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Histone H2A Ubiquitination Mediates the Establishment of Reactivation-

Competent HSV-1 Latent Infection.

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Abstract

Herpes simplex virus 1 (HSV-1) establishes a latent infection of neurons, where it persists for life with the capability to reactivate. During latent infection, heterochromatin is deposited onto the viral genome to presumably permit viral gene silencing. Repressive histone modifications associated with the latent genome include the constitutive heterochromatin modifications, H3K9me2/3, and the facultative heterochromatin modification, H3K27me3. The role of these different types during entry into latency and as substrates for reactivation is unknown. H3K27me3 is associated with Polycomb silencing, and a second modification also associated with Polycomb silencing, especially in pluripotent cells and during early development, is H2AK119ub1. Here we found that H2AK119ub1 is enriched on a sub-population of latent HSV genomes. We examined the contribution of both H2AK119ub1 and H3K7me3 deposition to HSV-1 gene silencing during entry into latency. We found that H2AK119ub1 was deposited prior to H3K27me3 and that only inhibition of H2AK119ub1 deposition plays a subtle role in latency establishment. Importantly, we found that inhibiting the enzymatic activity of Polycomb Repressive Complex 1 (PRC1) that deposits H2AK119ub1 during latency establishment prevented later reactivation. In contrast, inhibiting the activity of PRC2, which deposits H3K27me3, did not impact reactivation. Together, these data demonstrate the heterogenic nature of the epigenetic structure of latent HSV genomes and provide evidence that those associated with H2AK119ub1 are the template for reactivation.

Introduction

Human herpesviruses establish lifelong latent infections in specific host cells. During latency, viral genomes are typically associated with repressive heterochromatin, which likely maintains long-term gene silencing and enables viral persistence. However, heterochromatin is highly diverse and can vary in the mechanisms of formation, the fate of the chromatin structure, and its reversibility. Because herpesvirus genomes periodically reactivate from latency to produce infectious progeny, they must remain transcriptionally silent yet retain the capacity to revert to euchromatin and support lytic gene expression. As highly evolved viruses, herpesviruses have likely coevolved with their host species to exploit certain types of heterochromatin that permit such transitions. Therefore, investigating heterochromatin-based silencing mechanisms on herpesvirus genomes provides insights into the host mechanisms of gene silencing and reversal, and also informs strategies to prevent viral reactivation.

Herpes Simplex Virus-1 (HSV-1) establishes a latent infection in post-mitotic peripheral neurons, and reactivation can cause lesions, recurrent pain, blindness, and encephalitis. There is also accumulating evidence linking HSV-1 infection with late-onset Alzheimer's disease (1). The latent HSV-1 viral genome is known to be associated with heterochromatin, with the enrichment of certain histone post-translational modifications (PTMs) including H3K27me3, which is indicative of Polycomb silencing (2-5), as well as H3K9me3 (3), which is characteristic of more long-term constitutive heterochromatin. Although associated with repressive histone modifications, it is currently unknown whether heterochromatin deposition on the viral genome directly

drives gene silencing. An alternative is that heterochromatin is deposited on already

silenced genes to maintain long-term gene silencing and viral latency. This is important

to understand to gain insight into how the virus establishes a latent infection of neurons.

EZH1 and EZH2 catalyze the addition of K27 methylation onto histone H3 as part of

the Polycomb repressive complex 2 (PRC2) (6). Previously, PRC2 has been found

stably associated with the HSV-1 genome as latency is established, but only after lytic

gene silencing had already occurred (2), indicating that PRC2 activity may not directly

drive lytic gene repression during entry into latency. On the cellular genome, H3K27me3

can be accompanied by the mono-ubiquitination of H2AK119 (H2AK119ub) (7).

H2AK119ub is deposited by the Polycomb Repressive Complex 1 (PRC1) (8-10).

Although H2AK119ub was initially thought to be deposited after H3K27me3, multiple

recent studies have found that H2AK119ub is deposited first, followed by H3K27me3, at

least in embryonic stem cells and during early development (11-14). In this context,

H2AK119ub deposition by PRC1 but not H3K27me3 deposition drives gene silencing.

H3K27me3 can form in the absence of H2AK119ub1, and this may play a more

important role in maintaining gene silencing, often referred to as the primary form of

Polycomb silencing in terminally differentiated cells (15-17). HSV-1 establishes a latent

infection in non-mitotic, terminally differentiated neurons, where the contribution of

H2AK119ub1 to driving gene silencing is unknown. Therefore, the association of

H2AK119ub with HSV-1 is also unknown. Further, when the HSV-1 genome enters a

host cell nucleus, it does so as epigenetically naïve, meaning it does not contain a pre-

existing chromatin template (18, 19). Therefore, HSV-1 also serves as a tool to study

the basic mechanisms of Polycomb recruitment and silencing in terminally differentiated

neurons.

Understanding the contribution of heterochromatin silencing to HSV latency and

reactivation requires considering that epigenetic structures across individual genomes

may be heterogeneous, with different types of heterochromatin playing distinct roles in

the initiation of gene silencing. HSV-1 latent infection is heterogeneous, with altered

expression of the latency-associated transcript (LAT) and differential localization of viral

genomes to distinct subnuclear regions (20). Further, at any one time only a fraction of

viral genomes undergo reactivation (21-25). We recently reported that the association of

HSV-1 genomes with H3K9me3 and the reader protein ATRX results in a form of latent

infection that is restricted for reactivation (26). However, it is currently unknown whether

there is a specific HSV-1 epigenetic structure that is more reactivation-competent.

Because of its reversible nature, Polycomb silencing is a key candidate for

reactivation competent HSV-1 genomes (27). Accumulating evidence suggests that

H2AK119ub marks regions on the host genome that are silenced during early stages of

development, with the capacity to transition to euchromatin for gene expression upon

differentiation (28). Therefore, here we set out to determine whether H3AK119ub was

even associated with the latent viral genome. We found enrichment of this modification

on latent viral genomes. Further, we found that inhibiting the deposition of H2AK119ub1

during latency establishment had a mild impact on viral gene expression at early times,

whereas inhibiting PRC2 activity had a negligible impact. Importantly, though, PRC1

inhibition during latency establishment dramatically reduces the ability of latent viral

genomes to reactivate. These data, taken together, support a model in which PRC1

mediates lytic viral gene silencing in a manner amenable to later reactivation.

Results

H2AK119ub is enriched on the HSV-1 genome during latent infection established *in vivo.*

The latent HSV-1 genome is known to be associated with the H3K27me3. However, whether H2AK119ub can be targeted to a viral genome in terminally differentiated neurons was unknown. Therefore, we initially used a well-established *in vivo* model of HSV-1 latency to determine whether H2AK119ub was detectible on the viral genome (Fig. 1A). We infected mice via the ocular route and at 30 days post-infection, we harvested latently infected trigeminal ganglia (TG) and performed chromatin immunoprecipitation (ChIP) assays for H2AK119ub, as well as negative control IgG as previously described (2). We observed a significant enrichment of H2AK119ub on repressed lytic viral gene promoters during latent infection over IgG controls (Fig 1B), as determined by quantitative PCR (qPCR) with primers specific for the *ICP27* (an immediate-early gene) and *ICP8* (an early gene) promoter regions.

H2AK119ub and H3K27me3 associate with a population of latent HSV-1 genomes *in vitro.*

To mechanistically investigate the contribution of Polycomb Group proteins to HSV-1 latency, we turned to an in vitro system where we can more readily manipulate their function solely in neurons. First, we mapped the association of H2K119ub and H3K27me3 on the latent viral genome following neuronal infection in vitro. We infected primary neonatal sympathetic neurons isolated from the superior cervical ganglia of newborn mice with HSV-1 Stayput-GFP (21) at an MOI of 10 PFU/cell in the presence

of viral DNA replication inhibitor acyclovir (ACV) to promote latency establishment (Fig.

1C). Neurons were collected 30 days post-infection, a time point at which latency has

been fully established *in vitro* (21) and mirrors what is documented *in vivo* (2-4). Primary

neurons also have the advantage that they are more amenable to performing Cleavage

Under Target & Release Under Nuclease (CUT&RUN), which gives a higher resolution

than ChIP and it requires less input material. We performed CUT&RUN-sequencing

using control IgG, along with antibodies against H2AK119ub and H3K27me3. Both

H2AK119ub and H3K27me3 were broadly enriched over IgG controls, as well as input,

across the latent HSV-1 genome, although in more discernable peaks versus the ChIP-

Seq data (Fig 1D). Both H2AK119ub and H3K27me3 were enriched on lytic genes and

intergenic regions, including but not limited to immediate early genes ICP27 and ICP0

(Fig 1E). Both modifications were but were notably depleted at between CTCF sites in

the transcriptionally active LAT promoter region (Fig 1E). Therefore, both H3K27me3

and H2AK119ub are enriched on the latent HSV-1 genomes following latency

established either in vivo or in vitro.

<u>A sub-population of HSV-1 genomes associates with H2AK119ub1 and/or H3K27me3</u>

<u>during latent infection *in vitro.*</u>

Collectively, the data form Chip and CUT&RUN demonstrate genome-wide

enrichment of H2AK119ub on the repressed HSV-1 genome during latent infection.

However, there is known heterogeneity in HSV-1 latency based on prior lytic gene

expression, expression of the LAT, and the sub-nuclear localization of viral genomes

(20, 22, 26, 29). Therefore, we were interested in determining whether there is

heterogeneity in facultative heterochromatin associated with the latent HSV-1 genome.

Interestingly, only a subpopulation of neurons containing latent viral genomes will

reactivate at any one time (21, 22, 25, 26, 30-32). Therefore, we next used an additional

in vitro approach to investigate heterochromatin association with the latent viral genome

at the single-cell and individual viral-genome levels. We infected primary neurons with

an EdC-labelled HSV-1 virus at an MOI of 10 and fixed at 30 days post-infection. Using

a combination of click chemistry and immunofluorescence, we analyzed the 3-

dimensional co-localization of the latent HSV-1 genome with PRC1 and PRC2-mediated

histone modifications H3K27me2, H3K27me3 and H2Aub1. H3K27me2 was including in

the analysis as it is also correlated with gene repression and has recently been shown

to associate with the HSV-1 genome during early lytic infection of non-neuronal cells

(33). In agreement with the CUT&RUN and ChIP data, we observed viral genome foci

that colocalized with clusters of H3K27me3 and H2AK119ub (Fig 2A).

Histone modification stains are broadly nuclear, making it unclear whether overlaps

between the green and red signals are a random coincidence or genuine co-

localization. Therefore, to measure co-localization in a high-throughput and unbiased

fashion, we used our recently developed program NucSpotA (33). NucSpotA measures

the enrichment of the modification (based on the intensity of the immunofluorescence

signal) at the viral genome relative to the entire nucleus. By also measuring the

enrichment values of the same stain on a set of control images where the viral genome

is rotated 90 degrees, we can determine whether co-localization with the HSV-1

genome in original images is higher than that obtained for random placement of the viral

genome. We further assign a threshold for the enrichment values by eye and calculate

the proportion of viral genomes that meet this threshold to estimate what proportion of

latent viral genomes are co-localized with these Polycomb modifications and

components during latent infection. In agreement with our population-level findings, the

HSV-1 genome significantly co-localized with H3K27me3 at 30 days post-infection (Fig

2B). Specifically, we found that H3K27me3 co-localized with a subset of latent viral

genomes, approximately 30%. In contrast, only approximately 8% of genomes co-

localized with H3K27me2, and this was not significantly enriched at this time point

compared to the rotated control (Fig. 2B). Importantly, the latent HSV-1 genome also

significantly co-localizes with H2AK119ub in approximately 34% of the infected neurons

(Fig. 2B).

We also quantified the co-localization of different PRC1 components with the viral

genome, including the core components Ring1A and Ring1B, and the PRC2 component

Suz12. We observed staining puncta for all three proteins (Fig. 2C) and viral genes that

co-localized with these puncta in approximately 48% of neurons for Ring1A, 20% for

Ring1B, and 37% for Suz12. Therefore, using multiple methods, our data demonstrate

enrichment for H2AK119ub and H3K27me3 but not H3K27me2 on at least a sub-

population of viral genomes, and co-localization with components of the complexes that

can both read and write these modifications. For at least one of these (Ring1A), the

frequency was higher than either modification alone (48% compared to approximately

30% for H3K27me3 and H2Aub1).

<u>PRC1 associates with the viral genome during active stages of viral gene silencing.</u>

As previously determined by performing ChIP on ganglia in a mouse model,

PRC2 (specifically Suz12) and associated H3K27me3 are not enriched on the viral

genome until lytic viral genes have already been silenced (2). These observed kinetics

suggest that PRC2 may not play an active role in initiated lytic gene silencing in

neurons. Because of the known function of vPRC1-medited H2AK119ub in driving gene

silencing before H3K27me3 deposition (12, 34-37), at least in embryonic stem cells, we

posited that H2AK119ub may be deposited prior to H3K27me3. In addition, the dimethyl

form of H3K27me2 can precede H3K27me3 and can silence cellular genes

independently of H3K27me3 (38). Therefore, we investigated the kinetics of H3K27me2,

H3K27me3, and H2AK119ub following the infection of primary neurons at a single-

genome resolution. Several time points were preliminarily surveyed, but here we

present the acute time point of 3 days post-infection; a time point at which we have

previously observed a steep reduction in lytic viral gene expression during latency

establishment (21). In agreement with previous *in vivo* findings, H3K27me3 was not

found to be significantly co-localized with latent viral genomes at this 3-day post-

infection time point (Fig. 3 A&B). In addition, we did not observe co-localization of

H3K27me2 with the viral genome (Fig. 3 A&B). In contrast, we found that 20% of viral

genomes were positively co-localized with H2AK119ub and the enrichment was

significantly enriched compared to the rotation control images (Fig. 3A&B). The core

PRC1 components Ring1B and Ring1A, were also significantly co-localized at the early

three-day post-infection timepoint (Fig3. C& D). These data indicate that vPRC1-

mediated H2Aub1 deposition on the viral genome occurs prior to PRC2-mediated

H3K27me3.

<u>PRC1 but not PRC2 inhibition or depletion results in a modest increased in lytic gene</u>

<u>expression during acute infection of neurons.</u>

PRC1 association with repressed HSV-1 genomes, and the kinetics of this

association, suggests PRC1 may actively silence these genomes. However, our data on

binding, co-localization, and viral gene repression are thus far only correlative in nature.

We therefore took a functional approach to investigate the contribution of PRC1 and

PRC2 to the repression of viral lytic gene expression. We infected primary neurons with

HSV-1 Stayput-GFP in the presence of PRC1 and PRC2 inhibitors. We used PTC-209

(39) and RB-3 (40), which are both Ring1 inhibitors. PTC-209 has been demonstrated

to reduce H2AK119ub on cellular DNA in neurons, although the exact mechanism of

PTC-209 is unclear. RB-3 blocks the association of both Ring1A and Ring1B with

chromatin and, therefore, the ability to deposit H2AK119ub (40). We also used the

PRC2 inhibitors UNC1999, CPI-1205 and EPZ438. We observed a subtle but

statistically significant increase in all classes of viral transcripts upon PRC1 inhibition

with both PTC-209 and RB-3 (Fig. 4A, Supplemental Figure 1A and 1B). In contrast,

the PRC2 inhibitor-treated samples did not display a statistically significant

enhancement in the expression of immediate early, early, or late genes surveyed (Fig.

4A and Supplemental Figure 1A and 1B). We verified the loss of H2AK119ub or

H3K27me3 by quantifying the genome co-localization in the presence of the PRC1

inhibitor RB-3 or the PRC2 inhibitor EPZ6438. We found that RB-3 significantly reduced

the proportion of genomes associated with H2Aub1 from 26 to 17% positive co-

localization, which is a number similar to that observed for genome co-localization by

chance (Fig. 4B and Supplemental Figure 1C).

We also produced lentiviruses expressing shRNAs targeting the core components of

PRC1, Ring1A and Ring1B. Depletion was validated by both RT-qPCR and semi-

quantitative immunofluorescence (Fig. 4E and 4F, and Supplemental Figure 1 G and

1H). Neonatal sympathetic neurons were transduced 5 days prior to infection with HSV-

1 Stayput-GFP, and viral gene expression was analyzed at 3 days post-infection. The

co-depletion of both Ring1A and Ring1B was also carried out because previous studies

have shown redundancy between the two proteins in vPRC1 activity (41). For each

class of viral lytic genes, viral gene expression was enhanced with the single

knockdown of Ring1A (via two independent shRNA-targeting lentiviruses) and dual

Ring1A/B knockdown (Fig 4D, Supplemental Figure 1 E and F). These functional data in

combination with our kinetic data suggest that PRC1 and H2AK119ub are deposited

onto the HSV-1 genome early during latency establishment to silence viral lytic genes.

We also depleted the core component of PRC2, Suz12, using one of three

independent shRNA-targeting lentiviruses and subsequently infected cultures. It has

previously been demonstrated that Suz12 is required for PRC2 activity and that its

elimination prevents its function (42, 43). Upon Suz12 depletion, as validated at the

level of RNA during each individual replicate in Fig 4G, the expression of all three

classes of viral genes analyzed did not demonstrate a robust or significant

enhancement at 3 days post-infection (Fig 4D, Supplemental Figure 1E and 1F).

Therefore, our data suggest that, in contrast to PRC1, PRC2 does not promote HSV-1

lytic gene silencing during the establishment of a latent infection.

PRC1 inhibition reduces future reactivation *in vitro.*

Only a proportion of HSV-1 genomes associated with H2AK119ub, Ring1B, and

H3K27me3 during established latency. We and others have also demonstrated that only

a proportion of individual latent genomes initiate reactivation in response to different

stimuli (21, 22, 30-32, 44). Our recent study found that genomes associated with

H3K9me3 and read by ATRX are restricted for reactivation (26). Therefore, we

hypothesized that association with H2Aub1 and/or H3K27me3 may result in genomes

more primed for reactivation.

We therefore investigated the ability of viral genomes that undergo Polycomb

silencing to reactivate by inhibiting PRC1 or PRC2 during latency establishment in the

presence of viral DNA replication inhibitor acyclovir (ACV). The use of ACV here permits

the equal establishment of latent infection despite PRC1/2 inhibitor treatment because

viral DNA copy number remains stable (Fig. 5A). Following latency establishment in the

presence of PRC1/2 inhibitors, cultures were stimulated with LY294002, and the

number of GFP-positive neurons at 48 hours post-stimulus was quantified as a readout

of neurons that have undergone the full HSV-1 reactivation. Interestingly, when PRC1

activity was inhibited during the establishment of latency, future reactivation was

robustly decreased (Fig 5B). This phenotype was recapitulated using three independent

PRC1 inhibitors. As this reduction in reactivation occurred despite equivalent latent viral

DNA copy numbers, the reduced capacity to reactivate under PRC1 inhibition was not

due to fewer viral genomes that established a latent infection. Notably, this reactivation

defect was not observed when PRC2 was inhibited during this timeframe using three

independent inhibitors. Further, reactivation was not perturbed when RB-3 was added

instead for three days at 3-days post-infection (Fig. 5C), confirming that interrupting with

Ring1 activity as the H2Aub1 modification is being depositing within the first 3 days of

infection is required to impact later reactivation.

We verified the loss of H2AK119ub or H3K27me3 by quantifying the genome co-

localization in the presence of the PRC1 inhibitor RB-3 or the PRC2 inhibitor EPZ6438.

We found that RB-3 significantly reduced the proportion of genomes associated with

H2Aub1 from 32% to 18% positive co-localization, a value similar to that expected by

chance (Fig. 5D). H3K27me3 co-localization was reduced by EPZ-6838 treatment, from

35% to 19% (Fig. 5E). We also investigated the effects of PRC1 inhibition on

H3K27me3 levels and of PRC2 inhibition on H2Aub1 levels. RB-3 treatment resulted in

a small and consistent decrease in H3K27me3 levels, from 35 to 21%. However, this

decrease was not significant (Fig. 5E). This indicates that on the viral genome, either

H2Aub1 deposition is required for H3K27me3 deposition on a subpopulation of neurons,

or that H3K27me3 deposition is independent of vPRC activity. Inhibition of PRC2 also

had a small but non-significant effect on H2Aub1 co-localization, decreasing from 32%

to 21%. However, this was not fully convincing between individual experiments (Fig.

5D). Therefore, based on this data alone, it is hard to conclude for sure the impact of

PRC2 activity on H2Aub1. Importantly, our co-localization analysis verifies both the

reduced association of h2Aub1 with the genome in the presence of the PRC1 inhibitor

and demonstrates that Polycomb silencing, specifically H2AK119ub deposition, during

the early phase of latency establishment promotes later reactivation, possibly via the

formation of more reactivation-competent latent HSV-1 genomes.

Discussion

Here, we demonstrate that PRC1-mediated H2AK119ub is enriched on the HSV-

1 genome during latency established *in vivo* and *in vitro*. The direct contribution of

heterochromatin deposition on the viral genome to promoting gene repression and

establishment of latency has not been described. Here we dissect the contributions of

PRC1 and PRC2 and find that only the loss of inhibition affects viral gene expression,

and only mildly. These data suggest that H2Aub1 only moderately represses gene

expression, or, following PRC1 inhibition, alternative forms of heterochromatin can

participate in gene silencing instead. This latter explanation is most intriguing, as we

found that although PRC1 inhibition had only mild effects on latency establishment, its

inhibition had a drastic effect on the ability of genomes to reactivate. Therefore, the type

of heterochromatin on the viral genome may play a less important role in promoting

gene silencing but does impact the ability of viral genomes to reactivate.

Interestingly, the enrichment of PRC1 component BMI1 was previously explored

on the latent HSV-1 genome in a mouse model, but not found to be significant (2, 4).

However, it has become clear over time from studies on the cellular genome that

Polycomb repressor complexes can be found in a multitude of forms (45-47). BMI1

traditionally associates with one form of PRC1 (cPRC1) that does not efficiently carry

out H2AK119ub (13, 14, 48, 49). Therefore, the enrichment of H2AK119ub but not BMI

on the HSV-1 genome is not confounding. What is unexpected is that H2AK119ub is

actively targeted to the HSV-1 genome in the specialized cell type of a post-mitotic

peripheral neuron. Studies on the cellular genome have thus far suggested that vPRC1-

mediated H2AK119ub deposition and silencing is abundant in embryonic cells, like

neuronal progenitor cells/NPCs, but decreases in relevance to cellular genome silencing

and maintenance as cells differentiate (15). Our work now demonstrates that in

response to environmental stimuli, such as a viral infection, H2AK119ub deposition is a

relevant process in post-mitotic neurons. We also confirm the kinetics of H3K27me3

enrichment previously observed *in vivo*, where the HSV-1 genome becomes enriched in

H3K27me3 at later time points post-infection after lytic genes have been repressed (2,

4).

These are also the first functional studies that directly probe the contributions of

PRC1/2 to lytic viral gene silencing during latency establishment. Importantly, we find

that H2AK119ub is targeted to the viral genome and contributes to the repression of

lytic genes during the early stages of latency establishment. Consistent with its kinetics,

PRC2 does not actively repress viral gene expression, at least not during the time

points tested here. These data, taken together, suggest a model in which PRC1 is

targeted to the viral genome to initiate viral gene repression. H3K27me3, either

dependent on PRC1 or via PRC1-independent mechanisms, is subsequently recruited

to reinforce and maintain viral gene repression. On the cellular genome, PRC2 can be

recruited in a manner that is dependent on prior H2AK119ub deposition, or

independently of H2AK119ub (50-52). This can result in different types of Polycomb

silencing that can be either enriched or depleted for H2AK119ub. We observed a similar

proportion of co-localization between H2AK119ub and H3K27me3 and the latent

genome (one-third), suggesting that these modifications bind to the same viral

genomes; however, we cannot currently rule out that they are on separate genomes.

The role of H2AK119ub in promoting H3K27me3 has predominately been studied in

embryonic stem cells and during the early stages of differentiation (51-55). The protein

that promotes PRC2 recruitment to H2AK119ub to mediate H3K27me3 addition

(JARID2) is altered in differentiated cells in that a truncated version predominates that

lacks both the H3AK119ub reader domain and PRC2 binding domain (55, 56).

Therefore, it remains unresolved whether H2AK119ub1 can directly promote

H3K27me3 in neurons and on the HSV genome, and the contribution of JARID2.

The mechanisms of PRC1, as well as PRC2 recruitment to the HSV-1 genome

remain unresolved. One potential mechanism of recruitment and/or activation of PRC1

and PRC2 to mediate deposition of histone post-translational modifications is via

binding to long noncoding RNAs (lncRNAs). Notably, during the establishment and

maintenance of HSV-1 latent infection, the virus expresses a lncRNA LAT, at least in a

proportion of neurons. The exact molecular functions of the LAT are unknown, and

while it is not essential for the establishment of a latent infection, it may modulate lytic

gene expression and promote increased association with H3K27me3 (3). Viral LAT

expression may therefore actively promote a type of gene silencing that is capable of

reactivating. In fact, the LAT has been demonstrated to enhance reactivation in several

models, further supporting this theory (57, 58). PRC1 may alternatively be recruited

using host protein KDM2B, which recruits vPRC1 to cellular genomes by binding

unmethylated GC-rich sequences (13, 59), which comprise much of the HSV-1 and

other human herpesvirus genomes (60). In fact, fellow human herpesvirus KSHV uses

its intrinsic GC-richness and KDM2B to recruit Polycomb (61) (62). PRC1 may also be

recruited to the HSV-1 genome by host transcription factors, such as RUNX1, which is

abundantly expressed in in sensory neurons and sympathetic neurons, where HSV-1

readily establishes latency. RE1-silencing transcription factor (REST, also known as

neuron-restrictive silencing factor (NRSF)) can also interact with PRC1 (and PRC2) and

maintains Polycomb binding to neuronal genes in the human teratocarcinoma NT2-D1

cells and mESCs (63). The HSV-1 genome carries predicted REST binding sites and

may repress the ability of HSV-1 to transcribe lytic viral genes and reactivate (64) (65).

PRC1-mediated silencing may be more amenable to reactivation, but the

question of how repressive H2AK119ub is modified or removed following a reactivation

stimulus remains unresolved. Accumulating evidence increasingly suggests that HSV-1

reactivation is a biphasic process. Initial viral gene expression events during Phase I are

independent of the removal of repressive heterochromatin, which is instead modified to

enable viral gene expression. Repressive H3K9me3, for example, undergoes a

phospho-methyl switch, where histone phosphorylation on a neighboring serine allows

viral gene expression to occur despite the persistence of repressive H3K9 (66). During

Phase II, repressive heterochromatin is fully removed. One model is that during Phase II

reactivation, H2AK119ub would likely be fully removed from the viral genome by de-

ubiquitinase (DUB) BRCA1-associated protein 1, BAP1. Interestingly, BAP1 has

previously been demonstrated to associate with host cell factor 1 (HCF-1), which may

also be required for the progression to the full, Phase II reactivation by interacting with

viral protein VP16 (67-70).

Materials & Methods

Reagents

Compounds used in the study are described in Table 1. Compound concentrations were

used based on previously published IC50s and assessed for neuronal toxicity using the

cell body and axon health and degeneration index as previously published (30). All

compounds had an average score less than or equal to 1.

Table 1. Compounds used and concentrations			
Compound	**Supplier**	**Identifier**	**Concentration**
NGF 2.5S	Alomone Labs	N-100	50 ng/mL
GDNF	Peprotrech	450-44	50 ng/mL
Aphidicolin (APH)	AG Scientific	A-1026	3.3 µg/ml
L-glutamic acid	Millipore Sigma	G5638	3.7 µg/ml
Acycloguanosine	Millipore Sigma	A4669	10 µM, 50 µM
Primocin	Invivogen	ant-pm-1	100 µg/ml
PRIME-XV IS21 Neuronal Supplement	Irvine Scientific	91142	1:50
Neurobasal Medium™, minus phenol red	Gibco	12348017	
BrainPhys	STEMCELL Technologies	05791	
LY294002	Tocris	1130	20-40 µM
AFDye 555 Azide	Click Chemistry	1479-1	10 µM

Plus	Tools		
EdC	Sigma-Aldrich	T511307	10 µM
PTC-209	Tocris	5191	2 µM
RB-3			30 µM
PRT4165	Sigma-Aldrich	SML1013-5MG	40 µM
UNC1999	Cayman Chemical Company	14621	1 µM
CPI-1205	Selleck Chemicals	S8353	10 µM
EED226	Cayman Chemical Company	22031	25 µM
EPZ005687	Cayman Chemical Company	13966	0.5 µM

Preparation of HSV-1 virus stocks

Stayput-GFP, as well as SCgHZ, is propagated and titrated on previously constructed

gH-complementing F6 cells, which contain copies of the gH gene under the control of

an HSV-1 gD promoter, as described in (71). Vero F6 cells were maintained in

Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% FetaPlex (Gemini

BioProducts) and 250 µg/mL of G418/Geneticin (Gibco).

HSV-1 stocks of Syn17+, and KOS (for *in vivo* experiments) were grown and titrated on

Vero cells obtained from the American Type Culture Collection (Manassas, VA). Cells

were maintained in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with

10% FetalPlex (Gemini Bio-Products) and 2 mM L-Glutamine. eGFP-Us11 Patton

(HSV-1 Patton strain with eGFP reporter protein fused to true late protein Us11 (72)

was kindly provided by Dr. Ian Mohr at New York University.

Primary neuronal cultures

Sympathetic neurons from the superior cervical ganglia (SCG) of post-natal day 0–2

(P0-P2) CD1 Mice (Charles River Laboratories) were dissected as previously described

(66). Sensory neurons from the trigeminal ganglia (TG) or adult (P21-24) CD1 Mice

(Charles River Laboratories) were dissected as previously described (73). Rodent

handling and husbandry were carried out under animal protocols approved by the

Animal Care and Use Committee of the University of Virginia (UVA). Ganglia were

briefly kept in Leibovitz's L-15 media with 2.05 mM L-Glutamine before dissociation in

Collagenase Type IV (1 mg/mL) followed by Trypsin (2.5 mg/mL) for 20 min each at 37

⬚C. Dissociated ganglia were triturated, and approximately 5-10,000 neurons per well

were plated onto rat tail collagen in a 24-well plate. Sympathetic neurons were

maintained in CM1 (Neurobasal Medium supplemented with PRIME-XV IS21 Neuronal

Supplement (Irvine Scientific), 50 ng/mL Mouse NGF 2.5S, 2 mM L-Glutamine, and

Primocin). Aphidicolin (3.3 µg/mL) was added to the CM1 for the first 5 days post-

dissection to select against proliferating cells. Sensory neurons were maintained in the

same media supplemented with GDNF (50 ng/ml; Peprotech 450-44), and more

Aphidicolin (6.6 µg/mL) was used for the first 5 days as more non-neuronal cells tend to

be dissected with this neuron type.

Establishment and reactivation of latent HSV-1 infection in primary neurons

P6-8 SCG neurons were infected with Stayput Us11-GFP at MOI 7.5 PFU/cell,

assuming 10,000 cells per well in Dulbecco's Phosphate Buffered Saline (DPBS) +

$CaCl_2$ + $MgCl_2$ supplemented with 1% fetal bovine serum and 4.5 g/L glucose for 3.5 h

at 37°C. Post infection, inoculum was replaced with feeding media (as described

above). Acyclovir (ACV) was not utilized in these infections. Lytic infection was

quantified by performing reverse transcription–quantitative PCR (RT–qPCR) of HSV-1

lytic mRNAs isolated from the cells in culture.

Neonatal SCGs were infected at postnatal days 6-8 with either Us11-GFP, SCgHZ, or

Stayput-GFP at an MOI of 7.5 PFU/cell assuming 10,000 cells/well in DPBS +CaCl2

+MgCl2 supplemented with 1% Fetal Bovine Serum, 4.5 g/L glucose, and either with or

without 10 µM Acyclovir (ACV) for 3 hr at 37 C. Post infection, inoculum was replaced

with feeding media with or without 50 µM ACV. Reactivation was carried out in

BrainPhys (Stem Cell Technologies) supplemented with 2 mM L-Glutamine, 10% Fetal

Bovine Serum, Mouse NGF 2.5S (50 ng/mL) and Primocin. Reactivation was quantified

by counting number of GFP-positive neurons or performing Reverse Transcription

Quantitative PCR (RT-qPCR) for HSV-1 lytic transcripts.

Mouse Infections

Five-week-old male and female CD-1 mice (Charles River Laboratories) were

anesthetized by intraperitoneal injection of ketamine hydrochloride (80 mg/kg of body

weight) and xylazine hydrochloride (10 mg/kg) and inoculated with 1.5×10^6 PFU of

HSV-1 WT KOS/eye of virus (in a 5 µL volume) onto scarified corneas, as described

previously (2). Mice were housed in accordance with institutional and National Institutes

of Health guidelines on the care and use of animals in research, and all procedures

were approved by the Institutional Animal Care and Use Committee of the University of

Virginia. Criteria used for clinical scoring based on the formation of lesions and

neurological and eye symptoms are shown in Table 2 and were based on a previously

established scoring scale (74). Mice were randomly assigned to groups, and all

experiments included biological replicates from independent litters.

Table 2. Clinical scoring scale for HSV-infected mice.			
Score	Description of score by type		
	Lesion	Eye	Neurological
0	None	No symptoms	No symptoms
1	Small area of broken skin (<0.5cm)	Pus around edges	Hunched, normal movement
2	Area of broken skin 0.5-1cm	Pus and squint	Hunched, slow movement
3	Bleeding, scabbing, or pustules	Closed	Hunched, labored breathing and/or hind-leg paralysis
4	Broken skin >1cm with multiple pustules or scabbing	Scab formation	Hunched, ruffled fur, little/no movement
5	Severe scabbing or bleeding	Severe scabbing	Moribund or dead

	with pustules		

Western Blotting

Neurons were lysed in RIPA Buffer with cOmplete, Mini, EDTA-Free Protease Inhibitor

Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche) on ice for 2

hours with regular vortexing to aid lysis. Insoluble proteins were removed via

centrifugation, and lysate protein concentration was determined using the Pierce

Bicinchoninic Acid Protein Assay Kit (Invitrogen) using a standard curve created with

BSA standards of known concentration. Equal quantities of protein (15–50 μg) were

resolved on 4–20% gradient SDS-Polyacrylamide gels (Bio-Rad) and then transferred

onto Polyvinylidene difluoride membranes (Millipore Sigma). Membranes were blocked

in PVDF Blocking Reagent for Can Get Signal (Toyobo) for 1 hr. Primary antibodies

were diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo) and

membranes were incubated overnight at 4˚C. HRP-labeled secondary antibodies were

diluted in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo) and

membranes were incubated for 1 hr at room temperature. Antibody usage is recorded in

Table 4. Blots were developed using Western Lightning Plus-ECL Enhanced

Chemiluminescence Substrate (PerkinElmer) and ProSignal ECL Blotting Film

(Prometheus Protein Biology Products) according to manufacturer's instructions. Blots

were stripped for reblotting using NewBlot PVDF Stripping Buffer (Licor). Band density

was quantified in ImageJ.

Preparation of lentiviral vectors

Lentiviruses expressing shRNA against Ring1A (Ring1A-1 = TRCN0000040561

Ring1A-2 = TRCN0000040562), Ring1B (Ring1B-1 = TRCN0000226018, Ring1B-2=

TRCN0000226019), SUZ12 (Suz12-1 = TRCN0000234040, Suz12-2 =

TRCN0000218848, Suz12-3 =TRCN0000123893) or a control lentivirus shRNA (empty

pLKO.1 or pLKO.5 vector) were prepared by co-transfection with psPAX2 and pCMV-

VSV-G (75) using the 293LTV packaging cell line (Cell Biolabs). Supernatant was

harvested at 40- and 64-h post-transfection and filtered using a 45 µM PES filter.

Sympathetic neurons were transduced overnight in neuronal media containing 8 µg/ml

protamine sulfate and 50 µM ACV.

CUT&RUN Sequencing

A minimum of approximately 120,000 neurons were plated for each CUT&RUN

reaction. Neurons were collected following 10-minute incubation with trypsin at 37 □C

without fixation. Neurons were permeabilized with 0.01% digitonin (concentration

optimized for neurons) and CUT&RUN was performed using reagents included in the

CUT&RUN Kit (EpiCypher) according to attached protocol. Periodic quality checks as

recommended and detailed by protocol Appendix were performed and passed for

neurons. CUT&RUN libraries were prepared using CUT&RUN Library Prep Kit

(EpiCypher) and subject to pair-ended partial-lane sequencing and de-multiplexing

using NovaSeq (Novogene). Data analysis was performed using command line and R

code, and workflow, adapted from tutorial on IO protocols (Zheng Y et al (2020)

Protocol.io). The Rivanna high-performance computing environment (UVA Research

Computing) was used for command line data processing. The HSV-1 SC16 genome

sequence (NCBI NC_KX946970.1) was used in combination with the mm39 mouse

genome assembly (RefSeq NC_000071.7) to make a joint Bowtie2 index genome.

Sequence alignment was performed with bowtie2 with the following settings*: --end-to-*

end --very-sensitive --no-mixed --no-discordant --phred33 -I 10 -X 700. Separate

alignments was performed to spike-in *E. Coli* DNA (MG1655, Genbank U00096.3), from

which sequencing depth was calculated and reads normalized accordingly before

filtering into separate human and viral bedgaph files. Data quality control and

visualization were performed using R. Two experimental replicates were performed.

Click Chemistry

For EdC-labeled HSV virus infections, an MOI of 7.5 was used. EdC labelled virus was

prepared using a previously described method (76). Click chemistry was carried out as

described previously (77) with some modifications. Neurons were washed with CSK

buffer (10 mM HEPES, 100 mM NaCl, 300 mM Sucrose, 3 mM MgCl2, 5 mM EGTA)

and simultaneously fixed and permeabilized for 10 minutes in 1.8% methanol-free

formaldehyde (0.5% Triton X-100, 1% phenylmethylsulfonyl fluoride (PMSF)) in CSK

buffer, then washed twice with PBS before continuing to the click chemistry reaction and

immunostaining. Samples were blocked with 3% BSA for 30 minutes, followed by click

chemistry using EdC-labelled HSV DNA and the Click-iT EdU Alexa Flour 555 Imaging

Kit (ThermoFisher Scientific, C10638) according to the manufacturer's instructions. For

immunostaining, samples were incubated overnight with primary antibodies in 3% BSA.

Following primary antibody treatment, neurons were incubated for one hour in Alexa

Fluor® 488-, 555-, and 647-conjugated secondary antibodies for multi-color imaging

(Invitrogen). Nuclei were stained with Hoechst 33258 (Life Technologies). Images were

acquired at 60x using an sCMOS charge-coupled device camera (pco.edge) mounted

on a Nikon Eclipse Ti Inverted Epifluorescent microscope using NIS-Elements software

(Nikon). Images were analyzed and intensity quantified using ImageJ.

Immunofluorescence

Neurons were fixed for 15 min in 4% Formaldehyde and blocked in 5% Bovine Serum

Albumin and 0.3% Triton X-100 and incubated overnight in primary antibody. Following

primary antibody treatment, neurons were incubated for 1 hr in Alexa Fluor 488-, 555-,

and 647-conjugated secondary antibodies for multi-color imaging (Invitrogen). Nuclei

were stained with Hoechst 33258 (Life Technologies). Images were acquired using an

sCMOS charge-coupled device camera (pco.edge) mounted on a Nikon Eclipse Ti

Inverted Epifluorescent microscope using NIS-Elements software (Nikon). Images were

analyzed using ImageJ.

Table 3. Antibodies

Designation	Source or reference	Identifiers	Additional Information
Anti-a-tubulin (Mouse monoclonal)	Millipore sigma	Cat #T9026	WB (1:2500)
Anti-Rabbit IgG Antibody (H+L), Peroxidase (Goat polyclonal)	Vector Labs	Cat #PI-1000	WB (1:10,000)
Anti-mouse IgG Antibody (H+L), Peroxidase (Horse polyclonal	Vector Labs	Cat #PI-2000	WB (1:10,000)
Beta III Tubulin	EMD Millipore	Cat #9354	IF (1:500)
IgG	EMD Millipore	12-370	CUT&RUN ChIP-qPCR
H2A	Cell Signaling Technology	12349S	ChIP-qPCR
H2AK119ub	Cell Signaling Technology	8240S	ChIP (1:100) CUT&RUN IF (1:1600)
H3K27me3	Cell Signaling Technology	Cat #9733	CUT&RUN ChIP 1:50 IF (1:750)
H3K27me2	Active Motif	Cat #39245	IF 1:500
Ring1B	Cell Signaling Technology	5694S	IF, WB
ICP4	Abcam	ab6514	IF (1:850)
ICP5	Abcam	ab6508	IF
ICP8	Abcam	ab20194	IF
H3	Cell Signaling Technology		WB
Alpha-tubulin	Cell Signaling Technology		WB

Analysis of mRNA expression and viral genome copy number

To assess expression of HSV-1 lytic mRNA, total RNA was extracted from approximately 5,000 neurons using the Quick-RNA Miniprep Kit (Zymo Research) with an on-column DNase I digestion. mRNA was converted to cDNA using the Maxima cDNA synthesis kit (Thermo Fisher) using random hexamers for first-strand synthesis and equal amounts of RNA (20–30 ng/ reaction). To assess viral DNA load, total DNA was extracted from approximately 5,000 neurons using the Quick-DNA Miniprep Plus Kit (Zymo Research). qPCR was carried out using PowerUp™ SYBR Green Master Mix (Thermoscientific). Relative mRNA/DNA copy numbers were determined using the Comparative CT (ΔΔCT) method normalized to mRNA levels in latently infected samples. Viral RNAs were normalized to mouse reference gene 18s ribosomal RNA. All samples were run in duplicate on an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System and the mean fold change compared to the reference gene calculated. Exact copy numbers were determined by comparison to standard curves of known DNA copy number of viral genomes or plasmid containing 18S rRNA (p18S.1 tag was a gift from Vincent Mauro; Addgene plasmid # 51729 ; http://n2t.net/addgene:51729 ; RRID:Addgene_51729, (78).

Table 4. Sequences of Primers Used	
Primer	**Sequence 5' to 3'**
mGAPDH 1SF	CAT GGC CTT CCG TGT GTT CCT A
mGAPDH 1SR	GCG GCA CGT CAG ATC CA
ICP27 F	GCA TCC TTC GTG TTT GTC ATT CTG
ICP27 R	GCA TCT TCT CTC CGA CCC CG
ICP8 1SF	GGA GGT GCA CCG CAT ACC
ICP8 1SR	GGC TAA AAT CCG GCA TGA AC
gC F	GAG TTT GTC TGG TTC GAG GAC
gC R	ACG GTA GAG ACT GTG GTG AA
VP16 F	GGA CCG GAC GGA CCT TAT
VP16 R	GGT TGC TTA AAT GCG TGG TG
LAT F	TGT GTG GTG CCC GTG TCT T
LAT R	CCA GCC AAT CCG TGT CGG
UL30 F	CGC GCT GGC GGG TA TTA ACA T
UL30 R	TGG GTG TCC GGC AGA ATA AAG
Ring1A F (Set 2)	CAAGAGGACAAAGGAGGACAAA
Ring1A R (Set 2)	CAGGGTAGGAAACTAGGGTCTAT
Ring1B F	CAAGTATCTGGCTGTGAGGTTAG

(Set 1)	
Ring1B R (Set 1)	GACAGCGCACAGACAAGATA

Statistical Analysis

631   All statistical analyses were performed using Prism V10. The normality test and

632   statistical test used for each figure are denoted in the figure legends. Individual

633   biological replicates are plotted, each corresponding to a single well of cells/neurons. All

634   neuronal experiments were repeated from pooled neurons from at least 3 litters.

References

References

1.   R. F. Itzhaki, Herpes simplex virus type 1 and Alzheimer's disease: increasing evidence
for a major role of the virus. *Front Aging Neurosci* **6**, 202 (2014).

2.   A. R. Cliffe, D. M. Coen, D. M. Knipe, Kinetics of facultative heterochromatin and
polycomb group protein association with the herpes simplex viral genome during
establishment of latent infection. *mBio* **4** (2013).

3.   A. R. Cliffe, D. A. Garber, D. M. Knipe, Transcription of the herpes simplex virus latency-
associated transcript promotes the formation of facultative heterochromatin on lytic
promoters. *J Virol* **83**, 8182-8190 (2009).

4.   D. L. Kwiatkowski, H. W. Thompson, D. C. Bloom, The polycomb group protein Bmi1
binds to the herpes simplex virus 1 latent genome and maintains repressive histone
marks during latency. *J Virol* **83**, 8173-8181 (2009).

5.   M. P. Nicoll *et al.*, The HSV-1 Latency-Associated Transcript Functions to Repress Latent
Phase Lytic Gene Expression and Suppress Virus Reactivation from Latently Infected
Neurons. *PLoS Pathog* **12**, e1005539 (2016).

6.   R. Cao *et al.*, Role of histone H3 lysine 27 methylation in Polycomb-group silencing.
Science **298**, 1039-1043 (2002).

7.   P. Trojer, D. Reinberg, Facultative heterochromatin: is there a distinctive molecular
signature? *Mol Cell* **28**, 1-13 (2007).

8.   R. Cao, Y. Tsukada, Y. Zhang, Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox
gene silencing. *Mol Cell* **20**, 845-854 (2005).

9.   M. de Napoles *et al.*, Polycomb group proteins Ring1A/B link ubiquitylation of histone
H2A to heritable gene silencing and X inactivation. *Dev Cell* **7**, 663-676 (2004).

10.   H. Wang *et al.*, Role of histone H2A ubiquitination in Polycomb silencing. *Nature* **431**,
873-878 (2004).

11.   N. P. Blackledge *et al.*, PRC1 Catalytic Activity Is Central to Polycomb System Function.
Mol Cell **77**, 857-874.e859 (2020).

12.   S. Tamburri *et al.*, Histone H2AK119 Mono-Ubiquitination Is Essential for Polycomb-
Mediated Transcriptional Repression. *Mol Cell* **77**, 840-856 e845 (2020).

13.   N. P. Blackledge *et al.*, Variant PRC1 complex-dependent H2A ubiquitylation drives PRC2
recruitment and polycomb domain formation. *Cell* **157**, 1445-1459 (2014).

14.   L. Tavares *et al.*, RYBP-PRC1 complexes mediate H2A ubiquitylation at polycomb target
sites independently of PRC2 and H3K27me3. *Cell* **148**, 664-678 (2012).

15.   M. Tsuboi *et al.*, Ubiquitination-Independent Repression of PRC1 Targets during
Neuronal Fate Restriction in the Developing Mouse Neocortex. *Dev Cell* **47**, 758-772
e755 (2018).

16.   N. P. Blackledge, R. J. Klose, The molecular principles of gene regulation by Polycomb
repressive complexes. *Nat Rev Mol Cell Biol* **22**, 815-833 (2021).

17.   J. J. Kim, R. E. Kingston, Context-specific Polycomb mechanisms in development. *Nat Rev*
Genet 10.1038/s41576-022-00499-0 (2022).

18.   A. F. Lentine, S. L. Bachenheimer, Intracellular organization of herpes simplex virus type
1 DNA assayed by staphylococcal nuclease sensitivity. *Virus Res* **16**, 275-292 (1990).

19.   P. F. Pignatti, E. Cassai, Analysis of herpes simplex virus nucleoprotein complexes
extracted from infected cells. *J Virol* **36**, 816-828 (1980).

20. F. Catez *et al.*, HSV-1 genome subnuclear positioning and associations with host-cell
PML-NBs and centromeres regulate LAT locus transcription during latency in neurons.
PLoS Pathog **8**, e1002852 (2012).
21. S. Dochnal *et al.*, DLK-Dependent Biphasic Reactivation of Herpes Simplex Virus Latency
Established in the Absence of Antivirals. *J Virol* **96**, e0050822 (2022).
22. J. B. Suzich *et al.*, PML-NB-dependent type I interferon memory results in a restricted
form of HSV latency. *EMBO Rep* **22**, e52547 (2021).
23. N. M. Sawtell, R. L. Thompson, Comparison of herpes simplex virus reactivation in
ganglia in vivo and in explants demonstrates quantitative and qualitative differences. *J*
Virol **78**, 7784-7794 (2004).
24. S. R. Cuddy *et al.*, Neuronal hyperexcitability is a DLK-dependent trigger of herpes
simplex virus reactivation that can be induced by IL-1. *Elife* **9** (2020).
25. J. Y. Kim, A. Mandarino, M. V. Chao, I. Mohr, A. C. Wilson, Transient reversal of episome
silencing precedes VP16-dependent transcription during reactivation of latent HSV-1 in
neurons. *PLoS Pathog* **8**, e1002540 (2012).
26. A. L. Whitford *et al.*, Interferon Dependent Immune Memory during HSV-1 Neuronal
Latency Results in Increased H3K9me3 and Restriction of Reactivation by ATRX. *bioRxiv*
10.1101/2025.01.28.633013, 2025.2001.2028.633013 (2025).
27. S. A. Dochnal, A. K. Francois, A. R. Cliffe, De Novo Polycomb Recruitment: Lessons from
Latent Herpesviruses. *Viruses* **13** (2021).
28. L. Condemi, I. Mocavini, S. Aranda, L. Di Croce, Polycomb function in early mouse
development. *Cell Death Differ* **32**, 90-99 (2025).
29. J. T. Proença, H. M. Coleman, V. Connor, D. J. Winton, S. Efstathiou, A historical analysis
of herpes simplex virus promoter activation in vivo reveals distinct populations of
latently infected neurones. *J Gen Virol* **89**, 2965-2974 (2008).
30. S. R. Cuddy *et al.*, Neuronal hyperexcitability is a DLK-dependent trigger of herpes
simplex virus reactivation that can be induced by IL-1. *Elife* **9**, e58037 (2020).
31. H. L. Hu *et al.*, Single-cell transcriptomics identifies Gadd45b as a regulator of
herpesvirus-reactivating neurons. *EMBO Rep* **23**, e53543 (2022).
32. R. L. Thompson, C. M. Preston, N. M. Sawtell, De novo synthesis of VP16 coordinates the
exit from HSV latency in vivo. *PLoS Pathog* **5**, e1000352 (2009).
33. A. K. Francois *et al.*, Single-genome analysis reveals a heterogeneous association of the
herpes simplex virus genome with H3K27me2 and the reader PHF20L1 following
infection of human fibroblasts. *mBio* **15**, e0327823 (2024).
34. W. Zhou *et al.*, Histone H2A monoubiquitination represses transcription by inhibiting
RNA polymerase II transcriptional elongation. *Mol Cell* **29**, 69-80 (2008).
35. J. K. Stock *et al.*, Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase
II at bivalent genes in mouse ES cells. *Nat Cell Biol* **9**, 1428-1435 (2007).
36. L. Di Croce, K. Helin, Transcriptional regulation by Polycomb group proteins. *Nat Struct*
Mol Biol **20**, 1147-1155 (2013).
37. N. P. Blackledge *et al.*, PRC1 Catalytic Activity Is Central to Polycomb System Function.
Mol Cell **77**, 857-874 e859 (2020).
38. Y. Hou *et al.*, PHF20L1 as a H3K27me2 reader coordinates with transcriptional
repressors to promote breast tumorigenesis. *Sci Adv* **6**, eaaz0356 (2020).

39. A. Kreso *et al.*, Self-renewal as a therapeutic target in human colorectal cancer. *Nat Med*
20, 29-36 (2014).
40. S. Shukla *et al.*, Small-molecule inhibitors targeting Polycomb repressive complex 1 RING
domain. *Nat Chem Biol* **17**, 784-793 (2021).
41. Y. Zhu *et al.*, Functional redundancy among Polycomb complexes in maintaining the
pluripotent state of embryonic stem cells. *Stem Cell Reports* **17**, 1198-1214 (2022).
42. D. Pasini, A. P. Bracken, M. R. Jensen, E. Lazzerini Denchi, K. Helin, Suz12 is essential for
mouse development and for EZH2 histone methyltransferase activity. *EMBO J* **23**, 4061-
4071 (2004).
43. R. Cao, Y. Zhang, SUZ12 is required for both the histone methyltransferase activity and
the silencing function of the EED-EZH2 complex. *Mol Cell* **15**, 57-67 (2004).
44. J. B. Suzich, A. R. Cliffe, Strength in diversity: Understanding the pathways to herpes
simplex virus reactivation. *Virology* **522**, 81-91 (2018).
45. A. Piunti, A. Shilatifard, The roles of Polycomb repressive complexes in mammalian
development and cancer. *Nat Rev Mol Cell Biol* **22**, 326-345 (2021).
46. K. E. Connelly, E. C. Dykhuizen, Compositional and functional diversity of canonical PRC1
complexes in mammals. *Biochim Biophys Acta Gene Regul Mech* **1860**, 233-245 (2017).
47. Z. Gao *et al.*, PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1
family complexes. *Mol Cell* **45**, 344-356 (2012).
48. A. Lagarou *et al.*, dKDM2 couples histone H2A ubiquitylation to histone H3
demethylation during Polycomb group silencing. *Genes Dev* **22**, 2799-2810 (2008).
49. H. F. Moussa *et al.*, Canonical PRC1 controls sequence-independent propagation of
Polycomb-mediated gene silencing. *Nat Commun* **10**, 1931 (2019).
50. G. van Mierlo, G. J. C. Veenstra, M. Vermeulen, H. Marks, The Complexity of PRC2
Subcomplexes. *Trends Cell Biol* **29**, 660-671 (2019).
51. S. Cooper *et al.*, Jarid2 binds mono-ubiquitylated H2A lysine 119 to mediate crosstalk
between Polycomb complexes PRC1 and PRC2. *Nat Commun* **7**, 13661 (2016).
52. V. Kasinath *et al.*, JARID2 and AEBP2 regulate PRC2 in the presence of H2AK119ub1 and
other histone modifications. *Science* **371** (2021).
53. S. Sanulli *et al.*, Jarid2 Methylation via the PRC2 Complex Regulates H3K27me3
Deposition during Cell Differentiation. *Mol Cell* **57**, 769-783 (2015).
54. J. Son, S. S. Shen, R. Margueron, D. Reinberg, Nucleosome-binding activities within
JARID2 and EZH1 regulate the function of PRC2 on chromatin. *Genes Dev* **27**, 2663-2677
(2013).
55. D. Al-Raawi *et al.*, A novel form of JARID2 is required for differentiation in lineage-
committed cells. *EMBO J* **38** (2019).
56. E. Glancy *et al.*, PRC2.1- and PRC2.2-specific accessory proteins drive recruitment of
different forms of canonical PRC1. *Mol Cell* **83**, 1393-1411 e1397 (2023).
57. Z. L. Watson *et al.*, In Vivo Knockdown of the Herpes Simplex Virus 1 Latency-Associated
Transcript Reduces Reactivation from Latency. *J Virol* **92** (2018).
58. D. A. Leib *et al.*, A deletion mutant of the latency-associated transcript of herpes simplex
virus type 1 reactivates from the latent state with reduced frequency. *J Virol* **63**, 2893-
2900 (1989).

59. A. M. Farcas *et al.*, KDM2B links the Polycomb Repressive Complex 1 (PRC1) to
recognition of CpG islands. *Elife* **1**, e00205 (2012).
60. J. C. Brown, High G+C Content of Herpes Simplex Virus DNA: Proposed Role in Protection
Against Retrotransposon Insertion. *Open Biochem J* **1**, 33-42 (2007).
61. T. Gunther *et al.*, A comparative epigenome analysis of gammaherpesviruses suggests
cis-acting sequence features as critical mediators of rapid polycomb recruitment. *PLoS*
Pathog **15**, e1007838 (2019).
62. N. G. Naik *et al.*, Epigenetic factor siRNA screen during primary KSHV infection identifies
novel host restriction factors for the lytic cycle of KSHV. *PLoS Pathog* **16**, e1008268
(2020).
63. N. Dietrich *et al.*, REST-mediated recruitment of polycomb repressor complexes in
mammalian cells. *PLoS Genet* **8**, e1002494 (2012).
64. T. Du, G. Zhou, S. Khan, H. Gu, B. Roizman, Disruption of HDAC/CoREST/REST repressor
by dnREST reduces genome silencing and increases virulence of herpes simplex virus.
Proc Natl Acad Sci U S A **107**, 15904-15909 (2010).
65. G. Zhou, T. Du, B. Roizman, HSV carrying WT REST establishes latency but reactivates
only if the synthesis of REST is suppressed. *Proc Natl Acad Sci U S A* **110**, E498-506
(2013).
66. A. R. Cliffe *et al.*, Neuronal Stress Pathway Mediating a Histone Methyl/Phospho Switch
Is Required for Herpes Simplex Virus Reactivation. *Cell Host Microbe* **18**, 649-658 (2015).
67. Y. J. Machida, Y. Machida, A. A. Vashisht, J. A. Wohlschlegel, A. Dutta, The
deubiquitinating enzyme BAP1 regulates cell growth via interaction with HCF-1. *J Biol*
Chem **284**, 34179-34188 (2009).
68. M. E. Sowa, E. J. Bennett, S. P. Gygi, J. W. Harper, Defining the human deubiquitinating
enzyme interaction landscape. *Cell* **138**, 389-403 (2009).
69. S. Misaghi *et al.*, Association of C-terminal ubiquitin hydrolase BRCA1-associated protein
1 with cell cycle regulator host cell factor 1. *Mol Cell Biol* **29**, 2181-2192 (2009).
70. H. Yu *et al.*, The ubiquitin carboxyl hydrolase BAP1 forms a ternary complex with YY1
and HCF-1 and is a critical regulator of gene expression. *Mol Cell Biol* **30**, 5071-5085
(2010).
71. A. Forrester *et al.*, Construction and properties of a mutant of herpes simplex virus type
1 with glycoprotein H coding sequences deleted. *J Virol* **66**, 341-348 (1992).
72. L. Benboudjema, M. Mulvey, Y. Gao, S. W. Pimplikar, I. Mohr, Association of the herpes
simplex virus type 1 Us11 gene product with the cellular kinesin light-chain-related
protein PAT1 results in the redistribution of both polypeptides. *J Virol* **77**, 9192-9203
(2003).
73. S. A. Malin, B. M. Davis, D. C. Molliver, Production of dissociated sensory neuron
cultures and considerations for their use in studying neuronal function and plasticity.
Nat Protoc **2**, 152-160 (2007).
74. R. E. Riccio, S. J. Park, R. Longnecker, S. J. Kopp, Characterization of Sex Differences in
Ocular Herpes Simplex Virus 1 Infection and Herpes Stromal Keratitis Pathogenesis of
Wild-Type and Herpesvirus Entry Mediator Knockout Mice. *mSphere* **4** (2019).
75. S. A. Stewart *et al.*, Lentivirus-delivered stable gene silencing by RNAi in primary cells.
RNA **9**, 493-501 (2003).

76.   S. McFarlane *et al.*, The histone chaperone HIRA promotes the induction of host innate
immune defences in response to HSV-1 infection. *PLoS Pathog* **15**, e1007667 (2019).
77.   T. Alandijany *et al.*, Distinct temporal roles for the promyelocytic leukaemia (PML)
protein in the sequential regulation of intracellular host immunity to HSV-1 infection.
PLoS Pathog **14**, e1006769 (2018).
78.   L. G. Burman, V. P. Mauro, Analysis of rRNA processing and translation in mammalian
cells using a synthetic 18S rRNA expression system. *Nucleic Acids Res* **40**, 8085-8098
(2012).
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Figure 1.
<u>H2Aub1 associates with the latent HSV-1 genome.</u>
A) Schematic of in vivo experiments with HSV-1.
B) Mice were infected with WT HSV-1, and latent trigeminal ganglia (TG) were collected 30 days
post-infection. Chromatin immunoprecipitation (ChIP) was performed using an IgG control or
H2AK119ub, and quantitative PCR (qPCR) was performed to analyze binding to select
promoters of the immediate-early viral gene ICP27 and the early viral gene ICP8. Enrichment
over Input determined. 3 biological replicates; Welch's t-test. Normality determined by Shapiro-
Wilk test. Means and SEMs are represented. *, $P < 0.05$; **, $P < 0.01$. ns, not significant.
C) Schematic of infection of primary neurons with HSV-1
D) Primary neurons were infected with HSV-1 Stayput-GFP in the presence of viral DNA
replication inhibitor acyclovir/ACV (10 µM during inoculation period and 50 µM following
removal of inoculum). CUT&RUN was performed on neurons collected and permeabilized 30
days post-infection using IgG control (blue), H2AK119ub (orange), or H3K27me3 (green). Raw
reads from merged experimental replicates aligned across the HSV-1 genome.
E) Zoomed in region as marked.
A and C were Created in BioRender. Cliffe, A. (2025) https://BioRender.com/jdycuyb and Cliffe,
A. (2025) https://BioRender.com/ta6o69j
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Figure 2
<u>H2Aub1, H3K27me3, and Polycomb Proteins co-localize with a sub-population of HSV-1</u>
<u>genomes during latency.</u>
Primary neurons were infected with EdC-labeled HSV-1 in the presence of ACV. 30 days post-
infection, latently infected neurons were fixed and stained for Polycomb-associated histone
modifications H3K27me3, H3K27me2, or H2AK119ub (A & B) and Polycomb repressor complex
1 (PRC1) components Ring1A and Ring1B, and PRC2 core component Suz12 (C & D). The HSV-1
genome was visualized using Click Chemistry. Enrichment values of chromatin
mark/reader/writer with each individual HSV-1 genome analyzed using NucSpotA are shown. B
and D) Signal intensity of histone modifications (B) and Polycomb proteins (D) at the viral
genome at 30-days post-infection. Each data point represents one viral genome. Percentages
indicate the proportion of genomes with NucSpotA intensity ratios above the denoted co-
localization threshold (dashed line). Paired comparisons were performed for each image by
rotation of the genome channel 90 degrees to achieve random placement. Threshold N>75
cells from 3 biological replicates. Wilcoxon test. Normality determined by Kolmogorov-Smirnov
test. *, $P < 0.05$; **, $P < 0.01$. ns, not significant.
857
Figure 3.
<u>Single-viral-genome analysis demonstrates that HSV-1 genomes associate with PRC1-mediated</u>
<u>H2AK119ub during active viral gene silencing.</u>
A and B) Primary neurons were infected with EdC-labeled HSV-1 in the presence of ACV. 3 days
post-infection, neurons were fixed and stained for histone modifications H3K27me3,
H3K27me2 and H2AK119ub (A & B), and PRC1 core components Ring1A, Ring1B and PRC2 core
component Suz12 (C & D). The HSV-1 genome was visualized using Click Chemistry. Enrichment
values of chromatin mark/reader/writer with each individual HSV-1 genome are shown. 10 µm

scale, 1 μm scale for zoom. Signal intensity of histone modifications (B) and Polycomb proteins
(D) at the viral genome at 30-days post-infection. Each data point represents one viral genome.
Percentages indicate the proportion of genomes with NucSpotA intensity ratios above the
denoted co-localization threshold (dashed line). Paired comparisons were performed for each
image by rotation of the genome channel 90 degrees to achieve random placement. Threshold
N>75 cells from 3 biological replicates. Wilcoxon test. Normality determined by Kolmogorov-
Smirnov test. *, P□<□0.05; **, P□<□0.01. ns, not significant.

<u>Figure 4</u>
<u>PRC1 but not PRC2 mildly promotes HSV-1 lytic gene silencing during the establishment of</u>
<u>latent infection.</u>
(A-C) Primary neurons were treated with PRC1 inhibitors PTC-209, RB-3, UNC1999, CPI-12-5 or
EPZ6438 pre-infection for 1 hour, during inoculation with HSV-1 Stayput-GFP in the absence of
ACV and following the removal of inoculum for 3 days post-infection. Expression of lytic viral
immediate early gene ICP27 (A), 3 biological replicates, Wilcoxon Test.
B) Quantification of genome co-localization with H2Aub1 following RB-3 treatment.
C) Quantification of genome co-localization with H3K27me3 following EPZ6438 treatment.
D-G) Neonatal sympathetic neurons were transduced with a non-targeting shRNA control
lentivirus or one of two independent lentiviruses expressing shRNAs that target Ring1A (sh-
Ring1A 61, sh-Ring1 62) or Ring1B (sh-Ring1 18, sh-Ring1 290), or both Ring1A and Ring1B (sh-
Ring1 62 and sh-Ring1 180), or Suz12 (sh-Suz12 211, sh-Suz1 404). Following depletion, primary
neurons were infected with Stayput-GFP at a multiplicity of infection (MOI) of 5 in the absence
of ACV. (D-G) Relative viral expression at 3 days post-infection compared to shRNA control
quantified by RT-qPCR for viral genes ICP27 (D), and cellular Ring1A (E), Ring1B (F) or Suz12 (G)
normalized to cellular control mGAPDH. 3 biological replicates, Wilcoxon Test. Normality
determined by Kolmogorov-Smirnov test. *, P□<□0.05; **, P□<□0.01. ns, not significant.

Figure 5
<u>PRC1 activity during latency establishment is required to establish a reactivation-competent</u>
<u>HSV-1 latent infection.</u>
Primary neurons were treated with a vehicle control, or with inhibitors of PRC1 (warm colors;
PRT4165 40 μM, PTC-209 2 μM, RB-3 30 μM), or PRC2 (cool colors; UNC1999 1 μM, CPI-1205 10
μM, 1 μM EPZ6438) pre-infection for 1 hour, during inoculation with Stayput-GFP in the
presence of ACV, and following the removal of inoculum. 6 days following infection, ACV and
respective inhibitors were removed from cultures, and spontaneous reactivation, as indicated
by GFP-positive neurons, was monitored for two days. Latently infected cultures were
reactivated with LY294002 and GFP-positive neurons indicative of full reactivation events were
monitored over time. (A) Latent viral DNA copy number normalized to vehicle controls. (B) The
number of GFP-positive neurons at 48 hours post-stimulus presented as a fraction of vehicle
control reactivation output. 3 biological replicates; Wilcoxon test against respective controls.
Normality determined by Kolmogorov-Smirnov test. *, P□<□0.05; **, P□<□0.01. ns, not
significant.
D) Quantification of genome co-localization with H2Aub1 following RB-3 and EPZ6438
treatment

E). Quantification of genome co-localization with H3K27me3 following RB-3 and EPZ6438
treatment.

Figure 1.

A.

HSV EdC	H3K27me2	Merge	Zoom	0.96
HSV EdC	H3K27me3	Merge	Zoom	1.55
HSV EdC	H2AK119ub	Merge	Zoom	2.66

C.

HSV EdC	RING1A	Merge	Zoom	9.24
HSV EdC	RING1B	Merge	Zoom	6.51
HSV EdC	SUZ12	Merge	Zoom	3.59

B. Polycomb Modifications Colocalization
30 D.P.I

Relative Signal at Viral Genome

17.6% 27.4%   33.8% 11.3%   30.7% 14.5%

1.5

H3K27me2   H3K27me3   H2AK119ub1

Original / Rotated

ns   ****   **

D. Polycomb Readers/Writers Colocalization
30 D.P.I

Relative Signal at Viral Genome

48.1% 5.6%   20.4% 5.6%   37.5% 4.7%

1.8

Ring1A   Ring1B   Suz12

Original / Rotated

****   **   ****

Figure 2.

Figure 3.

Figure 4

Figure 5.