



Inhibition of the Super Elongation Complex Suppresses Herpes Simplex Virus Immediate Early Gene Expression, Lytic Infection, and Reactivation from Latency

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ABSTRACT Induction of herpes simplex virus (HSV) immediate early (IE) gene transcription promotes the initiation of lytic infection and reactivation from latency in sensory neurons. IE genes are transcribed by the cellular RNA polymerase II (RNAPII) and regulated by multiple transcription factors and coactivators. The HCF-1 cellular coactivator plays a central role in driving IE expression at multiple stages through interactions with transcription factors, chromatin modulation complexes, and transcription elongation components, including the active super elongation complex/P-TEFb (SEC-P-TEFb). Here, we demonstrate that the SEC occupies the promoters of HSV IE genes during the initiation of lytic infection and during reactivation from latency. Specific inhibitors of the SEC suppress viral IE expression and block the spread of HSV infection. Significantly, these inhibitors also block the initiation of viral reactivation from latency in sensory ganglia. The potent suppression of IE gene expression by SEC inhibitors indicates that transcriptional elongation represents a determining rate-limiting stage in HSV IE gene transcription and that the SEC plays a critical role in driving productive elongation during both phases of the viral life cycle. Most importantly, this supports the model that signal-mediated induction of SEC-P-TEFb levels can promote reactivation of a population of poised latent genomes.

IMPORTANCE HSV infections can cause pathologies ranging from recurrent lesions to significant ocular disease. Initiation of lytic infection and reactivation from latency in sensory neurons are dependent on the induced expression of the viral immediate early genes. Transcription of these genes is controlled at multiple levels, including modulation of the chromatin state of the viral genome and appropriate recruitment of transcription factors and coactivators. Following initiation of transcription, IE genes are subject to a key regulatory stage in which transcriptional elongation rates are controlled by the activity of the super elongation complex. Inhibition of the SEC blocks both lytic infection and reactivation from latency in sensory neurons. In addition to providing insights into the mechanisms controlling viral infection and reactivation, inhibitors of critical components such as the SEC may represent novel antivirals.

KEYWORDS transcription elongation, HSV reactivation, HCF-1, AFF4, herpes simplex virus, latency, super elongation complex, transcription

Following a primary infection, herpes simplex virus (HSV) establishes a quiescent/latent infection in sensory neurons. Upon stimulation, the virus can reenter the lytic replication cycle to produce recurrent disease that ranges from oral and genital lesions to severe ocular disease. In addition, neurological and developmental issues are linked to congenital infections (1, 2).

Regulation of viral infection occurs primarily at the level of transcription of viral

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genes that are expressed in defined classes in a sequential manner. For HSV, immediate early (IE) genes represent the first wave of viral genes to be expressed and the products of these genes are critical to promote lytic infection and reactivation. Transcription of IE genes is mediated by cellular RNA polymerase II (RNAPII) and is regulated by viral (VP16) and cellular (e.g., GAPB, SP1, Oct-1) transcription factors, transcriptional coactivators, and chromatin modulation complexes that are recruited to IE enhancer/promoters (3). A central component that is essential for IE gene expression is the cellular transcriptional coactivator HCF-1 (4). HCF-1 is a component of multiple chromatin modulation complexes and functions primarily to bridge IE transcription factors and the cellular chromatin modulation machinery. One key HCF-1 complex that controls IE gene expression contains histone demethylases (LSD1/KDM1A and JMJD2s/KDM4s) and histone methyltransferases (SETD1A/KMT2F and MLL1/KMT2A) that function to remove repressive heterochromatin or to prevent its accumulation on viral IE genes (5–9).

HCF-1 also plays a significant role in the control of viral reactivation from latency in sensory neurons. Upon stimulation that promotes viral reactivation, HCF-1 is rapidly transported from the cytoplasm to the nucleus and is assembled on viral IE gene enhancers/promoters (10–12). In addition, inhibitors of the HCF-1-associated histone demethylases (LSD1/KDM1A, JMJD2s/KDM4s) suppress the initiation of viral reactivation (6, 7, 13, 14). Thus, initiation of lytic infection and initiation of reactivation are both critically dependent on this coactivator and its associated components.

In addition to roles in mediating transcriptional initiation via interactions with TFs and chromatin modulation complexes, analyses of HCF-1 complexes in uninfected and infected cells revealed that the protein was associated with a network of transcriptional elongation components (15). For many genes, modulation of transcription elongation rate is a critical regulatory step that may poise genes for rapid activation in response to environmental signals, allow for appropriate transitions in chromatin states, or prevent epigenetic repression of genes (16–20).

Following initiation, RNAPII associates with negative elongation factors (NELF, DSIF), resulting in enhanced pausing and reduced elongation rates. A critical step in the control of pause release is mediated by recruitment of the P-TEFb complex containing cyclin T1 (CCNT1) and CDK9 kinase. CDK9 phosphorylates RNAPII (S2p), NELF, and DSIF, resulting in dissociation of NELF, association of PAF1, and stimulation of polymerase release and elongation (21–24). P-TEFb/CDK9 is itself regulated in a complex manner. More than 50% of P-TEFb is associated with the 7SK-snRNP complex and thus is sequestered in a catalytically inactive configuration. Upon stimulation by stress or environmental signals, P-TEFb is released and the active complex is recruited to specific genes in association with BRD4 or the super elongation complex (SEC) (25–35).

In addition to control of cellular signal-mediated transcriptional induction, regulated transcriptional elongation plays an important role in the life cycle of multiple viruses that utilize the host RNAPII machinery (e.g., adenovirus, human cytomegalovirus [hCMV], Kaposi's sarcoma-associated herpesvirus [KSHV], hepatitis B virus [HBV], human immunodeficiency virus [HIV], and human T cell leukemia virus [HTLV]) (36–46). In HSV infection, RNAPII pausing and induction of elongation critically regulate viral IE gene expression. Paused polymerase and associated pausing factors (NELF) occupy the promoter-proximal regions of IE genes, and the SEC is specifically required for induction of IE gene transcription (15). Most significantly, compounds that inhibit the competitive adaptor BRD4 or enhance the levels of active SEC-P-TEFb induce HSV reactivation in a mouse model of HSV latency (15). These observations led to the model that stress signal-mediated activation of SEC-P-TEFb can promote IE gene expression and reactivation of a population of latent viral genomes.

Here, the use of recently identified novel inhibitors of the SEC-P-TEFb (47) has allowed for direct probing of the role of this complex in control of HSV lytic infection and reactivation. Inhibition of the SEC suppresses viral IE gene expression, initiation of lytic infection, spread of infection, and viral reactivation from latency in sensory ganglia. These compounds function via reduction in levels of the SEC scaffold protein AFF4, resulting in reduction in SEC occupancy of viral IE promoters, further supporting the

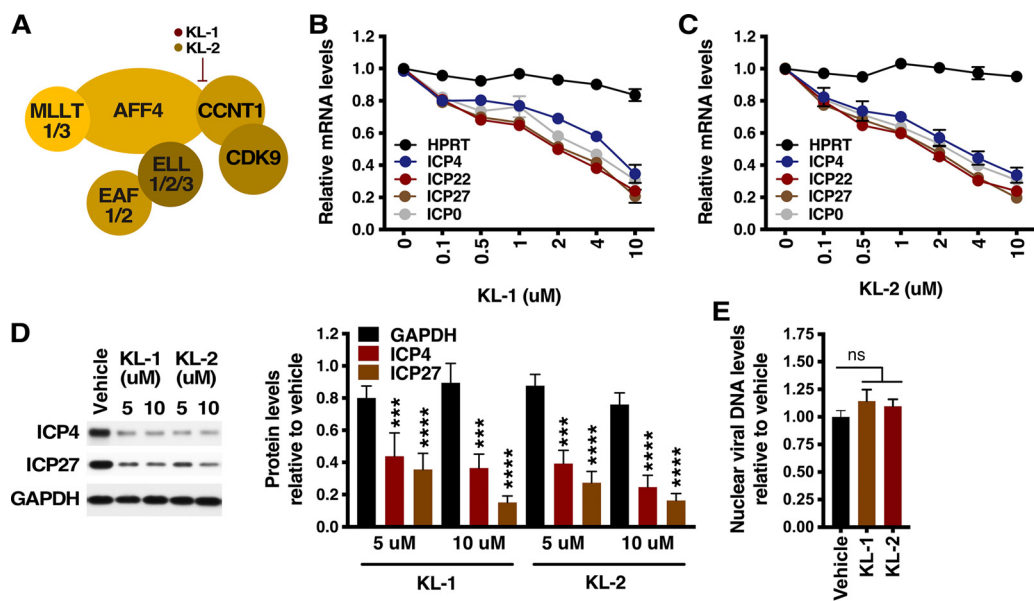


FIG 1 Inhibitors of the SEC suppress HSV IE gene expression. (A) KL-1 and KL-2 interfere with the interaction of CCNT1/P-TEFb with SEC scaffold subunit AFF4. (B to D) HFF cells were treated with the indicated concentrations of KL-1 (B) or KL-2 (C) and infected with HSV (MOI = 1) for 2.5 h. (B and C) mRNA levels of viral IE genes (ICP4, ICP22, ICP27, and ICP0) and a control cellular gene (HPRT) relative to those in vehicle-treated cells. Sample mRNA levels were normalized based on the levels of cellular GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNAs. Data are means \pm SEM of results from at least two experiments. (D) (Left) Western blot of viral IE (ICP4 and ICP27) and cellular control (GAPDH) protein levels. (Right) Quantitation of protein levels in cells treated with KL-1 or KL-2 relative to those in cells treated with vehicle. Data are means \pm SEM of results from four replicates (analysis of variance [ANOVA] with Dunnett's *post hoc* test). (E) HFF cells were treated with vehicle or 8 μ M KL-1 or KL-2 and infected with HSV (MOI = 1) for 2 h. Nuclear HSV DNA levels in cells treated with KL-1 or KL-2 relative to those in cells treated with vehicle are indicated. ns, not significant (ANOVA with Dunnett's *post hoc* test).

model that activation of SEC-P-TEFb directly promotes the initiation of lytic infection and reactivation. Interestingly, levels of the SEC scaffold protein AFF4 are enhanced in HSV-infected cells in an ICP0-dependent manner, suggesting a novel virus-mediated mechanism for modulation of SEC activity.

Translationally, compounds that target cellular components like the SEC that are critical for HSV infection have potential as antivirals, especially for those mutants that are resistant to canonical antivirals that inhibit viral replication machinery (48, 49).

RESULTS

Suppression of HSV IE gene expression and lytic infection by inhibition of the SEC. Previously, it was demonstrated that transcriptional elongation of the HSV IE genes was a critical rate-limiting step in IE gene expression. Stimulation of elongation specifically required the SEC which was found in association with the transcriptional coactivator HCF-1, an essential component of the HSV IE gene regulatory complex (15).

Recently, specific inhibitors of the SEC-P-TEFb (KL-1 and KL-2) that interfere with the assembly/association of P-TEFb subunit CCNT1 (cyclin T1) with the SEC scaffold AFF4 were identified (47) (Fig. 1A). Therefore, to probe the significance of the SEC in HSV IE transcription, primary human foreskin fibroblast (HFF) cells were treated with increasing concentrations of vehicle, KL-1, or KL-2 for 2 h followed by infection with HSV. As shown, treatment with KL-1 or KL-2 resulted in a dose-dependent decrease in levels of viral IE gene mRNAs (Fig. 1B and C) and protein (Fig. 1D) without significantly impacting the expression of the control cellular hypoxanthine phosphoribosyltransferase (HPRT) gene. The 50% inhibitory concentration (IC_{50}) values corresponding to inhibition of IE gene expression by KL-1 (2.25 to 6.98 μ M) and KL-2 (1.61 to 2.29 μ M) are shown in Table S1.

Neither compound affected the transport of the viral genome to the infected cell nucleus, as the levels of nuclear viral DNA in cells treated with KL-1 or KL-2 were not

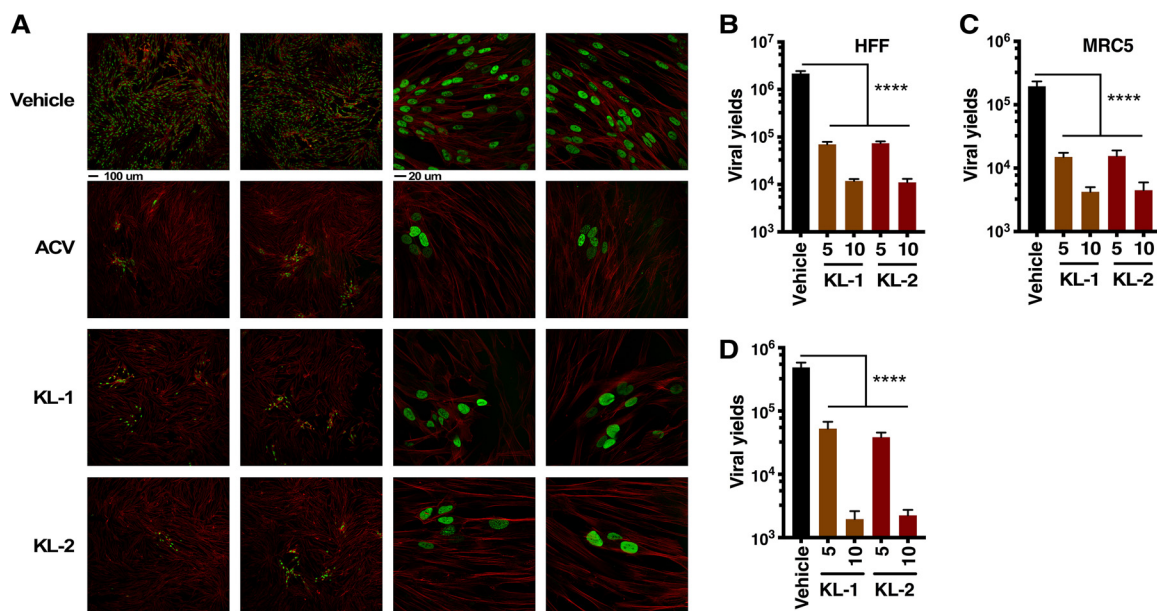


FIG 2 Inhibitors of the SEC suppress the spread of HSV lytic infection. (A to C) HFF and MRC5 cells were infected with HSV (MOI = 0.025) for 8 h and then treated with vehicle, acyclovir (ACV), or 5 or 10 μ M KL-1 or KL-2 for 16 h. (A) HFF cells were stained with anti-UL29 (green) and phalloidin-647 (F-actin, red). Scale bars, 100 μ m (left 2 columns) and 20 μ m (right two columns). (B and C) Viral yields from HFF-infected cells (B) and MRC5-infected cells (C). Data are means \pm SEM of results from 4 replicates (ANOVA with Dunnett's *post hoc* test). (D) Viral yields from HFF cells treated with vehicle or 5 or 10 μ M KL-1 or KL-2 and infected with HSV (MOI = 3) for 12 h. Data are means \pm SEM of results from 8 replicates (ANOVA with Dunnett's *post hoc* test).

significantly different from those seen in cells treated with vehicle (Fig. 1E). Importantly, treatment with KL-1 or KL-2 also did not result in apparent cell toxicity (Fig. S1). Thus, disruption of the active SEC results in potent suppression of viral IE gene transcription.

To determine if these compounds would effectively block the spread of HSV infection, HFF and MRC5 cells were infected at a low multiplicity of infection (MOI) for 8 h in the absence of drug to allow one round of replication. Vehicle, KL-1, KL-2, or the DNA replication inhibitor ACV was then added for an additional 16 h. The cultures were stained for the lytic replication protein UL29 or harvested for viral yields. As shown in Fig. 2A, KL-1 and KL-2 both suppressed the spread of infection in a manner analogous to that seen with the control replication inhibitor ACV. Consistent with this, both inhibitors reduced viral yields 1 to 2 logs in the spread assay (Fig. 2B and C) and in a one-step growth assay (Fig. 2D).

KL-1 and KL-2 SEC inhibitors reduce the number of transcriptionally active viral genomes. Transcriptionally active viral genomes can be detected by the association with the major viral transcriptional activator (ICP4) early in infection. To assess the impact of the SEC inhibitors on the number of transcriptionally active viral foci, HFF cells were pretreated with vehicle, KL-1, or KL-2 and infected with HSV for 2 h. Cells were stained and the number of ICP4 foci counted. As shown in Fig. 3, both KL-1 and KL-2 significantly reduced the number of transcriptionally active viral foci, a result consistent with early suppression of viral IE gene expression.

Inhibition of the SEC reduces the levels of SEC occupancy of viral IE gene promoters. Recruitment of the SEC to viral IE genes early in infection can be detected by association of the SEC scaffold protein AFF4 with viral IE promoters. Given the impact of SEC inhibitors on IE gene expression, the levels of AFF4 occupancy of viral IE promoters were assessed in cells treated with vehicle or KL-2. As shown in Fig. 4, chromatin immunoprecipitation (ChIP) assays illustrate that, in cells treated with vehicle, the SEC was associated primarily with the promoter-proximal regions of the viral IE genes (ICP0 and ICP4) and the positive-control HSPA8 cellular gene whereas little SEC was detected at the ICP0 enhancer region (ICP0 -374) or the viral negative-control promoter (LAT). In cells treated with KL-2, promoter occupancy was significantly

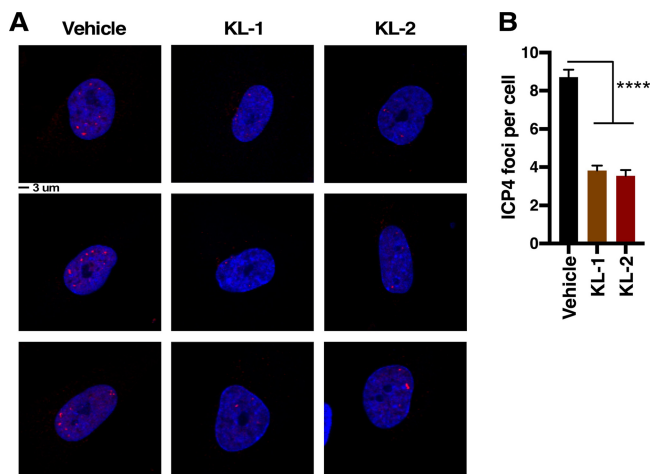


FIG 3 Inhibitors of the SEC reduce the number of transcriptionally active viral genomes. (A and B) HFF cells were treated with vehicle or with KL-1 (10 μ M) or KL-2 (10 μ M) and infected with HSV (MOI = 5) for 2 h. (A) Cells were stained with anti-ICP4 (viral transcriptional foci; red) and 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar = 3 μ m. Three representative images are shown for each condition. (B) The number of transcriptionally active foci per cell. Data are means \pm SEM ($n > 136$ cells per group) (ANOVA with Dunnett's *post hoc* test).

reduced at both viral IE and the SEC-responsive HPSA8 promoter-proximal regions. As treatment of cells with KL-1 or KL-2 had previously been shown to result in disruption in the active SEC complex and ultimately in reduced cellular levels of AFF4 (47), this likely accounts for the decreased SEC occupancy of IE promoters. Similar impacts of KL-2 were seen in ChIP assays monitoring the occupancy of a second component of the SEC (AF9/MLLT3; Fig. S2). Together, the data clearly implicate the SEC in mediating transcription of HSV IE genes during the initiation of lytic infection.

Suppression of HSV reactivation from latency by inhibition of the SEC. In addition to control of lytic infection, regulation of transcriptional elongation by the SEC is proposed to also impact the initiation of viral reactivation from latency in sensory neurons. This is based on the induction of viral reactivation by bromodomain and extraterminal (BET) inhibitors that inhibit the competitive adaptor BRD4 and enhance the levels of active SEC-P-TEFb (15). These data led to a model in which a population of poised genomes respond rapidly to signal-mediated induction of active SEC-P-TEFb to promote IE expression and the initiation of reactivation.

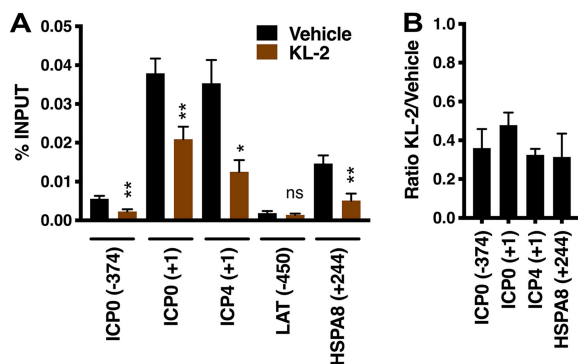


FIG 4 KL-2 reduces the recruitment of the SEC to viral IE promoters. (A and B) HFF cells were treated with vehicle or 8 μ M KL-2 and infected with HSV (MOI = 2) for 2 h. (A) ChIP assay data showing the levels of the SEC (AFF4) associated with viral IE (ICP0 and ICP4), control viral (LAT), or control cellular (HSPA8) genes in cells treated with vehicle or KL-2. Data are means \pm SEM of results from 4 experiments (paired two-tailed *t* tests). (B) Ratio of AFF4 occupancy levels in KL-2-treated versus vehicle-treated cells.

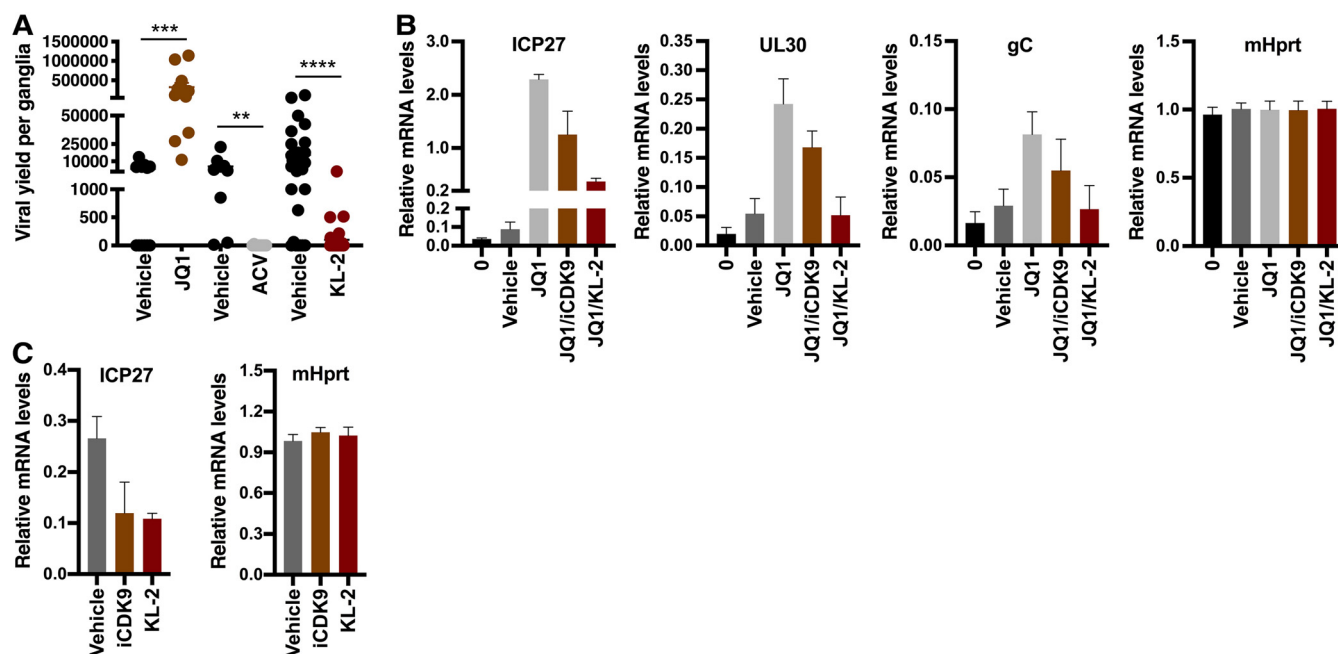


FIG 5 KL-2 suppresses the initiation of viral reactivation from latency. (A) Viral yields from latently infected trigeminal ganglia explanted in the presence of vehicle, JQ1 (1 μ M), KL-2 (10 μ M), or ACV (100 μ M) for 48 h. Data are from individual ganglia ($n \geq 9$; Wilcoxon matched-pairs 2-tailed signed rank test). (B) mRNA levels of viral (ICP27, UL30, and gC) genes and cellular control mHprt genes in latently infected trigeminal ganglia explanted in the presence of vehicle, JQ1 (1 μ M), JQ1/iCDK9 (1 μ M/150 nM), or JQ1/KL-2 (1 μ M/5 μ M) for 6 h. (C) mRNA levels of viral (ICP27) and cellular control (mHPRT) genes in latently infected trigeminal ganglia explanted in the presence of vehicle, iCDK9 (150 nM), or KL-2 (5 μ M) for 12 h. (B and C) Sample mRNA levels were normalized based on the levels of cellular murine GAPDH (mGapdh) mRNA. Data are means \pm SEM of results from 2 experiments ($n \geq 2$ pools of five ganglia per group).

In the mouse ganglia explant system, reactivation is stimulated by explant of latently infected mouse trigeminal ganglia into culture. This allows for the inclusion of compounds that target specific components to probe for mechanisms involved in reactivation. Therefore, to directly probe the significance of the SEC in viral reactivation, latently infected trigeminal ganglia were bisected followed by explant of one half of the ganglia in the presence of control vehicle and the other half in the presence of the replication inhibitor ACV or KL-2 for 48 h. JQ1 was included as a control compound that stimulates reactivation via inhibiting the SEC competitor BRD4 and increasing the levels of active P-TEFb (15, 26, 50). Post-explant, viral yields were determined as a measure of reactivation. As shown in Fig. 5A, JQ1 induced reactivation whereas ACV suppressed viral yields as anticipated. Strikingly, KL-2 significantly suppressed reactivation, indicating that the SEC is required for efficient reactivation.

To determine if inhibition of the SEC suppressed the initiation of reactivation via inhibition of expression of viral IE genes, latently infected ganglia were explanted in the presence of vehicle, JQ1, or JQ1 in combination with a CDK9/P-TEFb inhibitor (iCDK9 [51]) or the SEC inhibitor KL-2. The levels of viral lytic mRNAs were determined at 6 h post-explant. At this early time point, JQ1 was included to enhance the induction/frequency of reactivation (15). As shown in Fig. 5B, JQ1 effectively induced viral reactivation as evidenced by significant levels of a representative viral IE (ICP27) mRNA and low levels of viral E (UL30) and L (gC) mRNAs. Importantly, both iCDK9 and KL-2 reduced the ability of JQ1 to stimulate viral gene expression.

To determine the impact of KL-2 on viral reactivation in the absence of JQ1 stimulation, latently infected ganglia were explanted in the presence of vehicle, iCDK9, or KL-2 for 12 h prior to determining the levels of viral lytic mRNAs. At this point, in the absence of additional stimulus, only the viral IE gene mRNA was reliably detected and both iCDK9 and KL-2 significantly reduced the mRNA levels (Fig. 5C). No significant change in mRNA levels of control murine HPRT (mHprt) gene was seen at either 6 or 12 h post-explant under any conditions (Fig. 5B and C). Thus, inhibition of the SEC

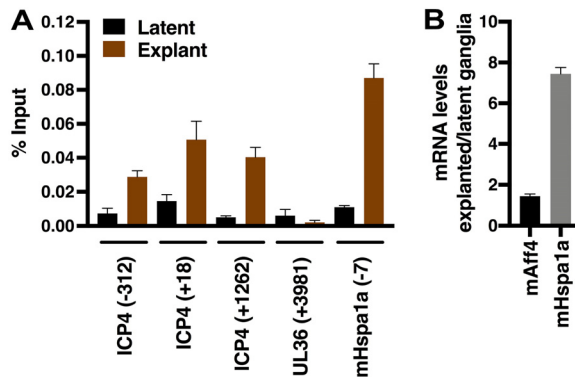


FIG 6 SEC recruitment to viral IE genes during initiation of viral reactivation from latency. (A) Data from ChIP assays showing the levels of the SEC (mAff4) associated with viral (ICP4 and UL36) and cellular (mHspa1a) genes in latently infected trigeminal ganglia at 0 h post-explant (latent) and 6 h post-explant (reactivation). Data are means \pm SEM of results from 2 experiments; each immunoprecipitation was performed with a pool of 10 ganglia. (B) mRNA levels of mAff4 and mHspa1a in explanted ganglia relative to latently infected ganglia. mRNA levels were normalized based on the levels of cellular mGapdh mRNA. Data represent means of results from 2 pools of 4 ganglia each.

suppresses the initiation of viral reactivation specifically by suppression of viral IE gene expression.

The SEC is recruited to viral IE promoters upon induction of viral reactivation.

During lytic infection, the SEC is recruited to viral IE gene promoters to stimulate IE transcription (Fig. 4). KL-2-mediated inhibition of IE gene expression during viral reactivation suggested that the SEC may also directly promote IE expression in this context. Therefore, latently infected trigeminal ganglia were harvested immediately (time zero; latent) or were explanted for 6 h and chromatin immunoprecipitations were done to assess SEC occupancy at viral IE and control regions. As shown in Fig. 6A, no significant levels of the SEC were associated with IE genes of the latent viral genome. However, upon explant induction, the SEC was recruited to a representative IE promoter (ICP4). Recruitment was specific, as the complex was not detected at a control region of the viral genome (late gene UL36 coding). Explant also resulted in SEC occupancy of the positive-control mHspa1a gene promoter and correlated with induced levels of mHspa1a mRNA.

Levels of the SEC scaffold AFF4 protein are regulated in HSV-infected cells.

Overall, the data presented here highlight the significance of AFF4/SEC as a regulatory complex that is important for the expression of HSV IE genes during lytic infection and reactivation from latency. Strikingly, in HFF cells infected with HSV, the levels of AFF4 protein were increased (Fig. 7A) in contrast to the levels of other related proteins (HEXIM1, BRD4, and CDK9). This increase in AFF4 protein levels was enhanced with increasing MOI (Fig. S3A) and was seen in several cell types (HFF, MRC5, and Vero; Fig. S3C). The increased levels of AFF4 protein were not due to enhanced transcription of the AFF4 gene as the relative mRNA levels did not change upon infection (Fig. 7B; see also Fig. S3B).

In cells, the levels of active SEC are modulated by targeted degradation of the AFF4 scaffold and ELL1/2 subunits by the E3 ubiquitin ligase SIAH1 (52–55). Consistent with this, depletion of SIAH1 in MRC5 cells resulted in strongly (~5-fold) enhanced levels of AFF4 protein and a concomitant increase in transcription of HSV IE (ICP4 and ICP27) genes (Fig. S4A–B). Similar increases in AFF4 and HSV IE (ICP4) protein levels were also seen in HFF cells depleted for SIAH1 (Fig. S4C).

Interestingly, previous studies identified SIAH1 as an interaction partner for the HSV IE protein ICP0 and demonstrated that a recombinant virus with a mutant ICP0 that did not interact with SIAH1 was defective in growth *in vitro* and *in vivo* (56, 57). Therefore, to determine if ICP0 was involved in the regulation of AFF4 protein levels upon infection, cells were infected with wild-type (WT) HSV or a recombinant lacking ICP0

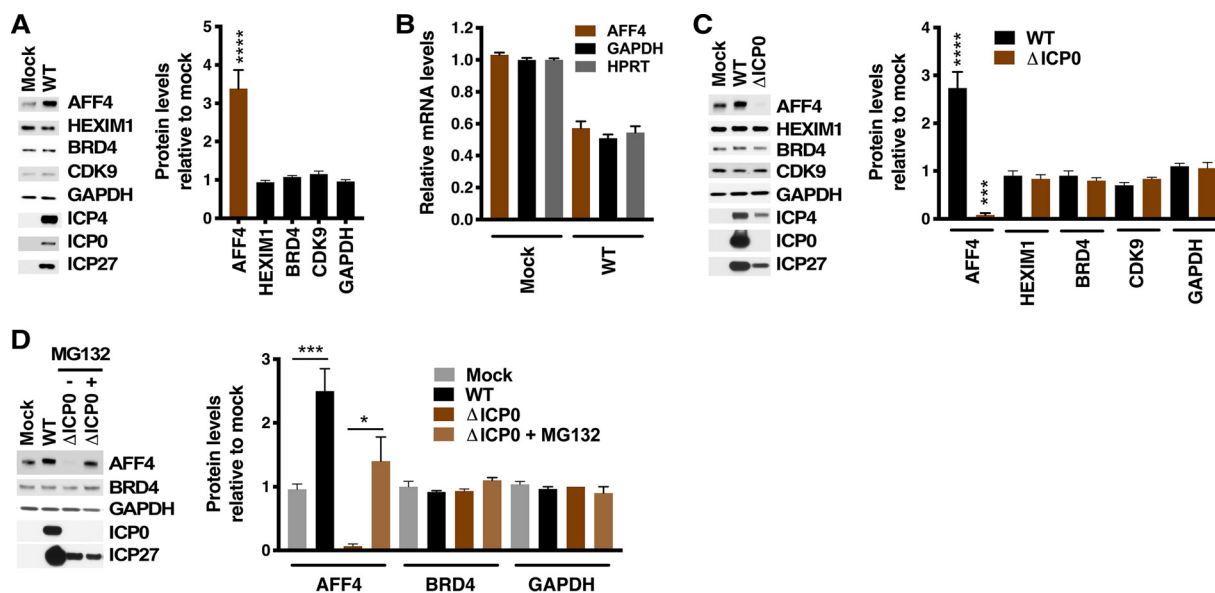


FIG 7 HSV infection increases the levels of AFF4 protein in an ICP0-dependent manner. (A) (Left) Western blot of AFF4 and control cellular proteins (HEXIM1, BRD4, CDK9, and GAPDH) and viral IE proteins (ICP4, ICP0, and ICP27) in extracts from mock-infected HFF cells or cells infected with wild-type HSV (MOI = 5) for 4 h. (Right) Quantitation of protein levels in HSV-infected relative to mock-infected cells. Data are means \pm SEM of results from 4 experiments (unpaired two-tailed *t* tests). (B) mRNA levels of AFF4 and cellular control genes (GAPDH and HPRT) in cells infected with HSV (MOI = 5) relative to those in mock-infected cells. Data are means \pm SEM of results from at least 2 experiments and 4 to 10 replicates (ANOVA with Dunnett's *post hoc* test). (C) (Left) Western blot of AFF4 and control cellular proteins (HEXIM1, BRD4, CDK9, and GAPDH) and viral IE proteins (ICP4, ICP0, and ICP27) in extracts from mock-infected cells or cells infected with wild-type (WT) HSV or Δ ICP0 HSV (MOI = 5) for 4 h. (Right) Quantitation of protein levels in HSV-infected relative to mock-infected cells. Data are means \pm SEM of results from 2 experiments and 3 to 6 replicates (ANOVA with Dunnett's *post hoc* test). (D) (Left) Western blot of AFF4 and control cellular proteins (BRD4 and GAPDH) and viral IE proteins (ICP0 and ICP27) in extracts from mock-infected cells or cells infected with wild-type (WT) HSV or Δ ICP0 HSV (MOI = 5) for 4 h in the absence or presence of 10 μ M MG132. (Right) Quantitation of protein levels in HSV-infected relative to mock-infected cells. Data are means \pm SEM of results from 3 replicates (unpaired two-tailed *t* tests).

(Δ ICP0 [58, 59]). In contrast to the stabilization of AFF4 seen upon infection with WT virus, infection with the Δ ICP0 virus resulted in reduced levels of AFF4 relative to the levels in mock-infected cells (Fig. 7C). The reduction in AFF4 protein levels in Δ ICP0-infected cells could be reversed, at least partially, by treatment with the proteasome inhibitor MG132, suggesting that turnover of AFF4 was enhanced in the absence of ICP0 in HSV-infected cells (Fig. 7D). Thus, HSV infection resulted in increased levels of the SEC scaffold AFF4 protein in an ICP0-dependent manner.

DISCUSSION

Expression of HSV IE genes is a critical stage that determines the progression of lytic infection and the initiation of reactivation from latency in sensory neurons. Regulation of IE expression has been extensively studied, but the mechanisms involved in induction of IE transcription early in infection and, more importantly, during the initiation of reactivation from latency remain to be fully described.

IE transcription is mediated by cellular RNAPII and is controlled primarily via a cellular transcriptional coactivator, HCF-1, that mediates activation of transcription factors assembled on the viral IE enhancer/promoter regions. One of key functions of this protein is to prevent the accumulation of repressive histone modifications (H3K9me3) and to promote the installation of activating marks (H3K4me3) on nucleosomes associated with viral IE genes. This is accomplished via recruitment of an HCF-1 complex that contains histone H3K9 demethylases (LSD1 and JMJD2s) and H3K4 methyltransferases (SETD1A/MLL1). Ultimately, the transition from repressed to active chromatin promotes the initiation of IE transcription. Importantly, HCF-1 and its associated chromatin modulation components also appear to be critical components of the viral latency-reactivation cycle. HCF-1 is rapidly transported to the nucleus upon induction of reactivation, and inhibition of the HCF-1-associated histone demethylases blocks the initiation of viral reactivation (Fig. 8).

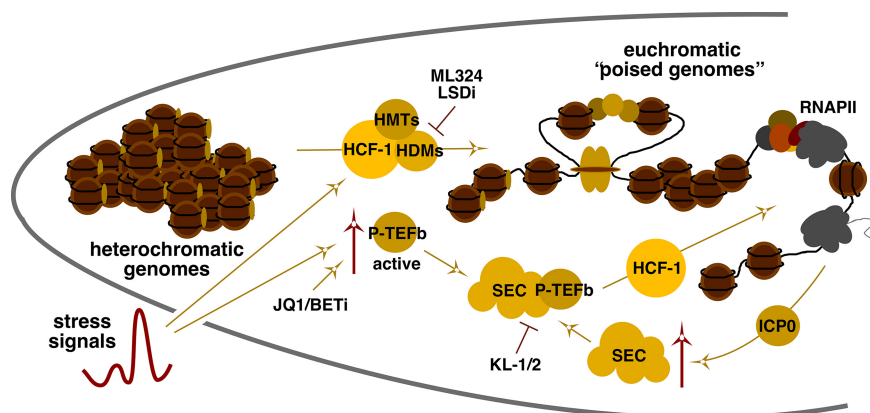


FIG 8 Model of epigenetic modulation and SEC-mediated induction of viral IE gene expression during initiation of HSV reactivation. Stress signaling promotes viral reactivation at multiple rate-limiting stages. Signaling induces the transport of HCF-1 to the nucleus of latently infected neurons where HCF-1 complexes containing histone H3K9 demethylases (LSD1 and JMJD2s) and histone H3K4 methyltransferases (SETD1A and MLLs) are involved in promoting the transition of heterochromatic viral genomes to accessible euchromatic genomes. This transition can be blocked by inhibitors (LSDi and ML324) of these HCF-1-associated H3K9 demethylases, resulting in suppression of viral reactivation. Signaling also results in the release of P-TEFb from the 7SK-snRNP complexes, which increases the levels of active SEC-P-TEFb. Along with HCF-1, recruitment of SEC-P-TEFb to viral IE gene promoters stimulates productive expression of IE genes and reactivation of a population of accessible genomes. Compounds such as BET inhibitors (JQ1) enhance the levels of active P-TEFb and induce reactivation, while SEC inhibitors (KL-1/2) disrupt the active SEC and suppress reactivation. Following induction, the accumulation of the viral IE ICP0 protein may further stabilize the SEC, enhance active SEC-P-TEFb levels, and contribute to reactivation. Note that while part of this model reflects the described roles of HCF-1 and its associated chromatin modulation factors in viral reactivation, the epigenetic regulation of latent viral genomes is complex. Other repressive histone marks (e.g., H3K27me3 [72, 77–81]) are also associated with latent viral genomes, and it is likely that additional pathways and components contribute to the epigenetic regulation of latency and reactivation.

For many genes, following RNAPII initiation, transcriptional elongation becomes a critical rate-limiting stage. Regulated RNAPII pausing may poise genes for rapid expression in response to signaling, allow for coordinated chromatin modulation and assembly of RNA processing complexes, and/or prevent genes from being inactivated during periods of low-level transcription. One important step in mediating pause release and efficient elongation is the recruitment of an active P-TEFb kinase complex which phosphorylates RNAPII and the pausing factors NELF and DSIF. This complex is itself regulated at several levels. It is sequestered in an inactive state by association with the 7SK-snRNP. Upon stress signaling, P-TEFb is released and the active enzyme associates with the specificity adaptors BRD4 or the SEC.

A number of viruses that utilize host cell RNAPII transcriptional machinery also exhibit regulated pausing and elongation. For HIV, this is a well-characterized mechanism that appears to play an essential role in the control of viral latency/reactivation. During latency, low-level transcription is mediated by BRD4-P-TEFb whereas reactivation is induced upon TAT-dependent recruitment and stabilization of the SEC that promotes enhanced transcription of the viral genome (60, 61).

For HSV, IE genes also exhibit characteristics of genes that are highly dependent upon regulated transcriptional elongation. RNAPII and associated pausing factors (NELF) are detected at the promoter-proximal regions of IE genes during the initiation of lytic infection, and, as in the case of HIV, the SEC is required for efficient pause-release and transcriptional elongation. Consistent with these results, PRO-Seq analyses defined distinct RNAPII pause sites in IE genes during infection (62). Interestingly, in addition to functions in the modulation of chromatin and initiation of IE gene transcription, the coactivator HCF-1 also associates with multiple transcriptional elongation components, including PAF1, SETD2, FACT, and the SEC-P-TEFb, suggesting that HCF-1 may coordinate multiple stages of IE transcription.

In addition to the control of lytic infection, regulation of elongation of HSV IE

transcription may also be a critical rate-limiting step for viral reactivation from latency as compounds (e.g., BET inhibitors) that enhance the levels of active P-TEFb also potently induce viral reactivation in sensory ganglia (15, 26, 27, 63). These data are consistent with a model where induction of active P-TEFb promotes IE expression and initiation of reactivation in a population of latent viral genomes (Fig. 8).

Recently, inhibitors of the SEC (KL-1, KL-2) were identified in a screen for compounds that disrupt the interaction of the SEC scaffold AFF4 with the cyclin T1 component of the p-TEFb kinase subcomplex. Disruption results in enhanced turnover of AFF4 and in reduced levels of active SEC, ultimately impacting the rate of transcriptional elongation of the population of genes that normally exhibit SEC occupancy (47). These compounds also suppressed Tat-mediated induction of HIV transcription in a reporter-based assay (47).

Given the indication that the SEC-P-TEFb plays a critical role in driving expression of HSV IE genes, the impact of inhibition of the SEC was investigated during lytic infection and reactivation from latency. These SEC inhibitors potently suppressed HSV IE gene expression and spread of a primary infection and reduced SEC occupancy of viral IE gene promoters during the initiation of viral lytic infection. However, as the SEC is required for IE expression and these IE gene products are required for expression of subsequent E/L genes, it remains unknown if the SEC is also involved in regulation of later classes of HSV genes or if alternative components or mechanisms are involved. The viral IE protein ICP22 has been shown to interact with and modulate P-TEFb/CDK9 activity as well as promote the recruitment of elongation factors FACT, SPT5, and SPT6 to the viral genome (64–67). Moreover, ICP22 is required for efficient transcriptional elongation of some HSV genes later in infection (65). With respect to the alternative P-TEFb adaptor BRD4, while this protein does not play a significant role in mediating IE expression, depletion of this adaptor does reduce the accumulation of viral proteins and viral yields late in infection (68). Nonetheless, the potential involvement of the SEC in regulation of multiple gene classes could account for the potent suppression of viral spread by the SEC inhibitors.

Importantly, inhibitors of specific components that may regulate viral IE gene expression allow probing of mechanisms that control the induction of IE expression during the initiation of viral reactivation. Here, inhibition of the SEC suppressed reactivation of HSV in explants of latently infected sensory ganglia as measured by viral yields and the reduction in viral IE gene expression at an early point in reactivation. Most significantly, the SEC was detected at viral IE genes during the early stage of reactivation and correlated with expression of viral IE mRNAs. Together, the data strongly support a model that, as in the case of HIV, the initiation of HSV lytic infection and reactivation from latency are critically regulated by SEC-dependent transcriptional elongation.

The levels of active SEC-P-TEFb are regulated by multiple mechanisms. In addition to signal-mediated modulation of P-TEFb availability, the levels of the SEC scaffold AFF4 and the ELL2 subunit proteins are regulated by the ubiquitin ligase SIAH1, which induces turnover of these critical SEC components. More recently, SIAH1 itself was shown to be targeted for degradation by poly(ADP-ribose) polymerase 1 (PARP1)-mediated PARylation-dependent ubiquitination, resulting in enhanced levels of ELL2 and induction of HIV transcription (55).

Strikingly, levels of AFF4 protein were induced in cells infected with HSV, suggesting that a virus-mediated mechanism might enhance SEC levels for HSV gene expression. Interestingly, reports from the Hauber laboratory have shown that the ICP0 viral IE protein interacts with SIAH1 through SIAH1-binding consensus motifs and that viruses with mutations in these motifs are defective for growth in culture and *in vivo* (56, 57). The authors propose several models, including one in which ICP0 functions to sequester SIAH1, thus blocking SIAH1's interactions with its targets. Consistent with this, infection with an ICP0 null virus resulted in the loss of AFF4 induction that was partially recovered by proteasomal inhibition (Fig. 7C and D). These results suggest that ICP0 may modulate SIAH1 activity and thus ultimately regulate the stability of AFF4. This

RNA isolation, cDNA synthesis, and quantification. Quantitation of RNA levels in tissue culture cells and trigeminal ganglia was done as detailed in Text S1.

Chromatin immunoprecipitations. ChIP assays of cultured cells and of trigeminal ganglia were done as detailed in Text S1. Primer sequences are in Table S3.

siRNA depletions. MRC5 or HFF cells (1×10^6) were transfected with 30 nM siSIAH1 small interfering RNA (siRNA) or siControl pools using TransIT-X2 reagent (Mirus) per the manufacturer's recommendations. The siRNAs used are listed in Table S2.

Statistical analyses. Results are presented as means \pm standard errors of the means (SEM). Parameters are stated in the figure legends and detailed in Table S4. Analyses were done using GraphPad Prism 8. Where indicated, asterisks denote statistical significance as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.02 MB.

FIG S1, PDF file, 0.05 MB.

FIG S2, PDF file, 0.5 MB.

FIG S3, PDF file, 0.1 MB.

FIG S4, PDF file, 0.08 MB.

TABLE S1, PDF file, 0.03 MB.

TABLE S2, PDF file, 0.05 MB.

TABLE S3, PDF file, 0.04 MB.

TABLE S4, PDF file, 0.05 MB.

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