# Innate Immune Activation in Support of Herpes Simplex Virus-1 Reactivation from Latency

By

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## Abstract

As a neurotropic virus, herpes simplex virus-1 (HSV) has evolved in close coordination with neuronal signaling pathways. While HSV can undergo productive lytic replication in mucosal epithelial cells and peripheral neurons during acute infection, the virus can only establish latency and undergo reactivation from peripheral neurons. The cellular mechanisms governing latency and reactivation reflect the dependence of HSV on neuronal signaling pathways. While our lab and others have defined critical steps in HSV reactivation, such as dual leucine zipper kinase (DLK) and c-Jun N-terminal kinase (JNK) activation, additional signaling mechanisms have yet to be elucidated. Using a combination of *in vivo* studies and an *in vitro* model of latency in primary peripheral neurons, we investigated the role of the innate immune signaling pathways in the context of HSV reactivation. The overall focus of this dissertation sought to define components of the neuronal innate immune system that support the transition from a transcriptionally silent latent infection to reactivation and the production of infectious virus. We found that HSV uses innate immune signaling to both trigger reactivation as well as activate pathways required to facilitate lytic gene transcription early in reactivation. After determining that neuronal hyperexcitability is a trigger of HSV reactivation, we found that the inflammatory cytokine interleukin 1β (IL-1β) induces DLK-dependent reactivation by prompting a hyperexcitable state in mature neurons. Additionally, we identified a role for the HSV protein UL12.5 in promoting lytic gene expression during reactivation by activating stimulator of interferon genes (STING), which regulates innate immunity downstream of DNA-sensing by cyclic GMP-AMP synthase (cGAS). UL12.5 is the first viral protein known to exclusively support HSV

reactivation and, importantly, to be required for lytic gene expression in Phase I. Our data also suggests that UL12.5 has the potential to activate RNA-sensing pathways in addition to the cGAS-STING DNA-sensing pathway. Together, these observations demonstrate the ability of HSV to co-opt neuronal immune pathways to support HSV reactivation and provide insight into the signaling events that mediate early events in reactivation.

## Dedication

This work, in truth, is a somewhat selfish endeavor in the pursuit of knowledge and thrill of discovery. From the early age of 13, I had dreamed of studying the nervous system – check my grade school yearbook for proof. The culmination of this dream lies within these pages and is due, in no small part, to the relentless efforts of those around me throughout my academic, professional, and personal life.

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# Herpes Simplex Virus-1 (HSV)

## Herpes simplex virus-1

Herpes simplex virus-1 (HSV) belongs to the extensive *Herpesviridae* family of dsDNA viruses. Herpesviruses are characterized by the presence of linear dsDNA held within a viral capsid that is surrounded by proteinaceous tegument and a lipid bilayer envelope. In addition to structural hallmarks, herpesviruses also exhibit the unique ability to establish latent infections within a host cell. These latent infections restrict lytic gene expression and therefore allow herpesviruses to effectively evade the host immune response. As a result, herpesviruses persist throughout the lifetime of the host. While productive lytic infection can occur in a broad range of cell types, *alphaherpesviruses* like HSV are capable of establishing these latent infections only in peripheral neurons.

The HSV life cycle can be defined by three distinct phases: lytic replication, latency, and reactivation. Lytic replication typically occurs at the epithelial mucosa where the virus replicates. The major steps of HSV lytic replication are described in **Figure 1**. Thereafter, HSV can infect peripheral neurons that innervate the face and neck where the virus can undergo more lytic replication or establish a latent infection. Lytic gene transcription is highly restricted during latency and latent infections are typically asymptomatic in the absence of a robust host immune response. Recurrent reactivation is associated with significant morbidities, as mentioned above, but the precise mechanisms mediating reactivation are unknown. Therefore, it is of great interest to elucidate the major steps within the pathway in order to provide insight into both disease etiologies as well as potential therapeutic targets.

## Lytic HSV infection

### *Cell Entry*

Although HSV can readily infect a variety of cell types [1], transmission typically occurs at the mucosal epithelia. The HSV envelope contains a range of glycoproteins on its surface that mediate interactions with host cell surface proteins to allow for cell entry. Interestingly, only four of these glycoproteins are required to mediate the fusion of the viral envelope and host cell membrane [2–6]. HSV entry into the cytosol appears to be mediated primarily by fusion events that allow for endocytosis of the viral capsid into the host cytosol [1,7,8]. Phagocytosis-like uptake and endosome acidification have also been implicated in HSV entry of some cell types, suggesting that HSV has adapted to different target cells [1]. Within the cytosol the vDNA is maintained with the viral capsid.

### *Cytosolic Transport*

Following cell entry, the viral capsid undergoes retrograde transport to the nuclear pore complex (NPC) of the host nucleus. Although passive diffusion of the viral capsid had been suggested as a means to reach the nucleus, the association of dynein and kinesin with the viral capsid suggest that trafficking to the nucleus is mediated by active transport along microtubules [9–12]. Viral tegument proteins, such as VP26 and VP1/2, in concert with some host factors, may play a role in this active directional transport[13–15]. Upon reaching the NPC, the viral genome is then injected into the nuclear space via the nuclear pore complex [16–18] by a combination of viral proteins and host factors[19–21].

*vDNA Replication*

Upon entry into the host cell nucleus, the HSV genome rapidly circularizes prior to replication. Some of the viral genomes may also become associated with histones that can have modifications promoting active transcription [22]. However, the HSV genome does not integrate with the host genome within the nucleus. vDNA lytic gene expression occurs in a highly ordered temporal cascade that proceeds through three classes of genes [23]. These genes are defined by their order of expression: immediate-early (IE; $\alpha$), early (E; $\beta$), or late (L; $\gamma$). To initiate lytic gene expression, VP16, a protein brought into the cell with the viral genome, complexes with host cell proteins to facilitate the initial transcription of IE genes [24]. IE genes encode for proteins mediating additional gene transcription and oppose intrinsic host defenses. Replication compartments can form around individual replicating genomes [25,26] during of E gene transcription. E proteins are predominantly involved in carrying out functions that support viral genome replication. In response to vDNA replication L gene transcription then initiates. L proteins encode a variety or proteins that are primarily involved in the assembly, packaging, and egress of the virus [27,28].

*Virion Assembly and Maturation*

The production of viral capsid proteins occurs in the cytosol, but nuclear localization sequences on the viral capsid proteins allow for its translocation to the nucleus for assembly [14,29] where vDNA replication continues to occur. Within the nucleus, viral capsids complex and mature with the addition of some tegument proteins

and a fraction receive newly synthesized viral genomes [30,31]. The HSV capsids exit the nucleus through the generation of a nuclear egress complex and a combination of fusion events via the inner and outer nuclear membrane [32–39]. After leaving the nucleus, additional tegument proteins are added to the capsid [31]. The viral capsids then fuse with endosomes containing the HSV glycoproteins translated in the endoplasmic reticulum and processed in the Golgi network [40–42]. After being trafficked to the plasma membrane, the mature virion buds in order to infect neighboring epithelial cells, shed from the skin surface, or spread internally to infect peripheral neurons [43].

## Models of latency and reactivation

The breadth of experimental models of HSV latency and reactivation permit interrogation of the difference phases of HSV as well systemic to intracellular responses. HSV is permissive within murine cells, thereby making mouse models an attractive system to understand systemic effects on HSV infection. The most common routes of HSV inoculation *in vivo* are via ocular scarification or via lip scarification. This allows the virus to undergo lytic replication at the epithelial surface and subsequently infect innervating axons from the peripheral nervous system. This paradigm allows for the full integration of systemic responses that affect the establishment and maintenance of HSV latency. HSV can establish latency in murine trigeminal ganglia (TG) following peripheral infection naturally. However, intracellular signaling pathways that engage with the viral genome and viral proteins during infection can difficult to discern during *in vivo* infection.

As a complement to *in vivo* assays, one of the most commonly used systems to study HSV is *in vitro* models of latency and reactivation. Given the inability to reliably and consistently utilize patient neurons, there is emerging interest in the use of human-derived neurons for use in culture to better recapitulate human infection. Embryonic stem cells that have the potential to differentiate into sensory-like neurons *in vitro*, have been recently used to study both HSV and varicella zoster virus (VZV), another alphaherpesvirus [44,45]. Lund human mesencephalic neuronal cell line (LUHMES) cells, which can be differentiated into dopamine-like neuron, as well as HD10.6 cells, which can be differentiated into sensory-like neurons, have been used in recent models of latency and reactivation [46,47]. Although shown to harbor quiescent HSV genomes that mimic latency and can later reactivate [48–50], the evidence using this model is still in its infancy. Another potential model of human-derived neurons will be directly differentiated neurons from human patient fibroblasts of different ages, which can be differentiated into neurons while leaving the epigenome intact. This model will be of particular interest to HSV latency and reactivation given the propose epigenetic mechanisms that drive the viral life cycle as well as how these mechanisms change with age. Altogether, human-derived neuronal models of HSV latency and reactivation represent a promising direction of the field, but will require further validation.

Some of the most tractable and widely used *in vitro* HSV model systems use primary murine sensory or sympathetic neurons dissected from pre-natal rats, post-natal mice, and adult mice [51–55]. Latency can be readily established in these neurons using acyclovir (ACV), phosphonoacetic acid (PAA), of type I IFN. Latent infection of

these murine neurons displays the characteristics of latent HSV infection *in vivo*: robust expression of the *latency associated transcript* (*LAT*), production of microRNAs (miRNA), and lack of detectable viral proteins or infectious virus production [51,53,54,56]. Importantly, quiescent viral genomes can reactivate from these latently-infected cells. Reactivation from latently infected rat and mouse neurons has been observed across a broad array of triggers. While the use of additional treatments is required to establish latent infections *in vitro*, Dochnal et al recently demonstrated that lytic gene expression patterns during reactivation using these *in vitro* systems recapitulated lytic gene expression patterns observed *in vivo* [57]. Use of these primary neuronal systems additionally permits the use of an extensive array of ready-made cellular biochemical tools that can be used to perturb or modulate infection. Modeling HSV latency and reactivation *in vitro* has been invaluable to the HSV field thus far, but it will be important to determine how findings within the homogenous cultures translate to *in vivo* infection and ultimately human pathology.

## Latent HSV infection

Following acute infection at the mucosal epithelia, HSV can infect axons of the peripheral nervous system. The viral genome is trafficked from the distal axon to the soma and ultimately nucleus, wherein the viral genome persists for the lifetime of the neuron. Although considerable debate over the specific nature of latency continues, there is consensus that a latent infection is characterized by the persistence of a viral genome in the absence of infectious virus that has the ability to re-initiate lytic gene transcription and reactivate. Some studies have suggested non-neuronal cell types can

harbor latent infections, but these studies focused on only *LAT* expression and not chromatin structure, a global analysis of viral gene expression, or whether viral genomes in these cells could reactivate [58]. A recent review [59] described latency as a multilayered system of repressive chromatin, *LAT* and miRNA expression, and cellular and systemic responses that control infection. Rather than being a passive process, it appears that latency is a dynamic interaction between host and virus that can vary depending on the context of infection. As a phase of the virus life cycle, latency permits HSV to last within post-mitotic neurons for the lifetime of the human host in the absence of a robust immune response typically observed with acute infection.

One of the main hallmarks of latency is the transcriptional repression of the nuclear HSV genome. During latency, lytic gene promoters are associated with repressive heterochromatin that likely facilitates the silencing of the viral genome [60]. In some of the initial characterizations of latency, Deshmane and Fraser found that typically transcriptionally active portions of the viral genome were associated with nucleosomal structures that mimicked cellular chromatin in the brainstem of infected mice [61]. Following this report, several studies have confirmed that the HSV genome associates with cellular histones in the TG of infected mice [62–64]. Moreover, viral lytic gene promoters are enriched with histone H3 di- and tri-methylated lysine 9 (H3K9me2/3) and H3K27me3, which are characteristic marks of repressive heterochromatin [62,64–67]. More recently, some reports have suggested that detectable viral proteins are produced during latency, including the observation that CD8+ T cells associate with latent neurons due to low levels of viral antigen, but it is impossible to

discern whether this is due to abortive reactivation characterized by sporadic bursts of viral proteins that do not progress to reactivation or sustained viral protein translation in latently infected cells [68].

Although lytic gene transcription is extremely restricted, the HSV genome is not totally silent during latency. LAT, a long non-coding RNA, is an 8.3kb transcript that can be spliced into multiple transcripts, including a stable 2kb intron and multiple miRNAs [69–71]. Even in human post-mortem ganglia, there was little evidence of activation HSV transcription outside of the LAT region [72]. Exclusive expression of the LAT is likely driven by an enrichment of chromatin modifications associated with euchromatin on *LAT* promoters and enhancer elements [65,73]. Interestingly, LAT in HSV-infected neurons regulates the association of heterochromatin on the HSV genome during latency and evidence suggests that LAT modulates levels H3K9me2/3 and H3K27me3 on the latent viral genome [62,64–66]. The *LAT* itself is enriched with euchromatin marks, including H3K4me3 as well as acetylation of histone H3 [63,65]. The contrasting chromatin states between lytic promoters and LAT may be attributed to differences in binding sites for the cellular insulator protein CCCTC-binding factor (CTCF) that can modulate H3K27me3 deposition on the latent genome [74,75]. Therefore, epigenetic silencing of the viral genome is a major factor in establishing and maintaining latency in neurons. Interestingly, LATs expressed during latent infection can modulate viral genome chromatin structure [65,66,76,77].The various miRNAs derived from the LAT have been suggested to similarly support the establishment and/or maintenance of latency in

neuronal infection [78]. However, the precise function of LAT, as well as its requirement for latency, is still being elucidated.

A critical aspect of HSV latency is the ability of the viral genome to re-initiate lytic gene transcription to produce infectious virus. One unique consideration about the viral genome during latency is the lack of epigenetic modification associated with stably repressive chromatin. The HSV genome does not contain detectable CpG methylation [63,79] and lytic promoters have not been found to associate with H4K20me3 [65], both of which are modifications classically attributed to stable, constitutive heterochromatin [80]. This suggests that the majority of the repressed viral genome can be considered facultative heterochromatin that, by definition, can convert to active euchromatin. RNA polymerase II promoter-proximal pausing is hypothesized to also contribute to the ability of the viral genome to activate transcription in that *ex vivo* activation of the super-elongation complex, a complex involved in transcription elongation, enhanced HSV reactivation [81]. Altogether, evidence suggests that epigenetic and regulatory factors associated with the HSV genome during latency permit a relatively easy transition from repression heterochromatin to active euchromatin in reactivation.

## Reactivation of HSV

### Reactivation is a dynamic process

Periodically, the latent HSV genome can reactivate from peripheral neurons to produce infectious virus that permits transmission to new hosts. Our lab and others have demonstrated that HSV reactivation is characterized by a two-phase pattern of

lytic gene expression that can ultimately result in the production of infectious virus [57,82,83]. The first stage, Phase I, consists of a transient yet synchronous burst of lytic gene expression that includes genes from IE, E, and L viral gene classes [84]. Importantly, this induction of lytic gene expression occurs independently of the viral transactivator VP16 and neither IE protein synthesis nor vDNA replication are required for E or L gene transcription [84]. While this gene expression pattern was first observed *in vitro*, other reports have demonstrated that *ex vivo* reactivation occurs in a similar manner [85,86] and independent of acyclovir-induced latency [57]. Activation of the neuronal stress pathway mediated by Dual Leucine Zipper Kinase (DLK) and c-Jun N-terminal kinase (JNK) are essential steps in initiating lytic gene transcription during Phase I, but induction of lytic gene transcription at this stage is independent of histone demethylase activity [54,57,85,87]. The persistence some lytic gene transcription despite no clinical reactivation events *in vivo* corroborate *in vitro* findings that some neurons may express lytic transcripts but not produce infectious virus. Therefore, it is possible for HSV genomes to initiate Phase I, but not progress to Phase II (full reactivation) wherein infectious virus is produced.

After about 20 hours after the induction of Phase I *in vitro*, the pattern of lytic gene expression transitions to Phase II. This second phase of reactivation closely resembles the ordered gene cascade of IE, E, and L gene transcription observed in *de novo* infection. Progression to Phase II therefore results in the vDNA replication and the production of infectious virus [84]. It is hypothesized that the transition from Phase I to Phase II is dependent on synthesis of essential viral proteins during Phase I, such as

VP16 [84]. Although Phase I does not require histone demethylase activity, viral gene expression in Phase II is dependent on histone demethylases and therefore the full removal of repressive heterochromatin marks on lytic promoters [54,57,84]. While biphasic HSV reactivation occurs across multiple models, certain robust experimental triggers such as axotomy, have demonstrated a more rapid induction of lytic gene expression dependent on VP16 that is not biphasic while still relying on histone demethylase activity [24].

## Neuronal stress signaling is required for HSV reactivation

Following a period of latency, host neuronal pathway activation can trigger HSV reactivation. Given that lytic gene transcription is restricted during latency, there is little to no detectable viral proteins produced. Therefore, HSV must co-opt host neuronal pathways to initiate lytic gene transcription in Phase I of reactivation.

Many experimental triggers of HSV infection have thus far been identified and, importantly, many converge on the neuronal stress signaling pathway mediated by dual leucine zipper kinase (DLK) and c-Jun N-terminal kinase (JNK). One of the earliest identified triggers of *in vitro* HSV reactivation is nerve growth factor (NGF) deprivation of latently-infected primary rat sensory neurons [51]. The NGF signaling pathway is a critical signaling system to development of both sympathetic and sensory peripheral neurons and has therefore been extensively described. NGF reception by TrkA is responsible for activating 3 primary pathways mediated by phosphoinositide phospholipase C (PLCγ), mitogen-activated protein kinase (MAPK), or phosphoinositide

3-kinase (PI3K), but only the PI3K pathway has been implicated in HSV reactivation. In brief, when the NGF receptor TrkA is not bound by NGF, PI3K is inhibited. PI3K inhibition results in the activation of Akt and subsequently DLK and JNK. In host neurons, DLK is a key modulator of apoptosis following NGF-deprivation and axon-specific pruning induced by axon-targeted NGF deprivation [88]. Neuronal JNK exists in several isoforms and is constitutively active under homeostatic conditions, contributing to synaptic plasticity and dendrite arborization [89]. However, in response to NGF deprivation, JNK becomes an essential signaling molecule in the neuronal stress response as a result of DLK activation [90]. The full extent of JNK function is this redirected in the context of reactivation is not fully understood.

Knowledge of NGF signaling pathways and TrkA signaling in development have helped our understanding of this pathway in HSV reactivation. PI3K inhibition alone has been found to trigger reactivation *in vitro* [54,91] and, even though PI3K inhibition activates multiple pathways, HSV reactivation seems to be specific to the downstream pathway mediated by Akt downstream of TrkA [53]. Multiple studies across different experimental systems, including *in vitro*, *in vivo*, and *ex vivo* latency and reactivation models have confirmed that DLK and JNK signaling constitute major signaling pathways that are critical to HSV reactivation [53,54,57,86,87,92–95]. Therefore, DLK and JNK activation are critical junctures in which multiple triggers of HSV reactivation converge to facilitate lytic gene expression. Our lab has previously shown that JNK activation is correlated with recruitment of JNK to viral promoters [54]. However, the precise downstream signaling

mechanisms of DLK and JNK activation that contribute to HSV reactivation and how ubiquitous DLK/JNK activation is across different triggers of reactivation are unknown.

*In vivo* and *in vitro* model systems have thus far demonstrated that a variety of different stimuli can trigger HSV reactivation from latently infected neurons. Some of the major experimental triggers of HSV reactivation include axotomy, heat shock, and UV radiation. One of the earliest clinical triggers of HSV reactivation was reported 1905 in patients undergoing surgical resection of trigeminal ganglia [96]. Reactivation following surgical resection is likely matched the axotomy of latently infected neurons *in vitro* that similarly induces reactivation. The direct cellular mechanisms linking axotomy to reactivation are currently unknown, but may rely on an increase in cAMP levels associated with other triggers of reactivation [97,98] or activation of DLK as a sensor of local axonal injury [99,100]. In clinical settings, fever is strongly associated with HSV reactivation in human patients and, in parallel, hyperthermia is used to triggers reactivation in *in vitro* as well as *in vivo* mouse models [101,102]. Exposure to both UV exposure is sufficient to induce *in vivo* reactivation [103,104] and is associated with increased DNA damage, which can similarly trigger reactivation *in vitro* [105]. Psychological stress as well as infections, such as SARS-CoV2, can induce reactivation in patients [106–108], though the mechanisms are unknown. Additional triggers of reactivation, such as protein synthesis inhibition [54,109] or epinephrine [110–112], work through currently undefined mechanisms, but are essential tools in elucidating the cellular processes governing HSV reactivation.

## HSV episome modifications contribute to reactivation

In addition to understanding the signaling pathways mediating HSV reactivation, it is critical that we understand how these pathways engage with the viral genome to induce lytic gene expression. These host pathways must overcome the lytic transcriptional repression imbued by epigenetic silencing and the formation of facultative heterochromatin during latency. There is great variability in the potential histone modifications on the viral genome and control of gene expression is often mediated by a combination of post-translational modification [113]. This variability may contribute to the differential ability of viral genomes to reactivate in response to the same stimulus[57,87,114,115]. One fascinating mechanism by which the HSV genome can rapidly initiate gene transcription is via the addition of a phospho/methyl switch. This phospho/methyl switch is characterized by the addition of phosphorylation marks on the serine residues neighboring repressive lysine modifications [116,117]. The addition of the phospho/methyl switch on lytic gene promoters during Phase I, specifically K3K9me3/pS10, is dependent on JNK activation and contributes to the burst of lytic gene expression in Phase I [54]. While the ubiquity of the phospho/methyl switch on lytic promoters remains to be determined, this mechanism allows the HSV genome to initiate lytic gene expression rapidly without the requirement for demethylase activity.

In order for the viral genome to enter Phase II of reactivation, more extensive remodeling of the viral episome must be accomplished. As already mentioned, accumulation of the viral transactivator VP16 is thought to accumulate to sufficient levels in Phase I to drive progression into Phase II. This is largely due to the recruitment

of VP16 along with host transcription factors, such as Oct 1 and HCF1, to IE promoters on the viral genome and facilitates the recruitment of additional proteins that remodel chromatin [118]. The role of VP16 and host transcription factors indicates that extensive chromatin remodeling is required for lytic gene expression in Phase II. The phospho/methyl switch likely permits the burst of lytic gene expression in Phase I, but the extensive chromatin opening in Phase II requires to activity of host demethylase enzymes that act on methylated H3K27 and H3K9 [24,54,57,93,118]. The drivers and consequences of interactions between the viral genome and host proteins that result in the progression from Phase I to Phase II represent persistent pressing questions in the field.

## Neuronal Innate Immunity in HSV Reactivation

### Innate immune signaling molecules

The outcome of HSV infection is determined by the host response to infection, including systemic and intracellular responses. Much of the host response to HSV infection is composed of the innate immune system promoting a type I interferon (IFN) response as well as the production of inflammatory cytokines, such as interleukin 1 (IL-1). In combination with nucleic acid-sensing pathways, the culmination of innate immune signals greatly affects HSV replication and persistence at each stage of the viral life cycle.

*The type I IFN response*

The type I IFN response represents a critical arm of host defense to viral infections. As one of the best studied classes of IFNs, type I IFNs are compose primarily of IFNα and IFNβ, though there are additional IFN proteins that are less defined [119]. Nearly all cells in the human body produce type I IFN in response to stimulation of pattern recognition receptors (PRRs) that are activated by damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs). DAMPs encompass foreign nucleic acids and aberrant nuclear DNA, while PAMPs are specifically viral proteins or material associated viral particles. Some of the major PRRs utilized in eukaryotic cells include RNA sensors, such as RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated gene5), cytosolic DNA sensors, such as cGAS-STING (cyclic GMP-AMP synthase, stimulator of interferon genes), and surface receptors, such as toll-like receptors (TLRs) [120]. Activation of these receptors is sufficient to initiate the production of type I IFN in most cells through a diverse set of downstream signaling pathways involving IFN-regulatory factors (IRFs) that ultimately lead to the transcription and translation of type I IFNs.

Upon release, type IFN can bind to receptors composed of interferon alpha and beta receptor subunit 1 or 2 (IFNAR1/2). Canonical activation of the type I IFN receptors leads to the activation of the Janus kinase 1 (JAK) and signal transducer and activator of transcription 1 or 2 (STAT1/2) [120]. Consequently, additional signaling molecules and recruited, translocated to the nucleus, and bind to IFN-stimulated gene (ISG) promoter to initiate the transcription of diverse signaling molecules involved in the antiviral response [121]. ISG transcription can give rise to a multitude of antiviral proteins and

mechanisms, which are dependent on the cell-type and context in which they are activated. These mechanisms range from recruitment and maturation of professional immune cells to inducing apoptosis [122].

*Neuronal type I IFN signaling*

Although generally considered immunologically silent, neurons can both sense and produce type I IFN. As with other cell types, neurons can upregulate ISG expression to mount an antiviral response to combat an invading pathogen [123]. However, the neuronal response to type I IFN is markedly different from other cells in the body and the production of type I IFN is limited compared to other cell types [124,125]. Additionally, the ISG profile observed in neurons following type I IFN sensing appears to be neuronal-specific that appears to be much more refined in scope [92,123,126]. Although not yet fully understood, the type I IFN response in neurons likely contributes to control of viral infection, but in a more restrained way than replicating cells.

In addition to its role in protecting neurons from viral infection, type I IFNs are also implicated in modulating otherwise homeostatic neuronal processes. In fact, type I IFN signaling plays an integral role in neurodevelopment, contributing to the synapse formation as well as neuronal autophagy [127]. Studies looking at excessive type I IFN signaling that ultimately leads to neurotoxicity have also provided insight into more nuanced effects type I IFN has on neurons. This includes modulation of neurogenesis [128], decreased neurotrophic signaling [129], and alterations to action potential firing capacity [130]. In contrast to replicating cells that rely on type I IFN to mount a robust

antiviral response, these reports suggest that neurons might take a more nuanced approach to sensing type I IFN.

*IL-1 inflammatory signaling*

IL-1 is a key mediator of inflammation in the innate immune system. Although there are two key forms of IL-1, α and β, the two molecules also similar in their signaling function and differ primarily in their processing mechanism [131].The primary sources of IL-1 are professional immune cells of the innate immune system, such as macrophages and monocytes [132–134], that synthesize IL-1 from its precursor protein using caspase-1 following inflammasome activation [135]. However, epithelial cells, keratinocytes, and fibroblasts are also recognized as sources of IL-1 that release the molecule in cell death as an alarmin [136–140]. Reception of IL-1 by the type I IL-1 receptor (IL-1R1), which is expressed throughout the entire body, results in the rapid induction of hundreds of genes, including additional cytokines, that mediate an inflammatory response. Signaling downstream of IL-1R1 is mediated by interleukin 1 receptor-activated protein kinases (IRAKs) that recruits tumor necrosis factor-associated factor 6 (TRAF6). The downstream events connecting TRAF6 activation and oligomerization and resulting gene expression are not fully understood, but have been shown to involve mitogen-activated protein kinase kinase kinase (MAPKKK), NF-κB, JNK, and p38 MAPK pathways [141]. Importantly, the downstream consequences of IL-1 signaling is highly cell-type dependent and reflects the complexity and variability of signaling proteins in this pathway.

*Neuronal response to IL-1 signaling*

In a similar way to type I IFN, the neuronal response to IL-1 lacks a robust inflammatory component while simultaneously affecting homeostatic functions. IL-1 contributes to long-term potentiation (LTP) and memory formation in central nervous system (CNS) neurons [142]. In line with its contribution to LTP, IL-1 can modulate neuronal action potential firing, leading to increased neuronal excitability and affect the development of synapses [143]. Neurogenesis is inhibited in neuronal precursors expressing certain subtypes of the IL-1 receptor [144,145], but interestingly IL-1 does not induce neuronal death, which is a common consequence of IL-1 signaling in other cell types [146]. While IL-1 may be responsible for an efficient and effective inflammatory response in most cell types, neurons display a unique phenotype in response to IL-1 that severely restricts induction of inflammation while still making use of the signaling molecule in other ways.

## Innate immune sensors of DNA

As a DNA virus, HSV infection is intimately related to the DNA-sensing innate immune response. The host response to HSV infection likely encompasses the TLRs, inflammasome activation, and induction of the type I IFN response from a variety of DNA sensors, depending on the cell type and degree of infection. An extensive array of HSV proteins that inhibit the cGAS-STING pathway have been well-described in the literature [147], suggesting that inhibition of this pathway is critical to viral replication. Therefore, the TLR9, AIM2-inflammasome, and IFI16 pathways will be discussed as

major signaling pathways briefly in this section, followed by an in-depth discussion of the cGAS-STING pathway.

*TLR9*

TLR9 receptors are primarily localized to intracellular compartments such as the ER, endosome, or lysosome within specialized innate immune cells. TLR9 is activated in response to hypomethylated CpG DNA sequences [148], a sequence relatively rare in mammals but common in microbial DNA. TLR9 activation results in the recruitment of myeloid differentiation primary response gene 88 (MyD88) [149]. MyD88 then forms a complex with IRAK kinase family members to activate TRAF6 [150] and downstream activation of nuclear factor κ-B (NF-κB) and interferon regulatory factor 7 (IRF7) [151]. NF-κB and IRF7 then translocate to the nucleus to induce transcription of proinflammatory cytokines and type I IFN expression.

*AIM2-Inflammasome*

Inflammasome activation is best characterized downstream of the cytosolic DNA-sensor absent in melanoma 2 (AIM2). AIM2 activation is mediated by the binding of dsDNA in the cytosol in a sequence-independent manner [152,153]. AIM2 clustering along DNA sequences allows for the recruitment of apoptosis-associated speck-like protein containing a caspase recruiting domain (ASC) and subsequent protease activation of caspase-1 [154]. This multiprotein complex is referred to as the inflammasome. Following its formation, the AIM2-inflammasome promotes the maturation of proinflammatory cytokines by caspase-1 and mediates pyroptosis via gasdermin-D, leading to a robust

immune response [155–157]. Interestingly, herpesvirus infection can induce inflammasome activation via NLRP3 independent of AIM2 [158], but the mechanisms mediating NLRP3-inflammasome activation remain controversial [159].

*IFI16*

IFI16 in humans (p204 is the murine orthologue) is an AIM2-like receptor that is responsible for sensing damaged host dsDNA [160] and vDNA[161,162] within the nucleus. Binding of DNA to IFI16 can result in activation of the ASC-dependent inflammasome pathway [163–165], induction of the type I IFN response via activation of STING and IRF3 [161,166], or cytokine production by NF-κB [167,168]. Importantly, IFI16 has been shown to synergistically activate STING with cGAS during DNA virus infection [169,170], but the induction of the IFN response during DNA virus infection is still reliant of activation of cGAS [171]. The interactions between IFI16 and the cGAS-STING pathway will be discussed further in **Section 3.2**. IFI16 can also sense single-stranded DNA (ssDNA) and induce a cytokine response independently from cGAS [171–173]. In addition to its role in immune pathway activation, IFI16 acts as a transcriptional restriction factor to repress expression of vDNA from numerous DNA viruses via mediating histone methyltransferase activity on lytic gene promoters via unknown mechanisms [174–179].

*cGAS-STING*

In response to cytosolic DNA, cGAS produces the secondary messenger molecule 2’3’ cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) to activate STING [180–182]. STING activation can result in the induction of three major

immune signaling pathways: the induction of a type I interferon (IFN) response via activation of TANK binding kinase 1 (TBK1) and IFN regulatory factor 3 (IRF3), cytokine expression via activation of NF-κB, or the induction of autophagy by an unknown mechanism [183]. Notably, cGAS-STING activation in different cell types can result in the activation of vastly different downstream responses. Activation of cGAS-STING and a description of these major downstream responses are described in **Figure 2**. This suggests a high degree of differential regulation between cell types that has yet to be comprehensively defined. Therefore, it is necessary to understand how cGAS-STING can be activated in a variety of cell types in addition to our broad understanding of the pathway.

## cGAS-STING is a major DNA-sensing pathway

In 2013, Sun and colleagues first identified cGAS as the DNA-sensor responsible for inducing the type I IFN response previously observed during viral infection[184]. cGAS functions as a nucleotidyl transferase enzyme that produces the secondary messenger molecule cGAMP [180,181,185–187]. cGAMP, in turn, activates STING and the requisite downstream response. The cGAS protein is approximately 520 amino acids (aa) in length with a poorly-conserved, positively charged N-terminal domain and C-terminal domain that partially overlaps with a central Mab-21 domain-containing 1 (MB21D1) domain. The central MB21D1 is responsible for directly sensing DNA and can bind to approximately 16-18 base pairs (bp) [185,187,188]. DNA binding triggers a conformational change in cGAS and subsequent dimerization of cGAS monomers. This dimerization is required for functional cGAS activity, as the two helices from the DNA molecule engage

separate DNA-binding sites on each cGAS molecule to cross-link the individual cGAS monomers. Longer DNA sequences enable cGAS to form higher-order oligomeric structures to promote a more robust production of cGAMP, but this effect was more pronounced in murine cGAS compared to human cGAS [180,189,190].

When discussing cGAS-STING activation, it is important to consider the role of IFI16 during DNA virus infection. IFI16 is an innate immune sensor of nuclear DNA and colocalizes to nuclear puncta that have been shown to be sites of HSV replication during lytic HSV infection [162,191]. Moreover, while IFI16 is not required for induction of the type I IFN response [171], the synergistic activation of STING by IFI16 and cGAS provides optimal activation of the downstream signaling cascades and both are required for the full potential IFN response [169]. While this synergistic function has been shown immortalized keratinocytes, THP-1 cells additionally utilize IFI16 to amplify production of cGAMP by cGAS [192]. Therefore, IFI16 function and its contribution to the cGAS-STING pathway may differ between cells depending on abundance of IFI16 or cell type.

*cGAS Phase Separation*

A unique feature of cGAS is its ability to undergo a liquid-liquid phase separation within the cytosol [193]. Upon reaching a certain threshold of DNA concentration in the cytosol, cGAS molecules and DNA molecules coalesce to exert a transition from liquid-phase movements in the cytosol to lattice-like structures of cGAS condensates. This interaction is mediated by a recently identified third binding site on cGAS, site ‘C’ [194]. The high concentrations of cGAS and DNA allow for rapid induction of robust cGAMP

production [195]. Given that cGAS is constitutively active in most cell types, this phase separation likely allows for a rapid switch to activate the pathway that is not reliant on upregulation of gene expression.

*Membrane-Bound and Nuclear cGAS*

As mentioned previously, cGAS was initially thought to be localized exclusively to the cytosol in order to detect cytosolic DNA. However, evidence indicates that cGAS interacts with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to specifically localize to lipids rafts within the plasma membrane in order to prevent recognition of aberrant host DNA in the cytosol even under steady state conditions [196–198]. However, whether there is a functional difference between cytosolic and membrane-bound cGAS is unclear.

In addition to being present in the cytosol and plasma membrane, cGAS has also been found to be present in the nucleus [198–201] which requires a tethering complex [201]. While nuclear cGAS is capable of sensing self-DNA in the nucleus, activation of nuclear cGAS is severely restricted through its association with interactions with the acidic patch of histone H2A that blocks all known DNA-binding sites of cGAS [202–204]. Although nuclear cGAS retains its ability to generate cGAMP in response to sensing DNA in the nucleus and induce immune activation, it produces cGAMP at lower levels than cytosolic cGAS [198]. This suggests additional regulation of nuclear cGAS that differentiates its function from cytosolic cGAS as well as serving a function independent of or in conjunction with innate immune signaling. Interesting, some studies suggest IFI16 but not cGAS is recruited to HSV viral genomes entering the nucleus during lytic

infection [170]. This is consistent with evidence that IFI16 recognizes nucleosome free DNA [175]. The mechanisms regulating the extent of cGAS activation in response to endogenous DNA appear to be regulated by associations with nucleosomal DNA, but potential non-canonical activation of nuclear cGAS or what role it plays, if any, in infection is not known.

cGAS has also been shown to have a role in the DNA damage response. After DNA damage, cGAS is shuttled to the nucleus and colocalizes with γH2A.X[199], a marker for double-stranded DNA breaks. Notably, this occurs even in post-mitotic cells, suggesting that there is a function independent of recognition of aberrant DNA replication. Interestingly, nuclear cGAS inhibits homologous recombination in response to DNA damage, but has not been shown to prevent non-homologous end-joining [199,205]. This selectivity of inhibition of DNA repair mechanisms suggests that cGAS primarily functions outside of mitosis. Moreover, nuclear cGAS has also been shown to interact with poly (ADP-ribose) polymerase 1 (PARP1), a chromatin remodeling protein implicated in DNA repair [199]. These interactions are indicative of a major role for cGAS in response to DNA damage, but the mechanisms mediating this pathway or the function of cGAS in this process is unknown.

*cGAS Regulation*

As an innate immune activator, cGAS activation is highly regulated. Regulation of cGAS is achieved by both post-translation modifications (PTMs) and unique accessory proteins [206,207]. It is interesting to note that cGAS can be differentially regulated in

response to infection. Under normal conditions cGAS is ubiquitinated by TRIM14 so that it can be targeted for p62-mediated selective autophagy. During infection, however, these interactions are inhibited by USP14, thus allowing for cGAMP production by cGAS [208]. Although the mechanisms upstream of USP14 that support cGAS during infection are unknown, cGAS has also been shown to interact with Beclin-1, a critical protein in mammalian autophagy. While cGAS interaction with Beclin-1 results in the inhibition of cGAS to prevent aberrant activation, Beclin-1 can also enhance the autophagy-mediated degradation of microbial DNA [209]. In addition to crosstalk with the autophagy pathway, there is regulatory cross-talk between the cGAS-STING pathway and inflammasome pathway. Following both canonical and non-canonical inflammasome activation, caspases have been shown to cleave cGAS and therefore inhibit its function [210–212]. These interactions highlight the extensive network of proteins responsible for mediating cGAS activation during normal and infection conditions.

*3.3 2'3'-cGAMP*

Following the discovery of cGAS, mass spectrometry was used to identify 2'3'-cGAMP (cGAMP) as the secondary messenger produced by cGAS that activates STING [213]. cGAMP is a cyclic di-nucleotide that is produced with a unique combination of 3'5' with non-canonical 2'5' phosphodiester bonds [214,215] that permit enhanced binding properties to its downstream receptor STING and results in more robust activation of STING [214,216]. This is in contrast to both mammalian and bacterial bonds within DNA molecules, that are able to activate STING but to a markedly lesser intensity.

In addition to activating STING, some reports indicate other novel functions of cGAMP. Interestingly, cGAMP facilitates the phosphorylation of serine-threonine kinase 1 and 2 (ULK1 and ULK2). ULK1/2 thereafter phosphorylate STING in order to inhibit induction of a type I IFN response[217]. Somewhat paradoxically, cGAMP has also been shown to directly activate STING transcription as well [218], suggesting a highly coordinated network that uses cGAMP to modulate STING activity independent of its direct activation.

*2'3'-cGAMP Regulation*

Although intracellular mechanisms to degrade or inhibit cGAMP are unknown in eukaryotes, cGAMP can be negatively regulated by ectonucleotide pyrophosphatase/ phosphodiesterase family member 1 (ENPP1). ENPP1 limits cGAMP stability, but its catalytic domain is in the extracellular space, suggesting that only extracellular cGAMP can be regulated this ENPP1 [219]. Interestingly, both bacterial and viral pathogens employ phosphodiesterases and poxins, respectively, to target cGAMP for destruction in order to inhibit immune response activation [220].

*STING*

STING functions as a major hub of intracellular innate immune signaling pathways in response to cGAMP-mediated activation. After being initially identified as a mediator of the immune response to RNA in 2008 [221,222] (prior to the discovery of cGAS as a DNA sensor), STING was found to be essential in the cytosolic DNA response by

inducing a type I IFN response in 2009 [223]. Subsequent to that discovery, STING was found to be essential for interpreting cyclic dinucleotide signals in 2011 [224,225], paving the way for the discovery of its agonist 2'3'-cGAMP in 2013 [213]. Since its discovery, STING has been studied extensively and is responsible for mediating immune response pathways through TBK1/IRF3 activation, NF-kB activation, and autophagy induction.

*STING in the ER*

The STING protein is 80kDa with four transmembrane helices that organize to form dimers with a ligand-binding pocket facing the cytosol [226]. Under normal conditions, the STING dimers are dispersed throughout the endoplasmic reticulum (ER) by the retention factor stromal interaction molecule 1 (STIM1). STIM1 is also a calcium sensor within the ER, suggesting that STING may react to calcium perturbation within the ER. Indeed, ER stress can result in activation of immune response pathways [227]. Mutations in STING have also been demonstrated to disrupt calcium homeostasis in the ER that induces apoptosis, as well [228]. Precisely how STING contributes to calcium homeostasis is unclear, but it represents another critical junction of cell signaling that STING is implicated.

*STING Activation*

cGAMP binding to STING induces a conformational change as one cGAMP molecule binds to a region between the two STING monomers. After binding, a β-sheet "lid" on STING forms around the binding pocket, a process thought to enhance binding affinity of cGAMP [226,229,230]. cGAMP binding is highly selective, especially in human

STING, with other variants of cGAMP resulting in less robust activation of cGAMP [214]. This conformational change exposes the STING C-terminal tail (CTT) residues which are responsible for inducing the type I IFN response and cytokine production pathways [230]. Moreover, the ligand-binding domains of STING move to facilitate side-by-side interactions with other STING dimers and ultimately allow for the generation of larger complexes [226,229]. These higher order complexes promote more downstream activity [231]. TRIM56 has been show to ubiquitinate K63-linked chains on the CTT to further facilitate dimerization of STING. This oligomerization is also required for the translocation of STING from the ER ultimately to the Golgi network where downstream activation events are mediated [232].

Subsequent to the its trafficking to the ER-Golgi intermediate complex (ERGIC) to induce autophagy (described below), STING is trafficked to the Golgi network. Within the Golgi, STING is able to interact with a variety of downstream mediator proteins that ultimately determine the downstream signaling response to cGAS-STING activation. These signaling pathways include autophagy induction, cytokine production, and the IFN response; the individual pathways are discussed in detail below. STING activation has also been linked to the activation of pathways mediated by ERK1/2, JNK, c-JUN, and p38 [233], but the role of these pathways downstream of STING during infection remains unclear. It will be particularly interesting to investigate the role of JNK and c-JUN after cGAS-STING activation in neurons give they are essential mediators of a major neuronal stress response pathway.

*STING Regulation*

Given that STING is a central hub of innate immune signaling pathways, it is actively regulated through both PTMs and accessory proteins [234–236]. The extensive list of regulators is described in **Tables 1** and **2** (Appendix). Similar to the cGAS studies, many of the regulatory mechanisms described for STING are derived from experiments with immortalized cell lines and undifferentiated cells. It will be of great interest to understand how STING activation is regulated in different cell types and how this potentially differential regulation contributes to the downstream immune response.

*Differential Activation of STING*

An essential aspect of STING activation is its differential activation. This differential activation can be due to cell type differences, different culture conditions, and other unknown factors. Fang et al found that STING activation following infection with vaccinia virus (VACV), a dsDNA virus, was differentially regulated across a number of cell lines. Moreover, STING activation can be modulated within each cell line depending on the available ion concentration in the media [237]. Notably, the gain or loss of STING function was not uniform across the cell lines tested after changing ion concentrations in the culture, suggesting that there are regulatory differences on STING intrinsic to each cell type. These results also highlight the impact on cGAS-STING activation of the extracellular environment composition which can vary greatly *in-vivo*.

Another important feature from the Fang study was the differential activation of STING after VACV infection across different cell types even under the same culture

conditions. While THP-1 cells and induced bone marrow-derived macrophages (BMDMs) demonstrated a type I IFN response after VACV infection, several other cell lines (L929, B16-F10, HeLa, HT 1080, and MCF7) did not produce any detectable type I IFN under the same conditions [237]. In another study, stimulation of B cells with 3'3'-cGAMP resulted in apoptosis resulting from less efficient degradation of STING while T cells exhibited no apoptosis in response to the same treatment [238]. Although 3'3'-cGAMP is a modified form of 2'3'-cGAMP to activate STING, these are experiments still demonstrate differential activation between cell types despite direct activation by the same ligand. In the same study, the authors also found that B cell apoptosis was mediated by caspase-3/7 and upregulation of proapoptotic genes, but these events were not observed in mouse embryonic fibroblasts (MEFs), monocyte-derived dendritic cells (MDDCs), or BMDMs [238]. Therefore, there is considerable variability in the downstream signaling events mediated by STING between different cell types. Given this variability among cell from immortalized cell lines or undifferentiated cells, it would be particularly interesting to see how STING activation occurs in fully differentiated or post-mitotic cell lines as well.

*Induction of interferon*

Induction of the type I IFN response mediated by cGAS-STING activation is the most extensively studied signaling pathway downstream of STING and widely viewed as the main immune signaling output from STING activation in mammals. The type I IFN response itself is the production of type I IFN that can be used to activate expression of IFN-stimulated genes (ISGs) that prime cells for infection.

After translocation of STING to the Golgi network by ATG8/5, STING is activated by TBK1 via phosphorylation within the CTT domain of STING [239]. This phosphorylation event exposes a binding site for IRF3 and induces the subsequent phosphorylation of TBK1 by STING. Thereafter, the CTT of STING acts as a scaffold to enhance the phosphorylation of a number of sites on the C-terminus of IRF3 by TBK1 [240,241]. Genetic manipulation studied have demonstrated that these interactions occur at very specific residues along the STING CTT and mutations of these residues can result in either aberrant activation of IRF3 signaling or a complete knockout of the downstream IFN response.

Following IRF3 activation by TBK1, IRF3 is trafficked to the nucleus. Under steady state conditions, both the nuclear localization signal (NLS) and nuclear export signal (NES) are constitutively active on IRF3 as it is shuttled between the cytosol and nucleus. Upon its activation, however, the NES becomes more dormant thereby allowing IRF3 to accumulate within the nucleus [242]. While in the nucleus, active IRF3 can bind to type I IFN promoter regions to induce transcription [243]. IRF3 also associates with CREB binding protein (CBP) to induce chromatin remodeling by acetyl transferase activity and increase access of transcription factors to the DNA to promote expression of type I IFN [242,244].

Although both TBK1 and IRF3 are expressed in nearly every mammalian tissue, there are major differences in functionality between cell types and these interactions

can be heavily modified. IRF3 activation has been observed in a number of cell lines, but its activation is not universal across eukaryotic cell types in response to cytosolic DNA. Also, tripartite motif containing 9 (TRIM9) has been shown to promote interferon production by facilitating the recruitment of glycogen synthase kinase-3 β (GSK3β) to TBK1. GSK3β initiates the oligomerization of TBK1 and inhibits cytokine production, thereby indirectly promoting the IFN response over cytokine response[245]. Conversely, IRF3 is targeted for degradation by tripartite motif containing 26 (TRIM26) [246]. Notably, TRIM9 and TRIM26 are differentially expressed in mammals, with relatively elevated expression of both in the central nervous system (CNS) [246–248]. Therefore, intrinsic cellular mechanisms within different body systems may influence the ability to induce a type I IFN response downstream of cGAS-STING activation.

*Cytokine Production*

In addition to IFN production, STING activation can mediate the production of inflammatory cytokines through the activation of the transcription factor NF-κB. NF-κB induces the transcription of proinflammatory cytokines and chemokines in order to combat infection [249]. In contrast to the IFN response, cytokines generate a proinflammatory response that can result in cell or tissue damage.

The NF-κB is a complex composed of a combination NF-κB family subunits. The mammalian NF-κB family contains five members: p105, p100, RelA, RelB, and c-Rel. These NF-κB family members form dimers of a complex that acts as a transcription factor, referred to as NF-κB. Although all of the members share the Rel homology

domain in the N-terminus, only RelA, RelB, and c-Rel have a transactivation domain in their C-terminus [250]. This transactivation domain allows for the NF-κB complex to bind to promoters for cytokine expression [251]. Therefore, the subunit composition of NF-κB helps determine which downstream genes will be expressed after activation. Although p105/RelA is the most common heterodimer in canonical NF-κB signaling activation that results in increased cytokine expression [252,253], the composition of these dimers (and therefore the downstream gene expression profile) can vary between cell types.

Activation of the NF-κB complex is regulated by the IKK complex. The I kappa B kinase (IKK) complex is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/ NF-κB essential modulator (NEMO) [254]. In the canonical NF-κB pathway, NF-κB is inhibited by the binding of Iκβα. Upon activation of the IKK complex, the Iκβα protein is phosphorylated and ubiquitinated by the IKK complex in order to promote the degradation Iκβα. This event induces the release of NF-κB [255]. NF-κB can then translocate to the nucleus and induce cytokine gene transcription. IKKβ is specifically required for cytokine production [256,257], in contrast to IKKα that promotes type I IFN induction after TLR9 activation [258]. Moreover, IKKβ and NEMO are required for type I IFN induction after cGAS-STING activation [237].

TBK1 is an IKK-related protein that can regulate the activation of NF-κB downstream of cGAS-STING. Although TBK1 activates NF-κB via the IKKαβ activation loop, NEMO-activated IKKβ is similarly required for the full activation of TBK1 to induce

type I IFN expression. This suggests a complex interaction and positive feedback loop of signaling to ensure a robust response. NF-κB can also contribute to IFN expression, indicating that both NF-κB and IRF3 likely contribute to IFN induction downstream of cGAS-STING activation. The precise mechanisms underlying this complex relationship are still unclear, but would provide great insight into differential downstream responses of cGAS-STING.

Interestingly, not all cells respond to cGAS-STING activation with robust cytokine production. Indeed, the majority of cells in the literature predominantly display an IFN response over a cytokine response. This is especially interesting give the emergence of cytokines predates the IFN response. Sequence analysis suggests that modifications to the CTT in STING over evolution likely contribute to the lower levels of activation by NF-κB than by IRF3 [241]. Given that IFI16 can preferentially activate NF-κB via STING compared to IRF3 [171,259], it is possible that IFI16 differences between cells contributes to these phenotypes as well. It is likely that it is more beneficial for some cell types to avoid an inflammatory cytokine response in favor of an IFN response in order to retain cellular integrity. However, STING activation independent of cGAS by etoposide-induced DNA damage prompted more robust NF-κB activation in cells compared to IRF3 activation after cGAS-STING activation [259]. This suggests that cytokine production can still be induced by STING and that cells can alter the effect of STING activation by modulating the upstream signaling response.

*Autophagy*

Autophagy induction is an ancestral function of STING activation that still persists in some eukaryotes [260]. Following cGAS-STING activation, autophagy can be used to degrade microbial DNA through the autophagosome formation and lysosome-mediated degradation. Additionally, autophagy induction has been suggested to restrain cGAS-STING signaling by degrading pathway components.

Prior to its migration to the Golgi network, STING is trafficked via endosomal transport to the ERGIC by autophagy related protein 5 (ATG5) [232], autophagy related protein 9 (ATG9) [261] and WIPI2 [260]. While at the ERGIC, STING activation is associated with the lipidation of microtubule-associated protein 1A/1B-light chain 3 (LC3) [262], a marker of autophagy induction. Moreover, STING colocalizes with the endosome marker early endosome antigen 1 (EEA1), the recycling endosome markers Tf receptor (TFR), and the exocyst complex subunit Sec5 [223]. Only a fraction of STING dimers remain in the ERGIC while the majority are trafficked to the Golgi, and the STING remaining at the ERGIC is subsequently trafficked to the lysosome in a Rab7-dependent manner transmembrane protein 203 (TMEM203) [263260]. Therefore, it has been suggested that STING activates its own autophagic pathway for both degradation of STING and for the degradation of invading pathogens.

Autophagy induction is independent of TBK1/IRF3 or NF-kB activation, but autophagy induction requires the presence of CTT [260]. The mechanism that facilitates autophagy from CTT is unknown, however. The core structure of STING has been highly conserved throughout the course of evolution and most variations in the STING

protein occur within the CTT [264,265]. These differences are demonstrated by the differential activation of the IFN response, autophagy, and cytokine production between species. *Drosophila melanogaster* and *nematostella vectensis* rely on autophagy induction downstream of STING activation that is independent of IRF3 or NF-κB activation [266,267]. This ancestral function of STING appears to be retained in mammalian cells. Mycobacterium tuberculosis and gram-negative bacteria similarly are cleared by autophagy in mammalian cells [268] and emerging evidence suggests that herpes simplex virus-1 infection is also cleared with autophagy. Although some proteins associated with this autophagy response have been identified as described briefly above, the mechanisms by which STING activates a non-canonical autophagy pathway remains unclear.

## Pathologies associated with neuronal cGAS-STING

### *Neuronal cGAS-STING Activation After Injury*

Several recent reports have implicated neuronal cGAS-STING activation in both infectious and non-infectious pathologies. In response to hypoxia-ischemia (HI) in which nuclear envelope integrity is destroyed by physical damage and DNA is released into the cytosol, both cGAS and STING can be detected colocalizing with the neuronal marker NeuN via immunofluorescence (IF) of HI rat brains tissue sections [269]. In another model of traumatic brain injury (TBI) in mice, Abdullah and colleagues observed an increase in STING IF staining in cortical neurons following TBI [270]. Although these studies demonstrate that cGAS and STING are present, it does not reliably determine that cGAS-STING was indeed activated in neurons because the IF staining does not

provide appropriate resolution or quantification demonstrating the translocation of STING, which is a commonly used marker of STING activation by IF.

*Neuronal cGAS-STING Contributes to Neurodegenerative Disease Pathology*

Recent studies have also implicated cGAS-STING signaling in neurodegenerative pathologies [271]. Sharma, et al demonstrated that immortalized striatal neuronal cells harboring Huntingtin's disease (HD) mutations have increased cGAS and cytokine expression. Given that the presence of micronuclei within the cytosol has been observed in HD pathology previously, the authors posit that micronuclei may be the source of increased cGAS activation [272]. However, the authors do not robustly demonstrate this. It will be interesting to see whether this same phenomenon occurs *in-vivo* or with primary neurons. These results suggest that at least in immortalized neuronal cell lines cGAS activation may be affected indirectly by pathological mutations that disrupt cellular function. Of particular interest is defining how cGAS-STING signaling is mediated in these cells and whether this response occurs *in-vivo* or with primary neurons.

Sliter and colleagues employed an elegant study to investigate the potential role of STING activation in Parkinson's disease (PD) pathology. They showed that chronic and acute mitochondrial stress can induce a STING-dependent type I IFN response that results in neurodegeneration in the substantia nigra, a hallmark of PD [273]. Although they did not show that mitochondrial stress specifically resulted in cGAS-STING activation in neurons, it is likely that a neuronal response to mitochondrial stress contributes to the

neurodegeneration they observe. Other cell types in the CNS are capable of apoptosis should the mitochondrial stress be too great, but post-mitotic neurons retain the stressed mitochondria they accumulate as mitophagy is impaired in their model. While the authors observe a STING-dependent increase in the cytokine IL-6 and type I IFN [273], it is possible that neurons induce paracrine signaling pathways to activate microglia or astrocytes that produce these inflammatory molecules. It will be interesting to examine cGAS-STING activation within these neurons *in-vitro* to determine what cellular pathways are specifically activated in neurons.

Neuronal cGAS-STING signaling has also been hypothesized contribute to amyotrophic lateral sclerosis (ALS) pathology. TDP-43, a hallmark protein of ALS, can trigger the release of mtDNA into the cytosol via the mitochondrial permeability transition pore (mPTP) to activate the neuronal cGAS-STING pathway [274]. In both patients with ALS as well as TDP overexpressing mice, the authors found elevated levels the cGAMP, the secondary messenger molecule responsible for activating STING downstream of cGAS activation [274]. These studies suggest that prolonged cGAS-STING activation can negatively affect nervous system health and contribute to additional neurodegenerative pathologies. However, it remains unclear how extended activation of STING affect neuronal health at the cellular level and if this form of activation differs in downstream signaling mechanisms from acute activation.

*Neuronal cGAS-STING in Infection*

In addition to the evidence supporting cGAS-STING activation in sterile environments, studies also provide insight into neuronal cGAS-STING during viral infections. The Reinert study mentioned earlier proposed that CNS neurons rely more on autophagy than an IFN response to clear viral infection [275]. This is because the type I IFN present in the brain following infection with HSV was largely derived from microglia and viral titers in the trigeminal ganglia (TG) are unaffected in *Goldenticket* mice [275] that harbor a mutation that prevents lack of a STING-induced IFN response [224]. Another study by Yamashiro, et al indicates that while the IRF3-binding residue is not required for the control of HSV infection in the CNS, unidentified portions of the CTT are required. This phenotype was specific to HSV infection as IFN production was still required to control listeria infection despite both pathogens presumably activating cGAS-STING [276]. However, a major consideration of these studies is that they are *in-vivo* and therefore it is difficult to attribute cGAS-STING activation or any variation therein specifically to neurons. Yordy, et al demonstrated that peripheral neurons from the dorsal root ganglia (DRG) also utilize autophagy rather than an IFN response to control HSV infection [277]. The mechanisms mediating this autophagy induction even generally are unknown, however.

Interestingly, RNA virus infection of NT2-N cells (a human post-mitotic neuron-derivative cell line) resulted in IFN and cytokine production, but HSV, a DNA virus, infection only induced upregulation of certain cytokines [278]. This is in contrast to literature supporting a non-cytolytic response to viral infection in neurons and the studies described above [279,280], however. Importantly, cell lines immortalized by

oncogenes may also confound results as numerous tumors demonstrate the development cGAS-STING inhibitory mechanisms to remain proliferative [281,282]. Another study found that in response to IFN, neurons upregulated a unique set of neuron-specific ISGs [92], which suggests that even during infection neurons respond differently than other cell types. The cellular factors contributing to these differences are not fully known, but warrant further investigation in order better understand how terminally differentiated cells respond to infection.

*Non-immunogenic activation of cGAS & STING*

Recent studies have demonstrated that either cGAS or STING activation can result in activation of pathways that are independent of an antiviral response [283]. Within cancer cells, cGAS can localize to mitochondria and inhibit the induction of cell death via ferroptosis though a mechanism that bypasses STING activation [284]. In this context, cGAS has a direct interaction with dynamin-1-like protein (DRP1), which plays a role in mitochondrial fission, redox, and the control of ferroptosis, but the precise drivers of DRP1 and cGAS engagement in this context is unknown. The authors suggest that cGAS therefore plays a role in regulating reactive oxygen species (ROS) in these cells [284], aligning cGAS activity with mitochondrial metabolism independent of known signaling pathways. This study shows the cGAS activation can actually support tumor growth [284], but whether this modified activation of cGAS plays a role in other pathologies will need to be determined.

As a cell type that typically avoids an inflammatory immune response, neurons also rely on a recently described non-immunogenic activation of STING. Although reliant on type I IFN signaling, STING regulates nociception in peripheral neurons and serves to suppress nociception following injury [285]. Moreover, direct activation of STING by the secondary messenger cGAMP after axotomy in peripheral neurons results in upregulation of genes that are associated axonal regeneration [286]. In contrast to some central nervous system neurons that can regulate axonal regeneration via STING intracellularly, cGAMP derived from neighboring cells induced by IFNγ signaling from the injured peripheral axons drive the axonal regeneration program by STING only in peripheral neurons [286]. These findings support a model in which peripheral neuron activation of STING and the use of immunostimulatory molecules is unique, even among different neuronal subtype, and the potential downstream consequences of STING activation in these cells is not understood.

## mtDNA activates innate immune pathways in HSV infection

Given that cGAS binds to DNA regardless of pattern recognition, DNA from many sources can activate the cGAS-STING pathway. The immunostimulatory potential of mtDNA was first described in 2004 [287] and numerous studies have since implicated mtDNA a potent DAMP [288,289]. mtDNA copy number is regulated by cell-specific mechanisms or in response to environment or intracellular cues [290]. Interestingly, the quantity of mtDNA present appears to be in excess of what is required to achieve oxidative phosphorylation and is more resistant to eukaryotic nuclease [289], making it a suitable activator of cGAS-STING signaling to initiate an immune response.

*mtDNA Release*

Activation of the cGAS-STING pathway by mtDNA requires its release from mitochondria into the cytosol. High rates of oxidative phosphorylation, IL-1β signaling, destabilization of mtDNA, and other forms of mitochondrial stress induce the release of mtDNA into the cytosol and subsequent activation of cGAS-STING [287,291–294]. Recent evidence shows that mtDNA is released through mitochondrial permeability transition pores (mPTPs) in the outer mitochondrial membrane (IMM) formed by Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist/killer (BAK) [295,296]. In order for mtDNA to reach the cytosol, IMM extrusion through the BAX/BAK pore likely permeabilizes the IMM to release mtDNA fragments into the cytosol [295,297].

mtDNA is also thought to be released into the cytoplasm during apoptosis, suggesting functional crosstalk between the apoptosis and cGAS-STING signaling pathways. While mtDNA release from mitochondria during apoptosis appears to occur independently of pro-apoptotic caspase activity [295], the release of mtDNA through the mPTP can trigger NLRP3-inflammasome activation [294,298]. mtDNA release after cholesterol buildup has been implicated in activation of the AIM2-inflammasome, as well, but it is unknown how mtDNA is released into the cytosol under these culture conditions [299]. Importantly, apoptotic protease activating factor 1 (APAF1) and activation of apoptotic caspases can inhibit IFN production during mitochondrial-mediated apoptosis to mute the immune response [300], suggesting complex regulatory mechanisms differentiating between cGAS-STING activation or inflammasome

activation by mtDNA. More recent work from Newman et al shows that mtDNA stress induced by knockdown of TFAM, a core mtDNA packaging protein, can trigger release of mtDNA through a novel mechanism that involves rupturing of mtDNA-containing endosomes [301].

*HSV Induces the Release of mtDNA*

The HSV gene ICP27 encodes a multifunctional IE protein that plays an critical role in the progression of E to L gene expression during lytic replication [302–305]. Recently, it was shown that during lytic HSV infection in HEK293T cells, ICP17 can induce apoptosis by inhibiting the interaction between BAX and its inhibitor, 14-3-3θ. As a result of this inhibition, BAX then localizes to the mitochondria where it can mediate the release of cytochrome c into the cytosol to induce apoptosis [306]. However, studies have also found that HSV can block apoptosis following the release of cytochrome c in human epithelial cells [307–309]. Therefore, it is likely that apoptosis induction is not the primary role of BAX translocation by ICP27. As noted earlier, mtDNA may be secreted into the cytosol as a result of BAX pore formation, which would in turn activate cGAS-STING. As studies of non-infectious activation of cGAS-STING have shown, different mitochondrial stressors can result in different pores mediating mtDNA release. In the instance of TFAM knockout, mtDNA is released via endosomal rupturing [301], while TDP-43 induces mtDNA release via through the mPTP [274]. Therefore, it is possible that ICP27 serves to induce the translocation of BAX to the mitochondria, open the mPTP, or trigger endosomal rupturing to promote the release of mtDNA. The functional

significance of mtDNA release from mitochondria during HSV infection remains to be elicited, however.

*UL12.5 Depletes mtDNA and mtRNA*

The HSV protein UL12.5 was first hypothesized by Costa et al in 1983 as a N-terminal truncation of the nuclear alkaline nuclease, UL12, given that the predicted 1.9 kb mRNA of UL12.5 was colinear with the 2.3kb mRNA of UL12. Although no available antibodies at the time reacted with UL12.5, the protein was predicted to retain the alkaline exonuclease activity of UL12 while being ~130 amino acids shorter at ~60 kDA [310]. UL12 and UL12.5 display similar expression kinetics and are considered E proteins given their expression in absence of vDNA replication, but UL12 and UL12.5 are under the control of different promoters [311]. This production of distinct viral proteins from that same open reading frame (ORF) is not unique to UL12 and UL12.5, but is actually a conserved hallmark among herpesvirus genomes. For instance, ICP47, US11, and US10 are collinear and share the same polyadenylation and termination sequence. Additional intragenic and epigenetic mechanisms mediating expression of UL12.5 have yet to be studied, however.

UL12.5 retains all 7 motifs from UL12 orthologs across all herpesviruses, including motif II that is required for the catalytic nuclease function. However, UL12.5 expression is not sufficient to compensate for a UL12 deletion during lytic replication [312,313]. While UL12.5 retains much of the same exonucleolytic and endonucleolytic properties of nuclear-resident UL12 [314], UL12.5 cannot compensate for UL12 because

the terminal truncation generates a 60 amino acid N-terminus residue that targets UL12.5 to the mitochondrial matrix [315]. During lytic infection, UL12.5 is therefore localized to the mitochondria there it depletes mtDNA and mtRNA [293,315–322]. Although the precise mechanisms mediating mtDNA and mtRNA depletion are unclear, UL12.5 does partly rely on the mitochondrial residence nucleases endonuclease G (ENDOG) and exonuclease G (EXOG) [318]. The interaction between UL12.5 and ENDOG/EXOG is unknown, but is hypothesized to be through interacting partners. Moreover, these interactions may contribute to mtRNA depletion by UL12.5, as ENDOG and EXOG display low levels of RNase activity [323,324] and the depletion mediated by UL12.5 is too rapid to be the result of transcriptional defects. Thus far, the only established function of UL12.5 is considered to be the depletion of mtDNA and mtRNA and the induction of mtDNA stress.

mtDNA stress is associated with the release of mitochondrial nucleic acids in to the cytosol and subsequent activation of innate immune pathways that respond to nucleic acids in the cytosol. Indeed, multiple inducers of mtDNA stress result in activation of the cGAS-STING pathway and upregulation of an antiviral response. Knockdown of TFAM results in chronic mtDNA stress and activation of cGAS-STING. Interestingly, activation of cGAS-STING in this context results in a unique set of ISGs that are not associated with NF-κB or IRF3 gene activation [325]. Instead, cGAS-STING activation in this context drive expression of DNA repair genes, such as PARP9. Mitochondrial nucleic acids are released after UL12.5 expression in a similar manner to TFAM KD in which enlarged nucleoids form on mitochondria rupture to release mtDNA

and are associated with cGAS [301]. Following activation of cGAS-STING by UL12.5 in mouse embryonic fibroblasts, IFN and ISGs are upregulated and hypothesized to restrict viral replication [293]. In certain specialized immune cells, such as THP-1 cells, restriction of viral growth by UL12.5 is attributed to preferential activation of RIG-I via RNA Pol3 activity in response to cytosolic mtDNA transcripts [321]. However, a role of RNA Pol3 in innate immune sensing of HSV remains controversial and not observed I many cell types. Therefore, UL12.5 expression results in the activation of innate immune pathways during lytic infection that ultimately restrict viral growth. Given that mtDNA depletion does not support lytic HSV replication *in vitro* [319], it is puzzling that HSV encodes a protein that directly inhibits its ability to undergo lytic replication. However, it is unknown if there are additional downstream effects of UL12.5 expression, especially in specialized cells.

While mtDNA and mtRNA depletion and the induction of an immune response are considered the primary function of UL12.5, there are some additional interactions with mitochondria that may be associated with unknown functions. In mouse embryonic fibroblasts infected with a UL12.5-GFP retrovirus, UL12.5 decreased cellular ATP levels and disrupted mitochondrial membrane potential [320]. While the mitochondrial membrane potential was rescued under homeostatic conditions, these experiments still demonstrate the ability of UL12.5 to perturb membrane polarization. Additionally, UL12.5 was found to prevent processing of the mitochondrial protein Opa1 [317], which regulates multiple cellular processes focused on the mitochondrial network, metabolism, and regulation of cytochrome C [326]. Interestingly, UL12.5 is also associated with

extracellular virions and is hypothesized to be a tegument protein [312]. UL12.5 was hypothesized to contribute to establishing or maintaining a latent infection, but the implications of this characterization are unknown.

## Figures

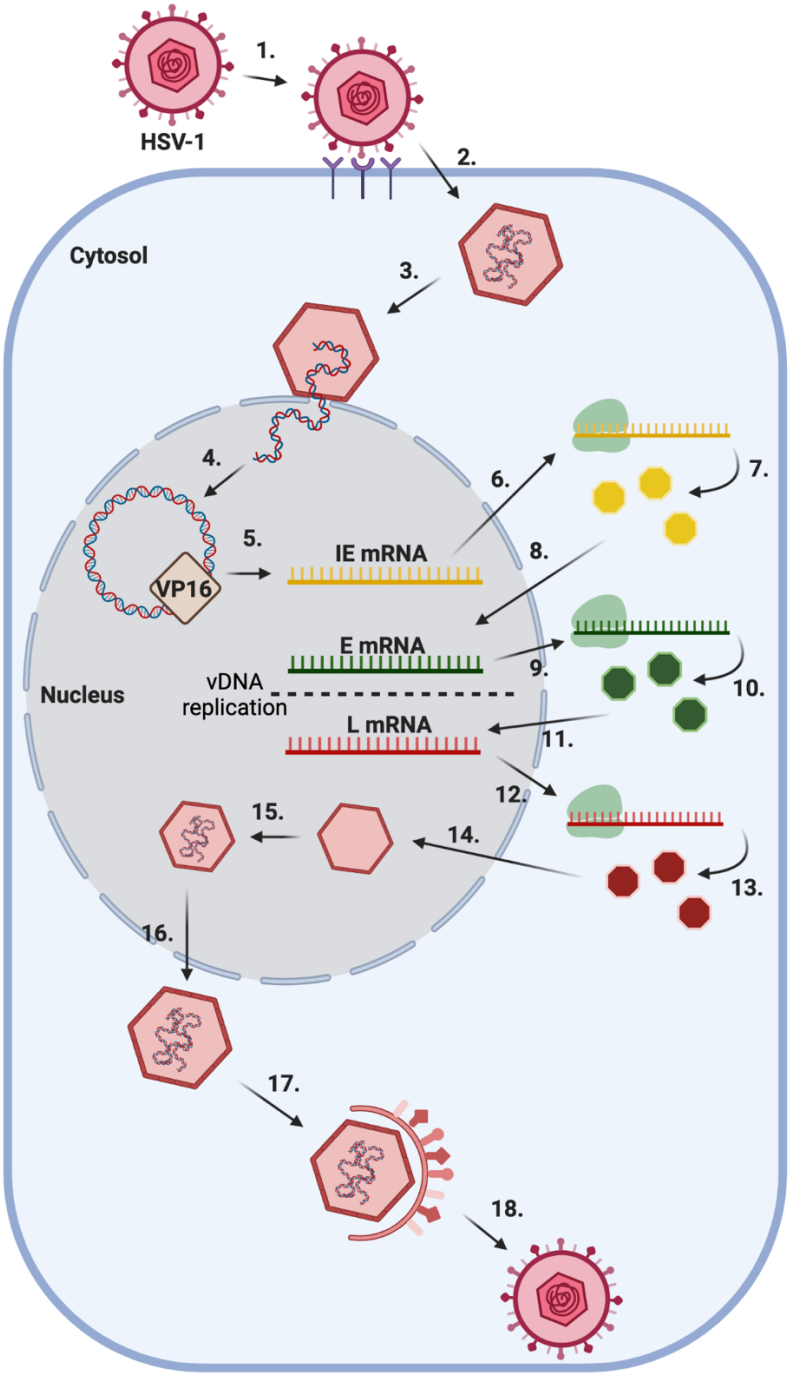


**Figure 1. HSV Lytic Replication.** Lytic HSV replication can occur in a range of cell types, including epithelial cells and peripheral neurons. 1. Glycoproteins embedded within the HSV envelope adhere to the host cell sur-face. 2. As the viral envelope fuses with the host cell membrane, the viral capsid containing the viral genome is release into the cytosol. 3. The viral capsid is trafficked via retrograde transport along microtubules to the host nucleus. 4. The vDNA is released into the nuclear space via the nuclear pore complex within the nuclear membrane. 5. The HSV protein VP16 initiates transcription of IE mRNA. 6/7. IE mRNA is translated into IE proteins within the cytosol. 8. IE proteins traffic to the nucleus to mediate transcription of E mRNA. 9/10. E mRNA is translated into E proteins in the cytosol. 11. E proteins traffic to the nucleus to facilitate vDNA replication and L gene transcription. 12/13. L mRNA is translated into L proteins in the cytosol. 14. L proteins traffic to the nucleus to assemble the viral capsid. 15. vDNA is pack-aged within the viral capsid. 16. The viral capsid leaves the nucleus via membrane fusion and the NEC. 17. The viral capsid is enveloped in the cytosol by membrane containing viral glycoproteins to the virion. 18. The mature virion is then released from the host cell. vDNA: viral DNA; NEC: nuclear egress complex; IE: immediate-early; E: early; L: late.

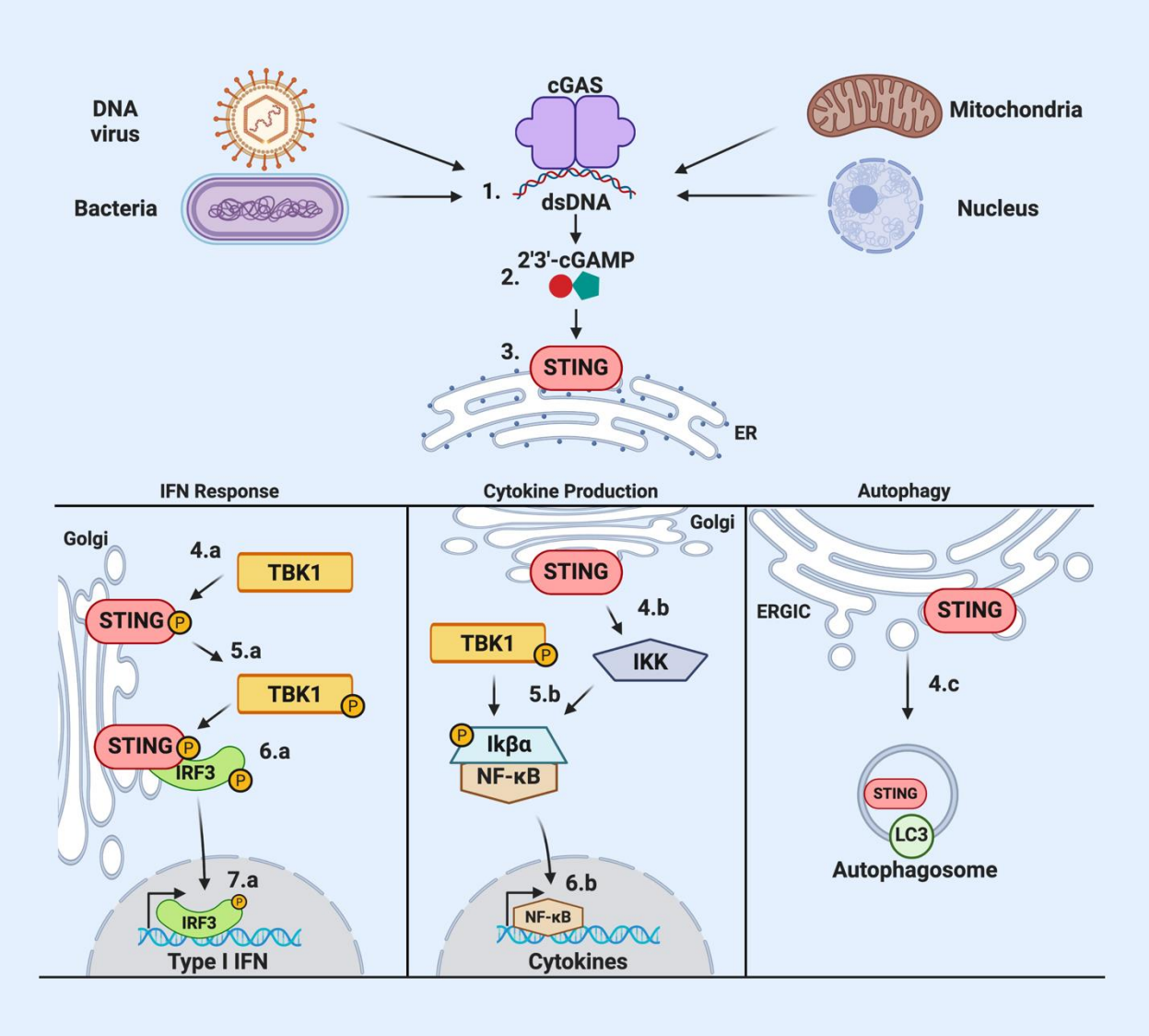


**Figure 2**. **cGAS-STING Pathway Activation**. Activation of the innate immune cGAS-STING pathway by cytosolic DNA can result in three primary downstream signaling cascades that result in the IFN response, cytokine production, or autophagy. 1. cGAS binding to DNA results in the dimerization and activation of cGAS. 2. Active cGAS produces the secondary messenger molecule 2'3'-cGAMP. 3. 2'3-cGAMP activates the downstream signaling protein STING on the ER. Following STING activation in the ER, the signaling pathway can be differentiated into the IFN response (a), cytokine production (b), or autophagy induction (c). For the IFN response, 4a. Active STING is trafficked to the Golgi network to be phosphorylated by TBK1. 5a. STING phosphorylates TBK1 (pTBK1). 6a. Using STING as a scaffold, pTBK1 mediates the phosphorylation of IRF3 (pIRF3). 7a. Active pIRF3 dimerizes and translocates to the nucleus to induce transcription of type I IFN. For cytokine production, 4b. active STING activates the IKK complex. 5b. IkB can be phosphorylated by the IKK complex and pTBK1 to release NF-kB. 6b. NF-kB translocates to the nucleus to induce transcription of proinflammatory cytokines. For autophagy induction, 4c. STING translocates to the ERGIC to produce LC3+ autophagosomes. dsDNA: double-stranded DNA; 2'3'-cGAMP: 2'3'-cyclic guanosine monophosphate-adenosine monophosphate; STING: stimulator if interferon genes; ER: endoplasmic reticulum; TBK1: TANK binding kinase 1; IRF3: interferon regulatory factor 3; ERGIC: ER-Golgi intermediate complex; IKK: inhibitor of Ikβα kinase; NF-kB: nuclear factor k-B; LC3: microtubule-associated protein 1A/1B-light chain 3.

*End of Chapter 1*

# *Chapter 2*

# Neuronal hyperexcitability is a DLK-dependent trigger of herpes simplex virus reactivation that can be induced by IL-1

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## Abstract

Herpes simplex virus-1 (HSV) establishes latent infections in peripheral neurons and periodically reactivates to re-initiate lytic gene transcription and produce infectious virus. Although HSV reactivation is associated with significant clinical morbidities, the cellular mechanisms underlying reactivation have not been fully elucidated. Previous studies have demonstrated that forskolin can trigger HSV reactivation through an unknown mechanism. Our study reports that forskolin induces a hyperexcitable state within peripheral neurons that triggers HSV reactivation. Importantly, forskolin-mediated reactivation induces Phase I gene expression that is associated with histone phosphorylation and is dependent on DLK and JNK activation. Moreover, we identify IL-1β as a physiological trigger of HSV reactivation via the induction of neuronal hyperexcitability. HSV reactivation mediated by IL-1β induced histone phosphorylation and was dependent on both DLK activity and neuronal excitation. Thus, neuronal hyperexcitability induces biphasic HSV reactivation that can be triggered by co-option of the IL-1 signaling pathway.

## Introduction

Herpes simplex virus-1 (HSV) is a ubiquitous human pathogen that can establish lifelong infections in neurons of the peripheral nervous system. Following a period of latency, the virus can periodically re-initiate lytic gene transcription through a process called reactivation. Although generally asymptomatic, HSV reactivation is associated with significant clinical morbidities such as herpes simplex encephalitis, keratitis, and painful oral and genital lesions [327]. More recently, epidemiological and cell biology studies have indicated that HSV infection is linked with the development of Alzheimer's disease and dementia [328–333]. This association suggests that HSV engages with canonically inflammatory neurodegenerative disease, but the triggers mediating HSV reactivation and how these reactivation events contribute to various pathologies is not clear.

During a latent infection, the viral genome persists as an episome within the neuronal nucleus by associating with host histones that allow for the formation of transcriptionally repressive heterochromatin [334–337]. In this this state, lytic promoters contain modifications that are known to maintain transcriptional silencing, such as di- and tri-methylation of lysine 9 on histone H3 (H3K9me2/3) as well as di-methylation of lysine 27 on histone H3 (H3K27me3) [65,66,77,338]. The re-initiation of lytic gene transcription during reactivation necessitates that these repressive modifications are altered or removed to permit lytic gene expression, viral DNA (vDNA) replication, and the production of infectious virus. Given that there is little to no detectable viral protein

during latency, initiation of reactivation relies on the activation of host pathways [339]. The cellular mechanisms mediating this exit from latency are not fully understood.

One of the earliest experimental triggers of *in vitro* HSV reactivation is nerve growth factor (NGF) deprivation, has been found to mediate reactivation through activation of the neuronal stress pathway mediated by dual leucine zipper kinase (DLK) and c-Jun N-terminal kinase (JNK) [51–53]. The activation of DLK and JNK represent a critical step in the reactivation process across multiple triggers [54,57,85,340,341] and is associated with additional triggers, but the role of JNK signaling in those contexts is not yet known. Our lab found that while JNK functions to generate histone phosphorylation marks proximal to repressive methylation modifications that lead to a rapid induction of lytic gene expression [54]. This histone modification is, importantly, independent of demethylase activity, but how DLK and JNK recruit these phosphorylation marks and how these phospho/methyl modifications is unknown.

Given the reliance on host pathways to activate JNK and trigger reactivation, the events mediating reactivation are incredibly complex and different that lytic transcription patterns observed during lytic replication. Lytic replication is characterized by an ordered viral gene cascade in which immediate-early (IE) proteins are required for early (E) gene transcription and translation. E gene products then mediate vDNA replication, which is required for late (L) gene expression. In contrast, JNK-mediated reactivation is biphasic [54,57,82,84,85]: Phase I is defined as a stochastic burst of lytic transcripts from all three gene classes (IE, E, L) independent of vDNA replication and can progress Phase

II during which viral gene transcription follows the ordered cascade seen in lytic replication and results in the production of infectious virus [54,84]. While Phase I generally lasts approximately 20 hours following a stimulus, Phase II occurs 48-72 hours after the stimulus and requires the viral transactivator VP16 as well as histone demethylase activity [24,54,57,93,118,209]. Although this biphasic pattern of gene expression has not been observed in all triggers, such as axotomy, whether JNK activation and biphasic gene expression patterns emerge following physiological triggers of reactivation is not yet known.

Fever, stress, and UV exposure are well-established clinical triggers of HSV reactivation [107,342–344] and the number of experimental triggers is incredibly diverse, but for many triggers the cellular mechanisms mediating reactivation are unknown. Forskolin, an activator of adenylyl cyclase, has previously been shown to induce HSV reactivation through an unknown mechanism [97,98,345,346]. Interestingly, forskolin also induces hyperstimulation and induces a global phospho/methyl switch in cortical neurons [347]. Neuronal hyperstimulation is correlated with cellular DNA damage and perturbations of the DNA damage response trigger reactivation via modulation of the JNK signaling pathway [105]. Therefore, we hypothesized that forskolin was triggering HSV reactivation via neuronal hyperexcitability as well as activation of DLK and JNK. In this study, we describe the biphasic lytic gene expression pattern following forskolin treatment and confirm that forskolin-induced reactivation is dependent on neuronal hyperexcitability as well as DLK/JNK activation.

In addition to forskolin, we sought to identify physiological triggers of HSV reactivation that similarly acted through neuronal hyperexcitability. Interleukin 1 (IL-1) is a major controller of the cellular inflammatory response and is released during conditions of psychological stress or fever [348–351]. Although IL-1 is involved in the innate immune response to viral infection [352], in neurons specifically IL-1 stimulates a hyperexcitable state within neurons [353–357]. Given that IL-1 can display markedly different downstream effects between cell types, we hypothesized that IL-1 could trigger HSV reactivation in peripheral neurons via hyperexcitability. Remarkably, we found that IL-1β was capable of triggering HSV reactivation from mature sympathetic neurons and that this reactivation was dependent on neuronal hyperexcitability in a manner similar to forskolin. Our results also found that activation of the DLK and JNK pathway is required for IL-1-mediated reactivation, identifying IL-1β as a novel HSV trigger and highlighting the significance of DLK signaling in HSV reactivation.

## Results

### Increased intracellular levels of cAMP induces reactivation of HSV from latent infection in murine sympathetic neurons

In addition to forskolin, cAMP mimetics can also trigger HSV reactivation [97,98,345,346]. To determine the mechanism mediating initiation of lytic gene transcription with forskolin, we infected primary murine neurons from the superior cervical ganglia (SCG) with a US11-GFP tagged HSV [358] and established a latent infection using acyclovir (ACV) (Figure 1A). Forskolin has two primary mechanisms of action: direct activation of ion channels and activation of adenylate cyclase [359–361] (Figure 1B). While we confirmed that forskolin triggered reactivation, dideoxy forskolin (dd-forskolin) [360,362], which is unable to pass through the cell membrane, did not induce HSV reactivation to comparable levels of forskolin (Figure 1C). Therefore, it is likely that the intracellular mechanisms of forskolin primarily contribute to HSV reactivation.

Treatment of latency-infected neurons with a cAMP mimetic, 8-bromo-cAMP, triggered reactivation (Figure 1D), suggesting that an increase in intracellular cAMP can trigger HSV reactivation. When forskolin was added to neuron in the presence of a adenylate cyclase inhibitor, SQ22, [363]HSV reactivation was significantly reduced (Figure 1E). Altogether, these data demonstrate that forskolin-mediated reactivation is associated with an increase of intracellular cAMP that is required for reactivation to occur.

## DLK and JNK activity are required for the early phase of viral gene expression in response to forskolin treatment

Previously, our lab found that DLK and JNK activation following NGF deprivation is critical for induction of Phase I [54]. In order to determine whether DLK and JNK activation contributed to forskolin-mediated reactivation, we added forskolin to latently-infected neurons in the presence of SP600125 (Figure 2A), a JNK inhibitor, or GNE-3511 (Figure 2B), a DLK-inhibitor. Both the DLK and JNK inhibitors drastically reduced forskolin-mediated reactivation 3 days post-stimulus. Therefore, activation of DLK and JNK represents an essential step downstream of forskolin in the induction of HSV reactivation.

Given that JNK-mediated reactivation has been shown to induce a biphasic wave of lytic gene expression [54,57,84], we hypothesized that forskolin treatment would undergo both Phase I and Phase II. To first determine whether forskolin triggers reactivation that culminates with Phase II and the production of infectious virus, we measured the production of infectious virus, lytic gene expression, and vDNA replication after treating latently-infected neurons with forskolin. Synthesis of US11-GFP was used to measure L gene expression following forskolin treatment, showing that US11-GFP appears around 48 hours post-reactivation and is robust around 72 hours post-stimulus (Figure 2C). Superinfection of latently-infected neurons with a replication competent virus was used as a control (Figure 2C). Production of infectious virus was confirmed following treatment of latently-infected neurons with forskolin (Figure 2, S1A&B). These data

suggest that forskolin triggers HSV reactivation within only a subset of neurons or that only of subset of neurons make it to full reactivation.

After confirming that full reactivation by forskolin is dependent on DLK and JNK and the production of infectious virus results from forskolin treatment, we hypothesized that forskolin triggers a lytic gene expression pattern characteristic of Phase I. To evaluate the expression kinetics of lytic gene expression following forskolin treatment, we measured viral transcripts throughout the first 20 hours following addition of forskolin. For all viral transcripts tested, we found that the transcripts were significantly upregulated at or before 20 hours post-stimulus (Figure 2D-F, S1C-E). Importantly, we observed *gC* expression by 20 hours, prior to vDNA replication was detected (Figure S1B). Therefore, lytic gene expression across all viral gene classes occurs within the first 20 hours post-forskolin and is independent, which is characteristic of Phase I.

To determine whether activation of DLK and JNK specifically contributed to Phase I, we reactivated neurons with forskolin in the presence of SP1600125 or GNE-3511. Importantly, we found that lytic gene expression during Phase I was robustly restricted in the presence of either the DLK or JNK inhibitor (Figure 2G-I). Next, we confirmed the role of DLK in Phase I induction through forskolin by transducing latently-infected neurons with DLK-targeting shRNA vectors. Following confirmation of knockdown of DLK protein (Figure 2J), we found that when DLK was knocked down lytic gene expression was significantly reduced during Phase I (Figure 2K-L). We further confirmed that additional downstream signaling proteins that interact with JNK, PKA,

CREB, and EPAC1/2 do not play a role in a forskolin induction of Phase I (Figure S2A-F). These studied demonstrate that forskolin induces Phase I of HSV reactivation that requires activation of DLK and JNK, but is independent of PKA, EPAC1/2, or CREB.

## Forskolin triggers a Phase I wave of viral gene expression that is independent of histone demethylase activity

Additional targets of cAMP include cyclic nucleotide-gated ion channels in neurons, which can modulate action potential firing. We hypothesized that forskolin promotes a hyperexcitable state in neurons that results with an accumulation of nuclear cFOS and specific histone modifications linked to physiological DNA damage [364,365]. We found that forskolin treatment was associated with an increase in γH2AX, a DNA damage marker, as well as cFOS (Figure S3A, C & D). Although previous reports showed that HSV reactivation can be triggered by loss of AKT phosphorylation following DNA damage [105], we did not find a loss of pAKT signal alongside the increase in γH2AX after forskolin treatment (Figure S3E). However, we did confirm that forskolin triggered a transient increase in the H3K9me3pS10 phospho/methyl switch in sympathetic neurons that was previously described in cortical neurons (Figure S3A & B) [347].

Given that the global phospho/methyl switch induced by forskolin is the same histone modification associated with lytic gene transcription during Phase I, we hypothesized that H3K9me3/pS10 co-localizes with the viral genome following forskolin treatment in order to promote lytic gene expression. Using Click-chemistry to label viral

genomes of HSV US11-GFP virus, we found that forskolin treatment resulted in a significant increase in the co-localization of H3K9me3/pS10 both 5- and 10-hours post-treatment (Figure 3A & B). This raised the possibility that the same phospho/methyl switch described following PI3K inhibition downstream of JNK activation [54] facilitates lytic gene expression during Phase I during forskolin-mediated HSV reactivation.

Previous reports have shown that histone demethylase activity is required for Phase II or full reactivation, including the H3K9me3 histone demethylase LSD1 [24,118]. Using two independent LSD1 inhibitors, we confirmed that LSD1 is required for full reactivation (Figure S3F), but is not required for Phase I lytic gene expression following forskolin treatment (Figure 3C & D). Moreover, inhibition of H3K27me histone demethylase activity by UTX/JMJD3 prevented full reactivation (Figure S3G), but not lytic gene expression in Phase I after forskolin treatment (Figure 3E & F). Therefore, forskolin induces a histone phospho/methyl switch that may permit lytic gene expression in Phase I and this lytic gene expression is independent of the removal of repressive methylation marks at H3K9 and H3K27.

## Forskolin-mediated reactivation requires neuronal excitability

The co-localization of the viral genome with histone modifications associated with neuronal hyperexcitability suggested that reactivation itself may be reliant on increased action potential firing. To investigate the link between neuronal hyperexcitability and HSV reactivation, we treated latently-infected neurons with forskolin in the presence of tetrodotoxin (TTX) or tetraethylammonium (TEA) which block voltage-gated sodium

channels or voltage-gated potassium channels, respectively. Inhibition of either channel is sufficient to substantially reduce action potential firing. Reactivation by forskolin was significantly reduced in neurons treated with either TTX or TEA (Figure 4A & B), indicating that action potential firing is required for forskolin-mediated reactivation.

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which are sodium and potassium channels that are activated by membrane hyperpolarization, can be activated directly by cAMP [366,367]. When cAMP levels are increased, HCN channels are more likely to open and therefore increase the propensity for action potential firing [367–369]. Inhibition of these channels with ZD 7288 concurrent with forskolin treatment resulted in a significant reduction in lytic gene transcripts during reactivation (Figure 4D & S4C & D). Additionally, cesium chloride, a non-selective cation channel blocker, as well as ivabradine, a broad HCN inhibitor, reduced reactivation induced by forskolin (Figure S4A & B).

To confirm that inhibition of HCN channels prevented neuronal hyperexcitability, we measured H3K9me3/pS10 and γH2AX staining after forskolin treatment with the addition of ZD 7288. We found that staining for both histone modifications was decreased in the presence of ZD 7288 (Figure 4E & F), suggesting that these ion channel facilitate hyperexcitability induced by forskolin. Therefore, activity of these HCN channels or voltage-gated sodium channels in response to elevated cAMP level results in histone modifications and lytic gene expression during Phase I of HSV reactivation.

## HSV reactivation can be induced by stimuli that directly increase neuronal excitability

Our previous results demonstrated that ion channel activity is an essential aspect of forskolin-mediated reactivation. We were interested in whether additional stimuli induce neuronal hyperexcitability to the point of HSV reactivation and whether hyperexcitability-induced reactivation required chronic or short-term action potential firing. Given that increases in extracellular KCl is well-established to increase action potential firing, we tested whether KCl treatment of latently-infected neurons is sufficient to trigger reactivation. We found that KCl robustly triggered HSV reactivation when added for 8 hours or more (Figure 5A), indicating that chronic neuronal hyperexcitability is important aspect in reactivation triggered by hyperexcitability.

In order to further delineate the role of hyperexcitability as a stimulus of HSV reactivation, evaluated the role of TTX block removal in promoting reactivation. The removal of a TTX is reported to promote neuronal entry into a hyperexcitable state following the TTX-mediated ion channel inhibition [370–373]. Washout of TTX of latently-infected neurons was sufficient to trigger reactivation (Figure 5B). Importantly, reactivation triggered in this way was blocked by DLK and JNK inhibitors (Figure 5B) or knockdown of DLK (Figure 5C). Together, these data show that direct induction of neuronal hyperexcitability triggers HSV reactivation via DLK and JNK.

## IL-1β triggers HSV reactivation in mature neurons in a DLK and voltage-gated sodium channel-dependent manner

Following our previous observations, we sought to identify a physiological trigger of HSV reactivation that could trigger reactivation *in vivo* via induction of neuronal hyperexcitability. Neuronal excitation is known to be induced by sensing of the inflammatory cytokine IL-1β [353–355]. As a major mediator of host inflammation, IL-1β is released systemically during periods of psychological stress as well as fever [348–351]. In contrast to post-natal neurons that showed no change in γH2AX and H3K9me3/pS10 staining, IL-1β treatment of sympathetic neurons from adults led to an increase in γH2AX and H3K9me3/pS10 staining (Figure 6A, S5A & B). Although the precise reasons for this differential response are unknown, it could be due to changes in cellular factors responding to IL-1β associated with aging. To determine whether these histone modifications were dependent on action potential firing downstream of IL-1 sensing, we measured the staining intensity of γH2AX and H3K9me3/pS10 following IL-1β treatment in the presence of TTX and a neutralizing antibody for the IL-1 receptor. We found that changes in γH2AX and H3K9me3/pS10 staining were directly linked with neuronal action potential firing as well as IL-1 receptor activation (Figure 6B-C).

Calcium ion levels provide a direct readout for neuronal hyperexcitability. To test the ability of IL-1β to prompt a hyperexcitable state within aged sympathetic neurons, we measured calcium ion levels in these neurons with the addition of acetylcholine after incubation with IL-1β. Neurons treated with IL-1β for 20 hours showed higher levels of cytosolic calcium ions compared to mock treated controls following the addition of

acetylcholine (Figure 6D-E). Ionomycin was added at the end of the recording to control for any intrinsic artifacts from dye loading ore retention. Importantly, the increase in cytosolic calcium ions was lost when the IL-1R neutralizing antibody was added, indicating that increased action potential firing was due to IL-1 receptor activation. Therefore, IL-1β induced a hyperexcitable state in aged sympathetic neurons.

Since IL-1β prompted neurons to enter a hyperexcitable state, we hypothesized that IL-1β was able to trigger HSV reactivation. Remarkably, treatment of latently-infected aged neurons with IL-1β triggered reactivation (Figure 7A). This induction of reactivation was both dependent on IL-1 receptor activation and mimicked levels observed with forskolin treatment of neurons from the same mice (Figure 7B). Inhibition of ion channels as well as DLK inhibition restricted reactivation via IL-1β (Figure 7C), suggesting that IL-1β functions through a mechanism similar to forskolin. However, HCN inhibition did not significantly affect reactivation by IL-1β and may be the result of activity that is not wholly reliant on cAMP levels. Importantly, knockdown of DLK prevented IL-1β-mediated HSV reactivation (Figure 7D). Therefore, we identified IL-1β as a novel physiological that's triggers HSV reactivation via neuronal hyperexcitability and DLK activation.

## Discussion

Given the lack of viral proteins during latency, HSV has evolved to co-opt host signaling pathways to re-initiate lytic gene transcription during reactivation. The pathways that HSV utilizes to facilitate reactivation have yet to be fully elucidated, but the requirement of DLK and JNK signaling in reactivation have shown how HSV can rely on essential pathways associated with neurotrophic signaling factors [54,57]. Another essential function of neurons is the firing of action potentials, with the potential to modulate the rate of action potential firing to induce states of hyperexcitability when needed. Our report builds on this idea to demonstrate that HSV takes advantage of this potential for hyperexcitability and its subsequent activation of DLK and JNK to mediate reactivation. Moreover, hyperexcitability induces a phospho/methyl switch in neurons that induces lytic gene expression without histone demethylase activity. Stress, UVA damage, and inflammation can result in hyperexcitability and are linked with the major signaling cytokine IL-1β [348–351,374]. We show that IL-1β stimulates DNA damage and histone phosphorylation as well as increased action potential firing in response to neurotransmitter stimulation. In a manner similar to forskolin, IL-1β stimulated HSV reactivation via neuronal hyperexcitability and DLK activation. Taken together, this study found a physiologically relevant stimulus that triggers HSV reactivation through an increase in neuronal excitation and highlights the essentiality of DLK/JNK activation and a histone phospho/methyl switch in HSV reactivation.

Primary neuron model systems have been indispensable in understanding the neuronal intracellular mechanisms governing HSV reactivation. Previous to this study,

the ubiquity of Phase I and Phase II and DLK/JNK activation downstream of stimuli separate from the NGF signaling pathway that trigger HSV reactivation was unknown [54,84,86]. In our report, we characterize a new mechanistic trigger of HSV reactivation that displays Phase I of reactivation reliant on JNK activation, but independent of histone demethylase activity. Our data showing that the H3K9me3/pS10 switch occurs on viral genomes following periods of increased excitability support that Phase I gene expression occurs without the removal of repressive methylation marks. Additionally, our results highlight that Phase I and Phase II patterns of gene expression are characteristic of HSV reactivation across different triggering stimuli. Other stimuli that trigger reactivation, such as axotomy or heat shock, that have been posited to bypass Phase I may be example of differential downstream expression kinetics between different triggers wherein Phase I may happen more rapidly [24,82,375]. Changes in these expression kinetics early in reactivation may be the result of different model systems, the impact of host immune responses on the potential for reactivation, or nuanced interactions between the viral epigenome and host signaling pathways.

HSV reactivation occurs across various tissues can be greatly affected by the host immune response to infection. The presence of both type I and II interferons can prevent reactivation when present prior to the end of Phase II [92] or during initial infection [115]. However, this study demonstrates that HSV can use immune signaling pathways to promote HSV reactivation. Neurons are unique in that they have muted responses to key cytokines [123,126,130] and, in fact, utilize inflammatory signaling molecules to modulate homeostatic functions, such as learning and memory formation [142,356,376]. Therefore,

HSV may take advantage of distinct immune signaling mechanisms that, instead of inducing a potentially toxic inflammatory response, promote the viability of a terminally differentiated cell type. The induction of neuronal hyperexcitability by IL-1β to reactivate HSV suggests that the link between inflammatory neurodegenerative diseases and the immune responses during reactivation are incredibly complex and warrant further investigation.

Previously, IL-1 has been shown to induce neuronal hyperexcitability and is increased during HSV reactivation *in vivo* [104,353]. Interestingly, high levels of both IL-1α and IL-1β, which can both activate the IL-1 receptor, are released by keratinocytes during HSV infection [377]. This release of IL-1 as an alarmin may affect signaling mechanisms that drive the establishment of latency or have prolonged effects from sensing at initial infection that modulate reactivation at a later time, as with IFN and the formation of PML-NBs [115]. In the case of reactivation, IL-1 can be implicated in the known clinical triggers of stress, fever, and UV damage. First, stress and inflammation are well-described in their increase in systemic IL-1 levels [348–351]. Second, keratinocytes and corneal epithelial cells release in response to UV radiation [374,378], which may link IL-1 to reactivation associated with sunburn. Additional signaling molecules, such as homeostatic release of NGF as well as immune-activated IL-6 and TNFα, can induce neuronal hyperexcitability [104,353,379]. It is therefore possible that the switch between latency and reactivation fits within balance determined by the milieu of immune and homeostatic molecules. The role of these signaling molecules in regulating HSV

reactivation *in vivo* will have to be delineated in order to further our understanding of this unique facet of immune signaling that HSV evolved to exploit.

In this study, we found that forskolin and IL-1β trigger HSV reactivation through activation of DLK, supporting the model that DLK and JNK represent critical signaling events across multiple triggers of HSV reactivation. Previously, the potential for DLK activation following neuronal excitability was not known and it will be important to delineate how hyperexcitability directly or indirectly leads to the activation of DLK. Moreover, understanding what transcription or pioneer factors facilitate the JNK-dependent phospho/methyl switch and induction of lytic gene transcription in Phase I as well as how the viral chromatin state engage with these signaling molecules will need to be elucidated. In addition, our report provides further evidence of the biphasic reactivation pattern across different stimuli, suggesting that HSV has evolved unique signaling patterns to address the distinct immune and cellular context of latency. Investigation into how HSV co-opts these pathways, including how differential activation in neurons in particular, will provide essential information in understanding clinical HSV reactivation and the development of novel therapeutics.

## Figures

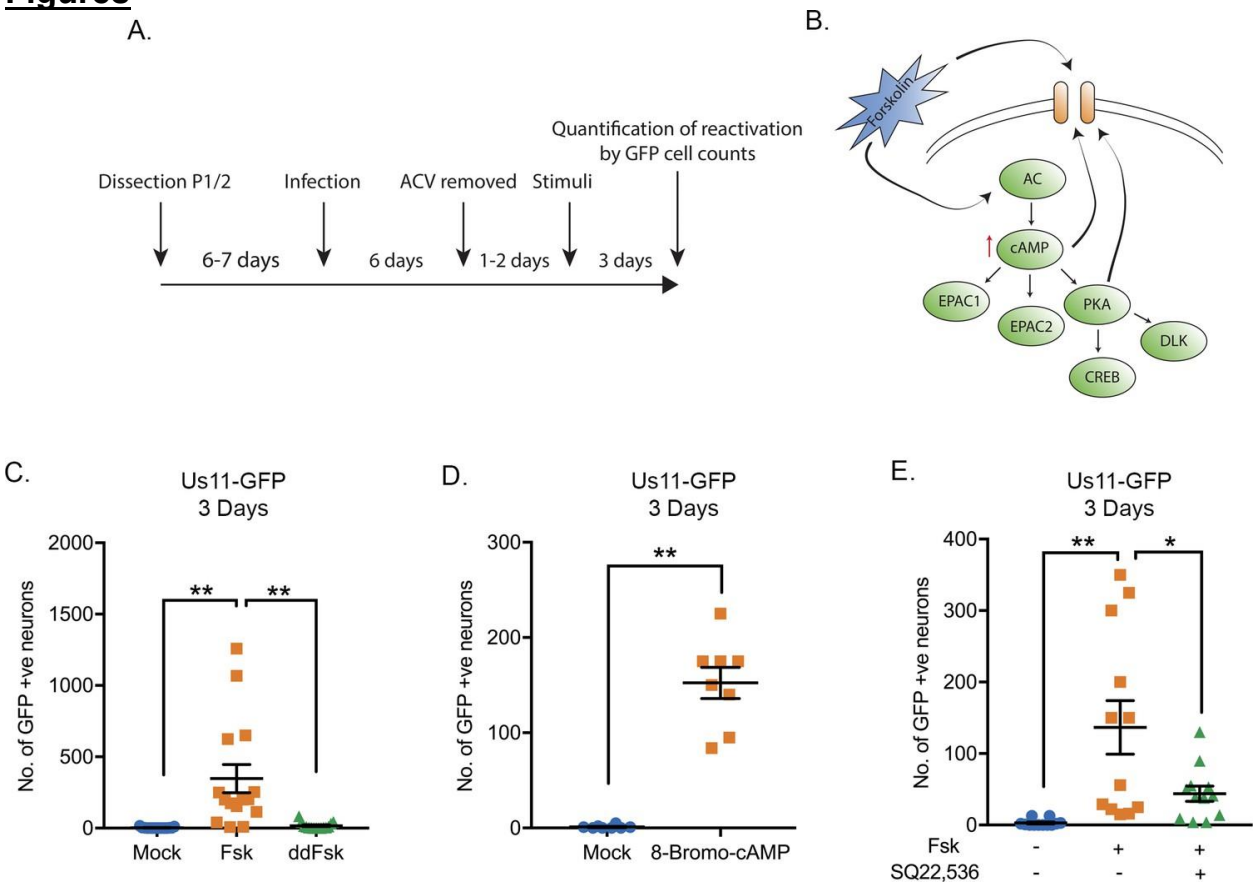


**Figure 1. HSV Reactivation from sympathetic neurons is induced by adenylate cyclase activation.** (A) Schematic of the primary sympathetic superior cervical ganglia (SCG)-derived model of HSV latency. Reactivation was quantified based on Us11-GFP-positive neurons in presence of WAY-150168, which prevents cell-to-cell spread. (B) Schematic of the cellular pathways activated by forskolin treatment. Forskolin can act both intracellularly to activate adenylate cyclase (AC) and increasing the levels of cAMP or extracellularly on ion channels. (C) Numbers of Us11-GFP-positive neurons following addition of either forskolin (60 μM) or cell-impermeable dideoxy-forskolin (60 μM) treatment of latently-infected sympathetic neurons. (D) Numbers of Us11-GFP-positive neurons following treatment with a cAMP mimetic 8-Bromo-cAMP (125 μM). (E) Reactivation, quantified by Us11-GFP-positive neurons, was induced by forskolin in the presence or absence of the adenylate cyclase inhibitor SQ22,536 (50 μM). In C-E each point represents a single biological replicate, and the mean and standard errors of the mean (SEM) are also shown. In D statistical comparisons were made using an unpaired t-test. In C and E statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparisons test. *p<0.05, **p<0.01. Experiments performed by Austin Schinlever, Parijat Kundu, and Anna Cliffe, PhD.

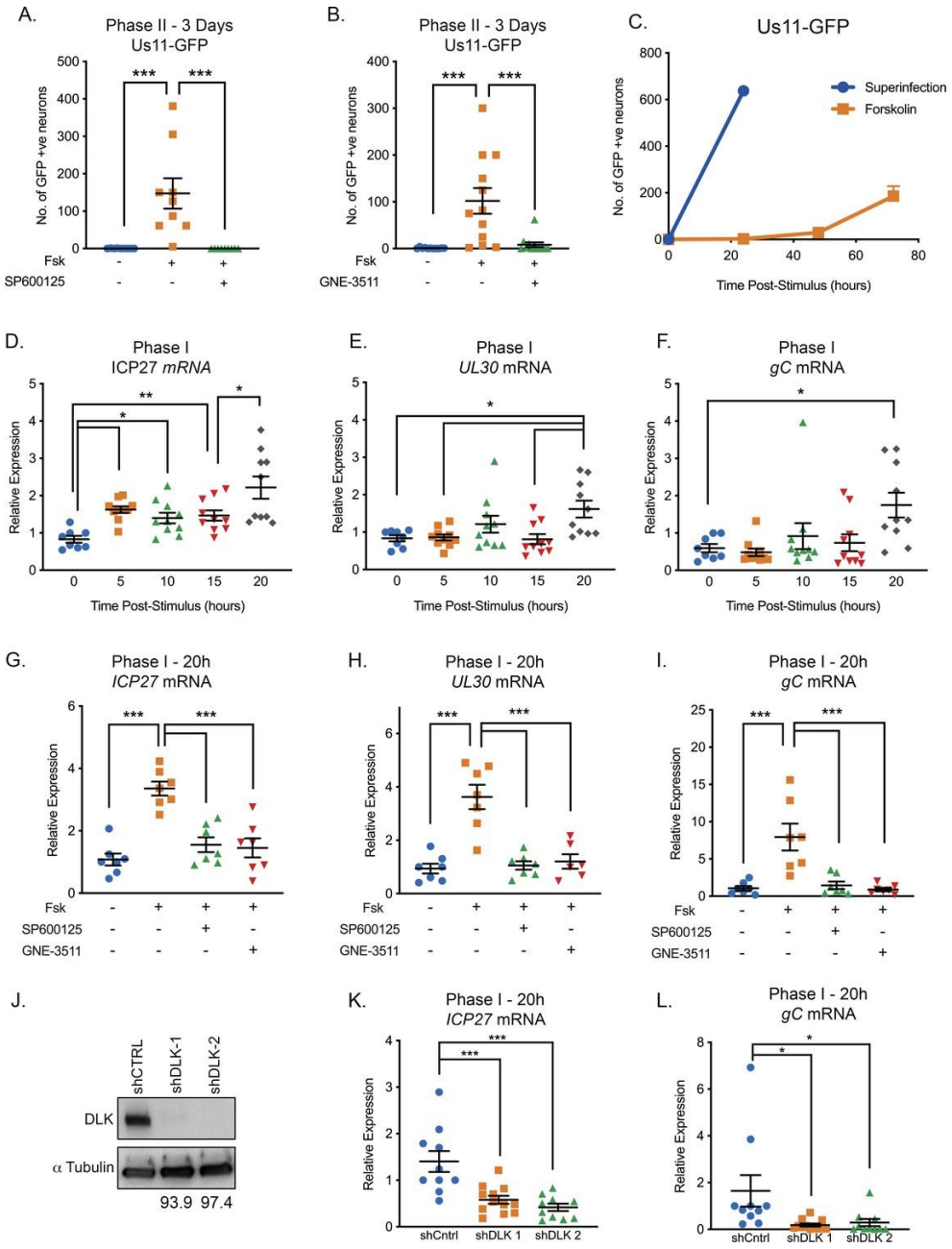


**Figure 2. Reactivation triggered by forskolin involves a DLK/JNK-dependent phase I of viral gene expression.** (A) Reactivation was induced by forskolin in the presence of JNK inhibitor SP600125 (20 μM). (B) Reactivation was induced by forskolin in the presence of the DLK inhibitor GNE-3511 (4 μM). In A and B each experimental replicate is shown. (C) Reactivation was induced by forskolin or superinfection with a wild-type (F strain) HSV (MOI of 10 PFU/cell) and qualified based on Us11-GFP-positive neurons (n = 3). (D–F) RT-qPCR for viral mRNA transcripts following forskolin treatment of latently infected SCGs. (G–I) RT-qPCR for viral lytic transcripts at 20 hr post-forskolin treatment and in presence of the JNK inhibitor SP600125 (20 μM) and the DLK inhibitor GNE-3511 (4 μM). (J) Neurons were transduced with a non-targeting shRNA control lentivirus or two independent lentiviruses expressing shRNAs that target DLK (shDLK-1, shDLK-2). Western-blotting for DLK or β-III tubulin was carried out 3 days post transduction. The percentage knock-down of DLK normalized to β-III tubulin is shown. (K and L) RT-qPCR for viral mRNA transcripts following forskolin treatment of latently infected SCGs that were either transduced with the shRNA control or shRNA DLK lentiviruses. In D-I, K, and L, each experimental replicate is represented. Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison. *p<0.05, **p<0.01, ***p<0.001. The mean and SEM are shown. D-F performed by Austin Schinlever, J-L by Sara Dochnal.

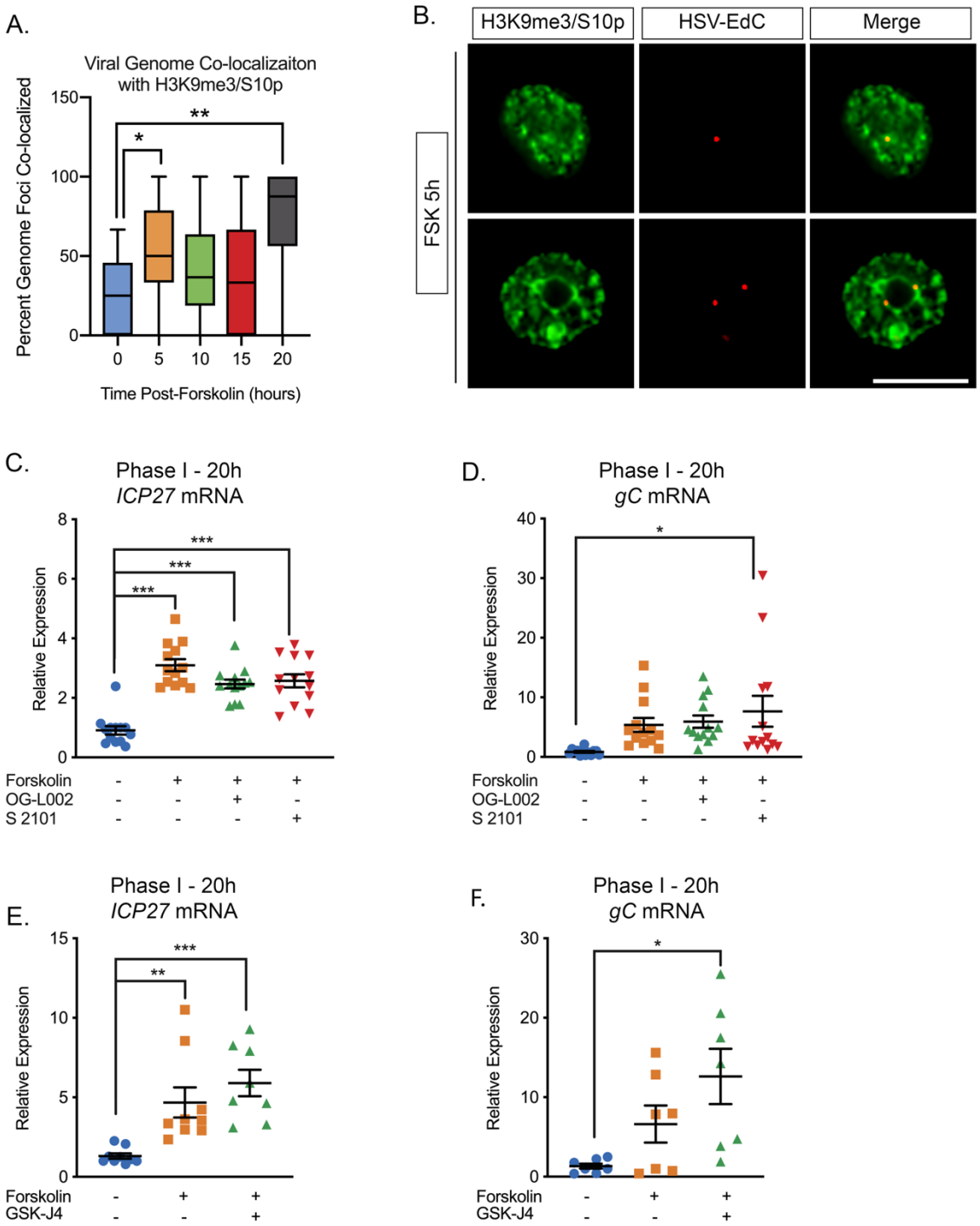
A.
Viral Genome Co-localizaiton with H3K9me3/S10p
Percent Genome Foci Co-localized
150
100
50
0
*
**
0
5
10
15
20
Time Post-Forskolin (hours)
B.
H3K9me3/S10p
HSV-EdC
Merge
FSK 5h
C.
Phase I - 20h
ICP27 mRNA
Relative Expression
8
6
4
2
0
***
***
***
Forskolin - + + +
OG-L002 - - + -
S 2101 - - - +
D.
Phase I - 20h
gC mRNA
Relative Expression
40
30
20
10
0
*
Forskolin - + + +
OG-L002 - - + -
S 2101 - - - +
E.
Phase I - 20h
ICP27 mRNA
Relative Expression
15
10
5
0
***
**
Forskolin - + +
GSK-J4 - - +
F.
Phase I - 20h
gC mRNA
Relative Expression
30
20
10
0
*
Forskolin - + +
GSK-J4 - - +

**Figure 3. The Initial wave of viral lytic gene expression during forskolin-mediated reactivation is independent on histone demethylase activity.** (A) Quantification of the percentage of genome foci stained using click-chemistry that co-localize with H3K9me3/S10p. At least 15 fields of view with 1–8 genomes per field of view were blindly scored from two independent experiments. Data are plotted around the median, with the boxes representing the 25th–75th percentiles and the whiskers the 1st-99th percentiles. (B) Representative images of click-chemistry based staining of HSV-EdC genomes and H3K9me3/S10p staining at 5 hr post-forskolin treatment. (C and D). Effect of the LSD1 inhibitors OG-L002 and S 2101 on forskolin-mediated Phase I of reactivation determined by RT-qPCR for ICP27 (C) and gC (D) viral lytic transcripts at 20 hr post-forskolin treatment and in the presence of 15 μM OG-L002 and 20 μM S 2102. (E) Effect of the JMJD3 and UTX inhibitor GSK-J4 (2 μM) on forskolin-mediated Phase I measured by RT-qPCR for viral lytic transcripts ICP27 (E) and gC (F) at 20 hr post-forskolin treatment and in the presence of GSK-J4. For C-F each experimental replicate along with the mean and SEM is represented. (C–F). Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

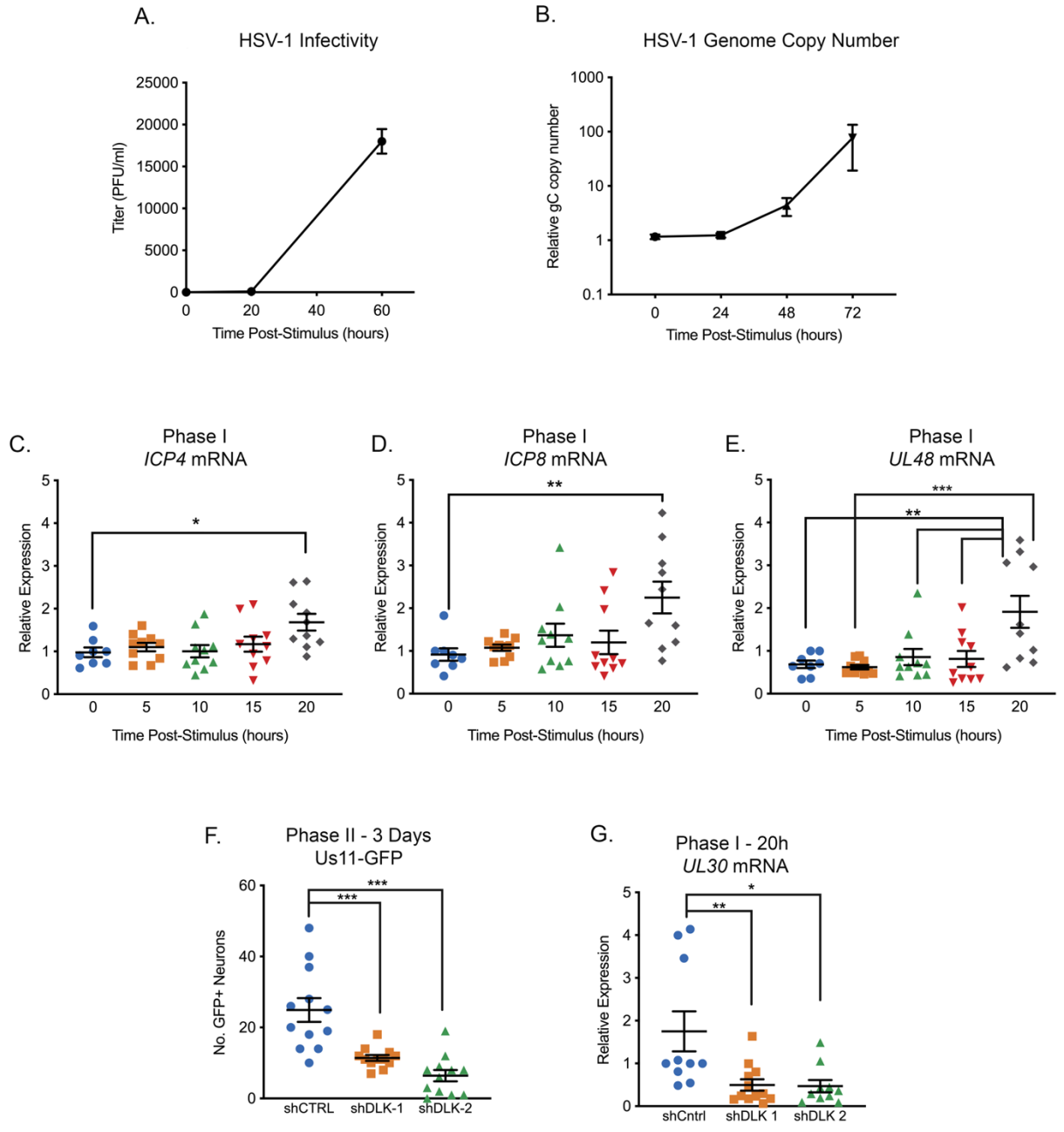


**Figure S1. Reactivation triggered by forskolin triggers a wave of lytic gene expression that precedes DNA Replication and infectious virus production.** (A) Titers of infectious virus detected from reactivating neurons induced with forskolin (n = 4). (B) Quantification of the relative viral genome copy number following forskolin-mediated reactivation based on gC copy number normalized to cellular GAPDH and expressed relative to the 0 hr time-point (n = 7). (C–E) RT-qPCR for viral mRNA transcripts following forskolin treatment of latently infected SCGs. (F) Quantification of Us11-GFP neurons and (G) RT-qPCR for UL30 mRNA transcript following forskolin treatment of latently infected SCGs that were either transduced with the shRNA control or shRNA DLK lentiviruses. Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison. *p<0.05, **p<0.01, ***p<0.001. (C–E) In C-G each biological replicate is represented. The means and SEMs are shown.

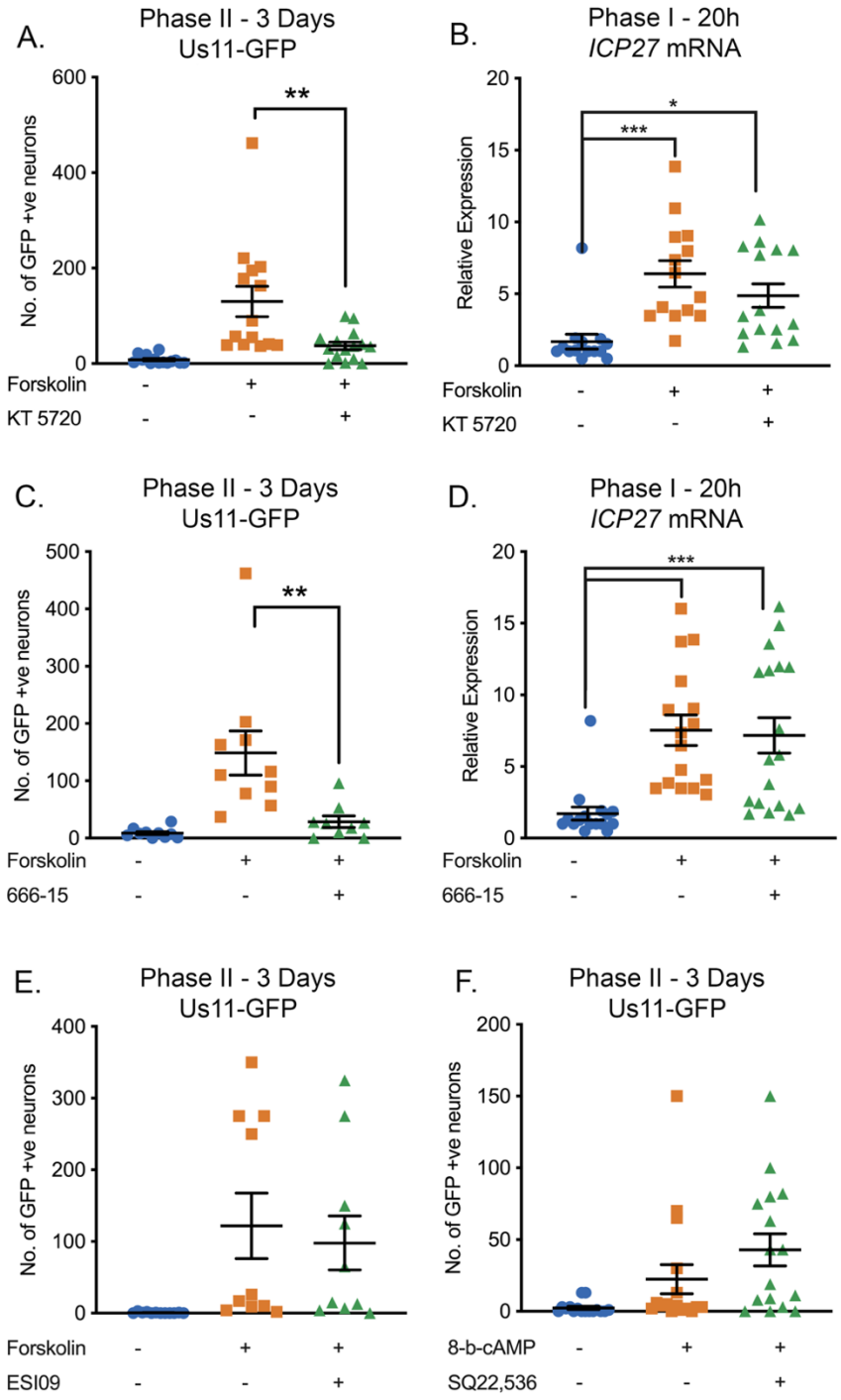


**Figure S2. Effect of PKA, CREB, Rapgef2 and EPAC Inhibition on HSV Reactivation.** (A)Latently infected cultures were reactivated with forskolin (60 μM) in the presence of the PKA inhibitor KT 5720 (3 μM) and the number of Us11-GFP positive neurons quantified at 3 days post-reactivation. (B) RT-qPCR for the viral lytic transcript ICP27 at 20h post-forskolin treatment and in the presence of KT 5720. (C) Latently infected cultures were reactivated with forskolin in the presence of the CREB inhibitor 666-15 (2 μM). (D) RT-qPCR for ICP27 at20h post-forskolin treatment and in the presence of 666-15. (E) Latently infected cultures were reactivated with forskolin (60μM) in the presence of the EPAC inhibitor ESI09 (10 μM). (F) Latently infected cultures were reactivated with 8-Bromo-cAMP (125μM) in the presence of the Rapgef2 inhibitor SQ22,536 (50 μM). Individual experimental replicates are represented. Statistical comparisons were made using a one-way ANOVA with a Tukey' s multiple comparison. *P<0.05, ** P<0.01, ***P<0.001. Performed by Austin Schinlever.

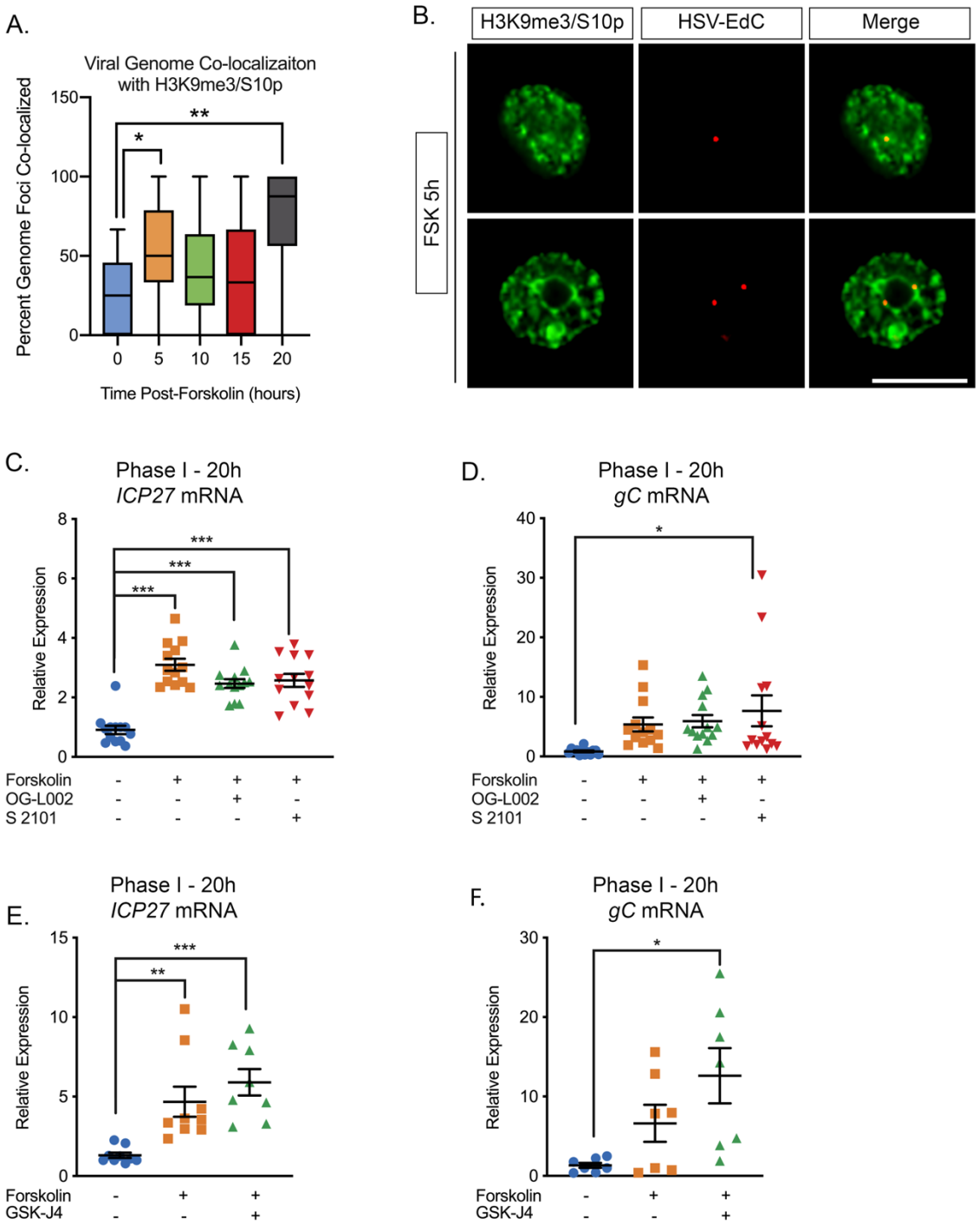
A.
Viral Genome Co-localizaiton with H3K9me3/S10p
Percent Genome Foci Co-localized
150
100
50
0
*
**
0
5
10
15
20
Time Post-Forskolin (hours)
B.
H3K9me3/S10p
HSV-EdC
Merge
FSK 5h
C.
Phase I - 20h
ICP27 mRNA
Relative Expression
8
6
4
2
0
***
***
***
Forskolin - + + +
OG-L002 - - + -
S 2101 - - - +
D.
Phase I - 20h
gC mRNA
Relative Expression
40
30
20
10
0
*
Forskolin - + + +
OG-L002 - - + -
S 2101 - - - +
E.
Phase I - 20h
ICP27 mRNA
Relative Expression
15
10
5
0
***
**
Forskolin - + +
GSK-J4 - - +
F.
Phase I - 20h
gC mRNA
Relative Expression
30
20
10
0
*
Forskolin - + +
GSK-J4 - - +

**Figure 3. The Initial Wave of Viral Lytic Gene Expression During Forskolin-mediated Reactivation is Independent on Histone Demethylase Activity.** (A) Quantification of the percentage of genome foci stained using click-chemistry that co-localize with H3K9me3/S10p. At least 15 fields of view with 1-8 genomes per field of view were blindly scored from two independent experiments. Data are plotted around the median, with the boxes representing the 25th-75th percentiles and the whiskers the 1st-99th percentiles. (B) Representative images of click-chemistry based staining of HSV-EdC genomes and H3K9me3/S10p staining at 5h post-forskolin treatment. (C and D). Effect of the LSD1 inhibitors OG-L002 and S 2101 on forskolin-mediated Phase I of reactivation determined by RT-qPCR for ICP27 (C) and gC (D) viral lytic transcripts at 20h post-forskolin treatment and in the presence of 15μM OG-L002 and 20μM S 2102. (E) Effect of the JMJD3 and UTX inhibitor GSK-J4 (2μM) on forskolin-mediated Phase I measured by RT-qPCR for viral lytic transcripts ICP27 (E) and gC (F) at 20h post-forskolin treatment and in the presence of GSK-J4. For C-F each experimental replicate along with the mean and SEM is represented. (C-F). Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison. *P<0.05, ** P<0.01, ***P<0.001.

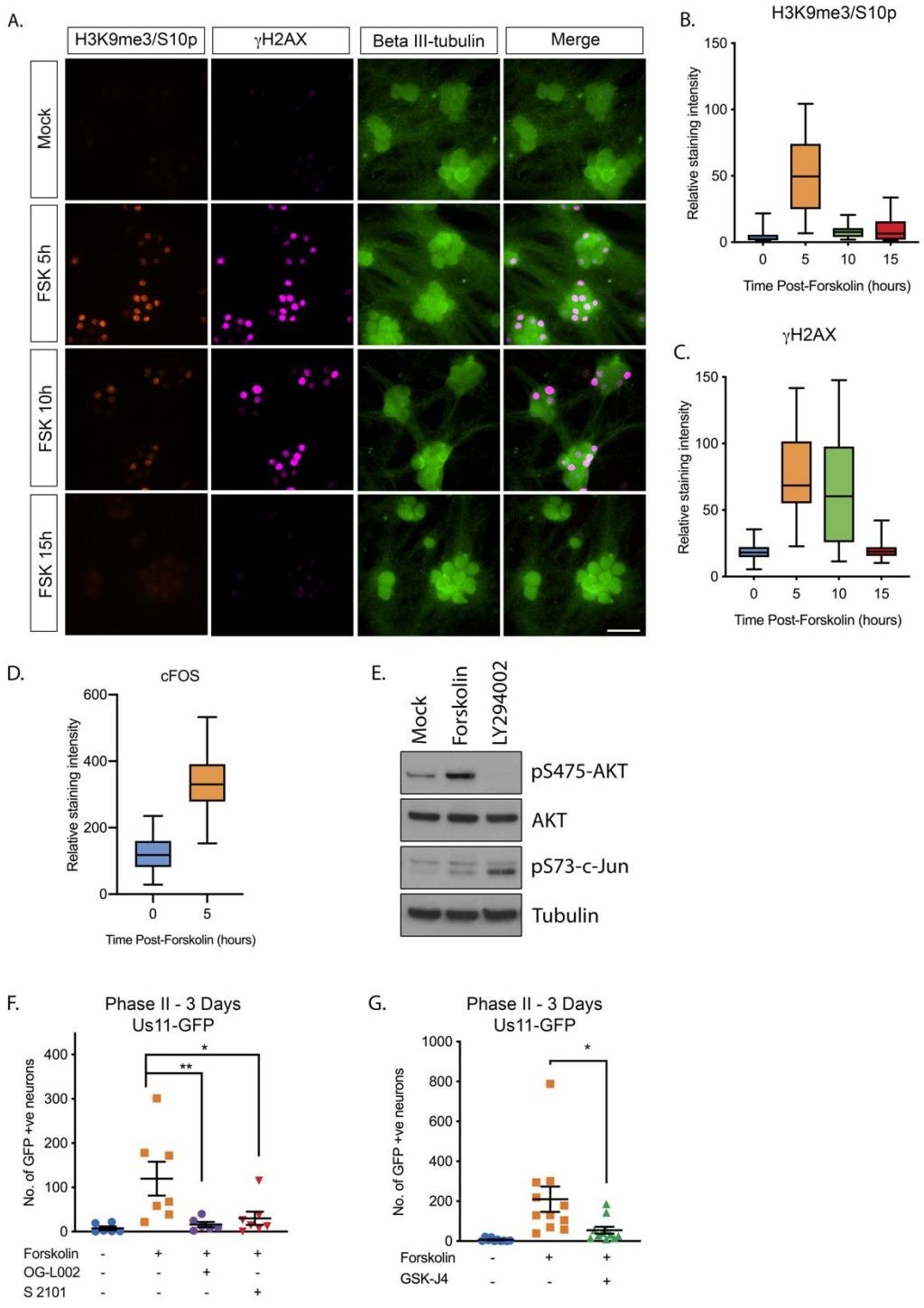
A.
H3K9me3/S10p
γH2AX
Beta III-tubulin
Merge
Mock
FSK 5h
FSK 10h
FSK 15h
B.
H3K9me3/S10p
Relative staining intensity
Time Post-Forskolin (hours)
C.
γH2AX
Relative staining intensity
Time Post-Forskolin (hours)
D.
cFOS
Relative staining intensity
Time Post-Forskolin (hours)
E.
Mock
Forskolin
LY294002
pS475-AKT
AKT
pS73-c-Jun
Tubulin
F.
Phase II - 3 Days
Us11-GFP
No. of GFP +ve neurons
Forskolin
OG-L002
S 2101
G.
Phase II - 3 Days
Us11-GFP
No. of GFP +ve neurons
Forskolin
GSK-J4

**Figure S3. Forskolin Induces Hyperexcitability-Associated Chromatin Changes, and HSV Reactivation that Requires Histone Demethylase (Supplement to Figure 3).** (A) SCG neurons were treated with forskolin and immunofluorescence staining was carried out for H3K9me3/S10p, the DNA damage marker γH2AX and the neuronal marker beta III-tubulin. (B) Quantification of neuronal nuclear staining intensity for H3K9me3 (>150 cells/condition). (C) Quantification of neuronal nuclear staining for γH2AX. In B and C data are plotted around the median and whiskers represent the 2.5-97.5 percentile range. (D). Western blotting for pS475-AKT, total AKT, pS73-c-Jun and tubulin at 15h post-treatment with the PI3-kinase inhibitor LY294002 (20μM) or forskolin (60 μM) (E). Effect of the LSD1 inhibitors OG-L002 (15μM) and S 2101 (20μM) on forskolin-mediated reactivation measured by Us11-GFP positive neurons (F). Effect of the JMJD3 and UTX inhibitor GSK-J4 (2μM) on forskolin-mediated reactivation measured by Us11-GFP positive neurons (G). Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison. *P<0.05, ** P<0.01, ***P<0.001 (E, F). In E and F individual experimental replicates are shown along with the mean and SEM.

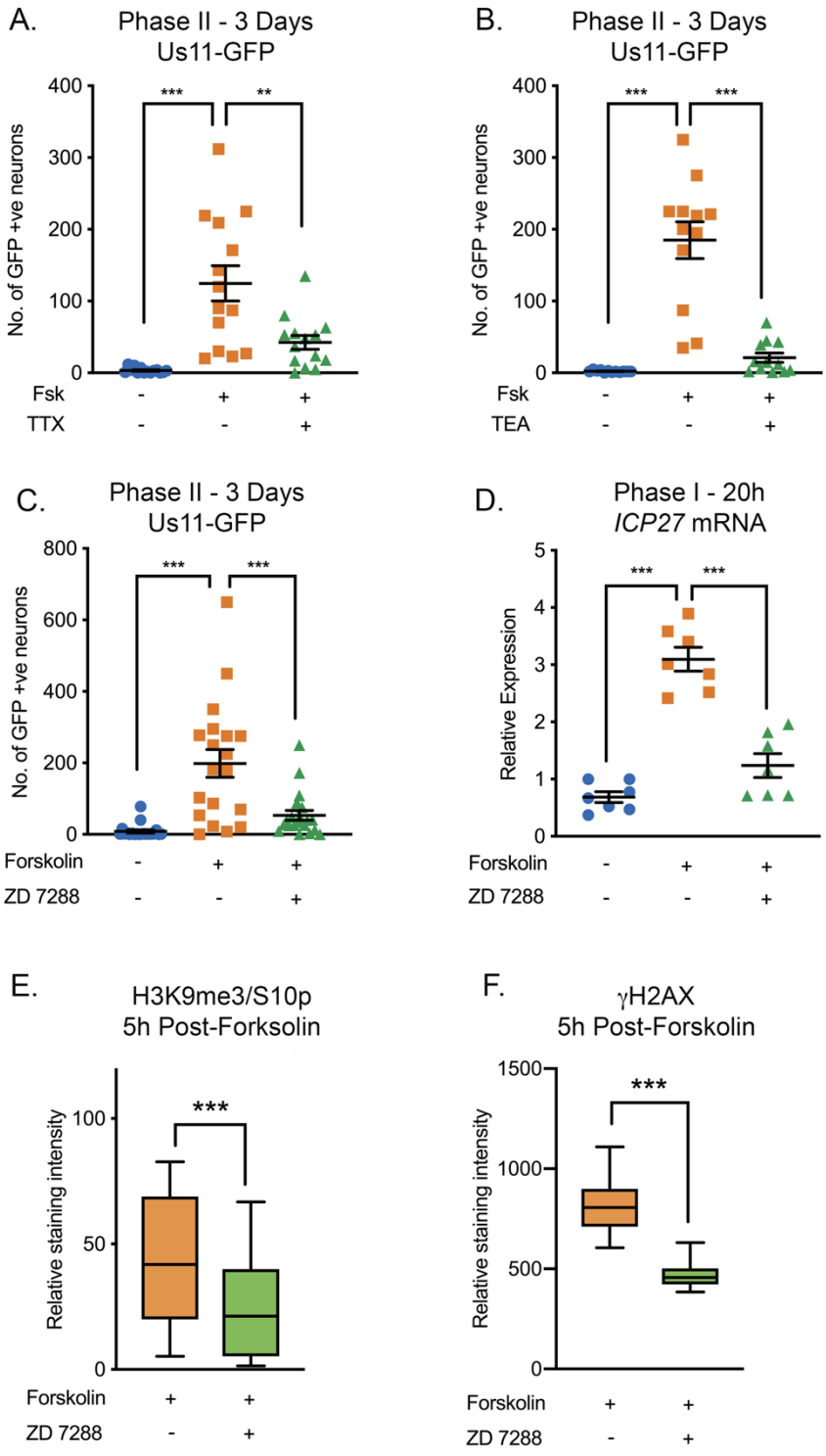
A.
Phase II - 3 Days
Us11-GFP
No. of GFP +ve neurons
400
300
200
100
0
***
**
Fsk - + +
TTX - - +
B.
Phase II - 3 Days
Us11-GFP
No. of GFP +ve neurons
400
300
200
100
0
***
***
Fsk - + +
TEA - - +
C.
Phase II - 3 Days
Us11-GFP
No. of GFP +ve neurons
800
600
400
200
0
***
***
Forskolin - + +
ZD 7288 - - +
D.
Phase I - 20h
ICP27 mRNA
Relative Expression
5
4
3
2
1
0
***
***
Forskolin - + +
ZD 7288 - - +
E.
H3K9me3/S10p
5h Post-Forksolin
Relative staining intensity
100
50
0
***
Forskolin + +
ZD 7288 - +
F.
γH2AX
5h Post-Forskolin
Relative staining intensity
1500
1000
500
0
***
Forskolin + +
ZD 7288 - +

**Figure 4. HSV Reactivation Mediated by Forskolin Requires Neuronal Excitability.** (A) Latently infected cultures were reactivated with forskolin in the presence of the voltage-gated sodium channel blocker tetrodotoxin (TTX; 1μM) and the number of Us11-GFP positive neurons quantified at 3 days post-reactivation. (B) Latently infected cultures were reactivated with forskolin in the presence of the voltage-gated potassium channel blocker tetraethylammonium (TEA; 10 mM) and the number of Us11-GFP positive neurons quantified at 3 days post-reactivation. (C) Forskolin-mediated reactivation in the presence of the HCN channel blockers ZD 7288 (10□M) quantified as the numbers of Us11-GFP positive neurons at 3 days post-reactivation. (D) The effect of ZD 7288 on the HSV lytic gene transcript ICP27 during Phase I reactivation measured at 20h post-forskolin treatment by RT-qPCR. In A-D individual experimental replicates are represented along with the mean and SEM. (E and F) Quantification of the relative nuclear staining for H3K9me3/S10p and □H2AX in SCG neurons at 5h post-forskolin treatment and in the presence of ZD 7288 from >800 cells/condition from two independent experiments. Data are plotted around the mean, with the boxes representing the 25th-75th percentiles and the whiskers the 5st-95th percentiles. Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison (A-D) or two-tailed unpaired t-test (E-F). *P<0.05, ** P<0.01, ***P<0.001. In A-D individual experimental replicates are represented.

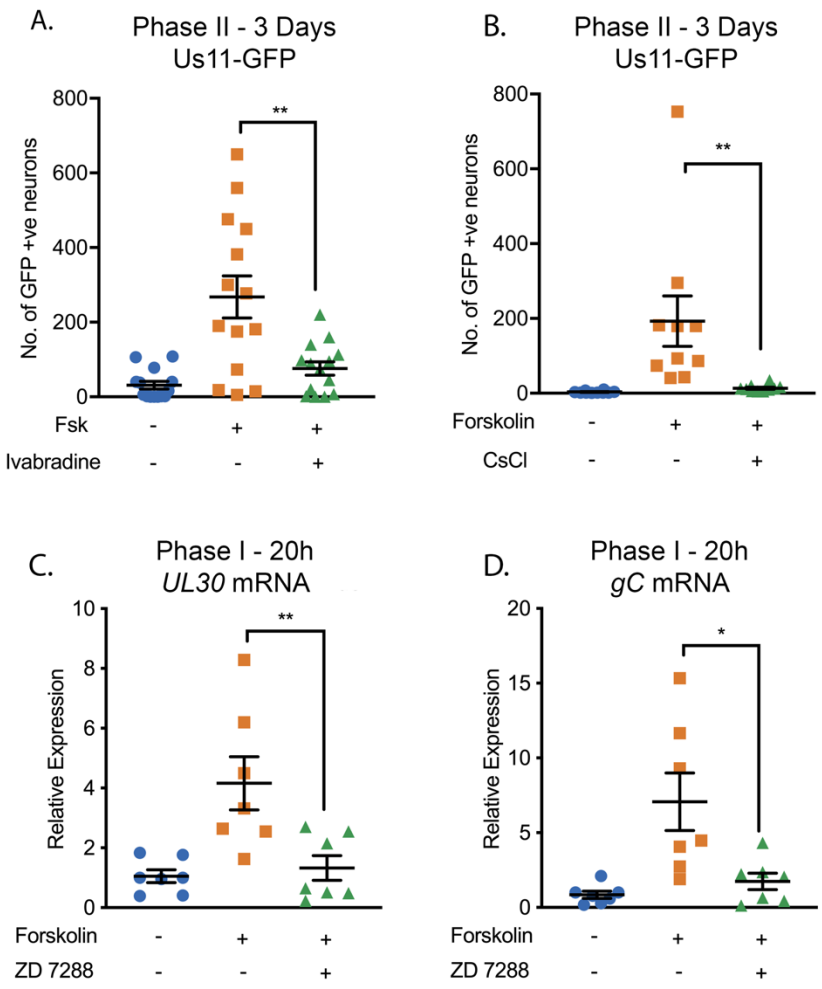


**Figure S4. HSV Reactivation Mediated by Forskolin Requires Neuronal Excitability** (Supplement to Figure 4). (A and B) Latently infected cultures were reactivated with forskolin in the presence of the HCN channel inhibitors ivabradine (20μM; A) and CsCl (3mM; B). Latently infected cultures were reactivated with forskolin in the presence of the HCN inhibitor ZD 7288 (10 μM) and viral lytic transcripts measured at 20h post-reactivation (C and D). Individual experimental replicates are represented in addition to the mean and SEM. Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison. *P<0.05, ** P<0.01.

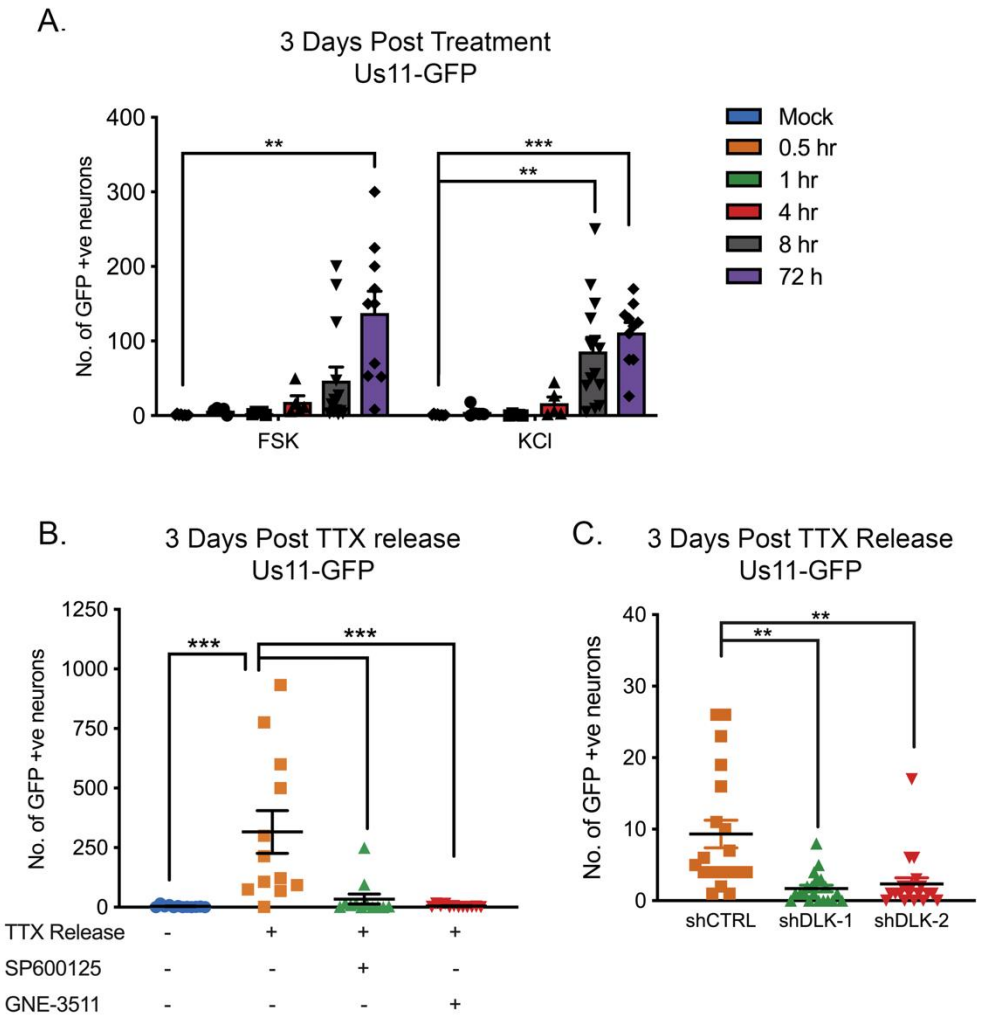


**Figure 5. HSV Reactivation Triggered by Prolonged Neuronal Hyperexcitability is DLK/JNK Dependent.** (A) Latently infected SCG cultures were treated with forskolin or KCl (55mM) for the indicated times followed by wash-out. Reactivation was quantified by number of Us11-GFP positive neurons at 3 days after the initial stimulus was added. (B) Latently infected neurons were placed in tetrodotoxin (TTX; 1μM) for 2 days and the TTX was then washed out. At the time of wash-out the JNK inhibitor SP600125 (20μM) or DLK inhibitor GNE-3511 (4μM) was added. (C) Latently infected neurons were transduced with either control non-targeting shRNA or shRNA targeting DLK for 3 days, then placed in tetrodotoxin (TTX; 1μM) for 2 days and the TTX was then washed out. Reactivation was quantified at 3-days post-wash-out. Individual experimental replicates, the mean and SEMs are represented. Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison. **P<0.01, *** P<0.001.

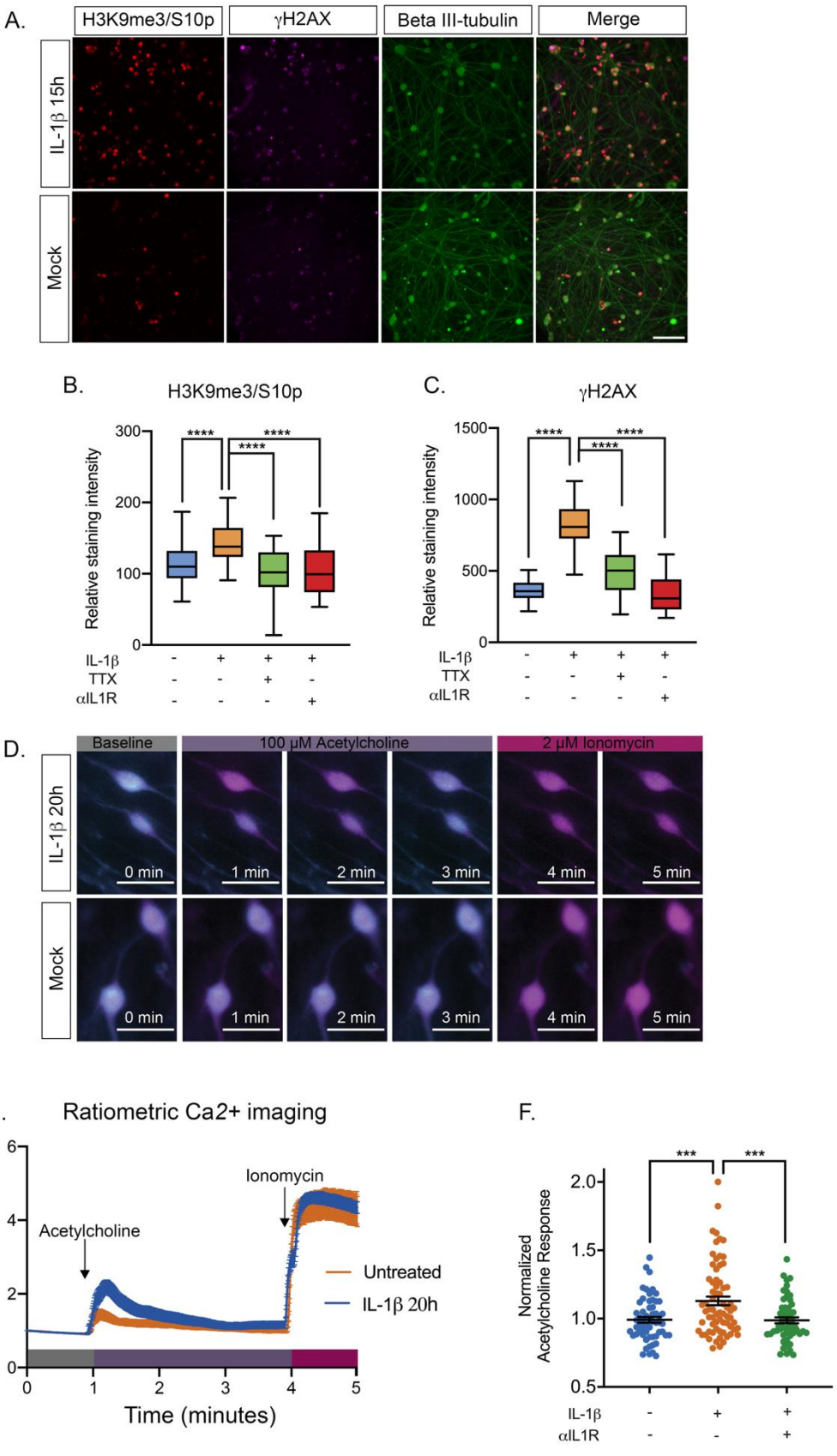
A.
H3K9me3/S10p
γH2AX
Beta III-tubulin
Merge
IL-1β 15h
Mock
B.
H3K9me3/S10p
Relative staining intensity
300
200
100
0
****
****
****
IL-1β - + + +
TTX - - + -
αIL1R - - - +
C.
γH2AX
Relative staining intensity
1500
1000
500
0
****
****
****
IL-1β - + + +
TTX - - + -
αIL1R - - - +
D.
Baseline
100 μM Acetylcholine
2 μM Ionomycin
IL-1β 20h
Mock
0 min
1 min
2 min
3 min
4 min
5 min
E.
Ratiometric Ca2+ imaging
ΔF/F0 Cytosolic Ca2+
Acetylcholine
Ionomycin
Untreated
IL-1β 20h
Time (minutes)
F.
Normalized Acetylcholine Response
***
***
IL-1β - + +
αIL1R - - +

**Figure 6. IL-1β Treatment of Sympathetic Neurons Results in Changes Consistent with Heightened Neuronal Excitability.** (A) Adult P36 SCG neurons were treated with IL-1β (30ng/mL) for 15 hrs and stained for H3K9me3/S10p, γH2AX and beta II-tubulin to mark neurons. (B & C) Quantification of the intensity of H3K9me3/S10p (B) and γH2AX (C) staining following 15 of IL-1β treatment and in the presence of tetrodotoxin (TTX; 1μM) or anti-IL1 receptor (IL-1R) blocking antibody (2μg/ml). Data are plotted around the median and whiskers represent the 5-95$^{th}$ percentiles. (D) Representative images of cytosolic $Ca^{2+}$ elevations measured using Fura-2-AM in neurons stimulated with 100 μM acetylcholine either pre-treated with IL-1β for 20 hours or mock treated. As a control the neurons were also treated with Ionomycin at the end of the protocol. Bar=100 μm. (E) Representative experiment for cytosolic $Ca^{2+}$ elevations in neurons stimulated with 100 μM acetylcholine. Cells were pretreated with IL-1β or vehicle for 20 hours prior to imaging. The plotted values were calculated as a change in fluorescence/initial fluorescence (ΔF/F0). Error bars represent SEM (IL-1β treatment, n=58 cells and vehicle control, n=25 cells) (F) Peak cytosolic $Ca^{2+}$ elevations normalized to untreated controls in neurons stimulated with 100 μM acetylcholine. Cells were pretreated with IL-1β (n= 70, wells) or vehicle (n=58, wells) for 20 hours prior to imaging. IL-1R blocking antibody (n=54, wells) was also added. Data points represent individual wells, horizontal line represents mean. Statistical comparisons were made using a one-way ANOVA with a Tukey's multiple comparison (B-D). *** P<0.001 ****P<0.0001.

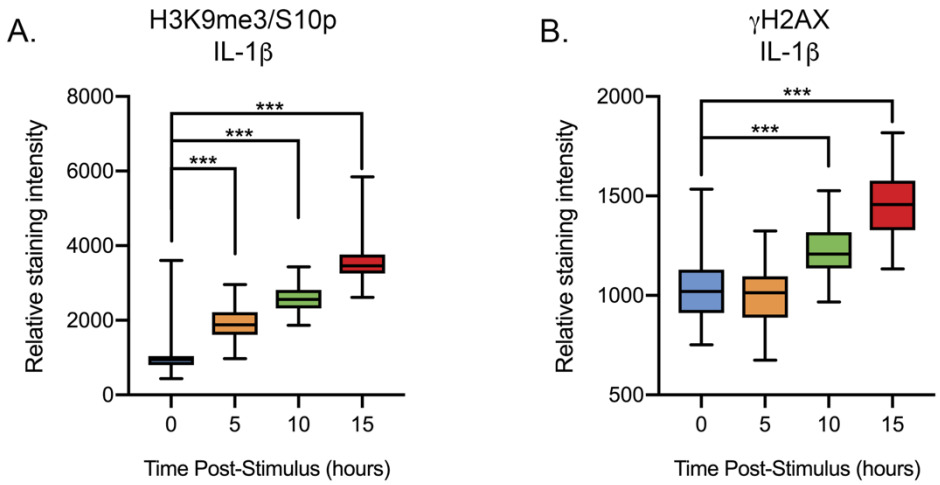


**Figure S5. IL-1β Treatment of Mature SCG Neurons Induces Excitability-Associated Histone Post-Translational Modifications (Supplement to Figure 6).** Quantification of the nuclear staining intensity in P36 sympathetic neurons for H3K9me3/S10 (A) and γH2AX (B) following treatment with IL-1β (30ng/mL) from 150 nuclei from two independent experiments. Data are plotted around the median and whiskers represent the 5-95$^{th}$ percentile.

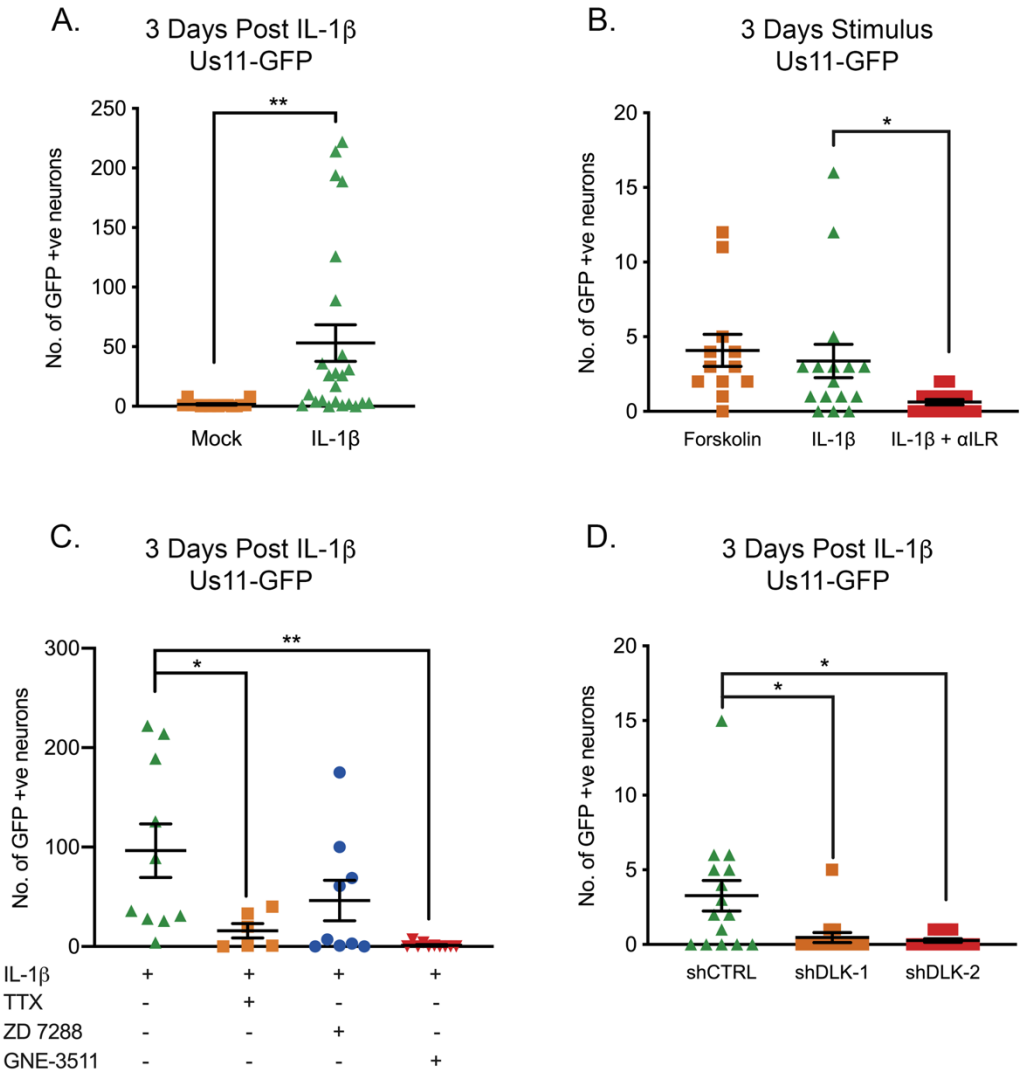


**Figure 7. IL-1β-Induced HSV Reactivation is Linked to Heightened Neuronal Excitability and DLK Activation.** (A). Quantification of Us11-GFP expressing neurons following addition of IL-1β to latently infected cultures of mature SCG neurons. (B) Numbers of Us11-GFP positive neurons following addition of forskolin or IL-1β to mature SCG neurons, and in the presence of an IL-1R-blocking antibody (2μg/ml). (C) Quantification of IL-1β induced reactivation in the presence of the voltage gated sodium channel blocker TTX (1μM), the HCN channel blocker ZD 7288 (10μM) and the DLK inhibitor GNE-3511 (4μM). (D) Latently infected SCG neurons were transduced with an shRNA control lentivirus or lentiviruses expressing shRNA against DLK. Three days later IL-1β was added to the cultures and the numbers of GFP-positive neurons quantified at 3-days later. Individual experimental replicates, means and SEMs are represented. Statistical comparisons were made using two-tailed unpaired t-test (A) or a one-way ANOVA with a Tukey's multiple comparison (B-D). *P<0.05, **P<0.01.

*End of Chapter 2*

# *Chapter 3*

# The Intersection of Innate Immune Pathways with the Latent Herpes Simplex Virus Genome

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*Parts of this chapter have been adapted from:*
Cuddy SR & Cliffe AR. The Intersection of Innate Immune Pathways with the Latent Herpes Simplex Virus Genome. Journal of Virology *in press* (2023)

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## Abstract

Innate immune responses can impact different stages of viral life cycles. Herpes simplex virus latent infection of neurons and subsequent reactivation provides a unique context for immune responses to intersect with different stages of infection. Here we discuss recent findings linking neuronal innate immune pathways with modulation of latent infection, acting at the time of reactivation and during initial neuronal infection to have a long-term impact on the ability of the virus to reactivate.

## Infection of neurons in the context of acute replication at the body surface

Herpes simplex virus-1 (HSV) is a ubiquitous human pathogen that establishes a latent infection in host peripheral nervous system (PNS) neurons. Host infection typically begins at the mucosal epithelia, in which the virus lytically replicates following an ordered cascade of immediate-early (IE), early (E), and subsequently late (L) gene expression that is stimulated by DNA replication, and ultimately infectious virus is produced. Following lytic replication at the mucosal epithelial layer, HSV infects the innervating distal axons of PNS neurons. Within these neurons, the viral capsid is trafficked to the neuronal cell body and the HSV genome enters the nucleus. HSV infection of PNS neurons can result in a range of outcomes, from proliferative lytic replication to the establishment of a latent infection. Once a latent infection is established, the viral DNA genome associates with cellular histone proteins to generate a heterochromatinized extrachromosomal episome [54,62,65,68]. During latency, lytic gene expression is restricted, whereas the *Latency Associated Transcript (LAT)*, a long noncoding RNA, is abundantly expressed [68,69,380].

When HSV gains access to peripheral neurons, it does so in the context of acute infection of the mucosal surface where the virus undergoes productive replication in epithelial cells and fibroblasts. This lytic replication prompts a robust innate immune response from both the infected cells and professional immune cells. *In vitro* and *in vivo* models of HSV infection, as well as clinical samples taken from patients (although largely samples from recurrent lesions), have demonstrated that an extensive array of cytokines are released in tissue during mucosal lytic infection, including interferon α/β

(IFNα/β), interferon γ (IFNγ), and inflammatory cytokines [381]. The response to these innate cytokines by neurons infected with HSV likely impacts the outcome of infection. However, for many of these cytokines, it is unknown how they impact neuronal HSV infection.

There is a strong rationale for understanding how neuronal innate immune pathways affect HSV productive replication as well as the establishment of and reactivation from latency. As highly specialized post-mitotic cells, the peripheral neuronal response during HSV infection is markedly unique. Some studies have suggested that neurons have a muted inflammatory response to key innate immune cytokines like type I IFN [123,126,130]. Whereas type I IFN signaling promotes cell death in many cell types, in neurons type I IFN has the opposite effect. This difference may be attributed to the distinct needs of a non-replenishing cell type [277,382]. Although the neuronal response to viral infection is often subdued compared to other cell types, neurons can sense and respond to immunostimulatory molecules, including inflammatory molecules, such as damage associated molecular patterns (DAMPs) and interleukins [383–385]. *Ifnar1*, the predominant type I IFN receptor subunit, is expressed in over 90% of neurons within peripheral dorsal root ganglia [386]. Inflammatory cytokines as well as type I & II IFNs can be released from epithelial cells in tissues innervated by axonal termini, myelinating Schwann cells or tissue-resident immune cells along the distal axon, or satellite glia around the soma [344,387,388]. Given the abundance of immune signaling molecules and the differential response to infection even between neuronal subtypes, it is likely that these molecules contribute to heterogeneity in HSV latency.

This heterogeneity during HSV latency is illustrated by the different levels of *LAT* expression, evidence of lytic gene expression prior to latency [389–391], the subnuclear distribution of viral genomes, and ultimately the ability of individual genomes within a neuron to reactivate [57,84,114,115,392–397]. The lifelong persistence and highly polarized morphology of neurons, diversity of neuronal types and subtypes, and varied exogenous insults have made delineating neuronal innate immune pathways in HSV infection difficult thus far.

## Effects of cytokines on HSV lytic replication in neurons

The exposure of neurons to different cytokines during initial infection of neurons suggests a potential role for innate immunity in modulating the outcome of HSV infection. Cytokines can induce different responses in different cell types, with terminally differentiated cells like neurons likely displaying divergent responses compared to mitotic cells. Neuronal infection with HSV, therefore, presents itself as an exceptional tool for understanding neuron-specific innate immune responses during both acute and prolonged infections. In addition, the extensive cellular structure of neurons means that acute HSV infection of neurons occurs across various tissues and cell types, from epithelial and immune cells at the skin surface to Schwann cells, satellite cells and infiltrating immune cells within peripheral ganglia [398–401]. Throughout both the mucosal epithelia and, importantly, infected PNS ganglia, acute infection is associated with increased inflammatory cytokines, including various interleukins (IL-1, IL-2, IL-4, IL-6, IL-10), tumor necrosis factor α (TNFα), and type I and type II IFNs [344,381,402]. Elevation of these cytokines can last for months following *in vivo* infection and is associated with

CD8+ cells that persist within infected ganglia, even in the absence of viral replication [403–405], presumably in response to limited HSV antigen expression during latency [406]. Neurons themselves, however, produce low levels of type I IFN during HSV infection [277]. Therefore, cytokine responses both systemically and within ganglia during acute infection have the potential to affect the initial outcome of HSV neuronal infection and likely play a role in modulating latent infection and reactivation.

Perhaps the most widely studied cytokines are the type I IFNs. Type I IFNs can be released from most cell types, with certain neuronal subtypes considered notable exceptions. Type I IFN expression occurs following the sensing of foreign or misplaced host nucleic acids, including viral genomes, as well as other pathogen-associated molecular patterns (PAMPs). Type I IFNs are then released and activate the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathways that mediate transcription of interferon-stimulated genes (ISGs) and activation of cellular processes that restrict viral replication in neighboring cells [407]. Studies investigating type I IFNs in the context of HSV neuronal infection have revealed that they can limit productive replication, promote latency establishment, and restrict reactivation [92,98].

Intriguingly, an inability to restrict viral replication in the forebrain of certain patients with herpes simplex encephalitis (HSE) has been linked to deficiencies in the toll-like receptor 3 (TLR3)-dependent type I IFN responses [408]. This demonstrates that type I IFN-dependent mechanisms are critical to the human response to HSV infection. In studies using sensory and central nervous system (CNS) differentiated neurons from

patient-derived induced pluripotent stem cells, the requirement for the TLR3 signaling pathway is both neuron-intrinsic and specific to CNS neurons, but not sensory neurons [408]. This subtype variability has been attributed to TLR3-dependent basal type I IFN release in CNS neurons. Although these studies come with the slight caveat of somewhat artificial *in vitro* differentiation protocols, they highlight potential neuronal subtype-dependent responses.

In addition to having differential roles in different neuronal subtypes, the neuronal response to IFN differs depending on whether IFN is sensed in the distal axon or soma [409], although both are sufficient to activate the JAK-STAT pathway and subsequently restrict viral replication during axonal HSV infection [409,410]. However, only type I IFN added to the soma itself can restrict HSV infection in the soma [410]. Intriguingly, following axon-specific type I IFN treatment, levels of phosphorylated STAT1 increase but there is no nuclear translocation of STAT, therefore indicating an axon-specific function that does not depend on the induction of ISG transcription. Instead, axon-specific type I IFN treatment induces local translation in axons [409]. Therefore, neurons have the potential to activate distinct responses depending on whether they receive type I IFN from the periphery or ganglia. Moreover, both type I and II IFNs induce a unique set of ISG expression that appears to be neuron-specific [92]. Together, these studies indicate that neurons have a tightly-controlled and tailored response to infection that likely considers their unique morphology and non-replicating nature. Given that *in vivo* and *de novo* infection occurs at the distal axon and reactivation initiates in the soma, the mechanistic outcome of type I IFN responses during initial, acute infection may differ from those

activated during reactivation. The precise impact of timing and localization of IFN responses on entry into latent infection and reactivation are currently undefined.

The full mechanistic role of type I IFN in limiting HSV lytic replication is unknown. Both HSV infection and type I IFN induce a selective antiviral autophagy and autophagic clustering in peripheral neurons and autophagy is considered to be the primary neuronal defense against HSV infection [260,277,411–414]. These autophagic clusters form almost exclusively in neurons that lack viral proteins while retaining viral genomes [413], a state reminiscent of latency. A conserved autophagy and ubiquitin-dependent receptor, optineuron (OPTN), similarly restricts expression of HSV proteins required for lytic gene expression, namely VP16, which are also targets of autophagy [415]. Following axonal infection, VP16 is trafficked as a tegument protein to the nucleus where it can then act as a viral transactivator [416,417]. VP16 has been found to regulate entry into productive neuronal infection *in vivo* [395] and the absence of nuclear VP16 in neurons, especially following axon-specific infection, is linked to decreased lytic gene expression, thereby promoting entry into a latent infection [394,417]. This raises the possibility that OPTN-mediated autophagy restricts the amount of VP16 reaching the neuronal nucleus, which in turn could affect whether the infection becomes productive or latent. However, whether OPTN is linked to autophagy in response to type I IFN is unclear and it is unknown whether OPTN can degrade incoming axonal VP16 before it reaches the nucleus. Precisely how the timing, localization, and viral effectors affect prolonged and latent neuronal HSV infection continues to represent an exciting and pressing question in the field.

The extent to which type I IFNs promote the establishment of latency is also not well understood. During HSV infection, treatment of peripheral neurons with type I IFN infection is sufficient to induce a latent infection and can support the establishment of latency *in vitro* [98,418–420]. A study from our lab showed that type I IFN did not impact latency establishment, but this study included the antiviral drug acyclovir that may lessen the requirement for type I IFN in promoting latency. Moreover, *in vivo* mutations of STAT1 in neurons demonstrated that HSV can establish a latent infection even in the absence of a functional neuronal IFN response [421]. However, fewer viral genomes were found to reside within ganglia during latency in these mutants [421]. While IFN signaling may support the establishment of a larger latent pool that can later reactivate, possibly by supporting neuronal survival during initial infection, it is likely that multiple factors during neuronal infection, in addition to type I IFN, stimulate a neuronal response that facilitates the establishment of latency [418,419]. Reactivation *in vivo* is more likely to occur in the context where there are a high number of neurons latently infected [422]. Therefore, it is possible that while IFN maintains its antiviral purpose, it may also support a larger accumulation of latent genomes to reactivate at a later time that increases the chances of HSV transmission. However, as noted below, type I IFN also acts to alter the nature of HSV latent infection and prevents reactivation from individual neurons. IFNs clearly represent an important host response that impacts neuronal HSV infection, with nuanced and differential consequences to IFN sensing that reflect the complex morphology and differentiation state of neurons, in addition to the nature of infection itself.

## Cytokine treatment of neurons can restrict reactivation

Progression from a repressive latent infection to the initiation of viral gene transcription in reactivation relies on activation of host pathways. The reactivation stimuli studied to date have been found to converge on the neuronal stress pathway involving Dual Leucine Zipper Kinase (DLK) mediated activation of c-Jun N-terminal kinase (JNK), which is also involved in the downregulation of mTOR (mammalian target of rapamycin) activity [423]. JNK-mediated reactivation is biphasic [54,57,82,84,85,87], beginning with an initial Phase I that is associated with a stochastic burst of lytic transcripts from all three gene classes (IE, E, and L), which is independent of viral DNA replication. Following Phase I, Phase II viral gene expression appears to follow the cascade of viral gene expression observed in lytic replication, where late gene expression is dependent on viral DNA replication to culminate in the release of infectious virus [54,84]. Phase II, unlike Phase I gene expression, requires the lytic transactivator VP16 and the activity of histone demethylase enzymes that remove methylated lysine residues [24,54,57,84,87,118]. Studies using *in vivo* models of HSV reactivation have found that cytokines maintain latency and/or prevent reactivation from occurring. Although not an innate inflammatory cytokine, a role for IFNγ release from CD8+ T cells within trigeminal ganglia in the maintenance of latency *in vivo* highlights the ability of cytokines to limit HSV reactivation [403]. Additional cytokines associated with immune cell infiltration, such as TNFα, CXCL10, and CCL5, are persistently found within latently infected ganglia in humans and potentially support the repression of reactivation [424].

The use of *in vitro* model systems of HSV latency and reactivation has permitted investigation into the effects of type I IFN and the adaptive immune response associated with type II IFN, specifically on reactivating neurons. Importantly, both cytokine types can act to prevent entry into Phase I of reactivation via mechanisms that are unknown [92,425,426]. In contrast, both cytokines fail to prevent reactivation if added after Phase I has already occurred [92]. The mechanistic role for type I and II IFNs in preventing Phase I reactivation are unknown but clearly warrant further investigation, especially as they could serve therapeutically to prevent entry into the earliest stage of reactivation. In addition, as discussed below, whether they have long-term effects on the later ability of viral genomes to reactivate will be especially important to elucidate. The inability of type I and II IFNs to prevent reactivation during Phase II could result from either the accumulation of viral proteins in Phase II that may either inhibit the downstream effects of interferon or instead relate to the mechanistic role of viral proteins in inhibiting gene expression. For example, type I or II IFN could interfere with host signaling pathways that mediate Phase I gene expression and/or act specifically on viral chromatin during Phase I, which is later remodeled when the virus enters Phase II. Overall, IFNs represent a comprehensive system to continually and actively repress lytic gene transcription throughout latent HSV infection of neurons, though much remains unknown how precisely IFN signaling ultimately interacts with the viral genome to limit reactivation.

## Impact of neuronal innate immune memory on HSV latent infection

Given the milieu of innate immune signaling molecules present during HSV infection at the mucosa and ganglia, these molecules likely affect the nature of neuronal infection by HSV. There is evidence that HSV latent infection is heterogenous, with different copy numbers of viral genomes per neuron, altered expression levels of the *LAT* between neurons, and different subnuclear localization patterns of the viral genome [64,115,393,422,427–429]. Similar heterogeneity exists in the ability of viral genomes to reactivate, with only a subpopulation of neurons containing reactivating genomes after a specific stimulus [57,84,87,395–397]. This heterogeneity may result from intrinsic differences in neurons themselves, the outcomes of neuronal responses to reactivation stimuli, or the chromatin state of the latent viral genomes. It is especially interesting and clinically relevant to investigate what the different chromatin states of individual viral genomes are, what constitutes a state more likely of reactivating, and how different types of chromatin form on the latent genome. It is tempting to speculate that the latent HSV genome may have a memory of a previous immune environment that results in different types of chromatin on the genome and an altered ability to reactivate. Innate immune memory is a concept that is most often associated with the adaptive immune responses. However, there is accumulating evidence that cells possess memory of previous innate immune stimulation, often resulting in exacerbated responses to subsequent stimuli. Innate immune memory has largely been studied in immune cells, such as monocyte macrophages and natural killer (NK) cells [430]. Given the long-lived nature of neurons, their ability to respond to innate immune cytokines, and the possibility of this cell type

adapt to previous stimulation, it is also likely that neurons retain memory of previous immune stimulation, which could modify the nature of HSV latent infection.

*In vitro* models provide a reductionist approach to HSV latent infection that allows the concept of innate immune memory of latent viral genomes to be investigated. Such models enable neuronal exposure to immune molecules at distinct times during infection, allowing the effects of these molecules on latency and reactivation to be quantified. In a recent study, our lab showed that exposure of neurons to type I interferon solely at the time of HSV infection resulted in a more restricted form of HSV latent infection that was less able to undergo reactivation [115]. Notably, reactivation was restricted even though expression of key ISGs had returned to baseline following initial infection, indicating that IFN signaling was no longer occurring. This *in vitro* model relies on the use of the viral DNA replication inhibitor acyclovir, thereby ensuring that viral genome copy numbers were also equivalent between IFN-treated and untreated neurons. Reactivation was not restricted when neurons were treated with IFN several days prior to infection however, indicating that restriction in this context did not result from changes in the neuronal responses to the reactivation stimulus due to previous IFN exposure [115]. Therefore, the observed effects were due to the inability of viral genomes to express lytic transcripts in response to a reactivation stimulus and indicated a long-term epigenetic change to the viral genomes. In line with these findings, the viral genomes in neurons treated with IFN at the time of infection co-localized with nuclear domains known as promyelocytic leukemia (PML) nuclear bodies (PML-NBs) (which were previously absent from untreated neurons) and depletion of PML prior to infection

restores the ability of viral genomes to reactivate [115]. The impact of immune memory and PML formation in response to cytokine sensing by neurons represents a critical arm of innate immunity that controls HSV infection long past acute infection with appreciable effects on latency and reactivation.

PML-NBs had long been implicated in repressing herpesvirus gene expression [393,431,432], although prior to this study there was no known role for PML-NBs in HSV latent infection. The mechanism of HSV gene repression by PML-NBs is currently not known, although additional heterochromatin associated proteins including DAXX (death domain associated protein) and ATRX (alpha-thalassemia/mental retardation, X-linked) have been implicated [115,433,434]. Moreover, there's evidence that PML-NBs may promote a particular type of heterochromatin via deposition and maintenance of histone H3 lysine 9 tri-methylation (H3K9me3) [347,435,436]. Hence, it is tempting to speculate that IFN pre-treatment results in association of viral genomes with PML-NBs and subsequent increased enrichment with H3K9me3. However, many questions remain as to why these genomes would be more restricted for reactivation. Importantly, this study demonstrated that viral genomes can possess a memory of previous type I IFN exposure. In the future, it will be especially intriguing to determine how other cytokines and innate immune molecules similarly impact the nature of HSV latency and the ability of the viral genome to reactivate.

## Innate immune pathways can promote HSV reactivation

Although many cytokines serve to maintain latency or restrict HSV reactivation, the downstream effect of particular cytokines can vary drastically depending on cell-type and the context of cytokine-sensing. Remarkably, IL-1, a key mediator of the inflammatory response, can directly induce reactivation from latently infected peripheral neurons [87]. Monocytes and macrophages are the primary sources of IL-1beta during infection or stress, but IL-1beta is expressed throughout the body and is released in response to inflammasome activation. Furthermore, IL-1alpha is present at high levels in keratinocytes [377,437] and can be released upon cellular damage, including from exposure to ultraviolet light [374,378]. In contrast to other cell types that induce an inflammatory response or anti-viral responses following IL-1 receptor type 1 (IL-1R1) activation [377], in neurons, IL-1 instead modulates the homeostatic function of neuronal action potential firing, likely to support long-term potentiation and memory formation [142,356,376]. Importantly, IL-1-mediated reactivation was dependent on action potential firing, suggesting that hyperexcitability induced by IL-1 induced HSV reactivation [87]. Additional stimuli that also induce neurons to enter a prolonged hyperactive state, such as forskolin or removal of a tetrodotoxin block, also induce HSV to reactivate from latent infection [87,97]. Therefore, increased action potential firing presents itself as a trigger of HSV reactivation and can be induced by to exposure to inflammatory cytokines such as IL-1.

Mechanistically, both neuronal hyperexcitability and IL-1 treatment result in increased histone phosphorylation, including an increase in histone phosphorylation on

S10 in the context of histone H3 lysine 9 trimethylation (H3K9me3p10) [87]. This same modification, known as a histone methyl/phospho switch, has been detected on viral lytic promoters during Phase I reactivation and is dependent on JNK activation [54]. Therefore, one role for neuronal hyperexcitability in HSV reactivation may be to induce a histone methyl/phospho switch on viral lytic gene promoters. However, it is likely that this alone is not sufficient to induce lytic gene expression and potentially other factors that may be dependent or independent of DLK/JNK signaling are likely required. In addition, there is currently no known mechanistic link between neuronal hyperexcitability and DLK activation. In the context of inflammatory pain, DLK is required for the intrinsic sensory neuron immune response [438] and cytokines are intimately connected with inflammatory pain by mediating nociception [386] as well as altering action potential firing [439]. Therefore, additional physiological and pathological processes may similarly intersect on mechanisms mediated by hyperexcitability that promote HSV reactivation.

Alongside IL-1, IL-6, and TNFα are additional inflammatory cytokines upregulated during reactivation that can promote neuronal hyperexcitability [104,353]. Given the redundant function of many viral proteins and synergistic nature of inflammatory cytokines, it would not be surprising if HSV in fact co-opts multiple immune pathways to facilitate viral gene expression during reactivation. However, conflicting reports about the roles of IL-6 and TNFα in reactivation and the lack of significant data on how IL-6 and TNFα affect intracellular neuronal pathways during latency and reactivation obscure any considerable consensus whether they in fact support HSV reactivation [440–446]. While together these new findings revealed a novel mechanism by which HSV can

manipulate host immune pathways to promote reactivation, significant questions remain about how additional innate immune pathways converge on the viral genome and, importantly, how these pathways seemingly antagonize each other in support of HSV reactivation.

## Conclusions and future directions

These recent advances in HSV virology, immunology, and neuroscience shed light on the complex relationship between innate immunity and HSV reactivation. Given the cell type specific nature of innate immunity, HSV benefits from a delicate balance between the unique type I IFN response and inflammatory cytokine response found only within terminally differentiated and morphologically distinct neurons. In both instances, these signaling molecules support neuronal and host viability while simultaneously promoting HSV persistence, a feature of HSV that is uniquely critical to lifelong latent infections of post-mitotic neurons. While we can speculate about additional proteins and pathways associated with PML-NBs or downstream of IL-1, the synergistic and redundant nature of innate immune pathways likely necessitates virus co-option of additional interacting partners to compensate for or contribute to the innate heterogeneity of neuronal populations and latent infections.

How downstream activation of these host pathways precisely interact with the viral genome to modulate viral gene transcription, especially to modulate the long-term nature of HSV latent infection and during early during reactivation in Phase I, are exciting areas of study that will benefit the field of HSV, epigenetics, and

neuroimmunology alike. The roles of additional host or viral proteins in signaling, transcription, and epigenetic modulation will have to be considered to fully understand this dynamic interaction, including what promotes latency differences between different neurons. Future questions can also address the potential role of intrinsic innate signaling pathways in addition to the extrinsic signaling molecules already described, especially given that IL-1 is associated with intracellular immune signaling molecules such as mitochondrial DNA [291]. When considering therapeutics for HSV infection, an important question will be whether IFN treatment after the establishment of latency or during Phase I significantly contributes to immune memory and affects future reactivation events. The intersection of innate immunity and HSV reactivation is a complex relationship and, in contrast to the notion that viruses serve to primarily inhibit host immunity, HSV has likely evolved nuanced and interesting ways to take advantage the host immune response to increase its ability for transmission.

## Figures

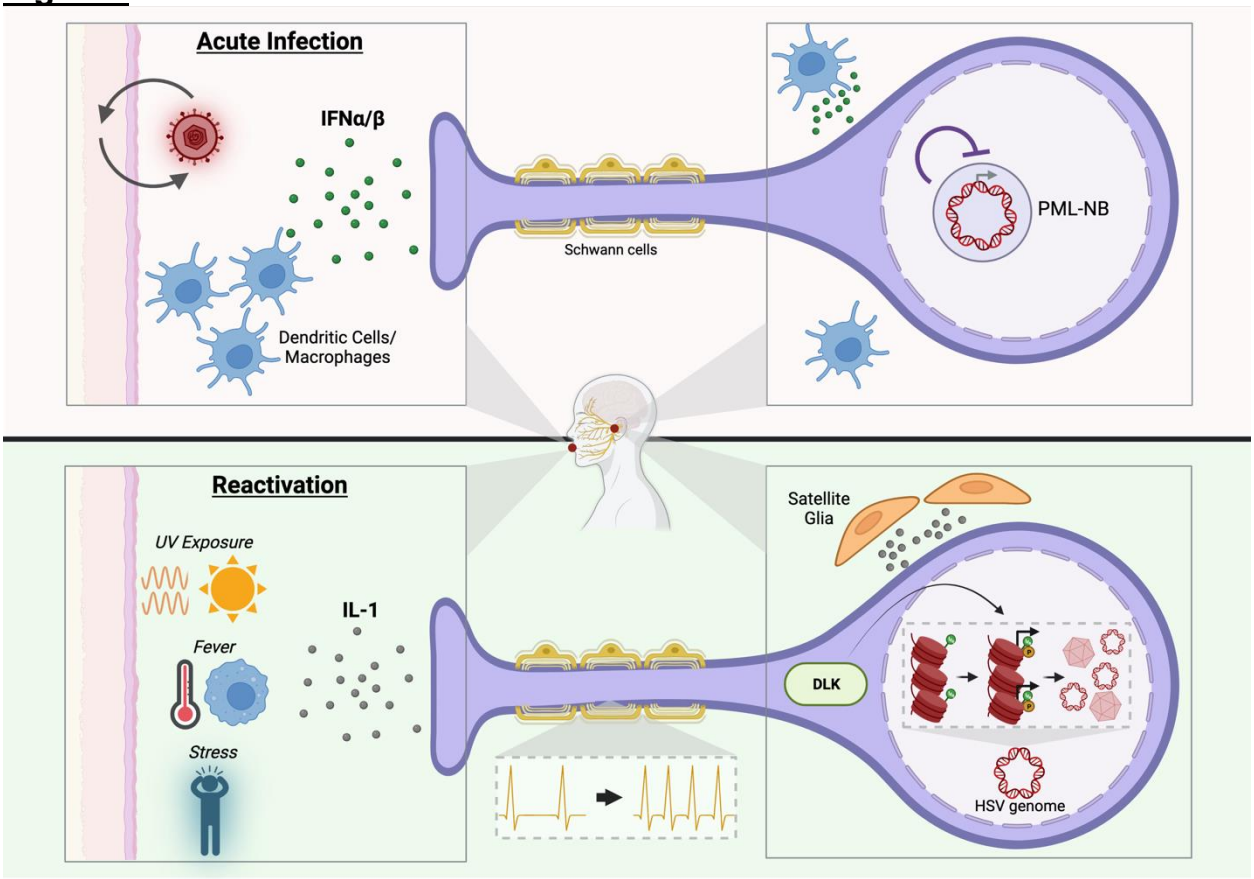


**Fig 1.** *Innate immune modulation of HSV latency and reactivation.* During acute infection, type I IFN is released from infected cells and tissue-resident immune cells at the body surface and ganglia. Top: Type I IFN signaling in neurons as they are infected with HSV promotes viral genome is nucleation at promyelocytic leukemia nuclear bodies (PML-NBs) to establish a restrictive latent infection. Bottom: UV exposure, fever, and stress can induce the release of the inflammatory cytokine interleukin-1 (IL-1). In peripheral neurons, IL-1 promotes a hyperexcitable state that results in a global phospho/methyl switch and HSV reactivation that is dependent on dual leucine zipper kinase (DLK) activation.

*End of Chapter 3*

# *Chapter 4*

# Co-option of mtDNA-mediated STING Activation by HSV UL12.5 for Reactivation from Latent Infection

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Cuddy SR, Krakowiak PA, Flores M, Dochnal SA, Babnis A, and Cliffe AR. Co-option of mtDNA-mediated STING Activation by HSV UL12.5 for Reactivation from Latent Infection. *In prep*. (2023)

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## Abstract

Successful viruses have persisted within human populations due to their capacity to co-opt or subvert host innate immune pathways. Both DNA and RNA viruses, including the ubiquitous herpes simplex virus-1 (HSV), encode viral proteins that are targeted to mitochondria and induce mitochondrial DNA (mtDNA) stress that is associated with the release of nucleic acids into the cytosol. These cytosolic nucleic acids can be recognized by a pathway mediated by cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) that, upon sensing aberrant DNA in the cytosol, trigger a robust antiviral immune response. HSV has been found to encode a protein, UL12.5, that activates the cGAS-STING pathway following depletion of mtDNA and mitochondrial RNA (mtRNA) which consequently restricts lytic viral replication replicating cells. In this study, we investigated the role of UL12.5 in neurons, a terminally differentiated cell-type, and in HSV reactivation, a unique stage of the HSV life cycle in which lytic gene transcription is re-starts following a period of quiescence (latency). Using a primary neuron model of HSV latency as well as *in vivo* studies, we found that UL12.5 has a unique function in neurons that supports the de-repression of lytic gene during the establishment of latency as well as during reactivation. Moreover, direct activation of STING was sufficient to trigger HSV reactivation and supports lytic gene expression early during reactivation. Altogether, these data identify a pro-viral consequence of mtDNA depletion and demonstrate how neuronal STING activation by UL12.5 drives lytic gene expression during reactivation.

## Introduction

Viruses and their hosts continually antagonize each other in a battle between host immunity and viral persistence. The success of persistent viruses can be attributed to the subversion or co-option of host innate immune pathways mediated by pattern-recognition receptors (PRRs) that recognize cytosolic DNA, RNA, or viral proteins. Cytosolic mitochondrial DNA (mtDNA) is also recognized by these nucleic acid-sensing pathways, resulting in an antiviral response to control infection. Somewhat paradoxically, flavivirus [291,447], influenza A virus [448], herpesvirus [449], and other highly evolved viruses express proteins that specifically induce mtDNA stress that is associated with mtDNA release and subsequent activation of innate immune pathways. Thus far, it has been difficult to determine how mtDNA stress induced by viruses and its impact on immune pathways contributes to viral persistence, if at all.

Of the PRRs that respond to viral infection and mtDNA, cGAS (cyclic GMP-AMP synthase) and STING (stimulator of interferon genes) mediate a cytosolic DNA-sensing pathway that is one of the most evolutionarily conserved nucleic acid-sensing pathways found in mammals and plays a central role in the cellular response to infection. Upon sensing cytosolic DNA, cGAS dimerizes and produces the secondary messenger 2'-3'-cyclic GMP-AMP (2'-3'-cGAMP) that activates the oligomerization and subsequent activation of STING. Depending on the cell type, STING activation typically leads to an inflammatory response mediated by nuclear factor κB (NF-κB), interferon (IFN) production and upregulation of interferon stimulated genes (ISGs), or activation of c-Jun

N-terminal kinase (JNK). In some cell types, such as neurons, STING activation primarily results in an upregulation of autophagy.

As a virus that has co-evolved with humans, it is not surprising that herpes simplex virus-1 (HSV) has concurrently evolved in response to nucleic acid-sensing pathways, including cGAS-STING. The dsDNA genome of HSV encodes many viral proteins that inhibit or modulate nucleic acid sensing pathways in an effort to minimalize their impact on viral replication. During lytic replication, viral gene expression occurs over a defined ordered cascade: viral immediate-early (IE) mRNAs dependent on host and viral transcription factors encode proteins that promote the expression of viral early (E) mRNA, followed by the transcription of viral late (L) genes that are dependent upon viral DNA (vDNA) replication. HSV proteins across gene classes restrict major innate immune pathways, with ICP27, ICP0, and ICP34.5 inhibiting STING signaling during lytic infection [450,451]. The breadth and redundancy of viral proteins from each gene class that either disrupt or inhibit the host immune response to cytosolic DNA underscore the importance of modulating these pathways to promote viral viability. It is therefore puzzling that HSV encodes a protein, UL12.5, that actively induces an innate immune response.

During lytic infection, the clear outcome of expression of UL12.5, a mitochondria-targeted nuclease, is restriction of viral growth. As a truncated isoform of the nuclear-resident nuclease UL12, UL12.5 instead localizes to the mitochondria and depletes host mtDNA and mitochondrial RNA (mtRNA) [293,315–318,320]. This depletion is associated with

the release of mitochondrial nucleic acids into the cytosol and subsequent activation of cGAS-STING that substantially restricts HSV replication [293,319,321]. However, previous studies on UL12.5 were performed in mitotic cells during lytic infection, not in immunologically-distinct and post-mitotic neurons that harbor latent and/or reactivating genomes. Therefore, we hypothesized that while UL12.5 restricts lytic replication in non-neuronal cells, HSV is evolutionarily conserved to support lytic gene expression specifically in neurons.

HSV engages with host innate immunity throughout its lifecycle, including following acute infection at the mucosal epithelia, wherein the virus can establish a latent infection in peripheral neurons. Periodically, HSV re-initiates lytic gene expression in response to host pathway activation. This reactivation event is biphasic: Phase I consists of a stochastic burst of lytic gene expression from all three gene classes (IE, E, L) independent of vDNA replication while the subsequent Phase II recapitulates the ordered viral gene cascade observed in lytic replication [57,84,85,87,397]. While Phase I may not necessarily progress to Phase II, Phase I represents a critical stage in initiating the reactivation program and progression to Phase II results in the production of infectious virus (full reactivation) [84,397]. Although latency is generally asymptomatic, reactivation can be associated with significant clinical morbidities, including herpes simplex keratitis, encephalitis, and mucosal lesions. The growing evidence for an association between HSV infection and inflammatory neurodegenerative diseases [328–333], however, suggests a latency may also contribute to a neurodegenerative diseases characterized by inflammation.

The absence of viral proteins during latency necessitates that HSV exploits existing neuronal signaling pathways to initiate and progress reactivation. Several stimuli have been identified that trigger reactivation, including nerve growth factor (NGF) deprivation [51], DNA damage [452], and neuronal hyperexcitability [87], with a notable convergence on the neuronal stress pathway mediated by Dual Leucine Zipper Kinase (DLK) and JNK [339]. Our recent work has identified interleukin-1β (IL-1β), a major inflammatory cytokine, in triggering HSV reactivation via hyperexcitability [87]. These findings demonstrate the ability of HSV to co-opt canonical immune pathways to support reactivation from peripheral neurons, as well. Many of these neuronal pathways, including cGAS-STING, have yet to be fully defined in peripheral neurons. Type I and II IFNs have been shown to prevent reactivation or prevent progression to Phase II [92,425], but interactions between innate immune pathways and HSV reactivation are poorly understood and it is unknown if viral proteins play a role early during Phase I. Therefore, we hypothesized that activation of specifically neuronal cGAS-STING by UL12.5 supports lytic gene expression during HSV reactivation.

We found that while UL12.5 expression does not affect lytic replication in peripheral neurons, UL12.5 expression is essential for optimal viral gene expression during Phase I & Phase II. Moreover, activation of the cGAS-STING pathway compensates for the lack of UL12.5 expression in order to rescue lytic gene expression during Phase I. STING activation is required for lytic gene expression in Phase I and can trigger full reactivation. We there identified UL12.5 as the first viral protein that

functions in Phase I gene expression to facilitate exit from latency and subsequently full HSV reactivation. These findings highlight the differential activation of STING pathways in peripheral neurons that promote lytic gene expression in HSV reactivation. Ultimately, we have described a novel mechanism by which HSV utilizes host mtDNA to activate neuron-specific innate immune pathways to promote lytic gene expression during HSV reactivation.

## Results

### UL12.5 does not affect lytic replication in peripheral primary neurons.

The conservation of UL12.5 through HSV evolution despite its activation of innate immune pathways implies that UL12.5 confers a benefit to the virus at some point in the viral life cycle. Thus far, the consequences of UL12.5 expression on viral replication have been investigated exclusively in mitotic cells, such as fibroblasts. However, HSV is a neurotropic virus that can undergo lytic replication in neurons as well as establish latent infections that are capable of reactivating. We therefore sought to determine whether UL12.5 was required for lytic replication or latent infection in peripheral neurons. To this end, we isolated primary neurons isolated from the superior cervical ganglia (SCG) of postnatal mice and infected them with virus lacking UL12.5 expression. Because UL12.5 is co-linear with the essential *UL12* open-reading frame (ORF), we used the previously described KOS-UL98 virus, in which the entire *UL12* ORF is replaced with *UL98* [319], an ortholog from cytomegalovirus that maintains the alkaline nuclease function of UL12 but prevents expression of UL12.5 [453]. Compared to the control virus KOS-SPA, we found no difference in viral replication in primary neurons based on assays for both infectious virus and vDNA replication (Figure 1A & B). This is in contrast to what we observed in primary murine dermal fibroblasts (DFs) isolated from the same mice, where the KOS-UL98 virus replicated to higher levels than the wild-type KOS-SPA virus (Figure 1C). Therefore, UL12.5 is neither detrimental nor required for HSV replication in peripheral neurons.

The absence of a discernible replication phenotype for HSV in neurons compared to DFs raised the possibility that UL12.5 functions differently in neurons. To confirm that the main function of UL12.5, to deplete mtDNA and mtRNA, is maintained during lytic infection of peripheral neurons, we used qPCR and RT-qPCR to evaluate levels of mitochondrial transcripts following infection with KOS-SPA or KOS-UL98. Importantly, we found that by 12 hours post-infection both mtDNA and mtRNA were significantly decreased in peripheral neurons when infected with virus expressing UL12.5 (Figure 1D & E). Therefore, UL12.5 mediates mtDNA and mtRNA depletion in peripheral neurons in a manner similar to non-neuronal cell types.

In contrast to replicating cells, such as epithelial cells and fibroblasts, neurons are reported to rely on autophagy rather than release type I IFN during neuronal HSV infection. While UL12.5 expression is typically associated with activation of innate immune pathways that results in an IFN response, activation of these pathways in neurons may be restricted or severely modified. To determine whether innate immune pathway activation occurs in neurons downstream of UL12.5 expression, primary neurons were transduced with lentiviral plasmids expressing either PGK-UL12.5-SPA. Neurons expressing PGK-UL12.5-SPA displayed a marked increase in STING staining intensity and localization with TGN46, a Golgi maker (Figure 1F), suggesting that UL12.5 expression alone is sufficient to activate innate immune pathways in peripheral neurons. Therefore, UL12.5 is expressed during lytic infection of neurons and can activate innate sensing pathways, but this does not result in an induction of type I IFN transcription and neither restricts or promote HSV lytic replication.

## UL12.5 supports initial de-repression of lytic HSV genes following *in vivo* infection.

Although UL12.5 had no significant role in lytic infection of peripheral neurons, we hypothesized that UL12.5 may instead be supporting another phase of the HSV life cycle specific to neurons, namely the establishment of or reactivation from a latent infection. To investigate the role of UL12.5 in the establishment of latency, we first performed *in vivo* infection with either KOS-SPA or KOS-UL98 via the ocular route, which results in an acute infection in peripheral ganglia followed by the establishment of a latent infection. After latency had been established 28 days post-infection, the trigeminal ganglia (TG) were collected from infected mice and viral genome copy numbers per ganglia was determined by qPCR. Interestingly, we found that KOS-UL98-infected TG had significantly smaller pool of viral genomes at latency compared to those infected with KOS-SPA (Figure 2A). This experiment was repeated using the same experimental paradigm with additional UL12.5 mutants, F/L and Rescue. While not significant, the results are trending towards a significant difference between F/L and Rescue, which may be attributed to low levels of UL12.5 expression that is observed in lytic infection with this mutant (Figure 2B). The F/L strain contains mutations on the two potential transcriptional start sites for UL12.5 that are associated with reduced expression of UL12.5 during lytic infection [319]. Therefore, UL12.5 expression supports the establishment of a latency *in vivo* by promoting a larger pool of latent viral genomes.

Prior to the establishment of latency in peripheral neurons *in vivo*, the viral genome undergoes an initial repression of lytic gene transcription early during infection that promotes a state of latency [389]. However, this initial repression can be overcome to drive lytic gene transcription during acute infection, resulting in low levels of infectious virus in TG for several days prior to the establishment of latency [390,391,454]. This is likely mediated in response to acute replication at the body surface and unknown communication mechanisms with neurons to promote lytic gene expression. We therefore hypothesized that UL12.5 expands the pool of latent genomes by promoting the de-repression of viral genomes early during neuronal infection that results in increased lytic replication and subsequently more viral dissemination throughout the TG. To investigate the effect of UL12.5 in lytic gene expression during the establishment of latency *in vivo*, we again infected mice via ocular scarification with either KOS-SPA or KOS-UL98 and measured lytic and latent RNA transcripts in TG early during infection. qPCR analysis of viral transcripts showed that lytic viral gene expression, measured by *ICP17* and *VP16* mRNA, was decreased in TG in the absence of UL12.5 expression throughout early infection with KOS-UL98 (Figure 2C & D). In contrast, *latency associated transcript* (*LAT*), a long noncoding RNA highly expressed during latency, was increased in mice infected with KOS-UL98 (Figure 2E). Thus, UL12.5 supports the initial de-repression of lytic genes early during infection *in vivo*, allowing for increased viral dissemination throughout the TG and the establishment of a larger latent pool of genomes that can reactivate.

## UL12.5 is required for optimal lytic gene expression during HSV reactivation.

Given that the viral genome undergoes de-repression to facilitate lytic gene expression during HSV reactivation, we hypothesized that UL12.5 may support lytic gene transcription and viral replication in reactivation *in vitro*. To investigate the role of UL12.5 in HSV reactivation, we isolated primary neurons from SCGs of postnatal mice and established latent infections using acyclovir (ACV) with either KOS-UL98 or KOS-SPA. Although equivalent amount of KOS-UL98 and KOS-SPA vDNA were present in neurons during latency, vDNA replication following reactivation triggered by PI3K inhibitor LY294002 (LY) was decreased in KOS-UL98-infected neurons by 60 hours post-trigger (Figure 1A). In agreement with these data, we observed that KOS-UL98 remarkably was unable to efficiently expression viral lytic genes through both Phase I and Phase II from across all gene classes following addition of LY: *ICP27* (IE), *UL30* (E), and *gC* (L) (Figure 3B-D).

To confirm the requirement of UL12.5 for lytic gene expression during Phase I of reactivation, we assessed reactivation from peripheral neurons using two additional HSV UL12.5 mutant viruses. Both F/L and AN-1 (*ICP6:lacz* insertion into the *UL12* ORF) displayed decreased lytic gene expression analysis during Phase I compared to their respective controls, Rescue and WT KOS, when measured by RT-qPCR (Figure 3E & F). These results place UL12.5 as a viral protein central to the optimal expression of lytic genes during Phase I and Phase II and identifies UL12.5 as the first viral protein known to mediate Phase I gene expression.

## UL12.5 is expressed early during Phase I of reactivation

Previous reports hypothesized that protein synthesis is not required for lytic gene expression in Phase I [84]. However, given that UL12.5 expression is required for lytic gene expression in Phase I, we hypothesized that functional UL12.5 protein is required for Phase I. To resolve our observation that lytic gene expression in Phase I requires UL12.5 expression and determine whether UL12.5 synthesis may be required for Phase I, primary neurons were latently infected with Stayput-GFP and reactivated with LY, LY and cycloheximide (CHX) (0hrs), or LY with CHX added 10 hours later (10hrs). Using RT-qPCR to measure viral transcripts, we confirmed that protein synthesis is not required in Phase I 10hrs post-trigger, but found that inhibition of protein synthesis by CHX at the start of reactivation (0hrs) does in fact restrict Phase I lytic gene expression (Figure 4A & B). These experiments show that a role for viral protein synthesis, including UL12.5, can no longer be ruled out in promoting Phase I gene expression.

Since UL12.5 depletes mtDNA and mtRNA during lytic infection of peripheral neurons, we hypothesized that UL12.5 would retain this function and deplete mtDNA during reactivation. Following a latent infection of neurons with Stayput-GFP, we triggered reactivation using LY and quantified mtDNA (*mtDLoop1*) transcripts. qPCR revealed that mtDNA were significantly decreased by end of Phase I, suggesting that mtDNA depletion is in fact occurring (Figure 4C). It is important to note that these transcripts are harvested from bulk samples in which many neurons are not infected and not all infected neurons reactivate in response to a single stimulus. The impact of

mtDNA depletion by the end of Phase I in those neurons undergoing reactivation may therefore be more appreciable than what is detected.

To confirm that UL12.5 was expressed in Phase I, we sought to detect UL12.5 expression using RT-qPCR immediately following the initiation of reactivation. Using primary neurons latently infected with Stayput-GFP, we measured viral transcripts of UL12.5 and UL12 following the addition of LY as a reactivation trigger. Given the collinearity of UL12 and UL12.5, we measured transcripts using primers exclusive to UL12 sequences (UL12) and primers that measured colinear sequences (UL12.5). We detected a significant increase in UL12.5, but not UL12 transcripts, indicating that UL12.5 is selectively upregulated by 3 hours post-trigger (Figure 4D & E). These results demonstrate that mtDNA depletion occurs throughout Phase I and may be attributed to UL12.5 function within the first 10 hours of reactivation.

## STING activation promotes lytic gene expression during Phase I

It is well-established that UL12.5 expression results in the downstream activation of nucleic acid-sensing pathways, most notably cGAS-STING. STING activation in peripheral neurons in the context of injury has recently been shown to result in axon regeneration [286], but the ultimate downstream consequences of cGAS-STING activation in neurons in the context of infection is largely undescribed. When considering the potential functional contribution of UL12.5 that supports lytic gene expression in Phase I, we therefore hypothesized that activation of cGAS-STING by UL12.5 indeed supports lytic gene transcription during Phase I. To test whether cGAS-STING activation

supports HSV reactivation, we tested various cGAS-STING agonists as triggers of reactivation for peripheral neurons latently infected with Stayput-GFP. The GFP tag on the late viral protein US11 allows for the visualization of individual neurons reactivating and undergoing full reactivation. Incredibly, agonists for both cGAS (ISD) and STING (DMXAA, Poly(dA-dT)/LyoVec, ADU-S100) triggered reactivation when using GFP fluorescence as a readout for full reactivation (Figure 5A-D). While the various cGAS-STING agonists induced varying degrees of reactivation, likely due to differences in activation mechanism, DMXAA induced comparable levels of reactivation to the well-established trigger LY. Therefore, activation of the cGAS-STING nucleic acid-sensing pathway is sufficient to initiate viral gene transcription and reactivation.

Activation of DLK and its downstream mediator JNK are critical steps in reactivation associated with numerous triggers *in vitro*. In order to determine whether DLK activation was required during DMXAA-mediated reactivation, we treated neurons latently infected with Stayput-GFP with DMXAA or DMXAA and the DLK inhibitor GNE-3511 (Figure 5E). Using GFP fluorescence as a readout for reactivation, GNE-3511 blocked reactivation induced by DMXAA. This suggests that HSV reactivation downstream of cGAS-STING activation is reliant in DLK activation.

Given that STING activation triggers HSV reactivation and is the primary result of UL12.5 expression, we hypothesized that STING activation by DMXAA will rescue lytic gene expression during Phase I in virus lacking UL12.5 expression. To test this hypothesis, we first established a latent infection in peripheral neurons *in vitro* with

either KOS-SPA or KOS-UL98. After the establishment of latency, neurons were treated with LY alone, DMXAA alone, or LY followed by DMXAA 2.5 hours later (Figure 5F). Using RT-qPCR to measure viral transcripts, we found that LY nor DMXAA alone was sufficient to induce lytic gene expression in KOS-UL98-infected neurons that was equivalent to reactivating KOS-SPA genomes following LY. However, when DMXAA was added 2.5 hours after LY treatment, lytic gene expression recovered relative to levels in KOS-SPA-infected neurons. Therefore, direct activation of STING facilitates lytic gene expression in Phase I even in the absence of UL12.5, suggesting that UL12.5 serves to induce lytic gene expression via STING activation.

To confirm the role of STING in Phase I lytic gene expression, we reactivated Stayput-GFP from latently infected peripheral neurons using LY in the presence of a robust STING inhibitor, H-151 (Figure 5G). Using RT-qPCR to evaluate lytic gene expression during Phase I, we found that STING inhibition significantly reduced the expression of lytic transcripts during Phase I. Although somewhat paradoxical, these data demonstrate that STING activation by UL12.5 during Phase I facilitates lytic gene expression and is therefore critical to HSV reactivation.

## Discussion

In addition to their role in host metabolism, mitochondria are increasingly appreciated for their role in coordinating innate immunity as central signaling hubs for nucleic acid-sensing pathways. It is puzzling, then, that both DNA and RNA viruses encode viral proteins that are known only to induce mtDNA stress, a process closely associated with mtDNA release and activation of innate immune pathways. While these virus-mitochondria interactions often result in activation of nucleic acid-sensing pathways and subsequent restriction of viral replication, we found that the HSV protein UL12.5 re-purposes activation of the innate immune pathway mediated by STING and takes advantage of the downstream consequences to promote lytic gene expression in reactivation from neurons. Moreover, we identify UL12.5 as the only known HSV protein to facilitate lytic gene expression during Phase I of reactivation as well as the only viral protein known to promote viral gene expression exclusively in reaction. The combined requirement of JNK and STING activation early in HSV reactivation suggests that reactivation is more complex than initially considered and is derived, at least in part, from the neurotropism of HSV latency. Importantly, these results also highlight how STING pathway activation within terminally differentiated and post-mitotic neurons can be specifically targeted and subverted by HSV.

Our results show that HSV expresses a protein that demonstrate cell type-specific behaviors, even at the expense of certain outcomes in others. The life cycle of HSV necessitates that the virus continually infects multiple different cell types. Therefore, HSV must constantly balance the need to inhibit innate immunity to support

lytic replication in epithelial cells while subsequently utilizing neuronal signaling pathways to initiate reactivation. Previous reports on UL12.5 clearly demonstrate that the only known function of UL12.5 is to activate innate immune pathways that restrict lytic replication [293,319,321]. Depending on the cell type infected, UL12.5 was shown to preferentially activated cGAS-STING, likely through mtDNA release into the cytosol, in highly replicative cell lines [293]. In more specialized cells lines, such as THP-1 cells that are highly sensitive to cytosolic RNA, UL12.5 activates RNA-sensing pathways mediated by RIG-I after mtRNA transcripts are generated by RNA Pol3 and mtDNA [321]. These previous reports hinted at the cell type-specific consequences of UL12.5, though the restriction of viral growth is the same in either case. We show that in neurons UL12.5 expression promotes the expression of lytic genes during reactivation *in vitro* as well as following *de novo* TG infection *in vivo*. UL12.5 expression has been shown to disrupt mitochondrial membrane potential and ATP production, suggesting that UL12.5 may activate pathways in addition to nucleic acid-sensing pathways [320], but the extent to which these pathways are activated and their relevance to viral viability are unknown. Therefore, UL12.5 is maintained in the HSV genome to provide a benefit to HSV that is specific to neurons. It will be important to determine if and how activation of additional nucleic acid-sensing, metabolic, and other intrinsic pathways are affected by UL12.5 or other viral proteins. HSV proteins, particularly those that engage with innate immunity, will also have to be considered across the spectrum of cell types that permit HSV infection to account for functional nuances between host cells, particularly in the context of infection *in vivo*.

The establishment of latency *in vivo* is a dynamic process involving a combination of host and viral signaling pathways that function in the context of a systemic immune response to infection. Following initial infection at the mucosal epithelia, the HSV genome is trafficked along the axon until reaching the neuronal nucleus within the soma residing in the TG [455]. TG infection *in vivo* initially leads to a silent infection that resembles latency in which the incoming viral genome is silenced [389]. Subsequent to this repression, the viral genome can be de-repressed in the presence of the viral transactivator VP16; when more VP16 is present, the genome is more likely to undergo lytic transcription and enter lytic replication prior to the establishment of latency *in vivo* [389]. Our findings show that UL12.5 similarly functions to promote lytic gene expression early in TG infection *in vivo*. Given that UL12.5 can be associated with the HSV tegument that is trafficked to the nucleus during initial infection and we observed VP16 expression was dependent on UL12.5 during reactivation *in vitro*, it is possible that UL12.5 supports VP16 expression during initial TG infection *in vivo* to drive more lytic replication that ultimately establishes a larger latent pool of HSV genomes. Interestingly, it is hypothesized that IFN released from neighboring cells promotes the establishment of a larger pool of HSV genomes in neurons by inhibiting neuronal cell death during initial infection [418,419]. The larger pool of latent genomes in the TG is considered to support HSV persistence as genomes are more likely to reactivate when more TG neurons are infected [422]. IFN sensing at the time of infection has also been show to induce a form of immune memory that restricts the ability of HSV to reactivate later [115]. It is possible, therefore, that early expression of UL12.5 may similarly play a role in the establishment of latency that affects the ability of individual

viral genomes to reactivate. While certain cytokines like IFNs are sufficient to induce latency *in vitro* [98], the transient mechanisms promoting latency as well as those prolonged systems that maintain latency *in vivo* are likely multifaceted. It will be important to delineate how activation of pathways downstream of UL12.5, both in neurons and other cells in the surrounding tissue, converge on the viral genome and the ultimate effect these pathways have on latency and reactivation.

Previous work from our lab and others have demonstrated that HSV relies on host signaling pathways to trigger HSV reactivation in the absence of viral proteins during latency [84,395]. Activation of the neuronal stress pathway mediated by DLK and JNK is an essential step in HSV reactivation across diverse triggers of reactivation, including reactivation mediated by IL-1 sensing [87,339]. However, the signaling pathways mediating early transcriptional events in Phase I, including the role of JNK signaling in those events, is unclear. Our findings that UL12.5 expression and STING activation are required for optimal lytic gene expression suggest that in addition to JNK activation, STING activation by UL12.5 similarly represents a critical step early in HSV reactivation. Moreover, the block of DMXAA-mediated reactivation with a DLK inhibitor suggests that the downstream events stimulating lytic gene expression by STING activation similarly rely on DLK and JNK activation. This places STING activation in a unique position in which JNK, in this case activated via PI3K inhibition by LY, must first stimulate expression of UL12.5 to activate STING and then STING subsequently relies on JNK activation for further upregulation of lytic HSV genes in Phase I. Although we show that additional agonists of cGAS-STING can trigger reactivation *in vitro*, it will be important to

determine whether off-target effects of DMXAA and whether those contribute to lytic gene expression. In light of this and that lytic gene expression in KOS-UL98 reactivation is rescued with DMXAA only when it is added 2.5 hours after LY, an appreciable pattern of combined host and viral signaling pathways activation emerges that drive lytic gene expression. Although our understanding of how both JNK and STING engage with the viral genome, particularly in the context of HSV reactivation, is relatively unknown, our findings add to the complexity of signaling mechanisms that mediate an escape from latency and provide the first known example of a viral protein integrating itself within host pathways to facilitate lytic gene expression early during Phase I.

The timing of viral gene expression appears to be crucial to the progression of Phase I and ultimately Phase II. Previous studies have demonstrated that IFN is sufficient to prevent reactivation when added prior to Phase I, but does not prevent reactivation when added at the end of Phase I, likely because viral proteins are present to restrict significant effects of IFN [92,425,426]. In activating STING within the first few hours of Phase I, HSV must account for viral proteins like ICP27 that can inhibit STING [450] and are similarly expressed to high levels early during Phase I [84]. When Phase I was initially characterized, the authors concluded that viral protein synthesis was not required for Phase I lytic gene expression because CHX did not prevent lytic gene expression when added 10 hours post-trigger [84]. After confirming these findings, however, we found that when CHX was added at the same time as LY, lytic gene expression in Phase I was not affected. Given that we found UL12.5 expression precedes peak lytic gene expression

in Phase I and UL12.5 is expressed by 3 hours post-trigger, UL12.5 therefore may function to support reactivation within the first 10 hours of reactivation. In a study from our lab, we observed that following neuronal hyperexcitability, HSV reactivation is associated with two distinct waves of a histone phospho/methyl switch that can rapidly induce lytic gene expression [87]. The first wave was ~5 hours post-trigger while the second wave was when lytic gene expression peaks during Phase I at ~20 hours post-trigger. This leaves open the possibility that following a trigger, UL12.5 is either transcribed as a result of this phospho/methyl switch or activates the switch to induce broader lytic gene expression. The rapid induction of lytic gene expression we observed during Phase I due to STING may be attributed to rapid changes in the viral epigenome, potentially via a phospho/methyl switch on viral lytic promoters by IKKα from the NF-κB pathway [456], recruitment of transcription or pioneer factors, such as JNK or c-Jun, or activation of other pathways that promote chromatin modifications, such as DNA repair pathways associated with reactivation [105]. Exactly where UL12.5 expression and function fit within these first hours of reactivation will have to be determined in order to better understand the sequential events leading to Phase I and Phase II.

HSV reactivation has evolved in direct response to the neuronal signaling pathways that consider the highly specific needs of a post-mitotic and morphologically distinct cell type, particularly in the context of innate immunity. During *de novo* neuronal HSV infection, STING-mediated upregulation of autophagy is considered to be the primary intracellular mechanisms by which neurons control HSV replication [277,411,412,414]. Induction of robust immune response, including an inflammatory or IFN response, by

neuronal STING in HSV infection is notably lacking [277], suggesting that neuronal STING activation is considerably different compared to other infected cell types. This could be expected given that the downstream consequences of STING activation vary by cell type and neurons likely avoid potentially damaging inflammation as a terminally differentiated cell. It is striking, however, that HSV activates STING to support lytic gene expression during reactivation. Apart from an upregulation of autophagy, the breadth of consequences from STING activation in peripheral neurons during infection are largely unknown. Reports showing that STING activation in peripheral neurons following injury results in upregulation of genes associated with axon regeneration [286] and that STING activation plays a role in nociception [285] indicate that neuronal STING activation is not exclusively associated with an antiviral response. While lytic infection occurs in the presence of cytokines, including inflammatory molecules and IFN, and across multiple tissues *in vivo* [381,398], Phase I is much more immunologically silent, as it is restrained exclusively to the soma and lacks the major inflammatory responses observed in acute infection. Moreover, neurons have distinct transcriptional profiles after sensing IFN compared with other cells [92] and mtDNA has been shown to induce unique ISG expression profiles that support DNA damage repair [325]. Therefore, it is possible that HSV relies on a non-immunostimulatory activation of STING by mtDNA to promote lytic HSV gene expression during reactivation. The scale and impact of neuronal STING activation during infection is not yet known, but our results show that HSV has evolved to highjack a very specific niche within the finely tuned and neuron-specific cGAS-STING pathway.

Our findings further substantiate that model that HSV highjacks neuronal pathways that are canonically immune to support reactivation from peripheral neurons. As a cell type that has limited an immunological response to HSV infection and, in fact, utilizes IFN signaling to withstand infection, neurons provide the ideal repository for latent viral genomes. Moreover, it is clear that while mtDNA is typically associated with an antiviral response to infection, HSV has co-opted mtDNA and host nucleic acid-sensing mechanisms to facilitate lytic gene expression during reactivation. The continued requirement of JNK activation alongside UL12.5-mediated activation of STING during Phase I clearly indicates that the initial events triggering the entry out of latency are more incredibly complex and warrant further investigation. The temporal kinetics and interactions between the JNK and STING pathways will need to be elucidated, under both homeostatic and infection conditions, in order to better understand how neurons respond to reactivation following latency and how HSV manipulates these hose pathways to promote viral persistence. STING activation as a trigger of reactivation suggests that HSV has evolved to detect the presence of other pathogens during latency, suggesting that reactivation in this context is in response to sensing that the host neuron is in danger. It will also be important to delineate any additional interacting partners or downstream mediators that are unique to neuronal signaling pathways. While our findings represent one example of mtDNA-induced signaling that supports viral replication, and future work will have to determine whether additional mitochondrial factors, such as mitochondrial membrane potential, affect viral replication and how cell type-specific pathways contribute to viral persistence.

## Figures

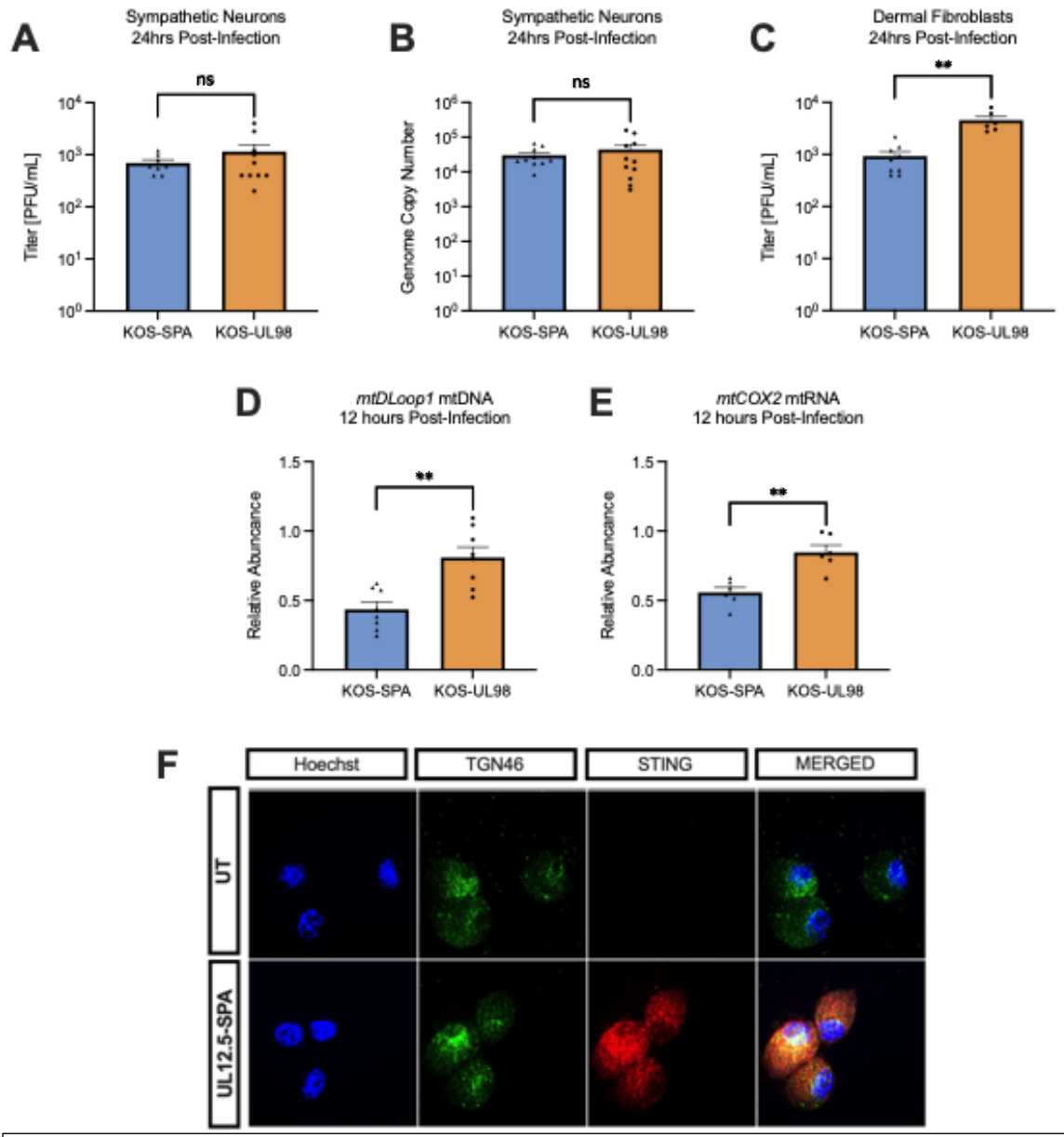



**Figure 1. UL12.5 does not affect lytic replication peripheral primary neurons.** (A-B) Titers of infectious virus and vDNA copy number measured by qPCR 24 hours after infection of primary sympathetic neurons with KOS-SPA or KOS-UL98. (C) Titers of infectious virus 24 hours after infection of dermal fibroblasts with KOS-SPA or KOS-UL98. (D-E) Relative abundance of *mtDLoop1* mtDNA and *mtCOX2* mtRNA transcripts by qPCR and RT-aPCR 12 hours after infection with KOS-SPA or KOS-UL98. (F) Representative immunofluorescence images of STING and TGN46 following 3-day transduction with PGK-UL12.5-SPA; 60x. All data are plotted as biological replicates from three independent experiments; shown as mean +/- standard errors of the mean (SEM). Shapiro-Wilk normality test; Mann-Whitney test between conditions; **, $p<0.01$.

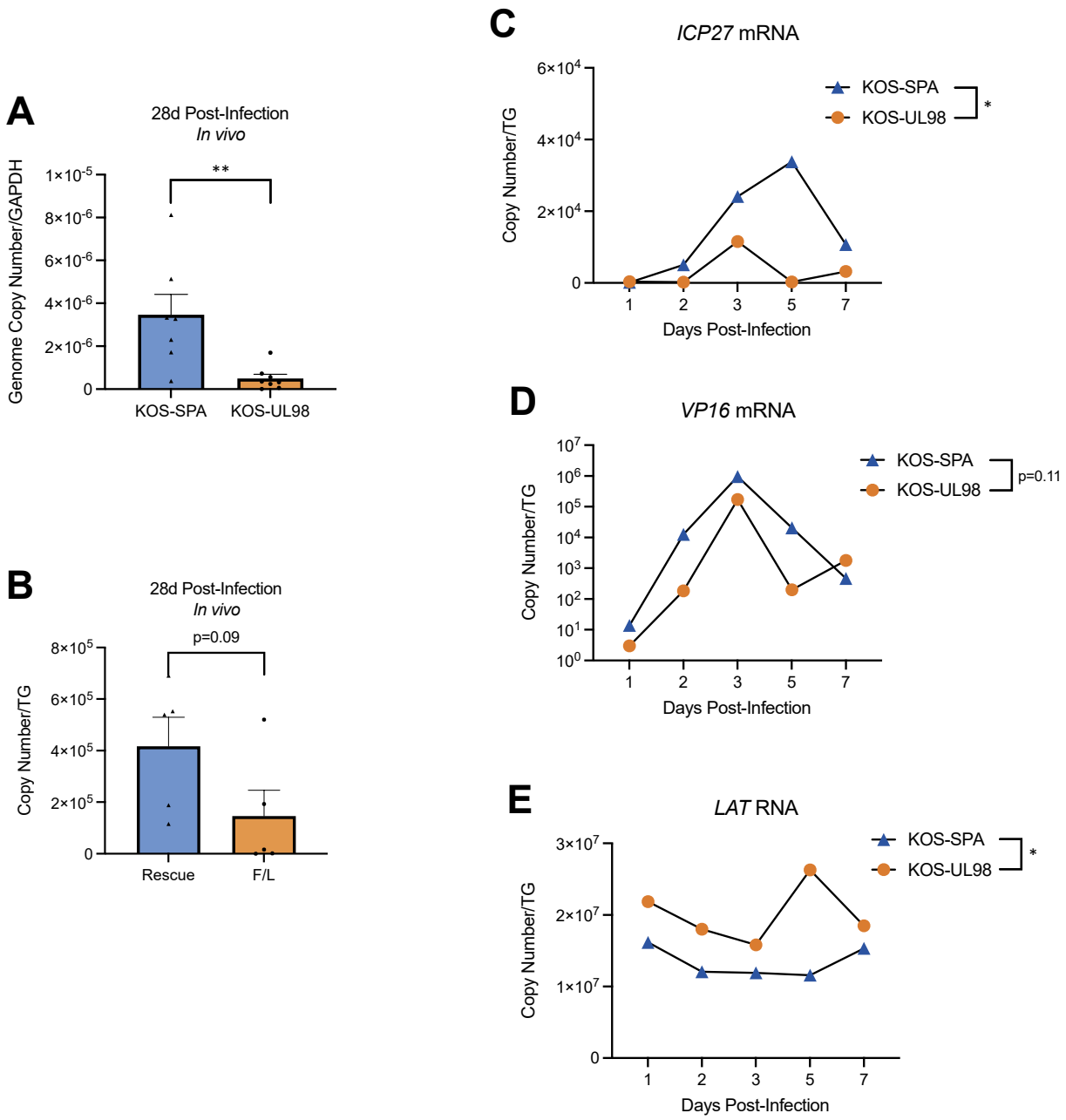
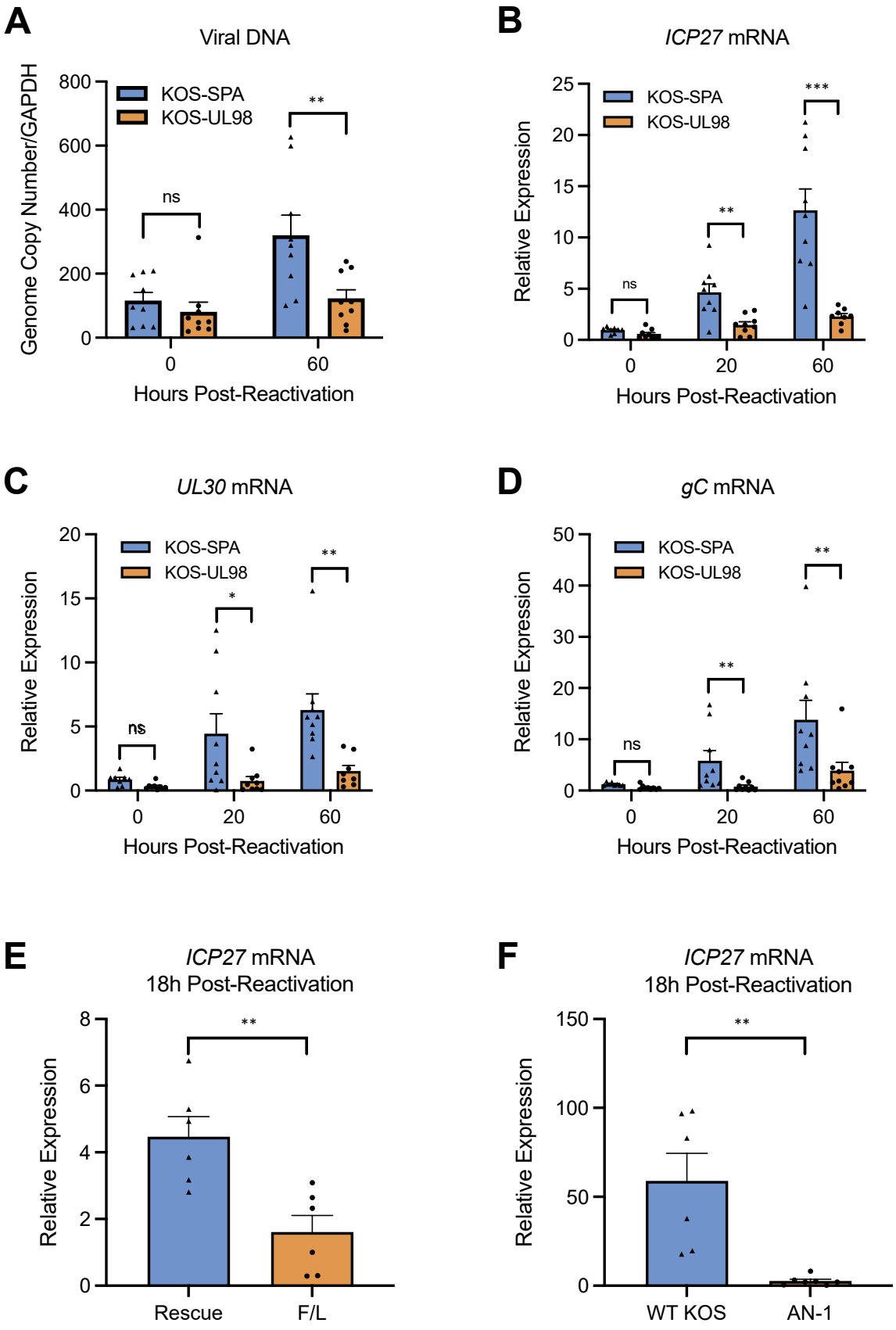



**Figure 2. UL12.5 supports initial de-repression of lytic HSV genes following *in vivo* infection.** (A) Quantification of vDNA by qPCR of *ICP8* transcripts in mouse trigeminal ganglia during latency following ocular infection with either KOS-SPA or KOS-UL98. (B) Quantification of vDNA by qPCR of *ICP8* transcripts in mouse trigeminal ganglia during latency following ocular infection with either F/L or Rescue. (C-E) Quantification lytic and latent viral transcripts by RT-qPCR during the establishment of latency with KOS-SPA or KOS-UL98. Data plotted are as biological replicates from three independent experiments +/-SEM or mean. Shapiro-Wilk normality test; Mann-Whitney test or two-way ANOVA; **p<0.05, * p<0.01.


A
Viral DNA
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Relative Expression
25
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***
**
ns
0
20
60
Hours Post-Reactivation
C
UL30 mRNA
Relative Expression
20
15
10
5
0
KOS-SPA
KOS-UL98
**
*
ns
0
20
60
Hours Post-Reactivation
D
gC mRNA
Relative Expression
50
40
30
20
10
0
KOS-SPA
KOS-UL98
**
**
ns
0
20
60
Hours Post-Reactivation
E
ICP27 mRNA
18h Post-Reactivation
Relative Expression
8
6
4
2
0
**
Rescue
F/L
F
ICP27 mRNA
18h Post-Reactivation
Relative Expression
150
100
50
0
**
WT KOS
AN-1

**Figure 3. UL12.5 is required for optimal lytic gene expression during HSV reactivation.** (A) Quantification of vDNA by qPCR of *ICP8* transcripts during latency (0hrs) and full reactivation (60hrs) of KOS-SPA and KOS-UL98 after addition of LY (20μM) in the presence of WAY-150168, which prevents cell-to-cell spread. (B-D) Quantification of KOS-SPA and KOS-UL98 reactivation by RT-qPCR of *ICP27* (IE), *UL30* (E), and *gC* (L) mRNA transcripts at latency and 20hrs and 60hrs after addition of LY (20μM) in the presence of WAY-150168. (E) Quantification of F/L and Rescue reactivation by RT-qPCR of *ICP27* mRNA transcripts of 20 hours after addition of LY (20μM) to latent neurons in the presence of WAY-150168. (F) Quantification of AN-1 and WT KOS reactivation by RT-qPCR of *ICP27* mRNA transcripts of 20 hours after addition of LY (20μM) to latent neurons in the presence of WAY-150168. All data are plotted as biological replicates

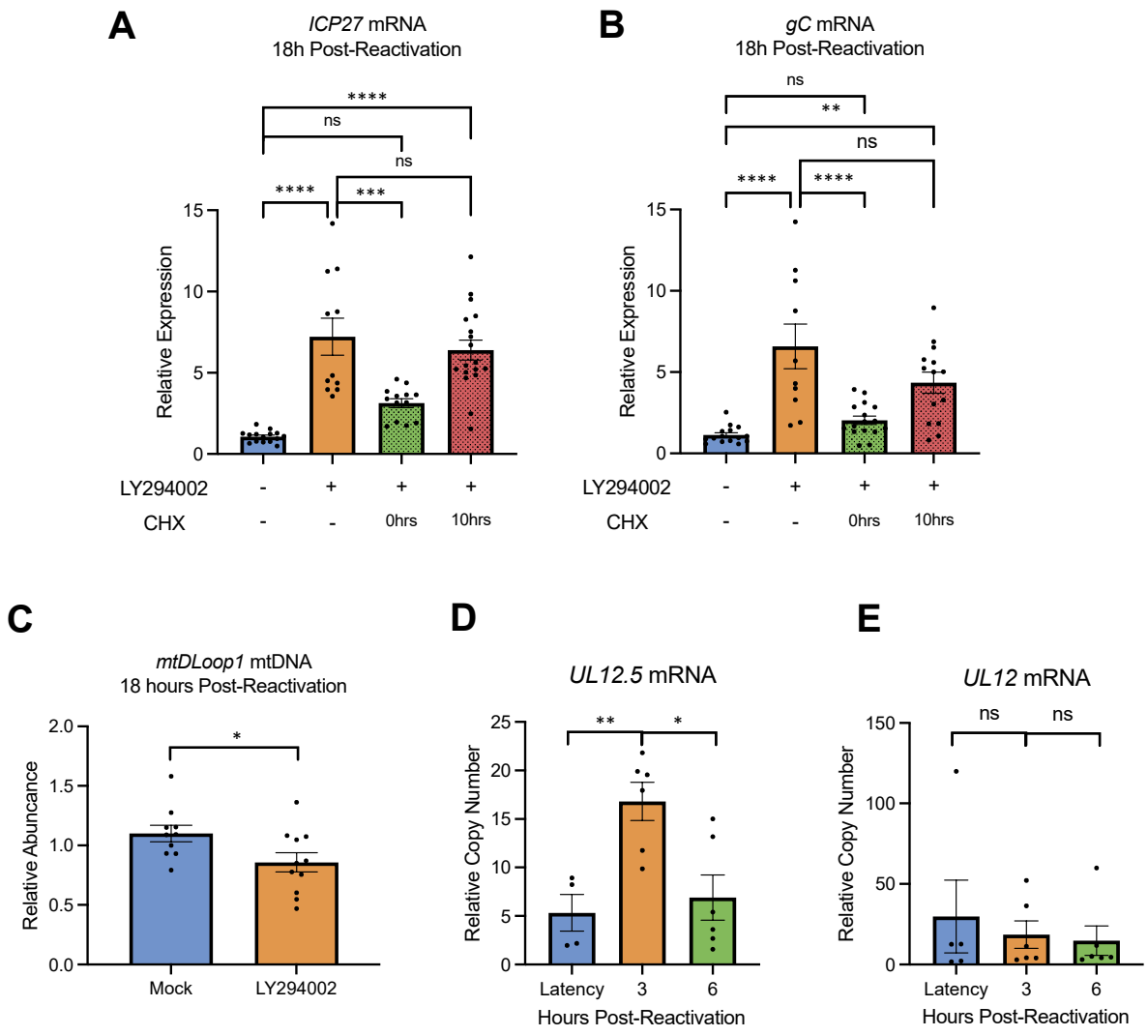


**Figure 4. UL12.5 is expressed early during Phase I of HSV reactivation.** (A-B) Quantification of Stayput-GFP reactivation by RT-qPCR of *ICP27* and *gC* mRNA transcripts after treatment of with LY (20µM) alone, LY (20µM) + CHX (10 µg/mL), or LY (20µM) with CHX (10 µg/mL) added 10 hours post-LY. (C) Relative abundance of *mtDLoop1* mtDNA transcripts in primary neuron cultures at latency (Mock) and after treatment with LY (20µM). (D-E) Quantification of Stayput-GFP reactivation by RT-qPCR of *UL12.5* and *UL12* mRNA transcripts after treatment of with LY (20µM). All data are plotted as biological replicates from three independent experiments; shown as mean +/- SEM. Shapiro-Wilk normality test; Mann-Whitney test or student's t-test; *p<0.05, **p<0.01, ***p<0.001.

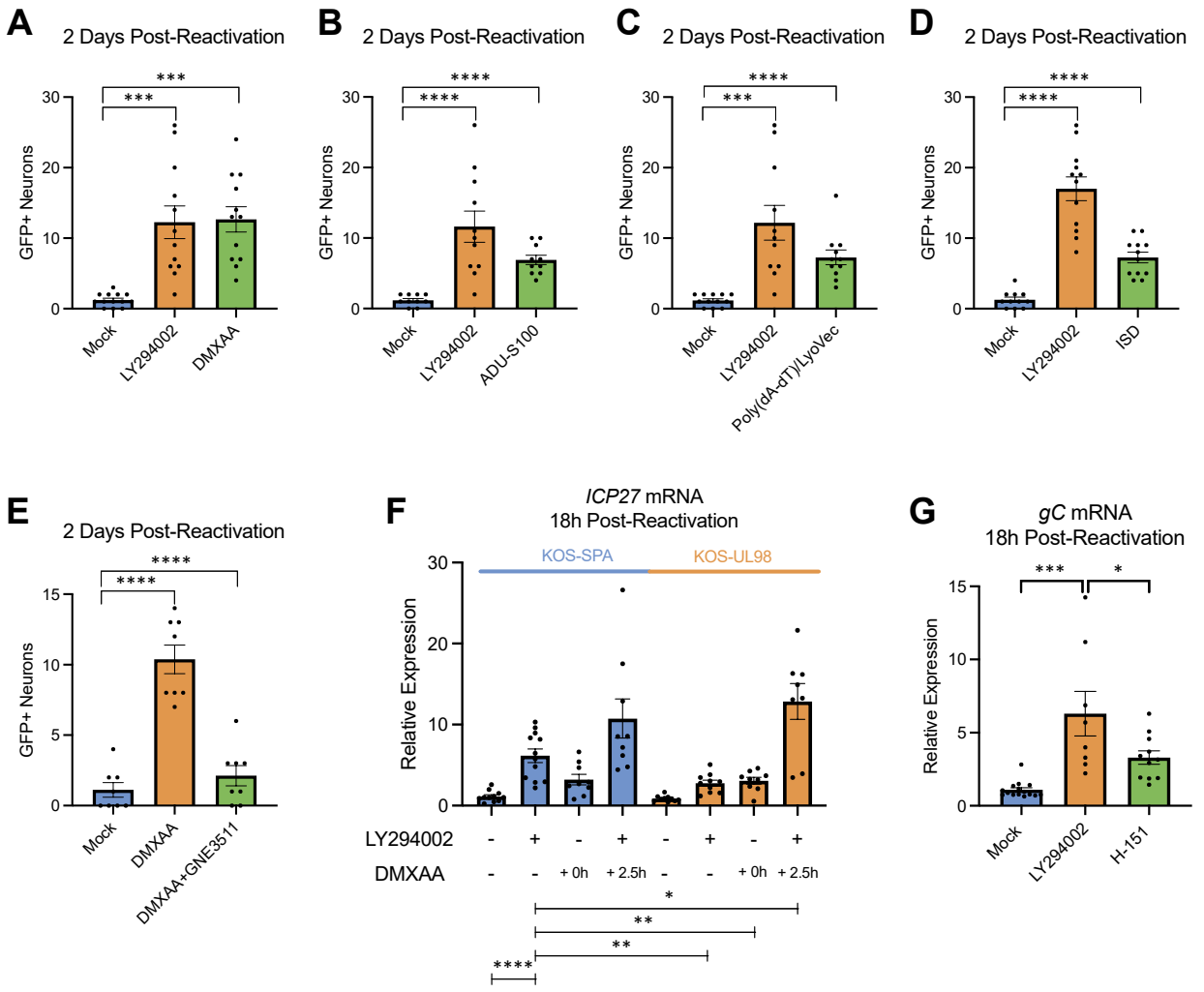


**Figure 5. STING activation promotes lytic gene expression during Phase I.** (A-D) Reactivation from latency is quantified by Us11-GFP expression neurons after addition of LY (20µM), DMXAA (50 µg/mL), Poly(dA-dT)/LyoVec (5 µg/mL), ADU-S100 (10 µg/mL), or ISD (5µg/mL). (E) Number of Stayput-GFP-infected neurons expressing GFP 2 days post-treatment with LY (20 µM), DMXAA (50 µg/mL), or DMXAA (50 µg/mL) + GNE-3511 (4 µM). (F) Quantification of KOS-SPA and KOS-UL98 reactivation by RT-qPCR of *ICP27* mRNA transcripts after treatment with LY (20µM), DMXAA (50 µg/mL), or LY (20 µM) with DMXAA (50 µg/mL) added 2.5hrs after LY. (G) Quantification of Stayput-GFP reactivation by RT-qPCR of *ICP27* and *gC* mRNA transcripts after treatment with LY (20µM) or LY (20µM) + H-151 (5 µM). All data are plotted as biological replicates from three independent experiments; shown as mean +/- SEM. Shapiro-Wilk normality test; Mann-Whitney test or student's t-test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

*End of Chapter 4*

*Chapter 5*

# UL12.5 can Activate MAVS-Mediated Pathways That Restrict HSV Growth

## Abstract

The Herpes simplex virus-1 (HSV) protein UL12.5 is well-described in its ability to deplete host mitochondrial DNA (mtDNA) and RNA (mtRNA). mtDNA-depletion by UL12.5 is associated with mitochondrial stress that results in the release of mitochondrial nucleic acids into the cytosol. These nucleic acids ultimately activate DNA-sensing pathway mediated by cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) and RNA-sensing pathway activated by retinoic acid-inducible gene I (RIG-I) following RNA Pol3 reception of mtDNA to restrict viral replication as a result of UL12.5 expression during lytic replication. However, the full extent of pathways activated by UL12.5 is unknown and it is unclear whether mtRNA can be sensed directly as a result of UL12.5 expression. In this study, we show that UL12.5 expression results in activation of mitochondrial antiviral-signaling protein (MAVS), but not RNA Pol3 to restrict lytic replication. Therefore, activation of the RNA-sensing pathways mediated by MAVS may directly sense mtRNA to trigger an immune response.

## Introduction

The only known function of the HSV protein UL12.5 is the depletion of both mtDNA and mtRNA during both lytic infection as well as reactivation from peripheral neurons. Recently, UL12.5 was shown to induce the release of mtDNA via bursting endosomes that associated with cGAS [301] and mtDNA transcripts have been detected in the cytosol following UL12.5 expression [321]. Ultimately, activation of the cGAS-STING pathway is largely considered the primary mechanism by which UL12.5 restricts lytic viral growth [293]. However, Berry et al demonstrated that while UL12.5 may induce the release of mtDNA into the cytosol, RNA transcripts can be generated from the mtDNA and subsequently activate the RNA-sensing pathway mediated by retinoic acid-inducible gene 1 (RIG-I) [321]. Although these experiments were performed in cells with a high reliance RNA Pol3, they show it is possible to activate RNA-sensing pathways following UL12.5 expression, albeit indirectly. The extent to which additional pathways are activated by UL12.5 is not known.

The release of mtRNA directly from mitochondrial has not been considered a major contributing factor to the immune pathway activation induced by UL12.5. However, mtRNA is sufficient to trigger innate immunity [457] and mtRNA is being increasingly appreciated as an important immunostimulatory molecule in response to viral infection [458]. The depletion of mtRNA by UL12.5 is too rapid to be attributed to defects in translation [316], suggesting that they may instead be targeted directly or indirectly by UL12.5. The mitochondrial-resident nucleases, endonuclease G (ENDOG) and exonuclease G (EXOG), contribute to the depletion of mtDNA and have been

shown to exhibit some RNase activity [323,324]. Therefore, we hypothesized that mtRNA may directly activate cytosolic RNA-sensors, such as RIG-I or melanoma differentiation-associated protein 5 (MDA5).

RIG-I recognizes short double-stranded RNA (dsRNA) while MDA5 senses relatively longer dsRNA [459], but the length of mtRNA species generated by UL12.5, if any, is unknown. Regardless, RIG-I and MDA5 are essential mediators of cytosolic RNA-sensing and converge on major signaling protein mitochondrial antiviral-signaling protein (MAVS). Upon activation, MAVS can activate an inflammatory response via NF-κB or IFN response via a TBK1 complex [460], suggesting that MAVS activation might contribute to the TBK1 activation observed downstream of UL12.5.

To further investigate the impact of UL12.5 in lytic replication, we sought to characterize additional downstream nucleic acid-sensing pathways that can be activated by UL12.5 expression during lytic replication. Using a human cell line reported to lack a functional cGAS-STING pathway, we show that UL12.5 expression restricts viral growth through a mechanism independent of DNA-sensing mediated by cGAS-STING. Furthermore, we demonstrate that UL12.5 mediates mtRNA depletion during lytic infection of human cells and that activation of the RNA-sensing pathway mediated by MAVS contributed to the growth restriction phenotype we observe. Ultimately, this study suggests that UL12.5 expression may be associated with activation of the MAVS-mediated RNA-sensing pathway and that this is sufficient to restrict viral growth independent of STING activation.

## Results

### UL12.5 depletes mtRNA in human cells lines.

Previously, UL12.5 has been shown to deplete mtRNA in cell lines derived from murine tissue [320]. To confirm that UL12.5 mediates mtRNA depletion in human tissue, we infected immortalized human foreskin fibroblasts (HFF-T) and immortalized ARPE-19 (ARPE-T) cells with either KOS-SPA or KOS-UL98. Both cells allow for studies using a physiologically relevant human cell type in lytic HSV infection, but ARPE-T cells generated and kindly provided by Dr. Agnel Sfeir are reported to lack a functional cGAS-STING pathway [461,462]. ARPE-T cells are therefore useful in investing viral replication that is unaffected by cGAS-STING activation. RT-qPCR of mtRNA transcripts 24 hours post-infection showed that mtRNA is significantly reduced during lytic infection in the presence of UL12.5 expression in both HFF-T and ARPE-T (Figure 1A & B). Therefore, mtRNA depletion by UL12.5 occurs in human cells and is not a species-specific phenotype.

### UL12.5 restricts viral growth in cells lacking cGAS-STING

Previous reports have demonstrated that the downstream consequences of UL12.5 during lytic infection can differ between cell types [293,321]. To determine whether UL12.5 restricts viral replication in our model systems, we again infected HFF-T and ARPE-T cells with KOS-SPA or KOS-UL98 and measured infectious virus production. A significant decrease in production of infectious virus was associated with expression of UL12.5 in both HFF-T and ARPE-T cell lines (Figure 2A & B). Interestingly, the degree of restriction was noticeably higher during infection of HFF-Ts compared to ARPE-Ts.

Given that mtDNA release has been hypothesized thus far to mediate the restriction of lytic HSV growth in replicating cells, it was surprising to see that UL12.5 restricted viral growth in a cell line lacking a functional cGAS-STING pathway. To confirm these results, we performed a single-step growth curve of both KOS-SPA and KOS-UL98 during lytic infection of ARPE-T cells. The virus lacking *UL12.5* was shown again to replicate better in ARPE-T cells compared to the WT virus (Figure 2C). Therefore, UL12.5 expression may restrict lytic viral growth independent of the cGAS-STING pathway.

## MAVS contributes to viral growth restriction downstream of UL12.5

To investigate the role of UL12.5 expression during lytic replication in ARPE-T cells, we utilized knockout (KO) cell lines for *STING* or *MAVS* that are derived from the immortalized parent ARPE-T line. Following a 24-hour infection with either KOS-SPA or KOS-UL98, we found that lytic viral growth was unaffected within the *MAVS* KO ARPE-T cells, but the restriction of viral growth by UL12.5 was maintained in the *STING* KO cells (Figure 3A). These data suggest that UL12.5 expression is capable of activating MAVS, which can restrict lytic viral growth.

In THP-1 cells, UL12.5 was shown to activate RIG-I using RNA transcripts derived from mtDNA and RNA Pol3 [321]. Given that THP-1 cells are highly sensitive to RIG-I activation as macrophage-derived cells, we hypothesized that RNA Pol3 activity downstream of UL12.5 is not required to restrict lytic viral growth. To examine the role of RNA Pol3 downstream of UL12.5, we infected ARPE-T cells in with KOS-SPA or KOS-

UL98 in presence of an RNA Pol3 inhibitor and found that RNA Pol3 inhibition did not affect the amount of infectious virus produced (Figure 3B). Altogether, these data suggest that RNA transcripts generated by UL12.5 activate MAVS to restrict viral replication.

## Discussion

It is well-established that UL12.5 expression activates innate immune signaling pathways to restrict viral replication. This was initially attributed to activation of the cGAS-STING pathway mediated directly by detection of mtDNA released into the cytosol [293]. A more recent report demonstrated that following UL12.5 expression, mtDNA can indirectly activate RIG-I sensing pathways via the generation of RNA transcripts from RNA Pol3 [321]. These studies and our recent reports demonstrate that UL12.5 can differentially activate pathways, especially depending on the cell type. In this chapter, we show that RNA-sensing pathways mediated by the signaling protein MAVS contribute to the growth restriction of lytic replication downstream of UL12.5. Moreover, these data highlight the ability for UL12.5 to differentially activate downstream signaling pathways as well as the ability to activate multiple pathways.

As noted in previous chapters, the differential activation of pathways during lytic infection is a unique feature of UL12.5. Given that both DNA- and RNA-sensing pathways have been shown to play a role in replication and that HSV encodes multiple proteins to inhibit both pathways, it is likely that multiple pathways are activated by UL12.5 during lytic replication. Indeed, Berry et al. showed that while cGAS activation contributed minimally innate immune activation in THP-1 cells by UL12.5, the primary response was via RIG-I [321]. Although not a direct comparison, the magnitude of viral growth restriction by UL12.5 was slightly higher in HFF-T compared to ARPE-T. This may be due to the activation of both cGAS-STING as well as MAVS in HFF-Ts, while only MAVS is activated in ARPE-Ts. While the RNA Pol3 inhibitor appeared to have no

effect on KOS-SPA or KOS-UL98 lytic replication, it will be important to validate inhibition has occurred to confirm this phenotype. The activation of multiple nucleic acid-sensing pathways suggests a combinatorial effect of different degrees of innate immune activation that can contribute to the heterogeneity of HSV infection.

Moving forward, it will be important to delineate what mtRNA species are produced by UL12.5 and how these mtRNA transcripts are sensed. While a recent report indicates that mtDNA is sensed by cGAS association with ruptured endosome, it is unclear when and how mtRNA transcripts might be released from mitochondria. One possible explanation is through the BAX/BAK pore, although UL12.5 expression is not associated with morphological changes consistent with apoptosis [316] that results from BAX/BAK pore formation in replicating cells. Other potential pores include the mitochondrial permeability transition pore (mPTP) or voltage-dependent anion channel (VDAC) pores, but how each of these pores form in response to mtDNA and/or mtRNA stress is unknown. Moreover, the release of mitochondrial nucleic acids might be facilitated differently between cell types, particularly as sympathetic neurons lack BAK [463].

Altogether, this chapter provides additional evidence that activation of the cytosolic RNA-sensing pathway likely contributes to the restriction of viral growth due to UL12.5 expression. Importantly, this is the first evidence that sensing mtRNA directly released from mitochondria maybe playing a role in activating these innate immune pathways. The downstream consequences may be incredibly varied between cell types

and contexts, and our work further demonstrates that UL12.5 can activate several different pathways through its interaction with the mitochondria.

## Figures

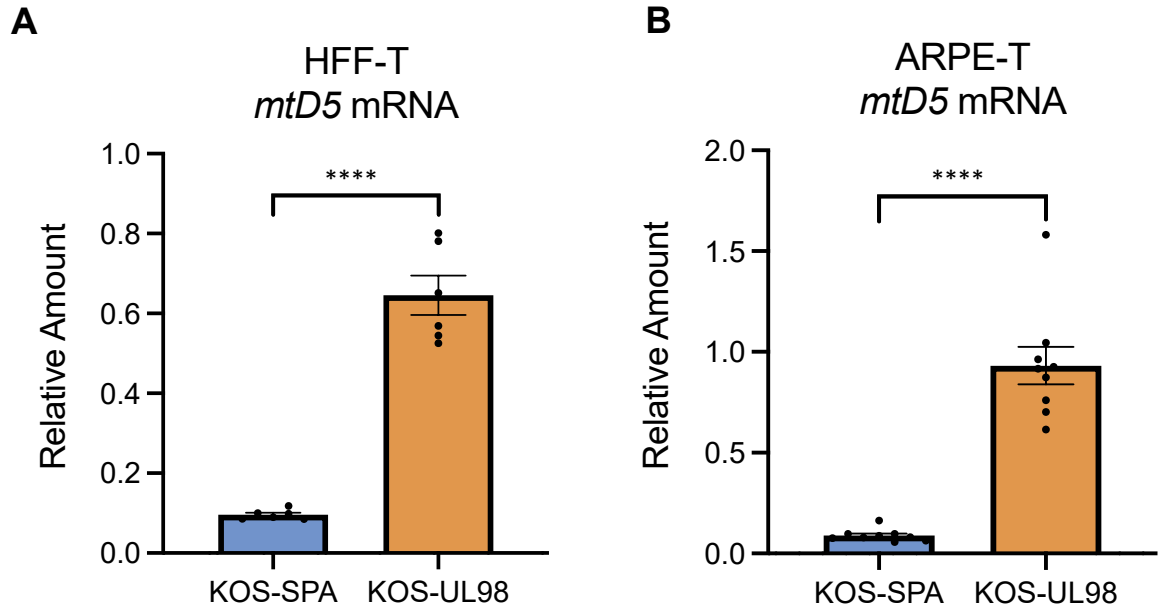


**Figure 1. UL12.5 depletes mtRNA in human cells lines.** (A-B) Quantification of mitochondrial RNA by RT-qPCR of *mtD5* transcripts after 24-hour infection of HFF-Ts or ARPE-Ts with KOS-SPA or KOS-UL98. All data are plotted as biological replicates from three independent experiments; shown as mean +/- standard errors of the mean (SEM). Shapiro-Wilk normality test; student t-test; ****, p<0.0001.

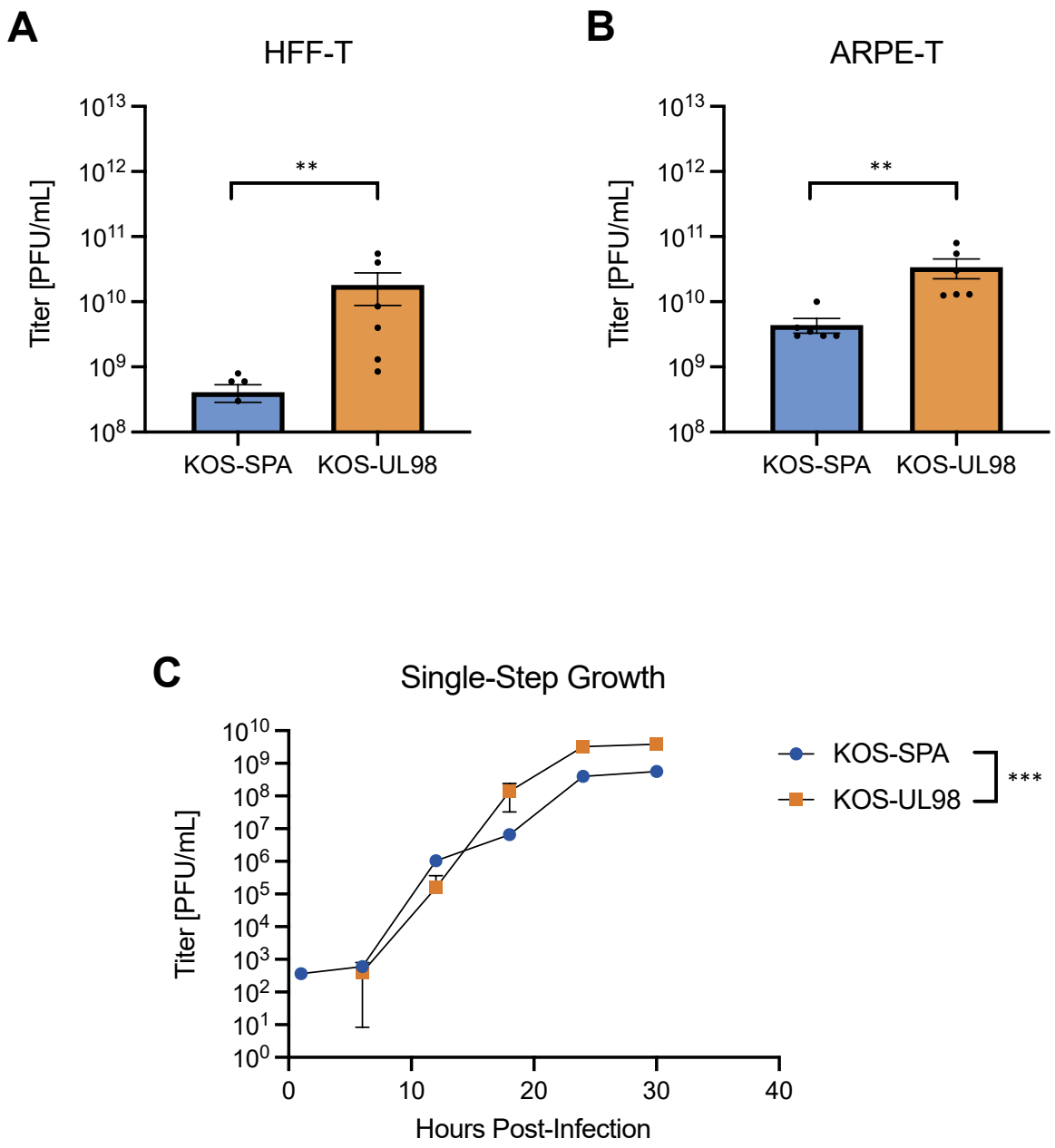


**Figure 2. UL12.5 restricts viral growth in cells lacking cGAS-STING.** (A-B) Titers of infectious virus 24 hours after infection of HFF-Ts or ARPE-Ts with KOS-SPA or KOS-UL98. (C) Titers of infectious virus through 28 hours of infection of ARPE-Ts with KOS-SPA or KOS-UL98. All data are plotted as biological replicates from three independent experiments; shown as mean +/- standard errors of the mean (SEM). Shapiro-Wilk normality test; Mann-Whitney; **, $p<0.01$.

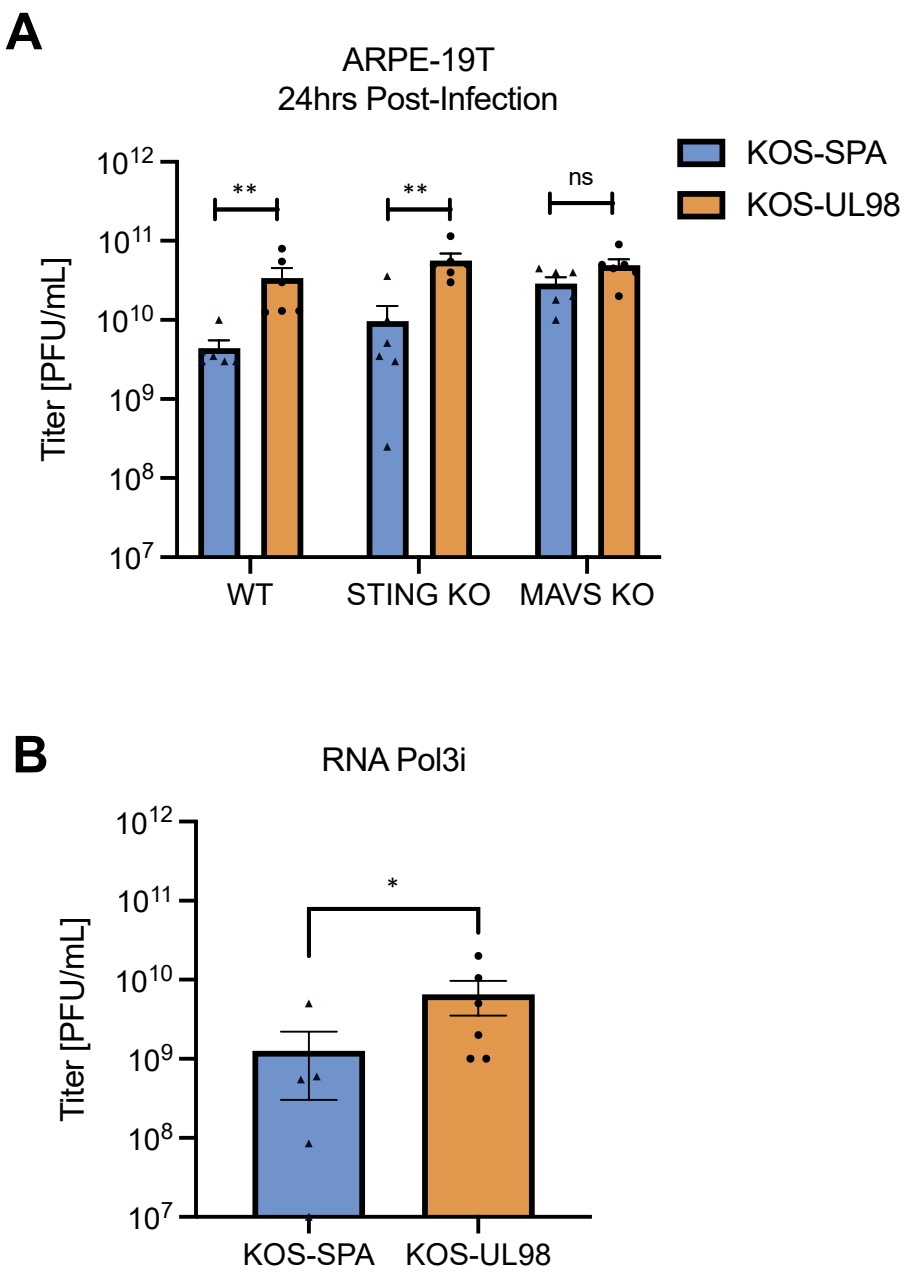


**Figure 3. MAVS contributes to viral growth restriction downstream of UL12.5.** (A) Titers of infectious virus 24 hours after infection of STING knockout (STING KO) or MAVS knockout (MAVS KO) ARPE-Ts with KOS-SPA or KOS-UL98. (B) Titers of infectious virus 24 hours after infection of ARPE-Ts with KOS-SPA or KOS-UL98 in the presence of an RNA Pol3 inhibitor. All data are plotted as biological replicates from three independent experiments; shown as mean +/- standard errors of the mean (SEM). Shapiro-Wilk normality test; Mann-Whitney; **, $p<0.01$; *$p<0.05$.

*End of Chapter 5*

# *Chapter 6*

# Conclusions and Future Directions

Thus far, I have presented new data from multiple model systems that contributes to our understanding of the role of innate immune activity in promoting HSV reactivation. Each respective chapter contains a discussion of conclusions and future directions relevant to the data therein. In this final chapter, I will discuss the impact of our findings in aggregate, propose additional hypotheses, and suggest further areas of study.

## HSV Relies on Specifically-Neuronal Pathways

From the initial characterization of NGF-deprivation as a trigger of HSV reactivation in 1987 [51], activation of the NGF-deprivation pathway has provided an invaluable model for interrogating the cellular responses that drive HSV reactivation [53,54,57,86,91–95]. Of similar significance is the physiological nature of NGF signaling and TrkA activation in peripheral neurons, both of which are essential signaling events guiding neurodevelopment and the maintenance of neuronal integrity in maturity. This essentiality of NGF signaling to neurons makes it a prime target for co-option by HSV, as it is a pathway that should be readily available for activation in a viable peripheral neuron. Downstream of NGF-deprivation, DLK and JNK act as core signaling hubs that engage with multiple different intracellular pathways, including expressly neuronal functions such as axon pruning and dendritic arborization [89]. Our results further substantiate the reliance of HSV reactivation on DLK and JNK activation, adding to a model in which HSV can respond to a variety of stimuli that converge on DLK while also being able to employ the multiple pathways downstream of DLK. In this way, HSV has

demonstrated the remarkable ability to utilize a signaling hub that is integral to neuronal physiology.

Our work builds upon the idea that HSV utilizes signaling pathways inherent to neuronal physiology to facilitate the transition from a latent infection to reactivation. Action potential firing is a homeostatic mechanism of neurons and a core function of both CNS and PNS neurons. In a way similar to NGF-deprivation, we show that HSV has evolved to use the intrinsic neuronal ability to fire action potentials to facilitate reactivation. Our results demonstrate that neuronal hyperexcitability, independent of the originating stimuli tested, is sufficient to drive the viral genome into reactivation. Moreover, our findings that IL-1β can trigger HSV reactivation via hyperexcitability shows that HSV has evolved to utilize the non-canonical response of neurons to inflammatory cytokines that promotes neuronal excitation [353]. IL-1β is reported to regulate sleep and memory formation in neurons under homeostatic conditions [143], indicating that IL-1β plays a critical role in neuronal physiology and making the IL-1 signaling pathway another reliable system through which reactivation can be triggered. It will be important for future work to elucidate the additional signaling proteins that mediate HSV reactivation by IL-1β and how these proteins may fit uniquely into neuronal signaling systems.

The activation of neuronal STING in HSV reactivation similarly supports a model in which HSV leverages intrinsically neuronal cellular mechanisms. Sympathetic neurons that harbor latent HSV genomes lack expression of BAK [464,465] and the ability of

neurons to avoid restrict apoptotic cell death is well-described [280]. The suppression of cell death pathways is largely attributed to an inability to activate caspases due to low levels of APAF-1, high levels of X-linked inhibitor of apoptosis (XIAP), and the rapid degradation of cytosolic cytochrome c [466–468]. These factors provide an ideal and unique context in which UL12.5 can manipulate mtDNA/mtRNA and activate STING with less risk of inducing cell death typically associated with pronounced mitochondrial dysfunction [469]. Although STING activation is primarily attributed with induction of a robust antiviral response, upregulation of autophagy by STING is considered the neuronal response to HSV infection rather than an IFN response or inflammatory response [277,411–414]. STING activation also provides unique non-immunogenic functions in neurons, including axon regeneration following injury [286] and the inhibition of nociception downstream of type I IFN signaling [285]. The differential activation of STING in peripheral neurons and our results demonstrating that HSV relies heavily on STING activation to facilitate reactivation adds to the evidence of HSV evolution that is highly-tailored to specifically neuronal systems. While not covered in this study, understanding the pathways that contribute to and arise from STING activation in neurons will be essential in understanding how neurons respond to infection and how HSV achieves lytic gene transcription in Phase I of reactivation.

While the induction of a hyperexcitable state within peripheral neurons by IL-1β was expected, our results showing for the first time that IL-1β can activate DLK in peripheral neurons was a novel finding. IL-1β signals through JNK to mediate neuronal differentiation of early precursor cells [470], but the role of DLK and JNK signaling in

mature neurons was previously unknown. In fact, previous reports have shown that hippocampal neurons do not activate JNK after IL-1β treatment [471], suggesting that IL-1β elicits a unique response in peripheral neurons compared to CNS neurons. However, it is unclear what steps downstream of IL-1R activation contribute of DLK activation and how DLK activation affects or is affected by hyperexcitability in these neurons. The convergence of both IL-1β and STING on the major DLK-JNK signaling hub highlight the ability of HSV to sense invading pathogens that might be detrimental to neuronal survival. Reactivation following these stimuli may therefore represent a critical moment in which HSV can exit latency prior to the induction of a robust and potentially damaging immune response. Moreover, our results place DLK and JNK as highly relevant players in innate immune pathways within peripheral neurons. The significance of DLK and JNK activation is well-described and we expand on that knowledge here, but it will be important to know the full extent to which DLK and JNK engage with neuronal immunity and how these relationships affect neuronal viability and HSV reactivation.

While not entirely surprising given the lack of viral proteins present during latency to drive the transition to reactivation, the extent to which HSV highjacks highly-specific and integral neuronal pathways represents an appreciable pattern of turning otherwise systematic signaling pathways into mechanisms that support lytic gene transcription. These themes may be important in understanding or identifying additional clinical triggers of HSV reactivation. Axotomy, which is unique also to neuronal cells, is a well-established trigger of reactivation [472] that may provide the stimulus for reactivation in patients following peripheral nerve surgery [96]. Future work investigating the intracellular

mechanisms regulating HSV will benefit from considering the specific physiological aspects of neuronal systems, especially with respect to the differential activation of immune pathways and how these pathways relate to clinical manifestations of HSV reactivation.

### Other inflammatory molecules have been implicated in HSV reactivation

While others have suggested that inflammatory cytokines upregulated systemically during reactivation may contribute to reactivation, our work was the first to confirm neuronal sensing of inflammatory cytokines can support HSV reactivation. Using our reductionist approach to study HSV reactivation that utilizes *in vitro* modeling of latency and reactivation, we were able to define specific effects allotted by IL-1β on sympathetic neurons and how these contribute to HSV reactivation. The induction of reactivation in the presence of IL-1β may seem paradoxical, but can provide some advances to HSV persistence. In contrast to CNS neurons that use IL-1β to mediate homeostatic functions [143], systemic inflammatory responses to infection expose peripheral neurons to high levels of immune signaling molecules, including both inflammatory cytokines as well as IFNs. Given the diversity of cells of the peripheral ganglia in which reactivating neurons reside, it is possible that variable release of IL-1β can trigger reactivation in some neurons while IFNs, which are known to both induce latency and restrict reactivation [92,98,409], can sustain or re-initiate latency in others in order to preserve a lasting population of infected cells. The heterogeneity observed in reactivation may be due to these subtle differences in signaling combined with potential differences in chromatin states of viral episomes. While our study focused on the

intracellular mechanisms governing IL-1β-mediated reactivation, it will be important for future work to determine how these pathways function *in vivo* in the presence of neighboring cells and professional immune cells.

As another major inflammatory cytokine upregulated in reactivation, the precise role of IL-6 in HSV reactivation has been contested since the 1990s. Although there is evidence of IL-6 upregulation during reactivation *in-vivo* [104,442,473], a considerable consensus is lacking in whether its action is pro-viral or antiviral. Initial reports demonstrated that anti-IL-6 antibodies reduced HSV reactivation *in-vivo* [443] and HSV lacking IL-6 response elements in the LAT promoter region reactivated less than controls [440], altogether suggesting a role of IL-6 in supporting reactivation. However, other studies showed no significant difference in HSV latency or reactivation in IL-6 knockout mice [441]. Additional *in-vivo* findings revealed a correlative dose-dependent decrease in IL-6 with decreased HSV reactivation in the presence of corticosterone inhibitor [442]. These results are further confounded by the interdependent and convergent nature of major inflammatory cytokines, which may compensate for each other or modulate the cellular response in the context of neuronal HSV infection. IL-6 is also reported to inhibit JNK in neurons [474,475], which would thereby necessitate a JNK-independent mechanism to mediate reactivation. Ultimately, significant data directly linking HSV reactivation to IL-6 and the effect of IL-6 in HSV-infected neurons is lacking and presents an interesting track to pursue given the available *in-vitro* cellular models that have been developed since these initial reports.

TNFα has been reported to enhance rates of HSV reactivation [444], though any mechanism remains unknown and others have reported that TNFα inhibited reactivation [445]. This discrepancy may be due to a difference in experimental system, a requirement for TNFα in the establishment of latency, or, given that TNFα is secreted around latently infected neurons *ex*-vivo, the removal of TNFα releases an inhibition of reactivation. However, in light of IL-1β, the potential for TNFα to support reactivation seems plausible, especially given that TNFα receptor activation can result in JNK phosphorylation in neurons [476,477]. Moreover, H4 hyperacetylation downstream of neuronal TNFα [477,478], which is associated with chromatin decompaction in lytic HSV infection [338], leaves open the possibility that TNFα has the potential to promote opening the closed chromatin of the viral episome during reactivation.

### The complete functional significance of UL12.5 is not yet known

The role of mtDNA depletion by UL12.5 is well-described across species and cell-type and mtDNA and mtRNA depletion is the only known function of UL12.5 [315–319,321,322]. Although this function results in reduced lytic replication of the virus, our work has shown that UL12.5 instead plays a critical role in promoting lytic gene transcription during Phase I of HSV reactivation. However, major questions remain about how mtDNA/mtRNA depletion is achieved and the full consequences of this action. Although UL12.5 retains much of the same nuclease activity as UL12 [314], others have shown that UL12.5 recruits the mitochondrial-resident nucleases ENDOG and EXOG to deplete mtDNA [318]. Some of the cleavage sites of ENDOG have been described, but the extent to which UL12.5 or ENDOG/EXOG contribute to mitochondrial stress and the release of

mtDNA into the cytosol in the context of HSV infection is unknown. We have identified STING activation as a critical step in HSV reactivation, but the potential for UL12.5 to activate other pathways should not be underestimated. One report using UL12.5 as a tool to remove mtDNA from cells showed that UL12.5 does induce minor mitochondrial membrane depolarization [320], but the extent to which this depolarization stresses mitochondria or how this depolarization modulates signaling pathways in neurons is unclear. Future work elucidating the extent of immune pathways activated by UL12.5 will benefit our understanding of the cellular mechanisms affecting both lytic infection and reactivation.

Additional DNA-sensing pathways have been shown to respond to mtDNA and therefore might also be activated downstream of UL12.5. Following activation of the cytosolic nucleic acid sensor ZBP1 (Z-DNA-binding protein 1) by mtDNA, TBK1 is activated to initiate an immune response [479]. Importantly, TBK1 activation is also an important step in mediating gene transcription downstream of STING activation. One major limitation of our current work is the lack of characterization of signaling events downstream of STING and which signaling molecules specifically contribute to the induction of lytic gene transcription. Given that both ZBP1 and STING can sense mtDNA and converge on activation of TBK1, we will have to determine what downstream effectors of each pathway can contribute to lytic gene transcription in Phase I. In the same way that DLK and JNK activation from multiple stimuli can trigger reactivation, different mtDNA or mtRNA species might be able to activate multiple pathways that can support lytic gene transcription. Although our work showed that

activation of RNA-sensing pathways let to low levels of reactivation, activation of these pathways by other RNA species remains a possibility and should be investigated further.

Although Berry et al has shown that RIG-I can be activated by UL12.5 expression following the generation of RNA transcripts from mtDNA via RNA Pol3 [321], our results suggest that UL12.5 can activate an RNA-sensing pathway independent of RNA Pol3 activity. Notably, our findings suggest a more direct interaction between MAVS or RIG-I and mtRNA transcripts. However, the cause of mtRNA depletion and extent to which mtRNA can be released into the cytosol after UL12.5 expression has not yet been described. ENDOG and EXOG display low levels of RNase activity [323,324], but future work will need to identify the types of RNA species generated by UL12.5, as these can differentially activate cytosolic nucleic acid sensors. For instance, MDA5 preferentially recognizes long doubles-stranded RNA (dsRNA) while RIG-I sense shorter species of dsRNA [459]. The significance of RNA-sensing and how it differs from DNA-sensing neurons will be an important question to consider when trying to understanding viral interactions with host neurons. Future studies will also have to elucidate the full repertoire of downstream signaling cascades activated by UL12.5 and, importantly, how differential activation of these pathways is achieved between different cell types.

Mitochondrial stress is hypothesized to cause the release of mtDNA into the cytosol following different stressors, including UL12.5. Newman et al recently described how endosomal rupture of mtDNA-containing vesicles associated with mitochondria

release mtDNA to activate cGAS following UL12.5 expression [301]. In line with previous reports that UL12.5 does not induce apoptosis [316], this would suggest that UL12.5 induces a level of mitochondrial stress that is not severe enough to activate a BAX/BAK pore or the mPTP, which would activate cell death pathways in many cell types. Given the potential differences in mitochondria between cell types as well as between subcellular neuronal compartments, it will be important to confirm that this mechanism of mtDNA downstream of UL12.5 expression occurs in neurons and how it may affect activation of cGAS, STING, or other sensors.

### STING activation can promote lytic transcription

Both DLK/JNK and STING make ideal candidates for manipulation by HSV in that activation of either pathway will not induce a potentially cytotoxic immune response. However, a major question that remains from our work is how STING activation leads to a rapid induction of lytic gene transcription in Phase I. cGAS-STING is reported to activate JNK [480], which could potentially mediate the lytic gene expression we see in Phase I and Phase II. Whether this occurs following neuronal STING activation or how it could contribute to lytic gene expression after STING activation remain open questions, however. Intriguingly, the timing difference required to rescue KOS-UL98 gene expression during reactivation, only occurring when DMXAA added 2.5 hours after LY, suggests that STING activation must follow JNK activation to initiate Phase I gene expression. Our results come with the caveat that LY was added to the reactivating culture the entire time, so we are unable to parse apart exactly how long and when JNK signaling is required for Phase I using this paradigm. Additional experiments showing

that reactivation triggered by DMXAA was reliant on JNK activity suggest that a second round or continuous activation of JNK may be required for lytic gene transcription in Phase I. In our system, this would mean that LY stimulates JNK-dependent expression of UL12.5, which in turn activates STING to activate JNK again to maintain transcriptional activation. As these systems are incredibly complex, it is possible that additional transcriptional or pioneer factors are recruited to facilitate the transition from a latent to transcriptionally active viral genome. Future work will have to consider the temporal kinetics governing both transcription as well as signaling pathways when investigating the cellular mechanisms responsible for Phase I.

Given the significance of the phospho/methyl switch in lytic transcription in Phase I, it is likely STING induces lytic gene expression by inducing a similar phospho/methyl switch that avoids the use of host histone demethylases. Although the full extent of activation downstream of neuronal STING is unknown, some studies suggest that the NF-κB pathway can be activated by STING in neurons [481]. Central to NF-κB signaling are the IκB kinases (IKKs), of which IKKα has been found to mediate phosphorylation of histone H3S10, in addition to the histone variant H3.3 at serine 31 in cortical neurons to promote gene transcription [456]. H3.3 is a histone variant that can regulate viral gene expression during lytic replication, but is reported to be replaced by histone 3.1 that is more stably repressive [482]. While viral genomes in our *in vitro* model system likely associate with H3.3 due to the use of ACV in establishing latency, whether H3.3 persists within a subset of lytic gene promoters *in vivo* is not known and how IKKα may be activated downstream of STING in neurons has not be defined. Although the

connection between STING activation and IKKα activation in this manner is yet unclear, it is possible that activation of IKKα downstream of STING phosphorylates histones associated with lytic gene promoters associated with histones. This hypothesis is in line with the reliance of HSV on rapid initiation of lytic transcription via a phospho/methyl switch, but determining whether IKKα can phosphorylate histones downstream of STING in neurons will be central to this idea.

It is possible that HSV co-opts other NF-κB signaling cascades downstream of STING activation to induce transcription of lytic genes. NF-κB can activate transcription directly by binding to promoters or inducing chromatin remodeling that permits gene transcription. HSV activates NF-κB during lytic replication [483] and NF-κB plays a well-established role in controlling inducible gene expression [250,484], but the ultimate consequences of this activity in peripheral neurons, particularly in the context of reactivation, is not yet known. Some HSV genes, such as the IE gene *ICP4*, contain consensus sequences for NF-κB and activated NF-κB can enhance expression of these genes during lytic replication [483,485,486]. ICP4, in particular, is a viral transcription factor that regulates viral gene expression [487] and the ICP4 promoter region undergoes chromatin remodeling to permit rapid transcription with 30 minutes of a reactivation stimulus *in vivo* [111]. Therefore, it is possible that activated NF-κB in the nucleus following STING activation may induce remodeling of vDNA chromatin, such as the ICP4 promoter, to facilitate lytic gene transcription.

Another possibility is that in response specifically to activation of cGAS-STING by mtDNA, DNA damage response proteins may facilitate the removal of repressive heterochromatic marks to allow for IE gene expression. This hypothesis is possible given the observation that PARP9, a chromatin remodeler that enhances ISG expression, is upregulated in response to DNA damage [488] or mtDNA-mediated cGAS-STING activation [325]. This possibility is underscored by the finding that mtDNA stress resulted in a specific pattern of ISG expression that is tailored to DNA damage repair [325]. Activation of the DNA damage response is a known trigger of HSV reactivation and HSV replication is impaired during lytic infection in the absence of a DNA damage response [489]. Peripheral neurons have neuronal-specific ISG patterns in response to IFN sensing [92], so it is possible that in peripheral neurons mtDNA induces a more unique subset of ISGs that can promote chromatin remodeling of the viral genome. Future investigations into the drivers of lytic gene transcription downstream of STING will be an essential next step in understanding the complexities underlying early events of Phase I of HSV reactivation.

### The mechanisms underlying Phase I are complex and still being uncovered

Phase I of HSV reactivation represents a critical stage in transitioning the latent viral episome into a transcriptionally active genome and is therefore key to viral transmission and persistence. In addition to the necessity of DLK and JNK activation to this process, our results show that STING activation by UL12.5 similarly is required for the induction of lytic gene transcription. In the absence of UL12.5, we found that peak lytic gene expression is drastically reduced during Phase I, likely causing the failure of lytic gene transcription and vDNA replication observed in Phase II. For reasons that are

not entirely known, Phase I may not always progress to Phase II (full reactivation) that produces infectious virus [84,397]. The activation of DLK/JNK and STING may therefore constitute a checkpoint in which full reactivation only occurs if the virus can effectively 1) initiate lytic gene expression via the neuronal stress response and 2) maintain lytic gene expression via STING activation. Accumulation of the viral transactivator VP16 has been suggested to enable the transition from Phase I to Phase II [84], but we show that VP16 transcription in Phase I is dependent on UL12.5. Therefore, VP16 accumulation in Phase I may represent a readout for these initial checkpoints. It will be interesting for future studies to determine the extent of redundancy between JNK, STING, and other signaling proteins and our understanding of the evolutionary advantage of biphasic HSV reactivation.

*In vivo* studies have suggested that continuous IFN released from immune cells infiltrating the TG contribute to the maintenance of HSV latency [114,399,405,424,490]. While many cytokines are upregulated in TG following acute infection, even for months [404,424], the sustained immune response to latent infection is small compared to acute infection. In the absence of robust detectable viral protein synthesis early in Phase I [84], it is likely that the systemic immune response during Phase I is also relatively limited. The utilization of IL-1β signaling and STING activation in transitioning the viral genome out of latency is therefore specifically fitted to the low level of immune activation in Phase I. These signaling mechanisms would likely be implausible for use in other stages of the HSV life cycle, including Phase II, due to the amount of systemic immune activation in response to viral PAMPs [491]. Thus, the evolutionary pressures on HSV helped establish

the unique features of Phase I in which the virus paradoxically relies on immune activation for its survival. It will be important for additional studies to determine how evolutionary pressures promote different aspects of host-viral interactions and how these interactions contribute to different stages of the viral life cycle.

Our analysis of the H3K9me3/pS10 phospho/methyl switch following neuronal hyperexcitability showed that there are two potential waves of the switch during Phase I of reactivation. Therefore, it is possible lytic gene promoters are targeted during the first phase (~5hrs post-stimulus) as well as the second phase (~20hrs post-stimulus). In agreement with the hypothesis of sequential activation of JNK and STING outlined above, it is possible that PI3K inhibition leads to the first wave of the phospho/methyl switch while STING activation by UL12.5 results in the second. Considering the sequential activation of JNK and STING and the two stages of the phospho/methyl switch, Phase I may be able to further divided into two sub-phases. The beginning of Phase I, termed Phase Ia, may be a burst of lytic gene transcription that permits UL12.5 expression and therefore STING activation. Phase Ib may then following STING activation, in which seemingly stochastic gene expression from all gene classes that is characteristic of Phase I is achieved. As an established trigger of HSV reactivation, axotomy has been hypothesized to bypass Phase I or go through Phase I too quickly to have been detected [339]. In this model, it is possible that axonal injury activates STING to more rapidly reach Phase Ib while skipping Phase Ia and consequently transition to Phase II more quickly. Overall, our work highlights the complexity in which multiple signaling pathways are responsible for Phase I and future studies will provide needed

insight into how these pathways interact to alleviate transcriptional repression of the viral genome.

### Mitochondria are key players in viral infection

Mitochondria are highly dynamic organelles, particularly within neurons that have a high degree of energy burden and long axons for trafficking. Perinuclear localization of mitochondria has been observed in HSV infection [492], but it is unclear whether UL12.5 differentially affects those mitochondria that are most proximal to the nucleus. Given that mitochondrial function is also hypothesized to function slightly differently between cell types in the nervous system [493], it will be important to discern whether aspects unique to peripheral or sympathetic neurons play a role in HSV reactivation. The morphological differences between mitochondria among different subcellular compartments (axon, dendrites, soma) of the same neurons [494] also suggests subtle differences in mitochondrial nature that may add nuance to UL12.5 function depending on the cellular compartment. Our results do not investigate how UL12.5 function is affected between different mitochondria, but these will be important considerations in our understanding of how UL12.5 functions in neurons.

The seemingly deliberate aggravation of mitochondria by viruses is a well-reported phenomenon across different virus types, including herpesviruses [495]. HSV-2 and murine gammaherpesvirus-68 (MHV-68), both related herpesviruses, contain proteins that mediate mtDNA depletion during infection [293]. While the HSV-2 protein is posited to be analogous to UL12.5, the protein promoting mtDNA stress in MHV-68 infection is unknown [293]. Another herpesvirus, Epstein-Barr virus (EBV), encodes a

transcriptional and regulatory factor, Zta, that re-localizes mitochondrial single-stranded binding (mtSSB) protein to the nucleus to supports EBV DNA replication [449]. The manipulation of mtDNA-associated factors is not ubiquitous to all herpesviruses, however, suggesting that this mechanism only survives evolutionarily in specific contexts. Our results describe how HSV utilizes mtDNA stress to promote lytic transcription in reactivation, but the potential benefits of the direct antagonism of host mitochondria by other viruses is largely undescribed.

In addition to UL12.5, HSV encodes at least 5 additional viral proteins that have been found to engage with host mitochondria during lytic replication. The role of these proteins includes modulating mitochondrial metabolism, altering mitochondrial dynamics, and BAX recruitment, but the ultimate functional significance of these proteins is yet undescribed in the context of reactivation [306,496–498]. This relatively high number of viral proteins associating with mitochondria would suggest that mitochondria play an important role in HSV infection. While we established a role for UL12.5 and mtDNA stress in reactivation, it will be important to determine how additional HSV proteins contribute to STING activation and whether these virus-mitochondrial interactions can similarly support lytic transcription in reactivation.

The manipulation of mtDNA and activation of DNA-sensing pathways is an emerging theme across both DNA and RNA viruses. Influenza A virus (IAV), an RNA virus, encodes the M2 protein that induces the release of mtDNA into the cytosol to activate both cGAS and DDX41 [448]. Importantly, downstream activation by M2 is

abrogated by the nonstructural protein 1 (NS1), suggesting a highly controlled manipulation of mtDNA by IAV [448]. However, it is unclear whether M2-induced mtDNA stress and/or cGAS activation provide any replicative benefit to IAV. Interestingly, HSV may employ a similar system in which UL12.5 mediates mtDNA stress and the activation of cGAS-STING, but this activation is later inhibited through in inhibition of STING with ICP27 [450]. A recent paper also found that norovirus, an RNA virus, encodes the NS4 protein which mediates the release of both nuclear and mtDNA into the cytosol to activate STING and trigger an IFN response [499]. In contrast to IAV and other RNA viruses that actively inhibit STING-dependent antiviral responses, it is unknown whether noroviruses encode proteins that inhibit or subvert these pathways. Despite the numerous viral proteins that induce mtDNA release into the cytosol, whether these virus-host interactions ultimately serve to benefit viral replication more broadly will be important to determine. While our results show how this signaling mechanism can support viral persistence, additional studies will have to identifying potential additional signaling partners and how nuanced or differential activation of STING-mediated pathways affect viral persistence.

### Innate immune pathways are a critical aspect of HSV reactivation

In all, our studies have demonstrated the delicate and nuanced relationship between neuronal innate immunity and HSV reactivation. Activation of these immune pathways via external or internal stimuli can now be considered as essential to HSV reactivation. The integration of neuronal stress signaling and innate immunity highlight the ability of HSV to adapt to neuronal systems and rely on the unique signaling

infrastructure within peripheral neurons to enable reactivate. Further investigation into the interactions between these simultaneously diverse and diverging pathways will be important in understanding what drives HSV reactivation and identifying potential therapeutic strategies to limit HSV reactivation and persistence.

*End of Chapter 6*

# *Chapter 7*

# Materials and Methods

**Reagents**

Compounds used in the study are described in Table 1. 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 concentrations were used based on previously published IC50s and assessed for neuronal toxicity using cell body and axon health and degeneration index as previously published [83]. All compounds used had an average score ≤1.

**Table 1. Compounds used and concentrations**

| Compound | Supplier | Identifier | Concentration |
|---|---|---|---|
| NGF 2.5S | Alomone Labs | N-100 | 50 ng/mL |
| Aphidicolin (APH) | AG Scientific | A-1026 | 3.3 µg/ml |
| L-glutamic acid | Millipore Sigma | G5638 | 3.7 µg/ml |
| Acycloguanosine | Millipore Sigma | A4669 | 10 µM, 50 µM |
| FUDR | Millipore Sigma | F-0503 | 50 µg/mL |
| Cycloheximide | Sigma-Aldrich | C4859 | 10 µg/mL |
| LY294002 | Tocris | 1130 | 20 µM |
| H-151 | Tocris | 6675 | 5 µM |
| Ethidium bromide | Thermo Fisher | 15585011 | 200 ng/mL |
| Sodium pyruvate | Thermo Fisher | 11360070 | 1 mM |
| DMXAA | Invivogen | tlrl-dmx | 50 µg/mL |
| ISD/LyoVec™ | Invivogen | tlrl-isdc | 5 µg/mL |
| ADU-S100 | Invivogen | | 10 µg/mL |

| | | | |
|---|---|---|---|
| Poly(I:C) LMW/LyoVec™ | Invivogen | tlrl-picwlv | 10 µg/mL |
| Poly(I:C) HMW/LyoVec™ | Invivogen | tlrl-piclv | 10 µg/mL |
| Poly(dA:dT)/LyoVec™ | Invivogen | tlrl-patc | 5 µg/mL |
| Primocin | Invivogen | ant-pm-1 | 100 µg/ml |
| G418/Geneticin | Gibco | 10131-035 | 250µg/mL |
| PRIME-XV IS21 Neuronal Supplement | Irvine Scientific | 91142 | |
| GDNF | Peprotrech | 450-44 | 50 ng/mL |
| WAY-150138 | Pfizer | N/A | 10 µg/mL |
| CyclosporinA | Sigma Aldrich | C3662 | |
| BAX channel inhibitor (CAS 335165-69-0) | Sigma Aldrich | 196805 | 15 µM |
| Bax inhibitor peptide V5 | Sigma Aldrich | B1436 | 100 µM |

**Preparation of HSV virus stocks**

HSV stocks of KOS-UL98, KOS-SPA, F/L, and F/L Rescue were propagated and titrated on Vero cells obtained from American Type Culture Collection (Manassas, VA). Vero cells were maintained in Dulbecco's modified Eagle's medium (Gibco)

supplemented with 10% FetalPlex (Gemini Bio-Products). KOS-UL98, KOS-SPA, F/L, and F/L-Rescue were kindly provided by James Smiley [319].

Stocks of AN-1 and KOS37 (WT) were propagated and titrated on UL12 complementing 6-5 cells. 6-5 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FetalPlex (Gemini Bio-Products). KOS37 AN-1 and KOS37 (WT) were kindly provided by Sandra Weller [500] and the 6-5 cells were provided by Renata Szcepaniak.

Stocks of Stayput Us11-GFP were propagated and titrated on gH-complementing F6 cells [57,501]. Vero F6 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FetalPlex (Gemini Bio-Products) and 250µg/mL of G418/Geneticin (Gibco).

**Measuring of infectious virus**

For *in vitro* infections, sterile milk was added to wells of a 24-well plate with infected neurons or dermal fibroblasts. Samples were then freeze-thawed at -80°C and 37°C three times to lyse cells. Homogenates from were simultaneously titrated on a monolayer of Vero cells at 34°C.

For *in vivo* infections, sterile milk was added to media containing dissected ganglia and samples were snap-frozen. Ganglia were thawed at 37°C and then homogenized using

a BeadBug™ microtube homogenizer. The homogenized ganglia were then sonicated at 25% for 30 s three times on ice in a Fisher brand model 120 sonic dismembrator. After sonication, homogenates of ganglia were frozen on dry ice and thawed at 37°C. Homogenates from all time points were simultaneously titrated on a monolayer of Vero cells at 34°C.

**Primary neuronal cultures**

Sympathetic neurons from the Superior Cervical Ganglia (SCG) of post-natal day 0-2 (P0-P2) CD1 Mice (Charles River Laboratories) were dissected as previously described [54]. Sensory neurons from TG of adults were dissected as previously described [502] with a modified purification protocol using Percoll [503]. Rodent handling and husbandry were carried out under animal protocols approved by the Animal Care and Use Committee of the University of Virginia (UVA). Ganglia were briefly kept in Leibovitz's L-15 media with 2.05 mM l-glutamine before dissociation in collagenase type IV (1 mg/ml) followed by trypsin (2.5 mg/ml) for 20 min; each dissociation step was 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 feeding media: 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 media for the first five days post-dissection to select against proliferating cells. Sensory neurons were maintained in the same media supplemented with GDNF (50 ng/ml; PeproTech 450-44).

## Lytic HSV infections

DF were maintained in DMEM + 10% FBS and infected with either KOS-SPA or KOS-UL98 at an MOI of 0.3 PFU/cell for 1 hour in DPBS + CaCl2 + MgCl2 supplemented with 1% fetal bovine serum, 4.5 g/L glucose. Neurons were infected with either KOS-SPA or KOS-UL98 at an MOI of 10 PFU/cell for 1 hour in DPBS + CaCl2 + MgCl2 supplemented with 1% fetal bovine serum, 4.5 g/L glucose.

## Mouse infections

Five-week-old male and female CD-1 mice (Charles River Laboratories) were anesthetized by intraperitoneal injection of ketamine hydrochloride (80 mg/kg of body weight) and xylazine hydrochloride (10 mg/kg) and inoculated with $1 \times 10^6$ PFU/eye of virus (in a 5 µL volume) onto scarified corneas, as described previously [65]. Mice were housed in accordance with institutional and National Institutes of Health guidelines on the care and use of animals in research, and all procedures were approved by the Institutional Animal Care and Use Committee of the University of Virginia. Criteria used for clinical scoring based on the formation of lesions and neurological and eye symptoms are shown in Table 2 and were based on a previously established scoring scale [504]. Mice were randomly assigned to groups, and all experiments included biological replicates from independent litters.

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

**Establishment and reactivation of latent HSV infection in primary neurons**

P6-8 SCG neurons were infected with eGFP-Us11 at MOI 7.5, Stayput Us11-GFP at MOI 10 PFU/cell, KOS-UL98/KOS-SPA at MOI 10 PFU/cell, F/L/F/L-Rescue at MOI 5 PFU/cell, or AN-1/WT at MOI 5 PFU/cell (assuming 10,000 cells per well) in Dulbecco's Phosphate Buffered Saline (DPBS) + $CaCl_2$ + $MgCl_2$ supplemented with 1% fetal

bovine serum, 4.5 g/L glucose, and 10 mM acyclovir (ACV) for 4 h at 37°C. Post infection, inoculum was replaced with feeding media (as described above) with 50 mM ACV. 7 days post-infection, ACV was washed out and replaced with feeding media alone. Reactivation was quantified by counting number of GFP-positive neurons or performing reverse transcription–quantitative PCR (RT–qPCR) of HSV lytic mRNAs isolated from the cells in culture. WAY-150138 (10 μg/ml) was added to the reactivation cocktail for eGFP-Us11, KOS-UL98, and KOS-SPA experiments to limit cell-to-cell spread.

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

To assess relative expression of HSV lytic mRNA, total RNA was extracted from approximately 1.0 × 104 neurons using the Quick-RNA™ Miniprep Kit (Zymo Research) with an on-column DNase I digestion. mRNA was converted to cDNA using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fisher Scientific), 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 1.0 × 104 neurons using the Quick-DNA™ Miniprep Plus Kit (Zymo Research). qPCR was carried out using PowerUp™ SYBR™ Green Master Mix (ThermoFish Scientific). The relative mRNA or DNA copy number was determined using the comparative CT (ΔΔCT) method normalized to mRNA or DNA levels in latently infected samples. Viral RNAs were normalized to mouse reference gene 18S rRNA. All samples were run in triplicate on an

Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System and the mean fold change compared to the calculated reference gene. Exact copy numbers were determined by comparison to standard curves of known DNA copy number of viral genomes.

**Preparation of lentiviral vectors**

Lentiviruses expressing shRNA against STING (STING-1 TRCN0000346321, STING-2 TRCN0000346266), DLK (DLK-1 TRCN0000022572, DLK-2 TRCN0000022573, or a control lentivirus shRNA (Everett et al, 2006) were prepared by co-transfection with psPAX2 and pCMV-VSV-G [505] using the 293LTV packaging cell line (Cell Biolabs). Supernatant was harvested at 40- and 64-h post-transfection and filtered using a 45 μM PES filter. Sympathetic neurons were transduced overnight in neuronal media containing 8μg/ml protamine and 50 μM ACV.

**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). Images were acquired using an sCMOS charge-coupled device camera (pco.edge) mounted on a

Nikon Eclipse Ti Inverted Epifluorescent microscope using NIS-Elements software (Nikon). Images were analyzed and intensity quantified using ImageJ.

**Western Blot Analysis**

Neurons were lysed in RIPA Buffer with cOmplete, Mini, EDTA-Free Protease Inhibitor Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche) on ice for one hour with regular vortexing to aid lysis. Insoluble proteins were removed via centrifugation, and lysate protein concentration was determined using the Pierce Bicinchoninic Acid Protein Assay Kit (Invitrogen) using a standard curve created with BSA standards of known concentration. Equal quantities of protein (20-50 µg) were resolved on 4-20% gradient SDS-Polyacrylamide gels (Bio-Rad) and then transferred onto Polyvinylidene difluoride membranes (Millipore Sigma). Membranes were blocked in PVDF Blocking Reagent for Can Get Signal (Toyobo) for one hour. Primary antibodies were diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo) and membranes were incubated overnight at 4$^{o}$C. HRP-labeled secondary antibodies were diluted in Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo) and membranes were incubated for one hour at room temperature. Blots were developed using Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate (PerkinElmer) and ProSignal ECL Blotting Film (Prometheus Protein Biology Products) according to manufacturer's instructions. Blots were stripped for reblotting using NewBlot PVDF Stripping Buffer (Licor). Band density was quantified in ImageJ.

## Click Chemistry

For EdC-labeled HSV virus infections, an MOI of 7.5 was used. EdC labelled virus was prepared using a previously described method[97]. Click chemistry was carried out a described previously[56] 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% methonal-free formaldehyde (0.5% Triton X-100, 1% phenylmethylsulfonyl fluoride (PMSF)) in CSK buffer, then washed twice with PBS before continuing to the click chemistry reaction and immunostaining. Samples were blocked with 3% BSA for 30 minutes, followed by click chemistry using EdC-labelled HSV DNA and the Click-iT EdU Alexa Flour 555 Imaging Kit (ThermoFisher Scientific, C10638) according to the manufacturer's instructions. For immunostaining, samples were incubated overnight with primary antibodies in 3% BSA. Following primary antibody treatment, neurons were incubated for one hour in Alexa Fluor® 488-, 555-, and 647-conjugated secondary antibodies for multi-color imaging (Invitrogen). Nuclei were stained with Hoechst 33258 (Life Technologies). Images were acquired at 60x using an sCMOS charge-coupled device camera (pco.edge) mounted on a Nikon Eclipse Ti Inverted Epifluorescent microscope using NIS-Elements software (Nikon). Images were analyzed and intensity quantified using ImageJ.

## Cytosolic $Ca^{2+}$ imaging using ratiometric Fura-2 (microscopy)

For ratiometric $Ca^{2+}$ imaging, neurons were seeded on coverslips and incubated for 30 min at RT with 5 μM Fura-2-AM, 0.02% pluronic acid in Ringer solution (in mM, 155 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 10 glucose, adjusted to pH 7.4). Excitations

of Fura-2 at 340 nm and 380 nm emissions were carried out using a DG4 Illuminator (Sutter Instruments). Emissions were collected at 510 nm using an ORCA-Flash 4.0 V2 CMOS camera (Hamamatsu). Cells were imaged every 500 milliseconds for the duration of the experiment. Acetylcholine (100 μM) and Ionomycin (2 μM) were applied at indicated timepoints. Data were acquired and processed using SlideBook 6 software.

**Cytosolic $Ca^{2+}$ imaging using ratiometric Fura-2 (FlexStation)**

For ratiometric $Ca^{2+}$ imaging, neurons were seeded on a 96-well black walled plate and incubated for 30 min at RT with 5 μM Fura-2-AM, 0.02% of pluronic acid in Ringer solution ([in mM] 155 NaCl, 4.5 KCl, 10 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 10 glucose, pH 7.4). Fura-2 emissions were collected at 510 nm and with 340/380 nm excitation. Plates were imaged using the FlexStation 3 (Molecular Devices). Cells were imaged every 5 seconds for the duration of the experiment.

**Statistical Analysis**

Power analysis was used to determine the appropriate sample sizes for statistical analysis. All statistical analysis was performed using Prism V8.4. An unpaired t-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.  EdC virus and H3K9me3S10/p

co-localization was quantified using ImageJ after sample blinding of at least 8 fields of view from 2 biological replicates. Mean fluorescence intensity of γH2AX and H3K9me3pS10 was quantified using ImageJ from at least 100 cells from at least 3 biological replicates.

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