, yet compartmental phosphorylation processes contribute to their individual molecular masses. Thereby, we hypothesized that K-RTA is mildly phosphorylated in the cytoplasm as indicated by the smaller, 98/100 kDa doublet polypeptides, and is further phosphorylated once it enters the nucleus as indicated by the mature 114 kDa protein.

IDENTIFICATION BY MASS SPECTROMETRY OF Thr-513 AND Thr-514 AS THE PRIMARY *IN VIVO* PHOSPHORYLATION MOTIF IN K-RTA

In order to conclusively identify which residues are phosphorylated *in vivo*, affinity purified 114 kDa K-RTA and 100 kDa NLSm derived from Dox-treated 293TetKR and 293TetNLSm cells, respectively, were subjected to LC/MS/MS analysis (**Figure 2B**). It was expected that there would be more phosphorylated residues identified in the 114 kDa nuclear K-RTA than in the 100 kDa cytoplasmic NLSm. Disappointedly, the same results were obtained from both analyses: Thr-513 or Thr-514 located in the tryptic peptides encompassing amino acids 502–516 (DSTAAATAAEATTPK) of K-RTA and NLSm were the only amino acids with phosphate potentially conjugated to them. Of these two residues, Thr-513 is more likely to serve as an alternative position for phosphate modification (**Figure 2B**, detailed in the legend). From these results, we can conclude that Thr-513 or Thr-514 is preferentially phosphorylated in the cytoplasm (NLSm), and this modified status is retained in the nucleus (K-RTA).

Next, the role of phosphorylated Thr-513 or Thr-514 in K-RTA activity was assessed by biological assays using three Ala substitution mutants, which mimic the non-phosphorylated state of 513 and 514. The three variants were primarily localized in the nucleus and the protein expression levels and molecular weights of the three variants were comparable to those of the wild type K-RTA by Western blot analysis (data not shown). A luciferase reporter assay using upstream sequences of KSHV PAN RNA was performed to quantitate the transactivation activity of each Ala variant. No significant difference was observed between any Ala mutant and the wild type K-RTA (not shown). These results suggested that that phosphorylation of Thr-513 and Thr-514 might play only an ancillary role in K-RTA activity. Interestingly, Gwack et al. (2003b) noted previously that Ser-503, Thr-504, Thr-508, Thr-513, and Thr-514 are five potential phosphorylation sites for a Ste20-like kinase hKFC, and that phosphorylation of K-RTA by hKFC resulted in a negative effect in K-RTA-mediated transactivation. Thus, our results excluded the possibility that Thr-513 or Thr-514 is an hKFC target site. Taken together, although located proximal to NLS and a DNA binding inhibitory/protein abundance regulatory signal (**Figure 2C**), defective in phosphorylation of Thr-513 or Thr-514 has little

influence in K-RTA nuclear localization, transcriptional activation, or protein abundance.

IDENTIFICATION OF AN NELF-B HOMOLOGOUS MOTIF IN THE C-TERMINUS OF K-RTA

In order to explore the existence of phosphorylated Ser or Thr residues that had been missed by mass spectrometry (MS; discussed below), computer-assisted Blast search was employed to look for known phosphorylated tryptic peptides that share sequence similarity with K-RTA. Briefly, amino acid sequences of several large tryptic peptides derived from K-RTA were used as queries to find hits using a pre-calculated position-specific score matrix in the NCBI Conserved Domain database as described by Marchler-Bauer et al. (2005). Amino acids 633–652 were found to share significant homology with an *in vivo* phosphorylated tryptic peptide encompassing amino acids 554–573 of NELF-B (**Figure 3A**). NELF-B is one of the five negative elongation factors that control the processivity of RNA Pol II on an active promoter. The *in vivo* phosphorylation sites of NELF-B have been identified previously to be located at Ser-557, Thr-564, and Ser-573 (Beausoleil et al., 2004; Olsen et al., 2006), which were aligned to K-RTA Ser-636, Ser-644, and Ser-652, respectively (**Figure 3A**). Although

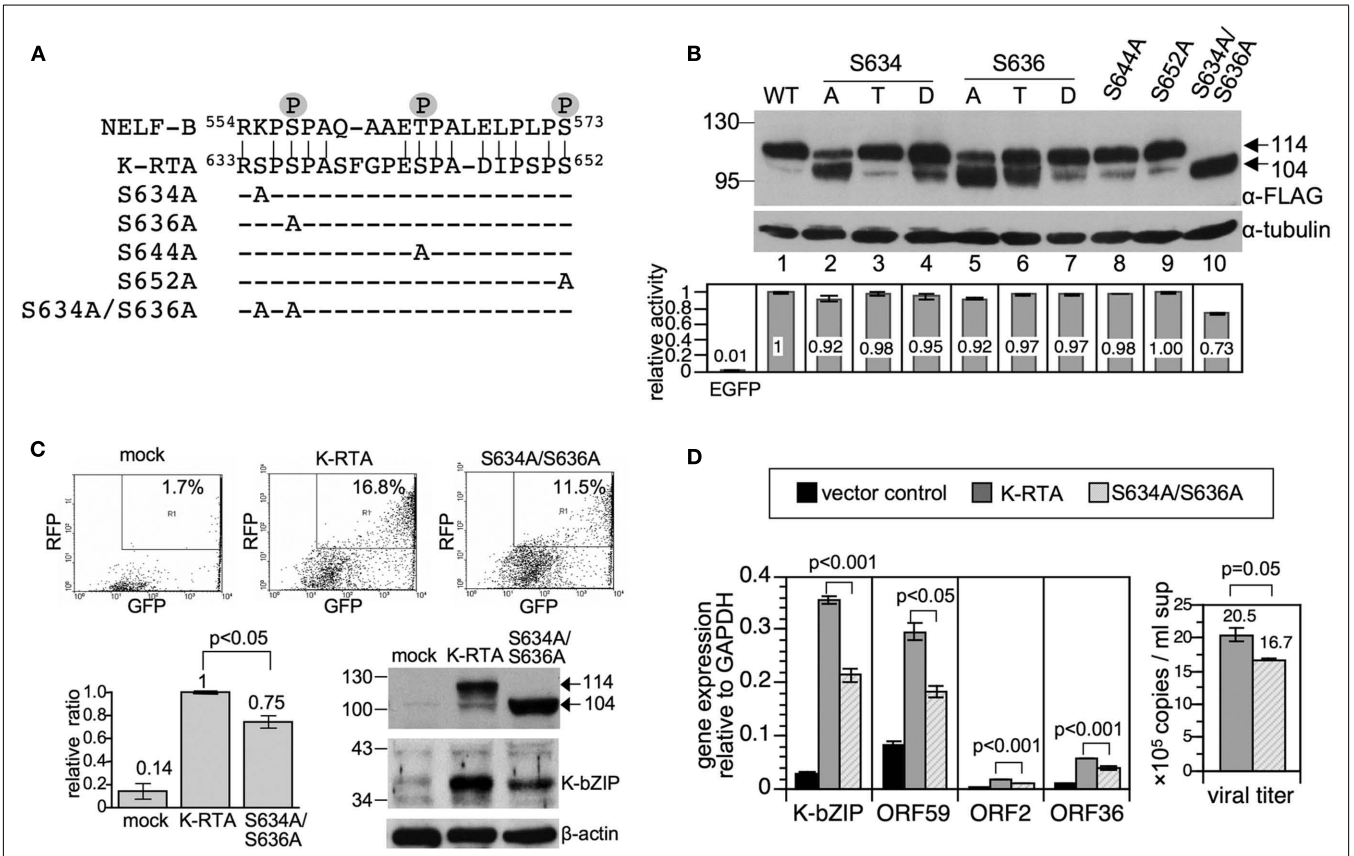

FIGURE 3 | Ser to Ala substitutions at Ser-634 and Ser-636 impaired K-RTA transactivation potency and reduced KSHV reactivation. (A) Alignment of a Ser-rich region (amino acids 633–652, 30% Ser) located in the C-terminus of K-RTA with an *in vivo* phosphorylated peptide derived from negative elongation factor B (NELF-B). *In vivo* phosphorylated residues in NELF-B identified previously are denoted by circled P (Beausoleil et al., 2004; Olsen et al., 2006). Accordingly, a series of K-RTA variants with mutations in Ser-634, -636, -644, and -652 were generated. The four Ser to Ala mutants and Ser-634 and Ser-636 double mutants are denoted. **(B)** (Upper) the expression levels and patterns of K-RTA and the nine phosphorylation site mutants were compared in parallel by Western blot analysis using M2 FLAG antibody. α-tubulin served as a loading control. (Lower) luciferase reporter assay of KSHV PAN promoter responding to the wild type and the phosphorylation mutants transiently expressed in HEK293 cells. Data are presented as the means ± SD for quadruplicate assays. Three independent experiments were performed; a representative result is shown. **(C)** (Upper) comparison by flow cytometry of potency for viral latency disruption of K-RTA and S634A/S636A. 293/rKSHV.219 cells transfected with K-RTA or S634A/S636A for 72 h were analyzed on a fluorescence-activated cell sorter followed by scoring the percentage of double positive (GFP and RFP) cells in each population, as indicated in the upper right of each panel. Untreated cells (mock) served as a control. (Lower left) results derived from four independent experiments are summarized in the schematic chart. Data are presented as the means ± SD with K-RTA group set to 1. Statistical evaluation was performed with Student's *t*-test. (Lower right) induction of expression of lytic protein K-bZIP by K-RTA and S634A/S636A in 293/rKSHV.219 cells was compared in parallel by Western blot analysis. β-actin served as a loading control. **(D)** (Left) total RNAs of 293/rKSHV.219 cells transfected with pLenti-FLAG-CPO (vector control), pLenti-FLAG-K-RTA, and pLenti-FLAG-S634A/S636A for 48 h were analyzed by quantitative RT-PCR for gene expressions of K-bZIP, ORF59, ORF2, and ORF36. Results from each reaction were normalized to cellular GAPDH. Statistical evaluations were performed by using Student's *t*-test. (Right) KSHV viral particles released from K-RTA or S634A/S636A-expressing cells at 72 h were determined by comparative quantitative PCR of KSHV DNA polymerase genes (ORF9). Data are presented as means ± SD for three PCR assays in an experiment. Similar results were obtained from two independent experiments; one set of data is shown.

the role of phosphorylated Ser-557, Thr-564, and Ser-573 in NELF-B is still unclear, it was tempting to presume that the conserved residues in K-RTA are also phosphorylated. To test our hypothesis, Ser to Ala point mutation was individually introduced into K-RTA Ser-636, Ser-644, and Ser-652 (**Figure 3A**). In addition, we initially included S634A as a control variant since no conserved Ser was matched in the corresponding site of NELF-B. We first confirmed that all four Ser to Ala mutants correctly resided in the nucleus of HEK293 cells (data not shown). Next, the protein expression level of each mutant was compared by Western blot analysis in parallel with that of wild type K-RTA. Noteworthy, the major bands in S634A and S636A, but not in S644A and S652A were estimated to be 104 kDa, approximately 10 kDa shorter than that of the wild type (**Figure 3B**, compare lanes 1, 2, 5, 8, 9). To investigate further, Ser to Thr, Ser to Asp, and double Ser to Ala substitutions were introduced into amino acids 634 and 636, yielding S634T, S634D, S636T, S636D, and S634A/S636A. Similarly, each mutant was shown by an immunofluorescence assay to localize in the nucleus (data not shown). Interestingly, the discrepancy in molecular mass in S634A and S636A could be partially or completely overcome by substitution of a functionally conserved residue (e.g., S634T and S636T) or with a phosphorylation mimetic residue (e.g., S634D and S636D), suggesting that phosphorylation of Ser-634 or Ser-636 is involved in migration mobility of K-RTA (**Figure 3B**, lanes 3, 4, 6, 7). To corroborate the molecular mass discrepancy with transactivation potential, a PAN promoter-driven luciferase reporter assay was performed to compare transactivation activity of K-RTA and the nine Ser-634 and Ser-636 variants. As summarized in **Figure 3B** (lower panel), the transactivation activities were 8–27% lower in S634A, S636A, and S634A/S636A relative to that of the wild type and other variants. Although the reductions were small, these results mirrored those observed for migration mobility, namely substitution of Ser with Ala at 634 or 636 produced a 104 kDa species of K-RTA that is less potent. Because S634A/S636A yielded the most apparent changes in migration pattern and transactivation activity, this mutant was chosen for further investigation.

To extend these studies to cells infected with KSHV, K-RTA, and S634A/S636A, respectively, were ectopically expressed in 293/rKSHV.219 cells (Vieira and O'Hearn, 2004), followed by scoring of the percentage of GFP and RFP double positive cells, an indicator of KSHV lytic reactivation (Vieira and O'Hearn, 2004). The results showed that lytic reactivation induced by K-RTA was impaired when Ser-634 and Ser-636 were replaced with Ala, either by scoring of PAN promoter activity or by detecting K-bZIP expression (**Figure 3C**). This defect in lytic cycle reactivation of S634A/S636A was further validated by quantitative RT-PCR analysis of additional lytic genes (ORF2, ORF36, and ORF59) and determination of viral particles released in the culture media (**Figure 3D**). Thereby, we provide evidence indicating that phosphorylation of Ser-634 and Ser-636 contributes to the full potency of K-RTA in lytic cycle reactivation and virus production.

Taken together, we found that amino acids 633–652 of K-RTA shares 70% homology with a phosphorylated tryptic peptide derived from NELF-B. Among the three conserved Ser residues between K-RTA and NELF-B, Ser-636 seemed to play a role in K-RTA migration mobility and transactivation activity. In conjunction with Ser-634, Ser to Ala substitutions resulted in ∼25% decrement in K-RTA activity and was ∼10 kDa smaller in molecular weight.

K-RTA ^{634}SPSP^{637} IS A POTENTIAL CDK9 TARGET SITE

At first, we hypothesized that the 10 kDa decrease in molecular mass of S634A/S636A was caused by a deficiency in phosphorylation, namely Ser-634 or Ser-636 may serve as a priming site to "dock" kinase(s), after which a successive, extensive phosphorylation is initiated. Thus, given a phosphate group is 80 Da, the 10 kDa difference could be attributed to as many as 125 phosphates conjugating onto K-RTA, but not to the S634A/S636A mutant; that is, the overall phosphorylation state in K-RTA should be higher than that in S634A/S636A. To validate this hypothesis, protein extracts of HEK293 cells transiently expressing FLAG-tagged K-RTA or S634A/S636A were treated with CIP and analyzed against their untreated counterparts by SDS-PAGE and Western blot analysis. As expected, CIP converted the majority of K-RTA and S634A/S636A to a 98 kDa species (**Figure 4A**). To determine the overall phosphorylation state in K-RTA vs. S634A/S636A, protein extracts were immunoprecipitated with M2 FLAG resin followed by Western blot analysis using anti-phospho Ser or anti-phospho Thr antibodies (**Figure 4B**). After normalized with the total protein (α-FLAG), the overall phosphorylation status of K-RTA and S634A/S636A was very similar. Taken together, these results refuted our hypothesis that the 10 kDa difference was caused by a deficiency in the degree of phosphorylation in S634A/S636A.

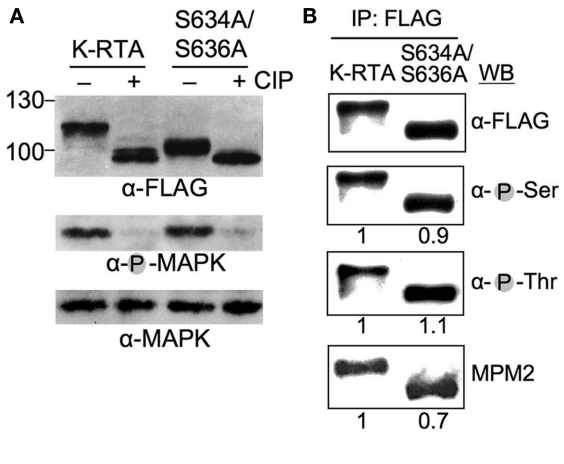

FIGURE 4 | The 10-kDa difference between K-RTA and S634A/S636A is not due to distinct global phosphorylation state. (A) Protein extract from HEK293 cells transfected with plasmids expressing K-RTA or S634A/S636A for 24 h was incubated with (+) or without (–) calf intestinal phosphatase (CIP) at 37°C for 45 min. Each reaction mixture was analyzed in parallel by Western blot analysis using specified antibodies. Phosphorylated MAPK served as a control for completeness of CIP treatment. **(B)** The overall Ser phosphorylation (α-pSer), Thr phosphorylation (α-pThr), and MPM2 reactivity of K-RTA and S634A/S636A were revealed by immunoprecipitation (IP)-Western blot analysis. Because the expression abundance of each species were varied, the intensity of each band was first normalized with the intensity of its corresponding FLAG polypeptide (α-FLAG), followed by comparing to the normalized K-RTA value that was set to 1.

On the other hand, we noticed that ^{634}SPSP^{637} of K-RTA represents a canonical motif of MPM2, an antibody that recognizes the phosphorylated Ser-Pro or phosphorylated Thr-Pro motifs present on a polypeptide, we tested whether mutation of this motif (^{634}APAP^{637} in S634A/S636A) would influence its immunoreactivity to MPM2 antibody. Indeed, as shown in **Figure 4B**, S634A/S636A retained ∼70% immuno-reactivity of MPM2 antibody relative to K-RTA by IP-Western blot analysis. This result suggested that ^{634}SPSP^{637} could be an *in vivo* phosphorylation site. Because ^{634}SPSP^{637} also represents a typical motif for Pro-directed kinases including various CDKs, an *in vitro* translation (IVT) coupled IP-kinase assay was performed to identify potential CDKs that phosphorylate Ser-634 or Ser-636. As a result, phosphorylation of K-RTA was detectable when CDK1, 2, or 9 were used as kinase source (not shown). Among these, we were particularly interested in CDK9 because it belongs to transcriptional CDK families that tightly control the conformation and processivity of RNA Pol II and negative elongation factor complex, NELF, and DSIF (Lolli, 2009).

First, by *in vitro* IP-kinase assay, we confirmed that CDK9, but not its dominant negative variant (CDK9-D167N) specifically phosphorylated K-RTA *in vitro* (**Figure 5A**). To inspect whether Ser-634 or Ser-636 were the target sites of CDK9, IP-kinase assays of K-RTA, and S634A/S636A were performed in parallel. As depicted in **Figure 5B**, although CDK9 phosphorylated both species, CDK9 phosphorylated higher fraction of K-RTA than that of S634A/S636A (1:0.4), strongly indicating that the ^{634}SPSP^{637} motif could be one of the multiple CDK9 sites present in K-RTA. If this were true, we envisioned that *in vivo*, interaction between CDK9 and K-RTA would be stronger than that between CDK9 and S634A/S636A. As anticipated, results of IP-Western blot analysis revealed that higher fraction of K-RTA associated with CDK9 relative to that of S634A/S636A (1: 0.4, **Figure 5C**). The involvement of CDK9 in Ser-634 and Ser-636 phosphorylation was further supported by using DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole) and roscovitine in a PAN promote-driven luciferase reporter assay. DRB and roscovitine are well-characterized CDK9 inhibitors (Taylor et al., 2004; Kapasi et al., 2009). When used as a dosage that did not significantly influence general transcription (evidenced by negligible changes in rellina activities of transfection controls, expressions of FLAG protein and β-actin between the control and the drugged groups, not shown), S634A/S636A was less sensitive to CDK9 inhibitors compared to K-RTA (**Figure 5D**), reinforcing that ^{634}SPSP^{637} is an *in vivo* target site of CDK9. To extend these studies to cells infected with KSHV, K-RTA expressing 293/rKSHV.219 cells were treated with vehicle control (DMSO), DRB, or roscovitine for 42 h before harvest. RNAs of each group were subjected to quantitative RT-PCR analysis for various K-RTA target genes. As shown in **Figure 5E**, while the expressions of β-actin or RNA Pol III-directed 18S ribosomal RNA were insensitive to CDK9 inhibitors, the expression of MYC was significantly suppressed by DRB or roscovitine treatment, which is consistent with previous finding that MYC is a prototypic RNA Pol II pausing gene whose expression is regulated by CDK9-related pathway (Romano and Giordano, 2008). Notably, DRB and roscovitine also down-regulated the expressions of known K-RTA target genes (cellular SERPINB1; Brown et al., 2010, PAN, K-bZIP, and ORF59) and decreased the amount of viral particles released in the culture media, reinforcing the positive regulatory role of CDK9 in K-RTA-mediated transactivation and virus production. Taken together, we showed that although both K-RTA and S634A/S636A were phosphorylated *in vitro* by CDK9, S634A/S636A only retained 40% intensity of phosphorylation compared to that in K-RTA; both K-RTA and S634A/S636A associated with CDK9 *in vivo*, yet S634A/S636A only preserved 40% recruitment capacity relative to that of K-RTA; CDK9 inhibitors, DRB, and roscovitine, displayed higher inhibition potency on K-RTA than that of S634A/S636A. We conclude that there are multiple CDK9 target sites on K-RTA and ^{634}SPSP^{637} motif is highly likely to be one of them.

DISCUSSION

In the present study, we demonstrated that phosphorylation of K-RTA can be subsidiary to (at Thr-513 and Thr-514) or influential in (at Ser-634 and Ser-636) the biological activity of K-RTA. We showed that mutation of ^{634}SPSP^{637} to ^{634}APAP^{637} in K-RTA reduced its capacity in CDK9 recruitment and potency in viral lytic cycle reactivation. Because CDK9 is central to regulating the RNA Pol II processivity on active promoters, we propose that similar to HIV Tat, by recruiting CDK9 to the viral genome K-RTA has evolved to potentiate the host transcription machinery for a robust viral transcriptome expression during lytic cycle replication.

Among the multiple mechanisms that control RNA Pol II activity in a transcription cycle, promoter-proximal pausing/stalling of RNA Pol II is considered as a rate-limiting step in the activation of hundreds of genes responding to dynamic environmental and developmental cues (Muse et al., 2007; Zeitlinger et al., 2007). In addition, genome-wide epigenetic analysis of human cells revealed that although only ∼30–45% of known genes have detectable transcripts, the majority of protein-coding genes are with active chromatin marks (K3K4me3) and bound with low level of RNA Pol II in the regions surrounding their promoters (Guenther et al., 2007). Interestingly, recent epigenetic studies revealed similar phenomena in the KSHV genome that it is also dynamically marked with both active and suppressive chromatin marks during latent infection, indicating that the KSHV genome is transcriptionally poised during latency, but can be rapidly activated in response to lytic cycle inducers (Gunther and Grundhoff, 2010; Toth et al., 2010).

The negative elongation factor (NELF) is a transcription regulatory complex comprises four subunits, NELF-A, B, C/D, and E. NELF exerts to interact stably with RNA Pol II and other elongation regulatory factors within the promoter-proximal region, which prevents further transcription elongation. Recently, a novel role of NELF was revealed: by residing at the pausing site, NELF prevents nucleosome formation and maintains a permissive chromatin architecture that allows a rapid transcription process upon induction. Thus, NELF-mediated RNA Pol II pausing can provide both negative and positive effects on gene expression (Gilchrist et al., 2008). Regardless of which effect, phosphorylation of NELF by CDK9 enables the disengagement of NELF from the promoter-proximal region that is followed by a productive elongation process.

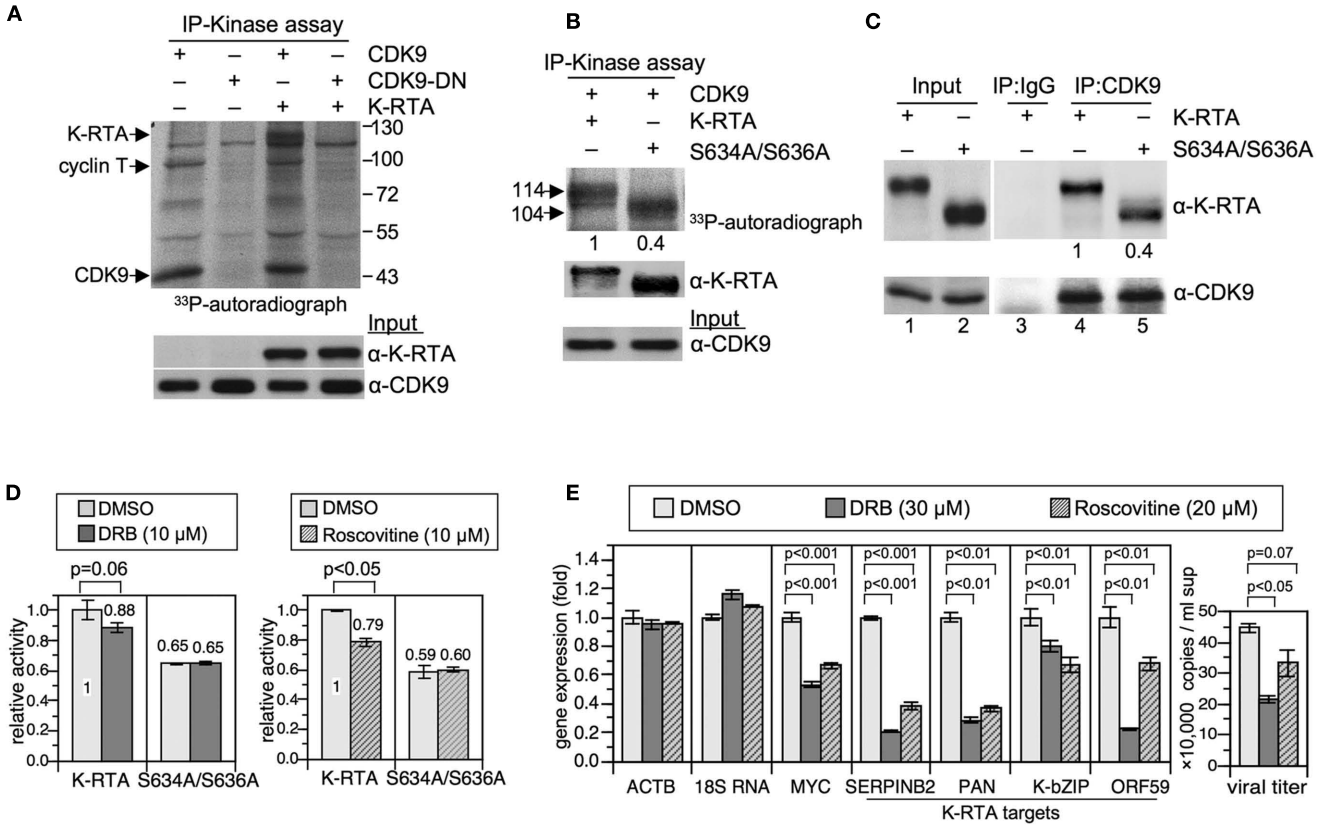

FIGURE 5 | CDK9 phosphorylated K-RTA *in vitro* and CDK9 associated with K-RTA *in vivo*. (A) Protein extracts of 293T cells expressing indicated plasmids were harvested and subjected to immunoprecipitation (IP)-kinase assay using M2 FLAG affinity resin. The resulting mixtures were resolved by 8% SDS-PAGE and revealed by autoradiography. A distinct 114 kDa band is detected only when both K-RTA and CDK9 are present, indicating that K-RTA is a CDK9 substrate. CDK9-DN, CDK9 dominant negative. **(B)** Quantitation of CDK9 activities toward K-RTA vs. S634A/S636A. The band intensity in the autoradiograph film was normalized with the band intensity in α-K-RTA followed by setting K-RTA's value to be 1. Although more S634A/S636A was captured in the immunocomplex, only 40% γ-^{33}P-ATP was labeled onto S634A/S636A relative to that in K-RTA, suggesting that wild type K-RTA is a better substrate for CDK9. **(C)** Phosphorylation of Ser-634 and Ser-636 may play a role in CDK9 recruitment. Association of K-RTA and S634A/S636A with cellular CDK9 was determined by IP-Western blot analysis using α-CDK9 bounded Dynabeads-protein G. After normalized with the input proteins (lanes 1 and 2), S634A/S636A only retained 40% in CDK9 association ability, suggesting that Ser-634 ad Ser-636 are involved in CDK9 recruitment. **(D)** The effects of DRB (5,6-dichloro-1-beta-d-ribofuranosylbenzimidazole) and roscovitine on K-RTA-mediated transactivation of PAN promoter were determined by a luciferase reporter assay. HEK293 cells were cotransfected with plasmids expressing K-RTA or S634A/S636A together with PANp-driven luciferase reporter plasmid for 6 h, followed by inhibitor treatments for 18 h. Each firefly luciferase value was normalized to an internal control plasmid phRL-TK. The normalized value of the untreated group in K-RTA was set to 1. Data are presented as the means ± SD for quadruplicate transfections. Similar pattern was observed in two independent experiments. One set of data is shown. Statistical evaluations were performed with Student's *t*-test. **(E)** (Left) quantitative RT-PCR analysis for gene expressions of β-actin (ACTB), 18S ribosomal RNA (18S RNA), known RNA Pol II pausing gene (MYC), and K-RTA target genes (cellular SERPINB2, KSHV PAN, K-bZIP, ORF59) in K-RTA expressing 293/rKSHV.219 cells treated with indicated inhibitors for 42 h. GADPH-normalized RNA value was quantified as fold change over the DMSO group. Statistical evaluations were performed by using Student's *t*-test. (Right) KSHV viral particles released in the culture media from each treated groups were determined by comparative quantitative PCR of KSHV ORF9, as described in Figure 3D. Data are presented as means ± SD for three PCR assays in an experiment. Similar results were obtained from two independent experiments; one set of data is shown.

Here, we found that amino acids 633–652 of K-RTA share sequence homology with amino acids 554–573 of NELF-B, which is located in an *in vivo* phosphorylated tryptic peptide (554–580). Although the kinase that phosphorylates this tryptic peptide in NELF-B has not been characterized, we found that Ser-634 and Ser-636 in K-RTA are involved in CDK9 recruitment/phosphorylation and the expressions of multiple K-RTA target genes were impaired when CDK9 inhibitors were present. In addition, de-phosphorylated Ser-634 and Ser-636 mimetic, namely S634A/S636A, is 10 kDa shorter than the wild type, however, this decrement in molecular mass was not due to deficiency in global Ser- or Thr-phosphorylation, which raises a possibility that phosphorylation-mediated conformational change might be involved. Along the same vein, S634A/S636A was less immunoreactive to MPM2 antibody, suggesting that phosphorylation of ^{634}SPSP^{637} could be targeted by PIN1, a peptidyl-prolyl cis/trans isomerase that regulates the conformation and function of numerous MPM2-recognized proteins (Stukenberg and Kirschner, 2001; Lu and Zhou, 2007; Shaw, 2007). More experiments are required to prove this hypothesis. Taken together, it

is envisioned that K-RTA possesses functional domain similar to those in NELF, which serves to recruit CDK9 and assures a productive gene expression program on the viral genome.

Although K-RTA has been known as a nuclear protein, the full maturation process of K-RTA may involve multiple cellular compartments. To distinguish various compartment-specific phosphorylation processes, we constructed a nuclear localization mutant of K-RTA, referred to as NLSm. While primarily located in the cytoplasm by immunofluorescence assays, NLSm retained 50% wild type transactivation activity (**Figure 1D**). There are two possibilities to account for this result. First, a proportion of NLSm may "sneak into" the nucleus in the overwhelming liposomal transfection used in the luciferase reporter assay. This possibility is supported by the presence of the 114 kDa nuclear polypeptide in protein extracts of long term (24 h) Dox-treated NLSm cells (**Figure 2A**, middle). Alternatively, NLSm could exert some yet-to-be-identified actions in the cytosol. For example, EBV Rta was documented to be functional in the cytoplasm (Hsu et al., 2005).

Originally, we anticipated to see more phosphorylated residues in the 114 kDa nuclear form of K-RTA because of its apparently larger molecular mass. To our surprise, the same Thr-513 and Thr-514 were shown to be the only phosphorylated amino acids identified in both K-RTA and NLSm by LC/MS/MS. Two possibilities could contribute to this result. One is that our MS analysis may exclude some larger tryptic peptides because of their size (e.g., amino acids 531–633, approximately 10.6 kDa, contain 25 Ser/Thr). The other possibility is that we did not obtain adequate quantity of phosphorylated K-RTA in the first place. We believe that further improvement of the purification procedure, or the use of alternative proteases such as chymotrypsin to supplement the trypsin digestion, will help to identify more phosphorylated sites in the mature 114 kDa K-RTA by MS. Nonetheless, in the present study we show that Thr-513 or Thr-514 is the preferred phosphorylation site in K-RTA that is modified in the cytoplasmic compartment and retained in the nucleus.

In summary, hijacking of host transcription machinery, including CDK9, by the virus to express its own transcriptome is increasingly documented (Garber et al., 2000; Zhou et al., 2000; Bark-Jones et al., 2006; Durand and Roizman, 2008; Kapasi and Spector, 2008; Kapasi et al., 2009). Our finding that K-RTA-mediated transcriptional activation is impaired by two CDK9 inhibitors provides a new example of this paradigm. Interestingly, the CDK inhibitor R-roscovitine (also known as seliciclib and CYC202) was used recently to reduce tumor size and plasma EBV DNA in patients with nasopharyngeal carcinoma (Hsieh et al., 2009). Thus, the application of CDK9 inhibitors to disturb virus replication may provide a promising direction for treatment of viral malignancies including Kaposi's sarcoma.

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REFERENCES

Bark-Jones, S. J., Webb, H. M., and West, M. J. (2006). EBV EBNA 2 stimulates CDK9-dependent transcription and RNA polymerase II phosphorylation on serine 5. *Oncogene* 25, 1775–1785.

Barlev, N. A., Emelyanov, A. V., Castagnino, P., Zegerman, P., Bannister, A. J., Sepulveda, M. A., Robert, F., Tora, L., Kouzarides, T., Birshtein, B. K., and Berger, S. L. (2003). A novel human Ada2 homologue functions with Gcn5 or Brg1 to coactivate transcription. *Mol. Cell. Biol.* 23, 6944–6957.

Barrasa, M. I., Harel, N. Y., and Alwine, J. C. (2005). The phosphorylation status of the serine-rich region of the human cytomegalovirus 86-kilodalton major immediate-early protein IE2/IEP86 affects temporal viral gene expression. *J. Virol.* 79, 1428–1437.

Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., Villen, J., Li, J., Cohn, M. A., Cantley, L. C., and Gygi, S. P. (2004). Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. U.S.A.* 101, 12130–12135.

Bhende, P. M., Seaman, W. T., Delecluse, H. J., and Kenney, S. C. (2005). BZLF1 activation of the methylated form of the BRLF1 immediate-early promoter is regulated by BZLF1 residue 186. *J. Virol.* 79, 7338–7348.

Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4, 1633–1649.

Boutell, C., Everett, R., Hilliard, J., Schaffer, P., Orr, A., and Davido, D. (2008). Herpes simplex virus type 1 ICP0 phosphorylation mutants impair the E3 ubiquitin ligase activity of ICP0 in a cell type-dependent manner. *J. Virol.* 82, 10647–10656.

Brown, H. J., Peng, L., Harada, J. N., Walker, J. R., Cole, S., Lin, S. F., Zack, J. A., Chanda, S. K., and Sun, R. (2010). Gene expression and transcription factor profiling reveal inhibition of transcription factor cAMP-response element-binding protein by gamma-herpesvirus replication and transcription activator. *J. Biol. Chem.* 285, 25139–25153.

Bu, W., Palmeri, D., Krishnan, R., Marin, R., Aris, V. M., Soteropoulos, P., and Lukac, D. M. (2008). Identification of direct transcriptional targets of the Kaposi's sarcoma-associated herpesvirus Rta lytic switch protein by conditional nuclear localization. *J. Virol.* 82, 10709–10723.

Chang, H., Gwack, Y., Kingston, D., Souvlis, J., Liang, X., Means, R. E., Cesarman, E., Hutt-Fletcher, L., and Jung, J. U. (2005). Activation of CD21 and CD23 gene expression by Kaposi's sarcoma-associated herpesvirus RTA. *J. Virol.* 79, 4651–4663.

Chang, P. C., and Li, M. (2008). Kaposi's sarcoma-associated herpesvirus K-cyclin interacts with Cdk9 and stimulates Cdk9-mediated phosphorylation of p53 tumor suppressor. *J. Virol.* 82, 278–290.

Chang, P. J., Shedd, D., and Miller, G. (2008). A mobile functional region of Kaposi's sarcoma-associated herpesvirus ORF50 protein independently regulates DNA binding and protein abundance. *J. Virol.* 82, 9700–9716.

Chen, J., Ueda, K., Sakakibara, S., Okuno, T., and Yamanishi, K. (2000). Transcriptional regulation of the Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor gene. *J. Virol.* 74, 8623–8634.

Chen, Y. J., Tsai, W. H., Chen, Y. L., Ko, Y. C., Chou, S. P., Chen, J. Y., and Lin, S. F. (2011). Epstein-Barr virus (EBV) Rta-mediated EBV and Kaposi's Sarcoma-associated herpesvirus lytic reactivations in 293 cells. *PLoS ONE* 6, e17809. doi:10.1371/journal.pone.0017809

Cokol, M., Nair, R., and Rost, B. (2000). Finding nuclear localization signals. *EMBO Rep.* 1, 411–415.

Durand, L. O., and Roizman, B. (2008). Role of cdk9 in the optimization of expression of the genes regulated by ICP22 of herpes simplex virus 1. *J. Virol.* 82, 10591–10599.

El-Guindy, A. S., and Miller, G. (2004). Phosphorylation of Epstein-Barr virus ZEBRA protein at its casein kinase 2 sites mediates its ability to repress activation of a viral lytic cycle late gene by Rta. *J. Virol.* 78, 7634–7644.

Fakhari, F. D., and Dittmer, D. P. (2002). Charting latency transcripts in Kaposi's sarcoma-associated herpesvirus by whole-genome real-time quantitative PCR. *J. Virol.* 76, 6213–6223.

Foskett, S. M., Ghose, R., Tang, D. N., Lewis, D. E., and Rice, A. P. (2001). Antiapoptotic function of Cdk9 (TAK/P-TEFb) in U937 promonocytic cells. *J. Virol.* 75, 1220–1228.

Francis, A. L., Gradoville, L., and Miller, G. (1997). Alteration of a single serine in the basic domain of the Epstein-Barr virus ZEBRA protein separates its functions of transcriptional activation and disruption of latency. *J. Virol.* 71, 3054–3061.

Fujinaga, K., Irwin, D., Geyer, M., and Peterlin, B. M. (2002). Optimized chimeras between kinase-inactive mutant Cdk9 and truncated cyclin T1 proteins efficiently inhibit Tat transactivation and human immunodeficiency virus gene expression. *J. Virol.* 76, 10873–10881.

Ganem, D. (2007). "Kaposi's sarcoma-associated herpesvirus," in *Fields Virology*, eds D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (Philadelphia, PA: Lippincott Williams and Wilkins), 2847–2888.

Garber, M. E., Mayall, T. P., Suess, E. M., Meisenhelder, J., Thompson, N. E., and Jones, K. A. (2000). CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. *Mol. Cell. Biol.* 20, 6958–6969.

Gilchrist, D. A., Nechaev, S., Lee, C., Ghosh, S. K., Collins, J. B., Li, L., Gilmour, D. S., and Adelman, K. (2008). NELF-mediated stalling of Pol II can enhance gene expression by blocking promoter-proximal nucleosome assembly. *Genes Dev.* 22, 1921–1933.

Gradoville, L., Gerlach, J., Grogan, E., Shedd, D., Nikiforow, S., Metroka, C., and Miller, G. (2000). Kaposi's sarcoma-associated herpesvirus open reading frame 50/Rta protein activates the entire viral lytic cycle in the HH-B2 primary effusion lymphoma cell line. *J. Virol.* 74, 6207–6212.

Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R., and Young, R. A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* 130, 77–88.

Gunther, T., and Grundhoff, A. (2010). The epigenetic landscape of latent Kaposi sarcoma-associated herpesvirus genomes. *PLoS Pathog.* 6, e1000935. doi:10.1371/journal.ppat.1000935

Gwack, Y., Baek, H. J., Nakamura, H., Lee, S. H., Meisterernst, M., Roeder, R. G., and Jung, J. U. (2003a). Principal role of TRAP/mediator and SWI/SNF complexes in Kaposi's sarcoma-associated herpesvirus RTA-mediated lytic reactivation. *Mol. Cell. Biol.* 23, 2055–2067.

Gwack, Y., Nakamura, H., Lee, S. H., Souvlis, J., Yustein, J. T., Gygi, S., Kung, H. J., and Jung, J. U. (2003b). Poly(ADP-ribose) polymerase 1 and Ste20-like kinase hKFC act as transcriptional repressors for gamma-2 herpesvirus lytic replication. *Mol. Cell. Biol.* 23, 8282–8294.

Gwack, Y., Byun, H., Hwang, S., Lim, C., and Choe, J. (2001). CREB-binding protein and histone deacetylase regulate the transcriptional activity of Kaposi's sarcoma-associated herpesvirus open reading frame 50. *J. Virol.* 75, 1909–1917.

Gwack, Y., Hwang, S., Lim, C., Won, Y. S., Lee, C. H., and Choe, J. (2002). Kaposi's Sarcoma-associated herpesvirus open reading frame 50 stimulates the transcriptional activity of STAT3. *J. Biol. Chem.* 277, 6438–6442.

Hsiao, H. H., Hsieh, H. Y., Chou, C. C., Lin, S. Y., Wang, A. H., and Khoo, K. H. (2007). Concerted experimental approach for sequential mapping of peptides and phosphopeptides using C18-functionalized magnetic nanoparticles. *J. Proteome Res.* 6, 1313–1324.

Hsieh, W. S., Soo, R., Peh, B. K., Loh, T., Dong, D., Soh, D., Wong, L. S., Green, S., Chiao, J., Cui, C. Y., Lai, Y. F., Lee, S. C., Mow, B., Soong, R., Salto-Tellez, M., and Goh, B. C. (2009). Pharmacodynamic effects of seliciclib, an orally administered cell cycle modulator, in undifferentiated nasopharyngeal cancer. *Clin. Cancer Res.* 15, 1435–1442.

Hsu, T. Y., Chang, Y., Wang, P. W., Liu, M. Y., Chen, M. R., Chen, J. Y., and Tsai, C. H. (2005). Reactivation of Epstein-Barr virus can be triggered by an Rta protein mutated at the nuclear localization signal. *J. Gen. Virol.* 86, 317–322.

Kang, H., and Lieberman, P. M. (2011). Mechanism of glycyrrhizic acid inhibition of Kaposi's sarcoma-associated herpesvirus: disruption of CTCF-cohesin-mediated RNA polymerase II pausing and sister chromatid cohesion. *J. Virol.* 85, 11159–11169.

Kapasi, A. J., Clark, C. L., Tran, K., and Spector, D. H. (2009). Recruitment of cdk9 to the immediate-early viral transcriptosomes during human cytomegalovirus infection requires efficient binding to cyclin T1, a threshold level of IE2 86, and active transcription. *J. Virol.* 83, 5904–5917.

Kapasi, A. J., and Spector, D. H. (2008). Inhibition of the cyclin-dependent kinases at the beginning of human cytomegalovirus infection specifically alters the levels and localization of the RNA polymerase II carboxyl-terminal domain kinases cdk9 and cdk7 at the viral transcriptosome. *J. Virol.* 82, 394–407.

Liang, Y., and Ganem, D. (2003). Lytic but not latent infection by Kaposi's sarcoma-associated herpesvirus requires host CSL protein, the mediator of Notch signaling. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8490–8495.

Lin, S. Y., Chen, Y. Y., Fan, Y. Y., Lin, C. W., Chen, S. T., Wang, A. H., and Khoo, K. H. (2008). Precise mapping of increased sialylation pattern and the expression of acute phase proteins accompanying murine tumor progression in BALB/c mouse by integrated sera proteomics and glycomics. *J. Proteome Res.* 7, 3293–3303.

Lolli, G. (2009). Binding to DNA of the RNA-polymerase II C-terminal domain allows discrimination between Cdk7 and Cdk9 phosphorylation. *Nucleic Acids Res.* 37, 1260–1268.

Lu, K. P., and Zhou, X. Z. (2007). The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease. *Nat. Rev. Mol. Cell Biol.* 8, 904–916.

Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999). Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. *J. Virol.* 73, 9348–9361.

Lukac, D. M., Renne, R., Kirshner, J. R., and Ganem, D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. *Virology* 252, 304–312.

Marchler-Bauer, A., Anderson, J. B., Cherukuri, P. F., DeWeese-Scott, C., Geer, L. Y., Gwadz, M., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Marchler, G. H., Mullokandov, M., Shoemaker, B. A., Simonyan, V., Song, J. S., Thiessen, P. A., Yamashita, R. A., Yin, J. J., Zhang, D., and Bryant, S. H. (2005). CDD: a conserved domain database for protein classification. *Nucleic Acids Res.* 33, D192–D196.

Muse, G. W., Gilchrist, D. A., Nechaev, S., Shah, R., Parker, J. S., Grissom, S. F., Zeitlinger, J., and Adelman, K. (2007). RNA polymerase is poised for activation across the genome. *Nat. Genet.* 39, 1507–1511.

Nakamura, H., Lu, M., Gwack, Y., Souvlis, J., Zeichner, S. L., and Jung, J. U. (2003). Global changes in Kaposi's sarcoma-associated virus gene expression patterns following expression of a tetracycline-inducible Rta transactivator. *J. Virol.* 77, 4205–4220.

Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127, 635–648.

Palermo, R. D., Webb, H. M., and West, M. J. (2011). RNA polymerase II stalling promotes nucleosome occlusion and pTEFb recruitment to drive immortalization by Epstein-Barr virus. *PLoS Pathog.* 7, e1002334. doi:10.1371/journal.ppat.1002334

Peterlin, B. M., and Price, D. H. (2006). Controlling the elongation phase of transcription with P-TEFb. *Mol. Cell* 23, 297–305.

Romano, G., and Giordano, A. (2008). Role of the cyclin-dependent kinase 9-related pathway in mammalian gene expression and human diseases. *Cell Cycle* 7, 3664–3668.

Sakakibara, S., Ueda, K., Chen, J., Okuno, T., and Yamanishi, K. (2001). Octamer-binding sequence is a key element for the autoregulation of Kaposi's sarcoma-associated herpesvirus ORF50/Lyta gene expression. *J. Virol.* 75, 6894–6900.

Sarid, R., Flore, O., Bohenzky, R. A., Chang, Y., and Moore, P. S. (1998). Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). *J. Virol.* 72, 1005–1012.

Saunders, A., Core, L. J., and Lis, J. T. (2006). Breaking barriers to transcription elongation. *Nat. Rev. Mol. Cell Biol.* 7, 557–567.

Shaw, P. E. (2007). Peptidyl-prolyl cis/trans isomerases and transcription: is there a twist in the tail? *EMBO Rep.* 8, 40–45.

Stukenberg, P. T., and Kirschner, M. W. (2001). Pin1 acts catalytically to promote a conformational change in Cdc25. *Mol. Cell* 7, 1071–1083.

Sun, R., Lin, S. F., Staskus, K., Gradoville, L., Grogan, E., Haase, A., and Miller, G. (1999). Kinetics

of Kaposi's sarcoma-associated herpesvirus gene expression. *J. Virol.* 73, 2232–2242.

Taylor, S. L., Kinchington, P. R., Brooks, A., and Moffat, J. F. (2004). Roscovitine, a cyclin-dependent kinase inhibitor, prevents replication of varicella-zoster virus. *J. Virol.* 78, 2853–2862.

Toth, Z., Maglinte, D. T., Lee, S. H., Lee, H. R., Wong, L. Y., Brulois, K. F., Lee, S., Buckley, J. D., Laird, P. W., Marquez, V. E., and Jung, J. U. (2010). Epigenetic analysis of KSHV latent and lytic genomes. *PLoS Pathog.* 6, e1001013. doi:10.1371/journal.ppat.1001013

Trester-Zedlitz, M., Burlingame, A., Kobilka, B., and von Zastrow, M. (2005). Mass spectrometric analysis of agonist effects on posttranslational modifications of the beta-2 adrenoceptor in mammalian cells. *Biochemistry* 44, 6133–6143.

Vieira, J., and O'Hearn, P. M. (2004). Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. *Virology* 325, 225–240.

Wang, J. T., Yang, P. W., Lee, C. P., Han, C. H., Tsai, C. H., and Chen, M. R. (2005). Detection of Epstein-Barr virus BGLF4 protein kinase in virus replication compartments and virus particles. *J. Gen. Virol.* 86, 3215–3225.

Wang, S. E., Wu, F. Y., Yu, Y., and Hayward, G. S. (2003). CCAAT/enhancer-binding protein-alpha is induced during the early stages of Kaposi's sarcoma-associated herpesvirus (KSHV) lytic cycle reactivation and together with the KSHV replication and transcription activator (RTA) cooperatively stimulates the viral RTA, MTA, and PAN promoters. *J. Virol.* 77, 9590–9612.

Xue, Y., Ren, J., Gao, X., Jin, C., Wen, L., and Yao, X. (2008). GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol. Cell. Proteomics* 7, 1598–1608.

Yang, Z., and Wood, C. (2007). The transcriptional repressor K-RBP modulates RTA-mediated transactivation and lytic replication of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 81, 6294–6306.

Yoo, S. M., Zhou, F. C., Ye, F. C., Pan, H. Y., and Gao, S. J. (2005). Early and sustained expression of latent and host modulating genes in coordinated transcriptional program of KSHV productive primary infection of human primary endothelial cells. *Virology* 343, 47–64.

Zeitlinger, J., Stark, A., Kellis, M., Hong, J. W., Nechaev, S., Adelman, K., Levine, M., and Young, R. A. (2007). RNA polymerase stalling at developmental control genes in the *Drosophila melanogaster* embryo. *Nat. Genet.* 39, 1512–1516.

Zhou, M., Halanski, M. A., Radonovich, M. F., Kashanchi, F., Peng, J., Price, D. H., and Brady, J. N. (2000). Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. *Mol. Cell. Biol.* 20, 5077–5086.

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APPENDIX

Table A1 | Sequences of primers used in Figures 3 and 5.

	Forward seq (5′ → 3′)	Reverse seq (5′ → 3′)
KSHV GENES		
K-bZIP	TGTGCCGTCGTCCGG	TGGATGGTTCCCCAGATGA
ORF2	TGCTCGCCAGGCTTGG	CGTGTTTCTCTCGCATGATAGC
ORF36	CACCGGCAAAGCCCAG	TGCTTCTGAAACGCCAGCT
ORF59	CGAGTCTTCGCAAAAGGTTC	AAGGGACCAACTGGTGTGAG
PAN	CTGGATGTGTATCTTATTGGTGC	CGCCTATGTCATTCAAATCG
CELLULAR GENES		
18S rRNA	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC
ACTB	CCTTGGCATCCACGAAACT	TCTCCTTCTGCATCCTGTCG
GAPDH	CAAGAAGGTGGTGAAGCAGG	GCTGTTGAAGTCAGAGGAGACC
MYC	AGCATACATCCTGTCCGTCC	CTCAGCCAAGGTTGTGAGGT
SERPINB2	GTGTTATGACAGGGAGAACTGG	GGTGAGGAAAATCTGCCG

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The Kaposi's sarcoma-associated herpesvirus ORF57 protein and its multiple roles in mRNA biogenesis

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Post-transcriptional events which regulate mRNA biogenesis are fundamental to the control of gene expression. A nascent mRNA is therefore steered through multimeric RNA–protein complexes that mediate its capping, splicing, polyadenylation, nuclear export, and ultimately its translation. Kaposi's sarcoma-associated herpesvirus (KSHV) mRNA transport and accumulation protein, or ORF57, is a functionally conserved protein found in all herpesviruses which plays a pivotal role in enhancing viral gene expression at a post-transcriptional level. As such, ORF57 has been implicated in multiple steps of RNA biogenesis, including augmenting viral splicing, protecting viral RNAs from degradation to enhancing viral mRNA nuclear export and translation. In this review, we highlight the multiple roles of KSHV ORF57 in regulating the post-transcriptional events which are fundamental to the control of virus gene expression.

Keywords: KSHV, ORF57, mRNA export, mRNA stability

KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) or human herpesvirus 8 is a gamma-2 herpesvirus associated with multiple AIDS-related malignancies (Chang et al., 1994), including KS, a highly vascular tumor of endothelial lymphatic origin (Ganem, 2006), and two lymphoproliferative disorders; primary effusion lymphoma and multicentric Castleman's disease (Chang et al., 1994; Cesarman et al., 1995; Soulier et al., 1995). In sub-Saharan Africa, widespread HIV infection has turned KS into an epidemic disease and KS is now amongst the most common of all diagnosed malignancies (Parkin et al., 2008). Moreover, solid-organ transplant recipients are at an increased risk of developing KS due to a pre-existing infection or recipients being infected by donors (Piselli et al., 2009).

Like other herpesviruses, KSHV has two distinct forms of infection, latency, and lytic replication (Ganem, 2006). However, in contrast to other oncogenic herpesviruses, where latent gene expression plays a prominent role in tumorigenesis, lytic replication plays an important part in the tumorigenicity, pathogenesis, and spread of KSHV infection (Cai et al., 2010). Specifically, lytic replication appears to be a necessary antecedent step in KS development from the primary target of viral infection, the B lymphocyte reservoir, to endothelial cells where tumors are observed. Moreover, lytic gene expression potentially contributes to the development of KS through the expression of lytic viral proteins which mediate paracrine secretion of growth and angiogenic factors that are essential for tumor growth and development (Ballon et al., 2011; Bottero et al., 2011). In addition, they sustain the population of latently infected cells that would otherwise be reduced due to the poor persistence of the KSHV episome during spindle cell division (Grundhoff and Ganem, 2004). Therefore, it is essential to study the molecular mechanisms which regulate lytic replication to fully understand KSHV pathogenesis. This in turn may lead to novel therapeutic interventions which could become an important strategy for the treatment of KSHV-associated diseases.

Post-transcriptional events which regulate mRNA biogenesis are fundamental to the control of gene expression. As a consequence, cells have evolved a "gene expression production line" that encompasses the routing of a nascent transcript through multimeric mRNA–protein complexes that mediate its splicing, polyadenylation, nuclear export, and translation (Hastings and Krainer, 2001; Proudfoot, 2011; Rodríguez-Navarro and Hurt, 2011). These pathways are particularly important for herpesviruses which replicate in the host cell nucleus and express numerous lytic intronless mRNAs. Due to the reliance of herpesviruses on the host cell machinery for efficient processing of their mRNAs, an immediate issue arises concerning the mechanism by which the viral intronless mRNAs are efficiently exported from the nucleus, given that the majority of cellular bulk mRNA nuclear export is intimately linked, and dependent upon, splicing (Luo and Reed, 1999; Valencia et al., 2008).

To circumvent the problem associated with efficient intronless viral mRNA nuclear export KSHV encodes a multifunctional protein, the mRNA transport and accumulation (MTA) protein, or ORF57, which functions in many aspects of RNA processing (Malik et al., 2004b). Many of these properties are also conserved in ORF57 homologs throughout herpesviruses including HSV-1 ICP27; HSV-2 UL54; EBV SM/EB2; CMV UL69, VZV ORF4, HHV-6 U42, HHV-7 U42, HVS ORF57 (Swaminathan, 2005; Boyne and Whitehouse, 2006a; Boyne et al., 2008a; Sandri-Goldin, 2008; Toth and Stamminger, 2008; Ote et al., 2009). The most widely characterized of these is the herpes simplex (HSV-1) homolog ICP27, which has been used as a prototype for herpes virus infection (Sandri-Goldin, 2008). Functionally, ORF57 and

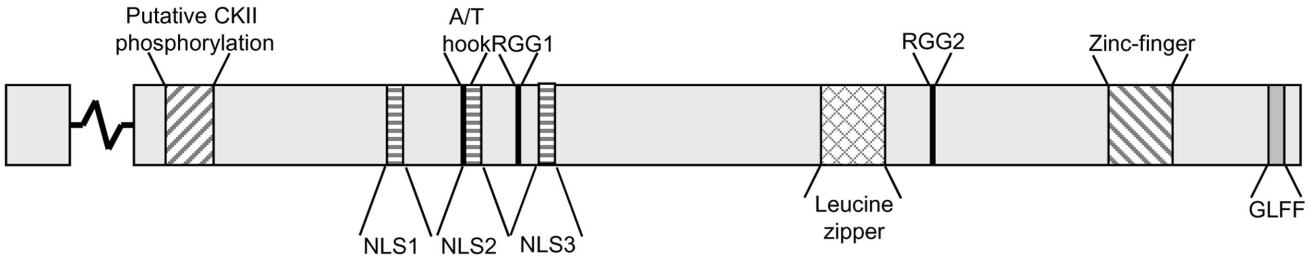

FIGURE 1 | Putative motifs within the KSHV ORF57 protein. KSHV ORF57 is a 455 amino acid protein with an exon junction within the codon for aa 17. The functional motifs comprise a putative CKII phosphorylation site (region 18–43); three nuclear localization signals (NLS1,2,3; regions 101–107, 121–131, 143–152); an A/T hook (region 119–131); two arginine–glycine–glycine motifs (RGG1,2; regions 138–140, 372–374); a leucine zipper (region 343–364); a zinc finger domain (region 423–432); and a hydrophobic glycine–leucine–phenylalanine–phenylalanine motif (GLFF; region 448–451). The putative phosphorylation sites were predicted using GPS V2.1 (Xue et al., 2008).

its homologs regulate viral and cellular RNA processing, which results in accumulation of target genes and may also contribute to host cell shut-off (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Ruvolo et al., 1998; Whitehouse et al., 1998). However, ORF57 homologs are evolutionarily diverged, with little sequence conservation and have adapted into different roles in the respective viruses. Therefore this review focuses on KSHV ORF57 and its role in the lytic replication cycle and mRNA processing.

THE KSHV ORF57 PROTEIN

ORF57 GENE AND PROTEIN STRUCTURE

Kaposi's sarcoma-associated herpesvirus lytic gene expression is initiated by the immediate early protein the replication and transcription activator, RTA, which is necessary and sufficient for complete reactivation into the lytic cascade (Lukac et al., 1998, 1999). RTA functions as a viral transcription factor to initiate transcription of various viral genes by multiple mechanisms (Dourmishev et al., 2003). Interestingly, although RTA has been shown to be able to recognize specific RTA response elements in promoters, it activates the ORF57 promoter indirectly via an interaction with RBP-Jκ (Liang et al., 2002; Chang et al., 2005). Additional cellular cofactors including HMGB1 are also thought to be necessary for efficient RTA-mediated activation of the ORF57 promoter (Song et al., 2004; Harrison and Whitehouse, 2008).

ORF57 is transcribed as a monocistronic pre-mRNA, containing a small intron comprising 109 nucleotides. It is interesting to note that both RTA and ORF57, as immediate early proteins contain introns enabling efficient mRNA processing prior to ORF57 action on numerous delayed early and late intronless transcripts. The ORF57 gene encodes a 455 aa protein, with only the first 16 aa translated from the first exon. Currently there are no solved structures for KSHV ORF57 or any of its homologs, but computer predictions indicate that the N-terminal region is mainly unstructured, whereas the C-terminal domain forms primarily alpha helices (Taylor et al., 2011). Nevertheless, several functional regions have been identified (**Figure 1**).

Three nuclear localization signals (NLS) are located in the N-terminal region of ORF57. Each NLS consists of a stretch of basic residues and each individual NLS is sufficient to localize ORF57 into the nucleus. Mutation of any two of these NLS is sufficient to severely inhibit ORF57 function (Majerciak et al., 2006). Furthermore, it has been shown that these NLS also confer nucleolar localization (**Figure 2**). Nucleolar trafficking is thought to be essential for KSHV ORF57 function, as well as the related HVS ORF57 (Boyne and Whitehouse, 2006b, 2009), however as yet the role of the nucleolus in ORF57 function is yet to be determined.

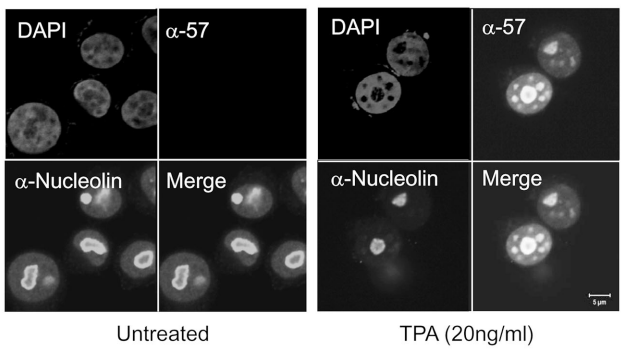

FIGURE 2 | Cellular localization of KSHV ORF57. BCBL-1 cells were reactivated with TPA to express the lytic KSHV genes. Cells were fixed and permeabilized and proteins immunostained using an ORF57-specific antibody and a monoclonal antibody to C23 and FITC and Texas Red secondary antibodies. ORF57 can be seen localizing in the nucleus, as well as the nuclear speckles and colocalizing in the nucleolus with C23.

ORF57 binds to both viral and cellular target RNAs, for example cellular IL-6 and viral vIL-6 have been shown to interact with ORF57 using crosslinking immunoprecipitation (CLIP) assays (Kang et al., 2011). Two RGG motifs, which are putative RNA binding sites are found in ORF57. However, deletion of the first RGG motif has no effect on ORF57 RNA binding, while deletion of the second C-terminal RGG motif results in a lack of RNA binding and consequently inefficient nuclear export of a viral target RNA. Although, this could be due to a deficiency in RNA binding or binding of cellular interacting proteins (Nekorchuk et al., 2007). However, more recent evidence using site-directed mutants of the RGG motifs suggests to defects in homodimerization and intranuclear trafficking of the RGG2 mutant (Taylor et al., 2011). Interestingly, the N-terminal domain is also thought to be sufficient to bind RNA (Majerciak et al., 2006). A similar observation

has also been seen in the highly conserved Herpesvirus saimiri ORF57 protein (Goodwin et al., 1999). Further work is necessary to fully elucidate the ORF57 RNA binding domain or indeed domains.

ORF57 also contains an uncharacterized AT-Hook motif. AT-Hook motifs are generally found in DNA binding proteins and are also found in a series of transcriptional cofactors (Aravind and Landsman, 1998). ORF57 has been shown to bind directly to DNA, with the AT-Hook domain implicated in this interaction (Palmeri et al., 2007). EMSA-based analysis shows a deletion of the AT-Hook region lead to a loss of ORF57 activation of the polyadenylated nuclear RNA (PAN) promoter and diminished ability of ORF57 to bind DNA. It is intriguing to note that ORF57 has been described to act as a cofactor for RTA-mediated transactivation (Kirshner et al., 2000), which will be discussed in more detail later. Additionally, a putative leucine zipper domain is located in the C-terminal domain of ORF57, however the function of this region is still unclear. Although, a leucine rich region in the ORF57 homolog ICP27 from HSV-1, has been shown to contain a functional nuclear export signal (Sandri-Goldin, 1998). Together with the NLS, this motif might therefore be responsible for the ability of ORF57 to shuttle between nucleus and cytoplasm (Bello et al., 1999).

Finally, the C-terminal domain of ORF57 also contains a zinc finger domain and a GLFF motif. The zinc finger domain is conserved throughout ORF57 homologs whereas the GLFF motif is restricted to gamma-herpesviruses (Goodwin et al., 2000). The functional relevance of the zinc finger domain of KSHV ORF57 is not fully characterized, however work on HSV-1 ICP27 highlighted a potential role in dimerization and binding of the nuclear export factor TAP (Hernandez and Sandri-Goldin, 2010, 2011). The GLFF motif plays a role in transactivation as well as repression properties of the HVS ORF57 protein and SM protein in EBV, however, again no role has been shown in KSHV to date (Goodwin et al., 2000; Ruvolo et al., 2004).

CASPASE-7 CLEAVAGE OF ORF57

An interesting recent study presented a hypothesis for a possible cellular mechanism against lytic KSHV virus infection (Majerciak et al., 2010). The authors showed that infection with KSHV or reactivation into the lytic cycle induces the caspase-8 mediated apoptosis pathway. This leads to the expression of caspase-7 which was shown to cleave ORF57 33 amino acids from the N-terminus. This cleaved form of ORF57 has been shown to be deficient in its functional roles of viral mRNA maturation and processing. Furthermore, cells expressing caspase-7 showed little lytic reactivation and underwent caspase-induced apoptosis, whereas cells expressing full-length ORF57 appeared to show diminished activity of caspase-7 and were able to enter into a full lytic cycle. The mechanisms underlying this delicate balancing act are yet to be defined, but understanding how caspase-7 is suppressed in a number of lytically active cells, and how caspase-8 is induced leading to apoptosis in the remainder of cells presents an interesting scenario for a potential antiviral mechanism against KSHV. It is, however, possible that this cleavage is simply a neutral event, or perhaps even beneficial to the virus. For example, KSHV could utilize this as a post-translational mechanism to control ORF57 activity. Alternatively, it maybe possible that the virus could utilize this cleavage event to prevent full lytic reactivation in the majority of cells maintaining the large latent pool of infected cells.

THE MULTIFUNCTIONAL ROLES OF ORF57 IN mRNA BIOGENESIS

THE ROLE OF ORF57 IN SPLICING

The mechanism of pre-mRNA splicing and the function of ORF57 and its homologs is closely linked. For example, HSV-1 ICP27 effectively inhibits splicing to reduce host cell gene expression, thereby eliciting a host cell shut-off (Smith et al., 2005). This is mediated by an interaction between ICP27 and SAP145, an essential pre-mRNA splicing factor (Bryant et al., 2001). No such effect has been reported for KSHV ORF57 to date, as KSHV encodes a number of spliced transcripts. On the contrary, KSHV ORF57 is able to enhance splicing of a number of viral genes (Majerciak et al., 2008). This enhancement effect on splicing is not limited to KSHV genes as a similar enhancement has been observed on non-viral mRNA constructs. ORF57 was able to enhance splicing of reporter constructs which are normally only poorly spliced due to a large exon prior to the spliced intron. ORF57 forms a complex with the spliceosome, as demonstrated by association with the small spliceosomal RNAs (U1, U2, U4, U5, and U6), as well as the splicing factors SF2/ASF and U2AF (Majerciak et al., 2008). Interestingly, association of ORF57 with unspliced pre-mRNA could only be observed in the presence of nuclear extract and not purified ORF57, indicating that the association with pre-mRNA is indirect and ORF57 is acting as a modulator of normal splicing rather than directly recruiting splicing factors to the mRNA. A similar effect has since also been reported for the EBV homolog of ORF57, SM, which acts as an alternative splicing factor, influencing the choice of splice site (Verma and Swaminathan, 2008). To this end, SM has been reported to interact directly with SRp20 (Verma et al., 2010) however it remains to be determined whether the same is true for KSHV ORF57.

ORF57 FUNCTIONS IN INTRONLESS VIRAL mRNA EXPORT

Spicing of cellular mRNAs is intrinsically linked to a number of upstream and downstream processes, including transcription, 3′ end formation, mRNA export, RNA stability, and translation (Moore and Proudfoot, 2009). This link is due to the recruitment of numerous proteins to an mRNA during the splicing process. One such complex of proteins is the human transport/export (hTREX) complex which comprises multiple proteins including UAP56, Aly, the multi-protein THO complex, and the recently discovered Tex1 and CIP29 (Masuda et al., 2005; Dufu et al., 2010). hTREX is deposited onto the 5′ end of the mRNA in a splicing-dependent manner through an interaction between Aly and the cap-binding complex (Cheng et al., 2006). Aly then recruits the remainder of the hTREX complex before interacting with the nuclear export receptor protein, TAP. The handover from Aly to TAP is promoted by arginine methylation of Aly within its TAP and RNA binding domains (Hung et al., 2010). Methylation of these sites reduces the RNA binding affinity of Aly and allows TAP to efficiently displace Aly during the export process. TAP then interacts with nucleoporins to facilitate the transport of the mRNP through the nuclear pore (Kohler and Hurt, 2007). However, whilst understanding of the export of intron-containing genes has

increased significantly over recent years, the mechanisms underlying intronless cellular mRNA export are poorly understood. This is of particular interest with regards to ORF57 as numerous KSHV mRNAs are intronless. However, a recent study has identified that some intronless cellular mRNAs can also be exported by hTREX and TAP, increasing our understanding of how intronless mRNAs are processed (Lei et al., 2011).

A pivotal function of ORF57 in viral mRNA metabolism is its role in nuclear export of viral mRNA. To efficiently export viral intronless mRNAs from the nucleus ORF57 is able to recruit the entire hTREX complex to intronless viral mRNAs (Boyne et al., 2008b; Tunnicliffe et al., 2010). ORF57 is able to bind directly to the RNA and recruit the export adapter, Aly to the 5′ end of the RNA, bypassing the requirement for splicing-dependent recruitment of Aly. Aly then recruits the remainder of the hTREX complex as well as TAP to form an export competent viral RNP which then allows efficient translocation through the nuclear pore complex. This function is also conserved in the HVS ORF57 homolog (Colgan et al., 2009).

However, a long-standing conundrum with this model has been the role of the exporter adapter Aly. Surprisingly, depletion studies in both mammalian and viral systems show that Aly is dispensable for mRNA export. The explanation for this appears to lie in an apparent redundancy in the eukaryotic hTREX components; specifically due to the discovery of a second export adapter protein, UAP56 interacting factor (UIF; Hautbergue et al., 2009). UIF binds to the mRNA in a splicing-independent reaction and is recruited to the mRNA via a direct interaction with the histone chaperone FACT. UIF can then interact with TAP to facilitate delivery of the mRNA to the nuclear pore. To this end, ORF57 is now known to interact directly with both Aly and UIF and to recruit the entire hTREX complex through these interactions (Jackson et al., 2011), as well as interacting with other cellular factors such as RBM15 and OTT3 (Majerciak et al., 2011; **Figure 3**). While our understanding of the proteins involved in the export of intronless KSHV mRNAs has improved, the exact mechanism remains elusive. For example, it has been demonstrated that ORF57 interacts directly with UIF and Aly, but it is not known whether these interactions occur simultaneously or independent of one another. siRNA depletion of both Aly and UIF is sufficient to severely impair ORF57-mediated mRNA export. However, depletion of Aly and UIF individually also causes a slight decrease in the level of mRNA export, perhaps suggesting that both proteins are required simultaneously for the efficient export KSHV mRNAs.

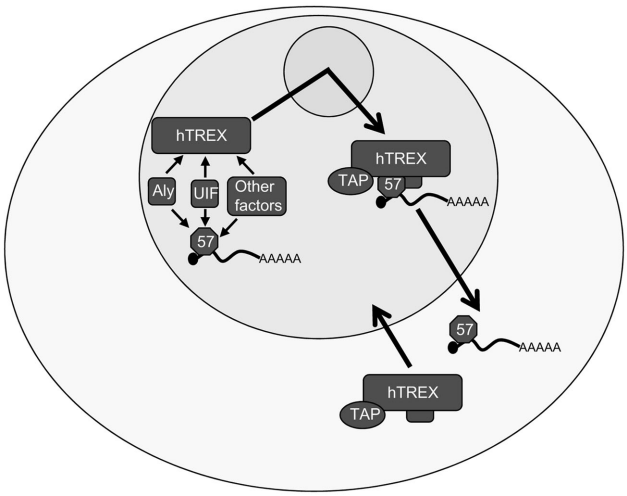

FIGURE 3 | Kaposi's sarcoma-associated herpesvirus ORF57-mediated intronless viral mRNA nuclear export. ORF57 binds to intronless viral mRNA in the nucleus and interacts directly with the export adapters Aly and UIF. Via the interaction with one or both of these export adapters ORF57 can then recruit the remainder of the hTREX complex, including the proteins UAP56 and the THO complex as well as the export receptor, TAP. ORF57 shuttles this complex through the nucleolus prior to the mRNA being exported into the cytoplasm in a TAP-dependent mechanism. The hTREX components are then recycled back into the nucleus while ORF57 remains bound to the viral mRNA for involvement in further downstream mechanisms.

THE ROLE OF ORF57 IN mRNA STABILITY

Another major role of KSHV ORF57 is to enhance RNA stability. Multiple studies have shown that a series of KSHV mRNAs are stabilized by ORF57 (e.g., ORF47, ORF59, and PAN), whereas others show no dependence on ORF57 (e.g., GCR and K5), suggesting that a specific RNA motif may be recognized by ORF57 to enhance the stability of target mRNAs (Kirshner et al., 2000; Boyne et al., 2008b). Recent analysis has now shed light on a possible ORF57 response element (ORE), although to date information is mainly limited onto the KSHV PAN. An ORE has been identified in the 5′ end of PAN and further analysis showed that the transfer of this element confers ORF57 responsiveness to an intronless reporter construct (Sei and Conrad, 2011). Deletion and mutational mapping have demonstrated that a nine nucleotide long core sequence is essential for the binding of ORF57 to PAN. A similar response element was independently confirmed, however the suggested minimal element of nine nucleotides is one position shifted compared to the sequence identified by Sei and Conrad (Massimelli et al., 2011). The binding of ORF57 to PAN via this RNA sequence is believed to enhance PAN RNA stability. In addition, two further proteins have been shown to interact with the ORF57 binding sequence, namely PABPC1 and EIB-AP5 (Massimelli et al., 2011). As the PABPC1 binding to the ORE is ORF57-dependent and specific to this binding site, it has been suggested that PABPC1 recruitment might be involved in stabilizing the PAN RNA in an ORF57-dependent manner (Massimelli et al., 2011; **Figure 4**).

An additional ORE has been identified within the KSHV vIL-6 mRNA (Kang et al., 2011). This sequence however only shares a 4-bp core (GGAU) with the PAN minimal ORE. Interestingly, investigations of ORF57 binding to this ORE highlighted a mechanism by which ORF57 stabilizes and protects vIL-6 from miRNA-mediated degradation. ORF57 protects the mRNA from degradation by a miRNA by a competitive binding mechanism. Moreover, the cellular IL-6 is also protected by ORF57 binding from the action of a second miRNA. How the other ORF57 target mRNAs are recognized remains still unclear, but considering the pace of recent findings, a better understanding of the underlying mechanism of ORF57 RNA binding and RNA stability seems closer than ever.

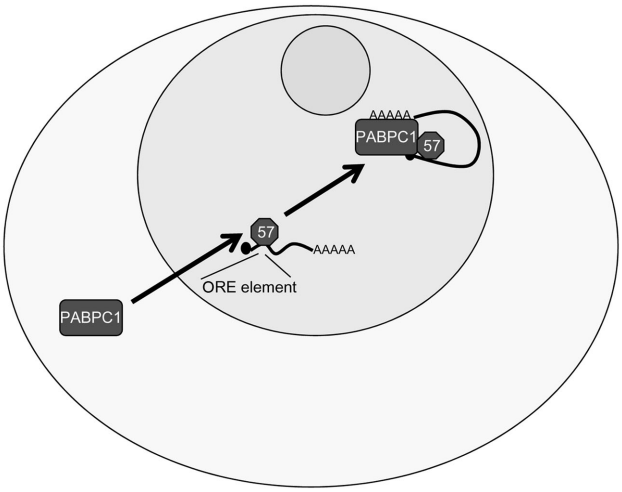

FIGURE 4 | Kaposi's sarcoma-associated herpesvirus ORF57 stabilizes PAN RNA in the nucleus. ORF57 binds to the ORF57 response element (ORE) in the 5′ of the KSHV PAN RNA, stabilizing PAN, and increasing expression. Additionally, ORF57 is able to redistribute PABPC1 to the nucleus, as well as PABPC1 being able to bind to the ORE, possibly explaining the stabilizing ability of the ORF57–PABPC1 interaction on PAN RNA.

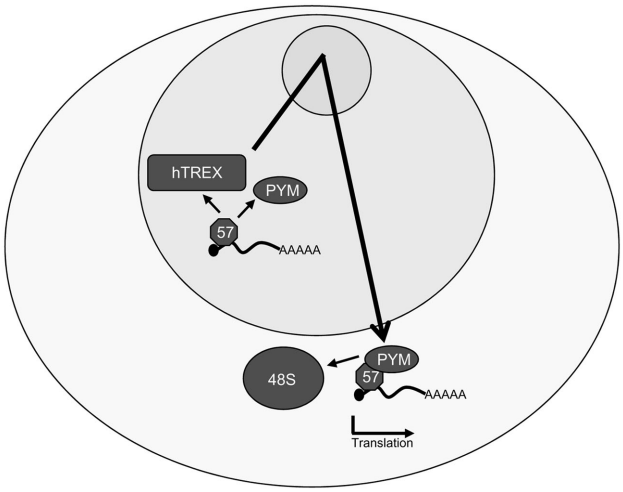

FIGURE 5 | Kaposi's sarcoma-associated herpesvirus ORF57 enhances the translation of intronless viral mRNAs. As well as interacting with the hTREX complex in the nucleus, ORF57 also binds directly to the cellular protein PYM and recruits it to intronless KSHV mRNAs. ORF57 then shuttles through the nucleolus in an mRNP complex with the hTREX components, the intronless viral mRNA, PYM, and possibly other factors before exporting the mRNA. PYM recruits the 48S preinitiation complex to the viral mRNA, enhancing the pioneer round of translation in an ORF57-dependent manner.

ORF57 FUNCTIONS TO ENHANCE THE TRANSLATION OF VIRAL TRANSCRIPTS

The multistep processes of mRNA biogenesis and maturation are not limited to the mechanisms that take place in the nucleus. A major protein complex deposited onto cellular mRNA during splicing is the exon junction complex (EJC; Bono and Gehring, 2011). The EJC is deposited ∼20 nts upstream of every exon–exon junction of spliced mRNA. The EJC functions in nonsense mediated decay targeting aberrant mRNAs which contain premature stop codons (Chang et al., 2007). In addition, the EJC plays a critical role in enhancing the translation of cellular mRNA transcripts, although the mechanisms by which it achieves this process have only been recently elucidated. It is believed that the EJC interacts with multiple translational enhancement proteins. For example, the EJC associated SKAR is able to recruit the 40S ribosomal subunit S6 protein kinase 1 (S6K1) to newly synthesized mRNA. This recruitment of S6K1 leads to an mTORC1 signaling cascade that results in enhancement of the pioneer round of translation (Ma et al., 2008). Additionally, the cellular protein PYM is able to bind to the EJC proteins Y14 and Magoh. PYM is then able to interact with the 48S preinitiation complex through a direct interaction with the small ribosomal subunit. This recruitment of the 48S preinitiation complex by PYM then acts to enhance the pioneer round of translation of cellular mRNAs (Diem et al., 2007).

Analysis of the complexes recruited to a viral mRNA by ORF57 has shown that ORF57 only recruits hTREX components to viral intronless mRNAs, not EJC components, which leads to the intriguing question of how the viral transcripts are efficiently translated, when lacking an EJC. The answer lies in that ORF57 is able to enhance translation of viral transcripts itself. To this end, ORF57 interacts directly with PYM *in vitro* and *in vivo*, bypassing the need for PYM to interact with the EJC, and recruit the 48S preinitiation complex to intronless KSHV mRNAs (**Figure 5**; Boyne et al., 2010a,b). The functional importance of the PYM–ORF57 interaction was confirmed through *in vivo* use of transdominant mutants of PYM lacking the C- and N-terminal domains that are essential for the interaction of PYM with both the EJC and the 48S preinitiation complex. Importantly, these transdominant PYM mutants are still able to interact with ORF57 and expression *in vivo* alongside ORF57 dramatically reduced expression levels of late KSHV proteins and, concurrently KSHV virion production highlighting the importance of the KSHV ORF57–PYM interaction for enhancement of KSHV mRNA translation.

To date no other ORF57 homolog has been shown to interact with PYM to enhance translation by a similar method, although translational enhancement is not something that is unique to KSHV ORF57. The ICP27 protein of HSV-1 has been shown to enhance the translation of several late proteins including VP16 and ICP5 (Fontaine-Rodriguez and Knipe, 2008), although the mechanism of this enhancement has not yet been defined. Interestingly, ICP27 does not affect the translation of all HSV-1 proteins as protein levels of the viral glycoprotein gD are not affected by the presence of ICP27, suggesting that whatever translational enhancement effect ICP27 is having, it is not a global effect on all mRNAs. Similarly, the SM protein of EBV has been shown to enhance translation of intronless viral transcripts. Moreover, insertion of an intron into intronless EBV viral transcripts negated the requirement for SM for efficient export and translation (Ricci et al., 2009). One possible explanation for this is that SM functions in a similar

manner to KSHV ORF57 and recruits translational enhancement proteins. Therefore inserting an artificial intron allows the formation of an EJC which can recruit PYM independently of SM thereby enhancing the translation of viral transcripts.

A POSSIBLE ROLE FOR ORF57 IN TRANSCRIPTIONAL ENHANCEMENT

The roles of ORF57 are not limited to post-transcriptional processes; ORF57 also interacts with the KSHV transcriptional activator, RTA (Malik et al., 2004a). RTA can transactivate a number of KSHV and cellular promoters by binding directly to promoter regions containing an RTA responsive element (RRE) or interact with other transcriptional control proteins (Dourmishev et al., 2003). Alternatively, RTA can target transcriptional repressors for degradation through the ubiquitin proteasome pathway through its E3 ubiquitin ligase activity (Yu et al., 2005; Gould et al., 2009). Importantly, ORF57 has been shown to interact directly with RTA through its N-terminal region and through this interaction synergistically transactivates a number of viral promoters, including its own promoter as well as promoters for PAN/nut-1, Kaposin, *ori-Lyt* (L), K-bZIP, and TK (Kirshner et al., 2000; Malik et al., 2004a; Palmeri et al., 2007).

The A/T hook domain in the ORF57 N-terminus has been shown to confer a DNA binding ability on ORF57, although deletion of this domain did not completely abrogate DNA binding (Palmeri et al., 2007). Moreover, ORF57 was able to transactivate the PAN/nut-1 promoter irrespective of whether there was an intact A/T hook domain. Furthermore, ORF57 has also been shown to have a low transactivation effect on other viral promoters, such as Kaposin and TK in the absence of RTA (Kirshner et al., 2000). However, transactivation by ORF57 in the context of a lytic infection appears to be dependent on the ORF57–RTA interaction (Malik et al., 2004a). Additionally, transactivation by the ORF57–RTA complex appears to be promoter-, transcript-, and cell line-specific (Palmeri et al., 2007). Spontaneous KSHV reactivation in lytic cells is an inefficient process that is limited by the expression of RTA. It is interesting to note that ORF57 is able to enhance the expression of RTA *in vivo*, and one possibility is that activation of the RTA promoter by the ORF57–RTA complex is one mechanism by which KSHV overcomes the initial hurdle of inefficient reactivation.

CONCLUSION AND FUTURE PROSPECTS

A number of recent studies have highlighted that ORF57 and its homologs are highly multifunctional with major roles in transcriptional activation, splicing, RNA stability, RNA nuclear export, and translational enhancement. The key function of this family of proteins, conserved amongst all α-, β-, and γ-herpesviruses, is the enhancement of viral mRNA transcript accumulation, although different homologs may have alternative ways they perform this function, as well as additional roles they play within the viral lifecycle.

A major question yet to be answered is how the multifunctional aspects of ORF57 are controlled. As all ORF57 homologs are designated as S/R proteins it seems highly likely that they are heavily posttranslationally modified, perhaps by multiple modifications. The ORF57 homolog of CMV, UL69, has been shown to be phosphorylated by CDKs, and colocalizes with CDK9 (Rechter et al., 2009). Inhibiting CDK activity in cell culture affects the nuclear localization of UL69 and causes it to form aggregates within the nucleus. Moreover, KSHV ORF57 is known to interact with the major cellular kinase, casein kinase II, and has been shown to be phosphorylated (Malik and Clements, 2004). This phosphorylation regulates the interaction between ORF57 and the cellular protein hnRNP K, a multifunctional protein involved in regulating gene expression. However, ORF57 has multiple protein partners, both cellular and viral, the majority of which presumably have their interaction controlled by some form of post-translational modification. Further analysis of which residues of ORF57 are phosphorylated, and what effect this has on the various protein–protein interactions, as well as export and import of ORF57, is therefore essential to understanding how the multiple roles of ORF57 are controlled. Moreover, arginine methylation is known to affect protein import and export within the cell as well as protein–protein interactions. The HSV-1 ICP27 protein has recently been shown to be methylated on three residues within its RGG box (Souki and Sandri-Goldin, 2009; Souki et al., 2009), and that this methylation affected the interaction between ICP27 and the cellular proteins Aly and SRPK1, as well as regulating its export; although import of ICP27 into the nucleus is not affected by methylation. It is therefore important to test whether KSHV ORF57 is methylated in a similar way within either RGG1 or RGG2, and what effect any potential methylation has on its function. Additionally, a protein-wide screen of which, if any, ORF57 residues are methylated could lead to an understanding of how various ORF57 interactions and functions are coordinated.

It is clear from recent data from a number of independent research groups that ORF57 binds specifically to PAN RNA in conjunction with PABPC1 to stabilize PAN and increase its expression (Borah et al., 2011; Kumar et al., 2011; Massimelli et al., 2011; Sei and Conrad, 2011). However, one major unanswered question relating to this is why does ORF57 stabilize PAN? It would be interesting to see in the context of the virus what effect a PAN lacking the MRE would have on virus replication. Presumably, as PAN constitutes ∼80% of all polyadenylated RNA in a KSHV lytically active cell, the binding to PAN is an important aspect of ORF57 function. Furthermore, the understanding of ORF57 RNA binding is currently very limited with little awareness of how ORF57 is able to distinguish between cellular and viral transcripts, as well as the mechanistic way in which ORF57 recognizes and binds to the RNA. Answering these questions is vital to continue our understanding of the role of ORF57 in viral mRNA biogenesis.

Current understanding of the mechanisms involved in both viral and cellular mRNA biogenesis is still in its infancy. The function of some of the key proteins in mRNA export, such as the THO complex, is still poorly understood, as is the way that mRNA is stabilized both by cellular and viral mechanisms. Elucidation of the functions that ORF57 has and the roles it plays in various aspects of mRNA biogenesis will not only improve our understanding of viral mechanisms, but it will act as a model for the wider field of RNA biogenesis and processing. Moreover, from a clinical perspective a major difference between KSHV and the other human oncogenic herpesviruses is that KSHV requires reactivation and lytic

expression for the majority of its tumorigenic properties. For this reason, studying the switch between latency and lytic replication is essential for both fully understanding the mechanism of KSHV tumorigenesis and also for developing treatments to prevent the onset or spread of KSHV-associated malignancies. Therefore, the study and understanding of the immediate early proteins that control this latent–lytic switch, including ORF57, is paramount for the future development of novel KSHV therapeutics.

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REFERENCES

Aravind, L., and Landsman, D. (1998). AT-hook motifs identified in a wide variety of DNA-binding proteins. *Nucleic Acids Res.* 26, 4413–4421.

Ballon, G., Chen, K., Perez, R., Tam, W., and Cesarman, E. (2011). Kaposi sarcoma herpesvirus (KSHV) vFLIP oncoprotein induces B cell transdifferentiation and tumorigenesis in mice. *J. Clin. Invest.* 121, 1141–1153.

Bello, L. J., Davison, A. J., Glenn, M. A., Whitehouse, A., Rethmeier, N., Schulz, T. F., and Barklie Clements, J. (1999). The human herpesvirus-8 ORF 57 gene and its properties. *J. Gen. Virol.* 80(Pt 12), 3207–3215.

Bono, F., and Gehring, N. H. (2011). Assembly, disassembly and recycling: the dynamics of exon junction complexes. *RNA Biol.* 8, 24–29.

Borah, S., Darricarrère, N., Darnell, A., Myoung, J., and Steitz, J. A. (2011). A Viral nuclear noncoding RNA binds re-localized poly(A) binding protein and is required for late KSHV gene expression. *PLoS Pathog.* 7, e1002300. doi:10.1371/journal.ppat.1002300

Bottero, V., Kerur, N., Sadagopan, S., Patel, K., Sharma-Walia, N., and Chandran, B. (2011). Phosphorylation and polyubiquitination of transforming growth factor β-activated kinase 1 are necessary for activation of NF-κB by the Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. *J. Virol.* 85, 1980–1993.

Boyne, J. R., Colgan, K. J., and Whitehouse, A. (2008a). Herpesvirus saimiri ORF57: a post-transcriptional regulatory protein. *Front. Biosci.* 13, 2928–2938.

Boyne, J. R., Colgan, K. J., and Whitehouse, A. (2008b). Recruitment of the complete hTREX complex is required for Kaposi's sarcoma-associated herpesvirus intronless mRNA nuclear export and virus replication. *PLoS Pathog.* 4, e1000194. doi:10.1371/journal.ppat.1000194

Boyne, J. R., Jackson, B. R., Taylor, A., Macnab, S. A., and Whitehouse, A. (2010a). Kaposi's sarcoma-associated herpesvirus ORF57 protein interacts with PYM to enhance translation of viral intronless mRNAs. *EMBO J.* 29, 1851–1864.

Boyne, J. R., Jackson, B. R., and Whitehouse, A. (2010b). ORF57: master regulator of KSHV mRNA biogenesis. *Cell Cycle* 9, 2702–2703.

Boyne, J. R., and Whitehouse, A. (2006a). Gamma-2 herpes virus post-transcriptional gene regulation. *Clin. Microbiol. Infect.* 12, 110–117.

Boyne, J. R., and Whitehouse, A. (2006b). Nucleolar trafficking is essential for nuclear export of intronless herpesvirus mRNA. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15190–15195.

Boyne, J. R., and Whitehouse, A. (2009). Nucleolar disruption impairs Kaposi's sarcoma-associated herpesvirus ORF57-mediated nuclear export of intronless viral mRNAs. *FEBS Lett.* 583, 3549–3556.

Bryant, H. E., Wadd, S. E., Lamond, A. I., Silverstein, S. J., and Clements, J. B. (2001). Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step. *J. Virol.* 75, 4376–4385.

Cai, Q., Verma, S. C., Lu, J., and Robertson, E. S. (2010). Molecular biology of Kaposi's sarcoma-associated herpesvirus and related oncogenesis. *J. Adv. Virus Res.* 78, 87–142.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Chang, P. J., Shedd, D., and Miller, G. (2005). Two subclasses of Kaposi's sarcoma-associated herpesvirus lytic cycle promoters distinguished by open reading frame 50 mutant proteins that are deficient in binding to DNA. *J. Virol.* 79, 8750–8763.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266, 1865–1869.

Chang, Y.-F., Imam, J. S., and Wilkinson, M. F. (2007). The nonsense-mediated decay RNA surveillance pathway. *Annu. Rev. Biochem.* 76, 51–74.

Cheng, H., Dufu, K., Lee, C.-S., Hsu, J. L., Dias, A., and Reed, R. (2006). Human mRNA export machinery recruited to the 5′ end of mRNA. *Cell* 127, 1389.

Colgan, K. J., Boyne, J. R., and Whitehouse, A. (2009). Uncoupling of hTREX demonstrates that UAP56 and hTHO-complex recruitment onto herpesvirus saimiri intronless transcripts is required for replication. *J. Gen. Virol.* 90, 1455–1460.

Diem, M. D., Chan, C. C., Younis, I., and Dreyfuss, G. (2007). PYM binds the cytoplasmic exon-junction complex and ribosomes to enhance translation of spliced mRNAs. *Nat. Struct. Mol. Biol.* 14, 1173–1179.

Dourmishev, L. A., Dourmishev, A. L., Palmeri, D., Schwartz, R. A., and Lukac, D. M. (2003). Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. *Microbiol. Mol. Biol. Rev.* 67, 175–212.

Dufu, K., Livingstone, M. J., Seebacher, J., Gygi, S. P., Wilson, S. A., and Reed, R. (2010). ATP is required for interactions between UAP56 and two conserved mRNA export proteins, Aly and CIP29, to assemble the TREX complex. *Genes Dev.* 24, 2043–2053.

Fontaine-Rodriguez, E. C., and Knipe, D. M. (2008). Herpes simplex virus ICP27 increases translation of a subset of viral late mRNAs. *J. Virol.* 82, 3538–3545.

Ganem, D. (2006). KSHV infection and the pathogenesis of Kaposi's sarcoma. *Annu. Rev. Pathol.* 1, 273.

Goodwin, D. J., Hall, K. T., Giles, M. S., Calderwood, M. A., Markham, A. F., and Whitehouse, A. (2000). The carboxy terminus of the herpesvirus saimiri ORF 57 gene contains domains that are required for transactivation and transrepression. *J. Gen. Virol.* 81, 2253–2265.

Goodwin, D. J., Hall, K. T., Stevenson, A. J., Markham, A. F., and Whitehouse, A. (1999). The open reading frame 57 gene product of herpesvirus saimiri shuttles between the nucleus and cytoplasm and is involved in viral RNA nuclear export. *J. Virol.* 73, 10519–10524.

Gould, F., Harrison, S. M., Hewitt, E. W., and Whitehouse, A. (2009). Kaposi's sarcoma-associated herpesvirus RTA promotes degradation of the Hey1 repressor protein through the ubiquitin proteasome pathway. *J. Virol.* 83, 6727–6738.

Grundhoff, A., and Ganem, D. (2004). Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. *J. Clin. Invest.* 113, 124–136.

Hardwicke, M. A., and Sandri-Goldin, R. M. (1994). The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. *J. Virol.* 68, 4797–4810.

Hardy, W. R., and Sandri-Goldin, R. M. (1994). Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. *J. Virol.* 68, 7790–7799.

Harrison, S. M., and Whitehouse, A. (2008). Kaposi's sarcoma-associated herpesvirus (KSHV) Rta and cellular HMGB1 proteins synergistically transactivate the KSHV ORF50 promoter. *FEBS Lett.* 582, 3080–3084.

Hastings, M. L., and Krainer, A. R. (2001). Pre-mRNA splicing in the new millennium. *Curr. Opin. Cell Biol.* 13, 302.

Hautbergue, G. M., Hung, M. L., Walsh, M. J., Snijders, A. P., Chang, C. T., Jones, R., Ponting, C. P., Dickman, M. J., and Wilson, S. A. (2009). UIF, a New mRNA export adaptor that works together with REF/ALY, requires FACT for recruitment to mRNA. *Curr. Biol.* 19, 1918–1924.

Hernandez, F. P., and Sandri-Goldin, R. M. (2010). Herpes simplex virus 1 regulatory protein ICP27 undergoes a head-to-tail intramolecular interaction. *J. Virol.* 84, 4124–4135.

Hernandez, F. P., and Sandri-Goldin, R. M. (2011). Head-to-tail

intramolecular interaction of herpes simplex virus type 1 regulatory protein ICP27 is important for its interaction with cellular mRNA export receptor TAP/NXF1. *MBio* 1, e00268-10.

Hung, M. L., Hautbergue, G. M., Snijders, A. P., Dickman, M. J., and Wilson, S. A. (2010). Arginine methylation of REF/ALY promotes efficient handover of mRNA to TAP/NXF1. *Nucleic Acids Res.* 38, 3351–3361.

Jackson, B. R., Boyne, J. R., Noerenberg, M., Taylor, A., Hautbergue, G. M., Walsh, M. J., Wheat, R., Blackbourn, D. J., Wilson, S. A., and Whitehouse, A. (2011). An interaction between KSHV ORF57 and UIF Provides mRNA-adaptor redundancy in herpesvirus intronless mRNA export. *PLoS Pathog.* 7, e1002138. doi:10.1371/journal.ppat.1002138

Kang, J. G., Pripuzova, N., Majerciak, V., Kruhlak, M., Le, S. Y., and Zheng, Z. M. (2011). Kaposi sarcoma-associated herpesvirus ORF57 promotes escape of viral and human IL6 from microRNA-mediated suppression. *J. Virol.* 85, 2620–2630.

Kirshner, J. R., Lukac, D. M., Chang, J., and Ganem, D. (2000). Kaposi's sarcoma-associated herpesvirus open reading frame 57 encodes a posttranscriptional regulator with multiple distinct activities. *J. Virol.* 74, 3586–3597.

Kohler, A., and Hurt, E. (2007). Exporting RNA from the nucleus to the cytoplasm. *Nat. Rev. Mol. Cell Biol.* 8, 761–773.

Kumar, G. R., Shum, L., and Glaunsinger, B. A. (2011). Importin α-mediated nuclear import of cytoplasmic poly(A) binding protein occurs as a direct consequence of cytoplasmic mRNA depletion. *Mol. Cell. Biol.* 31, 3113–3125.

Lei, H., Dias, A. P., and Reed, R. (2011). Export and stability of naturally intronless mRNAs require specific coding region sequences and the TREX mRNA export complex. *Proc. Natl. Acad. Sci. U.S.A.* 108, 17985–17990.

Liang, Y., Chang, J., Lynch, S. J., Lukac, D. M., and Ganem, D. (2002). The lytic switch protein of KSHV activates gene expression via functional interaction with RBP-Jkappa (CSL), the target of the Notch signaling pathway. *Genes Dev.* 16, 1977–1989.

Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999). Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. *J. Virol.* 73, 9348–9361.

Lukac, D. M., Renne, R., Kirshner, J. R., and Ganem, D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. *Virology* 252, 304–312.

Luo, M. J., and Reed, R. (1999). Splicing is required for rapid and efficient mRNA export in metazoans. *Proc. Natl. Acad. Sci. U.S.A.* 96, 14937–14942.

Ma, X. M., Yoon, S. O., Richardson, C. J., Julich, K., and Blenis, J. (2008). SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs. *Cell* 133, 303–313.

Majerciak, V., Kruhlak, M., Dagur, P. K., Mccoy, J. P. Jr., and Zheng, Z. M. (2010). Caspase-7 cleavage of Kaposi sarcoma-associated herpesvirus ORF57 confers a cellular function against viral lytic gene expression. *J. Biol. Chem.* 285, 11297–11307.

Majerciak, V., Uranishi, H., Kruhlak, M., Pilkington, G. R., Massimelli, M. J., Bear, J., Pavlakis, G. N., Felber, B. K., and Zheng, Z.-M. (2011). Kaposi's sarcoma-associated herpesvirus ORF57 interacts with cellular RNA export cofactors RBM15 and OTT3 to promote expression of viral ORF59. *J. Virol.* 85, 1528–1540.

Majerciak, V., Yamanegi, K., Allemand, E., Kruhlak, M., Krainer, A. R., and Zheng, Z. M. (2008). Kaposi's sarcoma-associated herpesvirus ORF57 functions as a viral splicing factor and promotes expression of intron-containing viral lytic genes in spliceosome-mediated RNA splicing. *J. Virol.* 82, 2792–2801.

Majerciak, V., Yamanegi, K., Nie, S. H., and Zheng, Z. M. (2006). Structural and functional analyses of Kaposi sarcoma-associated herpesvirus ORF57 nuclear localization signals in living cells. *J. Biol. Chem.* 281, 28365–28378.

Malik, P., Blackbourn, D. J., Cheng, M. F., Hayward, G. S., and Clements, J. B. (2004a). Functional co-operation between the Kaposi's sarcoma-associated herpesvirus ORF57 and ORF50 regulatory proteins. *J. Gen. Virol.* 85, 2155–2166.

Malik, P., Blackbourn, D. J., and Clements, J. B. (2004b). The evolutionarily conserved Kaposi's sarcoma-associated herpesvirus ORF57 protein interacts with REF protein and acts as an RNA export factor. *J. Biol. Chem.* 279, 33001–33011.

Malik, P., and Clements, J. B. (2004). Protein kinase CK2 phosphorylation regulates the interaction of Kaposi's sarcoma-associated herpesvirus regulatory protein ORF57 with its multifunctional partner hnRNP K. *Nucleic Acids Res.* 32, 5553–5569.

Massimelli, M. J., Kang, J.-G., Majerciak, V., Le, S.-Y., Liewehr, D., Steinberg, S., and Zheng, Z. M. (2011). Stability of a long noncoding viral RNA depends on a 9-nt core element at the RNA 5′ end to interact with viral ORF57 and cellular PABPC1. *Int. J. Biol. Sci.* 7, 1145–1160.

Masuda, S., Das, R., Cheng, H., Hurt, E., Dorman, N., and Reed, R. (2005). Recruitment of the human TREX complex to mRNA during splicing. *Genes Dev.* 19, 1512–1517.

Moore, M. J., and Proudfoot, N. J. (2009). Pre-mRNA processing reaches back to transcription and ahead to translation. *Cell* 136, 688–700.

Nekorchuk, M., Han, Z., Hsieh, T. T., and Swaminathan, S. (2007). Kaposi's sarcoma-associated herpesvirus ORF57 protein enhances mRNA accumulation independently of effects on nuclear RNA export. *J. Virol.* 81, 9990–9998.

Ote, I., Lebrun, M., Vandevenne, P., Bontems, S., Medina-Palazon, C., Manet, E., Piette, J., and Sadzot-Delvaux, C. (2009). Varicella-zoster virus IE4 protein interacts with SR proteins and exports mRNAs through the TAP/NXF1 pathway. *PLoS ONE* 4, e7882. doi:10.1371/journal.pone.0007882

Palmeri, D., Spadavecchia, S., Carroll, K. D., and Lukac, D. M. (2007). Promoter- and cell-specific transcriptional transactivation by the Kaposi's sarcoma-associated herpesvirus ORF57/Mta protein. *J. Virol.* 81, 13299–13314.

Parkin, D. M., Sitas, F., Chirenje, M., Stein, L., Abratt, R., and Wabinga, H. (2008). Part I: cancer in indigenous Africans – burden, distribution, and trends. *Lancet Oncol.* 9, 683–692.

Piselli, P., Busnach, G., Citterio, F., Frigerio, M., Arbustini, E., Burra, P., Pinna, A. D., Bresadola, V., Ettorre, G. M., Baccarani, U., Buda, A., Lauro, A., Zanus, G., Cimaglia, C., Spagnoletti, G., Lenardon, A., Agozzino, M., Gambato, M., Zanfi, C., Miglioresi, L., Di Gioia, P., Mei, L., Ippolito, G., and Serraino, D. (2009). Risk of Kaposi sarcoma after solid-organ transplantation: multicenter study in 4767 recipients in Italy, 1970–2006. *Transplant. Proc.* 41, 1227–1230.

Proudfoot, N. J. (2011). Ending the message: poly(A) signals then and now. *Genes Dev.* 25, 1770–1782.

Rechter, S., Scott, G. M., Eickhoff, J., Zielke, K., Auerochs, S., Müller, R., Stamminger, T., Rawlinson, W. D., and Marschall, M. (2009). Cyclin-dependent kinases phosphorylate the cytomegalovirus RNA export protein pUL69 and modulate its nuclear localization and activity. *J. Biol. Chem.* 284, 8605–8613.

Ricci, E. P., Mure, F., Gruffat, H., Decimo, D., Medina-Palazon, C., Ohlmann, T., and Manet, E. (2009). Translation of intronless RNAs is strongly stimulated by the Epstein–Barr virus mRNA export factor EB2. *Nucleic Acids Res.* 37, 4932–4943.

Rodríguez-Navarro, S., and Hurt, E. (2011). Linking gene regulation to mRNA production and export. *Curr. Opin. Cell Biol.* 23, 302–309.

Ruvolo, V., Sun, L., Howard, K., Sung, S., Delecluse, H. J., Hammerschmidt, W., and Swaminathan, S. (2004). Functional analysis of Epstein-Barr virus SM protein: identification of amino acids essential for structure, transactivation, splicing inhibition, and virion production. *J. Virol.* 78, 340–352.

Ruvolo, V., Wang, E., Boyle, S., and Swaminathan, S. (1998). The Epstein–Barr virus nuclear protein SM is both a post-transcriptional inhibitor and activator of gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 95, 8852–8857.

Sandri-Goldin, R. M. (1998). ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. *Genes Dev.* 12, 868–879.

Sandri-Goldin, R. M. (2008). The many roles of the regulatory protein ICP27 during herpes simplex virus infection. *Front. Biosci.* 13, 5241–5256.

Sei, E., and Conrad, N. K. (2011). Delineation of a core RNA element required for Kaposi's sarcoma-associated herpesvirus ORF57 binding and activity. *Virology* 419, 107–116.

Smith, R. W. P., Malik, P., and Clements, J. B. (2005). The herpes simplex virus ICP27 protein: a multifunctional post-transcriptional regulator of gene expression. *Biochem. Soc. Trans.* 33, 499–501.

Song, M. J., Hwang, S., Wong, W., Round, J., Martinez-Guzman, D., Turpaz, Y., Liang, J., Wong, B., Johnson, R. C., Carey, M., and Sun, R. (2004). The DNA architectural protein HMGB1 facilitates RTA-mediated viral gene expression in gamma-2 herpesviruses. *J. Virol.* 78, 12940–12950.

Souki, S. K., Gershon, P. D., and Sandri-Goldin, R. M. (2009). Arginine methylation of the ICP27 RGG box regulates ICP27 export and is required for efficient herpes simplex virus 1 replication. *J. Virol.* 83, 5309–5320.

Souki, S. K., and Sandri-Goldin, R. M. (2009). Arginine methylation of the ICP27 RGG box regulates the functional interactions of ICP27 with SRPK1 and Aly/REF during herpes simplex virus 1 infection. *J. Virol.* 83, 8970–8975.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'agay, M.-F., Clauvel, J. P., Raphael, M., Degos, L., and Sigaux, F. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86, 1276–1280.

Swaminathan, S. (2005). Post-transcriptional gene regulation by gamma herpesviruses. *J. Cell. Biochem.* 95, 698–711.

Taylor, A., Jackson, B. R., Noerenberg, M., Hughes, D. J., Boyne, J. R., Verow, M., Harris, M., and Whitehouse, A. (2011). Mutation of a C-terminal motif affects KSHV ORF57 RNA binding, nuclear trafficking and multimerisation. *J. Virol.* 85, 7881–7891.

Toth, Z., and Stamminger, T. (2008). The human cytomegalovirus regulatory protein UL69 and its effect on mRNA export. *Front. Biosci.* 13, 2939–2949.

Tunnicliffe, R. B., Hautbergue, G. M., Kalra, P., Jackson, B. R., Whitehouse, A., Wilson, S. A., and Golovanov, A. P. (2010). Structural basis for the recognition of cellular mRNA export factor REF by herpes viral proteins HSV-1 ICP27 and HVS ORF57. *PLoS Pathog.* 7, e1001244. doi:10.1371/journal.ppat.1001244

Valencia, P., Dias, A. P., and Reed, R. (2008). Splicing promotes rapid and efficient mRNA export in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 105, 3386–3391.

Verma, D., Bais, S., Gaillard, M., and Swaminathan, S. (2010). Epstein-Barr virus SM protein utilizes cellular splicing factor SRp20 to mediate alternative splicing. *J. Virol.* 84, 11781–11789.

Verma, D., and Swaminathan, S. (2008). Epstein-Barr virus SM protein functions as an alternative splicing factor. *J. Virol.* 82, 7180–7188.

Whitehouse, A., Cooper, M., and Meredith, D. M. (1998). The immediate-early gene product encoded by open reading frame 57 of herpesvirus saimiri modulates gene expression at a posttranscriptional level. *J. Virol.* 72, 857–861.

Xue, Y., Ren, J., Gao, X., Jin, C., Wen, L., and Yao, X. (2008). GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol. Cell Proteomics* 7, 1598–1608.

Yu, Y., Wang, S. E., and Hayward, G. S. (2005). The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteosome-mediated degradation. *Immunity* 22, 59–70.

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Post-translational modifications of Kaposi's sarcoma-associated herpesvirus regulatory proteins – SUMO and KSHV

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KSHV latency can be envisioned as an outcome that is balanced between factors that promote viral gene expression and lytic replication against those that facilitate gene silencing and establish or maintain latency. A large body of work has focused on the activities of the key viral regulatory proteins involved in KSHV latent or lytic states. Moreover, recent studies have also begun to document the importance of epigenetic landscape evolution of the KSHV viral genome during latency and reactivation. However, one area of KSHV molecular virology that remains largely unanswered is the precise role of post-translational modifications on the activities of viral factors that function during latency and reactivation. In this review, we will summarize the post-translational modifications associated with three viral factors whose activities contribute to the viral state. The viral proteins discussed are the two major KSHV encoded transcription factors, K-Rta (KSHV replication and transcriptional activator) and K-bZIP (KSHV basic leucine zipper) and the viral latency-associated nuclear antigen (LANA). A special emphasis will be placed on the role of the sumoylation pathway in the modulation of the KSHV lifecycle. Newly uncovered small ubiquitin-like modifier (SUMO)-associated properties of LANA and K-Rta will also be presented, namely LANA histone targeting SUMO E3 ligase activity and K-Rta SUMO-targeted ubiquitin ligase function.

Keywords: KSHV, LANA, K-bZIP, K-Rta, SUMO, post-translational modification, transcription

INTRODUCTION

Reactivation from a latent state is an important feature of infection and disease caused by many herpesviruses. Although the molecular mechanisms of reactivation have been extensively studied, gaps exist in our current knowledge concerning the processes by which these viruses establish, maintain, and emerge from latency. KSHV latency can be envisioned as the outcome balanced between factors that require viral gene expression and lytic replication against those that are essential for establishment and/or maintenance of latent infection. In this review, we will provide an update on three viral factors whose activities contribute to the viral state with an emphasis on post-translational modifications that modulate multiple functions of these factors. The viral proteins discussed are the two major KSHV encoded transcription factors, K-Rta and K-bZIP and the viral latency-associated nuclear antigen (LANA).

K-bZIP

K-bZIP (K8) is an early lytic gene and is among the earliest viral genes expressed after acute infection or during reactivation from latency. K-bZIP is a 237-amino acid protein containing a basic and leucine zipper domain and is the structural and positional analog of Epstein–Barr virus (EBV) BZLF1 (Lin et al., 1999). K-bZIP has been reported to be phosphorylated (Polson et al., 2001), sumoylated (Chang et al., 2010; Lefort et al., 2010), and acetylated (Lefort et al., 2010). K-bZIP directly associates with K-Rta in KSHV infected cells and represses K-Rta-mediated transactivation at a subset of KSHV promoters. This activity of K-bZIP is subject to post-translational modulation *via* phosphorylation (Izumiya et al., 2007) and sumoylation (Izumiya et al., 2005). As detailed below, K-bZIP repression activity on K-Rta-mediated transactivation is regulated in an opposing manner by these two post-translational modifications. While K-bZIP repression is largely dependent on sumoylation, phosphorylation serves as a negative regulator. A schematic diagram of K-bZIP and its post-translational modification sites are presented in **Figure 1**.

PHOSPHORYLATION

K-bZIP was reported to be phosphorylated on residues Thr 111 and Ser 167 (Polson et al., 2001; Izumiya et al., 2007). Interestingly, these sites are contained within cellular cyclin-dependent kinase (CDK) recognition sites with the consensus sequence (S/T)PXR suggesting that K-bZIP may be phosphorylated by CDKs. Indeed, K-bZIP was confirmed to be a substrate for several cellular CDK–cyclin complexes *in vitro*, including CDK-2/cyclin A, CDK-1/cyclin B, and CDK-2/cyclin E (Polson et al., 2001). K-bZIP was also found to interact with, and be a substrate for, the viral protein kinase (vPK, ORF36). vPK is a serine/threonine protein kinase expressed from mRNAs with early and early-late kinetics and the major vPK phosphorylation site on K-bZIP was determined to also be residue threonine (T) 111 (Izumiya et al., 2007). K-bZIP

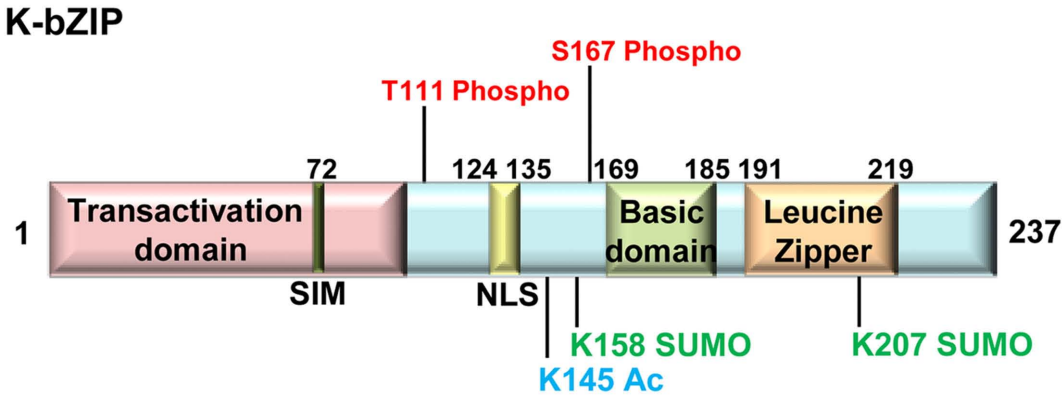

FIGURE 1 | Schematic representation of KSHV K-bZIP. K-bZIP protein and its post-translational modification sites as discussed in the text are depicted. Phosphorylation (Phospho), acetylation (Ac), and sumoylation (SUMO) sites are shown. The K-bZIP SUMO interaction motif (SIM) and nuclear localization signal (NLS) are also indicated. Numbers indicate amino acid position.

directly interacts with K-Rta and represses K-Rta activation of certain K-Rta responsive promoters such as ORF57 and K-bZIP, but not at other similarly responsive promoters such as PAN. The functional significance of K-bZIP T111 phosphorylation site with respect to repression of K-Rta-mediated transactivation was examined using a mutagenesis strategy in which T111 was changed to a non-phospho acceptor reside alanine, or to aspartic acid, a phospho-mimetic. In co-transfection experiments with K-Rta and a K-Rta responsive promoter construct, K-bZIP-T111D repressive function was similar to wild-type K-bZIP, whereas K-bZIP-T111A was a more potent repressor. Moreover, K-bZIP-T111A was found to be more heavily sumoylated than wild-type K-bZIP or K-bZIP-T111D and overexpression of vPK reduced the sumoylation of wild type K-bZIP. Taken together, these results are consistent with a model whereby phosphorylation at T111 has a negative effect on both the extent of sumoylation and the repressive activity of K-bZIP (Izumiya et al., 2007). Thus K-bZIP activities are modulated by phosphorylation and sumoylation in an antagonistic fashion similar to other cellular transcription factors such as ELK-1 (Girdwood et al., 2003), AP-1 (Bossis et al., 2005), AIB1 (Wu et al., 2006), progesterone receptor B (PR-B; Daniel et al., 2007; Daniel and Lange, 2009), STAT1 (Zimnik et al., 2009), and KAP-1 (Goodarzi et al., 2011). KSHV genome-wide reporter assays support the notion that K-bZIP preferentially represses early-lytic genes but not late gene promoters (Ellison et al., 2009), consistent with temporal regulation of viral gene expression, potentially mediated, in part, through a K-bZIP SUMO-phospho switch.

ACETYLATION

K-bZIP was reported to be acetylated on multiple residues and K145 was identified as one acetylation site (Lefort et al., 2010). K-bZIP was previously found associated with CBP (Hwang et al., 2001) and the ability of K-bZIP to repress transcription was reported to be mediated through sequestration of CBP. In co-transfection experiments, increasing amounts of CBP resulted in increased acetylation of K-bZIP and a reversal of K-bZIP-mediated repression of the IFNα-activated 2′, 5′-OAS gene. In addition to effects on chromatin, CBP may be capable of acetylating K-bZIP. However, the functional role of K-bZIP acetylation in the KSHV life cycle remains to be seen.

SUMOYLATION

Sumoylation (small ubiquitin-like modifier) was identified as a reversible post-translational protein modification in 1997 (reviewed in Geiss-Friedlander and Melchior, 2007). Three isoforms of SUMO exist: SUMO-1, -2, and -3. SUMO-2 and SUMO-3 are highly homologous (97% identity in the mature form), whereas SUMO-1 is more distantly related to both (∼50%). Hundreds of protein targets for SUMO-modification have been identified, and the majority of these are nuclear proteins. Similar to phosphorylation, sumoylation is a rapid and reversible modification. Moreover, in a manner similar to the binding of phosphorylated tyrosine residues by signaling molecules carrying phosphotyrosine (PTB) or Src homology 2 (SH2) domains, sumoylated proteins are specifically recognized by proteins containing one or more SUMO-interacting motifs (SIM). Analogous to ubiquitylation, conjugation of SUMO to target proteins is a multi-step process involving an E1 activating enzyme heterodimer SAE1/SAE2 (AOS1/UBA2), an E2 conjugating enzyme Ubc9 (ubiquitin-like protein SUMO-1 conjugating enzyme 9), and an E3 ligase, which is believed to provide specificity within the sumoylation pathway (Geiss-Friedlander and Melchior, 2007). Sumoylation is a highly dynamic process, and its outcomes are very diverse, ranging from changes in subcellular localization to altered activity and, in some cases, stability of the modified protein. The underlying principle of sumoylation is the alteration of inter- or intra-molecular interactions of the modified substrate (Prudden et al., 2007; Uzunova et al., 2007; Xie et al., 2007). For DNA tumor viruses, the immediate-early and early gene products, which are transcriptional factors, are often sumoylated. Examples include IE1 (immediate-early 1 protein) and IE2 (immediate-early 2 protein) of cytomegalovirus (CMV; Hofmann et al., 2000; Spengler et al., 2002; Hsu et al., 2004), E1 and E2 of human papillomavirus (HPV; Rosas-Acosta et al., 2005; Wu et al., 2007), BZLF1 of EBV (Adamson and Kenney, 2001), K-bZIP, and LANA2 of KSHV (Izumiya et al., 2005; Marcos-Villar et al., 2009). Viral proteins are also known to modulate the sumoylation

of specific cellular proteins. For example, HPV E7 protein and adenovirus E1A protein block sumoylation of RB (Ledl et al., 2005) and KSHV vPK inhibits the sumoylation of KAP-1 (Chang et al., 2009). Viruses can also affect global sumoylation of cellular proteins by directly modulating the SUMO machinery. Avian Adenovirus Gam1 is known to inhibit the SUMO E1 activating enzyme by targeting the SAE1/SAE2 (AOS1/UBA2) heterodimer to cullin RING ligases (CRLs) and promoting SAE1 ubiquitylation and degradation (Colombo et al., 2002; Boggio et al., 2007).

The repressive activity of K-bZIP requires sumoylation at lysine-158 and an intact leucine zipper region. Sumoylation at this site is required for the association of K-bZIP with the cellular E2 SUMO conjugation enzyme Ubc9. Both SUMO-1 and SUMO-2/3 were found conjugated to a significant fraction (∼10%) of endogenous K-bZIP in BCBL-1 cells. Mutation of lysine-158 to arginine eliminated SUMO-modification of K-bZIP *in vivo* with a concomitant loss of the majority of K-bZIP repressive function. Although SUMO-modification of K-bZIP may influence its repressive function through several mechanisms, including effects on the physical interaction between K-bZIP and K-Rta, it is likely that a major effect of SUMO is mediated by its ability to recruit Ubc9 to K-bZIP target promoters. Ubc9 binding to K-bZIP, as well as co-occupancy of K-bZIP, K-Rta, and SUMO at target viral promoters has been observed. Based on these results, we predicted that K-bZIP may function as a SUMO E3 ligase or SUMO adaptor which functions to deliver Ubc9 to potential substrates (Izumiya et al., 2005). In addition to lysine-158, Lefort et al. (2010) have also identified a previously unrecognized sumoylation site in a K-bZIP splice variant (K207).

Subsequently, Chang et al. (2010) have confirmed that K-bZIP functions as the prototypical viral SUMO E3 ligase. K-bZIP was found to be a SIM-containing poly-SUMO-specific E3 ligase with specificity for SUMO-2/3. As discussed above, K-bZIP had been previously known to associate with Ubc9 (Izumiya et al., 2005), Chang et al. further demonstrated that K-bZIP bound SUMO-2 and SUMO-3, but not SUMO-1. K-bZIP was found to contain a SIM at amino acid residues 72 to 76, which was identical to that of the cellular SUMO-ligases, PIAS1, and PIASx. The sumoylation activity of K-bZIP was dependent on an intact SIM, and K-bZIP could catalyze its auto-sumoylation and the sumoylation of two K-bZIP-interacting proteins, p53 and RB. In contrast, Lefort et al. (2010) have reported that K-bZIP repression of interferon-α signaling was SIM-independent but was dependent on K-bZIP K158 sumoylation site, a Ubc9 consensus binding site. As described more detail below, K-Rta preferentially degrades SUMO-modified proteins, similar to an activity ascribed to HSV-1 ICP0 (Boutell et al., 2011). This suggests that a balance between sumoylation and SUMO-dependent degradation may be important for the KSHV life cycle. As the assembly and disassembly of Promyelocytic leukemia (PML; ND10) bodies at herpesvirus replication complexes are SUMO-dependent, modulation of the SUMO environment by K-bZIP and K-Rta during lytic replication cycle may help dictate whether viral replication will proceed or if latency will be established. Another potential role of K-bZIP may be to mark proteins that are destined to be targeted by the SUMO-targeting ubiquitin ligase (STUbL) activity of K-Rta (see below). Although not fully established, this idea is supported by the report that KSHV LANA2 increases PML sumoylation, which facilitates its degradation by a cellular ubiquitin ligase (Marcos-Villar et al., 2009).

K-RTA

K-Rta is essential and sufficient to induce lytic reactivation of the latent KSHV genome in the BCBL-1 cell line model as well as *de novo* infection model (Lukac et al., 1998, 1999; Sun et al., 1998; Nakamura et al., 2003; Xu et al., 2005). K-Rta is a potent transcription factor, with an N-terminal putative DNA binding domain and a C-terminal transactivation domain (Lukac et al., 1999; West and Wood, 2003). K-Rta possesses broad target sequence specificity, and various K-Rta responsive promoter elements have been identified (Lukac et al., 1999; Chang et al., 2002; Deng et al., 2002; Song et al., 2002; West and Wood, 2003). K-Rta has been reported to be phosphorylated (Lukac et al., 1999; Gwack et al., 2003), poly(ADP-ribosyl)ated (Gwack et al., 2003), and ubiquitylated (Yu et al., 2005). A schematic diagram of K-Rta modification sites is presented in **Figure 2**, and the consequences of these modifications will be discussed.

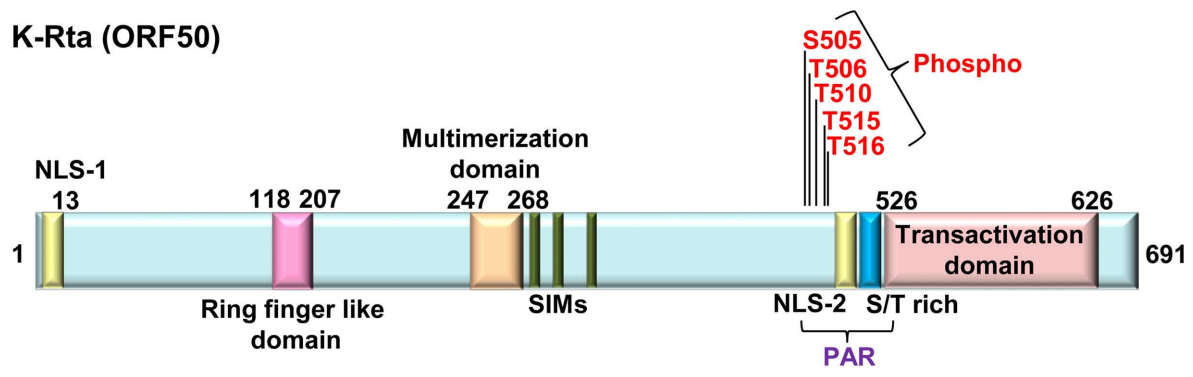

FIGURE 2 | Schematic representation of KSHV K-Rta. K-Rta protein and its post-translational modification sites as discussed in the text are depicted. Phosphorylation (Phospho) site numbering and PARylation (PAR) site localization of Gwack et al. (2003) is utilized. The K-Rta nuclear localization signals (NLS-1 and NLS-2), ring-finger like domain, SUMO interaction motifs (SIMs), and serine/threonine (S/T) rich regions are also indicated. Numbers indicate amino acid position.

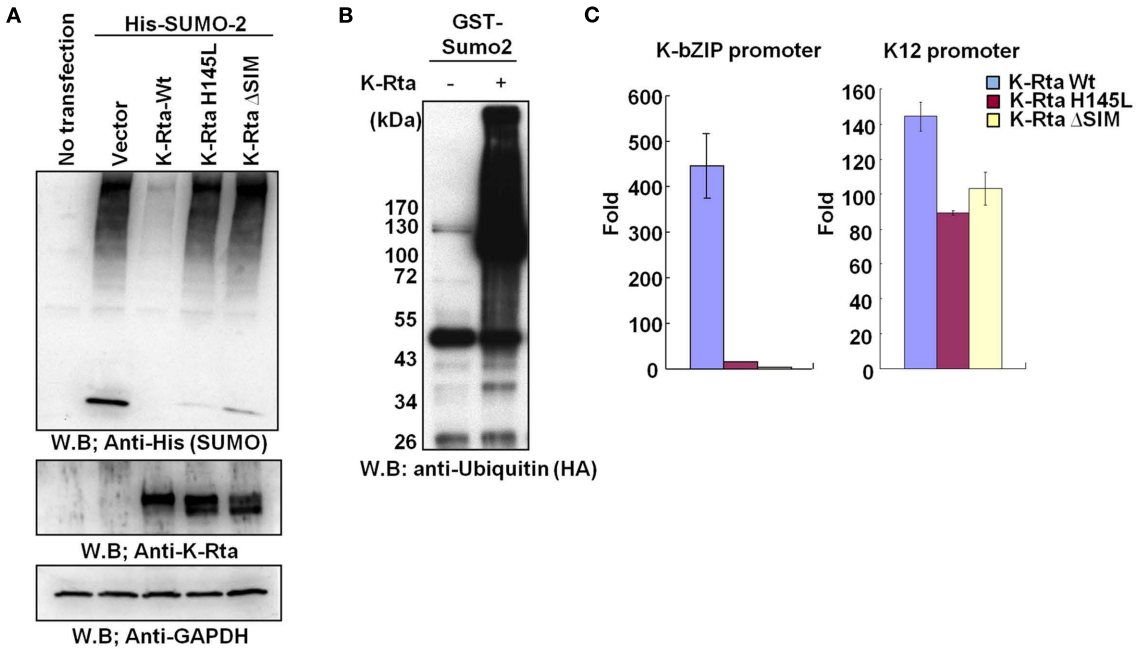

FIGURE 3 | SUMO-targeting ubiquitin ligase activity of KSHV K-Rta. **(A)** 293T cells were co-transfected with his-tagged SUMO-2 and the indicated K-Rta expression vector. Forty-eight hours later cellular lysates were prepared and analyzed by immunoblotting using the indicated antibodies. (H145L, ring-finger like domain mutant; ΔSIM, SIM deletion mutant). **(B)** *In vitro* ubiquitin conjugation reactions were reconstituted using purified components (E1, Ube1, 25 nM; E2, Ubc H5a, 50 nM; E3, K-Rta, 150 nM). Reaction products were probed by immunoblotting. In the presence of K-Rta, GST–SUMO was conjugated with ubiquitin. **(C)** KSHV promoter reporter assay. 293T cells were co-transfected with K-Rta wt or mutant and the indicated KSHV promoter luciferase reporter. Forty eight hour later cellular lysates were prepared and assayed for luciferase activity. Values represent the mean fold induction (mean ± SD, $n = 3$ determinations) relative to the value derived from reporter + empty vector set as = 1 (K-Rta wt, blue; H145L, red; ΔSIM, yellow).

PHOSPHORYLATION

As part of an initial characterization of various aspects of ORF50 RNA and protein expression, structure, and function, K-Rta was found to be highly phosphorylated in mammalian cells (Lukac et al., 1999). By immunoblot analysis, K-Rta protein migrated with an apparent molecular mass of ∼110 kDa, greater than the predicted value of 74 kDa. Analysis of the K-Rta primary amino acid sequence revealed several potential phosphorylation sites including a C-terminal S/T-rich region, as well as additional consensus phosphorylation sites for the cellular serine/threonine kinases casein kinase-II and protein kinase C (PKC). Phosphatase treatment of K-Rta protein partially reduced the slower electrophoretic mobility of K-Rta suggesting that phosphorylation accounted for most of the anomalous migration observed during SDS-PAGE analysis of K-Rta. Subsequently, Gwack et al. (2003) identified the Ste20-like kinase hKFC and poly(ADP-ribose) polymerase 1 (PARP-1, see below) as proteins that specifically interact with the S/T-rich region of K-Rta. There are greater than 30 different Ste20-related kinases in humans and three members of a novel subfamily exhibit a high degree of homology to a Ste-like kinase originally isolated from chick cells (Yustein et al., 2000, 2003). hKFC is identical to JIK (Tassi et al., 1999), a kinase reported to be negatively regulated by upstream tyrosine kinase receptors. hKFC was shown to directly interact with K-Rta and phosphorylate at serine (S505) and threonine (T506, T510, T515, and T516) of K-Rta. Together with poly(ADP-ribosylation), phosphorylation inactivates K-Rta transcriptional activity and was hypothesized to serve as a contributor to the maintenance of KSHV latency. As hKFC has been shown to localize to the cytoplasm (Yustein et al., 2003), whereas K-Rta is primarily nuclear (Lukac et al., 1998), the interaction and phosphorylation by hKFC was proposed to potentially affect K-Rta localization.

POLYADP-RIBOSYLATION (PARYLATION)

Although the historical focus of PARP-1 has been its role in the cellular DNA damage response, studies have also revealed important roles for PARP-1 in transcriptional regulation. Poly-ADP-ribose (PAR) is a large negatively charged polymer that functions in both its free form or in the context of protein post-translational modifications. In mammalian cells, the majority of PAR production is catalyzed by PARP-1 and targets of PARP-1 catalytic activity include histones, transcription factors, nuclear enzymes, and nuclear structural components (see Krishnakumar and Kraus, 2010; for a recent review). Similar to hKFC described above, PARP-1 was identified by mass spectrometry as a protein that interacted with the S/T-rich region of K-Rta (Gwack et al., 2003). The interaction was mapped to the NAD^+ binding domain of PARP-1 and this interaction mediated the PARylation of K-Rta. Synergistic effects between PARP-1 and hKFC on K-Rta binding and its subsequent modification were observed. The *in vitro* interaction between K-Rta and PARP-1 was increased by the addition of hKFC and conversely, *in vitro* interaction between K-Rta and hKFC was also

increased with the addition of PARP-1. The reciprocal increases in binding were accompanied by enhanced phosphorylation and PARylation of K-Rta by hKFC and PARP-1, respectively. Moreover, PARP-1 and hKFC were found to act as repressors of K-Rta activity in reporter assays and in the efficiency of K-Rta-mediated viral reactivation. A combination of K-Rta mutational analysis, ChIP assays, and reporter activity measurements in PARP-1 knockout cells suggested a model in which PARP-1 and hKFC, acting as cellular sensors of herpesvirus infection, interfere directly or indirectly with the recruitment of K-Rta onto target promoters, leading to a decrease in K-Rta-mediated transcriptional activation (Gwack et al., 2003).

SUMOYLATION

Herpesvirus infections are controlled by acquired and innate defenses involving cellular, humoral, and cytokine-mediated responses. In recent years, a concept of intrinsic antiviral resistance has emerged as an additional antiviral defense mechanism that operates within individual cells. Unlike cytokine-mediated responses, intrinsic antiviral resistance involves the actions of pre-existing cellular proteins that, in the case of herpesviruses, act to repress viral transcription. PML nuclear bodies (ND10 or PML–NBs) have been identified as an important factor of intrinsic antiviral resistance against herpesvirus. Components of PML–NBs are recruited to sites associated with the viral chromosome soon after genomes enter the nucleus. Both SUMO-modification and SIMs of PML are required for formation of PML–NBs as well as recruitment to the viral genome. Viral gene expression is silenced by PML–NBs through deacetylation of local histones, which inhibits viral replication. Importantly, herpesvirus infection often disrupts PML–NBs, resulting in antagonism of both innate and intrinsic immune responses in early infection (Everett et al., 2006, 2008; Lukashchuk et al., 2006; Tavalai et al., 2006; Boutell et al., 2011).

Promyelocytic leukemia–NBs are mobile organelles that form distinct sub-compartments in the cell nucleus as described in a number of reviews (Bernardi and Pandolfi, 2007; Borden, 2008; Nagai et al., 2011). Assembly of PML–NBs, "storage" sites for SUMO, depends on the sumoylation of PML and other components such as Daxx, SP-100, and ATRX (Ishov et al., 1999; Zhong et al., 2000; Seeler et al., 2001; Fu et al., 2005). In addition, chromatin can be an integral part of PML–NBs. For example, a particular class of PML–NBs exists in tumor cells that maintain their telomeres in the absence of telomerase activity by a process referred to as alternative lengthening of telomeres (ALT). Recent studies identified that ALT is caused by the genomic mutations in Daxx or ATRX, both of which are localized in PML–NBs in a SUMO- and SIM-dependent manner (Lin et al., 2006; Berube et al., 2008; Heaphy et al., 2011). Interestingly, an ATRX–Daxx complex was recently identified as a histone chaperone, which specifically deposits the histone H3 variant, H3.3 (Drane et al., 2010; Goldberg et al., 2010; Lewis et al., 2010), leading to a possibility that PML–NBs function as a center of epigenetic gene regulation.

Like HSV-1 ICP0, our recent studies identified that K-Rta contained multiple SIMs and bound to SUMO *in vitro*. In co-transfection experiments with SUMO-2, K-Rta was found to markedly decrease the level of total cellular SUMO-2 modified proteins and mutations in the K-Rta SIM or ring-finger like domain significantly impaired this activity (**Figure 3A**). Using purified components, K-Rta was shown to conjugate ubiquitin to SUMO and SUMO-chains (**Figure 3B**). In addition, K-Rta preferentially targets SUMO-modified proteins for ubiquitylation; thus K-Rta is able to function as a STUbL which is capable of ubiquitylation of SUMO and SUMO conjugates *in vitro* and *in vivo* (**Figures 3A,B**; Izumiya et al., unpublished). The prototypical STUbL, cellular RNF4, was first identified as a ubiquitin ligase that functions to maintain genomic stability, and was recently reported to be a ubiquitin ligase targeting SUMO-containing proteins, including sumoylated PML (Sun et al., 2007; Tatham et al., 2008). Members of this ubiquitin ligase family include mammalian RNF4 and the heterodimers Hex3–Slx8 and Rfp1–Slx8 in *S. cerevisiae* and *S. pombe*, respectively (Uzunova et al., 2007; Xie et al., 2007). Similar to HSV-1 ICP0 and cellular RNF4, KSHV K-Rta disrupts the PML body in ubiquitin ligase dependent fashion (data not shown). Sumoylation is often linked to gene repression due to its ability to serve as a platform to recruit strong co-repressors such as SETDB1, HDAC1, and HDAC2 *via* SUMO-SIM interactions. Accordingly, K-Rta's ability to remove SUMO is relevant to its transactivation functions. Mutations in SIM or ring-finger like domain significantly impaired K-Rta transactivation activity. We observed that the K-bZIP promoter, a well recognized promoter transactivated by K-Rta, is activated by wild-type K-Rta, but not by H145L (ring-finger) mutant or a K-Rta SIM mutant (**Figure 3C**). Interestingly, the K12 (**Figure 3C**) or PAN promoter (not shown) were relatively unaffected. Thus, it is likely that there are SUMO-dependent and SUMO-independent promoters, which may depend on local chromatin structure and mode of K-Rta recruitment. These results suggest that K-Rta, in addition to being a strong transactivator, has an ability to regulate the local SUMO environment; the latter may contribute to its former functions through disassembly of SIM-containing co-repressors.

UBIQUITYLATION

K-Rta was found to contain an E3 ubiquitin ligase activity which was associated with the regulation of the cellular levels of interferon regulatory factor 7 (IRF7; Yu et al., 2005). During the characterization of this activity, it was determined that that K-Rta was subjected to self-ubiquitylation. Three residues were identified in the Cys/His-rich region of K-Rta which was found to be critical for both K-Rta auto-ubiquitylation and for degradation of IRF7. These residues, Cys131, Cys141, and His145 were proposed to be part of a novel Cys-rich domain extending from K-Rta amino acids 118–207 (C3HC3). However, this ring-finger like domain shows no significant homology with any previously described sequences conserved among E3 ligases. Based on analogy to other transcriptional activators, the K-Rta auto-ubiquitylation function may serve as a "degron" to activate gene expression (Muratani and Tansey, 2003; Kodadek et al., 2006). Indeed, K-Rta mutants that harbor a mutation in ring-finger like domain are more stable than K-Rta Wt. This stability is associated with a concomitant loss of transactivation function (**Figure 3C**). Further studies are required to dissect the contribution of K-Rta auto-ubiquitylation and repressor degradation activity to K-Rta transactivation (Yang et al., 2008).

LANA

KSHV (LANA/ORF73) is key regulatory protein that is essential for the establishment and maintenance of viral latency. LANA is a DNA binding protein that is a functional homolog of EBV EBNA1. LANA binds to the viral latent origin of replication, located at the terminal repeat (TR) sequence of the KSHV genome, and is highly expressed in all KSHV-associated disorders (Kedes et al., 1997; Schulz, 2001; Hu et al., 2002; Komatsu et al., 2004; Verma et al., 2007; Kelley-Clarke et al., 2009; Ganem, 2010; Mesri et al., 2010). LANA functions as both a transcriptional activator and a repressor depending on the context of promoters and cell line interrogated (An et al., 2005; Kelley-Clarke et al., 2009). Accordingly, LANA has been shown to associate with a broad range of transcriptional regulators such as RBP-Jκ, CBP, Daxx, BRD2, RB, p53, and Sp-1 (Friborg et al., 1999; Platt et al., 1999; Radkov et al., 2000; Lim et al., 2001; Verma et al., 2004; Lan et al., 2005; Murakami et al., 2006). LANA function is regulated and/or mediated by protein partner interaction. In addition, LANA activity is regulated by post-translational modifications, including phosphorylation (Platt et al., 1999; Fujimuro et al., 2005; Bajaj et al., 2006; Varjosalo et al., 2008; Cheng et al., 2009; Cha et al., 2010), acetylation (Lu et al., 2006), PARylation (Ohsaki et al., 2004), sumoylation (Izumiya et al., unpublished), and arginine methylation (Campbell et al., 2011). These modification sites are depicted in **Figure 4**.

PHOSPHORYLATION

Latency-associated nuclear antigen interacts with and is phosphorylated by several kinases including glycogen synthase kinase 3 (GSK-3; Fujimuro and Hayward, 2003; Fujimuro et al., 2005; Liu et al., 2007), Pim-1 and Pim-3 (Bajaj et al., 2006; Varjosalo et al., 2008; Cheng et al., 2009), and DNA-PK/Ku (Cha et al., 2010). LANA is also phosphorylated by an unidentified kinase mediated through an interaction of LANA with RING3 (Platt et al., 1999). In studies designed to characterize the interaction of LANA with RING3, Platt et al. (1999) first described phosphorylation of LANA *in vitro*. The interaction between RING3 and LANA resulted in serine/threonine phosphorylation of LANA on residues located between LANA amino acids 951 and 1107, and binding of RING3 to LANA facilitated the phosphorylation of LANA *via* an unknown co-immunoprecipitating kinase. However, the identity of the kinase and its role in the KSHV life cycle remains to be determined.

Using yeast two-hybrid screening with LANA as the bait protein, GSK-3 was shown to interact with LANA and through co-immunoprecipitation LANA interacted with both GSK-3α and GSK-3β in transfected cells. This interaction resulted in increased nuclear accumulation of GSK-3β (Fujimuro and Hayward, 2003). Fujimuro et al. (2005) further showed that GSK-3β phosphorylated multiple N-terminal residues of LANA *in vitro* and in KSHV infected PEL cells. Phosphorylation of LANA by GSK-3β was necessary for the LANA/GSK-3β interaction. GSK-3β predominantly phosphorylates pre-phosphorylated (primed) substrates that contain the motif S/TxxxS/Tp, where the +4 position S or T residue has prior phosphorylation mediated by another priming kinase (reviewed in Forde and Dale, 2007). LANA was found to require priming phosphorylation *in vitro* in order to serve as a GSK-3β substrate; mitogen-activated protein kinase (MAPK) and casein kinase-I (CK-I) could function as priming kinases, while CK-II could not. Subsequently ERK1/2 was also found to phosphorylate and prime LANA for subsequent GSK-3β phosphorylation (Liu et al., 2007). A GSK-3β priming pocket mutant (R96A) could not bind to LANA. Interestingly, in co-transfection experiments, GSK-3β present in a LANA–GSK-3β complex was unable to phosphorylate a primed peptide substrate. These results were interpreted to suggest that GSK-3β phosphorylation sites on LANA function to increase the affinity of LANA binding to GSK-3β and simultaneously provide a competitive substrate that minimizes potential phosphorylation events on other non-complexed substrates (Fujimuro et al., 2005). Taken together, these results suggested a model in which LANA sequesters GSK-3β in the nucleus leading to dysregulation of the kinase function in both the cytoplasmic (β-catenin stability) and nuclear (gene reprogramming) compartments (Fujimuro and Hayward, 2003; Fujimuro et al., 2005; Liu et al., 2007). Aspects of this model have been challenged by a report, in which LANA overexpression showed no effect on endogenous β-catenin levels (Hagen, 2009); this report

FIGURE 4 | Schematic representation of KSHV LANA. LANA protein and its post-translational modification sites as discussed in the text are depicted. Phosphorylation (Phospho) sites and the arginine methylation (Me) site are shown. The N-terminal histone binding domain, nuclear localization signal (NLS) and SUMO interaction motif (SIM) are also indicated. Numbers indicate amino acid position.

demonstrates the complexity of the interaction and its context dependence.

Bajaj et al. (2006) identified LANA as a substrate for Pim-1 kinase. The *Pim-1* gene was first identified as a common proviral insertion site of the Moloney murine leukemia virus (proviral insertion site MuLV; Cuypers et al., 1984). Pim-1 encodes a Ser/Thr kinase, which belongs to the group of calcium/calmodulin-regulated kinases. This gene is expressed primarily in B-lymphoid and myeloid cell lines, and is overexpressed in hematopoietic malignancies. Pim-1 plays a role in signal transduction in blood cells, contributing to cell growth and survival, and thus may provide a selective advantage in tumorigenesis (Bachmann and Moroy, 2005). Pim-1 was shown to bind to the C-terminus of LANA and to phosphorylate LANA at its N-terminus, specifically serine residues 205 and 206. Using human kinome expression screening, Varjosalo et al. (2008) confirmed that Pim-1 could phosphorylate LANA and augment viral reactivation primed by low levels of K-Rta. Cheng et al. (2009) further showed that another Pim family kinase member, namely Pim-3, could also phosphorylate LANA at serines 205 and 206. Moreover, ectopic expression of Pim-1 or Pim-3 in rKSHV.219-infected Vero cells enhanced the level of reactivation when co-expressed with low levels of exogenous K-Rta. Mechanistically, Pim-1 and Pim-3 phosphorylation of LANA was shown to antagonize LANA-dependent transcriptional repression of a synthetic TR reporter as well as decrease the ability of LANA to inhibit K-Rta auto-activation of its own promoter. As Pim-1 and Pim-3 are up-regulated during viral reactivation (Cheng et al., 2009) a model was proposed in which these kinases participate in reactivation by negative modulation of LANA function *via* direct phosphorylation.

Latency-associated nuclear antigen-dependent latent episomal replication is also regulated by phosphorylation. The DNA-PK/Ku complex bound and phosphorylated LANA (Cha et al., 2010). Uninfected B cell extracts were fractionated over a GST affinity resin containing LANA amino acids 1–52. Among the proteins found to interact with this LANA fragment were DNA-dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs), Ku70, and Ku86. In agreement with Ohsaki et al., 2004; see below), Cha et al. also identified PARP-1 as a LANA interacting protein. The phosphorylation of LANA by the DNA-PK/Ku complex was wortmannin-sensitive and mapping data suggested that LANA was phosphorylated at multiple positions. Interestingly, transient replication of a TR-containing plasmid was reduced when a phosphorylation-competent N-terminal LANA fragment was fused to a C-terminal LANA fragment that supported TR-dependent replication. Two similarly designed mutants that were not phosphorylated by DNA-PK/Ku were not defective in this assay. Overexpression of Ku70 also impaired transient TR-dependent replication in co-transfection assays conducted in 293T cells. Although overexpression of Ku86 did not affect transient replication assays set up in 293T cells, the authors noted an increase in TR-dependent replication when the assays were conducted in Ku86 heterozygous HCT116 Ku86+/− cells when compared to a HCT116 Ku86+/+ background. Taken together, these results suggest that the DNA-PK/Ku complex negatively regulates latent episomal replication.

ACETYLATION

Latency-associated nuclear antigen was reported to be lysine acetylated in response to sodium butyrate or trichostatin A-mediated reactivation (Lu et al., 2006). Although the site(s) of lysine acetylation on LANA were not mapped, LANA acetylation was observed in the presence of HDAC inhibition using either ectopic LANA expression or endogenous LANA in BCBL-1 cells as substrate. LANA acetylation was observed in HDAC inhibitor-treated cells using immunological detection with antibodies specific for acetyl lysine or by metabolic labeling of cells with [^{14}C]-sodium acetate followed by fluorography. LANA acetylation occurred during reactivation and this was accompanied by disruption of the interaction between LANA and the cellular proteins Sp-1 and histone H2B. ChIP analysis of HDAC inhibitor-treated BCBL-1 cells also revealed a loss of LANA from the K-Rta promoter region that occurred within the same time frame as LANA acetylation. Together, these results suggest that LANA acetylation contributes to the ORF50 transcriptional de-repression during the early stage of KSHV lytic cycle reactivation.

PARYLATION

As mentioned previously, PARP has been historically studied in the context of the DNA damage response, but PARP-1 has more recently been linked to the regulation of transcription and chromatin structure. PARP-1 binds to a variety of DNA structures, nucleosomes, and chromatin-associated proteins (Krishnakumar and Kraus, 2010). Ohsaki et al. (2004) reported that LANA was a target of PARP-1 and was poly(ADP-ribosyl)ated. Using a TR affinity column and BC3 cell extracts, PARP-1 was isolated as a TR interacting protein. TR binding by PARP-1 was independent of LANA and was also observed with BJAB cell extracts; however the affinity column elution profiles of PARP-1 and LANA were similar. In addition, PARP-1 was found to co-localize with LANA in the nucleus, suggesting that LANA may be a potential PARP-1 substrate. A small fraction of LANA present in BC3 anti-LANA immunoprecipitates was found to react with anti-PAR antibody. Moreover, when LANA immunoprecipitates were incubated with purified human PARP-1 and NAD *in vitro*, a species consistent with NAD-incorporated LANA was observed. Intriguingly, PARP-1 activity appeared to control viral copy number in infected cells. When BC3 cells were treated with hydroxyurea, a compound that elevates PARP activity, a ∼4-fold decrease in KSHV copy number was observed over the course of a 5-day treatment period. Conversely, when cells were similarly treated with compounds that decreased PARP activity (niacinamide and 3-aminobenzamide), KSHV copy numbers increased ∼8-fold. Interestingly, for hydroxyurea-treated cells, the decrease in viral copy number was accompanied by an increase in the PARylation signal detected in LANA immunoprecipitates. Although mechanistic details remain to be elucidated, these results suggest that the viral replication and/or episome partitioning functions of LANA may be influenced by cellular PARylation activity. Similar results were reported for EBV EBNA1, which was found interact with both PARP-1 (Deng et al., 2002; Tempera et al., 2010) and telomere-associated PARPs, Tankyrases-1 and -2 (Deng et al., 2002, 2005). Both PARP-1 and Tankyrase-1 were found to catalyze PARylation of EBNA1 and negatively regulate EBNA1 origin of

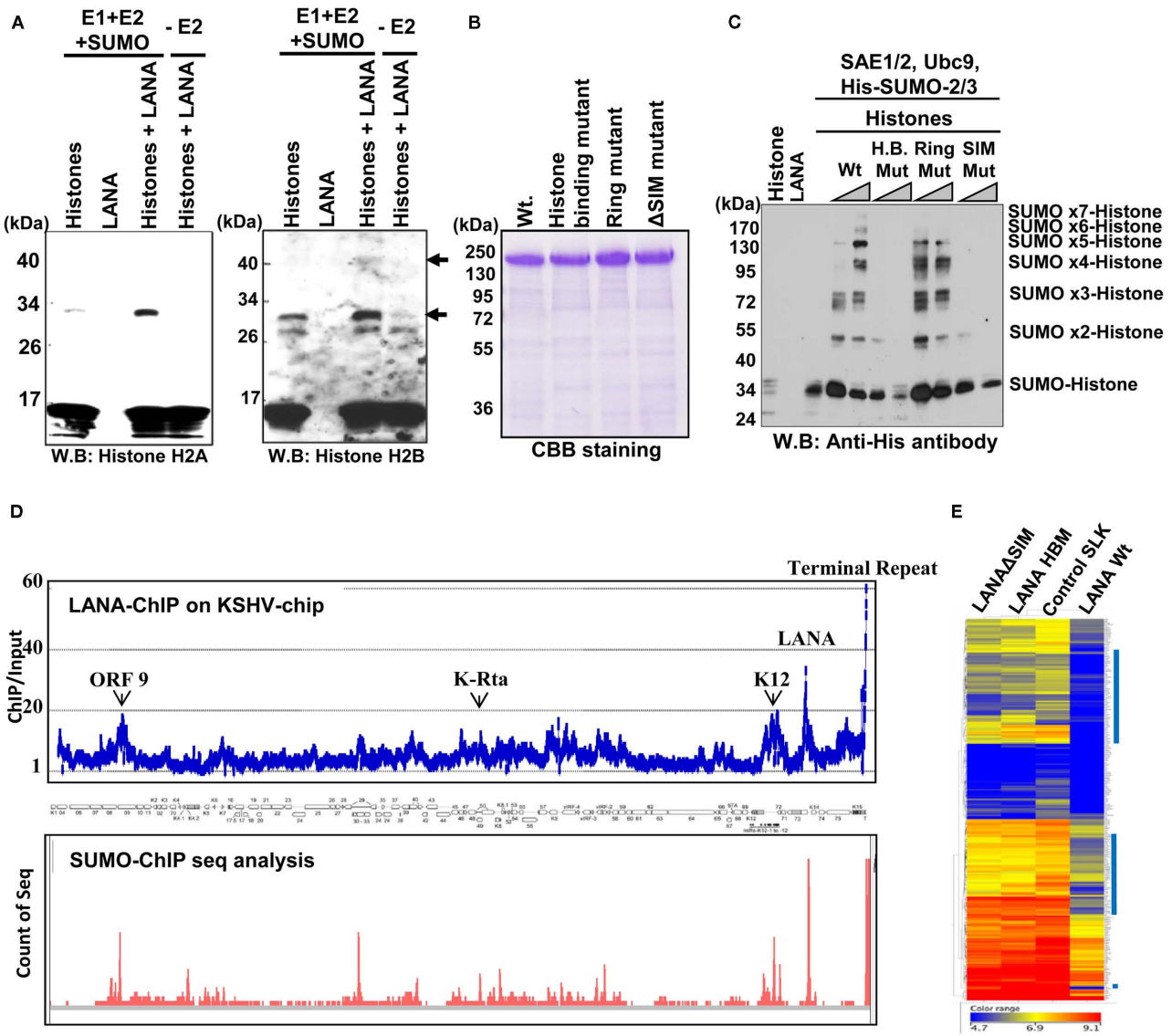

FIGURE 5 | Latency-associated nuclear antigen enhances histone sumoylation *in vitro* and *in vivo*. (A) LANA enhances histone H2A and H2B SUMO-modification *in vitro*. The *in vitro* SUMO conjugation reaction was performed with purified histone octamer (5 μg; Roche) as a substrate. SAE1/2 (E1, 50 nM), Ubc9 (E2, 50 nM), and HA-SUMO-2 (50 μM) were incubated in reaction buffer containing 1 mM ATP and $MgCl_2$ with or without a catalytic amount of LANA (50 nM). In the presence of LANA, H2A, and H2B SUMO-modification is enhanced. The arrows indicate SUMO-modified histones, which were increased in SUMO-reaction mixtures supplemented with LANA (three lanes from left). Full-length LANA Wt and mutant proteins were prepared with recombinant baculoviruses **(B)** and used as an E3 SUMO-ligase for *in vitro* SUMO conjugation reactions. CBB, Coomassie brilliant blue. **(C)** Histone binding domain and SUMO-interacting motif of LANA are necessary for its SUMO-ligase function. LANA Wt but neither a histone binding mutant (HB Mut) or SUMO-binding mutant (SIM Mut) can catalyze SUMO-modification of histones. The corresponding number of SUMO-chains is indicated on the right (20 kDa increments). LANA ring-finger like domain is dispensable for LANA-mediated SUMO conjugation of histones (Ring Mut). **(D)** Correlation between LANA recruitment sites and SUMO-enriched loci on the latent KSHV genome. ChIP analyses were performed with anti-LANA or anti-SUMO-2 antibodies using latent BCBL-1 cells. ChIP with LANA antibody was hybridized with custom-made KSHV tiling arrays and SUMO-ChIP was directly analyzed by sequencing (ChIP-seq). Enrichment over input DNA is shown (upper panel). Counts and position of sequence reads of SUMO-ChIP are shown in the bottom panel. SUMO-2 is enriched where LANA is recruited. Annotation of the KSHV genome is shown in the middle panel. The KSHV terminal repeat region is located at the extreme right of the annotated genome. **(E)** cDNA microarray analyses. RNA was prepared from indicated LANA stable cells or vector control stable cells and hybridized to Affymetrix human whole genome U133 plus 2.0 arrays. Raw data was subjected to baseline transformation and analyzed by hierarchical clustering using GeneSpring GX11 software. Genes highly repressed by LANA wt but not mutants are indicated by blue vertical lines to the right.

plasmid replication (OriP) activity in a PAR-dependent manner. Although the effects of the cellular DNA damage response on viral replication and gene expression are multi-faceted and enigmatic (Lilley et al., 2007; Weitzman et al., 2010) these result suggest that

current PARP-1 inhibitors (see Rouleau et al., 2010; for a recent review) under evaluation for treatment of certain malignancies might be worth examining for their efficacy as a treatment for KSHV infected individuals.

ARGININE METHYLATION

Our lab has determined that LANA is a substrate for protein arginine methyltransferase (PRMT)-1 *in vitro* and *in vivo*, and the major methylation site was mapped to the N-terminus of LANA (Arg 20; Campbell et al., 2011). Although, there was no effect of methylation on LANA nuclear localization or nuclear matrix association, methylation at this position did affect the interaction of LANA with histone octamers *in vitro* and with KSHV chromatin *in vivo*. *In vitro*, the histone octamer-LANA interaction was strengthened when the R20 site was replaced with a methyl-mimic residue, phenylalanine (R20F). Similarly, using LANA ChIP analysis, consistently higher amounts of KSHV DNA could be recovered from cells harboring latent KSHV bacmids containing the LANA R20F mutant. These results suggest that arginine methylation at position R20 may potentially influence a sub-population of LANA protein within infected cells resulting in increased interaction of LANA with KSHV-associated chromatin.

SUMOYLATION

Sumoylation-modification plays important roles in assembling protein complexes to regulate gene expression. Recent studies support a model in which the SUMO-modification has a significant impact on heterochromatin formation by recruiting SIM-containing co-repressors (Uchimura et al., 2006; Stielow et al., 2008). Accordingly, our lab has found that LANA is both heavily sumoylated and binds SUMO. By creating a SUMO-rich environment on local chromatin, we propose LANA may be able to condense chromatin, which leads to gene silencing mediated through its SIM domain. Interestingly, when we searched the LANA protein sequence for the SUMO-modification consensus motif (V/I/L-K-x-E), which is a well-established Ubc9 recognition sequence, there was no such motif. This observation indicates that LANA itself may bind SUMO to recruit Ubc9. Since LANA is heavily modified by SUMO in the absence of an E3 SUMO-ligase *in vitro*, LANA is likely to bind to the SUMO–Ubc9 complex ("active-SUMO") directly for its own SUMO-modification. Further detailed SUMO-binding analyses with a panel of GST–LANA deletion proteins confirmed that LANA possesses a SIM domain at its C-terminus. These result prompted us to investigate if LANA functions as an E3 SUMO-ligase, because the recruitment of SUMO–Ubc9 complex (active-SUMO) is one of the mechanisms for catalyzing the conjugation of SUMO. Although LANA associates with multiple proteins, we decided to focus on chromatin components and histone modifying enzymes involved in epigenetic gene regulation. We chose to use histone octamers as substrates, because LANA has been shown to associate with histones at the H2A–H2B acidic patch (Barbera et al., 2006). In addition, SUMO-modification of histones, especially histone H2B, is linked to chromatin condensation (Nathan et al., 2006). Our results confirmed that LANA can enhance SUMO-modification of histones *in vitro* (**Figure 5A**). The purified proteins that were used in the *in vitro* SUMO-reactions are shown in **Figure 5B**. Sumoylation reactions using two LANA mutants clearly show that both a histone binding mutation and SIM mutation significantly diminished histone targeting SUMO-ligase activity, supporting the idea that LANA needs to bind both substrate and SUMO for the activity (**Figure 5C**). Our results demonstrate for the first time that a viral protein acts as an E3 SUMO-ligase to increase SUMO-modification of histones, and which is likely to be involved in the formation of localized SUMO-rich chromatin. Next, we examined the distribution of SUMO and LANA across the latent KSHV episome. Comparison of the SUMO-ChIP assay with KSHV tiling array data indicated that the overlap of local SUMO occupancy with LANA binding sites within the KSHV genome was extensive (**Figure 5D**). Together, these experiments show that LANA can increase the content of histone SUMO-modification *in vitro* (isolated system without other proteins), and *in vivo* at the local chromatin level. Based on these data, we propose that LANA increases local SUMO-modification, and thus impacts and initiates epigenetic changes at these sites. These epigenetic changes may causally associate with the observed silencing of both the KSHV genome and at specific genomic loci of the host cell. We therefore examined whether assembly of a SUMO-mediated complex would translate into gene repression. If LANA SUMO-ligase function is important for such a complex formation, the LANA SUMO-ligase mutants [SUMO-binding mutant (ΔSIM) and histone binding mutant (HBM)] should have impaired gene repression function. To answer this question, cDNA microarray analyses were employed to probe the significance of histone targeting SUMO-ligase function in gene regulation. LANA wild-type and ΔSIM and HBM mutant stable SLK cells were generated. These cells

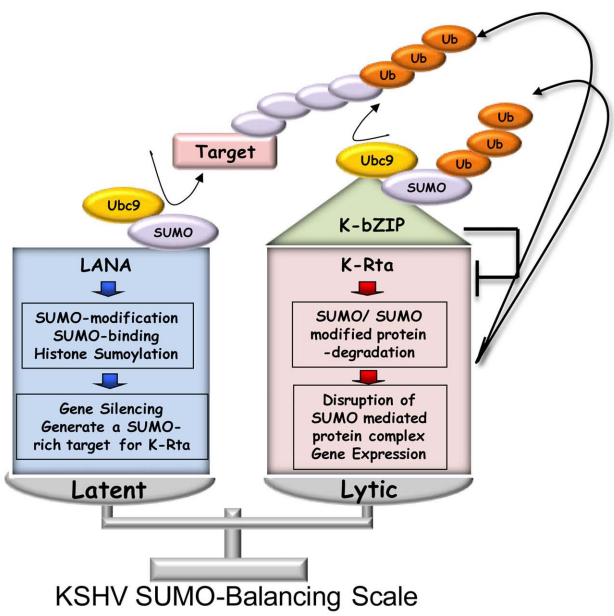

FIGURE 6 | KSHV SUMO-dependent balance. The outcome of the KSHV life cycle is depicted as a balance between the functions of the viral factors LANA, K-Rta, and K-bZIP which are mediated through their SUMO-dependent activities. The SUMO E3 ligase activities of K-bZIP and LANA and the STUbL activity of K-Rta are shown (see text for details).

lines expressed equivalent amounts of LANA or mutant LANA proteins when examined by western blotting with anti-Flag antibody. As shown in **Figure 5E**, both ΔSIM and HBM mutants had significantly impaired gene repression activity relative to wild-type LANA cells; these two cell lines showed gene expression patterns very similar to vector control SLK cells. Taken together, these results demonstrate that LANA recruits SUMO–Ubc9 complexes through SUMO-binding, and facilitates local histone SUMO-modification. Importantly, enrichment of SUMO at LANA binding sites may also prepare the KSHV genome for reactivation by generating a suitable target for K-Rta. Obviously, further studies are required to test this possibility. A putative SUMO-mediated KSHV latency-reactivation model is depicted in **Figure 6**.

SUMMARY

Although there are numerous studies describing post-translational modifications of several KSHV regulatory factors, there appears to be a fundamental gap in our knowledge as to precisely how, where and when these modifications affect the regulatory functions associated with each protein. These are very difficult questions to address for several reasons. The modifications affect only a small percentage of the total pool of a particular molecule, are transient in nature, and, in many cases, detection is technically demanding. Nevertheless, this area of herpesvirus research should be fruitful in yielding novel insights into both virus-host interactions and basic cellular signaling pathways. The recent advancement of proteomic approaches will certainly help to answer these questions.

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REFERENCES

Adamson, A. L., and Kenney, S. (2001). Epstein-Barr virus immediate-early protein BZLF1 is SUMO-1 modified and disrupts promyelocytic leukemia bodies. *J. Virol.* 75, 2388–2399.

An, F. Q., Compitello, N., Horwitz, E., Sramkoski, M., Knudsen, E. S., and Renne, R. (2005). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus modulates cellular gene expression and protects lymphoid cells from p16 INK4A-induced cell cycle arrest. *J. Biol. Chem.* 280, 3862–3874.

Bachmann, M., and Moroy, T. (2005). The serine/threonine kinase Pim-1. *Int. J. Biochem. Cell Biol.* 37, 726–730.

Bajaj, B. G., Verma, S. C., Lan, K., Cotter, M. A., Woodman, Z. L., and Robertson, E. S. (2006). KSHV encoded LANA upregulates Pim-1 and is a substrate for its kinase activity. *Virology* 351, 18–28.

Barbera, A. J., Chodaparambil, J. V., Kelley-Clarke, B., Joukov, V., Walter, J. C., Luger, K., and Kaye, K. M. (2006). The nucleosomal surface as a docking station for Kaposi's sarcoma herpesvirus LANA. *Science* 311, 856–861.

Bernardi, R., and Pandolfi, P. P. (2007). Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. *Nat. Rev. Mol. Cell Biol.* 8, 1006–1016.

Berube, N. G., Healy, J., Medina, C. F., Wu, S., Hodgson, T., Jagla, M., and Picketts, D. J. (2008). Patient mutations alter ATRX targeting to PML nuclear bodies. *Eur. J. Hum. Genet.* 16, 192–201.

Boggio, R., Passafaro, A., and Chiocca, S. (2007). Targeting SUMO E1 to ubiquitin ligases: a viral strategy to counteract sumoylation. *J. Biol. Chem.* 282, 15376–15382.

Borden, K. L. (2008). Pondering the puzzle of PML (promyelocytic leukemia) nuclear bodies: can we fit the pieces together using an RNA regulon? *Biochim. Biophys. Acta* 1783, 2145–2154.

Bossis, G., Malnou, C. E., Farras, R., Andermarcher, E., Hipskind, R., Rodriguez, M., Schmidt, D., Muller, S., Jariel-Encontre, I., and Piechaczyk, M. (2005). Down-regulation of c-Fos/c-Jun AP-1 dimer activity by sumoylation. *Mol. Cell. Biol.* 25, 6964–6979.

Boutell, C., Cuchet-Lourenco, D., Vanni, E., Orr, A., Glass, M., McFarlane, S., and Everett, R. D. (2011). A viral ubiquitin ligase has substrate preferential SUMO targeted ubiquitin ligase activity that counteracts intrinsic antiviral defence. *PLoS Pathog.* 7, e1002245. doi:10.1371/journal.ppat.1002245

Campbell, M., Chang, P.-C., Huerta, S., Izumiya, C., Davis, R., Tepper, C. G., Kim, K. Y., Shevchenko, B., Wang, D.-H., Jung, J. U., Luciw, P. A., Kung, H.-J., and Izumiya, Y. (2011). Protein arginine methyltransferase 1-directed methylation of Kaposi sarcoma-associated herpesvirus latency-associated nuclear antigen. *J. Biol. Chem.* doi: 10.1074/jbcM111.289496

Cha, S., Lim, C., Lee, J. Y., Song, Y.-J., Park, J., Choe, J., and Seo, T. (2010). DNA-PK/Ku complex binds to latency-associated nuclear antigen and negatively regulates Kaposi's sarcoma-associated herpesvirus latent replication. *Biochem. Biophys. Res. Commun.* 394, 934–939.

Chang, P. C., Fitzgerald, L. D., van Geelan, A., Izumiya, Y., Ellison, T. J., Wang, D.-H., Ann, D. K., Luciw, P. A., and Kung, H. J. (2009). Kruppel-associated box domain-associated protein-1 as a latency regulator for Kaposi's sarcoma-associated herpesvirus and its modulation by the viral protein kinase. *Cancer Res.* 69, 5681–5689.

Chang, P. C., Izumiya, Y., Wu, C. Y., Fitzgerald, L. D., Campbell, M., Ellison, T. J., Lam, K. S., Luciw, P. A., and Kung, H. J. (2010). Kaposi's sarcoma-associated herpesvirus (KSHV) encodes a SUMO E3 ligase that is SIM-dependent and SUMO-2/3-specific. *J. Biol. Chem.* 285, 5266–5273.

Chang, P. J., Shedd, D., Gradoville, L., Cho, M. S., Chen, L. W., Chang, J., and Miller, G. (2002). Open reading frame 50 protein of Kaposi's sarcoma-associated herpesvirus directly activates the viral PAN and K12 genes by binding to related response elements. *J. Virol.* 76, 3168–3178.

Cheng, F., Weidner-Glunde, M., Varjosalo, M., Rainio, E. M., Lehtonen, A., Schulz, T. F., Koskinen, P. J., Taipale, J., and Ojala, P. M. (2009). KSHV reactivation from latency requires Pim-1 and Pim-3 kinases to inactivate the latency-associated nuclear antigen LANA. *PLoS Pathog.* 5, e1000324. doi:10.1371/journal.ppat.1000324

Colombo, R., Boggio, R., Seiser, C., Draetta, G. F., and Chiocca, S. (2002). The adenovirus protein Gam1 interferes with sumoylation of histone deacetylase 1. *EMBO Rep.* 3, 1062–1068.

Cuypers, H. T., Selten, G., Quint, W., Zijlstra, M., Maandag, E. R., Boelens, W., van Wezenbeek, P., Melief, C., and Berns, A. (1984). Murine leukemia virus-induced T-cell lymphogenesis: integration of proviruses in a distinct chromosomal region. *Cell* 37, 141–150.

Daniel, A. R., Faivre, E. J., and Lange, C. A. (2007). Phosphorylation-dependent antagonism of sumoylation derepresses progesterone receptor action in breast cancer cells. *Mol. Endocrinol.* 21, 2890–2906.

Daniel, A. R., and Lange, C. A. (2009). Protein kinases mediate ligand-independent derepression of sumoylated progesterone receptors in breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 106, 14287–14292.

Deng, H., Song, M. J., Chu, J. T., and Sun, R. (2002). Transcriptional regulation of the interleukin-6 gene of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus). *J. Virol.* 76, 8252–8264.

Deng, Z., Atanasiu, C., Zhao, K., Marmorstein, R., Sbodio, J. I., Chi, N. W., and Lieberman, P. M. (2005). Inhibition of Epstein-Barr virus OriP function by tankyrase, a telomere-associated poly-ADP ribose polymerase that binds and modifies EBNA1. *J. Virol.* 79, 4640–4650.

Drane, P., Ouararhni, K., Depaux, A., Shuaib, M., and Hamiche, A. (2010). The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3. *Genes Dev.* 24, 1253–1265.

Ellison, T. J., Izumiya, Y., Izumiya, C., Luciw, P. A., and Kung, H. J. (2009). A comprehensive analysis of recruitment and transactivation potential of K-Rta and K-bZIP during reactivation of Kaposi's sarcoma-associated herpesvirus. *Virology* 387, 76–88.

Everett, R. D., Parada, C., Gripon, P., Sirma, H., and Orr, A. (2008). replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and sp100. *J. Virol.* 82, 2661–2672.

Everett, R. D., Rechter, S., Papior, P., Tavalai, N., Stamminger, T., and Orr, A. (2006). PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. *J. Virol.* 80, 7995–8005.

Forde, J. E., and Dale, T. C. (2007). Glycogen synthase kinase 3: a key regulator of cellular fate. *Cell. Mol. Life Sci.* 64, 1930–1944.

Friborg, J., Kong, W., Hottiger, M. O., and Nabel, G. J. (1999). p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* 402, 889–894.

Fu, C., Ahmed, K., Ding, H., Ding, X., Lan, J., Yang, Z., Miao, Y., Zhu, Y., Shi, Y., Zhu, J., Huang, H., and Yao, X. (2005). Stabilization of PML nuclear localization by conjugation and oligomerization of SUMO-3. *Oncogene* 24, 5401–5413.

Fujimuro, M., and Hayward, S. D. (2003). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus manipulates the activity of glycogen synthase kinase-3 beta. *J. Virol.* 77, 8019–8030.

Fujimuro, M., Liu, J., Zhu, J., Yokosawa, H., and Hayward, S. D. (2005). Regulation of the interaction between glycogen synthase kinase 3 and the Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen. *J. Virol.* 79, 10429–10441.

Ganem, D. (2010). KSHV and the pathogenesis of Kaposi sarcoma: listening to human biology and medicine. *J. Clin. Invest.* 120, 939–949.

Geiss-Friedlander, R., and Melchior, F. (2007). Concepts in sumoylation: a decade on. *Nat. Rev. Mol. Cell Biol.* 8, 947–956.

Girdwood, D., Bumpass, D., Vaughan, O. A., Thain, A., Anderson, L. A., Snowden, A. W., Garcia-Wilson, E., Perkins, N. D., and Hay, R. T. (2003). P300 transcriptional repression is mediated by SUMO modification. *Mol. Cell* 11, 1043–1054.

Goldberg, A. D., Banaszynski, L. A., Noh, K. M., Lewis, P. W., Elsaesser, S. J., Stadler, S., Dewell, S., Law, M., Guo, X., Li, X., Wen, D., Chapgier, A., DeKelver, R. C., Miller, J. C., Lee, Y. L., Boydston, E. A., Holmes, M. C., Gregory, P. D., Greally, J. M., Rafii, S., Yang, C., Scambler, P. J., Garrick, D., Gibbons, R. J., Higgs, D. R., Cristea, I. M., Urnov, F. D., Zheng, D., and Allis, C. D. (2010). Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* 140, 678–691.

Goodarzi, A. A., Kurka, T., and Jeggo, P. A. (2011). KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during DNA double-strand break response. *Nat. Struct. Mol. Biol.* 18, 831–839.

Gwack, Y., Nakamura, H., Lee, S. W., Souvis, J., Yustein, J. T., Gyri, S., Kung, H. J., and Jung, J. U. (2003). Poly(ADP-ribose) polymerase 1 and Ste20-like kinase hKFC act as transcriptional repressors for gamma-2 herpesvirus lytic replication. *Mol. Cell. Biol.* 23, 8282–8294.

Hagen, T. (2009). Characterization of the interaction between latency-associated nuclear antigen and glycogen synthase kinase 3 beta. *J. Virol.* 83, 6312–6317.

Heaphy, C. M., de Wilde, R. F., Jiao, Y., Klein, A. P., Edil, B. H., Shi, C., Bettegowda, C., Rodriguez, F. J., Eberhart, C. G., Hebbar, S., Offerhaus, G. J., McLendon, R., Rasheed, B. A., He, Y., Yan, H., Bigner, D. D., Oba-Shinjo, S. M., Marie, S. K., Riggins, G. J., Kinzler, K. W., Vogelstein, B., Hruban, R. H., Maitra, A., Papadopoulos, N., and Meeker, A. K. (2011). Altered telomeres in tumors with ATRX and DAXX mutations. *Science* 333, 425.

Hofmann, H., Floss, S., and Stamminger, T. (2000). Covalent modification of the transactivator protein IE2-p86 of human cytomegalovirus by conjugation to the ubiquitin-homologous proteins SUMO-1 and hSMT3b. *J. Virol.* 74, 2510–2524.

Hsu, C. H., Chang, M. D., Tai, K. Y., Yang, Y. T., Wang, P. S., Chen, C. J., Wang, Y. H., Lee, S. C., Wu, C. W., and Juan, L. J. (2004). HCMV IE2-mediated inhibition of HAT activity downregulates p53 function. *EMBO J.* 23, 2269–2280.

Hu, J., Garber, A. C., and Renne, R. (2002). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus supports latent DNA replication in dividing cells. *J. Virol.* 76, 11677–11687.

Hwang, S., Gwack, Y., Byun, H., Lim, C., and Choe, J. (2001). The Kaposi's sarcoma-associated herpesvirus K8 protein interacts with CREB-binding protein (CBP) and represses CBP-mediated transcription. *J. Virol.* 75, 9509–9516.

Ishov, A. M., Sotnikov, A. G., Negorev, D., Vladimirova, O. V., Neff, N., Kamitani, T., Yeh, E. T., Strauss, J. F., 3rd, and Maul, G. G. (1999). PML is critical for ND10 formation and recruits the PML-interacting protein Daxx to this nuclear structure when modified by SUMO-1. *J. Cell Biol.* 147, 221–234.

Izumiya, Y., Ellison, T. J., Yeh, E. T., Jung, J. U., Luciw, P. A., and Kung, H. J. (2005). Kaposi's sarcoma-associated herpesvirus K-bZIP represses gene transcription via SUMO modification. *J. Virol.* 79, 9912–9925.

Izumiya, Y., Izumiya, C., Van Geelen, A., Wang, D. H., Lam, K. S., Luciw, P. A., and Kung, H. J. (2007). Kaposi's sarcoma-associated herpesvirus-encoded protein kinase and its interaction with K-bZIP. *J. Virol.* 81, 1072–1082.

Kedes, D. H., Lagunoff, M., Renne, R., and Ganem, D. (1997). Identification of the gene encoding the major latency-associated nuclear antigen of the Kaposi's sarcoma-associated herpesvirus. *J. Clin. Invest.* 100, 2606–2610.

Kelley-Clarke, B., De Leon-Vazquez, E., Slain, K., Barbera, A. J., and Kaye, K. M. (2009). Role of Kaposi's sarcoma-associated herpesvirus C-terminal LANA chromosome binding in episome persistence. *J. Virol.* 83, 4326–4337.

Kodadek, T., Sikder, D., and Nalley, K. (2006). Keeping transcriptional activators under control. *Cell* 127, 261–264.

Komatsu, T., Ballestas, M. E., Barbera, A. J., Kelley-Clarke, B., and Kaye, K. M. (2004). KSHV LANA1 binds DNA as an oligmer and residues N-terminal to the oligomerization domain are essential for DNA binding, replication, and episome persistence. *Virology* 319, 225–336.

Krishnakumar, R., and Kraus, W. L. (2010). The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets. *Mol. Cell* 39, 8–24.

Lan, K., Kuppers, D. A., and Robertson, E. S. (2005). Kaposi's sarcoma-associated herpesvirus reactivation is regulated by interaction of latency-associated nuclear antigen with recombination signal sequence-binding protein J-kappa, the major downstream effector of the Notch signaling pathway. *J. Virol.* 79, 3468–3478.

Ledl, A., Schmidt, D., and Muller, S. (2005). Viral oncoproteins E1A and E7 and cellular LxCxE proteins repress SUMO modification of the retinoblastoma tumor suppressor. *Oncogene* 24, 3810–3818.

Lefort, S., Gravel, A., and Flamand, L. (2010). Repression of interferon-alpha stimulated genes expression by Kaposi's sarcoma-associated herpesvirus K-bZIP protein. *Virology* 408, 14–30.

Lewis, P. W., Elsaesser, S. J., Noh, K. M., Stadler, S. C., and Allis, C. D. (2010). Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14075–14080.

Lilley, C. E., Schwartz, R. A., and Weitzman, M. D. (2007). Using or abusing: viruses and the cellular DNA damage response. *Trends Microbiol.* 15, 119–126.

Lim, C., Gwack, Y., Hwang, S., Kim, S., and Choe, J. (2001). The transcriptional activity of cAMP rsponse element-binding protein is modulated by the latency associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. *J. Biol. Chem.* 276, 31016–31022.

Lin, D. Y., Huang, Y. S., Jeng, J. C., Kuo, H. Y., Chang, C. C., Chao, T. T., Ho, C. C., Chen, Y. C., Lin, T. P., Fang, H. I., Hung, C. C., Suen, C. S., Hwang, M. J., Chang, K. S., Maul, G. G., and Shih, H. M. (2006). Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol. Cell* 24, 341–354.

Lin, S. F., Robinson, D. R., Miller, G., and Kung, H. J. (1999). Kaposi's sarcoma-associated herpesvirus encodes a bZIP protein with homology to BZLF1 of Epstein-Barr virus. *J. Virol.* 73, 1909–1917.

Liu, J., Martin, H., Shamay, M., Woodard, C., Tang, Q.-Q., and Hayward, S. D. (2007). Kaposi's sarcoma-associated herpesvirus LANA protein downregulates nuclear glycogen synthase kinase 3 activity and consequently blocks differentiation. *J. Virol.* 81, 4722–4731.

Lu, F., Day, L., Gao, S. J., and Lieberman, P. M. (2006). Acetylation of the latency-associated nuclear antigen regulates repression of Kaposi's sarcoma-associated herpesvirus lytic transcription. *J. Virol.* 80, 5273–5282.

Lukac, D. M., Kirshner, J. R., and Ganem, D. (1999). Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. *J. Virol.* 73, 9348–9361.

Lukac, D. M., Renne, R., Kirshner, J. R., and Ganem, D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency

by expression of the ORF 50 transactivator, a homolog of the EBV R protein. *Virology* 252, 304–312.

Lukashchuk, V., McFarlane, S., Everett, R. D., and Preston, C. M. (2006). Human cytomegalovirus protein pp71 displaces the chromatin-associated factor ATRX from nuclear domain 10 at early stages of infection. *J. Virol.* 82, 12543–12554.

Marcos-Villar, L., Lopitz-Otsoa, F., Gallego, P., Munoz-Fontela, C., Gonzalez-Santamaria, J., Campagna, M., Shou-Jiang, G., Rodriguez, M. S., and Rivas, C. (2009). Kaposi's sarcoma-associated herpesvirus protein LANA2 disrupts PML oncogenic domains and inhibits PML-mediated transcriptional repression of the survivin gene. *J. Virol.* 83, 8849–8858.

Mesri, E. A., Cesarman, E., and Boshoff, C. (2010). Kaposi's sarcoma and its associated herpesvirus. *Nat. Rev. Cancer* 10, 707–719.

Murakami, Y., Yamagoe, S., Noguchi, K., Takebe, Y., Takahashi, N., Uehara, Y., and Fukazawa, H. (2006). Ets-1-dependent expression of vascular endothelial growth factor receptors is activated by latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus through interaction with Daxx. *J. Biol. Chem.* 281, 28113–28121.

Muratani, M., and Tansey, W. P. (2003). How the ubiquitin-proteasome system controls transcription. *Nat. Rev. Mol. Cell Biol.* 4, 192–201.

Nagai, S., Davoodi, N., and Gasser, S. M. (2011). Nuclear organization in genome stability: SUMO connections. *Cell Res.* 21, 474–485.

Nakamura, H., Lu, M., Gwack, Y., Souvlis, J., Zeichner, S. L., and Jung, J. U. (2003). Global changes in Kaposi's sarcoma-associated virus gene expression patterns following expression of a tetracycline-inducible Rta transactivator. *J. Virol.* 77, 4205–4220.

Nathan, D., Ingvarsdottir, K., Sterner, D. E., Bylebyl, G. R., Dokmanovic, M., Dorsey, J. A., Whelan, K. A., Krsmanovic, M., Lane, W. S., Meluh, P. B., Johnson, E. S., and Berger, S. L. (2006). Histone sumoylation is a negative regulator in *Saccharomyces cerevisiae* and shows dynamic interplay with positive-acting histone modifications. *Genes Dev.* 20, 966–976.

Ohsaki, E., Ueda, K., Sakakibara, S., Do, E., Yada, K., and Yamanishi, K. (2004). Poly(ADP-ribose) polymerase 1 binds to Kaposi's sarcoma-associated herpesvirus (KSHV) terminal repeat sequence and modulates KSHV replication in latency. *J. Virol.* 78, 9936–9946.

Platt, G. M., Simpson, G. R., Mittnacht, S., and Schulz, T. F. (1999). Latent nuclear antigen of Kaposi's sarcoma-associated herpesvirus interacts with RING3, a homolog of the *Drosophila* female sterile homeotic (fsh) gene. *J. Virol.* 73, 9789–9795.

Polson, A. G., Huang, L., Lukac, D. M., Blethrow, J. D., Morgan, D. O., Burlingame, A. L., and Ganem, D. (2001). Kaposi's sarcoma-associated herpesvirus K-bZIP protein is phosphorylated by cyclin-dependent kinases. *J. Virol.* 75, 3175–3184.

Prudden, J., Pebernard, S., Raffa, G., Slavin, D. A., Perry, J. J., Tainer, J. A., McGowan, C. H., and Boddy, M. N. (2007). SUMO-targeted ubiquitin ligases in genome stability. *EMBO J.* 26, 4089–4101.

Radkov, S. A., Kellam, P., and Boshoff, C. (2000). The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. *Nat. Med.* 6, 1121–1127.

Rosas-Acosta, G., Langereis, M. A., Deyrieux, A., and Wilson, V. G. (2005). Proteins of the PIAS family enhance the sumoylation of the papillomavirus E1 protein. *Virology* 331, 190–203.

Rouleau, M., Patel, A., Hendzel, M. J., Kaufmann, S. H., and Poirier, G. G. (2010). PARP inhibition: PARP1 and beyond. *Nat. Rev. Cancer* 10, 293–301.

Schulz, T. F. (2001). KSHV/HHV8-assoicated lymphoproliferations in the AIDS setting. *Eur. J. Cancer* 37, 1217–1226.

Seeler, J. S., Marchio, A., Losson, R., Desterro, J. M., Hay, R. T., Chambon, P., and Dejean, A. (2001). Common properties of nuclear body protein SP100 and TIF1alpha chromatin factor: role of SUMO modification. *Mol. Cell. Biol.* 21, 3314–3324.

Song, M. J., Li, X., Brown, H. J., and Sun, R. (2002). Characterization of interactions between RTA and the promoter of polyadenylated nuclear RNA in Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8. *J. Virol.* 76, 5000–5013.

Spengler, M. L., Kurapatwinski, K., Black, A. R., and Azizkhan-Clifford, J. (2002). SUMO-1 modification of human cytomegalovirus IE1/IE72. *J. Virol.* 76, 2990–2996.

Stielow, B., Sapetschnig, A., Wink, C., Kruger, I., and Suske, G. (2008). SUMO-modified Sp3 represses transcription by provoking local heterochromatic gene silencing. *EMBO Rep.* 9, 899–906.

Sun, H., Leverson, J. D., and Hunter, T. (2007). Conserved function of RNF4 family proteins in eukaryotes: targeting a ubiquitin ligase to SUMOylated proteins. *EMBO J.* 26, 4102–4112.

Sun, R., Lin, S. F., Gradoville, L., Yuan, Y., Zhu, F., and Miller, G. (1998). A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. *Proc. Natl. Acad. Sci. U.S.A.* 95, 10866–10871.

Tassi, E., Biesova, Z., Di Fiore, P. P., Gutkind, J. S., and Wong, W. T. (1999). Human JIK, a novel member of the STE20 kinas family that inhibits JNK and is negatively regulated by epidermal growth factor. *J. Biol. Chem.* 274, 33287–33295.

Tatham, M. H., Geoffroy, M. C., Shen, L., Plechanovova, A., Hattersley, N., Jaffray, E. G., Palvimo, J. J., and Hay, R. T. (2008). RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. *Nat. Cell Biol.* 10, 538–546.

Tavalai, N., Papior, P., Rechter, S., Leis, M., and Stamminger, T. (2006). Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. *J. Virol.* 80, 8006–8018.

Tempera, I., Deng, Z., Atanasiu, C., Chen, C.-J., D'Erme, M., and Lieberman, P. (2010). Regulation of Epstein-Barr virus OriP replication by poly(ADP-ribose) polymerase 1. *J. Virol.* 84, 4988–4997.

Uchimura, Y., Ichimura, T., Uwada, J., Tachibana, T., Sugahara, S., Nakao, M., and Saitoh, H. (2006). Involvement of SUMO modification in MBD1- and MCAF1-mediated heterochromatin formation. *J. Biol. Chem.* 281, 23180–23190.

Uzunova, K., Gottsche, K., Miteva, M., Weisshaar, S. R., Glanemann, C., Schnellhardt, M., Niessen, M., Scheel, H., Hofmann, K., Johnson, E. S., Praefcke, G. J., and Dohmen, R. J. (2007). Ubiquitin-dependent proteolytic control of SUMO conjugates. *J. Biol. Chem.* 282, 34167–34175.

Varjosalo, M., Bjorklund, M., Cheng, F., Synanen, H., Kivioja, T., Kilpinen, S., Sun, Z., Kallioniemi, O., Stunnenberg, H. G., He, W.-W., Ojala, P., and Taipale, J. (2008). Application of active and kinase-deficient kinome collection for identification of kinases regulating hedgehog signaling. *Cell* 133, 537–548.

Verma, S. C., Borah, S., and Robertson, E. (2004). Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus upregulates transcription of human telomerase reverse transcriptase promoter through interaction with transcription factor Sp1. *J. Virol.* 78, 10348–10359.

Verma, S. C., Lan, K., and Robertson, E. (2007). Structure and function of latency-associated nuclear antigen. *Curr. Top. Microbiol. Immunol.* 312, 101–136.

Weitzman, M. D., Lilley, C. E., and Chaurushiya, M. S. (2010). Genomes in conflict: maintaining genome integrity during virus infection. *Annu. Rev. Microbiol.* 64, 61–81.

West, J. T., and Wood, C. (2003). The role of Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8 regulator of transcription activation (RTA) in control of gene expression. *Oncogene* 22, 5150–5163.

Wu, H., Sun, L., Zhang, Y., Chen, Y., Shi, B., Li, R., Wang, Y., Liang, J., Fan, D., Wu, G., Wang, D., Li, S., and Shang, Y. (2006). Coordinated regulation of AIB1 transcriptional activity by sumoylation and phosphorylation. *J. Biol. Chem.* 281, 21848–21856.

Wu, Y. C., Deyrieux, A. F., and Wilson, V. G. (2007). Papillomaviruses and the host SUMOylation system. *Biochem. Soc. Trans.* 35, 1433–1435.

Xie, Y., Kerscher, O., Kroetz, M. B., McConchie, H. F., Sung, P., and Hochstrasser, M. (2007). The yeast Hex3.Slx8 heterodimer is a ubiquitin ligase stimulated by substrate sumoylation. *J. Biol. Chem.* 282, 34176–34184.

Xu, Y., AuCoin, D. P., Huete, A. R., Cei, S. A., Hanson, L. J., and Pari, G. S. (2005). A Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 ORF50 deletion mutant is defective for reactivation of latent virus and DNA replication. *J. Virol.* 79, 3479–3487.

Yang, Z., Yan, Z., and Wood, C. (2008). Kaposi's sarcoma-associated herpesvirus transactivator RTA promotes degradation of the repressors to regulate viral lytic replication. *J. Virol.* 82, 3590–3603.

Yu, Y., Wang, S. E., and Hayward, G. S. (2005). The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteasome-mediated degradation. *Immunity* 22, 59–70.

Yustein, J. T., Li, D., Robinson, D., and Kung, H. J. (2000). KFC, a Ste20-like kinase with mitogenic potential and

capability to activate the SAPK/JNK pathway. *Oncogene* 19, 710–718.
Yustein, J. T., Xia, L., Kahlenburg, J. M., Robinson, D., Templeton, D., and Kung, H. J. (2003). Comparative studies of a new subfamily of human Ste-20-like kinases: homodimerization, subcellular localization, and selective activation of MKK3 and p38. *Oncogene* 22, 6129–6141.
Zhong, S., Muller, S., Ronchetti, S., Freemont, P. S., Dejean, A., and Pandolfi, P. P. (2000). Role of SUMO-1-modified PML in nuclear body formation. *Blood* 95, 2748–2752.
Zimnik, S., Gaestel, M., and Niedenthal, R. (2009). Mutually exclusive STAT1 modifications identified by Ubc9/substrate dimerization-dependent SUMOylation. *Nucleic Acids Res.* 37, e30.

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The ubiquitin system and Kaposi's sarcoma-associated herpesvirus

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Ubiquitination is a post-translational modification in which one or more ubiquitin molecules are covalently linked to lysine residues of target proteins. The ubiquitin system plays a key role in the regulation of protein degradation, which contributes to cell signaling, vesicular trafficking, apoptosis, and immune regulation. Bacterial and viral pathogens exploit the cellular ubiquitin system by encoding their own proteins to serve their survival and replication in infected cells. Recent studies have revealed that Kaposi's sarcoma-associated herpesvirus (KSHV) manipulates the ubiquitin system of infected cells to facilitate cell proliferation, anti-apoptosis, and evasion from immunity. This review summarizes recent developments in our understanding of the molecular mechanisms used by KSHV to interact with the cellular ubiquitin machinery.

Keywords: Kaposi's sarcoma-associated herpesvirus, ubiquitin, proteasome, polyubiquitin, RING

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8, is classified as a rhadinovirus in the γ-herpesvirus subfamily and was the eighth human herpesvirus to be discovered from Kaposi's sarcoma (KS) specimens (Chang et al., 1994). KSHV is closely associated with KS and is also linked to AIDS-related lymphoproliferative disorders, such as primary effusion lymphoma (PEL, also known as body cavity-based lymphoma) and plasmablastic variant multicentric Castlemans disease (Russo et al., 1996; Ganem, 2007). The neoplastic potential of KSHV is well-established, especially in the context of immunosuppressed patients who are undergoing organ transplants, or who are co-infected with human immunodeficiency virus (HIV).

Like other herpesviruses, KSHV has two life cycles (i.e., latency and lytic replication). In latency, the KSHV genome circularizes and forms a double-stranded DNA, the episome, in the nucleus of a host cell. The KSHV genome contains several homologs of human genes such as viral cyclin D (v-cyclin), viral FLICE inhibitory protein (v-FLIP), viral G-protein coupled receptor (vGPCR), vIRF1, vBcl2, and vIL6 (Jenner and Boshoff, 2002), which contribute directly to KSHV-induced pathogenesis. KSHV establishes a latent infection in KS and PEL expressing several viral genes, including latency associated nuclear antigen (LANA), v-FLIP, v-cyclin, kaposin, and microRNAs. LANA (also called LANA1) encoded by ORF73 is required for replication and maintenance of the KSHV episomal DNA (Ballestas et al., 1999) and is expressed in all KSHV-associated malignancies (Järviluoma and Ojala, 2006). LANA also contributes to KSHV-associated oncogenesis through interaction with cellular molecules. KSHV alternates between lytic replication (productive infection) and latency (latent infection) by way of replication and transcription activator (RTA) expression. RTA, encoded by the immediate-early gene ORF50, is a critical switch molecule for initiating lytic replication (Deng et al., 2007). During lytic infection, progeny viruses are generated and released from host cells.

Kaposi's sarcoma-associated herpesvirus and other herpesviruses have a unique feature: KSHV can manipulate cellular machineries, such as cell proliferation, anti-apoptosis, and immune surveillance, by hijacking the cellular ubiquitin system, including polyubiquitin-dependent degradation by the 26S proteasome. The ubiquitin system plays fundamental roles in the regulation of cellular events, such as protein degradation, signal transduction, endocytosis, protein trafficking, and immune responses (Pickart, 2001; Ciechanover, 2005). It is well known that many viruses exploit the ubiquitin system in various ways for entry or release of progeny virus (Bieniasz, 2006) and also manipulate the system to overcome the cellular apoptosis machinery and immune responses. Additionally, viruses target deubiquitinating enzymes (DUBs) to stabilize cellular and viral proteins needed for viral processes (Sulea et al., 2006). In this review, we discuss how KSHV can hijack the cellular ubiquitin system to promote successful viral infection.

THE UBIQUITIN SYSTEM AND 26S PROTEASOME AS TARGETS OF KSHV'S STRATEGY

THE UBIQUITIN SYSTEM AND KSHV

In the ubiquitin system, a substrate protein is tagged with a polyubiquitin chain or a single ubiquitin molecule via isopeptide bonds that are formed between the carboxyl termini of ubiquitin molecules and either the ε-amino groups of the lysine residues in the target protein or the ubiquitin molecules themselves. Ubiquitin conjugation is catalyzed by the sequential actions of

ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). In this system, ubiquitin, a polypeptide of 76 amino acid residues, is first transferred to the E1 in an ATP-dependent manner (**Figure 1A**). This activated ubiquitin is then transferred to the E2. Finally, the ubiquitin is covalently attached to the target protein by E3 ubiquitin ligase, leading to the formation of a polyubiquitin chain (Pickart, 2001; Ciechanover, 2005; Weissman et al., 2011).

Ubiquitin has seven internal Lys residues, at positions 6, 11, 27, 29, 33, 48, and 63; Lys48 is most commonly used for the formation of a polyubiquitin chain. Ubiquitination consists of monoubiquitination and polyubiquitination, and ubiquitination can be classified into two types: a degradation signal for the 26S proteasome (**Figure 1B**), and a non-degradation signal (**Figures 1C,D**). Polyubiquitination can be reversed by the isopeptidase activities of DUBs, also called deubiquitinase and isopeptidase, and of the POH1/Rpn11 subunit of the 26S proteasome. DUBs disassemble polyubiquitin chains from polyubiquitinated substrates, and also replenish the pool of free ubiquitin. Monoubiquitination, the conjugation of a single ubiquitin molecule, is involved in DNA repair, protein–protein interactions, and endocytosis of membrane proteins (Hicke, 2001). The polyubiquitin chain, linked through Lys48 (K48-linked polyubiquitin), functions as a signal for degradation by the 26S proteasome. On the other hand, it has been thought that the polyubiquitin chain linked through Lys other than Lys48 (i.e., Lys6, 11, 27, 29, 33, and 63), plays a role in numerous cellular events. In particular, K63-linked polyubiquitin is involved in protein–protein interactions, cell signaling pathways, DNA repair, the stress response, and ribosomal function (Weissman et al., 2011; **Figure 1C**). Moreover, K63-linked polyubiquitination functions as a signal that induces internalization and lysosome-mediated degradation through endocytosis toward cell surface proteins (Hicke, 1999; **Figure 1D**).

Recent studies have shown that KSHV targets the ubiquitin system to manipulate cell signaling pathways, apoptosis, cell cycle regulation, IFN response, and antigen presentation. KSHV dysregulates the stabilities of regulator molecules p53, p27, IκB, Notch, and β-catenin. By hijacking regulator molecules, KSHV is able to alter viral and cellular gene expression. Additionally, KSHV can utilize the ubiquitin system by providing its own viral protein as an E3 enzyme or as a component protein of the E3 complex. It was reported that KSHV-encoded RTA acted as the E3 ubiquitin

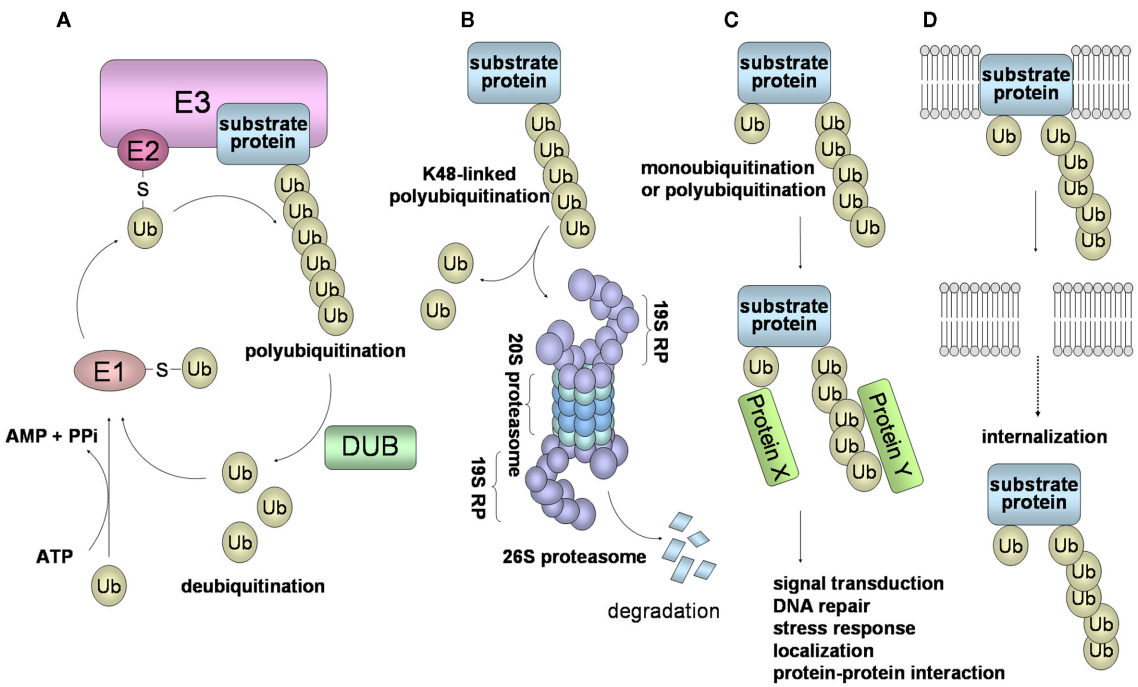

FIGURE 1 | Schematic of the ubiquitin system and 26S proteasome-mediated degradation. (A) Ubiquitin is first transferred to E1 in an ATP-dependent manner. This process requires the initial ATP-dependent activation of ubiquitin, with the release of PP_i, followed by binding of ubiquitin to a cysteine residue of E1 through a thioester linkage. Then, the activated ubiquitin is transferred to E2. Some selectivity for substrate occurs in this step, but the exquisite selectivity of intracellular proteolysis comes from the E3 ubiquitin ligases, which couple with E2 to bind substrates and catalyze the transfer of the activated ubiquitin from the E2 to the substrate, processively forming a chain of ubiquitins. **(B)** In the case of conjugation with K48-linked polyubiquitin, the polyubiquitinated protein is recognized by the 26S proteasome and is destroyed in an ATP-dependent manner. The 26S proteasome consists of the 19S regulatory complex (19RP) and a proteolytic core particle, the 20S proteasome. The 20S proteasome is arranged as a stack of four rings, composed of two outer α rings and two inner β rings. Three β subunits (β2, β5, β1) of a β ring are responsible for the distinct trypsin-like, chymotrypsin-like, and caspase-like proteolytic activities, respectively. **(C)** Modifications of a monoubiquitin and a polyubiquitin chain linked through Lys other than Lys48 (for example, Lys63, Lys11, and Lys29) play a role in many cellular processes, such as signal transduction, DNA repair, and localization through protein–protein interactions. **(D)** Monoubiquitination and K63-linked polyubiquitination are also involved in the internalization and endocytosis of membrane proteins.

ligase, and mediates polyubiquitination of IFN regulatory factor 7 (IRF7) for proteasomal degradation. KSHV-encoded LANA functions as a potential component of the E3 ubiquitin ligase complex for polyubiquitination of p53 and VHL (**Figure 1B**). KSHV also encodes ubiquitin ligases (K3 and K5) that induce the K63-linked polyubiquitination and internalization of major histocompatibility complex class I (MHC-I) molecules (**Figure 1D**). Furthermore, KSHV encodes a viral DUB which can disassemble polyubiquitin chains to stabilize the cellular and viral proteins. Thus, KSHV can create a favorable environment for the proliferation and survival of KSHV-infected host cells using these encoded proteins.

KSHV AND THE 26S PROTEASOME

Proteins conjugated with a K48-linked polyubiquitin chain are rapidly degraded by the 26S proteasome in an ATP-dependent manner (**Figure 1B**). Attachment of a chain composed of four or more ubiquitins is required for 26S proteasome-mediated protein degradation. The 26S proteasome consists of the 19S regulatory complex (19RP) and the 20S proteasome (Ciechanover, 2005; Weissman et al., 2011). The 20S proteasome is arranged as a stack of four rings, two outer α rings and two inner β rings, that have trypsin-like, chymotrypsin-like, and caspase-like proteolytic activities. The 19S RP can bind to a polyubiquitinated protein through the S5a/Rpn10 subunit, detach the polyubiquitin chain through the action of the Rpn11 subunit, and unfold the substrate and move it into the 20S proteasome for degradation. In the case of the globular proteins, the substrate undergoes ATP-dependent unfolding by the association with the 19S ATPases, followed by translocation into the entrance opening of the 20S proteasome. Finally, peptides ranging in length from 10 to 25 amino acid residues emerge from the 20S proteasome.

Kaposi's sarcoma-associated herpesvirus dysregulates cellular antiviral responses in various ways: (1) downregulation of MHC-I molecules, (2) suppression of the IFN response by IRF7 degradation, and (3) inhibition of the proteasomal processing process needed for antigen production. Virus-infected cells are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) upon display of virus-derived peptides by MHC-I. These virus-derived peptides are generated by the 26S proteasome, and then the viral peptides are translocated into the endoplasmic reticulum where they form a complex with MHC-I, with the assistance of peptide transporters (TAP) and chaperones. In particular, it is known that EBV prevents antigen presentation by inhibiting the 26S proteasome. EBV-encoded EBNA1 is required for the maintenance of EBV genomes. Furthermore, EBNA1 blocks the ability of the 26S proteasome to produce antigenic peptides (Levitskaya et al., 1995). EBNA1 is separated into N- and C-terminal domains by a Gly-Ala repeat domain, which disturbs the presentation of EBNA1 epitopes to MHC-I and also inhibits EBNA1 mRNA translation, thus minimizing protein levels in infected cells (Yin et al., 2003). EBNA1 is thus protected from endogenous presentation through the MHC-I pathway and this is also likely to be responsible for the long half-life of the EBNA1 protein. KSHV-encoded LANA, like EBNA1, may contribute to disturbing the antigen presentation by inhibition of the 26S proteasome activity. In fact, LANA also functions as a *cis*-acting inhibitor of antigen presentation (Zaldumbide et al., 2007). LANA has no sequence homology to EBNA1 but is apparently functionally analogous. LANA is a 220 kDa protein with unique N- and C-terminal regions separated by an acidic central repeat (CR) region, which contains three sets of repeats that are rich in Asp and Glu, Pro and Gln, and Pro and Glu, respectively. The CR region does not contain any proteolytic site for the proteasome. The LANA CR region can be further subdivided into three subdomains, based on differences in amino acid (aa) repeat sequences: CR1 (aa 321–428 in ORF73 of the BC-1 sequence, CR2 (aa 430–768), and CR3 (aa 769–937). It is known that the subdomain containing CR2CR3 contributes to LANA stability *in vitro* and *in vivo* and also retards LANA protein synthesis, minimizing LANA protein levels (Kwun et al., 2007). A recent study showed that the LANA CR1 subdomain inhibited MHC-I peptide antigen presentation in *cis* (Kwun et al., 2011). The CR2 subdomains retard LANA proteasomal processing but do not inhibit LANA peptide processing by MHC-I. These findings suggest that LANA physically inhibits the proteasome by insertion of the repeats into the proteolytic core particle of the 26S proteasome; however, KSHV and EBV use different mechanisms to evade host CTL-dependent surveillance.

KSHV AND E3 UBIQUITIN LIGASES

VIRAL PROTEINS AS COMPONENTS OF E3 UBIQUITIN LIGASES

Various combinations of E2 and E3 ubiquitin ligases determine the specificity toward target proteins and the types of ubiquitin linkages of the polyubiquitin chains. It is believed that there are in excess of 1000 different ubiquitin ligases in our genomes. E3 ubiquitin ligases are a large family of proteins that can be classified into three major and structurally distinct types: (1) HECT-type, (2) RING-finger-type, and (3) U-box-type E3 ubiquitin ligases (Pickart, 2001; Weissman et al., 2011). HECT-type E3s directly bind the substrate and contain a HECT domain that binds E2 and catalyzes the ubiquitin ligation (**Figure 2A**). Ubiquitin is transferred from the E2 to an active-site cysteine residue within the HECT domain, forming an E3 ubiquitin thioester complex. Ubiquitin is then transferred to the target protein. In humans, there are more than 50 kinds of HECT-type E3 enzymes. E6AP was the first HECT-type E3 identified, and this protein mediates K48-linked polyubiquitination of p53 in cells that express the E6 of human papillomavirus (HPV), types 16 and 18. The HPV E6 protein forms a complex with cellular E6AP, and increases the affinity for p53. RING-finger-type E3s contain RING-finger domains that interact with E2. RING-finger-type E3s can be sub-classified into monomeric RING-finger E3s (**Figure 2B**) and multimeric complex RING-finger E3s (**Figure 2C**), such as SCF and ECS (Lipkowitz and Weissman, 2011). RING-finger-type E3s function primarily as scaffolds, orienting the E2-ubiquitin thioester complex and the target protein for ubiquitin transfer. Mdm2 (**Figure 2B-2**) is monomeric RING-finger E3 for K48-linked polyubiquitination of p53 in normal cells. Multimeric RING-finger E3s [also called Cullin (Cul)-based E3s] constitute the largest subfamily and include the SKP1-Cul1-F-box protein (SCF) complex, the elongin C-elongin B-Cul5-SOCS-box (ECS) complex, and the anaphase-promoting complex/cyclosome (APC/C). The SCF complex (**Figure 2C-4**) is composed of Skp1 (adaptor protein), Cul1 (scaffold protein), Rbx1/Roc1 (E2-binding protein), and F-box protein. F-box protein, classified into Fbw (β-TrCP and

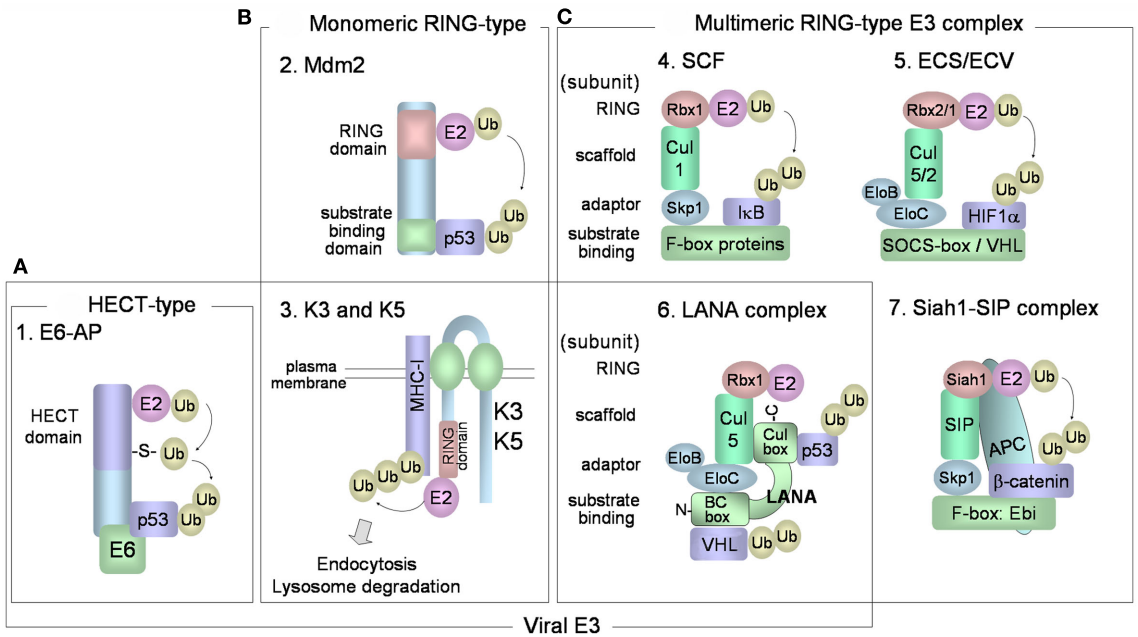

FIGURE 2 | E3 ubiquitin ligases. (A) HECT-type E3 has a HECT domain that binds to E2. E6-E6AP complex mediates polyubiquitination of p53. **(B,C)** RING-finger-type E3 enzymes have RING-finger domains that bind to E2 enzymes and can be categorized into monomeric **(B)** and multimeric RING-finger E3 enzymes **(C)**. **(B-2)** Mdm2 is monomeric RING-finger E3 for the K48-linked polyubiquitination of p53 in normal cells. **(B-3)** K3 and K5 bind to target substrates (MHC class I) via their transmembrane regions, and contain a RING-CH domain that interacts with E2. The cytoplasmic tails of the target substrates are mono- and poly-ubiquitinated, and the substrates are then endocytosed from the plasma membrane for degradation in lysosomes. **(C-4)** The SCF complex consists of Skp1 (adaptor protein), Cul1 (scaffold protein), Rbx1/Roc1 (E2-binding protein), and F-box protein that binds to Skp1 and recognizes the substrate. **(C-5)** The ECS complex is composed of elongin B, elongin C, Cul5, Rbx2, and substrate receptor SOCS-box protein. ECV is composed of elongin B, elongin C, Cul2, Rbx1, and VHL-box protein for binding HIF-1α. **(C-6)** KSHV LANA functions as a SOCS-box-like protein in the ECS/ECV complex, consisting of elongin B, elongin C, Cul5, and Rbx1. LANA SOCS-box-like motif consists of an elongin B/C box (N-terminus) and a Cul box (C-terminus). LANA binds to p53 protein through its C-terminus and binds to VHL protein through its N-terminus. **(C-7)** The human homolog of *Drosophila* Siah-1 has a RING-finger domain to bind E2 and forms an E3 complex composed of SIP, Skp1, and Ebi that recognizes β-catenin. Siah-1 binds to APC and this binding promotes degradation of β-catenin.

Fbw7), Fbl (Skp2), and Fbx types, binds through its F-box motif to Skp1 and recognizes the substrate. The F-box protein also binds to Skp1, which can link the F-box protein to Cul1. Cul1 functions as a scaffold, binding the RING-finger protein, Rbx1, and an E2. The ECS complex (**Figure 2C-5**) is composed of elongin B, elongin C, Cul5, Rbx2, and SOCS-box protein. SOCS-box binds to the Skp1 homologs elongin C and elongin B. ECV consists of Cul2 in place of Cul5 in ECS, Rbx1 in place of Rbx2 in ECS, and also the VHL-box protein that binds HIF-1α.

Kaposi's sarcoma-associated herpesvirus proteins may function as potential components of E3 ubiquitin ligase complexes. Because E3 ubiquitin ligase can directly destabilize target proteins, the cellular polyubiquitin machinery is dysregulated by a spurious E3 ubiquitin ligase containing viral components. KSHV-encoded LANA plays an important role in the maintenance of the KSHV genome, and also contributes to KSHV-associated oncogenesis through interaction with pRb (Radkov et al., 2000), p53 (Friborg et al., 1999), and GSK-3 (Fujimuro et al., 2003). LANA also functions as a potential component within the ECV/ECS complex, and targets the tumor suppressor, VHL, and p53 protein for polyubiquitination (Cai et al., 2006). LANA contains a putative SOCS-box motif, which is necessary for its assembly into the Rbx1-Cul5-elongin B-elongin C complex (**Figure 2C-6**). LANA binds to p53 protein through its C-terminus and binds to VHL protein through its N-terminus. VHL protein, a component of ECV, functions as a receptor for hydroxylated HIF-1α and induces K48-linked polyubiquitination of HIF-1α (**Figure 2C-5**). The oxygen-sensitive transcription factor, HIF-1α (or HIF-2α), binds to hypoxia-responsive DNA promoters involved in angiogenesis and cell proliferation. During normoxia, hydroxylated HIF-1α and 2α are recognized by VHL protein, resulting in their polyubiquitination and degradation. LANA stabilizes HIF-1α and increases HIF-1α-dependent transactivation by down-regulating VHL protein. Thus, LANA directly serves as a substrate receptor in ECS to promote degradation of the VHL and p53. It is known that multiple KSHV proteins, apart from LANA, are involved in p53 polyubiquitination for proteasomal degradation. KSHV encodes viral IFN regulatory factor 4 (vIRF4) that binds Mdm2, leading to the proteasomal degradation of p53 in KSHV-infected cells (Lee et al., 2009). The central region of vIRF4 interacts with MDM2, leading to inhibition of MDM2 autoubiquitination and, thereby, increased MDM2 stability. Moreover, KSHV-encoded vIRF1 also contributes to destabilizing the protein level of p53 by manipulating ATM kinase, which is activated by DNA damage. ATM can phosphorylate the Ser15 residues of p53, and this phosphorylation inhibits the interaction with Mdm2, resulting in p53 stabilization.

However, vIRF1 suppresses Ser15 phosphorylation of p53 by ATM, resulting in an increase of p53 ubiquitination and degradation by the proteasome (Shin et al., 2006).

Replication and transcription activator, a key regulator for KSHV reactivation from latent to lytic infection, may function as a E3 ubiquitin ligase for IRF7. In concert with UbcH5α as an E2 enzyme, RTA mediates the polyubiquitination of IRF7 depending on its Cys/His-rich domain, resulting in proteasomal degradation of IRF7 (Yu et al., 2005). RTA also autoregulates its own polyubiquitination and stability. The Cys/His-rich domain in the RTA N-terminal region is not categorized into a RING-finger domain and is composed of six Cys and one His residues (Cys3HisCys3). RAUL, a HECT-type E3, mediates polyubiquitination of IRF7 and IRF3. It is known that RTA induces the stabilization of RAUL and enhances the ability of RAUL to degrade IRF7 and IRF3 (Yu and Hayward, 2010). Furthermore, RTA induces the downregulation of "Toll-interleukin-1 receptor domain-containing adaptor-inducing beta-interferon" (TRIF; Ahmad et al., 2011), the cellular transcriptional repressor, Hey1 (Gould et al., 2009), and K-RBP (KSHV RTA binding protein)/human hypothetical protein MGC2663 (Yang et al., 2008). RTA interacts with Hey1 and K-RBP, and RTA mediates polyubiquitination, dependent on the Cys/His-rich domain of RTA, resulting in proteasomal degradation, while the Cys/His-rich domain of RTA is partially necessary for TRIF degradation, and RTA-mediated degradation of TRIF is partially mediated through the ubiquitin system. Thus, RTA manipulates the stabilities of various cellular proteins to dysregulate innate immunity and transcriptional regulation. However, it remains to be determined whether RTA Cys/His-rich domain directly binds E2. It is not known what kind of signal, such as protein–protein interaction, conformational switch or post-transcriptional modification of the substrate, is necessary to trigger interaction between RTA and it's substrates. These issues are of great importance to many researchers and are expected to be resolved in future studies.

K3 AND K5

Kaposi's sarcoma-associated herpesvirus has elaborate machineries to escape innate and adaptive antiviral immune responses. KSHV establishes a life-long infection in healthy human hosts, and it is fundamentally important for KSHV to evade the host immune response. KSHV thus exploits the cellular ubiquitin system by providing its own viral E3 enzymes. KSHV-encoded K3 and K5 proteins (**Figure 2B-3**), a class of viral RING-CH-finger E3 ubiquitin ligases, also called the "membrane-associated RING-CH" (MARCH) family E3 ubiquitin ligases, can downregulate the cellular surface display of MHC-I proteins, by inducing endocytosis (Brander et al., 2000; Coscoy and Ganem, 2000; Ishido et al., 2000a). Furthermore, K3 and K5 both target γ-interferon receptor 1 (IFN-γR1) and induce its ubiquitination, endocytosis, and degradation, resulting in downregulation of IFN-γR1 surface expression (Li et al., 2007). K3 and K5 proteins exhibit 40% amino acid homology to each other and localize to the plasma membrane. The RING-CH domains in their cytosolic N-termini are responsible for polyubiquitination of the cytoplasmic tail region of the MHC-I protein, and the two transmembrane regions are required for recognizing MHC-I. K3 and K5-mediated polyubiquitination of MHC-I leads to rapid internalization of MHC-I from the cell surface and subsequent endolysosomal degradation.

The RING-CH domain of K5 interacts with UbcH5a as an E2 enzyme (Coscoy et al., 2001), which is involved in K63-, K11-, and K48-linked polyubiquitination (Bosanac et al., 2011). K5 also downregulates other cell surface molecules, such as natural killer cell activators ICAM-1, B7-2 (Ishido et al., 2000b; Coscoy and Ganem, 2001), CD1d (Sanchez et al., 2005), MHC class I-related chain A/MICA (Thomas et al., 2008), HFE (Rhodes et al., 2010), and CD31/PECAM (Mansouri et al., 2006) by enhancing endocytosis and subsequent endolysosomal degradation, leading to the escape of infected cells from natural killer cells. K5 also targets the cytoplasmic tail of tetherin (also known as BST2 and CD317) for ubiquitination, leading to relocalization of tetherin to endosomal compartments (Mansouri et al., 2009; Pardieu et al., 2010). Tetherin is an interferon-induced antiviral membrane protein that prevents the release of many enveloped viral particles, including KSHV and HIV. Thus, K5 contributes to efficient KSHV particle release by down-regulating tetherin.

K3 mediates the K63-linked polyubiquitination of MHC-I, which is necessary for the efficient endocytosis and lysosomal degradation of MHC-I. K3 also mediates MHC-I monoubiquitination, and K3-mediated polyubiquitination through the K63-linkage requires both UbcH5 and Ubc13 as E2 enzymes for initiating monoubiquitination and subsequent K63-linked polyubiquitination, respectively, of MHC-I (Duncan et al., 2006). Ubc13 has been implicated in a variety of cellular processes, such as cell signaling and DNA repair, and has the ability to catalyze formation of K63-linked polyubiquitin chains on various substrates.

CELLULAR EVENTS MODULATED BY KSHV THROUGH UBIQUITINATION

SUBSTRATE RECOGNITION BY THE SCF COMPLEX

Many substrates of the 26S proteasome are modified by the post-translational modification before their K48-linked polyubiquitination. E3 ubiquitin ligases strictly recognize the post-translational modified forms of the target substrates: several different types of triggers for polyubiquitination – phosphorylation, proline hydroxylation and glycosylation – function as recognition signals for E3s. However, it is not clear how KSHV E3s (i.e., K3, K5, LANA, and RTA) recognize their substrates. There is very limited information available concerning LANA and RTA substrate recognition. It is not known what type of post-transcriptional modification of the substrate triggers interaction with LANA or RTA. Prospective studies are expected to resolve these important issues. Furthermore, it remains to be determined which amino acid residue of the substrate is modified and what type of polyubiquitin chain is conjugated with their substrate. These issues are also expected to be cleared in future studies.

In contrast to KSHV E3s, the substrate recognitions of cellular SCF-type E3s are well known. Many F-box proteins of SCF-type E3s can recognize phosphorylated forms of target substrates (Lipkowitz and Weissman, 2011). The SCF complex consists of Skp1, Cul1, Rbx1/Roc1, and F-box protein that binds the substrate (**Figure 2C-4**). More than 70 F-box proteins in humans can be categorized into three types (**Figure 3**): F-box proteins containing WD40 repeats domain (Fbw/FBXW), F-box proteins containing

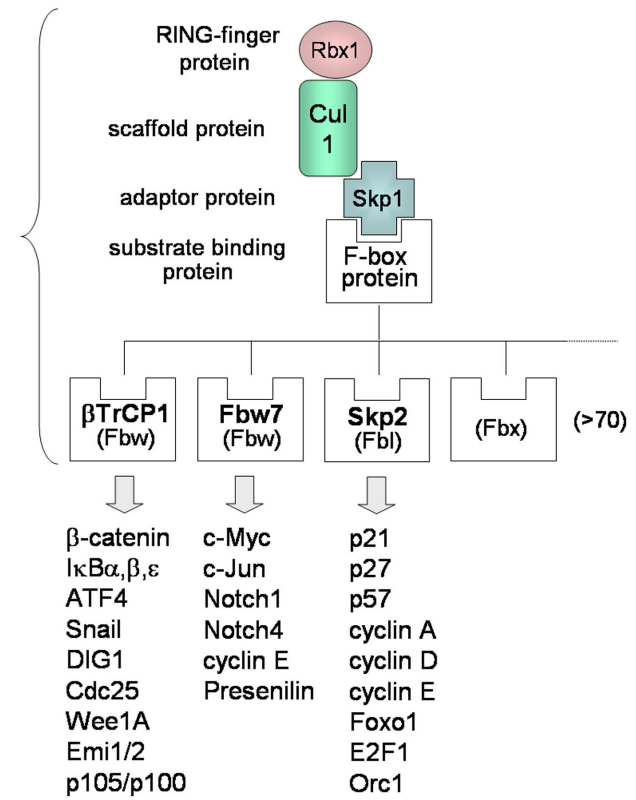

FIGURE 3 | Molecular diversity of SCF E3 complexes. The SCF complex consists of Skp1, Cul1, Rbx1 (E2-binding protein), and F-box protein that binds Skp1 and recognizes the substrate. There are more than 70 F-box proteins in humans and the F-box protein decides the substrate specificity of SCF. F-box proteins can be categorized into three types: F-box proteins containing a WD40-repeat domain (Fbw), F-box proteins containing a leucine-rich-repeat domain (Fbl), and F-box proteins containing other domains "X" (Fbx). Each F-box protein binds the phosphorylated consensus sequence of the specific substrate, resulting in K48-linked polyubiquitination.

leucine-rich repeats domain (Fbl/FBXL), and F-box proteins containing other domains (Fbx/FBXO; Nakayama and Nakayama, 2006). The $SCF^{\beta TrCP}$ complex, containing β-TrCP/Fbw1 as the Fbw-type F-box protein, binds to cellular signaling regulators, such as β-catenin, NF-κB inhibitors (IκBα, β, ε), ATF4, Snail, DIG1, Cdc25, and Wee1A. β-TrCP recognizes the conserved DpSGXXpS motif (with phosphorylated serine residues) in the substrate. The phosphorylation of Ser32 and Ser36 of IκBα is necessary for binding of β-TrCP, and the phosphorylation of Ser33 and Ser37 of β-catenin is necessary for binding of β-TrCP. As described in the following sections, KSHV can promote these phosphorylations of IκBα and β-catenin in order to up-regulate NF-κB and Wnt signaling. The SCF^{Fbw7} complex, containing Fbw7 as the Fbw-type F-box protein, recognizes c-Myc, c-Jun, Notch-1, Notch-4, cyclin E, and presenilin. The SCF^{Skp2} complex, containing Skp2/Fbl1 as the Fbl-type F-box protein, recognizes p21, p27, p57, p130, cyclin A, -D, -E, E2F, Foxo1, Orc1, and Cdk9. SCF^{Fbw7} complex is involved in downregulation of cell cycle accelerators, such as c-Myc, c-Jun, and Notch, whereas SCF^{Skp2} is involved in downregulation of cell cycle break molecules, such as p21, p27, p57, and p130. As described in the next section, KSHV can also promote the phosphorylation of Thr187 of p27, leading to polyubiquitination of p27. For the many substrates recognized by the SCF complex, phosphorylation is required for binding with the respective F-box proteins and for initiating K48-linked polyubiquitination.

As described previously, the ubiquitin system controls the stabilization of regulatory factors involved in cell signaling pathways, which serve in the progression of cell proliferation, anti-apoptosis, and control of the viral life-cycle. It is known that KSHV dysregulates recognition signals for cellular SCF complex E3s and exploits polyubiquitinating activities of SCF E3s. KSHV manipulates the upstream regulators of these cell signals in order to utilize cellular SCF E3s and control cell signalings. By targeting the specific recognition signals for the SCF complex – the phosphorylation of substrates such as IκB, β-catenin, and p27 – KSHV can affect cell cycle regulation, NF-κB signaling, and Wnt signaling (described in the following sections).

CELL CYCLE REGULATION

G1-S phase progression is strictly regulated by cyclin-dependent kinase (Cdk)-cyclin complexes. The cyclin D-Cdk4 (or Cdk6) and cyclin E-Cdk2 complexes phosphorylate pRb from mid-G1 to the late-G1 stage. These pRb phosphorylations lead to release, and activate E2F for the S phase progression. Cdk inhibitors (CKIs), negative-regulators of cyclin-Cdk complexes, can be classified into the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$) and the INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$). Cip/Kip family proteins bind cyclin-Cdk complexes, leading to inhibition of Cdk activity, and are targeted for K48-linked polyubiquitination by SCF^{Skp2} complex E3 (Nakayama and Nakayama, 2006).

Kaposi's sarcoma-associated herpesvirus mimics and uses these regulatory factors. KSHV v-cyclin/ORF72, a human cyclin D2 homolog, interacts with and activates Cdk4 and Cdk6 to achieve phosphorylation of pRb and cellular cyclin D, leading to G1-S phase progression (Chang et al., 1996; Mittnacht and Boshoff, 2000; Järviluoma and Ojala, 2006). Interestingly, unlike the cellular cyclins, v-cyclin-Cdk complex is resistant to the CKIs, p21, p27, and p16 (Swanton et al., 1997), and this complex thus functions as a constitutively active kinase. The v-cyclin-Cdk6 complex phosphorylates p21 on Ser130, and this phosphorylated p21 cannot inhibit Cdk2 (Järviluoma et al., 2006). Furthermore, v-cyclin-Cdk6 complex interacts with p27 and phosphorylates p27 at Thr187, leading to polyubiquitination of p27 for degradation (Ellis et al., 1999; Mann et al., 1999; Järviluoma et al., 2004). This regulation of p27 is normally performed by cellular cyclin E-Cdk2. Skp2 F-box protein of SCF^{Skp2} complex can bind Thr187-phosphorylated p27 and mediates its modification with a K48-linked polyubiquitin chain (**Figure 3**), leading to its degradation.

NOTCH SIGNALING

Kaposi's sarcoma-associated herpesvirus-encoded proteins manipulate upstream regulators in cell signaling and exploit the ubiquitin system to alter the stabilization of target proteins. Furthermore, KSHV proteins can directly bind a component of E3 ubiquitin

ligases to inhibit the E3 activity for polyubiquitination, leading to stabilization of target substrates. The SCF^{Fbw7} complex (**Figure 3**) is composed of Fbw7 as an F-box protein, which recognizes and binds the intracellular regions of Notch-1, -3, and -4 (Nakayama and Nakayama, 2006; Matsumoto et al., 2011). The F-box protein Fbw7, also known as Sel-10 in *C. elegans* and hCDC4 in humans, is thus a critical component of SCF^{Fbw7}, while it is a negative regulator of Notch signaling. Notch signaling plays roles in cell fate decisions, proliferation, and differentiation. When a Notch ligand, such as Jagged or Delta, binds the Notch receptor, a γ-secretase complex, including presenilin, cleaves the intramembrane region of the Notch receptor, Notch-1, 2, 3, and 4, resulting in release of intracellular Notch (ICN, also called the Notch intracellular domain, NICD). ICN, which functions as a transcriptional activator, enters the nucleus and binds "CBF1/Suppressor of Hairless/Lag-1" (CSL), upregulating transcription of its targets (Hayward et al., 2006).

It is known that, in addition to ICN, KSHV RTA interacts with CSL and activates target gene expression in cooperation with co-activators (Liang and Ganem, 2003). KSHV also has another way to activate Wnt signaling. KSHV LANA binds Fbw7/Sel-10, a component of SCF^{Fbw7}, to inhibit polyubiquitination of intracellular Notch-1 (ICN), which leads to stabilization of ICN. The carboxyl-terminus of LANA binds the F-box domain and the WD40 domain of Fbw7/Sel-10, whereas it competes with ICN for interaction with Fbw7/Sel-10, resulting in inhibition of ICN polyubiquitination and degradation (Lan et al., 2007). Stabilization of ICN by LANA is also related to the proliferation of KSHV-infected cells.

NF-κB SIGNALING

NF-κB signaling, regulated by the ubiquitin system, influences cell proliferation, anti-apoptosis, and inflammation. KSHV activates NF-κB pathways to promote host cell proliferation, survival, and angiogenesis. Additionally, constitutive activation of NF-κB signaling is essential for latent infection of KSHV and survival in KSHV-infected PEL cells. In normal resting cells, the canonical NF-κB signaling pathway is suppressed by IκBα protein. The NF-κB pathway is further regulated by IKK complex, consisting of IKKα, β, and a scaffold subunit (IKKγ/NEMO). A stimulus, such as TNF-α or IL-1β, induces activation of the IKK complex via TRAF6 and TRAF2. TRAF6 can modify NEMO by K63-linked polyubiquitination, in collaboration with Ubc13. K63-linked polyubiquitinated NEMO then activates the IKK complex, which phosphorylates IκBα. Phosphorylation of Ser32 and Ser36 IκBα can be a trigger for K48-linked polyubiquitination by $SCF^{\beta TrCP}$ (**Figure 3**). Phosphorylated IκBα is then subjected to $SCF^{\beta TrCP}$-mediated K48-linked polyubiquitination and subsequent proteasomal degradation. This releases active NF-κB (p50–p65/RelA heterodimer). There is another upregulation system for IKK complex: TGF-β-activated kinase (TAK1) also phosphorylates and activates IKK. TAK1 forms a complex with TAK1-binding protein 1 (TAB1) and TAB2 (or TAB3). TAB2 stimulates TAK1 kinase activity and binds to K63-linked polyubiquitinated NEMO (or TRAF6) through the Zn-finger domain of TAB2. In response to TNF-α stimuli, receptor-interacting protein (RIP) is modified with K63-linked polyubiquitin chain, and polyubiquitinated RIP recruits NEMO into the IKK complex.

Kaposi's sarcoma-associated herpesvirus-encoded v-FLIP, K7, and microRNA can induce NF-κB transcriptional activation by targeting the protein level of IκB. v-FLIP (ORF 71) achieves anti-apoptosis by activating NF-κB signaling (Chaudhary et al., 1999; Keller et al., 2000; Chugh et al., 2005). v-FLIP can activate the IKK complex through interaction with NEMO of the IKK complex, including Hsp90 (Field et al., 2003) and RIP (Chaudhary et al., 1999; Liu et al., 2002). Additionally, TRAF2 leads to the association of v-FLIP with the NEMO of the IKK complex (Guasparri et al., 2006). Activated IKK complex phosphorylates IκBα, leading to K48-linked polyubiquitination and proteasomal degradation. miR-K1 of the miRNA cluster, encoded by the KSHV genome, can decrease IκBα in a ubiquitin system-independent manner. miR-K1 reduces expression of IκBα protein by targeting the 3′-UTR of its transcript (Lei et al., 2010). Thus, miR-K1 upregulates NF-κB activity and inhibits viral lytic replication in PEL cells. KSHV small membrane protein, K7, inhibits caspase-3-induced apoptosis and interacts with ubiquitin/PLIC1 (Feng et al., 2004). Ubiquilin has an N-terminal ubiquitin-like (UBL) domain and a C-terminal ubiquitin-associated (UBA) domain. The UBA domain binds to the K7 protein and the polyubiquitin chain. The interaction between K7 and ubiquilin induces the degradation of IκB and concomitant activation of NF-κB signaling. Thus, to activate the NF-κB pathway, KSHV may induce the phosphorylation of IκBα for $SCF^{\beta TrCP}$-mediated K48-linked polyubiquitination, resulting in reduced IκBα protein. Interestingly, K7 induces the degradation of KSHV-encoded ORF74/vGPCR, which is a pirated version of the human IL-8 receptor gene. vGPCR is a ligand-independent, constitutively active signaling molecule that activates NF-κB signaling and promotes cell proliferation. K7 causes vGPCR to be retained in the endoplasmic reticulum and induces the proteasomal degradation of vGPCR. Furthermore, K7 expression reduces vGPCR tumorigenicity in nude mice (Feng et al., 2008).

Wnt SIGNALING

The Wnt signaling pathway, also regulated by ubiquitin and the proteasome, is involved in several key developmental processes and in tumorigenesis. The Wnt signaling cascade is triggered when Wnt ligands bind to Frizzled receptors and the signal is transmitted through Disheveled to the axin-APC-GSK-3β complex, resulting in the disruption of this complex. The β-catenin level is regulated by GSK-3β (GSK-3), Ser/Thr kinase, which phosphorylates β-catenin and targets it for K48-linked polyubiquitination and, subsequently, proteasomal degradation. Displacement of GSK-3 from the complex leads to stabilization of β-catenin, which translocates to the nucleus and forms a complex with Lef (or Tcf4) transcription factors, stimulating the expression of c-Myc, c-Jun, and cyclin D1. Phosphorylation of β-catenin is essential for binding $SCF^{\beta TrCP}$ complex, containing the β-TrCP that recognizes the DpSGXXpS motif in β-catenin. $SCF^{\beta TrCP}$ complex can mediate K48-linked polyubiquitination for β-catenin (**Figure 3**). GSK-3 kinase recognizes and phosphorylates serine and/or threonine residues in the context of the S/Txxx pS/pT motif. The downstream Ser or Thr residue must first be phosphorylated by a priming kinase. In β-catenin, phosphorylation of Ser45 by casein kinase I serves as the priming event for GSK-3 kinase, which then phosphorylates the Thr41, Ser37, and Ser33

residues. Phosphorylation of Ser33 and Ser37 of β-catenin is necessary for binding of the $SCF^{\beta TrCP}$ complex and K48-linked polyubiquitination.

Kaposi's sarcoma-associated herpesvirus dysregulates Wnt signaling by LANA (Fujimuro and Hayward, 2003; Fujimuro et al., 2003, 2005). The β-catenin protein accumulates in latently KSHV-infected PEL cells and KS cells. The mechanism of β-catenin dysregulation is related to the interaction of LANA and GSK-3 kinase. LANA associates with GSK-3, leading to nuclear translocation of GSK-3. Although GSK-3 is localized primarily in the cytoplasm, a small proportion of GSK-3 is known to enter the nucleus during S phase (Diehl et al., 1998), and LANA increases the number of cells in S phase (Fujimuro et al., 2003). Because LANA binds to nuclear GSK-3, LANA induces depletion of cytoplasmic GSK-3. In the absence of cytoplasmic GSK-3, β-catenin accumulates in the cytoplasm and enters the nucleus to stimulate transcriptional activation of downstream target genes. Additionally, LANA binds ERK1/2, which participates in the phosphorylation of Ser 9 of GSK-3, resulting in inactivation of GSK-3 (Liu et al., 2007). The stability of c-Myc is regulated by the c-Myc phosphorylation at Ser62, mediated by Erk, and at Thr58, mediated by GSK-3. This phosphorylation of c-Myc is necessary for the interaction with Fbw7 protein of SCF^{Fbw7} and K48-linked polyubiquitination (Yada et al., 2004). Inactivation of nuclear GSK-3 by LANA may increase the stability and activity of c-Myc and further contribute to LANA-mediated growth dysregulation. In fact, abnormally stable c-Myc protein can be observed, and LANA is responsible for this stability in PEL cells (Bubman et al., 2007).

EBV upregulates Wnt signaling through a different mechanism than that in KSHV (Hayward et al., 2006). EBV LMP1 represses expression of the transcripts for Siah-1 (Jang et al., 2005), the human homolog of *Drosophila seven in absentia*. Siah-1 binds E2 enzyme with its N-terminal RING-finger domain and forms an E3 ubiquitin ligase complex, consisting of Siah-1, SIP, Skp1, and Ebi that binds the β-catenin protein (**Figure 2C-7**). This E3 ubiquitin ligase promotes the degradation of β-catenin through a mechanism independent of GSK-3β-mediated phosphorylation; Siah-1 binds to APC and this binding directly promotes degradation of β-catenin. On the other hand, an E3 complex including Siah-1 targets KSHV protein for polyubiquitination. The KSHV-encoded ORF45 protein is capable of inhibiting virus-dependent interferon induction and appears to be essential for both early and late stages of infection. Siah-1 complex induces the destabilization of ORF45 protein through polyubiquitination (Abada et al., 2008).

KSHV AND DEUBIQUITINATING ENZYME

Polyubiquitination is reversed by the isopeptidase activities of DUBs. DUBs disassemble polyubiquitin chains from substrates, resulting in inhibition of the recognition by the 26S proteasome, and induce stabilization of substrate proteins. It is well known that human herpesviruses utilize activities of DUBs for stabilization of their viral proteins. DUBs disassemble polyubiquitin chains from polyubiquitinated proteins, resulting in stabilization of substrate proteins.

USP7 (also called HAUSP) is an evolutionary-conserved mammalian DUB, which was originally identified as a protein binding to the EBNA1 of EBV and ICP0 of HSV-1 (Canning et al., 2004; Holowaty and Frappier, 2004). USP7 can remove the K48-linked polyubiquitin from the EBNA1 and ICP0, and stabilize them. Interestingly, recent studies show that KSHV also targets DUBs to stabilize the cellular and viral proteins needed for viral processes. KSHV encodes a viral DUB (ORF64) that can disassemble both K48- and K63-linked polyubiquitin chains (González et al., 2009), while ORF64 protein suppresses RIG-I-mediated IFN signaling by reducing the polyubiquitination of RIG-I (Inn et al., 2011). Additionally, RTA targets the USP7 to stabilize the HECT-type E3, RAUL (Yu and Hayward, 2010). KSHV, directly or indirectly, may exploit DUBs to stabilize its own proteins and other cellular proteins involved in its survival and replication in infected cells.

CONCLUSION

Ubiquitination plays a key role in controlling the function and stability of target proteins. KSHV hijacks the ubiquitin system and the degradation-by-proteasome system for the purpose of cell proliferation, anti-apoptosis, and evasion from immune surveillance in infected cells. KSHV-encoded proteins and E3 enzymes take advantage of cellular ubiquitins and induce the degradation of host proteins that are antagonistic to infection. KSHV also utilizes the activities of DUBs for stabilization of their viral proteins. However, there are many questions about these KSHV dysregulating mechanisms. It remains to be determined how K3, K5, LANA, and RTA recognize and dock their substrates. Particularly, it is not known what type of signal (such as post-transcriptional modification of the substrate, conformation-changing of substrate, or other protein interaction with substrate) can trigger interactions with KSHV E3s. These questions need to be resolved in a future study. Additionally, a very interesting discovery has been made with RTA Cys/His-rich domain, which is not categorized into RING-finger domain, and which may be a new type of RING-finger. However, it is unclear whether or not RTA Cys/His-rich domain binds to E2. This important question also needs to be answered in a future study.

With the exception of ubiquitination, several post-translational modifications of cellular and viral proteins, such as glycosylation, methylation, acetylation, sumoylation, and *S*-nitrosylation, have been described by numerous studies. Because these modifications play critical roles in regulating functions of substrate proteins, we need to consider these modification as well as ubiquitination. Further study is expected to reveal how KSHV associates with these post-translational modifications.

To further elucidate KSHV pathogenesis, it should be resolved how KSHV dysregulates and exploits ubiquitin pathways through advanced proteomic approaches. A better understanding of the interaction between KSHV infection and the ubiquitin system will provide new insights into the viral evasion of host immunity and the process of carcinogenesis triggered by KSHV, and may provide the basis for theories for the development of novel therapeutic interventions against KSHV-related cancers.

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REFERENCES

Abada, R., Dreyfuss-Grossman, T., Herman-Bachinsky, Y., Geva, H., Masa, S. R., and Sarid, R. (2008). SIAH-1 interacts with the Kaposi's sarcoma-associated herpesvirus-encoded ORF45 protein and promotes its ubiquitylation and proteasomal degradation. *J. Virol.* 82, 2230–2240.

Ahmad, H., Gubbels, R., Ehlers, E., Meyer, F., Waterbury, T., Lin, R., and Zhang, L. (2011). Kaposi sarcoma-associated herpesvirus degrades cellular Toll-interleukin-1 receptor domain-containing adaptor-inducing beta-interferon (TRIF). *J. Biol. Chem.* 286, 7865–7872.

Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* 284, 641–644.

Bieniasz, P. D. (2006). Late budding domains and host proteins in enveloped virus release. *Virology* 344, 55–63.

Bosanac, I., Phu, L., Pan, B., Zilberleyb, I., Maurer, B., Dixit, V. M., Hymowitz, S. G., and Kirkpatrick, D. S. (2011). Modulation of K11-linkage formation by variable loop residues within UbcH5A. *J. Mol. Biol.* 408, 420–431.

Brander, C., Suscovich, T., Lee, Y., Nguyen, P. T., O'Connor, P., Seebach, J., Jones, N. G., van Gorder, M., Walker, B. D., and Scadden, D. T. (2000). Impaired CTL recognition of cells latently infected with Kaposi's sarcoma-associated herpes virus. *J. Immunol.* 165, 2077–2083.

Bubman, D., Guasparri, I., and Cesarman, E. (2007). Deregulation of c-Myc in primary effusion lymphoma by Kaposi's sarcoma herpesvirus latency-associated nuclear antigen. *Oncogene* 26, 4979–4986.

Cai, Q. L., Knight, J. S., Verma, S. C., Zald, P., and Robertson, E. S. (2006). EC5S ubiquitin complex is recruited by KSHV latent antigen LANA for degradation of the VHL and p53 tumor suppressors. *PLoS Pathog.* 2, e116. doi:10.1371/journal.ppat.0020116

Canning, M., Boutell, C., Parkinson, J., and Everett, R. D. (2004). A RING finger ubiquitin ligase is protected from autocatalyzed ubiquitination and degradation by binding to ubiquitin-specific protease USP7. *J. Biol. Chem.* 279, 38160–38168.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposis sarcoma. *Science* 266, 1865–1869.

Chang, Y., Moore, P. S., Talbot, S. J., Boshoff, C. H., Zarkowska, T., Godden-Kent, Paterson, H., Weiss, R. A., and Mittnacht, S. (1996). Cyclin encoded by KS herpesvirus. *Nature* 382, 410.

Chaudhary, P. M., Jasmin, A., Eby, M. T., and Hood, L. (1999). Modulation of the NF-(κ B pathway by virally encoded death effector domains-containing proteins. *Oncogene* 18, 5738–5746.

Chugh, P., Matta, H., Schamus, S., Zachariah, S., Kumar, A., Richardson, J. A., Smith, A. L., and Chaudhary, P. M. (2005). Constitutive NF-κB activation, normal Fas-induced apoptosis, and increased incidence of lymphoma in human herpes virus 8 K13 transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12885–12890.

Ciechanover, A. (2005). Proteolysis: from the lysosome to ubiquitin and the proteasome. *Nat. Rev. Mol. Cell Biol.* 6, 79–87.

Coscoy, L., and Ganem, D. (2000). Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8051–8056.

Coscoy, L., and Ganem, D. (2001). A viral protein that selectively downregulates ICAM-1 and B7–2 and modulates T cell costimulation. *J. Clin. Invest.* 107, 1599–1606.

Coscoy, L., Sanchez, D. J., and Ganem, D. (2001). A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. *J. Cell Biol.* 155, 1265–1273.

Deng, H., Liang, Y., and Sun, R. (2007). Regulation of KSHV lytic gene expression, *Curr. Top. Microbiol. Immunol.* 312, 157–183.

Diehl, J. A., Cheng, M., Roussel, M. F., and ana Sherr, C. J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev.* 12, 3499–3511.

Duncan, L. M., Piper, S., Dodd, R. B., Saville, M. K., Sanderson, C. M., Luzio, J. P., and Lehner, P. J. (2006). Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *EMBO J.* 25, 1635–1645.

Ellis, M., Chew, Y. P., Fallis, L., Freddersdorf, S., Boshoff, C., Weiss, R. A., Lu, X., and Mittnacht, S. (1999). Degradation of p27 (Kip) cdk inhibitor triggered by Kaposi's sarcoma virus cyclin-cdk6 complex. *EMBO J.* 18, 644–653.

Feng, H., Dong, X., Negaard, A., and Feng, P. (2008). Kaposi's sarcoma-associated herpesvirus K7 induces viral G protein-coupled receptor degradation and reduces its tumorigenicity. *PLoS Pathog.* 4, e1000157. doi:10.1371/journal.ppat.1000157

Feng, P., Scott, C. W., Cho, N. H., Nakamura, H., Chung, Y. H., Monteiro, M. J., and Jung, J. U. (2004). Kaposi's sarcoma-associated herpesvirus K7 protein targets a ubiquitin-like/ubiquitin-associated domain-containing protein to promote protein degradation. *Mol. Cell. Biol.* 24, 3938–3948.

Field, N., Low, W., Daniels, M., Howell, S., Daviet, L., Boshoff, C., and Collins, M. (2003). KSHV vFLIP binds to IKK-gamma to activate IKK. *J. Cell Sci.* 116, 3721–3728.

Friborg, J. J., Kong, W., Hottiger, M. O., and Nabel, G. J. (1999). p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* 402, 889–894.

Fujimuro, M., and Hayward, S. D. (2003). The latency-associated nuclear antigen of Kaposis sarcoma-associated herpesvirus manipulates the activity of glycogen synthase kinase-3beta. *J. Virol.* 77, 8019–8030

Fujimuro, M., Liu, J., Zhu, J., Yokosawa, H., and Hayward, S. D. (2005). Regulation of the interaction between glycogen synthase kinase 3 and the Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen. *J. Virol.* 79, 10429–10441.

Fujimuro, M., Wu, F. Y., Aprhys, C., Kajumbula, H., Young, D. B., Hayward, G. S., and Hayward, S. D. (2003). A novel viral mechanism for dysregulation of beta-catenin in Kaposis sarcoma-associated herpesvirus latency. *Nat. Med.* 9, 300–306.

Ganem, D. (2007). "Kaposi's sarcoma-associated herpesvirus," in *Fields' Virology*, 5th Edn, eds D. M. Knipe and P. M. Howley (Philadelphia, PA: Lippincott Williams and Wilkins), 2847–2888.

González, C. M., Wang, L., and Damania, B. (2009). Kaposi's sarcoma-associated herpesvirus encodes a viral deubiquitinase. *J. Virol.* 83, 10224–11033.

Gould, F., Harrison, S. M., Hewitt, E. W., and Whitehouse, A. (2009). Kaposi's sarcoma-associated herpesvirus RTA promotes degradation of the Hey1 repressor protein through the ubiquitin proteasome pathway. *J. Virol.* 83, 6727–6738.

Guasparri, I., Wu, H., and Cesarman, E. (2006). The KSHV oncoprotein vFLIP contains a TRAF-interacting motif and requires TRAF2 and TRAF3 for signalling, *EMBO Rep.* 7, 114–119.

Hayward, S. D., Liu, J., and Fujimuro, M. (2006). Notch and Wnt Signaling: Mimicry and manipulation by γ herpesviruses. *Sci. STKE* 335, re4.

Hicke, L. (1999). Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol.* 9, 107–112.

Hicke, L. (2001). Protein regulation by monoubiquitin. *Nat. Rev. Mol. Cell Biol.* 2, 195–201.

Holowaty, M. N., and Frappier, L. (2004). HAUSP/USP7 as an Epstein–Barr virus target. *Biochem. Soc. Trans.* 32, 731–732.

Inn, K. S., Lee, S. H., Rathbun, J. Y., Wong, L. Y., Toth, Z., Machida, K., Ou, J. H., and Jung, J. U. (2011). Inhibition of RIG-I-mediated signaling by Kaposi's sarcoma-associated herpesvirus-encoded deubiquitinase ORF64. *J. Virol.* 85, 10899–10904.

Ishido, S., Wang, C., Lee, B. S., Cohen, G. B., and Jung, J. U. (2000a). Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *J. Virol.* 74, 5300–5309.

Ishido, S., Choi, J. K., Lee, B. S., Wang, C., DeMaria, M., Johnson, R. P., Cohen, G. B., and Jung, J. U. (2000b). Inhibition of natural killer cell-mediated cytotoxicity by Kaposi's sarcoma-associated herpesvirus K5 protein. *Immunity* 13, 365–374.

Jang, K. L., Shackelford, J., Seo, S. Y., and Pagano, J. S. (2005). Up-regulation of beta-catenin by a viral oncogene correlates with inhibition of the seven in absentia homolog 1 in B lymphoma cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 18431–18436.

Järviluoma, A., Child, E. S., Sarek, G., Sirimongkolkasem, P., Peters, G., Ojala, P. M., and Mann, D. J. (2006). Phosphorylation of the cyclin-dependent kinase inhibitor p21Cip1 on serine 130 is essential for viral cyclin-mediated bypass of a p21Cip1-imposed G1 arrest. *Mol. Cell. Biol.* 26, 2430–2440.

Järviluoma, A., Koopal, S., Rasanen, S., Makela, T. P., and Ojala, P. M. (2004). KSHV viral cyclin binds to p27KIP1 in primary effusion lymphomas. *Blood* 104, 3349–3354.

Järviluoma, A., and Ojala, P. M. (2006). Cell signaling pathways engaged by KSHV. *Biochim. Biophys. Acta* 1766, 140–158.

Jenner, R. G., and Boshoff, C. (2002). The molecular pathology of Kaposis sarcoma-associated herpesvirus. *Biochim. Biophys. Acta* 1602, 1–22.

Keller, S. A., Schattner, E. J., and Cesarman, E. (2000). Inhibition of NF-kappaB induces apoptosis of KSHV-infected primary effusion lymphoma cells. *Blood* 96, 2537–2542.

Kwun, H. J., da Silva, S. R., Qin, H., Ferris, R. L., Tan, R., Chang, Y., and Moore, P. S. (2011). The central repeat domain 1 of Kaposi's sarcoma-associated herpesvirus (KSHV) latency associated-nuclear antigen 1 (LANA1) prevents cis MHC class I peptide presentation. *Virology* 412, 357–365.

Kwun, H. J., da Silva, S. R., Shah, I. M., Blake, N., Moore, P. S., and Chang, Y. (2007). Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mimics Epstein-Barr virus EBNA1 immune evasion through central repeat domain effects on protein processing. *J. Virol.* 81, 8225–8235.

Lan, K., Verma, S. C., Murakami, M., Bajaj, B., Kaul, R., and Robertson, E. S. (2007). Kaposi's sarcoma herpesvirus-encoded latency-associated nuclear antigen stabilizes intracellular activated Notch by targeting the Sel10 protein. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16287–16292.

Lee, H. R., Toth, Z., Shin, Y. C., Lee, J. S., Chang, H., Gu, W., Oh, T. K., Kim, M. H., and Jung, J. U. (2009). Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor 4 targets MDM2 to deregulate the p53 tumor suppressor pathway. *J. Virol.* 83, 6739–6747.

Lei, X., Bai, Z., Ye, F., Xie, J., Kim, C. G., Huang, Y., and Gao, S. J. (2010). Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. *Nat. Cell Biol.* 12, 193–199.

Levitskaya, J., Coram, M., Levitsky, V., Imreh, S., Steigerwald-Mullen, P. M., Klein, G., Kurilla, M. G., and Masucci, M. G. (1995). Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* 375, 685–688.

Li, Q., Means, R., Lang, S., and Jung, J. U. (2007). Downregulation of gamma interferon receptor 1 by Kaposi's sarcoma-associated herpesvirus K3 and K5. *J. Virol.* 81, 2117–2127.

Liang, Y., and Ganem, D. (2003). Lytic but not latent infection by Kaposi's sarcoma-associated herpesvirus requires host CSL protein, the mediator of Notch signaling. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8490–8495.

Lipkowitz, S., and Weissman, A. M. (2011). RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. *Nat. Rev. Cancer* 11, 629–643.

Liu, J., Martin, H., Shamay, M., Woodard, C., Tang, Q. Q., and Hayward, S. D. (2007). Kaposi's sarcoma-associated herpesvirus LANA protein downregulates nuclear glycogen synthase kinase 3 activity and consequently blocks differentiation. *J. Virol.* 81, 4722–4731.

Liu, L., Eby, M. T., Rathore, N., Sinha, S. K., Kumar, A., and Chaudhary, P. M. (2002). The human herpesvirus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the ikappa B kinase complex. *J. Biol. Chem.* 277, 13745–13751.

Mann, D. J., Child, E. S., Swanton, C., Laman, H., and Jones, N. (1999). Modulation of p27 (Kip1) levels by the cyclin encoded by Kaposi's sarcoma-associated herpesvirus. *EMBO J.* 18, 654–663.

Mansouri, M., Douglas, J., Rose, P. P., Gouveia, K., Thomas, G., Means, R. E., Moses, A. V., and Früh, K. (2006). Kaposi sarcoma herpesvirus K5 removes CD31/PECAM from endothelial cells. *Blood* 108, 1932–1940.

Mansouri, M., Viswanathan, K., Douglas, J. L., Hines, J., Gustin, J., Moses, A. V., and Früh, K. (2009). Molecular mechanism of BST2/tetherin downregulation by K5/MIR2 of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 83, 9672–9681.

Matsumoto, A., Onoyama, I., Sunabori, T., Kageyama, R., Okano, H., and Nakayama, K. I. (2011). Fbxw7-dependent degradation of Notch is required for control of "stemness" and neuronal-glial differentiation in neural stem cells. *J. Biol. Chem.* 286, 13754–13764.

Mittnacht, S., and Boshoff, C. (2000). Viral cyclins. *Rev. Med. Virol.* 10, 175–184.

Nakayama, K. I., and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. *Nat. Rev. Cancer* 6, 369–381.

Pardieu, C., Vigan, R., Wilson, S. J., Calvi, A., Zang, T., Bieniasz, P., Kellam, P., Towers, G. J., and Neil, S. J. (2010). The RING-CH ligase K5 antagonizes restriction of KSHV and HIV-1 particle release by mediating ubiquitin-dependent endosomal degradation of tetherin. *PLoS Pathog.* 6, e1000843. doi:10.1371/journal.ppat.1000843

Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.* 70, 503–533.

Radkov, S. A., Kellam, P., and Boshoff, C. (2000). The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene H-ras transforms primary rat cells. *Nat. Med.* 6, 1121–1127.

Rhodes, D. A., Boyle, L. H., Boname, J. M., Lehner, P. J., and Trowsdale, J. (2010). Ubiquitination of lysine-331 by Kaposi's sarcoma-associated herpesvirus protein K5 targets HFE for lysosomal degradation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 16240–16245.

Russo, J. J., Bohenzky, R. A., Chein, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc. Natl. Acad. Sci. U.S.A.* 93, 14862–14867.

Sanchez, D. J., Gumperz, J. E., and Ganem, D. (2005). Regulation of CD1d expression and function by a herpesvirus infection. *J. Clin. Invest.* 115, 1369–1378.

Shin, Y. C., Nakamura, H., Liang, X., Feng, P., Chang, H., Kowalik, T. F., and Jung, J. U. (2006). Inhibition of the ATM/p53 signal transduction pathway by Kaposi's sarcoma-associated herpesvirus interferon regulatory factor 1. *J. Virol.* 80, 2257–2266.

Sulea, T., Lindner, H. A., and Menard, R. (2006). Structural aspects of recently discovered viral deubiquitinating activities. *Biol. Chem.* 387, 853–862.

Swanton, C., Mann, D. J., Fleckenstein, B., Neipel, F., Peters, G., and Jones, N. (1997). Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. *Nature* 390, 184–187.

Thomas, M., Boname, J. M., Field, S., Nejentsev, S., Salio, M., Cerundolo, V., Wills, M., and Lehner, P. J. (2008). Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1656–1661.

Weissman, A. M., Shabek, N., and Ciechanover, A. (2011). The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. *Nat. Rev. Mol. Cell Biol.* 12, 605–620.

Yada, M., Hatakeyama, S., Kamura, T., Nishiyama, M., Tsunematsu, R., Imaki, H., Ishida, N., Okumura, F., Nakayama, K., and Nakayama, K. I. (2004). Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. *EMBO J.* 23, 2116–2125.

Yang, Z., Yan, Z., and Wood, C. (2008). Kaposi's sarcoma-associated herpesvirus transactivator RTA promotes degradation of the repressors to regulate viral lytic replication. *J. Virol.* 82, 3590–35603.

Yin, Y., Manoury, B., and Fahraeus, R. (2003). Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1. *Science* 301, 1371–1374.

Yu, Y., and Hayward, G. S. (2010). The ubiquitin E3 ligase RAUL negatively regulates type-I interferon through ubiquitination of IRF7 and IRF3. *Immunity* 33, 863–877.

Yu, Y., Wang, S. E., and Hayward, G. S. (2005). The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteosome-mediated degradation. *Immunity* 22, 59–70.

Zaldumbide, A., Ossevoort, M., Wiertz, E. J., and Hoeben, R. C. (2007). In cis inhibition of antigen processing by the latency-associated nuclear antigen I of Kaposi sarcoma herpes virus. *Mol. Immunol.* 44, 1352–1360.

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Tegument proteins of Kaposi's sarcoma-associated herpesvirus and related gamma-herpesviruses

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A herpesvirus virion is composed of a viral genomic DNA-containing capsid surrounded by a viral envelope with glycoprotein spikes on its surface. Located between the capsid and the outer viral envelope is the virion tegument layer. Though the majority of the virion proteins are located in the tegument, this layer is less studied and was thought to be an amorphous structure. Over the last decade, a number of studies have indicated the presence of organized tegument structures across the spectrum of herpesviruses, implicating tegument components in critical steps governing the viral life cycle. In the case of Kaposi's sarcoma-associated herpesvirus (KSHV), the etiological agent of Kaposi's sarcoma, several functions exerted by tegument proteins at different stages of the viral life cycle, inclusive of primary *de novo* infection and virion assembly, have been identified over the last several years. In this review, KSHV tegument components are cataloged and the occurrence of organized tegument structures in KSHV, built through interactions amongst the different virion proteins, is discussed in depth. The significant functional roles of the KSHV tegument proteins at different stages of the viral life cycle are elaborated under separate headings. Definitive functional roles exerted by tegument proteins of related gamma-herpesviruses are also discussed. Since tegument proteins play key roles during viral assembly, viral entry, and represent an important interface for virus–host interactions, further research in this area should provide detailed insights into the functional capacity of the KSHV tegument, resulting in a better understanding of the viral life cycle.

Keywords: Kaposi's sarcoma-associated herpesvirus, human herpesvirus type 8, tegument, virion, protein interaction, virion assembly

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiological agent of Kaposi's sarcoma (KS). Based on phylogenetic analysis, KSHV is placed in the *human herpesviridae* family and *gamma herpesvirinae* sub-family along with other closely related gamma-herpesviruses such as Epstein–Barr virus (EBV; Chang et al., 1994; Moore et al., 1996; Russo et al., 1996). KSHV being the last addition to the human herpesvirus group is thus also designated as the human herpesvirus-8 (HHV-8; Moore et al., 1996; Russo et al., 1996). KSHV was first identified in 1994 from a KS lesion (Chang et al., 1994) and thereafter has been found to be unequivocally associated with KS (Chang et al., 1994; Dupin et al., 1995; Moore and Chang, 1995; Schalling et al., 1995; Chuck et al., 1996). Prior to the AIDS epidemic, KS was a rare disease occurring predominantly in elderly men across the Mediterranean and Middle East regions (Dupin et al., 1995). KS is now recognized as a leading cause of death in AIDS patients (Moore and Chang, 1995; Antman and Chang, 2000; Ganem, 2010). The AIDS-associated KS (AIDS-KS) manifests frequently on the skin with high propensity to spread to multiple organs such as lungs and the gastrointestinal tract (Dezube, 1996; Ganem, 2010). AIDS-KS is the most common oral malignancy in HIV-infected individuals contributing to about 70–90% of all AIDS-associated oral tumors (Antman and Chang, 2000). In addition to KS, KSHV is also implicated in two other lymphoproliferative disorders, namely the primary effusion lymphoma (PEL; Cesarman et al., 1995a) and the plasma cell variant of multicentric Castleman's disease (MCD; Soulier et al., 1995).

Similar to other herpesviruses, KSHV exhibits two alternative phases in its life cycle, the lytic and latent. Primary infection of a host cell begins with the entry of KSHV viral particles into the cell which get transported to the nucleus (Akula et al., 2001; Wang et al., 2001; Chandran, 2010). The viral genomic DNA is released into the nucleus and establishes viral latency by default (Cesarman et al., 1995b; Ballestas et al., 1999). In latently infected cells, there is expression of only a limited number of viral (latent) genes and no infectious virions are produced (Zhong et al., 1996; Sarid et al., 1998). The latent viral genome undergoes periodic lytic reactivation followed by a temporally regulated cascade of viral gene expression (Renne et al., 1996; Miller et al., 1997; Sun et al., 1999). The newly expressed viral proteins assemble to form infectious mature virions which egress out of the cell and initiate primary infection of uninfected cells (reviewed in Ganem, 2007).

Unlike other herpesviruses where the latent life cycle is usually responsible for the disease potential, in KSHV, the lytic cycle is also responsible, contributing to tumorigenesis. The most compelling evidence for this notion is the observation that treatment of KS patients or AIDS patients at risk for KS with anti-herpesviral drugs, such as Foscarnet and ganciclovir that block lytic but not

latent KSHV replication, resulted in regression of KS lesions with a decrease in the incidence of KS development (Morfeldt and Torssander, 1994; Martin et al., 1999). Furthermore, the productive replication cycle directly contributes to viral tumorigenesis by spreading viruses to target cells and providing paracrine regulation for KS development (Cesarman et al., 2000). An early study by Grundhoff and Ganem (2004) suggests a role of lytic replication in sustaining the population of latently infected cells that otherwise would be quickly lost by segregation of latent viral episomes as spindle cells divide. Thus, KSHV lytic replication and constant primary infection to fresh cells are crucial for viral tumorigenicity. Given the importance of the KSHV lytic cycle in both viral propagation and tumorigenesis, a thorough understanding of viral lytic replication processes including virion assembly and egress becomes vital.

KSHV VIRION STRUCTURE AND PROTEINS

A typical herpesviral particle (or virion) consists of the following morphologically distinct components: (i) a core which contains a linear double stranded viral DNA enclosed within an icosahedral capsid, (ii) an outer envelope with viral glycoproteins appearing as spikes on the surface, and (iii) an electron-dense material defined as the tegument, located between the capsid and envelope (Pellet and Roizman, 2007). During the lytic cycle, almost the entire set of KSHV viral genes are expressed. Approximately one-third of these viral genes encode virion proteins that assemble into infectious virions. These virion proteins are thought to contain the functional information required for both *de novo* primary infection and for the KSHV virion assembly and egress. Employing proteomic approaches, two independent studies identified nearly thirty KSHV virion proteins from purified KSHV virions (Bechtel et al., 2005; Zhu et al., 2005).

Among these virion proteins, five are characterized as capsid proteins (Nealon et al., 2001). Specific interactions amongst these capsid proteins mediate capsid assembly processes (Sathish and Yuan, 2010). Seven virion proteins are specified as viral glycoproteins, mediating entry of KSHV into host cells through interactions with the host cell surface receptors (Baghian et al., 2000; Akula et al., 2001; Wang et al., 2001; Naranatt et al., 2002; Koyano et al., 2003; Chandran, 2010). Although the viral capsid and the envelope are well-characterized, the KSHV tegument remains largely undefined. The first step toward defining the KSHV tegument was an identification of its component proteins. The majority of earlier identified KSHV virion proteins were characterized as tegument components, on the basis of specific biochemical criteria. These criteria included (i) resistance to trypsin digestion in the absence of detergent and (ii) susceptibility to trypsin digestion following treatment with outer viral envelope dissolving detergents (Bechtel et al., 2005; Zhu et al., 2005). Based on these criteria, proteins encoded by ORFs 21, 33, 45, 64, and 50 (replication transcription activator/Rta) are identified as components of the KSHV tegument (Bechtel et al., 2005; Zhu et al., 2005). In addition, proteins encoded by ORFs 6, 7, 11, 52, 63, and 75, which belong to neither the capsid nor the viral envelope, are also considered putative tegument proteins (Bechtel et al., 2005; Zhu et al., 2005). Among these, (i) ORFs 11, 52, and 75 are identified as tegument components in closely related gamma-herpesviruses such as murine herpesvirus-68 (MHV-68) and murid herpesvirus 4 (MHV-4; Bortz et al., 2003, 2007; Gaspar et al., 2008). On the basis of considerable sequence similarity, the KSHV homologs of these ORFs are also considered tegument components (Bechtel et al., 2005; Zhu et al., 2005); (ii) the analog of ORF63 in HSV-1 is a well-characterized tegument protein and is predicted to encode for a tegument protein in KSHV as well (Russo et al., 1996; Zhu et al., 2005); (iii) ORFs 6 and 7 are yet to be characterized (May et al., 2005a,b). ORF19 and ORF67 are also predicted to encode for tegument proteins, based on their sequence homology to tegument proteins of other well-characterized herpesviruses and await further characterization (Russo et al., 1996). Taken together, proteins encoded by ORFs 11, 21, 33, 45, 50, 52, 63, 64, and 75 are currently considered as KSHV tegument proteins with several more to be characterized.

Though the tegument was earlier considered an amorphous layer of proteins, recent studies indicate the occurrence of ordered tegument structures built through specific protein–protein interactions in HSV-1 (Zhou et al., 1999; Vittone et al., 2005), human cytomegalovirus (HCMV; Chen et al., 1999; Phillips and Bresnahan, 2011), and MHV-68 (Dai et al., 2008). **Figure 1** illustrates a 3D structure of MHV-68 virion reconstructed by cryoET, which demonstrates a two-layered organization of the tegument. The inner tegument layer (green in **Figure 1B**) remains tethered to the capsid by directly interacting with it. The outer tegument (violet in **Figure 1B**) occupies the space between the inner layer and the envelope, and is much more loosely organized, as it conforms to the irregular shape of the viral envelope.

Further details regarding the tegument architecture were obtained through a systematic investigation of the KSHV tegument protein interactions (**Figure 2**). A total of 37 binary protein interactions were detected between the KSHV tegument proteins and the different virion proteins (inner capsid proteins/tegument proteins/outer envelope glycoproteins; Rozen et al., 2008). This observation pointed to the occurrence of organized tegument structures in KSHV also. A significant proportion of the above interactions are mediated by the tegument proteins encoded by ORFs 21, 33, 45, 52, 63, 64, and 75 (Rozen et al., 2008).

FUNCTIONAL ROLES OF KSHV TEGUMENT PROTEINS IN VIRAL ASSEMBLY PROCESSES

Herpesviral assembly is a multi-stage process consisting of the following events: (i) incorporation of the newly replicated DNA into the pre-formed capsid within the nucleus generating the viral nucleocapsid; (ii) exit of the nucleocapsid from the nucleus into the cytoplasm through a sequence of coordinated steps such as acquisition of a primary envelope (from the nuclear membrane) by the nucleocapsid and its subsequent loss; (iii) acquisition of tegument proteins by the nucleocapsid in the cytoplasm and transportation of tegumented capsids to sites of final envelopment, the Golgi-derived vesicles; (iv) final envelopment including acquisition of other tegument and envelope glycoproteins by budding into the vesicles; and (v) egress of mature virions from the infected cell following fusion of the vesicle membrane with the cell plasma membrane (reviewed in Mettenleiter, 2002; Mettenleiter et al., 2009). Although the above framework for herpesvirus assembly and egress may be common to all herpesviruses, the detailed steps and the mechanisms underlying these processes have been less studied in gamma-herpesviruses including KSHV.

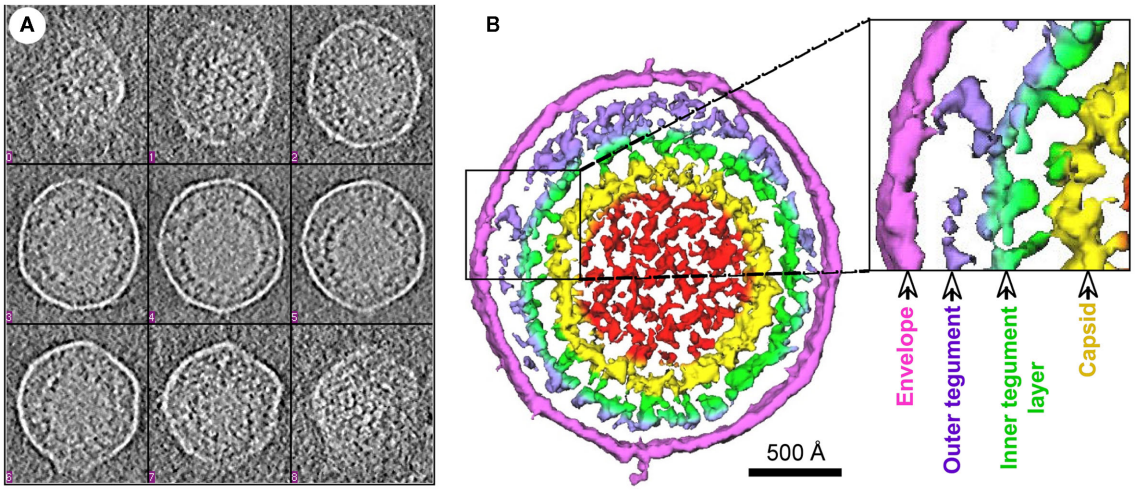

FIGURE 1 | 3D structure of an MHV-68 virion reconstructed by cryo-electron tomography. (A) Tomogram slices from a representative HMV-68 virion. **(B)** Shaded surface representation of central slabs of the virion segmented from the tomogram shown in **(A)**. Color coding: red, internal density in capsid; yellow, capsid shell; green, inner layer tegument; violet, outer layer tegument; magenta, viral envelope. Two layers of tegument are shown: an inner tegument layer tethered to the underlying capsid and an outer tegument layer conforming to the overlying envelope. This figure is adapted from Dai et al. (2008), courtesy of Z. Hong Zhou at UCLA, with permission from Elsevier Press, Oxford, UK.

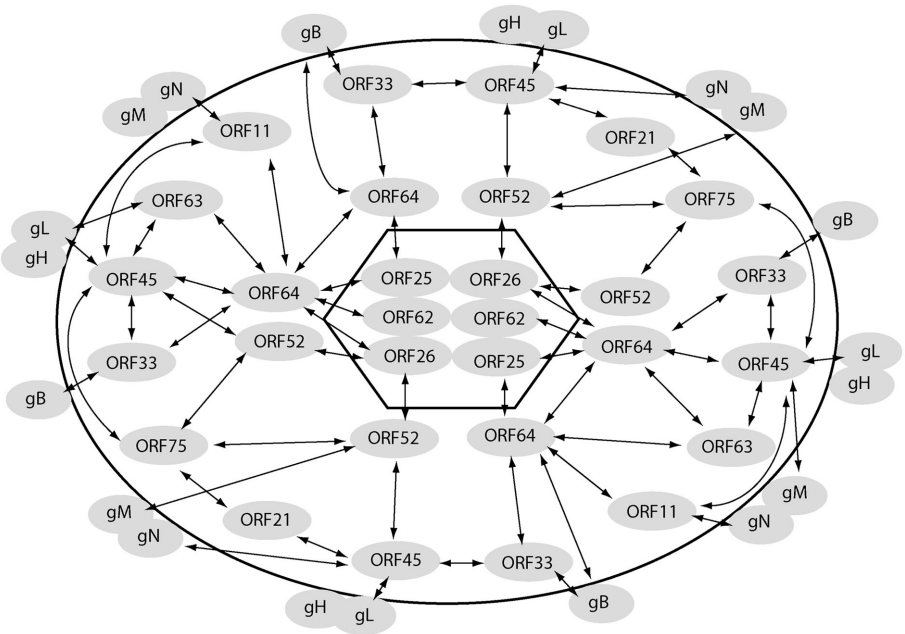

FIGURE 2 | Interactome map of KSHV virion proteins. Interactions amongst the virion proteins, capsid (inside the hexagon), tegument (outside the hexagon but within the oval), and envelope (outside oval) are indicated with arrows. The interaction pattern of the ORF64–ORF52–ORF33–ORF45 network with multiple virion proteins is evident from the figure.

Dynamic and intricate interactions mediated by the tegument proteins amongst themselves as well as with the inner capsid and the outer envelope proteins impacts both tegumentation and envelopment in related herpesviruses like HSV and HCMV thus influencing viral assembly processes (Mettenleiter, 2002; Mettenleiter et al., 2009). Given the homology amongst tegument proteins of herpesviruses, it is reasonable to assume that tegument proteins in KSHV could also influence virion assembly pathways. As a first step toward bridging the existing gap, interactions of the KSHV tegument proteins with the different virion proteins (capsid/tegument/envelope) have been systematically investigated (Rozen et al., 2008). This has led to a revelation of a virion-wide

protein interaction network (**Figure 2**). This network is expected to serve as a road map to gain insights into the less studied but critical KSHV tegumentation and viral assembly processes. Here we attempt to summarize the knowledge accumulated over the recent years regarding roles of KSHV tegument proteins in virion assembly and egress.

ORF64

This ORF encodes for a 290-kDa tegument protein and is the largest protein encoded by the KSHV genome. Homologues of ORF64 exist across the three herpesvirus sub-families (Zhu et al., 2005). The earlier established KSHV protein interaction map indicated a diverse interaction potential of ORF64. This protein was found to interact with three viral capsid proteins, namely ORFs 25 (major capsid protein/MCP), 26 (TRI-2), and 62 (TRI-1; Rozen et al., 2008), suggesting an attachment of ORF64 to the icosahedral capsid structure (**Figure 2**). This is likely to be true as the HSV-1 analog, VP1/2 encoded by UL36, is also involved in a tight interaction with the viral capsid (McNabb and Courtney, 1992; Newcomb and Brown, 2010). A three-dimensional image reconstructed from a cryo-electron microscopic study of HSV-1 particles revealed interactions of the ORF64 homolog of HSV-1 (VP1/2) with MCP, TRI-2, and TRI-1 (Zhou et al., 1999). There is no doubt that the association of ORF64 with three capsid proteins is conserved across the herpesvirus family.

KSHV ORF64 was also found to interact with several tegument proteins including ORFs 11, 21, 33, 45, 63, 75 and with itself (Rozen et al., 2008). The interactions of ORF64 with a plethora of tegument proteins has led to a hypothesis that ORF64 functions as a major hub or a scaffold protein, recruiting other tegument proteins during KSHV virion tegumentation (Rozen et al., 2008). ORF64 also interacted with several envelope glycoproteins (Rozen et al., 2008). This finding justifies a rather peculiar behavior of ORF64 wherein its degradation was noticed subsequent to treatment of intact virion particles with trypsin, despite the absence of envelope dissolving detergent, suggesting its association with the viral envelope as well (Zhu et al., 2005). The ability of ORF64 to interact with KSHV glycoproteins is consistent with a role of this protein in positioning the DNA-filled tegumented capsid at the trans-Golgi apparatus through association with envelope glycoproteins, promoting secondary envelopment processes. Delineating the interaction dynamics of KSHV ORF64 with the different virion components is needed to identify the step/s in which these interactions occur in the viral assembly pathways.

ORF45

This ORF encodes a protein of 407 amino acids (Zhu and Yuan, 2003; Zhu et al., 2005), homologues of which are present only in gamma-herpesviruses. An important functional role of ORF45 in KSHV assembly pathways was suggested in a study employing an ORF45-null recombinant KSHV (Zhu et al., 2006). A noticeably lowered yield of progeny virions was obtained from ORF45-null recombinant mutant virus reconstituted cells as compared to that obtained from cells reconstituted with the wild-type virus though viral gene expression and viral DNA replication remained unaffected in the absence of ORF45 (Zhu et al., 2006). A specific role of ORF45 in KSHV virion assembly was revealed by the finding that ORF45 interacts with the microtubule (MT) associated kinesin-2 motor protein, KIF3A, and mediates the docking of entire viral capsid–tegument complex on to the cargo-binding domain of KIF3A (Sathish et al., 2009). KIF3A is involved in transportation of cargo along the MTs from the nucleus toward the cell periphery (Yamazaki et al., 1995; Hirokawa, 2000). Inhibition of KIF3A–ORF45 interaction with a headless dominant negative (DN) mutant of KIF3A or by an shRNA-mediated silencing of endogenous KIF3A expression decreased KSHV particle release noticeably. These approaches, did not impact HSV-1 virion release, demonstrating the specificity of KIF3A in transport of KSHV particles. The above observations led to a proposed model (**Figure 3**) on the role of ORF45 in KSHV assembly and egress, wherein (i) subsequent to nuclear egress, KSHV capsids acquire tegument proteins such as ORFs 64, 63, and 45 in the cytoplasm, (ii) ORF45 recruits KIF3A and mediates loading of the viral particles onto KIF3A, and (iii) the viral particles are transported along the MTs from the peri-nuclear region to the cell periphery or trans-Golgi network (TGN) membrane for final envelopment and egress (Sathish et al., 2009). The KSHV envelope glycoproteins were not transported along with the KSHV tegument–capsid complexes, indicating that as newly assembled viral tegumented capsids are transported by kinesin-2 to sites of further envelopment, viral glycoproteins travel separately.

ORF52

This ORF encodes a small protein of 21 kDa, conserved across the gamma-herpesviruses with homologs in both the closely related MHV-68 and EBV (Bortz et al., 2003; Zhu et al., 2005). ORF52 has been characterized as a tegument protein in MHV-68 on the basis of its presence in the detergent-resistant pellet fraction subsequent to detergent treatment and centrifugation of virions and its absence in the capsid pellet fraction subsequent to treatment of virions with non-ionic detergent by high salt extraction and centrifugation (Bortz et al., 2007). MHV-68 ORF52 shares a 28% identity with the KSHV homolog, thus serving as a useful model to assess the functional roles of the less studied KSHV ORF52 (Zhu et al., 2005; Bortz et al., 2007).

An ORF52-null recombinant MHV-68 exhibited no apparent defects in viral DNA replication, viral lytic gene expression, and capsid assembly processes. A normal nuclear accumulation of viral capsid structures was noticed by TEM-based approaches (Bortz et al., 2007). Though partially tegumented nucleocapsids could be seen juxtaposed to the trans-Golgi vesicular membranous compartments, enveloped virions were not noticed within these membranous vesicles. Analysis of these partially tegumented capsids revealed the presence of capsid proteins and tegument proteins such as ORF64 and ORF67 but the absence of other tegument proteins such as ORF45 and ORF42 (Bortz et al., 2007). Structural studies performed on MHV-68 ORF52 have indicated the existence of an N-terminal α-helical domain which interacts with the other tegument protein partners (Benach et al., 2007). Failure of an ORF52 mutant (lacking the N-terminal α-helix) in compensating defective virion production seen with a MHV-68 ORF52-null mutant virus clearly demonstrated the necessity of the interactions between ORF52 and other tegument proteins (Benach et al., 2007). All the above observations suggested a vital role of ORF52

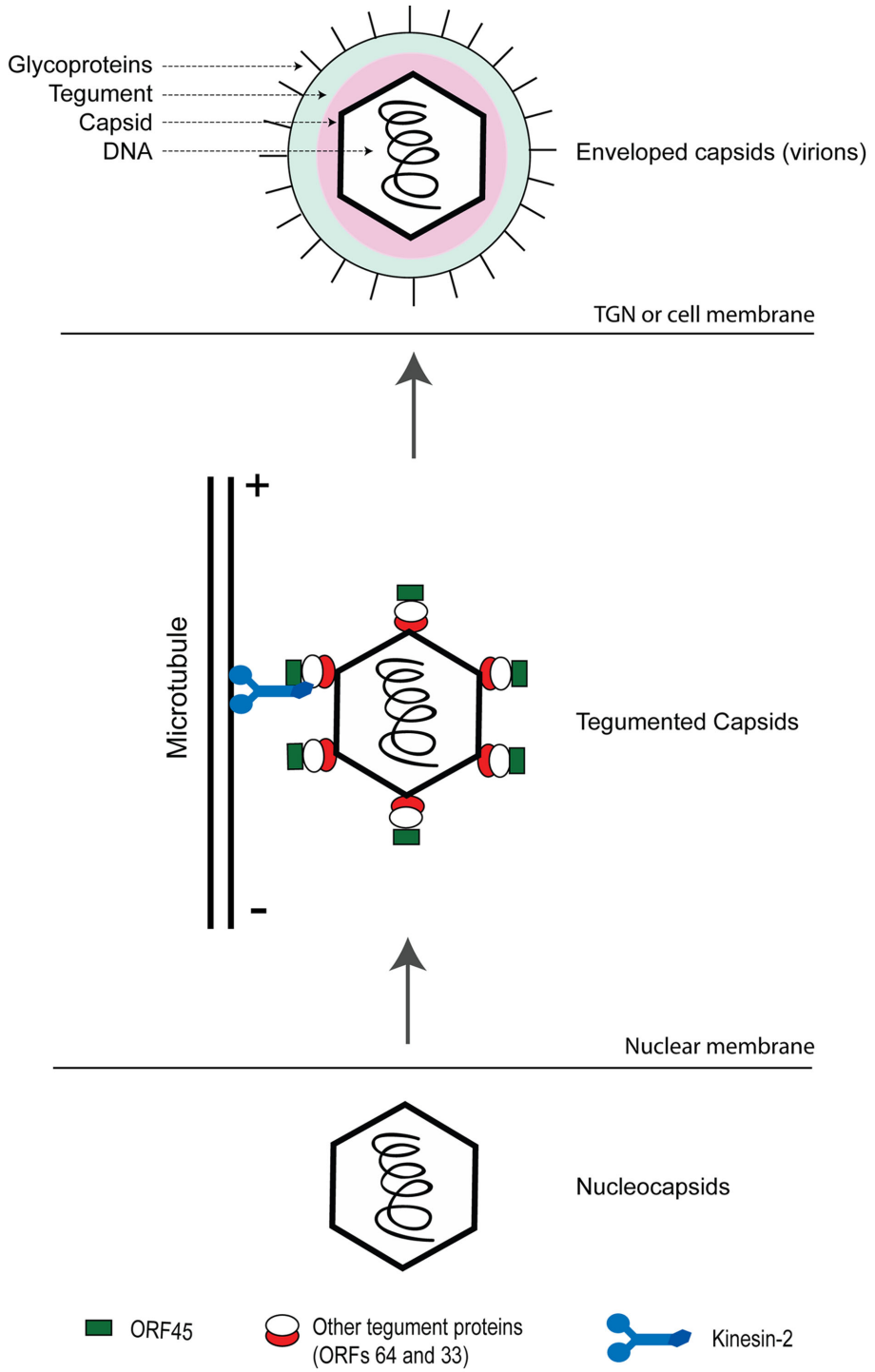

FIGURE 3 | Role of ORF45 in mediating transportation of assembled KSHV capsid–tegument complexes on microtubules toward sites of maturation/envelopment and egress. Newly synthesized nucleocapsids exit out of the nuclear compartment wherein they acquire the tegument proteins including ORFs 33, 45, and 64. ORF45 on the viral particles binds to KIF3A, docking the entire viral capsid–tegument complexes onto it. The complexes are then transported along microtubules either to trans-Golgi network (TGN) or the cell membrane for further envelopment and viral egress. This figure is adapted from Sathish et al. (2009).

in MHV-68 tegumentation during viral assembly. On the basis of considerable homology in ORF52 sequences between MHV-68 and KSHV, it is tempting to speculate that similar structural and functional characteristics could be exhibited by KSHV ORF52 also.

ORF33

This ORF encodes a tegument protein of 334 amino acids and homologs of this protein are present across all three herpesvirus sub-families (Jenner and Boshoff, 2002; Zhu et al., 2005; Guo et al., 2009). Though functional studies on ORF33 have not been undertaken in KSHV, such studies were performed in MHV-68. An ORF33-null recombinant MHV-68 has provided useful information on the functional role of this protein in virion assembly. This mutant virus did not exhibit any defects in viral DNA replication, viral gene expression and viral capsid assembly. However, a TEM-based analysis of thin sections obtained from ORF33-null MHV-68 virus carrying cells showed a defective release of nucleocapsids from the nucleus into the cytoplasm. Enveloped virions were not seen both within the cytoplasmic vesicles and in the extracellular space (Guo et al., 2009). These observations are consistent with the functional roles of ORF33 homologues in both HSV-1 (UL16; Meckes and Wills, 2007) and HCMV (UL94; Liu et al., 2009) in directing partially tegumented particles to the trans-Golgi vesicles. Analysis of the intracytoplasmic partially tegumented particles of ORF33-null MHV-68 revealed the presence of capsid proteins and some tegument proteins including ORF64 and ORF52 though ORF45 was absent (Guo et al., 2009). These observations indicate a pivotal role of ORF33 in MHV-68 tegumentation and viral assembly. It would be worthwhile to investigate if KSHV ORF33 also exerts a similar function.

PERSPECTIVES ON THE ROLES OF KSHV TEGUMENT PROTEINS IN VIRAL ASSEMBLY PATHWAYS

The above studies indicate the critical roles exerted by tegument proteins of KSHV and other related gamma-herpesvirus in viral assembly processes. The earlier established KSHV virion protein interaction network indicates the functional potential of an ORF64–ORF52–ORF33–ORF45 network (**Figure 2**) in both tegumentation and secondary envelopment processes (Rozen et al., 2008) and hence would need to be further investigated. Since virion assembly and egress are dynamic processes, interactions among virion proteins could be transiently exerted at different stages in the viral life cycle (Meckes and Wills, 2007). These dynamic interactions could either be environment-dependent (such as pH regulated) or could occur as the viral particles travel through the assembly and egress pathway. Thus the focus of the virion assembly studies would also need to shift toward identifying the step(s) in the KSHV viral assembly pathway at which specific protein interactions come into play. Though much remains to be discovered, certain approaches as above could result in a better understanding of the functional roles of the tegument proteins in KSHV assembly.

FUNCTIONAL ROLES OF KSHV TEGUMENT PROTEINS IN VIRAL ENTRY PATHWAYS

Kaposi's sarcoma-associated herpesvirus infects a broad range of cell types *in vitro* resulting in viral latency. Establishment of KSHV latency requires the delivery of the viral genome into the host cell nucleus which is preceded by a well connected sequence of events. The first step involves the binding of KSHV virions, specifically the viral envelope glycoproteins (e.g., gB and gpK8.1A), to the ubiquitously expressed heparan sulfate (HS) cell surface receptor (Akula et al., 2001; Birkmann et al., 2001; Wang et al., 2001; Hahn et al., 2009), followed by temporal interactions with integrin molecules and other transmembrane proteins such as xCT (CD98) (Akula et al., 2002; Kaleeba and Berger, 2006; Veettil et al., 2008). Subsequent to the initial binding, the viral particles enter the infected cells within endocytic vesicles, releasing tegumented capsids into the cytoplasm by fusion of the viral envelope with the endocytic vesicles (Akula et al., 2003; Raghu et al., 2009; reviewed in Chandran, 2010).

Transportation of the tegumented capsids to the nucleus involves a complex interplay between cellular and viral components. Confocal microscopic images of KSHV infected cultured cells revealed a close association of incoming KSHV viral particles with MTs. Treatment of these cells with MT depolymerizing agents disrupted the association resulting in an inhibition in nuclear delivery of the viral DNA (Naranatt et al., 2005; Raghu et al., 2007, 2009). Dyneins are minus-end directed motors that transport cargo along the MTs from the cell periphery to the nucleus (Mandelkow and Mandelkow, 1995; King, 2000). Interference with dynein activity through over expression of dynamitin (dynactin complex disruptor) or through treatment of cells with sodium orthovanadate (an inhibitor of dynein activity) resulted in reduced KSHV genomic DNA in the nucleus of infected cells (Naranatt et al., 2005). These observations indicated significant roles of MTs and dyneins in the transportation of incoming KSHV particles toward the nucleus. The above functional roles exerted by MTs and dyneins are in lines with similar roles exerted by them in related herpesviruses such as HSV-1 (Douglas et al., 2004; Döhner et al., 2006; Diefenbach et al., 2008) and HCMV (Ogawa-Goto et al., 2003).

In HSV-1, inner tegument proteins, VP16, VP1-3, UL37, recruit dynein and dynactin mediating transportation of viral particles along the MTs (Luxton et al., 2005; Wolfstein et al., 2006). Both untegumented naked capsids and completely tegumented capsids of HSV failed to recruit the dynein motors, indicating a role of inner tegument proteins in recruiting cellular cytoskeletal elements (Wolfstein et al., 2006). This gives rise to an interesting premise that tegument proteins of KSHV could also exert a similar role in dynein-mediated transport of viral particles on MTs. To date, KSHV virion protein/s mediating transportation of incoming KSHV particles to the nucleus remains unidentified thus providing a very fertile ground for research.

The tegument protein ORF75 of MHV-4 (a murine gamma-herpesvirus) has been hypothesized to exert a role in incoming viral particle transportation based on two experimental observations. The first was a significant reduction in the peri-nuclear capsid staining with majority of the particles scattered in the cytoplasm, during examination of ORF75$^-$ virus infected BHK-21 cells by immunofluorescence. The second observation was the defectiveness of ORF75$^-$ viral particles in establishing an infection subsequent to intranasal infection of mice. This was illustrated by a steady increase in the viral genome copy numbers in the lungs of wild-type virus infected mice, which was not noticed in the ORF75$^-$ virus infected animals. These observations suggest a role of ORF75 in associating the incoming MHV-4 viral particles to the host motor proteins (Gaspar et al., 2008). On similar lines, the ORF75 homolog in EBV, BNRF1, mediates the transportation of

incoming viral particles from the endosomal compartments to the nucleus (Feederle et al., 2006).

PERSPECTIVES ON THE ROLES OF KSHV TEGUMENT PROTEINS IN VIRAL ENTRY PATHWAYS

As the outermost components of incoming KSHV particles, the tegument proteins have the potential to mediate viral particle transportation to the nucleus through interactions with the cellular cytoskeleton. In the absence of comprehensive studies on this aspect, there are certain issues which would need to be resolved. One is the identification of KSHV protein(s) participating in the transport of incoming viral particles through interactions with the cellular cytoskeleton elements. KSHV inner tegument components may be involved in interaction with dyneins and MTs, as the outer tegument components are released into the cytosol subsequent to entry.

Yet another interesting aspect would be identifying if any of the KSHV tegument proteins play a role in the induction of host cell signaling pathway proteins that regulate cellular cytoskeleton dynamics. In fact, KSHV cellular entry has been shown to induce Rho GTPases leading to acetylation of MTs, promoting MT reorganization and transportation of incoming viral particles to the nucleus (Sharma-Walia et al., 2004; Naranatt et al., 2005; Veettil et al., 2006; Raghu et al., 2007; reviewed in Chandran, 2010).

REGULATORY ROLES EXERTED BY KSHV TEGUMENT PROTEINS

ROLES OF ORF45 IN EVASION OF IFN-MEDIATED ANTI-VIRAL RESPONSE

Primary KSHV infection gets initiated with the binding of viral glycoproteins to host cell surface receptors. This event elicits type I IFNs that constitute the first line of the innate anti-viral defense mechanism of host cell against the invading virus (reviewed in Sathish and Yuan, 2011). Induction of type I IFNs is influenced by a group of proteins, belonging to the family of IFN regulatory factors (IRFs). Among the nine different IRFs identified to date, IRFs 3 and 7 contribute significantly to the type I IFN induction (Honda et al., 2005a,b; Honda and Taniguchi, 2006; Hiscott, 2007). Subsequent to a viral infection, the toll-like receptors recognize viruses and through specific signaling pathways bring about the activation of IRFs. Activation of IRFs involves their phosphorylation and subsequent nuclear translocation, wherein they bind to the promoter regions of the type I IFN genes mediating their induction (Honda et al., 2005b). Recent observations have documented a greater potential of IRF-7 (as compared to IRF-3), in the induction of type I IFNs (Honda et al., 2005a,b). Type I IFNs thus induced, mediate the transcription of a diverse group of anti-viral effector proteins, the IFN stimulated genes (ISGs), which inhibit multiple stages of the viral life cycle (Goodbourn et al., 2000; Brierley and Fish, 2002; Sadler and Williams, 2008).

As type I IFNs are elicited immediately following a viral infection (Perry and Compton, 2006; Zhu et al., 2010), it is essential for incoming KSHV particles to effectively antagonize them for a successful establishment of a primary infection (reviewed in Sathish and Yuan, 2011). Noticeably reduced transcription of both type I IFN genes and their cognate receptors following primary KSHV infection of cultured cells has been documented (Perry and Compton, 2006). A similar observation witnessed subsequent to primary infection of cells with UV-irradiated KSHV virions, suggested an involvement of virion component proteins in disarming the type I IFN signaling initially triggered by viral glycoprotein attachment (Naranatt et al., 2004; Zhu et al., 2010).

Among the virion proteins, tegument proteins are the outermost components of incoming herpesvirus particles, exposed to the cellular milieu. Therefore the tegument proteins do have a potential to curtail the type I IFN responses. ORF45 was characterized as a tegument component mediating the type I IFN evasion in KSHV. Being a KSHV tegument protein, ORF45 is delivered to the cell cytosol at very early stages of KSHV infection (Zhu et al., 2010). ORF45 was shown to inhibit the activation of IRF-7, the master regulator of type I IFN responses, through inhibition of both its phosphorylation and nuclear translocation (Zhu et al., 2002; Sathish et al., 2011). The role of ORF45 in antagonizing type I interferon responses during primary infection was demonstrated using an ORF45-null recombinant virus. While cells infected with wild-type KSHV failed to induce anti-viral responses judged by their permissiveness to superinfection with vesicular stomatitis virus (VSV), infection of cells with an ORF45-null recombinant KSHV (BAC-stop45) resulted in increased transcription of type I IFN and downstream anti-viral effector genes that resisted VSV superinfection. In addition, a lentiviral mediated ectopic expression of ORF45 in human fibroblast cells diminished the host cell elicited type I IFN anti-viral responses (Zhu et al., 2010). Taken together, the inhibitory effect exerted by ORF45 on IRF-7 contributes to KSHV survival subsequent to a primary infection.

ROLE OF ORF63 IN INHIBITION OF INFLAMMATION REACTION

ORF63 has been identified as a KSHV virion tegument component (Russo et al., 1996; Zhu et al., 2005). A recent study revealed a vital role of this tegument protein in inhibiting the generation of the inflammasome. ORF63 shares amino acid sequence homology with NLRP1, specifically its nucleotide binding domain (NBD) and was found to interact with it (Gregory et al., 2011). NLRP1 is a protein of the nucleotide binding and oligomerization, leucine-rich repeat family (NLRs), which along with TLRs are placed under the broader group of pattern recognition receptors (Meylan et al., 2006). Activation pathways involving these NLRs culminate in the generation of inflammasomes, which are large multimeric protein complexes constituted by specific NLRs and procaspase-1 among others (Martinon et al., 2002). The inflammasomes induce the caspase-1 mediated proteolytic processing of proinflammatory cytokines such as IL-1β and IL-18 (Stutz et al., 2009). Induction of these cytokines triggers pyroptosis, an inflammatory process involving caspase-1 mediated death of infected cells.

The critical role of NLRP1 in combating KSHV was evidenced by the fact that knockdown of NLRP1 expression in BCBL-1 cells resulted in increased KSHV genomic DNA replication and infectious virion titers subsequent to lytic reactivation. Conversely, an siRNA-mediated knockdown of ORF63 resulted in reduced KSHV lytic gene expression and virion production along with significantly increased expression levels of IL-1β (Gregory et al., 2011). These observations suggested a role of ORF63 in mediating a potent reactivation of KSHV through inhibition of NLRP1 activity. ORF63 was found to inhibit both the oligomerization

and the association of NLRP1 with active components of the inflammasome (Gregory et al., 2011).

ORF64 ENCODES FOR A POTENT VIRAL DEUBIQUITINASE

A KSHV large tegument protein encoded by ORF64 functions as a potent viral deubiquitinase (DUB) (González et al., 2009), an enzyme that removes ubiquitin (Ub) from either the cellular E3 ligase or the target protein thus abrogating the Ub-mediated regulation (Amerik and Hochstrasser, 2004). ORF64 homologues in related herpesviruses such as HSV-1 (Kattenhorn et al., 2005), HCMV (Wang et al., 2006), EBV (Sompallae et al., 2008), and MHV-68 (Gredmark et al., 2007) exhibit DUB activity. The DUB activity of KSHV ORF64 was localized to its first 205 amino acids in the N-terminal domain. Among the K48- and K63-linked Ub chains commonly targeted by DUBs, KSHV ORF64 has no distinct specificity to either of them and was capable of targeting both (González et al., 2009). This behavior is in sharp contrast to the DUBs of other herpesviruses, which had exclusive specificity for the K48-linked Ub chains alone (Amerik and Hochstrasser, 2004; Kattenhorn et al., 2005; Wang et al., 2006).

An siRNA-mediated knockdown of ORF64 resulted in decreased reactivation of KSHV from latency accompanied with decreased levels of viral lytic replication, suggesting a possible role of ORF64 in influencing the KSHV lytic cycle through its deubiquitination function (González et al., 2009). A recent study has documented a function of the KSHV ORF64 deubiquitinase in inhibiting the retinoic acid-inducible gene 1 (RIG-1) mediated type I IFN signaling (Inn et al., 2011). Though RIG-1 is known to be a cytosolic RNA sensor recognizing viral RNA, recent studies do indicate a role of this protein in recognition of DNA viruses like herpesviruses (Samanta et al., 2006, 2008; Rasmussen et al., 2009). A persistent presence of KSHV was noticed in RIG-1 deficient cells indicating an important role of this protein in influencing the outcome of primary KSHV infection. On these lines it was also observed that KSHV ORF64 inhibits the tripartite motif protein 25 (TRIM25) mediated ubiquitination of RIG-1 (Inn et al., 2011), a critical step essential for the initiation of the type I IFN signaling cascade (Gack et al., 2007).

In addition, ORF64, by virtue of its being a tegument component (Zhu et al., 2005), is delivered into cells during *de novo* infection, hence is efficiently poised to exert its DUB activity toward modifying the cellular environment. In this context, HSV-1 DUB is known to be involved in multiple processes such as virion transportation along MTs, release of the viral nucleic acid into the host cell nucleus, tegumentation and viral egress pathways (Abaitua and O'Hare, 2008; Jovasevic et al., 2008; Shanda and Wilson, 2008). It remains to be clarified if KSHV ORF64 DUB also plays a similar role during *de novo* infection.

ORF75 HOMOLOGUES TARGET COMPONENTS OF PROMYELOCYTIC LEUKEMIA NUCLEAR BODIES (PML NUCLEAR BODIES)

MHV-68 tegument protein, ORF75c, has been shown to mediate the rapid degradation of promyelocytic leukemia protein (PML) nuclear bodies (NBs) through a proteasome-dependent mechanism (Gaspar et al., 2008; Ling et al., 2008). These PML NBs are dynamic nuclear organelles characterized by the presence of the PML and several other cellular proteins including Daxx, Sp100, and ATRX (Bernardi and Pandolfi, 2007; Everett and Chelbi-Alix, 2007). IFN treatment is known to directly trigger the transcription of several genes associated with PML NBs such as PML and Sp100, resulting in increased expression levels of these proteins and an increase in both the size and number of PML NBs (Chelbi-Alix et al., 1995; Lavau et al., 1995; Grotzinger et al., 1996). These observations have suggested the involvement of PML NBs and its constituents in host anti-viral defense mechanisms (Everett and Chelbi-Alix, 2007).

The EBV homologue of MHV-68 ORF75c, BNRF1, known to influence establishment of viral latency (Feederle et al., 2006), has also been characterized as a PML NB interacting protein. BNRF1 specifically binds with Daxx, abrogating the association of ATRX (a Daxx-interacting partner) with Daxx, preventing the localization of this complex to the PML-NBs. In addition to interfering with the PML-mediated anti-viral defenses, this process also regulates the chromatin organization toward establishment of latent infection (Tsai et al., 2011).

Targeting PML NBs or their constituent proteins is not a unique property of gamma-herpesviruses. Similar to EBV BNRF1, the pp71 tegument protein of HCMV also interacts with Daxx. Unlike the BNRF1, pp71 induces the degradation of Daxx subsequent to displacement of ATRX (Lukashchuk et al., 2008). Furthermore, HSV-1 ICP0 mediates a proteasomal degradation of PML (Maul et al., 1993; Everett et al., 1998; Boutell et al., 2002). Yet only the *de novo* synthesized form is capable of this activity (Maul et al., 1993) unlike the tegument delivered ORF75c which exerts its effect immediately following viral infection. With the significance of PML-NBs and its constituents in influencing the outcome of a primary herpesviral infection, it is not surprising that multiple herpesviruses target PML-NBs or its constituent proteins through multiple mechanisms. It would be interesting to investigate if KSHV ORF75 also exerts a similar action on the PML-NBs.

ROLES IN MANIPULATION OF CELLULAR SIGNALING

Several tegument proteins of KSHV have been reported to regulate or interfere with cellular signal transduction.

(i) A study employing reverse transcription cell microarray (RTCM) technology identified ORF75 as a viral gene activating NF-κB. ORF75 was involved in the inactivation of IκB complexes, thereby promoting the nuclear translocation of NF-κB (Konrad et al., 2009; de Oliveira et al., 2010). Increased levels of NF-κB are known to exert inhibitory effects on the activation of gamma-herpesvirus lytic promoters, lytic protein synthesis and virus replication (Brown et al., 2003). On the other hand, inhibition of NF-κB activity in latently infected cells facilitates KSHV lytic protein synthesis reflective of viral reactivation (Grossmann and Ganem, 2008). These observations do suggest the involvement of NF-κB in regulating the fine balance between the KSHV lytic and latent phases. In the context of a primary KSHV infection, the release of ORF75 tegument protein into cells subsequent to primary infection could activate NF-κB and help establish viral latency. Future experiments are needed to elucidate the role of ORF75 modulation of NF-κB in KSHV viral latency.

(ii) ORF45 was shown to stimulate the kinase activities of two serine–threonine kinase proteins, RSK1 and RSK2 (Kuang

et al., 2008). RSK1 and RSK2 belong to the family of 90-kDa ribosomal S6 kinases (RSKs) and are directly phosphorylated by mitogen-activated protein kinases (MAPKs) such as ERK1/ERK2. The activated RSKs in turn mediate the phosphorylation of many nuclear and cytoplasmic proteins influencing several biological processes such as gene expression, cell cycle and cell growth, cell survival and proliferation (Roux and Blenis, 2004; Hauge and Frodin, 2006). The RSKs are activated subsequent to both KSHV primary infection and lytic reactivation. A dramatic reduction in lytic gene expression and virion titers following lytic reactivation was seen in the cells exhibiting siRNA-mediated RSK knockdown, suggesting a role of these RSKs in pathways subsequent to KSHV lytic reactivation (Kuang et al., 2008).

Infection of cells with UV-irradiated KSHV resulted in activation of RSKs, indicating that a virion component protein participates in the activation (Sharma-Walia et al., 2005). ORF45-null mutant virus exhibited noticeably reduced activation of ERK/RSK in the cells infected in comparison to the levels with wild-type viral infection, indicating a role of ORF45 in the ERK/RSK activation (Kuang et al., 2008). A more recent study demonstrated that the binding of ORF45 to RSK also augments the association of ERK to RSK, resulting in the generation of high-molecular weight complexes constituted by ORF45, RSK, and ERK. These complexes help to stabilize the otherwise transient interactions between active phosphorylated pERK and pRSK, also protecting them against cellular phosphatases (Kuang et al., 2009). Thus, the high-molecular weight complex-associated RSK and ERK are activated and sustained at levels sufficient to mediate their biological effects. Since the ERK/RSK MAPK signaling cascade promotes cell survival and proliferation, the ORF45–RSK–ERK complex may lead to prolonged cell survival contributing to KSHV viral pathogenesis.

CONCLUDING REMARKS

It is evident that tegument components of KSHV, which were previously less studied and understood, are slowly catching the attention of herpesvirologists. KSHV tegument components are known to influence several phases of the viral life cycle such as assembly and transportation of viral particles toward egress, transportation of incoming viral particles toward the nucleus, immune evasion processes and a multitude of other regulatory roles (an overview of the functional roles of KSHV tegument proteins is depicted in **Table 1**). In spite of this, a number of issues remain to be addressed. For example, it is unknown if KSHV tegument components influence the nuclear egress of capsids into cytosol

Table 1 | Functional roles of KSHV tegument proteins.

Functional roles	ORF	Functional ability	Reference
In viral egress	ORF64	Inner tegument proteins tethering to the capsid; Hub/scaffolding protein mediating tegumentation and secondary envelopment processes	Rozen et al. (2008)
	ORF45	Recruitment of KIF3A motor onto newly assembled viral tegumented capsids for transportation along microtubules to trans-Golgi vesicles for secondary envelopment	Sathish et al. (2009)
	ORF52	Contributes to tegumentation and secondary envelopment processes	Bortz et al. (2007)
	ORF33	Contributes to tegumentation and secondary envelopment processes	Guo et al. (2009)
	To be identified	Possible role in nuclear egress of capsids	
In viral ingress	To be identified	Probable role in recruitment of dynein motors mediating transportation of incoming viral particles along microtubules to the nucleus	Naranatt et al. (2005)
	To be identified	Probable role in induction and modulation of host cell signaling molecules (e.g., RhoA) that promote acetylation and stabilization of microtubules and change cell physical status	Chandran (2010)
Regulatory roles			
Immune evasion	ORF45	Inhibition of type I IFN-mediated innate anti-viral responses through inhibition of IRF-7 activation	Zhu et al. (2010)
	ORF63	Prevention of caspase-1 activation and inhibition of induction of interleukins IL-1β and IL-18 through inhibition of inflammasome formation	Gregory et al. (2011)
Other regulatory roles	To be identified (ORF75 probably)	Inhibition of NF-κB activation helping to establish viral latency	Konrad et al. (2009)
	ORF64	A possible role of its deubiquitinase activity in multiple processes including virion transportation along microtubules, release of viral nucleic acid into the host cell nucleus, tegumentation, and viral egress	González et al. (2009)
		Inhibition of the retinoic acid-inducible gene 1 (RIG 1) mediated type IIFN signaling	Inn et al. (2011)
	ORF45	Generation of high-molecular complexes with RSK and ERK, activating and sustaining their activities probably contributing to prolonged cell survival and thus KSHV viral pathogenesis	Kuang et al. (2008, 2009)

during viral assembly. It would also be interesting to investigate if tegument proteins contribute to establishment of latency. Findings from these studies could serve as examples of the diverse functional capabilities of the tegument proteins.

Some cellular proteins have been found within the KSHV virions, presumably in the tegument layer (Zhu et al., 2005). Although the functions of these cellular proteins in KSHV virion have not been revealed, it is possible that some of these proteins are specifically recruited into virions to exert important roles in viral life cycle. Some of the cellular proteins identified in KSHV virions such as non-muscle β-actin, annexins, Hsp70, Hsp90, 14-3-3, and APOBEC3G have also been detected in other herpesviruses, other non-herpes DNA viruses and RNA viruses (Johannsen et al., 2004; del Rio et al., 2005; Chung et al., 2006; Loret et al., 2008; Kramer et al., 2011). Pharmacological inhibition of Hsp90 or its knockdown by siRNA has been shown to inhibit replication of several RNA viruses such as HCV and VSV (Connor et al., 2007; Nakagawa et al., 2007), indicating the significance of virus associated cellular proteins. Understanding functions of the cellular proteins in KSHV tegument and their interaction with viral tegument proteins or other virion proteins would lead to a better understanding of the multiple facets of the KSHV life cycle. To conclude, we expect to see a great increase in the number of studies addressing the biological functions of tegument components of KSHV, and of other herpesviruses. Future studies on these fascinating proteins should herald the onset of a new chapter in our ever evolving understanding of herpesvirus biology.

REFERENCES

Abaitua, F., and O'Hare, P. (2008). Identification of a highly conserved, functional nuclear localization signal within the N-terminal region of herpes simplex virus type 1 VP1-2 tegument protein. *J. Virol.* 82, 5234–5244.

Akula, S. M., Naranatt, P. P., Walia, N. S., Wang, F. Z., Fegley, B., and Chandran, B. (2003). Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) infection of human fibroblast cells occurs through endocytosis. *J. Virol.* 77, 7978–7990.

Akula, S. M., Naranatt, P. P., Wang, F. Z., and Chandran, B. (2001). Human herpesvirus envelope-associated glycoprotein B interacts with heparan sulfate-like moieties. *Virology* 284, 235–249.

Akula, S. M., Naranatt, P. P., Wang, F. Z., and Chandran, B. (2002). Integrin α3β1 (CD49c/29) is a cellular receptor for Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) entry into target cells. *Cell* 108, 407–419.

Amerik, A. Y., and Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. *Biochim. Biophys. Acta* 1695, 189–207.

Antman, K., and Chang, Y. (2000). Kaposi's sarcoma. *N. Engl. J. Med.* 342, 1027–1038.

Baghian, A., Luftig, M., Black, J. B., Meng, Y. X., Pau, C. P., Voss, T., Pellett, P. E., and Kousoulas, K. G. (2000). Glycoprotein B of human herpesvirus 8 is a component of the virion in a cleaved form composed of amino- and carboxyl-terminal fragments. *Virology* 269, 18–25.

Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* 284, 641–644.

Bechtel, J. T., Winant, R. C., and Ganem, D. (2005). Host and viral proteins in the virion of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79, 4952–4964.

Benach, J., Wang, L., Chen, Y., Ho, C. K., Lee, S., Seetharaman, J., Xiao, R., Acton, T. B., Montelione, G. T., Deng, H., Sun. R., and Tong, L. (2007). Structural and functional studies of the abundant tegument protein ORF52 from murine gammaherpesvirus 68. *J. Biol. Chem.* 282, 31534–31541.

Bernardi, R., and Pandolfi, P. P. (2007). Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. *Nat. Rev. Mol. Cell Biol.* 8, 1006–1016.

Birkmann, A., Mahr, K., Ensser, A., Yaguboglu, S., Titgemeyer, F., Fleckenstein, B., and Neipel, F. (2001). Cell surface heparan sulfate is a receptor for human herpesvirus 8 and interacts with envelope glycoprotein K8.1. *J. Virol.* 75, 11583–11593.

Bortz, E., Wang, L., Jia, Q., Wu, T. T., Whitelegge, J. P., Deng, H., Zhou, Z. H., and Sun, R. (2007). Murine gammaherpesvirus 68 ORF52 encodes a tegument protein required for virion morphogenesis in the cytoplasm. *J. Virol.* 81, 10137–10150.

Bortz, E., Whitelegge, J. P., Jia, Q., Zhou, Z. H., Stewart, J. P., Wu, T.-T., and Sun, R. (2003). Identification of proteins associated with murine gammaherpesvirus 68 virions. *J. Virol.* 77, 13425–13432.

Boutell, C., Sadis, S., and Everett, R. D. (2002). Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. *J. Virol.* 76, 841–850.

Brierley, M. M., and Fish, E. N. (2002). Review: IFN-alpha/beta receptor interactions to biologic outcomes: understanding the circuitry. *J. Interferon Cytokine Res.* 22, 835–845.

Brown, H. J., Song, M. J., Deng, H., Wu, T.-T., Cheng, G., and Sun, R. (2003). NF-κ-B inhibits gammaherpesvirus lytic replication. *J. Virol.* 77, 8532–8540.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995a). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Cesarman, E., Moore, P. S., Rao, P. H., Inghirami, G., Knowles, D. M., and Chang, Y. (1995b). In vitro establishment and characterization of two acquired immunodeficiency syndrome-related lymphoma cell lines (BC-l and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. *Blood* 86, 2708–2714.

Cesarman, E., Mesri, E. A., and Gershengorn, M. C. (2000). Viral G protein-coupled receptor and Kaposi's sarcoma: a model of paracrine neoplasia? *J. Exp. Med.* 191, 417–422.

Chandran, B. (2010). Early events in Kaposi's sarcoma-associated herpesvirus infection of target cells. *J. Virol.* 84, 2188–2199.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper J., Knowles, D. M, and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS associated Kaposi's sarcoma. *Science* 266, 1865–1869.

Chelbi-Alix, M. K., Pelicano, L., Quignon, F., Koken, M. H., Venturini, L., Stadler, M., Pavlovic, J., Degos, L., and de Thé, H. (1995). Induction of the PML protein by interferons in normal and APL cells. *Leukemia* 9, 2027–2033.

Chen, D., Jiang, H. H., Lee, M., Liu, F., and Zhou, Z. H. (1999). Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus. *Virology* 260, 10–16.

Chuck, S., Grant, R. M., Katongole-Mbidde, E., Conant, M., and Ganem, D. (1996). Frequent presence of a novel herpesvirus genome in lesions of human immunodeficiency virus-negative Kaposi's sarcoma. *J. Infect. Dis.* 173, 248–251.

Chung, C., Chen, C., Ho, M., Huang, C., Liao, C., and Chang, W. (2006). Vaccinia virus proteome: identification of proteins in vaccinia virus intracellular mature virion particles. *J. Virol.* 80, 2127–2140.

Connor, J. H., McKenzie, M. O., Parks, G. D., and Lyles, D. S. (2007). Antiviral activity and RNA polymerase degradation following Hsp90 inhibition in a range of negative strand viruses. *Virology* 362, 109–119.

Dai, W., Jia, Q., Bortz, E., Shah, S., Liu, J., Atanasov, I., Li, X., Taylor, K. A., Sun, R., and Zhou, Z. H. (2008). Unique structures in a tumor herpesvirus revealed by cryo-electron tomography and microscopy. *J. Struct. Biol.* 161, 428–438.

de Oliveira, D. E., Ballon, G., and Cesarman, E. (2010). NF-kappaB signaling modulation by EBV and KSHV. *Trends Microbiol.* 18, 248–257.

del Rio, T., DeCoste, C. J., and Enquist, L. W. (2005). Actin is a component of the compensation mechanism in pseudorabies virus virions lacking the major tegument protein VP22. *J. Virol.* 79, 8614–8619.

Dezube, B. J. (1996). Clinical presentation and natural history of AIDS – related Kaposi's sarcoma. *Hematol. Oncol. Clin. North Am.* 10, 1023–1029.

Diefenbach, R. J., Miranda-Saksena, M., Douglas, M. W., and Cunningham, A. L. (2008). Transport and egress of herpes simplex virus in neurons. *Rev. Med. Virol.* 18, 35–51.

Döhner, K., Radtke, K., Schmidt, S., and Sodeik, B. (2006). Eclipse phase of herpes simplex virus type 1 infection: efficient dynein-mediated capsid transport without the small capsid protein VP26. *J. Virol.* 80, 8211–8224.

Douglas, M. W., Diefenbach R. J., Homa, F. L., Miranda-Saksena, M., Rixon, F. J., Vittone, V., Byth, K., and Cunningham, A. L. (2004). Herpes simplex virus type 1 capsid protein VP26 interacts with dynein light chains RP3 and Tctex1 and plays a role in retrograde cellular transport. *J. Biol. Chem.* 279, 28522–28530.

Dupin, N., Grandadam, M., Calvez, V., Gorin, I., Aubin, J. T., Havard, S., Lamy, F., Leibowitch, M., Huraux, J. M., Escande, J. P., and Agut, H. (1995). Herpesvirus-like DNA sequences in patients with Mediterranean Kaposi's sarcoma. *Lancet* 345, 761–762.

Everett, R. D., and Chelbi-Alix, M. K. (2007). PML and PML nuclear bodies: implications in antiviral defence. *Biochimie* 89, 819–830.

Everett, R. D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M., and Parkinson, J. (1998). The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. *J. Virol.* 72, 6581–6591.

Feederle, R., Neuhierl, B., Baldwin, G., Bannert, H., Hub, B., Mautner, J., Behrends, U., and Delecluse, H. J. (2006). Epstein–Barr virus BNRF1 protein allows efficient transfer from the endosomal compartment to the nucleus of primary B lymphocytes. *J. Virol.* 80, 9435–9443.

Gack, M. U., Shin, Y. C., Joo C.-H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S., and Jung, J. U. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446, 916–920.

Ganem, D. (2007). "Kaposi's sarcoma-associated herpesvirus," in *Fields Virology*, 5th Edn, eds D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (Philadelphia, PA: Lippincott Williams & Wilkins), 2875–2888.

Ganem, D. (2010). KSHV and the pathogenesis of Kaposi sarcoma: listening to human biology and medicine. *J. Clin. Invest.* 120, 939–949.

Gaspar, M., Gill, M. B., Lösing, J. B., May, J. S., and Stevenson, P. G. (2008). Multiple functions for ORF75c in murid herpesvirus-4 infection. *PLoS ONE* 3, e2781. doi:10.1371/journal.pone.0002781

González, C. M., Wang, L., and Damania, B. (2009). Kaposi's sarcoma-associated herpesvirus encodes a viral deubiquitinase. *J. Virol.* 83, 10224–10233.

Goodbourn, S., Didcock, L., and Randall, R. E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *J. Gen. Virol.* 81, 2341–2364.

Gredmark, S., Schlieker, C., Quesada, V., Spooner, E., and Ploegh, H. L. (2007). A functional ubiquitin-specific protease embedded in the large tegument protein (ORF64) of murine gammaherpesvirus 68 is active during the course of infection. *J. Virol.* 81, 10300–10309.

Gregory, S. M., Davis, B. K., West, J. A., Taxman, D. J., Matsuzawa, S., Reed, J. C., Ting, J. P., and Damania, B. (2011). Discovery of a viral NLR homolog that inhibits the inflammasome. *Science* 331, 330–334.

Grossmann, C., and Ganem, D. (2008). Effects of NFκB activation on KSHV latency and lytic reactivation are complex and context-dependent. *Virology* 375, 94–102.

Grotzinger, T., Jensen, K., and Will, H. (1996). The interferon (IFN)-stimulated gene Sp100 promoter contains an IFN-gamma activation site and an imperfect IFN-stimulated response element which mediate type I IFN inducibility. *J. Biol. Chem.* 271, 25253–25260.

Grundhoff, A., and Ganem, D. (2004). Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. *J. Clin. Invest.* 113, 124–136.

Guo, H., Wang, L., Peng, L., Zhou, Z. H., and Deng, H. (2009). Open reading frame 33 of a gammaherpesvirus encodes a tegument protein essential for virion morphogenesis and egress. *J. Virol.* 83, 10582–10595.

Hahn, A., Birkmann, A., Wies, E., Dorer, D., Mahr, K., Stürzl, M., Titgemeyer, F., and Neipel, F. (2009). Kaposi's sarcoma-associated herpesvirus gH/gL: glycoprotein export and interaction with cellular receptors. *J. Virol.* 83, 396–407.

Hauge, C., and Frodin, M. (2006). RSK and MSK in MAP kinase signalling. *J. Cell Sci.* 119, 3021–3023.

Hirokawa, N. (2000). Stirring up development with the heterotrimeric kinesin KIF3. *Traffic* 1, 29–34.

Hiscott, J. (2007). Triggering the innate antiviral response through IRF-3 activation. *J. Biol. Chem.* 282, 15325–15329.

Honda, K., and Taniguchi, T. (2006). IRFs: master regulators of signaling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat. Rev. Immunol.* 6, 644–658.

Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., Shimada, N., Ohba, Y., Takaoka, A., Yoshida, N., and Taniguchi, T. (2005a). IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434, 772–777.

Honda, K., Hideyuki, Y., Takaoka, A., and Taniguchi, T. (2005b). Regulation of the type I IFN induction: a current view. *Int. Immunol.* 17, 1367–1378.

Inn, K. S., Lee, S. H., Rathbun, J. Y., Wong, L. Y., Toth, Z., Machida, K., Ou, J. H., and Jung, J. U. (2011). Inhibition of RIG-I-mediated signaling by Kaposi's sarcoma-associated herpesvirus-encoded deubiquitinase ORF64. *J. Virol.* 85, 10899–10904.

Jenner, R. G., and Boshoff, C. (2002). The molecular pathology of Kaposi's sarcoma-associated herpesvirus. *Biochim. Biophys. Acta* 1602, 1–22.

Johannsen, E., Luftig, M., Chase, M. R., Weicksel, S., Cahir-McFarland, E., Illanes, D., Sarracino, D., and Kieff, E. (2004). Proteins of purified Epstein–Barr virus. *Proc. Natl. Acad. Sci. U.S.A.* 101, 16286–16291.

Jovasevic, V., Liang, L., and Roizman, B. (2008). Proteolytic cleavage of VP1-2 is required for release of herpes simplex virus 1 DNA into the nucleus. *J. Virol.* 82, 3311–3319.

Kaleeba, J. A., and Berger, E. A. (2006). Kaposi's sarcoma-associated herpesvirus fusion-entry receptor: cystine transporter xCT. *Science* 311, 1921–1924.

Kattenhorn, L. M., Korbel, G. A., Kessler, B. M., Spooner, E., and Ploegh, H. L. (2005). A deubiquitinating enzyme encoded by HSV-1 belongs to a family of cysteine proteases that is conserved across the family Herpesviridae. *Mol. Cell* 19, 547–557.

King, S. M. (2000). The dynein microtubule motor. *Biochim. Biophys. Acta* 1496, 60–75.

Konrad, A., Wies, E., Thurau, M., Marquardt, G., Naschberger, E., Hentschel, S., Jochmann, R., Schulz, T. F., Erfle, H., Brors, B., Lausen, B., Neipel, F., and Stürzl, M. (2009). A systems biology approach to identify the combination effects of human herpesvirus 8 genes on NF-kappaB activation. *J. Virol.* 83, 2563–2574.

Koyano, S., Mar, E. C., Stamey, F. R., and Inoue, N. (2003). Glycoproteins M and N of human herpesvirus 8 form a complex and inhibit cell fusion. *J. Gen. Virol.* 84, 1485–1491.

Kramer, T., Greco, T. M., Enquist, L. W., and Cristea, I. M. (2011). Proteomic characterization of pseudorabies virus extracellular virions. *J. Virol.* 85, 6427–6441.

Kuang, E., Tang, Q., Maul, G. G., and Zhu, F. (2008). Activation of p90 ribosomal S6 kinase by ORF45 of Kaposi's sarcoma-associated herpesvirus and its role in viral lytic replication. *J. Virol.* 82, 1838–1850.

Kuang, E., Wu, F., and Zhu, F. (2009). Mechanism of sustained activation of ribosomal S6 kinase (RSK) and ERK by kaposi sarcoma-associated herpesvirus ORF45: multiprotein complexes retain active phosphorylated ERK AND RSK and protect them from dephosphorylation. *J. Biol. Chem.* 284, 13958–13968.

Lavau, C., Marchio, A., Fagioli, M., Jansen, J., Falini, B., Lebon, P., Grosveld, F., Pandolfi, P. P., Pelicci, P. G., and Dejean, A. (1995). The acute promyelocytic leukaemia-associated PML gene is induced by interferon. *Oncogene* 11, 871–876.

Ling, P. D., Tan, J., Sewatanon, J., and Peng, R. (2008). Murine gammaherpesvirus 68 open reading frame 75c tegument protein induces the degradation of PML and is essential for production of infectious virus. *J. Virol.* 82, 8000–8012.

Liu, Y., Cui, Z., Zhang, Z., Wei, H., Zhou, Y., Wang, M., and Zhang, X. E. (2009). The tegument protein UL94 of human cytomegalovirus as a binding partner for tegument protein pp28 identified by intracellular imaging. *Virology* 388, 68–77.

Loret, S., Guay, G., and Lippé, R. (2008). Comprehensive characterization of extracellular herpes simplex virus type 1 virions. *J. Virol.* 82, 8605–8618.

Lukashchuk, V., McFarlane, S., Everett, R. D., and Preston, C. M. (2008). Human cytomegalovirus protein pp71 displaces the chromatin-associated factor ATRX from nuclear domain 10 at early stages of infection. *J. Virol.* 82, 12543–12554.

Luxton, G. W., Haverlock, S., Coller, K. E., Antinone, S. E., Pincetic, A., and Smith, G. A. (2005). Targeting of herpesvirus capsid transport in axons is coupled to association with specific sets of tegument proteins. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5832–5837.

Mandelkow, E., and Mandelkow, E. M. (1995). Microtubules and

microtubule-associated proteins. *Curr. Opin. Cell Biol.* 7, 72–81.

Martin, D. F., Kuppermann, B. D., Wolitz, R. A., Palestine, A. G., Li, H. and Robinson, C. A. (1999). Oral ganciclovir for patients with cytomegalovirus retinitis treated with a ganciclovir implant. Roche Ganciclovir Study Group. *N. Engl. J. Med.* 340, 1063–1070.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Mol. Cell* 10, 417–426.

Maul, G. G., Guldner, H. H., and Spivack, J. G. (1993). Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). *J. Gen. Virol.* 74, 2679–2690.

May, J. S., Coleman, H. M., Boname, J. M., and Stevenson, P. G. (2005a). Murine gammaherpesvirus-68 ORF28 encodes a non-essential virion glycoprotein. *J. Gen. Virol.* 86, 919–928.

May, J. S., Walker, J., Colaco, S., and Stevenson, P. G. (2005b). The murine gammaherpesvirus 68 ORF27 gene product contributes to intercellular viral spread. *J. Virol.* 79, 5059–5068.

McNabb, D., and Courtney, R. (1992). Characterization of the large tegument protein (ICP1/2) of herpes simplex virus type 1. *Virology* 190, 221–232.

Meckes, D. G. Jr., and Wills, J. W. (2007). Dynamic interactions of the UL16 tegument protein with the capsid of herpes simplex virus. *J. Virol.* 81, 13028–13036.

Mettenleiter, T. C. (2002). Herpesvirus assembly and egress. *J. Virol.* 76, 1537–1547.

Mettenleiter, T. C., Klupp, B. G., and Granzow, H. (2009). Herpesvirus assembly: an update. *Virus Res.* 143, 222–234.

Meylan, E., Tschopp, J., and Karin, M. (2006). Intracellular pattern recognition receptors in the host response. *Nature* 442, 39–44.

Miller, G., Heston, L., Grogan, E., Gradoville, L., Rigsby, M., Sun, R., Shedd, D., Kushnaryov, V. M., Grossberg, S., and Chang, Y. (1997). Selective switch between latency and lytic replication of Kaposi's sarcoma herpesvirus and Epstein–Barr virus in dually infected body cavity lymphoma cells. *J. Virol.* 71, 314–324.

Moore, P. S., and Chang, Y. (1995). Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and without HIV infection. *N. Engl. J. Med.* 332, 1181–1185.

Moore, P. S., Gao, S. J., Dominguez, G., Cesarman, E., Lungu, O., Knowles, D. M., Garber, R., Pellett, P. E., McGeoch, D. J., and Chang, Y. (1996). Primary characterization of a herpesvirus agent associated with Kaposi's sarcoma. *J. Virol.* 70, 549–558.

Morfeldt, L., and Torssander, J. (1994). Long-term remission of Kaposi's sarcoma following foscarnet treatment in HIV-infected patients. *Scand. J. Infect. Dis.* 26, 749–752.

Nakagawa, S., Umehara, T., Matsuda, C., Kuge, S., Sudoh, M., and Kohara, M. (2007). Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. *Biochem. Biophys. Res. Commun.* 353, 882–888.

Naranatt, P. P., Akula, S. M., and Chandran, B. (2002). Characterization of gamma2-human herpesvirus-8 glycoproteins gH and gL. *Arch. Virol.* 147, 1349–1370.

Naranatt, P. P., Krishnan, H. H., Smith, M. S., and Chandran, B. (2005). Kaposi's sarcoma-associated herpesvirus modulates microtubule dynamics via RhoA-GTP diaphanous 2 signaling and utilizes the dynein motors to deliver its DNA to the nucleus. *J. Virol.* 79, 1191–1206.

Naranatt, P. P., Krishnan, H. H., Svojanovsky, S. R., Bloomer, C., Mathur, S., and Chandran, B. (2004). Host gene induction and transcriptional reprogramming in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8)-infected endothelial, fibroblast, and B cells: insights into modulation events early during infection. *Cancer Res.* 64, 72–84.

Nealon, K., Newcomb, W. W., Pray, T. R., Craik, C. S., Brown, J. C., and Kedes, D. H. (2001). Lytic replication of Kaposi's sarcoma-associated herpesvirus results in the formation of multiple capsid species: isolation and molecular characterization of A, B, and C capsids from a gammaherpesvirus. *J. Virol.* 75, 2866–2878.

Newcomb, W. W., and Brown, J. C. (2010). Structure and capsid association of the herpesvirus large tegument protein UL36. *J. Virol.* 84, 9408–9414.

Ogawa-Goto, K., Tanaka, K., Gibson, W., Moriishi, E., Miura, Y., Kurata, T., Irie, S., and Sata, T. (2003). Microtubule network facilitates nuclear targeting of human cytomegalovirus capsid. *J. Virol.* 77, 8541–8547.

Pellet, P. E., and Roizman, B. (2007). "The family Herpesviridae: a brief introduction," in *Fields' Virology*, 5th Edn, eds D. M. Knipe, P. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (New York, NY: Lippincott Williams & Wilkins), 2479–2499.

Perry, S. T., and Compton, T. (2006). Kaposi's sarcoma-associated herpesvirus virions inhibit interferon responses induced by envelope glycoprotein gpK8.1. *J. Virol.* 80, 11105–11114.

Phillips, S. L., and Bresnahan, W. A. (2011). Identification of binary interactions between human cytomegalovirus virion proteins. *J. Virol.* 85, 440–447.

Raghu, H., Sharma-Walia, N., Valiya Veettil, M., Sadagopan, S., Caballero, A., Sivakumar, R., Varga, L., Bottero, V., and Chandran, B. (2007). Lipid rafts of primary endothelial cells are essential for Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8-induced phosphatidylinositol 3-kinase and RhoA-GTPases critical for microtubule dynamics and nuclear delivery of viral DNA but dispensable for binding and entry. *J. Virol.* 81, 7941–7959.

Raghu, H., Sharma-Walia, N., Valiya Veettil, M., Sadagopan, S., and Chandran, B. (2009). Kaposi's sarcoma associated herpesvirus utilizes an actin polymerization-dependent macropinocytic pathway to enter human dermal microvascular endothelial and human umbilical vein endothelial cells. *J. Virol.* 83, 4895–4911.

Rasmussen, S. B., Jensen, S. B., Nielsen, C., Quartin, E., Kato, H., Chen, Z. J., Silverman, R. H., Akira, S., and Paludan, S. R. (2009). Herpes simplex virus infection is sensed by both Toll-like receptors and retinoic acid-inducible gene-like receptors, which synergize to induce type I interferon production. *J. Gen. Virol.* 90, 74–78.

Renne, R., Zhong, W., Herndier, B., McGrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat. Med.* 2, 342–346.

Roux, P. P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* 68, 320–344.

Rozen, R., Sathish, N., Li, Y., and Yuan, Y. (2008). Virion-wide protein interactions of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 82, 4742–4750.

Russo, J. J., Bohenzky, R. A., Chien, M. C., Chen, J., Yan, M., Maddalena, D., Parry, J. P., Peruzzi, D., Edelman, I. S., Chang, Y., and Moore, P. S. (1996). Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc. Natl. Acad. Sci. USA* 93, 14862–14867.

Sadler, A. J., and Williams, B. R. G. (2008). Interferon-inducible antiviral effectors. *Nat. Rev. Immunol.* 8, 559–568.

Samanta, M., Iwakiri, D., Kanda, T., Imaizumi, T., and Takada, K. (2006). EB virus-encoded RNAs are recognized by RIG-I and activate signaling to induce type I IFN. *EMBO J.* 25, 4207–4214.

Samanta, M., Iwakiri, D., and Takada, K. (2008). Epstein–Barr virus-encoded small RNA induces IL-10 through RIG-I-mediated IRF-3 signaling. *Oncogene* 27, 4150–4160.

Sarid, R., Flore, O., Bohenzky, R. A., Chang, Y., and Moore, P. S. (1998). Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). *J. Virol.* 72, 1005–1012.

Sathish, N., and Yuan, Y. (2010). Functional characterization of Kaposi's sarcoma-associated herpesvirus small capsid protein by bacterial artificial chromosome-based mutagenesis. *Virology* 407, 306–318.

Sathish, N., and Yuan, Y. (2011). Evasion and subversion of interferon-mediated antiviral immunity by Kaposi's sarcoma-associated herpesvirus: an overview. *J. Virol.* 85, 10934–10944.

Sathish, N., Zhu, F. X., Golub, E. E., Liang, Q., and Yuan, Y. (2011). Mechanisms of autoinhibition of IRF-7 and a probable model for inactivation of IRF-7 by Kaposi's sarcoma-associated herpesvirus protein ORF45. *J. Biol. Chem.* 286, 746–756.

Sathish, N., Zhu, F. X., and Yuan, Y. (2009). Kaposi's sarcoma-associated herpesvirus ORF45 interacts with kinesin-2 transporting viral capsid–tegument complexes along microtubules. *PLoS Pathog.* 5, e1000332. doi:10.1371/journal.ppat.1000332

Schalling, M., Ekman, M., Kaaya, E. E., Linde, A., and Biberfeld, P. (1995). A role for a new herpes virus (KSHV) in different forms of Kaposi's sarcoma. *Nat. Med.* 1, 707–708.

Shanda, S. K., and Wilson, D. W. (2008). UL36p is required for efficient transport of membrane-associated herpes simplex virus type 1 along microtubules. *J. Virol.* 82, 7388–7394.

Sharma-Walia, N., Krishnan, H. H., Naranatt, P. P., Zeng, L., Smith, M. S., and Chandran, B. (2005). ERK1/2 and MEK1/2 induced by Kaposi's sarcoma associated herpesvirus (human herpesvirus 8) early during infection of target cells are essential for expression of viral genes and for establishment of infection. *J. Virol.* 79, 10308–10329.

Sharma-Walia, N., Naranatt, P. P., Krishnan, H. H., Zeng, L., and Chandran, B. (2004). Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 envelope glycoprotein gB induces the integrin-dependent focal adhesion kinase-Src-phosphatidylinositol 3-kinase rho GTPase signal pathways and cytoskeletal rearrangements. *J. Virol.* 78, 4207–4223.

Sompallae, R., Gastaldello, S., Hildebrand, S., Zinin, N., Hassink, G., Lindsten, K., Haas, J., Persson, B., and Masucci, M. G. (2008). Epstein–Barr virus encodes three bona fide ubiquitin-specific proteases. *J. Virol.* 82, 10477–10486.

Soulier, J., Grollet, L., Okshendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J.-P., Raphael, M., Degos, L., and Sigaux, F. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86, 1276–1280.

Stutz, A., Golenbock, D. T., and Latz, E. (2009). Inflammasomes: too big to miss. *J. Clin. Invest.* 119, 3502–3511.

Sun, R., Lin, S. F., Staskus, K., Gradoville, L., Grogan, E., Hasse, A., and Miller, G. (1999). Kinetics of Kaposi's sarcoma-associated herpesvirus gene expression. *J. Virol.* 73, 2232–2242.

Tsai, K., Thikmyanova, N., Wojcechowskyj, J. A., Delecluse, H. J., and Lieberman, P. M. (2011). EBV tegument protein BNRF1 disrupts DAXX-ATRX to activate viral early gene transcription. *PLoS Pathog.* 7, e1002376. doi:10.1371/journal.ppat.1002376

Veettil, M. V., Sadagopan, S., Sharma-Walia, N., Wang, F.-Z., Raghu, H., Varga, L., and Chandran, B. (2008). Kaposi's sarcoma-associated herpesvirus forms a multimolecular complex of integrins (αvβ5, αvβ3, and α3β1) and CD98-xCT during infection of human dermal microvascular endothelial cells, and CD98-xCT is essential for the postentry stage of infection. *J. Virol.* 82, 12126–12144.

Veettil, M. V., Sharma-Walia, N., Sadagopan, S., Raghu, H., Sivakumar, R., Naranatt, P. P., and Chandran, B. (2006). RhoA-GTPase facilitates entry of Kaposi's sarcoma-associated herpesvirus into adherent target cells in a Src-dependent manner. *J. Virol.* 80, 11432–11446.

Vittone, V., Diefenbach, E., Triffett, D., Douglas, M. W., Cunningham, A. L., and Diefenbach, R. J. (2005). Determination of interactions between tegument proteins of herpes simplex virus type 1. *J. Virol.* 79, 9566–9571.

Wang, F. Z., Akula, S. M., Pramod, N. O., Zeng, L., and Chandran, B. (2001). Human herpesvirus 8 envelope glycoprotein K8.1 interaction with the target cells involves heparan sulfate. *J. Virol.* 75, 7517–7527.

Wang, J., Loveland, A. N., Kattenhorn, L. M., Ploegh, H. L., and Gibson, W. (2006). High-molecular-weight protein (pUL48) of human cytomegalovirus is a competent deubiquitinating protease: mutant viruses altered in its active-site cysteine or histidine are viable. *J. Virol.* 80, 6003–6012.

Wolfstein, A., Nagel, C. H., Radtke, K., Döhner, K., Allan, V. J., and Sodeik, B. (2006). The inner tegument promotes herpes simplex virus capsid motility along microtubules in vitro. *Traffic* 7, 227–237.

Yamazaki, H., Nakata, T., Okada, Y., and Hirokawa, N. (1995). KIF3A/B: a heterodimeric kinesin superfamily protein that works as a microtubule plus end-directed motor for membrane organelle transport. *J. Cell Biol.* 130, 1387–1399.

Zhong, W., Wang, H., Herndier, B., and Ganem, D. (1996). Restricted expression of Kaposi sarcoma-associated herpesvirus (human herpesvirus 8) genes in Kaposi sarcoma. *Proc. Natl. Acad. Sci. U.S.A.* 93, 6641–6646.

Zhou, Z., Chen, D., Jakana, J., Rixon, F. J., and Chiu, W. (1999). Visualization of tegument–capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J. Virol.* 73, 3210–3218.

Zhu, F. X., Chong, J. M., Wu, L., and Yuan, Y. (2005). Virion proteins of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79, 800–811.

Zhu, F. X., King, S. M., Smith, E. J., Levy, D. E., and Yuan, Y. (2002). A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5573–5578.

Zhu, F. X., Li, X., Zhou, F., Gao, S. J., and Yuan, Y. (2006). Functional characterization of Kaposi's sarcoma-associated herpesvirus ORF45 by bacterial artificial chromosome-based mutagenesis. *J. Virol.* 80, 12187–12196.

Zhu, F. X., Sathish, N., and Yuan, Y. (2010). Antagonism of host antiviral responses by Kaposi's sarcoma-associated herpesvirus tegument protein ORF45. *PLoS ONE* 5, e10573. doi:10.1371/journal.pone.0010573

Zhu, F. X., and Yuan, Y. (2003). The ORF45 protein of Kaposi's sarcoma-associated herpesvirus is associated with purified virion. *J. Virol.* 77, 4221–4230.

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Kaposi's sarcoma-associated herpesvirus genome replication, partitioning, and maintenance in latency

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Kaposi's sarcoma-associated herpesvirus (KSHV) is thought to be an oncogenic member of the γ-herpesvirus subfamily. The virus usually establishes latency upon infection as a default infection pattern. The viral genome replicates according to the host cell cycle by recruiting the host cellular replication machinery. Among the latently expressing viral factors, LANA plays pivotal roles in viral genome replication, partitioning, and maintenance. LANA binds with two LANA-binding sites (LBS1/2) within a terminal repeat (TR) sequence and is indispensable for viral genome replication in latency. The nuclear matrix region seems to be important as a replication site, since LANA as well as cellular replication factors accumulate there and recruit the viral replication origin in latency (ori-P) by its binding activity to LBS. KSHV ori-P consists of LBS followed by a 32-bp GC-rich segment (32GC). Although it has been reported that LANA recruits cellular pre-replication complexes (pre-RC) such as origin recognition complexes (ORCs) to the ori-P through its interaction with ORCs, this mechanism does not account completely for the requirement of the 32GC. On the other hand, there are few reports about the partitioning and maintenance of the viral genome. LANA interacts with many kinds of chromosomal proteins, including Brd2/RING3, core histones, such as H2A/H2B and histone H1, and so on. The detailed molecular mechanisms by which LANA enables KSHV genome partitioning and maintenance still remain obscure. By integrating the findings reported thus far on KSHV genome replication, partitioning, and maintenance in latency, we will summarize what we know now, discuss what questions remain to be answered, and determine what needs to be done next to understand the mechanisms underlying viral replication, partitioning, and maintenance strategy.

Keywords: Kaposi's sarcoma-associated herpesvirus, human herpesvirus 8, latency-associated nuclear antigen, ori-P, DNA replication, genome maintenance, pre-replication complex, nuclear matrix

INTRODUCTION

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) is a gamma-2 herpesvirus discovered from KS specimens in 1994 (Chang et al., 1994). KSHV is closely associated with KS and several non-Hodgkin lymphomas, including primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD; Cesarman et al., 1995, 1996; Soulier et al., 1995). While KS is the most common cancer in acquired immune deficiency syndrome patients (Potthoff et al., 2010), KSHV is detected in about 95% of all types of KS lesions by PCR analysis (Dupin et al., 1995; Huang et al., 1995; Moore and Chang, 1995). PEL is a rare B cell lymphoma originated from preterminal B cells, and PEL in AIDS patients is often associated with KSHV as well as EBV. Several KSHV-infected PEL cell lines have been established, and EBV is frequently lost in the course of establishment (Arvanitakis et al., 1996; Gaidano et al., 1996; Renne et al., 1996; Said et al., 1996; Carbone et al., 1997, 1998; Katano et al., 1999). MCD is a plasmacytic lymphadenopathy with polyclonal hyper-immunoglobulinemia and high levels of serum IL-6 (Frizzera et al., 1983; Yoshizaki et al., 1989).

Like all herpesviruses, KSHV has two life cycles: latent and lytic replication phases (for review, see Boshoff and Chang, 2001). Whereas KSHV is usually in latency when it infects KS and PEL cells, in MCD some cells express lytic genes (Katano et al., 2000; Parravicini et al., 2000). On the other hand, it has been reported that KSHV infection itself and/or viral lytic proteins promote cell proliferation and angiogenesis as well as lymphatic reprogramming (Ciufo et al., 2001; Gao et al., 2003; Carroll et al., 2004; Hong et al., 2004; Naranatt et al., 2004; Pan et al., 2004; Wang et al., 2004; Sharma-Walia et al., 2006; Qian et al., 2007, 2008; Sadagopan et al., 2007; Ye et al., 2007).

In latency, the KSHV genome is present as an episome, which is capable of autonomously replicating during S phase of the host cell cycle without integration into host chromosomes, and only limited genes are expressed during latency. Therefore, there is no generation of progeny virions. It is very important to elucidate and learn the virus's survival strategy in order to control infection and to formulate treatment for KSHV-related diseases.

In this review, we would like to focus on studies on the mechanisms underlying viral DNA replication, genome segregation and maintenance, and gene expression regulation in latency, and to discuss these topics in the light of studies on cellular mechanisms.

GENE EXPRESSION CONTROL IN KSHV LATENCY

The KSHV genome is a double-stranded linear DNA in the virion. It is circularized upon infection and is maintained as an episome in the infected nucleus. The complete genome is about 160–170 kbp,

including a 40∼50 times repeated sequence called a terminal repeat (TR), which is 801 bp as a unit, at each end of the genome (**Figure 1**). The viral genome encodes approximately 90 ORFs in the unique region (for reviews, see Moore and Chang, 2001; Dourmishev et al., 2003). In latency, the viral genome is maintained in a cell cycle-dependent manner, and extremely limited viral genes, such as latent gene clusters, are expressed. Although the establishment of latent infection could be this virus's default infection mode, it is not good for the virus to continue latent infection for a long time, since it will vanish from the earth without progeny virus production. Thus, latency is a state in which the virus waits for an opportunity for the explosive production of progeny virions. Nevertheless, it is very interesting how KSHV establishes latency and is maintained in infected host cells without losing the genomes.

As mentioned above, the limited region within the KSHV genome is transcriptionally active in latency, and this region contains only several genes, including the latency-associated nuclear antigen (Wang and Frappier, 2009), viral cyclin (*v-CYC*), viral FLICE-inhibitory protein (*v-FLIP*), *kaposin*, 17 microRNAs (miRNAs), and viral interferon regulatory factor 3 (*v-IRF-3*; Chang et al., 1996; Thome et al., 1997; Muralidhar et al., 1998; Lubyova and Pitha, 2000; Gomez-Roman et al., 2001; Rivas et al., 2001; Staudt and Dittmer, 2003; Cai et al., 2005; Pearce et al., 2005; Pfeffer et al., 2005; Samols et al., 2005). Such genes, except for *v-IRF-3*, are in that limited region, and *LANA*, v-*CYC*, and *v-FLIP* are in one of the unit's genes. This region forms an active locus for expression including miRNAs and *kaposin*. It is unclear why this region is active for the expression of genes and is insulated from inactive lytic genes, such as ORF69 and K14 just downstream and upstream, respectively.

Recently, it was reported that CTCF, which is the only insulator protein found in vertebrates, coupled with SMC3, endows this gene insulation. More interestingly, the binding sites are in the genes, i.e., downstream from the *LANA* transcription start sites, not at the boundary regions (Stedman et al., 2008; Kang and Lieberman, 2009). Transcriptional analysis using the KSHV-BAC system demonstrated that mutations of CTCF binding sites abolished latency-regulated transcription such as K14 and ORF74 during latency (Kang and Lieberman, 2009). CTCF usually binds at the boundary regions between active and inactive loci in mammalian genomes, forming locus control regions (LCRs; Tanimoto et al., 2003). A typical example is an LCR seen in the beta-globin locus. CTCF binds to several DNase I hypersensitivity sites (HS), called HS4 and HS5, and forms boundaries to insulate this locus from the outside locus (Tanimoto et al., 2003; Hou et al., 2008). Thus, latent gene expression in KSHV-infected cells might be regulated differently from the mechanism observed in the beta-globin locus.

Inversely, it is interesting how the viral lytic genes are tightly inactivated in latency. Epigenetic regulation seems to be essential for inactivation as well as activation of latent genes. LANA recruits heterochromatin components to the TR by the interaction between LANA and SUV39H1, which is a key factor that methylates histone H3, which in turn recruits heterochromatin protein 1 (HP1; Sakakibara et al., 2004). Because this mechanism contributes to the propagation and maintenance of heterochromatin, it appears that heterochromatin could spread over the KSHV genome during latency. The propagation of heterochromatin into the active latent gene zone might be blocked by the boundary effect and by the enhancer-blocking activity of an insulator, CTCF which has multiple functions such as gene activation or inactivation, X-chromosome inactivation, and gene imprinting (for review, see Zlatanova and Caiafa, 2009).

Thus, it is thought that not the overall lytic genes region, except for the latent gene clusters, forms heterochromatin during latency, because recent genome-wide analysis using ChIP-on-chip showed that not only latent gene clusters but also several regions of lytic genes are enriched in activating histone marks (acetylated H3 and H3K4me3). However, H3K27me3, which is a bivalent histone marker, is widely distributed through the KSHV genome (Toth et al., 2010), meaning that the genome is poised for reactivation. Furthermore, the treatment of specific histone demethylases of H3K27me3 such as JMJD3 and UTX could induce the lytic reactivation. Immunoprecipitation of methylated DNA assay showed that the KSHV genome was methylated during latency (Gunther and Grundhoff, 2010). Gunther and Grundhoff (2010) suggested that the CpG methylation process could take a long time to prevail over the genome, and thus could not control early latency. There are several reports that DNA methylation of viral genomes is related to the regulation of the gene expression of gammaherpesviruses such as EBV and herpesvirus saimiri (HVS; Minarovits, 2006). Heterochromatin formation on the viral genomes, however, seems to be inconvenient for the rapid induction of lytic replication. Further investigations are needed to clarify how viruses are ready for lytic induction if heterochromatin and/or DNA methylation was formed on the genome.

Viral factors play key roles in maintaining gene expression profiles in latency. Otherwise, modulation by viral and cellular factors maintains viral latency. In addition to the recruitment of

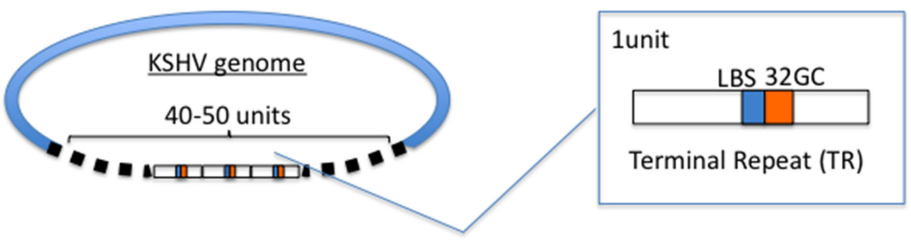

FIGURE 1 | Terminal repeat (TR) sequences. The KSHV genome has 40–50 units of TR sequences. TR contains LANA-binding site (LBS) and GC-rich sequences termed 32GC. It is thought that one of the LBS-32GC sequence is selected as DNA replication origin (ori-P).

heterochromatin factors on to the genome, LANA itself tends to repress viral lytic gene expression. LANA physically associates with recombination signal sequence-binding protein Jκ (RBP-Jκ) and represses the replication and transcription activator (RTA) promoter through the RBP-Jκ binding site existing within its promoter (Lan et al., 2005a).

Viral FLICE-inhibitory protein, also known as K13, interacts with several NF-κB-related signaling proteins and activates the NF-κB pathways, thus enhancing cell survival (Chaudhary et al., 1999; Field et al., 2003; Matta et al., 2003, 2007; Matta and Chaudhary, 2004). It should be noted that the effect of NF-κB signaling on reactivation depends on cellular context (Grossmann and Ganem, 2008) and seems to be regulated by an intricate balance within the cellular environment. Previous studies, however, demonstrated that v-FLIP repressed the RTA promoter by activating NF-κB binding to the cognate sequence, but in that case it deregulates vIL-6 and hIL-6 expression (Zhao et al., 2007). The reactivation is initiated by RTA, which is a lytic switch protein and a homolog of EBV BRLF1 (Liang et al., 2002). The RTA promoter region is highly responsive to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or phorbol 12-myristate 13-acetate (PMA), sodium butylate (NaB), and trichostatin A (TSA), and is associated with several histone deacetylase proteins such as HDAC, which leads to chromatin remodeling of a nucleosome and then regulates KSHV reactivation from latency (Lu et al., 2003). RTA activates various viral genes through direct binding with RTA-responsive elements existing within the K8 and ORF57 promoters (Byun et al., 2002) and also through indirect mechanisms on RTA itself and vIRF-1 (Nishimura et al., 2001; Sakakibara et al., 2001; Ueda et al., 2002). Although RTA is a strong transactivator and inducer of lytic replication, it also enhances LANA expression and then is involved in the establishment of latency in the early infection phase (Lan et al., 2005b). This feedback mechanism explains the low efficiency of lytic replication and the generation of complete viral particles in KSHV-infected cell lines.

MicroRNAs (miRNAs) are single-stranded and 20- to 23-nucleotide RNA molecules that are involved in gene expression (Bartel, 2004; Bartel and Chen, 2004). Recent studies have highlighted the critical role of viral microRNAs (miRNAs) in the maintenance of KSHV latency (for review, see Ganem and Ziegelbauer, 2008; Boss et al., 2009; Lei et al., 2010a). The KSHV genome contains 17 miRNAs that are clustered and located in the intragenic region between *kaposin* and *v-FLIP* (Cai et al., 2005; Pearce et al., 2005; Samols et al., 2005; Cai and Cullen, 2006). Surprisingly, a new proteomic approach suggests that a single miRNA can directly lead to the suppression of the synthesis of hundreds of proteins at both mRNA and translation levels, although the level of suppression is mild (Baek et al., 2008; Selbach et al., 2008).

Kaposi's sarcoma-associated herpesvirus miRNAs are reported to regulate, directly or indirectly, various factors including lytic genes (Murphy et al., 2008; Bellare and Ganem, 2009) and cellular factors such as NF-κB and IκBα, the latter of which is directly regulated by KSHV miR-K1 (Lei et al., 2010b) and Bcl-2 associated factor (*BCLAF1*) as a target of miR-K5 (Ziegelbauer et al., 2009). miR-K12-7 (Lin et al., 2011) and miR-K9 (Bellare and Ganem, 2009) directly target RTA and contribute to the maintenance of latency. miR-K12-11 shows remarkable homology to cellular miR-155;it inhibits a BACH-1 3′UTR-containing reporter and downregulates the expression of BACH-1, which is a broadly expressed transcriptional repressor that regulates genes involved in the hypoxia response (Gottwein et al., 2007; Skalsky et al., 2007). Thrombospondin 1 (*THBS1*), an inhibitor of angiogenesis, is targeted by multiple KSHV miRNAs, such as miR-K12-1, miR-K12-3-3p, miR-K12-6-3p, and miR-K12-11(Samols et al., 2007). miR-K1 represses the expression of p21 via the 3′UTR and attenuates p21-mediated cell cycle arrest during KSHV latency (Gottwein and Cullen, 2010).

CELLULAR DNA LICENSING AND VIRAL DNA REPLICATION IN LATENCY

Because eukaryotic DNA replication is strictly regulated by a licensing mechanism, the genome is replicated only once per cell cycle. DNA replication starts at multiple sites on a chromosome; these sites are called the replication origin, whose number is predicted to be 30,000 ∼ 50,000 (Huberman and Riggs, 1966). The genome size of eukaryotes is about 10^7 to 10^{11} bp (fungi to mammals), and the entire DNA must be replicated within a limited time (Wyrick et al., 2001). To achieve this, many proteins participate in DNA replication licensing, including origin recognition complex (ORC), Cdc6, Cdt1, and mini-chromosomal maintenance (MCM) helicase, and so on. First, ORC recognizes and binds to the origins and then recruits a Cdc6 followed by the association of another replication protein, called Cdt1. Finally, the MCM helicase is loaded onto the complex to establish a complete pre-replication complex (pre-RC) (reviews in Nishitani and Lygerou, 2002; DePamphilis, 2003, 2005).

KSHV ORIGIN OF REPLICATION IN LATENCY

The features of DNA replication origins have been reported. Although there are no consensus sequences for the replication origin, recent studies showed that CpG islands, promoter regions, DNA topology, and nucleosome positioning are involved in origin selection (Mechali, 2010). *Saccharomyces cerevisiae* (*S. cerevisiae*) has autonomous replication sequence (ARS) elements that are specific 12 bp consensus sequences and has origin activity (Stinchcomb et al., 1979; Bell and Stillman, 1992). *S. pombe* ARS also has been identified, but it does not share a consensus sequences as in *S. cerevisiae* (Segurado et al., 2003; Dai et al., 2005; Heichinger et al., 2006). Substitution experiments showed that the ARS region could be replaced with a 40-bp poly (dA/dT) fragment (Okuno et al., 1999).

In higher eukaryotes, no consensus sequences are identified, though known origin sequences have been reported (for review, see Aladjem, 2004). It is not yet known why there is no consensus sequence among ORC binding sites of higher eukaryotes, or how they are selected. Replication origins should be determined by different mechanisms, and recent genome-wide analyses show that the origin sequences are closely related with transcriptional regulatory elements and CpG islands but not sequence motifs (Cadoret et al., 2008; Sequeira-Mendes et al., 2009).

The KSHV genome appears to replicate once per cell cycle during latency, as cellular DNA replication. The number of the genome copies is supposed to be 50–100 per KSHV-infected PEL cell and the copy number is kept at the same number, at least

appears to be kept at the same copy number (Cesarman et al., 1995; Ballestas et al., 1999; Ueda et al., 2006). This observation suggests that the KSHV genome uses cellular replication machinery so that viral DNA replication synchronizes with the cell cycle. The KSHV origin of replication in latency, called ori-P, consists of two LANA-binding sites (LBS), in which LBS1 has a higher affinity with LANA (Garber et al., 2002), and the following 32 bp GC-rich segment (termed 32GC in this manuscript) and the ori-P is in the TR region of the KSHV episome (Hu and Renne, 2005; **Figure 1**). LANA directly binds to LBS and supports viral DNA replication (Garber et al., 2002; Hu et al., 2002; Fejer et al., 2003; Grundhoff and Ganem, 2003). The components of pre-RC, such as ORC, Cdc6, and MCM were recruited to the TR sequences in a LANA-dependent manner (Lim et al., 2002; Ohsaki et al., 2004; Stedman et al., 2004; Verma et al., 2006).

In the case of EBV ori-P, the dyad symmetry (DS) and family of repeats (FR) are essential for the ori-P activity (Reisman et al., 1985), though FR rather works for viral genome maintenance. The DS element contains two EBNA1 binding sites and is the functional replicator in the presence of EBNA1 (Wysokenski and Yates, 1989; Harrison et al., 1994). The FR element contains 20 copies of a 30-bp repeat sequence and has an essential role in the long-term maintenance of ori-P-containing plasmid (Krysan et al., 1989; Marechal et al., 1999). It is suggested that cellular replication factors bind to the sequences adjacent to EBNA1 binding sites through the interaction with EBNA1 (Yates et al., 2000; Koons et al., 2001). A chromatin immunoprecipitation assay suggested that the ORC complex and EBNA1 bound to chromatin and ori-P in G0-arrested cells (Ritzi et al., 2003). Nucleosome assembly proteins, such as NAP1 and TAF-I, interact with EBNA1 and are recruited to the ori-P regions. These proteins contribute to the activation of transcription, although TAF-I negatively regulates DNA replication (Wang and Frappier, 2009). The EBNA1 LR1 and LR2 domains are critical for the interaction with ORC and for disrupting this association by binding with G-rich RNA (Norseen et al., 2009). Thus, the latent replication of KSHV and EBV totally depends on cellular DNA replication machinery with the only exception of the requirement of LANA and EBNA1, respectively.

HOW LANA WORKS

LANA is a nuclear protein with 1162 amino acids. It shows a functional homology to EBNA1 of EBV and, in part, to E1/E2 of human papillomavirus and to SV40 large T antigen. Especially, the C-terminus of LANA and that of EBNA1 conserve secondary and tertiary structures (Han et al., 2010). The N-terminus of LANA contains a chromosome binding site (CBS) and a nuclear localization signal (NLS), and the C-terminus contains a DNA binding domain and a dimerization domain, called DBD. In the DBD, there appears to be another NLS, but this NLS is rather cryptic and weak, since an N-terminal deleted mutant, which contains 108–1162 aa, is localized in the cytoplasm (Ohsaki et al., 2009). The central region is composed of a proline-rich region, an aspartate (D)- and glutamate (E)-rich repetitious region, and a glutamine-rich domain (Garber et al., 2001; Piolot et al., 2001; **Figure 2**). DBD (923–1162 aa) is necessary and partially sufficient to support ori-P replication compared to the full-length LANA (Hu et al., 2002; Ohsaki et al., 2009), and when considering their expression levels in *in vitro* study.

Many studies show that LANA binds to LBS and recruits ORC to the origin as described above. What is the mechanism by which ORC is recruited to ori-P? One possible mechanism is that LANA directly interacts with ORC so that ORC is loaded to the origin (Lim et al., 2002; Stedman et al., 2004; Verma et al., 2006). If LANA could directly interact with ORC and recruit to the origin, we are confronted with this question: why is 32GC required for ori-P activity despite LANA's ability to bind to the ori-P? It could be that the 32GC is required to load ORC and the other pre-RC components to the region next to LBS, although the underlying molecular mechanism is not yet known. Further study is needed to resolve this question.

LANA is highly expressed in KSHV-related malignancies, and plays an essential in episomal maintenance. It interacts with multiple cellular proteins, including tumor suppressors such as p53 (Friborg et al., 1999) and Rb (Radkov et al., 2000), as well as transcription factors such as ATF4/CREB2 (Lim et al., 2000) and STAT3 (Muromoto et al., 2006), chromatin-associated proteins such as HP1 (Lim et al., 2003), histone H2A/B (Barbera et al., 2006b), MeCP2 (Krithivas et al., 2002; Matsumura et al., 2010), and Brd4 (Ottinger et al., 2006), in addition to signal transducers such as GSK-3b (Fujimuro and Hayward, 2003) and so on (**Figure 2**). Almost all of these proteins interact with the C-terminal domain of LANA, implying the functional importance of this domain. However, careful analysis of protein–protein interaction is required, because only DBD sometimes shows non-specific binding with other proteins and may exhibit different properties from the full-length LANA (our personal observation).

REGULATION OF KSHV ori-P ACTIVATION

The origin number varies from species to species. In mammals, it, 30,000–50,000 origins are thought to exist at each cell cycle (Huberman and Riggs, 1966). However, not all of these origins start DNA synthesis at the same time. Some origins are activated early in the S phase, whereas others are activated in the late S phase; that is, the DNA replication timing is controlled (Dimitrova and Gilbert, 1999; Cimbora et al., 2000). How is the timing of DNA replication determined? Some groups have provided possible answers to this question. It was reported that heterochromatin could change the timing of DNA replication by transgene insertion into a mammalian genome (Lin et al., 2003). Sir proteins, which are silencer proteins, can delay replication and correlate with transcriptional silencing (Zappulla et al., 2002). Thus, various studies suggest that heterochromatin modulates replication timing (Goren et al., 2008; Klochkov et al., 2009; Schwaiger et al., 2010) and EBV replication in latency occurs in mid-late S phase (Zhou et al., 2009). In the case of KSHV, the replication timing of the viral genome is not yet known. Considering that ori-P is present in the proximity of heterochromatin because of LANA-dependent accumulation of heterochromatin, DNA replication of the KSHV genome may start at the middle or late S phase.

The frequency of origin usage also differs from origin to origin; some origins are used in every cell cycle, whereas others are used rarely. Various studies including in *Drosophila*, *Xenopus,* and mammals demonstrate how specific origins are selected, but several

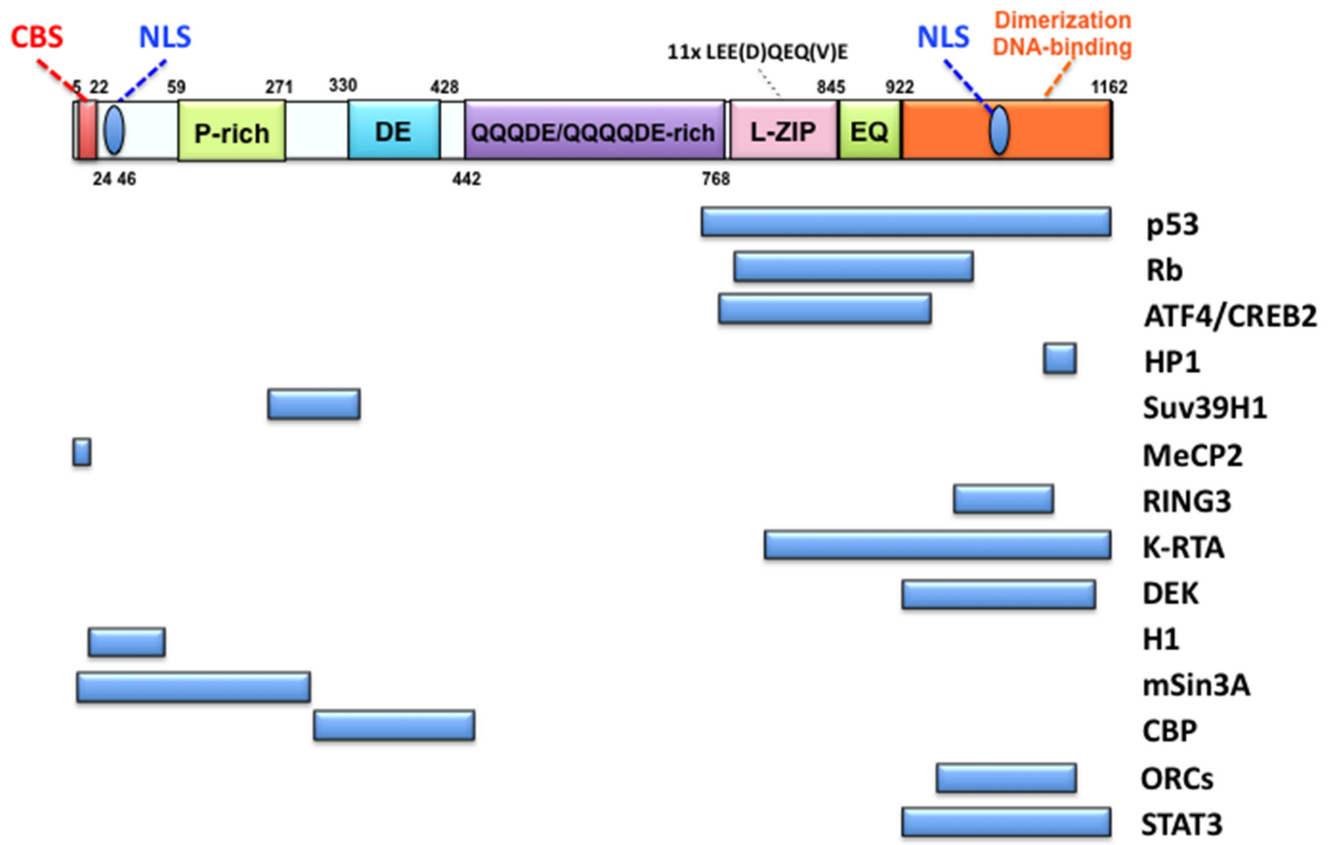

FIGURE 2 | Organization of LANA and its interacting proteins. The top Figure shows the organization of the LANA protein. LANA has two nuclear localization signals (NLS): one at the N-terminal region and the other at the C-terminal region. The former is a primary NLS and the latter is cryptic. A chromosome binding site (CBS) was reported to be present at the N-terminal end as a histone H1 binding region. The mid-part of LANA consists of a very peculiar amino acid composition called a proline-rich region (P-rich), an extremely aspartate (D)- and glutamate (E)-rich region (DE) and a glutamine (Q)-aspartate (D)-glutamate (E)-rich region (QQQDE/QQQQDE). LEE(D)QEQ(V)E looks like a leucine zipper but has not been recognized as a functional determinant. The C-terminal region has roles in both homo-dimerization and DNA binding to LBS. The main interacting proteins with LANA are listed below. The C-terminal region seems to be a region that interacts with many proteins.

questions about the decision mechanism of origins remain unanswered. Considering the genome size of this virus, one origin is enough to complete replication within the S phase; and because of the existence of "origin interference" by the ATR and ATM pathways (Shechter et al., 2004), a single origin must be chosen.

The micrococcal nuclease digestion pattern at TR in G1-arrested cells leads to change, suggesting that the chromatin structure became more accessible to enzymatic digestion (Stedman et al., 2004). Therefore, the chromatin structure may be changed by the recruitment of the replication machinery during the late G1 phase.

A recent study shows that the cellular deubiquitylating enzyme USP7 stimulates EBNA1 binding to its recognition sites so that histone modification at the EBV ori-P is changed by EBNA1 mediating the recruitment of USP7 (Sarkari et al., 2009).

THE ROLES OF NUCLEAR ARCHITECTURES

NUCLEAR MATRIX AS A SCAFFOLD FOR DNA REPLICATION AND TRANSCRIPTION

The nucleus consists of a well-organized structure and is highly complex. The structures of nuclear matrix proteins such as lamins, nuclear mitotic apparatus (NuMA), hnRNP, and so on, are important for the organization of chromatin, DNA replication, and transcription (Dechat et al., 2008). The nuclear matrix, isolated by Berezney and Coffey (1974), is believed to support the spatial distribution of several nuclear factors, such as DNA replication machinery and transcription factors. The nuclear matrix fraction contains DNase I-resistant and high salt-resistant proteins. Because the nuclear matrix can be visualized only after chromatin extraction, there has been a debate that such a nuclear matrix is an essential component of *in vivo* nuclear architectures. In previous studies, replication origins have come to the nuclear matrix (van der Velden et al., 1984; Amati and Gasser, 1990; Adom et al., 1992; Brylawski et al., 1993; Fallaux et al., 1996). Eukaryotic DNA is organized into DNA loops generated by the attachment of chromatin to the nuclear matrix via specific regions, referred to as scaffold/matrix attachment regions (Pardoll et al., 1980; Vogelstein et al., 1980; Laemmli et al., 1992; Roberge and Gasser, 1992). DNA loop formation is essential for DNA replication, transcription, and chromosomal packaging (Gasser and Laemmli, 1987; Berezney et al., 1995; Bode et al., 1995; Nickerson et al., 1995; Razin et al., 1995; Jackson, 1997; Volpi et al.,

2000; Mahy et al., 2002; Sumer et al., 2003; Marenduzzo et al., 2007).

In EBV, both the latent ori-P and the lytic ori-Lyt are bound to the nuclear matrix (Jankelevich et al., 1992; Mattia et al., 1999). EBV nuclear antigen leader protein (EBNA-LP) is a nuclear matrix-associated protein, and its nuclear matrix-targeting signal is a 10-aa segment, which also functions as a NLS (Yokoyama et al., 2001). However, this is not the case for LANA, because it was reported that C-terminal truncation up to 1128 aa could not be localized in a high salt-resistant fraction (nuclear matrix fraction), but the C-terminal region (1129–1143 aa) is high salt extractable (Viejo-Borbolla et al., 2003). We previously showed that the N-terminal region up to 107 aa is localized in nucleocytoplasmic and chromatin fractions (Ohsaki et al., 2009). The localization to the nuclear matrix fraction of LANA might depend on the conformation of LANA or post-translational modifications.

On the other hand, a cell fractionation assay has shown that cellular pre-RC components, such as ORC2, Cdc6, and Cdt1, preferentially localize in the nuclear matrix fraction in a cell cycle-dependent manner, and LANA itself also can localize in the nuclear matrix fraction. Accordingly, TR accumulates in the nuclear matrix fraction during the late G1 phase, suggesting that LANA recruits the ori-P to the nuclear matrix, so that cellular replication machinery is abundant and available for viral DNA replication during latency (Ohsaki et al., 2009; **Figure 3**).

GENOME SEGREGATION MECHANISMS OF KSHV IN LATENCY

The same copy number of KSHV genomes appears to be maintained in daughter cells after every cell division (Ballestas et al., 1999; Ueda et al., 2006), indicating that a strict genome maintenance mechanism is working. In the KSHV-infected PEL cell lines, LANA associates with a condensed mitotic chromatin (Ballestas et al., 1999; Cotter and Robertson, 1999; Tetsuka et al., 2004). It was reported that MeCP2, a methyl CpG-binding protein, interacts with the N-terminal of LANA and that DEK protein interacts with the C-terminal of LANA. These two independent interactions are involved in the tethering of LANA to chromosomes (Krithivas et al., 2002). Various studies have reported the interaction between LANA and multiple cellular proteins associated with chromatin.

Brd4, which is a member of the BET family that carries two bromodomains and associates with mitotic chromosomes, interacts with LANA on mitotic chromosomes (You et al., 2006). It has also been reported that core histones such as H2A and H2B are essential for LANA N-terminal chromosome binding (Barbera et al., 2006a,b). Furthermore, LANA interacts with Brd2/Ring3, which is a member of the BET family of double bromodomain-containing genes and contains two tandem bromodomains (Viejo-Borbolla et al., 2005).

Xiao et al. (2010) reported that LANA is associated with centromeres via the formation of complexes with Cenp-F and Bub1, which are kinetochore-associated proteins. This suggests that LANA preferentially interacts with kinetochore-associated proteins and that its association is critical for segregation into daughter cells. Though the interaction of LANA with kinetochore factors might interfere with correct spindle formation, it suggests that LANA should support viral genome segregation along with condensed chromatin.

A NuMA plays a critical role in the nuclear architecture in the interphase. After nuclear envelope breakdown in mitosis, NuMA is hyperphosphorylated by $p34^{cdc2}$ and is distributed at spindle poles, where it remains until the anaphase and plays an essential role in tethering spindle microtubules to each pole (Merdes et al., 1996; Gehmlich et al., 2004). Although NuMA drastically alters the localization and functions in the interphase and M phase, biochemical fractionation analysis shows that NuMA is localized in

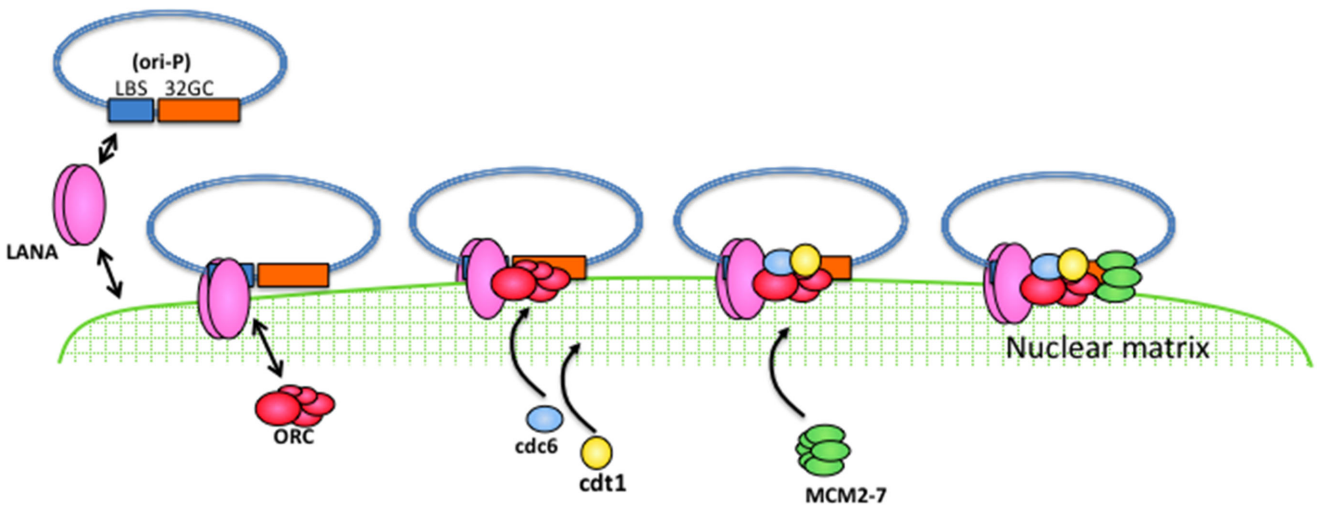

FIGURE 3 | A model for latent DNA replication of KSHV. LANA can associate with the nuclear matrix and can directly bind to the ori-P so that LANA can recruit the ori-P to the nuclear matrix. Cellular DNA replication machinery assembles to the nuclear matrix in a cell cycle-dependent manner, and is therefore available for viral DNA replication.

the insoluble nuclear matrix fraction in both phases. A recent study demonstrated that the C-terminus of LANA was co-localized with NuMA during the interphase and that the knockdown of NuMA expression caused the disruption of genome segregation and TR-containing plasmid maintenance (Bhaumik et al., 2008) and thus, KSHV genome segregation is disrupted in the absence of NuMA. Through siRNA and knockdown strategies in mice, NuMA has been shown to be an essential protein for early embryogenesis and cellular proliferation (Harborth et al., 2001; Silk et al., 2009), and it is thus unclear how the interaction with LANA actually works for viral genome segregation.

In either case, LANA has the capability of associating with various cellular proteins so that KSHV can maintain the genome stably if the cells are divided into two daughter cells. Nuclear matrix proteins function as a scaffold of DNA replication, transcription, and repair during the interphase, and also play an essential role in the segregation of condensed chromosomes in mitosis. Condensed chromosomes include, for example, NuMA, which can behave as a component of a spindle pole during mitosis, or Cenp-F, which is a nuclear matrix protein during the interphase and is distributed to kinetochores in mitosis. Taken together, the previous and present results suggest that nuclear architectures such as the nuclear matrix have essential roles not only in DNA replication and transcription but also in genome segregation during mitosis (**Figure 4**).

CONCLUSION AND PERSPECTIVES FOR FUTURE ANALYSIS

The importance of nuclear architecture is increasingly recognized as important in various nuclear events, such as DNA replication, transcription, and DNA repair. It is well accepted that the chromosomes are organized into distinct territories in the interphase. These distributions of chromosomes are closely related to the place for active or inactive transcription, the presence of DNA replication machinery, and the formation of higher-order structures of chromatin loops. DNA looping appears to be mediated by attachment to the nuclear matrix and thus achieves transcriptional control (Ostermeier et al., 2003). Other studies have suggested that the gene-rich chromosomes are frequently located in the nuclear interior. On the other hand, gene-poor chromosomes are located in the nuclear periphery (Tanabe et al., 2002; Reddy et al., 2008). A recent study shows that the transcriptional silencing might be accomplished by the binding of a specific promoter region to lamin type A (Lee et al., 2009). It is reported that replication foci at the middle to late S phase are also preferentially located toward the nuclear periphery, whereas early replication foci are located in the nuclear interior (Grasser et al., 2008) or throughout the nucleus (Izumi et al., 2004).

The spatial and temporal analyses using live-cell imaging revealed that replication forks are generated at the same origin and are closely associated during replication (Kitamura et al., 2006). Interestingly, two replication loci, located at the same distance from the origin, were in closer proximity when DNA replication took place at these loci, after which they moved apart from each other after replication. It is speculated that the replication factory anchors some region and that replicated DNA can move away from a replication factory immediately after DNA synthesis. This anchored region may be a nuclear scaffold such as the nuclear matrix.

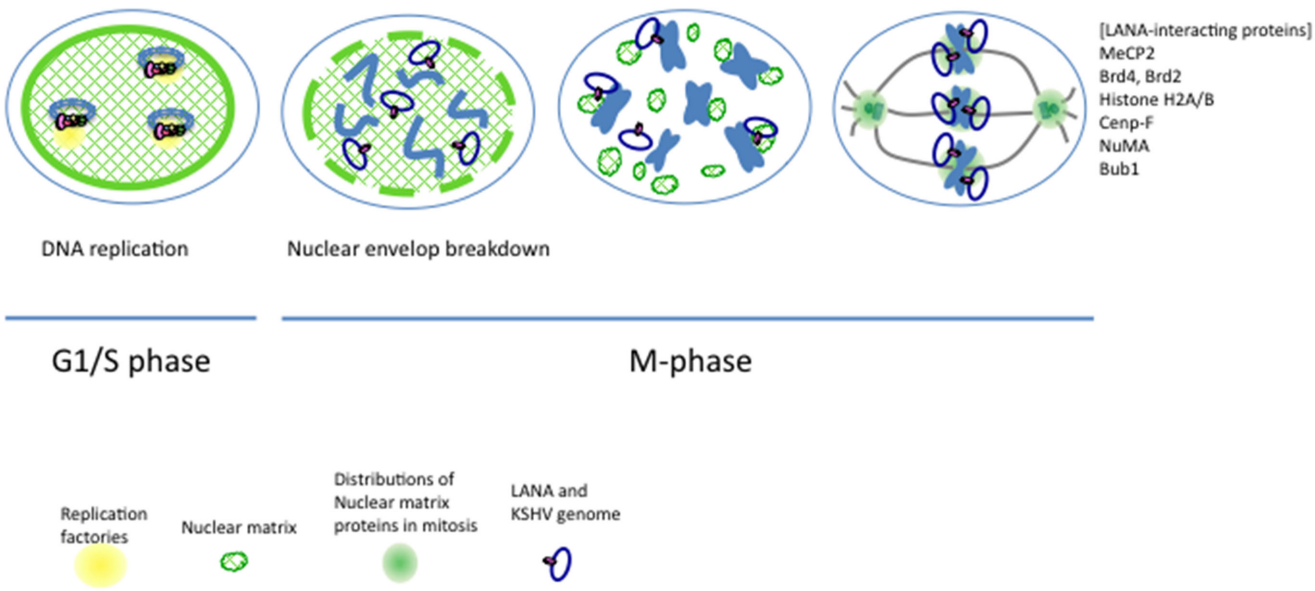

FIGURE 4 | A model for KSHV genome segregation. The KSHV genome associates with the nuclear matrix through LANA, which exhibits nuclear matrix localization activity. DNA replication factories are supposed to anchor to the nuclear matrix, and DNA replication of the KSHV genome occurs on the nuclear matrix at the G1/S phase. Nuclear architectures, including the nuclear matrix, are disrupted at the prophase (nuclear envelope breakdown), and nuclear matrix proteins drastically change these functions and distributions; such as spindle pole or kinetochore proteins. It has been reported that KSHV LANA interacts with various proteins such as MeCP2, Brd2, Brd4, Histone H2A/B, Cenp-F, NuMA, Bub1, and so on. These interactions can associate a sister-chromatid with LANA, and thus KSHV genomes can separate into two daughter cells.

The cellular environment is critical for viral survival, for which viruses make full use of the cellular machinery. The analysis of insoluble proteins, including nuclear matrix proteins, is difficult for the investigation of protein–protein and protein–DNA interactions *in vitro*, because the conditions of *in vitro* experiments always include soluble fractions. To overcome these problems, recent new approaches, such as genome-wide analysis using chromatin immunoprecipitation or live-cell imaging that reflects the *in vivo* environment, may be more powerful and accurate.

As for KSHV genome replication and maintenance, it would be first necessary to clarify how LANA is involved in both. Considering the real and actual cellular environments, we need to develop more powerful tools to know what the virus does in cells.

REFERENCES

Adom, J. N., Gouilleux, F., and Richard-Foy, H. (1992). Interaction with the nuclear matrix of a chimeric construct containing a replication origin and a transcription unit. *Biochim. Biophys. Acta* 1171, 187–197.

Aladjem, M. I. (2004). The mammalian beta globin origin of DNA replication. *Front. Biosci.* 9, 2540–2547.

Amati, B., and Gasser, S. M. (1990). *Drosophila* scaffold-attached regions bind nuclear scaffolds and can function as ARS elements in both budding and fission yeasts. *Mol. Cell. Biol.* 10, 5442–5454.

Arvanitakis, L., Mesri, E. A., Nador, R. G., Said, J. W., Asch, A. S., Knowles, D. M., and Cesarman, E. (1996). Establishment and characterization of a primary effusion (body cavity-based) lymphoma cell line (BC-3) harboring Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in the absence of Epstein-Barr virus. *Blood* 88, 2648–2654.

Baek, D., Villen, J., Shin, C., Camargo, F. D., Gygi, S. P., and Bartel, D. P. (2008). The impact of microRNAs on protein output. *Nature* 455, 64–71.

Ballestas, M. E., Chatis, P. A., and Kaye, K. M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. *Science* 284, 641–644.

Barbera, A. J., Chodaparambil, J. V., Kelley-Clarke, B., Joukov, V., Walter, J. C., Luger, K., and Kaye, K. M. (2006a). The nucleosomal surface as a docking station for Kaposi's sarcoma herpesvirus LANA. *Science* 311, 856–861.

Barbera, A. J., Chodaparambil, J. V., Kelley-Clarke, B., Luger, K., and Kaye, K. M. (2006b). Kaposi's sarcoma-associated herpesvirus LANA hitches a ride on the chromosome. *Cell Cycle* 5, 1048–1052.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297.

Bartel, D. P., and Chen, C. Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. *Nat. Rev. Genet.* 5, 396–400.

Bell, S. P., and Stillman, B. (1992). ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* 357, 128–134.

Bellare, P., and Ganem, D. (2009). Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation. *Cell Host Microbe* 6, 570–575.

Berezney, R., and Coffey, D. S. (1974). Identification of a nuclear protein matrix. *Biochem. Biophys. Res. Commun.* 60, 1410–1417.

Berezney, R., Mortillaro, M. J., Ma, H., Wei, X., and Samarabandu, J. (1995). The nuclear matrix: a structural milieu for genomic function. *Int. Rev. Cytol.* 162A, 1–65.

Bhaumik, D., Scott, G. K., Schokrpur, S., Patil, C. K., Campisi, J., and Benz, C. C. (2008). Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells. *Oncogene* 27, 5643–5647.

Bode, J., Schlake, T., Rios-Ramirez, M., Mielke, C., Stengert, M., Kay, V., and Klehr-Wirth, D. (1995). Scaffold/matrix-attached regions: structural properties creating transcriptionally active loci. *Int. Rev. Cytol.* 162A, 389–454.

Boshoff, C., and Chang, Y. (2001). Kaposi's sarcoma-associated herpesvirus: a new DNA tumor virus. *Annu. Rev. Med.* 52, 453–470.

Boss, I. W., Plaisance, K. B., and Renne, R. (2009). Role of virus-encoded microRNAs in herpesvirus biology. *Trends Microbiol.* 17, 544–553.

Brylawski, B. P., Tsongalis, G. J., Cordeiro-Stone, M., May, W. T., Comeau, L. D., and Kaufman, D. G. (1993). Association of putative origins of replication with the nuclear matrix in normal human fibroblasts. *Cancer Res.* 53, 3865–3868.

Byun, H., Gwack, Y., Hwang, S., and Choe, J. (2002). Kaposi's sarcoma-associated herpesvirus open reading frame (ORF) 50 transactivates K8 and ORF57 promoters via heterogeneous response elements. *Mol. Cells* 14, 185–191.

Cadoret, J. C., Meisch, F., Hassan-Zadeh, V., Luyten, I., Guillet, C., Duret, L., Quesneville, H., and Prioleau, M. N. (2008). Genome-wide studies highlight indirect links between human replication origins and gene regulation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 15837–15842.

Cai, X., and Cullen, B. R. (2006). Transcriptional origin of Kaposi's sarcoma-associated herpesvirus microRNAs. *J. Virol.* 80, 2234–2242.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B., and Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5570–5575.

Carbone, A., Cilia, A. M., Gloghini, A., Canzonieri, V., Pastore, C., Todesco, M., Cozzi, M., Perin, T., Volpe, R., Pinto, A., and Gaidano, G. (1997). Establishment of HHV-8-positive and HHV-8-negative lymphoma cell lines from primary lymphomatous effusions. *Int. J. Cancer* 73, 562–569.

Carbone, A., Cilia, A. M., Gloghini, A., Capello, D., Todesco, M., Quattrone, S., Volpe, R., and Gaidano, G. (1998). Establishment and characterization of EBV-positive and EBV-negative primary effusion lymphoma cell lines harbouring human herpesvirus type-8. *Br. J. Haematol.* 102, 1081–1089.

Carroll, P. A., Brazeau, E., and Lagunoff, M. (2004). Kaposi's sarcoma-associated herpesvirus infection of blood endothelial cells induces lymphatic differentiation. *Virology* 328, 7–18.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Cesarman, E., Nador, R. G., Aozasa, K., Delsol, G., Said, J. W., and Knowles, D. M. (1996). Kaposi's sarcoma-associated herpesvirus in non-AIDS related lymphomas occurring in body cavities. *Am. J. Pathol.* 149, 53–57.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266, 1865–1869.

Chang, Y., Moore, P. S., Talbot, S. J., Boshoff, C. H., Zarkowska, T., Godden, K., Paterson, H., Weiss, R. A., and Mittnacht, S. (1996). Cyclin encoded by KS herpesvirus. *Nature* 382, 410.

Chaudhary, P. M., Jasmin, A., Eby, M. T., and Hood, L. (1999). Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. *Oncogene* 18, 5738–5746.

Cimbora, D. M., Schubeler, D., Reik, A., Hamilton, J., Francastel, C., Epner, E. M., and Groudine, M. (2000). Long-distance control of origin choice and replication timing in the human beta-globin locus are independent of the locus control region. *Mol. Cell. Biol.* 20, 5581–5591.

Ciufo, D. M., Cannon, J. S., Poole, L. J., Wu, F. Y., Murray, P., Ambinder, R. F., and Hayward, G. S. (2001). Spindle cell conversion by Kaposi's sarcoma-associated herpesvirus: formation of colonies and plaques with mixed lytic and latent gene expression in infected primary dermal microvascular endothelial cell cultures. *J. Virol.* 75, 5614–5626.

Cotter, M. A. II, and Robertson, E. S. (1999). The latency-associated nuclear antigen tethers the Kaposi's sarcoma-associated herpesvirus genome to host chromosomes in body cavity-based lymphoma cells. *Virology* 264, 254–264.

Dai, J., Chuang, R. Y., and Kelly, T. J. (2005). DNA replication origins in the *Schizosaccharomyces pombe* genome. *Proc. Natl. Acad. Sci. U.S.A.* 102, 337–342.

Dechat, T., Pfleghaar, K., Sengupta, K., Shimi, T., Shumaker, D. K., Solimando, L., and Goldman, R. D. (2008). Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin. *Genes Dev.* 22, 832–853.

DePamphilis, M. L. (2003). The 'ORC cycle': a novel pathway for regulating eukaryotic DNA replication. *Gene* 310, 1–15.

DePamphilis, M. L. (2005). Cell cycle dependent regulation of the origin recognition complex. *Cell Cycle* 4, 70–79.

Dimitrova, D. S., and Gilbert, D. M. (1999). The spatial position and replication timing of chromosomal domains are both established in early G1 phase. *Mol. Cell* 4, 983–993.

Dourmishev, L. A., Dourmishev, A. L., Palmeri, D., Schwartz, R. A., and Lukac, D. M. (2003). Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. *Microbiol. Mol. Biol. Rev.* 67, 175–212.

Dupin, N., Gorin, I., Deleuze, J., Agut, H., Huraux, J. M., and Escande, J. P. (1995). Herpes-like DNA sequences, AIDS-related tumors, and Castleman's disease. *N. Engl. J. Med.* 333, 798.

Fallaux, F. J., Hoeben, R. C., Cramer, S. J., Van Den Wollenberg, D. J., Briet, E., Van Ormondt, H., and Van Der Eb, A. J. (1996). The human clotting factor VIII cDNA contains an autonomously replicating sequence consensus- and matrix attachment region-like sequence that binds a nuclear factor, represses heterologous gene expression, and mediates the transcriptional effects of sodium butyrate. *Mol. Cell. Biol.* 16, 4264–4272.

Fejer, G., Medveczky, M. M., Horvath, E., Lane, B., Chang, Y., and Medveczky, P. G. (2003). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus interacts preferentially with the terminal repeats of the genome in vivo and this complex is sufficient for episomal DNA replication. *J. Gen. Virol.* 84, 1451–1462.

Field, N., Low, W., Daniels, M., Howell, S., Daviet, L., Boshoff, C., and Collins, M. (2003). KSHV vFLIP binds to IKK-gamma to activate IKK. *J. Cell. Sci.* 116, 3721–3728.

Friborg, J. Jr., Kong, W., Hottiger, M. O., and Nabel, G. J. (1999). p53 Inhibition by the LANA protein of KSHV protects against cell death. *Nature* 402, 889–894.

Frizzera, G., Banks, P. M., Massarelli, G., and Rosai, J. (1983). A systemic lymphoproliferative disorder with morphologic features of Castleman's disease. Pathological findings in 15 patients. *Am. J. Surg. Pathol.* 7, 211–231.

Fujimuro, M., and Hayward, S. D. (2003). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus manipulates the activity of glycogen synthase kinase-3beta. *J. Virol.* 77, 8019–8030.

Gaidano, G., Cechova, K., Chang, Y., Moore, P. S., Knowles, D. M., and Dalla-Favera, R. (1996). Establishment of AIDS-related lymphoma cell lines from lymphomatous effusions. *Leukemia* 10, 1237–1240.

Ganem, D., and Ziegelbauer, J. (2008). MicroRNAs of Kaposi's sarcoma-associated herpes virus. *Semin. Cancer Biol.* 18, 437–440.

Gao, S. J., Deng, J. H., and Zhou, F. C. (2003). Productive lytic replication of a recombinant Kaposi's sarcoma-associated herpesvirus in efficient primary infection of primary human endothelial cells. *J. Virol.* 77, 9738–9749.

Garber, A. C., Hu, J., and Renne, R. (2002). Latency-associated nuclear antigen (LANA) cooperatively binds to two sites within the terminal repeat, and both sites contribute to the ability of LANA to suppress transcription and to facilitate DNA replication. *J. Biol. Chem.* 277, 27401–27411.

Garber, A. C., Shu, M. A., Hu, J., and Renne, R. (2001). DNA binding and modulation of gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 75, 7882–7892.

Gasser, S. M., and Laemmli, U. K. (1987). Improved methods for the isolation of individual and clustered mitotic chromosomes. *Exp. Cell Res.* 173, 85–98.

Gehmlich, K., Haren, L., and Merdes, A. (2004). Cyclin B degradation leads to NuMA release from dynein/dynactin and from spindle poles. *EMBO Rep.* 5, 97–103.

Gomez-Roman, J. J., Sanchez-Velasco, P., Ocejo-Vinyals, G., Hernandez-Nieto, E., Leyva-Cobian, F., and Val-Bernal, J. F. (2001). Human herpesvirus-8 genes are expressed in pulmonary inflammatory myofibroblastic tumor (inflammatory pseudotumor). *Am. J. Surg. Pathol.* 25, 624–629.

Goren, A., Tabib, A., Hecht, M., and Cedar, H. (2008). DNA replication timing of the human beta-globin domain is controlled by histone modification at the origin. *Genes Dev.* 22, 1319–1324.

Gottwein, E., and Cullen, B. R. (2010). A human herpesvirus microRNA inhibits p21 expression and attenuates p21-mediated cell cycle arrest. *J. Virol.* 84, 5229–5237.

Gottwein, E., Mukherjee, N., Sachse, C., Frenzel, C., Majoros, W. H., Chi, J. T., Braich, R., Manoharan, M., Soutschek, J., Ohler, U., and Cullen, B. R. (2007). A viral microRNA functions as an orthologue of cellular miR-155. *Nature* 450, 1096–1099.

Grasser, F., Neusser, M., Fiegler, H., Thormeyer, T., Cremer, M., Carter, N. P., Cremer, T., and Muller, S. (2008). Replication-timing-correlated spatial chromatin arrangements in cancer and in primate interphase nuclei. *J. Cell. Sci.* 121, 1876–1886.

Grossmann, C., and Ganem, D. (2008). Effects of NFkappaB activation on KSHV latency and lytic reactivation are complex and context-dependent. *Virology* 375, 94–102.

Grundhoff, A., and Ganem, D. (2003). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus permits replication of terminal repeat-containing plasmids. *J. Virol.* 77, 2779–2783.

Gunther, T., and Grundhoff, A. (2010). The epigenetic landscape of latent Kaposi sarcoma-associated herpesvirus genomes. *PLoS Pathog.* 6, e1000935. doi:10.1371/journal.ppat.1000935

Han, S. J., Hu, J., Pierce, B., Weng, Z., and Renne, R. (2010). Mutational analysis of the latency-associated nuclear antigen DNA-binding domain of Kaposi's sarcoma-associated herpesvirus reveals structural conservation among gammaherpesvirus origin-binding proteins. *J. Gen. Virol.* 91, 2203–2215.

Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T., and Weber, K. (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J. Cell. Sci.* 114, 4557–4565.

Harrison, S., Fisenne, K., and Hearing, J. (1994). Sequence requirements of the Epstein-Barr virus latent origin of DNA replication. *J. Virol.* 68, 1913–1925.

Heichinger, C., Penkett, C. J., Bahler, J., and Nurse, P. (2006). Genome-wide characterization of fission yeast DNA replication origins. *EMBO J.* 25, 5171–5179.

Hong, Y. K., Foreman, K., Shin, J. W., Hirakawa, S., Curry, C. L., Sage, D. R., Libermann, T., Dezube, B. J., Fingeroth, J. D., and Detmar, M. (2004). Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. *Nat. Genet.* 36, 683–685.

Hou, C., Zhao, H., Tanimoto, K., and Dean, A. (2008). CTCF-dependent enhancer-blocking by alternative chromatin loop formation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20398–20403.

Hu, J., Garber, A. C., and Renne, R. (2002). The latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus supports latent DNA replication in dividing cells. *J. Virol.* 76, 11677–11687.

Hu, J., and Renne, R. (2005). Characterization of the minimal replicator of Kaposi's sarcoma-associated herpesvirus latent origin. *J. Virol.* 79, 2637–2642.

Huang, Y. Q., Li, J. J., Kaplan, M. H., Poiesz, B., Katabira, E., Zhang, W. C., Feiner, D., and Friedman-Kien, A. E. (1995). Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. *Lancet* 345, 759–761.

Huberman, J. A., and Riggs, A. D. (1966). Autoradiography of chromosomal DNA fibers from Chinese hamster cells. *Proc. Natl. Acad. Sci. U.S.A.* 55, 599–606.

Izumi, M., Yatagai, F., and Hanaoka, F. (2004). Localization of human Mcm10 is spatially and temporally regulated during the S phase. *J. Biol. Chem.* 279, 32569–32577.

Jackson, D. A. (1997). Chromatin domains and nuclear compartments: establishing sites of gene expression in eukaryotic nuclei. *Mol. Biol. Rep.* 24, 209–220.

Jankelevich, S., Kolman, J. L., Bodnar, J. W., and Miller, G. (1992). A nuclear matrix attachment region organizes the Epstein-Barr viral plasmid in Raji cells into a single DNA domain. *EMBO J.* 11, 1165–1176.

Kang, H., and Lieberman, P. M. (2009). Cell cycle control of Kaposi's sarcoma-associated herpesvirus latency transcription by CTCF-cohesin interactions. *J. Virol.* 83, 6199–6210.

Katano, H., Hoshino, Y., Morishita, Y., Nakamura, T., Satoh, H., Iwamoto, A., Herndier, B., and Mori, S. (1999). Establishing and characterizing a CD30-positive cell line harboring HHV-8 from a primary effusion lymphoma. *J. Med. Virol.* 58, 394–401.

Katano, H., Sato, Y., Kurata, T., Mori, S., and Sata, T. (2000). Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. *Virology* 269, 335–344.

Kitamura, E., Blow, J. J., and Tanaka, T. U. (2006). Live-cell imaging reveals replication of individual replicons in eukaryotic replication factories. *Cell* 125, 1297–1308.

Klochkov, D. B., Gavrilov, A. A., Vassetzky, Y. S., and Razin, S. V. (2009). Early replication timing of the

chicken alpha-globin gene domain correlates with its open chromatin state in cells of different lineages. *Genomics* 93, 481–486.

Koons, M. D., Van Scoy, S., and Hearing, J. (2001). The replicator of the Epstein-Barr virus latent cycle origin of DNA replication, oriP, is composed of multiple functional elements. *J. Virol.* 75, 10582–10592.

Krithivas, A., Fujimuro, M., Weidner, M., Young, D. B., and Hayward, S. D. (2002). Protein interactions targeting the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus to cell chromosomes. *J. Virol.* 76, 11596–11604.

Krysan, P. J., Haase, S. B., and Calos, M. P. (1989). Isolation of human sequences that replicate autonomously in human cells. *Mol. Cell. Biol.* 9, 1026–1033.

Laemmli, U. K., Kas, E., Poljak, L., and Adachi, Y. (1992). Scaffold-associated regions: cis-acting determinants of chromatin structural loops and functional domains. *Curr. Opin. Genet. Dev.* 2, 275–285.

Lan, K., Kuppers, D. A., and Robertson, E. S. (2005a). Kaposi's sarcoma-associated herpesvirus reactivation is regulated by interaction of latency-associated nuclear antigen with recombination signal sequence-binding protein Jkappa, the major downstream effector of the Notch signaling pathway. *J. Virol.* 79, 3468–3478.

Lan, K., Kuppers, D. A., Verma, S. C., Sharma, N., Murakami, M., and Robertson, E. S. (2005b). Induction of Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen by the lytic transactivator RTA: a novel mechanism for establishment of latency. *J. Virol.* 79, 7453–7465.

Lee, D. C., Welton, K. L., Smith, E. D., and Kennedy, B. K. (2009). A-type nuclear lamins act as transcriptional repressors when targeted to promoters. *Exp. Cell Res.* 315, 996–1007.

Lei, X., Bai, Z., Ye, F., Huang, Y., and Gao, S. J. (2010a). Regulation of herpesvirus lifecycle by viral microRNAs. *Virulence* 1, 433–435.

Lei, X., Bai, Z., Ye, F., Xie, J., Kim, C. G., Huang, Y., and Gao, S. J. (2010b). Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. *Nat. Cell Biol.* 12, 193–199.

Liang, Y., Chang, J., Lynch, S. J., Lukac, D. M., and Ganem, D. (2002). The lytic switch protein of KSHV activates gene expression via functional interaction with RBP-Jkappa (CSL), the target of the Notch signaling pathway. *Genes Dev.* 16, 1977–1989.

Lim, C., Lee, D., Seo, T., Choi, C., and Choe, J. (2003). Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus functionally interacts with heterochromatin protein 1. *J. Biol. Chem.* 278, 7397–7405.

Lim, C., Sohn, H., Gwack, Y., and Choe, J. (2000). Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) binds ATF4/CREB2 and inhibits its transcriptional activation activity. *J. Gen. Virol.* 81, 2645–2652.

Lim, C., Sohn, H., Lee, D., Gwack, Y., and Choe, J. (2002). Functional dissection of latency-associated nuclear antigen 1 of Kaposi's sarcoma-associated herpesvirus involved in latent DNA replication and transcription of terminal repeats of the viral genome. *J. Virol.* 76, 10320–10331.

Lin, C. M., Fu, H., Martinovsky, M., Bouhassira, E., and Aladjem, M. I. (2003). Dynamic alterations of replication timing in mammalian cells. *Curr. Biol.* 13, 1019–1028.

Lin, X., Liang, D., He, Z., Deng, Q., Robertson, E. S., and Lan, K. (2011). miR-K12-7-5p encoded by Kaposi's sarcoma-associated herpesvirus stabilizes the latent state by targeting viral ORF50/RTA. *PLoS ONE* 6, e16224. doi:10.1371/journal.pone.0016224

Lu, F., Zhou, J., Wiedmer, A., Madden, K., Yuan, Y., and Lieberman, P. M. (2003). Chromatin remodeling of the Kaposi's sarcoma-associated herpesvirus ORF50 promoter correlates with reactivation from latency. *J. Virol.* 77, 11425–11435.

Lubyova, B., and Pitha, P. M. (2000). Characterization of a novel human herpesvirus 8-encoded protein, vIRF-3, that shows homology to viral and cellular interferon regulatory factors. *J. Virol.* 74, 8194–8201.

Mahy, N. L., Perry, P. E., Gilchrist, S., Baldock, R. A., and Bickmore, W. A. (2002). Spatial organization of active and inactive genes and noncoding DNA within chromosome territories. *J. Cell Biol.* 157, 579–589.

Marechal, V., Dehee, A., Chikhi-Brachet, R., Piolot, T., Coppey-Moisan, M., and Nicolas, J. C. (1999). Mapping EBNA-1 domains involved in binding to metaphase chromosomes. *J. Virol.* 73, 4385–4392.

Marenduzzo, D., Faro-Trindade, I., and Cook, P. R. (2007). What are the molecular ties that maintain genomic loops? *Trends Genet.* 23, 126–133.

Matsumura, S., Persson, L. M., Wong, L., and Wilson, A. C. (2010). The latency-associated nuclear antigen interacts with MeCP2 and nucleosomes through separate domains. *J. Virol.* 84, 2318–2330.

Matta, H., and Chaudhary, P. M. (2004). Activation of alternative NF-kappa B pathway by human herpes virus 8-encoded Fas-associated death domain-like IL-1 beta-converting enzyme inhibitory protein (vFLIP). *Proc. Natl. Acad. Sci. U.S.A.* 101, 9399–9404.

Matta, H., Mazzacurati, L., Schamus, S., Yang, T., Sun, Q., and Chaudhary, P. M. (2007). Kaposi's sarcoma-associated herpesvirus (KSHV) oncoprotein K13 bypasses TRAFs and directly interacts with the IkappaB kinase complex to selectively activate NF-kappaB without JNK activation. *J. Biol. Chem.* 282, 24858–24865.

Matta, H., Sun, Q., Moses, G., and Chaudhary, P. M. (2003). Molecular genetic analysis of human herpes virus 8-encoded viral FLICE inhibitory protein-induced NF-kappaB activation. *J. Biol. Chem.* 278, 52406–52411.

Mattia, E., Ceridono, M., Chichiarelli, S., and D'erme, M. (1999). Interactions of Epstein-Barr virus origins of replication with nuclear matrix in the latent and in the lytic phases of viral infection. *Virology* 262, 9–17.

Mechali, M. (2010). Eukaryotic DNA replication origins: many choices for appropriate answers. *Nat. Rev. Mol. Cell Biol.* 11, 728–738.

Merdes, A., Ramyar, K., Vechio, J. D., and Cleveland, D. W. (1996). A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. *Cell* 87, 447–458.

Minarovits, J. (2006). Epigenotypes of latent herpesvirus genomes. *Curr. Top. Microbiol. Immunol.* 310, 61–80.

Moore, P. S., and Chang, Y. (1995). Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and without HIV infection. *N. Engl. J. Med.* 332, 1181–1185.

Moore, P. S., and Chang, Y. (2001). Molecular virology of Kaposi's sarcoma-associated herpesvirus. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 356, 499–516.

Muralidhar, S., Pumfery, A. M., Hassani, M., Sadaie, M. R., Kishishita, M., Brady, J. N., Doniger, J., Medveczky, P., and Rosenthal, L. J. (1998). Identification of kaposin (open reading frame K12) as a human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) transforming gene. *J. Virol.* 72, 4980–4988.

Muromoto, R., Okabe, K., Fujimuro, M., Sugiyama, K., Yokosawa, H., Seya, T., and Matsuda, T. (2006). Physical and functional interactions between STAT3 and Kaposi's sarcoma-associated herpesvirus-encoded LANA. *FEBS Lett.* 580, 93–98.

Murphy, E., Vanicek, J., Robins, H., Shenk, T., and Levine, A. J. (2008). Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5453–5458.

Naranatt, P. P., Krishnan, H. H., Svojanovsky, S. R., Bloomer, C., Mathur, S., and Chandran, B. (2004). Host gene induction and transcriptional reprogramming in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8)-infected endothelial, fibroblast, and B cells: insights into modulation events early during infection. *Cancer Res.* 64, 72–84.

Nickerson, J. A., Blencowe, B. J., and Penman, S. (1995). The architectural organization of nuclear metabolism. *Int. Rev. Cytol.* 162A, 67–123.

Nishimura, K., Ueda, K., Sakakibara, S., Ishikawa, K., Chen, J., Okuno, T., and Yamanishi, K. (2001). Functional analysis of Kaposi's sarcoma-associated herpesvirus RTA in an RTA-depressed cell line. *J. Hum. Virol.* 4, 296–305.

Nishitani, H., and Lygerou, Z. (2002). Control of DNA replication licensing in a cell cycle. *Genes Cells* 7, 523–534.

Norseen, J., Johnson, F. B., and Lieberman, P. M. (2009). Role for G-quadruplex RNA binding by Epstein-Barr virus nuclear antigen 1 in DNA replication and metaphase chromosome attachment. *J. Virol.* 83, 10336–10346.

Ohsaki, E., Suzuki, T., Karayama, M., and Ueda, K. (2009). Accumulation of LANA at nuclear matrix fraction is important for Kaposi's sarcoma-associated herpesvirus replication in latency. *Virus Res.* 139, 74–84.

Ohsaki, E., Ueda, K., Sakakibara, S., Do, E., Yada, K., and Yamanishi, K. (2004). Poly(ADP-ribose) polymerase 1 binds to Kaposi's sarcoma-associated herpesvirus (KSHV) terminal repeat sequence and modulates KSHV replication in latency. *J. Virol.* 78, 9936–9946.

Okuno, Y., Satoh, H., Sekiguchi, M., and Masukata, H. (1999). Clustered adenine/thymine stretches are essential

for function of a fission yeast replication origin. *Mol. Cell. Biol.* 19, 6699–6709.

Ostermeier, G. C., Liu, Z., Martins, R. P., Bharadwaj, R. R., Ellis, J., Draghici, S., and Krawetz, S. A. (2003). Nuclear matrix association of the human beta-globin locus utilizing a novel approach to quantitative real-time PCR. *Nucleic Acids Res.* 31, 3257–3266.

Ottinger, M., Christalla, T., Nathan, K., Brinkmann, M. M., Viejo-Borbolla, A., and Schulz, T. F. (2006). Kaposi's sarcoma-associated herpesvirus LANA-1 interacts with the short variant of BRD4 and releases cells from a BRD4- and BRD2/RING3-induced G1 cell cycle arrest. *J. Virol.* 80, 10772–10786.

Pan, H., Zhou, F., and Gao, S. J. (2004). Kaposi's sarcoma-associated herpesvirus induction of chromosome instability in primary human endothelial cells. *Cancer Res.* 64, 4064–4068.

Pardoll, D. M., Vogelstein, B., and Coffey, D. S. (1980). A fixed site of DNA replication in eucaryotic cells. *Cell* 19, 527–536.

Parravicini, C., Chandran, B., Corbellino, M., Berti, E., Paulli, M., Moore, P. S., and Chang, Y. (2000). Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. *Am. J. Pathol.* 156, 743–749.

Pearce, M., Matsumura, S., and Wilson, A. C. (2005). Transcripts encoding K12, v-FLIP, v-cyclin, and the microRNA cluster of Kaposi's sarcoma-associated herpesvirus originate from a common promoter. *J. Virol.* 79, 14457–14464.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., Van Dyk, L. F., Ho, C. K., Shuman, S., Chien, M., Russo, J. J., Ju, J., Randall, G., Lindenbach, B. D., Rice, C. M., Simon, V., Ho, D. D., Zavolan, M., and Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nat. Methods* 2, 269–276.

Piolot, T., Tramier, M., Coppey, M., Nicolas, J. C., and Marechal, V. (2001). Close but distinct regions of human herpesvirus 8 latency-associated nuclear antigen 1 are responsible for nuclear targeting and binding to human mitotic chromosomes. *J. Virol.* 75, 3948–3959.

Potthoff, A., Brockmeyer, N. H., Stucker, M., Wieland, U., Kreuter, A., and Competence Network, H. A. (2010). Kaposi sarcoma in a HIV uninfected man who has sex with men. *Eur. J. Med. Res.* 15, 79–80.

Qian, L. W., Greene, W., Ye, F., and Gao, S. J. (2008). Kaposi's sarcoma-associated herpesvirus disrupts adherens junctions and increases endothelial permeability by inducing degradation of VE-cadherin. *J. Virol.* 82, 11902–11912.

Qian, L. W., Xie, J., Ye, F., and Gao, S. J. (2007). Kaposi's sarcoma-associated herpesvirus infection promotes invasion of primary human umbilical vein endothelial cells by inducing matrix metalloproteinases. *J. Virol.* 81, 7001–7010.

Radkov, S. A., Kellam, P., and Boshoff, C. (2000). The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. *Nat. Med.* 6, 1121–1127.

Razin, S. V., Gromova, I. I., and Iarovaia, O. V. (1995). Specificity and functional significance of DNA interaction with the nuclear matrix: new approaches to clarify the old questions. *Int. Rev. Cytol.* 162B, 405–448.

Reddy, K. L., Zullo, J. M., Bertolino, E., and Singh, H. (2008). Transcriptional repression mediated by repositioning of genes to the nuclear lamina. *Nature* 452, 243–247.

Reisman, D., Yates, J., and Sugden, B. (1985). A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. *Mol. Cell. Biol.* 5, 1822–1832.

Renne, R., Zhong, W., Herndier, B., Mcgrath, M., Abbey, N., Kedes, D., and Ganem, D. (1996). Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat. Med.* 2, 342–346.

Ritzi, M., Tillack, K., Gerhardt, J., Ott, E., Humme, S., Kremmer, E., Hammerschmidt, W., and Schepers, A. (2003). Complex protein-DNA dynamics at the latent origin of DNA replication of Epstein-Barr virus. *J. Cell. Sci.* 116, 3971–3984.

Rivas, C., Thlick, A. E., Parravicini, C., Moore, P. S., and Chang, Y. (2001). Kaposi's sarcoma-associated herpesvirus LANA2 is a B-cell-specific latent viral protein that inhibits p53. *J. Virol.* 75, 429–438.

Roberge, M., and Gasser, S. M. (1992). DNA loops: structural and functional properties of scaffold-attached regions. *Mol. Microbiol.* 6, 419–423.

Sadagopan, S., Sharma-Walia, N., Veettil, M. V., Raghu, H., Sivakumar, R., Bottero, V., and Chandran, B. (2007). Kaposi's sarcoma-associated herpesvirus induces sustained NF-kappaB activation during de novo infection of primary human dermal microvascular endothelial cells that is essential for viral gene expression. *J. Virol.* 81, 3949–3968.

Said, J. W., Tasaka, T., Takeuchi, S., Asou, H., De Vos, S., Cesarman, E., Knowles, D. M., and Koeffler, H. P. (1996). Primary effusion lymphoma in women: report of two cases of Kaposi's sarcoma herpes virus-associated effusion-based lymphoma in human immunodeficiency virus-negative women. *Blood* 88, 3124–3128.

Sakakibara, S., Ueda, K., Chen, J., Okuno, T., and Yamanishi, K. (2001). Octamer-binding sequence is a key element for the autoregulation of Kaposi's sarcoma-associated herpesvirus ORF50/Lyta gene expression. *J. Virol.* 75, 6894–6900.

Sakakibara, S., Ueda, K., Nishimura, K., Do, E., Ohsaki, E., Okuno, T., and Yamanishi, K. (2004). Accumulation of heterochromatin components on the terminal repeat sequence of Kaposi's sarcoma-associated herpesvirus mediated by the latency-associated nuclear antigen. *J. Virol.* 78, 7299–7310.

Samols, M. A., Hu, J., Skalsky, R. L., and Renne, R. (2005). Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79, 9301–9305.

Samols, M. A., Skalsky, R. L., Maldonado, A. M., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Identification of cellular genes targeted by KSHV-encoded microRNAs. *PLoS Pathog.* 3, e65. doi:10.1371/journal.ppat.0030065

Sarkari, F., Sanchez-Alcaraz, T., Wang, S., Holowaty, M. N., Sheng, Y., and Frappier, L. (2009). EBNA1-mediated recruitment of a histone H2B deubiquitylating complex to the Epstein-Barr virus latent origin of DNA replication. *PLoS Pathog.* 5, e1000624. doi:10.1371/journal.ppat.1000624

Schwaiger, M., Kohler, H., Oakeley, E. J., Stadler, M. B., and Schubeler, D. (2010). Heterochromatin protein 1 (HP1) modulates replication timing of the Drosophila genome. *Genome Res.* 20, 771–780.

Segurado, M., De Luis, A., and Antequera, F. (2003). Genome-wide distribution of DNA replication origins at A+T-rich islands in *Schizosaccharomyces pombe*. *EMBO Rep.* 4, 1048–1053.

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. *Nature* 455, 58–63.

Sequeira-Mendes, J., Diaz-Uriarte, R., Apedaile, A., Huntley, D., Brockdorff, N., and Gomez, M. (2009). Transcription initiation activity sets replication origin efficiency in mammalian cells. *PLoS Genet.* 5, e1000446. doi:10.1371/journal.pgen.1000446

Sharma-Walia, N., Raghu, H., Sadagopan, S., Sivakumar, R., Veettil, M. V., Naranatt, P. P., Smith, M. M., and Chandran, B. (2006). Cyclooxygenase 2 induced by Kaposi's sarcoma-associated herpesvirus early during in vitro infection of target cells plays a role in the maintenance of latent viral gene expression. *J. Virol.* 80, 6534–6552.

Shechter, D., Costanzo, V., and Gautier, J. (2004). ATR and ATM regulate the timing of DNA replication origin firing. *Nat. Cell Biol.* 6, 648–655.

Silk, A. D., Holland, A. J., and Cleveland, D. W. (2009). Requirements for NuMA in maintenance and establishment of mammalian spindle poles. *J. Cell Biol.* 184, 677–690.

Skalsky, R. L., Samols, M. A., Plaisance, K. B., Boss, I. W., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. *J. Virol.* 81, 12836–12845.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and Sigaux, F. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86, 1276–1280.

Staudt, M. R., and Dittmer, D. P. (2003). Viral latent proteins as targets for Kaposi's sarcoma and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) induced lymphoma. *Curr. Drug Targets Infect. Disord.* 3, 129–135.

Stedman, W., Deng, Z., Lu, F., and Lieberman, P. M. (2004). ORC, MCM, and histone hyperacetylation at the Kaposi's sarcoma-associated herpesvirus latent replication origin. *J. Virol.* 78, 12566–12575.

Stedman, W., Kang, H., Lin, S., Kissil, J. L., Bartolomei, M. S., and Lieberman, P. M. (2008). Cohesins localize with CTCF at the KSHV latency control region and at cellular c-myc and H19/Igf2 insulators. *EMBO J.* 27, 654–666.

Stinchcomb, D. T., Struhl, K., and Davis, R. W. (1979). Isolation and characterisation of a yeast chromosomal replicator. *Nature* 282, 39–43.

Sumer, H., Craig, J. M., Sibson, M., and Choo, K. H. (2003). A rapid method of genomic array analysis of scaffold/matrix attachment regions (S/MARs) identifies a 2.5-Mb region of enhanced scaffold/matrix attachment at a human neocentromere. *Genome Res.* 13, 1737–1743.

Tanabe, H., Habermann, F. A., Solovei, I., Cremer, M., and Cremer, T. (2002). Non-random radial arrangements of interphase chromosome territories: evolutionary considerations and functional implications. *Mutat. Res.* 504, 37–45.

Tanimoto, K., Sugiura, A., Omori, A., Felsenfeld, G., Engel, J. D., and Fukamizu, A. (2003). Human beta-globin locus control region HS5 contains CTCF- and developmental stage-dependent enhancer-blocking activity in erythroid cells. *Mol. Cell. Biol.* 23, 8946–8952.

Tetsuka, T., Higuchi, M., Fukushi, M., Watanabe, A., Takizawa, S., Oie, M., Gejyo, F., and Fujii, M. (2004). Visualization of a functional KSHV episome-maintenance protein LANA in living cells. *Virus Genes* 29, 175–182.

Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J. L., Schroter, M., Scaffidi, C., Krammer, P. H., Peter, M. E., and Tschopp, J. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 386, 517–521.

Toth, Z., Maglinte, D. T., Lee, S. H., Lee, H. R., Wong, L. Y., Brulois, K. F., Lee, S., Buckley, J. D., Laird, P. W., Marquez, V. E., and Jung, J. U. (2010). Epigenetic analysis of KSHV latent and lytic genomes. *PLoS Pathog.* 6, e1001013. doi:10.1371/journal.ppat.1001013

Ueda, K., Ishikawa, K., Nishimura, K., Sakakibara, S., Do, E., and Yamanishi, K. (2002). Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) replication and transcription factor activates the K9 (vIRF) gene through two distinct cis elements by a non-DNA-binding mechanism. *J. Virol.* 76, 12044–12054.

Ueda, K., Sakakibara, S., Ohsaki, E., and Yada, K. (2006). Lack of a mechanism for faithful partition and maintenance of the KSHV genome. *Virus Res.* 122, 85–94.

van der Velden, H. M., Van Willigen, G., Wetzels, R. H., and Wanka, F. (1984). Attachment of origins of replication to the nuclear matrix and the chromosomal scaffold. *FEBS Lett.* 171, 13–16.

Verma, S. C., Choudhuri, T., Kaul, R., and Robertson, E. S. (2006). Latency-associated nuclear antigen (LANA) of Kaposi's sarcoma-associated herpesvirus interacts with origin recognition complexes at the LANA binding sequence within the terminal repeats. *J. Virol.* 80, 2243–2256.

Viejo-Borbolla, A., Kati, E., Sheldon, J. A., Nathan, K., Mattsson, K., Szekely, L., and Schulz, T. F. (2003). A Domain in the C-terminal region of latency-associated nuclear antigen 1 of Kaposi's sarcoma-associated Herpesvirus affects transcriptional activation and binding to nuclear heterochromatin. *J. Virol.* 77, 7093–7100.

Viejo-Borbolla, A., Ottinger, M., Bruning, E., Burger, A., Konig, R., Kati, E., Sheldon, J. A., and Schulz, T. F. (2005). Brd2/RING3 interacts with a chromatin-binding domain in the Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 (LANA-1) that is required for multiple functions of LANA-1. *J. Virol.* 79, 13618–13629.

Vogelstein, B., Pardoll, D. M., and Coffey, D. S. (1980). Supercoiled loops and eucaryotic DNA replicaton. *Cell* 22, 79–85.

Volpi, E. V., Chevret, E., Jones, T., Vatcheva, R., Williamson, J., Beck, S., Campbell, R. D., Goldsworthy, M., Powis, S. H., Ragoussis, J., Trowsdale, J., and Sheer, D. (2000). Large-scale chromatin organization of the major histocompatibility complex and other regions of human chromosome 6 and its response to interferon in interphase nuclei. *J. Cell. Sci.* 113(Pt 9), 1565–1576.

Wang, H. W., Trotter, M. W., Lagos, D., Bourboulia, D., Henderson, S., Makinen, T., Elliman, S., Flanagan, A. M., Alitalo, K., and Boshoff, C. (2004). Kaposi sarcoma herpesvirus-induced cellular reprogramming contributes to the lymphatic endothelial gene expression in Kaposi sarcoma. *Nat. Genet.* 36, 687–693.

Wang, S., and Frappier, L. (2009). Nucleosome assembly proteins bind to Epstein-Barr virus nuclear antigen 1 and affect its functions in DNA replication and transcriptional activation. *J. Virol.* 83, 11704–11714.

Wyrick, J. J., Aparicio, J. G., Chen, T., Barnett, J. D., Jennings, E. G., Young, R. A., Bell, S. P., and Aparicio, O. M. (2001). Genome-wide distribution of ORC and MCM proteins in *S. cerevisiae*: high-resolution mapping of replication origins. *Science* 294, 2357–2360.

Wysokenski, D. A., and Yates, J. L. (1989). Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within oriP of Epstein-Barr virus. *J. Virol.* 63, 2657–2666.

Xiao, B., Verma, S. C., Cai, Q., Kaul, R., Lu, J., Saha, A., and Robertson, E. S. (2010). Bub1 and CENP-F can contribute to Kaposi's sarcoma-associated herpesvirus genome persistence by targeting LANA to kinetochores. *J. Virol.* 84, 9718–9732.

Yates, J. L., Camiolo, S. M., and Bashaw, J. M. (2000). The minimal replicator of Epstein-Barr virus oriP. *J. Virol.* 74, 4512–4522.

Ye, F. C., Blackbourn, D. J., Mengel, M., Xie, J. P., Qian, L. W., Greene, W., Yeh, I. T., Graham, D., and Gao, S. J. (2007). Kaposi's sarcoma-associated herpesvirus promotes angiogenesis by inducing angiopoietin-2 expression via AP-1 and Ets1. *J. Virol.* 81, 3980–3991.

Yokoyama, A., Kawaguchi, Y., Kitabayashi, I., Ohki, M., and Hirai, K. (2001). The conserved domain CR2 of Epstein-Barr virus nuclear antigen leader protein is responsible not only for nuclear matrix association but also for nuclear localization. *Virology* 279, 401–413.

Yoshizaki, K., Matsuda, T., Nishimoto, N., Kuritani, T., Taeho, L., Aozasa, K., Nakahata, T., Kawai, H., Tagoh, H., and Komori, T. (1989). Pathogenic significance of interleukin-6 (IL-6/BSF-2) in Castleman's disease. *Blood* 74, 1360–1367.

You, J., Srinivasan, V., Denis, G. V., Harrington, W. J. Jr., Ballestas, M. E., Kaye, K. M., and Howley, P. M. (2006). Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen interacts with bromodomain protein Brd4 on host mitotic chromosomes. *J. Virol.* 80, 8909–8919.

Zappulla, D. C., Sternglanz, R., and Leatherwood, J. (2002). Control of replication timing by a transcriptional silencer. *Curr. Biol.* 12, 869–875.

Zhao, J., Punj, V., Matta, H., Mazzacurati, L., Schamus, S., Yang, Y., Yang, T., Hong, Y., and Chaudhary, P. M. (2007). K13 blocks KSHV lytic replication and deregulates vIL6 and hIL6 expression: a model of lytic replication induced clonal selection in viral oncogenesis. *PLoS ONE* 2, e1067. doi:10.1371/journal.pone.0001067

Zhou, J., Snyder, A. R., and Lieberman, P. M. (2009). Epstein-Barr virus episome stability is coupled to a delay in replication timing. *J. Virol.* 83, 2154–2162.

Ziegelbauer, J. M., Sullivan, C. S., and Ganem, D. (2009). Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. *Nat. Genet.* 41, 130–134.

Zlatanova, J., and Caiafa, P. (2009). CCCTC-binding factor: to loop or to bridge. *Cell. Mol. Life Sci.* 66, 1647–1660.

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Modulation of immune system by Kaposi's sarcoma-associated herpesvirus: lessons from viral evasion strategies

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Kaposi's sarcoma-associated herpesvirus (KSHV), a member of the herpesvirus family, has evolved to establish a long-term, latent infection of cells such that while they carry the viral genome gene expression is highly restricted. Latency is a state of cryptic viral infection associated with genomic persistence in their host and this hallmark of KSHV infection leads to several clinical–epidemiological diseases such as KS, a plasmablastic variant of multicentric Castleman's disease, and primary effusion lymphoma upon immune suppression of infected hosts. In order to sustain efficient life-long persistency as well as their life cycle, KSHV dedicates a large portion of its genome to encode immunomodulatory proteins that antagonize its host's immune system. In this review, we will describe our current knowledge of the immune evasion strategies employed by KSHV at distinct stages of its viral life cycle to control the host's immune system.

Keywords: KSHV, modulation of immune system, viral evasion strategies

INTRODUCTION

Herpesviruses have co-evolved with their hosts for more than one hundred million years. During this coevolution, hosts have come to equip themselves with elaborate immune system to defend themselves from invading viruses and other pathogens. In order to establish infection and maintain latency, herpesviruses have acquired a number of genes that cause selective suppression of normal immune system functions and allow for an apathogenic, persistent infection.

Kaposi's sarcoma-associated herpesvirus [KSHV; also known as human herpesvirus 8 (HHV8)] is a large double-stranded, DNA oncogenic virus belonging to the gammaherpesvirus subfamily. Its genome consists of a long unique region (LUR) encoding over 87 open reading frames (ORFs), at least 17 microRNAs, and a variable number of GC-rich 801-bp large terminal repeat (TR) elements. Like all other herpesviruses, KSHV can adopt one of two lifestyles known as latency and lytic replication. In latency, the viral genome is retained as a circular episome in the nucleus, expressing only a minimal number of genes without producing progeny virions. Lytic replication can be induced from the latency state by a single master-switch viral gene, which encodes a transcription factor known as RTA. The switch to lytic replication from latency leads to the expression of the entire viral genome as well as the production of progeny virions. Interestingly, KSHV dedicates almost a quarter of its genome to immunomodulatory genes, most of which seem to be pirated from the host and are cellular orthologs. Through these proteins, KSHV has developed elaborate mechanisms for outsmarting and adapting to the host's immune responses, ultimately establishing a life-long, persistent infection within an immunocompetent host after primary infection.

Herein, we summarize recent advances in our understanding of how KSHV utilizes immunomodulatory genes to subvert the antiviral immune responses of its host (**Figure 1** and **Table 1**).

HOST IMMUNE SYSTEM OVERVIEW

The immune system provides numerous mechanisms of protection against invading pathogens like viruses. These immune responses include both broad spectrum, innate responses, and highly specific, adaptive responses. The innate immune system is our first line of defense against invading organisms while the adaptive immune system acts as a second line of defense that also affords protection against re-exposure to the same pathogen. In order to overcome innate responses, KSHV proteins deregulate interferon responses, the complement cascade, inflammatory cytokine production, natural killer (NK) cell immunity, Toll-like receptors (TLRs), apoptosis, and autophagy pathways. Furthermore, KSHV employs several strategies to block the two adaptive arms of the immune system: the humoral immune response arm (production of antibodies by B cells) and the cellular immune response arm (activities carried out by cytotoxic $CD4^+$ and $CD8^+$ T cells).

INNATE IMMUNITY

Interferon

Interferon (IFN) plays an important role as a primary defense mechanism against viral infection by inhibiting viral replication, suppressing cell growth, promoting apoptosis, upregulating antigen presentation, and modulating several signal transduction pathways. To date, three distinct classes of IFN designated as types I to III have been identified, classified according to their receptor complex and amino acid sequence (Pestka et al., 2004; Sadler and Williams, 2008). Type II IFN consists of a single gene, IFN-γ, that

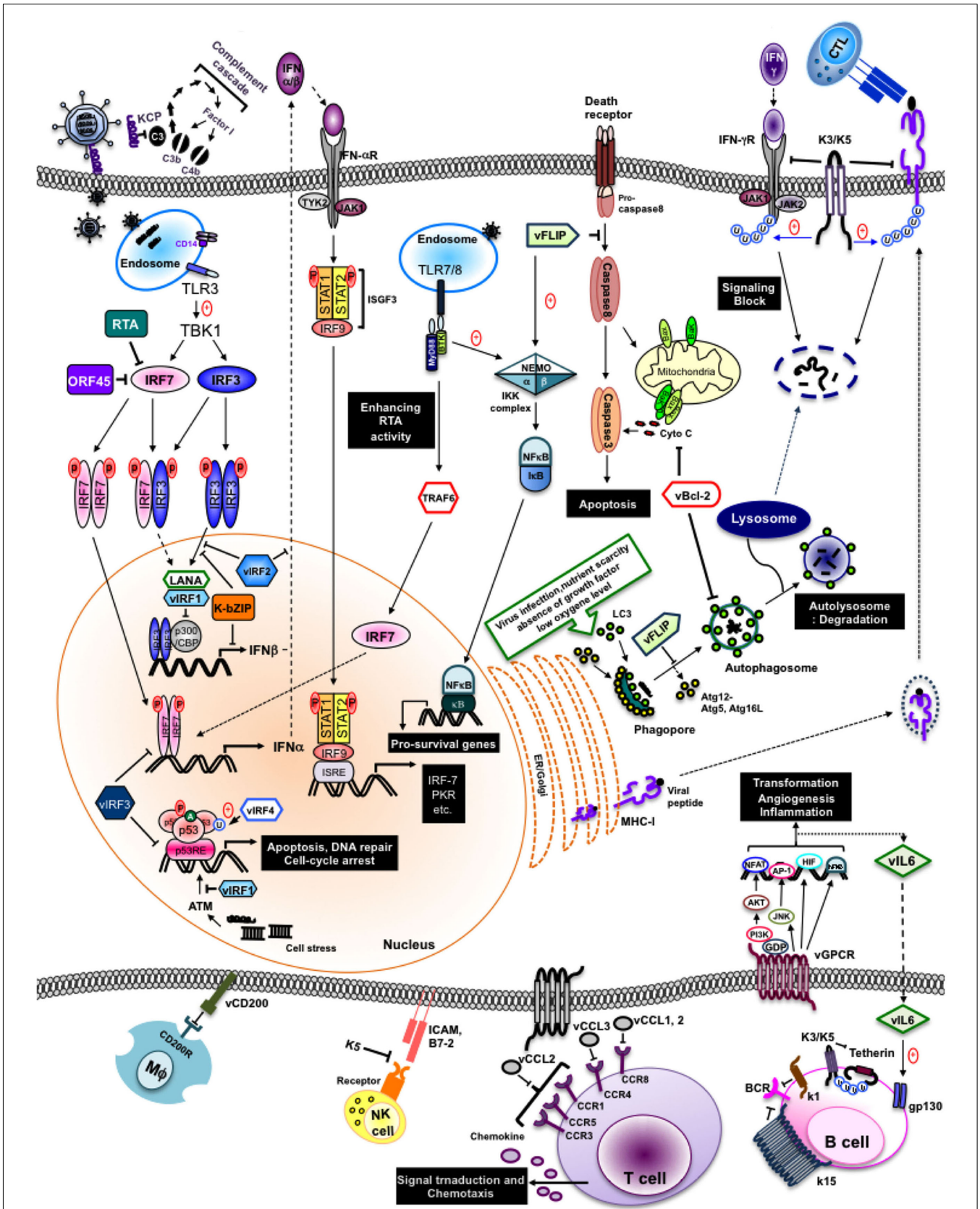

FIGURE 1 | Evasion of immune responses by individual KSHV products. Individual KSHV products are shown to circumvent immune response, both innate and adapt, during their life cycle.

can interact with IFN-γ receptor (IFNγR) complexes and induce broad immune responses to pathogens other than viruses. Type III IFNs (namely, IFN-λ1, -λ2, and -λ3, also known as IL-28/29), and type I IFNs (IFN-α/β) mainly regulate antiviral responses. Virus-activated IFN-regulatory factors (IRFs) undergo phosphorylation, dimerization, and nuclear translocation, ultimately turning on positive regulators of the transcription of type I IFN and type III IFN genes (Sadler and Williams, 2008; Trinchieri, 2010).

Remarkably, KSHV encodes four viral IRFs (vIRF1–4) that show homology with cellular IRFs in one cluster region of its genome, and employs them as negative regulators that subvert host IFN-mediated immune responses (Takaoka et al., 2008). They are all expressed during lytic reactivation, but vIRF1 and vIRF3 [also know as latency-associated nuclear antigen (LANA2)] have also been detected in latently infected cells. This suggests that although vIRFs may function redundantly, they may act independently depending on the cell type and the stage of the viral lifecycle to thereby elicit disparate and distinct mechanisms of IFN evasion (Cunningham et al., 2003; Lee et al., 2009a). A number of previous studies have shown that vIRFs associate with and regulate cellular IRFs, other transcriptional factors and cofactors rather than directly interacting with DNA to inhibit IFN-induced gene transcripts (Takaoka et al., 2008; Tamura et al., 2008; Lee et al., 2009a, 2010).

vIRF1 (K9). Viral IRF1 was the first vIRF found to effectively repress cellular IFN responses (Gao et al., 1997; Zimring et al., 1998). vIRF1 does not compete with IRF1 for DNA binding although it inhibits IRF1 transcriptional activity (Zimring et al., 1998). Alternatively, vIRF1 binds to transcriptional cofactor p300 and interferes with CBP/p300–IRF3 complex formation along with p300 histone acetyltransferase (HAT) activity, thus preventing IRF3-mediated transcriptional activation (Li et al., 2000; Lin et al., 2001).

vIRF2 (K11/K11.1). Full length vIRF2, translated from two spliced exons (K11 and K11.1), accelerates IRF3 degradation and inhibits IRF3 transactivation, thereby repressing IRF3-mediated IFN-β transcriptional activity (Areste et al., 2009). In addition, vIRF2 inhibits IFN-α/β driven signaling as well as signaling induced by IFN-λ (Fuld et al., 2006). The underlying mechanism, however, has yet to be defined. In a recent study, vIRF2 reduced the activation of the IFN-induced interferon-response element (ISRE) promoter through the deregulation of IFN-stimulated gene factor-3 (ISGF-3), (Mutocheluh et al., 2011). It is suggested that vIRF2 possesses pleiotropic activity of inhibiting early type I IFN (IFN enhanceosome-dependent) and delayed type I IFN (ISGF-dependent) responses. Previous studies have shown that the first exon of vIRF2 (K11.1) interacts with cellular IRF1, IRF2, IRF8, RelA, and p300 (Burysek and Pitha, 2001). Furthermore, vIRF2 prevents PKR kinase activity to overcome IFN-α/β-mediated antiviral effects (Burysek and Pitha, 2001).

vIRF3 (K10.5). Viral IRF3 interaction with cellular IRF7 suppresses IRF7 DNA binding activity and, therefore, inhibits IFN-mediated immunity through the inhibition of IFN-α production (Joo et al., 2007). Remarkably, a putative double α-helix motif of vIRF3 (residues 240–280) that has been shown to be responsible for the interaction of vIRF3 with IRF7 is also sufficient to bind to IRF5 (Wies et al., 2009). As a result of this interaction, vIRF3 inhibits IRF5-mediatd ISRE and IFN-β promoter activity (Wies et al., 2009). It was recently shown that vIRF3 is required for the survival of primary effusion lymphoma (PEL) cells. RNA interference (RNAi) knockdown of vIRF3 in PEL cells reduced cell proliferation by releasing IRF5 from p21 promoter transcription complexes.

While it remains to be discovered whether vIRF4, the most recently identified member of the vIRF family, affects IFN-mediated innate immunity, the downregulation of the IFN-regulatory pathway is a common characteristic of the vIRFs, whose functions have been well studied, as IRF3 and IRF7 are key initiation factors of the host immune surveillance program against viral infection. Accordingly, in addition to vIRFs, other viral proteins restrain the type I and II IFN signaling pathways. The LANA protein, expressed during viral latency, competes with IRF3 for binding to the IFN-β promoter, thus preventing the expression of the CREB-binding protein as well as inhibiting IFN-β transcription (Cloutier and Flamand, 2010). Three immediately early gene products, K8, ORF45, and RTA, are also involved in the inhibition of IFN pathway in distinctive manners. The K8 (K-bZIP) precludes efficient IFN-β gene expression by impeding IRF3 binding to the IFN-β promoter through a direct interaction with the IFN-β promoter (Lefort et al., 2007). ORF45 interacts with IRF7, preventing its phosphorylation and subsequent translocation into the nucleus, hence impairing this amplification loop in type I IFN production (Zhu et al., 2002). RTA antagonizes type I IFN-mediated antiviral response, enhancing the ability of RTA-associated ubiquitin ligases (RAUL) to degrade IRF7 and IRF3 (Yu et al., 2005; Yu and Hayward, 2010). Much less is known about the state of type I IFN signaling in infected cells. Bisson et al. (2009) demonstrated that lytically infected cells display profound defects in IFN-mediated STAT activation. More recent studies have demonstrated that an early protein RIF (the product of ORF10) forms complexes with Jak1, Tyk2, STAT2, and both IFNAR subunits, resulting in the inhibition of the kinase activities of both Jak1 and Tyk2 (Bisson et al., 2009). In addition, KSHV ORF64, a tegument protein with deubiquitinase (DUB) activity, suppresses retinoic acid-inducible gene I-mediated IFN signaling by reducing the ubiquitination of RIG-1 upon KSHV infection (Inn et al., 2011). In summary, KSHV dedicates almost 10% of its gene production to modulating host IFN responses, further indicating the importance of IFN-mediated innate immunity as a primary defense mechanism against viral infection. Recently, HIV-1 Vpu led to the discovery of the interferon-induced transmembrane protein, tetherin (BST-2, CD317), as a novel component of host innate defense against enveloped viruses. Thus far, six viral proteins have been reported to counteract tetherin: HIV-1 Vpu, HIV-2, SIV Env, SIV Nef, Ebola glycoprotein (GP), and KSHV K5 (Neil et al., 2008; Van Damme et al., 2008; Jia et al., 2009; Kaletsky et al., 2009; Le Tortorec and Neil, 2009; Mansouri et al., 2009; Zhang et al., 2009). KSHV K5 induces the proteasomal degradation of tetherin during primary infection and upon reactivation from latency in endothelial cells. Furthermore, tetherin reduces KSHV release upon inhibition of K5 expression by small interfering RNA (siRNA), suggesting that tetherin is part of the IFN-induced innate immune response against KSHV (Mansouri et al., 2009; Pardieu et al., 2010).

p53 tumor suppression regulation

p53 (also known as tumor protein 53, TP53) is a transcriptional factor often described as the "guardian of the genome" because of its major roles in cell cycle arrest and in inducing apoptosis in response to a myriad of cellular stresses such as DNA damage and viral infection (Vogelstein et al., 2000; Collot-Teixeira et al., 2004). Therefore, viruses openly employ products that overcome the action of p53 to circumvent its growth-suppressive and proapoptotic actions (Coscoy, 2007; Liang et al., 2008; Lee et al., 2009a).

p53 is activated in a specific manner by post-translational modifications that lead to either cell cycle arrest, a program that induces cell senescence, or cellular apoptosis. Activation of the p53 network by stressors including virus replication and DNA damage is marked by two major events. First, the half-life of the p53 protein is increased drastically, leading to a quick accumulation of p53 in stressed cells (Collot-Teixeira et al., 2004). Second, a conformational change activates p53 as a transcriptional regulator in these cells, stimulating the phosphorylation and acetylation of p53 to increase the levels of activated p53. Activated p53 then binds to its regulatory region, which activates the expression of several target genes leading to diverse cellular responses, such as apoptosis, cell cycle arrest, or DNA repair (Harris and Levine, 2005). When p53 is no longer needed, it is ubiquitinated by murine double minute 2 (MDM2) and translocates from the nucleus to be degraded (Michael and Oren, 2003; Bond et al., 2005). In order to hinder p53-mediated irreversible cell cycle arrest and apoptosis, viruses need to tightly regulate the expression and/or functions of p53.

Viral IRFs. Notably, mounting studies show that vIRFs have remarkable functional redundancy in inhibiting p53-mediated cell cycle arrest and apoptosis. vIRF1 suppresses its acetylation, impeding the transcriptional activation of p53, resulting in a decrease in p53 target gene expression and transcription (Nakamura et al., 2001; Seo et al., 2001). Moreover, vIRF1 blocks ATM kinase activity, thereby reducing p53 phosphorylation and increasing p53 ubiquitination (Shin et al., 2006). Like vIRF1, vIRF3, and vIRF4 also interfere with p53 signaling. The B cell-specific, latently expressed vIRF3 inhibits p53-mediated transcriptional activity and apoptosis through its interaction with p53, although the way in which it targets the p53 pathway is not clear (Rivas et al., 2001). vIRF4 targets two major components, HAUSP and MDM2, of the p53 pathway to effectively reducing p53 and suppressing p53-mediated apoptosis. Furthermore, vIRF4's actions with HAUSP and MDM2 are functionally and genetically separable and the vIRF4–MDM2 interaction has a more significant role in downregulating p53 than the vIRF4–HAUSP interaction (Lee et al., 2009b, 2011).

LANA (ORF73). Similar to vIRF3, the LANA also acts as negative regulator of the p53 pathway. LANA physically interacts with p53 to repress its transcriptional and translational activity (Friborg et al., 1999; Si and Robertson, 2006; Chen et al., 2010). Ultimately, LANA inhibits the ability of p53 to induce cell death, thereby, promoting chromosomal instability (Friborg et al., 1999; Si and Robertson, 2006). Initially, the modulation of p53 transcriptional activity by LANA was reported to be neither through p53 degradation nor inhibition of its DNA binding ability. However, a later report indicated that expression of LANA leads to decreased p53 protein levels due to LANA enhanced ubiquitination and degradation of p53 (Suzuki et al., 2010).

Cytokine regulation

Viral infection stimulates the production of cytokines and chemokines, secreted proteins that enable either the positive or negative regulation of the immune responses. They have crucial roles in inducing the migration of immune cells to areas of infection, immune regulation, antiviral defense, as well as the capacity of targets cells to support viral replication. Therefore, it is not surprising to find that many viruses benefit from an antagonism of cytokine activity by encoding proteins homologous to cellular cytokines (virokines), chemokine receptors (viroceptors), and/or secreting chemokine-binding proteins. Alternatively, viruses also utilize viral proteins to neutralize cytokine activities and may use cytokine pathways to induce cell proliferation, cell migration, or control homeostasis to enhance viral replication.

Viral cytokine (vIL-6). KSHV K2, an early lytic gene, encodes a viral interleukin, vIL-6, which shows homology to cellular IL-6 (Swanton et al., 1997). vIL-6 directly binds to and activates gp130 without the need of the gp80 α-subunit of the IL-6 receptor for IL-6 signal transduction (Molden et al., 1997; Hoischen et al., 2000; Chow et al., 2001; Chatterjee et al., 2002; Chen et al., 2009). As a functional consequence, vIL-6 mimics a number of IL-6 activities, including stimulation of IL-6 dependent proliferation of B cells, activation of the JAK/STAT pathway and stimulation of cells by intracellular signaling (Molden et al., 1997; Burger et al., 1998; Meads and Medveczky, 2004). Furthermore, vIL-6 plays important roles as an angiogenic factor through the induction of vascular endothelial growth factor (VEGF; Aoki et al., 1999), the promotion of Th2-cell development by increasing CCL2 expression, as well as blocking IFN response (Chatterjee et al., 2002; Diehl and Rincon, 2002). Moreover, secreted vIL-6 can be detected from latently infected KSHV-positive B cells and contributes to the survival and growth of PEL cells (Moore et al., 1996; Foussat et al., 1999; Mori et al., 2000; Chen et al., 2009). Hence, it is indicated that vIL-6 has distinctive functions in latency, where it promotes the growth and survival of infected cells for the maintenance of the latent virus within host cells, and lytic replication, where it protects virally infected cells from undergoing growth arrest and cell death.

Viral chemokines (vCCL-1, vCCL-2, vCCL-3). KSHV contains three virally encoded chemokines: vCCL-1 (K6/vMIP-I), vCCL-2 (K4/vMIP-II), and vCCL-3 (K4.1/vMIP-III), (Lee et al., 2010). vCCL-1, vCCL-2, and vCCL-3 can activate signal transduction through CCR3/CCR8, CCR8, and CCR4, respectively. Interestingly, they appear to be chemoattractants that specifically target Th2 lymphocytes as these chemokine receptors are preferentially expressed on Th2 cells, and not Th1 cells (Choi and Nicholas, 2008). It is indicated that KSHV CCLs act as Th2-cell chemokine-receptor agonists and can polarize the adaptive immune response toward a predominantly Th2-type response at sites of KSHV infection, potentially reducing the efficacy of host antiviral responses. Likewise, vCCL-2 acts as an antagonist by inhibiting a broad range of CC and CXC receptors expressed by Th1 cells and effectively blocks the RANTES-induced arrest of monocytes while promoting the arrest of eosinophils (Chen et al., 1998; Weber et al., 2001;

Choi and Nicholas, 2008). Aside from their immune modulatory functions, vCCLs may further contribute to KSHV-associated disease by enhancing angiogenic responses through the induction of VEGF (Masood et al., 1997; Nakamura et al., 1997; Dittmer et al., 1998).

vGPCR (ORF74). The KSHV-encoded vGPCR (ORF74) is a seven-transmembrane, IL-8 receptor homolog that possesses promiscuous chemokine-binding activity (Swanton et al., 1997; Bais et al., 2003). Moreover, it has been shown that vGPCR constitutively activates mitogen-activated protein kinase (MAPK) cascades, p38 MAPK (Sodhi et al., 2000), Akt/protein kinase B (Cannon, 2007), mTOR (Montaner, 2007), as well as some small GTPases, like RhoA and Rac1 (Bottero et al., 2010). Consequently, certain transcription factors, including AP-1, NFAT, NF-κB, HIF-α, and cyclic AMP response element binding protein (CREB) have been shown to be activated by vGPCR expression, which in turn, lead to the expression of growth factors, proinflammatory cytokines, as well as angiogenic factors (Sodhi et al., 2000; Cannon et al., 2003; Cannon and Cesarman, 2004; Montaner et al., 2004). Expression of vGPCR in transgenic mice causes KS-like lesions characterized by increased vascularization and the recruitment of inflammatory cells (Bais et al., 2003; Montaner et al., 2003). Among the multiple intracellular pathways stimulated by vGPCR mentioned above, the PI3K/Akt/mTOR pathway has recently drawn great attention for its contribution to KS development. Notably, vGPCR expression was detected in only a very few cells within the tumor mass and other tissues, indicating that there is a paracrine effect, perhaps due to the enhanced activation of PI3Kγ (Martin et al., 2011). The fact that expression of vGPCR is restricted to the lytic phase of KSHV replication has raised arguments against its role in pathogenesis and further examination will be required.

vCD200 (K14). Another negative regulator of inflammatory signaling is K14, a surface glycoprotein (vOX2) expressed during the early lytic phase. It shows significant homology with OX2, also named CD200, a member of the immunoglobulin superfamily that is broadly distributed on the surface of cells (Chung et al., 2002). It was initially thought that vCD200 promotes the secretion of proinflammatory cytokines upon stimulation of monocytes, macrophages and DCs through an undefined receptor, to promote cytokine-mediated proliferation of KSHV-infected cells (Chung et al., 2002). However, vCD200 was later shown to downmodulate proinflammatory cytokines via a direct interaction with cellular CD200R, inhibiting myeloid cell activation and reducing Th1-cell-associated cytokine production (Foster-Cuevas et al., 2004; Rezaee et al., 2005; Shiratori et al., 2005). Furthermore, vCD200 has also been shown to suppress neutrophil-mediated inflammation in mice (Rezaee et al., 2005), suggestive of vCD200-mediated immunosuppressive activities in KSHV infection. Overall, mounting data has led to the conclusion that vCD200 can cause immune dysfunction associated with persistent infection, but the immunomodulatory role of vCD200 is still controversial. Recently, Salata et al. (2009) reported that vCD200 acts as a proinflammatory stimulus in the early stage of viral infection (or reactivation), while contributing to the downregulation of macrophage immune response activities, from phagocytosis to antigen presentation, in later stages.

TLR signaling

Toll-like receptors are transmembrane proteins expressed by various immune and non-immune cells of the innate immune system that recognize invading infectious agents and initiate signaling pathways, culminating in the increased expression of immune and inflammatory genes. To date, 11 human TLRs and 13 murine TLRs have been identified. Each TLR contains 21–25 leucine-rich repeat regions that specifically recognize a variety of pathogen associated molecular patterns (PAMPs), (Akira et al., 2006). TLRs are expressed in distinct cellular compartments: TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are expressed in intracellular vesicles such as the endosome and ER (O'Neill and Bowie, 2010). Accumulated evidence in relation to TLR signal transduction has demonstrated that TLR3, TLR4, TLR7, TLR8, and TLR9 are involved in the recognition of viruses by way of binding to RNA, DNA, or viral glycoproteins (Akira and Takeda, 2004; Kawai and Akira, 2006). A recent growing body of information is beginning to shed light on TLR recognition of KSHV: KSHV infection of human monocytes enhance TLR3 expression and induction of cytokines including IFN-β1, CCL2, and CXCL10 (West and Damania, 2008). Infection of endothelial cells with KSHV causes suppression of TLR4 through the activation of the extracellular signal-regulated kinase (ERK) MAPK pathway via viral gene expression-independent mechanisms and viral gene expression dependent mechanisms by way of vIRF1 and vGPCR (Lagos et al., 2008). In addition, stimulation of TLR7 and TLR8 reactivates KSHV in latently infected B lymphocytes (Gregory et al., 2009). A later study has shown that KSHV infection of plasmacytoid dendritic cells (pDCs) activates the TLR9 signaling pathway, leading to the upregulation of CD83 and CD86, and secretion of IFN-α (West et al., 2011). Overall, there is evidence that KSHV has developed different ways to escape TLR-mediated detection throughout their lifecycle to limit KSHV lytic replication and facilitate the establishment of latency.

Modulation of inflammasome pathway

Inflammasomes are emerging as key regulators of host responses against infectious agents. When a pathogen invades sterile tissues or elicits cellular damage, it can cause the activation of NLRs (nucleotide binding and oligomerization, leucine-rich repeat) that then trigger inflammasomes, molecular platforms composed of oligomers of specific NLRs, pro-caspase 1, and an adaptor protein called ASC (apoptotic-associated speck-like; Martinon et al., 2002). These cytosolic multiprotein complexes activate cysteine protease caspase 1, leading to the processing and secretion of the proinflammatory cytokines, IL-1β, IL-18, and IL-33 (Masters et al., 2009; Schroder and Tschopp, 2010; Lamkanfi and Dixit, 2011). In addition, production of IL-1β and IL-18 is associated with a caspase 1-mediated inflammatory cell death program known as pyrotosis (Ting et al., 2008; Miao et al., 2010). Therefore, it is not surprising that viruses have evolved a variety of strategies to interfere with inflammasome activation and downstream signaling cascades (Lamkanfi and Dixit, 2011).

Recently, Gregory et al. found that KSHV ORF63 shows sequence similarity to a NLR family member, NLRP1, and hinders the interaction between NLRP1 and pro-caspase 1 through a direct interaction with NLRP1 oligomerization domains. Thus, ORF63

reduces caspase 1 activity and lowers production of IL-1β and IL-18. Interestingly, ORF63 also interacts with two additional NLR family members, NOD2 and NLRP3, and may thus broadly inhibit NLR inflammasome responses (Gregory et al., 2011). Taken together, KSHV deploys ORF63 to prevent inflammasome assembly and further enhance viral virulence.

Apoptosis and autophagy

A common cellular defense against pathogenic invasion is cell death by two programmed cell death (PCD) mechanisms: apoptosis ("self-killing") and autophagy ("self-eating"), (Maiuri et al., 2007). These two PCDs are characterized by distinctive morphological and biochemical changes and both are highly conserved and tightly regulated processes that are essential for cell homeostasis, disease, and development. Apoptosis is the best-described cellular suicide mechanism against invading pathogens that involves a cascade of internal proteolytic digestion that result in the rapid demolition of cellular infrastructure (Danial and Korsmeyer, 2004; Green, 2005). By contrast, autophagy has been historically recognized as a cellular survival program that is involved in homeostasis by removing damaged or superfluous organelles through lysosomal degradation and recycling of cytoplasmic material delivered to autophagosomes (Maiuri et al., 2007; Kim et al., 2010; Liang and Jung, 2010). Recently, a growing body of evidence suggests that apoptosis and autophagy are not mutually exclusive pathways even though these two pathways seem to act independently. The functional relationship between apoptosis and autophagy is complex, with autophagy serving as a cell survival pathway that suppresses apoptosis that can also lead to cellular demise called autophagic cell death (type II PCD) in other cellular settings. Currently, a number of pathways that link the apoptotic and autophagic machineries and polarize the cellular response to favor one over the other have been deciphered at the molecular level. For instance, several apoptosis signaling molecules including TNF-α, TRAIL, FADD, and p53, also mediated autophagy. Likewise, Atg5, an autophagy effector, triggers apoptosis through its interaction with FADD (Mills et al., 2004; Feng et al., 2005; Levine, 2005; Lum et al., 2005; Pyo et al., 2005; Thorburn et al., 2005), while the inhibition of the class I PI3K, AKT, and mTOR pathways suppress both apoptosis and autophagy (Arico et al., 2001). Hence, it is not surprising that KSHV employs some gene products that target the crosstalk between apoptotic and autophagic signaling to evade and subvert this part of the overall host surveillance mechanism directed at blocking viral replication and dissemination.

vFLIP (K13). The K13 (ORF71) latent viral gene encodes viral FLICE (Fas-associated death-domain like IL-1 β-converting enzyme) inhibitory protein (vFLIP), a truncated homolog of cellular FLIP (cFLIP), (Dittmer et al., 1998; Fakhari and Dittmer, 2002). The vFLIP gene is expressed in KS and PEL cells from a polycistronic mRNA encompassing the latency locus. As such, vFLIP can inhibit extrinsic death-receptor-mediated apoptosis pathway by preventing the activation of caspases, including caspase 8 (Djerbi et al., 1999), and additionally associates with the IKK complex and heat shock protein 90 (HSP90) to induce the expression of anti-apoptotic proteins via the activation of NF-κB (Chaudhary et al., 1999; Liu et al., 2002; Field et al., 2003; Bubman et al., 2007). The induction of NF-κB activity has been linked to at least two aspects of KSHV-infected pathogenesis: viral latency and oncogenesis. On the other hand, NF-κB activation by vFLIP is crucial for its inhibition of lytic replication via the AP-1 pathway (Zhao et al., 2007; Ye et al., 2008). vFLIP likely contributes to a proinflammatory microenvironment given that its expression was found to induce NF-κB-regulated cytokine expression and secretion (Grossmann et al., 2006; Sun et al., 2006). Taken together, vFLIP appears to directly contribute to KSHV pathogenesis with functions in viral infected cell survival, transformation, inflammatory activation and latency control. Lately, vFLIP has not only been recognized to possess anti-apoptotic activities, but additionally serves as an anti-autophagy protein through its prevention of Atg3 binding to and processing of LC3, a protein involved in the elongation step of autophagy (Lee et al., 2009c).

vBcl-2 (ORF16). KSHV ORF16 encodes the viral Bcl-2 protein that generally shares 15–20% amino acid identity with human cellular homologs and inhibits apoptosis induced by virus infection and the pro-death protein BAX (Cheng et al., 1997; Sarid et al., 1997). Furthermore, recent evidence shows that vBcl-2 suppresses the cellular autophagy pathway through its direct interaction with the autophagy protein Beclin-1 (Pattingre et al., 2005; Liang et al., 2006). Although vBcl-2 possesses low degrees of homology with most regions of cellular Bcl-2 except the Bcl-2 homology (BH) 1 and BH2 domains, vBcl-2 shares three-dimensional structural conservation with Bcl-2 family members such that they all contain a central hydrophobic cleft called the BH3-peptide binding groove (Cheng et al., 1997; Huang et al., 2002; Loh et al., 2005). Interestingly, the BH3 binding cleft of vBcl-2 binds with high affinity and specificity with BH3-containing molecules (BAX, BAK, BIM, PUMA, BID, and Noxa) as well as selective binding to Beclin-1. It is thus possible that vBcl-2 may direct apoptosis and autophagy through a coordinated control of the two pathways. vBcl-2 is transcribed during lytic viral infection (Sarid et al., 1997; Paulose-Murphy et al., 2001) so inhibition of apoptosis and autophagy by vBcl-2 may function to prolong the life-span of KSHV-infected cells, which in turn enhances viral replication and establishment of latency. In summary, KSHV has evolved to acquire viral versions of Bcl-2 and FLIP to avoid elimination by the host's apoptotic and autophagy-mediated immune responses.

COMPLEMENT

The complement system has long been considered to be a part of the innate immune defense system that also bridges innate and adaptive immune responses, activated spontaneously in response to pathogen surface components or antibodies to help clear pathogens from an organism (Blue et al., 2004; Morgan et al., 2005). The complement system is comprised of 30 different proteins that are either circulating in the serum or attached to the cell surface, and they shared a common structural motif known as the short consensus repeat (SCR). They orchestrate three distinct cascades, termed the classic, alternative, and mannose-binding lectin pathways, which contribute to four major functions: lysis of infectious organisms, activation of inflammation, opsonization, and immune clearance. All three cascades converge at C3 convertase

assembly, which can either initiate the opsonization of the foreign body or continue to activate C5 and thus propagate the cascade (Dunkelberger and Song, 2010).

KCP (ORF4)

It is not surprising that KSHV encodes its own inhibitor of the complement system in ORF4, designated as KSHV complement control protein (KCP). Three lytic protein isoforms are produced by alternative splicing (Spiller et al., 2003b). All three isoforms retain four SCR domains and a transmembrane region, and can regulate complement by accelerating the decay of the classical C3 convertase and acting as cofactors for the inactivation of C3b and C4b, components of the C3 and C5 convertases (Spiller et al., 2003a, 2006; Mark et al., 2008). KCPs are expressed and incorporated onto the surface of the virions during lytic infection, and have been detected at the surface of *de novo* infected endothelial cells after induction of lytic replication as well as at the surface of KSHV-infected cells from patients with PEL (Spiller et al., 2003b, 2006). Moreover, other γ-herpesviruses also encode KCP homologs indicating that an anti-complement defense system is essential during KSHV infection (Fodor et al., 1995; Kapadia et al., 1999; Okroj et al., 2009).

ADAPTIVE IMMUNITY

B cell-dependent immune response

B lymphocytes are the major cell type involved in the creation of antibodies, also known as humoral immunity. Antibodies (e.g., IgG, IgM, and IgA) are produced by plasma cells, have been stimulated by $CD4^+$ Th cells, which activate B cells through a signaling mechanism involving binding of CD40 on the B cell surface to CD40 ligand. In lymph nodes, naïve B cells recognize cognate antigen by their surface antibodies, become activated, switch from

Table 1 | Anti-immune strategies of KSHV.

Strategy	Gene product	Function
INNATE IMMUNE EVASION		
Inhibit interferon	vIRF1	Inhibition of IRF3-mediated transcription
	vIRF2	Suppression of IRF1 and IRF3
	vIRF3	Inhibition of IRF7 DNA binding activity
	LANA	Competing with IRF3
	K8 (K-bZIP)	Impede IRF3 binding on IFN-β promoter
	ORF45	Prevent IRF7 activation
	ORF50 (RTA)	Promotes IRF7 degradation
	ORF64	Suppression of RIG-1
Regulation of p53 tumor suppression	vIRFs	Suppression of p53
	LANA	Repress transcriptional activity of p53
Inhibit cytokines/chemokines	K6 (vCCL-1)	CCR8 agonist, Th2 chemoattractant
	K4 (vCCL-2)	CCR3 and CCR8 agonist
		C-, CC-, CXC-, and CX_3C-chemokine antagonist
	K4.1 (vCCL-3)	CCR4 agonist, Th2 chemoattractant
	K14 (vCD200)	Inhibition of myeloid cell activation, reduction of Th1-associated cytokine production
	K2 (vIL-6)	B cell growth factor
	ORF74 (vGPCR)	Homolog of the cellular IL-8 receptor
TLR signaling	vIRF1, vGPCR	Suppression of TLR4
Modulation of inflammasome pathway	ORF63	Decrease caspase 1 activity and lower production of IL-1β and IL-18
Inhibit apoptosis and autophagy	K13 (vFLIP)	Inhibition of caspase 8 activity
		Prevent Atg3 interaction
	ORF16 (vBcl-2)	Bind proapoptotic Bak and Bax proteins
		Direct bind with Beclin-1
Inhibit complement	ORF4 (KCP)	Inhibit complement activation
ADAPTIVE IMMUNE EVASION		
Inhibit humoral immune response	K5	Induces tetherin degradation
Inhibit MHC class I antigen presentation	K3	Downregulates MHC class I molecules as well as CD1d
	K5	Downregulates HLA-A and HLA-B as well as CDId
	vIRF1	Downregulates MHC class 1
	vIRF3	Downregulates MHC class II
Inhibit the co-stimulation	K5	Downregulates ICAM1, B7-2

IgM to IgG production (class-switch), increase their immunoglobulin specificity and affinity, and differentiate into plasma cells or memory B cells as the cell continues to divide in the presence of cytokines. As a general host defense mechanism, antibodies can directly neutralize viruses by sterically hindering the receptor–virus ligand interaction or by inducing conformational changes in viral receptor ligands. Other indirect effects caused by antibodies include the recruitment or activation of the innate immune effector system such as antibody-dependent cell cytoxicity (ADCC), engulfment of antibody-coated (opsonized) viruses by phagocytes, and complement activation. B cells are the likely cellular reservoir of KSHV infection and KSHV seems to influence several aspects of B cell biology through the modulation of humoral immune responses.

Mounting evidence shows that a B cell terminal differentiation factor, X-box binding protein 1 (XBP-1), can effectively initiate KSHV reactivation by activating the RTA promoter, thus providing a link between B cell development and KSHV pathogenesis (Wilson et al., 2007; Yu et al., 2007; Dalton-Griffin et al., 2009). Interestingly, PEL cells predominantly express the inactive form of XBP-1, XBP-1u, but when the active form, XBP-1s, is induced, the KSHV lytic cycle is activated (Reimold et al., 1996). This raises the possibility that in KSHV-infected B cells, latency is maintained until plasma cell differentiation occurs.

T cell-dependent immune response

Unlike B cells, T cells recognize antigenic determinants associated with self MHC molecules on the surface of antigen-presenting cells (APCs) instead of soluble antigens. Classically, during viral infection, the recognition of viral peptides presented by MHC class I molecules on cytotoxic T lymphocytes (CTLs) is a key event in the elimination of cells producing abnormal or foreign proteins, specifically during a virus infection. CTLs thus play a critical role in the control of a viral infection, especially as a long-term immune surveillance effector that can more quickly react against the same virus after a primary infection (Micheletti et al., 2002). CTL evasion is hence a prerequisite for the replication of persistent viruses particularly in the case of herpesviruses, which must establish a persistent, latent infection, and must then reactivate in immunologically primed hosts to shed infectious virions. All herpesviruses implement strategies that target key stages of the MHC class I antigen presentation pathway with the goal of preventing the presentation of viral peptide to CTLs (for review see Alcami and Koszinowski, 2000; Ambagala et al., 2005). For instance, KSHV encodes two well-known inhibitors of MHC class I cell surface molecules that effectively suppress CTL response to virus infected cells.

Inhibition of MHC class I antigen presentation. The K3 and K5 proteins, also known as modulator of immune recognition (MIR) 1 and MIR2, consist of an N-terminal RING-CH domain harboring ubiquitin E3 ligases activity and two transmembrane domains responsible for substrate recognition (Lehner et al., 2005). In contrast to other viral inhibitors of MHC class I, K3 and K5 do not affect assembly or transportation of MHC complexes to the cell surface. Instead, they interact with MHC class I molecules through transmembrane interactions and trigger endocytosis and proteasomal degradation of MHC class I molecules without affecting their assembly or transport by ubiquitinating its cytoplasmic tail (Coscoy et al., 2001). Interestingly, K3 downregulates the expression of both canonical and non-canonical MHC class I molecules in humans (HLA-A, -B, -C, and -E), whereas K5 primarily downregulates HLA-A and -B alleles due to substrate specificity arising from TM interactions (Coscoy and Ganem, 2000; Ishido et al., 2000; Sanchez et al., 2002; Wang et al., 2004). Additionally, vIRF1 has been implicated to interact with p300 to prevent basal transcription of MHC class I molecules and thereby downregulate MHC class I molecules on the cell surface of infected cells (Lagos et al., 2007). Notably, from the viral perspective, the virus must thwart both CTLs and NK cells because the downregulation of only MHC class I molecules renders infected cells sensitive to NK cell-mediated lysis. NK cells recognize infected cells in an antigen-independent manner and destroy such cells via cytotoxic activities. Furthermore, they can rapidly produce large amounts of IFN-γ, which induces diverse antiviral immune responses upon receptor activation, including the augmentation of antigen presentation and activation/polarization of $CD4^+$ T cells and macrophages (Goodbourn et al., 2000). Hence, K3 and K5 also target gamma interferon receptor 1 (IFN-γR1) by inducing its ubiquitination, endocytosis, and degradation, ultimately resulting in the inhibition of IFN-γR1-mediated activation of transcription factors (Li et al., 2007). Very recently, Schmidt et al. reported that vIRF3 also contributes to viral immunoevasion by downregulating of IFN-γ and class II transactivator (CIITA), thus MHC class II expression (Schmidt et al., 2011). Overall, it is suggested that the downregulation of surface MHC class I molecules and IFN- γR1 by K3- and K5-mediated ubiquitination and lysosomal degradation inhibits primary host immunity against viral infection.

Inhibition of co-stimulation. The generation of a robust adaptive immune response requires the engagement of T cells with professional APCs such as DCs, macrophages, and B cells. To sufficiently activate T cells, costimulatory signals such as the interaction between CD28 and its ligands, B7-1 and B7-2 on APC surfaces (Sperling and Bluestone, 1996; Chambers and Allison, 1999), and between intercellular adhesion molecules 1 (ICAM1) and lymphocyte function-associated antigen 1 (LFA1; Dustin and Springer, 1989) are essential. In addition to lowering the level of MHC class I molecules, K5 also downregulates other components of the immune synapse on the B cell surface such as ICAM (CD54) and B7-2 (CD86), two co-activating molecules for NK cell activation, by inducing their endocytosis and degradation (Ishido et al., 2000; Coscoy and Ganem, 2001).

Taken together, K3 seems to specifically target MHC class I molecules, whereas K5 additionally targets multiple receptors including MHC class I, CD86, and ICAM, with the overall effect of preventing detection by CTL and NK cells (Nathan and Lehner, 2009; Manes et al., 2010). Even in a single gene overexpression system, the resultant downregulation is sufficient to confer resistance to immune cells in culture, and therefore may protect KSHV-infected cells undergoing lytic replication *in vivo*. The contribution of K3 and K5 to immune evasion in the context of viral genome

during latency, however, is less clear. Their influence appears to be most important in the early stages of KSHV infection, when diminished detection by T cells can result in a reduced antiviral cytokine responses and an impaired production of CTLs, thereby allowing the virus to establish a persistent infection.

VIRAL microRNAs

MicroRNAs (miRNAs) are a copious class of evolutionarily conserved small non-coding RNAs that are thought to modulate gene expression post-transcriptionally by targeting mRNAs for degradation or translational repression. miRNAs are 21–23 nucleotide long RNAs that lack protein coding capacity and primarily regulate gene expression by binding to the 3′ untranslated region (3′UTR) of target mRNAs (Bartel, 2004). The genomic regions encoding miRNAs are generally transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II. The nuclease Drosha cleaves pri-miRNA into precursor miRNAs (pre-miRNAs), which are further processed by Dicer to produce mature miRNAs. miRNAs are loaded into a protein complex known as the RNA-induced silencing complex (RISC) to inhibit the translation of target mRNAs (Bartel, 2004). Through various methods of microRNA target identification, miRNA have been found to regulate diverse biological processes including cell cycle, development, differentiation, and metabolism. Given the propensity of viruses to co-opt cellular systems and activities for their benefit, it is perhaps not surprising that several viruses have now been shown to reshape the cellular environment by reprogramming the host's RNAi machinery. Indeed, most herpesviruses encode miRNAs and KSHV in particular encodes 12 pre-miRNAs genes (miR-K1–12) located in the latency locus (Ganem and Ziegelbauer, 2008; Cullen, 2009; Lei et al., 2010a; Ziegelbauer, 2011). These pre-miRNAs are ultimately processed into mature miRNAs. Interestingly, in some cases, mature miRNAs can undergo RNA editing along with variations at the 5′ end of the mature miRNA, leading to at least five different mature miRNAs arising from one pre-miRNA (Pfeffer et al., 2004; Lin et al., 2010; Umbach and Cullen, 2010). Very recently, Gottwein et al. revealed that KSHV miRNAs directly target numerous cellular mRNAs, including many involved in pathways relevant to KSHV pathogenesis and replication (**Table 2**). Notably, around 60% of these miRNAs are also target EBV miRNAs in PEL cells (Gottwein et al., 2011).

Table 2 | Summary of KSHV miRNAs.

Target	miRNAs	Biological effects
THBS1	miR-K12-1, -3, -6, -11	Angiogenesis
BCLAF1	miR-K12-5, -9, -10a, -10b	Viral replication, caspase inhibition
TWEAKR	miR-K12-10a	Anti-apoptosis, anti-inflammation
BACH-1	miR-K12-11	Viral replication
MICB	miR-K12-7	Immune evasion
p21	miR-K12-1	Suppress growth arrest
RBL2	miR-K12-4	DNA alteration
NFKBIA	miR-K12-1	NF-κB activation, maintain latency
KSHV RTA	miR-K12-9*, -7, -5	Maintain latency

Targeting of cellular mRNAs

As with other viral mechanisms of host interaction, viral miRNAs display redundancy in that multiple miRNAs target and repress an individual cellular gene. In a gene expression profile using microarray, Samols et al. identified a total of 81 genes whose expression were significantly changed in cells stably expressing KSHV-encoded miRNAs. Among them, thrombospondin 1 (THBS1), a major regulator of cell adhesion, migration, and angiogenesis showed a significant decrease by several KSHV miRNAs including miR-K12-1, miR-K12-3-3p, miR-K-12-6-3p, and miR-K-12-11. This consequently led to reduced TGF-β activity (Samols et al., 2007). Hence, it is indicated that these KSHV miRNAs potentially contribute to KS angiogenesis by repressing the expression of an angiogenesis inhibitor, THBS1. Another microarray study showed that at least four miRNAs (miR-K-12-5, -9, -10a, and -10b) target BCLAF1 (BCL-2 associated factor). Intriguingly, these miRNAs sensitized latently infected cells to stimuli that induce lytic reactivation (Ziegelbauer et al., 2009). This is a different phenotype from that proposed for the effect of KSHV miRNAs on its own viral proteins, RTA (Lei et al., 2010b; Lu et al., 2010). Thus, it is suggested that KSHV employs its miRNAs to fine-tune gene regulation to either stabilize or destabilize latency. miR-K12-10a, it causes the miR-K12-10a mediated knockdown of TWEAKR (Tumor necrosis factor-like weak inducer of apoptosis receptor) reduces the level of apoptosis and interleukin-8 (IL-8), (Abend et al., 2010). Remarkably, the seed sequence of KSHV miR-K12-11 is identical to that of cellular miR-155. These two miRNAs consequently function as orthologs that downregulate the same target genes including BACH-1(Gottwein et al., 2007; Skalsky et al., 2007). Suppressing BACH-1 by miR-K12-11 increases expression of an amino acid transport protein, xCT, which enables KSHV propagation by cell fusion (Qin et al., 2010). Like HCMV and EBV, KSHV miR-K12-7 has been shown to inhibit MICB (MHC class I polypeptide-related sequence B) expression, thereby preventing NK cell-mediated cell killing during KSHV infection (Nachmani et al., 2009). To investigate the role of KSHV miRNAs in viral latency, Lei et al. generated a mutant KSHV lacking its 14 miRNAs. Interestingly, KSHV miR-K12-1 targets the mRNA of NF-κB inhibitor IκBα, thus promoting viral latency by activating NF-κB (Lei et al., 2010b). miR-K12-1 was also found to inhibit the cellular dependent kinase inhibitor p21 through a different mechanism (Gottwein and Cullen, 2010). KSHV miR-K12-4-5P epigenetically regulates KSHV lytic replication by inhibiting a repressor of DNA methyl transferases, Rbl2 (retinoblastoma-like protein2), to increase the mRNA levels of DNA methyl transferase 1, 3a, and 3b (Iorio et al., 2007). In summary, KSHV miRNAs manipulate host survival pathways to regulate viral latency and lytic replication.

Targeting of viral mRNAs

Currently, a validated viral target of KSHV miRNAs is RTA, a well-known viral latent-lytic switch protein. In order to control the expression of viral lytic genes, KSHV miR-K12-9* directly targets RTA (ORF50, lytic immediately early transcription factor) to prevent viral reactivation (Lu et al., 2010). Another viral miRNA, miR-K12-5, also antagonizes KSHV reactivation by consistently inhibiting RTA expression (Lei et al., 2010b). Taken together, miRNAs are essential for controlling KSHV replication and latency by

either directly targeting the expression of key viral lytic genes or indirectly targeting cellular regulatory genes.

CONCLUDING REMARKS

As we have seen, viruses have learned to manipulate host immune control mechanisms to facilitate their propagation by using captured host genes or evolving genes to target specific immune pathways. Thus, viral genomes can be regarded as repositories of important information of immune processes, offering us a viral view of the host immune system. Hence, studies that add to our growing knowledge of viral immunomodulatory proteins might help us uncover new human genes that control immunity. Their characterization will increase our understanding of not only viral pathogenesis, but also normal immune mechanisms. Furthermore, mechanisms used by viral proteins suggest strategies of immune modulation that might have therapeutic potential. With regards to the discovery of potential therapeutic reagents through virus research, our recent study sets a notable precedent: two peptides derived from the KSHV vIRF4 can additively inhibit HAUSP, leading to p53-mediated tumor regression (Lee et al., 2011). Despite this significant advance, it is only the tip of the iceberg. Although numerous immune evasion mechanisms employed by KSHV seem to be well understood, it is too early to translate the knowledge we have obtained from basic science research into developing more effective clinical management and therapies due to the absence of tractable tissues culture or *in vivo* models for KSHV. Time after time, there is more to be learned from viruses.

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REFERENCES

Abend, J. R., Uldrick, T., and Ziegelbauer, J. M. (2010). Regulation of tumor necrosis factor-like weak inducer of apoptosis receptor protein (TWEAKR) expression by Kaposi's sarcoma-associated herpesvirus microRNA prevents TWEAK-induced apoptosis and inflammatory cytokine expression. *J. Virol.* 84, 12139–12151.

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. *Nat. Rev. Immunol.* 4, 499–511.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell* 124, 783–801.

Alcami, A., and Koszinowski, U. H. (2000). Viral mechanisms of immune evasion. *Trends Microbiol.* 8, 410–418.

Ambagala, A. P., Solheim, J. C., and Srikumaran, S. (2005). Viral interference with MHC class I antigen presentation pathway: the battle continues. *Vet. Immunol. Immunopathol.* 107, 1–15.

Aoki, Y., Jaffe, E. S., Chang, Y., Jones, K., Teruya-Feldstein, J., Moore, P. S., and Tosato, G. (1999). Angiogenesis and hematopoiesis induced by Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. *Blood* 93, 4034–4043.

Areste, C., Mutocheluh, M., and Blackbourn, D. J. (2009). Identification of caspase-mediated decay of interferon regulatory factor-3, exploited by a Kaposi sarcoma-associated herpesvirus immunoregulatory protein. *J. Biol. Chem.* 284, 23272–23285.

Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P. F., Meijer, A. J., Codogno, P., and Ogier-Denis, E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J. Biol. Chem.* 276, 35243–35246.

Bais, C., Van Geelen, A., Eroles, P., Mutlu, A., Chiozzini, C., Dias, S., Silverstein, R. L., Rafii, S., and Mesri, E. A. (2003). Kaposi's sarcoma associated herpesvirus G protein-coupled receptor immortalizes human endothelial cells by activation of the VEGF receptor-2/KDR. *Cancer Cell* 3, 131–143.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297.

Bisson, S. A., Page, A. L., and Ganem, D. (2009). A Kaposi's sarcoma-associated herpesvirus protein that forms inhibitory complexes with type I interferon receptor subunits, Jak and STAT proteins, and blocks interferon-mediated signal transduction. *J. Virol.* 83, 5056–5066.

Blue, C. E., Spiller, O. B., and Blackbourn, D. J. (2004). The relevance of complement to virus biology. *Virology* 319, 176–184.

Bond, G. L., Hu, W., and Levine, A. J. (2005). MDM2 is a central node in the p53 pathway: 12 years and counting. *Curr. Cancer Drug Targets* 5, 3–8.

Bottero, V., Kerur, N., Sadagopan, S., Patel, K., Sharma-Walia, N., and Chandran, B. (2010). Phosphorylation and polyubiquitination of transforming growth factor beta-activated kinase 1 are necessary for activation of NF-kappaB by the Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. *J. Virol.* 85, 1980–1993.

Bubman, D., Guasparri, I., and Cesarman, E. (2007). Deregulation of c-Myc in primary effusion lymphoma by Kaposi's sarcoma herpesvirus latency-associated nuclear antigen. *Oncogene* 26, 4979–4986.

Burger, R., Neipel, F., Fleckenstein, B., Savino, R., Ciliberto, G., Kalden, J. R., and Gramatzki, M. (1998). Human herpesvirus type 8 interleukin-6 homologue is functionally active on human myeloma cells. *Blood* 91, 1858–1863.

Burysek, L., and Pitha, P. M. (2001). Latently expressed human herpesvirus 8-encoded interferon regulatory factor 2 inhibits double-stranded RNA-activated protein kinase. *J. Virol.* 75, 2345–2352.

Cannon, M. (2007). The KSHV and other human herpesviral G protein-coupled receptors. *Curr. Top. Microbiol. Immunol.* 312, 137–156.

Cannon, M., Philpott, N. J., and Cesarman, E. (2003). The Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor has broad signaling effects in primary effusion lymphoma cells. *J. Virol.* 77, 57–67.

Cannon, M. L., and Cesarman, E. (2004). The KSHV G protein-coupled receptor signals via multiple pathways to induce transcription factor activation in primary effusion lymphoma cells. *Oncogene* 23, 514–523.

Chambers, C. A., and Allison, J. P. (1999). Costimulatory regulation of T cell function. *Curr. Opin. Cell Biol.* 11, 203–210.

Chatterjee, M., Osborne, J., Bestetti, G., Chang, Y., and Moore, P. S. (2002). Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* 298, 1432–1435.

Chaudhary, P. M., Jasmin, A., Eby, M. T., and Hood, L. (1999). Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. *Oncogene* 18, 5738–5746.

Chen, D., Sandford, G., and Nicholas, J. (2009). Intracellular signaling mechanisms and activities of human herpesvirus 8 interleukin-6. *J. Virol.* 83, 722–733.

Chen, S., Bacon, K. B., Li, L., Garcia, G. E., Xia, Y., Lo, D., Thompson, D. A., Siani, M. A., Yamamoto, T., Harrison, J. K., and Feng, L. (1998). In vivo inhibition of CC and CX3C chemokine-induced leukocyte infiltration and attenuation of glomerulonephritis in Wistar-Kyoto (WKY) rats by vMIP-II. *J. Exp. Med.* 188, 193–198.

Chen, W., Hilton, I. B., Staudt, M. R., Burd, C. E., and Dittmer, D. P. (2010). Distinct p53, p53:LANA, and LANA complexes in Kaposi's sarcoma – associated herpesvirus lymphomas. *J. Virol.* 84, 3898–3908.

Cheng, E. H., Nicholas, J., Bellows, D. S., Hayward, G. S., Guo, H. G., Reitz, M. S., and Hardwick, J. M. (1997). A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. *Proc. Natl. Acad. Sci. U.S.A.* 94, 690–694.

Choi, Y. B., and Nicholas, J. (2008). Autocrine and paracrine promotion of cell survival and virus replication by human herpesvirus 8 chemokines. *J. Virol.* 82, 6501–6513.

Chow, D., He, X., Snow, A. L., Rose-John, S., and Garcia, K. C. (2001). Structure of an extracellular gp130 cytokine receptor signaling complex. *Science* 291, 2150–2155.

Chung, Y. H., Means, R. E., Choi, J. K., Lee, B. S., and Jung, J. U. (2002). Kaposi's sarcoma-associated herpesvirus OX2 glycoprotein activates myeloid-lineage cells to induce inflammatory cytokine production. *J. Virol.* 76, 4688–4698.

Cloutier, N., and Flamand, L. (2010). Kaposi sarcoma-associated herpesvirus latency-associated nuclear antigen inhibits interferon (IFN) beta expression by competing with IFN regulatory (factor)-3 for binding to IFNB promoter. *J. Biol. Chem.* 285, 7208–7221.

Collot-Teixeira, S., Bass, J., Denis, F., and Ranger-Rogez, S. (2004). Human tumor suppressor p53 and DNA viruses. *Rev. Med. Virol.* 14, 301–319.

Coscoy, L. (2007). Immune evasion by Kaposi's sarcoma-associated herpesvirus. *Nat. Rev. Immunol.* 7, 391–401.

Coscoy, L., and Ganem, D. (2000). Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8051–8056.

Coscoy, L., and Ganem, D. (2001). A viral protein that selectively down-regulates ICAM-1 and B7-2 and modulates T cell costimulation. *J. Clin. Invest.* 107, 1599–1606.

Coscoy, L., Sanchez, D. J., and Ganem, D. (2001). A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. *J. Cell Biol.* 155, 1265–1273.

Cullen, B. R. (2009). Viral and cellular messenger RNA targets of viral microRNAs. *Nature* 457, 421–425.

Cunningham, C., Barnard, S., Blackbourn, D. J., and Davison, A. J. (2003). Transcription mapping of human herpesvirus 8 genes encoding viral interferon regulatory factors. *J. Gen. Virol.* 84, 1471–1483.

Dalton-Griffin, L., Wilson, S. J., and Kellam, P. (2009). X-box binding protein 1 contributes to induction of the Kaposi's sarcoma-associated herpesvirus lytic cycle under hypoxic conditions. *J. Virol.* 83, 7202–7209.

Danial, N. N., and Korsmeyer, S. J. (2004). Cell death: critical control points. *Cell* 116, 205–219.

Diehl, S., and Rincon, M. (2002). The two faces of IL-6 on Th1/Th2 differentiation. *Mol. Immunol.* 39, 531–536.

Dittmer, D., Lagunoff, M., Renne, R., Staskus, K., Haase, A., and Ganem, D. (1998). A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 72, 8309–8315.

Djerbi, M., Screpanti, V., Catrina, A. I., Bogen, B., Biberfeld, P., and Grandien, A. (1999). The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J. Exp. Med.* 190, 1025–1032.

Dunkelberger, J. R., and Song, W. C. (2010). Complement and its role in innate and adaptive immune responses. *Cell Res* 20, 34–50.

Dustin, M. L., and Springer, T. A. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature* 341, 619–624.

Fakhari, F. D., and Dittmer, D. P. (2002). Charting latency transcripts in Kaposi's sarcoma-associated herpesvirus by whole-genome real-time quantitative PCR. *J. Virol.* 76, 6213–6223.

Feng, Z., Zhang, H., Levine, A. J., and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8204–8209.

Field, N., Low, W., Daniels, M., Howell, S., Daviet, L., Boshoff, C., and Collins, M. (2003). KSHV vFLIP binds to IKK-gamma to activate IKK. *J. Cell. Sci.* 116, 3721–3728.

Fodor, W. L., Rollins, S. A., Bianco-Caron, S., Rother, R. P., Guilmette, E. R., Burton, W. V., Albrecht, J. C., Fleckenstein, B., and Squinto, S. P. (1995). The complement control protein homolog of herpesvirus saimiri regulates serum complement by inhibiting C3 convertase activity. *J. Virol.* 69, 3889–3892.

Foster-Cuevas, M., Wright, G. J., Puklavec, M. J., Brown, M. H., and Barclay, A. N. (2004). Human herpesvirus 8 K14 protein mimics CD200 in down-regulating macrophage activation through CD200 receptor. *J. Virol.* 78, 7667–7676.

Foussat, A., Wijdenes, J., Bouchet, L., Gaidano, G., Neipel, F., Balabanian, K., Galanaud, P., Coudercr, J., and Emilie, D. (1999). Human interleukin-6 is in vivo an autocrine growth factor for human herpesvirus-8-infected malignant B lymphocytes. *Eur. Cytokine Netw.* 10, 501–508.

Friborg, J. Jr., Kong, W., Hottiger, M. O., and Nabel, G. J. (1999). p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* 402, 889–894.

Fuld, S., Cunningham, C., Klucher, K., Davison, A. J., and Blackbourn, D. J. (2006). Inhibition of interferon signaling by the Kaposi's sarcoma-associated herpesvirus full-length viral interferon regulatory factor 2 protein. *J. Virol.* 80, 3092–3097.

Ganem, D., and Ziegelbauer, J. (2008). MicroRNAs of Kaposi's sarcoma-associated herpes virus. *Semin. Cancer Biol.* 18, 437–440.

Gao, S. J., Boshoff, C., Jayachandra, S., Weiss, R. A., Chang, Y., and Moore, P. S. (1997). KSHV ORF K9 (vIRF) is an oncogene which inhibits the interferon signaling pathway. *Oncogene* 15, 1979–1985.

Goodbourn, S., Didcock, L., and Randall, R. E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *J. Gen. Virol.* 81, 2341–2364.

Gottwein, E., Corcoran, D. L., Mukherjee, N., Skalsky, R. L., Hafner, M., Nusbaum, J. D., Shamulailatpam, P., Love, C. L., Dave, S. S., Tuschl, T., Ohler, U., and Cullen, B. R. (2011). Viral microRNA targetome of KSHV-infected primary effusion lymphoma cell lines. *Cell Host Microbe* 10, 515–526.

Gottwein, E., and Cullen, B. R. (2010). A human herpesvirus microRNA inhibits p21 expression and attenuates p21-mediated cell cycle arrest. *J. Virol.* 84, 5229–5237.

Gottwein, E., Mukherjee, N., Sachse, C., Frenzel, C., Majoros, W. H., Chi, J. T., Braich, R., Manoharan, M., Soutschek, J., Ohler, U., and Cullen, B. R. (2007). A viral microRNA functions as an orthologue of cellular miR-155. *Nature* 450, 1096–1099.

Green, D. R. (2005). Apoptotic pathways: ten minutes to dead. *Cell* 121, 671–674.

Gregory, S. M., Davis, B. K., West, J. A., Taxman, D. J., Matsuzawa, S., Reed, J. C., Ting, J. P., and Damania, B. (2011). Discovery of a viral NLR homolog that inhibits the inflammasome. *Science* 331, 330–334.

Gregory, S. M., West, J. A., Dillon, P. J., Hilscher, C., Dittmer, D. P., and Damania, B. (2009). Toll-like receptor signaling controls reactivation of KSHV from latency. *Proc. Natl. Acad. Sci. U.S.A.* 106, 11725–11730.

Grossmann, C., Podgrabinska, S., Skobe, M., and Ganem, D. (2006). Activation of NF-kappaB by the latent vFLIP gene of Kaposi's sarcoma-associated herpesvirus is required for the spindle shape of virus-infected endothelial cells and contributes to their proinflammatory phenotype. *J. Virol.* 80, 7179–7185.

Harris, S. L., and Levine, A. J. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene* 24, 2899–2908.

Hoischen, S. H., Vollmer, P., Marz, P., Ozbek, S., Gotze, K. S., Peschel, C., Jostock, T., Geib, T., Mullberg, J., Mechtersheimer, S., Fischer, M., Grötzinger, J., Galle, P. R., and Rose-John, S. (2000). Human herpes virus 8 interleukin-6 homologue triggers gp130 on neuronal and hematopoietic cells. *Eur. J. Biochem.* 267, 3604–3612.

Huang, Q., Petros, A. M., Virgin, H. W., Fesik, S. W., and Olejniczak, E. T. (2002). Solution structure of a Bcl-2 homolog from Kaposi sarcoma virus. *Proc. Natl. Acad. Sci. U.S.A.* 99, 3428–3433.

Inn, K. S., Lee, S. H., Rathbun, J. Y., Wong, L. Y., Toth, Z., Machida, K., Ou, J. H., and Jung, J. U. (2011). Inhibition of RIG-I-mediated signaling by Kaposi's sarcoma-associated herpesvirus-encoded deubiquitinase ORF64. *J. Virol.* 85, 10899–10904.

Iorio, M. V., Visone, R., Di Leva, G., Donati, V., Petrocca, F., Casalini, P., Taccioli, C., Volinia, S., Liu, C. G., Alder, H., Calin, G. A., Ménard, S., and Croce, C. M. (2007). MicroRNA signatures in human ovarian cancer. *Cancer Res.* 67, 8699–8707.

Ishido, S., Wang, C., Lee, B. S., Cohen, G. B., and Jung, J. U. (2000). Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins. *J. Virol.* 74, 5300–5309.

Jia, B., Serra-Moreno, R., Neidermyer, W., Rahmberg, A., Mackey, J., Fofana, I. B., Johnson, W. E., Westmoreland, S., and Evans, D. T. (2009). Species-specific activity of SIV Nef and HIV-1 Vpu in overcoming restriction by tetherin/BST2. *PLoS Pathog.* 5, e1000429. doi:10.1371/journal.ppat.1000429

Joo, C. H., Shin, Y. C., Gack, M., Wu, L., Levy, D., and Jung, J. U. (2007). Inhibition of interferon regulatory factor 7 (IRF7)-mediated interferon signal transduction by the Kaposi's sarcoma-associated herpesvirus viral IRF homolog vIRF3. *J. Virol.* 81, 8282–8292.

Kaletsky, R. L., Francica, J. R., Agrawal-Gamse, C., and Bates, P. (2009). Tetherin-mediated restriction of filovirus budding is antagonized by the Ebola glycoprotein. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2886–2891.

Kapadia, S. B., Molina, H., van Berkel, V., Speck, S. H., and Virgin, H. W. T. (1999). Murine gammaherpesvirus 68 encodes a functional regulator of complement activation. *J. Virol.* 73, 7658–7670.

Kawai, T., and Akira, S. (2006). Innate immune recognition of viral infection. *Nat. Immunol.* 7, 131–137.

Kim, H. J., Lee, S., and Jung, J. U. (2010). When autophagy meets viruses: a double-edged sword with functions in defense and offense. *Semin. Immunopathol.* 32, 323–341.

Lagos, D., Trotter, M. W., Vart, R. J., Wang, H. W., Matthews, N. C., Hansen, A., Flore, O., Gotch, F., and Boshoff, C. (2007). Kaposi sarcoma herpesvirus-encoded vFLIP and vIRF1 regulate antigen presentation in lymphatic endothelial cells. *Blood* 109, 1550–1558.

Lagos, D., Vart, R. J., Gratrix, F., Westrop, S. J., Emuss, V., Wong, P. P., Robey, R., Imami, N., Bower, M., Gotch, F., and Boshoff, C. (2008). Toll-like receptor 4 mediates innate immunity to Kaposi sarcoma herpesvirus. *Cell Host Microbe* 4, 470–483.

Lamkanfi, M., and Dixit, V. M. (2011). Modulation of inflammasome pathways by bacterial and viral pathogens. *J. Immunol.* 187, 597–602.

Le Tortorec, A., and Neil, S. J. (2009). Antagonism to and intracellular sequestration of human tetherin by the human immunodeficiency virus type 2 envelope glycoprotein. *J. Virol.* 83, 11966–11978.

Lee, H. R., Choi, W. C., Lee, S., Hwang, J., Hwang, E., Guchhait, K., Haas, J., Toth, Z., Jeon, Y. H., Oh, T. K., Kim, M. H., Jung, J. U. (2011). Bilateral inhibition of HAUSP deubiquitinase by a viral interferon regulatory factor protein. *Nat. Struct. Mol. Biol.* 18, 1336–1344.

Lee, H. R., Kim, M. H., Lee, J. S., Liang, C., and Jung, J. U. (2009a). Viral interferon regulatory factors. *J. Interferon Cytokine Res.* 29, 621–627.

Lee, H. R., Toth, Z., Shin, Y. C., Lee, J. S., Chang, H., Gu, W., Oh, T. K., Kim, M. H., and Jung, J. U. (2009b). Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor 4 targets MDM2 to deregulate the p53 tumor suppressor pathway. *J. Virol.* 83, 6739–6747.

Lee, J. S., Li, Q., Lee, J. Y., Lee, S. H., Jeong, J. H., Lee, H. R., Chang, H., Zhou, F. C., Gao, S. J., Liang, C., and Jung, J. U. (2009c). FLIP-mediated autophagy regulation in cell death control. *Nat. Cell Biol.* 11, 1355–1362.

Lee, H. R., Lee, S., Chaudhary, P. M., Gill, P., and Jung, J. U. (2010). Immune evasion by Kaposi's sarcoma-associated herpesvirus. *Future Microbiol.* 5, 1349–1365.

Lefort, S., Soucy-Faulkner, A., Grandvaux, N., and Flamand, L. (2007). Binding of Kaposi's sarcoma-associated herpesvirus K-bZIP to interferon-responsive factor 3 elements modulates antiviral gene expression. *J. Virol.* 81, 10950–10960.

Lehner, P. J., Hoer, S., Dodd, R., and Duncan, L. M. (2005). Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligases. *Immunol. Rev.* 207, 112–125.

Lei, X., Bai, Z., Ye, F., Huang, Y., and Gao, S. J. (2010a). Regulation of herpesvirus lifecycle by viral microRNAs. *Virulence* 1, 433–435.

Lei, X., Bai, Z., Ye, F., Xie, J., Kim, C. G., Huang, Y., and Gao, S. J. (2010b). Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. *Nat. Cell Biol.* 12, 193–199.

Levine, B. (2005). Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* 120, 159–162.

Li, M., Damania, B., Alvarez, X., Ogryzko, V., Ozato, K., and Jung, J. U. (2000). Inhibition of p300 histone acetyltransferase by viral interferon regulatory factor. *Mol. Cell. Biol.* 20, 8254–8263.

Li, Q., Means, R., Lang, S., and Jung, J. U. (2007). Downregulation of gamma interferon receptor 1 by Kaposi's sarcoma-associated herpesvirus K3 and K5. *J. Virol.* 81, 2117–2127.

Liang, C., Feng, P., Ku, B., Dotan, I., Canaani, D., Oh, B. H., and Jung, J. U. (2006). Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG. *Nat. Cell Biol.* 8, 688–699.

Liang, C., and Jung, J. U. (2010). Autophagy genes as tumor suppressors. *Curr. Opin. Cell Biol.* 22, 226–233.

Liang, C., Lee, J. S., and Jung, J. U. (2008). Immune evasion in Kaposi's sarcoma-associated herpes virus associated oncogenesis. *Semin. Cancer Biol.* 18, 423–436.

Lin, R., Genin, P., Mamane, Y., Sgarbanti, M., Battistini, A., Harrington, W. J. Jr., Barber, G. N., and Hiscott, J. (2001). HHV-8 encoded vIRF-1 represses the interferon antiviral response by blocking IRF-3 recruitment of the CBP/p300 coactivators. *Oncogene* 20, 800–811.

Lin, Y. T., Kincaid, R. P., Arasappan, D., Dowd, S. E., Hunicke-Smith, S. P., and Sullivan, C. S. (2010). Small RNA profiling reveals antisense transcription throughout the KSHV genome and novel small RNAs. *RNA* 16, 1540–1558.

Liu, L., Eby, M. T., Rathore, N., Sinha, S. K., Kumar, A., and Chaudhary, P. M. (2002). The human herpes virus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the Ikappa B kinase complex. *J. Biol. Chem.* 277, 13745–13751.

Loh, J., Huang, Q., Petros, A. M., Nettesheim, D., van Dyk, L. F., Labrada, L., Speck, S. H., Levine, B., Olejniczak, E. T., and Virgin, H. W. T. (2005). A surface groove essential for viral Bcl-2 function during chronic infection in vivo. *PLoS Pathog.* 1, e10. doi:10.1371/journal.ppat.0010010

Lu, F., Stedman, W., Yousef, M., Renne, R., and Lieberman, P. M. (2010). Epigenetic regulation of Kaposi's sarcoma-associated herpesvirus latency by virus-encoded microRNAs that target Rta and the cellular Rbl2-DNMT pathway. *J. Virol.* 84, 2697–2706.

Lum, J. J., DeBerardinis, R. J., and Thompson, C. B. (2005). Autophagy in metazoans: cell survival in the land of plenty. *Nat. Rev. Mol. Cell Biol.* 6, 439–448.

Maiuri, M. C., Zalckvar, E., Kimchi, A., and Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat. Rev. Mol. Cell Biol.* 8, 741–752.

Manes, T. D., Hoer, S., Muller, W. A., Lehner, P. J., and Pober, J. S. (2010). Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins block distinct steps in transendothelial migration of effector memory CD4+ T cells by targeting different endothelial proteins. *J. Immunol.* 184, 5186–5192.

Mansouri, M., Viswanathan, K., Douglas, J. L., Hines, J., Gustin, J., Moses, A. V., and Fruh, K. (2009). Molecular mechanism of BST2/tetherin downregulation by K5/MIR2 of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 83, 9672–9681.

Mark, L., Proctor, D. G., Blackbourn, D. J., Blom, A. M., and Spiller, O. B. (2008). Separation of decay-accelerating and cofactor functional activities of Kaposi's sarcoma-associated herpesvirus complement control protein using monoclonal antibodies. *Immunology* 123, 228–238.

Martin, D., Galisteo, R., Molinolo, A. A., Wetzker, R., Hirsch, E., and Gutkind, J. S. (2011). PI3Kgamma mediates Kaposi's sarcoma-associated herpesvirus vGPCR-induced sarcomagenesis. *Cancer Cell* 19, 805–813.

Martinon, F., Burns, K., and Tschopp, J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol. Cell* 10, 417–426.

Masood, R., Cai, J., Zheng, T., Smith, D. L., Naidu, Y., and Gill, P. S. (1997). Vascular endothelial growth factor/vascular permeability factor is an autocrine growth factor for AIDS-Kaposi sarcoma. *Proc. Natl. Acad. Sci. U.S.A.* 94, 979–984.

Masters, S. L., Simon, A., Aksentijevich, I., and Kastner, D. L. (2009). Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (*). *Annu. Rev. Immunol.* 27, 621–668.

Meads, M. B., and Medveczky, P. G. (2004). Kaposi's sarcoma-associated herpesvirus-encoded viral interleukin-6 is secreted and modified differently than human interleukin-6: evidence for a unique autocrine signaling mechanism. *J. Biol. Chem.* 279, 51793–51803.

Miao, E. A., Leaf, I. A., Treuting, P. M., Mao, D. P., Dors, M., Sarkar, A., Warren, S. E., Wewers, M. D., and Aderem, A. (2010). Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat. Immunol.* 11, 1136–1142.

Michael, D., and Oren, M. (2003). The p53-Mdm2 module and the ubiquitin system. *Semin. Cancer Biol.* 13, 49–58.

Micheletti, F., Monini, P., Fortini, C., Rimessi, P., Bazzaro, M., Andreoni, M., Giuliani, M., Traniello, S., Ensoli, B., and Gavioli, R. (2002). Identification of cytotoxic T lymphocyte epitopes of human herpesvirus 8. *Immunology* 106, 395–403.

Mills, K. R., Reginato, M., Debnath, J., Queenan, B., and Brugge, J. S. (2004). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3438–3443.

Molden, J., Chang, Y., You, Y., Moore, P. S., and Goldsmith, M. A. (1997). A Kaposi's sarcoma-associated herpesvirus-encoded cytokine homolog (vIL-6) activates signaling through the shared gp130 receptor subunit. *J. Biol. Chem.* 272, 19625–19631.

Montaner, S. (2007). Akt/TSC/mTOR activation by the KSHV G protein-coupled receptor: emerging insights into the molecular oncogenesis and treatment of Kaposi's sarcoma. *Cell Cycle* 6, 438–443.

Montaner, S., Sodhi, A., Molinolo, A., Bugge, T. H., Sawai, E. T., He, Y., Li, Y., Ray, P. E., and Gutkind, J. S. (2003). Endothelial infection with KSHV genes in vivo reveals that vGPCR initiates Kaposi's sarcomagenesis and can promote the tumorigenic potential of viral latent genes. *Cancer Cell* 3, 23–36.

Montaner, S., Sodhi, A., Servitja, J. M., Ramsdell, A. K., Barac, A., Sawai, E. T., and Gutkind, J. S. (2004). The small GTPase Rac1 links the Kaposi sarcoma-associated herpesvirus vGPCR to cytokine secretion and paracrine neoplasia. *Blood* 104, 2903–2911.

Moore, P. S., Boshoff, C., Weiss, R. A., and Chang, Y. (1996). Molecular mimicry of human cytokine and cytokine response pathway genes by KSHV. *Science* 274, 1739–1744.

Morgan, B. P., Marchbank, K. J., Longhi, M. P., Harris, C. L., and Gallimore, A. M. (2005). Complement: central to innate immunity and bridging to adaptive responses. *Immunol. Lett.* 97, 171–179.

Mori, Y., Nishimoto, N., Ohno, M., Inagi, R., Dhepakson, P., Amou, K., Yoshizaki, K., and Yamanishi, K. (2000). Human herpesvirus 8-encoded interleukin-6 homologue (viral IL-6) induces endogenous human IL-6 secretion. *J. Med. Virol.* 61, 332–335.

Mutocheluh, M., Hindle, L., Areste, C., Chanas, S. A., Butler, L. M., Lowry, K., Shah, K., Evans, D. J., and Blackbourn, D. J. (2011). Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor-2 inhibits type 1 interferon signalling by targeting interferon-stimulated gene factor-3. *J. Gen. Virol.* 92, 2394–2398.

Nachmani, D., Stern-Ginossar, N., Sarid, R., and Mandelboim, O. (2009). Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. *Cell Host Microbe* 5, 376–385.

Nakamura, H., Li, M., Zarycki, J., and Jung, J. U. (2001). Inhibition of p53 tumor suppressor by viral interferon regulatory factor. *J. Virol.* 75, 7572–7582.

Nakamura, S., Murakami-Mori, K., Rao, N., Weich, H. A., and Rajeev, B. (1997). Vascular endothelial growth factor is a potent angiogenic factor in AIDS-associated Kaposi's sarcoma-derived spindle cells. *J. Immunol.* 158, 4992–5001.

Nathan, J. A., and Lehner, P. J. (2009). The trafficking and regulation of membrane receptors by the RING-CH ubiquitin E3 ligases. *Exp. Cell Res.* 315, 1593–1600.

Neil, S. J., Zang, T., and Bieniasz, P. D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451, 425–430.

Okroj, M., Mark, L., Stokowska, A., Wong, S. W., Rose, N., Blackbourn, D. J., Villoutreix, B. O., Spiller, O. B., and Blom, A. M. (2009). Characterization of the complement inhibitory function of rhesus rhadinovirus complement control protein (RCP). *J. Biol. Chem.* 284, 505–514.

O'Neill, L. A., and Bowie, A. G. (2010). Sensing and signaling in antiviral innate immunity. *Curr. Biol.* 20, R328–R333.

Pardieu, C., Vigan, R., Wilson, S. J., Calvi, A., Zang, T., Bieniasz, P., Kellam, P., Towers, G. J., and Neil, S. J. (2010). The RING-CH ligase K5 antagonizes restriction of KSHV and HIV-1 particle release by mediating ubiquitin-dependent endosomal degradation of tetherin. *PLoS Pathog.* 6, e1000843. doi:10.1371/journal.ppat.1000843

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927–939.

Paulose-Murphy, M., Ha, N. K., Xiang, C., Chen, Y., Gillim, L., Yarchoan, R., Meltzer, P., Bittner, M., Trent, J., and Zeichner, S. (2001). Transcription program of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus). *J. Virol.* 75, 4843–4853.

Pestka, S., Krause, C. D., and Walter, M. R. (2004). Interferons, interferon-like cytokines, and their receptors. *Immunol. Rev.* 202, 8–32.

Pfeffer, S., Zavolan, M., Grasser, F. A., Chien, M., Russo, J. J., Ju, J., John, B., Enright, A. J., Marks, D., Sander, C., and Tuschl, T. (2004). Identification of virus-encoded microRNAs. *Science* 304, 734–736.

Pyo, J. O., Jang, M. H., Kwon, Y. K., Lee, H. J., Jun, J. I., Woo, H. N., Cho, D. H., Choi, B., Lee, H., Kim, J. H., Mizushima, N., Oshumi, Y., and Jung, Y. K. (2005). Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. *J. Biol. Chem.* 280, 20722–20729.

Qin, Z., Freitas, E., Sullivan, R., Mohan, S., Bacelieri, R., Branch, D., Romano, M., Kearney, P., Oates, J., Plaisance, K., Renne, R., Kaleeba, J., and Parsons, C. (2010). Upregulation of xCT by KSHV-encoded microRNAs facilitates KSHV dissemination and persistence in an environment of oxidative stress. *PLoS Pathog.* 6, e1000742. doi:10.1371/journal.ppat.1000742

Reimold, A. M., Ponath, P. D., Li, Y. S., Hardy, R. R., David, C. S., Strominger, J. L., and Glimcher, L. H. (1996). Transcription factor B cell lineage-specific activator protein regulates the gene for human X-box binding protein 1. *J. Exp. Med.* 183, 393–401.

Rezaee, S. A., Gracie, J. A., McInnes, I. B., and Blackbourn, D. J. (2005). Inhibition of neutrophil function by the Kaposi's sarcoma-associated herpesvirus vOX2 protein. *AIDS* 19, 1907–1910.

Rivas, C., Thlick, A. E., Parravicini, C., Moore, P. S., and Chang, Y. (2001). Kaposi's sarcoma-associated herpesvirus LANA2 is a B-cell-specific latent viral protein that inhibits p53. *J. Virol.* 75, 429–438.

Sadler, A. J., and Williams, B. R. (2008). Interferon-inducible antiviral effectors. *Nat. Rev. Immunol.* 8, 559–568.

Salata, C., Curtarello, M., Calistri, A., Sartori, E., Sette, P., de Bernard, M., Parolin, C., and Palu, G. (2009). vOX2 glycoprotein of human herpesvirus 8 modulates human primary macrophages activity. *J. Cell. Physiol.* 219, 698–706.

Samols, M. A., Skalsky, R. L., Maldonado, A. M., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Identification of cellular genes targeted by KSHV-encoded microRNAs. *PLoS Pathog.* 3, e65. doi:10.1371/journal.ppat.0030065

Sanchez, D. J., Coscoy, L., and Ganem, D. (2002). Functional organization of MIR2, a novel viral regulator of selective endocytosis. *J. Biol. Chem.* 277, 6124–6130.

Sarid, R., Sato, T., Bohenzky, R. A., Russo, J. J., and Chang, Y. (1997). Kaposi's sarcoma-associated herpesvirus encodes a functional bcl-2 homologue. *Nat. Med.* 3, 293–298.

Schmidt, K., Wies, E., and Neipel, F. (2011). Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor 3 inhibits gamma interferon and major histocompatibility complex class II expression. *J. Virol.* 85, 4530–4537.

Schroder, K., and Tschopp, J. (2010). The inflammasomes. *Cell* 140, 821–832.

Seo, T., Park, J., Lee, D., Hwang, S. G., and Choe, J. (2001). Viral interferon regulatory factor 1 of Kaposi's sarcoma-associated herpesvirus binds to p53 and represses p53-dependent transcription and apoptosis. *J. Virol.* 75, 6193–6198.

Shin, Y. C., Nakamura, H., Liang, X., Feng, P., Chang, H., Kowalik, T. F., and Jung, J. U. (2006). Inhibition of the ATM/p53 signal transduction pathway by Kaposi's sarcoma-associated herpesvirus interferon regulatory factor 1. *J. Virol.* 80, 2257–2266.

Shiratori, I., Yamaguchi, M., Suzukawa, M., Yamamoto, K., Lanier, L. L., Saito, T., and Arase, H. (2005). Down-regulation of basophil function by human CD200 and human herpesvirus-8 CD200. *J. Immunol.* 175, 4441–4449.

Si, H., and Robertson, E. S. (2006). Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen induces chromosomal instability through inhibition of p53 function. *J. Virol.* 80, 697–709.

Skalsky, R. L., Samols, M. A., Plaisance, K. B., Boss, I. W., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. *J. Virol.* 81, 12836–12845.

Sodhi, A., Montaner, S., Patel, V., Zohar, M., Bais, C., Mesri, E. A., and Gutkind, J. S. (2000). The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. *Cancer Res.* 60, 4873–4880.

Sperling, A. I., and Bluestone, J. A. (1996). The complexities of T-cell co-stimulation: CD28 and beyond. *Immunol. Rev.* 153, 155–182.

Spiller, O. B., Blackbourn, D. J., Mark, L., Proctor, D. G., and Blom, A. M. (2003a). Functional activity of the complement regulator encoded by Kaposi's sarcoma-associated herpesvirus. *J. Biol. Chem.* 278, 9283–9289.

Spiller, O. B., Robinson, M., O'Donnell, E., Milligan, S., Morgan, B. P., Davison, A. J., and Blackbourn, D. J. (2003b). Complement regulation by Kaposi's sarcoma-associated herpesvirus ORF4 protein. *J. Virol.* 77, 592–599.

Spiller, O. B., Mark, L., Blue, C. E., Proctor, D. G., Aitken, J. A., Blom, A. M., and Blackbourn, D. J. (2006). Dissecting the regions of

virion-associated Kaposi's sarcoma-associated herpesvirus complement control protein required for complement regulation and cell binding. *J. Virol.* 80, 4068–4078.

Sun, Q., Matta, H., Lu, G., and Chaudhary, P. M. (2006). Induction of IL-8 expression by human herpesvirus 8 encoded vFLIP K13 via NF-kappaB activation. *Oncogene* 25, 2717–2726.

Suzuki, T., Isobe, T., Kitagawa, M., and Ueda, K. (2010). Kaposi's sarcoma-associated herpesvirus-encoded LANA positively affects on ubiquitylation of p53. *Biochem. Biophys. Res. Commun.* 403, 194–197.

Swanton, C., Mann, D. J., Fleckenstein, B., Neipel, F., Peters, G., and Jones, N. (1997). Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. *Nature* 390, 184–187.

Takaoka, A., Tamura, T., and Taniguchi, T. (2008). Interferon regulatory factor family of transcription factors and regulation of oncogenesis. *Cancer Sci.* 99, 467–478.

Tamura, T., Yanai, H., Savitsky, D., and Taniguchi, T. (2008). The IRF family transcription factors in immunity and oncogenesis. *Annu. Rev. Immunol.* 26, 535–584.

Thorburn, J., Moore, F., Rao, A., Barclay, W. W., Thomas, L. R., Grant, K. W., Cramer, S. D., and Thorburn, A. (2005). Selective inactivation of a Fas-associated death domain protein (FADD)-dependent apoptosis and autophagy pathway in immortal epithelial cells. *Mol. Biol. Cell* 16, 1189–1199.

Ting, J. P., Willingham, S. B., and Bergstralh, D. T. (2008). NLRs at the intersection of cell death and immunity. *Nat. Rev. Immunol.* 8, 372–379.

Trinchieri, G. (2010). Type I interferon: friend or foe? *J. Exp. Med.* 207, 2053–2063.

Umbach, J. L., and Cullen, B. R. (2010). In-depth analysis of Kaposi's sarcoma-associated herpesvirus microRNA expression provides insights into the mammalian microRNA-processing machinery. *J. Virol.* 84, 695–703.

Van Damme, N., Goff, D., Katsura, C., Jorgenson, R. L., Mitchell, R., Johnson, M. C., Stephens, E. B., and Guatelli, J. (2008). The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell Host Microbe* 3, 245–252.

Vogelstein, B., Lane, D., and Levine, A. J. (2000). Surfing the p53 network. *Nature* 408, 307–310.

Wang, X., Lybarger, L., Connors, R., Harris, M. R., and Hansen, T. H. (2004). Model for the interaction of gammaherpesvirus 68 RING-CH finger protein mK3 with major histocompatibility complex class I and the peptide-loading complex. *J. Virol.* 78, 8673–8686.

Weber, K. S., Grone, H. J., Rocken, M., Klier, C., Gu, S., Wank, R., Proudfoot, A. E., Nelson, P. J., and Weber, C. (2001). Selective recruitment of Th2-type cells and evasion from a cytotoxic immune response mediated by viral macrophage inhibitory protein-II. *Eur. J. Immunol.* 31, 2458–2466.

West, J., and Damania, B. (2008). Upregulation of the TLR3 pathway by Kaposi's sarcoma-associated herpesvirus during primary infection. *J. Virol.* 82, 5440–5449.

West, J. A., Gregory, S. M., Sivaraman, V., Su, L., and Damania, B. (2011). Activation of plasmacytoid dendritic cells by Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 85, 895–904.

Wies, E., Hahn, A. S., Schmidt, K., Viebahn, C., Rohland, N., Lux, A., Schellhorn, T., Holzer, A., Jung, J. U., and Neipel, F. (2009). The Kaposi's sarcoma-associated herpesvirus-encoded vIRF-3 inhibits cellular IRF-5. *J. Biol. Chem.* 284, 8525–8538.

Wilson, S. J., Tsao, E. H., Webb, B. L., Ye, H., Dalton-Griffin, L., Tsantoulas, C., Gale, C. V., Du, M. Q., Whitehouse, A., and Kellam, P. (2007). X box binding protein XBP-1s transactivates the Kaposi's sarcoma-associated herpesvirus (KSHV) ORF50 promoter, linking plasma cell differentiation to KSHV reactivation from latency. *J. Virol.* 81, 13578–13586.

Ye, F. C., Zhou, F. C., Xie, J. P., Kang, T., Greene, W., Kuhne, K., Lei, X. F., Li, Q. H., and Gao, S. J. (2008). Kaposi's sarcoma-associated herpesvirus latent gene vFLIP inhibits viral lytic replication through NF-kappaB-mediated suppression of the AP-1 pathway: a novel mechanism of virus control of latency. *J. Virol.* 82, 4235–4249.

Yu, F., Feng, J., Harada, J. N., Chanda, S. K., Kenney, S. C., and Sun, R. (2007). B cell terminal differentiation factor XBP-1 induces reactivation of Kaposi's sarcoma-associated herpesvirus. *FEBS Lett.* 581, 3485–3488.

Yu, Y., and Hayward, G. S. (2010). The ubiquitin E3 ligase RAUL negatively regulates type i interferon through ubiquitination of the transcription factors IRF7 and IRF3. *Immunity* 33, 863–877.

Yu, Y., Wang, S. E., and Hayward, G. S. (2005). The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteosome-mediated degradation. *Immunity* 22, 59–70.

Zhang, F., Wilson, S. J., Landford, W. C., Virgen, B., Gregory, D., Johnson, M. C., Munch, J., Kirchhoff, F., Bieniasz, P. D., and Hatziioannou, T. (2009). Nef proteins from simian immunodeficiency viruses are tetherin antagonists. *Cell Host Microbe* 6, 54–67.

Zhao, J., Punj, V., Matta, H., Mazzacurati, L., Schamus, S., Yang, Y., Yang, T., Hong, Y., and Chaudhary, P. M. (2007). K13 blocks KSHV lytic replication and deregulates vIL6 and hIL6 expression: a model of lytic replication induced clonal selection in viral oncogenesis. *PLoS ONE* 2, e1067. doi:10.1371/journal.pone.0001067

Zhu, F. X., King, S. M., Smith, E. J., Levy, D. E., and Yuan, Y. (2002). A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 5573–5578.

Ziegelbauer, J. M. (2011). Functions of Kaposi's sarcoma-associated herpesvirus microRNAs. *Biochim. Biophys. Acta* 1809, 623–630.

Ziegelbauer, J. M., Sullivan, C. S., and Ganem, D. (2009). Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. *Nat. Genet.* 41, 130–134.

Zimring, J. C., Goodbourn, S., and Offermann, M. K. (1998). Human herpesvirus 8 encodes an interferon regulatory factor (IRF) homolog that represses IRF-1-mediated transcription. *J. Virol.* 72, 701–707.

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KSHV induction of angiogenic and lymphangiogenic phenotypes

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Kaposi's sarcoma (KS) is a highly vascularized tumor supporting large amounts of neoangiogenesis. The major cell type in KS tumors is the spindle cell, a cell that expresses markers of lymphatic endothelium. KSHV, the etiologic agent of KS, is found in the spindle cells of all KS tumors. Considering the extreme extent of angiogenesis in KS tumors at all stages it has been proposed that KSHV directly induces angiogenesis in a paracrine fashion. In accordance with this theory, KSHV infection of endothelial cells in culture induces a number of host pathways involved in activation of angiogenesis and a number of KSHV genes themselves can induce pathways involved in angiogenesis. Spindle cells are phenotypically endothelial in nature, and therefore, activation through the induction of angiogenic and/or lymphangiogenic phenotypes by the virus may also be directly involved in spindle cell growth and tumor induction. Accordingly, KSHV infection of endothelial cells induces cell autonomous angiogenic phenotypes to activate host cells. KSHV infection can also reprogram blood endothelial cells to lymphatic endothelium. However, KSHV induces some blood endothelial specific genes upon infection of lymphatic endothelial cells creating a phenotypic intermediate between blood and lymphatic endothelium. Induction of pathways involved in angiogenesis and lymphangiogenesis are likely to be critical for tumor cell growth and spread. Thus, induction of both cell autonomous and non-autonomous changes in angiogenic and lymphangiogenic pathways by KSHV likely plays a key role in the formation of KS tumors.

Keywords: Kaposi's Sarcoma, HHV-8, angiogenesis, lymphangiogenesis, viral oncogenesis, Kaposi's Sarcoma-associated herpesvirus

KAPOSI'S SARCOMA AND KSHV

Kaposi's sarcoma (KS) was first described in the 1800s as a rare, fairly indolent tumor of specific populations. This form of KS, now referred to as classic KS, usually presents on the skin of the lower extremities of elderly men of specific European regions and religious origins. In the middle of the twentieth century KS became endemic in parts of Africa and is currently one of the most common tumors in parts of central Africa (Wabinga et al., 1993). In the late twentieth century KS became one of the first AIDS defining illnesses and is the most common tumor of AIDS patients world-wide. AIDS associated KS is generally far more aggressive than classic KS, arising on the skin in many parts of the body as well as in the oral cavity and can occur on internal organs where it is often fatal.

While they differ in aggressiveness, all forms of KS are relatively indistinguishable at the histological level. Grossly, the tumors have a characteristic red to purple hue, indicative of the high vascularization of the tumor. Histologically, the tumor exhibits large vascular slits lined by flattened endothelium; the slits are often, but not always, filled with blood cells. There are discernable levels of extravasated red blood cells and infiltrating lymphocytes in the tumor. While a number of cell types are present, the tumor is predominantly made up of elongated spindle cells. The spindle cells express endothelial cell markers on their surface including CD31 and CD34, but express low levels of factor VIIIRa (Russell Jones et al., 1995). Recent expression data and array studies have found that spindle cells most closely resemble lymphatic endothelium, expressing VEGF receptor 3 (VEGFR3), a specific marker of lymphatic endothelial cells (Jussila et al., 1998; Skobe et al., 1999; Weninger et al., 1999; Wang et al., 2004a). Other lymphatic endothelial cell specific markers, including LYVE-1, podoplanin, and Prox-1, are also expressed by the spindle cells (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004a).

Based on the epidemiology and the multicentric nature of the tumor, KS was predicted to have an infectious cause (Beral et al., 1990). In 1994, KSHV was discovered associated with all KS tumors and is now considered to be the etiologic agent (Chang et al., 1994). KSHV was the eighth human herpesvirus discovered and is subclassified as a gamma herpesvirus. Like all herpesviruses KSHV has both a lytic and latent lifecycle. During lytic replication all of the greater than 90 viral genes are expressed, the virus replicates rapidly, produces infectious virions and ultimately causes cell death likely due to a combination of host cell shut off and virus production. During viral latency in endothelial cells, a limited number of genes are expressed from a single locus. This locus includes LANA, vCyc, vFLIP, a family of proteins from a repeat region called the Kaposins, and 12 pre-microRNAs encoding 17 or more mature miRNAs. These latent genes are responsible for the

maintenance of the latent viral episome as well as the survival of latently infected cells.

In later-stage KS tumors, all of the spindle cells maintain infection with KSHV (Boshoff et al., 1995; Staskus et al., 1997; Dupin et al., 1999). As expected the virus is predominantly found in the latent state in spindle cells where the limited number of latent genes and miRNAs are expressed (Staskus et al., 1997; Marshall et al., 2007). However, approximately 1–5% of the spindle cells express lytic viral genes and produce infectious virus. In addition to spindle cells, KSHV is also found in other cell types in the KS lesion including monocytes (Blasig et al., 1997). However, only very low levels of these cells are infected in the tumor. KSHV can only sporadically be detected in the endothelium lining the vascular slits in the KS tumor (Dupin et al., 1999). KSHV is also associated with two lymphoproliferative diseases, primary effusion lymphoma (PEL) a pleural cavity solid B-cell lymphoma, and plasmablastic multicentric Castleman's disease, a lymph node B-cell growth (Cesarman et al., 1995; Soulier et al., 1995).

Because KS is an angioproliferative disease and the KS tumors are highly vascularized even at early stages, it has been proposed that KSHV may directly induce angiogenesis. Angiogenesis is a tightly regulated process. Endothelial cells of the vascular system are normally maintained in a quiescent, non-proliferating state. However, during solid tumor formation, the secretion of pro-angiogenic cytokines by tumor cells can activate nearby endothelial cells to induce new blood vessel formation. Many of the vascular slits identified in histological sections of early stage KS lesions are lined by uninfected endothelium, suggesting they are formed by endothelial cells activated in a paracrine fashion (Dupin et al., 1999). These uninfected cells may later become infected, as KSHV has, in some cases, been detected in the cells surrounding the vascular spaces of later-stage nodular KS (Boshoff et al., 1995; Dupin et al., 1999).

Despite the evidence for paracrine activation of uninfected endothelial cells, KSHV also likely activates infected endothelial cells in an autocrine or cell autonomous fashion. Because KS spindle cells are endothelial in origin, induction of the KS tumor cell is similar to the processes of angiogenesis. Many of the characteristics of activated endothelial cells and angiogenesis are also associated with oncogenesis, including proliferation, migration, and metalloprotease expression. These same phenotypes are induced in KSHV-infected endothelial cells. This review discusses the recent evidence that suggests that (1) KSHV promotes continual neovascularization through paracrine factors and (2) KSHV may drive tumor cell growth through autocrine and cell autonomous activation of angiogenic phenotypes.

PARACRINE INDUCTION OF ANGIOGENESIS BY KSHV

The vascular endothelial growth factor (VEGF) family of cytokines plays a prominent role in regulation of angiogenesis (Breen, 2007). VEGF-A and its receptors are required for embryonic vascular development and are important for vascular permeability, proliferation, and survival of newly formed vasculature. Several studies have explored the role of VEGF-A in KS pathogenesis. VEGF-A expression is detected in spindle cells of KS lesions, and its secretion is known to be increased by inflammatory cytokines that are present in the KS lesions (Samaniego et al., 1998). VEGF-A is also expressed by KSHV-infected PEL cell lines and conditioned media from these cells is sufficient to induce capillary morphogenesis by endothelial cells (Liu et al., 2001; Akula et al., 2005; Subramanian et al., 2010). Infection of endothelial cells with KSHV directly leads to increased expression of VEGF-A (Masood et al., 2002; Sivakumar et al., 2008; Wang and Damania, 2008). Further, KSHV conditioned media has been shown to regulate angiogenic phenotypes in endothelial cells (Sharma-Walia et al., 2010). Therefore, KSHV induction of VEGF-A is likely to be critical for both the induction of angiogenesis as well as the activation of infected spindle cells.

Although the mechanisms by which KSHV induces VEGF-A expression and secretion are still unclear, several potential pathways have been uncovered. Hypoxia induced factor (HIF)-1α is a transcription factor that has been shown to be important for upregulation of VEGF-A (Sodhi et al., 2000; Shin et al., 2008). HIF-1α is readily degraded during normal oxygen conditions. However, during hypoxia, HIF-1α is stabilized and can induce expression of genes through hypoxia responsive elements, including VEGF-A. Interestingly, KSHV infection of endothelial cells induces the expression of HIF-1α during normoxia which leads to increased HIF transcriptional activity (Carroll et al., 2006). Other studies have shown that KSHV encodes proteins that can lead to increased stability of HIF-1. The KSHV latency associated nuclear protein (LANA-1) can stabilize HIF-1α, through both degradation of its suppressors, von Hippel–Lindau protein and p53 (Cai et al., 2006), and through direct interaction between HIF-1α and LANA-1 (Cai et al., 2007). Additionally, the virally encoded interferon response factor (vIRF3) can, like LANA-1, interact with and stabilize HIF-1α, leading to increased VEGF-A expression (Shin et al., 2008). The KSHV viral G-protein coupled receptor (vGPCR) is able to increase the activity of HIF-1α as a transcription factor through activation of the MAPK and p38 signaling pathways and subsequent phosphorylation of HIF-1α (Sodhi et al., 2000). These pathways are depicted in **Figure 1**. While induction of HIF mRNA expression by KSHV infection has been shown, stabilization of HIF directly by KSHV infection of endothelial cells has yet to be clearly shown.

Other host proteins have been shown to be involved in the induction of VEGF-A during KSHV infection of endothelial cells as well. For example, Emmprin is a membrane-associated glycoprotein that promotes matrix metalloproteinase expression. Its expression in KSHV-infected cells promotes cell invasiveness through activation of the PI3K/Akt and MAPK pathways (Qin et al., 2010; Dai et al., 2011). These pathways are also necessary for emmprin-induced VEGF-A expression. Further work is ongoing in multiple labs to determine the cellular pathways essential for KSHV induction of VEGF-A.

Several KSHV genes expressed during lytic replication have been implicated in regulation of VEGF-A expression (**Table 1**). In BCBL-1 cells (a pleural effusion lymphoma cell line), glycoprotein B (gB) and K8.1 are required for enhanced VEGF expression (Subramanian et al., 2010). Treatment of these cells with siRNA or neutralizing antibodies to gB or K8.1 significantly reduced VEGF-A production. vGPCR is a constitutively active signaling receptor that has been linked to a variety of cell survival and pro-angiogenic signaling pathways (Arvanitakis et al., 1997; Bais

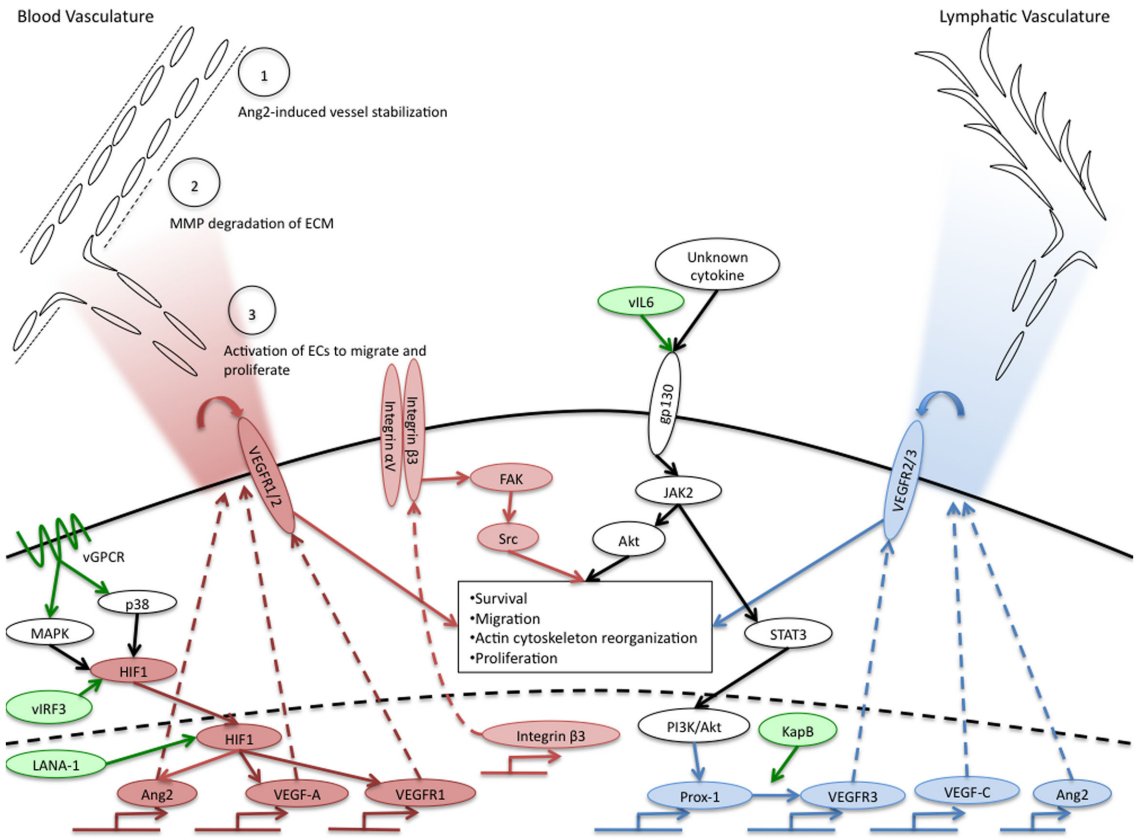

FIGURE 1 | Overview of KSHV induction of angiogenic and lymphangiogenic pathways. Pro-angiogenic factors that are induced by KSHV are indicated in red ovals, pro-lymphangiogenic factors induced by KSHV are indicated in blue ovals. White ovals indicate the activated pathway necessary for KSHV induction of blood to lymphatic endothelial cell differentiation. KSHV genes known to induce angiogenic or lymphangiogenic pathways are indicated in green ovals. The upper left corner indicates phenotypes of angiogenesis that are activated in normal endothelium by KSHV-infected cells and the gradient of cytokines secreted by KSHV-infected cells is indicated by the red gradient. The blue gradient in the upper right hand corner indicates the gradient of lymphangiogenic cytokines induced by KSHV-infected cells that could induce lymphangiogenesis. The central box indicates angiogenic phenotypes induced by KSHV in a cell autonomous fashion.

Table 1 | KSHV genes involved in the induction of angiogenesis.

KSHV gene	Pro-angiogenic effect
Glycoprotein B	Increased VEGF-A secretion
K8.1	Increased VEGF-A secretion
KI	Increased VEGF-A secretion; Disrupted VE-cadherin signaling
LANA-1	HIF-1 stability
vIRF3	HIF-1 stability
vGPCR	HIF-1 stability; Increased VEGF-A secretion; Increased angiopoietin-1 secretion; Increased angiopoietin-like 4 secretion; Disrupted VE-cadherin signaling
vIL-6	Increased VEGF-A secretion; Increased angiopoietin-1 secretion
Viral miRNAs	Downregulation of thrombospondin-1
vMIPs I–III	Chemoattraction
K5	Degradation of VE-cadherin; Degradation of PECAM-1

et al., 1998, 2003; Sodhi et al., 2000; Montaner et al., 2001; Shan et al., 2007). When injected into mice, NIH3T3 cells expressing vGPCR form highly vascularized tumors with some similarities to KS and this may be due, at least in part, to increased VEGF-A secretion (Bais et al., 1998; Guo et al., 2003). vGPCR upregulates VEGF-A through activation of MAPK and p38, which, as described above, promotes HIF-1α activity (Sodhi et al., 2000). Transgenic mice expressing vGPCR also form highly vascularized tumors that are reminiscent of KS tumors. However, cell lines derived from these tumors expressed the lymphatic growth factor VEGF-C, rather than VEGF-A (Guo et al., 2003). Increased VEGF-A expression in cells expressing vGPCR is associated with constitutive activation of its receptor, VEGFR2/KDR and downstream activation of PI3K/Akt, contributing to endothelial cell survival (Montaner et al., 2001; Bais et al., 2003). However, gB, K8.1, and vGPCR have only been detected in cells supporting lytic KSHV infection whereas the bulk of the tumor cells are latently infected. The KSHV glycoprotein K1 also induces increased VEGF-A expression in endothelial cells and is capable of immortalizing primary endothelial cells (Wang et al., 2004b, 2006). While there is evidence that K1 is expressed at very low levels during latency, the majority of its expression occurs during lytic infection (Chandriani and Ganem, 2010). In summary, the lytic phase of

KSHV infection might play a role in the paracrine induction of angiogenesis through increased secretion of VEGF-A into the tumor milieu.

In addition to VEGF-A, KSHV-infected endothelial cells also express other angiogenic cytokines. Angiopoietin-1 and -2 are ligands for the receptor tyrosine kinase Tie2. Although less is known about the functions of angiopoietins and Tie2, their signaling is required for proper vascular development during embryogenesis (Dumont et al., 1994). Angiopoietin-1 is an agonist for the Tie2 receptor, promoting endothelial cell survival and stability. In contrast, Ang2 acts as an antagonist for Tie2, although its role is context-dependent. Ang2 is upregulated during pathologic angiogenesis and this expression is thought to destabilize endothelial cells, allowing them to be activated by other pro-angiogenic stimuli, such as VEGF, see **Figure 1**, circle 1 (Gale et al., 2002). Interestingly, Ang2 is expressed in KS lesions, and is upregulated in endothelial cells infected with KSHV (Brown et al., 2000; Wang et al., 2004a; Vart et al., 2007; Ye et al., 2007). This induction can be activated by the KSHV genes vGPCR and vIL-6, and can occur through a paracrine mechanism (Vart et al., 2007). Another study suggests that the MAPK pathway activation of transcription factors AP-1 and Ets-1 is involved (Ye et al., 2007). In addition to Ang2, cells transfected with the vGPCR gene expressed increased levels of angiopoietin-like 4, a member of the angiopoietin-like proteins that may play a role in vascular permeability and angiogenesis (Ma et al., 2010).

KSHV induces a number of other cytokines known to be involved in angiogenesis in other systems. These include interleukin 6 (IL-6), Monocyte chemoattractant protein-1 (MCP-1), PAX-1, and prostaglandin E2 (Schwarz and Murphy, 2001; Polson et al., 2002; Xie et al., 2005; Caselli et al., 2007; Fonsato et al., 2008). Cyclooxygenase enzymes catalyze the rate limiting step in the conversion of arachidonic acid into prostaglandins. Prostaglandins signal through g-protein coupled receptors to regulate a variety of functions, including metabolic, neuronal, and immune functions. Cyclooxygenase-2 (COX-2) expression is induced early during KSHV infection of endothelial cells and plays a role in the establishment of latency (Naranatt et al., 2004; Sharma-Walia et al., 2006). This expression is associated with increased secretion of prostaglandin E2, which promotes inflammatory cytokine expression, cell survival, and angiogenesis (Sharma-Walia et al., 2010). An additional cellular factor associated with angiogenesis, angiogenin, is induced in endothelial cells by the latent protein, LANA-1. Angiogenin was recently shown to aid in induction of angiogenesis by both VEGF and basic fibroblast growth factor (Sadagopan et al., 2009). KSHV-induced angiogenin was able to promote endothelial cell migration and capillary morphogenesis (Sadagopan et al., 2009). Since angiogenin is internalized by both infected and uninfected cells, the authors suggested angiogenin may work in both paracrine and autocrine fashions. In fact, all KSHV-induced cytokines that act on endothelial cells have the potential to promote angiogenesis-like phenotypes on the endothelial-derived spindle cells.

Regulation of angiogenesis involves coordinated expression of both pro- and anti-angiogenic factors. KSHV not only upregulates pro-angiogenic cytokines, it may also promote angiogenesis through repression of angiogenic inhibitors. The KSHV latent locus encodes for 17 miRNAs which may play a role in downregulation of angiogenic gene expression (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005). Expression of 10 of these miRNAs in 293 cells altered the expression of 81 genes (Samols et al., 2007). Interestingly, one of these genes is the natural angiogenic inhibitor thrombospondin-1. Thrombospondin-1 plays multiple roles in the repression of angiogenesis, however one of its main functions is activation of the anti-angiogenic growth factor transforming growth factor-β (TGF-β). This study found that thrombospondin-1 contains 34 putative miRNA binding sites, and can be downregulated by multiple KSHV miRNAs (Samols et al., 2007). Downregulation of thrombospondin-1 by KSHV miRNAs corresponds to decreased TGF-β signaling. Therefore, downregulation of anti-angiogenic factors may be an important way by which KSHV promotes continual neovascularization.

The KSHV genome itself encodes for cytokine and chemokine-like factors that activate endothelial cells and stimulate angiogenesis (**Table 1**). Among these factors are three genes with homology to the cellular chemokine macrophage inflammatory protein, the vMIPs I–III (Boshoff et al., 1997; Stine et al., 2000). In addition to having chemoattractant properties, these proteins promoted neovascularization in the chick chorio-allantoic membrane angiogenesis assay (Boshoff et al., 1997; Stine et al., 2000). KSHV also encodes a viral homolog of interleukin 6 (IL-6), a pro-inflammatory and pro-angiogenic cytokine. This cytokine, when expressed in NIH3T3 cells, promoted secretion of VEGF-A (Aoki et al., 1999). Furthermore, when these cells were injected into mice, they gave rise to tumors more quickly than control cells and the tumors were more highly vascularized (Aoki et al., 1999). Expression of the vMIPs has been predominantly shown to occur during lytic infection. The viral IL-6 (vIL-6) is mostly detected in endothelial cells and spindle cells undergoing lytic replication but like K1 it has been shown to be expressed at very low levels in latently infected PEL cells and to be induced during latency only under specific conditions (Chatterjee et al., 2002; Chandriani et al., 2010).

In summary, conditioned media from KSHV-infected cells can induce angiogenic phenotypes in uninfected endothelial cells as indicated by the red gradient in **Figure 1**. KSHV infection of endothelial cells induces expression of a number of cytokines that are capable of inducing angiogenesis in a paracrine fashion. Paramount among these is VEGF-A, an angiogenic cytokine that is induced by KSHV infection of endothelial cells. While the predominant viral mechanism of VEGF-A induction is unknown, a number of lytic KSHV genes are sufficient to induce VEGF-A when overexpressed. KSHV-infected cells also produce a number of other angiogenic cytokines of cellular and viral origin that likely play a role in the induction of angiogenesis. Taken together, all of the cytokines and induced pathways likely create a milieu that is beneficial to the induction of new blood vessels and play a significant role in the high vascularization of KS tumors.

KSHV INDUCTION OF ANGIOGENIC PHENOTYPES WITHIN THE INFECTED CELL

The predominant tumor cell of KS lesions is the endothelial-derived spindle cell. Oncogenesis in endothelial cells and

angiogenesis have many phenotypes in common. Therefore KS tumor formation is likely to include increased angiogenic capacity of the spindle cells. There is growing evidence demonstrating the manipulation of host cell phenotypes by KSHV and the role of these changes in the promotion of angiogenesis related phenotypes. These infected cell phenotypes include increased stability of tubules formed by macrovascular endothelial cells, induction of capillary morphogenesis in low growth factor conditions, and enhanced migration and invasion (Qian et al., 2007; Sadagopan et al., 2007, 2009; Wang and Damania, 2008; Couty et al., 2009; DiMaio et al., 2011). Additionally, KSHV induces the expression of VEGF receptors on the surface of infected endothelial cells as discussed below.

Endothelial cells lining the vasculature form coordinated junctions to maintain barrier function. Breakdown of these junctions is necessary for initiation of angiogenesis, immune cell extravasation, and tumor cell metastasis. Interestingly, several studies have evaluated the adherens junctions of KSHV-infected endothelial cells and found them to be perturbed (Mansouri et al., 2008; Qian et al., 2008; Guilluy et al., 2011). This may result from the degradation of VE-cadherin (Mansouri et al., 2008; Qian et al., 2008) as well as disruption of VE-cadherin/beta-catenin signaling (Guilluy et al., 2011). Therefore, KSHV infection can directly initiate a key angiogenic step, the breakdown of cell–cell adherence. While the direct mechanism of KSHV-induced disruption of adherens junctions during latency is not known, there are a number of candidate KSHV genes that could be involved (**Table 1**). The KSHV-encoded ubiquitin ligase protein, K5, targets VE-cadherin for degradation (Mansouri et al., 2008). Overexpression of the KSHV vGPCR induces endothelial cell permeability and downregulation of cell surface VE-cadherin as well (Dwyer et al., 2011). K5 also targets other cellular proteins, including platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31), a transmembrane protein important for endothelial cell–cell communication, which could contribute to barrier dysfunction and increased permeability (Tomescu et al., 2003; Mansouri et al., 2006). K1, a primarily lytic protein that may also be expressed at low levels during latency was also shown to initiate signaling similar to that required for disruption of Cadherin signaling (Guilluy et al., 2011). While the exact viral mechanism of disruption of adherens junctions by KSHV infection is not known, the virus encodes multiple genes capable of altering Cadherin.

During angiogenesis, endothelial cells migrate from the preexisting vasculature toward the site of angiogenic stimulus. Endothelial cells exhibit enhanced migration and invasion following latent KSHV infection (DiMaio et al., 2011; Wu et al., 2011). This has been demonstrated by more rapid motility through transwell dishes. KSHV-infected cells also express increased levels of the matrix metalloproteinases MMP-1, -2, and -9 (Qian et al., 2007). MMP proteins break down the extracellular matrix supporting stable vasculature to allow for invasion and migration of endothelial cells during angiogenesis (**Figure 1**, circle 2). Expression of MMP proteins induced by KSHV allows for increased invasion of both infected and uninfected endothelial cells (Wang et al., 2004b; Qian et al., 2007; Shan et al., 2007). While these processes constitute one component of angiogenesis, they are also known to play roles in oncogenesis (Gialeli et al., 2011) indicating that KSHV activation of angiogenic phenotypes in endothelial cells may lead to enhanced oncogenesis as well.

Endothelial cells grown in three-dimensional culture will migrate and organize into capillary-like structures. This activity is dependent, at least in part, on growth factors and cytokines present in the matrix or growth media. KSHV-infected cells are able to undergo capillary morphogenesis in low growth factor conditions to a greater extent than uninfected cells (Wang and Damania, 2008). This could be partially due to increased cytokine secretion from KSHV-infected cells. In fact, when endothelial cells are cultured in the presence of conditioned media from KSHV-infected BCBL-1 cells, their ability to organize into capillary-like structures is increased (Wang and Damania, 2008). However, the effect of BCBL-1 conditioned media was greater on KSHV-infected endothelial cells than on mock-infected cells, suggesting that infected cells are more receptive to angiogenic growth factors. In addition, this same study found that capillary-like structures formed by KSHV-infected endothelial cells are more persistent than mock-infected cells, indicative of the promotion of cell survival and continual angiogenesis by KSHV (Wang and Damania, 2008 and our unpublished results).

KSHV latent infection of endothelial cells also induces VEGF receptor expression, which may allow infected cells to respond more robustly to VEGFs. There are three main receptors for VEGFs. VEGF receptors 1 and 2 play roles in angiogenesis while 2 and 3 play a role in lymphangiogenesis (described below). While KSHV infection has not been reported to alter the expression levels of VEGFR2 (KDR), VEGFR1 expression is significantly increased following KSHV endothelial cell infection (Carroll et al., 2004). Drugs that inhibited HIF-1 activation and signaling also inhibited VEGFR1 upregulation (Carroll et al., 2006). VEGFR1 has been described as both a positive and negative regulator of angiogenesis depending on the context. VEGFR1 mouse knockouts have higher levels of angiogenesis (Fong et al., 1995). However, in cell culture models, VEGFR1 has been shown to potentiate angiogenesis (Cao, 2009). More studies will be needed to determine the importance of increased VEGFR1 expression in KSHV infection and KS tumor formation. Interestingly, expression of VEGFR3, the main receptor for VEGF-C and D is also significantly increased by KSHV infection (Carroll et al., 2004; Hong et al., 2004). VEGFR3, a receptor specific to lymphatic endothelium and critical for lymphangiogenesis will be discussed below. Importantly, endothelial tip cells at the leading edge of new vascular protrusions are the only main adult cell type known to express both the blood endothelial cell receptor, VEGFR1, and the lymphatic endothelial cell receptor, VEGFR3 (Tammela et al., 2008): KSHV infection of endothelial cells directly induces expression of both of these receptors.

The mechanisms by which KSHV induces angiogenic phenotypes in latently infected cells are largely unknown. A number of angiogenic phenotypes are likely to be a direct result of the cytokine milieu of the infected cells. As described above, KSHV-infected cells secrete both viral and host cytokines that are sufficient to induce angiogenic phenotypes. These paracrine factors surely play a role in the induction of tumor cells. However, it is also apparent that some of the angiogenic effects seen in latently infected cells are cell autonomous, independent of either

paracrine or autocrine factors. As described above, conditioned media from PEL cells had stronger effects on tubule formation of KSHV-infected endothelial cells (Akula et al., 2005). We have also recently found that KSHV infection induces the pro-angiogenic integrin, integrin β3, during latent infection (DiMaio et al., 2011). Induction of integrin β3 leads to increased cell surface expression of the αVβ3 integrin heterodimer. We have further shown that latently infected endothelial cells become more adherent to the integrin ligands fibronectin and vitronectin, and are more migratory than mock-infected cells. These induced phenotypes require RGD-binding integrins, specifically integrin β3. Although both uninfected and infected cells organize in three-dimensional cultures in complete media, infected cells are more sensitive to inhibitors of integrin β3 and its downstream signaling molecules, such as Src kinase (DiMaio et al., 2011). This suggests that during latent KSHV infection there is a shift in endothelial cell signaling that results in a more angiogenic phenotype dependent on αVβ3 expression on the surface of the cell (**Figure 1**, center). Therefore, KSHV alteration of endothelial cell signaling pathways can dramatically affect how the cell responds to intra- and extra-cellular signals. These changes that lead to alterations in angiogenic properties are likely to play a role in the growth and cell–cell interactions of infected cells, thereby playing a role in KS tumor formation.

ANGIOGENESIS VS. LYMPHANGIOGENESIS

During development of the vascular system, a subset of endothelial cells in the cardinal vein begin to express markers of lymphatic differentiation, including the master regulatory gene, prox-1. These cells then bud from the cardinal vein, differentiate into lymphatic endothelial cells, and form the lymphatic vascular system (Wigle and Oliver, 1999). The mechanisms regulating lymphangiogenesis are in general less well understood when compared to angiogenesis. Immunohistochemistry of KS tumors showed that spindle cells express markers of lymphatic endothelium, suggesting these cells may arise from primary infection of lymphatic endothelial cells, rather than blood endothelial cells (Jussila et al., 1998; Skobe et al., 1999; Weninger et al., 1999; Pyakurel et al., 2006). An alternative hypothesis is that KSHV infection of blood endothelial cells drives differentiation toward a more lymphatic phenotype. This idea is supported by evidence that KSHV infection of blood endothelial cells promotes expression of lymphatic-specific genes, including prox-1, VEGFR3, podoplanin, and LYVE-1, effectively driving the reprogramming of blood endothelial cells to become lymphatic endothelium (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004a). Microarray analysis comparing KSHV-infected blood endothelial cells to blood and lymphatic endothelial cells indicate that KSHV-infected blood endothelial cells have gene expression profiles that align more closely to lymphatic endothelial cells than that of blood endothelial cells (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004a).

The mechanism by which KSHV induces lymphatic differentiation is not completely clear. The KSHV latent gene Kaposin B can directly promote the stability of Prox-1 mRNA (Yoo et al., 2010) leading to increased expression of Prox-1. However, this effect was not sufficient to induce Prox-1 expression in blood endothelial cells. We recently found that induction of blood to lymphatic endothelial cell reprogramming requires signaling through the cellular receptor gp130. Endothelial cells that are latently infected with KSHV have increased expression and signaling of gp130 (Morris et al., 2008). This leads to activation of the JAK/STAT3 pathway and the PI3K/AKT pathway leading to expression of lymphatic-specific genes starting with Prox-1. Inhibition of this pathway by siRNAs that target gp130 or AKT or pharmacological inhibitors that block PI3 kinase or Jak2/STAT3 signaling is sufficient to block lymphatic differentiation (see **Figure 1**, center). The cytokine responsible for activating gp130 is currently not known. KSHV vIL-6 is sufficient to induce gp130 activation and we recently found that vIL-6 is sufficient to induce lymphatic differentiation (Morris et al., 2012). However, KSHV lacking vIL-6 is still able to cause blood to lymphatic endothelial cell differentiation, indicating that KSHV has evolved multiple strategies to activate gp130 and induce blood to lymphatic endothelial cell differentiation (Morris et al., 2008).

Induction of lymphatic differentiation by KSHV is only part of the story, however. Despite the expression of lymphatic-specific genes, blood endothelial cells infected with KSHV retain expression of some blood specific markers (Wang et al., 2004a). Additionally, infection of lymphatic endothelial cells with KSHV induces expression of blood specific markers (Wang et al., 2004a). KSHV miRNAs were found to target the transcription factor MAF (Hansen et al., 2010). Downregulation of MAF in lymphatic endothelial cells by siRNA restored expression of blood endothelial markers, such as VEGFR1 and CXCR4. Thus, infection of blood or lymphatic endothelial cells by KSHV alters host gene expression to an intermediate state between the two cell types. As described above, this intermediate phenotype with both VEGFR1 and R3 expression is only present in the leading tip of endothelial cells involved in active neo-angiogenesis. In the KS lesions only LANA+ cells expressed Prox-1, indicating that this effect requires KSHV gene expression (Hong et al., 2004). This suggests that differentiation toward lymphatic endothelial cells may specifically allow the spindle cells to respond to lymphangiogenic growth factors. In fact, KSHV infection of endothelial cells induces both VEGF-A and VEGF-C (Sivakumar et al., 2008). VEGF-C is a key regulator of lymphangiogenesis. Therefore, induction of both VEGFR1 and R3 allow KSHV-infected cells to respond to key angiogenic and lymphangiogenic factors in the tumor environment. The direct role of KSHV reprogramming of blood endothelial cells to lymphatic in induction of angiogenic and lymphangiogenic phenotypes is still under investigation.

SUMMARY

The highly vascular nature of KS tumors and the large amounts of neo-angiogenesis in the tumor led to the proposal that the etiologic agent of the tumor might directly induce angiogenesis. In accordance with this hypothesis KSHV infection of endothelial cells, the main tumor cell type, induces host cell cytokines involved in angiogenesis. In particular, KSHV induces the expression of VEGF-A and -C and other cytokines as well as encoding angiogenic cytokines from its own genome (Boshoff et al., 1997; Aoki et al., 1999; Brown et al., 2000; Stine et al., 2000; Schwarz and Murphy, 2001; Masood et al., 2002; Polson et al., 2002; Wang et al., 2004b; Xie et al., 2005; Caselli et al., 2007; Vart et al., 2007; Ye et al.,

2007; Fonsato et al., 2008; Sivakumar et al., 2008; Wang and Damania, 2008; Sadagopan et al., 2009; Ma et al., 2010; Sharma-Walia et al., 2010). Therefore, KSHV may induce seeding of new blood vessels to the tumor milieu. Additionally, because the tumor cell is endothelial in nature, induction of angiogenic cytokines may also activate the tumor cells and aid in the growth of KS tumors. KSHV also induces angiogenic phenotypes directly in latently infected cells in a cell autonomous fashion, indicating that angiogenic activation of the infected endothelial cell may directly play a role in tumor formation.

While KSHV activates many growth-signaling properties and in general the induction of angiogenic phenotypes supports endothelial cell proliferation, in most cultures KSHV does not induce increases in endothelial cell proliferation. It is possible that the cell culture milieu simply does not match the tumor milieu. KS spindle cells are not fully transformed *ex vivo* and, except in very rare cases, have a limited life span indicating that factors in the tumor environment that come from other cells types could be necessary to maintain KS spindle cell growth. The increase in growth could also be masked by the fact that endothelial cells in culture are rapidly dividing and therefore do not need additional growth signals. Along those lines, mature endothelium *in vivo* is relatively quiescent. That being said, the endothelial cell transforming potential of KSHV in culture can be unmasked given specific conditions. Dermal microvascular endothelial cells that were immortalized with the E6 and E7 genes from papillomavirus are readily transformed by KSHV, including increased proliferation (Moses et al., 1999). Therefore, KSHV activation of endothelial cells can induce a proliferative advantage in the correct genetic environment. However, it is unknown if viral induction of angiogenic phenotypes is necessary for the growth in the E6/E7 immortalized endothelial cells.

In general, viruses do not evolve to cause cancer, as it is likely a dead end for transmission. KSHV likely evolved to activate the cell where it is maintained to ensure survival and spread of the virus. A major side effect of this activation may be providing an ideal environment for angiogenesis leading to increased vascularization of small tumor growths and expansion of KS tumors. While the study of viral induction of angiogenesis can lead to a better understanding of how KSHV causes endothelial cell tumors, information gleaned from the study of viral mechanisms of induction of angiogenesis and lymphangiogenesis will also lead to a better understanding of endothelial cell activation and tumor angiogenesis in general. Thus, the study of KSHV infection of endothelial cells provides a controlled system for analyzing the regulation and induction of angiogenic phenotypes that will likely shed light on the field of tumor angiogenesis.

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REFERENCES

Akula, S. M., Ford, P. W., Whitman, A. G., Hamden, K. E., Bryan, B. A., Cook, P. P., and McCubrey, J. A. (2005). B-Raf-dependent expression of vascular endothelial growth factor-A in Kaposi sarcoma-associated herpesvirus-infected human B cells. *Blood* 105, 4516–4522.

Aoki, Y., Jaffe, E. S., Chang, Y., Jones, K., Teruya-Feldstein, J., Moore, P. S., and Tosato, G. (1999). Angiogenesis and hematopoiesis induced by Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6. *Blood* 93, 4034–4043.

Arvanitakis, L., Geras-Raaka, E., Varma, A., Gershengorn, M. C., and Cesarman, E. (1997). Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. *Nature* 385, 347–350.

Bais, C., Santomasso, B., Coso, O., Arvanitakis, L., Raaka, E. G., Gutkind, J. S., Asch, A. S., Cesarman, E., Gershengorn, M. C., Mesri, E. A., and Gerhengorn, M. C. (1998). G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. *Nature* 391, 86–89.

Bais, C., Van Geelen, A., Eroles, P., Mutlu, A., Chiozzini, C., Dias, S., Silverstein, R. L., Rafii, S., and Mesri, E. A. (2003). Kaposi's sarcoma associated herpesvirus G protein-coupled receptor immortalizes human endothelial cells by activation of the VEGF receptor-2/KDR. *Cancer Cell* 3, 131–143.

Beral, V., Peterman, T. A., Berkelman, R. L., and Jaffe, H. W. (1990). Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? *Lancet* 335, 123–128.

Blasig, C., Zietz, C., Haar, B., Neipel, F., Esser, S., Brockmeyer, N. H., Tschachler, E., Colombini, S., Ensoli, B., and Stürzl, M. (1997). Monocytes in Kaposi's sarcoma lesions are productively infected by human herpesvirus 8. *J. Virol.* 71, 7963–7968.

Boshoff, C., Endo, Y., Collins, P. D., Takeuchi, Y., Reeves, J. D., Schweickart, V. L., Siani, M. A., Sasaki, T., Williams, T. J., Gray, P. W., Moore, P. S., Chang, Y., and Weiss, R. A. (1997). Angiogenic and HIV-inhibitory functions of KSHV-encoded chemokines. *Science* 278, 290–294.

Boshoff, C., Schulz, T. F., Kennedy, M. M., Graham, A. K., Fisher, C., Thomas, A., McGee, J. O., Weiss, R. A., and O'Leary, J. J. (1995). Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. *Nat. Med.* 1, 1274–1278.

Breen, E. C. (2007). VEGF in biological control. *J. Cell. Biochem.* 102, 1358–1367.

Brown, L. F., Dezube, B. J., Tognazzi, K., Dvorak, H. F., and Yancopoulos, G. D. (2000). Expression of Tie1, Tie2, and angiopoietins 1, 2, and 4 in Kaposi's sarcoma and cutaneous angiosarcoma. *Am. J. Pathol.* 156, 2179–2183.

Cai, Q., Murakami, M., Si, H., and Robertson, E. S. (2007). A potential alpha-helix motif in the amino terminus of LANA encoded by Kaposi's sarcoma-associated herpesvirus is critical for nuclear accumulation of HIF-1alpha in normoxia. *J. Virol.* 81, 10413–10423.

Cai, Q. L., Knight, J. S., Verma, S. C., Zald, P., and Robertson, E. S. (2006). EC5S ubiquitin complex is recruited by KSHV latent antigen LANA for degradation of the VHL and p53 tumor suppressors. *PLoS Pathog.* 2, e116. doi:10.1371/journal.ppat.0020116

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B., and Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5570–5575.

Cao, Y. (2009). Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Sci. Signal.* 2, re1.

Carroll, P. A., Brazeau, E., and Lagunoff, M. (2004). Kaposi's sarcoma-associated herpesvirus infection of blood endothelial cells induces lymphatic differentiation. *Virology* 328, 7–18.

Carroll, P. A., Kenerson, H. L., Yeung, R. S., and Lagunoff, M. (2006). Latent Kaposi's sarcoma-associated herpesvirus infection of endothelial cells activates hypoxia-induced factors. *J. Virol.* 80, 10802–10812.

Caselli, E., Fiorentini, S., Amici, C., Di Luca, D., Caruso, A., and Santoro, M. G. (2007). Human herpesvirus 8 acute infection of endothelial cells induces monocyte chemoattractant protein 1-dependent capillary-like structure formation: role of the IKK/NF-kappaB pathway. *Blood* 109, 2718–2726.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Chandriani, S., and Ganem, D. (2010). Array-based transcript profiling and limiting-dilution reverse transcription-PCR analysis identify additional latent genes in Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 84, 5565–5573.

Chandriani, S., Xu, Y., and Ganem, D. (2010). The lytic transcriptome of Kaposi's sarcoma-associated herpesvirus reveals extensive transcription of noncoding regions, including regions antisense to important genes. *J. Virol.* 84, 7934–7942.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266, 1865–1869.

Chatterjee, M., Osborne, J., Bestetti, G., Chang, Y., and Moore, P. S. (2002). Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* 298, 1432–1435.

Couty, J. P., Lupu-Meiri, M., Oron, Y., and Gershengorn, M. C. (2009). Kaposi's sarcoma-associated herpesvirus-G protein-coupled receptor-expressing endothelial cells exhibit reduced migration and stimulated chemotaxis by chemokine inverse agonists. *J. Pharmacol. Exp. Ther.* 329, 1142–1147.

Dai, L., Bratoeva, M., Toole, B. P., Qin, Z., and Parsons, C. (2011). KSHV activation of VEGF secretion and invasion for endothelial cells is mediated through viral upregulation of emmprin-induced signal transduction. *Int. J. Cancer.* doi:10.1002/ijc.26428

DiMaio, T. A., Gutierrez, K. D., and Lagunoff, M. (2011). Latent KSHV infection of endothelial cells induces integrin beta3 to activate angiogenic phenotypes. *PLoS Pathog.* 7, e1002424. doi:10.1371/journal.ppat.1002424

Dumont, D. J., Gradwohl, G., Fong, G. H., Puri, M. C., Gertsenstein, M., Auerbach, A., and Breitman, M. L. (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* 8, 1897–1909.

Dupin, N., Fisher, C., Kellam, P., Ariad, S., Tulliez, M., Franck, N., van Marck, E., Salmon, D., Gorin, I., Escande, J. P., Weiss, R. A., Alitalo, K., and Boshoff, C. (1999). Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4546–4551.

Dwyer, J., Le Guelte, A., Galan Moya, E. M., Sumbal, M., Carlotti, A., Douguet, L., Gutkind, J. S., Grange, P. A., Dupin, N., and Gavard, J. (2011). Remodeling of VE-cadherin junctions by the human herpes virus 8 G-protein coupled receptor. *Oncogene* 30, 190–200.

Fong, G. H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376, 66–70.

Fonsato, V., Buttiglieri, S., Deregibus, M. C., Bussolati, B., Caselli, E., Di Luca, D., and Camussi, G. (2008). PAX2 expression by HHV-8-infected endothelial cells induced a proangiogenic and proinvasive phenotype. *Blood* 111, 2806–2815.

Gale, N. W., Thurston, G., Hackett, S. F., Renard, R., Wang, Q., McClain, J., Martin, C., Witte, C., Witte, M. H., Jackson, D., Suri, C., Campochiaro, P. A., Wiegand, S. J., and Yancopoulos, G. D. (2002). Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. *Dev. Cell* 3, 411–423.

Gialeli, C., Theocharis, A. D., and Karamanos, N. K. (2011). Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. *FEBS J.* 278, 16–27.

Guilluy, C., Zhang, Z., Bhende, P. M., Sharek, L., Wang, L., Burridge, K., and Damania, B. (2011). Latent KSHV infection increases the vascular permeability of human endothelial cells. *Blood* 118, 5344–5354.

Guo, H. G., Sadowska, M., Reid, W., Tschachler, E., Hayward, G., and Reitz, M. (2003). Kaposi's sarcoma-like tumors in a human herpesvirus 8 ORF74 transgenic mouse. *J. Virol.* 77, 2631–2639.

Hansen, A., Henderson, S., Lagos, D., Nikitenko, L., Coulter, E., Roberts, S., Gratrix, F., Plaisance, K., Renne, R., Bower, M., Kellam, P., and Boshoff, C. (2010). KSHV-encoded miRNAs target MAF to induce endothelial cell reprogramming. *Genes Dev.* 24, 195–205.

Hong, Y. K., Foreman, K., Shin, J. W., Hirakawa, S., Curry, C. L., Sage, D. R., Libermann, T., Dezube, B. J., Fingeroth, J. D., and Detmar, M. (2004). Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. *Nat. Genet.* 36, 683–685.

Jussila, L., Valtola, R., Partanen, T. A., Salven, P., Heikkilä, P., Matikainen, M. T., Renkonen, R., Kaipainen, A., Detmar, M., Tschachler, E., Alitalo, R., and Alitalo, K. (1998). Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res.* 58, 1599–1604.

Liu, C., Okruzhnov, Y., Li, H., and Nicholas, J. (2001). Human herpesvirus 8 (HHV-8)-encoded cytokines induce expression of and autocrine signaling by vascular endothelial growth factor (VEGF) in HHV-8-infected primary-effusion lymphoma cell lines and mediate VEGF-independent antiapoptotic effects. *J. Virol.* 75, 10933–10940.

Ma, T., Jham, B. C., Hu, J., Friedman, E. R., Basile, J. R., Molinolo, A., Sodhi, A., and Montaner, S. (2010). Viral G protein-coupled receptor up-regulates angiopoietin-like 4 promoting angiogenesis and vascular permeability in Kaposi's sarcoma. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14363–14368.

Mansouri, M., Douglas, J., Rose, P. P., Gouveia, K., Thomas, G., Means, R. E., Moses, A. V., and Früh, K. (2006). Kaposi sarcoma herpesvirus K5 removes CD31/PECAM from endothelial cells. *Blood* 108, 1932–1940.

Mansouri, M., Rose, P. P., Moses, A. V., and Früh, K. (2008). Remodeling of endothelial adherens junctions by Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 82, 9615–9628.

Marshall, V., Parks, T., Bagni, R., Wang, C. D., Samols, M. A., Hu, J., Wyvil, K. M., Aleman, K., Little, R. F., Yarchoan, R., Renne, R., and Whitby, D. (2007). Conservation of virally encoded microRNAs in Kaposi sarcoma – associated herpesvirus in primary effusion lymphoma cell lines and in patients with Kaposi sarcoma or multicentric Castleman disease. *J. Infect. Dis.* 195, 645–659.

Masood, R., Cesarman, E., Smith, D. L., Gill, P. S., and Flore, O. (2002). Human herpesvirus-8-transformed endothelial cells have functionally activated vascular endothelial growth factor/vascular endothelial growth factor receptor. *Am. J. Pathol.* 160, 23–29.

Montaner, S., Sodhi, A., Pece, S., Mesri, E. A., and Gutkind, J. S. (2001). The Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor promotes endothelial cell survival through the activation of Akt/protein kinase B. *Cancer Res.* 61, 2641–2648.

Morris, V. A., Punjabi, A. S., and Lagunoff, M. (2008). Activation of Akt through gp130 receptor signaling is required for Kaposi's sarcoma-associated herpesvirus-induced lymphatic reprogramming of endothelial cells. *J. Virol.* 82, 8771–8779.

Morris, V. A., Punjabi, A. S., Wells, R. C., Wittkopp, C. J., Vart, R., and Lagunoff, M. (2012). The KSHV Viral IL-6 homolog is sufficient to induce blood to lymphatic endothelial cell differentiation. *Virology.* (in press).

Moses, A. V., Fish, K. N., Ruhl, R., Smith, P. P., Strussenberg, J. G., Zhu, L., Chandran, B., and Nelson, J. A. (1999). Long-term infection and transformation of dermal microvascular endothelial cells by human herpesvirus 8. *J. Virol.* 73, 6892–6902.

Naranatt, P. P., Krishnan, H. H., Svojanovsky, S. R., Bloomer, C., Mathur, S., and Chandran, B. (2004). Host gene induction and transcriptional reprogramming in Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8)-infected endothelial, fibroblast, and B cells: insights into modulation events early during infection. *Cancer Res.* 64, 72–84.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grässer, F. A., van Dyk, L. F., Ho, C. K., Shuman, S., Chien, M., Russo, J. J., Ju, J., Randall, G., Lindenbach, B. D., Rice, C. M., Simon, V., Ho, D. D., Zavolan, M., and Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nat. Methods* 2, 269–276.

Polson, A. G., Wang, D., DeRisi, J., and Ganem, D. (2002). Modulation of host gene expression by the constitutively active G protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus. *Cancer Res.* 62, 4525–4530.

Pyakurel, P., Pak, F., Mwakigonja, A. R., Kaaya, E., Heiden, T., and Biberfeld, P. (2006). Lymphatic and vascular origin of Kaposi's sarcoma spindle cells during tumor development. *Int. J. Cancer* 119, 1262–1267.

Qian, L. W., Greene, W., Ye, F., and Gao, S. J. (2008). Kaposi's sarcoma-associated herpesvirus disrupts adherens junctions and increases endothelial permeability by inducing degradation of VE-cadherin. *J. Virol.* 82, 11902–11912.

Qian, L. W., Xie, J., Ye, F., and Gao, S. J. (2007). Kaposi's sarcoma-associated herpesvirus infection promotes invasion of primary human umbilical vein endothelial cells by inducing matrix metalloproteinases. *J. Virol.* 81, 7001–7010.

Qin, Z., Dai, L., Slomiany, M. G., Toole, B. P., and Parsons, C. (2010). Direct activation of emmprin and associated pathogenesis by an oncogenic herpesvirus. *Cancer Res.* 70, 3884–3889.

Russell Jones, R., Orchard, G., Zelger, B., and Wilson Jones, E. (1995). Immunostaining for CD31 and CD34 in Kaposi sarcoma. *J. Clin. Pathol.* 48, 1011–1016.

Sadagopan, S., Sharma-Walia, N., Veettil, M. V., Bottero, V., Levine, R., Vart, R. J., and Chandran, B. (2009). Kaposi's sarcoma-associated herpesvirus upregulates angiogenin during infection of human dermal microvascular endothelial cells, which induces 45S rRNA synthesis, antiapoptosis, cell proliferation, migration, and angiogenesis. *J. Virol.* 83, 3342–3364.

Sadagopan, S., Sharma-Walia, N., Veettil, M. V., Raghu, H., Sivakumar, R., Bottero, V., and Chandran, B. (2007). Kaposi's sarcoma-associated herpesvirus induces sustained NF-kappaB activation during de novo infection of primary human dermal microvascular endothelial cells that is essential for viral gene expression. *J. Virol.* 81, 3949–3968.

Samaniego, F., Markham, P. D., Gendelman, R., Watanabe, Y., Kao, V., Kowalski, K., Sonnabend, J. A., Pintus, A., Gallo, R. C., and Ensoli, B. (1998). Vascular endothelial growth factor and basic fibroblast growth factor present in Kaposi's sarcoma (KS) are induced by inflammatory cytokines and synergize to promote vascular permeability and KS lesion development. *Am. J. Pathol.* 152, 1433–1443.

Samols, M. A., Hu, J., Skalsky, R. L., and Renne, R. (2005). Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79, 9301–9305.

Samols, M. A., Skalsky, R. L., Maldonado, A. M., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Identification of cellular genes targeted by KSHV-encoded microRNAs. *PLoS Pathog.* 3, e65. doi:10.1371/journal.ppat.0030065

Schwarz, M., and Murphy, P. M. (2001). Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor constitutively activates NF-kappa B and induces proinflammatory cytokine and chemokine production via a C-terminal signaling determinant. *J. Immunol.* 167, 505–513.

Shan, B., Morris, C. A., Zhuo, Y., Shelby, B. D., Levy, D. R., and Lasky, J. A. (2007). Activation of proMMP-2 and Src by HHV8 vGPCR in human pulmonary arterial endothelial cells. *J. Mol. Cell. Cardiol.* 42, 517–525.

Sharma-Walia, N., Paul, A. G., Bottero, V., Sadagopan, S., Veettil, M. V., Kerur, N., and Chandran, B. (2010). Kaposi's sarcoma associated herpes virus (KSHV) induced COX-2: a key factor in latency, inflammation, angiogenesis, cell survival and invasion. *PLoS Pathog.* 6, e1000777. doi:10.1371/journal.ppat.1000777

Sharma-Walia, N., Raghu, H., Sadagopan, S., Sivakumar, R., Veettil, M. V., Naranatt, P. P., Smith, M. M., and Chandran, B. (2006). Cyclooxygenase 2 induced by Kaposi's sarcoma-associated herpesvirus early during in vitro infection of target cells plays a role in the maintenance of latent viral gene expression. *J. Virol.* 80, 6534–6552.

Shin, Y. C., Joo, C. H., Gack, M. U., Lee, H. R., and Jung, J. U. (2008). Kaposi's sarcoma-associated herpesvirus viral IFN regulatory factor 3 stabilizes hypoxia-inducible factor-1 alpha to induce vascular endothelial growth factor expression. *Cancer Res.* 68, 1751–1759.

Sivakumar, R., Sharma-Walia, N., Raghu, H., Veettil, M. V., Sadagopan, S., Bottero, V., Varga, L., Levine, R., and Chandran, B. (2008). Kaposi's sarcoma-associated herpesvirus induces sustained levels of vascular endothelial growth factors A and C early during in vitro infection of human microvascular dermal endothelial cells: biological implications. *J. Virol.* 82, 1759–1776.

Skobe, M., Brown, L. F., Tognazzi, K., Ganju, R. K., Dezube, B. J., Alitalo, K., and Detmar, M. (1999). Vascular endothelial growth factor-C (VEGF-C) and its receptors KDR and flt-4 are expressed in AIDS-associated Kaposi's sarcoma. *J. Invest. Dermatol.* 113, 1047–1053.

Sodhi, A., Montaner, S., Patel, V., Zohar, M., Bais, C., Mesri, E. A., and Gutkind, J. S. (2000). The Kaposi's sarcoma-associated herpes virus G protein-coupled receptor up-regulates vascular endothelial growth factor expression and secretion through mitogen-activated protein kinase and p38 pathways acting on hypoxia-inducible factor 1alpha. *Cancer Res.* 60, 4873–4880.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d'Agay, M. F., Clauvel, J. P., Raphael, M., and Degos, L. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86, 1276–1280.

Staskus, K. A., Zhong, W., Gebhard, K., Herndier, B., Wang, H., Renne, R., Beneke, J., Pudney, J., Anderson, D. J., Ganem, D., and Haase, A. T. (1997). Kaposi's sarcoma-associated herpesvirus gene expression in endothelial (spindle) tumor cells. *J. Virol.* 71, 715–719.

Stine, J. T., Wood, C., Hill, M., Epp, A., Raport, C. J., Schweickart, V. L., Endo, Y., Sasaki, T., Simmons, G., Boshoff, C., Clapham, P., Chang, Y., Moore, P., Gray, P. W., and Chantry, D. (2000). KSHV-encoded CC chemokine vMIP-III is a CCR4 agonist, stimulates angiogenesis, and selectively chemoattracts TH2 cells. *Blood* 95, 1151–1157.

Subramanian, R., Sehgal, I., D'Auvergne, O., and Kousoulas, K. G. (2010). Kaposi's sarcoma-associated herpesvirus glycoproteins B and K8.1 regulate virion egress and synthesis of vascular endothelial growth factor and viral interleukin-6 in BCBL-1 cells. *J. Virol.* 84, 1704–1714.

Tammela, T., Zarkada, G., Wallgard, E., Murtomäki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellström, M., Schomber, T., Peltonen, R., Freitas, C., Duarte, A., Isoniemi, H., Laakkonen, P., Christofori, G., Ylä-Herttuala, S., Shibuya, M., Pytowski, B., Eichmann, A., Betsholtz, C., and Alitalo, K. (2008). Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* 454, 656–660.

Tomescu, C., Law, W. K., and Kedes, D. H. (2003). Surface downregulation of major histocompatibility complex class I, PE-CAM, and ICAM-1 following de novo infection of endothelial cells with Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 77, 9669–9684.

Vart, R. J., Nikitenko, L. L., Lagos, D., Trotter, M. W., Cannon, M., Bourboulia, D., Gratrix, F., Takeuchi, Y., and Boshoff, C. (2007). Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6 and G-protein-coupled receptor regulate angiopoietin-2 expression in lymphatic endothelial cells. *Cancer Res.* 67, 4042–4051.

Wabinga, H. R., Parkin, D. M., Wabwire-Mangen, F., and Mugerwa, J. W. (1993). Cancer in Kampala, Uganda, in 1989-91: changes in incidence in the era of AIDS. *Int. J. Cancer* 54, 26–36.

Wang, H. W., Trotter, M. W., Lagos, D., Bourboulia, D., Henderson, S., Mäkinen, T., Elliman, S., Flanagan, A. M., Alitalo, K., and Boshoff, C. (2004a). Kaposi sarcoma herpesvirus-induced cellular reprogramming contributes to the lymphatic endothelial gene expression in Kaposi sarcoma. *Nat. Genet.* 36, 687–693.

Wang, L., Wakisaka, N., Tomlinson, C. C., DeWire, S. M., Krall, S., Pagano, J. S., and Damania, B. (2004b). The Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) K1 protein induces expression of angiogenic and invasion factors. *Cancer Res.* 64, 2774–2781.

Wang, L., and Damania, B. (2008). Kaposi's sarcoma-associated herpesvirus confers a survival advantage to endothelial cells. *Cancer Res.* 68, 4640–4648.

Wang, L., Dittmer, D. P., Tomlinson, C. C., Fakhari, F. D., and Damania, B. (2006). Immortalization of primary endothelial cells by the K1 protein of Kaposi's sarcoma-associated herpesvirus. *Cancer Res.* 66, 3658–3666.

Weninger, W., Partanen, T. A., Breiteneder-Geleff, S., Mayer, C., Kowalski, H., Mildner, M., Pammer, J., Stürzl, M., Kerjaschki, D., Alitalo, K., and Tschachler, E. (1999). Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells. *Lab. Invest.* 79, 243–251.

Wigle, J. T., and Oliver, G. (1999). Prox1 function is required for the development of the murine lymphatic system. *Cell* 98, 769–778.

Wu, Y. H., Hu, T. F., Chen, Y. C., Tsai, Y. N., Tsai, Y. H., Cheng, C. C., and Wang, H. W. (2011). The manipulation of miRNA-gene regulatory networks by KSHV induces endothelial cell motility. *Blood* 118, 2896–2905.

Xie, J., Pan, H., Yoo, S., and Gao, S. J. (2005). Kaposi's sarcoma-associated herpesvirus induction of AP-1 and interleukin 6 during primary infection mediated by multiple mitogen-activated protein kinase pathways. *J. Virol.* 79, 15027–15037.

Ye, F. C., Blackbourn, D. J., Mengel, M., Xie, J. P., Qian, L. W., Greene, W., Yeh, I. T., Graham, D., and Gao, S. J. (2007). Kaposi's sarcoma-associated herpesvirus promotes angiogenesis by inducing angiopoietin-2 expression via AP-1 and Ets1. *J. Virol.* 81, 3980–3991.

Yoo, J., Kang, J., Lee, H. N., Aguilar, B., Kafka, D., Lee, S., Choi, I., Lee, J., Ramu, S., Haas, J., Koh, C. J., and Hong, Y. K. (2010). Kaposin-B enhances the PROX1 mRNA stability during lymphatic repro-

gramming of vascular endothelial cells by Kaposi's sarcoma herpes virus. *PLoS Pathog.* 6, e1001046. doi:10.1371/journal.ppat.1001046

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Kaposi's sarcoma-associated herpesvirus microRNAs

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Kaposi's sarcoma-associated herpesvirus (KSHV) is a human pathogenic γ-herpesvirus strongly associated with the development of Kaposi's Sarcoma and B cell proliferative disorders, including primary effusion lymphoma (PEL). The identification and functional investigation of non-coding RNAs expressed by KSHV is a topic with rapidly emerging importance. KSHV miRNAs derived from 12 stem-loops located in the major latency locus have been the focus of particular attention. Recent studies describing the transcriptome-wide identification of mRNA targets of the KSHV miRNAs suggest that these miRNAs have evolved a highly complex network of interactions with the cellular and viral transcriptomes. Relatively few KSHV miRNA targets, however, have been characterized at a functional level. Here, our current understanding of KSHV miRNA expression, targets, and function will be reviewed.

Keywords: KSHV, herpesvirus, microRNA

INTRODUCTION

Shortly after the discovery of miRNAs as an abundant class of small RNAs in animals (Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), miRNAs of viral origin were identified in Epstein–Barr virus (EBV) infected cells (Pfeffer et al., 2004). Since then, Kaposi's sarcoma-associated herpesvirus (KSHV) and most other herpesviruses were found to encode numerous viral microRNAs (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005; Grundhoff et al., 2006; Grundhoff and Sullivan, 2011; Skalsky and Cullen, 2011).

miRNAs are ∼22 nucleotide (nt) long non-coding RNAs that mediate target mRNA recognition by the miRNA-induced silencing complex (miRISC, referred to as RISC from hereon), which results in mRNA repression. miRNA biogenesis and RISC loading are reviewed in detail elsewhere (Kim et al., 2009) and summarized briefly in **Figure 1A**. The biogenesis of the large majority of cellular and viral miRNAs, including the KSHV miRNAs, begins with the transcription of capped and polyadenylated primary RNA (pri-miRNA) precursors by RNA polymerase II. miRNA maturation involves two sequential cleavage events. In the nucleus, the endonuclease Drosha excises the ∼65 nt pre-miRNA stem-loop, which is exported to the cytoplasm. In the cytoplasm, the pre-miRNA is further processed by the endonuclease Dicer to produce an imperfect ∼20 nt duplex, with 2 nt 3′ overhangs. The strand with the weaker 5′ base pairing is preferentially incorporated as a mature miRNA into the RISC. The non-incorporated strand, which is unstable and degraded, is termed the star or passenger strand. In cases where mature miRNAs are derived from both arms of the pre-miRNA, these miRNAs are designated -5p and -3p to reflect their location in the pre-miRNA.

In the RISC complex, miRNAs are loaded into one of four Argonaute proteins (Ago1–4). Imperfect base pairing of the miRNA with sites in mRNAs, most commonly within the 3′ UTR, results in mRNA repression, caused by mRNA destabilization and/or inhibition of translation (Huntzinger and Izaurralde, 2011). Canonical miRNA binding sites minimally exhibit perfect base pairing of the miRNA seed region, i.e. nts 2–7 from the 5′ end of the miRNA, together with base pairing at nt 8 (7mer-m8site), an adenine (A) across from nt 1 of the miRNA (7mer-A1 site), or both (**Figure 1B**; Bartel, 2009). Less commonly, non-canonical sites with suboptimal seed interactions and 3′ compensatory base pairing or extensive central base pairing confer regulation (Bartel, 2009; Shin et al., 2010). In addition, functional miRNA binding sites in 5′ UTRs and coding sequences (CDS) have also been reported (Grey et al., 2010; Lin and Ganem, 2011). While individual interactions typically have subtle regulatory outcomes, miRNA-mediated regulation is frequently cooperative with multiple sites for the same or different miRNAs in a given mRNA. RISC complexes containing Ago2 can mediate endonucleolytic cleavage of target mRNAs upon perfect base pairing between the entire miRNA sequence and its target (Hutvagner and Zamore, 2002). While this property of Ago2 is commonly exploited for miRNA activity assays, recognition of perfect targets by mammalian miRNAs occurs only rarely. Several hundred conserved human miRNAs have been identified and each miRNA can target hundreds of mRNAs, suggesting that most mRNAs and biological pathways are subject to miRNA-mediated regulation.

The human γ-herpesvirus KSHV is the etiological agent of Kaposi's Sarcoma, a complex neoplasm driven by KSHV-infected endothelial cells. KSHV is also strongly associated with the B cell proliferative disorder primary effusion lymphoma (PEL) and some cases of multicentric Castleman's disease (MCD). Like all herpesviruses, KSHV can enter a latency phase with highly restricted protein expression, thus limiting immune exposure while allowing persistence of the virus. During latency, KSHV expresses viral miRNAs from 12 pre-miRNAs in addition to a handful of latent proteins (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005; Grundhoff et al., 2006). Possible advantages of using miRNA-mediated regulation for herpesviruses may be that miRNAs are not

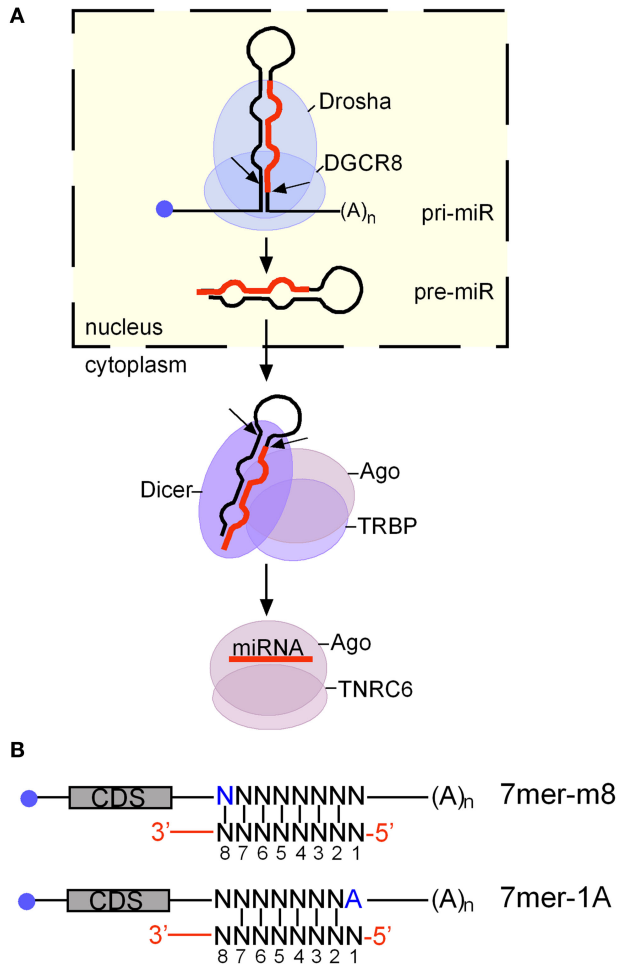

FIGURE 1 | miRNA biogenesis and target recognition. (A) Schematic of the canonical miRNA biogenesis pathway. pri-miRNAs are typically capped and polyadenylated and contain one or more miRNA stem-loops at exonic or intronic locations. The pre-miRNA stem-loop is excised by the microprocessor complex, containing the endonuclease Drosha and its cofactor DiGeorge syndrome critical region gene 8 (DGCR8), which helps position Drosha. Following nuclear export, the pre-miRNA is processed by Dicer to a miRNA:passenger strand duplex. Dicer processing is closely coupled to loading of the mature miRNA into the RISC. RISC complexes contain one of four Ago proteins (Ago1–4), which bind the miRNA, and associated proteins, including TNRC6. $(A)_n$ indicates the position of the polyA tail and arrows indicate cleavage sites. **(B)** Shown are the minimal 7mer-m8 (top) and 7mer-1A (bottom) seed-mediated interactions between a miRNA (nts are numbered from the 5′ end of the miRNA) and its mRNA target.

antigenic and their precursors can easily fit into multifunctional transcripts (Cullen, 2006). In addition, the regulatory potential of miRNAs differs fundamentally from that of proteins, i.e., they are commonly multifunctional and may allow viruses to regulate combinations of mRNAs and pathways that would be hard to access otherwise. Here, I review what is currently known about KSHV miRNA expression, targets and functions. miRNAs encoded by other viruses are reviewed elsewhere (Cullen, 2011; Grundhoff and Sullivan, 2011).

THE KSHV miRNAs

LOCUS

Kaposi's sarcoma-associated herpesvirus produces mature miRNAs from 12 stem-loops, all of which are encoded in the latency locus (**Figure 2A**). The latency region is complex and allows for the coordinated expression of the KSHV miRNAs with the viral proteins latency-associated nuclear antigen (LANA), FADD-like interleukin-1-β-converting enzyme (FLICE)/caspase-8-inhibitory protein (v-FLIP), v-cyclin, and Kaposins A–C (**Figure 2A**). The viral latent proteins mediate the episomal maintenance of the KSHV genome (LANA), stimulate NFκB signaling (v-FLIP), and modulate cell cycle progression (v-cyclin), among other functions. The mature KSHV miRNAs are called miR-K12-1 to miR-K12-12, based on their proximity to the kaposin (K12) gene, or simply miR-K1 to miR-K12. The transcripts from the latency region that serve as pri-miRNAs for the KSHV miRNAs are shown in **Figure 2B** (Cai and Cullen, 2006). All of these transcripts presumably also function as mRNAs for one or more of the Kaposin proteins, whose coding sequences are located in the common 3′ portion of these transcripts. The promoters with start sites at 127880/86 and 123751/60 are active during latency and the resulting pri-miRNAs contain miR-K1-K9 and miR-K11 within a ∼4.8-kb intron and miR-K10/miR-K12 in their 3′ terminal exon. At least in the PEL cell line BC-1, a singly spliced mRNA with a ∼9-kb intron containing miR-K1-K9 and miR-K11 also exists (**Figure 2B**, bottom). The latency promoters also drive the expression of mRNAs encoding LANA, v-cyclin, and v-FLIP (not shown in **Figure 2B**), which terminate downstream of the v-FLIP coding region and do not contain the miRNA hairpins (Cai and Cullen, 2006). In addition, there is a predominantly lytic promoter which gives rise to an unspliced ∼1.3 kb transcript that was shown to minimally express Kaposin B and also contains the miR-K10 and miR-K12 stem-loops, resulting in increased levels of miR-K10 and miR-K12 expression during lytic replication.

Virtually all of the KSHV pre-miRNAs overlap multifunctional regions. The entire miR-K10 stem-loop is embedded within the open reading frame of the Kaposin A and C proteins at a location that is also in the 3′ UTR of Kaposin B and the miR-K12 stem-loop is located in the 3′ UTRs of all Kaposin transcripts. Because the processing of these two exonic miRNAs and Kaposin translation are mutually exclusive events, the miR-K10 and miR-K12 stem-loops can be viewed as negative *cis*-regulatory RNA elements that mediate the nuclear destabilization of Kaposin mRNAs and consequently reduce protein expression from these transcripts (Lin and Sullivan, 2011). The miR-K9 stem-loop is located within a ∼1.7-kb sequence that can function as an origin of lytic genome replication (ORI-lytB; AuCoin et al., 2002; Lin et al., 2003). Much of this sequence represents an inverted repeat of a second origin of lytic replication (Ori-lytA), between ORF K4.2 and K5. In contrast to ORI-lytA, which is essential for replication, ORI-lytB is not required for genome replication and the function of this sequence in the context of the virus remains unknown (Xu et al., 2006). The miR-K1-K7 and miR-K11 stem loops are antisense to the recently identified 10 kb antisense to latency transcripts (ALT) RNA (**Figure 2A**; Chandriani et al., 2010). ALT is a non-coding RNA of unknown function expressed during lytic replication.

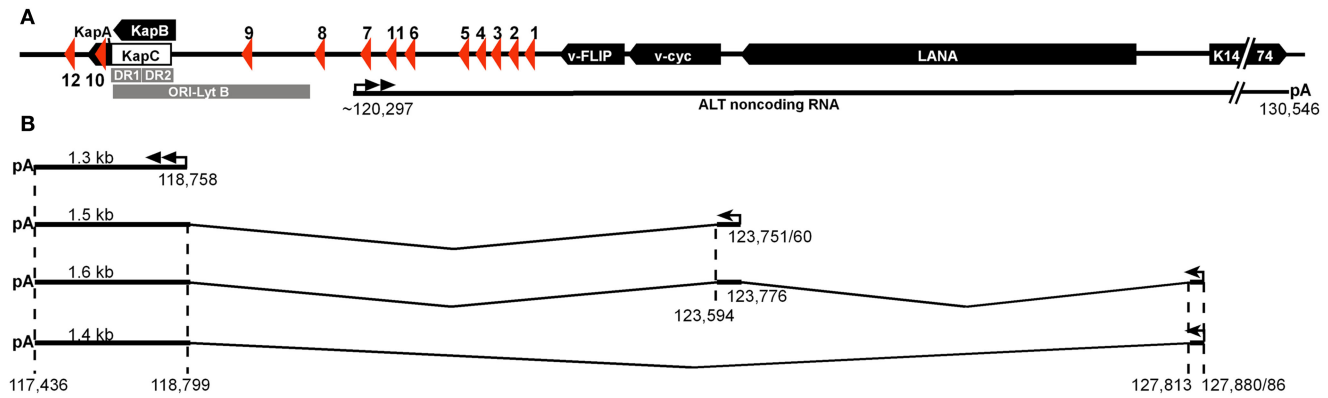

FIGURE 2 | The KSHV miRNAs are expressed from the major latency locus. (A) Schematic of the latency locus and the adjacent K14 and ORF74 genes. The LANA, v-cyclin, v-FLIP, and Kaposin A and B open reading frames (ORFs) are indicated by black arrows. The Kaposin C ORF includes the ORF of Kaposin A and an N-terminal extension (white box). Pre-miRNA sequences are indicated by numbered red arrows. Overlapping DNA elements are indicated by gray boxes. Also shown is the antisense to latency locus transcript (ALT). **(B)** Schematic of the KSHV pri-miRNAs, showing exons (thick lines), introns, transcription start sites. In both panels, nucleotide coordinates refer to positions in the KSHV genome (GenBank accession number: U75698.1). Double arrows indicate transcription start site of lytic RNAs, single arrows start sites of latent RNAs. The bottom transcript was detected in BC-1 cells, but not in BCBL-1 cells. **(A,B)** are drawn approximately to scale and aligned to each other.

While the ALT promoter has not been mapped, it may well overlap the miR-K8 stem-loop, which begins only 300 nts away from the ALT start site. Finally, the expression of sequences antisense to the miR-K8 stem-loop has been reported, although an antisense transcript in this region has not been identified (Lin et al., 2010; Umbach and Cullen, 2010).

SEQUENCES OF THE KSHV miRNAs

The KSHV miRNAs were first identified by cDNA cloning and traditional sequencing (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005) and by an approach that combined the computational prediction of candidate pre-miRNA stem-loops with high-throughput validation of miRNA expression (Grundhoff et al., 2006). These findings were further extended by small RNA deep-sequencing on the Illumina and ABI SOLiD platforms (Lin et al., 2010; Umbach and Cullen, 2010; Gottwein et al., 2011). **Table 1** lists the sequences of the mature KSHV miRNAs that are likely to be expressed at physiologically relevant levels under most conditions. This list is based on the number of reads obtained in deep-sequencing experiments, miRNA activity assays and detection of the miRNAs using qRT-PCR, primer extension analysis, or northern blotting. The actual number of KSHV miRNAs exceeds that of the 12 stem-loop precursors, due to the expression of relatively abundant 5p and 3p miRNAs from several of the pre-miRNAs and other sources of miRNA sequence variation discussed below. While 5p and 3p sequences from all stem-loop precursors have been detected at high sequencing depth (Lin et al., 2010; Umbach and Cullen, 2010), it remains to be seen if all of these sequences are expressed at functionally significant levels in every context. Expression of 5p and 3p miRNAs was consistently observed for some KSHV miRNAs (i.e., miR-K4, miR-K6, miR-K7, miR-K8, and miR-K9). In contrast, other sequences that were recovered by deep-sequencing (e.g., miR-K1-3p and miR-K3-3p) probably reflect true star strands without functional relevance in most settings. The KSHV miRNA repertoire is further expanded by differential processing at the 5′ end of miR-K10, which results in two distinct seed sequences for this miRNA (Umbach and Cullen, 2010), and through adenosine (A) to inosine (I) editing by RNA-specific adenosine deaminase (ADAR) of each of the miR-K10 seeds. Unedited and A to I edited miR-K10 are designated miR-K10a and miR-K10b, respectively (**Table 1**; Pfeffer et al., 2005). The longer 5′ processing variants of miR-K10 are designated miR-K10 + 1_5 (**Table 1**; Gottwein et al., 2011). During latency, expression of miR-K10b is low, but RNA editing at this location is induced during lytic replication and it is possible that a substantial fraction of miR-K10 expressed during lytic reactivation has undergone A to I editing (Gandy et al., 2007). Because I base-pairs like guanosine (G), miR-K10b is expected to target a different pool of mRNAs than miR-K10a. An interesting case is miR-K3, with several PEL cell lines encoding a novel miR-K3 sequence with an additional 5′ adenosine (miR-K3 + 1_5; Gottwein et al., 2011). As in the case of miR-K10, acquisition of the additional miRNA seed sequence is expected to extend the target pool of miR-K3.

CELLULAR ANALOGS OF THE KSHV miRNAs

Importantly, several of the KSHV miRNAs share seed homology with cellular miRNAs suggesting that they function as viral analogs of these miRNAs (**Figure 3A**). Such functional analogy has been demonstrated for miR-K11, which mimics miR-155 (Gottwein et al., 2007; Skalsky et al., 2007), and for the two miR-K10a miRNAs, which together mimic one of two miR-142-3p derived miRNAs (Gottwein et al., 2011). Interestingly, miR-K3 + 1_5 shares 7mer seed homology with the miR-23 miRNA family, but functional analogy between these miRNAs has not been demonstrated yet. A final analogy with likely functional significance is the 6mer seed homology between miR-K6-5p and the miR-214 family of miRNAs. Interestingly, miR-K6-5p also shares extended homology with the miR-15/miR-16 family of miRNAs (Skalsky et al., 2007), but the seed is offset by 1 nt (**Figure 3A**). For 6mer

Table 1 | Sequences of the KSHV miRNAs.

miRNA	Arm	Sequence
miR-K1-5p	5p	AUUACAGGAAACUGGGUGUAAG(CUG)
miR-K2-5p	5p	AACUGUAGUCCGGGUCGAU(CUGA)
miR-K3-5p	5p	UCACAUUCUGAGGACGGCAGCGA(CG)
miR-K3_+1_5[a]	5p	AUCACAUUCUGAGGACGGCAGCGA
miR-K4-3p	3p	UAGAAUACUGAGGCCUAGCUG(A)
miR-K4-5p	5p	AGCUAAACCGCAGUACUCUAGG
miR-K5-3p	3p	UAGGAUGCCUGGAACUUGCCGG(U)
miR-K6-3p	3p	UGAUGGUUUUCGGGCUGUUGAG(C)
miR-K6-5p	5p	CCAGCAGCACCUAAUCCAUCGG
miR-K7-3p	3p	UGAUCCCAUGUUGCUGGCGC(UCA)
miR-K7-5p	5p	AGCGCCACCGGACGGGGAUUUAUG
miR-K8-3p	3p	CUAGGCGCGACUGAGAGAGC(AC)
miR-K8-5p	5p	ACUCCCUCACUAACGCCCCGCU
miR-K9-3p	3p	CUGGGUAUACGCAGCUGCGU(AA)
miR-K9-5p	5p	ACCCAGCUGCGUAAACCCCG(CU)
miR-K10a (-3p)[c]	3p	UAGUGUUGUCCCCCCGAGUGG(C)
miR-K10b- (-3p)[b,c]	3p	U**I**GUGUUGUCCCCCCGAGUGG(C)
miR-K10a_+1_5 (-3p)[c]	3p	UUAGUGUUGUCCCCCCGAGUGG(C)
miR-K10b_+1_5 (-3p)[b,c]	3p	UU**I**GUGUUGUCCCCCCGAGUGG(C)
miR-K11-3p	3p	UUAAUGCUUAGCCUGUGUCCG(AU)
miR-K12-3p[c]	3p	UGGGGGAGGGUGCCCUGGUUG(A)
miR-K12-5p[c]	5p	AACCAGGCCACCAUUCCUCUCCG

Sequences of the KSHV miRNAs with significant expression under most experimental conditions are listed. Nucleotides in brackets are not always present in deep-sequencing reads. Bold letters highlight the A to I editing event within miR-K10. [a]miR-K3+ 1_5 is only present in a subset of KSHV strains. [b]miR-K10b may reach functionally relevant levels of expression only during lytic replication. [c]miR-K10 and miR-K12 expression is increased during lytic replication. In the case of miR-K10, it is unclear if the expression of unedited and/or edited miRNAs is increased.

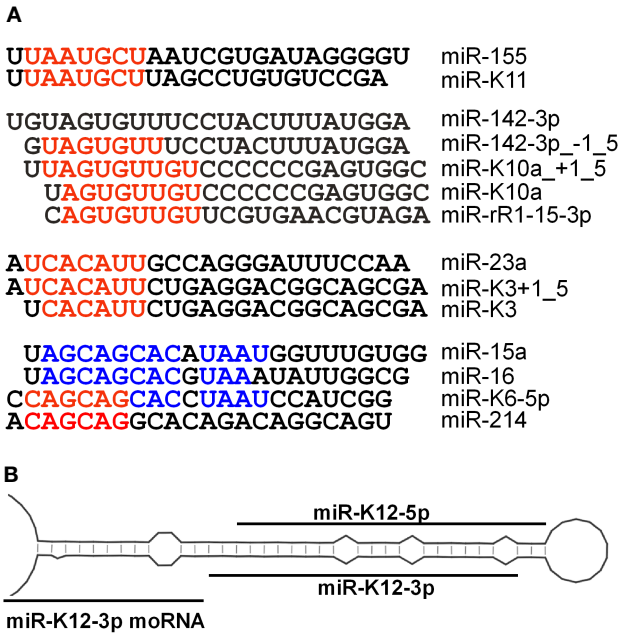

FIGURE 3 | (A) KSHV miRNAs with known or potential cellular analogs. miRNAs are aligned to show seed (red) and non-seed (blue) homologies. **(B)** Schematic illustrating the origin of moRNAs.

seed and offset homology, resulting functional analogies would be expected to be less complete than for 7mer seed analogs. Other potential analogs have been listed (Gottwein et al., 2011), but in most cases the cellular miRNAs in question have not undergone stringent validation. Findings in mice suggest that many of the small RNAs currently annotated as miRNAs may not represent true miRNAs (Chiang et al., 2010). It is therefore possible that many of the >1000 human miRNAs listed in miRBase have been misclassified as miRNAs. The significance of analogies with such uncharacterized miRNAs is therefore unclear and could be co-incidental (Grundhoff and Sullivan, 2011).

BIOLOGICAL SIGNIFICANCE OF KSHV miRNA EXPRESSION LEVELS

The regulatory impact of miRNA expression depends on the relative abundance of the miRNA and its targets. Consequently, the level of miRNA expression is of critical relevance for miRNA function. Methods used for miRNA detection have intrinsic biases and a careful analysis of the absolute and relative expression levels of individual KSHV miRNAs against oligonucleotide standards has not been reported. However, from deep-sequencing data, real-time PCR experiments and activity assays, it appears that individual KSHV miRNAs are expressed at dramatically different copy numbers. While some KSHV miRNAs are always readily detectable (especially miR-K1, miR-K3, miR-K4-3p, miR-K6-3p, miR-K11), others are unlikely to be abundant (e.g., miR-K9; Cai et al., 2005; Pfeffer et al., 2005; Gottwein et al., 2006, 2011; O'Hara et al., 2009; Hansen et al., 2010; Lin et al., 2010; Umbach and Cullen, 2010). Because all KSHV miRNAs are expressed from common pri-miRNAs, their differential expression must result from differences in processing efficiency, RISC loading, and/or stability. In addition, the expression level of the KSHV miRNAs also varies among PEL cell lines and *de novo* infection models (Hansen et al., 2010; Gottwein et al., 2011). While some PEL cell lines express relatively low levels of the KSHV miRNAs (~20–30% of all miRNA reads in deep-sequencing experiments, e.g., BC-1 and BCBL-1), other PEL cell lines express very high levels of the KSHV miRNAs (>80% of all miRNA reads, BC-3 cells). The KSHV miRNAs are constitutively expressed during latency and their expression is maintained upon entry into lytic replication (Cai et al., 2005; Pfeffer et al., 2005). miR-K10 and miR-K12 are induced several-fold during lytic replication due to their expression from both latent and lytic transcripts (see above). In addition, RNA editing of the miR-K10 stem-loop is likely induced during lytic replication, but it is unclear if this results in increased expression of miR-K10b over miR-K10a (Gandy et al., 2007). Finally, the expression levels of the KSHV miRNAs during natural KSHV infection remain unknown.

EVOLUTIONARY CONSERVATION AND VARIATION BETWEEN KSHV STRAINS

Like most herpesvirus miRNAs (Cai et al., 2006; Walz et al., 2010), the KSHV miRNA seed sequences are not conserved between

KSHV and evolutionary distant herpesviruses. One notable exception is the 6mer seed homology between KSHV miR-K10a and miR-rR1-15-3p of the closely related rhesus rhadinovirus (RRV, **Figure 3A**; Umbach and Cullen, 2010), which may be the result of convergent evolution rather than true evolutionary conservation. Despite this lack of evolutionary conservation of the miRNA seeds between evolutionary distant viruses, the mature sequences of the KSHV miRNAs appear to be highly conserved between KSHV isolates (Marshall et al., 2007, 2010). Maybe surprisingly, Marshall et al. also reported a high degree of conservation of sequences outside the mature miRNAs. For example the terminal loop sequences of the pre-miRNA stem-loops were generally conserved, despite the fact that these sequences are expected to be functionally irrelevant as long as the stem-loop structure is maintained. Because of the multifunctional nature of the latency region, it is possible that yet unappreciated selective pressures act on these sequences. Despite this caveat, the observed degree of miRNA conservation strongly argues that the KSHV miRNAs are important for KSHV. Several polymorphisms that change the KSHV miRNA repertoire have been described. An A to G polymorphism in the miR-K5 passenger strand (position 121,315) was found in >20% of all KSHV sequences analyzed (Marshall et al., 2007, 2010). This single nucleotide polymorphism (SNP) was reported elsewhere to alter the structure of the miR-K5 stem, which results in reduced processing of pri-miR-K5 by Drosha and lower levels of miR-K5 expression (Cai et al., 2005; Gottwein et al., 2006, 2011). The miR-K9 stem-loop appears to be the most variable between isolates and has been lost from at least one PEL cell line (BC-3; Marshall et al., 2007; Umbach and Cullen, 2010), suggesting that this miRNA is dispensable for the maintenance of latency, for lytic reactivation and possibly for lymphomagenesis by KSHV. Other reported variants of pre-miR-K9 are likely to also dramatically alter either miR-K9 sequence or expression (Marshall et al., 2007, 2010). Other frequent polymorphisms are located outside mature or passenger strand sequences and consequences on miRNA expression have not been reported (Marshall et al., 2007, 2010).

OTHER SMALL RNAs EXPRESSED FROM THE KSHV LATENCY REGION

Two reports described the detection of miRNA offset RNAs (moRNAs or moRs) and small RNAs antisense to the KSHV miRNAs (Lin et al., 2010; Umbach and Cullen, 2010). moRNAs were first described in the sea squirt *Ciona intestinalis*, which serves as a model organism for simple chordates (Shi et al., 2009). moRNAs are processed from sequences adjacent to the pre-miRNA stem-loop (**Figure 3B**) and are both frequent and abundant in *C. intestinalis*. moRs have also been detected in human small RNA deep-sequencing libraries, but at levels consistently below that of miRNAs and passenger strand sequences (Langenberger et al., 2009), suggesting that these RNAs are potentially only byproducts of miRNA biogenesis. KSHV moRNAs were detected for 9 of 12 KSHV miRNA stem-loops (Lin et al., 2010; Umbach and Cullen, 2010), but like human moRs, these sequences were of very low abundance. While RRV moRs have shown activity in cleavage assays for miRNA activity when expressed at high enough levels (Umbach et al., 2010), the low expression of KSHV moRs during KSHV infection suggests that these sequences might not be expressed at high enough levels to be functionally relevant.

Similarly, small RNAs antisense to miRNA hairpins for miR-K2, miR-K4, and miR-K8 were recovered at frequencies lower than for passenger strand miRNAs (Lin et al., 2010; Umbach and Cullen, 2010). These sequences arise due to bi-directional transcription of a miRNA locus. The expression of KSHV antisense miRNAs appears to be restricted to lytic replication and the recovery of -5p and -3p sequences suggests that they are products of the miRNA biogenesis machinery. A small RNA antisense to miR-K4 (miR-K4-AS-3p) was readily detectable and active in miRNA indicator assays when this region was placed into an expression vector (Lin et al., 2010). It is unclear, however, if this is also the case at physiological levels of expression in PEL. miR-K4-AS and miR-K2-AS could be derived from the ALT RNA. No transcript encompassing miR-K8-AS sequences has been identified to date.

TARGETS AND FUNCTIONS OF THE KSHV miRNAs

The constitutive expression and conservation of the KSHV miRNAs suggests they are functionally important during natural KSHV infection and may also contribute to the oncogenic properties of KSHV. Thus, identifying the targets and functions of the KSHV miRNAs is clearly important for our understanding of KSHV biology and pathogenesis. In principle, the KSHV miRNAs may have evolved to target cellular or viral mRNAs and to regulate cellular properties or viral gene expression. As discussed above, KSHV has evolved a number of novel miRNA seed sequences and also appears to access pre-existing, evolutionary conserved regulatory networks by encoding miRNAs with seed homology to evolutionary conserved cellular miRNAs. While cellular miRNAs are thought to repress many mRNAs, perhaps even hundreds, through seed interactions, there is no good reason to assume that novel viral miRNAs would also target multiple imperfect sites and predominantly engage in seed-mediated interactions. However, no cleavage targets of the KSHV miRNAs have been identified to date and it appears all KSHV miRNAs have many seed targets much like typical cellular miRNAs. It is noteworthy that the KSHV miRNAs must have undergone selection to be functionally compatible with all cell types relevant to KSHV infection, including endothelial cells and B cells, each with a different transcriptome. Consequently, it is possible that one or more targets with a strong selective advantage in one cell type or the latent or lytic stages of KSHV infection may come at a cost in other cell types or at other stages of the life cycle.

KSHV miRNA TARGETOME

Understanding the KSHV miRNA targetome is a critical step toward identifying the functions of the KSHV miRNAs. One of the most successful methods for the identification of functionally relevant targets of evolutionary conserved cellular miRNAs is the computational identification of evolutionary conserved seed matches in 3′ UTR sequences (Bartel, 2009). The assumption underlying this approach is that miRNA and target sequences co-evolve and that functionally important interactions will be conserved. With the exception of the viral analogs of cellular miRNAs, the KSHV miRNAs are unique to KSHV, and therefore not expected to preferentially target 3′ UTR sequences that are evolutionary conserved between species. Consequently, computational target prediction is generally not suitable for the identification of targets of the KSHV miRNAs with novel seeds, which

has mostly relied on experimental target identification. Several studies combined gene expression profiling upon ectopic delivery of individual or several KSHV miRNAs (Gottwein et al., 2007; Samols et al., 2007; Skalsky et al., 2007; Ziegelbauer et al., 2009; Hansen et al., 2010; Suffert et al., 2011) or their antagonism in the context of KSHV infection (Ziegelbauer et al., 2009) with the detection of seed matches to the expressed miRNA(s) among differentially expressed mRNAs in order to identify candidate targets. While such analyses can also give insight into the functional consequences of miRNA expression, the identification of direct targets from differentially expressed mRNAs is confounded by indirect consequences of miRNA expression. In addition, while miRNA-induced changes of mRNA expression are often predictive of the effect on protein expression (Baek et al., 2008; Selbach et al., 2008), the effect of individual interactions is typically small and many targets may be missed using this approach. To overcome these limitations, Dölken et al. (2010) used Ago2-immunoprecipitation coupled with microarray analysis of associated RNAs (RIP-Chip) to identify mRNAs that are enriched in RISC complexes in cells expressing KSHV miRNAs compared to control cells. This work resulted in a list of 114 high confidence candidate targets of the KSHV miRNAs. While RIP-Chip can measure the degree of mRNA association with RISC, it cannot confidently assign the targeting miRNA or map its binding site. This caveat of RIP-CHIP is overcome by the transcriptome-wide identification of miRNA binding sites using photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) and high-throughput sequencing of RISC protected fragments (Hafner et al., 2010; Gottwein et al., 2011). In contrast to other technologies, PAR-CLIP directly identifies RISC-binding sites, which are examined for miRNA seed matches. Applying PAR-CLIP to PEL cell lines has yielded thousands of experimentally identified Ago2 binding sites with seed matches to KSHV miRNAs, in more than 2000 candidate target mRNAs. Validation of a subset of these candidate targets by reporter assays suggested that $\geq$75% of the identified sites can be expected to cause measurable regulation by the assigned miRNA. Thus, extensive lists of candidate targets of the KSHV miRNAs and their likely binding sites exist, but further validation of individual interactions will be required. Because most of these experiments were performed in B cells and none of the datasets is expected to have captured all interactions of the KSHV miRNAs, future work will have to refine our understanding of the KSHV miRNA targetome in other cell types and to address which of the identified interactions impact gene expression and are functionally relevant. Despite these caveats, pathway analyses of high confidence candidates can already give insight into potential functions of the KSHV miRNAs (Dölken et al., 2010; Gottwein et al., 2011). While such analyses are still hampered by the incomplete functional annotation of the human transcriptome and may be biased by the cell type candidates were derived from, current data strongly suggest that KSHV miRNAs are highly multifunctional. Candidate targets of the KSHV miRNAs were found to be significantly enriched for those with roles as transcription factors and those that participate in signaling and vesicular trafficking, among others. The notion that the KSHV miRNAs have diverse functions is also supported by functional validation of diverse targets (below). PAR-CLIP and/or candidate approaches, have also resulted in a number of known or suspected viral targets of the KSHV miRNAs, including the mRNA encoding replication and transcription activator (RTA; Bellare and Ganem, 2009; Gottwein et al., 2011). Finally, a surprisingly high overlap between PAR-CLIP targets of the KSHV and EBV miRNAs, suggests that the KSHV and EBV miRNAs share many functions as a result of convergent evolution (Gottwein et al., 2011). While this cannot be said for sure until such common targets and functions are studied in detail, this hypothesis is already supported by the finding that KSHV, human cytomegalovirus (HCMV) and EBV all target MICB, an important mediator of NK cell recognition, through different sites (Nachmani et al., 2009). The overlapping target range of EBV and KSHV miRNAs may have functional significance in PEL, because >80% of PELs carry both EBV and KSHV (Cesarman et al., 1995) and co-express >30 EBV BART miRNAs with the KSHV miRNAs (Cai et al., 2006; Gottwein et al., 2011). It is therefore likely that the KSHV and EBV miRNAs synergize in PEL to repress important targets. Indeed, PAR-CLIP data predicts that several of the already validated KSHV miRNA targets may also be targeted by EBV miRNAs, including for example caspase 3 (Suffert et al., 2011). In summary, while a significant amount of work remains, we are beginning to understand the complexity of the KSHV miRNA targetome. An even higher degree of complexity is certain to emerge once additional cell types are analyzed at comparable depth.

The functional validation of miRNA targets typically includes several steps: (1) The 3′ UTR sequence of the candidate target is placed 3′ to a luciferase reporter and the specific regulation of indicator activity by the targeting miRNA is established by 3′ UTR reporter assays. The loss of reporter regulation upon mutation of the miRNA binding site is currently the only way to demonstrate the potential of a direct regulatory interaction in human cells. However, due to the artificiality of this assay, target regulation in a 3′ UTR reporter assay cannot prove that the interaction does indeed take place in the context of infection or is functionally relevant. (2) Repression of the endogenous protein by the miRNA is established. This is demonstrated by reduced target expression following the ectopic expression of physiological levels of the targeting miRNA in cells lacking this miRNA and by increased target expression following the inhibition of the miRNA in the context of infection. Because miRNA-induced changes are often subtle, quantitative western blotting is emerging as one of the best tools to accurately establish the level of regulation at the protein level (Abend et al., 2010). (3) The contribution of target regulation to observed functional consequences is established. Because it is often hard to unequivocally demonstrate that observed functional consequences are indeed due to the targeting of a particular mRNA, several lines of indirect evidence are usually accumulated to support this idea. Upon ectopic miRNA expression, rescue of a miRNA-induced phenotype can be performed by re-expression of the target. In addition, RNA interference (RNAi) of the target should phenocopy miRNA expression. Conversely, upon miRNA inhibition, siRNA of the target mRNA is expected to reverse the phenotype and overexpression of the target is expected to phenocopy miRNA knockdown. The few KSHV miRNA targets that have undergone fairly stringent functional validation are listed in **Table 2**. In the following paragraphs, key concepts and open questions that have emerged are highlighted.

Table 2 | KSHV miRNA targets.

Gene Symbol	miRNA(s)	Reference	Functional consequences
BACH1	miR-K11 (PC)	Gottwein et al. (2007), Skalsky et al. (2007), Qin et al. (2010a)	Upregulation of SCL7A11/xCT expression by the KSHV miRNAs, increased viability under oxidative stress
BCLAF1	K5 K9-3p K10a (PC), K10b	Ziegelbauer et al. (2009)	Prime cells for KSHV reactivation, modulate caspase activity
CASP3	miR-K1 (PC) miR-K3 (PC) miR-K4-3p (PC)	Suffert et al. (2011)	This interaction may contribute to the inhibition of apoptosis by KSHV
CDKN1A/p21	miR-K1 (PC)	Gottwein and Cullen (2010)	Attenuation of p21-dependent cell cycle arrest
IKBKE/IKKε	miR-K11	Gottwein et al. (2007), Liang et al. (2011)	Attenuation of interferon induction by pattern recognition receptors, stabilize KSHV latency
MAF	miR-K1 miR-K6-5p miR-K11	Hansen et al. (2010)	Downregulation of LEC marker expression, de-differentiation
MICB	miR-K7	Nachmani et al. (2009)	Reduced NK cell recognition and activation
NFIB	miR-K3	Lu et al. (2010a)	Stabilization of KSHV latency by regulation of RTA promoter activity
NFKBIA/IκBα	miR-K1	Lei et al. (2010)	Stabilization of KSHV latency by regulation of RTA promoter activity
RBL2	miR-K4-5p (PC)	Lu et al. (2010b)	De-repression of DNA methyl transferases Dnmt1, 3a and 3b?
SMAD5	miR-K11 (PC)	Liu et al. (2012)	Attenuation of TGF-beta signaling
TNFRSF12A/TWEAKR	miR-K10a (PC)	Abend et al. (2010)	Reduce cellular responses to TWEAK signaling, including the induction of inflammatory cytokines and the induction of apoptosis
KSHV RTA (ORF50)	miR-K9-5p	Bellare and Ganem (2009)	Reduce RTA expression and consequently inhibit KSHV reactivation

Only KSHV miRNA targets that have undergone stringent validation and at least some functional analyses are listed. The gene symbol, the targeting miRNA(s), original reports, and proposed functional consequences are listed. In the miRNA(s) column (PC) indicates that the proposed interaction was also recovered by PAR-CLIP (Gottwein et al., 2011).

KSHV ANALOGS OF CELLULAR miRNAs

Kaposi's sarcoma-associated herpesvirus gains access to a number of pre-existing regulatory networks by encoding viral analogs of cellular miRNAs. As mentioned above, miR-K11 shares 7mer seed homology with miR-155 and their overlapping target range has been extensively validated: ectopically expressed miR-K11 and miR-155 regulate similar sets of mRNAs and result in equivalent regulation of 3′ UTR reporters in all cases tested to date (Gottwein et al., 2007; Skalsky et al., 2007). Similar target regulation by miR-K11 and miR-155 was also observed at the level of protein expression for the transcription factors BACH1 and FOS in side-by-side experiments. In addition, it was demonstrated that miR-K11 regulates protein expression of known miR-155 targets IκB kinase epsilon (IKKε) and SMAD5 (Gottwein et al., 2007; Lu et al., 2008; Yin et al., 2008, 2010; Liang et al., 2011; Liu et al., 2012). More recently, the analogy between miR-K11 and miR-155 was further confirmed by the recovery of ∼40% of all known miR-155 targets as candidate targets of miR-K11 in PEL cells using PAR-CLIP (Gottwein et al., 2011). While a role of miR-K11 in KSHV-induced cancer has not been demonstrated, such a role appears likely, because (1) the constitutive expression of its analog miR-155 in B cells or hematopoietic stem cells causes B cell- or myelo-proliferative disease (Costinean et al., 2006; O'Connell et al., 2008), (2) EBV-induced cellular miR-155 is essential for the survival and growth of EBV-transformed lymphoblastoid cells (Linnstaedt et al., 2010), (3) a viral miR-155 analog encoded by the oncogenic chicken α-herpesvirus Marek's disease virus (MDV) was found to be essential for lymphomagenesis by MDV in chickens (Zhao et al., 2011), and (4) the constitutive expression of miR-K11 in human hematopoietic cells that were reconstituted into a humanized mouse model phenocopied expression of miR-155 and lead to an expansion of splenic B cells (Boss et al., 2011). Which of the many miR-155 targets are responsible for these observations is not understood in detail, but it is tempting to speculate that at least some of the relevant targets may be conserved in humans, mice, and chicken. In addition to a potential role in lymphomagenesis, miR-K11 contributes to the KSHV-induced transcriptional reprogramming of lymphatic endothelial cells (Hansen et al., 2010), promotes cell survival under oxidative stress (Qin et al., 2010a), and inhibits TGF-beta signaling (Liu et al., 2012). These functions of miR-K11 are discussed below.

Recently, a more complex analogy has been demonstrated between KSHV miR-K10a and the cellular miRNA miR-142-3p (Gottwein et al., 2011). miR-K10a and miR-142-3p both have two 5′ processing variants, which are expressed at close to a 1:1 ratio (Umbach and Cullen, 2010; Gottwein et al., 2011). The longer miR-K10a_+1_5 shares 7mer seed homology with the shorter miR-142-3p-1_5p (**Figure 3A**). Because the 5′ nt of miR-K10a is a uridine, 7mer-1A sites for miR-K10a are also 7mer sites for miR-K10a + 1_5 and both miRNAs expressed by miR-K10a are predicted to effectively mimic miR-142-3p-1_5. In fact, only 15% of the PAR-CLIP sites predicted as targets of the two miR-K10a variants were not also predicted to be targets of miR-142-3p-1_5. miR-142-3p is expressed in all cells of the hematopoietic lineage and largely absent in non-hematopoietic cells, but very few targets and functions of miR-142-3p have been identified to date. In PEL cell lines, miR-142-3p is likely significantly more abundant than miR-K10, suggesting that these miRNAs may be redundant in B cells. Because miR-142-3p is absent from many other cell types, including endothelial cells, it is possible that KSHV uses miR-K10 to introduce miR-142-3p-like functions into other cell types. Interestingly, PAR-CLIP sites assigned to miR-K10/miR-142-3p were also reported in latent viral mRNAs (Gottwein et al., 2011), including the 3′ UTRs of v-cyclin/LANA, the KSHV homolog of human IL-6 (v-IL-6), and latent nuclear antigen 2/viral interferon regulatory factor 3, a protein that interferes with cellular signaling and is essential for the survival of PEL cells in culture (Wies et al., 2008). While these interactions remain to be validated at a functional level, their identification raises the possibility that KSHV uses miR-142-3p and/or miR-K10 to fine tune latent protein expression. In conclusion, while KSHV clearly encodes functional mimics of cellular miRNAs, it is also important to point out that this analogy extends only to the seed region. Like in the case of viral homologs of cellular proteins, it appears likely that viral analogs of cellular miRNAs also differ in key aspects from their cellular counterparts. Although such differences remain to be identified, these may include differences in non-seed target interactions, miRNA stability, and miRNA regulation.

DO THE KSHV miRNAs REGULATE REACTIVATION?

The establishment of latency, a highly restricted gene expression pattern that mediates the maintenance of the viral episome and minimizes immune recognition, is universal among herpesviruses and critical for their life-long persistence. Expression of herpesviral immediate early (IE) genes serves as a "switch" that triggers reactivation from latency and initiates the lytic gene expression cascade that ultimately results in expression of all viral genes and the production of progeny virus. RTA is both necessary and sufficient for the initiation of lytic KSHV replication. RTA expression is tightly regulated to respond to a variety of physiological triggers. While the default gene expression program of KSHV in culture is latency, spontaneous reactivation occurs at a low rate (typically ∼1–2% of the cells). Latently expressed α-herpesviral miRNAs were found to regulate IE genes (Umbach et al., 2008; Jurak et al., 2011) and it is an attractive hypothesis that viral miRNAs in general function in this manner to stabilize latency (Murphy et al., 2008). Potentially, regulation of RTA could take place at the level of transcription, through epigenetic modification and the availability of repressors and activators of transcription, at the level of mRNA stability and translation and at the level of protein stability and activity. Thus, in order to affect RTA levels, the KSHV miRNAs may directly target RTA mRNAs or regulate cellular factors that control RTA expression and activity. Two independent studies reported that deletion of the intronic miRNAs, miR-K1-9 and miR-K11, from KSHV bac36 causes a two to fourfold increase in RTA transcript levels, a similarly modest increase in downstream viral lytic gene expression and an approximately twofold increase in virus yield from 293T cells, suggesting that the intronic miRNAs modulate the latent to lytic switch in favor of latency (Lei et al., 2010; Lu et al., 2010b). This phenotype was accompanied by a modest, 1.5- to 2-fold, increase in RTA promoter reporter activity in these cells (Lei et al., 2010), suggesting that regulation is at the level of promoter activity. While Lei et al. (2010) reported that this effect could be reversed by re-expression of miR-K1, Lu et al. (2010b) observed no restoration of RTA inhibition by re-expression of miR-K1 and presented data suggesting that the expression of miR-K5, and perhaps miR-K4, was able to restore lower RTA transcript levels. Given that a similar cell type was used by both groups, it is possible that differences in the level of the re-expressed miRNAs may account for the discrepancies between these studies. In a complementary approach, Bellare and Ganem (2009) transfected antisense inhibitors against individual miRNAs into human foreskin fibroblasts (HFFs) infected with rKSHV.219, a recombinant KSHV containing an RFP reporter under control of the lytic PAN promoter (Vieira and O'Hearn, 2004). Interestingly, no effect on the spontaneous reactivation of KSHV was observed upon individual inhibition of either miR-K1, miR-K4-5p, miR-K4-3p, or miR-K5, suggesting that, at least in HFF, inhibition of these miRNAs alone is not sufficient to cause an increase in spontaneous reactivation. In HFF, only the inhibition of miR-K9-5p resulted in a ∼2.5-fold increase in RFP expression, likely due to a direct interaction of this miRNA with a site in the 3′ UTR of RTA (Bellare and Ganem, 2009), but expression of miR-K9 was reported to be unable to restore RTA inhibition to the miRNA-deleted virus in 293 cells (Lu et al., 2010b). Another study reported that overexpression of miR-K3 in the PEL cell line BC-3 results in decreased RTA mRNA expression (Lu et al., 2010a), but the contribution of endogenously expressed miR-K3 to the maintenance of latency in PEL remains unclear. The re-expression of miR-K3 was unable to restore RTA inhibition to the miRNA-deleted virus in 293 cells (Lu et al., 2010b) and its inhibition did not result in reactivation of the virus in HFF (Bellare and Ganem, 2009). In addition to the study by Bellare and Ganem (2009), several laboratories have tested RTA-3′ UTR indicators for inhibition by the KSHV miRNAs, with different results (Lei et al., 2010; Lu et al., 2010b; Lin et al., 2011). Additional miRNAs that were reported to inhibit RTA indicator expression include miR-K5 and miR-K7-5p (Lu et al., 2010b; Lin et al., 2011). Lei et al. (2010), however, did not observe RTA indicator repression by any of the KSHV miRNAs. It appears that differences between studies are most likely due to the use of different approaches to miRNA expression, using mimics or from expression vectors, which may either overexpress or inadequately express 5p and 3p miRNAs. Another source of experimental discrepancies could be the folding of the 3′ UTR-indicator transcript which depends on the exact sequence of the

transcript. It is conceivable that unnatural 3′ UTR folding could result in both false positive and false negative results. While the above studies generally agree that the KSHV miRNAs modestly stabilize latency, Ziegelbauer et al. (2009) showed that the combined inhibition of miR-K5, miR-K9-3p, and miR-K10a/b in PEL and SLK cells had the opposite effect and reduced the percentage of cells undergoing spontaneous reactivation by about 40%, likely through de-repression of the cellular protein bcl2-associated factor (BCLAF1). Thus, several of the miRNAs may also help tip the balance in favor of lytic reactivation. In conclusion, while much work remains to be done to establish which individual or collaborating KSHV miRNAs affect KSHV reactivation in different cell types, existing data point to a subtle function of the intronic miRNAs in favor of maintaining latency. The observed effects were modest and may present additional controls to KSHV gene expression. It is plausible that the KSHV miRNAs are under evolutionary pressure to be compatible with the latent and lytic replication modes, and even possible that they have evolved to avoid strong interactions with RTA and other lytic mRNAs.

TRANSCRIPTIONAL AND EPIGENETIC REPROGRAMMING

While direct recognition by miRNAs often results in only subtle changes of target expression, indirect functional consequences can be more dramatic if regulated mRNAs encode transcription factors and other regulators of gene expression. Transcriptional regulation was the most highly enriched function among candidate targets of the KSHV miRNAs (Gottwein et al., 2011), and several transcription factors have already been validated as targets of KSHV miRNAs. KSHV-infected spindle cells are the predominant cell type in advanced KS lesion. Spindle cells are poorly differentiated with expression of lymphatic and blood vessel endothelial cell (LEC and BEC) as well as mesenchymal markers. This transcriptional reprogramming is recapitulated upon infection of LECs by KSHV *in vitro* (Boshoff et al., 1995; Hong et al., 2004; Wang et al., 2004; Cheng et al., 2011; Gasperini et al., 2012). Several KSHV miRNAs contribute to the transcriptional reprogramming of LEC by KSHV through targeting of the transcription factor c-Maf (MAF; Hansen et al., 2010). Large Maf transcription factors, including c-Maf, can act as activators or repressors and regulate the terminal differentiation of a number of tissues. c-Maf is among the proteins that are specifically induced in LEC by the transcriptional master regulator of lymphatic endothelial differentiation PROX1 (Petrova et al., 2002; Hong et al., 2004). c-Maf was downregulated in LEC stably expressing the intronic KSHV miRNAs and found to be directly repressed by KSHV miR-K6 and miR-K11. Downregulation of c-Maf following the expression of the intronic miRNAs or by RNAi induced the expression of BEC marker genes in LEC, suggesting that c-Maf is important to maintain LEC differentiation. Thus, regulation of MAF by the KSHV miRNAs at least partially accounts for the transcriptional reprogramming observed upon KSHV infection of LEC. Other examples of transcription factors targeted by KSHV miRNAs include the transcriptional repressors RBL2 (retinoblastoma-like 2), BACH1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1), and BCLAF1 (discussed above and below). RBL2 is downregulated by miR-K4-5p, potentially resulting in increased expression of DNA methyl transferases DNMT1, 3a, and 3b (Lu et al., 2010b). Consistent with a function of the KSHV miRNAs in the epigenetic modification of the KSHV and cellular genomes, deletion of the intronic miRNAs resulted in an overall reduction of CpG methylation of the viral and cellular genomes. BACH1 is among the most strongly regulated targets of the KSHV miRNAs validated to date (Gottwein et al., 2007; Skalsky et al., 2007). The best characterized target of BACH1-mediated repression is hemeoxygenase 1 (HO-1, HMOX1), the limiting enzyme in heme catabolism, which is strongly upregulated in KS (McAllister et al., 2004). More recently, Qin et al. (2010a) showed that BACH1 also represses SLC7A11 (solute carrier family 7, xCT), a transporter which imports cystine required for the synthesis of glutathione (GSH), a critical antioxidant that protects cells from reactive oxygen species (ROS). Consequently, the resulting upregulation of SLC7A11 protects infected cells from oxidative stress. BACH1-ChIP-seq in 293 cells (Warnatz et al., 2011), which express endogenous BACH1, confirmed HO-1 and SLC7A11 as BACH1 target genes and identified additional targets of BACH1, including additional factors involved in redox and transport processes, and those involved in cell cycle and apoptosis. Thus, several transcription factors have already been shown to be important targets of the KSHV miRNAs and many more are likely candidates, suggesting that transcriptional reprogramming is an important function of the KSHV miRNAs.

EVASION FROM CELL CYCLE ARREST AND CELL DEATH

The deregulation of the cell cycle, survival signaling, and cell death pathways contributes to the oncogenic properties of KSHV and other oncogenic viruses. Not surprisingly, several of the already validated targets of the KSHV miRNAs are predicted to facilitate viral escape from cell cycle arrest and apoptosis. KSHV miR-K1 targets p21, a cyclin-dependent kinase inhibitor (CDKI) induced by several growth inhibitory signals, and thereby attenuates p21-mediated cell cycle arrest (Gottwein and Cullen, 2010). More recently, several other miRNAs were reported to also bind the p21 3′ UTR and the 3′ UTR of a second CDKI, p27, but the functional impact of these interactions in the context of KSHV infection remains to be established (Gottwein et al., 2011). As discussed above, the downregulation of BACH1 by miR-K11 promotes cellular survival under oxidative stress, through de-repression of xCT (Qin et al., 2010a). KSHV miR-K10 was shown to allow the escape from TNFSF12/TWEAK-induced apoptosis by repression of TNRSFR12A/TWEAKR, its receptor (Abend et al., 2010), and miRNAs miR-K1, miR-K3, and miR-K4-3p target effector caspase 3 (CASP3; Suffert et al., 2011). The co-expression of the intronic KSHV miRNAs inhibited apoptosis following treatment with staurosporine, while the joint inhibition of miR-K1, miR-K3, and miR-K4-3p in KSHV-infected E6/E7-immortalized LEC enhanced etoposide-induced apoptosis by about twofold (Suffert et al., 2011). Attenuation of staurosporine-induced apoptosis was also observed in DG-75 cells in the absence of caspase 3 activation, which is defective in these cells, suggesting that caspase 3 is unlikely to be the functionally relevant target in DG-75 cells. An important remaining question is whether an approximately twofold reduction of procaspase 3 expression can indeed protect cells from cell death or even delay its progression, given that caspase 3 is an effector rather than an inducer of apoptosis and may

not be limiting to the progression of apoptosis. TGF-β is a multifunctional cytokine that induces cell cycle arrest and apoptosis in B cells. Importantly, PEL cell lines are generally resistant to the effects of TGF-β, at least in part due to inhibition of TGF-β receptor 2 subunit expression by LANA (Di Bartolo et al., 2008). The stable expression of the intronic KSHV miRNAs in 293T resulted in strongly reduced activity of a SMAD-responsive element reporter (Samols et al., 2007), suggesting that the KSHV miRNAs may also contribute to the inhibition of TGF-β signaling by KSHV or at least serve as an additional safeguard. The mechanism underlying this observation was not addressed, but, more recently it was shown that miR-K11, like its analog miR-155 (Yin et al., 2008, 2010; Rai et al., 2010), antagonizes SMAD5 and this interaction likely contributes to the observed resistance to TGF-beta induced growth inhibition and apoptosis in PEL (Liu et al., 2012). Other candidate targets with known roles in the regulation of cell cycle and cell death have been validated (Dölken et al., 2010; Gottwein et al., 2011), but are of unknown relevance in the context of KSHV infection.

OTHER FUNCTIONS OF THE KSHV miRNAs

Immune evasion is critical for the life-long persistence of herpesviral infection and herpesviruses employ multiple mechanisms to ensure the escape of infected cells from both the innate and the adaptive arms of the immune system. Latency and the widespread use of viral miRNA expression during latency themselves can be viewed as mechanisms of passive immune evasion. One important target with a role in evasion from natural killer (NK) cells is the miR-K7 target MICB (Nachmani et al., 2009), whose surface expression triggers recognition by the NK cell activating receptor NKG2D and subsequent killing by NK cells. MICB is also repressed by the miRNAs of other herpesviruses, including EBV miR-Bart2-5p and HCMV miR-UL112, through different sites (Stern-Ginossar et al., 2007; Nachmani et al., 2009), which further underscores the likely importance of this target in herpesviral infection. A final important emerging concept is the regulation of cellular signaling by the KSHV miRNAs and many of the validated targets discussed above participate in one or more signaling cascades (**Table 1**). Given the critical role cytokines play in the pathogenesis of KS, deregulation of cytokine expression by KSHV miRNAs is perhaps expected. Abend et al. (2010) demonstrated that the ectopic expression of miR-K10a in human umbilical vein endothelial cells (HUVECs) inhibited the TWEAK-induced expression of the proinflammatory cytokines IL-8 and MCP-1 approximately two to threefold. Another report has implicated several KSHV miRNAs in the induction of IL-6 and IL-10 secretion by murine and human myeloid-derived cells, potentially through downregulation of the C/EBPβ LIP isoform (Qin et al., 2010b). However, this effect is unlikely to be entirely due to a direct interaction of the proposed KSHV miRNAs with the C/EBPβ mRNA, because C/EBPβ isoforms other than LIP, which are expressed from the same mRNA through differential translational initiation (Calkhoven et al., 2000), were not affected. In addition, the murine and human C/EBPβ mRNAs lack canonical miRNA binding sites for miR-K3 and miR-K7. C/EBPβ, however, is a well validated target of cellular miR-155 (O'Connell et al., 2008; Costinean et al., 2009) and is also regulated by its analog miR-K11 (Boss et al., 2011). Lei et al. (2010) demonstrated that KSHV miR-K1 inhibits IκBα expression and consequently activates NFκB signaling, which may help stabilize KSHV latency. Finally, PAR-CLIP data from PEL cells identified numerous candidates for direct targets of the KSHV miRNAs with roles in signaling (Gottwein et al., 2011).

CONCLUSION AND OUTLOOK

An important open question in the miRNA field is whether miRNAs have a small number of critical targets or whether many, possibly hundreds of interactions of each individual miRNA have functional importance. The KSHV miRNAs have undoubtedly evolved a very complex set of regulatory relationships through viral analogs of cellular miRNAs and also novel miRNA sequences. Future functional experiments will have to clarify how many and which of these interactions are important for the KSVH life cycle and pathogenesis. Particularly relevant will be to elucidate functions of the KSHV miRNAs that contribute to the development of KSHV-induced disease, which could become targets for therapeutic intervention.

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REFERENCES

Abend, J. R., Uldrick, T., and Ziegelbauer, J. M. (2010). Regulation of tumor necrosis factor-like weak inducer of apoptosis receptor protein (TWEAKR) expression by Kaposi's sarcoma-associated herpesvirus microRNA prevents TWEAK-induced apoptosis and inflammatory cytokine expression. *J. Virol.* 84, 12139–12151.

AuCoin, D. P., Colletti, K. S., Xu, Y., Cei, S. A., and Pari, G. S. (2002). Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) contains two functional lytic origins of DNA replication. *J. Virol.* 76, 7890–7896.

Baek, D., Villen, J., Shin, C., Camargo, F. D., Gygi, S. P., and Bartel, D. P. (2008). The impact of microRNAs on protein output. *Nature* 455, 64–71.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233.

Bellare, P., and Ganem, D. (2009). Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation. *Cell Host Microbe* 6, 570–575.

Boshoff, C., Schulz, T. F., Kennedy, M. M., Graham, A. K., Fisher, C., Thomas, A., Mcgee, J. O., Weiss, R. A., and O'Leary, J. J. (1995). Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. *Nat. Med.* 1, 1274–1278.

Boss, I. W., Nadeau, P. E., Abbott, J. R., Yang, Y., Mergia, A., and Renne, R. (2011). A Kaposi's sarcoma-associated herpesvirus-encoded ortholog of microRNA miR-155 induces human splenic B-cell expansion in NOD/LtSz-scid IL2Rgammanull mice. *J. Virol.* 85, 9877–9886.

Cai, X., and Cullen, B. R. (2006). Transcriptional origin of Kaposi's sarcoma-associated herpesvirus microRNAs. *J. Virol.* 80, 2234–2242.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B., and Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5570–5575.

Cai, X., Schafer, A., Lu, S., Bilello, J. P., Desrosiers, R. C., Edwards, R., Raab-Traub, N., and Cullen, B. R. (2006). Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. *PLoS Pathog.* 2, e23. doi:10.1371/journal.ppat.0020023

Calkhoven, C. F., Muller, C., and Leutz, A. (2000). Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes Dev.* 14, 1920–1932.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Chandriani, S., Xu, Y., and Ganem, D. (2010). The lytic transcriptome of Kaposi's sarcoma-associated herpesvirus reveals extensive transcription of noncoding regions, including regions antisense to important genes. *J. Virol.* 84, 7934–7942.

Cheng, F., Pekkonen, P., Laurinavicius, S., Sugiyama, N., Henderson, S., Gunther, T., Rantanen, V., Kaivanto, E., Aavikko, M., Sarek, G., Hautaniemi, S., Biberfeld, P., Aaltonen, L., Grundhoff, A., Boshoff, C., Alitalo, K., Lehti, K., and Ojala, P. M. (2011). KSHV-initiated notch activation leads to membrane-type-1 matrix metalloproteinase-dependent lymphatic endothelial-to-mesenchymal transition. *Cell Host Microbe* 10, 577–590.

Chiang, H. R., Schoenfeld, L. W., Ruby, J. G., Auyeung, V. C., Spies, N., Baek, D., Johnston, W. K., Russ, C., Luo, S., Babiarz, J. E., Blelloch, R., Schroth, G. P., Nusbaum, C., and Bartel, D. P. (2010). Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. *Genes Dev.* 24, 992–1009.

Costinean, S., Sandhu, S. K., Pedersen, I. M., Tili, E., Trotta, R., Perrotti, D., Ciarlariello, D., Neviani, P., Harb, J., Kauffman, L. R., Shidham, A., and Croce, C. M. (2009). Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. *Blood* 114, 1374–1382.

Costinean, S., Zanesi, N., Pekarsky, Y., Tili, E., Volinia, S., Heerema, N., and Croce, C. M. (2006). Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7024–7029.

Cullen, B. R. (2006). Viruses and microRNAs. *Nat. Genet.* 38(Suppl.), S25–S30.

Cullen, B. R. (2011). Viruses and microRNAs: RISCy interactions with serious consequences. *Genes Dev.* 25, 1881–1894.

Di Bartolo, D. L., Cannon, M., Liu, Y. F., Renne, R., Chadburn, A., Boshoff, C., and Cesarman, E. (2008). KSHV LANA inhibits TGF-beta signaling through epigenetic silencing of the TGF-beta type II receptor. *Blood* 111, 4731–4740.

Dölken, L., Malterer, G., Erhard, F., Kothe, S., Friedel, C. C., Suffert, G., Marcinowski, L., Motsch, N., Barth, S., Beitzinger, M., Lieber, D., Bailer, S. M., Hoffmann, R., Ruzsics, Z., Kremmer, E., Pfeffer, S., Zimmer, R., Koszinowski, U. H., Grasser, F., Meister, G., and Haas, J. (2010). Systematic analysis of viral and cellular microRNA targets in cells latently infected with human gamma-herpesviruses by RISC immunoprecipitation assay. *Cell Host Microbe* 7, 324–334.

Gandy, S. Z., Linnstaedt, S. D., Muralidhar, S., Cashman, K. A., Rosenthal, L. J., and Casey, J. L. (2007). RNA editing of the human herpesvirus 8 kaposin transcript eliminates its transforming activity and is induced during lytic replication. *J. Virol.* 81, 13544–13551.

Gasperini, P., Espigol-Frigole, G., Mccormick, P. J., Salvucci, O., Maric, D., Uldrick, T. S., Polizzotto, M. N., Yarchoan, R., and Tosato, G. (2012). Kaposi sarcoma herpesvirus promotes endothelial-to-mesenchymal transition through Notch-dependent signaling. *Cancer Res.* 72, 1157–1169.

Gottwein, E., Cai, X., and Cullen, B. R. (2006). A novel assay for viral microRNA function identifies a single nucleotide polymorphism that affects Drosha processing. *J. Virol.* 80, 5321–5326.

Gottwein, E., Corcoran, D. L., Mukherjee, N., Skalsky, R. L., Hafner, M., Nusbaum, J. D., Shamulailatpam, P., Love, C. L., Dave, S. S., Tuschl, T., Ohler, U., and Cullen, B. R. (2011). Viral microrna targetome of KSHV-infected primary effusion lymphoma cell lines. *Cell Host Microbe* 10, 515–526.

Gottwein, E., and Cullen, B. R. (2010). A human herpesvirus microRNA inhibits p21 expression and attenuates p21-mediated cell cycle arrest. *J. Virol.* 84, 5229–5237.

Gottwein, E., Mukherjee, N., Sachse, C., Frenzel, C., Majoros, W. H., Chi, J. T., Braich, R., Manoharan, M., Soutschek, J., Ohler, U., and Cullen, B. R. (2007). A viral microRNA functions as an orthologue of cellular miR-155. *Nature* 450, 1096–1099.

Grey, F., Tirabassi, R., Meyers, H., Wu, G., Mcweeney, S., Hook, L., and Nelson, J. A. (2010). A viral microRNA down-regulates multiple cell cycle genes through mRNA 5′UTRs. *PLoS Pathog.* 6, e1000967. doi:10.1371/journal.ppat.1000967

Grundhoff, A., and Sullivan, C. S. (2011). Virus-encoded microRNAs. *Virology* 411, 325–343.

Grundhoff, A., Sullivan, C. S., and Ganem, D. (2006). A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses. *RNA* 12, 733–750.

Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., Rothballer, A., Ascano, M. Jr., Jungkamp, A. C., Munschauer, M., Ulrich, A., Wardle, G. S., Dewell, S., Zavolan, M., and Tuschl, T. (2010). Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 141, 129–141.

Hansen, A., Henderson, S., Lagos, D., Nikitenko, L., Coulter, E., Roberts, S., Gratrix, F., Plaisance, K., Renne, R., Bower, M., Kellam, P., and Boshoff, C. (2010). KSHV-encoded miRNAs target MAF to induce endothelial cell reprogramming. *Genes Dev.* 24, 195–205.

Hong, Y. K., Foreman, K., Shin, J. W., Hirakawa, S., Curry, C. L., Sage, D. R., Libermann, T., Dezube, B. J., Fingeroth, J. D., and Detmar, M. (2004). Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. *Nat. Genet.* 36, 683–685.

Huntzinger, E., and Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat. Rev. Genet.* 12, 99–110.

Hutvagner, G., and Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. *Science* 297, 2056–2060.

Jurak, I., Griffiths, A., and Coen, D. M. (2011). Mammalian alphaherpesvirus miRNAs. *Biochim. Biophys. Acta* 1809, 641–653.

Kim, V. N., Han, J., and Siomi, M. C. (2009). Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.* 10, 126–139.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. *Science* 294, 853–858.

Langenberger, D., Bermudez-Santana, C., Hertel, J., Hoffmann, S., Khaitovich, P., and Stadler, P. F. (2009). Evidence for human microRNA-offset RNAs in small RNA sequencing data. *Bioinformatics* 25, 2298–2301.

Lau, N. C., Lim, L. P., Weinstein, E. G., and Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* 294, 858–862.

Lee, R. C., and Ambros, V. (2001). An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* 294, 862–864.

Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75, 843–854.

Lei, X., Bai, Z., Ye, F., Xie, J., Kim, C. G., Huang, Y., and Gao, S. J. (2010). Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. *Nat. Cell Biol.* 12, 193–199.

Liang, D., Gao, Y., Lin, X., He, Z., Zhao, Q., Deng, Q., and Lan, K. (2011). A human herpesvirus miRNA attenuates interferon signaling and contributes to maintenance of viral latency by targeting IKKepsilon. *Cell Res.* 21, 793–806.

Lin, C. L., Li, H., Wang, Y., Zhu, F. X., Kudchodkar, S., and Yuan, Y. (2003). Kaposi's sarcoma-associated herpesvirus lytic origin (ori-Lyt)-dependent DNA replication: identification of the ori-Lyt and association of K8 bZip protein with the origin. *J. Virol.* 77, 5578–5588.

Lin, H. R., and Ganem, D. (2011). Viral microRNA target allows insight into the role of translation in governing microRNA target accessibility. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5148–5153.

Lin, X., Liang, D., He, Z., Deng, Q., Robertson, E. S., and Lan, K. (2011). miR-K12-7-5p encoded by Kaposi's sarcoma-associated herpesvirus stabilizes the latent state by targeting viral ORF50/RTA. *PLoS ONE* 6, e16224. doi:10.1371/journal.pone.0016224

Lin, Y. T., Kincaid, R. P., Arasappan, D., Dowd, S. E., Hunicke-Smith, S. P., and Sullivan, C. S. (2010). Small RNA profiling reveals antisense transcription throughout the KSHV genome and novel small RNAs. *RNA* 16, 1540–1558.

Lin, Y. T., and Sullivan, C. S. (2011). Expanding the role of Drosha to the regulation of viral gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11229–11234.

Linnstaedt, S. D., Gottwein, E., Skalsky, R. L., Luftig, M. A., and Cullen, B. R. (2010). Virally induced cellular microRNA miR-155 plays a key role in B-cell immortalization by Epstein-Barr virus. *J. Virol.* 84, 11670–11678.

Liu, Y., Sun, R., Lin, X., Liang, D., Deng, Q., and Lan, K. (2012). KSHV-encoded miR-K12-11 attenuates transforming growth factor beta signaling through suppression of SMAD5. *J. Virol.* 86, 1372–1381.

Lu, C. C., Li, Z., Chu, C. Y., Feng, J., Sun, R., and Rana, T. M. (2010a). MicroRNAs encoded by Kaposi's sarcoma-associated herpesvirus regulate viral life cycle. *EMBO Rep.* 11, 784–790.

Lu, F., Stedman, W., Yousef, M., Renne, R., and Lieberman, P. M. (2010b). Epigenetic regulation of Kaposi's sarcoma-associated herpesvirus latency by virus-encoded microRNAs that target Rta and the cellular Rbl2-DNMT pathway. *J. Virol.* 84, 2697–2706.

Lu, F., Weidmer, A., Liu, C. G., Volinia, S., Croce, C. M., and Lieberman, P. M. (2008). Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J. Virol.* 82, 10436–10443.

Marshall, V., Martro, E., Labo, N., Ray, A., Wang, D., Mbisa, G., Bagni, R. K., Volfovsky, N., Casabona, J., and Whitby, D. (2010). Kaposi sarcoma (KS)-associated herpesvirus microRNA sequence analysis and KS risk in a European AIDS-KS case control study. *J. Infect. Dis.* 202, 1126–1135.

Marshall, V., Parks, T., Bagni, R., Wang, C. D., Samols, M. A., Hu, J., Wyvil, K. M., Aleman, K., Little, R. F., Yarchoan, R., Renne, R., and Whitby, D. (2007). Conservation of virally encoded microRNAs in Kaposi sarcoma – associated herpesvirus in primary effusion lymphoma cell lines and in patients with Kaposi sarcoma or multicentric Castleman disease. *J. Infect. Dis.* 195, 645–659.

McAllister, S. C., Hansen, S. G., Ruhl, R. A., Raggo, C. M., Defilippis, V. R., Greenspan, D., Fruh, K., and Moses, A. V. (2004). Kaposi sarcoma-associated herpesvirus (KSHV) induces heme oxygenase-1 expression and activity in KSHV-infected endothelial cells. *Blood* 103, 3465–3473.

Murphy, E., Vanicek, J., Robins, H., Shenk, T., and Levine, A. J. (2008). Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5453–5458.

Nachmani, D., Stern-Ginossar, N., Sarid, R., and Mandelboim, O. (2009). Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. *Cell Host Microbe* 5, 376–385.

O'Connell, R. M., Rao, D. S., Chaudhuri, A. A., Boldin, M. P., Taganov, K. D., Nicoll, J., Paquette, R. L., and Baltimore, D. (2008). Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J. Exp. Med.* 205, 585–594.

O'Hara, A. J., Chugh, P., Wang, L., Netto, E. M., Luz, E., Harrington, W. J., Dezube, B. J., Damania, B., and Dittmer, D. P. (2009). Pre-micro RNA signatures delineate stages of endothelial cell transformation in Kaposi sarcoma. *PLoS Pathog.* 5, e1000389. doi:10.1371/journal.ppat.1000389

Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., Hayward, D. C., Ball, E. E., Degnan, B., Muller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E., and Ruvkun, G. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature* 408, 86–89.

Petrova, T. V., Makinen, T., Makela, T. P., Saarela, J., Virtanen, I., Ferrell, R. E., Finegold, D. N., Kerjaschki, D., Yla-Herttuala, S., and Alitalo, K. (2002). Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J.* 21, 4593–4599.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., Van Dyk, L. F., Ho, C. K., Shuman, S., Chien, M., Russo, J. J., Ju, J., Randall, G., Lindenbach, B. D., Rice, C. M., Simon, V., Ho, D. D., Zavolan, M., and Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nat. Methods* 2, 269–276.

Pfeffer, S., Zavolan, M., Grasser, F. A., Chien, M., Russo, J. J., Ju, J., John, B., Enright, A. J., Marks, D., Sander, C., and Tuschl, T. (2004). Identification of virus-encoded microRNAs. *Science* 304, 734–736.

Qin, Z., Freitas, E., Sullivan, R., Mohan, S., Bacelieri, R., Branch, D., Romano, M., Kearney, P., Oates, J., Plaisance, K., Renne, R., Kaleeba, J., and Parsons, C. (2010a). Upregulation of xCT by KSHV-encoded microRNAs facilitates KSHV dissemination and persistence in an environment of oxidative stress. *PLoS Pathog.* 6, e1000742. doi:10.1371/journal.ppat.1000742

Qin, Z., Kearney, P., Plaisance, K., and Parsons, C. H. (2010b). Pivotal advance: Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded microRNA specifically induce IL-6 and IL-10 secretion by macrophages and monocytes. *J. Leukoc. Biol.* 87, 25–34.

Rai, D., Kim, S. W., Mckeller, M. R., Dahia, P. L., and Aguiar, R. C. (2010). Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3111–3116.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 403, 901–906.

Samols, M. A., Hu, J., Skalsky, R. L., and Renne, R. (2005). Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79, 9301–9305.

Samols, M. A., Skalsky, R. L., Maldonado, A. M., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Identification of cellular genes targeted by KSHV-encoded microRNAs. *PLoS Pathog.* 3, e65. doi:10.1371/journal.ppat.0030065

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. *Nature* 455, 58–63.

Shi, W., Hendrix, D., Levine, M., and Haley, B. (2009). A distinct class of small RNAs arises from pre-miRNA-proximal regions in a simple chordate. *Nat. Struct. Mol. Biol.* 16, 183–189.

Shin, C., Nam, J. W., Farh, K. K., Chiang, H. R., Shkumatava, A., and Bartel, D. P. (2010). Expanding the microRNA targeting code: functional sites with centered pairing. *Mol. Cell* 38, 789–802.

Skalsky, R. L., and Cullen, B. R. (2011). Reduced expression of brain-enriched microRNAs in glioblastomas permits targeted regulation of a cell death gene. *PLoS ONE* 6, e24248. doi:10.1371/journal.pone.0024248

Skalsky, R. L., Samols, M. A., Plaisance, K. B., Boss, I. W., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. *J. Virol.* 81, 12836–12845.

Stern-Ginossar, N., Elefant, N., Zimmermann, A., Wolf, D. G., Saleh, N., Biton, M., Horwitz, E., Prokocimer, Z., Prichard, M., Hahn, G., Goldman-Wohl, D., Greenfield, C., Yagel, S., Hengel, H., Altuvia, Y., Margalit, H., and Mandelboim, O. (2007). Host immune system gene targeting by a viral miRNA. *Science* 317, 376–381.

Suffert, G., Malterer, G., Hausser, J., Viiliainen, J., Fender, A., Contrant, M., Ivacevic, T., Benes, V., Gros, F., Voinnet, O., Zavolan, M., Ojala, P. M., Haas, J. G., and Pfeffer, S. (2011). Kaposi's sarcoma herpesvirus microRNAs target caspase 3 and regulate apoptosis. *PLoS Pathog.* 7, e1002405. doi:10.1371/journal.ppat.1002405

Umbach, J. L., and Cullen, B. R. (2010). In-depth analysis of Kaposi's sarcoma-associated herpesvirus microRNA expression provides insights into the mammalian microRNA-processing machinery. *J. Virol.* 84, 695–703.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M., and Cullen, B. R. (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* 454, 780–783.

Umbach, J. L., Strelow, L. I., Wong, S. W., and Cullen, B. R. (2010). Analysis of rhesus rhadinovirus microRNAs expressed in virus-induced tumors from infected rhesus macaques. *Virology* 405, 592–599.

Vieira, J., and O'Hearn, P. M. (2004). Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. *Virology* 325, 225–240.

Walz, N., Christalla, T., Tessmer, U., and Grundhoff, A. (2010). A global analysis of evolutionary conservation among known and predicted gammaherpesvirus microRNAs. *J. Virol.* 84, 716–728.

Wang, H. W., Trotter, M. W., Lagos, D., Bourboulia, D., Henderson, S., Makinen, T., Elliman, S., Flanagan, A. M., Alitalo, K., and Boshoff, C. (2004). Kaposi sarcoma herpesvirus-induced cellular reprogramming contributes to the lymphatic endothelial gene expression in Kaposi sarcoma. *Nat. Genet.* 36, 687–693.

Warnatz, H. J., Schmidt, D., Manke, T., Piccini, I., Sultan, M., Borodina, T., Balzereit, D., Wruck, W., Soldatov, A., Vingron, M., Lehrach, H., and Yaspo, M. L. (2011). The BTB and CNC homology 1 (BACH1) target

genes are involved in the oxidative stress response and in control of the cell cycle. *J. Biol. Chem.* 286, 23521–23532.

Wies, E., Mori, Y., Hahn, A., Kremmer, E., Sturzl, M., Fleckenstein, B., and Neipel, F. (2008). The viral interferon-regulatory factor-3 is required for the survival of KSHV-infected primary effusion lymphoma cells. *Blood* 111, 320–327.

Xu, Y., Rodriguez-Huete, A., and Pari, G. S. (2006). Evaluation of the lytic origins of replication of Kaposi's sarcoma-associated virus/human herpesvirus 8 in the context of the viral genome. *J. Virol.* 80, 9905–9909.

Yin, Q., Mcbride, J., Fewell, C., Lacey, M., Wang, X., Lin, Z., Cameron, J., and Flemington, E. K. (2008). MicroRNA-155 is an Epstein-Barr virus-induced gene that modulates Epstein-Barr virus-regulated gene expression pathways. *J. Virol.* 82, 5295–5306.

Yin, Q., Wang, X., Fewell, C., Cameron, J., Zhu, H., Baddoo, M., Lin, Z., and Flemington, E. K. (2010). MicroRNA miR-155 inhibits bone morphogenetic protein (BMP) signaling and BMP-mediated Epstein-Barr virus reactivation. *J. Virol.* 84, 6318–6327.

Zhao, Y., Xu, H., Yao, Y., Smith, L. P., Kgosana, L., Green, J., Petherbridge, L., Baigent, S. J., and Nair, V. (2011). Critical role of the virus-encoded microRNA-155 ortholog in the induction of Marek's disease lymphomas. *PLoS Pathog.* 7, e1001305. doi:10.1371/journal.ppat.1001305

Ziegelbauer, J. M., Sullivan, C. S., and Ganem, D. (2009). Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. *Nat. Genet.* 41, 130–134.

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Looking at Kaposi's sarcoma-associated herpesvirus–host interactions from a microRNA viewpoint

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Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8, belongs to the gamma herpesviruses and is the etiological agent of Kaposi's sarcoma, primary effusion lymphoma, and some types of multicentric Castleman's disease. *In vivo,* KSHV mainly infects B cells and endothelial cells. The interactions between KSHV and its host cells determine the outcome of viral infection and subsequent viral pathogenesis. MicroRNAs (miRNAs) are small, non-coding RNAs that are important in fine-tuning cellular signaling. During infection, KSHV modulates the expression profiles and/or functions of a number of host miRNAs, for example hsa-miR-132 and hsa-miR-146a. Meanwhile, KSHV itself encodes 12 pre-miRNAs, including miR-K12-11, which is the functional ortholog of the host miR-155. A number of cellular and viral targets of deregulated cellular miRNAs and viral miRNAs are found in KSHV-infected cells, which suggests that miRNAs may be important in mediating KSHV–host interactions. In this review, we summarize our current understanding of how KSHV modulates the expression and/or functions of host miRNAs; we review in detail the functions of miR-K12-11 as the ortholog of miR-155; and we examine the functions of viral miRNAs in KSHV life cycle control, immune evasion, and pathogenesis.

Keywords: KSHV, microRNA

INTRODUCTION

The human tumor virus Kaposi's sarcoma-associated herpesvirus (KSHV), also called human herpesvirus 8 (HHV8), is the etiological agent of Kaposi's sarcoma (KS), the most common malignance in AIDS patients, primary effusion lymphoma (PEL), and some types of multicentric Castleman's disease (MCD; Chang et al., 1994; Cesarman et al., 1995; Soulier et al., 1995). The outcomes of viral infection and viral pathogenesis are determined by interactions between KSHV and host cells. Like all other herpesviruses, KSHV has two life cycle phases: latency and lytic replication. Only a few viral genes, such as latency-associated nuclear antigen [LANA, open reading frame (ORF) 73], viral cyclin (vCyclin, ORF72), and viral FLIP (vFLIP, ORF71), are expressed during latent infection and drive host cells proliferation and prevent apoptosis. Lytic transcripts, such as ORFK1, viral interleukin-6 (vIL-6, ORFK2), and viral G protein-coupled receptor (vGPCR) contribute to initiating angiogenesis and inflammatory lesions in host cells, and ultimately KS (Mesri et al., 2010). Both latent and lytic genes contribute to KSHV pathogenesis. Although a large number of diverse microRNAs (miRNAs) with potential regulatory functions exist in cell types from several different species (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), their role in virus–host interactions was not investigated until the recent discovery of virus-encoded miRNAs (Pfeffer et al., 2004). Here we review our current understanding of the roles of both virally encoded and cellular miRNAs in KSHV virus–host interactions. Because of conserved similarities among the herpesvirus family, some of these examples might also apply to other viruses in this family.

THE BIOGENESIS OF HOST AND VIRAL miRNAs

MicroRNAs are approximately 22 nucleotide (nt)-long non-coding RNAs with regulatory functions that are expressed by all multicellular eukaryotes (Bartel, 2009). In the canonical pathway for miRNAs biogenesis, primary miRNAs (pri-miRNAs) that form an imperfect stem-loop with a hairpin bulge are first transcribed by RNA Pol II from miRNA genes. These pri-miRNAs are then processed by microprocessor which is composed of the RNase III enzyme Drosha and its co-factor DGCR8. The processed products called precursor miRNAs (pre-miRNAs) have a hairpin structure of ∼70 nts and are exported from the nucleus to the cytoplasm by a Ran GTPase called Exportin-5. In the cytoplasm, the stem-loops of pre-miRNAs are further cleaved by another RNase III, Dicer, and the double-stranded RNA (dsRNA)-binding protein TRBP leaving a ∼22-nt RNA duplex. Often, one strand of the duplex is preferentially incorporated into the RNA-induced silencing complex (RISC). The other strand, known as miRNA star (miRNA*) or the passenger strand, is often degraded. RISC loaded with mature miRNA is subsequently guided by the miRNA to pair with target transcripts at the 3′ untranslated region (3′UTR) and induces posttranscriptional silencing (Bartel, 2009). In addition to the canonical pathways for generating miRNAs, some non-canonical ways to produce miRNA also exist (see review Yang and Lai, 2011).

Since Epstein–Barr virus (EBV) was first shown to encode several miRNAs (Pfeffer et al., 2004), many other viruses, especially herpesviruses including KSHV, have also been demonstrated to encode their own miRNAs. Most known viral miRNA genes are expressed using the canonical pathways of miRNA biogenesis with

only two exceptions for γ-herpesvirus. In murine γ-herpesvirus type 68 (also known as murid herpesvirus 4), miRNA genes embedded in tRNA-like transcripts are transcribed by RNA Pol III instead of Pol II (Bogerd et al., 2010; Diebel et al., 2010). Another exception is from *Herpesvirus saimiri* (HVS)-encoded miRNAs. Three of the seven HVS-encoded Sm-class U RNAs (HSURs) give rise to six mature miRNAs derived from hairpin structures located immediately downstream of the 3′ end processing signals. The processing of these miRNAs is not dependent on the microprocessor complex. Instead, the Integrator complex is required to generate the 3′ end of HSURs and the pre-miRNA hairpins. Pre-miRNAs are subsequently exported to the cytoplasm by Exportin-5, and Dicer is required to generate mature viral miRNAs (Cazalla et al., 2011).

Kaposi's sarcoma-associated herpesvirus encodes 12 pre-miRNAs that produce 25 mature miRNAs (Summarized in **Table 1**) through the canonical pathway for miRNA biogenesis (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005; Grundhoff et al., 2006; Lin et al., 2010; Umbach and Cullen, 2010). Ten of these miRNA precursors are organized into clusters within the latent genes locus. The other two pre-miRNAs (miR-K12-10, miR-K12-12) are located within the 3′UTR of ORF K12. All these viral miRNAs share two common promoters with the viral latent transcripts (Pearce et al., 2005; Cai and Cullen, 2006). Interestingly, the primary sequences of both miR-K12-10 and miR-K12-12 can be cleaved by Drosha in *cis*, which results in reduced transcript *K12* and decreased K12 protein expression (Lin and Sullivan, 2011). KSHV-encoded miRNAs were initially discovered in viral latency (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005; Grundhoff et al., 2006), but are also detected in lytic replication (Lin et al., 2010; Umbach and Cullen, 2010). The expression levels of some viral miRNAs are even higher in the lytic phase compared to the latent phase (Lin et al., 2010; Umbach and Cullen, 2010). It will be interesting in the future to determine the reason for this differential expression pattern in the viral life cycle.

miRNAs AS A MEDIATOR IN THE VIRUS–HOST INTERACTION NETWORK

Since the discovery of miRNA, we have known that it modulates gene expression post-transcriptionally through targeting the 3′UTRs of target genes or through inducing degradation of target gene transcripts (Lee et al., 1993). The expression of miRNAs themselves is also strictly regulated through different mechanisms. The discovery of virus-encoded miRNAs in herpesviruses provides us with an opportunity to reevaluate the interactions between the virus and its host from a new perspective. Based on the profound regulatory effects of miRNAs, we propose a four-component model to depict viral–host interactions. In this model, viral genes, viral miRNAs, host genes, and host miRNAs are four mediators of cellular signaling that regulate each others' expression by various means (**Figure 1**). This complicated and elaborate regulatory network mediating virus–host interactions determines the outcome of virus infection. Since herpesviruses establish long-term latency in the cell, they have multiple strategies to dominate the signaling network to favor long-term infection. The importance of virally hijacked host miRNAs and deregulated viral miRNAs in herpesvirus infection and pathogenesis has turned out to be beyond our expectations. In the following section, we review the critical roles of both cellular and viral miRNAs in KSHV infection (Summarized in **Table 2**).

KSHV MODULATES THE EXPRESSION PROFILE AND/OR FUNCTION OF HOST miRNAs

To date, more than 1400 mature miRNAs have been identified in humans (Kozomara and Griffiths-Jones, 2011), forming a delicate miRNA system that is critical for fine-tuning the cellular signaling network. The expression profile of human miRNAs varies significantly among different cell types and can be modulated by many cellular events. Increasing evidence suggests that virus infection can influence the expression profile of host miRNAs, resulting from either defensive host signaling against virus infection or viral hijacking to favor virus infection. Therefore, host miRNAs are important in balancing virus–host interactions. Viral gene products regulate host miRNA expression through gene alteration, transcription regulation or processing, directly or indirectly. In some cases they even modulate the function of host miRNAs. When herpesviruses establish long-term latency in the host cells, virus-specific host miRNAs expression pattern, or miRNAs signature, is established. Thus, the host miRNA system is an important tool that is hijacked by the herpesvirus for viral latency maintenance and viral pathogenesis.

Kaposi's sarcoma-associated herpesvirus mainly infects human endothelial cells (ECs) and B cells *in vivo*. Therefore, knowing the effects of KSHV infection on the host miRNA expression profiles of these two cell types will be interesting. Using quantitative PCR (qPCR)-based arrays, O'Hara et al. (2008) identified 68 miRNAs specifically expressed in PEL cell lines rather than in KSHV non-related lymphoma cell lines and tonsil tissues. However, the question remains whether these PEL-specific miRNAs are regulated directly by KSHV gene products or are the consequence of long-term infection and oncogenesis. To answer this question, in a later study, O'Hara et al. profiled PEL cell lines, KSHV-infected ECs, uninfected ECs, and primary KS biopsies for host miRNAs expression using a qPCR-based array. After adjustment for multiple comparisons, they identified 18 KSHV-regulated miRNAs. Moreover, they demonstrated that the tumor suppressor miRNAs in the let-7 family and miR-220/221 are downregulated in KSHV-associated cancers, including PEL and KS (O'Hara et al., 2009).

To determine the host miRNA profile during early KSHV infection, Lagos et al. detected host miRNA expression at 6 and 72 h post-KSHV infection of lymphatic endothelial cells (LECs) using an miRNA microarray. They identified two groups of miRNAs induced during primary KSHV infection. The first group consisted of nine miRNAs that reached their peak expression at 6 h post-infection, including hsa-miR-146a, hsa-miR-31, and hsa-miR-132. Expression levels of five miRNAs from the second group, including hsa-miR-193a and hsa-let-7i, steadily increased over the 72 post-infection hours (Lagos et al., 2010).

Although the functions of human miRNAs have been extensively studied and KSHV clearly regulates host miRNA expression, the roles of host miRNAs in KSHV infection are less well understood than the functions of the viral miRNAs. Based on a limited

Table 1 | List of KSHV-encoded miRNAs

Name	Sequence	Genome position[¶]
KSHV-miR-K12-1-5p	AUUACAGGAAACUGGGUGUAAG	122138–122159
	AUUACAGGAAACUGGGUGUAAGC	
	AUUACAGGAAACUGGGUGUAAGCU	
KSHV-miR-K12-1-3p	GCAGCACCUGUUUCCUGCAACC	122100–122121
KSHV-miR-K12-2-5p	AACUGUAGUCCGGGUCGAUCU	121978–121998
	AACUGUAGUCCGGGUCGAUCUG	
KSHV-miR-K12-2-3p	GAUCUUCCAGGGCUAGAGCUG	121935–121955
	GAUCUUCCAGGGCUAGAGCUGC	
KSHV-miR-K12-3-5p	UCACAUUCUGAGGACGGCAGC	121836–121856
	UCACAUUCUGAGGACGGCAGCG	
	UCACAUUCUGAGGACGGCAGCGA	
KSHV-miR-K12-3-3p	UCGCGGUCACAGAAUGUG	121797–121814
	GUCGCGGUCACAGAAUGU	
	UCGCGGUCACAGAAUGUGACA	
KSHV-miR-K12-4-5p	AGCUAAACCGCAGUACUCUAG	121704–121724
	AGCUAAACCGCAGUACUCUAGG	
KSHV-miR-K12-4-3p	UAGAAUACUGAGGCCUAGCUG	121666–121686
	UAGAAUACUGAGGCCUAGCUGA	
KSHV-miR-K12-5-5p	AGGUAGUCCCUGGUGCCCUAA	121554–121574
	AGGUAGUCCCUGGUGCCCUAAG	
	UAGGUAGUCCCUGGUGCCCUAA	
	UAGGUAGUCCCUGGUGCCCUAAG	
KSHV-miR-K12-5-3p	UAGGAUGCCUGGAACUUGCC	121516–121535
	UAGGAUGCCUGGAACUUGCCGG	
	UAGGAUGCCUGGAACUUGCCGGU	
KSHV-miR-K12-6-5p	CCAGCAGCACCUAAUCCAUCG	121045–121065
	CCAGCAGCACCUAAUCCAUCGG	
KSHV-miR-K12-6-3p	UGAUGGUUUUCGGGCUGUUGAG	121012–121033
	UGAUGGUUUUCGGGCUGUUGAGC	
KSHV-miR-K12-7-5p	AGCGCCACCGGACGGGGAUUUA	120646–120667
	AGCGCCACCGGACGGGGAUUUAU	
	AGCGCCACCGGACGGGGAUUUAUG	
KSHV-miR-K12-7-3p	UGAUCCCAUGUUGCUGGCGC	120608–120627
	UGAUCCCAUGUUGCUGGCGCU	
	UGAUCCCAUGUUGCUGGCGCUC	
KSHV-miR-K12-8-5p	ACUCCCUCACUAACGCCCCGC	120234–120254
	ACUCCCUCACUAACGCCCCGCU	
KSHV-miR-K12-8-3p	CUAGGCGCGACUGAGAGAG	120196–120214
	CUAGGCGCGACUGAGAGAGC	
	CUAGGCGCGACUGAGAGAGCA	
	CUAGGCGCGACUGAGAGAGCAC	
KSHV-miR-K12-9-5p	ACCCAGCUGCGUAAACCCCGC	119587–119607
	ACCCAGCUGCGUAAACCCCGCU	
KSHV-miR-K12-9-3p	CUGGGUAUACGCAGCUGCGUA	119553–119573
	CUGGGUAUACGCAGCUGCGUAA	
KSHV-miR-K12-10a-5p	GGCUUGGGGCGAUACCACCACU	118116–118137
KSHV-miR-K12-10a-3p	UAGUGUUGUCCCCCCGAGUGG	118077–118097
	UAGUGUUGUCCCCCCGAGUGGC	
	UUAGUGUUGUCCCCCCGAGUGG	
	UUAGUGUUGUCCCCCCGAGUGGC	
KSHV-miR-K12-10b-3p	UGGUGUUGUCCCCCCGAGUGGC	118077–118097
	UUGGUGUUGUCCCCCCGAGUGG	

(Continued)

Table 1 | Continued

Name	Sequence	Genome position¶
	UUGGUGUUGUCCCCCCGAGUGG	
	UUGGUGUUGUCCCCCCGAGUGGC	
KSHV-miR-K12-11-5p	GGUCACAGCUUAAACAUUUC	120868–120887
	GGUCACAGCUUAAACAUUUCUA	
	GGUCACAGCUUAAACAUUUCUAG	
	GGUCACAGCUUAAACAUUUCUAGG	
KSHV-miR-K12-11-3p	UUAAUGCUUAGCCUGUGUCCGA	120827–120848
KSHV-miR-K12-12-5p	AACCAGGCCACCAUUCCUCUC	117837–117857
	AACCAGGCCACCAUUCCUCUCC	
	AACCAGGCCACCAUUCCUCUCCG	
	UCAACCAGGCCACCAUUCCUC	
KSHV-miR-K12-12-3p	UGGGGGAGGGUGCCCUGGUUGA	117798–117819

Most abundant forms of KSHV mature miRNAs were listed here.

¶All positions indicated here were the positions of the sequences listed in the first lines and were referenced in KSHV genome (accession number: AF148805).

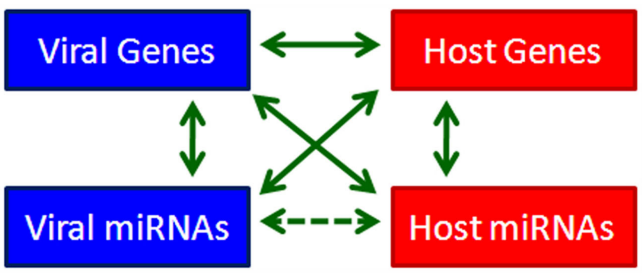

FIGURE 1 | A four-component model for viral–host interaction.

number of studies, we infer that KSHV hijacks the host miRNA system to favor infection and pathogenesis.

Lagos et al. showed that KSHV-induced hsa-miR-132 expression via a CREB-dependent pathway. The miRNA hsa-miR-132 negatively regulates interferon pathways by targeting the p300 transcriptional co-activator to facilitate viral replication. Interestingly, they show a similar function for hsa-miR-132 during infection of monocytes with herpes simplex virus-1 (HSV-1) and human cytomegalovirus (HCMV). Therefore, induction of hsa-miR-132 might be a common strategy for herpesvirus to control innate immunity at early stage of infection (Lagos et al., 2010). However, more extensive studies are needed to clarify the common signaling pathway that is responsible for CREB-mediated hsa-miR-132 activation.

Another host miRNA upregulated by KSHV is hsa-miR-146a (Lagos et al., 2010; Punj et al., 2010). Punj et al. showed that vFLIP is responsible for hsa-miR-146a upregulation. They identified two NF-κB sites in the promoter of hsa-miR-146a that are essential for its activation by vFLIP. Upregulation of hsa-miR-146a suppresses its target CXCR4; downregulation of CXCR4 might contribute to KS development by promoting premature release of KSHV-infected endothelial progenitors into circulation (Punj et al., 2010). Indeed, hsa-miR-146a is also reported to be upregulated by EBV LMP1, and hsa-miR-146a might function in a negative feedback loop to modulate the intensity and/or duration of the interferon response (Cameron et al., 2008). Therefore, hsa-miR-146a might be another common host miRNA target of herpesvirus infection.

Tsai et al. showed that K15M (the minor form of KSHV K15) induces expression of hsa-miR-21 and hsa-miR-31. Knocking down both of these miRNAs eliminates K15M-induced cell motility. Therefore, K15M might contribute to KSHV-mediated tumor metastasis and angiogenesis via regulation of hsa-miR-21 and hsa-miR-31 (Tsai et al., 2009). In another study, Wu et al. explored the genes and miRNAs involved in KSHV-induced cell motility by combining gene and miRNA profile data. They showed that KSHV induces global changes of miRNA expression in LECs. Specifically, the hsa-miR-221/hsa-miR-222 cluster is downregulated, whereas hsa-miR-31 is upregulated. Both LANA and Kaposin B repress the expression of the hsa-miR-221/hsa-miR-222 cluster, which results in upregulation of their target gene ETS1 or ETS2 and is sufficient to induce EC migration. In contrast, upregulated hsa-miR-31 stimulates EC migration by reduction its target gene FAT4 (Wu et al., 2011).

Kaposi's sarcoma-associated herpesvirus not only modulates host miRNAs expression, but also modulates the function of some host miRNAs. Kang et al. showed direct repression of vIL-6 by hsa-miR-1293 and repression of hIL-6 by hsa-miR-608. They also found that KSHV ORF57 and hsa-miR-1293/hsa-miR-608 compete for the same binding site on vIL-6/hIL-6 mRNA. ORF57 binding results in escape of viral and human IL-6 from miRNA-mediated suppression, contributing to KSHV pathogenesis (Kang et al., 2010, 2011).

Cazalla et al. (2010) showed that the HVS-encoded non-coding RNA, HSUR1, directs host miR-27 degradation and subsequent reduction of miR-27 target genes. This evidence also supports the hypothesis that viral genes can regulate host miRNA expression and function.

The function of host miRNAs in KSHV infection is increasingly a topic of interest in the field. More KSHV-regulated host miRNAs are being identified, and their function is usually clearer than that of viral miRNAs. We can therefore apply knowledge about other host miRNAs to the KSHV field to help understand the functions

Table 2 | Kaposi's sarcoma-associated herpesvirus-encoded miRNAs as mediators in virus–host interactions.

Category	miRNA	Gene	Function	Reference
Viral miRNAs target viral genes	KSHV-miR-K12-9-5p, KSHV-miR-K12-7-5p, KSHV-miR-K12-5	*RTA*	Repress RTA to maintain latency	Bellare and Ganem (2009), Lu et al. (2010b), Lin et al. (2011)
Viral miRNAs target cellular genes	KSHV-miR-K12-1	*IκB*	Inhibit RTA through NF-κB signaling to maintain latency	Lei et al. (2010)
		p21	Repress growth arrest	Gottwein and Cullen (2010)
		MAF	Facilitate cell trans-differentiation	Hansen et al. (2010)
	KSHV-miR-K12-3	*NFIB*	Suppress RTA through NFIB to maintain latency	Lu et al. (2010a)
		CEBPB	Induce IL-6 and IL-10 expression via inhibiting CEBPB	Qin et al. (2009)
	KSHV-miR-K12-4-5p	*RBL2*	Alter epigenetics by derepressing RBL2-DNMT pathway	Lu et al. (2010b)
	KSHV-miR-K12-6-5p	*MAF*	Facilitate cell trans-differentiation	Hansen et al. (2010)
	KSHV-miR-K12-7	*MICB*	Suppress cell-mediated immunity	Nachmani et al. (2009)
		CEBPB	Induce IL-6 and IL-10 expression via inhibiting CEBPB	Qin et al. (2009)
	KSHV-miR-K12-10a	*TWEAKR*	Anti-apoptosis/inflammation	Abend et al. (2010)
	KSHV-miR-K12-11	*IKKε*	Repress IFN response	Liang et al. (2011)
		SMAD5	Promote cell survival through repressing SMAD5 mediated TGF-β signaling	Liu et al. (2011)
		MAF	Facilitate cell trans-differentiation	Hansen et al. (2010)
		BACH1		Gottwein et al. (2007), Skalsky et al. (2007)
	KSHV-miR-cluster	*THBS1*	Angiogenesis	Samols et al. (2007)
		BCLF1	Inhibit caspase and promote viral replication	Ziegelbauer et al. (2009)
Cellular miRNAs target viral genes	hsa-miR-1293	*vIL-6*	Restrict viral pathogenesis	Kang et al. (2011)
Cellular miRNAs target cellular genes	hsa-miR-608	*hIL-6*	Restrict viral pathogenesis	Kang et al. (2011)
	hsa-miR-132	*p300*	Immune evasion	Lagos et al. (2010)
Viral genes target viral miRNAs	KSHV-miR-K12-9-5p	*PAN*		Predicted
Viral genes target cellular miRNAs	hsa-miR-146a	*vFLIP*	Repress CXCR4	Cazalla et al. (2010), Punj et al. (2010)
Cellular genes target viral miRNAs	KSHV-miR-K12-10, KSHV-miR-K12-12	*Drosha*	Regulate miRNAs expression and facilitate cell survival via promoting kaposin B expression	Lin and Sullivan (2011)
Cellular genes target cellular miRNAs	hsa-miR-132	*CREB/p300*	Induce hsa-miR-132 expression during early viral infection	Lagos et al. (2010)

of KSHV-hijacked host miRNAs in KSHV infection and related pathogenesis.

KSHV ENCODES ORTHOLOGS OF HOST miRNAs

Many KSHV-encoded ORFs are pirated from the host genome. An interesting question is whether KSHV also pirates pre-miRNA genes from the host genome. Early in the identification of KSHV miRNAs, two independent studies reported that miR-K12-11 is the ortholog of host hsa-miR-155 and these miRNAs have identical seed sequence (Gottwein et al., 2007; Skalsky et al., 2007). Hsa-miR-155 is a multifunctional miRNA that is important in immunity, hematopoiesis, inflammation, and oncogenesis (McClure and Sullivan, 2008). Not only miR-K12-11, but also miR-M4, which is encoded by the highly oncogenic Marek's disease virus of chickens, is a functional ortholog of hsa-miR-155(Zhao et al., 2009). EBV does not encode a hsa-miR-155 ortholog. Instead, EBV-induced hsa-miR-155 expression by LMP1 and hsa-miR-155 is important in EBV viral pathogenesis (Motsch et al., 2007; Gatto et al., 2008; Lu et al., 2008). Of note, hsa-miR-155 is downregulated in KSHV-infected cells (Gottwein et al., 2007; Skalsky et al., 2007); therefore KSHV might encode miR-K12-11 to replace the function of its ortholog, hsa-miR-155.

Since miR-K12-11 shares an identical seed sequence with hsa-miR-155, these two miRNAs may have similar functions and target

the same genes. Gottwein et al. (2007) used a range of assays to show that expression of physiological levels of miR-K12-11 or hsa-miR-155 results in the downregulation of an extensive set of common mRNA targets, including genes with known roles in cell growth regulation, for example BACH1. Moreover, Qin et al. (2010) found that KSHV-encoded microRNAs upregulate xCT expression in macrophages and ECs, largely through miR-K12-11 suppression of BACH1, a negative regulator of transcription that recognizes antioxidant response elements within gene promoters.

To compare miR-K12-11 and hsa-miR-155 functions *in vivo*, Boss et al. used a foamy virus vector to express the miRNAs in human hematopoietic progenitors and performed immune reconstitutions in NOD/LtSz-scid IL2Rγ (null) mice. They found that ectopic expression of miR-K12-11 or hsa-miR-155 targeting C/EBPβ, leads to a significant expansion of the CD19 (+) B-cell population in the spleen. This *in vivo* study validates miR-K12-11 as a functional ortholog of hsa-miR-155 in the context of hematopoiesis (Boss et al., 2011).

Our study indicated that miR-K12-11 is involved in attenuating interferon signaling and contributing to KSHV latency maintenance through targeting I-kappa-B kinase epsilon (IKKε). We demonstrated that miR-K12-11 attenuated IFN signaling by decreasing IKKε-mediated IRF3/IRF7 phosphorylation. We also demonstrated that IKKε enhances KSHV reactivation synergistically with 12-*O*-tetradecanoylphorbol 13-acetate treatment. Moreover, inhibition of miR-K12-11 enhances KSHV reactivation induced by vesicular stomatitis virus infection. Taken together, our findings suggest that miR-K12-11 can contribute to maintenance of KSHV latency by targeting IKKε (Liang et al., 2011).

More recently, we demonstrated that ectopic expression of miR-K12-11 downregulates TGF-β signaling and facilitates cell proliferation upon TGF-β treatment by directly targeting SMAD5. Our findings highlight a novel mechanism in which miR-K12-11 downregulates TGF-β signaling, and suggests that viral miRNAs and proteins may exert a dichotomous regulation in virus-induced oncogenesis by targeting the same signaling pathway (Liu et al., 2011).

Kaposi's sarcoma-associated herpesvirus exploits miR-K12-11 to tap into a host miRNA regulatory network (McClure and Sullivan, 2008). Based on the important functions of hsa-miR-155, we believe that miR-K12-11 is of particular importance in KSHV infection and pathogenesis.

Using Target Scan5.0 (Lewis et al., 2005), we found that miR-K12-2 shares the same seed sequence as miR-1183 (unpublished data). The function of miR-1183 is poorly understood. Further study is needed to confirm whether miR-K12-2 is the true ortholog of miR-1183, and to determine the function of these two miRNAs in KSHV infection.

FUNCTIONS OF VIRAL miRNA IN KSHV INFECTION

IDENTIFYING TARGETS OF KSHV-ENCODED miRNA

Studying the function of a particular miRNA can begin by simply predicting targets using software such as TargetScan, PicTar, or DIANA MicroT (Krek et al., 2005; Lewis et al., 2005; Maragkakis et al., 2009). These programs are based on the qualitative and quantitative properties and the thermodynamic nature of the miRNA/target heteroduplex (Lieber and Haas, 2011). However, most of these algorithms are not optimized for viral miRNAs and can yield many false-positive viral miRNA targets.

Laganà and colleagues developed the program miRiam, modified for prediction of viral miRNA targets. miRiam uses both thermodynamic features and empirical constraints to predict interactions between viral miRNAs and human targets. It exploits target mRNA secondary structure accessibility and interaction rules, inferred from validated miRNA/mRNA pairs. A set of target genes for EBV and KSHV miRNAs that are involved in apoptosis and cell-cycle regulation was identified using miRiam (Lagana et al., 2010).

In addition to bioinformatic methods, high throughput gene profile technologies such as cDNA microarrays can be used to identify viral miRNA targets. In an early study, Samols et al. (2007) performed gene expression profiling in cells stably expressing KSHV-encoded miRNAs, and identified a set of 81 genes whose expression was significantly changed in the presence of miRNAs. To increase the accuracy of this method, Ziegelbauer et al. developed a tandem array-based expression screening method to identify KSHV miRNA targets. Their approach is based on multiple screens that examine small changes in transcript abundance under different conditions of miRNA expression or inhibition, followed by searching the identified transcripts for seed sequence matches. They identified Bcl2-associated factor *BCLAF1* as a target for multiple KSHV miRNAs (Ziegelbauer et al., 2009). In addition to cDNA microarrays, protein profiling technologies such as stable isotope-labeling by amino acids, or SILAC, in cell culture are also being used to identify miRNA targets (Vinther et al., 2006).

A more direct identification of miRNA targets can be achieved by immunoprecipitation (IP) of Argonaute protein-containing complexes followed by microarray analysis of the associated mRNAs, in a method called RIP-chip (Keene et al., 2006; Beitzinger et al., 2007). Dolken et al. (2010) used Ago2-based RIP-chip to identify transcripts targeted by KSHV miRNAs ($n = 114$), EBV miRNAs ($n = 44$), and cellular miRNAs ($n = 2337$) in six latently infected or stably transduced human B-cell lines. Now, high throughput sequencing of RNA isolated by cross-linking IP of Argonaute-containing complexes, or Ago-HITS-CLIP; (Chi et al., 2009) and photoactivatable, ribonucleoside-enhanced *c*rosslinking and IP, or PAR-CLIP (Hafner et al., 2010), have been developed to identify targets for host and viral miRNA more precisely. A summary of methods to identify miRNA targets is in **Figure 2**.

KSHV miRNAs AND VIRAL LIFE CYCLE CONTROL

The idea that viral miRNA is involved in viral latency regulation first came from the finding that SV40-encoded miR-S1 targets the viral large T-antigen (Sullivan et al., 2005). This results in reduced expression of viral T-antigen but not infectious virus relative to that generated from a miRNA deletion mutant (Sullivan et al., 2005). In HSV-1, studies showed that two miRNAs encoded by HSV-1, miR-H2-3p, and miR-H6, facilitate the establishment and maintenance of viral latency by targeting viral immediate early transactivators *ICP0* and *ICP4.5*, respectively (Umbach et al., 2008). Similar results in HSV-2, found that miR-β contributes to viral latency control through silencing *ICP0* expression (Tang et al.,

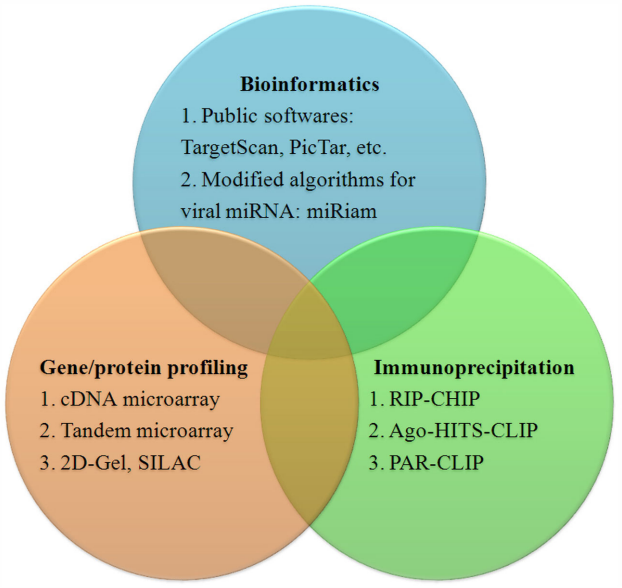

FIGURE 2 | Methods to identify miRNA targets.

2009). Studies in HCMV showed that virus-encoded miR-UL112-1 controls viral latency by inhibiting the viral immediate early gene 72 (IE72; Murphy et al., 2008). The authors predicted that other herpesviruses might use a similar strategy to control viral latency.

Studies from KSHV-encoded miRNAs confirmed this hypothesis. Several miRNAs have been found to affect the expression level of the viral immediate early gene replication and transcription activator (RTA), either directly (Bellare and Ganem, 2009; Lu et al., 2010b; Lin et al., 2011) or indirectly (Lei et al., 2010; Lu et al., 2010a). Bellare et al. using miRNA mimics or specific inhibitors for KSHV-encoded miRNAs and reporter constructs containing the RTA 3′UTR, found that miR-K12-9-5p targets RTA directly, and depends on the canonical 6-mer seed match site. When this miRNA was inhibited by a specific antagomir, a moderate increase in lytic replication was observed (Bellare and Ganem, 2009). A second study by Lu et al. (2010b) using constructs expressing KSHV-encoded miRNAs and a reporter containing the RTA 3′UTR demonstrated that miR-K12-5 represses RTA expression, although the RTA 3′UTR lacks a canonical miR-K12-5 seed sequence. In another study by us, a reporter containing the RTA 3′UTR and constructs expressing all 12 pre-miRNAs, miR-K12-9, and miR-K12-7-5p were found to target RTA directly. miR-K12-7-5p, targeting RTA, was shown to be mediated by a 7-mer seed match site. Additionally, endogenous RTA expression level was reduced by ectopically overexpressing miR-K12-7 and derepressed using an miR-K12-7-5p inhibitor. A decrease in viral particles was observed when miR-K12-7 was overexpressed (Lin et al., 2011). Lei et al. used an miR-cluster deletion mutant virus to determine that miR-K12-1 represses IκB, an inhibitor of NFκB. Inhibition of IκB leads to NFκB activation, which suppresses RTA to facilitate viral latency control (Lei et al., 2010). Lu et al. (2010a), using a lentivirus expressing individual KSHV miRNA, found that miR-K12-3 reduces RTA mRNA levels by targeting NFIB directly. Further studies showed a putative NFIB-binding site is located in the RTA promoter and shRNA knockdown of NFIB resulted in decreased RTA expression.

Taken together, the evidence suggests that multiple KSHV-encoded miRNAs are involved in viral latency maintenance and herpes viral latency control is important and a complex process. These data support the hypothesis that conservation among herpesviruses allows them to use viral miRNA(s) to target immediate early genes to control viral latency (Murphy et al., 2008).

KSHV miRNA AND IMMUNE REGULATION

To establish long-term latent infection in the host cell, KSHV has developed multiple strategies to evade the host innate and adaptive immunity (Areste and Blackbourn, 2009). Some studies suggest that KSHV-encoded miRNAs are involved in immune regulation and favor KSHV infection. For example, several herpesviruses, including KSHV, encode an miRNA that targets the MHC class I-related chain B (MICB), which is a stress-induced ligand recognized by the NKG2D receptor expressed by NK cells and CD8+ T-cells. In 2007, Stern-Ginossar et al. identified that HCMV miRNA miR-UL112-1 targets MICB. They showed that HCMV-miR-UL112 specifically downregulates MICB expression during viral infection, leading to decreased binding of NKG2D and reduced killing by NK cells (Stern-Ginossar et al., 2007). They further showed that both EBV miR-BART2-5p and KSHV-miR-K12-7 regulate MICB expression (Nachmani et al., 2009). Moreover, Thomas et al. showed that the KSHV immune evasion gene, K5, reduces cell-surface expression of the NKG2D ligands MHC class I-related chain A (MICA) and MICB, probably by K5-mediated ubiquitylation, which signals internalization and causes a potent reduction in NK cell-mediated cytotoxicity. These studies suggested that NKG2D ligands are common targets for both KSHV miRNA and ORF for evading NK cell antiviral function (Thomas et al., 2008). Another example is that miR-K12-11 directly targets IKKε, an important modulator of IFN signaling.

Kaposi's sarcoma-associated herpesvirus miRNAs also regulate the host immune response by modulating expression of cytokines. Abend et al. demonstrated that miR-K12-10a robustly downregulates the expression of tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) receptor (TWEAKR). The downregulation of TWEAKR by miR-K10a in primary human ECs results in a decrease in expression of the proinflammatory cytokines IL-8 and monocyte chemo attractant protein 1 in response to TWEAK. This protects cells from apoptosis and suppresses a proinflammatory response (Abend et al., 2010). Qin et al. (2009) demonstrated that miR-K12-3 and miR-K12-7 target C/EBPβ p20 (LIP), a negative transcriptional regulator of IL-6 and IL-10, and induces expression of IL-6 and IL-10 in macrophages. These cytokines have broad functions in oncogenesis and immune suppression. The function of miR-K12-3 and miR-K12-7 in immune regulation needs to be further studied (Boss and Renne, 2011).

KSHV miRNAs AND VIRAL PATHOGENESIS

In addition to viral life cycle control and viral immune evasion, KSHV-encoded miRNAs have other functions directly related to pathogenesis.

Samols et al. (2007) identified THBS1 as a target of several KSHV miRNAs. THBS1 is a strong tumor suppressor and anti-angiogenic factor. They proposed that KSHV-encoded miRNAs contribute directly to pathogenesis by downregulation of THBS1, promoting cell adhesion, migration, and angiogenesis. Gottwein et al. reported that miR-K1 directly targets the cellular cyclin-dependent kinase inhibitor p21, strongly attenuating the cell-cycle arrest that normally occurs upon p53 activation. They suggested that this KSHV miRNA likely contributes to the oncogenic potential of KSHV (Gottwein and Cullen, 2010). Hansen et al. demonstrated that multiple KSHV miRNAs contribute to virally induced reprogramming by silencing the cellular transcription factor MAF, which prevents expression of blood vascular endothelial cells (BECs) specific genes, thereby maintaining the differentiation status of LECs. These findings demonstrate that KSHV miRNAs could influence the differentiation status of infected cells, contributing to KSHV-induced oncogenesis (Hansen et al., 2010).

Using developing systems biology methods, the target list of KSHV miRNAs is rapidly increasing (Lieber and Haas, 2011). The functions of KSHV miRNAs include, but are not limited to, viral life cycle control, immune regulation, and pathogenesis. KSHV miRNAs are important mediators of viral–host interactions. Using viral miRNA knockout viruses will allow studies that lead to an overall understanding of the functions of unique KSHV miRNAs.

CONCLUSION

MicroRNAs are small, regulatory, non-coding RNAs with diverse functions in fine-tuning cellular signaling. KSHV modulates host miRNA expression and also encodes 25 mature viral miRNAs. Increasing evidence suggests that both virally hijacked host miRNAs and dysregulated viral miRNAs are important in KSHV life cycle control, immune evasion, and pathogenesis. In this review, we propose a four-component model for viral–host interactions (**Figure 1**). Existing evidence supports this model (**Table 2**), with examples for almost every action mode in this model of virus–host interactions. Using RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), we predict, according to our model, that KSHV-encoded polyadenylated nuclear RNA (PAN, also known as nut-1) functions as an miRNA sponge to inhibit KSHV-encoded miR-K12-9-5p. Based on the profound effects of miRNAs as mediators in virus–host interactions, we believe they will become emerging therapy targets for treating KSHV infection and KSHV-related malignancies.

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REFERENCES

Abend, J. R., Uldrick, T., and Ziegelbauer, J. M. (2010). Regulation of tumor necrosis factor-like weak inducer of apoptosis receptor protein (TWEAKR) expression by Kaposi's sarcoma-associated herpesvirus microRNA prevents TWEAK-induced apoptosis and inflammatory cytokine expression. *J. Virol.* 84, 12139–12151.

Areste, C., and Blackbourn, D. J. (2009). Modulation of the immune system by Kaposi's sarcoma-associated herpesvirus. *Trends Microbiol.* 17, 119–129.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233.

Beitzinger, M., Peters, L., Zhu, J. Y., Kremmer, E., and Meister, G. (2007). Identification of human microRNA targets from isolated argonaute protein complexes. *RNA Biol.* 4, 76–84.

Bellare, P., and Ganem, D. (2009). Regulation of KSHV lytic switch protein expression by a virus-encoded microRNA: an evolutionary adaptation that fine-tunes lytic reactivation. *Cell Host Microbe* 6, 570–575.

Bogerd, H. P., Karnowski, H. W., Cai, X., Shin, J., Pohlers, M., and Cullen, B. R. (2010). A mammalian herpesvirus uses noncanonical expression and processing mechanisms to generate viral microRNAs. *Mol. Cell* 37, 135–142.

Boss, I. W., Nadeau, P. E., Abbott, J. R., Yang, Y., Mergia, A., and Renne, R. (2011). A Kaposi's sarcoma-associated herpesvirus-encoded ortholog of microRNA miR-155 induces human splenic B-cell expansion in NOD/LtSz-scid IL2Rgammanull mice. *J. Virol.* 85, 9877–9886.

Boss, I. W., and Renne, R. (2011). Viral miRNAs and immune evasion. *Biochim. Biophys. Acta.* 1809, 623–630.

Cai, X., and Cullen, B. R. (2006). Transcriptional origin of Kaposi's sarcoma-associated herpesvirus microRNAs. *J. Virol.* 80, 2234–2242.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B., and Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5570–5575.

Cameron, J. E., Yin, Q., Fewell, C., Lacey, M., Mcbride, J., Wang, X., Lin, Z., Schaefer, B. C., and Flemington, E. K. (2008). Epstein-Barr virus latent membrane protein 1 induces cellular MicroRNA miR-146a, a modulator of lymphocyte signaling pathways. *J. Virol.* 82, 1946–1958.

Cazalla, D., Xie, M., and Steitz, J. A. (2011). A primate herpesvirus uses the integrator complex to generate viral microRNAs. *Mol. Cell* 43, 982–992.

Cazalla, D., Yario, T., and Steitz, J. A. (2010). Down-regulation of a host microRNA by a herpesvirus saimiri noncoding RNA. *Science* 328, 1563–1566.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culpepper, J., Knowles, D. M., and Moore, P. S. (1994). Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266, 1865–1869.

Chi, S. W., Zang, J. B., Mele, A., and Darnell, R. B. (2009). Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature* 460, 479–486.

Diebel, K. W., Smith, A. L., and Van Dyk, L. F. (2010). Mature and functional viral miRNAs transcribed from novel RNA polymerase III promoters. *RNA* 16, 170–185.

Dolken, L., Malterer, G., Erhard, F., Kothe, S., Friedel, C. C., Suffert, G., Marcinowski, L., Motsch, N., Barth, S., Beitzinger, M., Lieber, D., Bailer, S. M., Hoffmann, R., Ruzsics, Z., Kremmer, E., Pfeffer, S., Zimmer, R., Koszinowski, U. H., Grasser, F., Meister, G., and Haas, J. (2010). Systematic analysis of viral and cellular microRNA targets in cells latently infected with human gamma-herpesviruses by RISC immunoprecipitation assay. *Cell Host Microbe* 7, 324–334.

Gatto, G., Rossi, A., Rossi, D., Kroening, S., Bonatti, S., and Mallardo, M. (2008). Epstein-Barr virus latent membrane protein 1 trans-activates miR-155 transcription through the NF-kappaB pathway. *Nucleic Acids Res.* 36, 6608–6619.

Gottwein, E., and Cullen, B. R. (2010). A human herpesvirus microRNA inhibits p21 expression and attenuates p21-mediated cell cycle arrest. *J. Virol.* 84, 5229–5237.

Gottwein, E., Mukherjee, N., Sachse, C., Frenzel, C., Majoros, W. H., Chi, J. T., Braich, R., Manoharan, M., Soutschek, J., Ohler, U., and Cullen, B. R. (2007). A viral microRNA functions as an orthologue of cellular miR-155. *Nature* 450, 1096–1099.

Grundhoff, A., Sullivan, C. S., and Ganem, D. (2006). A combined computational and microarray-based approach identifies novel microRNAs encoded by human gamma-herpesviruses. *RNA* 12, 733–750.

Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., Rothballer, A., Ascano, M. Jr., Jungkamp, A. C., Munschauer, M., Ulrich, A., Wardle, G. S., Dewell, S., Zavolan, M., and Tuschl, T. (2010). Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell* 141, 129–141.

Hansen, A., Henderson, S., Lagos, D., Nikitenko, L., Coulter, E., Roberts, S., Gratrix, F., Plaisance, K., Renne, R., Bower, M., Kellam, P., and Boshoff, C. (2010). KSHV-encoded miRNAs target MAF to induce endothelial cell reprogramming. *Genes Dev.* 24, 195–205.

Kang, J. G., Majerciak, V., Uldrick, T. S., Wang, X., Kruhlak, M., Yarchoan, R., and Zheng, Z. M. (2011). Kaposi's sarcoma-associated herpesviral IL-6 and human IL-6 open reading frames contain miRNA binding sites and are subject to cellular miRNA regulation. *J. Pathol.* 225, 378–389.

Kang, J. G., Pripuzova, N., Majerciak, V., Kruhlak, M., Le, S. Y., and Zheng, Z. M. (2010). Kaposi's sarcoma-associated herpesvirus ORF57 promotes escape of viral and human interleukin-6 from microRNA-mediated suppression. *J. Virol.* 85, 2620–2630.

Keene, J. D., Komisarow, J. M., and Friedersdorf, M. B. (2006). RIP-Chip: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts. *Nat. Protoc.* 1, 302–307.

Kozomara, A., and Griffiths-Jones, S. (2011). miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39, D152–D157.

Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., Macmenamin, P., Da Piedade, I., Gunsalus, K. C., Stoffel, M., and Rajewsky, N. (2005). Combinatorial microRNA target predictions. *Nat. Genet.* 37, 495–500.

Lagana, A., Forte, S., Russo, F., Giugno, R., Pulvirenti, A., and Ferro, A. (2010). Prediction of human targets for viral-encoded microRNAs by thermodynamics and empirical constraints. *J. RNAi Gene Silencing* 6, 379–385.

Lagos, D., Pollara, G., Henderson, S., Gratrix, F., Fabani, M., Milne, R. S., Gotch, F., and Boshoff, C. (2010). miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. *Nat. Cell Biol.* 12, 513–519.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. *Science* 294, 853–858.

Lau, N. C., Lim, L. P., Weinstein, E. G., and Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* 294, 858–862.

Lee, R. C., and Ambros, V. (2001). An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* 294, 862–864.

Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75, 843–854.

Lei, X., Bai, Z., Ye, F., Xie, J., Kim, C. G., Huang, Y., and Gao, S. J. (2010). Regulation of NF-kappaB inhibitor IkappaBalpha and viral replication by a KSHV microRNA. *Nat. Cell Biol.* 12, 193–199.

Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120, 15–20.

Liang, D., Gao, Y., Lin, X., He, Z., Zhao, Q., Deng, Q., and Lan, K. (2011). A human herpesvirus miRNA attenuates interferon signaling and contributes to maintenance of viral latency by targeting IKKepsilon. *Cell Res.* 21, 793–806.

Lieber, D., and Haas, J. (2011). Viruses and microRNAs: a toolbox for systematic analysis. *Wiley Interdiscip. Rev. RNA* 2, 787–801.

Lin, X., Liang, D., He, Z., Deng, Q., Robertson, E. S., and Lan, K. (2011). miR-K12-7-5p encoded by Kaposi's sarcoma-associated herpesvirus stabilizes the latent state by targeting viral ORF50/RTA. *PLoS ONE* 6, e16224. doi:10.1371/journal.pone.0016224

Lin, Y. T., Kincaid, R. P., Arasappan, D., Dowd, S. E., Hunicke-Smith, S. P., and Sullivan, C. S. (2010). Small RNA profiling reveals antisense transcription throughout the KSHV genome and novel small RNAs. *RNA* 16, 1540–1558.

Lin, Y. T., and Sullivan, C. S. (2011). Expanding the role of Drosha to the regulation of viral gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 108, 11229–11234.

Liu, Y., Sun, R., Lin, X., Liang, D., Deng, Q., and Lan, K. (2011). KSHV-encoded miR-K12-11 attenuates transforming growth factor beta signaling through suppression of SMAD5. *J. Virol.* [Epub ahead of print].

Lu, C. C., Li, Z., Chu, C. Y., Feng, J., Sun, R., and Rana, T. M. (2010a). MicroRNAs encoded by Kaposi's sarcoma-associated herpesvirus regulate viral life cycle. *EMBO Rep.* 11, 784–790.

Lu, F., Stedman, W., Yousef, M., Renne, R., and Lieberman, P. M. (2010b). Epigenetic regulation of Kaposi's sarcoma-associated herpesvirus latency by virus-encoded microRNAs that target Rta and the cellular Rbl2-DNMT pathway. *J. Virol.* 84, 2697–2706.

Lu, F., Weidmer, A., Liu, C. G., Volinia, S., Croce, C. M., and Lieberman, P. M. (2008). Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J. Virol.* 82, 10436–10443.

Maragkakis, M., Reczko, M., Simossis, V. A., Alexiou, P., Papadopoulos, G. L., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, K., Vergoulis, T., Koziris, N., Sellis, T., Tsanakas, P., and Hatzigeorgiou, A. G. (2009). DIANA-microT web server: elucidating microRNA functions through target prediction. *Nucleic Acids Res.* 37, W273–W276.

McClure, L. V., and Sullivan, C. S. (2008). Kaposi's sarcoma herpes virus taps into a host microRNA regulatory network. *Cell Host Microbe* 3, 1–3.

Mesri, E. A., Cesarman, E., and Boshoff, C. (2010). Kaposi's sarcoma and its associated herpesvirus. *Nat. Rev. Cancer* 10, 707–719.

Motsch, N., Pfuhl, T., Mrazek, J., Barth, S., and Grasser, F. A. (2007). Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) induces the expression of the cellular microRNA miR-146a. *RNA Biol.* 4, 131–137.

Murphy, E., Vanicek, J., Robins, H., Shenk, T., and Levine, A. J. (2008). Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5453–5458.

Nachmani, D., Stern-Ginossar, N., Sarid, R., and Mandelboim, O. (2009). Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells. *Cell Host Microbe* 5, 376–385.

O'Hara, A. J., Vahrson, W., and Dittmer, D. P. (2008). Gene alteration and precursor and mature microRNA transcription changes contribute to the miRNA signature of primary effusion lymphoma. *Blood* 111, 2347–2353.

O'Hara, A. J., Wang, L., Dezube, B. J., Harrington, W. J. Jr., Damania, B., and Dittmer, D. P. (2009). Tumor suppressor micro RNAs are underrepresented in primary effusion lymphoma and Kaposi sarcoma. *Blood* 113, 5938–5941.

Pearce, M., Matsumura, S., and Wilson, A. C. (2005). Transcripts encoding K12, v-FLIP, v-cyclin, and the microRNA cluster of Kaposi's sarcoma-associated herpesvirus originate from a common promoter. *J. Virol.* 79, 14457–14464.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., Van Dyk, L. F., Ho, C. K., Shuman, S., Chien, M., Russo, J. J., Ju, J., Randall, G., Lindenbach, B. D., Rice, C. M., Simon, V., Ho, D. D., Zavolan, M., and Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nat. Methods* 2, 269–276.

Pfeffer, S., Zavolan, M., Grasser, F. A., Chien, M., Russo, J. J., Ju, J., John, B., Enright, A. J., Marks, D., Sander, C., and Tuschl, T. (2004). Identification of virus-encoded microRNAs. *Science* 304, 734–736.

Punj, V., Matta, H., Schamus, S., Tamewitz, A., Anyang, B., and Chaudhary, P. M. (2010). Kaposi's sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 suppresses CXCR4 expression by upregulating miR-146a. *Oncogene* 29, 1835–1844.

Qin, Z., Freitas, E., Sullivan, R., Mohan, S., Bacelieri, R., Branch, D., Romano, M., Kearney, P., Oates, J., Plaisance, K., Renne, R., Kaleeba, J., and Parsons, C. (2010). Upregulation of xCT by KSHV-encoded microRNAs facilitates KSHV dissemination and persistence in an environment of oxidative stress. *PLoS Pathog.* 6, e1000742. doi:10.1371/journal.ppat.1000742

Qin, Z., Kearney, P., Plaisance, K., and Parsons, C. H. (2009). Pivotal advance: Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded microRNA specifically induce IL-6 and IL-10 secretion by macrophages and monocytes. *J. Leukoc. Biol.* 87, 25–34.

Samols, M. A., Hu, J., Skalsky, R. L., and Renne, R. (2005). Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. *J. Virol.* 79, 9301–9305.

Samols, M. A., Skalsky, R. L., Maldonado, A. M., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Identification of cellular genes targeted by KSHV-encoded

microRNAs. *PLoS Pathog.* 3, e65. doi:10.1371/journal.ppat.0030065

Skalsky, R. L., Samols, M. A., Plaisance, K. B., Boss, I. W., Riva, A., Lopez, M. C., Baker, H. V., and Renne, R. (2007). Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155. *J. Virol.* 81, 12836–12845.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., D'agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and Sigaux, F. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86, 1276–1280.

Stern-Ginossar, N., Elefant, N., Zimmermann, A., Wolf, D. G., Saleh, N., Biton, M., Horwitz, E., Prokocimer, Z., Prichard, M., Hahn, G., Goldman-Wohl, D., Greenfield, C., Yagel, S., Hengel, H., Altuvia, Y., Margalit, H., and Mandelboim, O. (2007). Host immune system gene targeting by a viral miRNA. *Science* 317, 376–381.

Sullivan, C. S., Grundhoff, A. T., Tevethia, S., Pipas, J. M., and Ganem, D. (2005). SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature* 435, 682–686.

Tang, S., Patel, A., and Krause, P. R. (2009). Novel less-abundant viral microRNAs encoded by herpes simplex virus 2 latency-associated transcript and their roles in regulating ICP34.5 and ICP0 mRNAs. *J. Virol.* 83, 1433–1442.

Thomas, M., Boname, J. M., Field, S., Nejentsev, S., Salio, M., Cerundolo, V., Wills, M., and Lehner, P. J. (2008). Down-regulation of NKG2D and NKp80 ligands by Kaposi's sarcoma-associated herpesvirus K5 protects against NK cell cytotoxicity. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1656–1661.

Tsai, Y. H., Wu, M. F., Wu, Y. H., Chang, S. J., Lin, S. F., Sharp, T. V., and Wang, H. W. (2009). The M type K15 protein of Kaposi's sarcoma-associated herpesvirus regulates microRNA expression via its SH2-binding motif to induce cell migration and invasion. *J. Virol.* 83, 622–632.

Umbach, J. L., and Cullen, B. R. (2010). In-depth analysis of Kaposi's sarcoma-associated herpesvirus microRNA expression provides insights into the mammalian microRNA-processing machinery. *J. Virol.* 84, 695–703.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M., and Cullen, B. R. (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* 454, 780–783.

Vinther, J., Hedegaard, M. M., Gardner, P. P., Andersen, J. S., and Arctander, P. (2006). Identification of miRNA targets with stable isotope labeling by amino acids in cell culture. *Nucleic Acids Res.* 34, e107.

Wu, Y. H., Hu, T. F., Chen, Y. C., Tsai, Y. N., Tsai, Y. H., Cheng, C. C., and Wang, H. W. (2011). The manipulation of miRNA-gene regulatory networks by KSHV induces endothelial cell motility. *Blood* 118, 2896–2905.

Yang, J. S., and Lai, E. C. (2011). Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. *Mol. Cell* 43, 892–903.

Zhao, Y., Yao, Y., Xu, H., Lambeth, L., Smith, L. P., Kgosana, L., Wang, X., and Nair, V. (2009). A functional microRNA-155 ortholog encoded by the oncogenic Marek's disease virus. *J. Virol.* 83, 489–492.

Ziegelbauer, J. M., Sullivan, C. S., and Ganem, D. (2009). Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. *Nat. Genet.* 41, 130–134.

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Treatment of Kaposi sarcoma-associated herpesvirus-associated cancers

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Kaposi sarcoma (KS) is the most frequent AIDS-defining cancer worldwide. KS-associated herpesvirus (KSHV) is the etiological agent of KS, and the virus is also associated with two lymphoproliferative diseases. Both KS and KSHV-associated lymphomas, are cancers of unique molecular composition. They represent a challenge for cancer treatment and an opportunity to identify new mechanisms of transformation. Here, we review the current clinical insights into KSHV-associated cancers and discuss scientific insights into the pathobiology of KS, primary effusion lymphoma, and multicentric Castleman's disease.

Keywords: KSHV, lymphoma, sarcoma, viral cancer, therapy

KAPOSI SARCOMA

Kaposi sarcoma (KS) is an endothelial cell lineage tumor that is caused by KS-associated herpesvirus (KSHV). Clinically, multiple manifestations of KS have been observed. It is unclear whether these different clinical forms have the same molecular make-up and whether they would respond to the same treatment. The clinical forms of KS are classic KS, endemic KS, transplant-associated KS, and AIDS-associated KS. In the era of effective antiretroviral therapy (ART), we now also encounter a new type of KS that is HIV associated, but no longer AIDS-defining as it appears in individuals with near-normal CD4 counts.

Classic KS was described by M. Kaposi. He identified five patients exhibiting hemorrhagic sarcoma of the skin as well as sarcoma of internal organs upon postmortem examination (Kaposi, 1872; Abada et al., 2008). Note that Kaposi described an aggressive tumor in older – HIV-negative – men, whereas to date the classic form of KS is considered to be an indolent disease restricted to the skin. It also predominantly occurs in older men of Mediterranean and Eastern European origin. One report suggests a hereditary component for classic KS (Guttman-Yassky et al., 2004).

Endemic KS was described as occurring in sub-Saharan Africa before the emergence of HIV (Maclean, 1963). African KS is histologically indistinguishable from other forms of KS. It tends to be aggressive, and it is regularly seen in children (Slavin et al., 1970). Endemic KS is the leading cancer of children in some African countries owing to near universal infection with KSHV and other yet to be identified co-factors. It is as frequent, and as characteristic for the "Malaria belt" as Epstein–Barr-Virus (EBV)-associated endemic Burkitt lymphoma (BL). The only treatment, if available, is radiation and/or cytotoxic chemotherapy. In severe cases of KS that are localized to the limbs amputation is indicated.

Transplant-associated KS is seen in KSHV seropositive patients receiving immunosuppressive agents. Thus the incidence rates of transplant-associated KS track the seroprevalences of KSHV. Transplant-associated KS is a significant cause of morbidity and mortality in Italy, Turkey, Saudia-Arabia, e.g., in developed countries with substantial KSHV seroprevalence (8–18%). KS seen in solid organ transplant recipients is called iatrogenic KS (Harwood et al., 1979). The first-line therapy is tapering of the immune suppressive regimen. This often leads to lesions resolution (or immune-mediated tumor regression), though it risks organ rejection. Lately, rapamycin has emerged as an effective therapy for transplant-associated KS (Stallone et al., 2005), either as second-line substitutive immune suppressive regimen or as addition to cyclosporine mediated immune maintenance regimens. Transplant-associated KS tends to occur later (after the first year) than viremic herpesvirus-associated complications. Either the donor organ or the recipient can be the source of KSHV in this setting, as well as *de novo* exposure post transplantation when immunosuppressive therapies are administered (Barozzi et al., 2003).

AIDS–KS is found with greatly increased frequency in HIV-positive men. It is an AIDS-defining disease and was initially called epidemic KS. Note that KS can become an epidemic disease only in those HIV-positive populations that have high prevalence rates of KSHV. In the US and other developing countries, these are men who have sex with men, not individuals who acquired HIV through blood transfusion prior to the introduction of mandatory HIV screening of blood supplies (Gao et al., 1996a,b; Kedes et al., 1996). In regions of endemic KS, where initial exposure happens during childhood, these are all individuals – children, men, and women (though even here KS is twice as frequent in men as in women). In the context of substantial immune suppression, i.e., terminal AIDS,

KS can be highly aggressive, and spreads quickly to internal organs (Gottlieb et al., 1981). The clinical presentation of AIDS–KS has become more diverse with the advent of ART. First, approximately 30% of HIV-associated KS cases in the US now occur in the setting of successfully ART-suppressed HIV viral load. These cases tend to have a milder clinical presentation (Maurer et al., 2007; Krown et al., 2008). Second, terminal AIDS–KS-associated with ART failure remains the leading malignancy of HIV-positive men. Third, KS is often still the first indication of HIV infection, particularly in Sub-Saharan Africa. Like transplant-associated KS the lesions often regress after immune-restoration post initiation of ART. Fourth, the opposite clinical scenario has also been seen: HIV-infected individuals who newly start ART develop KS within the first months of therapy, a manifestation that defines immune reconstitution inflammatory syndrome (IRIS; Connick et al., 2004; Bower et al., 2005; Crane et al., 2005; Leidner and Aboulafia, 2005).

How do we treat these different forms of KS? Is there a molecular mechanism or signature that may be utilized as the basis of rational therapy choice? And how do we measure KS responses to compare different therapeutic modalities? As mentioned above KS can be limited or can present as fulminant disease, with internal organ involvement. Persons with severe KS may also develop primary effusion lymphoma (PEL) or suffer from concurrent KSHV-associated multicentric Castleman disease (MCD; described in detail in later sections; see below). There is a wide range in clinical presentations. Cutaneous lesions tend to be most common at the lower extremities, but can appear anywhere (Cheung et al., 2005). Oral KS, concurrent with cutaneous KS or as a single lesion absent cutaneous involvement, has been reported in the context of HIV infection. Whereas cutaneous lesions are a cause of stigma as much as physical pain, the more insidious presentation is that of KS in internal organs (lung, liver) without overt extensive cutaneous involvement, as is seen, e.g., in some patient populations in Brazil (deSouza, personal communication).

Cutaneous KS lesions are typically dark and can present as patches, papules, plaques, or nodules. There is a suspicion that these clinical forms are part of a disease progression. However, the different forms can present simultaneously on a single individual. No formal studies have explicitly linked these broad descriptors to survival or to response to therapy. KS can affect all internal organs including but not limited to the lungs, liver, lymph nodes, and the gastrointestinal tract. KS presents with lymphedema quite frequently. As with many carcinomas, lymph node involvement signifies poor prognosis in children. In adults, lymph node involvement does not always portend the same poor prognosis as other cancers (Myskowski et al., 1988). The current staging system of AIDS-associated KS is based on the AIDS Clinical Trials Group (ACTG) Oncology Committee (Krown et al., 1989, 1997). Akin to the standard TNM staging for solid tumors (Sobin et al., 2009), it incorporates extent of tumor, where T0 signifies that the KS is restricted to the skin and/or lymph nodes and/or minimal oral disease (i.e., non-nodular KS confined to the palate), and T1 signifies tumor-associated edema or ulceration, gastrointestinal KS, extensive oral KS, or KS in other non-nodal internal organs. Other criteria in this classification include the severity of immunodeficiency (I0 or I1) as measured by CD4 count and various systemic symptoms/illnesses (S0 or S1). Both poor immune response (I), as well as the stage of the tumor (T), most accurately predicted survival of AIDS–KS in the pre-ART era (Krown et al., 1997). Both tumor stage and systemic illness also predicts dismal prognosis in the post-ART era (Nasti et al., 2003). Of note this classification system was developed for AIDS–KS prior to ART. Whether this classification is also applicable to endemic KS is currently under study. Notably this classification does not consider KSHV viral load, as this classification system predates the discovery of KSHV. It does not consider molecular markers of the tumor, such as proliferative index of the lesions or circulating biomarkers, such as IL-6.

Regardless of clinical acumen, lesional biopsy is required to establish KS, as lesions can be mistaken for hemangiomas, dermatofibromas, hematomas, purpuras etc., and in developing countries, tuberculosis (skin and internal). Other diseases that may be mistaken for KS due to similar presentations include bacillary angiomatosis, lymphangioma, angiosarcoma, and hemangioendothelioma to list just a few.

Histologic diagnosis of KS is required prior to cytotoxic therapy. Most individuals with KS lesions will have detectable KSHV in blood and KS viral load is prognostic for KS. However, the degree of viremia is highly variable. A study of classic KS reported an average of <100 copies/ml (Guttman-Yassky et al., 2007), whereas AIDS–KS is almost always associated with >1000 copies/ml. Compared to other herpesvirus infections (Epstein–Barr-Virus or human Cytomegalovirus) in the setting of AIDS or transplant-associated immune suppression, the level of viremia is low, reducing the sensitivity of KSHV viral load based assays. By contrast specificity of KSHV viral load assays is high, as a high viral load is almost always associated with overt or imminent KS.

Seropositivity for KSHV latent nuclear antigen (LANA), which at present is the most specific assay, cannot be used to diagnose KS. It establishes exposure to the risk factor KSHV, however exposure precedes disease by a long time. A rise in serum antibody titers may happen in some instances, but not others, particularly in heavily immune deficient individuals. Early studies estimated a median latency of 7 years between exposure and disease for pre-ART AIDS–KS (Gao et al., 1996a). For classic KS, the latency may be even longer; for endemic KS in children the latency can be months.

The diagnosis of KS is based on histologic features of the H&E stain. As of late, immunohistochemistry for the KSHV antigen, LANA, has been added to the diagnostic repertoire. As another alternative, KSHV detection by PCR for viral DNA or RNA may be informative. The problem here is that sensitivity of the PCR depends on the accuracy and location of the excisional biopsy; for instance, to minimize bleeding the most aggressive lesion is typically not biopsied. A confounding factor for RNA-based diagnosis has been the RNAse-rich environment of the lesions, which prevented RNA analyses by traditional methods (e.g., Northern blot) until the introduction of more sensitive methods such as quantitative real-time PCR (Renne, Lagunoff, Dittmer, unpublished).

Lesions are composed of vascular spaces comprised of large endothelial cells that protrude into the vessel lumen due to thinning of the blood vessel wall. Spindle cell proliferation can be sparse or significant. These proliferating spindle cells are the KS tumor cells and the target for anti-KS therapy. Sometimes the lesions resemble a fibrosarcoma. The presence of extravascular erythrocytes and narrow irregular, angulated slits is a classic hallmark

of KS. Inflammatory mononuclear cell infiltrates are seen consistently and are generally comprised of infiltrating lymphocytes, macrophages, and plasma cells. Exactly how they contribute to lesion development (or if at all) is unclear.

TREATMENT

As there are many forms of KS, one must separately consider treatment options for each form. Clinical trial data in KS, especially phase III studies, are limited by declining numbers of patients in developed countries in the post-ART era. Therefore, published treatment recommendations are based on both older trial data, limited (phase I/II) trials, and consensus opinion. Of note, this review by no means offers specific recommendations. These fall into the purview of the treating physician. Rather, we will focus on those regimens for which a reasonable rational basis exists and these regimens are often arrived at post facto. With rare exceptions, phase III efficacy data are usually not available for the more modern therapies.

Kaposi sarcoma, as an AIDS-presenting manifestation in ART naïve patients, often responds to ART and the ensuing immune reconstitution alone. However, typically no more than half of patients achieve lesion resolution with successful ART (Nguyen et al., 2008). At present there are no clinical or molecular parameters that distinguish between responders and non-responders. A phase III clinical trial comparing ART alone, or with delayed chemotherapy to ART, with immediate adjunctive chemotherapy for limited AIDS–KS in resource-limited settings (REACT-KS) has recently started enrolling. Whether some ART regimens are better suited to treat AIDS–KS than others is also currently under investigation. Some HIV protease inhibitors have direct anti-tumor activity (Monini et al., 2004), and some also exhibit anti-viral KSHV activity (Gantt et al., 2011). Antiretroviral combinations with non-protease-inhibitors can also induce KS regression (Bower et al., 2006).

Systemic chemotherapy, e.g., Doxil™, can be required in AIDS–KS if lesions do not regress with primary ART therapy, and can be useful in other situations as well. KS as an indicator of ART failure requires second and/or third line therapy for HIV, again with concurrent or delayed chemotherapy. Early systemic chemotherapy may help to suppress IRIS-associated flares (Leidner and Aboulafia, 2005). KS that develops in the presence of successful ART also requires KS-targeted chemotherapy.

In addition to ART, radiotherapy or surgery can be used to treat isolated lesions. Cytotoxic chemotherapy includes vincristine, bleomycin, doxorubicin, and etoposide, as single agents or in combination. In particular the liposomal formulations of pegylated-doxorubicin (Doxil™) or daunorubicin have shown clinical efficacy and are often the first-line agent used. Taxol is considered as a second-line option. Its efficacy is, within a small margin, comparable to doxil. These chemotherapy options are part of the standard repertoire of cytotoxic therapy for solid tumors, including sarcomas, and were developed and clinically evaluated in the pre-ART and pre-KSHV era. Many of these drugs have significant toxicities and a lifetime limit of exposure. While these agents are efficacious, they were not chosen because of unique insights into the biology of the disease and they do not take into account any novel targets that the tumor-associated virus may present. Future improvements in KS treatment (i.e., higher efficacy and lower toxicity) will depend on better exploitation of these disease-specific targets.

In patients afflicted with transplant-associated KS, complete regression of cutaneous KS was seen when immunosuppressive therapy was switched from cyclosporin to rapamycin (Campistol et al., 2004; Stallone et al., 2005). These results have been recapitulated in immune deficient animal models of KS (Roy, Dittmer, unpublished observation), and similar encouraging response rates have been seen by others (Gutierrez-Dalmau et al., 2005; Zmonarski et al., 2005; Lebbe et al., 2006). Of course, exceptions have also been reported (Guenova et al., 2008). A recent study showed rapamycin (sirolimus) to be well tolerated over a long period of time (10 months) and to induce encouraging molecular responses and disease stabilization in a limited study of AIDS–KS (Krown et al., 2012).

Rapamycin is an allosteric inhibitor of mTORC1. Rapamycin binds to FK506-binding protein 12 (FKB12). The rapamycin–FKB12 complex inhibits mammalian target of rapamycin (mTOR) kinase activity (Sabers et al., 1995). Rapamycin typically inhibits cell proliferation with IC50s of 0.5–5 μM. Rapamycin is established clinically since 1999 as a second-generation immunosuppressive agent for organ transplantation, because it inhibits IL-2 translation and secretion and thus T-cell proliferation. In this context the cell autonomous G_1 arrest phenotype is augmented by inhibition of IL-2, which is a paracrine and autocrine growth factor for T cells. PEL are dependent on secreted autocrine growth factors. This has been established for the B-cell survival factors hIL-6 and hIL-10 (Komanduri et al., 1996; Asou et al., 1998; Aoki and Tosato, 1999; Drexler et al., 1999; Foussat et al., 1999; Jones et al., 1999; Chatterjee et al., 2002). IL-6, IL-10, IFNgamma, and IL12p40 secretion is inhibited by rapamycin (Sin et al., 2007). Other cytokines [IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-12(p70), IL-13, IL-15, IP-10, Eotaxin, IFNγ, GM-CSF, MCP-1, MIP-1α, RANTES, and TNFα] are not affected. VEGF-1 secretion is also inhibited by rapamycin (Damania, Dittmer, unpublished).

Clinically, both direct and indirect mechanisms may contribute to these responses. On one hand, tumor regression coincided with the recovery of T-cell memory responses against KSHV latent (Orf73) and lytic (K8.1) antigens (Barozzi et al., 2008). On the other hand, rapamycin is directly toxic to KSHV-infected cells (Sin et al., 2007), and KSHV-associated tumors, i.e., KS and PEL, depend on the mTOR signaling pathway, which is directly targeted by rapamycin (Sodhi et al., 2006; Wang and Damania, 2008). KS lesions almost universally exhibit phosphorylated Akt, which activates mTOR kinase and phosphorylated S6, which is a biomarker for mTOR kinase activity (Roy and Dittmer, 2011). This is expected since the PI3K/Akt pathway is activated upon of VEGF–VEGFR stimulation in endothelial cells and by the activation of the B-cell receptor (BCR) complex in B cells.

Active Akt kinase promotes multiple cellular survival mechanisms: (i) Akt enhances protein synthesis through increasing the phosphorylation of mTOR (Gingras et al., 1998), (ii) Akt counteracts apoptosis by directly phosphorylating and inactivating pro-apoptotic factors such as BAD (Datta et al., 1997; Del Peso et al., 1997; Cardone et al., 1998), (iii) Akt phosphorylates a family of transcription factors known as the forkhead (FKHR) or FOXO

transcription factors; Brunet et al., 1999; Kops and Burgering, 1999; Tang et al., 1999). Members of this family include FKHR (FOXO1a), FKHRL1 (FOXO3), and AFX (FOXO4). The net result of phosphorylation of the downstream targets of Akt is cell survival via inactivation of the FKHR family, GSK-3b, Caspase-9, and Bad (Cross et al., 1995; Datta et al., 1997; Del Peso et al., 1997; Cardone et al., 1998). Rapamycin blocks focus formation induced by oncogenic alleles of the upstream mTOR regulators, PI3K, or Akt (Aoki et al., 2001). Prolonged rapamycin treatment has been shown to lead to increased Akt phosphorylation by the mTOR–Rictor complex (Sarbassov et al., 2005, 2006), though this was not seen in KS. Thus, the efficacy of rapamycin (and more modern rapamycin analogs) in KS may plausibly be the result of a slightly different wiring of the PI3K/Akt/mTOR pathway in KSHV-associated cancers.

PRIMARY EFFUSION LYMPHOMA

The discovery of KSHV in KS prompted a frantic search for other cancers that may be associated with this new virus. Following the paradigm of EBV, which is seen in nasopharyngeal carcinoma, a solid organ cancer, as well as in lymphomas, KSHV sequences were rapidly identified in an uncommon type of B-cell lymphoma (Cesarman et al., 1995): PEL. KSHV infection is necessary for PEL development. The first cell lines for this lymphoma were obtained from body cavity effusions of what was hitherto called AIDS-associated lymphohematopoietic neoplasms. They carried EBV as well as KSHV (Knowles et al., 1989; Cesarman et al., 1995). PEL occurs with increased frequency in HIV-infected individuals; including those with concurrent KS. PEL account for 3% of AIDS-related lymphomas. This may be a skewed estimate, since it is may be quite common that AIDS patients die of other complications, including KS, before a diagnosis of PEL can be made (Nador et al., 1996; Gaidano et al., 1998). PEL is exceedingly rare in HIV-negative individuals (Walts et al., 1990; Green et al., 1995). The recognition that KSHV was always found in PEL, and EBV in 50–80% of PEL, led to their classification as a new sub-type of non-Hodgkin lymphoma (Nador et al., 1996). PEL cell lines contain many copies of the KSHV genome as nuclear plasmids, which greatly facilitates molecular studies for this virus.

Primary effusion lymphoma can also occur in unusual sites (Said et al., 1996; Moatamed et al., 2011) and solid variants have been described as well. These variants also carry the KSHV genome and express at least the KSHV latent genes (Nador et al., 1996; Said et al., 1996; Engels et al., 2003; Chadburn et al., 2004; Carbone et al., 2005; Deloose et al., 2005). The diagnostic criteria for PEL (Nador et al., 1996; Cesarman and Knowles, 1999; Raphaël et al., 2008) include immunoblastic–anaplastic large-cell morphology, null-cell phenotype with no B-cell-associated antigen and immunoglobulin expression, and B-cell genotype as ascertained by BCR rearrangement. High CD138/Syndecan-1 (Gaidano et al., 1997) expression and hypermutation of immunoglobulin genes (Matolcsy et al., 1998) established that PELs are post-germinal center (GC) tumors at a pre-terminal stage prior to plasma-cell differentiation (this does not exclude the possibility that PEL arrived at this stage through extra-GC maturation). Transcript profiling confirmed this genesis (Palarcik, 1991; Jenner et al., 2003; Klein et al., 2003; O'Hara et al., 2008, 2009).

Survival on conventional chemotherapy is very poor (Nador et al., 1996; Simonelli et al., 2003), but of course PEL patients tend to have multiple comorbidities at presentation. The effusions may be managed by repeated drainage but eventually solid nests of PEL abolish vital organ functions. In PEL the p53 and PTEN genes are rarely mutated. P53 mutations accumulate after chemotherapy (Petre et al., 2007; Chen et al., 2010). FHIT and WWOX, two fragile site tumor suppressor genes, are deleted in many PEL cell lines (Roy et al., 2011), however these and other genome-wide association studies are limited by the scarcity of cases. Individual case reports document responses to anti-viral therapy, bortezomib, rapamycin (Sin et al., 2007), rituximab-containing chemotherapy regimens (Oksenhendler et al., 1998; Boulanger et al., 2001; Hocqueloux et al., 2001; Ghosh et al., 2003; Lim et al., 2005; Siddiqi and Joyce, 2008), or pleurodesis with bleomycin (Yiakoumis et al., 2011). Bortezomib's (Velcade™) primary mechanism of action is inhibition of the 26S proteasome and nuclear factor B (NFκB) activity. Rapamycin (Sirolimus™) inhibits mTOR signaling, rituximab (Rituxan™) is a humanized monoclonal antibody against CD20, and bleomycin induces DNA breaks and eventually apoptosis in rapidly growing cells.

MULTICENTRIC CASTLEMAN DISEASE

One other disease entity has been convincingly associated with KSHV. This is a sub-type of Castleman disease, specifically the plasma-cell type (Keller et al., 1972). Castleman disease can be localized to just one, or multiple lymph nodes can be involved. The latter presentation is called MCD. MCD has mostly a plasma-cell type morphology and is accompanied by systemic symptoms (Waterston and Bower, 2004). These systemic symptoms may be a result of cytokines, and in particular interleukin-6 (IL-6). The viral homolog, vIL-6 is expressed in scattered plasmablasts surrounding the lymphoid follicles in MCD (Berti et al., 1997; Cannon et al., 1999; Staskus et al., 1999). Thus, both KSHV-induced human IL-6 and virus-encoded vIL-6 are found at high levels in MCD. Recently, cases of an inflammatory syndrome with clinical symptoms similar to MCD have been described in AIDS patients with KS, but no diagnosis of MCD (Uldrick et al., 2010). Here too, high amounts of vIL-6 in the serum have been noted. This suggests excess cytokine production is one of the systemic features of MCD (Hasson, 1985).

Multicentric Castleman disease patients may also develop concurrent frank cancer, such as KS and NHL. KSHV is invariably present in AIDS-associated MCD (Soulier et al., 1995; Larroche et al., 2002) and the presence of KS and MCD in the same lymph node is not rare. In some cases high angiosclerosis, GC, and perifollicular vascular proliferation is seen, while plasmacytosis was less pronounced (Suda et al., 2001). Studies are ongoing to better understand this disease and to arrive at a more succinct clinical description. The KSHV-positive cases may represent a distinct morphologic variant from KSHV-negative MCD (Dupin et al., 2000; Brousset et al., 2001). MCD lymph nodes can contain multiple KSHV-infected B cells, which may form microlymphomas or even frank lymphomas. Here, KSHV-infected plasmablasts are B-monotypic but polyclonal. They almost invariably express IgM-lambda (Du et al., 2001). The infected B-lineage plasmablasts in MCD differ from PEL. They lack somatic hypermutation and

expression of CD138. They express cytoplasmic Ig and may also express CD27 (Du et al., 2001; Chadburn et al., 2008).

Median survival of MCD in HIV-positive individuals in the pre-ART era was approximately 14 months (Oksenhendler et al., 1996; Bower, 2010). Evidence of KSHV lytic viral protein expression led to testing of ganciclovir, with mixed results in small case series (Corbellino et al., 2001; Casper et al., 2004). This is not unexpected if for instance vIL-6 expression is independent, rather than coincident with viral lytic gene expression (Chatterjee et al., 2002). Under such a scenario the viral kinases orf36 and tk, which are required for ganciclovir efficacy would not be expressed. Alternatively, the drug regimen of just one anti-viral may be insufficient as a two-drug regimen combining valganciclovir and zidovudine (AZT), showed promising results (Uldrick et al., 2011).

In MCD rational therapy has shown remarkable responses. Treatment with tocilizumab, an anti-human interleukin-6 (IL-6) receptor monoclonal antibody, resulted in clinical responses in MCD patients that did not have AIDS (Nishimoto et al., 2005; Song et al., 2010); and the anti-CD20 antibody, rituximab, has shown responses in up to 70% of patients (Corbellino et al., 2001; Gerard et al., 2007; Bestawros et al., 2008; Bower, 2010; Bower et al., 2011). The mechanism here is not clear. In MCD, the KSHV-positive B cells frequently lack expression of CD20 (Chadburn et al., 2008; Naresh et al., 2009), so the source of systemic symptoms may be virus-negative plasma cells or other immune cells that respond to infection.

CHALLENGES

Antiretroviral therapy has increased the life expectancy of the HIV-infected population. However, as these individuals age, there is likely to be a corresponding increase in the incidence of cancers in the HIV-positive population. Most of the current therapies do not target the unique viral etiology of cancers linked to KSHV infection. One exception are the anti-herpes viral drugs which target lytic virus but do are not effective against latent virus. The interactions of chemotherapy with HIV protease inhibitors is also another consideration that needs to be addressed. In the future, it will be important to determine whether traditional chemotherapies are safe in the context of currently prescribed HIV protease inhibitors, and to devise newer therapies that directly target the viral etiology of these cancers.

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REFERENCES

Abada, R., Dreyfuss-Grossman, T., Herman-Bachinsky, Y., Geva, H., Masa, S. R., and Sarid, R. (2008). SIAH-1 interacts with the Kaposi's sarcoma-associated herpesvirus-encoded ORF45 protein and promotes its ubiquitylation and proteasomal degradation. *J. Virol.* 82, 2230–2240.

Aoki, M., Blazek, E., and Vogt, P. K. (2001). A role of the kinase mTOR in cellular transformation induced by the oncoproteins P3k and Akt. *Proc. Natl. Acad. Sci. U.S.A.* 98, 136–141.

Aoki, Y., and Tosato, G. (1999). Role of vascular endothelial growth factor/vascular permeability factor in the pathogenesis of Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphomas. *Blood* 94, 4247–4254.

Asou, H., Said, J. W., Yang, R., Munker, R., Park, D. J., Kamada, N., and Koeffler, H. P. (1998). Mechanisms of growth control of Kaposi's sarcoma-associated herpes virus-associated primary effusion lymphoma cells. *Blood* 91, 2475–2481.

Barozzi, P., Bonini, C., Potenza, L., Masetti, M., Cappelli, G., Gruarin, P., Whitby, D., Gerunda, G. E., Mondino, A., Riva, G., Vallerini, D., Quadrelli, C., Bosco, R., Ciceri, F., Bordignon, C., Schulz, T. F., Torelli, G., and Luppi, M. (2008). Changes in the immune responses against human herpesvirus-8 in the disease course of posttransplant Kaposi sarcoma. *Transplantation* 86, 738–744.

Barozzi, P., Luppi, M., Facchetti, F., Mecucci, C., Alu, M., Sarid, R., Rasini, V., Ravazzini, L., Rossi, E., Festa, S., Crescenzi, B., Wolf, D. G., Schulz, T. F., and Torelli, G. (2003). Post-transplant Kaposi sarcoma originates from the seeding of donor-derived progenitors. *Nat. Med.* 9, 554–561.

Berti, E., Marzano, A. V., Decleva, I., Bestetti, G., Pizzuto, M., Poirel, L., Capra, M., Parravicini, C., and Corbellino, M. (1997). Simultaneous onset of primary cutaneous B-cell lymphoma and human herpesvirus 8-associated Kaposi's sarcoma. *Br. J. Dermatol.* 136, 924–929.

Bestawros, A., Michel, R., Seguin, C., and Routy, J. P. (2008). Multicentric Castleman's disease treated with combination chemotherapy and rituximab in four HIV-positive men: a case series. *Am. J. Hematol.* 83, 508–511.

Boulanger, E., Agbalika, F., Maarek, O., Daniel, M. T., Grollet, L., Molina, J. M., Sigaux, F., and Oksenhendler, E. (2001). A clinical, molecular and cytogenetic study of 12 cases of human herpesvirus 8 associated primary effusion lymphoma in HIV-infected patients. *Hematol. J.* 2, 172–179.

Bower, M. (2010). How I treat HIV-associated multicentric Castleman disease. *Blood* 116, 4415–4421.

Bower, M., Nelson, M., Young, A. M., Thirlwell, C., Newsom-Davis, T., Mandalia, S., Dhillon, T., Holmes, P., Gazzard, B. G., and Stebbing, J. (2005). Immune reconstitution inflammatory syndrome associated with Kaposi's sarcoma. *J. Clin. Oncol.* 23, 5224–5228.

Bower, M., Newsom-Davis, T., Naresh, K., Merchant, S., Lee, B., Gazzard, B., Stebbing, J., and Nelson, M. (2011). Clinical features and outcome in HIV-associated multicentric Castleman's disease. *J. Clin. Oncol.* 29, 2481–2486.

Bower, M., Palmieri, C., and Dhillon, T. (2006). AIDS-related malignancies: changing epidemiology and the impact of highly active antiretroviral therapy. *Curr. Opin. Infect. Dis.* 19, 14–19.

Brousset, P., Cesarman, E., Meggetto, F., Lamant, L., and Delsol, G. (2001). Colocalization of the viral interleukin-6 with latent nuclear antigen-1 of human herpesvirus-8 in endothelial spindle cells of Kaposi's sarcoma and lymphoid cells of multicentric Castleman's disease. *Hum. Pathol.* 32, 95–100.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96, 857–868.

Campistol, J. M., Gutierrez-Dalmau, A., and Torregrosa, J. V. (2004). Conversion to sirolimus: a successful treatment for posttransplantation Kaposi's sarcoma. *Transplantation* 77, 760–762.

Cannon, J. S., Nicholas, J., Orenstein, J. M., Mann, R. B., Murray, P. G., Browning, P. J., Digiuseppe, J. A., Cesarman, E., Hayward, G. S., and Ambinder, R. F. (1999). Heterogeneity of viral IL-6 expression in HHV-8-associated diseases. *J. Infect. Dis.* 180, 824–828.

Carbone, A., Gloghini, A., Vaccher, E., Cerri, M., Gaidano, G., Dalla-Favera, R., and Tirelli, U. (2005). Kaposi's sarcoma-associated herpesvirus/human herpesvirus type 8-positive solid lymphomas: a tissue-based variant of primary effusion lymphoma. *J. Mol. Diagn.* 7, 17–27.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282, 1318–1321.

Casper, C., Nichols, W. G., Huang, M. L., Corey, L., and Wald, A. (2004). Remission of HHV-8 and HIV-associated multicentric Castleman disease with ganciclovir treatment. *Blood* 103, 1632–1634.

Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. *N. Engl. J. Med.* 332, 1186–1191.

Cesarman, E., and Knowles, D. M. (1999). The role of Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in lymphoproliferative diseases. *Semin. Cancer Biol.* 9, 165–174.

Chadburn, A., Hyjek, E., Mathew, S., Cesarman, E., Said, J., and Knowles, D. M. (2004). KSHV-positive solid lymphomas represent an extracavitary variant of primary effusion lymphoma. *Am. J. Surg. Pathol.* 28, 1401–1416.

Chadburn, A., Hyjek, E. M., Tam, W., Liu, Y., Rengifo, T., Cesarman, E., and Knowles, D. M. (2008). Immunophenotypic analysis of the Kaposi sarcoma herpesvirus (KSHV; HHV-8)-infected B cells in HIV+ multicentric Castleman disease (MCD). *Histopathology* 53, 513–524.

Chatterjee, M., Osborne, J., Bestetti, G., Chang, Y., and Moore, P. S. (2002). Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* 298, 1432–1435.

Chen, W., Hilton, I. B., Staudt, M. R., Burd, C. E., and Dittmer, D. P. (2010). Distinct p53, p53:LANA, and LANA complexes in Kaposi's sarcoma – associated herpesvirus lymphomas. *J. Virol.* 84, 3898–3908.

Cheung, M. C., Pantanowitz, L., and Dezube, B. J. (2005). AIDS-related malignancies: emerging challenges in the era of highly active antiretroviral therapy. *Oncologist* 10, 412–426.

Connick, E., Kane, M. A., White, I. E., Ryder, J., and Campbell, T. B. (2004). Immune reconstitution inflammatory syndrome associated with Kaposi sarcoma during potent antiretroviral therapy. *Clin. Infect. Dis.* 39, 1852–1855.

Corbellino, M., Bestetti, G., Scalamogna, C., Calattini, S., Galazzi, M., Meroni, L., Manganaro, D., Fasan, M., Moroni, M., Galli, M., and Parravicini, C. (2001). Long-term remission of Kaposi sarcoma-associated herpesvirus-related multicentric Castleman disease with anti-CD20 monoclonal antibody therapy. *Blood* 98, 3473–3475.

Crane, H. M., Deubner, H., Huang, J. C., Swanson, P. E., and Harrington, R. D. (2005). Fatal Kaposi's sarcoma-associated immune reconstitution following HAART initiation. *Int. J. STD AIDS* 16, 80–83.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785–789.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell- intrinsic death machinery. *Cell* 91, 231–241.

Del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 278, 687–689.

Deloose, S. T., Smit, L. A., Pals, F. T., Kersten, M. J., Van Noesel, C. J., and Pals, S. T. (2005). High incidence of Kaposi sarcoma-associated herpesvirus infection in HIV-related solid immunoblastic/plasmablastic diffuse large B-cell lymphoma. *Leukemia* 19, 851–855.

Drexler, H. G., Meyer, C., Gaidano, G., and Carbone, A. (1999). Constitutive cytokine production by primary effusion (body cavity-based) lymphoma-derived cell lines. *Leukemia* 13, 634–640.

Du, M. Q., Liu, H., Diss, T. C., Ye, H., Hamoudi, R. A., Dupin, N., Meignin, V., Oksenhendler, E., Boshoff, C., and Isaacson, P. G. (2001). Kaposi sarcoma-associated herpesvirus infects monotypic (IgM lambda) but polyclonal naive B cells in Castleman disease and associated lymphoproliferative disorders. *Blood* 97, 2130–2136.

Dupin, N., Diss, T. L., Kellam, P., Tulliez, M., Du, M. Q., Sicard, D., Weiss, R. A., Isaacson, P. G., and Boshoff, C. (2000). HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. *Blood* 95, 1406–1412.

Engels, E. A., Pittaluga, S., Whitby, D., Rabkin, C., Aoki, Y., Jaffe, E. S., and Goedert, J. J. (2003). Immunoblastic lymphoma in persons with AIDS-associated Kaposi's sarcoma: a role for Kaposi's sarcoma-associated herpesvirus. *Mod. Pathol.* 16, 424–429.

Foussat, A., Wijdenes, J., Bouchet, L., Gaidano, G., Neipel, F., Balabanian, K., Galanaud, P., Couderc, J., and Emilie, D. (1999). Human interleukin-6 is in vivo an autocrine growth factor for human herpesvirus-8-infected malignant B lymphocytes. *Eur. Cytokine Netw.* 10, 501–508.

Gaidano, G., Carbone, A., and Dalla-Favera, R. (1998). Pathogenesis of AIDS-related lymphomas: molecular and histogenetic heterogeneity. *Am. J. Pathol.* 152, 623–630.

Gaidano, G., Gloghini, A., Gattei, V., Rossi, M. F., Cilia, A. M., Godeas, C., Degan, M., Perin, T., Canzonieri, V., Aldinucci, D., Saglio, G., Carbone, A., and Pinto, A. (1997). Association of Kaposi's sarcoma-associated herpesvirus-positive primary effusion lymphoma with expression of the CD138/syndecan-1 antigen. *Blood* 90, 4894–4900.

Gantt, S., Carlsson, J., Ikoma, M., Gachelet, E., Gray, M., Geballe, A. P., Corey, L., Casper, C., Lagunoff, M., and Vieira, J. (2011). The HIV protease inhibitor nelfinavir inhibits Kaposi's sarcoma-associated herpesvirus replication in vitro. *Antimicrob. Agents Chemother.* 55, 2696–2703.

Gao, S. J., Kingsley, L., Hoover, D. R., Spira, T. J., Rinaldo, C. R., Saah, A., Phair, J., Detels, R., Parry, P., Chang, Y., and Moore, P. S. (1996a). Seroconversion to antibodies against Kaposi's sarcoma-associated herpesvirus-related latent nuclear antigens before the development of Kaposi's sarcoma. *N. Engl. J. Med.* 335, 233–241.

Gao, S. J., Kingsley, L., Li, M., Zheng, W., Parravicini, C., Ziegler, J., Newton, R., Rinaldo, C. R., Saah, A., Phair, J., Detels, R., Chang, Y., and Moore, P. S. (1996b). KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. *Nat. Med.* 2, 925–928.

Gerard, L., Berezne, A., Galicier, L., Meignin, V., Obadia, M., De Castro, N., Jacomet, C., Verdon, R., Madelaine-Chambrin, I., Boulanger, E., Chevret, S., Agbalika, F., and Oksenhendler, E. (2007). Prospective study of rituximab in chemotherapy-dependent human immunodeficiency virus associated multicentric Castleman's disease: ANRS 117 CastlemaB Trial. *J. Clin. Oncol.* 25, 3350–3356.

Ghosh, S. K., Wood, C., Boise, L. H., Mian, A. M., Deyev, V. V., Feuer, G., Toomey, N. L., Shank, N. C., Cabral, L., Barber, G. N., and Harrington, W. J. Jr. (2003). Potentiation of TRAIL-induced apoptosis in primary effusion lymphoma through azidothymidine-mediated inhibition of NF-kappa B. *Blood* 101, 2321–2327.

Gingras, A. C., Kennedy, S. G., O'leary, M. A., Sonenberg, N., and Hay, N. (1998). 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev.* 12, 502–513.

Gottlieb, G. J., Ragaz, A., Vogel, J. V., Friedman-Kien, A., Rywlin, A. M., Weiner, E. A., and Ackerman, A. B. (1981). A preliminary communication on extensively disseminated Kaposi's sarcoma in young homosexual men. *Am. J. Dermatopathol.* 3, 111–114.

Green, I., Espiritu, E., Ladanyi, M., Chaponda, R., Wieczorek, R., Gallo, L., and Feiner, H. (1995). Primary lymphomatous effusions in AIDS: a morphological, immunophenotypic, and molecular study. *Mod. Pathol.* 8, 39–45.

Guenova, E., Metzler, G., Hoetzenecker, W., Berneburg, M., and Rocken, M. (2008). Classic mediterranean Kaposi's sarcoma regression with sirolimus treatment. *Arch. Dermatol.* 144, 692–693.

Gutierrez-Dalmau, A., Sanchez-Fructuoso, A., Sanz-Guajardo, A., Mazuecos, A., Franco, A., Rial, M. C., Iranzo, P., Torregrosa, J. V., Oppenheimer, F., and Campistol, J. M. (2005). Efficacy of conversion to sirolimus in posttransplantation Kaposi's sarcoma. *Transplant. Proc.* 37, 3836–3838.

Guttman-Yassky, E., Abada, R., Kra-Oz, Z., Sattinger, J., Perelman, A., Bergman, R., and Sarid, R. (2007). Relationship between human herpesvirus 8 loads and disease stage in classic Kaposi sarcoma patients. *Diagn. Microbiol. Infect. Dis.* 57, 387–392.

Guttman-Yassky, E., Cohen, A., Kra-Oz, Z., Friedman-Birnbaum, R., Sprecher, E., Zaltzman, N., Friedman, E., Silbermann, M., Rubin, D., Linn, S., Whitby, D., Gideoni, O., Pollack, S., Bergman, R., and Sarid, R. (2004). Familial clustering of classic Kaposi sarcoma. *J. Infect. Dis.* 189, 2023–2026.

Harwood, A. R., Osoba, D., Hofstader, S. L., Goldstein, M. B., Cardella, C. J., Holecek, M. J., Kunynetz, R., and Giammarco, R. A. (1979). Kaposi's sarcoma in recipients of renal transplants. *Am. J. Med.* 67, 759–765.

Hasson, H. M. (1985). Open laparoscopy for tubal occlusion in the female. *Adv. Contracept.* 1, 181–189.

Hocqueloux, L., Agbalika, F., Oksenhendler, E., and Molina, J. M. (2001). Long-term remission of an AIDS-related primary effusion lymphoma

with antiviral therapy. *AIDS* 15, 280–282.

Jenner, R. G., Maillard, K., Cattini, N., Weiss, R. A., Boshoff, C., Wooster, R., and Kellam, P. (2003). Kaposi's sarcoma-associated herpesvirus-infected primary effusion lymphoma has a plasma cell gene expression profile. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10399–10404.

Jones, K. D., Aoki, Y., Chang, Y., Moore, P. S., Yarchoan, R., and Tosato, G. (1999). Involvement of interleukin-10 (IL-10) and viral IL-6 in the spontaneous growth of Kaposi's sarcoma herpesvirus-associated infected primary effusion lymphoma cells. *Blood* 94, 2871–2879.

Kaposi, M. (1872). Idiopathisches multiples Pigmentsarkom der Haut. *Arch. Dermatol. Syph.* 4, 265–273.

Kedes, D. H., Operskalski, E., Busch, M., Kohn, R., Flood, J., and Ganem, D. (1996). The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. *Nat. Med.* 2, 918–924.

Keller, A. R., Hochholzer, L., and Castleman, B. (1972). Hyaline-vascular and plasma-cell types of giant lymph node hyperplasia of the mediastinum and other locations. *Cancer* 29, 670–683.

Klein, U., Gloghini, A., Gaidano, G., Chadburn, A., Cesarman, E., Dalla-Favera, R., and Carbone, A. (2003). Gene expression profile analysis of AIDS-related primary effusion lymphoma (PEL) suggests a plasmablastic derivation and identifies PEL-specific transcripts. *Blood* 101, 4115–4121.

Knowles, D. M., Inghirami, G., Ubriaco, A., and Dalla-Favera, R. (1989). Molecular genetic analysis of three AIDS-associated neoplasms of uncertain lineage demonstrates their B-cell derivation and the possible pathogenetic role of the Epstein-Barr virus. *Blood* 73, 792–799.

Komanduri, K. V., Luce, J. A., Mcgrath, M. S., Herndier, B. G., and Ng, V. L. (1996). The natural history and molecular heterogeneity of HIV-associated primary malignant lymphomatous effusions. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 13, 215–226.

Kops, G., and Burgering, B. (1999). Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. *J. Mol. Med.* 77, 656–665.

Krown, S. E., Lee, J. Y., and Dittmer, D. P. (2008). More on HIV-associated Kaposi's sarcoma. *N. Engl. J. Med.* 358, 535–536.

Krown, S. E., Metroka, C., and Wernz, J. C. (1989). Kaposi's sarcoma in the acquired immune deficiency syndrome: a proposal for uniform evaluation, response, and staging criteria. AIDS Clinical Trials Group Oncology Committee. *J. Clin. Oncol.* 7, 1201–1207.

Krown, S. E., Roy, D., Lee, J. Y., Dezube, B. J., Reid, E. G., Venkataramanan, R., Han, K., Cesarman, E., and Dittmer, D. P. (2012). Rapamycin with antiretroviral therapy in AIDS-associated Kaposi sarcoma: an AIDS malignancy consortium study. *J. Acquir. Immune Defic. Syndr.* 59, 447–454.

Krown, S. E., Testa, M. A., and Huang, J. (1997). AIDS-related Kaposi's sarcoma: prospective validation of the AIDS Clinical Trials Group staging classification. AIDS Clinical Trials Group Oncology Committee. *J. Clin. Oncol.* 15, 3085–3092.

Larroche, C., Cacoub, P., Soulier, J., Oksenhendler, E., Clauvel, J. P., Piette, J. C., and Raphael, M. (2002). Castleman's disease and lymphoma: report of eight cases in HIV-negative patients and literature review. *Am. J. Hematol.* 69, 119–126.

Lebbe, C., Euvrard, S., Barrou, B., Pouteil-Noble, C., Garnier, J. L., Glotz, D., Legendre, C., and Frances, C. (2006). Sirolimus conversion for patients with posttransplant Kaposi's sarcoma. *Am. J. Transplant.* 6, 2164–2168.

Leidner, R. S., and Aboulafia, D. M. (2005). Recrudescent Kaposi's sarcoma after initiation of HAART: a manifestation of immune reconstitution syndrome. *AIDS Patient Care STDS* 19, 635–644.

Lim, S. T., Rubin, N., Said, J., and Levine, A. M. (2005). Primary effusion lymphoma: successful treatment with highly active antiretroviral therapy and rituximab. *Ann. Hematol.* 84, 551–552.

Maclean, C. M. (1963). Kaposi's sarcoma in Nigeria. *Br. J. Cancer* 17, 195–205.

Matolcsy, A., Nador, R. G., Cesarman, E., and Knowles, D. M. (1998). Immunoglobulin VH gene mutational analysis suggests that primary effusion lymphomas derive from different stages of B cell maturation. *Am. J. Pathol.* 153, 1609–1614.

Maurer, T., Ponte, M., and Leslie, K. (2007). HIV-associated Kaposi's sarcoma with a high CD4 count and a low viral load. *N. Engl. J. Med.* 357, 1352–1353.

Moatamed, N. A., Song, S. X., Apple, S. K., and Said, J. W. (2011). Primary effusion lymphoma involving the cerebrospinal fluid. *Diagn. Cytopathol.*

Monini, P., Sgadari, C., Toschi, E., Barillari, G., and Ensoli, B. (2004). Antitumour effects of antiretroviral therapy. *Nat. Rev. Cancer* 4, 861–875.

Myskowski, P. L., Niedzwiecki, D., Shurgot, B. A., Kaufman, D., Krown, S. E., Nisce, L., and Safai, B. (1988). AIDS-associated Kaposi's sarcoma: variables associated with survival. *J. Am. Acad. Dermatol.* 18, 1299–1306.

Nador, R. G., Cesarman, E., Chadburn, A., Dawson, D. B., Ansari, M. Q., Sald, J., and Knowles, D. M. (1996). Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. *Blood* 88, 645–656.

Naresh, K. N., Trivedi, P., Horncastle, D., and Bower, M. (2009). CD20 expression in the HHV-8-infected lymphoid cells in multicentric Castleman disease. *Histopathology* 55, 358–359.

Nasti, G., Talamini, R., Antinori, A., Martellotta, F., Jacchetti, G., Chiodo, F., Ballardini, G., Stoppini, L., Di Perri, G., Mena, M., Tavio, M., Vaccher, E., D'arminio Monforte, A., and Tirelli, U. (2003). AIDS-related Kaposi's sarcoma: evaluation of potential new prognostic factors and assessment of the AIDS Clinical Trial Group Staging System in the Haart Era – the Italian Cooperative Group on AIDS and Tumors and the Italian Cohort of Patients Naive From Antiretrovirals. *J. Clin. Oncol.* 21, 2876–2882.

Nguyen, H. Q., Magaret, A. S., Kitahata, M. M., Van Rompaey, S. E., Wald, A., and Casper, C. (2008). Persistent Kaposi sarcoma in the era of highly active antiretroviral therapy: characterizing the predictors of clinical response. *AIDS* 22, 937–945.

Nishimoto, N., Kanakura, Y., Aozasa, K., Johkoh, T., Nakamura, M., Nakano, S., Nakano, N., Ikeda, Y., Sasaki, T., Nishioka, K., Hara, M., Taguchi, H., Kimura, Y., Kato, Y., Asaoku, H., Kumagai, S., Kodama, F., Nakahara, H., Hagihara, K., Yoshizaki, K., and Kishimoto, T. (2005). Humanized anti-interleukin-6 receptor antibody treatment of multicentric Castleman disease. *Blood* 106, 2627–2632.

O'Hara, A. J., Vahrson, W., and Dittmer, D. P. (2008). Gene alteration and precursor and mature microRNA transcription changes contribute to the miRNA signature of primary effusion lymphoma. *Blood* 111, 2347–2353.

O'Hara, A. J., Wang, L., Dezube, B. J., Harrington, W. J. Jr., Damania, B., and Dittmer, D. P. (2009). Tumor suppressor microRNAs are underrepresented in primary effusion lymphoma and Kaposi sarcoma. *Blood* 113, 5938–5941.

Oksenhendler, E., Clauvel, J. P., Jouveshomme, S., Davi, F., and Mansour, G. (1998). Complete remission of a primary effusion lymphoma with antiretroviral therapy. *Am. J. Hematol.* 57, 266.

Oksenhendler, E., Duarte, M., Soulier, J., Cacoub, P., Welker, Y., Cadranel, J., Cazals-Hatem, D., Autran, B., Clauvel, J. P., and Raphael, M. (1996). Multicentric Castleman's disease in HIV infection: a clinical and pathological study of 20 patients. *AIDS* 10, 61–67.

Palarcik, J. (1991). Clavicular fractures (group of patients treated in the Traumatological Research Institute in 1986–1989). *Czech. Med.* 14, 184–190.

Petre, C. E., Sin, S. H., and Dittmer, D. P. (2007). Functional p53 signaling in Kaposi's sarcoma-associated herpesvirus lymphomas: implications for therapy. *J. Virol.* 81, 1912–1922.

Raphaël, M., Said, J., Borisch, B., Cesarman, E., and Harris, N. L. (2008). "Lymphomas associated with HIV infection," in *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, eds S. H. Swerdlow, E. Campo, N. L. Harris, E. S. Jaffe, S. A. Pileri, H. Stein, and J. Thiele (Lyon: IARC Press), 340–342.

Roy, D., and Dittmer, D. P. (2011). Phosphatase and tensin homolog on chromosome 10 is phosphorylated in primary effusion lymphoma and Kaposi's sarcoma. *Am. J. Pathol.* 179, 2108–2119.

Roy, D., Sin, S. H., Damania, B., and Dittmer, D. P. (2011). Tumor suppressor genes FHIT and WWOX are deleted in primary effusion lymphoma (PEL) cell lines. *Blood* 118, e32–e39.

Sabers, C. J., Martin, M. M., Brunn, G. J., Williams, J. M., Dumont, F. J., Wiederrecht, G., and Abraham, R. T. (1995). Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J. Biol. Chem.* 270, 815–822.

Said, J. W., Tasaka, T., Takeuchi, S., Asou, H., De Vos, S., Cesarman, E., Knowles, D. M., and Koeffler, H. P. (1996). Primary effusion lymphoma in women: report of two cases of Kaposi's

sarcoma herpes virus-associated effusion-based lymphoma in human immunodeficiency virus-negative women. *Blood* 88, 3124–3128.

Sarbassov, D. D., Ali, S. M., Sengupta, S., Sheen, J. H., Hsu, P. P., Bagley, A. F., Markhard, A. L., and Sabatini, D. M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell* 22, 159–168.

Sarbassov, D. D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307, 1098–1101.

Siddiqi, T., and Joyce, R. M. (2008). A case of HIV-negative primary effusion lymphoma treated with bortezomib, pegylated liposomal doxorubicin, and rituximab. *Clin. Lymphoma Myeloma* 8, 300–304.

Simonelli, C., Spina, M., Cinelli, R., Talamini, R., Tedeschi, R., Gloghini, A., Vaccher, E., Carbone, A., and Tirelli, U. (2003). Clinical features and outcome of primary effusion lymphoma in HIV-infected patients: a single-institution study. *J. Clin. Oncol.* 21, 3948–3954.

Sin, S. H., Roy, D., Wang, L., Staudt, M. R., Fakhari, F. D., Patel, D. D., Henry, D., Harrington, W. J. Jr., Damania, B. A., and Dittmer, D. P. (2007). Rapamycin is efficacious against primary effusion lymphoma (PEL) cell lines in vivo by inhibiting autocrine signaling. *Blood* 109, 2165–2173.

Slavin, G., Cameron, H. M., Forbes, C., and Mitchell, R. M. (1970). Kaposi's sarcoma in East African children: a report of 51 cases. *J. Pathol.* 100, 187–199.

Sobin, L. H., Gospodarowicz, M. K., and Wittekind, C. (eds). (2009). *TNM Classification of Malignant Tumours*, 7th Edn. Wiley-Blackwell.

Sodhi, A., Chaisuparat, R., Hu, J., Ramsdell, A. K., Manning, B. D., Sausville, E. A., Sawai, E. T., Molinolo, A., Gutkind, J. S., and Montaner, S. (2006). The TSC2/mTOR pathway drives endothelial cell transformation induced by the Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. *Cancer Cell* 10, 133–143.

Song, S. N., Tomosugi, N., Kawabata, H., Ishikawa, T., Nishikawa, T., and Yoshizaki, K. (2010). Down-regulation of hepcidin resulting from long-term treatment with an anti-IL-6 receptor antibody (tocilizumab) improves anemia of inflammation in multicentric Castleman disease. *Blood* 116, 3627–3634.

Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., D'agay, M. F., Clauvel, J. P., Raphael, M., Degos, L., and Sigaux, F. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86, 1276–1280.

Stallone, G., Schena, A., Infante, B., Di Paolo, S., Loverre, A., Maggio, G., Ranieri, E., Gesualdo, L., Schena, F. P., and Grandaliano, G. (2005). Sirolimus for Kaposi's sarcoma in renal-transplant recipients. *N. Engl. J. Med.* 352, 1317–1323.

Staskus, K. A., Sun, R., Miller, G., Racz, P., Jaslowski, A., Metroka, C., Brett-Smith, H., and Haase, A. T. (1999). Cellular tropism and viral interleukin-6 expression distinguish human herpesvirus 8 involvement in Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. *J. Virol.* 73, 4181–4187.

Suda, T., Katano, H., Delsol, G., Kakiuchi, C., Nakamura, T., Shiota, M., Sata, T., Higashihara, M., and Mori, S. (2001). HHV-8 infection status of AIDS-unrelated and AIDS-associated multicentric Castleman's disease. *Pathol. Int.* 51, 671–679.

Tang, E. D., Nunez, G., Barr, F. G., and Guan, K. L. (1999). Negative regulation of the forkhead transcription factor FKHR by Akt. *J. Biol. Chem.* 274, 16741–16746.

Uldrick, T. S., Polizzotto, M. N., Aleman, K., O'Mahony, D., Wyvill, K. M., Wang, V., Marshall, V., Pittaluga, S., Steinberg, S. M., Tosato, G., Whitby, D., Little, R. F., and Yarchoan, R. (2011). High-dose zidovudine plus valganciclovir for Kaposi sarcoma herpesvirus-associated multicentric Castleman disease: a pilot study of virus-activated cytotoxic therapy. *Blood* 117, 6977–6986.

Uldrick, T. S., Wang, V., O'Mahony, D., Aleman, K., Wyvill, K. M., Marshall, V., Steinberg, S. M., Pittaluga, S., Maric, I., Whitby, D., Tosato, G., Little, R. F., and Yarchoan, R. (2010). An interleukin-6-related systemic inflammatory syndrome in patients co-infected with Kaposi sarcoma-associated herpesvirus and HIV but without multicentric Castleman disease. *Clin. Infect. Dis.* 51, 350–358.

Walts, A. E., Shintaku, I. P., and Said, J. W. (1990). Diagnosis of malignant lymphoma in effusions from patients with AIDS by gene rearrangement. *Am. J. Clin. Pathol.* 94, 170–175.

Wang, L., and Damania, B. (2008). Kaposi's sarcoma-associated herpesvirus confers a survival advantage to endothelial cells. *Cancer Res.* 68, 4640–4648.

Waterston, A., and Bower, M. (2004). Fifty years of multicentric Castleman's disease. *Acta Oncol.* 43, 698–704.

Yiakoumis, X., Pangalis, G. A., Kyrtsonis, M. C., Vassilakopoulos, T. P., Kontopidou, F. N., Kalpadakis, C., Korkolopoulou, P., Levidou, G., Androulaki, A., Siakantaris, M. P., Sachanas, S., and Andreopoulos, A. (2011). Primary effusion lymphoma in two HIV-negative patients successfully treated with pleurodesis as first-line therapy. *Anticancer Res.* 30, 271–276.

Zmonarski, S. C., Boratynska, M., Rabczynski, J., Kazimierczak, K., and Klinger, M. (2005). Regression of Kaposi's sarcoma in renal graft recipients after conversion to sirolimus treatment. *Transplant. Proc.* 37, 964–966.

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