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Neuronal Stress Pathway Mediating a Histone Methyl/Phospho Switch is Required for Herpes Simplex Virus Reactivation

Anna R. Cliffe[1,2,*], **Jesse H. Arbuckle**[3], **Jodi L.Vogel**[3], **Matthew J. Geden**[1,2], **Scott B. Rothbart**[4,7], **Corey L. Cusack**[2,5], **Brian D. Strahl**[4,6], **Thomas M. Kristie**[3], and **Mohanish Deshmukh**[1,2,6,*]

[1] Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, North Carolina 27599, USA.

[2] Neuroscience Center, University of North Carolina, Chapel Hill, North Carolina 27599, USA.

[3] National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

[4] Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA.

[5] Neurobiology Curriculum, University of North Carolina, Chapel Hill, North Carolina 27599, USA.

[6] Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, USA.

SUMMARY

Herpes simplex virus (HSV) reactivation from latent neuronal infection requires stimulation of lytic gene expression from promoters associated with repressive heterochromatin. Various neuronal stresses trigger reactivation, but how these stimuli activate silenced promoters remains unknown. We show that a neuronal pathway involving activation of c-Jun N-terminal kinase (JNK), common to many stress responses, is essential for initial HSV gene expression during reactivation. This JNK activation in neurons is mediated by dual leucine zipper kinase (DLK) and JNK-interacting protein 3 (JIP3), which direct JNK towards stress responses instead of other cellular functions. Surprisingly, JNK-mediated viral gene induction occurs independently of histone demethylases that remove repressive lysine modifications. Rather, JNK signaling results in a histone methyl/phospho switch on HSV lytic promoters, a mechanism permitting gene expression in the presence of repressive lysine methylation. JNK is present on viral promoters

[*]Correspondence to: Cliffe@email.unc.edu and Mohanish@med.unc.edu.

[7]Current address: Center for Epigenetics, Van Andel Research Institute, Grand Rapids, Michigan 49503, USA.

AUTHOR CONTRIBUTION

A.R.C and M.D. designed the study, A.R.C., J.H.A., J.L.V., M.J.G., C.J.C. and T.M.K. performed the experiments, S.B.D and B.D.S designed and carried out the histone-peptide array, A.R.C. analyzed and interpreted the data, A.R.C and M.D. wrote the manuscript and C.J.C., J.H.A. and T.M.K. edited the manuscript.

during reactivation, thereby linking a neuronal-specific stress pathway and HSV reactivation from latency.

INTRODUCTION

Herpes simplex virus (HSV) persists for the life-time of the host in the form of a latent infection in peripheral neurons (Knipe and Cliffe, 2008; Roizman et al., 2013). Periodically, HSV must re-enter the lytic phase of replication in order to produce progeny virus for dissemination, a process known as reactivation. However, during latent infection, the viral lytic genes are extensively down-regulated and their promoters assembled into repressive heterochromatin (Cliffe et al., 2009; Kwiatkowski et al., 2009; Wang et al., 2005). Therefore, reactivation requires viral lytic gene expression to be induced from silenced promoters in the absence of viral proteins.

The earliest events in HSV reactivation are poorly understood but recent work suggests that while similarities exist, there are several differences in the mechanisms of HSV gene expression during reactivation *versus* de novo lytic infection (Roizman et al., 2013). During lytic replication, over 70 viral gene products are expressed in a cascade dependent fashion. Recruitment of the cellular transcriptional machinery is dependent on both cellular and viral (HSV immediate-early activator, VP16) transcriptional transactivators to promote expression of the immediate-early (IE) mRNAs. Viral early (E) gene expression occurs following the synthesis of the IE proteins and finally late (L) gene expression is dependent upon viral DNA replication (Roizman et al., 2013). In contrast, during the early stages of reactivation the initial wave of lytic gene expression is not necessarily dependent upon VP16 expression (Kim et al., 2012). In addition, E and L gene expression can occur in the absence of viral protein synthesis (Du et al., 2011; Kim et al., 2012; Thompson et al., 2009) and L gene expression is not dependent on viral DNA replication (Kim et al., 2012). This initial phase of viral gene expression appears to represent an event that is distinct from full reactivation (i.e. the production of infectious virus), and has been termed Phase I or animation (Kim et al., 2012; Penkert and Kalejta, 2011). During Phase I, the observation that all three classes of viral genes are induced in the absence of viral protein synthesis suggests that host cell proteins initiate this process.

Although cellular proteins, including histone demethylases, have been found to be required for HSV reactivation (Hill et al., 2014; Liang et al., 2012; 2013; 2009; Messer et al., 2015), as yet no direct link has been identified between a reactivation stimulus and the earliest induction of lytic gene expression. Reactivation of HSV can be trigged by different forms of neuronal stress including nerve growth factor (NGF)-deprivation through inhibition of Phosphoinositide 3-kinase (PI3K) signaling (Camarena et al., 2010; Du et al., 2011; Wilcox and Johnson, 1987), axotomy (Carton and Kilbourne, 1952) and heat shock (Miller et al., 2009; Sawtell and Thompson, 1992). These stimuli also induce activation of the c-Jun N-terminal kinase (JNK) signaling pathway (Dorion and Landry, 2002; Estus et al., 1994; Kenney and Kocsis, 1998; Maroney et al., 1999; Tsui-Pierchala et al., 2000). We therefore hypothesized that activation of JNK is a key event in HSV reactivation.

JNKs are members of the MAP kinase family that in mice are encoded by three different genes, *Jnk1*, *Jnk2* and *Jnk3*. In the majority of cells types, JNKs are activated in response to cellular stress and cytokines. Neurons however have high levels of constitutive JNK activity that is required to regulate neuronal growth and homeostasis (Bjorkblom, 2005; Chang et al., 2003). The interaction of JNKs with different accessory proteins regulates whether they perform physiological or stress-inducible functions. For example, following a neuronal stress stimuli including NGF-deprivation or axotomy, the mixed lineage kinase protein dual leucine kinase (DLK) along with the JNK scaffold protein, JNK-interacting protein-3 (JIP-3), redirect JNK to induce a stress response, characterized by phosphorylation of c-Jun (Miller et al., 2009; Sengupta Ghosh et al., 2011; Welsbie et al., 2013). Activation of JNK by DLK/JIP-3 can result in cell death, axon degeneration or regeneration depending on the nature of the signal and maturation state of the neurons (Tedeschi and Bradke, 2013).

To investigate the role of JNK in HSV reactivation, we developed a model of latency in primary mouse sympathetic neurons similar to that described previously using neurons isolated from rats (Camarena et al., 2010; Wilcox and Johnson, 1987). Primary neuronal models are ideal for defining the cellular signaling pathways involved as robust reactivation can be induced in pure populations of intact neurons. Using this model, we show that JNK activity is critical for reactivation of HSV. Specifically, we found that the neuronal stress pathway of JNK activation, which is dependent upon DLK and JIP-3, is required to trigger the earliest detectable induction of lytic gene expression during Phase I of reactivation.

Because JNK-dependent Phase I of reactivation requires the up-regulation of gene expression from promoters associated with repressive histone modifications, we investigated whether histone demethylase activity was required. We found that neither LSD1 (H3K9-demethylase) nor UTX/JMJD3 (H3K27-demethylases) activities were necessary for Phase I gene expression. Therefore, reversal of these repressive modifications was not required in this initial wave of gene expression. While the presence of H3K27me3 or H3K9me3 is typically associated with gene silencing, it is becoming increasingly appreciated that additional histone modifications, such as phosphorylation, can modulate this silencing function (Gehani et al., 2010; Karch et al., 2013; Rothbart and Strahl, 2014). Indeed, we detected a JNK-dependent increase in phosphorylation on histone H3 that still maintained the K9me3 modification on nucleosomes associated viral lytic gene promoters. JNK itself was also enriched on lytic promoters during Phase I. We therefore provide a direct link between activation of a neuronal stress response that would permit an increase in viral lytic gene expression from an epigenetically repressed state during Phase I of HSV reactivation.

RESULTS

JNK activity is required for HSV reactivation

To investigate the role of the JNK in HSV reactivation, we utilized primary neurons isolated from the superior cervical ganglia (SCG) of postnatal mice to develop a system that would allow us to easily manipulate cellular signaling pathways in pure populations of neurons. Neurons were pre-treated with type I and type II interferons and infected at an MOI of 2 plaque forming units (PFU)/cell with HSV-1 strain KOS expressing GFP-tagged version of the immediate-early activator protein (VP16) (Ottosen et al., 2006) in the presence of

acyclovir (Fig. 1A). After 6 days the acyclovir was removed. At this point there was an absence of GFP expression (Fig. 1B) and increased expression of the latency associated transcript (LAT) compared to the lytic ICP8 mRNA (Fig. S1A). Reactivation was triggered by PI3K inhibition using LY294002, as previously described (Camarena et al., 2010; Kim et al., 2012; Kobayashi et al., 2012) (Fig. 1B). Reactivation was quantified based on the number of VP16-GFP expressing neurons in the presence of WAY-150138 (van Zeijl et al., 2000) which blocks packaging of the progeny genomes and therefore cell-to-cell virus spread (Fig. 1B and 1C). WAY-150138 was effective in inhibiting the HSV-1 KOS replication in neurons (Fig. S1B), confirming that we were quantifying reactivation and not viral spread.

PI3K inhibition resulted in the activation of JNK signaling, which is known to result in the up-regulation and phosphorylation of the JNK target protein, c-Jun (Eilers et al., 1998). JNK activation could be blocked by addition of the established JNK inhibitors SP600125 and AS601245 (Fig. 1D). We found that inhibition of JNK signaling by either SP600125 or AS601245 completely blocked HSV reactivation triggered by PI3K inhibition as shown by the suppression of GFP positive neurons at 72h post-reactivation (Fig. 1E and F). JNK-inhibition also prevented the expression of the immediate-early protein, ICP4 (Fig. S1C and S1D). In addition to PI3K inhibition, reactivation has previously been found to be triggered by dexamethasone (Cook et al., 1991; Du et al., 2012) or AKT inhibition (Camarena et al., 2010). We found that inhibition of JNK activity by SP600125 also prevented reactivation in the presence of dexamethasone (Fig. S1E) or AKT VIII inhibitor (Fig. S1F). Latent cultures of primary neurons isolated from the dorsal root ganglia (DRG) of mice were also established by infection with GFP-VP16 HSV in the presence of ACV (Fig. 1G). Reactivation of HSV from DRG neurons could be triggered by PI3K inhibition, which we also found to be inhibited by addition of the JNK inhibitor, SP600125 (Fig. 1G).

We next investigated the effect of JNK-inhibition on reactivation of HSV from neurons that were infected *in vivo*. Latency was established in mice following corneal infection and reactivation was triggered explant/axotomy of the trigeminal ganglia (Liang et al., 2009). Axotomy of the trigeminal ganglia resulted in JNK activation, as determined by c-Jun phosphorylation, by 4h post-explant (Fig. S2). c-Jun phosphorylation could be reduced by addition of SP600125, although the level of inhibition was variable, likely due to incomplete penetrance of the JNK inhibitor into the explanted ganglia (Fig. S2). Following explant/axotomy induced reactivation, addition of SP600125 reduced ICP27 mRNA expression at 6 hours post-explant (Fig. 2A). Additionally, SP600125 also inhibited viral DNA replication (Fig. 2B) and the production of infectious virus at 48h post-explant (Fig. 2C). Thus, activation of JNK was found to be essential for reactivation from both sympathetic and sensory neurons, triggered by multiple stimuli in both *in vitro* and *in vivo* models of HSV latency.

Neuronal apoptosis is dispensable for HSV reactivation

Immature post-natal day 5 neurons are known to undergo apoptosis in response to NGF-deprivation and PI3K inhibition (Kristiansen and Ham, 2014; Orike et al., 2001). This apoptosis is dependent upon activation of JNK signaling (Besirli and Johnson, 2003; Eilers

et al., 1998). Neurons will also undergo apoptosis in response to dexamethasone (Du et al., 2012). Therefore HSV reactivation could be either directly triggered by JNK signaling or a consequence of neuronal cell death. However, previous studies have demonstrated that immature neurons develop resistance to apoptosis as they mature both *in vitro* and *in vivo* (Kole et al., 2013). Consistent with this observation, neither PI3K inhibition nor dexamethasone resulted in neuronal cell death in these postnatal-day 18 (the same age of neurons at the time of reactivation) mature neurons (Fig. 3A). To definitively rule out a potential role for apoptosis in this reactivation assay, we utilized neurons that are deficient in the pro-apoptotic protein, Bax. Since SCG neurons do not expression Bak, deletion of Bax is sufficient to completely inhibit apoptosis in these neurons (Kristiansen and Ham, 2014). HSV reactivation was found to be equivalent in Bax knock-out and wild-type neurons (Fig. 3B). These results indicate that the neuronal stress pathway of JNK signaling, and not cell death, was critical for triggering HSV reactivation.

JNK-activation by DLK/JIP-3 is required for HSV Phase I of reactivation

We next examined whether activation of the JNK cell stress pathway was directly required to induce the transcription of the initial wave of viral lytic mRNAs during Phase I. In our system, the representative lytic mRNAs ICP27 and ICP8 were induced between 15-20h post-reactivation (Figs. 4A and S3A). Strikingly, inhibition of JNK by SP600125 blocked both ICP27 and ICP8 mRNAs induction at 18h post-reactivation (Figs. 4B and S3B). In contrast, inhibition of JNK had no effect on ICP27 gene expression following *de novo* lytic infection of neurons (Fig. S3C). Therefore, JNK activation is required for Phase I gene expression during reactivation but not IE gene expression during lytic replication in neurons.

In neurons, stress signaling including both local and axon-specific NGF-deprivation and axotomy activates JNK *via* DLK and JIP3 (Miller et al., 2009; Sengupta Ghosh et al., 2011; Welsbie et al., 2013). To determine whether DLK and JIP-3 were required for HSV reactivation we depleted either DLK or JIP3 protein using lentivirus-mediated delivery of shRNAs. We found that depletion of either protein blocked the induction of ICP27 gene expression at 18h post-reactivation (Fig. 4C-E). Hence, activation of the neuronal specific pathway of JNK by DLK/JIP-3 was required for the induction of viral lytic gene expression during Phase I of reactivation. Depletion of DLK or JIP3 did not reduce ICP27 mRNA levels following *de novo* infection (Fig. S3D-F), indicating that distinct mechanisms regulate lytic gene expression during Phase I of reactivation *versus* lytic replication.

Phase I of reactivation is independent of histone demethylase activity

During latent infection HSV promoters are associated with histone H3 trimethylated at lysine 27 (H3K27me3) and histone H3 di- and tri- methylated at lysine 9 (H3K9me2/3) (Cliffe et al., 2009; Kwiatkowski et al., 2009; Wang et al., 2005). Therefore, we investigated whether removal of these repressive histone modifications was required for the induction of lytic gene expression during Phase I. We first confirmed that the representative viral lytic gene promoters (*ICP27* and *ICP8)* were assembled into heterochromatin in the primary neuronal model by chromatin immunoprecipitation (ChIP) assays. Both the *ICP27* and *ICP8* promoters were found to be associated with H3K27me3 (Figs. 5A and 5B) and H3K9me3 (Figs. 6A and 6B) in latently infected primary neurons.

GSK-J4 is a compound that blocks both of the histone lysine 27 demethylases: UTX and JMJD3 (Kruidenier et al., 2012) and has been shown to inhibit HSV reactivation (Messer et al., 2015). Addition of GSK-J4 blocked HSV reactivation as determined by the detection of GFP positive neurons (Fig. 5C). However, GSK-J4 did not inhibit the induction of ICP27 or ICP8 mRNA during Phase I (Fig. 5D and 5E). In contrast, we found GSK-J4 reduced ICP27 expression by approximately 70% during lytic infection in neurons (Fig. 5F), consistent with the requirement for UTX for maximal IE gene expression during lytic infection (Oh et al., 2013). These results highlight the observation that while H3K27me3 demethylase activity is required for gene expression during lytic replication, it does not appear to be required for the Phase I of reactivation.

During HSV reactivation, the histone demethylases (HDMs) LSD1 and JMJD2 are required for the removal of H3K9 methylation (Liang et al., 2009; 2013). Inhibition of LSD1 activity using monoamine oxidase inhibitors (MAOIs) such as tranylcypromine (TCP) (Lee et al., 2006; Metzger et al., 2005) blocks reactivation in the mouse explant model system (Liang et al., 2009) and prevents recurrence *in vivo* (Hill et al., 2014). As shown in Fig. 6A and B, histone H3K9me3 was associated with lytic gene promoters in latently infected neurons. In a manner comparable to treatment with GSK-J4, addition of TCP inhibited HSV reactivation (Fig. 6C) but did not prevent ICP27 or ICP8 mRNA induction during Phase I (Fig. 6D and 6E). TCP reduced ICP27 expression by over 70% during lytic infection of neurons (Fig. 6F), once again highlighting the difference between lytic replication and Phase I of reactivation. Taken together, these results show that the first phase of gene expression during HSV reactivation is dependent on the JNK cell stress pathway but independent of H3K27 and H3K9 histone demethylase activity.

JNK-signaling triggers a methyl/phospho switch on lytic promoters

We next examined how JNK signaling could permit increased viral gene expression during Phase I without the removal of the repressive heterochromatin modifications. One mechanism by which cellular gene expression can be initiated even in the presence of repressive lysine methylation is through histone phosphorylation on a neighboring serine (i.e. H3S10 and H3S28). This is known as a histone methyl/phospho switch and has been demonstrated to occur following the activation of kinase signaling pathways (Fischle et al., 2005; 2003; Gehani et al., 2010; Hirota et al., 2005). To investigate the potential of such a mechanism in HSV reactivation, we first tested the specificities of several commercially antibodies raised against H3K9me3, H3K27me3 and the neighboring phosphorylation marks using a histone peptide microarray platform (Fuchs et al., 2011; Rothbart et al., 2012) (Fig. S4A-F). Generating these antibody specificity profiles was especially important since binding of an antibody to a single histone modification can be occluded by a combination of modifications (Fuchs et al., 2011). We identified an antibody that was specific for the dually modified histone H3K9me3/pS10 but not for either of the single S10 or K9 modifications (Fig. S4C). Using this antibody, we detected a robust increase in the enrichment of H3K9me3/pS10 on the ICP27 and ICP8 viral lytic gene promoters, during Phase I of reactivation (Fig. 7A and 7B). In contrast, we did not detect an increase in H3K9me3/pS10 enrichment at the latency-associated transcript (LAT) 5′ exon, which is depleted of H3K9me3 during latency (Cliffe et al., 2009) (Fig. 7C). Importantly, the increase in

H3K9me3/pS10 seen on viral lytic promoters was blocked when JNK activity was inhibited (Fig. 7A and 7B), indicating that JNK signaling resulted in phosphorylation of histone H3 at S10 while still maintaining H3K9 methylation.

The kinases that have been demonstrated to phosphorylate histone H3S10 in the context of lysine methylation include mitogen and stress activated kinases (MSKs) (Gehani et al., 2010; Sabbattini et al., 2014) and Aurora B kinase (Fischle et al., 2005; Hirota et al., 2005; Sabbattini et al., 2014). However, we found no role for these kinases in reactivation (Fig. 7D and 7E). Recently, JNK itself was shown to have the ability to of phosphorylate H3S10 *in vitro* (Tiwari et al., 2011). To examine the possibility that JNK directly phosphorylates histone H3 during HSV reactivation, we examined JNK occupancy on lytic promoters during reactivation. Our results show that JNK was enriched on viral lytic promoters, but not on the control LAT 5′ exon, during reactivation (Fig. 7F). Taken together, these data suggest that JNK occupancy of viral promoters mediates the histone methyl/phospho switch that would permit viral gene expression from repressed heterochromatin during HSV reactivation.

DISCUSSION

It has been long hypothesized that neuronal cell stress results in reactivation of HSV, yet the stress-signaling pathways mediating reactivation have remained undiscovered. Using a primary neuronal model of HSV latency and reactivation, we found that the DLK/JIP-3 JNK stress pathway is a key mediator of HSV reactivation in neurons and triggers the earliest detectable up-regulation in lytic gene expression. Importantly, we found that activation of the JNK pathway resulted in phosphorylation of histone H3 associated with HSV lytic promoters, which could allow viral lytic gene expression to occur without removal of repressive lysine modifications.

The pathway of JNK activation by DLK and JIP3 has been described for various types of neuronal insults that occur on either the whole neuron and localized only to the axon, including local or global NGF-deprivation and axotomy (Miller et al., 2009; Sengupta Ghosh et al., 2011; Welsbie et al., 2013). We hypothesize that activation of the DLK/JIP3 JNK pathway is a major trigger of HSV reactivation in response to various stimuli *in vivo*. Recent work has suggested that latency may be significantly dynamic with low levels of lytic gene expression in individual neurons and/or infrequent abortive reactivation events (Ma et al., 2014; Margolis et al., 2007). Increased viral gene expression in this context was associated with changes in cellular gene expression, including up-regulation of Bim mRNA (Ma et al., 2014), which is a key target of the DLK/JIP3 JNK pathway (Harris and Johnson, 2001). We propose that this low level of lytic gene expression could result from limited activation of the neuronal specific JNK cell stress pathway, perhaps following small insults to neurons. Viral reactivation could be completed when these signaling pathways reach full threshold. As DLK and JIP3 are expressed almost exclusively in neurons (Hirai et al., 2005; Kelkar et al., 2000), targeting them would be an effective mechanism to prevent HSV reactivation in response to multiple triggers.

We have further defined Phase I of reactivation as being dependent upon JNK signaling but independent of histone demethylase activity. Previous work has also found that Phase I occurs independently of viral protein synthesis (Du et al., 2011; Kim et al., 2012) and expression of the lytic transactivator VP16 (Kim et al., 2012). Although Phase I occurred even in the absence of histone demethylase activity, we found that lytic gene expression following *de novo* infection in neurons was dependent upon histone demethylase activity, supporting previous observations in non-neuronal cells (Liang et al., 2012; 2009; 2013; Oh et al., 2013). Therefore, the mechanisms of lytic gene expression during Phase I and *de novo* infection are distinct. Perhaps this is not surprising given that gene expression needs to be induced from promoters with different chromatin structures in reactivation versus *de novo* infection (Deshmane and Fraser, 1989; Kent et al., 2004; Lentine and Bachenheimer, 1990; Wang et al., 2005). Additionally, in contrast to *de novo* infection, gene expression during reactivation needs to be induced in the absence of viral transcriptional activators that can promote the recruitment of coactivator complexes containing chromatin remodeling proteins and histone demethylases.

Our results also support previous observations that full reactivation requires the activity of histone demethylases, most likely once a threshold for lytic gene expression is reached. Therefore, targeting histone demethylase activity along with the JNK pathway would be an effective, multi-step approach to prevent HSV reactivation. Previous work identifying a role for the histone demethylases LSD1 and JMJD2s in explant/axotomy induced reactivation found that they were required for lytic gene expression at 6h post-reactivation (Liang et al., 2009; 2013). Explant is known to induce rapid changes in cellular and viral lytic gene expression (Rishal and Fainzilber, 2013; Sawtell and Thompson, 2004); therefore it is possible that histone phosphorylation and demethylation are more tightly coupled following explant induced reactivation compared to reactivation in intact neurons. The viral lytic transactivator VP16 is required for full reactivation following thermal stress *in vivo* (Thompson et al., 2009) and PI3K inhibition in primary neurons (Kim et al., 2012) but not following explant induced reactivation (Sears et al., 1991; Steiner et al., 1990). Therefore an alternative hypothesis is that there are differences in the pathways to reactivation in axotomized ganglia versus intact neurons. Irrespective, in this study JNK activity was found to be required for the earliest stages of reactivation in both explanted and intact neurons.

Our results identify a direct link between activation of the DLK/JIP-3 JNK neuronal stress pathway and histone methyl/phospho switch that could allow gene expression to occur even without the removal of repressive lysine modifications (Fig. 4G). A histone methyl/phospho switch is thought to result in the eviction of histone readers that bind the methylated histones but are unable to do so when the neighboring serine residue is phosphorylated (Fischle et al., 2003; 2005; Gehani et al., 2010; Hirota et al., 2005; Sabbattini et al., 2014). Specifically, we detected enrichment in the H3K9me3/pS10 modification during the Phase I phase of reactivation that was dependent upon JNK activity. A histone methyl/phospho switch occurring at S10 has been found to result in the loss of HP1 binding to H3K9me3 during mitosis and loss of Polycomb group protein EZH1 binding to developmentally regulated genes in embryonic stem cells (Fischle et al., 2005; Hirota et al., 2005; Sabbattini et al., 2014). Histone phosphorylation can result in either transcriptional silencing or activation

depending upon the context (Sawicka and Seiser, 2014). Our results suggest that a JNK-mediated H3K9me3/pS10 switch in neurons results in HSV transcriptional activation during Phase I of reactivation.

Thus far, the kinases found to be responsible for an H3K9me3/pS10 switch are the MSKs and Aurora B kinase (Fischle et al., 2005; Hirota et al., 2005; Sabbattini et al., 2014). As we found no role for these kinases in HSV reactivation we conclude that they likely do not play a major role in mediating the methyl/phospho switch during Phase I. Furthermore, JNK mediates histone S10 phosphorylation during neuronal development, which is associated with transcriptional activation (Tiwari et al., 2011). However, in this context phosphorylation was not found to occur in the presence of H3K9-methylation. Our results now link JNK activity with an H3K9me3/pS10 switch.

In the context of HSV reactivation, maintaining the repressive histone-lysine modifications through the use of a methyl/phospho switch may allow the viral genome to become easily re-repressed if the threshold for full reactivation is not reached. Previous work from Kim *et al* (2012) has suggested only a sub-population of neurons that undergo Phase I progress to full reactivation, indicating that this step is indeed reversible. Importantly, by highjacking a neuronal signaling pathway, HSV has evolved a mechanism that allows the earliest gene expression to occur from repressed chromatin in the absence of viral-encoded activators. While our results show that this histone methyl/phospho switch allows for HSV reactivation, a similar mechanism may also permit cellular gene expression from repressed chromatin in other situations of neuronal stress.

EXPERIMENTAL PROCEDURES

Primary neuronal cultures

Sympathetic neurons were dissected from the superior cervical ganglia of post-natal day 1-3 (P1-3) CD1 mice (Charles River Laboratories) or Bax knock-out/WT litter mate controls (Knudson et al., 1995) as previously (Deshmukh et al., 2002). Sensory neurons were isolated from the dorsal root ganglia (DRG) of P0-1 CD-1 mice. Briefly, ganglia were placed in Leibovitz's L-15 before incubation in collagenase (1 mg/ml) followed by trypsin (2.5 mg/ml) for 20 min each at 37°C. and plated onto rat tail collagen. Sympathetic neurons were maintained in AM50 media (MEM with the addition of 50 ng/ml 2.5S NGF, 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine and 20 μM uridine). Aphidicolin (3.3μg/ml) was also added to the media for 3 days post plating to remove any proliferating cells. Sensory neurons were maintained in DRG media (NeuroCult Neurobasal media (Stem Cell Technologies), SM1 (Stem Cell Technologies), 4.5g/L glucose, 50ng/ml 2.5S NGF, 10ng/ml glial derived neurotropic factor, 2mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 20 μM fluorodeoxyuridine and 20 μM uridine). Aphidicolin (3.3μg/ml) was also added to the media for 2 days post plating, followed by cytosine arabinoside (3μM) for 2 days to remove proliferating cells.

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

To establish HSV-latently infected cultures in neurons isolated from SCGs, P9 neurons were pre-treated with 100u/ml mouse IFN-alpha (Millipore) and 250u/ml mouse IFN-gamma (Millipore) in AM50 without fluorodeoxyuridine and uridine (AM50-FUDR). After 24 hours, the cultures were infected with HSV-DG1 (KOS recombinant HSV expressing a VP16-GFP fusion protein) (Ottosen et al., 2006). Neurons were infected at a multiplicity of infection (MOI) of 2 PFU/cell (assuming 10^4 neurons/well/24 well plate or at 10^4 PFU/ml) in PBS containing 1% FBS, 4.5g/L glucose and 100μM acyclovir (ACV) for 2 hrs. Post infection, the media was changed to AM50-FUDR containing 100μM (ACV) for 6 days, and then AM50-FUDR. For the establishment of latently infected cultures of neurons isolated from DRGs, P5 neurons were infected at an MOI of 1 PFU/cell and maintained in DRG media without FUDR containing 100μM (ACV) for 4 days to allow for the establishment of latent infection, and then DRG media without FUDR. WAY-150138 (10 μg/ml) was added to limit cell-to-cell spread. Reactivation was quantified by counting the numbers of GFP-positive neurons. Analyses of the distributions were carried out by KS normality tests and statistical comparisons were made using two-tailed paired students T-test (Prism V5.0c)

Mouse infections and explant-induced *ex vivo* reactivation

Establishment and reactivation of HSV in murine trigeminal ganglia was carried out as described previous (Liang et al., 2012). Mice were infected by corneal scarification with 2×10^5 PFU/eye HSV-1 strain F. At 30-45 days post-infection, latently infected trigeminal ganglia were bisected and each half was explanted into media containing vehicle (DMSO) or SP600125 (10μM) for 48 hrs. The resulting viral yields were determined by titers of the ganglia homogenates on Vero cell monolayers. DNA was prepared from aliquots of the paired ganglia using ZR Genomic DNA-Tissue Miniprep Kit (Zymo) and HSV DNA levels were determined by qPCR using FastStart Universal SYBR Green Master Mix (Roche) in an Eppendorf Realplex^4. HSV (UL30) and control GAPDH primer sets are as described (Hill et al., 2014). All animal care and handling were done in accordance with the NIH Animal Care and Use Guidelines and as approved by the NIAID Animal Care and Use Committee. Statistical comparisons were made using two-tailed Wilcoxon signed-rank test (Prism V5.0c).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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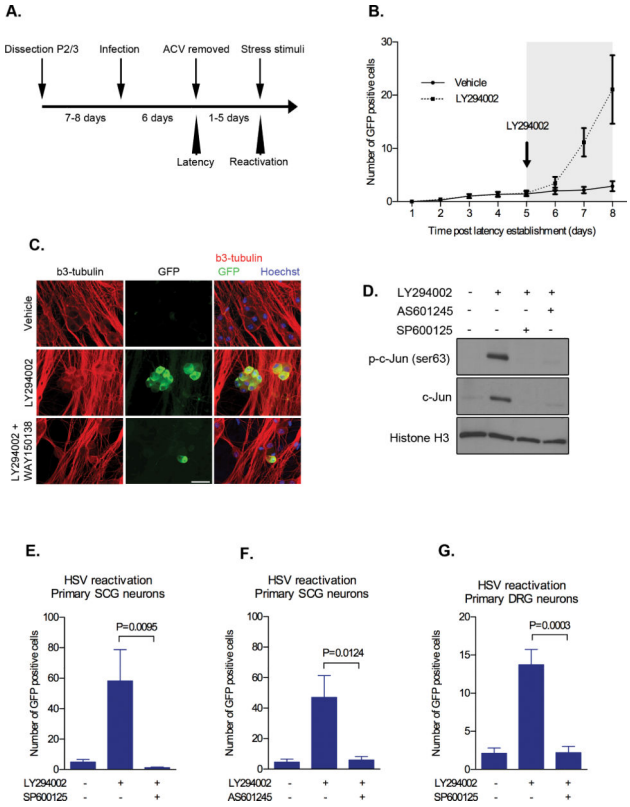

Figure 1.

Inhibiting JNK activation prevents HSV reactivation in primary neurons. (**A.**) Schematic of the primary superior sympathetic ganglia (SCG) derived neuronal system used to investigate HSV reactivation. (**B.**) The numbers of VP16-GFP expressing cells are shown following the establishment of latency in primary neurons isolated from the SCG and after the addition of the PI3K inhibitor LY294002 (20μM). Data represent the means +/− SEM, n=18. (**C.**) Immunofluorescence of VP16-GFP and β3-tubulin in the presence of LY294002 and WAY-150138. Bar = 50μm (**D.**) Western blots showing total c-Jun protein levels and phosphorylated c-Jun following treatments of primary SCG neurons with either LY294002 alone or in combination with AS601245 (20μM) or SP600125 (20μM). (**E.**) Reactivation of HSV from primary neurons isolated from the SCG triggered by LY294002 is blocked by SP600125 as determined by the numbers of VP16-GFP expressing cells 72h post-reactivation (mean +/− SEM, n=8). (**F.**) Reactivation from primary neurons isolated from the SCG triggered by LY294002 is blocked by AS601245 as determined by the numbers of VP16-GFP expressing cells 72h post-reactivation (mean +/− SEM, n=9). (**G.**) Reactivation of HSV from primary neurons isolated from the dorsal root ganglia (DRG) triggered by LY294002 is blocked by SP600125 as determined by the numbers of VP16-GFP expressing cells 72h post-reactivation (mean +/− SEM, >=9). SP600125 and AS601245 were both added at the time of reactivation. All reactivation experiment were carried out in the presence of WAY-150138 (10μg/ml) to block cell-to-cell spread. See also figure S1.

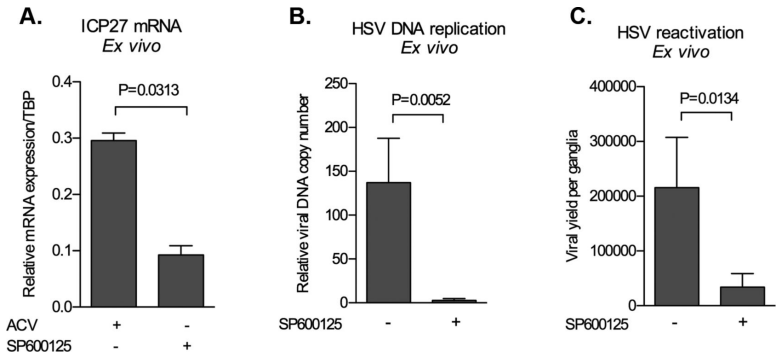

Figure 2.

Inhibiting JNK activity suppresses HSV reactivation in an *ex vivo* explant model (**A.**) ICP27mRNA expression 6h after explant induced reactivation from the TG in the presence of acyclovir (ACV) or SP600125 (10μM) (mean +/− SEM, n=6) (**B.**) The relative viral DNA copy number 48h post-explant in the presence and absence of SP600125. (**C.**) Total plaque-forming units (PFU) 48h after explant induced reactivation in the presence and absence of SP600125 (10μM). Data for B and C represents the mean +/− SEM, n=14. See also figure S2.

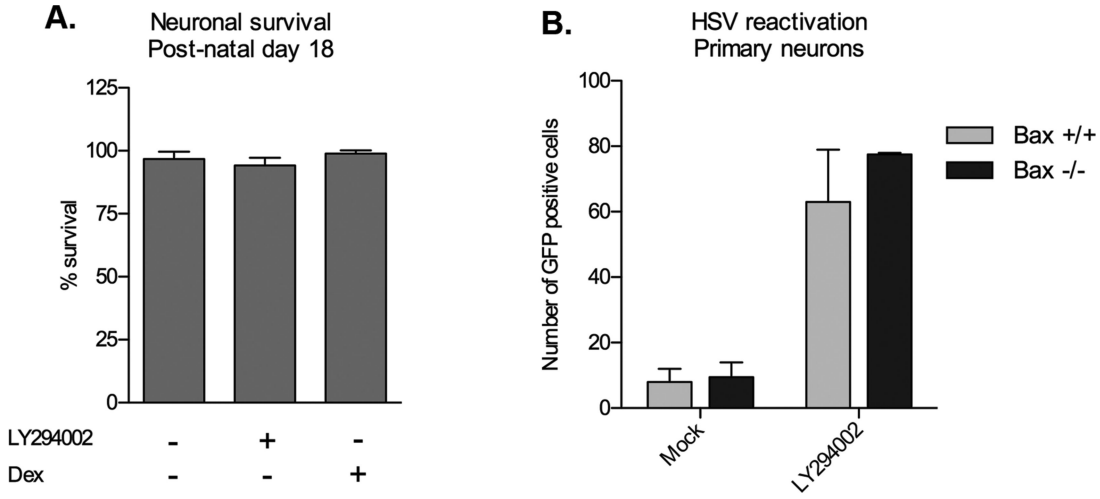

Figure 3.

Neuronal apoptosis is not required for HSV reactivation. (**A.**) Neuronal survival at 72h post-treatment with LY294002 (20μM) or dexamethasone (50μM). (**B.**) Reactivation triggered by LY294002 in wild-type or Bax knock-out neurons as determined by VP16-GFP expression at 72h in the presence of WAY-150138. Data are means +/− SEM n=3.

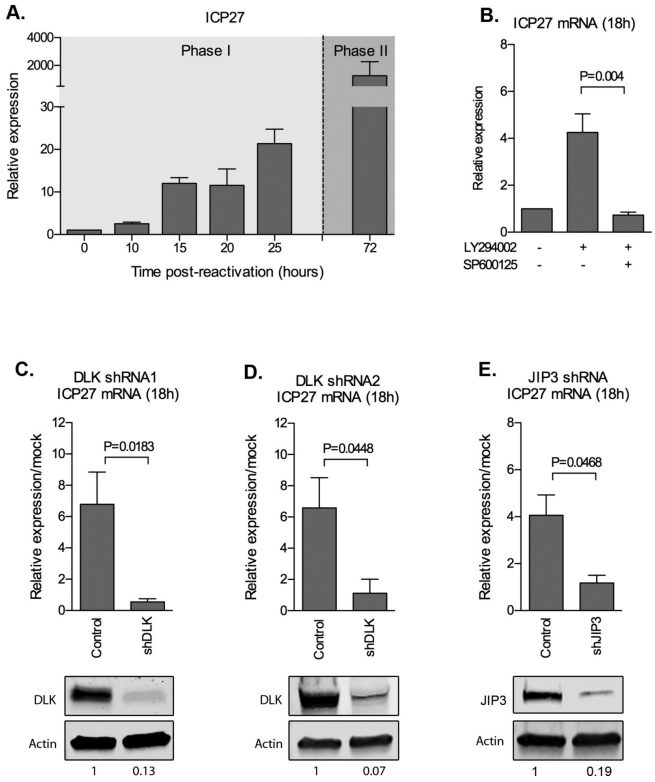

Figure 4.

Activation of the DLK/JIP3-JNK pathway is required for Phase I gene expression during HSV reactivation. (**A.**) ICP27 mRNA levels at different time points following reactivation stimulated by LY294002 as determined by RT-qPCR. The relative copy number of ICP27 mRNA was normalized to the relative GAPDH copy number in the same sample. The two previously characterized phases of HSV reactivation are highlighted (Means +/− SEM, n=3). Reactivation was carried out in the presence of WAY-150138. (**B.**) ICP27 mRNA levels at 18h post-reactivation in the presence and absence of SP600125. (**C-E.**) ICP27 mRNA levels at 18h post-reactivation following lentivirus shRNA-mediated depletion of DLK or JIP3, or infection with the control lentivirus (pLKO.1). Western blots of DLK and JIP3 protein levels with the relative levels of DLK and JIP3 normalized to β-actin (below). B-E represent the mean +/− SEM n>=5. See also figure S3.

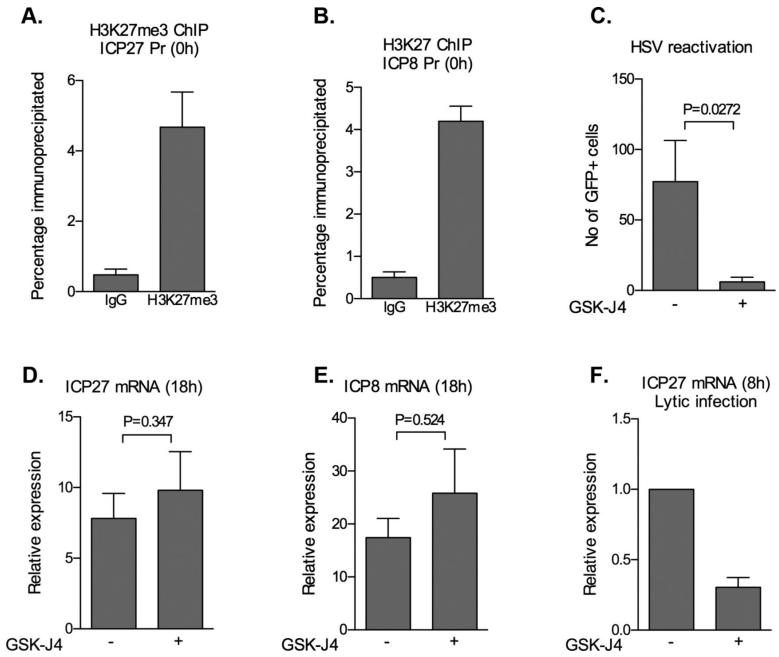

Figure 5.

Inhibition of H3K27me3 histone demethylase activity prevents reactivation but not Phase I gene expression. (**A-B**) ChIP assay for H3K27me3 association with *ICP27* (A) and *ICP8* (B) promoter during latency shown as the percentage of input. (**C.**) Effect of GSK-J4 (2μM) on HSV reactivation as determined by VP16-GFP expression at 72h in the presence of WAY-150138. (**D-E.**) ICP27 (D) and ICP8 (E) mRNA levels at 18h post-reactivation in the presence and absence of GSK-J4. (**F.**) ICP27 mRNA levels at 8h post-infection with HSV at an MOI of 10PFU/cell. Neurons were treated with GSK-J4 at the time of infection. Data are means +/− SEM, n>=4.

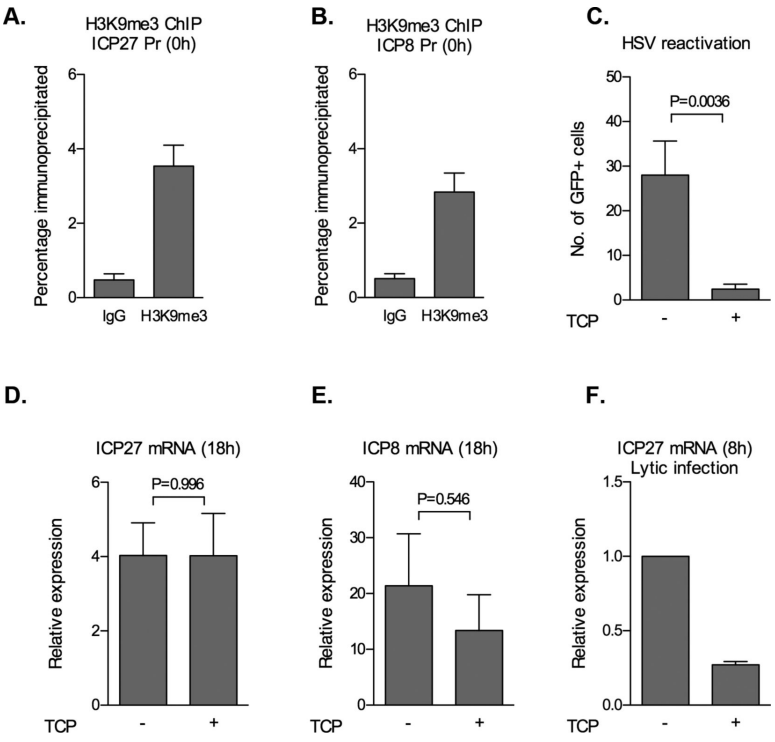

Figure 6.

Inhibition of H3K9me2 histone demethylase activity prevent reactivation but not phase I gene expression (**A-B**.) ChIP assay for H3K9me3 association with ICP27 (**A.**) and ICP8 (**B.**) promoter during latency. (**C.**) Effect of TCP (1mM) on HSV reactivation as determined by VP16-GFP expression at 72h in the presence of WAY-150138. ICP27 (**D**.) and ICP8 (**E.**) mRNA levels at 18h post-reactivation in the presence and absence of TCP. (**F.**) ICP27 mRNA levels at 8h post-infection with HSV at an MOI of 10PFU/cell. Neurons were treated with TCP at the time of infection. Data are means +/− SEM, n>=4.

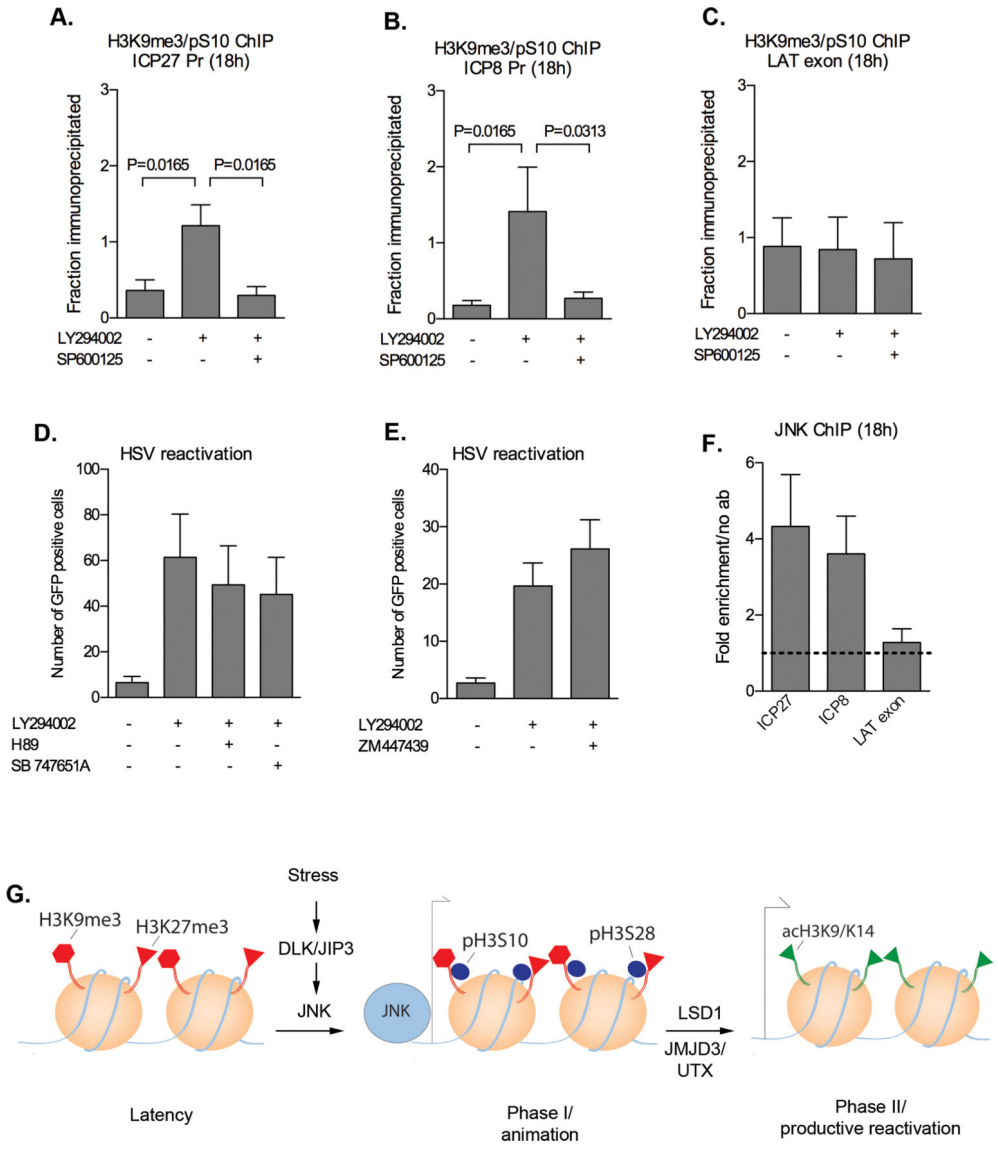

Figure 7.

JNK mediates a histone methyl/phospho switch during reactivation. (**A-C.**) ChIP using antibodies against H3K9me3/pS10 18h following LY294002-mediated reactivation in the presence and absence of SP600125. The relative amount of viral ICP27 promoter (**A.**), ICP8 promoter (**B.**) or LAT 5′ exon (**C.**) DNA immunoprecipitated with the indicated antibody is shown as a percentage of input after subtraction of background (control IgG). Data represent the mean +/− SEM, n=5. (**D.**) Reactivation in the presence of the MSK inhibitors H89 (5μM) or SB747651A (2μM) determined by the numbers of VP16-GFP expressing neurons at 72h post-reactivation. (**E.**) Reactivation in the presence of the Aurora B kinase inhibitor ZM 447439 (2 μM). Both (**D**) and (**E**) n=6. Reactivation was carried out in the presence of WAY-150138 to block cell-to-cell spread of HSV. (**F.**) ChIP assay for JNK recruitment at 18h post-reactivation. To determine positive enrichment, the amount of viral DNA immunoprecipitated with a JNK-specific antibody was normalized to the amount precipitated with a non-specific control antibody. Data represent the mean +/− SEM, n=5.

(**G.**) Model depicting histone modifications on HSV lytic promoters during latency and following neuronal cell stress stimuli triggering DLK/JIP-3 mediated activation of JNK and the contribution of histone demethylase activity. We hypothesize that a histone methyl/phospho switch also occurs at H3K27me3/pS28. Euchromatin associated marks enriched on lytic promoters during reactivation (e.g. acH3K9/14 (Neumann et al., 2007)) are also represented in phase II, although whether histone acetylation is required for Phase I or Phase II has not been fully established in a neuronal stress model. See also figure S4.