# The Role of Interferons in Regulating the Subnuclear Positioning of Latent Herpes Simplex Virus Genomes and Reactivation

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**Abstract**

Herpes simplex virus-1 (HSV-1) is a ubiquitous pathogen that establishes lifelong latent infections in post-mitotic neurons, most commonly in the peripheral ganglia. Latent HSV-1 infection is asymptomatic, but the virus periodically reactivates, which can lead to significant disease, including a life-threatening encephalitis or recurrent HSV-1 ocular infection, a leading cause of infectious blindness worldwide. Recent evidence suggests that recurrent HSV-1 infection may also impact the progression of Alzheimer's disease. Therefore, there is a need to understand how the virus remains latent in neurons to ultimately prevent reactivation and recurrent infection. During latency, the promoters of viral lytic genes are associated with heterochromatin, which is thought to maintain long-term gene silencing. However, HSV latency is heterogenous and latent viral genomes associate with different subnuclear structures and cellular proteins, which may result in different forms of latency that are more or less capable of reactivation. Additionally, this can be further impacted by the inflammatory environment and the presence of cytokines or other signaling molecules. In Chapter 2, we explore the role of type I interferons (IFNs) and promyelocytic leukemia nuclear bodies (PML-NBs) in promoting a restricted form of HSV latency. We first characterized PML distribution in primary peripheral neurons isolated from adult and postnatal mice and found that they are largely devoid of detectable PML-NBs. Treatment of these primary murine neurons with type I IFN induces robust formation of PML-NBs that continue to persist following cessation of IFN signaling. A large proportion of HSV-1 genomes are stably entrapped by PML-NBs throughout latency when IFNα is present during initial infection, and reactivation is restricted under these conditions. However, the ability of HSV to reactivate is rescued if PML is depleted either prior to or following infection, suggesting that IFNα-induced PML-NBs are required for the restriction of HSV-1 reaction. In Chapter 3, we further investigated the localization of latent viral genomes in primary murine neurons and found

they colocalize not only to alpha-thalassemia/mental retardation syndrome x-linked protein (ATRX) in the context of PML-NBs, but also to regions of dense ATRX staining localized outside of PML-NBs, even in the absence of IFNs. Furthermore, depletion of ATRX in latently infected neurons *in vivo* decreased the latent viral load in trigeminal ganglia (TG) and increased lytic gene expression during reactivation of superior cervical ganglia (SCG). Interestingly, we show that the subnuclear positioning of a latent viral genome modulates not only its chromatin structure and association with histones, but also its compaction state, as superresolution microscopy revealed that PML-NB-associated genomes are significantly less compact than non-PML-NB-associated genomes. Lastly, we characterized PML and ATRX distribution in human neuronal cell models of HSV-1 latency and found that IFNα increases PML-NB number and colocalization of PML-NBs to latent HSV-1 genomes in these cells. Together, these studies have expanded our understanding of heterogeneity in latency and provide key insight into what form of latency is the most repressive (also known as deep latency), why it is more repressive and how different forms of latency arise so that we may be able to develop therapies that manipulate latency and drive it into its deepest form.

**Dedication Page**

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# CHAPTER 1

*Introduction to the role of interferons and repressive nuclear bodies in herpes simplex virus infection*

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**Abstract**

Herpes simplex virus (HSV) establishes a latent infection in peripheral neurons and can periodically reactivate to cause disease. There is considerable heterogeneity in HSV latency, including at the neuronal level in the co-localization of latent HSV-1 viral genomes with different subnuclear structures. How this heterogeneity arises and whether certain genomes are able to reactivate more readily than others is not known. Furthermore, the interferon (IFN) system has been identified as a crucial component of the host innate immune response to control HSV infection. However, how neuronal interferon signaling impacts the establishment and maintenance of latency is not well characterized. Here, we demonstrate that the presence of IFNs during *de novo* infection of peripheral neurons can impact the subnuclear positioning of latent genomes. We further characterize how the association of latent viral genomes with promyelocytic nuclear bodies (PML-NBs) or host heterochromatin-associated proteins, including ATRX, can alter the heterochromatic signature, compaction state and, ultimately, the capacity to reactivate following a physiological stimulus of reactivation. In this introduction, we review HSV-1 biology, the role of IFNs in HSV-1 infection and the nuclear structures associated with HSV-1 viral genomes.

## Herpes Simplex Virus

Herpes simplex virus (HSV) 1 and 2 are ubiquitous pathogens that persist for the life of infected individuals. The ability of these viruses to develop lifelong infections is due to the presence of a latent pool of virus in terminally differentiated neurons, most commonly in the peripheral ganglia. It is estimated that approximately 90% of individuals worldwide are infected with HSV-1, HSV-2 or both of the viruses (Arvin *et al*, 2007). In the United States, the estimated prevalence rate of HSV-1 and HSV-2 in people aged 14–49 is 47.8% and 11.9% respectively, with higher prevalence in women and Mexican-American and non-Hispanic black persons (McQuillan *et al*, 2018). HSV infection is often clinically silent. However, HSV periodically re-enters a lytic replication cycle in a process known as reactivation. In immunocompetent persons, reactivation events result in replication at the body surface that can give rise to recurrent blisters or sores, which are typically self-limiting and resolve rapidly (Roizman *et al*, 2013). These lesions most commonly occur at oral, nasal or ocular sites with HSV-1 infection and at the genital skin and mucosa with HSV-2 infection. HSV reactivation can also lead to significant morbidity and mortality in immunocompromised individuals, and, in rare cases, infection of the central nervous system can lead to acute viral encephalitis or a recurrent lymphocytic meningitis.

### Lytic Infection

The ability of HSV to establish a lifelong infection can be attributed to its capacity to undergo contrasting infectious events, termed the lytic and latent stages. Primary infection at the body surface results in productive replication in epithelial cells (Roizman *et al.*, 2013) Infection is initiated when HSV surface glycoproteins bind to a number of receptors on the host cell surface. Two HSV surface glycoproteins, gB and/or gC, first attach to heparin sulfate proteoglycans. This initial attachment allows gB, the

heterodimer gH/gL and gD to then bind additional membrane receptors and trigger fusion of the viral lipid envelope with the cell membrane (WuDunn & Spear, 1989; Herold *et al*, 1991; Turner *et al*, 1998; Shukla *et al*, 1999; Pertel *et al*, 2001). The viral nucleocapsid is released into the cytoplasm along with a number of tegument proteins and is efficiently transported across the cytosol to the nuclear pore complex by a microtubule-mediated mechanism to release the linear 152kb double-stranded DNA viral genome into the host cell nucleus (Sodeik *et al*, 1997; Roizman *et al.*, 2013). Following nuclear entry, the viral genome is rapidly circularized and transcribed (Strang & Stow, 2005). During this initial lytic stage of infection, over 80 viral gene products are expressed in a cascade-dependent manner. Initial viral gene expression is enhanced by delivery of viral tegument proteins, including the viral transactivator, VP16, into the nucleus. This potent transcriptional activator, induces the formation of a transcriptional regulatory complex with multiple cellular co-activators to promote transcription of the viral immediate early (IE) or α genes. Products of the IE genes include proteins required for transcription of the early (E) or β genes that encode proteins required for viral DNA replication, leading to the reorganization of the nucleus and formation of replication compartments (RCs) (Quinlan *et al*, 1984; Monier *et al*, 2000). The final group of genes is the late (L) or γ genes, whose expression is dependent on viral DNA replication. L genes encode structural proteins required for assembly, egress and release of the infectious HSV particle. Progeny nucleocapsids are assembled in the nucleus and acquire tegument proteins and an envelope from the inner nuclear membrane, but it is thought that virions are de-enveloped at the outer nuclear membrane and then re-enveloped by budding at membranes of the *trans* Golgi network (Mettenleiter, 2002).

During primary infection, HSV is able to enter the terminal axons of neurons that innervate tissue at the initial site of infection. The virus fuses to the membrane at axonal

termini and the nucleocapsid undergoes dynein-mediated axonal retrograde transport to the neuronal cell body located within peripheral ganglia (Miranda-Saksena *et al*, 2018). Although viral genomes are most frequently detected in sensory ganglia, particularly the trigeminal ganglia (HSV-1) and lumbar-sacral ganglia (HSV-2), viral DNA and reactivation-competent virus can also be isolated from sympathetic and parasympathetic neurons (Baringer & Swoveland, 1973; Warren *et al*, 1978; Baringer & Pisani, 1994; Richter *et al*, 2009). Furthermore, HSV DNA can be detected in the central nervous system, with the frequency of detection increasing with age (Fraser *et al*, 1981; Gordon *et al*, 1996; Beffert *et al*, 1998). Following neuronal infection, the virus can enter latent infection. However, neurons can also support lytic replication, which may be associated with neuronal death (Thompson & Sawtell, 2001). There is also evidence of prior lytic promoter activity in latently infected neurons (Proenca *et al*, 2008). The mechanisms that regulate entry into lytic replication versus latent infection in neurons remain largely undefined.

Models of Latency and reactivation

A strength of the HSV field is the diversity of the model systems used to investigate the pathways to reactivation. Although there may be differences in the interpretation of the data based on the system or stimuli, all these systems will ultimately have relevance to basic science and human health. To elucidate the cellular signaling pathways involved in the reactivation processes, models of latency that allow faithful establishment of latency, robust reactivation and easy manipulation of signal transduction pathways are required. The most commonly used model organism in HSV research is the mouse. Infection of mice with HSV-1 results in initial lytic replication at the body surface and entry of the virus into innervating sensory and autonomic neurons. Following an initial period of acute replication in the ganglia, HSV establishes latency,

and reactivation can be triggered by explant of the ganglia (Sawtell & Thompson, 2004), hyperthermic stress (Sawtell & Thompson, 1992), UV irradiation (Shimeld *et al*, 1996) or hormone treatment (Cook *et al*, 1991; Vicetti Miguel *et al*, 2010). Overall, *in vivo* models of reactivation have the advantage of more accurately recapitulating the natural course of infection and incorporate host antiviral responses that may impact the state and population of latent genomes or modulate viral reactivation. However, the manipulation of cellular pathways *in vivo* can be challenging.

To elucidate the molecular pathways involved in HSV reactivation, *in vitro* models have proven to be invaluable (Wilson & Mohr, 2012; Thellman & Triezenberg, 2017). The optimal model system would utilize mature, human neurons. However, while sensory neurons can be isolated and maintained from human donors (Valtcheva *et al*, 2016), access to this material is limited, consistency is difficult to achieve, and the tissue may already be latently infected with HSV or varicella zoster virus (VZV). Human sensory neurons differentiated from embryonic stem cells have also been used to investigate latency and reactivation for both HSV and VZV (Markus *et al*, 2015; Pourchet *et al*, 2017). A recent study utilizing human differentiated neurons achieved latently infected cultures that could be reactivated with sodium butyrate, a histone deacetylase inhibitor (Pourchet *et al.*, 2017). There is also an emerging interest in human cell lines that can be easily differentiated into neurons. The HD10.6 cell line can be differentiated into sensory neurons with nociceptive properties (Raymon *et al*, 1999; Thellman *et al*, 2017), and Lund human mesencephalic neuronal cell line (LUHMES) can be differentiated into mature dopamine-like neurons (Lotharius *et al*, 2005). Quiescent infection can be established in both of these cell lines, and reactivation can be triggered from a sub-population of neurons following depletion of nerve growth factor (Thellman *et al.*, 2017; Edwards & Bloom, 2019).

Perhaps one of the best characterized *in vitro* systems to study HSV reactivation utilizes primary sensory or sympathetic neurons isolated from the peripheral ganglia of pre-natal rats and post-natal or adult mice (Wilcox & Johnson, 1987; Wilcox *et al*, 1990; Camarena *et al*, 2010; Cliffe *et al*, 2015; Ives & Bertke, 2017). Infection of these primary neuronal cultures in the presence of acyclovir or phosphonoacteic acid (PAA) results in a quiescent infection that resembles latency. Importantly, to accurately define a quiescent infection in these model systems, it is imperative to show that replicating virus remains undetectable following the removal of viral DNA replication inhibitors. When a quiescent infection is properly established, these systems exhibit all of the known molecular hallmarks of latency, including accumulation of the LAT intron, expression of latency-associated miRNAs, absence of replicating virus and undetectable levels of viral proteins (Wilcox & Johnson, 1987; Camarena *et al.*, 2010; Jurak *et al*, 2014; Cliffe *et al.*, 2015). Furthermore, these models maintain the capacity to undergo reactivation triggered by a variety of stimuli, including NGF-deprivation, suppression of DNA damage/repair pathways, dexamethasone, inhibition of protein synthesis or high intracellular levels of cAMP (Wilcox & Johnson, 1987; Colgin *et al*, 2001; Camarena *et al.*, 2010; Kobayashi *et al*, 2012; Cliffe *et al.*, 2015; Linderman *et al*, 2017; Hu *et al*, 2019). Recently, Cuddy *et al* (2020) used this model to show that reactivation can also be triggered by neuronal hyperexcitability induced by a number of stimuli, including forskolin, KCl, TTX-release, and Interleukin-1β. Thus, these systems provide a powerful tool to study the molecular features of latency and reactivation, such as the role of cell stress pathways or chromatin modulation, in primary neuronal populations. A caveat to these model systems is the absence of support cells that may also impact the nature of the latent infection and/or reactivation. The use of DNA replication inhibitors to promote latency is instead utilized to compensate for missing immune components. Whether DNA

replication inhibitors impact the nature of latency or reactivation mechanisms is not known. However, it is worth noting that symptomatic primary HSV-1 is often treated with anti-viral compounds (James & Whitley, 2010). Moving forward, it will be important to determine if the mode infection, age of neurons, presence of immune mediators or addition of DNA replication inhibitors alters the nature of latency or impacts events that occur during reactivation, as this will have relevance to both the model systems used and human disease.

Latent Infection

HSV latency is defined as the persistence of viral DNA in the absence of detectable infectious virus that retains the ability to reactivate following an appropriate stimulus. While expression of the viral lytic genes is largely repressed during latent infection, there is active transcription of the latency-associated transcript (LAT), which is composed of a primary 8.3kb unstable transcript that is spliced to give rise to a stable intron of approximately 2kb in length and multiple miRNAs (Stevens *et al*, 1987; Umbach *et al*, 2008; Kramer *et al*, 2011). Latency is usually defined at the level of the ganglia, but within a ganglion only a sub-population of latently infected cells will reactivate at any one time (Sawtell & Thompson, 2004). In addition, there is evidence that different stimuli can result in reactivation from different subtypes of neurons (Yanez *et al*, 2017). Therefore, the definition of a “latently” infected neuron may depend not only on the neuronal subtype, but also on the nature of the reactivating trigger.

Following the establishment of latent infection, viral lytic gene expression is silenced, and the lytic gene promoters are associated with repressive heterochromatin (Knipe & Cliffe, 2008). Key experiments performed in the 1980’s indicated that latent genomes in the brain stems of infected mice have a nucleosomal structure (Deshmane

& Fraser, 1989). Later studies confirmed that the latent viral genome associates with cellular histones in the trigeminal ganglia of mice (Kubat *et al*, 2004b; Wang *et al*, 2005; Cliffe *et al*, 2013). Coinciding with the silencing of lytic transcripts, the viral lytic gene promoters become enriched with characteristic heterochromatic histone modifications, namely histone H3 di- and tri-methylated at lysine 9 (H3K9me2/3) and H3K27me3 (Wang *et al.*, 2005; Cliffe *et al*, 2009; Kwiatkowski *et al*, 2009; Cliffe *et al.*, 2013; Nicoll *et al*, 2016). While it appears that factors intrinsic to neurons play a key role in the transcriptional silencing of the virus (Cliffe *et al.*, 2013), viral gene products expressed during latent infection can also modulate the chromatin structure (Wang *et al.*, 2005; Cliffe *et al.*, 2009; Kwiatkowski *et al.*, 2009; Raja *et al*, 2016). This modulation likely promotes long-term latency, while priming the genome for reactivation following the appropriate stimuli (Leib *et al*, 1989; Trousdale *et al*, 1991).

In contrast to the lytic gene promoters, the region encompassing the LAT promoter and enhancer elements are enriched with euchromatin-associated modifications (Kubat *et al*, 2004a; Cliffe *et al.*, 2009). This apparent demarcation in the nature of the chromatin likely arises due to binding sites for the cellular insulator protein CCCTC-binding factor (CTCF) on the viral genome. Interestingly, CTCF eviction coincides with reactivation (Ertel *et al*, 2012; Washington *et al*, 2018b), and depletion of CTCF *in vivo* promotes reactivation (Washington *et al*, 2018a). Furthermore, one important binding site of CTCF, known as CTRL2, lies downstream of the LAT enhancer and separates it from the nearby lytic ICP0 gene (Amelio *et al*, 2006). Deletion of this site from the viral genome results in increased heterochromatin formation on the LAT enhancer region and a paradoxically small increase in LAT gene expression (Lee *et al*, 2018). The CTRL2 binding site deletion also decreases the mutant virus's ability to reactivate from latency. These studies suggest that the organization of chromatin

domains may play a role in both the establishment and maintenance of latency and potentially poise the viral genome for reactivation.

Although regions of the latent viral genome are associated with heterochromatin, there is evidence to suggest that it exists in a state that is primed for reactivation. In mouse models of latency, the viral genome does not contain detectible canonical CpG methylation (Dressler *et al*, 1987; Kubat *et al.*, 2004b), which is associated with a particularly stable form of gene silencing. In addition, lytic promoters do not appear to be associated with H4K20me3 (Cliffe *et al.*, 2009), which is a modification that is classically associated with transcriptionally silenced regions of stable, constitutive heterochromatin in mammalian cells (Jorgensen *et al*, 2013). A component of the repressive PRC1 complex, which is a histone reader often found enriched at sites of cellular H3K27me3, is present at very low levels, if at all, on the latent viral genome (Kwiatkowski *et al.*, 2009; Cliffe *et al.*, 2013). Furthermore, a recent study by Alfonso-Dunn *et al* (2017) suggests that activation of the super-elongation complex by treatment with BET domain inhibitors enhances reactivation *ex vivo*, implicating RNA polymerase II promoter-proximal pausing as a rate-limiting step in HSV reactivation. Whether this is due to 'poised' RNA polymerase II on the latent viral genome clearly deserves more investigation. Taken together, these data indicates that portions of the viral genome are enriched with a form of heterochromatin that may be readily remodeled to permit rapid gene expression.

Heterogeneity in latency

There is now a growing body of evidence demonstrating heterogeneity in latently infected ganglia in terms of localization of viral genomes, copy numbers of viral DNA and expression of lytic and latent transcripts (Sawtell, 1997; Proenca *et al.*, 2008; Catez *et al*,

2012; Ma *et al*, 2014). This likely results in different viral chromatin states that are more or less primed to reactivate depending on the stimulus. How this heterogeneity arises is unclear, but it could occur due to a variety of factors, including the exposure of the neuron to different cytokines and signaling molecules, the amount of infecting virus, or heterogeneity in neuronal populations themselves. There is great diversity in sensory and sympathetic neurons, which can differ in their physiological, anatomical, structural and molecular properties (DeLeón *et al*, 1994; Gold *et al*, 1997; Liu & Ma, 2011; Lallemend & Ernfors, 2012), and this may have significant implications for HSV infection. Certain sensory neuron subtypes, characterized by the presence of neurofilaments (NefH) and calcitonin gene related peptide α (CGRP), as well as positive staining with the A5 antibody, have the highest levels of LAT promoter activity, are less permissive for HSV-1 productive infection, and preferentially undergo early-phase reactivation (Bertke *et al*, 2009; Bertke *et al*, 2011; Cabrera *et al*, 2018). Sympathetic and sensory neurons also vary in regards to their expression of different stress hormone receptors, neurotrophin receptors, and ion channels, which also influence how these neurons respond to cues during both acute infection and reactivation from latency (DeLeón *et al.*, 1994; Gold *et al.*, 1997; Liu & Ma, 2011; Lallemend & Ernfors, 2012). Neurons acquire differences in their epigenetic signatures resulting from experience dependent activity (Mo *et al*, 2015; Stroud *et al*, 2017), and there is even evidence of DNA copy number variations, at least in the central nervous system (McConnell *et al*, 2013). In addition, there is evidence of heterogeneity in how individual neurons respond to axotomy (Hu *et al*, 2016), and this is likely the case for other triggers of reactivation. Following a given stimulus, only a subpopulation of neurons fully reactivates, perhaps suggesting that certain viral genomes or populations of neurons are not capable of progressing through to full reactivation. Understanding how heterogeneity contributes to reactivation potential of latently infected neurons will have significant therapeutic and basic science value.

Reactivation

The ability to study the kinetics of lytic gene expression following interruption of NGF-signaling in tractable *in vitro* systems has highlighted key differences in the mechanism of viral gene expression from silenced latent genomes, as compared to *de novo* lytic infection. In this context, reactivation occurs as a two-stage program that overcomes a more compact viral chromatin structure and the absence of tegument factors, such as VP16 (Cliffe & Wilson, 2017). During the first stage, termed phase I, there is a transient burst of lytic gene transcription. This first wave of synchronous lytic gene expression leads to the simultaneous synthesis of many lytic transcripts with the potential to encode IE, E, and L viral proteins (Kim *et al*, 2012). A similar synchronous wave of gene expression has been observed following *ex vivo* reactivation when explant was combined with NGF-deprivation (Du *et al*, 2011) and with neuronal hyperstimulation-induced reactivation (Cuddy *et al.*, 2020), indicating that the specific stimuli, and not experimental system, impacts the mechanism of reactivation. Importantly, phase I is not dependent on the viral transactivator VP16 in primary neurons (Kim *et al.*, 2012). Furthermore, neither the synthesis of IE proteins nor viral DNA replication is necessary for the expression of viral E or L genes (Kim *et al.*, 2012). Viral gene expression in phase I is dependent on activation of c-Jun N-terminal kinase (JNK) cell stress response via activation of dual leucine zipper kinase (DLK), but it is independent of histone demethylases that have been implicated in the removal of repressive H3K9 and H3K27 methylation marks (Cliffe *et al.*, 2015; Cuddy *et al.*, 2020). DLK functions as a key regulator of the axon response, promoting different outcomes depending on the nature of the stimulus (Tedeschi & Bradke, 2013; Geden & Deshmukh, 2016), and may also be a key mediator of HSV reactivation, as one of its downstream targets, JNK, is required for HSV reactivation following explant (Cliffe *et al.*, 2015).

Histones associated with viral lytic promotors are phosphorylated on S10 in a JNK-dependent manner, resulting in a H3K9me3pS10 histone methyl/phospho switch. This switch could permit increased viral gene expression without the need to recruit histone demethylases and allow gene silencing to re-occur if the genome does not progress to full reactivation (Cliffe *et al.*, 2015; Cuddy *et al.*, 2020). The second phase of reactivation closely resembles *de novo* infection and is hypothesized to occur only if threshold amounts of key viral proteins are synthesized during phase I (Kim *et al.*, 2012). Similar to *de novo* infection, viral gene expression during phase II is dependent on histone demethylase enzyme activity and may require the viral transactivator VP16 (Kim *et al.*, 2012; Cliffe *et al.*, 2015). Ultimately, phase II results in the amplification of viral DNA and production of infectious viral particles (Kim *et al.*, 2012). It is important to note that other models of reactivation, including axotomy, are dependent on VP16 and the activities of LSD-1 and JMJD2, two histone K9 demethylases, and result in much more rapid viral gene expression that is not bi-phasic (Liang *et al*, 2009).

**The role of interferons in HSV infection**

Interferons (IFNs) are a broad group of cytokines elicited in response to the sensing of pathogen-derived products that act in an autocrine or paracrine manner to induce intracellular antimicrobial responses, as well as activate and modulate the innate and adaptive immune responses (Ivashkiv & Donlin, 2014). Interferons are named for shared ability to ‘interfere’ with viral replication and are divided into three classes, Type I, II and III, that signal through distinct heterodimeric receptors to activate the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway (Isaacs & Lindenmann, 1957; McNab *et al*, 2015).

<u>Type I IFN</u>

Type I IFNs are comprised of IFNα and IFNβ, the most broadly expressed and best characterized subtypes, as well as IFNε, IFNω, IFNκ, IFNτ, IFNζ and IFNδ (Pestka *et al*, 2004). Although plasmacytoid dendritic cells are considered a primary source of type I IFNs, most cell types can produce type I IFNs (Ali *et al*, 2019). Other critical sources include epithelial cells, fibroblasts and macrophages depending on the pathogen (Ali *et al.*, 2019). Type I IFNs all bind and act through the same heterodimeric cell-surface receptor, IFNAR, which is composed of IFNAR1 and IFNAR2 and found on most, if not all, nucleated cells (Schreiber, 2017). Differential activation and signaling can result from differences in binding affinity, binding length and cell-type specific variations such as number of surface receptors, but on engagement, the IFN-IFNAR receptor complex activates JAK1 and tyrosine kinase 2 (TYK2) (Schreiber, 2017). This leads to phosphorylation of STAT1 and STAT2 which form complexes, including the interferon-stimulated gene factor 3 (ISGF3) complex (composed of STAT1, STAT2 and IFN-regulatory factor 9) and STAT1 homodimers, that translocate to the nucleus and bind to IFN-stimulated response elements (ISREs) and gamma-activated sequences (GASs), respectively (Stark & Darnell, 2012). Binding of these promoter elements ultimately leads to transcription of hundreds of genes involved in antiviral responses, including interferon-stimulated genes (ISGs) (Schoggins *et al*, 2011; MacMicking, 2012; Stark & Darnell, 2012).

Type II and type III IFN

Type II IFN or IFNγ is predominantly secreted by activated immune cells, including natural killer (NK) and T-cells, and production can be directly stimulated by exposure to cytokines, including type I and III IFN and interleukin (IL)-12, 15 and 18 (Ank *et al*, 2006; Pegram *et al*, 2011; Ivashkiv, 2018). Type II IFN binds to the interferon gamma receptor (IFNGR) consisting of the IFNGR1 and IFNGR2 subunits, which

activate JAK1 and JAK2 respectively. The activation of these kinases leads to the phosphorylation and homodimerization of STAT1 which then translocates to the nucleus and binds GASs (Gotthardt & Sexl, 2016; Ivashkiv, 2018). In contrast to type I IFNs, type II IFN does not induce the formation of the ISGF3 complexes and, thus, does not induce transcription of genes with ISREs. The major target cells of type II IFN are antigen presenting cells (APCs) to enhance T-cell activation and stimulation of the adaptive immune response, as well as macrophages to induce nitric oxide (NO) production (Lee & Ashkar, 2018). Type II IFN has also been shown to promote an antiviral state in a number of different cell types, often by working in concert with and amplifying type I IFN activity (Lee & Ashkar, 2018).

Type III IFN or IFNλs bind to a distinct heterodimeric receptor complex, IFNLR, consisting of the IFNLR1 (IL-28RA) and shared IL-10R2 subunits but possess many similarities with type I IFNs (Lazear *et al*, 2015). Similar to that induced by type I IFNs, the type III receptor signaling cascade activates JAK1 and TYK2 which leads to the phosphorylation of STAT1 and STAT2 and formation of the ISGF3 complex. After translocating to the nucleus, the ISGF3 complex then binds to ISREs in the promoters of various ISGs to induce gene expression and activate antiviral activities (Lazear *et al.*, 2015). Production of type III IFN is inducible in a number of cell types, including both immune and non-immune cells, following infection with a number of viruses or following treatment with type I IFNs (Ank *et al.*, 2006). However, unlike type I IFN receptors, type III IFN receptors are largely restricted to epithelial cells highlighting their importance at barrier surfaces including the skin and mucosal membranes that line the respiratory, digestive and reproductive tracts (Durbin *et al*, 2013; Mesev *et al*, 2019).

Specificity of interferon responses

Type I, II and III IFNs are unrelated from a sequence and structural perspective and signal through three distinct receptors, but there are functional similarities due to a high overlap in types of induced-genes. However, there is a growing body of evidence to suggest significantly different functions and modes of actions for these different types of IFNs. First, transcriptional profiling highlights unique gene expression signatures following treatment with different types of IFN (Der *et al*, 1998; Levy *et al*, 2011; Odendall & Kagan, 2015). In addition, the canonical and non-canonical signaling pathways emanating from the receptor complexes is impacted by a number of variables, including levels of receptors on the plasma membrane, the binding affinities of different IFNs and the presence, activation and regulation of downstream signaling components (Jaks *et al*, 2007; Lavoie *et al*, 2011; Green *et al*, 2017; Mendoza *et al*, 2017). However, how different IFNs promote unique antiviral environments and how the specificity of IFN responses is maintained is still largely unknown.

Role of interferons in acute HSV infection

A role for type I IFNs in restricting HSV replication and disease was first identified in mouse strains that are either resistant or susceptible to HSV-1 infection and supported by studies that found higher mortality in resistant mice treated with anti-interferon serum (Lopez, 1975; Gresser *et al*, 1976; Zawatzky *et al*, 1982; Gill *et al*, 2006). Type I IFNs have now been shown to restrict HSV viral replication and spread both *in vitro* and *in vivo* (Hendricks *et al*, 1991; Mikloska *et al*, 1998; Mikloska & Cunningham, 2001; Sainz & Halford, 2002; Jones *et al*, 2003), and additional studies utilizing knockout mouse models and cell lines have implicated multiple components of the type I interferon pathway as important in the control of HSV infection, including toll-like receptor (TLR)-3 and 6, RIG-1, cGAS and IFI16 in the sensing of HSV (Jacquemont & Roizman, 1975; Krug *et al*, 2004; Malmgaard *et al*, 2004; Li *et al*, 2013; Johnson *et al*, 2014; Ma & He,

2014; Orzalli *et al*, 2015), the type I IFN receptor complex IFNAR (Luker *et al*, 2003; Conrady *et al*, 2011; Wilcox *et al*, 2016), and the downstream effectors STAT1 (Halford *et al*, 2006) and interferon regulator factor (IRF) 3 and 7 (Murphy *et al*, 2013). The role of type I IFN signaling in controlling HSV infection is further supported by studies that have investigated HSV immune evasion strategies and found a number of HSV proteins that act to subvert the antiviral effects of type I IFNs (Wilcox & Longnecker, 2016). The viral proteins ICP0 and ICP27 have been shown to inhibit Nf-kB and IRF-3 activation (Melroe *et al*, 2007; Kim *et al*, 2008; van Lint *et al*, 2010), and γ34.5, a major HSV virulence factor, has been shown to inhibit TANK binding kinase 1 (TBK1) and reverse host shutoff of protein synthesis by mediating dephosphorylation of eIF2α and IKKα (Chou & Roizman, 1992, 1994; He *et al*, 1997; Verpooten *et al*, 2009; Wilcox *et al*, 2015). Furthermore, genetic studies have found that increased susceptibility to HSV-1 infection and higher incidence of herpes simplex encephalitis (HSE) in children and adults, where only the brain is affected, is correlated to mutations in components of the type I IFN pathway (Casrouge *et al*, 2006; Zhang *et al*, 2007; Sancho-Shimizu *et al*, 2011; Herman *et al*, 2012; Andersen *et al*, 2015). HSE has an estimated prevalence of 1 in 500,000 individuals per year (Whitley, 2006), but inborn errors of single genes of the Toll-like receptor-3 (TLR3) signaling pathway that impair CNS type I IFN production in response to HSV-1 are causal of HSE and the likelihood of developing HSE is thought to be significantly higher in these individuals than in the general population (Zhang *et al.*, 2007; Andersen *et al.*, 2015).

Type II IFN also plays a critical role in controlling acute HSV infection. Similar to IFNAR knockout mice, IFNGR knockout mice are unable to adequately control HSV replication, leading to increased viral dissemination and mortality (Cantin *et al*, 1999a; Cantin *et al*, 1999b; Lekstrom-Himes *et al*, 2000). IFNγ-ligand-null mutant mice (Cantin

*et al.*, 1999b) and mice treated with neutralizing anti-IFNγ antibodies *in vivo (Smith et al, 1994)* also have a significantly diminished ability to clear infectious HSV. Interestingly, type I and type II interferons seem to work synergistically in limiting HSV infection, as treatment with both type I and type II IFNs more potently inhibits HSV replication *in vitro* and *in vivo* than with either type I IFN or type II IFN alone (Sainz & Halford, 2002), and dual knockout of IFNAR and IFNGR leads to uncontrollable dissemination of HSV and mortality *in vivo* (Luker *et al.*, 2003). There is also growing evidence to suggest a role for type III IFNs in the response to acute HSV infection. Similar to IFNα, IFNλ-treatment alone decreases the transcription of HSV-1 ICP27, and IFNλ in combination with IFNα further amplifies the anti-HSV-1 response (Melchjorsen *et al*, 2006). This suggests that IFNλ and IFNα mediate similar antiviral responses to HSV-1. However, although IFNλ has been shown to restrict HSV replication and cytotoxic activity *in vitro*, it potently blocks viral replication and disease in vaginal mucosa in an *in vivo* model of localized HSV-2 infection, even more so that IFNα, indicating that type III IFNs could be exerting its antiviral effects through stimulation of the immune response versus induction of an antiviral state (Ank *et al.*, 2006).

Role of interferons in latent HSV infection and reactivation

During HSV infection, interferons are elevated within peripheral ganglia (Cantin *et al*, 1995; Shimeld *et al*, 1995; Halford *et al*, 1996, 1997; Carr *et al*, 1998; Chen *et al*, 2000; Lucinda *et al*, 2017). Because sensory neurons are reported to have an impaired intrinsic immune response to HSV, it is thought that they rely on exogenous sources of IFN acting via a paracrine mechanism to potentiate an antiviral response (Liu *et al*, 2001; Van Opdenbosch *et al*, 2011; Low-Calle *et al*, 2014; Rosato & Leib, 2014). Although there is evidence to suggest that interferon signaling could be impaired in peripheral neurons to promote cell survival (Yordy *et al*, 2012), a number of studies have shown

that treatment of cultured peripheral neurons with type I IFNs leads to STAT1 nuclear localization and induction of ISGs (Wang & Campbell, 2005; Rosato & Leib, 2015; Song *et al*, 2016b; Linderman *et al.*, 2017). It is important to note that neuronal antiviral signaling can depend on neuronal subtype and differentiation, which could lead to these discrepancies (Cho *et al*, 2013; Farmer *et al*, 2013; Schultz *et al*, 2015). Importantly, treatment of cultured peripheral neurons with type I IFNs has been shown to restrict HSV-1 replication (Svennerholm *et al*, 1989; Carr *et al*, 2003; Low-Calle *et al.*, 2014; Rosato & Leib, 2015; Linderman *et al.*, 2017). Restriction of HSV replication occurs with isolated treatment of cell bodies through canonical IFN signaling, as well as with isolated treatment of axons through mechanisms independent of IFN signaling at the soma (Rosato & Leib, 2015). Additional studies have further demonstrated that both type I and type II IFNs can specifically limit HSV-1 retrograde axonal transport (Song *et al.*, 2016b). Treatment with type I IFNs can also promote the establishment of a quiescent HSV infection in cultured neurons (De Regge *et al*, 2010) and has been shown to influence the nuclear distribution of latent genomes during the establishment of latency *in vivo (*Maroui *et al, 2016)*, but neuronal IFN signaling is dispensable for latency establishment *in vivo (*Rosato *et al, 2016)*. Interestingly, Rosato *et al.* (2016) also showed reduced reactivation in TG explant cultures from mice lacking IFN signaling, suggesting that IFN signaling helps resolve acute infection and supports neuronal survival. However, a study in latently infected neuronal cultures showed that inducible reactivation of HSV-1 is transiently sensitive to type I IFNs (Linderman *et al.*, 2017).

Type II IFN is secreted from CD4+ and CD8+ lymphocytes and macrophages in latently infected ganglia *in vivo (*Cantin *et al., 1995)*. Type II IFN is dispensable for latency establishment (Ellison *et al*, 2000) but could regulate the number of latently infected neurons (Lekstrom-Himes *et al.*, 2000) and has a critical role in rapid

suppression of HSV replication following reactivation events (Cantin *et al.*, 1999a; Liu *et al.*, 2001; Knickelbein *et al*, 2008). Type II IFN has also been shown to limit HSV replication in peripheral neurons *in vitro* in a neuron-intrinsic manner *(*Li *et al, 2010; Linderman et al., 2017)*. Although type I and II IFN signal through distinct receptors, the cellular responses to both types of interferons depend on JAK activity and the STAT1 signaling molecule. Furthermore, type I and type II IFNs have overlapping signaling pathways downstream of their respective receptors, including the formation of STAT1 homodimers that bind ISREs. RNA-sequence analysis of IFNβ- or IFNγ-treated neurons undergoing reactivation showed a number of commonly upregulated host genes, including Mx1/2, Daxx and other known antiviral genes. However, they also expressed IFNβ- or IFNγ-specific transcript profiles that could modify the antiviral response (Linderman *et al.*, 2017). Interestingly, although both IFNβ and IFNγ have been shown to suppress HSV-1 reactivation, sensitivity to IFNγ is selectively lost when ICP0, a HSV-1 IE viral protein, is present prior to reactivation (Linderman *et al.*, 2017). This is unlike IFNβ, which can block HSV-1 reactivation even in the presence of ICP0 (Linderman *et al.*, 2017). Although specificity of type I- and type II IFN-mediated responses have not been described, it could be reflected by the unique gene expression signatures of type I or type II-treated neurons. Interestingly, type I and II IFNs have been shown to inhibit axonal transmission of HSV from cultured peripheral neurons to epithelial cells (Mikloska & Cunningham, 2001), highlighting differences in IFN responses in neuronal versus non-neuronal cells. Although not as well characterized, type III IFN has also been shown to limit replication of HSV in human neurons *in vitro (Li et al., 2010)*, and a single nucleotide polymorphism in the promoter of IFNλ3 has been associated with increased disease severity of recurrent HSV herpes labialis (Griffiths *et al*, 2013), and severity of recurrent herpes labialis disease inversely correlated with IFNλ serum levels (Pica *et al*, 2010).

**The role of promyelocytic leukemia nuclear bodies in HSV infection**

Promyelocytic leukemia nuclear bodies (PML-NBs) are heterogenous, phase-separated nuclear condensates made up of the PML tumor suppressor protein that is necessary for PML-NB assembly and a growing number of either constitutively- or transiently-associated proteins that share the ability to be SUMOylated (Maul *et al*, 2000; Lallemand-Breitenbach & de The, 2010; Van Damme *et al*, 2010).

PML-NB dynamics

PML-NBs have been described in most mammalian cells, but PML-NB number can differ significantly depending on cell type, differentiation stage and cell cycle phase (Bernardi & Pandolfi, 2007; Lallemand-Breitenbach & de The, 2010). Furthermore, PML-NBs are incredibly dynamic, heterogenous structures, and their number, size and composition can undergo significant changes in response to cellular stress and soluble factors (Bernardi & Pandolfi, 2007; Lallemand-Breitenbach & de The, 2010). Agents that cause DNA damage, heat shock, heavy metals and inhibition of transcription or the proteosome can promote either the aggregation or dispersal of PML-NBs (Lallemand-Breitenbach & de The, 2010). Furthermore, type I and II IFN treatment directly induces the transcription of PML, Daxx, Sp100 and other PML-NB constituents, which leads to elevated protein synthesis and a robust increase in both size and number of PML-NBs (Chelbi-Alix *et al*, 1995; Stadler *et al*, 1995; Grotzinger *et al*, 1996a; Greger *et al*, 2005; Shalginskikh *et al*, 2013).

Function of PML-NBs

PML-NBs have been implicated in a number of cellular processes. They have been associated with the transcriptional activation of cellular genes (Wang *et al*, 2004;

Bernardi & Pandolfi, 2007; Lallemand-Breitenbach & de The, 2010; Kim & Ahn, 2015; McFarlane *et al*, 2019), but also can recruit repressor proteins, including ATRX, Daxx and Sp100, that promote transcriptional repression and inhibition of both DNA and RNA virus replication (Zhong *et al*, 2000; Garrick *et al*, 2004; Bishop *et al*, 2006; Everett & Chelbi-Alix, 2007; Xu & Roizman, 2017). PML-NBs are also hypothesized to play a role in DNA damage responses, as they have been shown to increase in size and number following DNA double-stranded breaks (Dellaire *et al*, 2006), colocalize to sites of DNA repair and single-stranded breaks (Xu *et al*, 2003) and recruit DNA repair factors in a temporally regulated manner (Dellaire *et al.*, 2006). PML has also been shown to play a role in cell senescence (Ferbeyre *et al*, 2000; Pearson *et al*, 2000), as well as cell death through the induction of both p53-dependent and -independent apoptotic pathways (Quignon *et al*, 1998; Wang *et al*, 1998; Guo *et al*, 2000). Whether PML-mediated regulation of these pathways occurs in the context of PML-NBs or by PML itself is unclear, but interestingly, the pro-apoptotic functions of Daxx, a PML-NB-associated protein, may require localization to PML-NBs in certain cell types (Croxton *et al*, 2006). Importantly, there is growing evidence implicating a role for PML-NBs and PML-NB-associated proteins in host antiviral defenses, as they have been shown to associate with viral genomes and are disrupted by DNA and RNA viruses through a variety of mechanisms (reviewed in Everett & Chelbi-Alix (2007), Geoffroy & Chelbi-Alix (2011), Komatsu *et al* (2016); Scherer & Stamminger (2016)).

Role in lytic HSV infection

In the context of lytic infection of non-neuronal cells, PML-NBs have been shown to closely associate with HSV-1 genomes (Ishov & Maul, 1996; Maul *et al*, 1996; Maul, 1998; Everett *et al*, 2006; Everett *et al*, 2008; Lukashchuk & Everett, 2010; Glass & Everett, 2013; Merkl *et al*, 2018). This was first shown in plaque-edge recruitment

studies, where PML-NB components relocated to the viral DNA entry site in newly infected cells at the edge of a developing plaque (Everett *et al.*, 2006; Everett *et al.*, 2008), and later confirmed using 5-Ethynyl-2'-deoxyuridine (EdU) labelling of HSV-1 DNA in combination with click chemistry (Alandijany *et al*, 2018). Interestingly, the HSV-1 immediate-early and viral regulatory protein ICP0 also colocalizes with PML-NBs very early during infection (Everett & Maul, 1994) and is known to disrupt the integrity of these structures by targeting PML and other PML-NB associated proteins, including SP100, for degradation (Everett & Maul, 1994; Chelbi-Alix & de The, 1999; Muller & Dejean, 1999; Boutell *et al*, 2002; Everett *et al.*, 2008). Importantly, replication of ICP0-null HSV-1 mutants is restricted, and PML, SP100, ATRX and Daxx have been shown to directly contribute to the cellular repression of ICP0-null mutant viruses (Lium & Silverstein, 1997; Everett *et al.*, 2006; Everett *et al.*, 2008; Lukashchuk & Everett, 2010; Glass & Everett, 2013; Gu *et al*, 2013), indicating that PML-NBs entrapment of HSV-1 genomes during lytic infection create a transcriptionally repressive environment for viral gene expression. It should be noted that studies have suggested a possible pro-viral role for PML-NBs in the context of lytic infection. Merkl *et al.* (2018) showed a decrease in ICP0-null HSV replication in fibroblasts depleted of PML. The authors note this could be a result of the siRNA-mediated knockdown of PML, as shRNA knockdown of PML led to increased ICP0-null virus replication in the same study, but also hypothesized that different PML isoforms could differentially impact viral replication. Two additional studies found significantly reduced replication of WT HSV-1 in PML-/- cells (Xu *et al*, 2016; Xu & Roizman, 2017), further suggesting a more complex role for PML-NBs in lytic HSV infection.

Role in latent infection

Latent HSV-1 genomes have been shown to associate with Promyelocytic leukemia nuclear bodies (PML-NBs) in mouse models of infection, as well as in human autopsy material (Catez *et al.*, 2012; Maroui *et al.*, 2016). In the context of latency, neurons containing PML-encased latent genomes exhibit decreased expression levels of the LAT (Catez *et al.*, 2012), suggesting that they are more transcriptionally silent than latent genomes localized to other nuclear domains. Previous work by Cohen *et al* (2018) and Everett *et al* (2007) showed that quiescent genomes associated with PML-NBs in fibroblasts can be transcriptionally reactivated by induced expression of ICP0. However, these previous studies did not address the capability of viral genomes to reactivate in the absence of viral lytic protein (e.g. during reactivation from latency in neurons). In a separate study using cultured adult TG neurons, treatment of quiescently-infected neurons with the histone deacetylase inhibitor, trichostatin A (TSA), led to disruption of PML-NBs and induced active viral transcription in a subset of PML-NB-associated genomes (Maroui *et al.*, 2016). However, the mechanisms of reactivation following TSA treatment are not known, and may be direct via altering the HSV chromatin structure or indirect via increasing the acetylation levels of histones or non-histone proteins, including PML.

# CHAPTER 2

*A Restricted Form of HSV Latency Is Mediated by PML-NB-Dependent Memory of Type I Interferon Treatment*

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**Abstract**

Herpes simplex virus (HSV) establishes latent infection in long-lived neurons. During initial infection, neurons are exposed to multiple inflammatory cytokines but the effects of immune signaling on the nature of HSV latency are unknown. We show that initial infection of primary murine neurons in the presence of type I interferon (IFN) results in a form of latency that is restricted for reactivation. We also find that the subnuclear condensates, promyelocytic leukemia-nuclear bodies (PML-NBs), are absent from primary sympathetic and sensory neurons but form with type I IFN treatment and persist even when IFN signaling resolves. HSV-1 genomes colocalize with PML-NBs throughout a latent infection of neurons only when type I IFN is present during initial infection. Depletion of PML prior to or following infection does not impact the establishment of latency; however, it does rescue the ability of HSV to reactivate from IFN-treated neurons. This study demonstrates that viral genomes possess a memory of the IFN response during *de novo* infection, which results in differential subnuclear positioning and ultimately restricts the ability of genomes to reactivate.

**Introduction**

Herpes simplex virus-1 (HSV-1) is a ubiquitous pathogen that persists in the form of a lifelong latent infection in the human host. HSV-1 can undergo a productive lytic infection in a variety of cell types; however, latency is restricted to post-mitotic neurons, most commonly in sensory, sympathetic and parasympathetic ganglia of the peripheral nervous system (Baringer & Swoveland, 1973; Warren *et al.*, 1978; Baringer & Pisani, 1994; Richter *et al.*, 2009). During latent infection, the viral genome exists as an episome in the neuronal nucleus, and there is considerable evidence that on the population level viral lytic gene promoters assemble into repressive heterochromatin (Wang *et al.*, 2005; Cliffe & Knipe, 2008; Cliffe *et al.*, 2009; Kwiatkowski *et al.*, 2009). The only region of the HSV genome that undergoes active transcription, at least in a fraction of latently infected cells, is the latency associated transcript (LAT) locus (Stevens *et al.*, 1987; Kramer & Coen, 1995). Successful establishment of a latent gene expression program requires a number of molecular events, likely influenced by both cellular and viral factors, and is not uniform (Efstathiou & Preston, 2005). Significant heterogeneity exists in expression patterns of both lytic and latent transcripts in latently-infected neurons, as well as in the ability of latent genomes to reactivate in response to different stimuli (Sawtell, 1997; Proenca *et al.*, 2008; Catez *et al.*, 2012; Ma *et al.*, 2014; Maroui *et al.*, 2016; Nicoll *et al.*, 2016). This heterogeneity could arise from viral genome copy number, exposure to different inflammatory environments or intrinsic differences in the neurons themselves. Furthermore, there is growing evidence that heterogeneity in latency may ultimately be reflected in part by the association of viral genomes with different nuclear domains or cellular proteins (Catez *et al.*, 2012; Maroui *et al.*, 2016). However, what determines the subnuclear distribution of latent viral genomes is not known. In addition, it is currently unclear whether viral genome association with certain nuclear domains or cellular proteins results in an increased or decreased ability of the

virus to undergo reactivation. The aim of this study was to determine whether the presence of interferon during initial HSV-1 infection can intersect with the latent viral genome to regulate the type of gene silencing and ultimately the ability to undergo reactivation. Because the fate of viral genomes and their ability to undergo reactivation can be readily tracked, latent HSV-1 infection of neurons also serves as an excellent system to explore how exposure to innate immune cytokines can have a lasting impact on peripheral neurons.

PML expression is induced by both type I and II IFNs, as the PML promoter contains both ISRE and GAS elements, and this leads to a marked increase in size and number of PML-NBs (Chelbi-Alix *et al.*, 1995; Lavau *et al*, 1995; Stadler *et al.*, 1995). Importantly, binding of IFN-inducible transcription factors to these elements appears to be strong for the PML IFNα/β-stimulated response element ISRE, but weak for the PML IFNγ-activation site GAS, suggesting the importance PML as a primary target gene of type I IFNs. In addition to PML, type I and II IFNs induce the synthesis of other PML-NB associated proteins, including SP100, ISG20, SP120, SP140 and PA28 (Guldner *et al*, 1992; Lavau *et al.*, 1995; Grotzinger *et al.*, 1996a; Grotzinger *et al*, 1996b; Gongora *et al*, 1997). Furthermore, type I IFNs have been shown increase global SUMOylation, which is necessary for the formation of PML-NBs and for that localization of many PML-NB-associated proteins (Maroui *et al*, 2018).

Although *in vivo* models are incredibly powerful tools to investigate the contribution of the host immune response to HSV infection, they are problematic for investigating how individual components of the host's immune response specifically regulate neuronal latency. Conversely, *in vitro* systems provide a simplified model that lack many aspects of the host immune response. Therefore, to investigate the role of

type I IFN on HSV-1 latency and reactivation, we utilized a model of latency in primary murine sympathetic neurons (Cliffe *et al.*, 2015), which allowed us to manipulate conditions during initial HSV-1 infection and trigger synchronous robust reactivation. Using this model, we show that primary neurons isolated from mouse peripheral ganglia are largely devoid of detectable PML-NBs but PML-NBs form following type I IFN exposure and persist even when ISG gene expression and production of other antiviral proteins have returned to baseline. Neither exogenous type I IFN nor detectable PML-NBs are required for HSV gene silencing and entry into latency in this model system, but, importantly, the presence of IFNα specifically at the time of initial infection results in the entrapment of viral genomes in PML-NBs and a more restrictive form of latency that is less able to undergo reactivation. This study therefore demonstrates how the viral latent genome has a long-term memory of the innate response during *de novo* HSV infection that results in entrapment of genomes in PML-NBs and a more repressive form of latency.

**Results**

Interferon induces the formation of detectable PML-NBs in primary sympathetic and sensory neurons isolated from postnatal and adult mice.

We initially set out to investigate the contribution of PML-NBs to HSV latency and reactivation using primary sympathetic and sensory neurons that have been well characterized as *in vitro* models of HSV latency and reactivation (Wilcox & Johnson, 1987; Wilcox *et al.*, 1990; Camarena, 2011; Cliffe *et al.*, 2015; Ives & Bertke, 2017; Cuddy *et al.*, 2020). In addition, primary neuronal systems allow for much more experimental control of specific conditions during *de novo* infection and can be easily manipulated either immediately prior to or following infection. Peripheral neurons were isolated from the superior cervical ganglia (SCG) or trigeminal ganglia (TG) from young

(post-natal day; P1) or adult (>P28) mice and cultured for 6 days prior to staining. PML-NBs were defined as detectable punctate nuclear structures by staining for PML protein. Strikingly, we observed that both SCG and TG neurons were largely devoid of detectable PML-NBs (Fig. 2-1A).

In certain cell types, the transcription of certain PML-NB associated proteins, including PML, can be induced by either type I or type II interferon (IFN) treatment, which is correlated with an increase in PML-NB size and/or number per cell (Chelbi-Alix *et al.*, 1995; Stadler *et al.*, 1995). Therefore, we were interested in determining whether exposure of primary sensory or sympathetic neurons to different types of IFN resulted in PML-NB formation. Type I IFN treatment using IFN-alpha (IFNα) (Fig. 2-1B, 2-1C-F) or IFN-beta (Fig. 2-2A) led to a significant induction of detectable PML-NBs in both sensory and sympathetic neurons isolated from postnatal and adult mice. Representative images of IFNα-treated neurons are shown (Fig. 2-1B) and number of detectable PML-NBs per neurons are quantified (Fig. 2-1C-F). The increase in detectable PML-NBs was comparable for both 150 IU/ml and 600 IU/ml of IFNα. Type II IFN (IFNγ) led to a more variable response with a small but significant increase in detectable PML-NBs in a subpopulation of sympathetic neurons. However, IFNγ treatment of sensory neurons did not result in the formation of detectable PML-NBs. Exposure of neurons to IFN-lambda 2 (IFN-λ2), a type III IFN, did not induce the formation of detectable PML-NBs in either sympathetic or sensory neuron cultures (Fig. 2-1C-F; Fig. 2-2B,C). Therefore, PML-NBs are largely undetectable in primary sympathetic and sensory neurons but can form upon exposure to type I IFNs.

<u>Type I IFN treatment specifically at time of infection restricts reactivation of HSV-1 from primary sympathetic neurons without affecting initial infectivity or LAT expression.</u>

Because we observed that primary SCG neurons are largely devoid of PML-NBs and that PML-NBs form upon treatment with type I IFN treatment, we wanted to investigate whether type I IFN treatment at the time of infection impacted the ability of HSV to establish latency or reactivate in this model system. SCG neurons were pre-treated with IFNα (600 IU/ml) for 18h and during the initial 2h HSV inoculation (Fig. 2-3A). Following inoculation, IFNα was washed out and an IFNAR1 blocking antibody was used to prevent subsequent type I IFN signaling through the receptor. To confirm the effectiveness of the IFNAR1 ab to block detectable IFN signaling, we validated it by its ability to block ISG expression (ISG15) in cultured SCG neurons by RT-qPCR (Fig. 2-4A). Reactivation was induced and initially quantified based on the number of GFP positive neurons at 3-days post-stimuli. We found that full reactivation was restricted in neurons exposed to type I IFN just prior to and during *de novo* infection (Fig. 2-3B). We further confirmed this IFNα-mediated restriction of latency by the induction of lytic mRNAs upon reactivation. IFNα treatment at the time of infection significantly decreased the expression of immediate early gene (ICP27), early gene (ICP8) and late gene (gC) at 3 days post-reactivation (Fig. 2-3C, 2-4B, C). There were very few GFP-positive neurons and little to no viral gene expression in mock reactivated controls, further indicating that latency can be established in the presence and absence of IFN.

Reactivation of HSV in this system proceeds over two phases. GFP-positive neurons is a readout for full reactivation or Phase II. However, we and others have observed an initial wave of lytic gene expression that occurs prior to and independently of viral DNA replication at around 20 hours post-stimulus, termed Phase I (Du *et al.*, 2011; Kim *et al.*, 2012; Cliffe *et al.*, 2015; Cliffe & Wilson, 2017). Therefore, to determine if IFNα treatment at the time of infection restricted the Phase I wave of lytic we carried out RT-qPCR to detect representative immediate-early (ICP27), early (ICP8), and late

(gC) transcripts at 20 hours post addition of LY294002. We found significantly decreased expression in the IFNα-treated neurons (Fig. 2-3D, 2-4D, E). This is interesting as exogenous type I IFNs have previously been shown to suppress reactivation in murine neurons by preventing Phase I and are rendered ineffective once Phase I viral products accumulate (Linderman *et al.*, 2017). Therefore, type I IFN treatment solely at the time of infection has a long-term effect on the ability of HSV to initiate lytic gene expression and undergo reactivation.

Because IFN treatment could reduce nuclear trafficking of viral capsids during initial infection or impact infection efficiency, we next determined whether equivalent numbers of viral genomes were present in the neuronal cultures. At 8dpi, we measured relative viral DNA genome copy numbers in SCG neurons that were treated with IFNα compared to untreated controls and found no significant difference (Fig 2-3E). To further confirm that equivalent genomes were present in the neuronal nuclei, we infected neurons with HSV-1 containing EdC-incorporated genomes and performed click chemistry to detect vDNA foci. At 8 dpi, we found no significant difference in the average number of vDNA foci per nucleus of neurons treated with IFNα at the time of initial infection compared to untreated controls (Fig. 2-3F). Therefore, the restricted reactivation phenotype mediated by IFNα was not due to a decrease in the number of latent viral genomes.

The decreased reactivation observed with IFNα treatment could be secondary to changes in expression of the LAT and/or directly as a result of decreased viral genome accessibility. The HSV LAT, one of the only highly expressed gene products during latent infection, has been shown to modulate several features of latency, including the viral chromatin structure, lytic gene expression, and neuronal survival, as well as the

efficiency of latency establishment and reactivation (Leib *et al.*, 1989; Hill *et al*, 1990; Trousdale *et al.*, 1991; Gordon *et al*, 1995; Chen *et al*, 1997; Garber *et al*, 1997; Thompson & Sawtell, 1997; Perng *et al*, 2000; Thompson & Sawtell, 2001; Knipe & Cliffe, 2008). Therefore, the ability of HSV to undergo reactivation could be due to changes in LAT expression following IFNα treatment. However, when we evaluated LAT expression levels at 8 dpi by RT-qPCR, we found no detectable difference between IFNα-treated and untreated cultures of neurons. This suggests that the IFNα-mediated restriction in reactivation does not appear to occur as a result of changes in expression of the LAT (Fig. 2-3G). Therefore, it is possible that the type I IFN-mediated restriction of HSV latency is due to changes to the latent genome that results in a decreased ability to undergo reactivation following PI3-kinase inhibition.

Primary neurons have a memory of prior IFNα exposure characterized by persistence of PML-NBs

Because we observed a restriction in the ability of HSV to reactivate that occurred 7-8 days following type I IFN exposure, we went on to examine any long-term changes resulting from IFNα exposure. First, we investigated the kinetics of representative ISG expression. As expected, we saw a robust induction of *Isg15* and *Irf7* in IFNα-treated (600 IU/ml) neurons that persisted for at least 42 hours post-treatment post-addition of IFNα (this represents 1-day post-infection (dpi)). However, by 8 dpi, the time at which neurons were induced to reactivate, there was no detectable difference in *Isg15* or *Irf7* expression in IFNα treated neurons vs untreated controls (Fig. 2-5A, B), indicating that these representative ISGs were not detectably elevated at the time of reactivation. We also found similar *Isg15* and *Irf7* expression in HSV-1 infected neurons compared to uninfected controls, suggesting that HSV-1 infection was not impacting IFN signaling pathways at a population level. PML has been previously characterized as an

ISG product in non-neuronal cells (Chelbi-Alix *et al.*, 1995; Stadler *et al.*, 1995) and is responsive to both IFNβ and IFNγ in latently-infected rat sympathetic neurons induced to reactivate (Linderman *et al.*, 2017), and we found an approximate 5-fold-increased expression of *Pml* in primary sympathetic neurons following IFNα treatment which was less than the increased expression of *Irf7* and *Isg15* (approximate 250-fold- and 100-fold-increased expression respectively). *Pml* expression returned to untreated levels by 1 dpi (Fig. 2-5C).

Although we did not detect maintained induction of IFN stimulated gene expression including *Pml*, we were intrigued as to whether PML-NBs persisted throughout the course of infection. To assess this, we first established whether PML-NBs persist even in the absence of sustained ISG expression. Quantifying the number of PML-NBs following IFNα (600 IU/ml) treatment, we found that the number of bodies remain elevated through 15 days post-treatment (Fig. 2-5D). We went on to investigate additional products of ISGs including STAT1 and Mx1 because of the availability of specific antibodies against these proteins. We observed robust STAT1 staining following IFNα exposure for 18 hours. However, by 8 days post infection we could not detect STAT1 staining in primary neurons indicating that accumulation of this IFNα-induced protein had returned to baseline (Fig. 2-5E). Similarly, we found induction of punctate Mx1 staining in neurons exposed to IFNα for 18 hours that was undetectable by day 6 post-treatment (Fig. 2-5F). Therefore, exposure of primary neurons to type I IFN led to a modest induction of *Pml* mRNA but resulted in long-term persistence of PML-NBs, even in the absence of continued IFN signaling and when antiviral protein products of other ISGs were undetectable.

PML-NBs Persist and Stably Entrap Latent HSV-1 Genomes only if IFNα is Present at the Time of Initial Infection

The persistence of PML-NBs following IFN exposure raised the possibility that viral genomes are maintained within PML-NBs only in type I IFN-treated neurons. This would also suggest that PML-NB-associated genomes are less permissive for reactivation and provide us with an experimental system to investigate the contribution of PML-NBs to the maintenance of HSV latency. To determine whether viral genomes localize with PML-NBs in type I IFN-treated neurons, SCG neurons were pretreated with IFNα (600 IU/ml) then infected with HSV-$1^{EdC}$ at an MOI of 5 PFU/cell in the presence of ACV and IFNα as described above. By co-staining for PML, we found that a large proportion of vDNA foci colocalized with PML-NBs in the IFNα-treated neurons over the course of infection. In untreated neurons that are largely devoid of detectable PML-NBs, very few genomes were colocalized to PML puncta as expected. Representative images are shown (Fig. 2-6A) and the percent of genome foci colocalized to PML-NBs is quantified (Fig. 2-6B). Furthermore, high-resolution Airy scan-based 3D confocal microscopy of IFNα-treated neurons revealed that vDNA foci were entrapped within PML-NBs (Fig. 2-6C, D), as has also been reported upon lytic infection of non-neuronal cell lines (Alandijany *et al.*, 2018) and in latently infected TG *in vivo* (Catez *et al.*, 2012), and interestingly, we found that the volume of PML-NBs associated with vDNA is greater than PML-NBs not associated with vDNA (Fig. 2-6E). Previous studies have found that colocalization of viral DNA by PML-NBs during lytic HSV-1 infection of human fibroblasts occurs independently of type I IFN exposure (Maul *et al.*, 1996; Everett *et al*, 2004; Everett & Murray, 2005; Alandijany *et al.*, 2018), and we confirmed this was also the case in dermal fibroblasts isolated from postnatal mice (Fig. 2-7A, B), Therefore, the presence of IFNα during initial infection can impact the long-term subnuclear localization

of latent viral genomes in neurons by inducing PML-NBs that persist and stably entrap latent viral genomes.

Thus far, our data indicate that the presence of IFNα during initial infection determines subnuclear positioning of latent viral genomes and the ability of genomes to reactivate in response to inhibition of PI3 kinase activity. We considered that type I IFN treatment could have a long-term effect on cell signaling pathways which could impact the ability of HSV to reactivate. Therefore, to determine the direct versus indirect effects on the viral genome itself, we next investigated whether the timing of IFNα exposure had a differential effect on the ability of viral genomes to reactivate. We treated postnatal SCG neurons with IFNα (600 IU/ml) for 18h and during the 2h HSV inoculation (-18hpi) or exposed neurons to IFNα for 18h at 3 days prior to infection (-3dpi). Following pretreatment at -3dpi or -18hpi, IFNα was washed out and an IFNAR1 blocking antibody was used. As expected, IFNα during initial infection significantly inhibited HSV reactivation, but surprisingly, IFNα treatment at -3dpi did not restrict reactivation as shown by the similar number of GFP-positive neurons at 72 hours post-reactivation when compared to untreated neurons (Fig. 2-8A). Consistent with the reactivation data, we found that vDNA foci did not localize to PML-NBs in SCG neurons treated with IFNα at -3dpi (Fig. 2-8B). We confirmed that PML-NBs were present at the time of infection in neurons treated 3 day prior to infection (Fig. 2-8C), although we did detect slightly fewer PML-NBs per nucleus in neurons treated -3dpi compared to -18hpi (a mean of 17.57 versus 12.47 per nucleus respectively). We also confirmed comparable recruitment of known PML-NB-associated proteins ATRX, Daxx and SUMO-1 at 3 days post-IFNα treatment (Fig 2-9A-C). When IFNα treatment of SCG neurons is continued from -3dpi through infection, or if SCG neurons treated at -3dpi receive a second treatment of IFNα during infection, then a similar proportion of latent viral genomes colocalize with PML-

NBs as with a single treatment during infection (Fig. 2-9D). Together with the previous data on genome entrapment in dermal fibroblasts (Fig. 2-7A, B), these data indicate that type I IFN must be present during infection of neurons, but not necessarily non-neuronal cells, for vDNA to colocalize with PML-NBs.

The HSV Infected Cell Protein 0 (ICP0) is a RING-finger E3 ubiquitin ligase that is synthesized at very early stages of HSV-1 infection (Boutell *et al.*, 2002). During lytic infection, it localizes to PML-NBs and disrupts their integrity by targeting PML and other PML-NB associated proteins for degradation (Everett *et al*, 1998; Muller *et al*, 1998; Chelbi-Alix & de The, 1999; Boutell *et al.*, 2002; Boutell *et al*, 2011; Cuchet-Lourenco *et al*, 2012; Alandijany *et al.*, 2018). This activity is required for promoting the efficient onset of HSV-1 lytic replication, and ICP0-null mutants exhibit a defect in viral gene expression in certain cell types at low multiplicities of infection (Everett *et al.*, 2008). ICP0 mRNA is also known to be expressed during the establishment of latency (Cliffe *et al.*, 2013). Therefore, the colocalization of latent viral genomes to PML-NBs and ultimately the ability of HSV to undergo reactivation could be due the presence of IFNα during initial infection and its effect on the localization or amount of ICP0. To investigate the distribution of ICP0 at early time points post-infection, SCG neurons were treated with IFNα at either -3dpi or -18hpi and infected at a MOI of 7.5 PFU/cell with HSV-1 Us11-GFP in the presence of acyclovir (ACV). In both treatment groups, ICP0 staining similarly colocalized with puncta of ATRX, a correlate for PML-NBs, at 3-, 6- and 9-hours post-infection (Fig. 2-9E, F). Interestingly, foci of ATRX still remained even with the presence of ICP0, suggesting that ICP0 is not disrupting the integrity of PML-NBs in this system. To further investigate the effect of ICP0 on the colocalization of latent viral genomes to PML-NBs, we generated an EdC-labeled ICP0-null mutant strain n212 (Cai & Schaffer, 1989) and rescue (Lee *et al*, 2016) verified by immunofluorescence (Fig 2-

9G) and found that the presence or absence of ICP0 had no detectable impact on the ability of vDNA foci to colocalize to PML-NBs (Fig. 2-8D). Taken together, these data demonstrate that association of latent viral genomes with PML-NBs in peripheral neurons is dependent on the formation of type I IFN-induced PML-NBs and the presence of type I IFN during initial infection and is independent of ICP0 expression.

PML is Required for the IFNα-dependent Restriction of HSV-1 Latency

To determine whether the stable association of viral genomes with PML-NBs directly contributes to the IFNα-dependent restriction of HSV reactivation, we investigated whether PML depletion was sufficient to restore the ability of the latent viral genomes to reactivate. A previous study has shown that PML-dependent recruitment of HIRA to ISG promoters contributes to the up-regulation of gene expression as a result of cytokine release in response to HSV infection (McFarlane *et al.*, 2019). Although carried out in non-neuronal cells, this study and others (Ulbricht *et al*, 2012; Chen *et al*, 2015; Kim & Ahn, 2015; Scherer *et al*, 2016) suggest that PML itself may contribute to ISG upregulation, so to determine whether PML was indeed required for ISG stimulation in SCG neurons we carried out RNA deep sequence analysis in IFNα-treated neurons depleted of PML. Postnatal SCG neurons were transduced with lentiviral vectors expressing non-targeting control or PML-targeting shRNAs (shCtrl and shPML, respectively) and then mock treated or treated with IFNα (600 IU/ml) for 18h prior to RNA extraction for next generation sequencing**.** High confidence reads were used for gene expression and gene ontology (GO) analysis. As expected, treatment of shCtrl transduced neurons with IFNα caused large changes in differentially regulated gene expression, with an enrichment of upregulated genes involved in immune system regulation. Similar to control neurons, PML depleted neurons also significantly upregulated the expression of genes involved in the response to IFNα stimulation. We

found that of the total of 248 genes upregulated >1.5-fold following IFNα treatment, 83.47% of these genes were shared between the shCtrl- and shPML-treated groups (Fig. 2-116A). Furthermore, we found similar ISG expression (Fig. 2-116B) and GO pathway enrichment (Fig. 2-11C). Therefore, in primary SCG neurons, the expression of ISGs in response to exogenous IFNα is largely independent of PML expression.

Because PML depletion did not detectably prevent the induction of type I IFN response genes in SCG neurons, we were able to examine the effect of PML depletion prior to infection on the IFNα-mediated restriction of HSV-1 reactivation. SCG neurons were transduced with lentiviral vectors expressing different PML-targeting shRNA or control non-targeting shRNA. PML depletion was confirmed by average number of PML-NBs per nucleus (Fig. 2-10A) and *Pml* mRNA expression level (Fig. 2-11D, E) in neurons transduced for 3 days then treated with IFNα (600 IU/ml). As expected, we found a significant decrease in the percent of vDNA foci stably colocalizing with PML-NBs at 8 dpi in the shPML-treated neurons compared to shCtrl-treated neurons (Fig. 2-10B). Furthermore, we assessed reactivation in neurons infected with HSV-1 in the presence or absence of IFNα (150 IU/ml) at 3 days post-transduction. In these experiments, neurons were infected with a Us11-GFP gH-null virus, which is defective in cell-to-cell spread and eliminates the need for WAY-150138 during reactivation. In untreated neurons, we found no difference in reactivation following treatment with LY294002 (Fig. 2-10C, D). In addition, PML depletion had no effect on the number of GFP-positive neurons in the non-reactivated samples, indicating that in this system that PML was not required for the establishment of latency. However, in neurons treated with IFNα at the time of initial infection, depletion of PML using either of the three PML shRNAs increased the ability of HSV to reactivate as indicated by a 2.97-, 2.69- and 3.49- fold increase in GFP-positive neurons following treatment with LY294002,

respectfully (Fig. 2-10E, F). Moreover, there was no significant difference between the PML depleted, IFNα-treated neurons and the non-IFNα treated neurons, indicating that PML depletion fully restored the ability of HSV to reactivate from type I IFN treated neurons. Taken together, these data demonstrate that type I IFN exposure solely at the time of infection results in entrapment of viral genomes in PML-NBs and restricts reactivation. This suggests that genome entrapment by PML promotes a more restrictive or deeper form of latency where reactivation is limited.

Depletion of PML After the Establishment of Latency Enhances Reactivation in IFNα-treated Neurons

To explore the long-term effect of stable PML-NB-association on the latent viral genome, we next tested whether PML depletion after the establishment of latency was sufficient to restore the ability of the latent viral genomes to reactivate following treatment with a trigger that may directly disrupt PML-NBs. Arsenic trioxide (ATO) has been shown to bind directly to PML and disrupt PML-NBs (Lallemand-Breitenbach *et al*, 2008; Zhang *et al*, 2010; Sides *et al*, 2011), and we confirmed that ATO (1μM) fully disrupted IFNα-induced PML-NBs in our peripheral neurons by 18h post-treatment (Fig. 2-13A). When we investigated reactivation in neurons that were latently infected in the presence or absence of IFNα, then treated with arsenic trioxide (ATO) at 8 dpi, we found that ATO is a very potent stimulator of reactivation independent of IFNα-treatment, indicating that ATO is capable of triggering reactivation of genomes that are either PML-NB-associated or not (Fig. 2-13B). This is likely because ATO is a potent activator of the cell stress response and can result in robust histone phosphorylation (Gehani *et al*, 2010), which we have previously linked to reactivation (Cliffe *et al.*, 2015). Although ATO could also induce reactivation in the presence of IFNα-induced PML-NBs, this

reactivation was still less robust than mock treated neurons, likely reflecting the time required for disruption of PML-NBs by ATO.

Therefore, to more specifically determine whether PML-depletion restored the ability of neurons to reactivate following treatment with a physiological stimulus of reactivation, Neurons were infected with Us11-GFP gH null HSV-1 virus in the presence or absence of IFNα (150 IU/ml) and subsequently transduced with lentiviral vectors expressing PML-targeting shRNA or control non-targeting shRNA at 1 dpi. Under these experimental conditions, PML knockdown post-infection did not impact LY294002-induced reactivation in untreated neurons (Fig. 2-12A, C), but did increase the ability of HSV to reactivate from IFNα treated neurons in response to treatment with LY294002, as indicated by a 1.3-fold increase in GFP-positive neurons, albeit reactivation was not restored to levels seen in untreated neurons (Fig. 2-12B, D). As expected, we found that only a small proportion of vDNA foci stably colocalize with PML-NBs at 8 dpi in the shPML-treated neurons compared to vDNA foci in the shCtrl-treated neurons (Fig. 2-12E). Therefore, PML depletion post-infection does not result in detectable spontaneous reactivation of PML-NB-associated viral genomes, indicating that they are still in a repressed state and/or lack the necessary factors required to initiate gene expression. However, depletion of PML does partially restore the ability of HSV to enter the lytic from IFN-treated neurons in response to a reactivation stimulus.

Previously we have shown that reactivation in response to LY294002 is dependent on activation of the neuronal stress pathway involved dual-leucine zipper kinase (DLK) and JNK activation (Cliffe *et al.*, 2015). To test whether the same cell stress stimuli is required to induce reactivation from genomes released from PML-NBs upon shRNA-mediated knockdown of PML, we reactivated in the presence of the DLK

inhibitor GNE-3511 (Patel *et al*, 2015). GNE-3511 inhibited LY294002-mediated reactivation of latent genomes following PML depletion post-infection (Figure 2-12F). Therefore, PML-NBs maintain a restricted form of latency that is more refractory to reactivation, and following PML depletion, viral genomes do not undergo detectable spontaneous reactivation and are still dependent on activation of neuronal cell stress signaling pathways for reactivation.

**Discussion**

The considerable heterogeneity observed at the neuronal level in the colocalization of viral genomes with different nuclear domains likely results in different types of latency that are more or less susceptible to reactivation. The determinants of this heterogeneity and a direct link between the subnuclear localization of a latent genome and its ability to reactivate following a given stimulus was not known. Using a primary neuronal model of HSV latency and reactivation, we found that the presence of type I IFN solely at that time of initial infection acts as a key mediator of the subnuclear distribution of latent viral genomes in neurons and promotes a more restricted form of latency that is less capable of reactivation following disruption of NGF-signaling. Importantly, we show that activation of the type I IFN signaling pathway in peripheral neurons induces the detectable formation of PML-NBs, which stably entrap a proportion of latent genomes. Importantly, we show that this IFN-dependent restriction is mediated by PML, suggesting that PML-NBs are directly responsible for the observed restriction of reactivation.

PML-NBs typically number 1-30 bodies per nucleus in non-neuronal cells (Bernardi & Pandolfi, 2007). In the mouse nervous system, however, *Pml* mRNA expression levels have previously been found to be low as measured by *in situ*

hybridization (Gray *et al*, 2004). PML protein is enriched in neural progenitor cells, but the induction of differentiation results in the downregulation of PML both at a transcriptional and protein level, and *P*ml mRNA expression is undetectable in post-mitotic neurons in many regions of the developing brain (Regad *et al*, 2009). Our findings in postnatal peripheral neurons further support these observations. *Pml* expression in adult mouse neurons varies considerably between brain regions but is generally confined to the gray matter (Hall *et al*, 2016). Although implicated to play a role in regulating circadian rhythms (Miki *et al*, 2012), synaptic plasticity (Bloomer *et al*, 2007) and the response to toxic proteins that cause neurodegenerative disorders (Yamada *et al*, 2001; Kumada *et al*, 2002; Mackenzie *et al*, 2006; Chort *et al*, 2013), PML regulation and function in the adult nervous system is still largely unknown. In our study, we could not detect PML-NBs in adult primary neurons isolated from the SCG or the TG. In contrast to our findings, PML-NBs have previously been shown to be present in adult mouse and human TG neurons by FISH and immunofluorescence (Catez *et al.*, 2012; Maroui *et al.*, 2016). However, no quantification was done in these studies, and Catez *et al.* (2012) describes subpopulations of adult TG neurons that did not display any PML signal in the nucleus. In addition, characterization of PML distribution in adult TG neurons by IF-FISH of ganglia isolated *in vivo* may reflect prior exposure to type I IFNs or other signaling molecules. The functional significance of peripheral neurons lacking PML-NBs is unclear, but could be linked to the capacity of neurons to undergo dynamic rearrangement of local and global nuclear architecture during maturation or neuronal excitation. An absence of PML-NBs in neurons could also contribute to their resistance to apoptosis, as PML has also been shown to play a role in cell death through the induction of both p53-dependent and -independent apoptotic pathways (Quignon *et al.*, 1998; Wang *et al.*, 1998; Guo *et al.*, 2000). Whether PML-mediated regulation of these pathways occurs in the context of PML-NBs or by PML itself is unclear, but interestingly,

the pro-apoptotic functions of Daxx, a PML-NB-associated protein, may require localization to PML-NBs in certain cell types (Croxton *et al.*, 2006). Furthermore, our *in vitro* model using pure populations of intact neurons is devoid of the immune responses and complexities of intact animals, and we cannot rule out the possibility that axotomy or the processing of the neurons *ex vivo* could lead to PML-NB disruption or dispersal. However, notwithstanding these caveats, primary neurons provide an excellent model system to understand the impact of extrinsic immune factors and PML-NBs to the altering the nature of HSV latency.

Peripheral neurons are capable of responding to type I IFN signaling, given the robust induction of ISG expression and formation of PML-NBs following treatment with IFNα, and this is supported by a number of previous studies (Yordy *et al.*, 2012; Katzenell & Leib, 2016; Song *et al*, 2016a; Linderman *et al.*, 2017; Barragan-Iglesias *et al*, 2020). It will be important to delineate if the inflammatory environment at the initial site of infection acts on neuronal axons to prime the neuron for a more repressed latent infection or if inflammatory cytokines in the ganglia are crucial for promoting a more repressive state. Although responsive to IFN, primary peripheral and cortical mouse neurons have previously been shown to have inefficient type I IFN-mediated anti-viral protection compared to non-neuronal mitotic cells (Yordy *et al.*, 2012; Kreit *et al*, 2014). One study showed that DRG neurons are less responsive to type I IFN signaling and used an absence of cell death upon IFN treatment as one of their criteria (Yordy *et al.*, 2012). It should be noted that different cell types display specific responses to type I IFN signaling and peripheral neurons have even been reported to be more protected from cell death stimuli following IFN treatment (Chang *et al*, 1990). Furthermore, a previous study found that inducible reactivation of HSV-1 from latently infected neuronal cultures is transiently sensitive to type I IFNs (Linderman *et al.*, 2017). Our model of HSV-1

latency and reactivation in primary sympathetic neurons highlights a type I IFN response that is PML-dependent and suggests a role for neuronal IFN signaling in promoting a more restricted latent HSV-1 infection.

Prior to this study, it was not clear whether viral genomes associated with PML-NBs were capable of undergoing reactivation. In response to inhibition of NGF-signaling, our data demonstrate that PML-NB associated genomes are more restricted for reactivation given that 1) IFN induces PML-NB formation and increased association of viral genomes with PML-NBs, 2) IFN pretreatment promotes restriction of viral reactivation and 3) the ability of viral genomes to reactivate from IFN-treated neurons increases with PML knock-down either prior to or following infection. How increased acetylation relates to the physiological triggers that induce HSV reactivation is not clear. In contrast, loss of neurotrophic signaling can occur in response to known physiological stimuli that trigger HSV reactivation (Suzich & Cliffe, 2018). Although we cannot rule out the possibility that different stimuli have the potential for PML-NB associated genomes to undergo reactivation, this study clearly demonstrates that at least one well characterized trigger of reactivation cannot efficiently induce PML-NB associated genomes to undergo transcription.

Our results identify a persistence of PML-NBs, an IFN-mediated innate immune response that allows for long-term restriction of latent viral genomes in the absence of continued ISG expression. Interestingly, type I IFN-induced PML-NBs persisted for up to 15 days post-treatment both in the presence and absence of viral infection. Given the absence of PML-NBs in our untreated peripheral neurons, this induction and persistence could represent neuron-specific innate immune memory. Importantly, exposure to IFN and other cytokines has also been shown to generate innate immune memory or ‘trained

immunity' in fibroblasts and immune cells (Kamada *et al*, 2018; Moorlag *et al*, 2018), and PML-NBs themselves are potentially important in the host innate immune response. A previous study found that the histone chaperone HIRA is re-localized to PML-NBs in response to the innate immune defenses induced by HSV-1 infection, and in this context, PML was required for the recruitment of HIRA to ISG promoters for efficient transcription (McFarlane *et al.*, 2019). Prior exposure to type I interferons has also been shown to promote a transcriptional memory response in fibroblasts and macrophages (Kamada *et al.*, 2018). This interferon memory led to faster and more robust transcription of ISGs following restimulation and coincided with acquisition of certain chromatin marks and accelerated recruitment of transcription and chromatin factors (Kamada *et al.*, 2018). The persistence of PML-NBs in neurons may alter the subsequent response to IFN and/or viral infection, and it will be interesting to determine whether there is trained immunity in neurons such that subsequent responses differ from the first exposure. What is clear from our results however is the role of PML and IFN exposure in sustained repression of the latent HSV genome. Even in the absence of known chromatin changes that occur on the PML associated viral genome, this long-term effect on the ability of the HSV-1 genome to respond to an exogenous signal and restriction of reactivation is reminiscence of the classical definition of an epigenetic change (of course in the case of post-mitotic neurons in the absence of inheritance).

Here we demonstrate that there are different types of HSV latency dependent on the subnuclear positioning of the viral genome and ability to reactivate. Genomes associated with PML-NBs are one form of restricted latency in our system. PML-NBs are known to play a role in the restriction of viral gene expression in non-neuronal cells, but the potential mechanism of PML-NB-mediated HSV gene silencing in neurons is unknown. Investigating the identity, mechanism of targeting and role of these proteins in

the induction and maintenance of latency will ultimately facilitate the development of antiviral therapeutics that target the latent stage of infection to prevent reactivation.

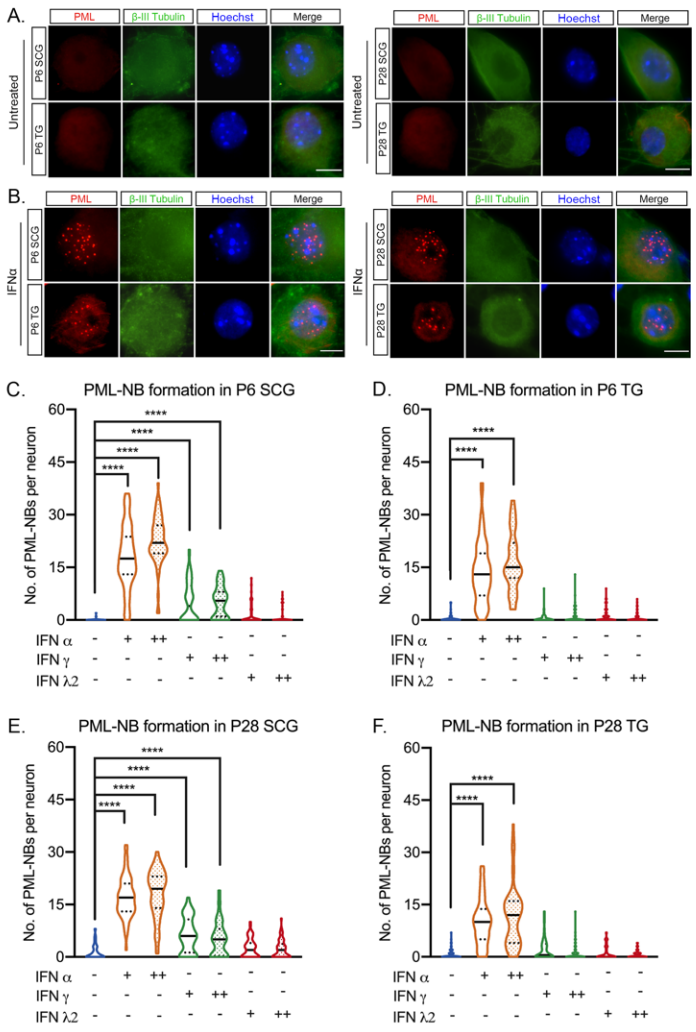


**Fig. 2-1.** Type I IFN induces the formation of PML-NBs in primary peripheral neurons. (**A**) Representative images of primary neurons isolated from superior cervical ganglia (SCG) and sensory trigeminal ganglia (TG) of postnatal (P6) and adult (P28) mice stained for PML and the neuronal marker BIII-tubulin. (**B**) SCG and TG neurons isolated from P6 and P28 mice were treated with interferon (IFN)α (600 IU/ml) for 18h and stained for PML and BIII-tubulin. (**C-F**) Quantification of detectable PML puncta in P6 and P28 neurons following 18h treatment with IFNα (150 IU/ml, 600 IU/ml), IFNγ (150 IU/ml, 500 IU/ml) and IFNλ2 (100 ng/ml, 500 ng/ml). Data represent the mean ± SEM. n=60 from 3 independent experiments. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (**** P<0.0001). Scale bar, 20μm.

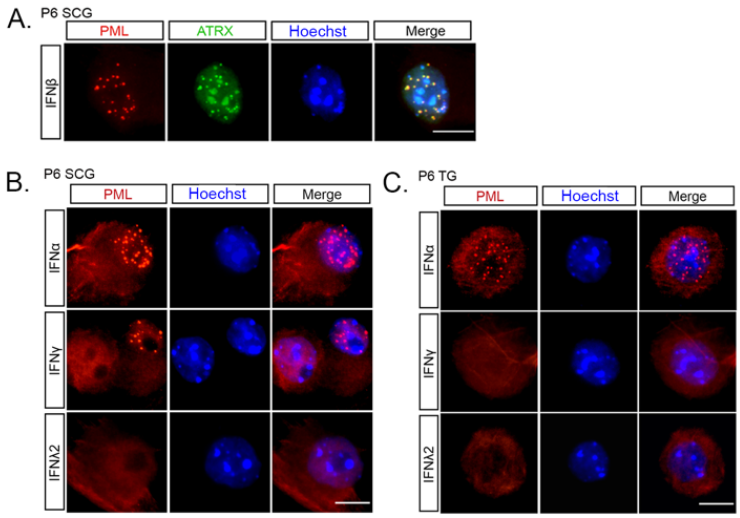


**Fig. 2-2.** Type I IFN alters the sub-cellular localization of ATRX, Daxx and SUMO-1 in primary peripheral neurons. (**A**) Representative images of P6 SCG neurons treated with IFNβ (150 IU/ml) and stained for PML and ATRX. (**B**) Representative images of P6 SCG treated with IFNα (600 IU/ml), IFNγ (500 IU/ml) and IFNλ2 (500 ng/ml) and stained for PML. (**C**) Representative images of P6 TG treated with IFNα (600 IU/ml), IFNγ (500 IU/ml) and IFNλ2 (500 ng/ml) and stained for PML. Scale bar, 20μm.

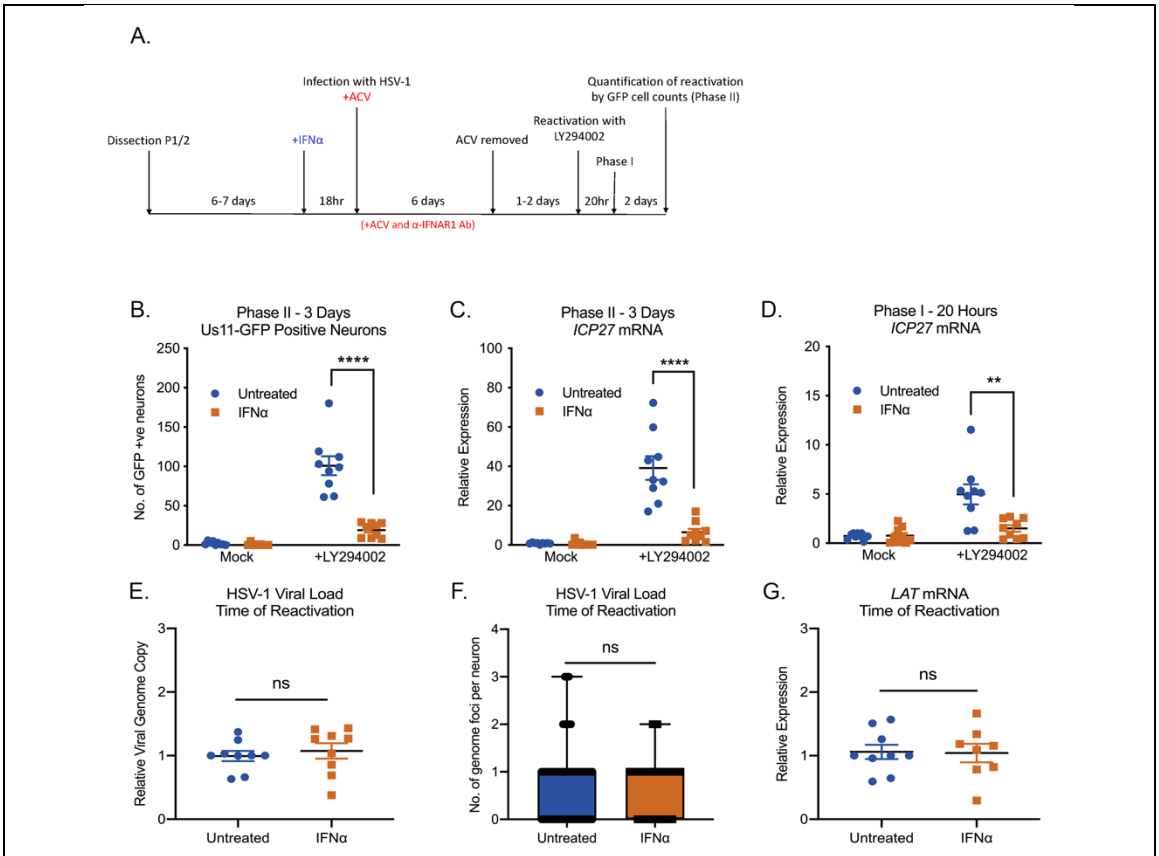


**Fig. 2-3.** Type I IFN treatment solely at time of infection inhibits LY294002-mediated reactivation of HSV-1 in primary sympathetic SCG neurons. (**A**) Schematic of the primary postnatal sympathetic neuron-derived model of HSV-1 latency. (**B**) Number of Us11-GFP expressing neurons at 3 days post-LY294002-induced reactivation in P6 SCG neuronal cultures infected with HSV-1 in the presence or absence of IFNα (600 IU/ml), then treated with an α-IFNAR1 neutralizing antibody. n=9 from 3 independent experiments. (**C**) RT-qPCR for viral mRNA transcripts at 3 days post- LY294002-induced reactivation of SCGs infected with HSV-1 in the presence or absence of IFNα. n=9 from 3 independent experiments. (**D**) RT-qPCR for viral mRNA transcripts at 20 hours post-LY294002-induced reactivation in SCGs infected with HSV-1 in the presence of absence of IFNα. n=9 from 3 independent experiments. (**E**) Relative amount of viral DNA at time of reactivation (8dpi) in SCG neurons infected with HSV-1 in the presence or absence of IFNα (600 IU/ml). n=9 from 3 independent experiments (**F**) Quantification of vDNA foci detected by click chemistry at time of reactivation (8dpi) in SCG neurons infected with HSV-1 in the presence or absence of IFNα (600 IU/ml). n=60 from 3 independent experiments (**H**) *LAT* mRNA expression at time of reactivation (8dpi) in neurons infected with HSV-1 in the presence or absence of IFNα (600 IU/ml). n=9 from 3 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a Mann-Whitney test (ns not significant, ** P<0.01, **** P<0.0001).

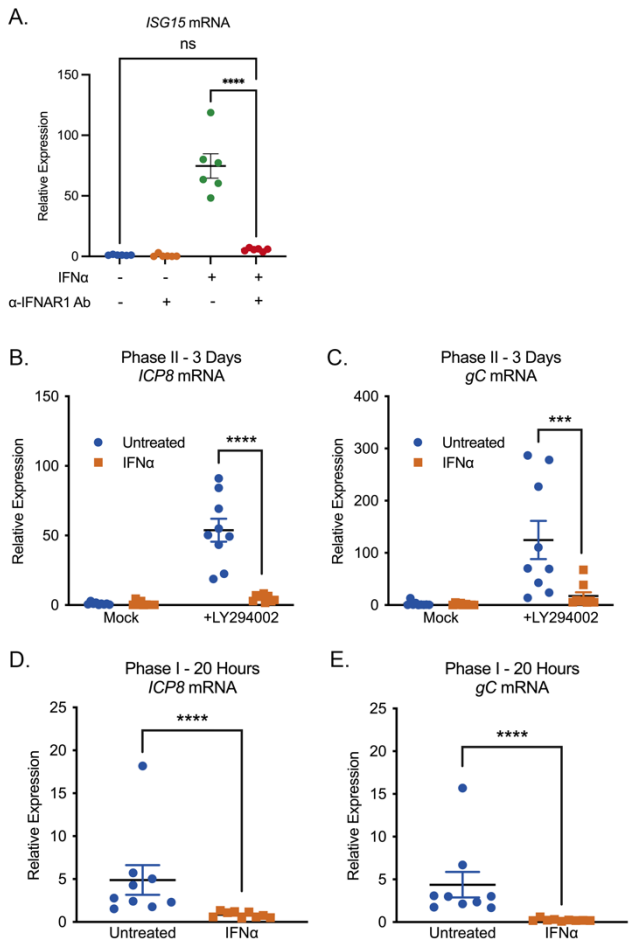


**Fig 2-4.** Type I IFN treatment solely at time of infection inhibits LY294002-mediated reactivation of HSV-1 in primary sympathetic SCG neurons. (**A**) RT-qPCR for *ISG15* mRNA expression in SCG neurons treated with IFNα (600IU/ml) in the presence or absence of anti-mouse IFNAR-1 antibody (1:1000). n=6 from 3 independent experiments. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (**** P<0.0001). (**B, C**) RT-qPCR for viral mRNA transcripts at 3 days post-LY294002-induced reactivation of SCGs infected with HSV-1 in the presence or absence of IFNα (600 IU/ml). n=9 from 3 independent experiments. (**D, E**) RT-qPCR for viral mRNA transcripts at 20 hours post- LY294002-induced reactivation in SCGs infected with HSV-1 in the presence or absence of IFNα (600 IU/ml). n=9 from 3 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a Mann-Whitney test (ns not significant, *** P<0.001, **** P<0.0001).

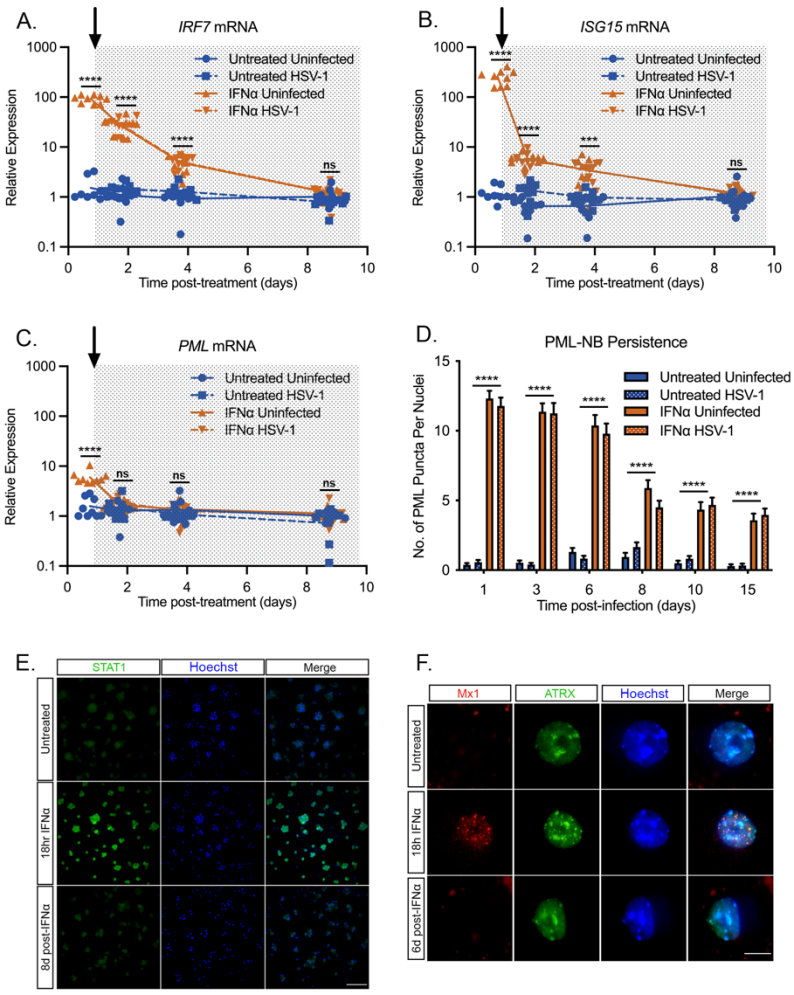


**Fig. 2-5.** Type I IFN-induced PML-NBs persist in primary sympathetic neurons despite resolution of IFN signaling. (**A-C**) Kinetics of *ISG15*, *IRF7* and *PML* mRNA expression at 0.75, 1.75, 3.75 and 8.75 days post-IFNα (600 IU/ml) treatment. Arrow indicates the time of HSV-1 infection at 18hr post-interferon treatment. n=9 from 3 independent experiments. (**D**) Quantification of PML puncta at 1-, 3-, 6-, 8-, 10- and 15-days post-infection with HSV-1 in untreated and IFNα (600 IU/ml)-treated SCG neurons. n≥60 from 3 independent experiments. (**E**) Representative images of P6 SCG neurons treated with IFNα (600 IU/ml) and stained for STAT1 at 18 hours and 8 days post-treatment. Scale bar, 100μm. (**F**) Representative images of P6 SCG neurons treated with IFNα (600 IU/ml) and stained for Mx1 at 18 hours and 6 days post-treatment. Scale bar, 20μm. Data represent the mean ± SEM. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (ns not significant, *** P<0.001, **** P<0.0001)

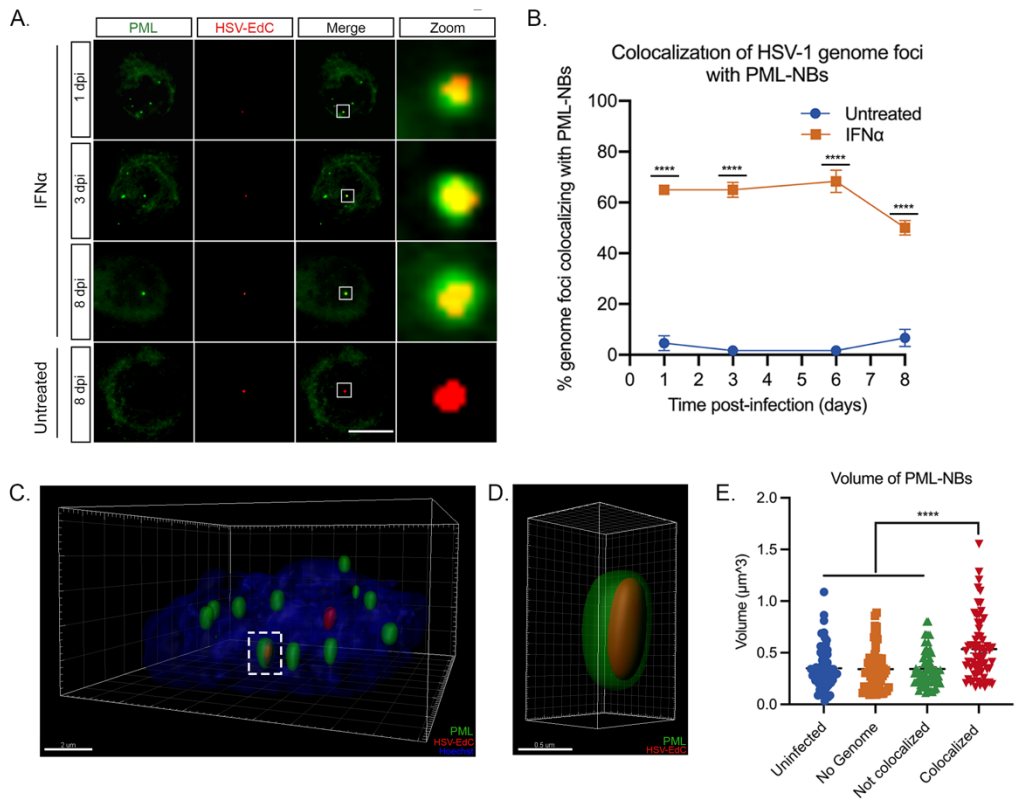


**Fig. 2-6.** Type I IFN induced PML-NBs stably entrap vDNA throughout a latent HSV-1 infection of primary sympathetic neurons. (**A**) Representative images of vDNA foci detected by click chemistry to PML at 1, 3, 6 and 8 dpi in P6 SCG neurons infected with HSV-1$^{EdC}$ in the presence or absence of IFNα (600 IU/ml). Z-stack images of individual vDNA foci were acquired. Scale bar, 20µm. (**B**) Percent colocalization of vDNA foci detected by click chemistry to PML at 1, 3, 6 and 8 dpi in SCG neurons infected with HSV-1$^{EdC}$ in the presence or absence of IFNα (600 IU/ml). n=60 genomes from 3 independent experiments. Statistical comparisons were made using a Mann-Whitney test. (**C**) 3D reconstruction of a high-resolution Z-series confocal image showing PML entrapment of an HSV-1$^{EdC}$ vDNA foci. Scale bar, 2µm. (**D**) Enlargement of PML entrapped vDNA outlined by white dashed box. Scale bar, 0.5µm. (**E**) Quantification of PML-NB volume in SCG neurons infected with HSV-1$^{EdC}$ in the presence or absence of IFNα (600 IU/ml). n=60 PML-NBs from 3 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (**** P<0.0001).

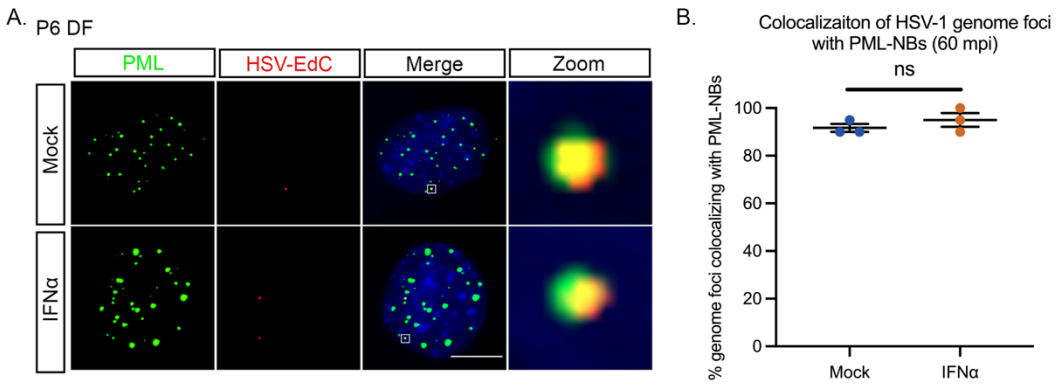


**Fig. 2-7.** PML-NBs entrap vDNA in the absence of type I IFN during lytic HSV-1 infection of murine dermal fibroblasts. (**A**) Representative images of vDNA foci detected by click chemistry to PML at 60 minutes post-infection in P6 dermal fibroblasts lytically infected with HSV-$1^{EdC}$ in the presence or absence of IFNα (600 IU/ml). Scale bar, 20μm. (**B**) Percent colocalization of vDNA foci detected by click chemistry to PML at 60mpi in P6 dermal fibroblasts infected with HSV-$1^{EdC}$ in the presence or absence of IFNα (600 IU/ml). n=60 genomes from 3 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a Mann-Whitney test. (ns not significant)

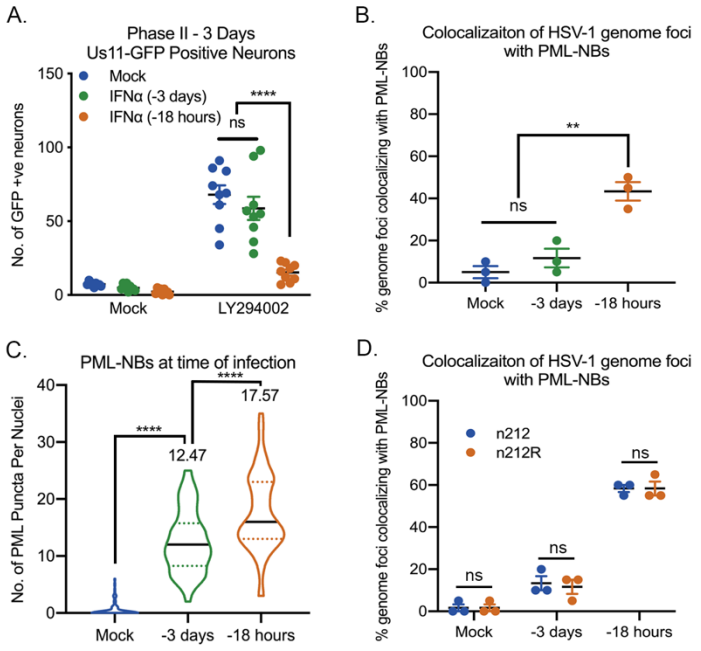


**Fig. 2-8.** HSV-1 genomes only associate with PML-NBs when type I IFN is present during initial infection. (**A**) Number of Us11-GFP expressing P6 SCG neurons infected with HSV-1 following IFNα treatment for 18 hours prior to infection or for 18 hours at 3 days prior to infection. n=9 from 3 independent experiments. (**B**) Percent colocalization of vDNA foci detected by click chemistry to PML at 8 dpi in SCG neurons infected with HSV-1$^{EdC}$ following IFNα treatment for 18 hours prior to infection or for 18 hours at 3 days prior to infection. n=60 genomes from 3 independent experiments. (**C**) Quantification of PML puncta at time of infection in P6 SCG neurons treated with IFNα (600 IU/ml) for 18 hours prior to infection or for 18hours at 3 days prior to infection. n=60 from 3 independent experiments. (**D**) Percent colocalization of vDNA foci detected by click chemistry to PML at 3 dpi in SCG neurons with HSV-1$^{EdC}$ infected with ICP0-null mutant HSV-1, n212, or a rescued HSV-1 virus, n212R, in P6 SCG neurons treated with IFNα for 18 hours prior to infection or for 18hours at 3 days prior to infection. n=60 genomes from 3 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (ns not significant, ** P<0.01, **** P<0.0001).

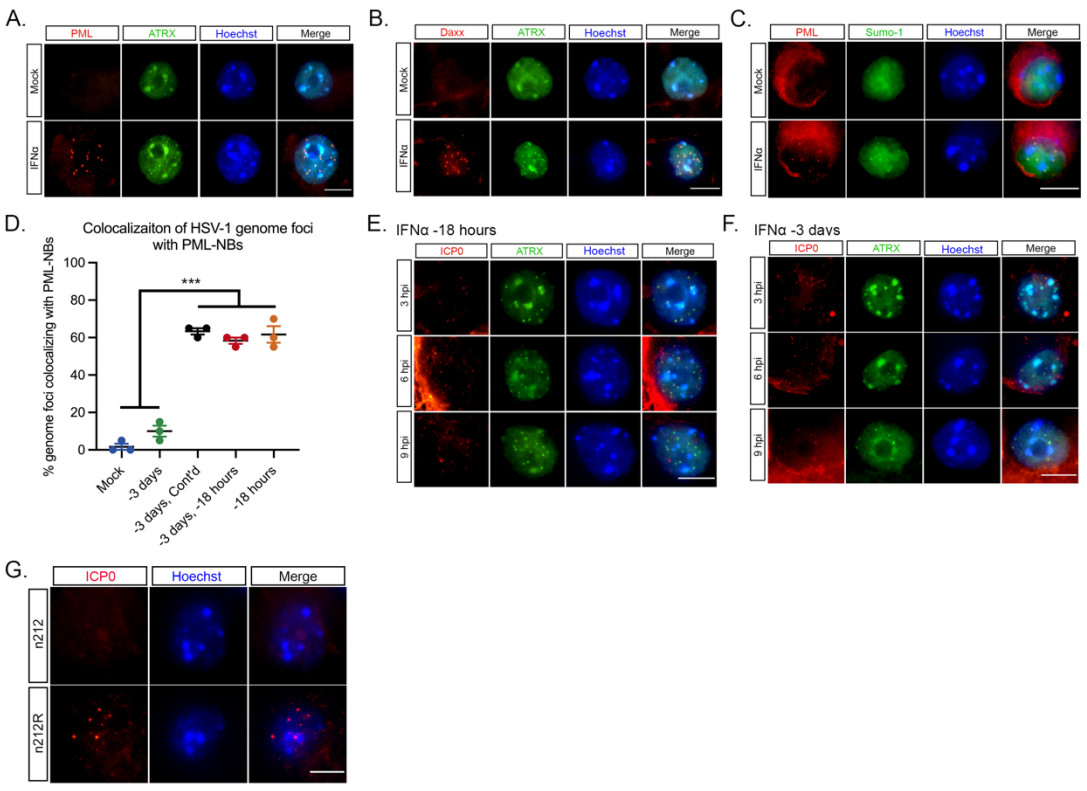


**Fig 2-9.** HSV-1 genomes only associate with PML-NBs when type I IFN is present during initial infection. (**A**) Representative images of untreated or IFNα-treated (600 IU/ml) P6 SCG neurons stained for PML and ATRX at 3 days post-treatment. (**B**) Representative images of untreated or IFNα-treated (600 IU/ml) P6 SCG neurons stained for Daxx and ATRX at 3 days post-treatment. (**C**) Representative images of untreated or IFNα-treated (600 IU/ml) P6 SCG neurons stained for PML and SUMO-1 at 3 days post-treatment. (**D**) Percent colocalization of vDNA foci detected by click chemistry to PML at 3 dpi in SCG neurons infected with HSV-1$^{EdC}$ with or without IFNα (600 IU/ml) present at the time of infection. n=60 genomes from 3 independent experiments. (**E, F**) Representative images of HSV-1-infected P6 SCG neurons treated with IFNα (600 IU/ml) for 18 hours prior to infection or for 18hours at 3 days prior to infection and stained for ICP0 and ATRX at 3, 6 and 9 hours post-infection. (**G**) Representative images of P6 SCG neurons infected with n212 or n212R for 8 hours and stained for HSV-1 ICP0. Data represent the mean ± SEM. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (***P<0.001). Scale bar, 20μm.

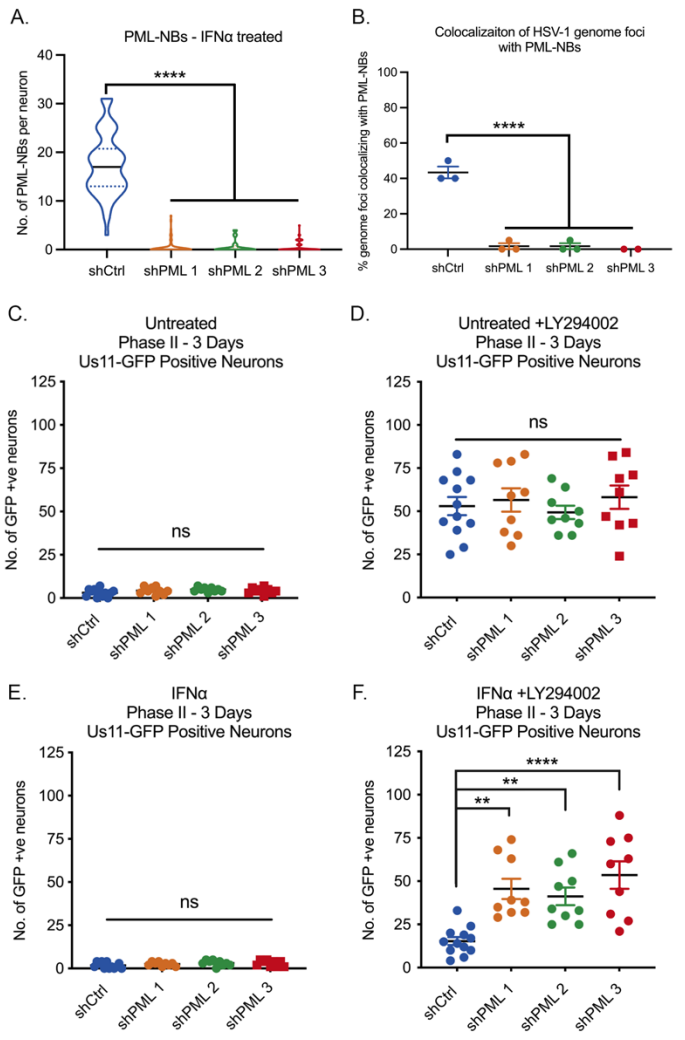


**Fig. 2-10.** Depletion of PML with shRNA-mediated knockdown prior to infection restores HSV-1 reactivation in type I interferon-treated primary sympathetic neurons. (**A**) Quantification of PML puncta in P6 SCG neurons transduced with either control non-targeting shRNA or shRNA targeting PML for 3 days, then treated with IFNα (600 IU/ml) for 18 hours. n=60 from 3 independent experiments. (**B**) Quantification of colocalization of vDNA foci detected by click chemistry to PML in primary SCG neurons transduced with shRNA targeting PML for 3 days prior to being infected with HSV-1$^{EdC}$ in the presence of IFNα (150 IU/ml). n=60 genomes from 3 independent experiments. (**C-F**) Number of Us11-GFP expressing neurons at 3 days post-LY294002-induced reactivation in P6 SCG neuronal cultures transduced with shRNA targeting PML for 3 days prior to infection with HSV-1 in the presence or absence of IFNα (150 IU/ml). n=9 from 3 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (ns not significant, ** P<0.01, **** P<.0001).

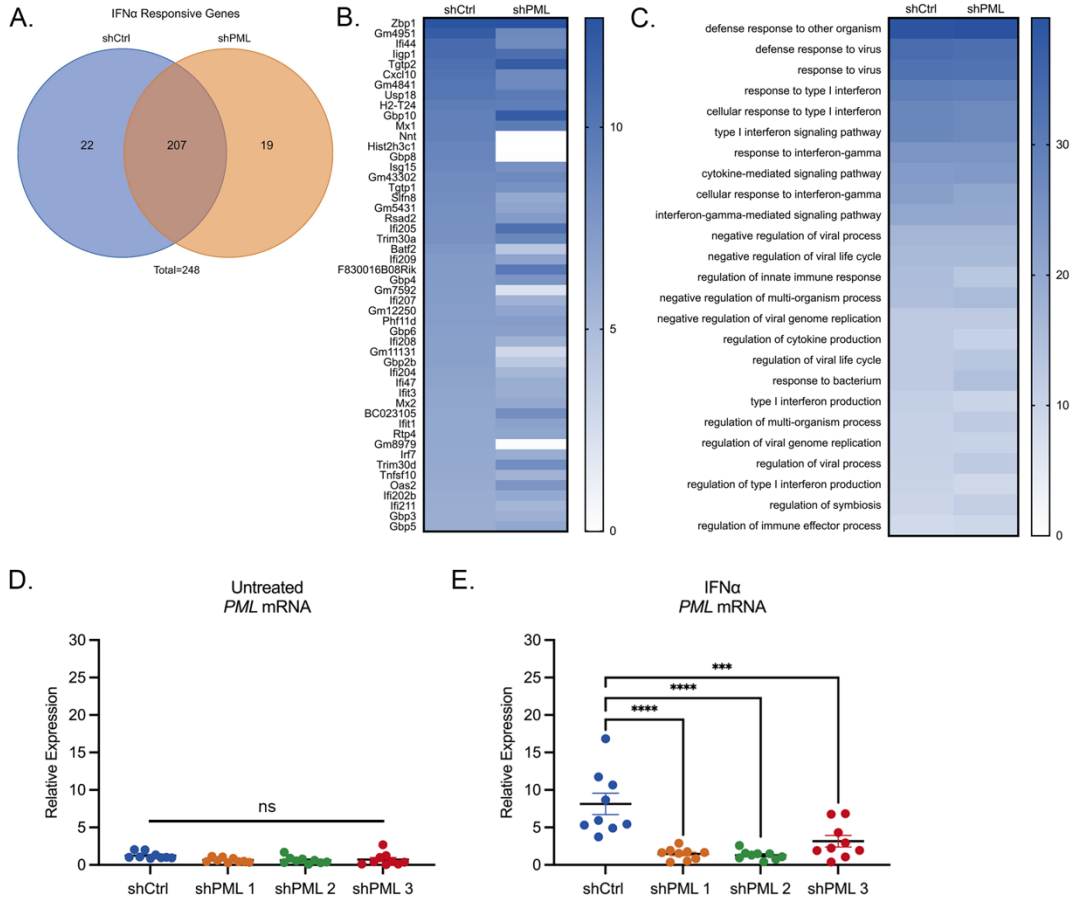


**Fig. 2-11.** PML is not required for ISG induction in primary postnatal sympathetic neurons. (**A**) P6 SCG neurons were transduced with either control non-targeting shRNA or shRNA targeting PML for 3 days, then treated with IFNα (600 IU/ml) for 18 hours. Total genes >1.5-fold higher in IFNα (600 IU/ml) treated cells than untreated cells were subdivided into 3 groups: shCtrl-treated neurons only. shPML-treated neurons only; and shCtrl and shPML neurons (shared). (**B**) Gene expression heat map of top 50 most upregulated genes in P6 neurons transduced with control non-targeting shRNA or shRNA targeting PML for 3 days, then treated with IFNα (600 IU/ml) for 18 hours. (**C**) Heat map of the top 25 shared upregulated GO terms in P6 neurons transduced with control non-targeting shRNA or shRNA targeting PML for 3 days, then treated with IFNα for 18 hours. (**D, E**) RT-qPCR for *Pml* mRNA expression in SCG neurons transduced with either control non-targeting shRNA or shRNA targeting PML for 3 days, then treated with IFNα (600 IU/ml) for 18 hours. Data represent the mean ± SEM. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (ns not significant, *** P<0.001, **** P<.0001).

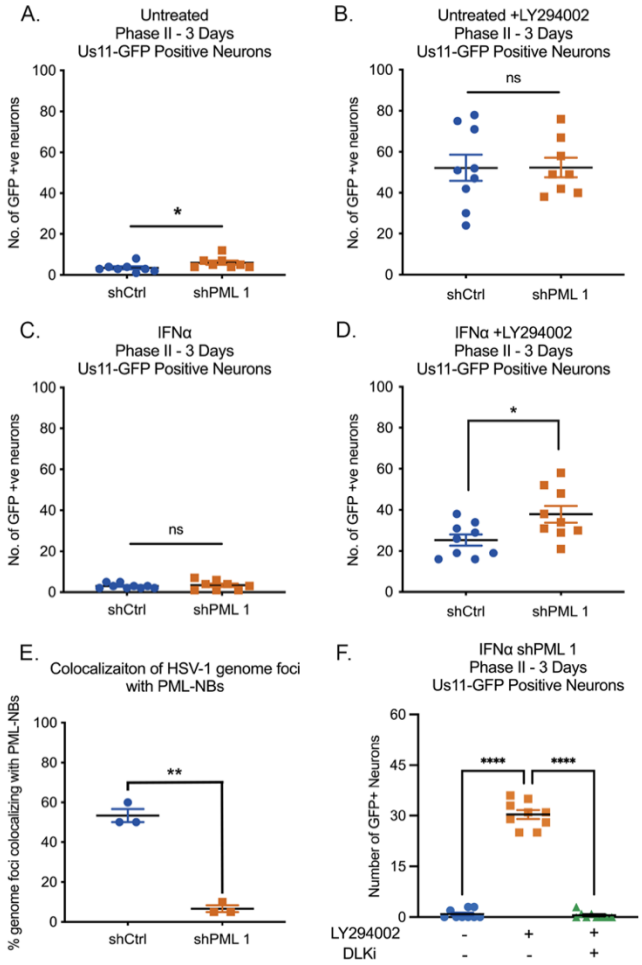


**Fig. 2-12.** Depletion of PML with shRNA mediated knockdown post-infection partially restores LY294002-mediated HSV-1 reactivation in type I interferon-treated primary sympathetic neurons. (**A-D**) Number of Us11-GFP expressing neurons at 3 days post-LY294002-induced reactivation in P6 SCG neuronal cultures transduced with shRNA targeting PML at 1 day post-infection with HSV-1 in the presence or absence of IFNα (150 IU/ml). n=9 from 3 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a Mann-Whitney test (ns not significant, * P<0.05). (**E**) Quantification of colocalization of vDNA foci detected by click chemistry to PML in primary SCG neurons transduced with shRNA targeting PML at 1 day post-infection with HSV-1$^{EdC}$ in the presence or absence of IFNα (150 IU/ml). n=60 genomes from 3 independent experiments. Statistical comparisons were made using a Mann-Whitney test (**F**) Number of Us11-GFP expressing neurons at 3 days post-reactivation in P6 SCG neuronal cultures transduced with shRNA targeting PML at 1 day post-infection with HSV-1 in the presence of IFNα (150 IU/ml). Reactivation was induced by LY294002 in the presence of the DLK inhibitor GNE-3511 (4μM). n=9 from 3 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (ns not significant, * P<.05, ** P<.01, **** P<.0001). Scale bar, 20μm.

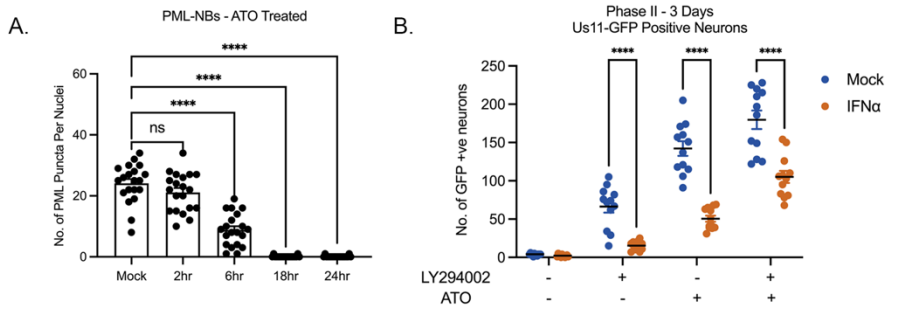


**Fig. 2-13.** ATO treatment induces HSV-1 reactivation in primary sympathetic SCG neurons. (**A**) Quantification of detectable, IFNα (600 IU/ml)-induced PML puncta in P6 SCG neurons treated with ATO (1μM) for 2, 6, 18 and 24 hours. (**B**) Number of Us11-GFP expressing neurons at 3 days post-treatment with LY294002 (20μM), ATO (1μM) or LY294002 (20μM) + ATO (1μM) in P6 SCG neuronal cultures infected with HSV-1 in the presence or absence of IFNα (600 IU/ml). n=12 from 4 independent experiments. Data represent the mean ± SEM. Statistical comparisons were made using a 2way ANOVA (ns not significant, **** P<.0001).

# CHAPTER 3

*HSV-1 subnuclear positioning and interactions with repressive cellular domains*

**Abstract**

HSV-1 only establishes latency in neurons, where the viral genome associates with repressive heterochromatin, but the cellular proteins and molecular mechanisms that mediate this chromatinization are largely unknown. We have found that primary murine peripheral neurons have abundant nuclear ATRX staining, and, in contrast to non-neuronal cells, this ATRX staining is localized outside of PML-NBs. We also found that latent viral genomes colocalize to regions of ATRX staining both in the context of PML-NBs where they associate with Daxx and the histone variant H3.3 or outside of PML-NBs where they associate with the histone variant H3.1. Furthermore, we show preliminary evidence that indicates when ATRX is specifically knocked out in latently infected neurons *in vivo*, the latent viral load in peripheral ganglia is decreased. Interestingly, latent genomes entrapped by PML-NBs are thought to be repressed but superresolution microscopy has revealed that they are in a less compact state than genomes that are not associated with PML-NBs. When we investigated PML-NBs in human neuronal cell models of HSV-1 latency, we found that they are present in the absence of IFNα, unlike our primary murine neurons, and colocalize with latent viral genomes. However, IFNα treatment leads to an increase in PML-NBs and a significant increase in colocalization with latent viral genomes. In sum, this study demonstrates that subnuclear localization can impact heterochromatic signature and compaction of viral genomes and implicates ATRX as a key heterochromatin-associated protein in the establishment and maintenance of HSV-1 latency.

**Introduction**

HSV-1 only establishes latency in neurons, where the viral genome associates with repressive heterochromatin, including H3K9me3 and H3K27me3, but the cellular proteins and molecular mechanisms that mediate this chromatinization are largely unknown (Wang *et al.*, 2005; Cliffe *et al.*, 2009; Kwiatkowski *et al.*, 2009; Bloom, 2016). Furthermore, HSV-1 latency in ganglia is heterogenous, with some neurons being more susceptible to reactivation than others (Sawtell, 1997; Proenca *et al.*, 2008; Bertke *et al.*, 2011), and this heterogeneity may ultimately be reflected in the association of viral genomes with different cellular proteins, histone variants or histone post-translational modifications (PTMs).

The SWI/SNF chromatin remodeler alpha thalassemia mental retardation x-linked protein (ATRX) is a 280kDa protein that is mutated in severe neurological disorders (Clynes *et al*, 2013). It contains multiple domains that allow it to interact with a variety of proteins, including methyltransferases and other heterochromatin-associated proteins, to promote transcriptional repression (Lewis *et al*, 2010; Clynes *et al.*, 2013; Noh *et al*, 2015). ATRX can act as a histone chaperone, forming a complex with the death-associated protein 6 (Daxx) to catalyze the deposition of non-canonical histone variant H3.3 (Lewis *et al.*, 2010). ATRX also can target chromatin through direct interactions with specific histone posttranslational modifications (PTMs), including H3K9me3-containing peptides, a histone mark associated with repressive heterochromatin (Noh *et al.*, 2015). Furthermore, it has recently been shown that ATRX can recruit the polycomb repressive complex 2 (PRC2) to specific genomic sites to contribute to their silencing (Sarma *et al*, 2014; Ren *et al*, 2020).

Importantly, ATRX and Daxx have been shown to be repressive to lytic HSV transcription and replication (Lukashchuk & Everett, 2010). More recent studies have further implicated ATRX in the maintenance and stability of viral heterochromatin during chromatin stress, as well as in reducing HSV viral DNA accessibility by altering the structure of the H3-loaded viral chromatin (Cabral *et al*, 2018; Cabral *et al*, 2021). In a quiescent infection of fibroblasts using a HSV IE-deficient virus, ATRX/Daxx and another H3.3-chaperone, HIRA, colocalized to viral genomes and chromatinized viral genes, including the LAT, with H3.3 modified by the repressive H3.3K9me3 mark, thereby rendering them transcriptionally silent (Cohen *et al.*, 2018). This function was dependent on PML, as depletion of PML reduced association of ATRX/Daxx with latent genomes, as well as H3.3 load on multiple viral loci (Cohen *et al.*, 2018). This is consistent with additional studies that show intrinsic epigenetic silencing of viral gene expression through deposition of variant histone H3.3 onto viral genomes at PML-NBs (Placek *et al*, 2009; Rai *et al*, 2017). In contrast to the canonical histone H3.1, which is deposited by chromatin assembly factory-1 (CAF-1) onto replicating DNA, the histone variant H3.3 is deposited in a replication-independent manner (Tagami *et al*, 2004; Szenker *et al*, 2011). ATRX/Daxx is thought to preferentially promote H3.3 deposition at telomeres and pericentric repeats (Lewis *et al.*, 2010), while the HIRA complex is thought to mediate H3.3 loading at actively transcribed genes and in a nucleosome gap-filling function (Tagami *et al.*, 2004; Ray-Gallet *et al*, 2011). In the context of HSV-1 lytic infection, there is replication-independent deposition of H3.3 onto HSV-1 genomes shortly after nuclear entry, and H3.1 is only incorporated during replication of the HSV-1 genome (Placek *et al.*, 2009; Conn *et al*, 2013; Cabral *et al.*, 2018). Although the histone variant H3.3 only differs from H3.1 by 4 residues, this can have a significant impact on transcriptional activity and compaction of the chromatin.

To further investigate the role of ATRX in HSV-1 latency and reactivation, we characterized the distribution of ATRX and Daxx in primary murine sympathetic neurons and found abundant nuclear ATRX staining in the absence of detectable PML-NBs, which is in contrast to dermal fibroblasts and other non-neuronal cells (Alandijany *et al.*, 2018). Interestingly, we have preliminary data that latent viral genomes co-localize to these regions of dense ATRX staining and associate with histone variant H3.1, while latent viral genomes that colocalize to IFNα-induced PML-NBs associate with ATRX, Daxx and histone variant H3.3. Furthermore, ATRX knockout specifically in latently infected neurons *in vivo* reduces viral load in peripheral ganglia, although this is also a preliminary result. Surprisingly, we show that non-PML-NB-associated viral genomes are more compact than genomes that are entrapped by IFNα-induced PML-NBs. Furthermore, we explored the distribution of PML in two human neuronal cell models of HSV-1 latency and found that both have detectable PML-NBs in the absence of type I IFN, but colocalization of latent viral genomes to PML-NBs significantly increased with IFNα treatment. Ultimately, this study demonstrates how the subnuclear positioning of the latent viral genome can impact its heterochromatic signature and begins to correlate the association of histone variants and cellular proteins with the latent genome radius of gyration and ability to undergo induced reactivation.

**Results**

HSV-1 latency can be established in the absence of type I IFN

Because we observed that primary SCG neurons are largely devoid of PML-NBs and that PML-NBs form upon treatment with type I IFN treatment, we first wanted to clarify that latency was maintained in the absence of IFN and presumably without PML-NB formation, consistent with our previous data (Cuddy *et al.*, 2020). SCG neurons were infected at a multiplicity of infection (MOI) of 7.5 plaque forming units (PFU)/cell with

HSV-1 Us11-GFP presence of acyclovir (ACV). The ACV was removed after 6 days and the neuronal cultures were monitored to ensure the no GFP-positive neurons were present. We found that latency could be established and maintained for up to 5 days following removal of ACV (Fig. 3-1A). Reactivation was triggered by PI3K inhibition using LY294002, as previously described (Camarena, 2011; Kim *et al.*, 2012; Kobayashi *et al.*, 2012; Cliffe *et al.*, 2015), and quantified based on the number of Us11-GFP neurons in the presence of WAY-150138 which blocks packaging of progeny genomes and thus cell-to-cell spread (van Zeijl *et al*, 2000). These data therefore indicate that exogenous IFN is not required to induce a latent state in this model system.

We have observed that treatment of primary peripheral neurons isolated from postnatal and adult mice with type II IFN (IFNγ) and type III IFN (IFNλ2) led to a more variable response in PML-NB formation. Treatment with IFNγ induced a small but significant increase in detectable PML-NBs in a subpopulation of sympathetic neurons but did not result in the formation of detectable PML-NBs in sensory neurons. Exposure of neurons to IFN-lambda 2 (IFN-λ2), a type III IFN, did not induce the formation of detectable PML-NBs in either sympathetic or sensory neuron cultures. However, we were interested if treatment with these well-known antiviral cytokines at the time of initial infection could also impact reactivation in this model. SCG neurons were pre-treated with IFNγ (150 IU/ml) or IFNλ (100ng/ml) for 18 hours and during the initial 2hour HSV inoculation. Reactivation was induced and quantified base on the number of GFP positive neurons. We found that full reactivation was restricted in neurons exposed to type II IFN just prior to and during de novo infection. However, reactivation was not impacted by type III IFN treatment at the time of infection (Fig. 3-1B). Therefore, treatment of sympathetic neurons with type II IFN solely at the time of infection has a long-term effect on the ability of HSV to undergo reactivation.

Because IFN treatment could reduce nuclear trafficking of viral capsids during initial infection or impact infection efficiency, we next determined whether equivalent numbers of viral genomes were present in the neuronal cultures. At 8dpi, we measured relative viral DNA genome copy numbers in SCG neurons that were treated with IFNγ (150 IU/ml) and IFNλ2 (100 ng/ml) compared to untreated controls and found no significant difference (Fig. 3-1C). Therefore, type II IFN can mediate restriction of HSV latency without decreasing the number of latent viral genomes or inducing robust formation of detectable PML-NBs.

The multi-functional, chromatin remodeler protein ATRX has abundant nuclear staining in neurons and colocalizes with latent viral genomes outside the context of PML-NBs

Given that we can establish long-term latency in SCG neurons in the absence of type I IFN and detectable PML-NBs, we wanted to investigate additional cellular proteins, including ATRX and Daxx, that could associate with latent viral genomes and maintain latency. In untreated neurons, we observed abundant ATRX staining throughout the nucleus in regions that also stained strongly with Hoechst (Fig. 3-2A, B). This potential co-localization of ATRX with regions of dense chromatin is consistent with a previous study demonstrating that in neurons ATRX binds certain regions of the cellular genome associated with the constitutive heterochromatin modification H3K9me3 (Noh *et al.*, 2015). Importantly, this distribution of ATRX differs from what is seen in murine dermal fibroblasts (Fig. 3-2A, B) and other non-neuronal cells, where there is a high degree of colocalization between ATRX and PML (Alandijany *et al.*, 2018). Following treatment with IFNα, we found a redistribution of ATRX staining and colocalization between ATRX and the formed PML-NBs, but the majority of ATRX staining remained outside the context of PML-NBs (Fig. 3-2A, B). Similar to PML,

sympathetic SCG and sensory TG neurons isolated from both postnatal and adult mice were devoid of detectable puncta of Daxx staining (Fig. 3-2B), and we did not observe extensive Daxx staining in untreated neurons as we did for ATRX. We were unable to directly co-stain for Daxx and PML; however, treatment of neurons with IFNα did induce punctate Daxx staining that strongly colocalized with puncta of ATRX (Fig. 3-2B), which given our previous observation of ATRX co-localization with PML following type I IFN treatment we used as a correlate for PML-NBs. Primary sympathetic neurons are largely devoid of detectable PML and Daxx signal but have very abundant ATRX containing domains, highlighting a key difference between neurons and non-neuronal cells

Given the robust nuclear staining of ATRX in the absence of PML-NBs, we next wanted to explore if latent viral genomes co-localized to regions of ATRX staining in primary SCG neurons. To determine whether viral genomes localize with ATRX in untreated neurons, SCG neurons were infected with HSV-$1^{EdC}$ at an MOI of 5 PFU/cell in the presence of ACV as described above. By co-staining for ATRX, we found that a proportion of vDNA foci in untreated neurons colocalized with regions of dense ATRX staining. Representative images are shown (Fig. 3-2C). Furthermore, there is a proportion of latent genomes that do not colocalize with PML-NBs in neurons treated with IFNα during initial infection, and we found that a subset of latent genomes in IFNα-treated neurons similarly colocalized with regions of dense ATRX staining outside the context of PML-NBs (Fig. 3-2C). Therefore, a subset of latent viral genomes colocalize with regions of dense ATRX that functions outside the context of PML-NBs.

Depletion of ATRX during the establishment of latency *in vivo* decreases latent viral load

ATRX has been shown to associate with viral genomes in latently infected trigeminal ganglia *in vivo* (unpublished data, Dr. Anna Cliffe), so we wanted to directly

investigate the role of ATRX in the establishment and maintenance of latency. Transgenic ATRX$^{fl/fl}$ mice (a gift from Dr. David Picketts, Garrick *et al* (2006)) were infected by corneal scarification with SC16 Lat Cre, an HSV-1 strain encoding Cre-recombinase under the control of latency associated transcript (LAT) promoter (dose: 2x106 PFU virus/eye), which allowed for deletion of ATRX only in latently infected neurons (Proenca *et al.*, 2008). Because ATRX could play a role in the establishment or maintenance of latency, we monitored HSV-1 clinical disease (Han *et al*, 2001) and mortality following infection and found no significant difference (Fig 3-3A-D). We next assessed relative viral DNA genome copy numbers in latently infected trigeminal and superior cervical ganglia at 28 dpi, and we found that TG infected with SC16 LAT Cre had a significantly lower viral load than TG infected with SC16 WT (Fig 3-3E). Although we saw a similar trend in SCG, it was not significant (Fig 3-3F). We next assessed reactivation by lytic gene expression in SCG neurons following explant/axotomy and treatment with LY294002 for 15 hours. Although not statistically significant, we found higher levels of *UL29* mRNA in SCG neurons infected with SC16 LAT Cre (Fig 3-3G) Therefore, although these are very preliminary findings, our data suggests that infection of ATRX$^{fl/fl}$ mice with SC16 LAT Cre led to a significantly lower HSV-1 latent viral load in mouse TG and higher lytic gene expression in SCG neurons following induced reactivation.

PML-NB-associated latent viral genomes preferentially colocalize with H3.3, while non-PML-NB-associated genomes preferentially colocalize with H3.1

The considerable heterogeneity at the neuronal level in the co-localization of viral genomes with different subnuclear domains and its effect on reactivation raised the possibility that reactivation is impacted by the association with different repressive cellular proteins, histones and/or histone modifications. To begin to explore this

possibility, we pretreated SCG neurons with IFNα (600 IU/ml) then infected with HSV-$1^{EdC}$ at an MOI of 5 PFU/cell in the presence of ACV and IFNα as described above. By co-staining for total H3, we found that vDNA foci colocalized to total H3 in both untreated and IFNα-treated neurons over the course of infection (Fig 3-4A, B). Given the colocalization of Daxx, an H3.3-specific histone chaperone, with IFNα-induced PML-NBs, we next investigated the colocalization of vDNA foci with Daxx and H3.3 and found that Daxx puncta and H3.3 only colocalized with genomes in IFNα-treated neurons (Fig 3-4C). We similarly investigated the colocalization of latent viral genomes with H3.1 and found that in untreated neurons, H3.1 colocalized to viral genomes that were associated with ATRX-dense regions (Fig 3-4D). This is very preliminary data and will need further investigation and quantification. However, these results suggest that latent viral genomes colocalize to regions of total H3 throughout a latent infection but specifically colocalize to H3.3 when associated with IFNα-induced PML-NBs and to H3.1 when associated with ATRX-dense regions in untreated neurons.

Latent viral genomes colocalized with PML-NBs have less compact packaging

Because entrapment of viral genomes by IFNα-induced PML-NB leads to differences in histone association and a repressive form of latency, we next wanted to investigate the nanoarchitecture of viral genomes that are either associated with IFNα-induced PML-NBs or not. SCG neurons were pretreated with IFNα (600 IU/ml) then infected with HSV-$1^{EdC}$ at an MOI of 5 PFU/cell in the presence of ACV and IFNα as described above. We performed click chemistry to detect vDNA foci, co-stained for PML and applied direct stochastic optical reconstruction microscopy (dSTORM) to produce 3D projections of viral genomes. In order to calculate the dimensions of each viral genome and the radii of gyration ($R_g$), we performed image analysis as previously described (Boettiger *et al*, 2016; Grant *et al*, 2018) and found that the PML-NB-

associated vDNA foci are less compact than non-PML-NB-associated vDNA foci. Representative images are shown in Fig 3.5 and mean $R_g$ are reported in Fig 3B. Thus, our preliminary data indicates that vDNA that is physically associated with IFNα-induced PML-NBs shows less dense chromatin packaging than vDNA that is not associated with PML-NBs.

PML-NBs are constitutively present in human neurons.

To complement our work in peripheral murine neurons, we aimed to utilize human neuronal cell line models. The HD10.6 immortalized cell line derived from human dorsal root ganglia proliferate by virtue of a transduced tetracycline-regulated v-*myc* oncogene (Raymon *et al.*, 1999), and the Lund human mesencephalic neuronal cell line (LUHMES) is a subclone of the tetracycline-controlled, v-*myc*-overexpressing human mesencephalon-derived cell line MESC2.10 (Lotharius *et al.*, 2005). Both HD10.6 and LUHMES cells have previously been characterized and shown to support HSV-1 latency (Thellman *et al.*, 2017; Edwards & Bloom, 2019). We initially set out to investigate the contribution of PML-NBs to HSV latency and reactivation using these models and stained them for PML protein. In contrast to our primary mouse neurons, we observed that mature, fully differentiated HD10.6 and LUHMES neurons possess detectable PML-NBs, or puncta of PML, in the absence of IFNα. However, there is a small, but significant increase in PML-NBs following 18hr treatment with IFNα (600 IU/ml) (Fig 3-6A-D). Interestingly, ATRX showed diffuse nuclear staining in both HD10.6 and LUHMES cells and did not form detectable puncta at PML-NBs in the presence or absence of type I IFN (Fig 3-6A, B). Therefore, two human neuronal cell lines have PML-NBs in the absence of IFNα, but treatment with IFNα increases the number of PML-NBs.

Given the presence of detectable PML-NBs in untreated LUHMES and HD10.6 cells, we investigated if HSV-1 genomes colocalized with PML-NBs. To determine whether viral genomes localize with PML-NBs in untreated and type I IFN-treated cells, HD10.6 and LUHMES cells were pretreated with IFNα (600 IU/ml) then infected with HSV-$1^{EdC}$ at an MOI of 5 PFU/cell in the presence of ACV and IFNα as described above. By co-staining for PML, we found that a proportion of vDNA foci colocalized with PML-NBs in absence of IFNα, and this proportion increases with IFNα treatment (Fig 3-6C, D).

**Discussion**

During latency, the viral genome is enriched with histone post-translational modifications (PTMs) consistent with repressive heterochromatin, including H3K9me2/3 and H3K27me3 (Wang *et al.*, 2005; Cliffe *et al.*, 2009; Kwiatkowski *et al.*, 2009; Cohen *et al.*, 2018). How this heterogeneity arises, whether it results in heterogeneity at the chromatin level or with the binding of different repressive histone readers and whether populations of viral genomes differ in their abilities to undergo reactivation is not known. Here, we show preliminary evidence that the subnuclear positioning of latent viral genomes correlates with colocalization of different heterochromatin-associated proteins and core histone variants, as well as with different physical compaction states of the viral genome.

Interestingly, we have found that the multi-functional, chromatin remodeler protein ATRX has abundant nuclear staining in neurons and, in contrast to non-neuronal cells, is localized outside of PML-NBs. ATRX staining overlapped with Hoechst DNA staining in our primary neurons, suggesting its localization with AT-rich heterochromatin regions (Bucevicius *et al*, 2019). ATRX can act as a histone chaperone, forming a

complex with Daxx to catalyze the deposition of the histone variant H3.3 (Lewis *et al.*, 2010). However, in the absence of type I IFN, we only saw faint staining of Daxx in our primary neurons, indicating it could be functioning in additional roles. ATRX has also been shown to interact with a variety of proteins, including methyltransferases and other heterochromatin-associated proteins, to promote transcriptional repression (Lewis *et al.*, 2010; Clynes *et al.*, 2013; Noh *et al.*, 2015), and it can target chromatin through direct interactions with specific histone PTMs, including H3K9me3-containing peptides (Noh *et al.*, 2015). With type I IFN treatment, ATRX redistributes and strongly colocalizes to puncta of PML and Daxx, but the majority of ATRX staining remains outside these IFNα-induced PML-NBs.

In a model of quiescence utilizing human primary fibroblasts and a replication deficient virus, HSV genomes associated with PML-NBs were almost exclusively enriched with H3.3, mediated by ATRX/Daxx and HIRA, and the H3.3K9me3 chromatin mark (Cohen *et al.*, 2018). Although preliminary, we similarly found that PML-NB-associated genomes strongly colocalize to Daxx and H3.3. Therefore, it is tempting to speculate that PML-associated latent genomes are specifically enriched for H3K9me3 and not H3K27me3. However, in a previous study, we have found that H3S10 becomes phosphorylated during transcriptional activation following a reactivation stimulus (Cliffe *et al.*, 2015) and viral genomes co-localize with regions of H3K9me3S10p in neurons that were not pre-treated with IFN (Cuddy *et al.*, 2020). In addition, removal of H3K9 methylation is required for HSV reactivation (Liang *et al.*, 2009; Liang *et al*, 2013). Together, these studies suggest that H3K9me3 is present on reactivation component genomes, but different combinations of modifications may exist on reactivation competent versus repressive genomes. It is also possible that viral genomes outside of PML-NBs are associated with both H3K9me3 and H3K27me3 and are more permissive

than genomes not associated with H3K27me3 within PML-NBs or PML-NB-associated genomes are completely devoid of histone PTMs, as regions of cellular DNA lacking histone modifications have previously been characterized as the most inactive regions of chromatin (Boettiger *et al.*, 2016).

Furthermore, PML-NB-associated genomes could associate with different histone readers. ATRX and HP1 are histone readers that have been found to be to be associated with PML-NBs in non-neuronal cells (Seeler *et al*, 1998; Ishov *et al*, 2004). Interestingly, ATRX is a H3K9me3 reader that is not evicted with the neighboring serine (S10) is phosphorylated (Noh *et al.*, 2015), a dual modification that we have previously linked to HSV reactivation (Cliffe *et al.*, 2015). This is in contrast to HP1 and Suz12, which are evicted from H3K9me3 and H3K27me3 respectively when the neighboring serine is phosphorylated (Fischle *et al*, 2005; Gehani *et al.*, 2010; Noh *et al.*, 2015). However, our preliminary data shows that latent viral genomes can also colocalize to regions of ATRX staining outside the context of IFNα-induced PML-NBs, but these genomes preferentially colocalize with histone variant H3.1, which could be more efficiently phosphorylated by JNK to trigger reactivation. Additionally, one of our most exciting preliminary findings is that PML-NB-associated genomes are less compact than non-PML-NB-associated genomes. This is a surprising result, as we had originally hypothesized that physical compaction of the latent viral genome by PML-NBs could make it less accessible to cellular proteins, such as Pol II, and lead to a more repressive state. However, the radius of gyration has previously been used to characterize regions of activate, inactive and Polycomb associated chromatin, and Polycomb associated chromatin was found to be the most compact, even more so than inactive chromatin, so it is possible that this is the case with non-PML-NB-associated genomes (Boettiger *et al.*, 2016). Again, it will be important to further characterize the colocalization of latent viral

genomes with BM1or Ring1A/B, core components of PRC1, and Suz12, a core component of PRC2.

In this study, we present preliminary evidence that could implicate ATRX as a heterochromatin-associated protein responsible for maintaining HSV-1 latency. An important caveat to our *in vivo* findings is that LAT is not expressed in the SC16 LAT Cre virus. This further complicates our findings, as we have latently infected neurons that lack both ATRX and the LAT transcripts. Previous work has shown that the LAT promotes lytic gene silencing and increases association of heterochromatin markers, including H3K9me2/3 and H3K27me3, with viral lytic genes during latency (Wang *et al.*, 2005; Cliffe *et al.*, 2009). Furthermore, the LAT gene serves to promote efficient establishment of latency by increasing the number of latently infected neurons (Thompson & Sawtell, 1997), and there is evidence to suggest that LAT promotes neuronal survival and frequency of reactivation (Leib *et al.*, 1989; Perng *et al.*, 2000; Thompson & Sawtell, 2001). Although our results do not resemble a LAT phenotype, the loss of LAT transcripts could be confounding our interpretations, and it will be important to specifically test the effect of ATRX knockdown in the context of wild type HSV-1 latent infection.

In order to establish more clinically relevant models of HSV-1 latency and reactivation, we wanted to complement our work in murine neurons with experiments in human neuronal cell lines. When we explored PML protein distribution in LUHMES and HD10.6 cells, we found that both cell lines had ~5 PML-NBs/nucleus in the absence of IFN, which is in contrast to our findings in mouse peripheral neurons that are largely devoid of PML-NBs. This discrepancy could be a result of multiple factors, in addition to species difference. First LUHMES are CNS neurons (Lotharius *et al.*, 2005) and

although HD10.6 are derived from DRG neurons (Raymon *et al.*, 1999), they could represent a different subtype of sensory neurons than sensory neuron cultures that we derived from mouse trigeminal ganglia. Furthermore, LUHMES and HD10.6 are embryonic (Raymon *et al.*, 1999; Lotharius *et al.*, 2005), and we only assessed postnatal and adult murine neurons. It is well characterized that PML-NBs can differ based on developmental stage and decrease in number with aging (Bernardi & Pandolfi, 2007; Lallemand-Breitenbach & de The, 2010). Moving forward, it will be beneficial to continue to characterize and evaluate HD10.6 and LUHMES cells as supplemental models in HSV-1 latency research.

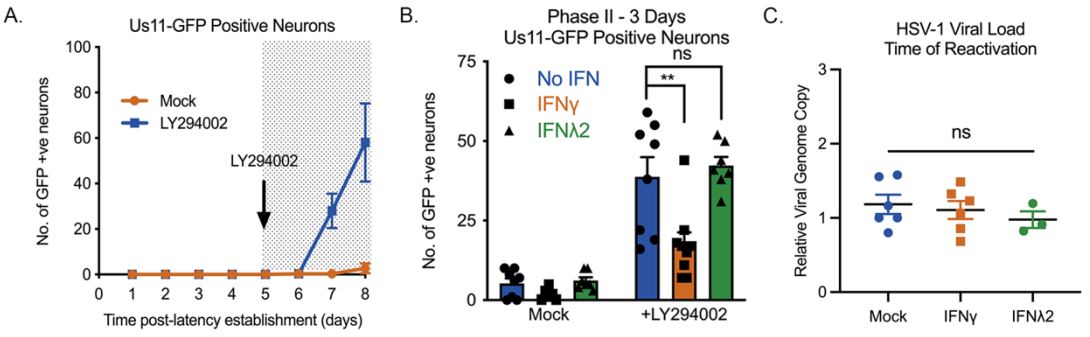


**Fig 3-1**. Type I IFN is not required for the establishment of latency. (**A**) Reactivation from latency is quantified by Us11-GFP expressing neurons following addition of the PI3K inhibitor LY294002 (20μM) in the presence of WAY-150168, which prevents cell-to-cell spread. (**B**) Number of Us11-GFP expressing neurons at 3 days post-LY294002-induced reactivation in P6 SCG neuronal cultures infected with HSV-1 in the presence or absence of IFNγ (150 IU/ml) or IFNλ2 (100 ng/ml). (**C**) Relative amount of viral DNA at time of reactivation (8dpi) in SCG neurons infected with HSV-1 in the presence or absence of IFNγ (150 IU/ml) or IFNλ2 (100 ng/ml). Data represent the mean ± SEM. Statistical comparisons were made using a one way ANOVA with a Tukey's multiple comparison (ns not significant, ** P<0.01)

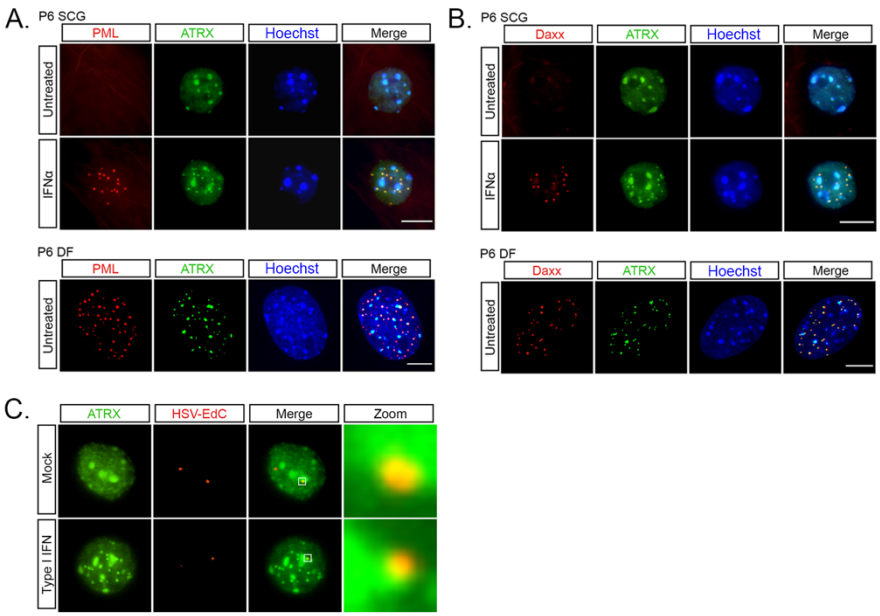


**Fig 3-2.** Type I IFN alters the sub-cellular localization of ATRX and Daxx in primary peripheral neurons. (**A**) Representative images of untreated or IFNα (600 IU/ml)-treated P6 SCG neurons stained for PML and ATRX. (**B**) Representative images of untreated or IFNα (600 IU/ml)-treated P6 SCG neurons stained for Daxx and ATRX. (**C**) Representative images of vDNA foci detected by click chemistry to ATRX at 3 dpi in P6 SCG neurons infected with HSV-1$^{EdC}$ in the presence or absence of IFNα (600 IU/ml). Scale bar, 20μm.

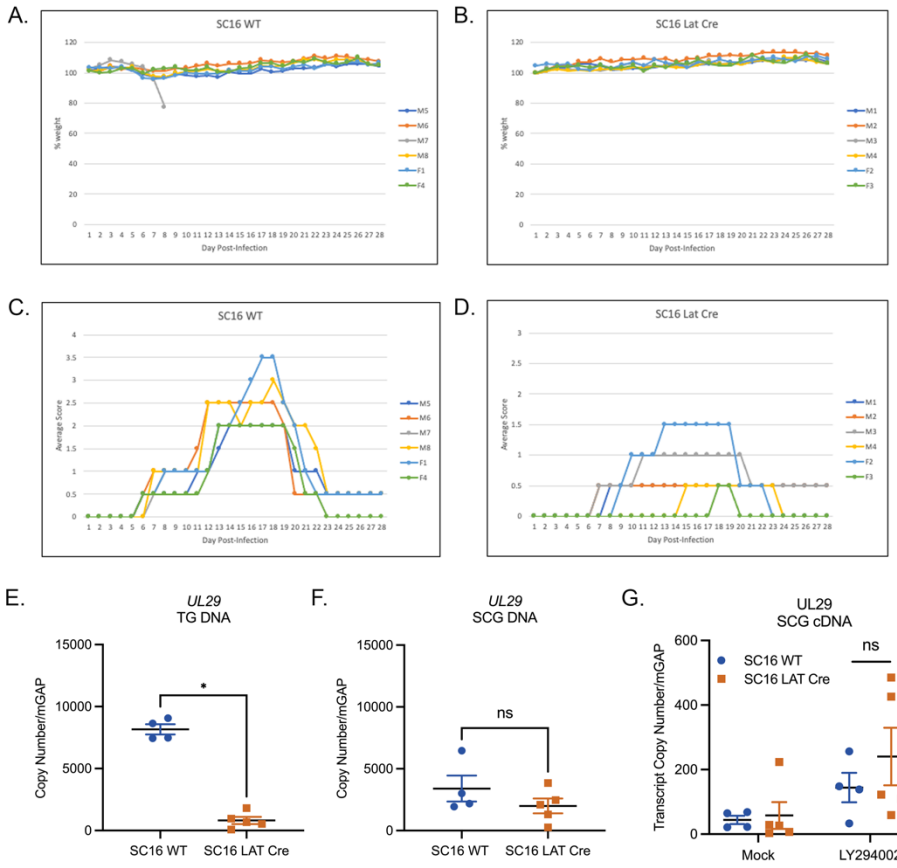


**Fig 3-3.** TG latently infected with SC16 LAT Cre have lower viral loads at 28dpi. (**A, B**) Percent weight loss in mice infected with SC16 WT or SC16 LAT Cre (**C, D)** Clinical disease scores in mice infected with SC16 WT or SC16 LAT Cre. (**E, F)** Relative amount of viral DNA at 28dpi in TG and SCG neurons infected with SC16 WT or SC16 LAT Cre. (**G**) RT-qPCR for viral mRNA transcripts at 15 hours post-LY294002-induced reactivation of SCGs infected with SC16 WT or SC16 LAT Cre. Data represent the mean ± SEM. Statistical comparisons were made using a Mann-Whitney test (ns not significant, * $p<0.05$)

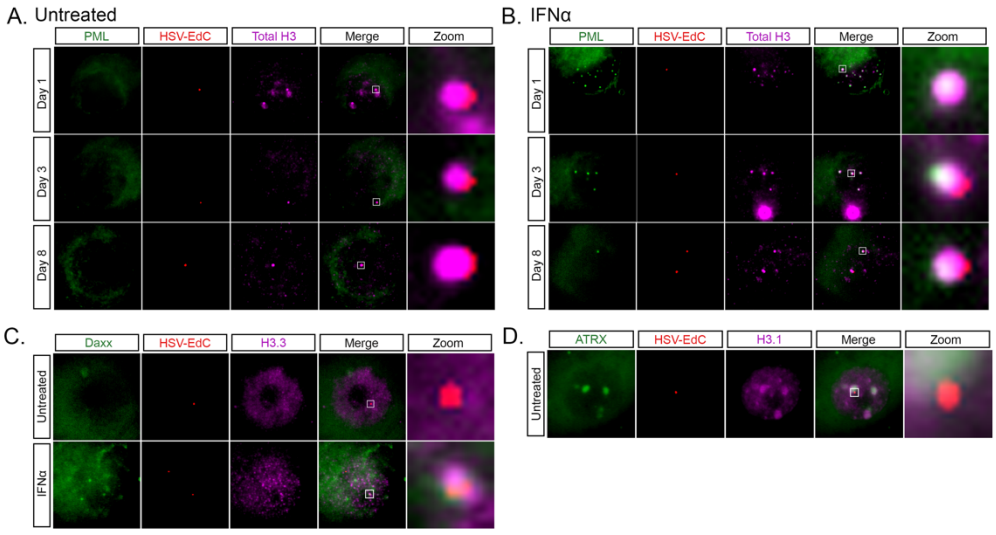


**Fig 3-4.** The latent viral genome colocalizes with H3 histone variants throughout latent infection. (**A, B**) Representative images of vDNA foci detected by click chemistry to PML and total H3 at 1, 3, 8 dpi in P6 SCG neurons infected with HSV-1$^{EdC}$ in the presence or absence of IFNα (600 IU/ml). (**C**) Representative images of vDNA foci detected by click chemistry to Daxx and H3.3 at 8 dpi in P6 SCG neurons infected with HSV-1$^{EdC}$ in the presence or absence of IFNα (600 IU/ml). (**D**) Representative images of vDNA foci detected by click chemistry to ATRX and H3.1 at 8 dpi in P6 SCG neurons infected with HSV-1$^{EdC}$ in the presence or absence of IFNα (600 IU/ml).

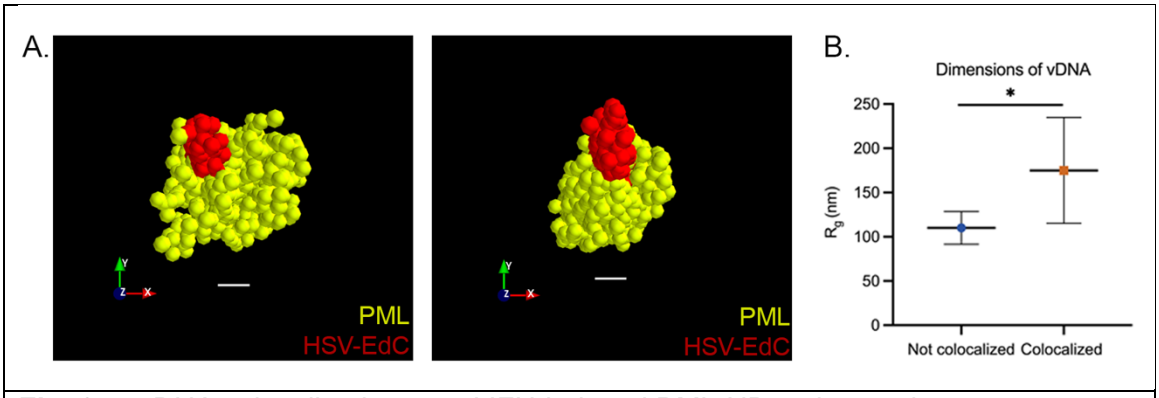


**Fig. 3-5.** vDNA colocalized to type I IFN-induced PML-NBs adopts a less compact packaging configuration. (**A**) The 3D projections of two representative vDNA foci (red) colocalized to PML (yellow) in IFNα-treated (600 IU/ml) SCG neurons at 1dpi. Spheres depict the Cartesian coordinates of individual photon emissions, with their radii scaled to the median axial localization precision of 100nm. (**B**) Mean $R_g$ data of vDNA foci in untreated and IFNα-treated SCG neurons at 1dpi. Data represent the mean ± SD. Statistical comparisons were made using a t-test (* $p<0.05$) Scale bar, 200nm.

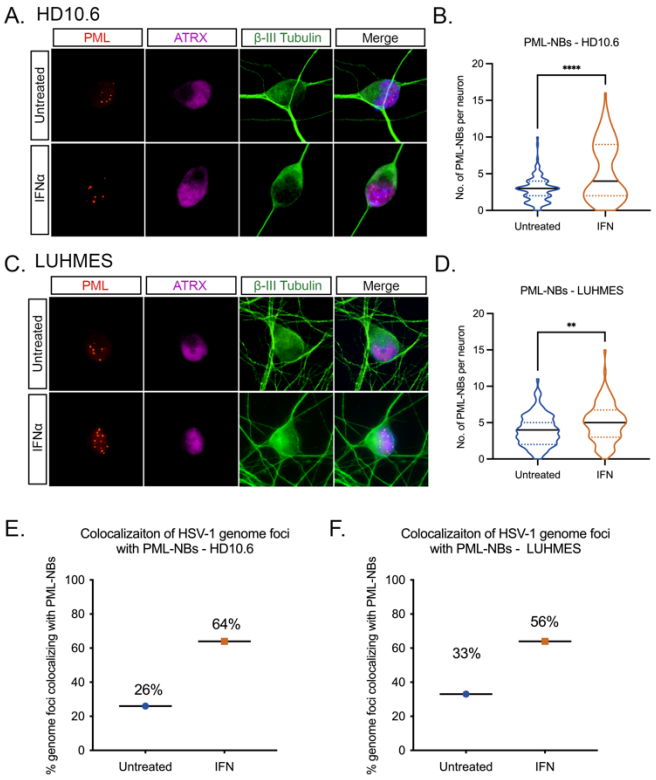


**Fig. 3-6.** Human neuronal cell models of HSV-1 latency possess PML-NB that colocalize to latent viral genomes. (**A, B**) Representative images and quantification of HD10.6 cells treated with interferon (IFN)α (600 IU/ml) for 18h and stained for PML, ATRX and the neuronal marker BIII-tubulin. Data represent the mean ± SEM. Statistical comparisons were made using a Mann-Whitney test (** p<0.01, **** P,0.0001) (**C, D**) Representative images and quantification of LUHMES cells treated with interferon (IFN)α (600 IU/ml) for 18h and stained for PML, ATRX and the neuronal marker BIII-tubulin. (**E, F**) Percent colocalization of vDNA foci detected by click chemistry to PML at 1 dpi in HD10.6 and LUHMES cells infected with HSV-$1^{EdC}$ in the presence or absence of IFNα (600 IU/ml). Significance not calculated as results represent values obtained from 1 experimental replicate.

# CHAPTER 4

*Conclusions and Future Directions*

In this dissertation, I have presented new sets of data which have advanced our understanding of the role of IFNs in latent HSV-1 infection. Each chapter was presented with a discussion and conclusions. In this chapter, I will expand on the implications to the field and suggest future studies to further advance the knowledge base.

Role of PML-NBs in HSV Latency

Prior to our work, the presence, composition and function of PML-NBs in peripheral neurons had not been fully explored. Although prior studies have concluded that PML-NBs are present in mouse and human sensory neurons by Immuno-FISH, these results are limited by the poor quality of staining, lack of quantification and the acknowledgment of a population of neurons that completely lack PML-NBs (Catez *et al.*, 2012; Maroui *et al.*, 2016). Using simple, reductionist approaches, we were able to perform high-quality, repeatable staining to more thoroughly characterize PML-NBs in murine peripheral neurons. More specifically, we were able to isolate and culture a number of different peripheral neuron populations (SCG, TG and DRG) from both postnatal and adult mice, and we found that these peripheral neurons lack detectable PML-NBs. We do acknowledge that axotomy or the processing of the neurons *ex vivo* could lead to PML-NB disruption or dispersal. However, this could be tested by performing axotomy in neurons cultured in microfluidic chambers, where axonal and soma compartments are physically and fluidically isolated. Additionally, we show that PML-NBs are present in HD10.6 and LUHMES cells, two human neuronal cell lines. Given that these are embryonic, it would be interesting to assess PML-NBs in similarly aged/developed murine peripheral neurons. Additionally, our lab is working to produce induced CNS neurons from that are directly differentiated from human fibroblasts that maintain age-related signatures. The lab will be able produce induced neurons from

fibroblasts isolated from normal individuals of different ages and could investigate the presence and distribution of PML-NBs in these differently aged neurons.

Very little is known about how IFN signaling in peripheral neurons impacts PML-NBs. In our work, we were able to show that PML-NBs were robustly induced by type I IFNs in all peripheral neuron populations assessed and to a lesser degree by type II IFN but just in a subset of SCG neurons. We were also able to show that previously characterized components of PML-NBs, including SUMO-1, ATRX, Daxx, and Mx1 associate with IFNα-induced PML-NBs in murine peripheral neurons. Interestingly, we used microfluidic chambers to show that the localization of IFNα treatment is important, as PML-NBs were induced following isolated treatment of the neuronal soma but not following isolated treatment of axons. Given the unique structure of peripheral neurons and potential that IFN secreted at a mucosal surface could act on axon terminals of innervating neurons, this is important, as it suggests that inflammatory cytokines in the ganglia are crucial for PML-NB formation. These data also supports evidence from Rosato et al (2015) that axon-specific treatment of mature sensory neurons with IFNβ did not induce STAT1 re-localization or upregulation of ISGs. This data is preliminary, however, and should be repeated. Given that our *in vitro* cultures of pure populations of intact neurons are not representative of the complex cellular makeup of peripheral ganglia, we also acknowledge that basal levels of IFNs *in vivo* could provide enough signaling to account for the discrepancies in baseline number of PML-NBs, and it would be interesting to assess PML-NB number in peripheral ganglia isolated from IFNAR- and IFNGR-knockout mice.

Our studies have further contributed to the understanding of IFN signaling in peripheral neurons: First, we show that PML is not required for induction of ISGs in

sympathetic neurons, which has been suggested in a number of non-neuronal cells (Ulbricht *et al.*, 2012; Chen *et al.*, 2015; Kim & Ahn, 2015; Scherer & Stamminger, 2016; McFarlane *et al.*, 2019). However, we acknowledge the limitations of our study and appreciate that PML could play a more nuanced role in ISG induction that was undetectable or outside the scope of our experimental parameters. Second, we have shown that IFNα-induced PML-NBs persist for at least 15 days following cessation of IFN exposure when elevated ISG expression and the protein products of known ISGs were undetectable. Importantly, this could represent neuron-specific innate immune memory that has only been demonstrated previously in fibroblasts and immune cells (Kamada *et al.*, 2018; Moorlag *et al.*, 2018). We do note that that average number of PML-NBs per nucleus does decrease over time, but there is evidence in non-neuronal cells to suggest that PML-NBs can fuse particularly when colocalized to viral genomes (Everett, 2016; Maroui *et al.*, 2016). We also show that vDNA-associated PML-NBs have significantly greater volumes than non-vDNA-associated PML-NBs at 3dpi, but it will be important to further assess PML-NB volume at different times post-IFNα treatment, both in the presence and absence of HSV-1 infections.

Although prior studies have shown that 1) type I IFNs can restrict HSV-1 replication in peripheral neurons (Svennerholm *et al.*, 1989; Carr *et al.*, 2003; Low-Calle *et al.*, 2014; Rosato & Leib, 2015; Linderman *et al.*, 2017) and 2) type I IFNs can alter the distribution pattern of viral genomes in peripheral neurons (Maroui *et al.*, 2016), our studies provide the first evidence that PML-NBs are directly responsible for an IFNα-dependent restriction of HSV-1 latency. Furthermore, we show that IFNα must be present during initial infection for 1) latent viral genomes to colocalize with PML-NBs and 2) IFNα to repress HSV-1 reactivation. It will be very interesting to continue to investigate how the timing IFNα signaling is impacting this colocalization. Although most PML-NBs

are thought to be relatively static (Wiesmeijer *et al*, 2002; Eskiw *et al*, 2003; Eskiw *et al*, 2004), studies have discovered a highly dynamic subset of PML-NBs that show rapid ATP-dependent movement (Muratani *et al*, 2002; Wiesmeijer *et al.*, 2002). Furthermore, experiments in fibroblasts show significant nuclear redistribution and asymmetric distribution of IFI16, Daxx and PML towards incoming viral genomes at the edge of plaques (Everett, 2016). Utilizing the chamber model, we have preliminary evidence to suggest that PML-NBs do not redistribute at early time points post-axonal infection with HSV-1, but this was assessed using static imaging. It would be interesting to further assess the dynamic movement of IFNα-induced PML-NBs in peripheral neurons utilizing live-cell, time-lapse microscopy with fluorescently labelled PML or Daxx at different time points post-axonal infection. Furthermore, fluorescence recovery after photobleaching (FRAP) experiments could also be very informative, as they would indicate the effect of the timing of IFNα treatment on the exchange of PML or PML-NB components, such as Daxx, from the nucleoplasm to PML-NBs or between PML-NBs, which could provide evidence why IFNα must be present during initial infection to have colocalization between viral genomes with PML-NBs.

Although we show that IFNα-induced PML-NBs entrap vDNA foci throughout a latent infection to promote a deeper form of latency, we need to further characterize how PML-NBs might influence the chromatin structure of viral genomes and the ability of the viral genomes to reactivate. We have preliminary evidence to show that viral genomes at PML-NBs are enriched in total H3 and the histone variant H3.3 specifically, but it will be important to determine if the genomes are also enriched with H3K9me3, H3K27me3 or both, as these modifications have previously been shown to be highly enriched on the latent genomes (Cliffe *et al.*, 2009). These experiments would assess co-localization by click chemistry to label viral genomes combined with IF in mock and IFN treated $PML^{fl/fl}$

neurons that are either transduced with cre-nanopod vector or mock transduced, which we have already successfully validated. It will be important to supplement these experiments with chromatin immunoprecipitation (ChIP) assays using the same antibodies and conditions. Interestingly, a prior study found that PML-NB-associated genomes were only enriched for H3K9me3 (Cohen *et al.*, 2018). Although there is evidence to suggest H3K9me3 is present on reactivation-competent genomes, Catez *et al* (2014) also show that PML-NB associated genomes are transcriptionally dead, including transcription of the LAT locus. In order to assess this in our system, RNA-fish could be combined with Click-chemistry and IF to evaluate the transcriptional activity of genomes that are PML-NB-associated vs those that are not associated. This technique could also be utilized to look at transcriptional activity of latent viral genomes at early time points post-reactivation to see if PML-NB associated genomes undergo phase I or phase II of reactivation.

Our studies have begun to explore the function of PML-NBs in neurons and the heterogeneity in HSV latency but in order to define how one population of viral genomes is less permissive for reactivation, we need to further investigate how association with PML-NBs or other nuclear domains influences the chromatin structure of the viral DNA. Ultimately, by using a variety of techniques to analyze the viral chromatin structure in the presence and absence of PML-NBs, we will acquire key data on how PML-NBs influence the latent genome structure to potentially promote a deeper form of latency, as well as learn more about the contribution of PMLNBs to altering the chromatin structure of other associated regions of DNA.

<u>Role of ATRX and other heterochromatin-associated proteins in HSV latency</u>

In addition to lacking detectable PML-NBs, we found that cultured murine peripheral neuronal populations have abundant ATRX-containing nuclear domains in the absence of PML-NBs. The results of our ATRX staining highlight a key difference between neurons and non-neuronal cells, which show a high-degree of colocalization between ATRX and PML puncta. Interestingly, our microscopy studies using primary peripheral neurons *in vitro*, although preliminary, has also suggested that ATRX associates with latent viral genomes both in the context of PML-NBs and outside of PML-NBs. Our lab also has evidence that ATRX is enriched on viral genomes in latently infected TG *in vivo.* Prior to our work, a study indicated that ATRX acts in complex with Daxx as a H3.3-specific chaperone to almost exclusively chromatinize PML-NB-associated quiescent HSV-1 genomes with H3.3K9me3 (Cohen *et al.*, 2018). We show similar evidence that PML-NB-associated latent genomes colocalize with ATRX, Daxx and H3.3, but we also define a separate population of latent genomes that colocalize with ATRX outside of PML-NBs and are colocalized with histone variant H3.1. It will be important to further characterize the association of different viral genomes with histone modifications known to be enriched on the latent genome, including H3K27me2/3 and H3K9me2/3.

Once this is better characterized, we can then determine how ATRX affects the chromatin structure of viral genomes by knocking out ATRX. These experiments would combine click chemistry to detect vDNA and immuno-labelling to detect histones and PTMs in mock and IFN treated $ATRX^{fl/fl}$ neurons that are either transduced with cre-nanopod vector or mock transduced. One caveat to examining changes in the chromatin structure following ATRX knockout is that the re-synthesis of viral proteins can also result in changes to the viral chromatin. To account for this, we will take advantage of our primary neuronal system where we can treat with cycloheximide to inhibit protein

synthesis. In addition, we will also infect primary neurons with a recombinant virus, d109, that does not express immediate-early viral mRNA (Samaniego *et al*, 1998). ChIP and Re-ChIP should also be performed to further supplement the microscopy experiments. If ATRX is functioning as a histone chaperone to deposit histones, such as H3.3, on the viral genome or to maintain repressive heterochromatin marks (i.e. H3K9me3), we would expect to see significantly less enrichment of histones and repressive PTMs on the viral genomes (decreased colocalization) following ATRX knockdown. If ATRX is acting as a histone reader and represses translation directly, we may not observe changes in histone PTMs. Ultimately, this aim will provide important mechanistic data on the regulation of gene expression following knockdown of a key heterochromatin-associated protein.

Moreover, it will be important to analyze association of latent genomes with other reader proteins, including HP1 and components of the PRC1 and PRC2 complexes. Both ATRX and HP1 have been found to be to be associated with PML-NBs in non-neuronal (Seeler *et al.*, 1998; Ishov *et al.*, 2004). Interestingly, a H3K9me3/pS10 histone phospho/methyl switch may permit transcription during HSV-1 reactivation (Cliffe *et al.*, 2015; Cuddy *et al.*, 2020), but ATRX is one of the few histone readers identified as not being evicted from H3K9me3 when the neighboring serine is phosphorylated (Noh *et al.*, 2015), and may prevent ATRX-associated genomes from re-entering the lytic program following neuronal stress. This is in contrast to HP1 and Suz12, which are evicted from H3K9me3 and H3K27me3 respectively when the neighboring serine is phosphorylated (Fischle *et al.*, 2005; Gehani *et al.*, 2010; Noh *et al.*, 2015). Therefore, we may expect to observe increased ATRX association in the presence of PML-NBs and increased Suz12 association in the absence of PML-NBs. Furthermore, different histone readers could also account for our preliminary data that suggests PML-NB-associated genomes are

less compact than non-PML-NB-associated genomes, as the radius of gyration has previously been used to characterize regions of activate, inactive and Polycomb associated chromatin, and polycomb associated chromatin was found to be the most compact, even more so than inactive chromatin (Boettiger *et al.*, 2016).

Our *in vivo* studies provide preliminary evidence that knockout of ATRX in latently infected neurons results in reentry into lytic gene expression. It is important to note that there are limitations to these results. First, the SC16 LAT Cre virus does not express LAT, so TG latently infected with SC16 LAT Cre lack both ATRX and LAT. Furthermore, the decrease in latent viral load seen in mice infected with SC16 LAT Cre could be a result of a lower initial viral inoculum dose, particularly given that we did not see any difference in morbidity or mortality between the two groups. In order to further implicate ATRX as the first heterochromatin-associated protein shown to be responsible for maintaining HSV-1 latency. I would suggest first examining if HSV-1 latency is disrupted in primary cultures of sympathetic neurons following depletion or knockout of ATRX. After establishing a latent HSV-1 infection in sympathetic neurons, reactivation can be evaluated following depletion of ATRX using lentivirus-mediated delivery of shRNA following the establishment of latency  We will also deplete Daxx to determine whether ATRX acts in complex with Daxx as a histone chaperone to maintain latency. We anticipate that depletion of ATRX will result in escape from latency. The contribution of Daxx at this stage is unknown. If Daxx depletion results in the same phenotype as ATRX depletion, it would indicate that the ATRX is functioning in complex with Daxx as a histone chaperone to maintain latency. This in itself would be interesting because it would suggest that the latent viral genome is fluid and requires the exchange of histones to maintain latency. Conversely, if Daxx is not required to maintain latency, ATRX may function to restrict gene expression either directly as a histone reader or indirectly by

altering the histone PTMs. It would also be important to repeat the effect of ATRX knockout *in vivo.* However, instead of utilizing the SC16 LAT Cre virus, I would suggest latently infecting TG with a wild type virus and carrying out Cre delivery by utilizing an AAV-Cre. It will also be important to confirm loss of ATRX binding to latent genomes by ChIP.

A greater understanding of the cellular proteins and molecular events regulating HSV infection in neurons and how this is impacted by the IFN-mediated immune response is critical for the development of novel therapies that target the latent stage of infection. Results obtained from my work and these future directions will provide mechanistic insights into the contribution of heterochromatin-based silencing in maintaining HSV-1 latency and preventing recurrent reactivation. Furthermore, the proposed studies will shed light on a long-standing question in the field as to how heterogeneity in HSV-1 latency arises and how a more repressive form of latency, as seen with type I IFN treatment, could result from a viral chromatin state that is less primed to reactivate. Ultimately, this would have important clinical implications and could advance therapeutic strategies to manipulate latency into a form that is completely refractory to reactivation stimuli.

## Materials and Methods

### Reagents

Compounds used in the study are as follows: Acycloguanosine, FUDR, LY 294002, Nerve Growth Factor 2.5S (Alomone Labs), Primocin (Invivogen), Aphidicolin (AG Scientific), IFN-α (EMD Millipore IF009) , IFN-β (EMD Millipore IF011), IFN-γ (EMD Millipore IF005), IFN-λ2 (PeproTech 250-33); WAY-150138 was kindly provided by Pfizer, Dr. Jay Brown and Dr. Dan Engel at the University of Virginia, and Dr. Lynn Enquist at Princeton University. Compound information and concentrations used can be found below in Table S1.

### Preparation of HSV-1 Virus Stocks

HSV-1 stocks of eGFP-Us11 Patton 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 (Benboudjema *et al*, 2003)) was kindly provided by Dr. Ian Mohr at New York University.

Stayput Us11-GFP was created by inserting an eUs11-GFP tag into the previously created gH-deficient HSV-1 SCgHZ virus (strain SC16) through co-transfection of SCgHZ viral DNA and pSXZY-eGFP-Us11 plasmid (Forrester *et al*, 1992). Stayput Us11-GFP is propagated and titrated on previously constructed Vero F6 cells, which contain copies of the gH gene under the control of an HSV-1 gD promoter, as described in Forrester *et al.* (1992). Vero F6s are maintained in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% FetaPlex (Gemini BioProducts). They are selected with the supplementation of 250 ug/mL of G418/Geneticin (Gibco).

**Primary Neuronal Cultures**

Sympathetic neurons from the Superior Cervical Ganglia (SCG) of post-natal day 0-2 (P0-P2) or adult (P21-P24) CD1 Mice (Charles River Laboratories) were dissected as previously described (Cliffe *et al.*, 2015). Sensory neurons from Trigeminal Ganglia (TG) of post-natal day 0-2 (P0-P2) CD1 mice (Charles River Laboratories) were dissected using the same protocol. Sensory neurons from TG of adult were dissected as previously described (Bertke *et al.*, 2011) with a modified purification protocol using Percoll from the protocol published by Malin *et al* (2007). 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 minutes each at 37 ˚C. Dissociated ganglia were triturated, and approximately 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 five days post-dissection to select against proliferating cells. Sensory neurons were maintained in the same media supplemented with GDNF (50ng/ml; Peprotech 450-44)

**Establishment and Reactivation of Latent HSV-1 Infection in Primary Neurons**

Latent HSV-1 infection was established in P6-8 sympathetic neurons from SCGs. Neurons were cultured for at least 24 hours without antimitotic agents prior to infection. The cultures were infected with eGFP-Us11 (Patton recombinant strain of HSV-1 expressing an eGFP reporter fused to true late protein Us11) or StayPut. Neurons were

infected at a Multiplicity of Infection (MOI) of 7.5 PFU/cell with eGFP-Us11 and at an MOI of 5 PFU/cell with StayPut (assuming $1.0x10^4$ neurons/well/24-well plate) in DPBS +$CaCl_2$ +$MgCl_2$ supplemented with 1% Fetal Bovine Serum, 4.5 g/L glucose, and 10 μM Acyclovir (ACV) for 2-3 hours at 37 ˚C. Post-infection, inoculum was replaced with CM1 containing 50 μM ACV and an anti-mouse IFNAR-1 antibody (Leinco Tech I-1188, 1:1000) for 5-6 days, followed by CM1 without ACV. Reactivation was carried out in DMEM/F12 (Gibco) supplemented with 10% Fetal Bovine Serum, Mouse NGF 2.5S (50 ng/mL) and Primocin. WAY-150138 (10 μg/mL) was added to reactivation cocktail to limit cell-to-cell spread. Reactivation was quantified by counting number of GFP-positive neurons or performing Reverse Transcription Quantitative PCR (RT-qPCR) of HSV-1 lytic mRNAs isolated from the cells in culture.

**Analysis of mRNA expression by reverse-transcription quantitative PCR (RT-qPCR)**

To assess relative expression of HSV-1 lytic mRNA, total RNA was extracted from approximately $1.0x10^4$ neurons using the Quick-RNA™ Miniprep Kit (Zymo Research) with an on-column DNase I digestion. mRNA was converted to cDNA using the SuperScript IV First-Strand Synthesis system (Invitrogen) 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 $1.0x10^4$ neurons using the Quick-DNA™ Miniprep Plus Kit (Zymo Research). qPCR was carried out using *Power* SYBR™ Green PCR Master Mix (Applied Biosystems). The relative mRNA or DNA copy number was determined using the Comparative $C_T$ ($\Delta\Delta C_T$) method normalized to mRNA or DNA levels in latently infected samples. Viral RNAs were normalized to mouse reference gene GAPDH. 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. Primers used are described in Table S2.

**Immunofluorescence**

Neurons were fixed for 15 minutes 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 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). Unless indicated otherwise, z-stack images of entire nuclei were acquired using an sCMOS charge-coupled device camera (pco.edge) mounted on a Nikon Eclipse Ti Inverted Epifluorescent microscope and processed into 2D projection images using the NIS-Elements software (Nikon) Extended Depth of Focus (EDF) plug-in. Images were further analyzed and processed using ImageJ.

**Click Chemistry**

For EdC-labeled HSV-1 virus infections, an MOI of 5 was used. EdC labelled virus was prepared using a previously described method (McFarlane *et al.*, 2019). Click chemistry was carried out a described previously (Alandijany *et al.*, 2018) with some modifications. Neurons were washed with CSK buffer (10 mM HEPES, 100 mM NaCl, 300 mM Sucrose, 3 mM $MgCl_2$, 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-1 DNA and the Click-iT EdU Alexa Flour 555 Imaging Kit (ThermoFisher Scientific,

C10638) according to the manufacturer's instructions with AFDye 555 Picolyl Azide (Click Chemistry Tools, 1288). 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- and 647-conjugated secondary antibodies for multi-color imaging (Invitrogen). Nuclei were stained with Hoechst 33258 (Life Technologies). Epifluorescence microscopy 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 processed using ImageJ. Confocal microscopy images were acquired using a Zeiss LSM 880 confocal microscope using the 63x Plan-Apochromat oil immersion lens (numerical aperture 1.4) using 405 nm, 488 nm, 543 nm, and 633 nm laser lines. Zen black software (Zeiss) was used for image capture, generating cut mask channels, and calculating weighted colocalization coefficients. Exported images were processed with minimal adjustment using Adobe Photoshop and assembled for presentation using Adobe Illustrator.

**Preparation of Lentiviral Vectors**

Lentiviruses expressing shRNA against PML (PML-1 TRCN0000229547, PML-2 TRCN0000229549, PML-3 TRCN0000314605), or a control lentivirus shRNA (Everett *et al.*, 2006) were prepared by co-transfection with psPAX2 and pCMV-VSV-G (Stewart *et al*, 2003) using the 293LTV packaging cell line (Cell Biolabs). Supernatant was harvested at 40- and 64-hours post-transfection. Sympathetic neurons were transduced overnight in neuronal media containing 8μg/ml protamine and 50μM ACV.

**RNA Sequence Analysis**

Reads were checked for quality using FASTQC (v0.11.8), trimmed using BBMAP (v3.8.16b), and aligned to the mouse genome with GENCODE (vM22) annotations using STAR (v2.7.1a). Transcripts per million calculations were performed by RSEM (v1.3.1), the results of which were imported into R (v4.0.2) and Bioconductor (v3.12) using tximport (v1.18.0). Significant genes were called using DESeq2, using fold change cutoffs and pvalue cutoffs of 0.5 and 0.05 respectively. Results were visualized using Heatplus (v2.36.0), PCAtools (v2.2.0), and UpSetR (v1.4.0). Functional enrichment was performed using GSEA and Metascape.

**Statistical Analysis**

Power analysis was used to determine the appropriate sample sizes for statistical analysis. All statistical analysis was performed using Prism V8.4. A Mann-Whitney test was used for all experiments where the group size was 2. All other experiments were analyzed using a one-way ANOVA with a Tukey's multiple comparison. Specific analyses are included in the figure legends. For all reactivation experiments measuring GFP expression, viral DNA, gene expression or DNA load, individual biological replicates were plotted (an individual well of primary neurons) and all experiments were repeated from pools of neurons from at least 3 litters.

**Data Availability**

1. RNA-Seq data: Gene expression

GEO GSE166738 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE166738)

**Supplemental Materials and Methods Tables**

**Table S1: Compounds Used and Concentrations**

| Compound | Supplier | Identifier | Concentration |
|---|---|---|---|
| Acycloguanosine | Millipore Sigma | A4669 | 10 μM, 50 μM |
| FUDR | Millipore Sigma | F-0503 | 20 μM |
| L-Glutamic Acid | Millipore Sigma | G5638 | 3.7 μg/mL |
| LY 294002 | Tocris | 1130 | 20 μM |
| IFNα | EMD Millipore | IF009 | 150 IU/ml, 600 IU/ml |
| IFNβ | EMD Millipore | IF011 | 150 IU/ml |
| IFNγ | EMD Millipore | IF005 | 150 IU/ml, 500 IU/ml |
| IFNλ2 | PeproTech | 250-33 | 100 ng/ml, 500 ng/ml |
| NGF 2.5S | Alomone Labs | N-100 | 50 ng/mL |
| Primocin | Invivogen | ant-pm-1 | 100 μg/mL |
| Aphidicolin | AG Scientific | A-1026 | 3.3 μg/mL |
| WAY-150138 | Pfizer | N/A | 10 μg/mL |
| AFDye 555 Azide Plus | Click Chemistry Tools | 1479-1 | 10μM |

**Table S2: Primers Used for RT-qPCR**

| Primer | Sequence 5' to 3' |
|---|---|
| mGAP 1SF | CAT GGC CTT CCG TGT GTT CCT A |
| mGAP 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 #1 F | GAG TTT GTC TGG TTC GAG GAC |
| gC #1R | ACG GTA GAG ACT GTG GTG AA |
| PML F | GGG AAA CAG AGG AGC GAG TT |
| PML R | AAG GCC TTG AGG GAA TTG GG |
| ISG15 F | CAA GCA GCC AGA AGC AGA CT |
| ISG15 R | CCC AGC ATC TTC ACC TTT AGG |
| IRF7 F | CCA GTT GAT CCG CAT AAG GT |
| IRF7 R | GAG GCT CAC TTC TTC CCT ATT T |
| LAT F | TGT GTG GTG CCC GTG TCT T |
| LAT R | CCA GCC AAT CCG TGT CGG |

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