Determination of KSHV lytic DNA replication origin using SMARD and its control mechanism

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by

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ABSTRACT

While it is clear that Kaposi’s Sarcoma-associated Herpesvirus (KSHV) is the etiologic agent of Kaposi’s sarcoma (KS) lesions and non-Hodgkins lymphomas such as Primary Effusion Lymphoma (PEL) and Multicentric Castleman’s Disease (MCD), the molecular mechanism of lytic replication is still unclear. It is during lytic phase of viral life cycle virions are produced and tumorigenesis is induced in the host, thereby demonstrating the importance of discerning the mechanism of lytic replication in order to advance anti-viral treatments.

KSHV lytic replication is initiated by ORF50/RTA, an immediate early lytic protein, which acts as a transcription factor activating downstream lytic factors. Previous research has indicated that RTA, along with other lytic proteins, binds to KSHV origin of lytic replication (oriLyt) in order to initiate viral DNA synthesis essential for virion production. One of these proteins is the KSHV processivity factor, ORF59, which has been shown to be crucial for the synthesis of viral transcripts by viral polymerase, ORF9. As part of this study, we have determined that although an ex-vivo assay suggests that KSHV possess two fully functional origins of lytic replication (oriLyt-L and oriLyt-R), the virus primarily uses oriLyt-L for initiating replication. Here, we also demonstrate that ORF59 is phosphorylated by a viral kinase, ORF36, which modulates its processivity function required for virion production. We also analyzed the interaction of cellular factors with ORF59 in initiating lytic replication and as a result determined that PRMT1 and PRMT5, protein arginine methyltransferase 1 and 5, to be involved in modifying chromatin confirmation at oriLyt and thereby playing an important role in initiating lytic replication.
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<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BAC36</td>
<td>KSHV recombinant BAC</td>
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<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<tr>
<td>CoIP</td>
<td>Co-immunoprecipitation</td>
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<td>CTL</td>
<td>Cytotoxic T cells</td>
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<td>DM1-3</td>
<td>ORF59 deletion mutants 1 through 3</td>
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<td>DMVECs</td>
<td>Dermal microvascular endothelial cells</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dnmt</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DOG</td>
<td>2-Deoxygalactose</td>
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<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
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<tr>
<td>E</td>
<td>Early gene</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>FISH</td>
<td>Fluorescent in situ Hybridization</td>
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<tr>
<td>galK</td>
<td>Galactokinase gene</td>
</tr>
<tr>
<td>GCV</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>H4R3me2as</td>
<td>Asymmetrically dimethylated arginine 3 of histone 4</td>
</tr>
<tr>
<td>H4R3me2s</td>
<td>Symmetrically dimethylated arginine 3 of histone 4</td>
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<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>hCMV</td>
<td>Human Cytomegalovirus</td>
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<td>Human herpesvirus 8</td>
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<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<td>HREs</td>
<td>Hypoxia response elements</td>
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<td>HSV</td>
<td>Herpes simplex virus</td>
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<td>HUVECs</td>
<td>Human umbilical vascular endothelial cells</td>
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<tr>
<td>IE</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kd</td>
<td>Kinase dead</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KLIP-1</td>
<td>KSHV LANA-interacting protein 1</td>
</tr>
<tr>
<td>KS</td>
<td>Kaposi's sarcoma</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s Sarcoma-associated Herpesvirus</td>
</tr>
<tr>
<td>LANA</td>
<td>Latency-associated nuclear antigen</td>
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<tr>
<td>MCD</td>
<td>Multicentric Castleman’s disease</td>
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<tr>
<td>MLL</td>
<td>Mixed Lineage Leukaemia</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N-MCT</td>
<td>N-methanocarbathymidine</td>
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<tr>
<td></td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NF-kB</td>
<td>Natural killer cells</td>
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<td>NuMA</td>
<td>Nuclear mitotic apparatus protein</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>oriLyt</td>
<td>Origin of lytic replication</td>
</tr>
<tr>
<td>oriP</td>
<td>Origin of latent replication</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PCAF</td>
<td>CBP/p300–associated factor</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PEL</td>
<td>Primary effusion lymphoma</td>
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<td>PF-8</td>
<td>KSHV processivity factor, ORF59</td>
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<tr>
<td>PFA</td>
<td>Fascarnet</td>
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<td>PM1-3</td>
<td>ORF59 point mutants 1 through 3</td>
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<tr>
<td>PRMT1</td>
<td>Protein arginine methyltransferase 1</td>
</tr>
<tr>
<td>PRMT5</td>
<td>Protein arginine methyltransferase 5</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RBP-Jk</td>
<td>Recombination signal binding protein Jk</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RRE</td>
<td>RTA response element</td>
</tr>
<tr>
<td>RRF</td>
<td>Region replicated first</td>
</tr>
<tr>
<td>RRL</td>
<td>Region replicated last</td>
</tr>
<tr>
<td>RRV</td>
<td>Rhesus Rotavirus</td>
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<tr>
<td>RTA</td>
<td>Replication and transcriptional activator</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SMARD</td>
<td>Single molecule analysis of the replicated DNA</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TR</td>
<td>Terminal repeat</td>
</tr>
<tr>
<td>UNG2</td>
<td>Uracil DNA glycosylase 2</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel Lindau</td>
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<tr>
<td>vIRF</td>
<td>Viral interferon regulatory factor</td>
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INTRODUCTION

Viruses and Cancer

Carcinogenesis is a complex, multi-step process during which a series of mutations take place in a number of cellular molecules, which have been shown to regulate cell metabolism, proliferation, and cell apoptosis (70). Infectious cancer agents, such as viruses, bacteria and parasites, express or promote the expression of oncogenes that directly contribute to cell transformation and the maintenance of tumor cell phenotype thereby accelerating this process. Not surprisingly, DNA tumor viruses have been implicated in 15-20% of all human cancers (12, 78). These cancers constitute a considerable public health burden in developing and low-income areas of developed nations with lengthy treatment regiments and eventual death (73, 78). A proportion of these cancers are caused by the members of γ-herpesviruses family including Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV).

KSHV, also known as Human herpesvirus 8 (HHV8), is the etiologic agent of Kaposi’s sarcoma (KS), Primary Effusion Lymphoma (PEL) and Multicentric Castleman’s Disease (MCD), which are more common in individuals with deficient immune systems. Although KS lesions were documented more than 100 years ago as a skin cancer effecting elderly, mainly Jewish men of Ashkenazi origin in Vienna, Austria, KSHV was not identified until mid 1990s in an AIDS-associated Kaposi’s sarcoma (53).

The concern for KS rose in the western world along with the onset of AIDS epidemic in the 1980s. In 1994, a group at Columbia University, New York used representational difference analysis, a PCR-based technique, to identify the DNA sequence of a new γ-herpesvirus from a KS lesion thereby coining the term KSHV (27, 66). Its prevalence was
noted in drug injection users, sexually active homosexual men, and women with multiple sex partners while KSHV transmission through blood transfusion was estimated to be low. Although defining risk factors of KSHV transmission have been somewhat controversial, its associated diseases have been statistically linked with the onset of AIDS and immune suppression. Interestingly, high frequency of KSHV detection in tonsil swabs from HIV-negative KS patients suggests an oral route to be the predominant route of infection (18).

Although in the United States successful reduction in KS has been accomplished with highly active anti-retroviral therapy (HAART), in some cases remission has been noted within 6 months post treatment termination (88). It is clear that immune restoration via HAART is vital in improving the prognosis of AIDS-KS patients as is evident by their increase in CD4+ and CD8+ anti-KSHV cytotoxic T-lymphocytes (CTL) counts as well as anti-KSHV antibody titers (120). HAART has been also shown to restore anti-PEL natural killer (NK) cell’s activity in these patients, which has been associated with the loss of KSHV DNA detection in patient’s peripheral blood mononuclear cells (PBMCs) (104). However, as indicated by a case report, in some circumstances an interruption of HAART after years of treatment may result in a rapid rebound of KSHV DNA load and patient’s seropositivity (89). Additionally, some patients show a lack of response to HAART, which is associated with AIDS-associated KS progression (20). With current therapeutics, the fate of KS-infected patients will depend on the continued success of HAART treatment.
KSHV genome structure

Gammaherpesvirus subgroup, like other herpesviruses, has a relatively large, double-stranded DNA genome that is 140-160 kilobases (kb) long. The KSHV encodes more than 90 open reading frames (ORFs) and is flanked by GC-rich terminal repeats (53, 66). The size of the genome may vary amongst different KSHV isolates depending on the number of these terminal repeats. The genetic structure of KSHV is similar to herpesvirus saimiri (HVS) and a γ-2-herpesvirus, which infects squirrel monkeys, and other non-human γ-herpesviruses such as rhesus rhadinovirus (RRV) and murine gamma herpesvirus (MHV-68) (31, 34).

Similar to other herpesviruses, KSHV genes have been further categorized into lytic and latent genes and also into immediate-early (IE), early (E), and late (L) genes depending on their sequence of expression. In general, KSHV has three classes of genes: 1) genes that are highly conserved among all herpesviruses, 2) KSHV specific genes, which are to KSHV, designated with “K” (K1-K15), and 3) genes that are classified to have cellular homologs and have been pirated from the host genome over time (122). Interestingly, KSHV has at least 11 open reading frames (ORFs) that encode for cellular homologs involved in signal transduction, cell cycle regulation and inhibition of apoptosis and immune response (21). This ability to acquire host genes undoubtedly enables KSHV to hijack many of the host processes to promote tumorigenesis.

KSHV Pathogenesis

After its identification in KS lesions, KSHV was subsequently identified in patient samples with PEL and MCD (22, 106). Although KSHV infection is necessary for its associated diseases but is not sufficient for inducing pathologies. An HIV infection and
immunosuppression significantly increases the risk of KSHV-associated diseases. It is important to note that in addition to these three typical KSHV-associated diseases, other conditions, such as hemophagocytic lymphohistiocytosis have been associated with KSHV (42).

*Kaposi’s Sarcoma (KS)*

Thus far, four types of KS have been classified—classic, endemic, iatrogenic, and epidemic. Classic KS is a nonaggressive disease noted in elderly Mediterranean men as cutaneous lesions on their lower extremities. Endemic KS affects people living in sub-Saharan African countries and is known for its aggressive nature. Iatrogenic KS develops in individuals undergoing immunosuppressive therapy following an organ transplant and can range from chronic, relatively harmless or a rapid disease involving lymph nodes, mucosa and inner organs. None of these classifications are thought to be associated with HIV. On the other hand, epidemic KS, which is AIDS-associated, is the most common cause of tumor development in this group of individuals and has been the basis for further studies (36).

Despite its various classifications, KS lesions are indistinguishable from each other. The tumor is histologically complex with spindle-like KSHV-infected endothelial cells embedded with reticular and collagen fibers and infiltrated with various inflammatory cells including B and T cells and CD14+/CD68+ monocytes. The tumor is highly vascular and contains unusually dense and irregular blood vessels, which leak red blood cells into the surrounding tissue resulting with the defining dark color of the KS lesion (112). Although classic KS is found in the lower extremities, AIDS-associated KS
usually involves not only the skin on the face, extremities and torso, but also the mucosal membrane of the oral cavity and gastrointestinal tract (28).

**Primary Effusion Lymphoma (PEL)**

Although PEL is encountered in less than two percent of HIV positive individuals and is even more rare in general population, it is considered to be associated with high mortality because of its nature as a recurring malignant effusion localized in the pericardial and peritoneal space. PEL is divided into two variants - classic and solid - where classic is characterized by lymphomatous involvement in the serous membrane and solid starts out as a tissue-based tumor with no malignant effusions. Both are similar in their morphology, immunophenotype and molecular characteristics with invariable detection of KSHV (15, 23, 79).

PEL tumor cells have been frequently identified to have not only the presence of KSHV but also elevated levels of interleukin, such as IL-6. Despite its B cell origin, the phenotypic cell surface markers common among this cancer are generally neither B cell (CD19 and CD20) nor T cell (CD2, CD3, CD5 and CD7) markers, but are more akin to multiple myeloma cell lines (CD45, CD30, CD38, CD138 and MUM1) (79). Additional studies are being conducted to further explore the role of KSHV in development of PEL and its role in the aggressive nature of this lymphoma.

**Multicentric Castleman’s Disease (MCD)**

MCD is considered to be localized and aggressive lymphoproliferative disorder characterized by expanded germinal and B cell proliferation as well as vascular expansion. Unlike in KS and PEL, KSHV can be detected in only a subset of MCD patients. However, KSHV is frequently detected in HIV-positive MCD patients (3, 83,
In all MCD, B cell hyperproliferation is thought to be due to autocrine and paracrine signaling in addition to increased levels of IL-6. Reports have shown the production of KSHV-encoded IL-6 and upregulation of NF-κB by viral FLICE accompanied by overall cytokine abnormalities to be important for disease manifestations (3, 110). Little is known about MCD and its KSHV-related pathogenesis and further studies are underway. Interestingly, with further analysis of KSHV genome sequence and the individual viral open reading frames (ORFs), additional mechanisms for oncogenesis were discovered (97). These include transiently expressed viral genes and cellular orthologs, such as viral interferon regulatory factors (vIRFs 1-4) and viral BCL-2 (ORF16) that are involved in modulating the immune response as well as cell growth and apoptotic pathways (11, 51, 77). Furthermore, recent studies suggest that KSHV drives transcriptional reprogramming in infected blood endothelial cells shifting them toward a lymphatic phenotype (50, 80, 116, 119). This remains controversial but has been suggested to create a microenvironment favorable for KSHV tumorigenesis (1).

KSHV persists as a multicopy episome in the nucleus of latently infected cells. Although tethering the viral genome to the host chromosome is slightly controversial, latency-associated nuclear antigen (LANA) is believed to play a crucial role in securing KSHV episome to histones thereby allowing for the transfer of the episome into each of the daughter cells during cell division. Despite success seen with HAART treatment, LANA is likely to be the reason for viral persistence in the host as up to 50% of AIDS-KS co-infected patients still experience some symptoms and there is no cure (13). KS is still the leading cause of death among African population and prevalent in communities of developing nations (33, 72). With over a decade of research, it is logical to assume that
the complicated nature of KSHV is further magnified by additional co-factors, which we may not be aware of. Additional research is vital in discerning these co-factors and controlling the disease.

**Viral life cycles**

Similar to other herpviruses, KSHV has a bi-phasic life cycle consisting of latent phase where a limited number of genes are expressed and the virus is subdued, and lytic replication phase where most of the genes are expressed and virions are produced (14). Latency is essential for persistent KSHV infection, evasion of the host immune system and the resulting malignancies. Upon reactivation, the virus is capable of modulating several diverse cellular pathways to promote lytic replication and tumorigenesis. The balance between latency and lytic reactivation is regulated by a complex mechanism and undoubtedly involves viral as well as cellular factors.

**Latent Phase**

In immunocompetent individuals, KSHV establishes latency after an initial acute infection. Since only few of the viral genes are expressed and no virions are produced, KSHV is able to evade host immune surveillance and facilitate the establishment of a life-long latent infection. These cells are viewed as a reservoir of chronic viral infection tightly regulated by the host’s immune system allowing for the virus to emerge when the host faces additional physical stress.

During latency, KSHV persists as a circular episome associated with histone in the nucleus via viral latency-associated nuclear antigen (LANA). KSHV latent replication initiates at oriP and proceeds in a bidirectional manner in synchronous with cellular replication due to its dependence on host DNA polymerase and accessory factors. LANA
allows for KSHV genome to remain tethered to mitotic chromatin, which enables it to maintain steady number of viral copies per host cell while avoiding being detected by the host immune system since majority of viral genes are silenced.

Recent studies have illustrated the importance of histone modifications in silencing lytic gene expression and promoting viral latency (44, 109). During a de-novo infection in endothelial cells, KSHV genome is heavily methylated at CpG islands followed by specific chromatin-condensing histone modifications that promote a rapid onset of latency. These repressive histone marks are not uniform throughout KSHV genome and in fact parts of the genome contain activation marks. In particular, H3K9/K14-ac, activating histone marks remain at several promoters of lytic genes including RTA (44). Chromatin Immunoprecipitation assays in latent BCBL-1 cells have detected both activating marks of H3K9/K27-ac and H3K4-me3 as well repressive marks of H3K27-me3 and H3K9 with vast majority of the genome displaying the repressive marks. Promoters of RTA as well as several other lytic proteins show both activating, H3K4-me3 and repressive marks H3K27-me3 (109). Presence of these mutually exclusive chromatin modifiers throughout the KSHV genome is significant as it shows the delicate balance between latency and lytic reactivation. With an appropriate trigger, lytic reactivation can occur quickly and efficiently as the repressive marks are eliminated and activation marks are free to open up the chromatin for transcription and replication.

Interestingly, although most of the KSHV genome displays repressive mark H3K27-me3, there is a significant reduction in the latency locus, which encodes LANA, vCyclin (ORF72), vFLIP (ORF71), and a cluster of 25 matured microRNAs (miR-K1-12) (32, 111). LANA, vCyclin, and vFLIP are transcribed as multiple polycistronic mRNAs.
Based on epigenetic studies conducted so far, these as well as miRs mentioned above are the only viral genes expressed in KS cells during latency. Latently infected B cells, PEL and MCD, have shown an additional expression of LANA-2/vIRF3 (ORF-K10.5), which suggests that KSHV latent gene expression is managed by cell or host specific factors and the differential expression may drive the development of different diseases within the host.

LANA

LANA, or the latency-associated nuclear antigen, is encoded by ORF73. It was first identified as a doublet of 220-230 kDa by Western blot and is considered to be the most immunogenic latent antigen in KSHV infected individuals (39). As previously mentioned, LANA is sufficient and necessary for KSHV genome maintenance in host cells. Both C- and N-terminus of LANA mediate the binding to terminal repeats (TRs) in KSHV genome tethering the KSHV episome to host chromosome during mitosis allowing for proper segregation of the viral DNA into daughter cells (7, 8, 30, 40). Several chromatin-binding proteins have been described to interact with LANA assisting its tethering function. For example, LANA has been shown to interact with bromodomain protein Brd4 through its carboxyl-terminal domain as well as methyl-CpG-binding protein MeCP2, DEK, and nuclear mitotic apparatus protein (NuMA) (58, 75, 103, 128). Their co-localization during different stages of the cell cycle reveals the numerous mechanisms utilized by the virus to maintain its genome in host cells.

Recently, we have also shown that LANA recruits TopoIIβ to the TR region where TopoIIβ induces double-stranded breaks required for replication (92). Despite the absence of viral DNA polymerase, KSHV manages to replicate its genome along with the
host cells. To do this, LANA is thought to recruit poly (ADP-ribose) polymerase 1, or PARP1, a cellular DNA polymerase and additional factors, such as origin recognition complex 2 (ORC2), CDC6, uracil DNA glycosylase 2 (UNG2) and Mcm7 to the TR region where latent viral replication is initiated (64, 82, 107, 113-115). Indeed, the association of ORCs with TR is LANA dependent where both N- and C-terminus are required (10, 55, 114).

Furthermore, LANA promotes latency by inhibiting viral lytic gene expression. Specifically, LANA interacts with mSin3 corepressor complex and KSHV LANA-interacting protein 1 (KLIP-1), a nuclear transcription repressive protein, to downregulate lytic gene transcription of thymidine kinases (59, 87). Through DNA methylation via DNA methyltransferase (Dnmt) recruitment, LANA also mediates chromatin compactness at RTA promoter thereby regulating its transcription (62, 68, 100). Evidence also suggests that LANA directly binds to RTA inhibiting its transactivating activity effectively disrupting the lytic gene cascade resulting in lytic reactivation (61).

Additionally, LANA advances latency by promoting survival and cell growth through its interaction with p53 and pRb tumor suppressors (16, 37, 54, 95). Although the functional as well as transcriptional inhibition of p53 by LANA is partial, PEL cells display a higher tolerance for DNA damage (37). Cell cycle progression is also altered in KSHV-infected cells as LANA acts to stabilize c-Myc oncogene by preventing its phosphorylation at T58 and activate it by independently stimulating its phosphorylation at S62 (67). Evidence shows that a knockdown of c-Myc reactivates the viral gene expression and increases apoptosis while its overexpression inhibits lytic replication (63). It is likely that LANA-
mediated manipulation of c-Myc contributes to KSHV-associated tumorigenesis by activating c-Myc regulated genes and stimulating cell cycle progression.

Lytic Phase

KSHV is readily activated when the host’s immune system is impaired or damaged by immune suppressants or disease. In these immunocompromised individuals, KSHV transcription and replication activator (RTA or ORF50) is expressed first as an immediate early (IE) gene, followed by early (E) genes such as MTA (ORF57) and viral processivity factor (ORF59), and late genes that complete the viral capsid and aid in viral egress from the cell. For oncolytic viruses, these genes not only drive the synthesis of new virions but also promote cell proliferation, angiogenesis, and local inflammation, which lead to tumorigenesis (18, 29, 38, 50, 65, 80, 86, 93, 94, 98, 102, 126). The importance of KSHV lytic replication is substantiated by clinical observations that KS tumor progression is strongly correlated with titers of KSHV lytic proteins (19, 20, 35, 43, 71, 91, 105). In addition, although a majority of the cells in KS tumors are latent, a small number undergoes lytic replication. Furthermore, regression in these mostly latent KS tumors has been reported following a treatment with valacyclovir, a molecule that targets viral polymerase used to inhibit KSHV lytic replication (52, 74, 76).

Lytic replication originates at a specific site (oriLyt) and proceeds in a rolling circle manner. KSHV has been documented to have oriLyt-L located between K4.2 and K5, and oriLyt-R located between K12 and ORF71 (6). The sequences for these two origins are virtually identical and both have been shown to support lytic replication ex-vivo (6). Three essential sequence motifs are conserved in both oriLyts: 1) 18 bp AT-palindrome,
2) eight C/EBP binding motifs, arranged as four spaced palindromes, and 3) an RTA response element (RRE) followed by a TATA box (118).

It is important to note that KSHV lytic replication is independent of cellular replication due to all the necessary proteins being encoded and expressed by the virus. A number of viral proteins, including RTA (transactivator), ORF59 (processivity factor), ORF9 (viral DNA polymerase), ORF6 (a single-stranded DNA binding protein), ORF40/41 (primase-associated factor), ORF44 (helicase), and ORF56 (primase) have been shown to be the part of replication complex, which binds to oriLyt and initiate lytic replication (96).

**RTA**

Expression of RTA is both necessary and sufficient to induce KSHV lytic replication (41, 69, 108). RTA acts as its own transcription factor to promote its expression as well as activate further downstream genes required for lytic replication and virion production such as vIL-6, polyadenyated nuclear RNA (PAN), MTA, K8, vIRF1, ORF-K1, small viral capsid protein (ORF65), ORF56, SOX (ORF37), vOX, and ORF52 (125-132). RTA binds to the promoters of these genes through either a specific DNA sequence designated as the RTA response element (RRE) or via direct interaction with DNA-binding proteins such as Sp-1, octamer-binding protein-1 (Oct-1) and XBP-1 (17, 26, 99, 121, 127, 130).

In addition, RTA-mediated activation of viral promoters depends on its ability to recruit CBP, the SWI/SNF chromatin remodeling complex, and the TRAP/mediator coactivator. RTA binds to the Brg1 subunit of SWI/SNF and TRAP230 subunit of TRAP/mediator via a short acidic segment in the carboxyl region unwinding the chromatin in a specific and sequential manner for lytic gene expression (46).
MTA

All members of the herpesvirus family encode multiple lytic cycle transactivators that cooperate with other proteins to promote lytic replication. KSHV encodes for MTA, which synergizes with RTA to not only elevate the expression levels of RTA mRNA but also to enhance transcription of nut-1/PAN and kaposin (56). MTA boosts accumulation of viral and well as cellular mRNA in a gene-specific manner based on the sequence within the promoter (45, 47, 56, 81). A putative A/T hook domain within MTA has been identified as a contributor to DNA binding and transactivation function of MTA. Initial expression of RTA is critical as MTA alone has little effect on PAN or kaposin; therefore, the data suggests that synergism between MTA and RTA strictly depends on RTA’s ability to activate transcription (84).

ORF59

Identified in BCBL-1 cells, ORF59 or the KSHV processivity factor (PF-8) is an essential lytic replication early-late protein localized to KSHV-infected cell nuclei (24). ORF59 is of great interest, as it is known to bind to viral DNA polymerase Pol-8 encoded by ORF9 enabling it to remain attached to the DNA strand and synthesize full-length fragments (65). Upon further characterization of ORF59, it was determined that its function is dependent on its ability to form a homodimer, which enhances its binding to double-stranded DNA and enables it to shuttle viral DNA polymerase into the nucleus for lytic replication (123). Crystal structure analysis of ORF59 indicate the formation of homodimer to be head-to-head resulting in a C clamp. The concave face of the clamp contains a number of basic residues important for ORF59’s binding to DNA (4, 9).
ORF59 binding sites to dsDNA and ORF9 as well as its processivity domain have been mapped (25).

Interestingly, ppUL44, an ORF59 human cytomegalovirus (hCMV) homolog, also binds to the viral polymerase and acts as a processivity factor. During viral infection, ppUL44 is phosphorylated by the viral kinase pUL97 as well cellular protein kinase CK1 and CK2. Specifically, the differential phosphorylation of ppUL44 modulates its ability for nuclear translocation. Phosphorylation of S413 residue by CK2 triggers further phosphorylation cascade resulting in a negative charge at S418 and S415, which facilitates optimal nuclear translocation. Conversely, a negative charge on T427 results in significant decrease in nuclear location of ppUL44 thereby diminishing its function as a processivity factor (2). ORF59 has been recognized as a phosphoprotein, but little is known about the role of this modification and its effect on KSHV lytic replication (25).

**Reactivation Triggers**

While various host and environmental factors can trigger KSHV reactivation, most of them achieve this indirectly by activating certain signaling pathways. For example, hypoxia is thought to induce lytic replication by promoting transcription of hypoxia response elements (HREs) that further bind to RTA and ORF34 promoters to activate their transcription (48, 49). HIV-1 transactivator, Tat, has been shown to be sufficient in inducing RTA expression, which would vindicate the accelerated aggressive nature of AIDS-KS tumors (129). Oxidative stress and reactive oxygen species (ROS) have also been shown to induce lytic replication in KSHV-infected BCBL-1 cells by activating the MAPK pathways. More importantly, reactivation of KSHV appears to be dependent upon the balance between AP-1 and NF-κB pathways. With primary infection in human
umbilical vascular endothelial cells (HUVECs), KSHV has been shown to activate MEK/ERK, JNK and p38 MARK pathways, which results in activation of AP-1. This promotes lytic replication as RTA, MTA and K8 contain functional DNA-binding sites for AP-1 and therefore are responsive to AP-1 activation (5, 117, 124). On the other hand, there is an immediate shift to latency in dermal microvascular endothelial cells (DMVECs) following a primary infection (57, 60). Although primary infection in DMVECs upregulates the ERK pathway, it also strongly upregulates the NF-κB pathway, which promotes survival and cell growth favorable for latency (85, 98, 101, 125).

Scope of the Thesis

Almost two decades of KSHV research has identified two origins of lytic replication, oriLyt-L and oriLyt-R, as well as the major viral factors involved in initiation of lytic replication. Recent research has indicated the involvement of cellular factors, such as chromatin modifiers, in regulating latency and playing a role in the switch to lytic replication. However, although the origins of lytic replication have been compared and analyzed ex-vivo, little has been done to explore their function and modification in the context of the whole KSHV genome. Considering the importance of lytic replication in driving tumorigenesis, it is critical to further investigate the control mechanism for the initiation of lytic replication and the factors involved. In this thesis, I identify the preferentially used origin of lytic replication in the context of entire viral genome using BCBL-1 cells. I also investigate the histone modifications associated with chromatin modulation at oriLyt during lytic reactivation to show the delicate balance between chromatin activating and repressing marks. My research also considers the impact of
KSHV processivity factor, ORF59, phosphorylation in regards to lytic replication and viral production.

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Chapter 1.

Single Molecule Analysis of Replicated DNA Determines oriLyt -L as the Functional Origin for Lytic DNA Replication in the Context of Viral Genome.

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ABSTRACT

Kaposi’s sarcoma associated herpesvirus (KSHV) also called human herpesvirus 8 (HHV8) is linked to various human malignancies including Kaposi’s sarcoma, Primary Effusion Lymphoma (PEL) and Multicentric Castleman’s Disease (MCD). KSHV, like other herpesviruses establishes life long latent infection with the expression of minimal genes to escape the host immune surveillance. Latency Associated Nuclear Antigen (LANA), detected in every KSHV infected cells, helps in suppressing virus reactivation from latency but a fraction of cells remains in lytic phase and produce viral progenies to infect fresh cells. During reactivation, virus undergoes a cascade of events to produce viral replication proteins, which accumulate on oriLyt (origin of lytic DNA replication) in sequence specific manner. KSHV genome has two origins, oriLyt-L and oriLyt-R, in the left and right ends of the genome, respectively. These sites have 95% sequence similarity and can support replication in transient replication assays when the trans acting viral proteins were expressed. However, the roles of these origins in the context of viral genomes were not determined. Using a single molecule analysis of the replicated DNA (SMARD) approach on KSHV infected BCBL-1 cells (TRExBCBL1-Rta) and lytic KSHV BAC (BJAB-lyt-CR2), we determined that oriLyt-L is preferentially used for lytic DNA replication. Analysis of sufficient number of DNA molecules confirmed that replication forks progresses in a bi-directional as well as uni-directional manner from the oriLyt-L. This suggests that replication starts in a bi-directional manner before turning into a rolling circle method to synthesize concatameric copies of the genome.
INTRODUCTION

Kaposi’s sarcoma associated herpesvirus (KSHV), is the recent member of the human herpesvirus family and is tightly linked with various human malignancies including, Kaposi’s Sarcoma (KS), Primary Effusion Lymphomas (PELs) and Multicentric Castleman’s Disease (MCD) [1,2,3]. KSHV establishes life long latent infection by expressing a handful of genes required for viral genome tethering and persistence [2,3]. Latency Associated Nuclear Antigen (LANA) is one of the predominantly expressed proteins in every KSHV infected cells, which helps in viral genome persistence and deregulation of various cellular and viral pathways for the benefits of the virus [3,4,5]. LANA deregulate tumor suppressor pathways by degrading pRb, p53 and von Hippel Lindau (VHL) proteins through ubiquitylation [6,7,8]. LANA also recruits various cellular kinases, PIM1, GSK-3b and AuroraA kinase to phosphorylate itself as well as various cellular targets to induce cell proliferation [9,10,11]. Modulation of these pathways by the latency proteins helps in cell proliferation and tumor growth [3,11]. However, lytic replication has also been shown to play important role in inducing tumorigenesis as several studies have found that the risk of KS was significantly reduced in AIDS patients who received lytic replication blocking drugs, ganciclovir (GCV), Foscarnet (PFA) and N-methanocarbathymidine (N-MCT) [12,13,14,15,16]. Indeed, a small fraction (2-5%) of latently infected cells continuously undergo lytic reactivation and produces cytokines, which acts in a paracrine manner to promote tumorigenesis [17,18]. Also, the virion produced from the fraction of lytic cells in latent tumors infects fresh cells in order to enhance tumor progression [17,19].
Various stimuli and cellular stresses can trigger reactivation of the virus from latency. Replication and Transcriptional Activator (RTA), an immediate early protein, is required for lytic reactivation [20]. LANA and RTA control the expression of each other to maintain a homeostasis but a shift towards RTA expression leads to the reactivation and expression of viral genes, including early and late genes, required for viral DNA synthesis and virion production [20,21]. The genes required for DNA replication, include ORF9 (DNA polymerase), ORF6 (single stranded DNA binding protein), ORF40/41 (primase-associated factor), ORF44 (helicase), ORF56 (primase), ORF59 (a processivity factor), K-bZIP and RTA (major replication and transcriptional activator) [20]. Among these proteins, K-bZIP is shown to be dispensable for lytic DNA replication as K-bZIP deleted KSHV BACs were able to replicate efficiently [22]. Additional studies demonstrated that K-bZIP plays a role in modulating latent and lytic phases of the virus life cycle by directly interacting with LANA [23]. Since RTA was able to bind oriLyt through the C/EBPα in the absence of K-bZIP and is critical for replication, RTA is considered to be a replication initiator [23]. RTA also binds to the RTA Response Element (RRE) to activate promoter and facilitate transcription of a short oriLyt transcript required for lytic DNA replication [24,25].

Previous studies mapped the lytic replication origins in the left and right end of the genome, oriLyt-L and oriLyt-R, respectively with almost identical sequence [25,26,27]. Both of these sites are capable of replicating plasmid in transient replication assay when the accessory proteins are expressed in trans [25,27]. Although the transient replication assays are great tool for detecting the replication of plasmids, they cannot be used for determining the roles of each origin in the context of the viral genome. Recombinant
virus approach, where point mutations and deletions are introduced, has proven to be a useful tool for determining the roles of replication origins [28]. KSHV recombinant BAC (BAC36) in which either or both the origins were deleted showed that oriLyt-L deleted BACmid were defective in lytic DNA replication and virion production [28]. Since these studies were performed on a deleted KSHV genome that may have affected the expression of viral genes required for DNA replication, we decided to use in cell labeling and replication origin detection on wild type KSHV genomes [29]. Additionally, the replication based on DpnI sensitivity assays used in earlier studies determines the extent of replicated DNA but not the replication initiation sites [28]. Therefore, we used a very sensitive and accurate method to detect the replication initiation sites and the replication fork progression on individual DNA molecules using SMARD approach. SMARD is a powerful technique, which relies on the fact that replicating DNA incorporates halogenated nucleotides in a steady state manner therefore tracking the incorporated nucleotides on individual molecules determines the replication origin and replication fork progression [29,30]. We used this approach to determine the replication origins of KSHV genome during latent infection and our data showed that latent replication could initiate at multiple sites, which most likely is determined based on the epigenetic modification of the chromatin [29].

In this study, we used SMARD for the detection of functional lytic origin in wild type KSHV genomes using BCBL-1 and the KSHV genome cloned in a Bacterial Artificial Chromosomes (BAC) [31]. Since reactivation is required for the replication initiation at the oriLytss, we took advantage of a TREX system where RTA expression is controlled by tetracycline inducer [32]. We also used recombinant BAC36 in which the
expression of RTA, the main lytic switch regulator, was under control of constitutive promoter, Ppgk to robustly induce lytic replication [31]. Analysis of dually labeled molecules suggested that oriLyt-L is preferential used for genome replication. Additionally, analyzing the progression of replication forks showed movement of forks in both directions respective to the origin and thus suggests that replication at these origins initiates in a bi-directional manner.

**MATERIALS AND METHODS**

**Cell culture and antibodies:**

KSHV infected, TRExBCBL1-Rta, a generous gift from Dr. Jae Jung (University of Southern California), and BJAB with BAC36-lyt-CR2 (BJAB-lyt-CR2) were grown in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin-streptomycin (5 U/ml and 5 mg/ml, respectively). TRExBCBL1-Rta cells were induced for lytic replication with 1 µg/ml of doxycycline (Dox; BD Clontech, Palo Alto, CA). Antibodies, rat anti-LANA (Advanced Biotechnologies, Inc), rabbit anti-RTA and mouse anti-GAPDH (US Biological) were used as previously described [33]. Primary antibodies to detect IdU, and CldU were mouse anti-IdU and rat anti-CldU as described previously [33]. Secondary antibodies, Alexa Fluor 594 conjugated goat anti-mouse, Alexa Fluor 488 chicken anti-rat, were used to detect IdU and CldU as recommended (Life Technologies).

**Labeling of the replicating DNA:**

TRExBCBL1-Rta cells growing at approximately 5x10^5 cells/ml were induced for 24 hr with doxycycline followed by labeling them for 3 hr each with 25 µM 5’-ido-2’-deoxyuridine (IdU; first label) and 25 µM 5’-chloro-2’-deoxyuridine (CldU; second
IdU was directly added into the media as first label followed by low speed centrifugation (600xg) at RT to remove IdU. BJAB-lyt-CR2 was labeled with IdU and CldU as such without induction. Labeled cells were washed twice with warm PBS and resuspended in pre-warmed media with a second label, CldU at the density of 5x10^5 cells/ml. At the end of both labeling, cells were collected, washed and mixed with molten InCert agarose (Lonza Inc. Rockland, ME) to pour into the molds to make plugs at approximately 10^6 cells/plug.

**Isolation of replicated KSHV genome by Pulse Field Gel Electrophoresis (PFGE):**

Plugs containing TRExBCBL1-RTA and BJAB-lyt-CR2 were treated with cell lysis buffer (0.5 M EDTA+1% Sarcosine and Proteinase K) for 96 hr at 50°C as described previously [33]. Plugs were washed with TE containing PMSF (1mM) to inactivate Proteinase K followed by thorough washing with TE to remove cell debris and PMSF. Plugs containing KSHV genome were digested with 50 U of Pmel to linearize the genome. Digested plugs were loaded onto 0.6% low melt agarose (Seplaque, Lonza Inc., Rockland, ME) to resolve the linear KSHV genome from the cellular DNA in a pulse field gel electrophoresis using CHEF-DRII (Bio-Rad Laboratories, Hercules, CA). The location of KSHV genome banding on the gel was identified by transferring a part of the gel onto a nylon membrane (Hybond N+, GE Healthcare) and hybridizing with KSHV specific probe in a Southern blot. 1 mm agarose slivers containing the linear KSHV genome were excised and treated with gelase (Epicenter Biotechnologies Inc., Madison, WI) as per manufacturer’s recommendations to isolate entire length KSHV genome.

**Stretching, hybridization and immunostaining of individual DNA molecules:**

Linearized KSHV genomes were stretched on a silanized glass slides by capillary
method as described previously [30,34]. Solution containing DNA was gently deposited at the interface between silanized microscope slides and non-silanized coverslips and the DNA starched along with liquid meniscus due to the capillary force. The stretched DNA was visualized under a fluorescent microscope by staining with YOYO-1. Appropriate dilutions of DNA were used to get well-separated DNA molecules. The coverslips were removed gently followed by denaturing and fixing the DNA with 0.1 M NaOH and glutaraldehyde, respectively.

Fluorescent in-situ hybridization, to detect KSHV genome, was performed using biotin-16-dUTP (Roche Inc. Indianapolis, IA) labeled three asymmetric probed as described earlier [29]. These probes hybridize between regions, 36883-47193 bp (p10), 85820-100784 bp (p15) 26937-33194 bp (p6) on the KSHV genome (accession number NC_93331). The above indicated fragments were cloned into plasmid/cosmid vectors to amplify followed by purifying and labeling with biotin using Bio-nick labeling kit (Invitrogen, Inc., Carlsbad, CA) as per manufacturer’s recommendations. The probes were mixed with pre-hybridization buffer (40% Formamide and 10% Dextran sulfate) and incubated with stretched DNA at 37°C overnight. Slides were washed to remove non-specific signals followed by detection of hybridization signals with NeutrAvidin conjugated to Alexa Fluor 350 (Molecular probes, Eugene, OR) as described earlier [33]. Hybridization signals were amplified by adding five layers of Alexa Fluor 350 conjugated with NeutrAvidin and Biotinylated anti-Avidin antibodies (Vector Laboratories Inc., Burlingame, CA) after washing with PBS containing 0.03% NP40 (Sigma Aldrich, St. Louis, MO). The KSHV molecules were aligned in same orientation, post-image capture, by aligning the probes. Since the molecules were fully labeled it was
easy to detect the KSHV genome even in the presence of hybridization backgrounds (blue dots in hybridization probes panel of figure 2). The background hybridization signals do not affect the detection of red and green signal, therefore digitally removed in subsequent images (Figures 3, 4 and 6).

IdU and CldU were detected by immuno-staining of the stretched and hybridized DNA by using mouse anti-IdU and rat anti-CldU primary antibodies. Alexa Fluor 594 conjugated goat anti-mouse and Alexa Fluor 488 chicken anti-Rat were used as secondary antibodies to detect IdU and CldU, respectively. These antibodies are highly specific and do not show any cross hybridization as demonstrated earlier [33]. The slides were mounted with ProLong Gold (Life Technologies) and stored in the dark. The slides were observed and the images were captured using an automated fluorescent microscope (Axio Observer, Zeiss Inc., Thornwood, NY) with oil objective (63x). A large number of slides were scanned for the detection of probes, which ensured that captured images were of KSHV genome. We also made sure that all the three probes (blue color) were detected on DNA molecules to ensure the intactness of the molecules. Images of DNA molecules with all three colors (blue, green and red) were captured to determine the replication fork. The lengths of the molecules were measured post-image capture using an inbuilt feature of the image capture and analysis software (Axiovision, Zeiss In., Thornwood, NY). Molecules with all three colors and correct sizes (~65 µm) were cropped and aligned based on increasing red signal from top to bottom (molecule number 1 through 21 in figure 4 and 1 through 17 in figure 6).

**Determining the regions replicating first (RRF):**

Aligned molecules were marked at every 5 kb, which generated 32 segments in
KSHV genome. The molecules stained with red (first label) were given a score of 1 and percent red molecules in every interval were calculated based on the total number of molecules. The intervals with highest number of molecules stained in red shows that replication initiated in that region and therefore replicated first (RRF).

**Dual labeling to detect lytic DNA replication initiation:**

We previously used SMARD for the detection of latent origins of KSHV genome where replication proceeded in a bi-directional manner from an origin and the genome replicated only once per cell cycle [33]. However, the lytic replication does not follow the cell cycle pattern. Therefore, we modified the DNA labeling approach and reduced the first and second labeling periods with IdU and CldU, respectively for 3 hr each. This period was sufficient to label the entire length of the KSHV genome with halogenated nucleotides. Cells undergoing lytic replication will have replication forks at different locations on the genome at the time of halogenated nucleotide pulsing and thus will starts to incorporate (first label, IdU) from there to substitute rest of the molecule. However, if the nucleotide analogs are changed and replaced with different nucleotide analog (CldU, second label) then that starts to incorporate and the rest of the molecules are substituted with the second analog. The location of transition from the first to second label will indicate the site of replication forks at the time of nucleotide analog switch. Since we analyzed molecules, which are fully substituted, we determined three species of molecules. First sets are the molecules where replication started at the time of first pulse therefore it would be labeled with the first nucleotide analog throughout the length. The second sets are the molecules where replication has started but was not completed before the time of second pulse and therefore they would have both the first and second label
with transition sites marking the replication forks. Molecules with small portion of the first label indicate that those molecules started replication at the end of first label therefore the transition sites also mark the replication initiation site. The third sets are the molecules where replication starts during the second labeling period and therefore are labeled with only second nucleotide analog. Analyzing fully substituted molecules eliminates the bias of looking the replication fork at any particular region of the molecule thus faithfully represents the distribution of replication forks in a steady state population of replicating molecules [33].

Labeling of the molecules to fully substitute with either or both the nucleotide analogs are advantageous because it ensures that we are looking at the entire length of the molecule, which cannot be achieved by short labeling periods. Additionally, fully substituted molecules are easy to align with the genomic map, which helps in eliminating molecules that are broken or unevenly stretched from the final analysis. It also helps in detecting any overlaps with another molecule, which may affect the outcome of our analysis.

**Generation of recombinant KSHV-lyt-CR2:**

KSHV BACmid (BAC36) was recently determined to have 10 kb region duplicated within the terminal repeats along with the BAC cassette [35]. Duplication has partial ORF19, which contains the Pmel site, on the left side of the BAC cassette and ORF18 to K5 region on the right sides of the BAC cassette. Duplicated ORF18-K5 of the TR region was deleted by galK positive/counter selection (recombineering protocol, NCI). However, the removal of major duplicated region (ORF18-K5) did not remove the second Pmel site, which was located on the left side of the BAC cassette in the partial
ORF19 sequence. Since a unique restriction site is needed for SMARD, to linearize the genome, a recombinant BAC36 with deleted partial ORF19 sequence was generated by homologous recombination and counter selection. Briefly, the \( \text{galK-Kan} \) cassette was PCR amplified containing 50 bp-flanking sequence at the end of ORF19 on the left side in the sense primer and 50 bp BAC sequence in the anti-sense primer. PCR amplification yielded 2278 bp long PCR product containing the targeting sequence. PCR product was electroporated into a modified \( \text{E.coli} \) strain, SW102. This strain does not have galactokinase gene (\( \text{galK} \)) therefore only galK cassette containing bacteria after homologous recombination are able to grow on a minimal media with galactose as carbon source (positive selection). The \( \text{galK-Kan} \) cassette was removed by an oligo (double stranded) with homology to the flanking site after recombination and selection on a minimal media containing 2-Deoxygalactose (DOG). DOG is harmless unless phosphorylated by functional galK. Therefore, the colonies with removed galK after recombination were able to grow. The insertion of galK-Kan at the targeted site was confirmed by restriction digestion with \( \text{BamHI} \) and Southern detection of fragment containing the galK cassette. The expected size of the band hybridizing with galK was 3700 bp as detected in the galK-Kan intermediate lane. Removal of the galK-kan cassette was further confirmed by the absence of a hybridizing band. These recombinants were also confirmed by hybridization with GFP probe of the BAC cassette, which yielded the expected band in CR1, Intermediate, and the \( \text{PmeI} \) deleted BAC36-CR2. The removal of the ORF19 sequence was also confirmed by PCR amplification of the junction and sequence analysis. We confirmed BAC36-CR2 for any deletion or duplication by performing real-time PCR analysis at every 1.5 kb on the genome. Presence of unique
PmeI site was also confirmed by digestion of the purified BAC36-CR2 DNA with PmeI and resolving on a pulse field gel electrophoresis.

RESULTS

Herpesviruses replicate their genomes using latent and lytic origins during respective phases of their life cycle [1,3,20,26,40]. KSHV uses host cellular replication machinery to initiate replication on latently persisting viral genomes [40,41,42,43]. In contrast, lytic DNA replication is exclusively dependent on viral proteins, which accumulate on the viral DNA in sequence specific manner (oriLyts) for the replication of viral genome [20,25]. Studies have identified two replication origins in the KSHV genome [26,27]. These origins are termed as oriLyt-L, located in the left end between ORFs 4.2 and K5 (23069-24619) and the right end between ORF69 and vFLIP (118133-119878) of the genome [26,27]. These two origins are almost identical with respect to DNA sequence and contain A+T rich sequence, C/EBPα binding motifs, AP-1 binding sites and RTA response element (RRE) [26,27]. Both of these sites are able to support replication in transient replication assay upon expression of core proteins of lytic replication [26,27]. However, their role in viral genome amplification during lytic replication was not determined in the context of entire genome. Recombinant BAC approach with deletions removes a large portion of the genome, thus might have modulated the replication in earlier study [28]. Therefore, we used SMARD to delineate origin usage in KSHV genome during lytic replication. A schematic of SMARD approach is shown in figure 1.

In this approach, actively growing cells, in this case TRExBCBL1-Rta induced with doxycycline are labeled with IdU (first label, detected in red) for 3 hr, which gets incorporated into the replicating DNA to label the entire length of the genome.
These cells are switched from IdU to CldU (second label detected in green) thus replicating DNA transition from IdU to CldU. Transition sites, representing the
replication forks at the time of nucleotide switch, are easily detected in immunostaining using specific antibodies (Fig. 1). Dually labeled cells are digested with PmeI to linearize after lysing the cells in agarose plugs. Linear KSHV genomes are enriched in pulse field gel electrophoresis followed by detecting the band of interests in a Southern blot with KSHV specific probe. Linear DNA is excised by treating the agarose slice with gelase to isolate the DNA. Replication forks (transition from red to green) detected on various regions of the KSHV genome are due to the fact that cells used for labeling are asynchronous therefore replication forks are located on various regions of the genome at the time of transition. Aligning the molecules with minimal red (first label) flanked by green (second label) on top shows the

Figure 2: TRExBCBL1-Rta induced with doxycyclin and PFGE separated KSHV genome. A. TRExBCBL1-Rta cells were induced with 1.0 µg/ml of doxycyclin for 24 hr before pulsing them with halogenated nucleotide analogs for SMARD. Immunofluorescence assay to detect RTA (green) and immuno blot showed robust induction. Cell nuclei were stained with TO–PRO-3 and imaged using Zeiss confocal microscope. B. Labeled TRExBCBL1-Rta cells were embedded into agarose plugs followed by lysis and digestion of the DNA with PmeI. Linear KSHV DNA was resolved on pulse field gel electrophoresis using Bio-Rad CHEF II DR system. Lambda DNA (λ) and yeast chromosome (yc) was also loaded onto the gel to determine the size of linear KSHV band after Southern blot using 32P labeled KSHV specific probe. Both sides of the PFGE gel are excised to stain with ethidium bromide (EtBr) and Southern blot to mark the area of KSHV genome. The central portion of the gel was used for isolation of linear KSHV genome after gelase treatment. DNA solution was added between silanized glass slides and non-silanized cover slip to stretch DNA on positively charged glass slides. Stretched DNA molecules (green) were visualized under fluorescent microscope after staining with YOYO-1.
presence of replication initiation site (Fig. 1). This is due to the fact that these molecules started initiating replication at the end of the first labeling period therefore have small first (red) label. Aligning the molecules with varying length of red and green signals also determines the directions of replication fork progression.

**KSHV genome from TRExBCBL1-Rta incorporated halogenated nucleotides after induction of lytic cycle:**

TRExBCBL1-RTA cells were shown to robustly undergo lytic reactivation after induction with 1 μg/ml doxycycline [32]. We induced these cells with 1 μg/ml doxycycline for different time points to ensure that almost all the cells were undergoing lytic DNA replication by detecting RTA in an immunofluorescence as well as in Western blot assay using anti-RTA antibody (Fig. 2A). The induced cells were labeled for 3 hr each, first with IdU then CldU to label the replicating DNA and determine the location of replication forks. Labeling of cells, with the halogenated nucleotide analogs, undergoing lytic DNA replication did not affect viral DNA synthesis determined by virion production from labeled and unlabeled cells (data not shown). Labeled cells were washed to remove any unincorporated nucleotides followed by embedding them in agarose plugs. The cells were lysed followed by digesting the DNA with *Pme*I to linearize in order to obtain a monomeric length of the viral genome. The linearized KSHV genomes were enriched and separated from cellular DNA by Pulse Field Gel Electrophoresis. Ethidium bromide stained gel showed digestion of cellular and viral DNA and the location of linear KSHV DNA was detected in a Southern blot (Fig. 2B). Linear KSHV genome was detected at around 165 kb as determined based on the lambda PFGE DNA marker (New England Biolabs) (Fig. 2B, λ). Gel slice containing KSHV genome was treated with gelase to
Figure 3: Immuno-staining and fluorescent in-situ hybridization (FISH) of KSHV genome. A. Image of a microscopic optical field showing merged image with all three colors as well as the individual panels. Immuno-staining with anti-IdU and CldU detected replicated DNA including the cellular and viral DNA. FISH signals, detected in blue color, distinguished the viral DNA from the cellular counterpart. Three FISH signals, p6, p15 and p10 are marked with white asterisks. Immunostaining to detect IdU and CldU are shown in different pseudocolors (IdU=red and CldU=green). One of the molecules in the above panel is Pmel linearized KSHV genome which showed three characteristic (bar code) FISH signals. The transition sites from IdU to CldU, which also represent the location of replication forks, are indicated with white arrow. B. KSHV episomes with recognizable hybridization signals (specific bar code with three distinct FISH signals) were analyzed for their size and the fully substituted DNA molecules were aligned with KSHV schematic to ensure that FISH signals correspond to the target sequence (probe regions are marked in blue, p6, p15 and p10 on the schematic). Images of six Pmel linearized KSHV episomes, three each with first and second labels (red and green) aligned with KSHV genome showing proper alignment with the probe region (marked by blue line). Yellow lines show the position of Pmel site used to linearize the KSHV episome. C. Average size of the imaged molecules determined by an inbuilt feature in the Axiovision software (Zeiss Inc.). Majority of the molecules were 65 µm (±5 µm) after stretching, which correspond to about 2.5 kb/µm based on the 165 kb genome size.
extract DNA followed by staining with YOYO-1. An aliquot of DNA was stretched between the positively charged (3-aminopropyl-triethoxysilane-coate) glass slides and non-silanized cover slip through capillary action. DNA molecules, visualized by fluorescent microscopy, showed uniform stretching on the glass slide (Fig. 2B YOYO-1). Since the molecules stretched by capillary action varies in their orientation, we used three asymmetric probes, which generated distinctive ‘bar code’ to orient the molecules in same orientation. The halogenated nucleotides detected using specific monoclonal antibodies and alexafluor conjugated secondary antibodies (Alexa Fluor 594 for IdU shown in red and Alexa Fluor 488 for CldU shown in green; throughout the manuscript). Labeling of cells with IdU and CldU labeled all replicating DNA (including cellular), which was detected with anti-IdU and anti-CldU antibodies (Fig. 3A). Substitution of DNA molecules with the nucleotide analogs throughout their length helped in detecting the viral genome even in the presence of substantial hybridization background signals. The molecules lacking FISH signal (probe hybridization signal shown in blue) were the DNA of cellular origin thus discarded from the analysis. Additionally, the signals not on a continuous line, may have been due to background signals or broken piece of DNA, were also discarded. In order to determine the average length of linear KSHV genome, fully substituted molecules, either with IdU (red) or CldU (green), were aligned with schematic of Pmel linearized genome based on the length of FISH signals (Fig. 3B). The approx. length of the probes varied from 2.5 μm (left end; p6), 6.0 μm (middle; p15) and 4 μm (right end; p10) depending on the size of these probes. Average length of the fully substituted molecules in this study was 65 μm (Fig. 3C), which was consistent with our previous study [29].
KSHV of BCBL1 initiates replication at the oriLyt-L during lytic replication:

In order to identify the replication initiation site of the KSHV genome, we aligned photographic images of dually labeled fully substituted DNA molecules using FISH signals as the alignment template. The molecules were arranged in a non-subjective manner however, based on increasing incorporation of IdU (red signals) in randomly selected representative DNA molecules from a pool of imaged molecules. Aligning the molecules based on increasing signal eliminates any bias of replication initiation on the
molecule. The movement of replication fork in single direction within the examined region is represented by an increase in red signal from one end. A replication initiation site is characterized by the presence of a small IdU (red) signal flanked by CldU (green) signals on both sides. Increasing red signal to both sides from an origin represents bi-directional replication fork progression. However, region on the genome with red signal flanked by green signals are the replication termination site, which was not detected on KSHV lytic DNA replication. Analysis of 21 molecules, dually labeled with IdU and CldU, showed the location of transition sites (marked with horizontal yellow arrows) progressed in the rightward direction (Fig. 4A). We also detected the location of replication initiation, defined by a small IdU (red) staining region flanked on both sides by CldU (green) staining (Fig. 4, molecules 1-5). Alignment of the red region with linear KSHV genome schematic and sequence analysis showed that replication initiation site falls in the area of oriLyt-L (Fig. 4A). Transition of red to green signals respective to the replication initiation site showed progression of replication forks in both directions suggesting a bidirectional replication initiation at oriLyt-L (Fig. 4A and B, molecule 1-5).

In order to define the progression of replication forks throughout the genome, replication profile of these molecules were generated by dividing the genome at every 5 kb intervals and calculating the percentage of molecules stained with red in each fragment (Fig. 4C). Replication profiles are important in determining the regions of the genome, which replicated first, RRF (regions replicated first) and most frequently contains the replication initiation site. Replication profile based on the imaged molecules confirmed that replication initiated in the left end of the genome (Fig. 4C). Replication profile with lower percentage of molecules stained in red, right end of the genome in this case showed
Figure 5: Deletion of the duplicated region in lytic BAC36 KSHV-lyt.
A. Duplicated region of ORF18-K5 on the right side of the BAC cassette were deleted using primers set described in Rossetto and Pari to obtain KSHV BAC36-lyt-CR1 (CR1) [36].
B. Duplicated region on the left side of the BAC cassette, which contains the partial ORF19 sequence and Pmel site were removed by homologous recombination with primer pairs outside of the ORF19. C. galK-Kan cassette containing intermediate after the removal of ORF19 sequence. GalK probe was used to confirm the insertion of galK-Kan cassette at the targeted site in a Southern blot. D. Duplex oligo used for removing the galK-Kan cassette after homologous recombination and negative selection. E. BAC36-lyt-CR2 with deleted duplicated sequence on both sides of the BAC cassette. Sequence of the junction region was confirmed by sequence analysis. galK-Kan recombination and negative selection completely removes the cassette without leaving any scar sequence. F. Restriction digestion of BAC36-lyt-CR1, galK-Kan intermediate and final BAC36-lyt-CR2 with complete removal of duplicated sequence. Southern detection of galK-Kan cassette in the intermediate using 32P labeled galK probe. G. BJAB cells stably maintaining BAC36-lyt-CR2 detected by GFP signals as the BAC cassette in the BACmid contain genes expressing GFP. H. Detection of LANA to confirm that these BJAB-BAC36-lyt-CR2 maintained KSHV genome and expressed latent and lytic proteins. Detection of RTA shows that these cells were spontaneously undergoing lytic reactivation. Comparison of LANA and RTA with TRExBCBL1-Rta shows that these BJAB-BAC36-lyt-CR2 were maintaining lower viral copies.
the regions, which replicates last also termed as RRL (regions replicated last).

**Replications forks move without pausing sites throughout the KSHV genome:**

Replication profile based on the number of molecules stained in red also represents the position and direction of the replication fork at the time of switching the first label to the second label [29]. Accumulation of replication forks in any particular region indicates that replication may have paused while progressing through that region thus suggesting a replication pausing site. Replication fork movement is affected by the complexity in the chromatin/genome structure [29]. Replication profile of these molecules showed uniform movement of the replication fork throughout the genome suggesting there were no replication pausing site in the KSHV genome (Fig. 4C).

**Generation of KSHV BACmid with removed duplicated region and *PmeI* sites:**

KSHV BACmid (BAC36) has been very useful in investigating the role of various viral

![Figure 6: BAC36-lyt-CR2 showed replication initiation at oriLyt-L. A. Schematic of *PmeI* linearized BAC36-lyt-CR2 showing the location of BAC cassette along with characteristic FISH probes location. oriLyt-L and R are indicated with red circle flanked by green. Dually stained, *PmeI* linearized KSHV BACmids were arranged with increasing red staining region towards the bottom. Transition sites from red to green are marked with horizontal yellow arrows showing the locations of the replication forks at the time of change of nucleotide from IdU to CldU. B. Schematic representing the BAC36-lyt-CR2 molecules in panel A. Molecules 1-3 show bi-directional replication fork progression confirming replication initiation at oriLyt-L. Rest of the molecules (4-17) show replication fork movement in single direction confirming that these molecules were from rolling circle replication. C. Replication profile of the IdU and CldU immunostained molecules of panel A. Replication profile generated based on the number of molecules having red staining regions show that left of end of genome, which contains the *oriLyt*-L, replicates first.](image-url)
genes in KSHV biology [37]. Therefore, we wanted to use BAC36 for investigating lytic DNA replication in SMARD assay. We took advantage of the modified BAC in which the expression of replication and transcriptional activator, RTA (main lytic switch regulator) was constitutively expressed through PGK promoter [31]. This recombinant BAC36 enters the lytic replication cycle by default without the need for further induction. Use of this BACmid ensured that the replication of the genome was exclusively through lytic origins [31]. However, it is important to mention that a recent study showed a duplication of 9 kb fragment of the long unique region in the terminal repeats [35]. This region includes partial sequence for ORF19 and complete sequences of ORF18, 17, 16, K7, K6 and K5 [35]. Duplication of these genes may not matter for the replication of the virus but SMARD requires unique restriction site to linearize the genome for alignment and origin mapping but the duplicated region carried additional Pmel sites, which cleaved the genome into fragments thus hampering the assay. Therefore, we used recombineering approach to remove the duplicated region in the TR of KSHV-lyt (Fig 5A). Deletion of major duplication (ORF 18- K5) removed one Pmel site located in the right side duplicated region yielding BAC36-lyt-CR1 (Fig. 5A). Sequence analysis and restriction mapping showed the presence of an additional Pmel site in the partial ORF19 duplicated region (Fig. 5B). The partial ORF19 region was subsequently removed by recombineering approach and schematic is shown in figure 5B-E. Briefly, partial ORF19 was replaced with galK-Kan cassette and the insertion of galK-Kan cassette was confirmed by a Southern blot (Fig. 5F). galK-Kan was further removed with an oligo duplex shown in figure 5D. The repaired BAC36-lyt-CR2 was confirmed by Southern blot as well as sequence analysis (Fig. 5 E and F).
Purified BAC-lyt-CR2 was transfected into BJAB and selected with hygromycin to obtain pure population maintaining the KSHV genome. The BAC cassette contains GFP signal therefore the cells maintaining KSHV genome were easily determined by fluorescence microscopy (Fig. 5G). In order to confirm that these stable cells were constitutively encoding RTA and undergoing lytic reactivation, level of RTA was detected in a Western blot (Fig. 5D). Not surprisingly, the level of RTA was lower when compared to the levels in TRExBCBL1-Rta Dox induced cells (Fig. 5H) This was primarily because the BJAB maintained lower copies of the viral genome as confirmed by the levels of a latent protein, LANA, which was also lower (Fig. 5H). These cells were labeled for 3 hr with the first label (IdU) followed by the second label (CldU) for the detection of replication initiation site.

**KSHV lytic BACmid initiated replication at oriLyt-L similar as BCBL1:**

Initiation of DNA replication sites on the KSHV BACmid was determined by capturing photomicrographic images of DNA molecules stained with the first (IdU) and second (CldU) labels. KSHV genome molecules were distinguished from the other cellular DNA by the presence of specific bar coding patterns of blue staining (three asymmetric and different sizes FISH signals). These molecules were aligned with increasing red staining towards the bottom in the alignment panel (Fig. 6A). As mentioned earlier, replication initiation sites are detected as a small red signal flanked by green signals as seen at oriLyt-L similar to the BCBL1 virus (Fig. 6A). We could also detect the progression of red to green transition sites on both sides of the red signal confirming the movement of replication forks in both directions thus suggesting for a bi-directional replication initiation at the oriLyt-L (Fig. 6A, molecules 1-3). Transition sites detected on the rest of
the molecules showed movement of the replication forks in single (rightward) direction (Fig. 6A, molecules 4-17). Schematic of molecules with transition sites marked as horizontal black arrows show the location of replication forks (Fig. 6B). Replication profile generated based on the percentage of molecules stained with red and the location of replication forks at every 5 kb region showed that left end of the genome replicated first with replication initiating at oriLyt-L (Fig. 6B and C). The replication forks moved almost uniformly throughout the genome with little exceptions of slightly more forks accumulating in the region with BAC cassette suggesting replication fork may have moved slightly slower than the rest of the genome (Fig. 6A and B). The data of BCBL1 lytic virus and the BACmid spontaneously undergoing lytic DNA replication confirms that oriLyt-L initiate replication in the context of viral genome in a bi-directional manner. Based on the progression of replication forks around the origin (oriLyt-L) we concluded that replication initiated as theta model with replication fork moving in both directions (Fig. 4 and 6). A schematic of replication initiation and replication fork progression is depicted in figure 7. Depending on the number of molecules containing only one transition site we conclude that the genome copies were amplified primarily by the rolling circle replication. In this method the first label start incorporating from the origin (shown in red). Transition sites from red to green were detected at various locations in different genome copies as the lytic replication may have started at different times in those molecules (Fig. 7, Rolling Circle). For instance, the bottom molecule in figure 7D had started replication first therefore have longer first label (red) as compared to the top molecules which started replication right before the transition of IdU (first) to CldU (second) analog thus have short first label (red).
DISCUSSION

KSHV establishes latent infection in the infected host with frequent reactivation to produce viral progenies [3]. During latency viral genome replicates in conjunction with cellular DNA, which is tightly coordinated so that virus can undergo only one round of replication per cell cycle [40,41,42]. However, during reactivation viral genome
replication is exclusively dependent on the viral proteins, which have been well characterized [20,24,25,27]. These viral proteins accumulate on well-defined cis acting DNA elements, termed as origin of DNA replication, to initiate DNA synthesis [25,37]. Studies have identified two origins of lytic DNA replication (oriLyt-L and R) in the KSHV genome, which can support replication of a plasmid in transient assays [26,27]. Studies have identified several elements within the oriLyt including two A+T rich sequences, C/EBPα binding motifs, AP-1 binding sites and a RTA response element (RRE) [27]. Mutational analyses of the oriLyt have established that the A+T rich regions cannot be mutated or deleted [24,26]. Although these origins are capable of supporting DNA replication of a plasmid in transient assays but their roles in the context of viral genome were not fully understood [27]. In an effort to address their function in viral genome replication, recombinant BACs with deleted oriLyt (oriLyt-L and oriLyt-R, individually or together) were used which showed that oriLyt-L deleted BACmid was unable to replicate [28]. In contrast, the absence of oriLyt-R in the BACmid increased the production of infectious virus suggesting that oriLyt-R have negative regulatory elements of replication [28]. Since deletion of oriLyt-R modulated the replication mediated by oriLyt-L compared to the wild type, it does not reflect the origin usage in its native state [28]. Therefore, we used a novel in cell labeling of replicating DNA approach to identify the functional replication origin in the context of wild type virus. SMARD detects the replication initiation site (origin) and replication fork progression in steady state replicating molecules [29].

In this study, we determined how the DNA replication initiated and replication fork progressed throughout the KSHV genome during lytic DNA replication using
BCBL1 lytic virus as well as KSHV BACmid spontaneously undergoing lytic replication. Viral genome molecules from both the cell lines showed almost similar pattern of nucleotide incorporation in the oriLyt-L region. Based on the number of molecules with replication fork progressing in both directions we propose that replication initiates as replication bubble at the oriLyt-L. Since we detected the bidirectional replication forks only in the close proximity of the origin suggests that replication of genome initiate through theta model but may replicate all the way using this method to complete the replication of the circular episomes before turning into rolling circle the method. Lower number of molecules with bi-directional fork may also be due to the fact that labeling of the cells was done at the time when most of the episomes were already in the rolling circle phase thus the number of molecules with single fork were enriched in the analysis. Labeling of cells with halogenated nucleotide analogs with varying time post-induction may be useful in obtaining more molecules with bi-directional replication forks, if the KSHV episomes duplicate by the theta model during early phases of lytic replication as suggested for the herpes simplex virus 1 [39]. However, labeling the cells during early time points post-infection may cause IdU and CldU incorporation through the latent origin. Therefore, in this study we ensured that almost all the cells were undergoing lytic cycle by detecting RTA in immunofluorescence and Western blot analysis. Lack or replication initiation sites other than oriLyt confirms that all the cells were in lytic phase during labeling with IdU and CldU.

Since the primary aim of this study was to determine the functional replication origin in the context of viral genome and the regulatory mechanism controlling replication, we did not focus on the rate of DNA synthesis from the oriLyt. However, it
would be interesting to determine the rate of replication fork progression and DNA synthesis of BACmid with deleted oriLyt-R, which are undergoing studies in the laboratory. Additionally, KSHV can be used as a model system to study the effect of transcription on DNA replication and fork progression as most of the viral genes are transcribed during lytic DNA replication. A recent study on bacterial genome replication showed that head on collision of the replication complex with RNA polymerase of transcription results in replication fork arrest but little or no effect on co-directional collision [38]. Since the KSHV genome appears to be replicating uniformly throughout the genome it suggests that there may be little or no transcription of the viral gene during replication.

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REFERENCES


Chapter 2.

Phosphorylation of KSHV processivity factor, ORF59, by a viral kinase modulates its ability to associate with RTA and oriLyt for DNA replication.

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Unpublished Data
**ABSTRACT**

Kaposi’s sarcoma-associated herpesvirus (KSHV) encoded ORF59, plays an essential role in viral lytic replication by providing DNA processivity activity to the viral DNA polymerase, ORF9. It forms a homodimer in the cytoplasm, binds and translocates ORF9 into the nucleus where it secures ORF9 to the origin of replication (oriLyt) allowing it to synthesize long DNA fragments. ORF59 binds to the oriLyt through an immediate early protein, replication and transcription activator (RTA). Our research shows that viral kinase, ORF36, phosphorylated ORF59 at Ser376, Ser378, and Ser379. Although mutating these serine residues has no effect on the binding between ORF59 and ORF9 viral polymerase or the viral kinase, it does significantly reduce the ability of ORF59 to bind to RTA. Consequently, Ser376, Ser378, and Ser379 residues are critical for binding of ORF59 to oriLyt and its processivity function. Ablation of these phosphorylation sites reduced the production of virion particles, in the context of viral genome, suggesting that phosphorylation is critical for viral DNA synthesis and viral life cycle.

**INTRODUCTION**

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), is a member of the gammaherpesvirus family. KSHV is associated with Kaposi’s sarcoma (KS) as well as several lymphoproliferative diseases including Primary Effusion Lymphoma (PEL) and Multicentric Castleman’s Disease (MCD) (8, 31). Similar to other herpesviruses, KSHV establishes a lifelong latency following a primary infection. During latency, the KSHV genome is maintained as a non-integrated episome tethered to the host chromosome via viral latency associated nuclear antigen (LANA) protein, encoded by ORF73. During latency limited number of genes are expressed and
replication of the virus is dependent on host cell replication machinery (4). KSHV latency can be disrupted by cis- and trans-acting signals resulting in viral lytic gene expression and production of infectious virions. KSHV replication and transcription activator (RTA), encoded by ORF50, is necessary and sufficient for the activation of KSHV lytic replication and production of viral particles (17, 20, 32). Several chemical inducers, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and sodium butyrate (NaB), have also been successfully used to trigger lytic replication (21).

In KSHV-induced malignancies, majority of the tumor cells are latently infected with the virus; however, a small proportion of cells (2-5%) of the latent tumor cells undergo lytic replication (11, 16). This spontaneous reactivation is proposed to produce homologs of cellular cytokines, which acts in a paracrine manner for tumor progression, as well as generation virions for further spread of infection (11). These cytokines are capable of promoting tumorigenesis by activating pathways involved in inflammation, angiogenesis, and enhanced proliferation, invasion and dissemination of tumor cells (7, 14, 15, 18, 22, 23, 25, 26, 28, 30, 34, 38). Consequently, the study of lytic replication is not only essential for the prevention of KSHV-associated malignancies, but also for developing new treatments.

KSHV lytic replication requires eight viral proteins including: ORF9 (DNA polymerase), ORF6 (single-stranded DNA binding protein), ORF40/41 (primase-associated factor), ORF44 (helicase), ORF56 (primase), ORF59 (processivity factor), ORF50 (replication and transcription activator or RTA), and ORF K8 (K-bZIP) (2). Similar to other hepresviruses, RTA is accepted as one of the most important proteins required for lytic replication. RTA binds to the C/EBPα and the RTA response element (RRE) binding
motifs in oriLyt thereby activating lytic replication through transcription activation as well as recruitment of additional factors (6, 10, 28, 29, 35, 39, 41).

One of the factors recruited by RTA is the viral processivity factor, ORF59. Rossetto et al. have shown that the binding of ORF59 to C/EBPα binding motif within oriLyt is crucial for its function and is dependent on the presence of RTA (27). Similar to other herpesviruses, ORF59 forms a homodimer, which translocates viral polymerase, ORF9, into the nucleus for efficient synthesis (processivity function) of DNA fragments (13). ORF59 homolog in other herpesvirus, human cytomegalovirus (hCMV), ppUL44 is phosphorylated by a viral Ser/Thr kinase, ppUL97, which modulates ppUL44’s ability to localize to the nucleus (1). BMRF1, Epstein-Barr virus (EBV) processivity factor, is phosphorylated by BGLF4 viral kinase within a hinge region-like domain known to be important for transmitting conformational changes (33). As a result BGLF4 phosphorylation enhances the transactivation activity of Zta (RTA homolog) and the synergistic activation of lytic replication at oriLyt (37). Similarly, ORF59 is a phosphoprotein, which is phosphorylated by KSHV viral Ser/Thr kinase, ORF36, but the consequences of this modification were not been determined previously (9). Here, we show that ORF36 phosphorylates ORF59 at Ser376, Ser378, and Ser379, which is critical for ORF59’s ability to bind to RTA and oriLyt. Furthermore, the replacing phosphorylating serines of these sites with alanines significantly reduced viral production.
MATERIALS AND METHODS

DNA constructs:

ORF36-Flag/Myc, ORF59-GST full length and ORF59-GST segments were constructed by PCR amplification and cloning using wild type KSHV genome as template. ORF59 deletion mutants were assembled from two PCR reactions: 1) one using sense ORF59 primer and anti-sense ORF59 mutant primer, 2) second using sense ORF59 mutant primer and anti-sense ORF59 primer. The two PCR amplicons were ligated, transformed and screened for correct mutation. ORF59 point mutants were constructed using QuickChange Lightning Multi Site-Directed Mutagenesis Kit as directed (Agilent Technologies Inc., Santa Clara, CA). These ORF59 deletion and point mutants were cloned into GST, pxi-HA and Flag-pLVX vectors using full-length PCR primers. ORF36 kinase dead mutant (ORF36 K108Q) was generated in a two-step approach, similar to ORF59 deletion mutants. The resulting PCR product was ligated, transformed and screened for K108Q mutation. All primers used for cloning and targeted mutagenesis are listed in Table 1. BAC36 ΔORF59 BACmid, ORF9-Flag, K8-Flag and 8088sc plasmids were a generous gift from Dr. Greg Pari’s lab (University of Nevada, Reno).

Table 1. Primer sequences

<table>
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<th>Mutant</th>
<th>Primer Oligonucleotide Sequence 5'-3'</th>
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<tr>
<td>ORF36-K108Q-pA3F</td>
<td>S  GTGTGCAGCAGTTGTGATAGGCCGCGGG</td>
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<tr>
<td></td>
<td>AS  AACTGCTGACACAGACTCCGAGGAGA</td>
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<tr>
<td>ORF59-d304-318 (DM1)</td>
<td>S  CCATCTGATACCCAGGACTCCGAGGCA</td>
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<td>AS  AGTCCGCTGACAGTCCGAGGACTCATA</td>
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<td>ORF59-d333-349 (DM2)</td>
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<tr>
<td>ORF59-d376-379 (DM3)</td>
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<tr>
<td>ORF59-S305A, S312A, S318A (PM1)</td>
<td>S  AACCAGCTCTGGAGCCGAGGAGGGCTGCTGCACAAAGGGTCGTTCCCGATGGAATCCGAGACTC</td>
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<tr>
<td>ORF59-S333A, S349A (PM2)</td>
<td>S  TGGAGCTCCGAGTACCGACTCCATTGACCCACACAGGCT</td>
</tr>
<tr>
<td>ORF59-S376A, S378A, S379A (PM3)</td>
<td>S  CCTCACAGAGGCGGCACGACGCGGCCAGTCCAGGGATCGT</td>
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Cell culture, transfection and transduction by lentivirus:

293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine growth serum (HyClone, Logan, UT), 2 mM l-glutamine, 25 U/ml penicillin, and 25 µg/ml streptomycin. All cultures were incubated at 37°C in a humidified environment supplemented with 5% CO2.

Cells were transfected by calcium phosphate method as previously described (24), with minor modification. Briefly, 500 µl 1xHBS (140 mM NaCl, .75 mM Na2HPO4·2H2O, 25 mM HEPES, 5 mM KCl, 6 mM Dextrose pH 7.1) was added to DNA and mixed. Thirty microliters of 2.5 M CaCl2 was added to the solution and mixed. After 20 min at room temperature, the solution was evenly added drop wise onto the 60-70% confluent 293T cells.

For generating lentivirus, 10 µg of lentiviral vector, 7.5 µg CMV-dR8.2 packaging plasmid and 2.5 µg pCMV-VSVG envelope plasmid were transfected into 100 mm dish of 293T cells using 1xHBS. Twenty-four hours post-transfection, virus was induced with 1 mM sodium butyrate (NaB) in 5% bovine growth serum DMEM with 100 mM HEPES for 10 hr. The supernatant was collected three times thereafter at 12-16 hr intervals. The supernatant was filtered with 0.45 µm pore-size filter and ultracentrifuged at 25,000 rpm for 1.5 hr using Beckman Coulter Optima L-90K ultracentrifuge (Beckman Coulter, Inc. Brea, CA). The virus pellet was resuspended in DMEM and added to the target cells.

BACmid DNA was transfected with Metafectene Pro (Biontex Laboratories GmbH, San Diego, CA) due to the larger size of the plasmid and fragility of DNA. Following the developed protocol, 90% confluent 6-well plate was transfected with a combination of
two solutions: 1) 5-10 µg BACmid DNA in 200 µl serum and antibiotic free medium, 2) 6 µl Metafectene Pro in 200 µl serum and antibiotic free medium. Following the combination of the two solutions, the mixture was allowed to sit at room temperature for 15-20 min and added onto the cells drop-wise. The BACmid contained GFP, which allowed for efficient evaluation of transfection efficiency and monitoring of selection.

**Generation of BAC36 ΔORF59:**

Mutagenesis of BAC36 was performed using the Red Recombination method as previously described (36, 40). Briefly, kanamycin resistance cassette flanked by FRT sequences was PCR amplified with primers containing sequence homologous to ORF59. The cassette was transformed into BAC36-containing *E. coli* EL350. These bacteria carry a bacteriophage lambda prophage with the genes *exo*, *bet*, and *gam*. These genes are under a temperature-sensitive repressor only allowing their expression at 42°C. Gam inhibits RecBCD nuclease from degrading linear DNA, while Exo and Beta provide double stranded break repair recombinase.

During cassette transformation, EL350 bacteria were induced at 42°C for 15 min to activate Gam, Exo, Beta and λ recombinase allowing for the PCR product to be inserted into a site of ORF59 homologous with the ends of the product. Transformed bacteria were plated on kanamycin plates and grown at 32°C to prevent further recombination. Southern blot was used screening the BAC36 ΔORF59 intermediate. Successful integration of kanamycin cassette gave a 5,883 bp fragment when the BACmid was digested with *PstI*. The kanamycin cassette was removed using the FRT sites flanking the cassette. FLIP recombinase was activated with 0.1% final concentration of arabinose, which used the FRT sites to loop out the kanamycin cassette, but as a result leaving one
of the sites as a “scar”. This region of ORF59 was PCR amplified and the presence of FRT scar was verified by sequencing. Consequently, the resulting BAC36 contained an interrupted ORF59 protein.

**Immunoprecipitation, Western blotting and antibodies used:**

Transfected cells were harvested, washed with ice-cold PBS, and lysed in 0.5 ml ice-cold RIPA buffer (1% Nonidet P-40 [NP-40], 10 mM Tris [pH 7.5], 2 mM EDTA, 150 mM NaCl), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin). Cell debris was removed by centrifugation at 13,000×g (10 min and 4°C), and lysates were then precleared for 1 hr rotation at 4°C with 30 µl of Protein A-Protein G-conjugated Sepharose beads. After approximately 5% of the lysate was saved for input control, the protein of interest was captured by rotating the remainder of the lysate with 1 µg of the appropriate antibody overnight at 4°C. Immune complexes were captured with 30 µl of Protein A-Protein G-conjugated Sepharose beads by rotating for 2 hr at 4°C. The beads were pelleted and washed three times with RIPA buffer. Proteins immunoprecipitated for kinase assay were washed with RIPA buffer containing 300 mM NaCl to reduce any contaminating proteins. For Western blot assay, input lysates and immunoprecipitated (IP) complexes were boiled for 5-10 min in Laemml1 buffer and resolved on SDS-PAGE. The nitrocellulose membrane was probed with appropriate antibodies followed by incubation with infrared-dye tagged secondary antibody, and viewed on an Odyssey imager (LiCor Inc., Lincoln, NE).

The following antibodies were used: mouse anti-Flag (M2, Sigma-Aldrich, St. Louis, MO), rabbit anti-Flag (F7425, Sigma-Aldrich, St. Louis, MO), mouse anti RTA (mouse
Purification of GST fusion proteins:

Escherichia coli BL21 (DE3) cells were transformed with the plasmid constructs for each GST fusion protein. Bacterial culture was incubated until the optical density at 600 nm was approximately 0.6, at which the cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hr at 37°C. The bacteria were pelleted, washed once with 5 ml STE buffer (100 mM NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5), resuspended in 5 ml NETN buffer (0.5% NP-40, 100 mM NaCl, 20 mM Tris, 1 mM EDTA, pH 8.0), supplemented with protease inhibitors, and incubated on ice for 15 min. A volume of 75 µl of 1 M dithiothreitol (DTT) and 900 µl of a 10% solution of Sarkosyl in STE buffer was added, and the suspension was sonicated (for 2 min at 40% amplitude 20 sec on and 20 sec off on ice) to solubilize the proteins. The lysates were centrifuged (13,000×g, 10 min, 4°C) to separate the unsolubilized fraction. The clear supernatant was transferred to a fresh tube, to which 1.5 ml of 10% Triton X-100 in STE buffer and 200 µl of Glutathione-Sepharose beads were added. The tube was rotated overnight at 4°C, after which the purified protein bound to Glutathione was collected by centrifugation (2 min, 600×g, 4°C) and washed five times with NETN buffer supplemented with protease inhibitors. The level of purification was determined by SDS-PAGE, and the purified proteins were stored at 4°C.
**In vitro translation and binding assay:**

For *in vitro* translated ORF36, we used TNT T7 Quick Coupled Transcription-Translation System (Promega, Fitchburg WI). Briefly, 50 µl reaction was set up with 2 µg ORF36-Flag plasmid, 40 µl TNT T7 Quick Master Mix, 1 mM $^{35}$S Methionine, and 1 µl T7 TNT Enhancer. The mixture was incubated at 30°C for 90 min after which the radioactively labeled protein was used for further binding experiments.

*In vitro* translated ORF36 was incubated with GST, GST precipitated ORF59 and its segments by rotating overnight at 4°C. As a control, TNT Luciferase DNA was also *in vitro* translated and rotated with GST and ORF59-GST. The beads were collected and washed with NETN buffer (0.5% NP-40, 100 mM NaCl, 20 mM Tris, 1 mM EDTA, pH 8.0), supplemented with protease inhibitors. The proteins were then visualized with a Coomassie stain and the gel was dried using Bio-Rad Gel Air Dryer (Hercules, CA). The radioactive gel was exposed to a phosphoimager plate and the phosphorylated proteins were imaged using Storm 820 Amersham Biosciences (GE Healthcare Inc., Waukesha,WI).

**In vitro kinase assay:**

Approximately 20 µg purified kinase protein and 20 µg of substrate protein per sample were washed with the Kinase Wash buffer (20 mM HEPES pH 7.5, 5 mM MnCl$_2$, 10 mM β-mercaptoethanol 14.2 M) and resuspended in 20 µl of Kinase Wash buffer for the reaction. Kinase and the control proteins were resuspended in 10 µl Kinase Wash buffer with 10 mM cold ATP (3.5 µl of 100 mM stock) and 0.2 U Ci/µl $^{32}$P γATP (0.7 µl of stock) and added to the substrate. The mixture was incubated at 37°C water bath for 30
min after which the reaction was stopped with 15 µl Laemmli buffer. The samples were heated to 95°C for 5-10 min and loaded on a 10% SDS-PAGE gel to resolve the proteins. The proteins were then visualized with a Coomassie stain and the gel was dried using Bio-Rad Gel Air Dryer (Hercules, CA). The radioactive gel was exposed to a phosphoimager plate and the phosphorylated proteins were imaged using Storm 820 Amersham Biosciences (GE Healthcare Inc., Waukesha, WI).

**Chromatin Immunoprecipitation (ChIP) analysis:**

Cells were cross-linked with 3% formaldehyde by rocking for 10 min at room temperature, followed by addition of 125 mM glycine to stop the cross-linking reaction. Cells were washed with cold PBS containing protease inhibitors (1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Cells were resuspended in 1 ml cell lysis buffer [5 mM piperazine-\(N\),\(N\prime\)-bis (2-ethanesulfonic acid) (PIPES)-KOH (pH 8.0)-85 mM KCl-0.5% NP-40] containing protease inhibitors and were incubated on ice for 10 min. Cells were subjected to Dounce homogenization for efficient lysis, followed by centrifugation at 2,500 rpm for 5 min at 4°C. Nuclei were resuspended in nuclear lysis buffer (50 mM Tris [pH 8.0]-10 mM EDTA-1% SDS containing protease inhibitors), followed by incubation on ice for 10 min. Chromatin was sonicated to an average length of 700 bp, and cell debris was removed by centrifugation at high speed for 10 min at 4°C. The supernatant containing sonicated chromatin was diluted fivefold with ChIP dilution buffer (0.01% SDS-1.0% Triton X-100-1.2 mM EDTA-16.7 mM Tris [pH 8.1]-167 mM NaCl including protease inhibitor). Samples were precleared with a salmon sperm DNA-protein A-protein G Sepharose slurry for 1 hr at 4°C with constant rotation. The supernatant was collected after a brief centrifugation
(2,000 rpm at 4°C). Ten percent of the total supernatant was saved for input in Western blotting, and the remaining 90% was divided into three fractions, to which was added 1 µg of (i) a control antibody, (ii) anti-Flag (M2, Sigma-Aldrich), or (iii) anti-RTA antibodies, respectively. Reaction complexes were rotated overnight at 4°C, followed by precipitation of the immune complex by using a salmon sperm DNA-protein A-protein G slurry. Beads were then washed sequentially with a low-salt buffer (0.1% SDS-1.0% Triton X-100-2 mM EDTA-20 mM Tris [pH 8.1]-150 mM NaCl), a high-salt buffer (0.1% SDS-1.0% Triton X-100-2 mM EDTA-20 mM Tris [pH 8.1]-500 mM NaCl), and a LiCl wash buffer (0.25 M LiCl-1.0% NP-40-1% deoxycholate-1 mM EDTA-10 mM Tris [pH 8.0]) and twice in Tris-EDTA. Ten percent of the immunoprecipitated chromatin was taken for Western blot assay. Chromatin was eluted using an elution buffer (1% SDS-0.1 M NaHCO₃) and reverse cross-linked by adding 0.3 M NaCl at 65°C overnight. Eluted DNA was precipitated, treated with proteinase K at 45°C for 2 hr, and purified. Purified DNA was used as a template for PCR amplification of the RRE region of KSHV oriLyt.

Quantitative real-time (RT) PCR:

Quantitative real-time PCR was performed in a total volume of 20 µl, including 10 µl of SYBR green PCR 2× Master mix (Applied Biosystems). For viral production, we used 0.5 µM each KSHV LANA primer (forward, 5’-TTGCCTATACCAGGAAGTCCACA-3’, and reverse, 5’-GGAGGAAGACGTGGTTACGGG-3’). Relative copies of viral particles were quantified using a standard curve and the ΔΔCₜ method. To obtain a fair comparison of the KSHV genome copy number, we used GAPDH (forward, 5’-CAGCAAGAGCACAAGAGGAAGA-3’, and reverse, 5’-TTGATGGTACATGACAAGGTGCGG-3’) primers to compare the calculated copy
number with relative number of cells analyzed. Overexpression ChIP samples were analyzed using oriLyt RRE primers (forward, 5’-CTCTGGGTGTTTCGGTAGA-3’, and reverse, 5’-TTTACACAAGGCGCCAGAA-3’). Purified DNA samples of the Hirt’s extraction, ChIP fraction and the input DNA samples were amplified with 3 min of 95°C, 40 cycles of 15 sec 95°C, 30 sec at 51°C, and 30 sec at 72°C on an ABI StepOne plus real-time PCR machine (Applied Biosystems). A melting curve analysis was performed to verify the specificity of the amplified product and each sample was tested in triplicate.

**Indirect immunofluorescence microscopy:**

To check the co-localization of ORF59 and its point mutants with ORF36, ORF9, and RTA, we transfected 293L cells that were plated on glass cover slips using 1xHBS. At 24 hr post transfection, cells were fixed with 3% paraformaldehyde for 20 min at room temperature, washed twice with 1xPBS and then permeabilized with 0.2% Triton X-100 for 10 min at room temperature. The cells were washed twice with 1xPBS and blocked in 0.4% Fish Skin Gelatin and 0.05% Triton X-100 for 40 min at room temperature. The cells were washed twice with 1xPBS and probed with primary antibody (0.2% Fish Skin Gelatin, 0.05% Triton X-100 and 0.5 µg antibody) for 1 hr at room temperature. The cells were washed with PBS and treated with secondary antibody (0.2% Fish Skin Gelatin, 0.05% Triton X-100 and 1:20,000 dilution of stock antibody). Alexa Fluor 488 conjugated chicken anti-mouse was used for ORF59 and Alexa Fluor 594 goat anti-Rabbit was used for RTA and ORF9. The cells were then washed with PBS and the nuclei were stained with TO-PRO-3 (Molecular Probe) for 10 min. Images were obtained using a laser scanning confocal microscope (Carl Zeiss, Inc.).
RESULTS

As part of the replication complex, ORF59, the KSHV processivity factor plays a critical role in initiating KSHV lytic DNA replication. It forms a homodimer in the cytoplasm, binds to viral polymerase (ORF9) to transport it into the nucleus and helps in synthesizing longer DNA fragments. It has a high affinity for double-stranded (ds) DNA and is found at the origin of lytic replication (oriLyt) (9). ORF59 is recruited to the origin through the viral replication and transcription activator (RTA) demonstrated previously using chromatin immunoprecipitation assay. In other herpesviruses, phosphorylation plays an important role in maintaining processivity function. A homolog of ORF59 in hCMV, ppUL44, is shown to be phosphorylated by viral kinase, ppUL97, to modulate its ability to localize to the nucleus. BMRF1, EBV processivity factor, is phosphorylated by BGLF4 viral kinase facilitating optimal oriLyt-dependent viral DNA replication (37).

KSHV viral kinase, ORF36, binds to ORF59 between 1-264aa and phosphorylates it between 265-396aa:

Previous studies have indicated that ORF59 is a phosphoprotein, but the significance of phosphorylation was not been explored (9). Although detectable binding between a kinase and its substrate is not necessary for phosphorylation to occur, we determined that ORF36 does in fact bind to ORF59 in an overexpressed system (Fig. 1A lane 4). This binding is specific due to the control vector not bringing down ORF59 (Fig. 1A lane 3). In vitro analysis of ORF59-GST and three ORF59 GST-tagged segments (1-132aa, 133-264aa, 265-396aa, see Fig. 1B-C) identified the binding site between 1-264aa with a higher affinity between residues 133-264aa (Fig. 1D lane 7). As a control, we used in
vitro translated luciferase, which showed no binding to ORF59-GST and in vitro translated ORF36, which confirmed its binding with ORF59-GST (Fig. 1D lane 3). Furthermore, *in vitro* analysis also confirmed that no other proteins are necessary for the binding between ORF36 and ORF59 to occur.

**Figure 1: KSHV viral kinase, ORF36, binds to ORF59 between 1-264aa.** A. Binding between full length ORF59 and ORF36 was established by cotransfecting ORF36-Flag and ORF59-HA plasmids and immunoprecipitating with Flag antibody. The Western blot shows specific binding between ORF36 and ORF59 as the vector does not bring down any ORF59. B. Schematic of ORF59 GST-tagged segments used for *in vitro* binding assay. C. Full length and ORF59 GST-tagged segments were synthesized as stated in Materials and Methods section and visualized with a coomassie-stained gel. A red asterisk indicates each of the purified proteins. D. In order to identify the site of interaction between ORF36 and ORF59, ORF59 full length as well as its GST-tagged segments (1-132aa, 133-264aa, 265-396aa) were expressed, purified, and used for an *in vitro* binding assay with *in vitro* translated ORF36. As a control, Luciferase was also *in vitro* translated, but it did not bind to either the GST or ORF59-GST. Radioactively labeled ORF36 was detected with ORF59-GST and with ORF59 1-132aa GST segment; however, ORF36 binding was stronger with ORF59 133-264aa GST segment.

In order to narrow down the site of phosphorylation, we used the same ORF59 GST-tagged segments for a kinase assay with ORF36 viral kinase and Flag vector as control. Our results indicate that ORF59 is highly phosphorylated by ORF36 (Fig. 2B lane 3). Phosphorylation of ORF59 fragments identified that the region between 265-396aa is the site of phosphorylation (Fig. 2B lane 6). Since ORF36 requires autophosphorylation for its kinase activity, detection of ORF36 phosphorylation in all the lanes confirmed the presence of active kinase (Fig. 2B lane 2-6).

As a negative control, we performed a kinase assay on ORF59-GST with Flag vector as
well as ORF36 with GST for a substrate. Consequently, we detected no phosphorylation of ORF59-GST when assayed with Flag vector (Fig. 2B lane 1) and although there is autophosphorylation of ORF36, there is no GST phosphorylation detected by the assay (Fig. 2B lane 2).

**ORF36 phosphorylates ORF59 at Ser376, Ser378, Ser379:**

In order to identify the probable phosphorylation sites on ORF59 between 265-396aa, we used the KinasePhos program ([http://kinasephos.mbc.nctu.edu.tw/](http://kinasephos.mbc.nctu.edu.tw/), National Chiao Tang University, Taiwan). The program predicted one Threonine and several serine residues as possible targets of ORF36 (Fig. 3A). We grouped these residues to make three ORF59 deletion mutants (DM1, DM2, DM3) (Fig. 3B). The red sequences in figure 3B denote the sequence deleted from full length ORF59. ORF59 deletion mutants were constructed.

![Figure 2: ORF36 phosphorylates ORF59 between 265-396aa.](image)

A. Schematic of ORF59 GST-tagged fragments used for *in vitro* kinase assay. B. Autoradiography and coomassie image of the *in vitro* kinase assay done with ORF59 and its GST-tagged segments and immunoprecipitated Flag and ORF36-Flag. Top row lane 1 is a negative control showing no phosphorylation when ORF36 is not present, while lane 3 shows ORF59-GST phosphorylated by the viral kinase, ORF36. Row two lane 6 shows ORF59 265-396aa segment to be phosphorylated by ORF36. Row three lanes 2-6 show ORF36 autophosphorylation, which is essential for its kinase function. Row four and five are excerpts of the coomassie stained kinase gel. Lanes 1 and 3 show ORF59-GST while lanes 4-6 show ORF59 GST 1-132aa, 133-264aa and 265-396aa segments respectively. The amount of protein used for the *in vitro* kinase assay was relatively similar.
using two PCR products, one was from amplification using sense ORF59 primer and anti-sense ORF59 mutant primer, and the second was from amplification using sense ORF59 mutant primer and anti-sense ORF59 primer. These deletion mutants were cloned into a GST vector.

The ORF59 GST-tagged DM1, DM2 and DM3 proteins were purified and used for *in vitro* kinase assay. ORF36-Flag and Flag vector were immunoprecipitated and washed with RIPA buffer containing higher salt concentration (300 mM NaCl). This was done to ensure no additional protein carry-over for the kinase assay as cellular kinases may also be pulled down, which may convolute the results. Consequently, the results of the kinase assay showed that ORF36 failed to phosphorylate ORF59 DM3 (Fig. 3C Lane 6) but not DM1 or DM2 (Fig. 3C lane 4-5). Specific phosphorylation was confirmed by performing a kinase assay with ORF59 deletion mutants and Flag vector, which showed no phosphorylation (Fig. 3C lane 1-3). This strongly suggested that ORF36 phosphorylates ORF59 at residues, Ser376, Ser378, or Ser379.

For a positive control, we used K8, a known ORF36 substrate, to test the activity of kinase used in this assay and as seen in figure 3E, ORF36 was able to autophosphorylate as well as phosphorylate K8 (Fig. 3E). We also tested the specificity of phosphorylation by ORF36 by using a kinase dead mutant of ORF36 (K108Q). Kinase dead (kd) mutant of ORF36 was generated by a two-step approach similarly to that used to generate ORF59 deletion mutants. Kinase assay with ORF36 K108Q showed that ORF59 was specifically phosphorylated by the viral kinase as kd mutant was not able to phosphorylate ORF59 (Fig. 3D lane 2). Abolition of kinase activity in kd mutant was evidenced by the lack of autophosphorylation of ORF36 (Fig. 3D lane 3).
Figure 3: ORF36 phosphorylates ORF59 between 376-379aa. A. Results of KinasePhos program analysis identifying possible phosphorylation sites on ORF59 between 364-396aa. The sites were grouped as indicated by red lines to generate three ORF59 deletion mutants. B. Schematic of ORF59 deletion mutants used to narrow down the site of ORF36 phosphorylation and the sequences that were deleted for the three mutants. C. In vitro kinase assay done with ORF59 GST-tagged deletion mutants. Top row shows the autoradiography image of ORF59 DM1, DM2, DM3 with Flag vector (Lane 1-3), and with immunoprecipitated ORF36-Flag (Lane 4-6). As seen in lane 6, ORF59 DM3 is not phosphorylated by ORF36, which identifies 376-379aa as the specific site phosphorylated by ORF36. Row two shows autophosphorylation of ORF36 (Lane 4-6), which is essential for its kinase function. Row three and four are an excerpt of the coomassie stained kinase gel. Row three lanes 1-3 show ORF59-GST DM1, DM2, DM3 with Flag and lanes 4-6 show DM1, DM2, DM3 used with ORF36. The amount of ORF59 deletion mutant proteins used for all reactions were relatively the same. Row four lanes 4-6 show the amount of ORF36 used for the in vitro kinase assay. D. As a control, ORF59 was used for an in vitro kinase assay with ORF36-Flag and kinase dead mutant ORF36 (K108Q). The results show that ORF59 is specifically phosphorylated by ORF36 due to no phosphorylation seen with just the vector (top row lane 1). When ORF36 kinase activity is terminated with a specific K108Q mutation, ORF59 is no longer phosphorylated (top row lane 3). E. Additional positive control was included with a known ORF36 substrate, K8. The top two rows show ORF36 autophosphorylation and K8 phosphorylation by ORF36. Bottom two rows show the coomassie-stained gel of the two proteins.
Moreover, we were concerned that large deletions in ORF59 deletion mutants may have an affect on protein confirmation and its tertiary structure; therefore, we generated ORF59 point mutants (PM1, PM2, PM3) to verify our findings and conduct further experiments. The point mutants, where each individual serine residue was mutated to an alanine, were generated using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit as directed (Fig. 4A). As with ORF59 deletion mutants, ORF59 PM1, PM2 and PM3 were used for *in vitro* kinase assay with immunoprecipitated ORF36 or just the Flag vector, as control. As expected, ORF59 PM3 was not while ORF59 PM1 and PM2 were phosphorylated by ORF36 (Fig. 4B lane 4-6). ORF36 autophosphorylation is shown in figure 4B as a control of kinase activity. Positive control, K8 was phosphorylated by ORF36 as demonstrated previously (Fig. 4C).

**ORF59 S376A, S378A, S379A kinase mutant does not bind to RTA or oriLyt:**

Considering the functions of ORF59 and how the absence of phosphorylation may affect the virus, we evaluated the binding between ORF59, its point mutants and the already known binding partners. First, we determined that ORF36 co-localizes with ORF59 point mutants in an overexpression system allowing for phosphorylation to take place (Fig. 6A). Then, we determined that the binding between ORF59 PM1, PM2, PM3 and ORF36 is not affected by the point mutations (Fig. 5A). The vector control did not bring down ORF36 confirming specific association (Fig. 5A lane 6).

Due to the proximity of these point mutations to ORF9 binding domain, we wanted to determine if these point mutations in ORF59 affect its ability to bind or co-localize with the viral polymerase, ORF9. We co-transfected ORF9-Flag plasmid, with HA vector, HA-tagged ORF59 and its point mutants. The co-localization of ORF9 and ORF59 was
similar to that of ORF9 and ORF59 point mutants suggesting that ORF59 point mutants were still able to bind to the viral polymerase and transport it into the nucleus (Fig. 7). The co-immunoprecipitation (CoIP) was done with HA antibody to precipitate ORF59.
Figure 5B shows that ORF9 was brought down by ORF59 and its point mutants but not the empty, HA vector. This indicates that phosphorylation does not affect the ability of ORF59 to bind to the viral polymerase.

Furthermore, we also considered the ability of ORF59 to dimerize despite the lack of phosphorylation at Ser376, Ser378, or Ser379. To do this, we co-transfected GFP-Flag-tagged lentivirus vector with PM3-HA, ORF59-pLVX-Flag with ORF59-HA, ORF59-pLVX-Flag with PM3-HA, and PM3-pLVX-Flag with PM3-HA into 293T cells and immunoprecipitated with anti-Flag (M2) antibody. If dimerization takes place, the Flag-tagged protein would be able to bring down its HA-tagged counterpart. We demonstrated that ORF59 was able to dimerize at a similar level with PM3 as it did with the wild type ORF59 (Fig. 5C lane 6-7). We also showed that PM3 dimerizes with itself at relatively the same level as wild type ORF59, which further verifies that dimerization was not affected by phosphorylation at serine residue 376, 378, or 379 (Fig. 5C lane 8). The vector control did not bring down ORF59 PM3 confirming specific association (Fig. 5C lane 5).

As previously stated, the interaction between ORF59 and RTA is critical for the function of ORF59 as a processivity factor. We looked at the binding and co-localization between ORF59 point mutants and RTA by co-transfecting RTA expression plasmid with lentivirus GFP-Flag-tagged vector, ORF59, ORF59 PM1, PM2 or PM3 pLVX-Flag. Immunofluorescence assay showed ORF59 and its point mutants still localizing with RTA (Fig. 6B); however, Western blot analysis of Flag IP indicated that although PM1 and PM2 are still able to bind with RTA (Fig. 5D lane 8-9), the S376A, S378A, S379A mutations in PM3 significantly hindered its binding with RTA (Fig. 5D lane 10). These
Figure 5: ORF59 S376A, S378A, S379A kinase mutant does not bind to RTA or oriLyt. A. Binding between ORF36 and ORF59 point mutants was determined by co-immunoprecipitation (CoIP). ORF36-Myc plasmid was co-transfected with either empty pLVX vector, ORF59-pLVX-Flag, PM1-pLVX-Flag, PM2-pLVX-Flag or PM3-pLVX-Flag. Flag IP complex was ran on a Western blot and probed for Myc and Flag. Lanes 1-5 show the inputs while lanes 7-10 show the immunoprecipitated ORF59 and its point mutants bringing down ORF36-Myc. B. Binding between ORF9 and ORF59 point mutants was determined by co-transfecting ORF9-Flag with either HA vector, ORF59-HA, PM1-HA, PM2-HA or PM3-HA. HA IP complex was ran on a SDS-PAGE and probed with Flag and HA. Lanes 1-5 show the inputs while lanes 7-10 show the immunoprecipitated ORF59 and its point mutants bringing down ORF9-Flag. C. Due to ORF36 targeting Serine residues near ORF59 dimerization domain, we tested ORF59 PM3’s ability to dimerize. We co-transfected GFP-Flag-tagged lentivirus vector with PM3-HA, ORF59-pLVX-Flag with ORF59-HA, ORF59-pLVX-Flag with PM3-HA, and PM3-pLVX-Flag with PM3-HA into 293T cells and immunoprecipitated with Flag antibody. The immunoprecipitated complex was resolved on a SDS-PAGE and stained with Flag and HA antibodies. Lanes 1-4 show the inputs and lanes 6-8 show that dimerization is not effected by S376A, S378A, S379A mutations. D. Binding between RTA and ORF59 point mutants was determined by CoIP. RTA plasmid was co-transfected with either empty pLVX vector, ORF59-pLVX-Flag, PM1-pLVX-Flag, PM2-pLVX-Flag or PM3-pLVX-Flag. Flag IP complex was resolved on a SDS-PAGE and probed for RTA and Flag. Lanes 1-5 show the inputs while lanes 7-10 show the immunoprecipitated ORF59. The blot shows ORF59, PM1 and PM2 binding to RTA, but PM3 is not able to bind to RTA (lanes 7-9 and 10). E-F. The ability of PM3 to bind to origin of lytic replication (oriLyt) was evaluated and compared with wild type ORF59 with overexpression chromatin immunoprecipitation (ChIP). Following a transfection of 293L cells with RTA-GFP, 8088sc plasmid, which contains oriLyt, and either GFP-Flag-lentivirus vector, ORF59-pLVX-Flag or PM3-pLVX-Flag, ChIP was done with Flag and RTA antibodies. Fraction of the precipitated complex was resolved on a SDS-PAGE to check efficiency. G-H. ChIP DNA product was analyzed with quantitative PCR using RRE primers to amplify specific region bound by RTA and ORF59 in oriLyt. The results were analyzed using ΔΔC_{T} and presented as a fold change. We see a significant decrease of PM3 binding to oriLyt when compared to the wild type ORF59 while RTA binding remains relatively unchanged.
findings are significant as they show that ORF36-mediated phosphorylation of ORF59 plays an important role in the interaction of RTA with ORF59.

Furthermore, we wanted to investigate ORF59 PM3 efficiency to binding at oriLyt with overexpression chromatin immunoprecipitation (ChIP). Following a transfection of 293L cells with RTA-GFP, 8088sc plasmid, which contains oriLyt, and either GFP-Flag-lentivirus vector, ORF59-pLVX-Flag or PM3-pLVX-Flag, chromatins were precipitated with Flag, RTA and control antibodies. A fraction of ChIP sample was analyzed in a Western blot, which showed Flag and RTA efficiently precipitated chromatins (Fig. 5E and F). Purified DNA was used for quantitative (q)PCR with RRE primers, which is the target of RTA-mediated ORF59 binding in oriLyt sequence. The results, analyzed using the ΔΔCt method, show a 16-fold decrease in the ability of ORF59 to bind to oriLyt when ORF36 was unable to phosphorylate at Ser376, Ser378, or Ser379 (Fig. 5G). Since ORF59 binds to RRE through RTA, we quantified the binding of RTA in this overexpression ChIP, as control. Binding of RTA to RRE was comparable in all three samples but the levels of ORF59 with point mutant 3 containing serines, 376, 378 and 379 mutated to alanines, had severely compromised binding to RRE (Fig. 5H).

**KSHV BAC36 ΔORF59 reconstituted with ORF59 PM3 was defective in virion production:**

In order to determine the effect of ORF59 S376A, S378A, S379A (PM3) point mutations on life cycle of KSHV, we used lentivirus vectors, ORF59 or ORF59 PM3 to reconstitute ORF59 deletion in a BAC36 ΔORF59 BACmid. The BACmid has proven to be as a useful tool for determining the role of individual viral genes or mutations in the gene in the context of the viral genome.
Figure 6: ORF59, PM1, PM2, PM3 co-localize with ORF36 and RTA. A. To test co-localization of ORF36 with ORF59 and its point mutants, we transfected 293L cells growing on cover slips with ORF36 DS-Red and GFP-tagged ORF59 and its point mutants PM1, PM2 and PM3. The cells were then fixed, permeabilized and stained with TO-PRO-3 nuclear stain. The figure shows ORF36 co-localizing with ORF59 and its point mutants. B. To test co-localization of RTA with ORF59 and its point mutants, 293L cells were transfected with RTA and ORF59-pLVX-Flag, PM1-pLVX-Flag, PM2-pLVX-Flag or PM3-pLVX-Flag. Cells were fixed, permeabilized and stained with mouse anti-RTA and rabbit anti-Flag primary antibodies. We then used 594 (red) anti mouse and 488 (green) anti rabbit to visualize the two proteins. The overlap of red and green (yellow) indicates co-localization of RTA and ORF59 as well as its point mutants.
BAC36 ΔORF59 BACmid was generated by site-directed homologous recombination using Kan cassette and FLIP recombination (Fig. 8A) as previously stated (36, 40). The correct site of Kan cassette insertion was verified by digesting the intermediate BACmid with PstI and analyzing it via Southern blot with a Kan probe. The resulting fragment containing Kan cassette yielded expected 5,883 bp size fragment with Kan probe (Fig. 8B). FLIP recombinase was then activated to remove the Kan cassette leaving, which
leaves one FRT site (5’-GAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC-3’) at the mutation site. Although the BACmid contains entire ORF59 sequence but the insertion of the remaining FRT site interrupts ORF59 open reading frame due to stop
codon thus blocks ORF59 expression. Furthermore, the absence of Kan cassette was verified with a Southern blot probing for Kan cassette. The junction sequence with FRT insertion was PCR amplified and sequenced to confirm the FRT insertion. The remainder of ORF59 was detected with ORF59 probe in a Southern blot to further validate the clone (Fig. 8B).

Figure 9: KSHV BAC36 ΔORF59 reconstituted with ORF59 PM3 produces less virions. A.-B. BAC36 ΔORF59 transduced with either lentivirus vector, wild type ORF59 or PM3 were tested for expression of LANA, RTA, HA-tagged ORF59 or PM3. Uninduced cells were probed for LANA, HA and GAPDH as a control and TPA and sodium butyrate cells were probed for RTA, HA and GAPDH as a control. These cell lines show to have similar KSHV genome and RTA expression upon reactivation. The lentivirus plasmids are not controlled by TPA or sodium butyrate and therefore their expression does not change. C. DNA was extracted from uninduced cells to examine genome copy maintenance in the three cell lines. LANA (ORF73) primers were used to calculate the KSHV genome copy number via qPCR. The results show the genome copy number to be virtually identical between the three cell lines. D. The three cell lines were induced with 20ng TPA and 1.5 mM sodium butyrate for 5 days after which the supernatant was collected and virion DNA was extracted. Number of virion particles was calculated with qPCR and LANA (ORF73) primers to show a significant decrease in virion production when ORF59 is mutated at Ser376, Ser378, or Ser379.

In order to reconstitute BAC36 ΔORF59, we first transduced 293L cells with pLVX, ORF59-HA-pLVX or ORF59-PM3-HA-pLVX lentivirus. Following a puromycin selection for transduced cells, we used Metafectene Pro to transfect the cells with BAC36 ΔORF59 BACmid. A dual selection with puromycin and hygromycin ensured that all surviving cells contained both the lentivirus and BAC36 ΔORF59. In addition, we were able to detect the BAC using the GFP tag inserted into the backbone (Data not shown).
We also verified the stable maintenance of the BACmid with LANA and RTA Western blots as well as the expression of ORF59 and PM3 with HA-probed Western blots (Fig. 9A-B).

Approximately $5 \times 10^6$ BAC36 ΔORF59 and pLVX, ORF59-HA-pLVX or PM3-HA-pLVX transduced cells collected for a modified Hirt’s extraction to compare the copies of KSHV genome latently maintained in these cells. The purified DNA was used for qPCR with LANA (ORF73) primers, which determined that the genome copy number of latently persisting genome were almost identical between these three cell lines.

The same number of cells were induced with 20 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA) per ml and 1.5 mM sodium butyrate (Sigma, St. Louis, MO) for 5 days. The supernatant was collected and virion DNA was extracted with modified Hirt’s extraction. Purified DNA was then used for qPCR with LANA (ORF73) primers and virion numbers were calculated using the standard curve. As a result, we determined that virion production was significantly decreased when ORF59 was mutated at Ser376, Ser378, or Ser379 (Fig. 9C). Since the virion production levels of PM3 reconstituted BAC36 ΔORF59 was so similar to that of non-reconstituted BAC36 ΔORF59, we may conclude that the function of ORF59 is dependent on the phosphorylation of Ser376, Ser378, or Ser379 residues by viral kinase, ORF36.

**DISCUSSION**

Kaposi’s sarcoma-associated herpesvirus is closely associated with multiple human malignancies including Kaposi’s sarcoma (KS), Primary Effusion Lymphomas (PELs) and Multicentric Castleman’s Disease (MCD). KSHV establishes a lifelong latency following a primary infection with the help of viral and cellular factors. During latency,
viral replication is dependent on cellular replication machinery. Upon reactivation, most of the viral genes are expressed including those necessary and sufficient for KSHV lytic replication (ORF9, ORF6, ORF40/41, ORF44, ORF56, ORF59 and ORF50) (2). Interestingly, although most of the cells in KSHV tumors are latent, approximately 2-5% of cells undergo spontaneous lytic reactivation to produce virions, which are implicated in tumorigenesis (11, 16).

Furthermore, similar to other herpesviruses, the replication and transcription activator (RTA) is accepted as one of the most important proteins required for lytic replication. RTA binds to the C/EBPα and the RTA response element (RRE) binding motifs within oriLyt and as a result activates lytic replication through transcription activation as well as recruitment of additional factors (17, 20, 32). RTA recruits the viral processivity factor, ORF59, to the origin of lytic replication (oriLyt) where it acts as an accessory factor or sliding clamp, stabilizing the binding between ORF9, viral polymerase, and DNA. ORF59 forms a homodimer in the cytoplasm and binds to ORF9 directly and specifically recruiting it to the nucleus, which is crucial for long-chain DNA synthesis in vitro (9, 12, 13, 19). Rossetto et al. have shown that ORF59’s binding to C/EBPα binding motif within oriLyt is necessary for its function and is dependent on its binding to RTA (27). Furthermore, the processivity function of ORF59 is dependent upon its interaction with RTA; consequently, the function of the viral polymerase is also dependent upon this interaction.

Although there is variability in the structures of processivity factor among herpesviruses, their functions are thought to be fairly conserved. Human cytomegalovirus (hCMV) processivity factor, ppUL44 is phosphorylated by viral Ser/Thr kinase, ppUL97, which
modulates its ability to localize to the nucleus (1). BMRF1, Epstein-Barr virus (EBV) processivity factor, is phosphorylated by BGLF4 viral kinase within a hinge region-like domain known to be important for transmitting conformational changes (33). Consequently, phosphorylation by BGLF4 enhances the transactivation activity of Zta (RTA homolog) and the synergistic activation of lytic replication at oriLyt (37). Similarly, ORF59 is a phosphoprotein, which is phosphorylated by the KSHV viral Ser/Thr kinase, ORF36, but the consequences of phosphorylation on the DNA processivity or virion production were not evaluated (9).

Phosphorylation of viral processivity factors is a frequently observed phenomenon in the human herpesviruses. Our aim was to identify the ORF36 target sites on the KSHV processivity factor, ORF59 and the possible effects of phosphorylation on viral production. In this study, three residues, Ser376, Ser378 and Ser379, were identified as the major ORF36 kinase targets on ORF59 through KinasePhos program (http://kinasephos.mbc.nctu.edu.tw/) (Fig. 4A). We mapped the binding region of ORF36 to ORF59 to be most prevalent between 133-264aa on ORF59 (Fig. 1), which did not correspond with the identified phosphorylation sites Ser376, Ser378 and Ser379. Nonetheless, as indicated by ORF59 crystal structure, these regions are in close proximity allowing ORF36 to phosphorylate ORF59 at its C-terminus (3). In fact, detectable binding between kinase and its substrate is not required for phosphorylation to take place. For instance, no binding was detected between the EBV processivity factor, BMRF1, and viral kinase, BGLF4; however, phosphorylation of BMRF1 by BGLF4 revealed to be necessary for the initiation of lytic replication at oriLyt (37).

Due to close proximity of identified phosphorylation sites with ORF59 dimerization
domain, ORF9-interacting domain and nuclear localization domain, we investigated the consequences these mutations could have on lytic replication by evaluating the effects of S376A, S378A and S379A (ORF59-PM3) on known functions of ORF59. Binding between ORF9, viral polymerase, and ORF59 is crucial for viral replication; therefore, we determined that binding between these two proteins and localization of ORF9 was not affected by phosphorylation at Ser376, Ser378 and Ser379 (Fig. 5B, Fig. 7). The binding between ORF59 and ORF9 is dependent upon the ability of ORF59 to dimerize; therefore, we supposed phosphorylation played no role on dimerization of ORF59 (5). Nevertheless, we confirmed the negligent role of phosphorylation on dimerization (Fig. 5C).

Notably, upon examination of binding between ORF59 point mutants and RTA, we determined that S376A, S378A, S379A mutations of ORF59 PM3 inhibited its binding to RTA (Fig. 5D). Previous data suggests that dimerization of ORF59 is not necessary for its interaction with RTA; therefore, it is not surprising that despite ORF59 PM3’s ability to dimerize, it is still not able to bind to RTA. Interestingly, a similar interaction was observed between BMRF1, EBV processivity factor, and BZLF1, RTA homolog, which plays an important role in lytic replication (42). Our finding is significant due to the detrimental affects reduced binding between PM3 and RTA may have on the function of ORF59 PM3 as a processivity factor. We expect this phenomenon to not only hamper the recruitment of ORF59 to oriLyt, but also significantly diminish the function of ORF9 polymerase.

Moreover, we used overexpression chromatin immunoprecipitation (ChIP) assay and reconstituted bacterial artificial chromosome (BAC36) ΔORF59 to demonstrate
downstream effects of S376A, S378A and S379A ORF59 mutations on viral production of KSHV. We have established that phosphorylation at Ser376, Ser378 and Ser379 has no effect on the ability of ORF59 to form a homodimer or bind to ORF9 viral polymerase; however, its hindered binding to RTA raises questions as to whether ORF59 PM3 is recruited and is capable of binding to oriLyt. We used overexpression ChIP to compare the efficiency of ORF59 and its PM3 direct binding to oriLyt in presence of RTA. The results indicated a significant decrease in ORF59 PM3 binding at oriLyt when compared with the wild type (Fig. 5G). This proved to have deleterious effects on viral production when we reconstituted BAC36 ΔORF59 with PM3 versus ORF59 lentivirus (Fig. 9). In fact, the virion production of PM3 reconstituted cell line was similar to that of the control, which contained a fully deleted ORF59 mutant suggesting the function of ORF59 to be largely dependent upon its binding with RTA and phosphorylation of ORF59 at serines 376, 378 and 379.

The precise mechanism by which RTA interacts with ORF59 is not yet clear. RTA-interaction domain of ORF59 has been mapped to 266-396aa and its binding to oriLyt is dependent upon its interaction with RTA. These data demonstrate the binding between RTA and ORF59 to be crucial for KSHV propagation. We propose that phosphorylation of ORF59 by ORF36 viral kinase at serines 376, 378 and 379 may promote formation of certain stabilizing structures, which foster the binding between ORF59 and RTA at oriLyt. The phosphorylation of ORF59 at serines 376, 378 and 379 by ORF36 is fundamental to KSHV propagation as in the absence of this interaction few virions are produced. Furthermore, this provides a possible model for herpesvirus kinases possibly regulating viral lytic replication originating at oriLyt.
ACKNOWLEDGEMENTS

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REFERENCES


Chapter 3.

ORF59 binds to PRMT5 through its catalytic site, displacing it from the chromatin and allowing for initiation of lytic replication at oriLyt.

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Unpublished Data
ABSTRACT

Kaposi’s sarcoma associated herpesvirus (KSHV), an etiologic agent of Kaposi’s sarcoma, body cavity based lymphoma, and multicentric Castleman’s disease, establishes a lifelong latency in infected cells. During latency, majority of the genome is silenced by repressive histone marks, such as H3K27me3. However, upon reactivation the landscape of viral chromatin changes to an open form with methylation of histone-activating marks (H3K4me3). Besides methylation on lysine (K) residues, arginines methylation plays role in determining the compactness of the chromatin. We identified that viral processivity factor binds to protein arginine methyl transferases (PRMTs) to alter chromatin structure. PRMTs dimethylate arginine 3 (R3) of histone, H4 tail in a symmetric (condense chromatin) and asymmetric (open chromatin) manner. We show that symmetric and asymmetric H4R3me2-mediated dimethylated chromatins at KSHV origin of lytic replication (oriLyt) maintained by the differential binding of protein arginine methyltransferase 1 (PRMT1) and protein arginine methyltransferase 5 (PRMT5). Upon viral reactivation, ORF59, a KSHV early lytic protein, binds to PRMT5 at its catalytic site blocking its methyltransferase function allowing for PRMT1 to open up the chromatin for lytic replication. This further promotes KSHV-related pathogenesis and progression of disease.

INTRODUCTION

Kaposi’s sarcoma-associated herpesvirus (KSHV) is a member of the gammaherpesvirus family and has been closely linked with Kaposi’s sarcoma (KS), a B cell lymphoproliferative disorder- Primary Effusion Lymphoma (PEL) and a plasmablastic subset of Multicentric Castleman’s Disease (8, 28). KSHV has a large genome and
encodes for more than 87 open reading frames (ORFs) including genes that encode for capsid, tegument, envelope, DNA replication and regulatory proteins. Among these genes, several of them are dedicated to mimic cellular proteins functions suggesting their significance in KSHV-associated tumorigenesis (1, 2, 6, 7, 9, 19-21). These cellular homologs include host cell cytokines and chemokines, chemokine receptors, anti-apoptotic factors, cyclins and transactivators that are involved in transformation of the host cell (12, 14).

Furthermore, KSHV has not only pirated cellular genes within its genome, but also has demonstrated the ability to pirate promoter regulatory sites, which are homologous to known cellular regulatory sequences or that bind to host cell factors such as AP-1, CCAAT/enhancer binding protein alpha (C/EBPα), NF-κB, and recombination signal binding protein Jκ (RBP-Jκ) (13-17). Treating KSHV-infected cells with agents targeting host cell regulatory mechanisms that affect chromatin and histone acetylation, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), triggers viral replication (25).

Previous report has shown the presence of histone repressive and activating marks throughout the viral genome (13). Thus far, three subgroups of histone modification patterns have been classified. Group I: the regulatory regions of some KSHV genes appear to have a bivalent chromatin structure. Upon virus reactivation, this balance shifts towards activating mark, such as H3K4me3 to form a euchromatin. Group II: the promoter region is marked with AcH3 and H3K4me3 with no repressive marks during latency. Group III: group of genes enriched for H3K27me3 and H3K9me3 are depleted of activation marks with no change upon reactivation (30).
Epigenetic modifications of KSHV genome are region specific and each subgroup is there to modify gene expression in a precise manner best suited for the virus. For example, RTA promoter appears to have a bivalent chromatin as it possesses, both activating, H3K4me3, and repressive marks, H3K27me3. The balance between the two is dependent upon the replication state of the virus, higher activating mark during lytic replication and higher repressive mark during latency (30). These studies support our hypothesis of additional histone marks utilized by the virus to change the chromatin structure upon induction of lytic replication when faced with favorable conditions.

One of the less studied epigenetic modifiers is the methyltransferases. PRMT5, a type II N-methyltransferase, has been described to symmetrically modify arginine 3 of histone H4, H4R3me2s. In embryonic stem cells, where high levels of H3K4me3 and H3K27me3 coexist on majority of promoters readily available to shift the balance in the direction necessary during differentiation. In these cells, Grg4 recruits PRMT5 to histone H4 in order to symmetrically dimethylate arginine 3 (H4R3me2s), which further recruits Polycomb proteins and silences the chromatin. Furthermore, H4R3me2s promotes H3K27 transcription-repressive mark at specific sites to further condense the chromatin (22). There appears to be cooperation between various repressive histone marks for close monitoring of critical promoters.

Specific methyltransferase activity of PRMT5 was first determined by the Pestka lab in 1999. Through extensive sequencing and comparison with other known methyltransferases, they identified Motif I (GXGRPGP) to be essential for the methyltransferase activity. In fact, PRMT5 point mutants containing substitutions of the
central glycine and arginine of the invariant GXGRPGP region showed little to no methyltransferase activity (24).

PRMT1, a type I methyltransferase, is found in every adult and embryonic tissue examined and is thought to contribute up to 85% of all cellular PRMT activity (23, 29). Its major target is the histones as it mediates replication and transcription by asymmetrically methylating H4R3me2s. As a result, H4R3me2as provides a binding surface for CBP/p300–associated factor (PCAF) and directly enhances subsequent acetylation of histones H3 (16, 32). PRMT1 expression in cancer cells may be altered depending on the tumor type. For instance, PRMT1 is an essential component of Mixed Lineage Leukaemia (MLL) transcriptional complex that asymmetrically dimethylates H4R3, which suggests its direct role in tumor initiation and progression (11).

Bivalent chromatin is thought to exist when both activating and repressive chromatin modulators (such as H3K4 and H3K27) are present at the site. In this case, for a specific shift towards euchromatin, a demethylase is required to remove a methyl group returning the histone residue to its monomethyl state where it is available to be altered in favor of open chromatin. Although there are multiple lysine demethylases but only a handful of arginine demethylase have been implicated in binding to methylated arginine residues in histone tails with JMJD6 being the only methyltransferase known to demethylate H4R3me2 (10).

In this study, we show that during latency PRMT1 and PRMT5 are located at KSHV origin of lytic replication (oriLyt) contributing to both H4R3me2 symmetric and asymmetric marks. During lytic reactivation there is a shift towards PRMT1 an25d H4R3me2as mark thereby opening the chromatin. ORF59, a KSHV processivity factor in
the replication complex, directly binds to PRMT5 at its catalytic domain (GXGRPGP), which allows PRMT1 to open up the chromatin for lytic replication. In EBV, the balance between PRMT1 and PRMT5, as post-translational modifiers, has been shown to play an important role in initiation of replication at the latent origin (oriP). Their modifications are critical for localization of EBNA1, a protein required for initiation of latent replication (27). Interestingly, H4R3me2s transcription repressive marks have been shown to upregulate H3K27me3 and antagonize H3K4me3 (27). This further suggests that PRMT5 may be the underlying cause to KSHV chromatin silencing at oriLyt.

We propose that PRMT5 symmetrically dimethylates histone H4 (H4R3me2s) during latency thereby condensing the chromatin at oriLyt to prevent initiation of lytic replication while the presence of ORF59 during reactivation diminishes these affects allowing for PRMT1 to asymmetrically dimethylate histone H4 (H4R3me2as) to open the chromatin at oriLyt (17).

MATERIALS AND METHODS

DNA constructs, transfection and transduction by the lentivirus:

The following plasmids were generated by PCR amplification and cloning: ORF59-pLVX-Flag, PRMT5-pA3M (Myc), PRMT5-pA3F (Flag) and ORF59-HA. Primers used for cloning are listed in Table 1.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Primer Oligonucleotide Sequence 5’-3’</th>
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<tr>
<td>ORF59-Flag-pLVX</td>
<td>S TTTGAATTCTCTGTGGATTTTCACTATGGGGTC</td>
</tr>
<tr>
<td></td>
<td>AS TCAGCGCGCGGAAAAACAAAGTCCCGGTTGGGG</td>
</tr>
<tr>
<td>ORF59-HA-pxi</td>
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<td>AS</td>
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<tr>
<td>PRMT5-pA3M/pA3F</td>
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<tr>
<td></td>
<td>AS</td>
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<td>JMJD6-pA3F</td>
<td>S</td>
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<td>AS</td>
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<td>GXGRPGP-pLVX-GFP</td>
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Cells were transfected by calcium phosphate method as previously described (7), with minor modification. Briefly, 500 µl 1xHBS (140 mM NaCl, 0.75 mM Na₂HPO₄·2H₂O, 25 mM HEPES, 5 mM KCl, 6 mM Dextrose pH 7.1) was added to DNA and mixed. Thirty microliters of 2.5 M CaCl₂ was added to the solution and mixed. After 20 min at room temperature, the solution was added to 60-70% confluent 293T cells drop wise.

For lentivirus, 10 µg of lentivirus plasmid, 7.5 µg CMV-dR8.2 packaging plasmid and 2.5 µg pCMV-VSVG envelope plasmid were transfected into 100 mm dish of 293T cells using 1xHBS. Twenty-four hours post-transfection, virus was induced with 1 mM sodium butyrate (NaB) in 5% bovine growth serum DMEM with 100 mM HEPES for 10 hr. The supernatant was collected three times thereafter at 12-16 hr intervals. The supernatant was filtered with 0.45 µm pore-size filter and ultracentrifuged at 25,000 rpm for 1.5 hr using Beckman Coulter Optima L-90K ultracentrifuge (Beckman Coulter, Inc. Brea, CA). The virus pellet was resuspended in DMEM and added to 293L cells.
BACmid DNA was transfected with Metafectene Pro (Biontex Laboratories GmbH, San Diego, CA) due to the larger size of the plasmid and fragility of DNA. Cells were transfected with a combination of two solutions: 1) 5-10 µg BACmid DNA in 200 µl serum and antibiotic free medium, 2) 6 µl Metafectene Pro in 200 µl serum and antibiotic free medium. Following the combination of two solutions, the mixture was allowed to sit at room temperature for 15-20 min and added onto the cells drop-wise. The BACmid contained GFP, which allowed for efficient evaluation of transfection efficiency and monitoring of selection.

**Cell culture and cell lines:**

293T and 293L cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine growth serum (HyClone, Logan, UT), 2 mM l-glutamine, 25 U/ml penicillin, and 25 µg/ml streptomycin. TRExBCBL1-RTA (Jae Jung, University of Southern California) was used for majority of the experiments due to the high induction efficiency of lytic replication with doxycycline. Treating TRExBCBL1-RTA with 1 µg/ml of doxycycline induces the RTA promoter, which then initiates lytic replication in a more natural manner. TRExBCBL1-RTA, JSC-1 and BC3 cells were grown in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% bovine growth serum (HyClone, Logan, UT), 2 mM l-glutamine, 25 U/ml penicillin, and 25 µg/ml streptomycin. All cultures were incubated at 37°C in a humidified environment supplemented with 5% CO₂.

Constituently expressing ORF59-Flag cells lines were generated using a lentivirus system and ORF59-Flag expressing BACmid. ORF59-pLVX-Flag lentivirus clone was generated and transfected into 293T cells along with packaging and envelope proteins (CMV-
dR8.2, pCMV-VSVG) as stated above. The vector alone was also transfected into 293T cells to serve as a control. The collected virus was added to 293L cells, media was changed 24 hr later, and transduced cells were selected with puromycin (2 mg/ml). BAC36 ORF59-Flag was transfected into 293L cells with Metafectene Pro (Biontex Laboratories GmbH, San Diego, CA) as previously described and hygromycin was used for positive selection. The selection in both cell lines was monitored with GFP signal encoded by the lentivirus and the BACmid.

**Generation of BAC36 ORF59-Flag:**

BAC modifications were done using modified bacterial strain, SW102, derived from DY380, which contains the λ prophage recombineering system. Briefly, a galK-Kan cassette containing homologous ends to the ORF59 C-terminus was PCR amplified (forward, 5′-

GACTCGAGCCAGTCGGATCGGGAAGTGCCCCAAAACCACATTATACCC

CCCTGATCTCTGGACAATTAATCATC -3′, and reverse, 5′-

TGACACCATGTGCGCCCTGGACAGTGAGCGCTCTGCTGCTCTTCTTCAGTTATC

TGAGCGGCTCAGCAAAAGTTCGATTATA

-3’) with 30-50 ng pgalK-Kan as template with 94°C for 2 min and 30 cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 1.5 min, following by 72°C for 3 min. Two microliters of DpnI per 50 µl reaction was added and incubated at 37°C for 1 hr to cleave any remaining template DNA. The DNA was purified and 150-200 ng was used for heat shock transformation into SW102 KSHV-containing cells. The intermediate was grown at 32°C and selected with kanamycin (10 µg/ml) and chloramphenicol (30 µg/ml) plates. The colonies were screened by digesting with BamHI followed by a Southern blot
analysis expecting to see a 6.2 kb fragment when probed with Kan probe. The correct intermediate clone was then used to generate competent cells for further recombination. To do this, the bacterial culture was grown at 32°C until OD$_{600}$ was approximately 0.6, incubated at 42°C for 15 min and then quickly placed on ice. The bacterial culture was washed 3 times with ice-cold MilliQ water. The $gal$K-Kan cassette was then substituted by a double-stranded oligo containing the same homologous ends and a flag tag sequence (GACTACAAAGACGATGACGACAAG). Approximately 1 µg of the oligo was electroporated into the intermediate ($gal$K-Kan-containing) SW102 cells using 0.1 cm cuvette at 25 µF, 1.75 kV, and 200 ohms. The bacteria were recovered in 10 ml LB at 32°C for 4.5 hr. The culture was then washed 10-12 times with 1xM9 medium (10 g/l Na$_2$HPO$_4$, 5 g/l KH$_2$PO$_4$, 5 g/l NH$_4$Cl, 2.5 g/l NaCl), and 1:10 dilution was plated on M63 minimal media plates with glycerol (0.2% Fisher), leucine (45 mg/l), d-biotin (1 mg/l), 2-deoxy-galactose (DOG) (0.2% Ferro Pfanstiehl), and chloramphenicol (10 µg/ml). The plates were incubated for 3 days at 32°C before any colonies were detected.

**Immunoprecipitation, Western blotting and antibodies used:**

Transfected cells were harvested, washed with ice-cold PBS, and lysed in 0.5 ml ice-cold RIPA buffer (1% Nonidet P-40 [NP-40], 10 mM Tris [pH 7.5], 2 mM EDTA, 150 mM NaCl), supplemented with protease inhibitors (1 mM phenylmethyisulfonfluoride, 1 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin). Cell debris was removed by centrifugation at 13,000×g (10 min and 4°C), and lysates were then precleared for 1 hr with rotation at 4°C with 30 µl of a 1:3 mixture of Protein A-Protein G-conjugated Sepharose beads. Approximately 5% of the lysate was saved for input control, the protein of interest was captured by rotating the remainder of the lysate with 1 µg of the
appropriate antibody overnight at 4°C. Immune complexes were captured with 30 µl of a 1:3 mixture of Protein A-Protein G-conjugated Sepharose beads with rotation for 2 hr at 4°C. The beads were pelleted and washed three times with RIPA buffer.

For Western blot assay, input lysates and immunoprecipitated (IP) complexes were boiled 5-10 min in Laemmli buffer and resolved on SDS-PAGE. The protein was transferred to a .45 µm nitrocellulose membrane, probed with appropriate antibodies followed by incubation with infrared-tagged secondary antibody, and viewed on an Odyssey imager (LiCor Inc., Lincoln, NE).

The following antibodies were used: mouse anti-Flag (M2, Sigma-Aldrich, St. Louis, MO), rabbit anti-Flag (F7425, Sigma-Aldrich, St. Louis), mouse anti-RTA (mouse hybridoma), mouse anti-LANA (mouse hybridoma), rabbit anti-HA (6908, Sigma-Aldrich, St. Louis, MO), mouse anti-GFP (G1546, Sigma-Aldrich, St. Louis), mouse anti-GAPDH (G8140, US Biological, Salem MA), and rabbit anti-Myc (SAB4300605, Sigma-Aldrich, St. Louis, MO), goat anti-PRMT5 (C-20, sc-22132, Santa Cruz Biotechnology), mouse anti-PRMT1 (B-2, sc-166963, Santa Cruz Biotechnology, Santa Cruz CA), mouse anti-ORF59 (11D1, Advanced Biotechnologies Inc., Columbia MD), rabbit anti-H3K4me3 (MC315, Millipore, Temecula, CA), rabbit anti-H4K27me3 39156, Active Motif), rabbit anti-H4R3me2s (61187, Active Motif, Carlsbad CA), rabbit anti-H4R3me2-asymmetric (39705, Active Motif, Carlsbad CA).

**Chromatin Immunoprecipitation (ChIP) analysis:**

Cells were cross-linked with 3% formaldehyde by rocking for 10 min at room temperature, followed by addition of 125 mM glycine to stop the cross-linking reaction. Cells were washed with cold PBS containing protease inhibitors (1 µg/ml leupeptin, 1
µg/ml aprotinin, 1 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Cells were resuspended in 1 ml cell lysis buffer [5 mM piperazine-\(N,N'\)-bis (2-ethanesulfonic acid) (PIPES)-KOH (pH 8.0)-85 mM KCl-0.5% NP-40] containing protease inhibitors and were incubated on ice for 10 min. Cells were subjected to Dounce homogenization for efficient lysis, followed by centrifugation at 2,500 rpm for 5 min at 4°C. Nuclei were resuspended in nuclear lysis buffer (50 mM Tris [pH 8.0]-10 mM EDTA-1% SDS containing protease inhibitors), followed by incubation on ice for 10 min. Chromatin was sonicated to an average length of 700 bp, and cell debris was removed by centrifugation at high speed for 10 min at 4°C. The supernatant containing sonicated chromatin was diluted fivefold with ChIP dilution buffer (0.01% SDS-1.0% Triton X-100-1.2 mM EDTA-16.7 mM Tris [pH 8.1]-167 mM NaCl including protease inhibitor). Samples were precleared with a salmon sperm DNA-protein A-protein G Sepharose slurry for 1 hr at 4°C with constant rotation. The supernatant was collected after a brief centrifugation (2,000 rpm at 4°C). Ten percent of the total supernatant was saved for input in Western blotting, and the remaining 90% was used for immunoprecipitation (IP). Reaction complexes were rotated overnight at 4°C, followed by precipitation of the immune complex by using a salmon sperm DNA protein A/G slurry. Beads were then washed sequentially with a low-salt buffer (0.1% SDS-1.0% Triton X-100-2 mM EDTA-20 mM Tris [pH 8.1]-150 mM NaCl), a high-salt buffer (0.1% SDS-1.0% Triton X-100-2 mM EDTA-20 mM Tris [pH 8.1]-500 mM NaCl), and a LiCl wash buffer (0.25 M LiCl-1.0% NP-40-1% deoxycholate-1 mM EDTA-10 mM Tris [pH 8.0]) and twice in Tris-EDTA. Ten percent of the immunoprecipitated chromatin was taken for Western blot detection. Chromatin was eluted using an elution buffer (1% SDS-0.1 M NaHCO₃) and reverse
cross-linked by adding 0.3 M NaCl at 65°C overnight. Eluted DNA was precipitated, treated with proteinase K at 45°C for 2 hr, and purified. Purified DNA was used as a template for amplification of the RRE region of KSHV oriLyt or RTA promoter.

**Quantitative real-time (RT) PCR:**
Quantitative real-time PCR was performed in a total volume of 20 µl, including 10 µl of SYBR green PCR 2× Master mix (Applied Biosystems). ChIP samples were analyzed using oriLyt RRE primers (forward, 5′-CTCTGGGTTTCCGGT-3’, and reverse, 5′-TTACACAAGGGCCGCAAA-3’) and RTA promoter primers (forward, 5′-GGTACCGAATGACAAATCTGTCGCT-3’, and reverse, 5′-TTTGTGGCTGCTGGCACAGTATCC-3’). Purified ChIP DNA samples and the input DNA samples were amplified with 3 min of 95°C, 40 cycles of 15 sec 95°C, 30 sec at 51°C, and 30 sec at 72°C on an ABI StepOne plus real-time PCR machine (Applied Biosystems). A melting curve analysis was performed to verify the specificity of the amplified product and each sample was tested in triplicate.

**Indirect immunofluorescence microscopy:**
BAC36 ORF59-Flag cells were plated on glass cover slips and non-adherent cells (TRExBCBL1-RTA, JSC-1 and BC3) were first induced with 20 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA) per ml and 1.5 mM sodium butyrate (Sigma, St. Louis, MO) for 24 hr and then spun down, resuspended in minimal amount of 1xPBS and spread on glass cover slips for further staining. The cells were fixed with 3% paraformaldehyde for 20 min at room temperature, washed twice with 1xPBS and then permeabilized with 0.2% Triton X-100 for 10 min at room temperature. The cells were washed twice with 1xPBS and blocked in 0.4% Fish Skin Gelatin and 0.05% Triton X-
100 for 40 min at room temperature. The cells were washed twice with 1xPBS and probed with primary antibody (0.2% Fish Skin Gelatin, 0.05% Triton X-100 and 0.5 µg antibody) for 1 hr at room temperature. The cells were washed twice with 1xPBS and treated with secondary antibody containing the fluorescent tag (0.2% Fish Skin Gelatin, 0.05% Triton X-100 and 1:20,000 dilution of stock antibody). PRMT5 was stained with Alexa Fluor 488 and ORF59 was stained with Alexa Fluor 594. The cells were then washed twice with 1xPBS and the nuclei were stained with TO-PRO-3 (Molecular Probe) for 10 min. Images were obtained using a laser scanning confocal microscope (Carl Zeiss, Inc.).

RESULTS

KSHV genome is managed by both suppressive and activating histone marks during latency (13). At this stage, RTA promoter appears to have a bivalent chromatin, as it possesses activating mark, H3K4me3, and repressive mark, H3K27me3. Upon reactivation, the activating mark is upregulated to create euchromatin at the promoter allowing for successful transcription of the immediate early lytic protein (30).

**ORF59-pLVX-Flag immunoprecipitation brought down endogenous PRMT5:**

In order to identify possible cellular binding factors for ORF59, we utilized the lentivirus system to generate a 293L constitutently expressing cell line. After verifying efficient lentivirus transduction with GFP signal (Fig. 1A) and ORF59-Flag expression with flag immunoprecipitation (IP) (Fig. 1B), we ran the protein complex on a gradient gel and stained with coomassie dye to visualize proteins. Comparing the control (pLVX-Flag vector, Fig. 1C lane 1) with ORF59-pLVX-Flag IP (Fig. 1C lane 2), we identified several
Figure 1: ORF59 brings down protein arginine methyl transferase 5 (PRMT5).
Screening for ORF59 cellular binding factors was done with ORF59 pLVX-Flag constitutively expressing cell line and its vector control. ORF59-pLVX-Flag and its vector control cell lines were generated by first producing a lentivirus by co-transfecting 293T with packaging and envelope proteins (CMV-dR8.2, pCMV-VSVG). The virus was collected, spun down and used to transduce 293L cells, which were then placed under selection with puromycin. A. The lentivirus vector contains a GFP tag, allowing for easy detection. B. The flag tag synthesized by the vector allowed for efficient immunoprecipitation by flag antibody of both the vector (lane 1) and ORF59 (lane 2). C. Immunoprecipitated complex was analyzed on a gradient gel via coomassie stain. Lane 1 shows the proteins brought down by vector alone and lane 2 shows proteins brought down by ORF59-Flag. Since the vector encodes for a GFP tag, we were able to detect the vector band (red star, lane 1). Red star in lane 2 indicates OF59-Flag. Unique bands to lane 2 were cut out and subjected for MALDI-TOF and peptide sequencing. Arrows indicate several of the proteins identified (MRP4, PRMT5, C1orf49, H2A/H2B).
<table>
<thead>
<tr>
<th>Name</th>
<th>Accession No.</th>
<th>Peptides</th>
</tr>
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<tbody>
<tr>
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<td>MRP4_HUMAN</td>
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<tr>
<td>Protein arginine N-methyltransferase 5 isoform a (Homo)</td>
<td>gi</td>
<td>20070220</td>
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<tr>
<td>Uncharacterized protein C1orf49 (Homo sapiens)</td>
<td>CA049_HUMAN</td>
<td>CLLCALK, LMGKTHR, QIKDLMDK, GNIPSEASGLYK, CLLCALKNNYNYR, LHEFVEIMKEMQ, DFDKILLFHEIMK, QGSLDLPJHCGTCCEK, TKQGSLDPLHICGTCCEK, GGEEMPQTQPSVHGAVPAPKQTEGR</td>
</tr>
<tr>
<td>Histone H2A (Homo sapiens)</td>
<td>gi</td>
<td>603553</td>
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</table>

Table 2: Partial list of peptide sequences of ORF59 interacting proteins. Four unique bands pulled down by ORF59 versus its vector are identified as MRP4, PRMT5, C1 orf49 and H2A/H2B.
unique bands, which were submitted for MALDI-TOF and peptide sequence at Applied Biomics (Hayward, CA). GFP-ORF59-Flag and GFP-Flag bands are marked with red stars while the arrows pointing out four unique bands were identified as MRP4, PRMT5, C1 orf49 and H2A/H2B proteins via sequencing.

Although there were several proteins of interest, we decided to follow up on protein arginine methyl transferase 5 (PRMT5) due to its role in chromatin modification. We hypothesized that ORF59 may have an additional role in initiating lytic replication by interacting with cellular epigenetic modulators and opening up the chromatin at oriLyt.

**ORF59 binds to PRMT5:**

We confirmed the binding between ORF59 and PRMT5 by transfecting ORF59-HA/Flag and PRMT5-Flag/Myc plasmids for co-immunoprecipitation (CoIP) and reverse CoIP. The protein complex was immunoprecipitated with flag antibody and analyzed on a Western blot. In both cases, ORF59 and PRMT5 were shown to bind in an overexpression system (Fig. 2A-B). We then used KSHV-infected B cell lines, TRExBCBL1-RTA, JSC-1, and BC3, to affirm this finding in an endogenous system. Each cell line was induced for lytic replication with 20 ng of 12-O-tetradecanoylphorbol-13-acetate (TPA) per ml and 1.5 mM sodium butyrate (Sigma, St. Louis, MO) and PRMT5 was immunoprecipitated with goat anti-PRMT5. The complex was analyzed on a Western blot to show that ORF59 was co-immunoprecipitated in each case (Fig. 2C).

**ORF59 co-localizes with PRMT5:**

Both, ORF59 and PRMT5, are nuclear proteins. We used KSHV-infected B cells, TRExBCBL1-RTA, JSC-1, and BC3, to reaffirm that they co-localize endogenously. As described in Materials and Methods, we induced the B cells for lytic replication, collected
uninduced as well as the induced cells 24 hr post induction and dried them on glass cover slips for staining. Both, uninduced as well as induced cells were stained for ORF59 and PRMT5 using specific antibodies. As expected, uninduced cells did not show ORF59 expression (red) (latent cells do not express ORF59), but a steady expression of PRMT5 (green). Upon induction of lytic replication, robust expression of ORF59 was evident (red) and distinct yellow punctuation marked co-localization of PRMT5 (green) with ORF59 (red) (Fig. 3).

**KSHV oriLyt showed H4R3me2s and H4R3me2as marks:**

Comprehensive study of DNA methylation and histone modifications across the entire KSHV genome during latency by Gunther and Grundhoff (2010) suggested the existence of a delicate balance between repressive and activating marks, which regulate gene expression and the switch to lytic replication (13, 30). Interestingly, despite latent KSHV
replication state, activating histone marks occupied several lytic promoters. However, accompanying these activating marks, majority of the viral genome was subjected to heavy tri-methylation of lysine 27 histone H3 (H3K27), a modification known to suppress transcription regardless of the presence of activating marks (4, 5, 26). In addition, RTA promoter appears to have a bivalent chromatin as it displays, activating, H3K4me3, and repressive marks, H3K27me3. Upon reactivation, the repressive mark (H3K27me3) decreases along with increase in the activation mark (H3K4me3) (30).

Figure 3: ORF59 co-localized with PRMT5. We used uninduced and TPA induced KSHV-infected cells, TREXBCBL1-RTA, JSC-1, and BC3 for immunofluorescence assay. All cells were stained for PRMT5 (green) and ORF59 (red) as well as the nuclear stain (blue). Uninduced cells show the absence of ORF59 because it is a lytic protein. In induced cells, the localization of ORF59 and PRMT5 is shown by a yellow color.
Similarly, we show that oriLyt is simultaneously under suppressive and activating histone modifications. Following our previous findings, we used TRExBCBL1-RTA cell line, which is easily induced for lytic replication (Fig. 4A), to examine oriLyt for repressive and activating histone marks related to PRMT5. Through chromatin immunoprecipitation (ChIP) analysis done on uninduced and 24 hr post induction TRExBCBL1-RTA cells, we identified repressive (anti-H4R3me2s) and activating (anti-H4R3me2as) histone marks at oriLyt (Fig. 4B). During latency, similar levels of symmetric and asymmetric dimethylation at Histone H4 were present; however, upon reactivation with doxycycline, there was a strong shift towards asymmetric dimethylation, which signifies open chromatin for lytic replication.

Concurrently, we considered the repressive and activating mark on H3 histone lysine residues with respect to H4R3me2. TRExBCBL1-RTA cells were used for ChIP analysis with anti-H3K4me3 and H3K27me3 antibodies (Fig. 4E) to show a reduction in repressive H3K27 mark and a significant increase in H3K4me3 mark upon reactivation (Fig. 4C). This is consistent with previous findings and is supportive of our hypothesis that specific histone modifications are required for the initiation of KSHV lytic replication. Additionally, we used the same ChIP samples to determine the mark at RTA promoter. We found a significant increase in the activating H3K4me3 mark, which is expected with an increase in RTA protein synthesis (Fig. 4A and 4F).
Accumulation of PRMT1 at oriLyt is not dependent on ORF59:

The balance between symmetric and asymmetric dimethylation of H4R3 is managed by interaction between several proteins. We have identified PRMT5 being responsible for symmetric methylation or repression of replication. We determined PRMT1 to be the

**Figure 4: KSHV oriLyt shows H4R3me2s and H4R3me2as marks.** Doxycycline inducible TRExBCBL1-RTA cells were used for chromatin immunoprecipitation (ChIP) assays. A. TRExBCBL1-RTA cells were robustly induced by doxycycline, which provided a good model to study KSHV lytic replication. B. Chromatin at oriLyt shows both symmetric and asymmetric dimethylation of H4R3. This was tested with uninduced and induced TRExBCBL1-RTA cells. Upon induction, an influx of PRMT1 is shown. C. Histone modification marks, such as H3K4me3 and H3K27me3, were present throughout the KSHV genome. We evaluated the presence of H3K4me3, an activating mark, and H3K27me3, repressive mark, at oriLyt. Here we show the upregulation of H3K4me3 and downregulation of H3K27me3 when TRExBCBL1-RTA cells are induced. D. The same cell lysate was used to assess the histone mark at RTA promoter. As expected we see an upregulation of H3K4 when the virus is induced for lytic replication. E. As a control for the ChIP analysis, fraction of immunoprecipitated protein was resolved on a Western blot to show an efficient pull-down.
Figure 5: Presence of PRMT1 at KSHV oriLyt is not ORF59 dependent. A. We used the overexpression system to determine that PRMT1 does not bind to ORF59. We co-transfected ORF59-HA with either PRMT1-Flag or Flag vector. Following a Flag IP, the protein complex was resolved on a Western blot and probed with anti-Flag and anti-HA antibodies. B. We confirmed this finding in an endogenous system by immunoprecipitating ORF59 from a stable cell line and probing for PRMT1 and PRMT5. The results confirmed that there was no binding between PRMT1 and ORF59; however, ORF59 bound to PRMT5. C. Immunofluorescence assay was used to visualize the localization between ORF59 (red) and PRMT1 (green). These two proteins co-localized as indicated by the yellow color. D.- E. Chromatin immunoprecipitation (ChIP) analysis was done with uninduced and induced TRExBCBL1-RTA cells using PRMT1 and PRMT5 specific antibodies. The purified DNA was used for qPCR with RRE primers. Here we show that uninduced KSHV-infected cells had both PRMT1 and PRMT5 bound at oriLyt; however, after induction PRMT5 was removed but PRMT1 remain bound there.
methyltransferase to asymmetrically dimethylate H4R3. PRMT1 is a type I methyltransferase and has been extensively linked with formation of euchromatin as well as transcriptional activation of several ER regulated genes (3, 31). Additionally, asymmetric dimethylation of H4R3 facilitates histone H3 acetylation on Lys9/Lys14 and as a result leads to efficient recruitment of transcription preinitiation complexes to active promoters (18).

For the initiation of lytic replication, the balance between PRMT1 and PRMT5 has to tip towards PRMT1 to form euchromatin. This may happen by two mechanisms: 1) PRMT5 may be displaced by an early lytic protein or a cellular factor recruited by a lytic protein, or 2) a KSHV lytic protein may shuttle additional PRMT1 to oriLyt. We have already established that ORF59, a KSHV lytic protein, binds to PRMT5 and shifts the balance

![Figure 6: ORF59 displaces PRMT5 from oriLyt in overexpression ChIP.](image)

293L cells were transfected with 8088sc plasmid, RTA, PRMT5 and ORF59pLVX-Flag or vector control. A.-B. ORF59-pLVX-Flag and PRMT5-Myc were immunoprecipitated with high efficiency. C. The purified DNA was used for qPCR with RRE primers to determine the binding of PRMT5 to oriLyt in presence of ORF59. The results indicate the binding of PRMT5 is greatly diminished when co-transfected with ORF59.
towards asymmetrically dimethylated H4R3me2as.

Next, we assessed the interaction between PRMT1 and ORF59. We co-transfected PRMT1-Flag or Flag vector with ORF59-HA into 293T cells, immunoprecipitated with anti-Flag antibody, and ran the protein complex on a Western blot. The results identified that ORF59 was unable to bind to PRMT1 in an overexpression system (Fig. 5A). We confirmed this result in an endogenous system by using ORF59-Flag constitutently expressing cell line generated with a lentivirus. GFP-ORF59-Flag or the GFP-Flag was pulled down using anti-Flag antibody resolved on a Western blot and probed for PRMT1 and PRMT5. Figure 5B shows that ORF59-Flag was able to bring down PRMT5 but not PRMT1 (Lane 4). In addition, we looked at the co-localization between ORF59 and PRMT1 in TRExBCBL1 RTA cells. We induced the B cells for 24 hr and stained them for ORF59 and PRMT1 along with uninduced cells as control. Because these proteins being nuclear, we expected to see co-localization but nowhere near the levels seen with PRMT5 (Fig. 3). Uninduced TRExBCBL1 RTA cells did not express any ORF59 as it is a lytic protein; however, ORF59 was robustly expressed in induced cells (red) and the overlap with PRMT1 (green) is displayed by yellow color (Fig. 5C).

We used ChIP analysis of uninduced and induced TRExBCBL1 RTA with antibodies specific for PRMT1 and PRMT5. Purified DNA was then used for qPCR analysis with RRE primers to examine the presence of both epigenetic modulators at oriLyt. Figure 5D shows the ethidium bromide gel of the amplified product with similar levels of PRMT1 and PRMT5 in uninduced TREx cells and a complete absence of PRMT5 after induction. Relative density comparison of these bands with respect to their inputs indicates a small increase in PRMT1 levels at oriLyt and complete elimination of PRMT5 upon induction.
In order to evaluate the interaction between PRMT1, PRMT5 and ORF59 with respect to the entire KSHV, we tagged ORF59 with a Flag tag in BAC36 CR1 KSHV containing BACmid. A. A galK-Kan cassette was inserted into the KSHV genome using homologous recombination. B. A double-stranded oligo containing a Flag tag and homologous ends was used to displace the galK-Kan cassette. C. Final clone contained a BACmid with intact ORF59 with a Flag tag. D. BAC36 CR1 ORF59-Flag
intermediate colonies were screened for Kan cassette using Southern blot and expecting to see a band at 6.2 kb. The Kan cassette was displaced by a double-stranded oligo; therefore, the signal is eliminated in the final clone. **E.** BACmid encodes for GFP allowing for easy detection and monitoring of transfection efficiency. **F.** BAC36 CR1 ORF59-Flag was used to generate a stable cell line. We were able to IP ORF59-Flag, which subsequently brought down endogenous PRMT5. **G.** Reverse IP with PRMT5 antibody brought down ORF59-Flag. **H.** Immunofluorescence assay with uninduced and induced BAC36 CR1 ORF59-Flag cells showed co-localization between PRMT5 and ORF59 Flag in induced cells. As expected, no ORF59 was detected in uninduced cells due to ORF59 being a lytic protein.

(Fig. 5E). ORF59 is a lytic protein, expressed primarily during lytic replication; therefore, despite the increase of PRMT1 at oriLyt during lytic replication, presence of PRMT1 at oriLyt during latency suggests that this binding is not ORF59 dependent.

**PRMT5 is displaced by ORF59 during lytic reactivation:**

In order to determine the significance of the interaction between ORF59 and PRMT5, we first evaluated the binding of PRMT5 to oriLyt in the presence or absence of ORF59 in an overexpression system using ChIP analysis. We co-transfected 293 cells with 8088sc plasmid containing KSHV oriLyt, PRMT5-Myc with either ORF59-pLVX or empty

![Figure 8](image_url)  
**Figure 8:** During lytic reactivation binding of PRMT5 to oriLyt was downregulated while PRMT1 upregulated in BAC36 CR1 ORF59-Flag. BAC36 CR1 ORF59-Flag cell line was induced for lytic replication by transfecting with RTA plasmid. Uninduced and induced cells were collected for chromatin immunoprecipitation (ChIP). **A.** ChIP efficiently immunoprecipitated chromatin bound proteins. Uninduced cells showed no expression of lytic proteins (RTA and ORF59); therefore, no protein was brought down in the IP. **B.** Purified DNA was used for qPCR analysis with RRE primers. We observed that lytic reactivation of KSHV in the BACmid system was similar to that seen in TRExBCBL1-RTA. Upregulation in PRMT1 binding to oriLyt was accompanied by downregulation of PRMT5.
vector, as a control. Flag and Myc IP of these proteins were done with high efficiency (Fig. 6A-B) and purified DNA was used for qPCR with RRE primers. Figure 6C indicates a strong binding of PRMT5 to oriLyt in the absence of ORF59, which in endogenous system would signify condensed chromatin unable to initiate DNA replication. On the contrary, introduction of ORF59 reduced the amount of PRMT5 at oriLyt, which signifies its displacement from the oriLyt.

Applying this observation to a more natural system, we used a BACmid containing KSHV genome with Flag-tagged ORF59. It was generated in our lab using the galK-Kan recombineering technique as stated in Materials and Methods (Fig. 7A-C). Although this system is not as easily induced as TRExBCBL1-RTA, it was convenient to use due to the Flag-tagged ORF59, which allowed efficient precipitation of ORF59 (Fig. 7F). Following

**Figure 9: Expanding on the mechanism of interaction between PRMT1, PRMT5, and ORF59.** The switch between symmetric and asymmetric methylation of H4R3, a demethylase is required for the intermediate monomethylated form. Thus far, only one arginine demethylase has been identified-JMJD6. **A.** We examined the binding of JMJD6 to ORF59 in an overexpression system by co-transfecting the plasmids and immunoprecipitating with Flag antibody. However, we could not detect the binding between PRMT5 and ORF59. **B.** We located the interaction between ORF59 and PRMT5 to be at PRMT5 catalytic domain (motif I). PRMT5 motif 1, included in the GXGRPGP-GFP plasmid, was used for a CoIP assay with ORF59-pLVX-Flag. Immunoprecipiation with Flag antibody showed GXGRPGP plasmid was brought down as part of the immune complex.
the substitution of *galK*-Kan cassette with the double stranded oligo, the clone was digested with *BamH*I and analyzed with a Southern blot in order to verify successful recombination (Fig. 7D). BAC36 CR1 ORF59-Flag also encoded GFP, which allowed for easy monitoring of selection (Fig. 7E). To further verify the binding between ORF59 and PRMT5, we immunoprecipitated ORF59 using the anti-Flag antibody (Fig. 7F) and PRMT5 using goat anti-PRMT5 (Fig. 7G). In both cases, the reciprocal protein was brought down in the protein complex. Immunofluorescence assay was used to visualize the co-localization of ORF59 and PRMT5 (Fig. 7H).

ChIP analysis was done on BAC36 CR1 ORF59-Flag cell line, which was induced by transfecting RTA plasmid via 1xHBS. For an accurate assessment of chromatin structure, we could not use TPA or sodium butyrate as they non-specifically alter the chromatin structure. Furthermore, BAC36 CR1 ORF59-Flag allowed us to analyze binding of not only PRMT1 and PRMT5 cellular epigenetic modifiers, but also the binding of RTA and ORF59-Flag to *oriLyt*. IP of these proteins was done with high efficiency (Fig. 8A) with no RTA or ORF59-Flag expressing in uninduced cells. Upon reactivation, we observed strong expression of lytic proteins (RTA and ORF59-Flag) as evidence of successful induction of lytic replication. Lytic reactivation was also evident in qPCR analysis as more RTA and ORF59-Flag bound to *oriLyt*. With induction, additional PRMT1 was recruited to *oriLyt* while PRMT5 was reduced slightly (Fig. 8B).

To further confirm the phenomenon, we analyzed the involvement of a demethylation, which is required for generating a monomethylated arginine residue prior to asymmetric dimethylation by PRMT1. We used the overexpression system to test binding between
ORF59 and JMJD6, a known arginine demethylase, but saw no binding between the two proteins (Fig. 9A). This is not surprising as limited studies have been done on arginine methylation and only recently has JMJD6 been identified as one of the arginine demethylases (36).

Nonetheless, we located the site of interaction between ORF59 and PRMT5 to be at PRMT5 catalytic domain or motif I (GXGRPGP). According to previous work, PRMT5

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**Figure 10: Schematic of the proposed mechanism of symmetric to asymmetric dimethylation switch of H4R3.**

A. Overall schematic of the role of ORF59 in unwinding of heterochromatin at oriLyt. PRMT1 and PRMT5 are present at oriLyt during latency. Upon reactivation, ORF59 binds to PRMT5, displaces it, allowing for PRMT1 to asymmetrically dimethylate H4R3 residue. This opens up the chromatin for lytic replication. B. Histone 3 tail is symmetrically dimethylated by PRMT5. Histone 4 tail is both symmetrically and asymmetrically dimethylated by PRMT1 and PRMT5. The repressive chromatin marks are shown in red while activating chromatin marks are shown in green. C. Chemical structure of arginine residue symmetrically and asymmetrically dimethylated. The repressive chromatin marks are shown in red while activating chromatin marks are shown in green.

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catalytic domain is critical for its function as a methyltransferase. Crystallography placed motif I within the AdoMet binding pocket; however, point mutations introduced into this sequence significantly limited if not completely abolished the function of PRMT5. The binding of ORF59 at motif I may block PRMT5’s methyltransferase activity allowing for PRMT1 to asymmetrically dimethylate H4R3. Taken together, these data suggest that accompanying induction of lytic replication, ORF59 binds to GXGRPGP sequence of PRMT5 thereby displacing it from oriLyt and allowing PRMT1 to asymmetrically methylate H3R3me2.

**DISCUSSION**

Kaposi’s sarcoma-associated herpesvirus is closely linked with multiple malignancies including Kaposi’s sarcoma, Primary Effusion Lymphoma and Multicentric Castleman’s Disease. Upon infection and a short period of lytic replication, the virus establishes a lifelong latency, which allows it to replicate along with the host cell and maintain its genome in each of the daughter cells. During latency, protein expression is tightly regulated by transcriptional repression and the expression of limited number of genes.

In fact for herpesviruses, the initiation of DNA replication is emerging as a complex and highly regulated event. In EBV, the balance between post-translational modifiers, PRMT1 and PRMT5, has been shown to play an important role in initiation of replication at the latent origin (oriP). Their specific modifications are critical for localization of EBNA1, a protein required for initiation of latent replication (27).

Methyltransferases are also known to be involved in the initiation of lytic replication. During latency, KSHV genome is compacted into a tight heterochromatin structure with the help of several repressive marks such as H3K27me3, and H3K9me3 (30). However,
promoters of proteins involved in lytic replication, such as RTA, are closely regulated by bivalent chromatin, which shows the presence of both repressive and activating marks. This allows for accelerated and efficient loosening of chromatin when lytic replication is initiated by paracrine signaling (30).

Similar to RTA promoter, we have determined that KSHV origin of lytic replication (oriLyt) is closely monitored by bivalent chromatin. Symmetric and asymmetric dimethylation of H4R3 are present at oriLyt during latency (Fig. 4B). Interestingly, upon reactivation, there is an upregulation of H4R3me2as indicating the formation of euchromatin at oriLyt, which allows for the initiation of lytic replication. Furthermore, this epigenetic change is merited by two chromatin modifiers - PRMT1 and PRMT5. Protein arginine methyltransferase 1 is a type I methyltransferase known for its function in opening up chromatin for replication and transcription by asymmetrically dimethylating H4R3. On the other hand, PRMT5 is a type II methyltransferase that symmetrically dimethylates H4R3 leading to more compact chromatin (15). Presence of these epigenetic modifiers at oriLyt verifies this bivalent chromatin structure (Fig. 5E).

We considered the interaction between PRMT1, an activating chromatin modifier, and ORF59. We determined that despite their colocalization (Fig. 5C), ORF59 does not bind to PRMT1 in vitro or in vivo (Fig. 5A-B). Consequently, although it is improbable that ORF59 recruits PRMT1 to oriLyt during lytic replication, this does not eliminate other factors from performing this function.

Furthermore, we identified PRMT5 as a binding partner of ORF59 through protein pull down assay and sequencing (Fig. 1). Their interaction was verified in an overexpression as well as an endogenous system (Fig. 2). ORF59 is a KSHV processivity factor essential
for lytic replication. It is one of the first proteins expressed during reactivation and is a component of the replication complex found at oriLyt. We were not surprised to find ORF59 binding with PRMT5; however, due to the repressive nature of PRMT5 we were unsure of the role of their interaction.

To assess the significance of ORF59 binding to PRMT5, we first used the overexpression chromatin immunoprecipitation (ChIP) assay to evaluate the binding of PRMT5 to oriLyt in the presence and absence of ORF59. When co-transfected with vector control, we show that PRMT5 binds to KSHV oriLyt; however, when it is accompanied by ORF59, its binding to oriLyt is significantly diminished (Fig. 6). This negative interaction between ORF59 and PRMT5 suggests an additional role for the KSHV processivity factor.

Additionally, we applied this observation to a more natural system -a BACmid containing KSHV genome- BAC36 with Flag-tagged ORF59. After generating the BAC36 CR1 ORF59-Flag stable cell line in 293L cells (Fig. 7), we induced KSHV lytic replication via transfection of RTA plasmid. Comparing the binding of PRMT1, PRMT5, ORF59-Flag and RTA to oriLyt in uninduced and induced cells, we saw an increase in PRMT1 and a decrease in PRMT5 mark (Fig. 8). Due to PRMT5 being a chromatin condensing modulator, its removal from the oriLyt likely signifies the dismissal of negative regulator of lytic replication. Simultaneously PRMT1 is free to symmetrically methylate H4R3, which is a well-known mark for euchromatin formation, further promoting KSHV lytic replication.

Furthermore, we were interested in discerning the mechanism of interaction between ORF59 and PRMT5. We have shown that ORF59, a lytic protein, binds to PRMT5,
which inevitably inhibits its function as a methyltransferase. Assuming this binding is sustained throughout lytic reactivation, displacing PRMT5 from the chromatin and allowing for formation of euchromatin, we were interested to see if ORF59 accomplishes this by binding to the catalytic site of PRMT5. Indeed, we determined that ORF59 binds to motif I of PRMT5 (GXGRPGP) (Fig. 9B). Further competitive binding experiments are being done to show a decrease in binding between ORF59 and PRMT5 with an increase in GXGRPGP plasmid.

In conclusion, we have shown that during latency oriLyt exists as a bivalent chromatin with both H4R3me2 symmetric and asymmetric marks being present. This balance is maintained by PRMT1 and PRMT5 binding to oriLyt. Differential binding of PRMT1 and 5 are regulated by KSHV processivity factor, ORF59, which binds to PRMT5 at its catalytic site thereby disrupting methyltransferase activity as well as its association with the chromatin. As a result of PRMT5 displacement, PRMT1 is able to asymmetrically methylate H4R3 to loosen the chromatin for lytic replication.

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REFERENCES


