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Inhibition of the lytic cycle of Kaposi's sarcoma-associated herpesvirus by cohesin factors following *de novo* infection

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ABSTRACT

Establishment of Kaposi's sarcoma-associated herpesvirus (KSHV) latency following infection is a multistep process, during which polycomb proteins are recruited onto the KSHV genome, which is crucial for the genome-wide repression of lytic genes during latency. Strikingly, only a subset of lytic genes are expressed transiently in the early phase of infection prior to the binding of polycomb proteins onto the KSHV genome, which raises the question what restricts lytic gene expression in the first hours of infection. Here, we demonstrate that both CTCF and cohesin chromatin organizing factors are rapidly recruited to the viral genome prior to the binding of polycombs during *de novo* infection, but only cohesin is required for the genome-wide inhibition of lytic genes. We propose that cohesin is required for the establishment of KSHV latency by initiating the repression of lytic genes following infection, which is an essential step in persistent infection of humans.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) is a member of the gamma-2 herpesvirus (Rhadinovirus) subfamily (Chang et al., 1994). Persistent KSHV infection in humans can lead to the development of various KSHV-associated cancers such as Kaposi's sarcoma, primary effusion lymphoma (PEL) or a subset of multicentric Castleman's disease in immunocompromised individuals (Cesarman, 2011; Mesri et al., 2010). KSHV can establish latency in a number of different cell types (e.g. B cells, endothelial cells, fibroblasts), which is characterized by the inhibition of lytic viral genes and constitutive expression of latent viral genes (Bechtel et al., 2003). During latency the 165-kb DNA genome of KSHV adopts a stable chromatin structure similar to that of the host genome and persists in the nuclei of infected cells as a chromatinized, non-integrating episome, which replicates synchronously with the host genome (Ballestas et al., 1999; Renne et al., 1996; Tempera and Lieberman, 2010).

Following infection the KSHV genome undergoes a biphasic euchromatin-to-heterochromatin transition in infected cells before the establishment of latency (Toth et al., 2013b). The stepwise chromatinization of the viral DNA is regulated by chromatin modifying host factors that interact with the KSHV genome and deposit specific histone marks on it in a spatially and temporally ordered manner during infection (Gunther et al., 2014; Toth et al., 2013b). Two of the major cellular transcription repressors involved in the inhibition of lytic KSHV genes during latency are the Polycomb repressive complexes 1 and 2 (PRC1 and 2) (Gunther and Grundhoff, 2010; Simon and Kingston, 2009; Toth et al., 2013b, 2010). During *de novo* infection the PRCs are recruited to lytic viral promoters by the latent KSHV factor LANA 24 h postinfection (hpi), which is required for the inhibition of the expression of lytic genes so that KSHV can establish latency (Toth et al., 2016). Strikingly, a limited number of lytic genes are known to be induced transiently during *de novo* KSHV infection despite neither PRC binding to the KSHV genome nor the viral DNA being heterochromatinized in the first 24 h of KSHV infection (Gunther et al., 2014; Krishnan et al., 2004; Toth et al., 2013b). This implies that there must be transcription repressors other than PRCs, which can globally inhibit KSHV lytic gene expression immediately following *de novo* infection. However, nuclear factors that can be recruited onto the viral DNA prior to the heterochromatinization of the KSHV genome and can repress lytic gene expression during *de novo* infection have not yet been identified.

Several nuclear factors such as the 11-zinc finger protein CTCF and the cohesin complex have been shown to be involved in the regulation of the 3D chromatin structure of the host genome, which can influence gene expression (Kagey et al., 2010; Phillips and Corces, 2009). The

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cohesin complex is composed of the core subunits RAD21, SMC1, SMC3, STAG1, STAG2, and several transiently associated regulatory factors (Peters et al., 2008). The interaction of cohesin with chromatin is determined by several nuclear factors. While NIPBL and MAU2 proteins act as loading factors, which can recruit cohesin onto the chromosomes, the histone deacetylase 8 (HDAC8) functions as a recycling enzyme of acetylated cohesin factors releasing cohesin from chromatin. Both cohesin and NIPBL have been implicated not only in the regulation of chromosome segregation during cell division but also in transcriptional regulation where NIPBL can control gene transcription in a cohesin-independent manner (Schaaf et al., 2013a; Zuin et al., 2014). Importantly, several mutations in NIPBL and cohesin subunits have been linked to the developmental disorder Cornelia de Lange syndrome (CdLS), which is likely due to the misexpression of developmentally important genes rather than a defect in chromosome segregation (Strachan, 2005). In addition, CdLS patients have been shown to be more susceptible to infection indicating a role for cohesin factors in restricting microbial infection (Jyonouchi et al., 2013).

Recent studies revealed that CTCF and cohesin can act as restriction factors of KSHV lytic replication by suppressing lytic reactivation of KSHV in different cell types (Chen et al., 2012; Li et al., 2014). Here, we investigated the role CTCF and cohesin in the inhibition of the lytic cycle of KSHV during *de novo* infection. We found that CTCF, cohesin and NIBPL can rapidly bind to the KSHV genome within a few hours postinfection and both cohesin and NIBPL can inhibit the expression of lytic genes and KSHV replication following infection. We also show that the restriction of lytic viral replication after infection is mainly due to cohesin-mediated inhibition of the expression of the viral gene RTA, which is required for the induction of lytic genes and viral replication. Importantly, our data indicate that cohesin binds to the RTA promoter and can repress the expression of lytic genes prior to the recruitment of PRCs and heterochromatinization of the viral genome, suggesting that cohesins can function as global restriction factors of KSHV lytic gene expression and replication not only during latency but also following primary infection. Thus, we propose that cohesin factors are essential for the establishment of KSHV latency.

2. Materials and methods

2.1. Cells, KSHV and de novo KSHV infection

293T, SLK, and HFF cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (P/S). The CdLS patient primary fibroblast cells designated as GM00045 and GM03478 were obtained from the Coriell Institute, which were cultured similarly to the primary fibroblast HFF. [Note: while NIPBL mutation has been identified in GM00045, it is not known which cohesin factor is mutated in GM03478 (Vrouwe et al., 2007)]. For *de novo* KSHV infection, we used the recombinant KSHV clone BAC16, which was produced using iSLK cell line that is described elsewhere (Myoung and Ganem, 2011; Toth et al., 2013b). The iSLK cell lines carrying WT KSHV (BAC16) or RTA KO KSHV (BAC16RTAstop) were maintained in DMEM medium supplemented with 10% FBS, P/S, 1 μg/ml puromycin, 250 μg/ml G418, and 1 mg/ml hygromycin. The construction of BAC16 and BAC16RTAstop viruses has been described previously (Brulois et al., 2012; Toth et al., 2012). KSHV was prepared from iSLKBAC16 and iSLKBAC16RTAstop cell cultures by treating the cells with 1 μg/ml doxycycline and 3 mM sodium butyrate for 84 h. Media were collected from virus-producing cells, filtered through a 0.45 μM PES filter, and KSHV was concentrated by ultracentrifugation of the collected media by 24,000 rpm for 3 h at 10 °C. The concentrated virus was resuspended in DMEM and stored at − 80 °C. The viral titer was calculated as described previously (Brulois et al., 2012; Toth et al., 2016). *De novo* infection was performed by spin-infection (2000 rpm, 45 min at 30 °C). After infection the media was changed and the infected cells were harvested at the indicated time points.

2.2. Antibodies

The following antibodies were used in ChIPs and/or immunoblots: anti-histone H3 (Abcam ab1791), anti-H3K27me3 (Active Motif #39155), anti-H3K4me3 (Active Motif #39159), anti-EZH2 (Active Motif #39875), anti-RING1B (Abcam ab3832), anti-LANA (Advanced Biotechnologies #13-210-100), anti-CTCF (Millipore, #07-729), anti-RAD21 (Abcam, ab992), anti-SMC3 (Abcam, ab9263), anti-NIPBL (Bethyl Laboratories, A301-779A), anti-K8 (Abcam ab36617), anti-RNA polymerase II (RNAPII) (Abcam), and anti-actin (Abcam). Anti-K3 and anti-RTA antibodies were generous gifts from Drs. Jae U. Jung (University of Southern California) and Yoshihiro Izumiya (University of California, Davis).

2.3. Chromatin immunoprecipitation (ChIP) assay

KSHV-infected cells (2–4 × 10^6) were fixed with 1% formaldehyde for 10 min at RT followed by adding 2 M glycine to the cells to stop crosslinking (final concentration of glycin 125 mM) for 5 min at RT. Cells were washed three times with cold PBS and then resuspended in cell lysis buffer (5 mM Tris-HCl, pH 8.0, 85 mM KCl, 0.5% NP40, 1x protease inhibitor cocktail (Roche)) and incubated on ice for 10 min. After centrifugation (5 min, 5000 rpm at 4 °C), the pellet was resuspended in 1.2 ml of RIPA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 140 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF, 1x protease inhibitor cocktail), sonicated and centrifuged by 13,000 rpm for 10 min at 4 °C. Aliquots of the supernatant containing both cellular and viral chromatin were stored at − 80 °C.

To prepare input DNA, 20 ul of chromatin was incubated in 100 ul of TE buffer containing 50 μg/ml RNase A for 30 min at 37 °C. The samples were then adjusted to contain 0.5% SDS and 0.5 mg/ml Proteinase K (Invitrogen) and incubated for 1 h at 37 °C. Formaldehyde crosslinks were reversed by adding sodium chloride (final concentration 300 mM) to the samples and incubated overnight at 65 °C. DNA was extracted first by one volume of phenol/chloroform/isoamyl alcohol (25:24:1) saturated with 10 mM Tris, pH8.0 and 1 mM EDTA and then purified once by one volume of chloroform. DNA was precipitated by cold absolute ethanol, 10% 3 M sodium acetate, pH 5.2 and 1.5 μl of 15 mg/ml Glycogen Blue (Ambion) at − 80 °C at least for 1 h following by wash with 70% ethanol. The input DNA was dried at RT and resuspended in 20 μl of water.

For ChIPs, chromatin containing 2–5 μg of DNA was first diluted in 500 μl of RIPA buffer and precleared by Sepharose A beads. Immunoprecipitation was carried out with 1–2 μg of antibodies overnight at 4 °C. Next day, Protein-A/G agarose was added for 4 h to pull down the DNA/protein complexes. ChIP was washed sequentially with RIPA buffer once briefly and once for 10 min followed by washing with LiCl buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate) once for 10 min and with TE buffer two times for 10 min. The ChIP-Protein A/G agarose complex was resuspended in 100 μl of TE buffer containing 50 μg/ml RNase A and incubated for 30 min at 37 °C. The Proteinase K treatment, crosslink reversal and DNA purification was performed exactly as described for the preparation of input DNA. Both input and ChIP DNAs were measured by SYBR green-based qPCR (BioRad). Based on the standard curves for each primer pairs the enrichment of proteins and histone modifications on specific genomic regions were calculated as percentage of the immunoprecipitated DNA compared to input DNA. Each data points in ChIP figures were the averages of at least two independent ChIPs using independent chromatins. The following forward (Fw) and reverse (Rev) primers were used in ChIP-qPCR for HS1 (Fw-TTCCTATTTGCCAAGGCAGT and Rev- CTCTTCAGCCATCCCAAGAC), OSBP (Fw- GCTGCTGTTTCCGCCATTCATTTC and Rev- GCTGATACCAACCACCAATCCATGAG), and Neg (Fw- CAGGATCTCCGAGAATCAGC and Rev- GAGTTGGGAGAGCTGTCAGG). The sequences of the other