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Chromatin Organization of Gammaherpesvirus Latent Genomes

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Abstract

The gammaherpesviruses are a subclass of the herpesvirus family that establish stable latent infections in proliferating lymphoid and epithelial cells. The latent genomes are maintained as multicopy chromatinized episomes that replicate in synchrony with the cellular genome. Importantly, most of the episomes do not integrate into the host chromosome. Therefore, it is essential that the viral “minichromosome” establish a chromatin structure that is suitable for gene expression, DNA replication, and chromosome segregation. Evidence suggests that chromatin organization is important for each of these functions and plays a regulatory role in the establishment and maintenance of latent infection. Here, we review recent studies on the chromatin organization of the human gammaherpesviruses, Epstein-Barr Virus (EBV) and Kaposi’s Sarcoma-Associated Herpesvirus (KSHV). We discuss the potential role of viral origins of DNA replication and viral encoded origin-binding proteins like EBNA1 and LANA in establishment of viral chromosome organization during latent infection. We also discuss the roles of host cell factors, like CTCF and Cohesins, that contribute to higher order chromosome structures that may be important for stable gene expression programs during latent infection in proliferating cells.

Introduction

Epstein -Barr Virus (EBV) and Kaposi’s Sarcoma-Associated Herpesvirus (KSHV) are prototypical members of the human gammaherpesvirus family. These viruses establish long-term latent infection in B-lymphocytes that can lead to lymphoproliferative disorders and cancers, especially in immune-compromised individuals. During latent infection the viral genomes are maintained as circular minichromosomes. The epigenetic modifications and chromatin structure of the viral genome plays a critical role in the establishment and maintenance of latent infection. The gammaherpesvirus genomes have distinct survival strategies that include the ability to replicate and segregate faithfully each cell cycle, similar to a cellular chromosome. The ability of the viral genomes to adopt variant gene expression programs reflects another level of virus-host interactions that integrates cellular growth and differentiation signal with viral gene expression and chromatin organization. Here, we review some of recent experimental findings and emerging concepts that help to understand the epigenetic mechanisms that control gammaherpesvirus latency.

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I. EBV

In latently infected cells, most EBV genomes persist as multicopy nuclear episomes with chromatin structure similar to that of the cellular chromosome (reviewed in [1–3]). The virus can adopt at least four distinct gene expression patterns that are referred to as latency types [4–6]. The different latency types correlate with the cellular differentiation state of the host cell or the tumor-type from which the virus has been isolated. In normal memory B-cells, EBV can persist in quiescent cells without expressing any viral coding RNAs. This most silent form of infection is referred to as type 0 latency. In cycling B-lymphocytes, EBV adopt a type I latency where viral gene expression is limited to a single protein coding gene, EBNA1, and several non-coding RNAs (e.g. EBERs). Type I latency is most commonly observed in EBV positive Burkitt lymphomas. In epithelial cell malignancies, like nasopharyngeal carcinoma, the virus adopts a type II latency gene expression program where EBNA1, LMP1, and LMP2 are the only protein coding genes detected. Type III latency is defined by the expression of all known latency gene products, including EBNA1, 2, 3A, 3B, 3C, LP, the latency membrane proteins LMP1 and LMP2, the non-coding RNAs EBERs, microRNAs, and BARTs. Type III latency is found in primary infection of naïve B-lymphocytes, AIDS-lymphomas, and lymphoblastoid cell lines that grow in the absence of reactive T-cells. In addition to these different latency types, the virus can express nearly 100 viral gene products during lytic cycle infection required for productive infections. Sporadic lytic gene expression is detected in most EBV carriers, and chronic lytic gene activity has been linked to oral hairy leukoplakia seen in AIDS [7,8]. Both genetic and epigenetic mechanisms have been implicated in regulating these reversible and irreversible switches in viral gene expression programs [6,9].

Control of Latency Type Transcription

Transitions between latency types are essential for viral and host survival. There are several lines of argument to suggest that latency type transcription is programmed and coordinated with host-cell differentiation, and in particular B-cell germinal center reactions [10]. Upon primary infection of resting B-lymphocytes EBV stimulates B-cell proliferation by establishing a type III latency transcription program [3]. The type III program is initiated through B-cell specific factors that activate the W promoter (Wp), which controls expression of EBNA2 [11]. EBNA2 is a transcriptional activator that stimulates cellular and viral gene promoters, including Cp, which supercedes Wp to expresses a multicistronic transcript encoding the entire family of EBNA genes, including EBNA2 [12]. The type III viral genes efficiently stimulate viral proliferation and survival, which can lead to viral-associated malignancies when unchecked. In healthy hosts, this proliferation is limited by T-cell response to immunogenic viral proteins. Nevertheless, EBV genomes persist in healthy immune systems by down regulating type III gene transcription, and switching to a type I latency transcription program. This down regulation of type III promoters correlates with epigenetic silencing, including DNA methylation and loss of histone acetylation at key promoter elements [13,14]. It is not known if this transcription silencing is initiated by cellular differentiation factors, T-cell cytokines, or stochastic selection due to T-cell elimination. It is also not known what spares silencing of type I promoters, like Qp, during this transition.

Chromatin Modifications and Latency Type

Histone modifications have been examined for several of the latency promoters in different latency types (Fig. 1). As might be expected, histone H3 and H4 acetylation at Cp is elevated in cells where Cp transcription is elevated [15]. Genome-wide ChIP analysis of EBV latent episomes revealed some additional information about the viral chromatin profile [16,17]. The chromatin region between the highly expressed RNA Pol III transcribed EBERs and Cp is marked by high level of H3mK4, H3acK9 and low level of H3meK9 both in type I and type III latency. This region also encompasses OriP, which remains bound by EBNA1 in all latency

types. Latency type-associated differences in histone modification patterns were noted in the regions between Cp and Qp, perhaps reflecting the different usage of these promoters for transcription. In type I cells, the histone modifications around the Qp are enriched for euchromatic histone marks (e.g. H3meK4m and AcH4), while these modifications are depleted at Cp. On the contrary, in the type III latency the Cp region is enriched with high levels of both H3meK4 and H3acK9, while the Qp promoter shows a reduced level of these modifications. Heterochromatic marks, especially those associated with histone H3 K9me3 were found elevated in type I latency, but only within the W repeat region and Wp control element. The relative scarcity of H3 mK9 throughout the EBV genome suggests that the viral genomes are mostly euchromatic during all forms of latency, with some isolated regions that have H3 mK9 associated transcription repression. At present, there is no strong evidence to suggest that the latent viral genome is highly heterochromatic as may have been envisioned for latent infection.

Role of EBNA1 and OriP in EBV Chromatin Organization

Genetic and biochemical studies suggest that OriP regulates latency transcription, as well as functions as an origin of replication and plasmid maintenance element [18,19]. OriP is situated between the EBERS and Cp, and falls within a euchromatic zone as indicated by the elevated levels of histone H3 me3K4. OriP consists of two clusters of EBNA1 binding sites, the family of repeats (FR) and the Dyad Symmetry (DS). The FR confers episomal maintenance while the DS confers initiation of DNA replication. In addition, OriP functions as a transcriptional enhancer of EBNA2 and LMP1 [20–24]. EBNA1 binding to OriP is required for all of the known functions of OriP, including cell cycle-dependent DNA replication, plasmid maintenance, and transcriptional enhancer activity. OriP also interacts with cellular replication and licensing factors that include components of the origin recognition complex (ORC) and replication licensing machinery [25–28]. ORC is required for the DNA replication function of OriP. ORC is also known to influence chromatin structure [17], and ChIP analysis indicates that ORC can bind to an extended region surrounding OriP [29]. However, it is not yet known if ORC regulates EBV chromatin structure nor whether it contributes to the regulation of EBNA2 and LMP1 gene expression. Earlier studies found that OriP and EBNA1 can influence the DNA methylation patterns at the EBNA2 promoter region (Cp) by a DNA replication-independent mechanism [30], suggesting that this region coordinates epigenetic changes at latency promoters. EBNA1 also binds to the transcription initiation site in Qp, where it functions to negatively auto-regulate its own transcription. It has been proposed that EBNA1 functions like a rheostat that activates Cp and inhibits Qp at high EBNA1 levels in type III latency, while low EBNA1 levels in type I latency promote Qp transcription activity [31]. It is not known if the switch from Cp usage to Qp usage that occurs in type III to type I latency switch is coordinated with changes in chromatin structure or OriP function.

ORC and Chromatin Regulation

Although OriP is thought to function primarily as an origin of DNA replication, EBV DNA synthesis can initiate outside of OriP, and frequently from a zone within the EBV BamHI A region over ~30kB from OriP [32–34]. Even when OriP does not function as an origin of replication, it still recruits ORC subunits. It has become increasingly apparent that ORC can interact with many sites in the cellular genome that do not initiate DNA replication. What is the function of these “inactive” origins? One possibility is that they function as reserve origins that fire only if necessary in cases of excessive replication stress. However, ORC has other functions besides its role in initiation of DNA replication [35]. One well-characterized alternative function is its involvement in transcriptional silencing at the mating type loci in budding yeast. ORC-associated transcription silencing is mediated by the SIR complex, that includes that NAD-dependent histone deacetylase Sir2 [36]. Mammalian ORC interacts with heterochromatin protein 1 (HP1) suggesting that transcription silencing by ORC is conserved in higher eukaryotes, and may be mediated by heterochromatin formation [37]. ORC has also

been implicated in sister chromatid cohesion, but the mechanism for this function is not yet known [38]. Alternatively, ORC can associate with the histone acetylase HBO1, which is associated with open chromatin and replication origin activation [39,40]. ORC can also play a role in nucleosome positioning and phasing that may influence local gene activity and chromatin structures important for genome stability [41]. These various functions of ORC suggest that OriP may play a more central role in chromatin organization and dynamics, rather than in replication initiation. ORC recruitment may provide a mechanism for EBV to coordinate chromatin structure and histone modifications with changes in cell cycle and DNA replication.

Cell cycle control of OriP and chromatin dynamics—There is some evidence that EBNA1 binding to OriP functions as an organizer of chromatin structure. Biochemical studies with purified components revealed that EBNA1 could displace nucleosomes from the FR binding sites and alter DNA conformation [42–44]. Micrococcal nuclease studies have revealed that OriP has an unusual nucleosome organization, probably due to the displacement of nucleosomes from the FR region [43,44]. In contrast, we and others have found that nucleosomes are strongly positioned immediately adjacent to the EBNA1 and TRF2 binding sites in the DS region [45]. The DS-positioned nucleosomes are remodelled in a cell-cycle dependent manner, consistent with the idea that nucleosome positioning can regulate DNA replication [38]. The chromatin-remodelling protein SNF2h associated with DS in early S phase suggesting it plays a role in the cell cycle remodelling of the DS-positioned nucleosome [38]. The nucleosome reorganization at DS correlated with an increase in binding of the cellular replicative helicase, as measured by ChIP assay with antibodies to Minichromosome Maintenance Complex (MCM) binding subunits. Remarkably, we found that the DS nucleosomes were subject to histone deacetylation in early S phase, followed by an increase in acetylation in mid-late S phase. This delay in histone acetylation corresponded to a delay in the onset of DNA replication as measured by BrdU incorporation [46]. These observations suggest that the chromatin remodelling and the histone modifications can provide a mechanism for the regulation of the viral genome replication by modulating the DNA accessibility for the replication machinery. The programmed delay in replication initiation at OriP may be important for some type of S phase checkpoint regulation at OriP, or may contribute to the establishment of a chromatin structure that promotes episome stability. It is also possible that this delay in replication may be important for coordinate transcription control of Cp.

Epigenetic Silencing of Cp

EBNA1 binding to FR also functions as a transcriptional enhancer of the Cp promoter in type III latency [20,21]. This activity is dependent on the UR1 domain of EBNA1 and is essential for EBNA2 expression during B-cell immortalization [47]. In type I latency, Cp transcription is extinguished. As mentioned above, both programmed changes in cellular transcription factors and stochastic epigenetic factors may contribute to this process. Epigenetic factors like CpG DNA methylation and histone modifications are known to contribute to Cp and LMP1 promoter silencing in type I latency [48]. DNA methylation of proximal promoter elements correlates with transcription repression of EBNA2 and LMP1 [49–52]. Inhibitors of DNA methylation can relieve this repression and induce a switch from type I to type III latency patterns of EBV gene expression in cell culture [51,53]. Post-translational modifications of histone tails are also known to be important for EBNA2 regulation since transcription activation correlates with promoter-specific histone hyperacetylation [54–56] and ectopic expression of EBNA2 stimulates H4 and H3 acetylation at Cp [15]. EBNA2 binds to the RBP-Jk (also referred to as CSL and CBF1) protein and functions as a viral orthologue of cellular NotchIC protein [57]. EBNA2 also interacts with transcriptional coactivators, including CBP and p300, which have histone acetyltransferase activity [58], and Swi/Snf components, which have nucleosome remodelling activity [59]. In addition to these well-characterized interactions at Cp, we have found that Cp can also be regulated by Rb and the E2F-family of transcription

factors [60]. An E2F binding site was identified in the promoter proximal region of Cp. E2F consists of a complex family of proteins, several of which can bind to the retinoblastoma protein Rb, which functions as a cell cycle-dependent repressor of transcription. Rb bound Cp and repressed Cp transcription in a cell cycle-dependent manner. This regulatory region of Cp also bound to a histone demethylase, LSD1, in a cell cycle-dependent manner, and cell cycle changes in histone H3 me3K4 were observed in type III latency. EBNA2 mRNA expression was also found to be cell cycle regulated. Since EBNA2 is a potent regulator of cellular proliferation, it is likely that its expression is subject to cell cycle control. It is also possible that E2F-Rb- LSD1 interactions may promote the long-term silencing of Cp during the development of type I latency from type III cells, perhaps as a consequence of long-term withdrawal from the cell cycle. This is consistent with the known role of Rb in epigenetic silencing and heterochromatin formation at other cellular location, including telomeres [61]. This hypothesis will need to be investigated in further detail since many other possible mechanisms may also control Cp silencing (Fig. 2).

Chromatin Boundaries, Enhancer-Blockers and CTCF

Chromatin domains, like that surrounding the EBV OriP region, may be organized by boundary elements that can change in different latency programs. One notable chromatin boundary factors is the 11- Zn finger, nuclear phosphoprotein CTCF [62,63]. CTCF can function as an insulator of transcriptional enhancers and a regulator of genomic imprinting through differential binding to unmethylated cytosine [64]. Genome wide studies have identified CTCF binding sites at several key regulatory elements in the EBV genome [16]. These include major binding sites immediately upstream of Qp, Cp, and EBER transcription start site, as well as downstream of Wp. CTCF binding at these regions could provide several functions, including insulation of neighbouring viral lytic promoters from activation during latent infection, and the regulation of enhancer-promoter interactions. We have found evidence to suggest that the CTCF binding site upstream of Cp is important for regulating the interaction of the EBNA1-FR enhancer with Cp. Genetic disruption of this Cp associated CTCF binding site led to an increase in Cp transcription levels in type I latently infected cells [65]. Moreover, CTCF binding at Cp is elevated in type I latency relative to type III latency, strengthening the idea that CTCF inhibits EBNA1-FR enhancer interactions with Cp in type I [65]. On the other hand the CTCF binding on the Qp and EBER I promoters was similar in both latency types, suggesting that CTCF may block the spreading of active histone modifications over adjoining lytic gene promoters. Since CTCF may also contribute to DNA loop formation, it is tempting to speculate that CTCF could also regulate the viral gene expression by long-range chromatin regulatory interactions (Fig. 3). Given that CTCF binds differentially to unmethylated DNA, it will be important to examine in more detail the CpG methylation pattern at CTCF binding sites in type I and type III latency.

Chromatin, B -cell Differentiation, and Latency Type Switch

The physiological signals that induce the EBV latency type switch from type III to type I are not known. Several lines of evidence suggest that elevated c-myc expression is incompatible with EBNA2 gene expression [66], and can lead to down regulation or deletion of EBNA2 from the viral genome [67]. However, elevated c-myc expression is not sufficient to block transcription from Cp, nor initiate a switch from type III to type I latency [68]. CTCF also functions in a critical regulatory loop of cellular signaling pathways that determine B-cell fate [69]. Conditional expression of CTCF in immature B- lymphocytes can lead to inhibition of proliferation without apoptosis [70]. CTCF has been implicated in the transcription repression of c-myc and Rb [71,72]. The CTCF binding site in the c-myc promoter is commonly disrupted by the Burkitt lymphoma-associated chromosomal translocation, and its loss by translocation or cytosine methylation may be sufficient to deregulate myc expression [73]. CTCF protein and mRNA levels can be upregulated by stimulation with TGF- β or by inhibition of the mTOR

and PI-3K pathways in B-lymphocytes [69]. This is interesting since inhibition of mTOR by rapamycin can inhibit EBV-associated B-cell proliferation [74]. Thus, it remains possible that chromatin regulatory factors, like CTCF and Rb, may integrate cell growth and proliferation signals with changes in EBV chromatin structure and programming of latency type gene expression.

EBV summary—The various patterns of latent cycle gene expression that are observed in different EBV-associated tumors suggest that EBV has evolved a sophisticated strategy to stimulate host-cell proliferation and evade immune system rejection. Suppression of antigenic viral gene products in type I latency is thought to be essential for escape from immune system detection, and long-term survival in latency. Stimulation of host-cell proliferation without inducing apoptosis, replication stress, or telomere dysfunction, requires coordinate action of several viral genes, depending on the cell type and differentiation stage. Coordinate control of viral gene expression is orchestrated largely through the multi-cistronic nature of latency transcripts. However, additional fine-tuning of the viral promoters by cell cycle control factors, like the E2F-Rb-LSD1 complex, may also be required for integrating viral gene expression with cellular proliferation and growth status. The mechanisms that suppress Cp expression as EBV-infected cells progress from type III to type I latency remains enigmatic. Changes in cellular proliferation signals and increase CpG methylation of Cp are clearly important for this process. Changes in chromatin organization may also play a key role in this process. Chromatin organizing factors, like CTCF, may suppress the OriP-dependent activation of Cp during type I latency and promote the transition from type III to type I latency. CTCF may also organize higher-order chromosomal structures, including those that allow long-distance interactions between the OriP enhancer and its target promoters at Cp and possibly Qp. Whether epigenetic modifications and chromatin organizing factors initiate changes in viral gene expression or merely stabilize events dictated by transcription factor regulation remains an important area of future investigation.

II. KSHV

Kaposi's sarcoma (KS) is among the most prevalent malignancies found in HIV- infected individuals, but can also affect HIV negative individuals in endemic areas [75]. Kaposi's Sarcoma- Associated Herpesvirus (KSHV) has been identified as the causative agent of both HIV-associated and endemic forms of KS [76,77]. KSHV is also associated with several lymphoproliferative disorders, including primary effusion lymphoma (PELs) and multicentric Castleman's disease [78–80]. KSHV has many structural and functional homologues to EBV, but also has numerous unique genes and different biological properties (reviewed in [81–86]). Like EBV, KSHV persists as a multicopy episome in the nucleus of latently infected cells. KSHV also has different gene expression patterns during latent infection, but these are not as well defined as the latency types in EBV [87,88]. Both latent and lytic cycle replication are essential for the long-term persistence of the virus, and gene products from both expression programs have been implicated in the pathogenesis of KSHV-associated disease [82,84].

KSHV Latency

Several cell lines carrying latent episomal genomes of KSHV have been isolated from PEL cells, and have provided a valuable model for understanding KSHV gene expression during latency [88–91]. In latently infected PEL cells, KSHV transcription is restricted to a few viral genes, including the latency associated nuclear antigen LANA (ORF73), v-CycD (ORF72), vFLIP (K13, ORF71), Kaposin (K12), and vmiRNAs [92–97]. Several other viral genes can be detected in latently infected tumor derived cells, including vGPCR (ORF74) and K14 [98]. The latency-associated viral products have growth transforming and cell cycle deregulating properties that are likely to contribute to KSHV-associated malignancies [81,

82,99]. LANA1, v-CycD, and v-FLIP are expressed as a multicistronic transcript from a single promoter that remains active during latent infections in most cell types examined. The Kaposin transcript can be expressed from one of two promoters downstream of ORF73, or within the tandem repeats upstream of K12 [100]. ORF74 and K14 is expressed as a bicistronic transcript on the complementary strand of LANA-vCyclin-vFLIP RNA. The ORF74- K14 transcript initiates from site within the 5' UTR of the LANA-vCyclin-vFLIP message and it remains enigmatic how ORF74- K14 can be expressed in latently infected cells that co-express LANA-vCyclin-vFLIP from the same genomes [101]. Both K14 and ORF74 have been detected in latently infected tumor biopsies and KS-lesions, suggesting that these genes can be expressed in some forms of latency [102]. Thus, it is possible that KSHV may have multiple latency types similar to EBV, but identification of these types is hindered by their inability to be maintained in laboratory tissue culture models.

LANA and KSHV episome maintenance

LANA is a multifunctional protein that is required for stable episomal maintenance and DNA replication of the latent viral genome [87,103–107]. LANA, like EBNA1 from EBV, is a DNA binding protein that stimulates plasmid-based DNA replication [87,104,108–110]. Deletion of the KSHV LANA or the LANA-related protein from herpesvirus saimiri (HVS) caused a complete loss of episomal genomes and failure to establish latent infection [106,107,111]. KSHV and HVS LANA bind to a ~60 bp sequence within the ~800 bp GC-rich terminal repeats (TR) [87,104,112,113]. At least two repeats were required for stable episomal maintenance [109,110], but a single LANA binding site was sufficient to support transient DNA replication of plasmids [104]. This resembles some of the properties of EBV OriP which can replicate transiently with a minimal two EBNA1 sites [114], but requires at least seven additional binding sites in the family of repeats to support stable plasmid maintenance [115–117]. Most viral genomes have from 2–20 copies of the TR and it is not clear how these tandem repeats influence LANA binding site conformations to support plasmid maintenance of the viral genome during latency.

Stimulation of DNA replication by LANA shares several common features with EBV encoded EBNA1, as well as with cellular origins. Like EBNA1, LANA can coimmunoprecipitate from soluble nuclear extracts with several subunits of ORC [28,110]. ORC binding is thought to be an essential step in the establishment of a bidirectional origin of replication [118,119]. ORC components associate with the LANA Binding Sites (LBS) within the KSHV TR in a LANA-dependent manner [120]. MCM components associate with the LBS in a cell cycle-dependent manner, and that siRNA depletion of MCM or ORC components inhibits LANA-dependent replication of TR-containing plasmids. However, like EBV, replication can initiate outside of the TR in latently infected cells. These studies suggest that the KSHV LBS functions similar to EBV DS both as an initiator of DNA replication, and an organizer of the viral chromosome.

Chromatin Structure and DNA Replication at the KSHV TR

During latent infection, KSHV DNA is episomal, associated with nuclear matrix fraction, and attaches to metaphase chromosomes [44,121,122]. ChIP assays suggest that most of the genome is associated with nucleosomes [120], but a detailed nucleosome density study has yet to be performed. Some regions of the genome have been examined in more detail. The latent cycle replication origin is embedded in the GC-rich sequence of the TR [87,104]. GC-rich tandem repeats have been shown to have a high tendency to form repressive heterochromatin that may exclude binding of cellular factors required for replication preinitiation complex assembly [123,124]. We have found that the KSHV TR is associated with stable positioned nucleosomes during latent infections [120]. The nucleosomes adjacent to the LBS were enriched for acetylated histone H3 and H4, and H3 me3K4. Similar to the positioned nucleosomes at DS, the nucleosomes at the KSHV LBS were subject to cell cycle regulation,

including nucleosome remodelling and modification changes. The major change at the LBS was an increase in histone H3 me3K4 during S phase, but no apparent changes in histone acetylation like was observed at the EBV DS. LANA has been shown to interact with numerous proteins associated with histone modifications, including the histone acetyltransferase CBP [125] and the double bromodomain containing protein BRD2 (RING3) [126,127]. We found evidence that HBO1, a member of the Myst family histone acetyltransferases that interacts with ORC1 and MCM2, was also enriched at the LBS in a LANA-dependent manner. LANA also associates with several other chromatin regulatory proteins, including core histone H2B [128], the methyl cytosine binding protein (MeCP2), DEK [129], KLIP1 [130], and mSin3 [131], suggesting that LANA may coordinate complex changes of chromatin at the TR and other regions of the viral and cellular chromosome.

Chromosome Dynamics, Cohesins and CTCF—One of the most challenging tasks of the viral episome is to persist through multiple mitotic cell divisions. Tethering to the mitotic chromosome as a passenger is the prevailing model for explaining how viral episomes, like KSHV, EBV, and HPV, are maintained during cellular division. LANA provides this function by tethering the viral genome to a metaphase chromosome component. The LANA N-terminal domain binds viral DNA (at TR) while motifs in the C-terminus tether to histone H2B, as well as to other chromatin factors like BRD2, DEK, and MeCP2. While these interactions with metaphase chromosome components are necessary for nuclear retention of the genome, it is not clear if these interactions are sufficient for faithful segregation of sister chromosomes to daughter cells. While it is debatable whether these viral genomes require an active segregation mechanism, recent studies suggest that the EBV episomes do partition symmetrically [132, 133]. In more complex host genomes, faithful chromosome segregation depends on a family of proteins involved in sister chromatid cohesion and condensation [134]. The cohesin and condensin proteins induce structural changes in the chromosome during mitosis and are essential for chromosome stability and segregation [135,136]. Cohesins and condensins are thought to bind every ~10 kb of cellular DNA and no specific DNA recognition sites have been identified [137]. To determine if cohesins or condensins bind to KSHV genome, we performed a genome-wide ChIP analysis [138]. Remarkably, we found that cohesin subunits, SMC1, SMC3, and Rad21, were highly enriched at a single location immediately upstream of the LANA open reading frame. The cohesin binding site colocalized with a cluster of CTCF binding sites at the same position. Deletion of the CTCF binding sites in the KSHV bacmid led to the loss of cohesin binding, indicating that cohesins were recognizing CTCF as a docking site on the viral chromosome. Deletion of the CTCF-cohesin binding site led to genome instability in 293 cell colonies, as well as to the deregulation of viral transcription from the latency control region. More recent studies indicate that this CTCF cluster is important for the cell cycle control of latency transcription [139]. At least two models could be envisioned based on these observations. The Cohesin -CTCF cluster may provide some type of chromosome organizing center that promotes stable episomal maintenance, perhaps by promoting sister-chromatid cohesion and chromosome segregation. Alternatively, the CTCF-cohesin cluster may provide a gene regulatory control element that helps regulate the cell cycle expression of LANA and vCyclin, and the repression of vGPCR-K14. Deregulation of these genes may lead to toxic viral gene expression, and therefore explain the selective loss of viral episomes. Members of the cohesins have been implicated in transcription regulation by facilitating long distance interactions between promoters and enhancers [140,141]. Human cohesin subunits have been implicated in the genetic instability associated with Cornelia de Lange syndrome [142,143]. CTCF-cohesin clusters have been mapped at numerous overlapping sites throughout the cellular genome, most notably at the c-Myc promoter and the H19/Igf2 imprinted locus. The precise function of these cohesin -CTCF clusters remains under investigation, but it seems likely that these sites coordinate chromosome structure with gene expression changes during mitotic cell division (Fig. 4).

KSHV Viral Micro-RNA and Gene Regulation—KSHV is known to encode at least 12 microRNAs that are encoded by the major latency transcript [144–146]. At least one KSHV encoded microRNA has been shown to function as a viral homologue of the cellular miR-155 [147,148]. The miR-155 is upregulated in many B-cell lymphomas and by EBV infection, but its precise function in lymphomagenesis and B-cell development are not completely understood. We have recently found that the miR-155 can modulate NF- κ B pathway and affect the stability of latent EBV genomes in B-lymphocytes [149]. Viral encoded microRNAs have also been found to down-regulate viral encoded proteins and down-modulate expression levels that may provoke immune recognition, as in SV40, or may limit lytic replication as is seen in HSV latent infections [150,151]. In addition to targeting and modulating translational efficiency and RNA stability of target mRNAs, microRNAs and other non-coding RNAs have been suspected of playing a more direct role in chromatin regulation. In *S. pombe* and plants, small non-coding RNAs have been implicated in the establishment of heterochromatin through the recruitment of HP1-orthologues [152]. Similarly, larger non-coding RNAs, like Xist have been implicated in the heterochromatin formation of the inactive X chromosome [153]. MicroRNA clusters have also been shown to indirectly affect chromatin structure by targeting global epigenetic regulators, like DNA methyltransferases [154,155]. The precise function of the KSHV microRNA cluster remains obscure. It will be interesting to determine if the KSHV microRNA have a function in regulating KSHV epigenetic modifications during latent infection.

Epigenetic regulation of KSHV latency and viral pathogenesis—The persistence of the KSHV genome during latency is central to understanding the pathogenesis in AIDS-associated malignancies. The molecular mechanisms that allow KSHV genomes to persist as multicopy episomes in latently infected B-lymphocytes are poorly understood. It also remains enigmatic that KSHV genomes do not readily establish stable episomal infections in non-lymphoid tissue culture cell models, including endothelial cells that are the resident host cell for KSHV in KS [156]. A similar observation has been made for EBV genome maintenance in several epithelial tumors, like NPC, which do not efficiently maintain viral genomes when cultured ex vivo [157]. One possible explanation for this is that the tumor microenvironment provides some selection to episomal maintenance. It is also known that stable episomes are established through rare epigenetic events, as was demonstrated for EBV episomes [158]. Maintenance of the KSHV or EBV chromatin structure is likely to be a component of this rare epigenetic event that allows a heritable pattern of gene expression and genome replication. Why this occurs more efficiently in B-lymphocytes, like BL or PEL cells, will be of great value to understand.

Chromatin organization and epigenetic modifications may also explain some of the pathogenesis observed in KS. Elevated expression of vGPCR in most KS lesions is thought to drive endothelial cell growth and proliferation. In latently infected PEL cells, vGPCR expression is limited during latent infection, but can be expressed at high levels during lytic reactivation. A loss of CTCF-cohesin repression at the latency control region may contribute to the elevated expression of vGPCR in KS lesions. This has not been formally tested, but it raises the question of whether defective chromatin control mechanisms can lead to aberrant activation of vGPCR, as well as other lytic genes, to promote pathogenic viral gene expression. Further understanding of the epigenetic control of viral latency will provide important insights into the mechanism of KSHV (and EBV) persistence, gene expression patterns, and pathogenesis.

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Figure 1. Epigenetic control mechanisms of EBV latency type gene expression

Type III latency observed in lymphoblastoid cell lines and non-Hodgkin's lymphomas is characterized by OriP enhancer activation of Cp and euchromatic H3me3K4 marks extending into the Cp and Qp regions. Type I latency observed in Burkitt lymphoma's is characterized by an increase in CTCF binding between OriP and Cp, and a decrease in H3me3K4 at Cp. Type I latency is also characterized by an increase in CpG methylation and H3 me3K9 methylation at the Cp and Wp regions. Rb is proposed to promote the silencing of Cp in type I latency.

Figure 2. Potential mechanisms of Rb-mediated epigenetic silencing of EBV Cp

The epigenetic silencing of Cp blocks expression of the multicistronic transcript encoding EBNA-LP, 2, 3A, 3B, and 3C. This process allows EBV infected cells to complete B-cell maturation and evade immune detection during long-term latent infection. E2F, Rb, and LSD1 associate with Cp and regulate the cell cycle transcription of EBNA2 in type III latency. Rb also bound to Cp in type I latency where EBNA2 transcription is silenced throughout the cell cycle. Rb has been implicated in epigenetic silencing and is known to associate with proteins involved in histone modification and DNA methylation.

Figure 3. Potential role of higher-order chromatin dynamics in the epigenetic silencing of EBV Cp Type III latency EBNA1-OriP enhancer stimulates Cp. In type I latency, Cp is not transcribed. This may be a consequence of (A) that CpG DNA methylation and Rb binding at Cp, (B) the failure of EBNA1 enhancer to activate Cp due to a loss of an essential co-factor or CTCF-enhancer blocking activity, or (C) a conformational change in the viral chromosome that prohibits activation of Cp.

Figure 4. Potential roles of chromatin organizing factors in the regulation of KSHV latency
 LANA binds to TR and recruits ORC. The major latency transcript encoding the multicistronic LANA-vCyclin-vFLIP transcript and also the viral microRNA and Kaposin are indicated in green. The lytic associated K14- vGPCR transcript is indicated in red. Cohesin-CTCF binding site is indicated in purple and blue. A) Cohesin-CTCF complex may restrict the expression of K14- vGPCR during latent infection. Loss of Cohesin-CTCF may lead to pathogenic expression of K14- vGPCR in KS lesions. B) Cohesin complex may function in sister-chromatid cohesion to promote faithful segregation of KSHV genomes after DNA replication. C) Cohesin complex may function in intramolecular looping that is important for viral gene expression during latency.