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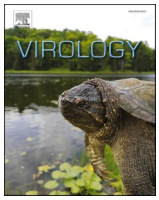

Invited Review

Mechanisms of HSV gene regulation during latency and reactivation

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ABSTRACT

Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) are prevalent human pathogens associated with many diseases. After productive (lytic) infection in peripheral tissues, HSV establishes lifelong latent infection in neurons of the peripheral nervous system. Periodic reactivation from latency, triggered by certain stimuli, can resume the lytic cycle. Lytic infection, latent infection and reactivation follow distinct viral gene expression patterns. The switch between the different infection programs is controlled by complicated regulatory mechanisms involving numerous viral and host molecules. Recent studies integrating cutting-edge technologies including neuronal culture techniques have greatly improved our understanding of the molecular details of latency and reactivation but many questions remain. This review summarizes the current knowledge about how HSV gene expression is regulated during latency and reactivation and discusses the important questions remaining to be addressed in future.

1. Introduction

Herpes simplex virus (HSV), including HSV-1 and HSV-2, is a double-stranded enveloped DNA virus with a tegument layer between the envelope and capsid. HSV-1 and HSV-2 are ubiquitous human pathogens with global seroprevalence of ~70% and ~10%, respectively (Knipe et al., 2021). HSV-1 can cause common cold sores and herpetic keratitis as well as rare but deadly encephalitis, HSV-2 can cause genital herpes, and both HSV-1 and HSV-2 are also major pathogens of neonatal herpes.

After productive (lytic) infection in mucosal epithelial cells of peripheral tissues, HSV enters neurons of the peripheral nervous system by membrane fusion at the axonal termini. Along the axon, the nucleocapsid is retrograde transported to the nucleus in the neuronal cell body where lifelong latent infection is established. HSV latency is generally characterized by undetectable production of infectious virus, an episomal viral genome in the nucleus, silencing of virtually all genes required for the lytic cycle (referred to as lytic genes) but relatively high expression of latency-associated transcripts (LATs) and some LAT-derived microRNAs (miRNAs). Of note, only a subset of neurons in latently infected tissues contain the HSV genome and these neurons exhibit a wide range of viral genome copy numbers (Sawtell et al., 1998). Also, laser-capture microdissection of latently infected mouse and human ganglia showed that not all latently infected neurons accumulate LATs to detectable levels (Chen et al., 2002b; Wang et al., 2005a). Therefore, HSV latent infection is heterogenous in different neurons. In response to certain stimuli such as heat shock, axotomy, changes in hormone levels, UV irradiation and psychological stress, the latent virus can reactivate to resume lytic replication (Suzich and Cliffe, 2018), after which the nucleocapsid newly produced in the neuronal cell body is anterograde transported to the peripheral tissues. Reactivation can sometimes cause recurrent disease, although many HSV reactivation events result in asymptomatic virus shedding, a phenomenon particularly frequent for HSV-2 genital infections (Sacks et al., 2004). Nucleoside analogs such as acyclovir can alleviate HSV acute disease but have no effect on latent infection and do not prevent reactivation. Nor can the immune system eliminate the latent virus. Therefore, the latency-reactivation cycle is a major obstacle to cure of HSV disease.

The human neurotropic nature of HSV latency presents a challenge for studies of HSV latency and reactivation. The natural sites of HSV latency, human ganglia, are not readily available. Researchers of this field traditionally relied on animal models. Mouse models of ocular inoculation are most commonly used for HSV-1 although other

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inoculation routes and rabbit models are available (reviewed in (Hussain et al., 2024)). Tree shrew models have emerged as new models for HSV-1 latency with an advantage of the host being closely related to primates (Li et al., 2016; Wang et al., 2020). HSV-2 latency and reactivation are mostly studied with guinea pig models of intravaginal inoculation. Primary neuronal culture models derived from mouse and rat trigeminal ganglia (TG), dorsal root ganglia and superior cervical ganglia (SCG) have been developed with the advantage of being easier to manipulate genetically (reviewed in (Wilson, 2022)). Still, the non-human in vivo and in vitro models are faced with the caveat that the mechanisms may not be conserved between the animals and humans. In terms of human cells, quiescent infection models have been developed using human fibroblasts infected with HSV-1 mutants (Cohen et al., 2018; Everett et al., 2007). Such models are helpful for understanding latency too because although the paradigm of the field is that HSV latency is a neuron-specific process, there are examples of quiescent infections in nonneuronal cells that can reactivate at least in culture (Cohen et al., 2020) suggesting that there are mechanisms in common between quiescence in non-neuronal cells and latency in neurons. In addition, latency models using human neurons derived from neuronal cell lines or stem cells are being developed (e.g.(Grams et al., 2023; Sodroski et al., 2024)). The human neuronal models are promising in providing the cellular environment closest to that of natural latent infection. However, they are artificially developed so they may not have the same chromatin environment, cell death restriction pathways and immune pathways, etc. as naturally developed mature neurons. Also, all cell culture models may lack certain aspects of the immune response important for latency. Given that no single model is perfect, it would be best to draw conclusions based on results from multiple models.

HSV latency and reactivation are complicated processes involving multiple factors including delivery of viral capsids along axons, survival of infected neurons as well as the interactions between the virus and host immune system. There is a large amount of literature in this field. Given the space limitation, this review builds on previous reviews regarding mechanisms of HSV latency (Bloom et al., 2010; Nicoll et al., 2012; Perng and Jones, 2010; Preston and Efstathiou, 2007; Roizman and Whitley, 2013; Sawtell and Thompson, 2021) and primarily focuses on recent findings in one aspect of the multifaceted issue: how viral gene expression is regulated during latency and reactivation.

2. HSV gene expression patterns at different stages of the life cycle

HSV lytic infection, latent infection and reactivation display distinct gene expression profiles. During lytic infection, HSV genes are expressed in a cascade fashion and classified into immediate-early (IE or α), early (E or β) and late (L or γ) genes. These kinetic classes are experimentally defined groups that roughly reflect the order of expression but lytic gene expression is more of a spectrum without clear boundaries for times of expression. Expression of the IE genes do not require prior protein synthesis but is stimulated by the VP16-induced complex. The IE gene products then stimulate the expression of E genes, whose products are involved in viral DNA synthesis. The L genes mainly encode structural proteins. Unlike IE and E genes, L genes depend on viral DNA synthesis for optimal expression. The L genes whose expression is partially blocked by DNA replication inhibitors are classified as leaky-late (γ1) genes while those whose expression is completely inhibited are classified as true-late (γ2) genes. During latent infection, lytic genes are globally silenced whereas the LAT gene locus shows high transcription activity. The silencing is not absolute as lytic transcripts are still detectable at low levels in latently infected ganglia (Feldman et al., 2002; Ma et al., 2014; Pesola et al., 2005). The 8.3 kb polyadenylated primary LAT is spliced into 1.5 and 2 kb stable introns that are abundant during latency, as well as a 6.3 kb exon, which itself is hard to detect but is processed into multiple miRNAs that are easily detectable (Fig. 1) (Chen et al., 2022b). miRNAs readily detected during latency include miR-H2, miR-H3, miR-H4, miR-H5, miR-H6 and miR-H7 of HSV-1 (Du et al., 2015; Jurak et al., 2010; Pan et al., 2019; Umbach et al., 2008), and miR-I (miR-H3), miR-II (miR-H4), miR-III (miR-H2), and miR-H6 of HSV-2 (Tang et al., 2008, 2009, 2011). The kinetics of reactivation is different from that of de novo lytic infection. A TG explant model of reactivation showed simultaneous accumulation of mRNAs of all kinetic classes during the first few hours of explant concomitant with a decrease in the accumulation of LATs and LAT-derived miRNAs (Du et al., 2011). A study in a primary neuronal culture model identified two phases of reactivation from latency (Kim et al., 2012). The first phase (also called the animation phase) is characterized by genome-wide de-repression of lytic genes without viral DNA synthesis or particle production. The second phase exhibits the ordered gene expression program similar to de novo lytic infection. The presence of two reactivation phases has been

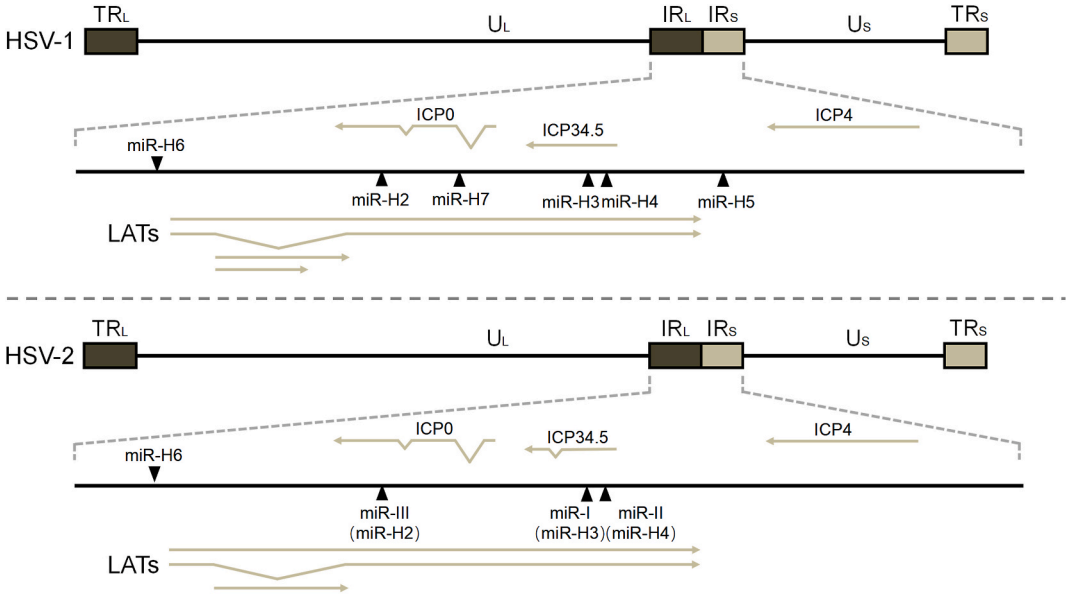

Fig. 1. Map of the LAT gene locus showing the positions of LATs and miRNAs readily detectable during latency. HSV-1 and HSV-2 genomes are shown in the prototype orientation. TR_L, IR_L, TR_S, IR_S, U_L and U_S denote long terminal repeat, long internal repeat, short terminal repeat, short internal repeat, long unique and short unique regions, respectively. The internal repeat regions are expanded to show the genomic positions of the different transcripts and miRNAs.

substantiated by multiple follow-up studies using primary neuronal models under a variety of conditions (Cliffe et al., 2015; Cuddy et al., 2020; Dochnal et al., 2022).

3. Mechanisms of gene activation during lytic replication

The HSV lytic and latent programs are interchangeable. Elucidating how viral genes are activated is crucial for understanding how they are repressed during latency. After HSV enters the nucleus, the free viral DNA is rapidly associated with histones to form chromatin (Deshmane and Fraser, 1989; Herrera and Triezenberg, 2004; Kent et al., 2004; Kubat et al., 2004; Oh and Fraser, 2008; Wang et al., 2005b). The characteristic of the initial viral chromatin is largely unknown but it has been reported to be located within PML nuclear bodies (PML-NBs) (Alandijany et al., 2018). The chromatin is likely to be dynamic and readily modulated by viral proteins as infection progresses (Hu et al., 2019b). The ordered gene activation process during lytic infection reflects the stepwise de-compaction of the viral chromatin (Fig. 2). To start, the viral tegument protein VP16 from the incoming virion forms a complex with host HCF-1 and OCT-1 proteins. After binding to the TAATGARATT response element in IE gene promoters, the VP16-induced complex recruits histone modifying enzymes such as LSD1, SETD1A, KMT2A, CBP and P300 to reduce heterochromatin modifications (such as H3K9me3) while increasing activating chromatin modifications (such as H3K4me3 and histone acetylation) (Herrera and Triezenberg, 2004; Kent et al., 2004; Liang et al., 2009). Components of the ATP-dependent chromatin remodeling complex SNF/SWI such as BRG1 and hBRM are also recruited by VP16 (Herrera and Triezenberg, 2004). HCF-1 is also associated with transcription elongation components including the super elongation complex that further drives transcription (Alfonso-Dunn et al., 2017). The IE gene products create favorable conditions for later gene expression through various activities. For example, the viral E3 ubiquitin ligase ICP0 induces the degradation of host restriction factors and immune molecules such as PML and IFI16, the reduction of heterochromatin at lytic gene promoters as well as the removal of HDAC1 from the CoREST-REST complex (Alandijany et al., 2018; Cliffe and Knipe, 2008; Gu and Roizman, 2007; Hou et al., 2022; Lee et al., 2016; Rodríguez et al., 2020). Another IE protein, ICP4, binds to numerous sites on the viral genome and recruits RNA polymerase II (Pol II), TATA-binding protein and the Mediator complex to form a transcription preinitiation complex (Dremel and DeLuca, 2019b). Binding of ICP4 and host transcription factors to E gene promoters induces E gene transcription. E proteins, many of which are enzymes responsible for DNA synthesis, then initiate viral DNA replication. Viral DNA replication results in the binding of TBP, TAF1 and RNA Pol II, in addition to ICP4, to previously silent L gene promoters (Dremel and DeLuca, 2019a), thereby stimulating L gene expression.

4. Formation of latent viral chromatin

In contrast to the loosely assembled lytic viral chromatin, the latent viral genomes are assembled into nucleosomes (Deshmane and Fraser, 1989) bearing abundant histone modifications but no detectable DNA methylation (Kubat et al., 2004). Concomitant with the establishment of latency is progressive enrichment of H3K9me3 and H3K27me3 modifications on viral lytic promoters (Fig. 3) (Cliffe et al., 2009; Kwiatkowski et al., 2009; Wang et al., 2005b). H3K9me3 is a marker of constitutive heterochromatin while H3K27me3 is a hallmark of facultative heterochromatin and might contribute to a “poised” viral chromatin ready for reactivation. Interestingly, H3K27me3 is not enriched on lytic promoters until 14 days post-infection, a time when lytic genes have been largely repressed, suggesting that other mechanisms repress lytic genes at earlier times (Cliffe et al., 2013). In contrast to lytic promoters, the LAT promoter is enriched with histone H3 acetylation and H3K4me3 modifications indicative of active chromatin during latency (Cliffe et al., 2009; Kubat et al., 2004). The differential chromatin modifications between the promoters of lytic genes and LAT might have to do with CTCF binding to clusters of CTCF binding motifs hypothesized to have insulator activity (Amelio et al., 2006). The CTRL2 site is particularly interesting because it is located between the LAT enhancer and ICP0 promoter. CTRL2 deletion mutant viruses showed altered enrichment of H3K27me3 at some genomic loci in latently infected mice or rabbits although the phenotypes were not all consistent between the mutants from different strains (Lee et al., 2018; Singh et al., 2022; Washington

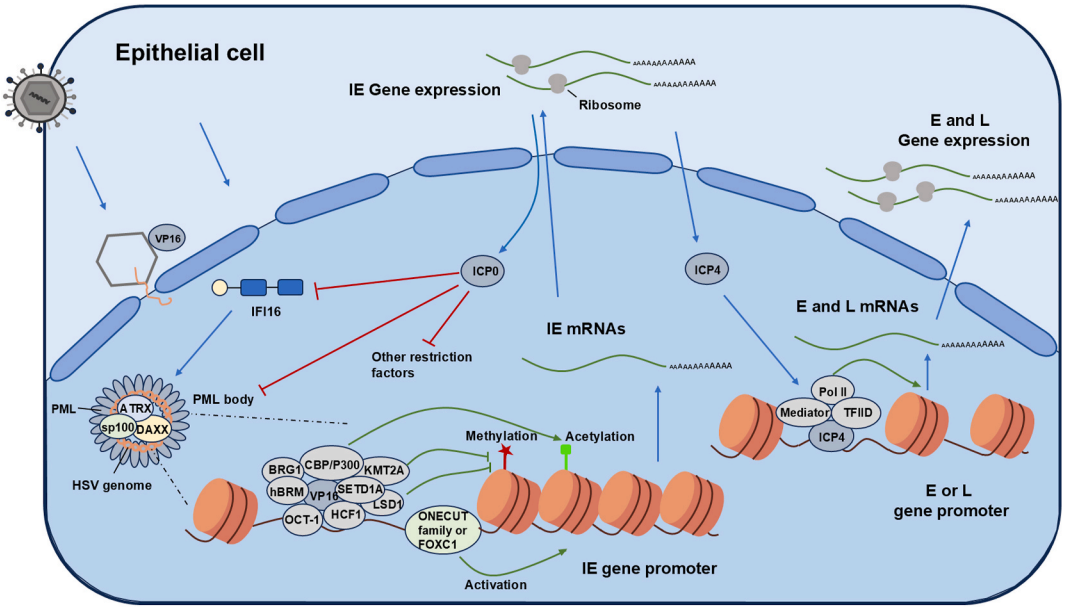

Fig. 2. Mechanisms of viral gene activation during lytic infection in epithelial cells. After nuclear entry, the viral genome froms heterochromatin within PML-NBs containing restriction factors. The VP16-induced complex recruits host chromatin modifying and remodeling proteins to the IE gene promoters to activate IE gene expression. Host ONECUT family and FOXC1 also bind to viral genes to facilitate activation. The IE protein ICP0 counteracts host restrictive mechanisms through the E3 ligase activity. ICP4 binds to the viral genome to recruit cellular general transcription factors to stimulate transcription of E and L genes. Some graphic elements were obtained from Server Medical Art (SMART) (https://smart.servier.com).

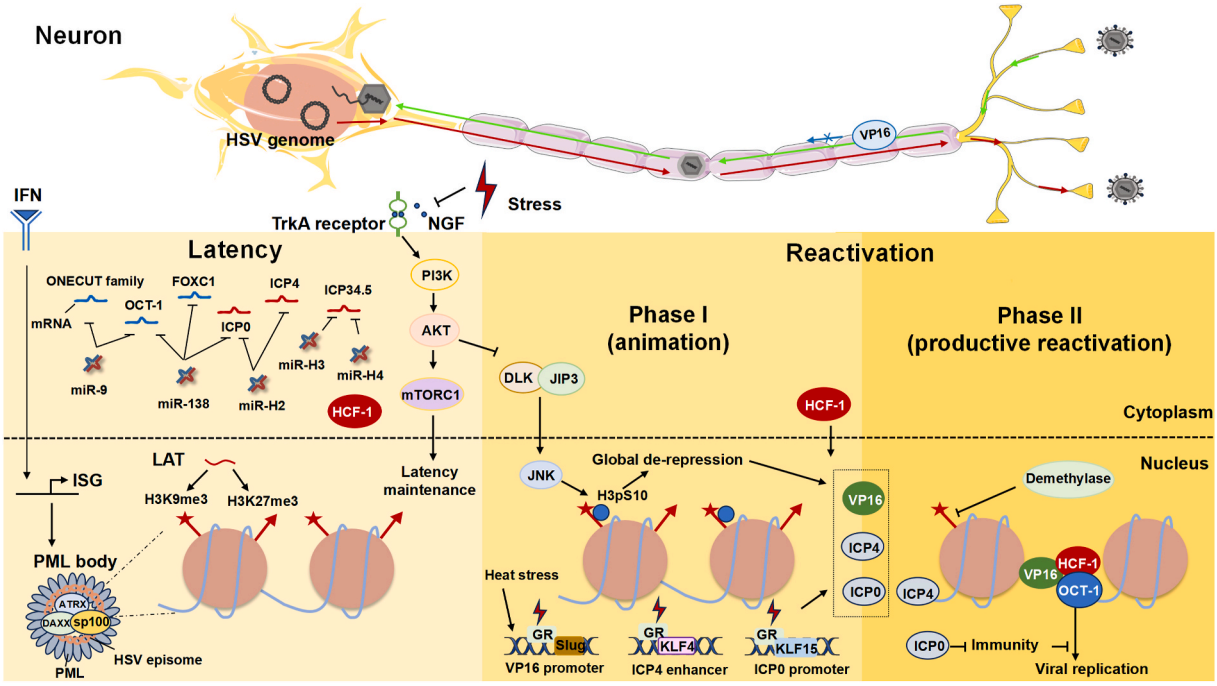

Fig. 3. Mechanisms of regulation of viral genes during latency and reactivation in neurons. At the top a sensory neuron is depicted with the movements of capsids indicated by arrows. A blocked arrow indicates dissociation of the VP16 during entry along the exon. The lower parts depict events during latency and the two phases of reactivation. During latency, highly expressed viral and host miRNAs targets viral and host genes important for the lytic cycle while LAT, interferon and PML-NB components promote formation of viral heterochromatin. The AKT-mTORC1 pathway is important for latency maintenance. Some stress signals deprive NGF resulting in the inhibition of the AKT pathway leading to phase 1 of reactivation through the DLK/JNK pathway. JNK causes a methyl/phosphor switch favorable for global de-repression. Meanwhile heat stress and hormones may specifically activate certain lytic gene promoters. The lytic proteins expressed during phase 1 then act together with host demethylases to stimulate phase 2. Some graphic elements were obtained from Server Medical Art (SMART) (https://smart.servier.com).

et al., 2019).

Current evidence that the modifications play an important role in regulation of the lytic-latent balance came mostly from experiments using inhibitors of the related enzymes. Treatment of latently infected mice with a histone deacetylase inhibitor caused reactivation (Neumann et al., 2007) suggesting that histone acetylation favors reactivation. Regarding histone methylation, pharmacological inhibition of the histone demethylase LSD1 or JMJD2 reduced HSV lytic replication and reactivation from latency in mouse, rabbit and guinea pig models (Hill et al., 2014; Liang et al., 2009, 2013a, 2013b). Likewise, inhibition of H3K27me3-specific histone demethylases JMJD3 and UTX reduced reactivation in multiple models (Cliffe et al., 2015; Cuddy et al., 2020; Dochnal et al., 2022; Messer et al., 2015). These results are consistent with a repressive role of histone methylation favorable for latency. However, it was reported that inhibition of the histone H3K27 methyltransferases EZH2 and EZH1 unexpectedly suppressed viral replication and gene expression in human fibroblasts as well as reactivation in mouse TG possibly due to the observed enhanced antiviral immunity caused by the inhibitors (Arbuckle et al., 2017) although another study showed no impact of an EZH2/1 inhibitor on lytic gene expression (Francois et al., 2024). The enzymes and small molecule inhibitors may not be specific for a particular modification or for the viral chromatin. Therefore, more work is needed to dissect the exact roles of individual modifications and their related enzymes in HSV latency and reactivation.

5. Reduced availability of the VP16-induced complex in latently infected neurons

One mechanism of gene silencing during latency is insufficient IE gene activation due to reduced availability of the VP16-induced complex in the nuclei of latently infected neurons (Fig. 3). The tegument protein VP16 itself dissociates from incoming virions in the long axons of DRG neurons (Aggarwal et al., 2012). Consistently, in a double chamber system, adding HSV-1 to distal axons of chicken embryonic TG explants favored nonproductive infection (Hafezi et al., 2012). Additionally, HCF-1 is mainly localized to the Golgi apparatus in normal or latently-infected neurons and re-localized to the nucleus only upon stimulation that results in reactivation (Whitlow and Kristie, 2009). Furthermore, OCT-1 is poorly expressed in sensory neurons at least in part due to high expression of neuronal miRNAs miR-138 and miR-9 that target Oct-1 mRNA (see below) (Deng et al., 2024; Sun et al., 2021). Given the importance of the VP16-induced complex for expression of IE genes, the lack of this complex would be favorable for silencing of lytic genes. It is worth noting that besides VP16, some other tegument proteins have been observed to dissociate from the viral capsid during axonal transport into the neuronal cell body (Aggarwal et al., 2012). Since multiple tegument proteins have been shown to facilitate the lytic environment by counteracting intrinsic and innate immune pathways (Kelly et al., 2009; Zhu and Zheng, 2020), the possibility that inefficient delivery of additional tegument proteins contributes to the establishment of the silencing state deserves consideration.

6. Viral RNA mediated repression of lytic genes during latency

Non-coding RNAs derived from the LAT locus have long been hypothesized to contribute to latency since they are the only gene products highly expressed during latent infection. However, the role of LATs has not been fully elucidated. The bulk of the literature supports the hypothesis that LATs promote neuronal survival and reactivation (Perng et al., 2000; Thompson and Sawtell, 2001; Watson et al., 2018) and suppress lytic gene expression. With regard to gene regulation, HSV-1 LAT-deletion mutants exhibited increased lytic gene expression during acute and latent infections (Chen et al., 1997; Garber et al., 1997) and increased frequencies of reporter gene expression from latent viral genomes (Nicoll et al., 2016). However, the mechanism of this repression

is elusive. One hypothesis is related to epigenetic regulation since LAT deletion mutant viruses showed decreased H3K9me2, H3K9me3 and H3K27me3 heterochromatin marks but increased H3K4me3 euchromatin marks on the latent viral genome (Cliffe et al., 2009; Wang et al., 2005b). Another hypothesis is antisense repression by the LAT itself or LAT-derived miRNAs, since the LAT introns and miR-H2 are antisense to ICP0 mRNA, and miR-H3 and miR-H4 are antisense to ICP34.5 mRNAs (Pan et al., 2017; Tang et al., 2008, 2009). The miRNA-target interactions and their repressive effects have been validated by PAR-CLIP and co-transfection experiments (Flores et al., 2013). Moreover, HSV-1 miR-H2 exhibits specific adenosine to inosine hyperediting in latently infected human TG and the edited miR-H2 can repress expression of ICP4 in addition to ICP0 in co-transfection assays (Zubković et al., 2023). However, neither ectopic expression of the LAT 2 kb stable intron nor deletion of LAT from the virus affected ICP0 expression (Burton et al., 2003; Chen et al., 2002a). HSV-1 miR-H2 mutants also showed no de-repression of ICP0 in acutely or latently infected mouse TG (Pan et al., 2017). Furthermore, HSV-2 mutants with lesions in miR-H2, H3 or H4 showed no phenotypic difference from wild type in guinea pig models of latency and reactivation (Kawamura et al., 2018). Thus, while the LATs seem to be important for efficient repression of lytic gene expression, these viral miRNAs appear to be redundant at least in current experimental models.

7. Viral gene silencing mediated by host neuronal miRNAs

Besides viral miRNAs, host neuronal miRNAs also participate in HSV gene regulation during latency (Fig. 3). The 3' UTR of ICP0 mRNA contains miR-138 binding sites partially conserved between HSV-1 and HSV-2. miR-138 is neuron-specific in that its high expression is observed only in neuronal cells and tissues. An HSV-1 mutant with altered sequences in the two miR-138 binding sites showed increased virulence as well as enhanced expression of ICP0 and other lytic genes in mouse TG during both acute and latent infections (Pan et al., 2014). miR-138 also represses HSV gene expression independent of ICP0 through other targets. Another target of miR-138 is host FOXC1, which can globally stimulate HSV gene expression at the IE stage. In neuronal cells, both ICP0 and FOXC1 can reduce heterochromatin occupancy on lytic gene promoters, coinciding with opposite effects of miR-138 (Sun et al., 2021). Another neuron-specific miRNA, miR-9, can repress viral replication and reactivation in neurons. miR-9 targets all three members of the host ONECUT family. The representative member of the family ONECUT2 can broadly stimulate HSV-1 gene expression, reduce heterochromatin on viral lytic genes, and globally increase the accessibility of the viral chromatin (Deng et al., 2024). Interestingly, both miR-138 and miR-9 also repress OCT-1 expression correlating with low OCT-1 expression in neurons, which would weaken VP16 mediated IE gene activation. Furthermore, all these regulatory events mediated by miR-138 and miR-9 appear to be conserved between HSV-1 and HSV-2 (Chen et al., 2022a; Deng et al., 2024). Therefore, the neuronal miRNAs promote formation of repressive viral chromatin by jointly targeting viral and host genes thereby providing an intracellular environment favorable for latency.

8. Role of intrinsic and innate immunity in viral gene repression during latency

Like other viruses, before adaptive immunity is elicited, HSV is controlled by an intricate antiviral defense network including components of both intrinsic immunity that is constitutively active and innate immunity that is induced by infection. HSV has evolved to coopt some of these mechanisms to form and stabilize latent infection (Fig. 3). HSV-1 genomes have been reported to be positioned within PML-NBs in acutely and latently infected mouse TG (Catez et al., 2012). In fibroblasts infected with a replication-defective HSV-1 mutant, PML is essential for the association of histone H3.3 with the viral genome (Cohen et al., 2018). Multiple PML body proteins, PML, ATRX, DAXX and SP100 can restrict viral replication and gene expression in ICP0-null virus infected fibroblasts (Everett et al., 2008; Lukashchuk and Everett, 2010). ATRX can also promote the maintenance of ICP0-null HSV-1 heterochromatin in fibroblasts (Cabral et al., 2018). Therefore, these cellular restriction factors may contribute to the establishment and maintenance of latency. Indeed, depletion of PML in the presence of type I interferon (IFN) increased reactivation in mouse SCG neuronal culture (Suzich et al., 2021). However, to our knowledge, the functions of the other restriction factors have yet to be investigated in the context of neuronal latent infection. Besides intrinsic immunity represented by epigenetic silencing and constitutively expressed restriction factors, IFN-inducible intracellular innate immunity also plays an important role in latency. Although IFN production from neurons is limited, HSV infected neurons can respond to antiviral cytokines produced by infiltrated immune cells in ganglia. In a rat SCG neuronal culture model, both exogenous IFN-β and IFN-γ suppressed lytic gene expression during phase 1 of reactivation (Linderman et al., 2017). IFN-stimulated levels of the nuclear DNA sensor IFI16 can stabilize virus-associated heterochromatin and repress viral gene expression in wild type HSV-1 infected fibroblasts (Sodroski and Knipe, 2023). Interestingly, although many host restriction factors are constitutively expressed, expression of some PML-NB proteins such as PML and Sp100 is further stimulated by IFN (Grötzinger et al., 1996; Stadler et al., 1995). Accordingly, type I IFN treatment promotes the formation of PML-NBs, which persist for days after IFN removal and colocalize with HSV-1 genomes to restrict reactivation in a neuronal culture model (Suzich et al., 2021).

9. Mechanisms of gene de-repression during reactivation from latency

The repressive mechanisms pivotal to the establishment and maintenance of latency become obstacles that the virus has to overcome during reactivation (Fig. 3). A major obstacle is the lack of the viral activators that can initiate the activating process in latently infected neurons. One hypothesis argues that certain viral proteins like VP16 are expressed first before they activate other lytic genes. Consistent with this, in a mouse hyperthermia model of reactivation, de novo expression of VP16 is required for viral protein expression at 22 h after hyperthermic stress, and activation of the VP16 promoter was detected in latently infected neurons following heat stress (Thompson et al., 2009; Thompson and Sawtell, 2006). In line with this hypothesis, in transient transfection experiments, the VP16 and ICP0 promoters as well as the ICP4 enhancer are activated by glucocorticoid receptor in collaboration with some other transcription factors whose expression is induced by stress hormones that can trigger reactivation (El-Mayet et al., 2022; Ostler et al., 2021; Santos et al., 2023). Interestingly, HSV-1 reactivated less efficiently in explanted TG from female mice expressing a glucocorticoid receptor mutant compared to male mice with the same mutation or wild-type parental mice suggesting that glucocorticoid receptor has female-specific effects on reactivation (Harrison et al., 2023).

Another hypothesis argues that reactivation starts with global de-repression of the viral genome instead of specific induction of certain viral genes. This hypothesis is based on experiments in a rat SCG neuronal culture model, which was initially used for investigating how nerve growth factor (NGF) deprivation can trigger reactivation (Wilcox and Johnson, 1987). The results showed that continuous signaling through the PI3-K-AKT pathway triggered by NGF binding to the TrkA receptor tyrosine kinase is essential for latency maintenance (Camarena et al., 2010). The mTORC1 pathway downstream of AKT has also been shown to be required to maintain HSV-1 latency (Kobayashi et al., 2012). Interestingly, the AKT-mTORC1 pathway requires endogenous DNA damage signals as well as external signals through the TrkA receptor (Hu et al., 2019a). Importantly, after reactivation is induced by a PI3-K inhibitor, two kinetically distinct phases of reactivation were identified. Phase 1 represents widespread de-repression of viral genes

irrespective of kinetic classes. Meanwhile, HCF-1 relocalizes from the cytoplasm of sensory neurons to the nucleus (Whitlow and Kristie, 2009). The VP16 synthesized during phase 1 and the nuclear HCF-1 then stimulates phase 2, ultimately leading to the production of new viral DNA and infectious particles (Kim et al., 2012).

Stepwise reactivation makes sense in that viral activators essential for the lytic program probably need to accumulate to certain levels before they can help the virus break through the multitude of repressive mechanisms. Therefore, the two hypotheses differ only in whether certain genes are stimulated ahead of others in phase 1. It is possible that certain viral genes are preferentially activated in the background of broad gene activation at the initial stage of reactivation. Also, different stimuli might activate viral genes in different ways. Regardless, it is clear that multiple host functions are required for reactivation. The importance of HCF-1 in reactivation has been demonstrated by in vivo experiments showing that its deletion in sensory neurons caused suppressed reactivation and a defect in the removal of repressive chromatin from latent viral genomes (Arbuckle et al., 2023). Work in the neuronal culture model further showed that phase 1 requires JNK signaling which is probably activated by DLK following inhibition of the PI3-K-AKT pathway since AKT suppresses DLK activity. Interestingly, phase 2, rather than phase 1, requires histone demethylases that remove repressive lysine modifications. Phase 1 appears to act through a histone methyl/phospho switch on lytic promoters caused by JNK signaling since this switch permits gene expression in the presence of repressive lysine methylations (Cliffe et al., 2015). Furthermore, JNK and DLK activities have been shown to be required for efficient reactivation in an ex vivo model as well as neuronal culture models involving reactivation induced by multiple other triggers including DNA damage, forskolin and IL-1β (Cuddy et al., 2020; Dochnal et al., 2022; Hu et al., 2019a; Whitford et al., 2022). In the absence pre-existing viral proteins, the initial de-repression step must involve host signaling pathways that transduce external cues to the viral genome in the nucleus. We are just beginning to learn about the pathways involved. More work is needed to gain deeper insight into the molecular details of reactivation.

10. Conclusions and future perspectives

Among the various mechanisms regulating HSV gene expression, chromatin-mediated control of transcription plays a central role. After nuclear entry, the viral genome is bound by histones to form repressive heterochromatin, which is capsulated by PML-NBs containing multiple restriction factors that help maintain the heterochromatin. In non-neuronal cells, these host intrinsic defense measures are counteracted by viral proteins like VP16 in collaboration with their host partners to remodel the chromatin in favor of transcription activation. In neurons, however, these viral and host activators are limited in the nucleus such that the host epigenetic silencing machinery predominates. After IFN production is induced, the repression is corroborated by IFN-mediated innate immunity. Thus, the host intrinsic and innate immunity are hijacked by the virus to induce latency. Additional support may be provided by LATs which also promote viral heterochromatin and viral gene repression. The latent viral chromatin is largely repressive but it also confers reactivation potential since it is enriched with both constitutive and facultative heterochromatin modifications. During reactivation, certain external signals are transmitted to the neuronal nucleus leading to changes in chromatin modifications in favor of viral gene activation.

Besides transcriptional regulation, miRNA mediated post-transcriptional regulation also contributes significantly to gene repression during latency. Multiple viral and neuronal host miRNAs highly expressed during latency can either directly target important viral lytic genes or indirectly repress viral genes through host targets. In particular, the important viral E3 ligase ICP0 is targeted by neuron-specific miR-138 resulting in attenuated antagonism against epigenetic silencing. Moreover, miR-138 and miR-9 collectively repress multiple cellular transcription factors involved in the initiation of HSV gene transcription including OCT-1, FOXC1 and ONECUT family proteins thereby preventing efficient onset of the lytic program. Interestingly, these miRNA targets are also modulators of viral chromatin highlighting the complexity of the regulatory network that involves both chromatin and miRNA machineries.

Despite the great progress toward understanding how HSV genes are regulated during latency and reactivation, multiple key questions remain unanswered or partly answered. First, the role of epigenetic regulation in latency and reactivation is only partly understood. The exact states of the viral chromatin at various stages of the viral life cycle as well as the role of various histone modifications and chromatin remodeling proteins remain obscure. Second, we have just started to learn about the intrinsic features of neurons that can help explain why HSV latency occurs specifically in neurons. There must be more neuron-specific molecules that contribute to latency and reactivation. Third, we know even less about how different reactivation stimuli transduce the signals to the latent viral genome in the nucleus. Recent work has shed light on the role of certain signaling pathways. However, many gaps remain in our understanding of the molecular details including how the signals lead to viral chromatin remodeling and whether different stimuli act through divergent or common pathways. Fourth, after decades of research, the role of LATs, the hallmark of HSV latency, remains puzzling. Even though some functions of LATs have been identified, we still know almost nothing about the underlying mechanisms. Lastly, the mechanisms of latency and reactivation have been mostly investigated for HSV-1. Alphaherpesviruses generally undergo latency in neurons. Whether these mechanisms are conserved in HSV-2 or other alphaherpesviruses is worth investigating because answers to this question may help us understand the basic principles of viral latency.

Future efforts to address these questions will be assisted by continuously developing technologies. Advanced proteomic technologies (Dembowski and DeLuca, 2018; Kim et al., 2021), combined with high-resolution imaging (Roberts et al., 2024), can facilitate the determination of the components and spatial features of the viral chromatin. Genome-wide approaches for analysis of gene expression, protein-DNA binding, and chromatin accessibility can efficiently provide large amounts of information that can help us systematically understand the binding or regulatory events (Deng et al., 2024). These approaches are being further boosted by the rapidly advancing single cell sequencing technologies that can provide additional information about heterogeneity of latency in different cells as well as cell-type specific factors involved in latency or reactivation (Hu et al., 2022; Ouwendijk et al., 2024; Wang et al., 2023). Experimental models of latent infection have also advanced significantly in recent years. New models using differentiated human neurons, powered by stem cell technology, will not only help to understand events in the natural host, but also enable experiments requiring large numbers of neurons, such as proteomics analysis and CRISPR screening. Finally, there have been reports of successful reduction of the latent viral reservoir that can reactivate by targeting the viral genome with gene-editing nucleases (Aubert et al., 2020; Yin et al., 2021). Therefore, we are entering an era where multiple cutting-edge tools are available for our attempts to elucidate the complex mechanisms of HSV latency and reactivation and to explore intervention strategies. Given that the latency-reactivation cycle is the major reason why HSV infection cannot yet be cured, such attempts should prompt development of therapeutics that hopefully will lead to cure of HSV disease.

CRediT authorship contribution statement

Hui Fu: Writing – original draft, Visualization. **Dongli Pan:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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