Selective recruitment of host factors by HSV-1 replication centers

Feng-Chao LANG[1,2], Xin LI[1,2], Olga VLADMIROVA[3], Zhuo-Ran LI[1,2], Gui-Jun CHEN[1], Yu XIAO[1], Li-Hong LI[1], Dan-Feng LU[1,2], Hong-Bo HAN[4], Ju-Min ZHOU[1,*]

[1] *Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming Yunnan* 650223, *China*

[2] *University of Chinese Academy of Sciences, Beijing* 100049, *China*

[3] *The Wistar Institute, Gene Expression and Regulation Program, Philadelphia PA* 19104, *USA*

[4] *Biology & Chemistry Engineering College, Panzhihua University, Panzhihua Sichuan* 617000, *China*

ABSTRACT

Herpes simplex virus type 1 (HSV-1) enters productive infection after infecting epithelial cells, where it controls the host nucleus to make viral proteins, starts viral DNA synthesis and assembles infectious virions. In this process, replicating viral genomes are organized into replication centers to facilitate viral growth. HSV-1 is known to use host factors, including host chromatin and host transcription regulators, to transcribe its genes; however, the invading virus also encounters host defense and stress responses to inhibit viral growth. Recently, we found that HSV-1 replication centers recruit host factor CTCF but exclude γH2A.X. Thus, HSV-1 replication centers may selectively recruit cellular factors needed for viral growth, while excluding host factors that are deleterious for viral transcription or replication. Here we report that the viral replication centers selectively excluded modified histone H3, including heterochromatin mark H3K9me3, H3S10P and active chromatin mark H3K4me3, but not unmodified H3. We found a dynamic association between the viral replication centers and host RNA polymerase II. The centers also recruited components of the DNA damage response pathway, including 53BP1, BRCA1 and host antiviral protein SP100. Importantly, we found that ATM kinase was needed for the recruitment of CTCF to the viral centers. These results suggest that the HSV-1 replication centers took advantage of host signaling pathways to actively recruit or exclude host factors to benefit viral growth.

Keywords: HSV-1; CTCF; γH2AX; Viral replication cente; RNA Pol II

INTRODUCTION

Herpes simplex virus type I (HSV-1) belongs to the herpes family of DNA viruses, and infects numerous cell types during the productive phase of infection, but enters latency in neuronal cells. HSV-1 infects more than 80% of the population (Roizman & Whitley, 2013), and when activated from latency, it is responsible for oral and genital herpes, keratitis and in rare but often fatal cases, herpes encephalitis (Knipe & Howley, 2007). HSV-2 is similar to HSV-1 in genome and there is common co-infection in HIV-1 infected people (Lai et al, 2003; Zhou et al, 2014).

The outcome of HSV-1 infection is determined by complex interactions between the virus and the host cell and immune system. At a cellular level, the incoming virus first releases its linear DNA into the nucleus, which quickly circularizes and becomes chromatinized (Conn & Schang, 2013). With the help of tegument proteins ICP0 and VP16 (Wysocka & Herr, 2003) and host transcription machinery, HSV-1 starts transcribing the viral genome, first the immediate early genes (IE) and then the early genes (E), to prepare viral DNA synthesis and modify host responses. With the onset of viral DNA replication, the virus also makes late genes and finally assembles viral particles (Everett, 2014; Knipe & Howley, 2007). Viral replication usually occurs about 6 hours post infection (hpi). Replicating viral genomes first appear at pre-replication centers marked by viral protein ICP8 (Knipe et al, 1982), a single strand DNA binding

Received: 09 March 2015; Accepted: 09 April 2015

Foundation items: This work was supported by grants from the Yunnan Provincial Government (2013FA051; 2011HA005), the National Science Foundation of China (NSFC 81471966 to JZ and NSFC 31200964 to YX), and the common project of the Panzhihua Science and Technology Bureau from China (2012CY-S-22(9) to HH)

*Corresponding author, E-mail: zhoujm@mail.kiz.ac.cn

protein, and then gradually form distinct replication centers or compartments. These centers grow quickly in size and finally merge into larger regions occupying much of the cellular nucleus. Unlike the viral genome during the pre-replication stage, the rapidly replicating HSV-1 genome is only partially chromatinized (Lacasse & Schang, 2010, 2012).

The incoming virus triggers a number of host responses, including activation of the interferon pathway (Griffiths et al, 2013; Lafaille et al, 2012; Shen et al, 2014), DNA damage response (Lilley et al, 2011; Smith et al, 2014; Volcy & Fraser, 2013), apoptosis (Prasad et al, 2013; Wang et al, 2011) and other host defense mechanisms that limit viral growth. Many viral genes are designed to deal with these host responses to ensure viral transcription, genome synthesis and assembly. For example, the viral protein ICP34.5 is a key viral factor that interferes with the interferon γ pathway (Pasieka et al, 2006; Rasmussen et al, 2011; Wang et al, 2014). Likewise, the viral IE protein ICP0 can quickly counter host transcription silencing of PML, SP100, RNF8 and RNF168 activity by degrading these proteins, and prevent host silencing complex CoREST from inhibiting viral genes (Ferenczy et al, 2011; Roizman, 2011; Wang et al, 2012). HSV-1 can also produce viral-host shutoff (VHS) factor to degrade host mRNAs (Barzilai et al, 2006; Esclatine et al, 2004; Taddeo et al, 2006, 2013), while viral protein ICP27 can inhibit host mRNA splicing (Nojima et al, 2009; Sedlackova et al, 2008), thus reducing cellular protein synthesis.

During lytic infection, the interactions between the incoming virus and the host are dynamic and complex, with well-organized viral replication centers. Indeed several proteins have been implicated in the organization of the replication centers. Nuclear lamin A, a structure protein playing an essential role in the organization of the host nucleus, is important for HSV-1 replication center organization (Silva et al, 2008) and host transcription regulator HCF-1 is needed for proper replication center formation (Peng et al, 2010). The host DNA damage response (DDR), in particular, has an intriguing interaction with replicating HSV-1. The incoming virus firstly activates the host DDR, probably due to the linear ends of its genome, and then reactivates the host DDR during viral genome replication, which exhausts host DNA replication factors (Burke et al, 2005). The DDR recruits a number of factors to the viral replication centers, some of which are beneficiary to viral growth, including RAD51 (Wilkinson & Weller, 2004), Fanconi Anemia factor FANCD2 (Karttunen et al, 2014) and ATM (Weitzman & Weitzman, 2014), and some of which inhibit viral transcription and are harmful to viral growth (Everett & Murray, 2005; Lilley et al, 2011; Parkinson et al, 1999; Song et al, 2000; Weitzman et al, 2004), including RNF8, RNF168 and host silenced chromatin. Recent research has shown that the host chromatin mark of DNA damage sites, γH2A.X, is recruited towards the replication centers but is prevented from entering, instead forming a cage-like structure surrounding the ICP4 or ICP8 marked viral genomes (Wilkinson & Weller, 2006). This finding strongly suggests that replicating viral genomes selectively recruit or exclude host factors. Thus, examination of the selective recruitment or exclusion of host factors by the HSV-1 replication center can help illuminate new regulatory mechanisms of viral-host interactions.

Although many important details of viral-host interaction are known, how the quickly replicating virus interacts with host chromatin and host DDR is still not well understood. Thus, we analyzed the interaction of HSV-1 replication centers with host chromatin and host DDR factors. We found that host modified histones H3K9me3 and H3K4me3 were excluded from the replication centers and host RNA polymerase II (RNA Pol II) was recruited, though dynamic changes in phosphorylated RNA Pol II recruitment were observed as small replication centers began to merge. Host DDR factors 53BP1 and BRCA1 were recruited but host RNF8 was excluded. Importantly, the recruitment of host organizer CTCF was enhanced by the ATM kinase pathway. Taken together, these findings provide further evidence that viral replication centers are highly organized and actively recruit or exclude host factors to facilitate viral growth.

MATERIAL AND METHODS

Cells and virus

The BJ, HeLa, 293T and Vero cells were obtained from American Type Culture Collection. ATM+/+ and ATM-/- cells were kindly provided by Matthew Weitzman from University of Pennsylvania Perelman School of Medicine, Philadelphia. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified 5% CO_2 atmosphere at 37 °C. The HSV-1 was gifted by Professor Qi-Han LI from the Institute of Medical Biology, Chinese Academy of Medicine Science. The virus was grown and titrated on Vero cells. Viral infections were done according to standard protocols. Briefly, cultured cells were replaced with serum free DMEM, followed by adding the virus and incubating for 1 hour with occasional rotation to get an even spread. The culture medium was then replaced by regular DMEM with 10% FBS and 1% antibiotics. Cell treatment was with ATM inhibitor KU55933, with cells treated with 20 uM KU55933 for 1 hour before HSV-1 infection. During the HSV-1 infection process, KU55933 was not removed from the medium.

Antibodies

CTCF polyclonal antibodies, H3K4me3 and SP100 were made by GLS Biochem (Shanghai, China), CTCF monocle antibodies were from Millipore, Germany. Antibodies against γH2A.X, RNA Pol II Ser2P, RNA Pol II Ser5P, H3, H3K9me3, 53BP1, BRCA1, RNF8 and RNF168 were obtained from Abcam Cambridge, UK. Monoclonal antibody against ICP4 was a gift from Gerd Maul's laboratory at the Wistar Institute (Everett et al, 2004; Showalter et al, 1981). The H3S10p antibody was presented from Ping ZHENG's laboratory at the Kunming Institute of Zoology, Chinese Academy of Sciences. Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L) Antibody and Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibody were from Life Technologies, USA.

Immunofluorescence

The BJ, HeLa, ATM+/+ and ATM-/- cells were seeded on glass

coverslips in 24-well plates one day before infection and used for infections at a multiplicity of infection (MOI) of 5 PFU/cell. At 5 or 6 hpi, cells were fixed with 4% paraformaldehyde at 4 °C for 60 min and extracted with 0.2% Triton X-100 in PBS for 10 min. Nuclei were visualized by staining with Hoechst 33342. Images were acquired using a Nikon 80i, Japan.

RESULTS

HSV-1 replication centers excluded modified histones

To determine how the replicating HSV-1 genomes interacted with the host chromatin, we conducted double immunostaining using an antibody against the viral protein ICP4 to label the viral replication centers and antibodies against histones in infected cells. In human primary fibroblast BJ cells, we infected with the 17+ strain of HSV-1 at an MOI of 5 and let the infection go for 6 hours, a time point when HSV-1 replication centers are well organized and recruitment or exclusion of host proteins is obvious. Due to the heterogeneity of cells and variation in the number of incoming viruses in each cell, we saw viral replication at various stages in different cells, from early small but distinct replication centers to large fused replication centers occupying most of the cellular nucleus.

We tested the localization of unmodified histone H3, modified histones H3 lysine 4 trimethylation (H3K4me3), H3 lysine 9 trimethylation (H3K9me3) and H3 serine 10 phosphorylation (H3S10p). Representative staining results are shown in Figures 1A-1D. Histone H3 did not show particular recruitment or exclusion in HSV-1 replication centers (Figure 1A). We highlighted a large replication center by dashed lines based on ICP4 staining, and the corresponding area on the H3 staining showed no difference in intensity from the surrounding areas outside the replication center (arrows). However, active chromatin mark H3K4me3 was clearly excluded from these centers (Figure 1B). We highlighted two centers with dashed circles (arrows), which indicated that the circled H3K4me3 (red) signal areas were obviously weaker than the surrounding area. In the merged image, ICP4 positive areas had little H3K4me3, confirming its exclusion. Likewise, H3K9me3, a mark of heterochromatin, was also excluded by replicating HSV-1 genomes. In Figure 1C, the dashed circle of the fused replication center (arrow) shows a reduction in the H3K9me3 signal, and the merged image shows mostly ICP4. Finally, we tested the serine10 phosphorylated form of histone H3, and found that in infected cells, H3S10p appeared to form clusters of staining signals and did not usually overlap with the ICP4 signals, although they appeared in close proximity in many cases, as shown in Figure 1D (arrows).

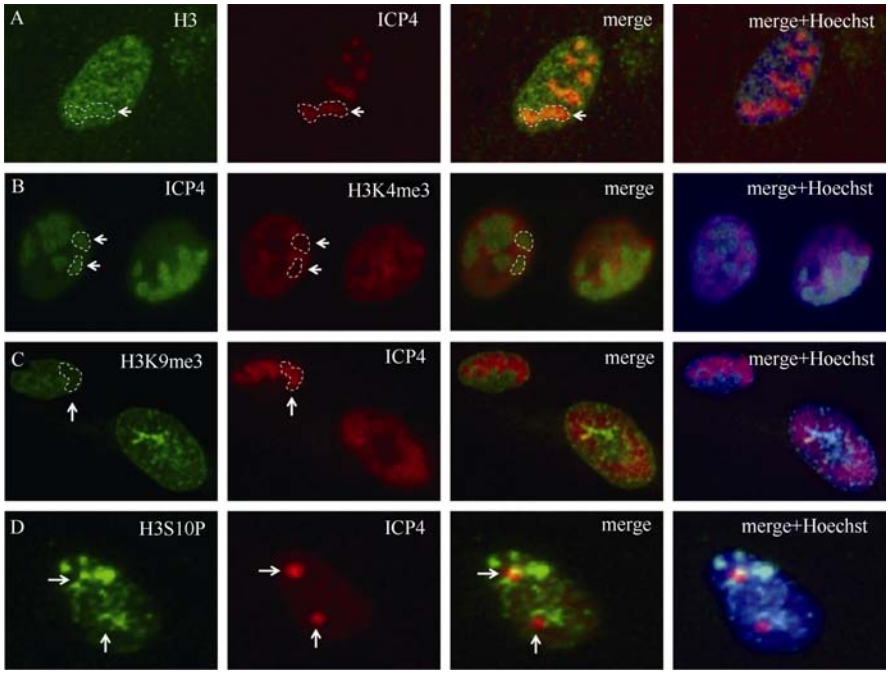

Figure 1 Recruitment or exclusion of histone and modified histone with HSV-1 replication centers

All cells were infected with HSV-1 at MOI=5 or 6 hours before fixing for immunofluorescent staining. In each row, cells were stained with two different antibodies, and images were merged to examine how the staining signals related to viral replication centers. A: Double staining with histone H3 (green) and ICP4 (red) antibodies. B: Double staining using ICP4 (red) and modified histone H3K4me3 (green). White arrows point to two of the viral replication centers, where H3K4me3 is very weak compared with the surrounding area. Merged images show green viral foci areas, suggesting a lack of red signal in the same area. C: Double staining with modified histone H3K9me3 (green) and ICP4 (red). Dashed circle shows part of a large fused replication center, where H3K9me3 is excluded. D: Cells double stained by modified histone H3S10P (green) and ICP4 (red). Arrows point to two distinct replication centers. H3S10P shows large clustered staining, which does not overlap with ICP4. Magnification ratio: 400X.

RNA polymerase II showed dynamic interaction with replicating viral genome

RNA Pol II is known to interact with replicating viruses and is highly phosphorylated in virally infected cells (Egloff & Murphy, 2008; Jenkins & Spencer, 2001). However, how dynamic RNA Pol II interacts with the HSV-1 genome as the virus transits from transcription to replication at 6 hpi is not known. To understand this dynamic interaction, we conducted double staining of ICP4 and RNA Pol II (Figure 2A). The ICP4 labeled early replication centers were colocalized with the RNA Pol II signal (orange arrows), with merged staining showing colocalization. We then stained antibodies specifically recognizing the serine 2 phosphorylated (Ser2P) form of RNA Pol II, which marks the elongating form of RNA polymerase (Kwak & Lis, 2013; Zhou et al, 2012). Figure 2B shows two infected cells, the left cell contains several distinct replication centers and the right cell contains large well-developed centers. Ser2P was recruited by smaller to intermediate sized replication centers (orange arrows), but not by large, well-developed centers (white arrows). This demonstrated that the elongating form of RNA Pol II associated more with early replicating viral genomes, but less with genomes in fully developed replication centers. We next examined the recruitment of serine 5 phosphorylated RNA Pol II. Similar to RNA Pol II Ser2P, the RNA Pol II Ser5P signal was also recruited to viral centers, but much more strongly (comparing orange arrows in Figures 2B and 2C), an observation consistent with reports that viral factor ICP22 degrades RNA Pol II Ser2P (Fraser & Rice, 2007; Zaborowska et al, 2014). In the present study, RNA Pol II Ser5P was recruited into viral centers in both early individual foci and late fused foci, although there was a drop in intensity in the RNA Pol II Ser5P signal in later fused replication centers. This could be seen by comparing orange arrows (marking individual viral center) with white arrows (well-developed, fused center), and the merged images in Figure 2C. The merged image shows that the smaller individual replication centers were mostly green and the large fused foci were orange, suggesting that the former had more RNA Pol II Ser5P than the latter. This result showed a reduction in RNA Pol II recruitment in fully developed replicating centers. Taken together, these findings suggest that active transcription occurred in smaller replication centers, but not in large fused ones.

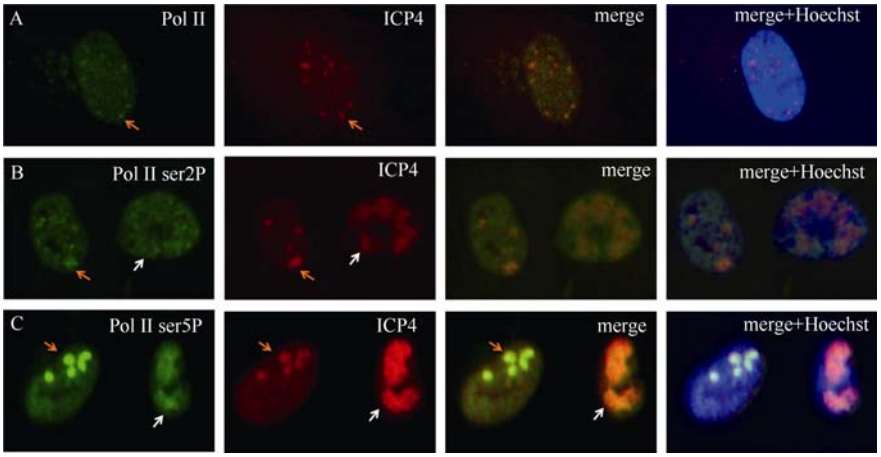

Figure 2 Recruitment of RNA Pol II by replicating HSV-1

BJ cells were infected with HSV-1 for 6 hours at MOI=5 and fixed for immunostaining. A. Double staining using ICP4 (red) and RNA Pol II (green) antibodies showing recruitment of total RNA Pol II to ICP4 labeled HSV-1 replication centers. B: Double staining using ICP4 (red) and Ser2 phosphorylated RNA Pol II (green) antibodies. Orange arrows show a well-defined replication center. White arrows show large fused replication centers. C: Immunostaining with ICP4 antibody (red) highlighting viral replication centers and Ser5 phosphorylated RNA Pol II antibody (green) showing paused RNA polymerase. Orange arrows indicate a cluster of well-defined replication centers, white arrows indicate late stage fused replication centers. Magnification ratio: 400X.

Interaction between HSV-1 replication centers and DDR factors

To determine the potential interaction of DDR factors with the replicating viral genome, we surveyed four DDR factors, 53BP1, BRCA1, RNF8 and RNF168, using immunofluorescent staining. 53BP1 is a key DNA damage repair factor involved in nonhomologous end joining (NHEJ) double strand break (DSB) repair (Taylor & Knipe, 2004; Weitzman & Weitzman, 2014). Only a few studies have explored whether 53BP1 plays a role in DNA virus infection (Bailey et al, 2009; Salsman et al, 2012). BRCA1 is another key regulator of host DDR and mediates a homologous recombination type of DSB repair (Yun & Hiom, 2009), though its role in HSV-1 infection has not yet been established. RNF8 and RNF168 are two essential ubiquitin ligases needed to modify histones and other DDR factors during DNA damage repair (Lilley et al, 2010; Mattiroli et al, 2012). They are reported to restrict HSV-1 infection (Mattiroli et al, 2012), however, how RNF8 and RNF168 relate to HSV-1 replication centers is not known. To characterize the relationship between viral replication centers and these DDR factors, we double stained using CTCF or ICP4 to label the viral replication centers.

CTCF can be recruited to the HSV-1 replication compartment and colocalized with ICP4 (Figures 4C and 4E). However,

because some commercial antibodies are not compatible with ICP4 (for example, mouse derived 53BP1 antibody and ICP4), we used rabbit derived CTCF antibody to label HSV-1 replication centers in certain experiments. As shown in Figure 3A, the 53BP1 and CTCF signals colocalized quite well, suggesting that 53BP1 was recruited by the viral centers. Similarly, BRCA1 was also recruited by these centers (Figure 3B, arrows), although the staining signals were weaker. Conversely, RNF8 was excluded by the HSV-1 replication centers. We circled two well-defined replication centers with dashed lines (Figure 3C, white arrows), which shows much weaker RNF8 signals compared with ICP4. RNF168 staining was too weak to determine recruitment or exclusion. The weak RNF168 signal was probably due to degradation by the viral ICP0 protein (Chaurushiya et al, 2012; Lilley et al, 2010). In contrast to the results of Lilley et al (2010), RNF8 showed less degradation than RNF168, as seen in Figures 3C and 3D. This may be due to differences between the cell line, antibody and infection period. Taken together, our results demonstrated that HSV-1 replication centers selectively recruited two DDR factors, 53BP1 and BRCA1, and excluded RNF8, which suggests that 53BP1 and BRCA1 may be beneficial to viral growth.

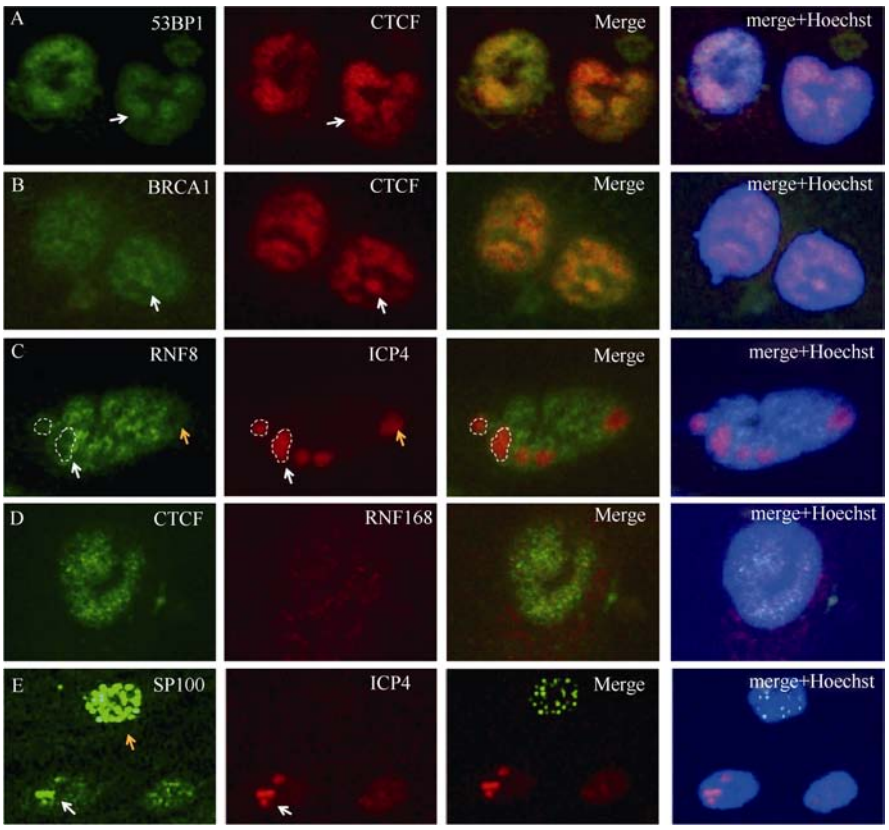

Figure 3 Recruitment or exclusion between HSV-1 replication centers and cellular DDR factors

Each row of panels was double stained using two antibodies. Red and green signals were merged to show how the two signals relate to each other (overlap or mutual exclusion). Nucleus was stained with Hoechst33342 to show nucleus outline. A: Double staining using 53BP1 (green) and CTCF antibodies (red). White arrows show a viral replication center, highlighting overlap between two proteins. B: Double staining using CTCF (red) and BRCA1 (green) antibodies. Arrows show a viral replication center. Merged signals show overlap of these two proteins in the replication center. C: Double staining using ICP4 (red) and RNF8 (green). Dashed circles show two HSV-1 replication centers highlighting apparent exclusion of RNF8 by replication centers. Orange arrows show a large replication center and the absence of RNF8 staining. D: Double staining of CTCF (green) and RNF168 proteins (red). E: Double staining of SP100 (green) and ICP4 (red). White arrows show a cluster of three replication centers and co-localization of SP100 with these centers. Orange arrow show an uninfected cell where SP100 shows punctate staining. Magnification ratio: 400X.

Cellular antiviral defense factor SP100 interacted with HSV-1 replication centers

SP100 is a key cellular antiviral defense protein normally stored in the promyelocytic leukemia protein (PML) bodies. HSV-1 protein ICP0 specifically targets the PML bodies and degrades their components (Everett & Murray, 2005; Gu et al, 2013; Negorev et al, 2009). This can be seen in the uninfected cell in Figure 3E (orange arrow), where SP100 staining exhibited a strong, punctate pattern. In infected cells, however, this pattern disappeared in Figure 3E (white arrow). To determine if SP100 was recruited by the viral replication centers, we increased the amount of exposure to offset the effect of degradation of SP100

by ICP0. The staining results clearly showed that SP100 was recruited by the viral replication centers (Figure 3E, white arrows), which suggests a possible direct interaction of SP100 with the HSV-1 genome to inhibit viral gene transcription.

CTCF recruitment was facilitated by the ATM kinase pathway

HSV-1 lytic infection activates the host DDR, as marked by the activation of ATM kinase, a key signaling kinase (Lilley et al, 2005). In the present study, several members of the host DDR were recruited to or towards the HSV-1 replication foci, including ATM, γH2A.X, 53BP1 (Figure 3A) and BRCA1 (Figure 3B). ATM kinase has been shown to affect HSV-1 replication (Lilley et al, 2011). To investigate whether CTCF recruitment was affected by the host DDR, we tested the effect of ATM inhibitor (ATMi) KU55933 on CTCF recruitment by the replicating viral foci (Hickson et al, 2004). As a control experiment, we monitored the behavior of γH2A.X. As shown in Figures 4A-4B, ATMi slightly inhibited the recruitment of γH2A.X to the HSV-1 replication center. Before adding ATMi, more γH2A.X was recruited to the ICP4 foci and it was distributed in a broad area around the foci, while in ATMi-treated cells, less γH2A.X was recruited and it was located in a much tighter area, which overlapped almost exactly with the ICP4 foci. The reduction in γH2A.X recruitment indicated that ATMi indeed inhibited the host DDR. Similarly, recruitment of

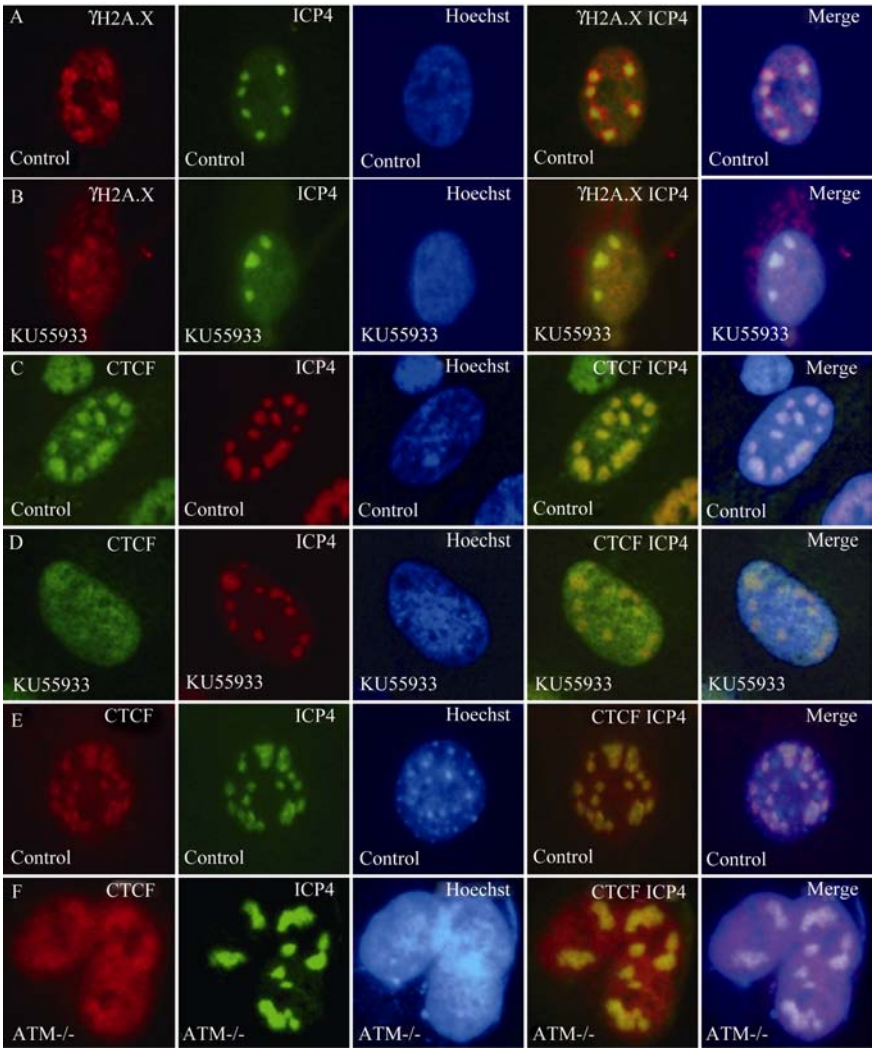

Figure 4 CTCF recruitment into HSV-1 replication centers facilitated by ATM pathway

To investigate whether CTCF recruitment was affected by the ATM pathway, we tested the effect of ATM inhibitor (ATMi) KU55933. A: BJ cells infected with HSV-1 17+ and fixed for immunostaining with either polyclonal antibodies against γH2A.X (red) or monoclonal antibody against viral protein ICP4 (green). Merged image shows γH2A.X recruitment to the viral replication centers and occupation of large areas around the viral foci. B: Reduced recruitment of γH2A.X, and colocalization with ICP4 when ATM inhibitor was added 1 hour prior to infection. C: CTCF (red) and ICP4 (green) showing clear colocalization at 6 hpi. D: Significant inhibition of CTCF recruitment after addition of ATMi. E: CTCF (red) and ICP4 (green) colocalization in mouse MEF cells. F: Less prominent CTCF recruitment and less defined staining in MEF cells deficient of ATM. Magnification ratio: 400X.

CTCF by the viral foci was also noticeably reduced by ATMi, as the inhibitor led to only slight thickening of the CTCF signal around viral replication centers, while the control showed a well-defined CTCF staining pattern (Figures 4C and 4D).

We quantified the effect of ATMi on overall HSV-1 foci appearance as well as the degree of CTCF recruitment by counting the HSV-1 infected cells that exhibited different types ICP4 staining: that is, forming foci, no foci but with high levels of diffused staining, and low levels of diffused ICP4 staining (Figure 5A). Ku55933 treatment of cells reduced the number of ICP4 positive cells by a third, or increased uninfected cells by a half, indicating inhibition of HSV-1 infection by the ATMi (Figure 5B). Consistent with this, the percentage of ICP4 cells with ICP4 foci was also reduced by about a third, while the proportion of diffused ICP4 staining increased. In ICP4 foci positive cells, about 90% of cells also recruited CTCF; however, in Ku55933-treated cells, only about 40% of cells recruited CTCF (Figure 5B).

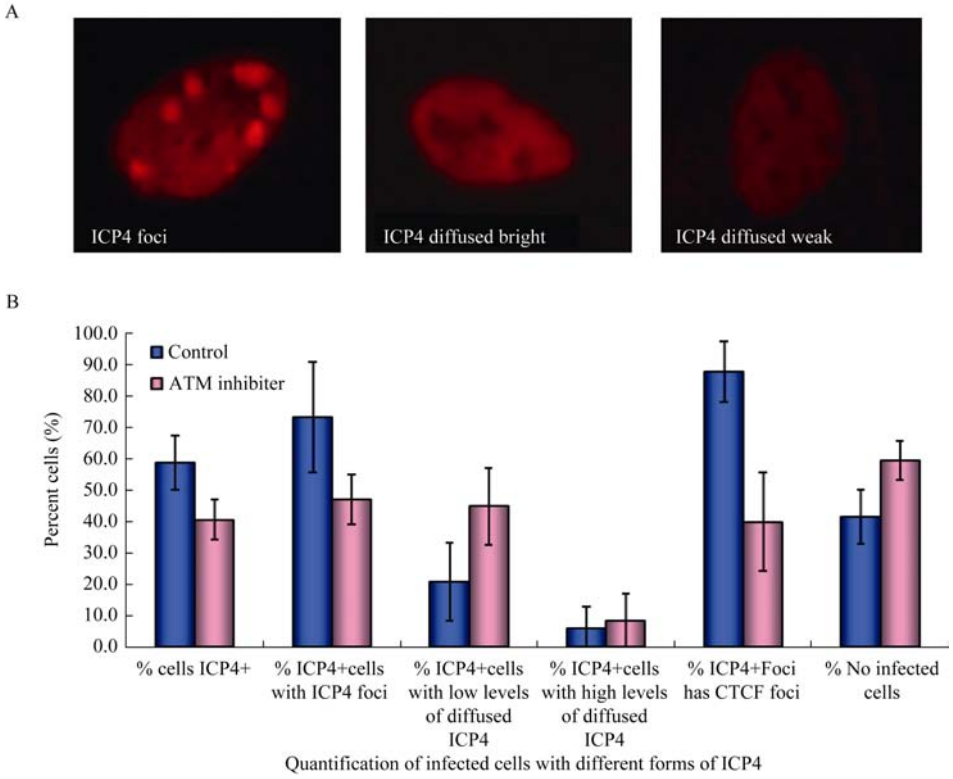

Figure 5 Inhibition of HSV-1 foci formation and CTCF recruitment into viral replication centers by ATMi

BJ cells were infected with HSV-1 17+ at 1 MOI and were fixed and stained with ICP4 antibody at 6 hpi. A**:** ICP4 staining is classified as "*ICP4 foci"* to represent clearly formed, defined viral foci; "*ICP4 diffused, bright"* to designate high levels of ICP4 staining but no foci formation; "*ICP4 diffused, weak"* to denote detectable ICP4 staining without foci formation. B**:** In control DMSO treated cells, a majority of infected cells display ICP4 foci while a smaller portion contain diffused staining. In cells treated with ATM inhibitor KU55933, the portion of foci forming cells is much smaller, about 20% of infected cells, while about half the infected cells show weak diffused staining and a third show strong diffused staining. At 1 MOI, ATMi also inhibited the number of cells infected by HSV-1, about a third drop percentage wise. At the same time, γH2A.X and CTCF recruitment into ICP4 foci were reduced by the inhibitor. Magnification ratio: 400X.

We also tested the effect of mouse MEF cells deficient of ATM (Lilley et al, 2011). The control MEF cells (Figure 4E) displayed a similar pattern of HSV-1 foci and CTCF recruitment to that of human BJ cells (Figures 4C and 4E). In the mutant cells, recruitment was significantly reduced (Figure 4F) compared with that observed in Figure 4E. These results strongly suggest that the ATM pathway facilitated CTCF recruitment into the HSV-1 replication centers.

DISCUSSION

We surveyed the interactions between HSV-1 replication centers and host chromatin, host RNA Pol II and host DDR factors. We found that viral replication centers selectively excluded modified histone H3, but not unmodified H3 (Figure 1). RNA Pol II was highly recruited to the centers, but there was a dynamic shift in the amount of recruitment as viral replication centers transited from small distinct foci to large fused centers (Figure 2). The host DDR factors also exhibited selective recruitment or exclusion from viral centers. BRCA1 and 53BP1 were recruited, but RNF8 was excluded (Figure 3). We found that the recruitment of host epigenetic regulator CTCF was regulated by ATM kinase (Figure 4 and 5), suggesting that recruiting host factors was an active process.

Interaction of host chromatin with HSV-1 replication centers

Immunostaining of histone H3 and modified histone H3 (H3K9me3, H3K4me3 and H3S10p) showed differential staining results. H3 interacted with the viral replication centers, but was not enriched in these centers (Figure 1A), while H3K9me3, H3K4me3 and H3S10p were all excluded by the replication centers (Figures 1B-D). H3K9me3 is a heterochromatin mark, and its exclusion was expected as the replicating virus was poorly chromatinized and unlikely to form heterochromatin. In contrast, the exclusion of H3K4me3, an active chromatin mark interacting with highly transcribed gene promoters, was rather unexpected. The functional implication of this exclusion is interesting and merits further investigation.

We also observed strong recruitment of RNA Pol II (Figure 2A), consistent with a previous study (Dai et al, 2006). However, we found that as the viral replication centers grew in size, RNA Pol II Ser2P quickly disappeared from these centers (Figure 2B). Similarly, RNA Pol II Ser5P also became weaker as small viral foci merged into large ones (Figure 2C). This suggests that as the virus began genome replication, the transcription of the viral genes was gradually reduced. Since transcription and DNA replication are incompatible, it is possible that as more viral genomes started rapid DNA synthesis, transcription and thus RNA Pol II recruitment was inhibited. How this process is regulated is an interesting and important question.

Replicating HSV-1 genome and host DDR

HSV-1 has a complex interaction with host responses. HSV-1 lytic infection activates the host DDR, either due to replicative stress resulting from depletion of host DNA replication factors, or from exposed double strand DNA ends from the linear genome (Smith et al, 2014). Host DDR will trigger apoptosis and transcription silencing, which are both deleterious to HSV-1 growth. However, some DDR components are needed for viral replication (Karttunen et al, 2014). Clearly, HSV-1 has successfully dealt with host DDR, i.e., taking advantage of host DDR factors that are beneficial, such as ATM, and inhibiting or degrading host DDR factors that are harmful for viral growth. For example, RINF8 and RNF168 are destroyed by ICP0 (Chaurushiya et al, 2012; Lilley et al, 2010). Although it is not clear how many DDR factors affect HSV-1 lytic infection, the recruitment or exclusion of certain DDR factors clearly indicates an active choice by the HSV-1 replication center. Previous research showed that γH2A.X was recruited by HSV-1 replication centers, but it did not co-localize with the replication centers exactly (Wilkinson & Weller, 2006). In this study, we found that cellular 53BP1 and BRCA1 were recruited by the viral replication center, while RNF8 was excluded. This suggests that 53BP1 and BRCA1 may play positive roles in viral growth, while RNF8 (and RNF168) may play a restrictive one. Consistent with this analysis, the positive role of 53BP1 and the inhibitory roles of RNF8 and RNF168 have been reported earlier (Bailey et al, 2009; Salsman et al, 2012).

Recruitment of CTCF and its implications

CTCF interacts with a consensus sequence through its zinc finger DNA binding domain (Burke et al, 2005; Klenova et al, 1993; Moon et al, 2005). Other than DNA methylation, which interrupts CTCF binding to its target, no other reported mechanism can regulate CTCF binding to DNA (Filippova et al, 2001; Teif et al, 2014). However, various studies, especially whole genome ChIP-seq studies, have revealed that CTCF binding to genomic sites is dynamic and regulated in a tissue specific manner, not all of which can be explained by DNA methylation of its binding sites (Shukla et al, 2011). In an earlier study (submitted, results in “CTCF interacts with the lytic HSV-1 genome to promote viral transcription and replication center organization”), we provided evidence that CTCF was recruited by HSV-1 replication centers and played a role in keeping γH2A.X from entering viral replication centers. In the present study, we showed that CTCF recruitment was facilitated by the ATM kinase pathway. The inhibition of ATM by Ku55933 also reduced γH2A.X aggregation around the HSV-1 foci, and inhibition may have caused infiltration of γH2A.X, a possibility consistent with the reduced recruitment of CTCF (Figure 4D). This ATM-assisted CTCF recruitment is reminiscent of the recruitment of host DDR factors to double strand DNA breaks (Matsuoka et al, 2007). Can CTCF participate in the host DDR? In a separate study, we found evidence that CTCF was recruited through the ATM pathway to double strand DNA breaks and participated in host DDR (unpublished). This new property of CTCF, if established, further suggests that HSV-1 could take advantage of host DDR to quickly recruit CTCF to organize the viral genome and replication centers, and facilitate viral transcription. Indeed, our CTCF ChIP-seq data (unpublished) suggested that 6 hour after HSV-1 infection, even though the total HSV-1 DNA per cell was less than that of the host, the recruitment of CTCF by HSV-1 had led to the loss of approximately 90% of CTCF binding peaks in the host genome, underscoring dramatic host genome reorganization.

REFERENCES

Bailey SG, Verrall E, Schelcher C, Rhie A, Doherty AJ, Sinclair AJ. 2009. Functional interaction between Epstein-Barr virus replication protein Zta and host DNA damage response protein 53BP1. *Journal of Virology,* **83**(21): 11116-11122.

Barzilai A, Zivony-Elbom I, Sarid R, Noah E, Frenkel N. 2006. The herpes simplex virus type 1 vhs-UL41 gene secures viral replication by temporarily evading apoptotic cellular response to infection: Vhs-UL41 activity might require interactions with elements of cellular mRNA degradation machinery. *Journal of Virology,* **80**(1): 505-513.

Burke LJ, Zhang R, Bartkuhn M, Tiwari VK, Tavoosidana G, Kurukuti S, Weth C, Leers J, Galjart N, Ohlsson R, Renkawitz R. 2005. CTCF binding and higher order chromatin structure of the H19 locus are maintained in mitotic chromatin. *The EMBO Journal,* **24**(18): 3291-3300.

Chaurushiya MS, Lilley CE, Aslanian A, Meisenhelder J, Scott DC, Landry S, Ticau S, Boutell C, Yates JR, 3rd, Schulman BA, Hunter T, Weitzman MD. 2012. Viral E3 ubiquitin ligase-mediated degradation of a cellular E3: viral mimicry of a cellular phosphorylation mark targets the RNF8 FHA domain. *Molecular Cell,* **46**(1): 79-90.

Conn KL, Schang LM. 2013. Chromatin dynamics during lytic infection with herpes simplex virus 1. *Viruses,* **5**(7): 1758-1786.

Dai-Ju JQ, Li L, Johnson LA, Sandri-Goldin RM. 2006. ICP27 interacts with the C-terminal domain of RNA polymerase II and facilitates its recruitment to herpes simplex virus 1 transcription sites, where it undergoes proteasomal degradation during infection. *Journal of Virology,* **80**(7): 3567-3581.

Egloff S, Murphy S. 2008. Cracking the RNA polymerase II CTD code. *Trends in Genetics,* **24**(6): 280-288.

Esclatine A, Taddeo B, Evans L, Roizman B. 2004. The herpes simplex virus 1 *U_L41* gene-dependent destabilization of cellular RNAs is selective and may be sequence-specific. *Proceedings of the National Academy of Sciences of the United States of America,* **101**(10): 3603-3608.

Everett RD. 2014. HSV-1 biology and life cycle. *Methods in Molecular Biology,* **1144**: 1-17.

Everett RD, Murray J. 2005. ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection. *Journal of Virology,* **79**(8): 5078-5089.

Everett RD, Sourvinos G, Leiper C, Clements JB, Orr A. 2004. Formation of nuclear foci of the herpes simplex virus type 1 regulatory protein ICP4 at early times of infection: localization, dynamics, recruitment of ICP27, and evidence for the de novo induction of ND10-like complexes. *Journal of Virology,* **78**(4): 1903-1917.

Ferenczy MW, Ranayhossaini DJ, Deluca NA. 2011. Activities of ICP0 involved in the reversal of silencing of quiescent herpes simplex virus 1. *Journal of Virology,* **85**(10): 4993-5002.

Filippova GN, Thienes CP, Penn BH, Cho DH, Hu YJ, Moore JM, Klesert TR, Lobanenkov VV, Tapscott SJ. 2001. CTCF-binding sites flank CTG/CAG repeats and form a methylation-sensitive insulator at the *DM1* locus. *Nature Genetics,* **28**(4): 335-343.

Fraser KA, Rice SA. 2007. Herpes simplex virus immediate-early protein ICP22 triggers loss of serine 2-phosphorylated RNA polymerase II. *Journal of Virology,* **81**(10): 5091-5101.

Griffiths SJ, Koegl M, Boutell C, Zenner HL, Crump CM, Pica F, Gonzalez O, Friedel CC, Barry G, Martin K, Craigon MH, Chen R, Kaza LN, Fossum E, Fazakerley JK, Efstathiou S, Volpi A, Zimmer R, Ghazal P, Haas J. 2013. A systematic analysis of host factors reveals a Med23-interferon-λ regulatory axis against herpes simplex virus type 1 replication. *PLoS Pathogens,* **9**(8): e1003514.

Gu HD, Zheng Y, Roizman B. 2013. Interaction of herpes simplex virus ICP0 with ND10 bodies: a sequential process of adhesion, fusion, and retention. *Journal of Virology,* **87**(18): 10244-10254.

Hickson I, Zhao Y, Richardson CJ, Green SJ, Martin NM, Orr AI, Reaper PM, Jackson SP, Curtin NJ, Smith GCM. 2004. Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. *Cancer Research,* **64**(24): 9152-9159.

Jenkins HL, Spencer CA. 2001. RNA polymerase II holoenzyme modifications accompany transcription reprogramming in herpes simplex virus type 1-infected cells. *Journal of Virology,* **75**(20): 9872-9884.

Karttunen H, Savas JN, Mckinney C, Chen YH, Yates JR, 3rd, Hukkanen V, Huang TT, Mohr I. 2014. Co-opting the fanconi Anemia genomic stability pathway enables herpesvirus DNA synthesis and productive growth. *Molecular Cell,* **55**(1): 111-122.

Klenova EM, Nicolas RH, Paterson HF, Carne AF, Heath CM, Goodwin GH, Neiman PE, Lobanenkov VV. 1993. CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-myc gene, is an 11-Zn-finger protein differentially expressed in multiple forms. *Molecular and Cellular Biology,* **13**(12): 7612-7624.

Knipe DM, Quinlan MP, Spang AE. 1982. Characterization of two conformational forms of the major DNA-binding protein encoded by herpes simplex virus 1. *Journal of Virology,* **44**(2): 736-741.

Kwak H, Lis JT. 2013. Control of transcriptional elongation. *Annual Review of Genetics,* **47**: 483-508.

Lacasse JJ, Schang LM. 2010. During lytic infections, herpes simplex virus type 1 DNA is in complexes with the properties of unstable nucleosomes. *Journal of Virology,* **84**(4): 1920-1933.

Lacasse JJ, Schang LM. 2012. Herpes simplex virus 1 DNA is in unstable nucleosomes throughout the lytic infection cycle, and the instability of the nucleosomes is independent of DNA replication. *Journal of Virology,* **86**(20): 11287-11300.

Lafaille FG, Pessach IM, Zhang SY, Ciancanelli MJ, Herman M, Abhyankar A, Ying SW, Keros S, Goldstein PA, Mostoslavsky G, Ordovas-Montanes J, Jouanguy E, Plancoulaine S, Tu E, Elkabetz Y, Al-Muhsen S, Tardieu M, Schlaeger TM, Daley GQ, Abel L, Casanova JL, Studer L, Notarangelo LD. 2012. Impaired intrinsic immunity to HSV-1 in human iPSC-derived TLR3-deficient CNS cells. *Nature,* **491**(7426): 769-773.

Lai W, Chen CY, Morse SA, Htun Y, Fehler HG, Liu H, Ballard RC. 2003. Increasing relative prevalence of HSV-2 infection among men with genital ulcers from a mining community in South Africa. *Sexually Transmitted Infections,* **79**(3): 202-207.

Lilley CE, Carson CT, Muotri AR, Gage FH, Weitzman MD. 2005. DNA repair proteins affect the lifecycle of herpes simplex virus 1. *Proceedings of the National Academy of Sciences of the United States of America,* **102**(16): 5844-5849.

Lilley CE, Chaurushiya MS, Boutell C, Everett RD, Weitzman MD. 2011. The intrinsic antiviral defense to incoming HSV-1 genomes includes specific DNA repair proteins and is counteracted by the viral protein ICP0. *PLoS Pathogens,* **7**(6): e1002084.

Lilley CE, Chaurushiya MS, Boutell C, Landry S, Suh J, Panier S, Everett RD, Stewart GS, Durocher D, Weitzman MD. 2010. A viral E3 ligase targets RNF8 and RNF168 to control histone ubiquitination and DNA damage responses. *The EMBO Journal,* **29**(5): 943-955.

Matsuoka S, Ballif BA, Smogorzewska A, Mcdonald ER, 3rd, Hurov KE, Luo J, Bakalarski CE, Zhao ZM, Solimini N, Lerenthal Y, Shiloh Y, Gygi SP, Elledge SJ. 2007. ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science,* **316**(5828): 1160-1166.

Mattiroli F, Vissers JHA, Van Dijk WJ, Ikpa P, Citterio E, Vermeulen W, Marteijn JA, Sixma TK. 2012. RNF168 ubiquitinates K13-15 on H2A/H2AX to drive DNA damage signaling. *Cell,* **150**(6): 1182-1195.

Moon H, Filippova G, Loukinov D, Pugacheva E, Chen Q, Smith ST, Munhall A, Grewe B, Bartkuhn M, Arnold R, Burke LJ, Renkawitz-Pohl R, Ohlsson R, Zhou JM, Renkawitz R, Lobanenkov V. 2005. CTCF is conserved from *Drosophila* to humans and confers enhancer blocking of the *Fab-8* insulator. *EMBO Reports,* **6**(2): 165-170.

Negorev DG, Vladimirova OV, Maul GG. 2009. Differential functions of interferon-upregulated Sp100 isoforms: herpes simplex virus type 1 promoter-based immediate-early gene suppression and PML protection from ICP0-mediated degradation. *Journal of Virology,* **83**(10): 5168-5180.

Nojima T, Oshiro-Ideue T, Nakanoya H, Kawamura H, Morimoto T, Kawaguchi Y, Kataoka N, Hagiwara M. 2009. Herpesvirus protein ICP27 switches PML isoform by altering mRNA splicing. *Nucleic Acids Research,* **37**(19): 6515-6527.

(Roizman BK DM, Whitley RJ) Knipe DM, Howley PM. 2007. Fields Virology. 5nd ed. Philadelphia, PA: Lippincott Williams & Wilkins.

Parkinson J, Lees-Miller SP, Everett RD. 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. *Journal of Virology,* **73**(1): 650-657.

Pasieka TJ, Baas T, Carter VS, Proll SC, Katze MG, Leib DA. 2006. Functional genomic analysis of herpes simplex virus type 1 counteraction of the host innate response. *Journal of Virology,* **80**(15): 7600-7612.

Peng H, Nogueira ML, Vogel JL, Kristie TM. 2010. Transcriptional coactivator HCF-1 couples the histone chaperone Asf1b to HSV-1 DNA replication components. *Proceedings of the National Academy of Sciences of the United States of America,* **107**(6): 2461-2466.

Prasad A, Remick J, Zeichner SL. 2013. Activation of human herpesvirus replication by apoptosis. *Journal of Virology,* **87**(19): 10641-10650.

Rasmussen SB, Horan KA, Holm CK, Stranks AJ, Mettenleiter TC, Simon AK, Jensen SB, Rixon FJ, He B, Paludan SR. 2011. Activation of autophagy by α-herpesviruses in myeloid cells is mediated by cytoplasmic viral DNA through a mechanism dependent on stimulator of IFN genes. *The Journal of Immunology,* **187**(10): 5268-5276.

Roizman B. 2011. The checkpoints of viral gene expression in productive and latent infection: the role of the HDAC/CoREST/LSD1/REST repressor complex. *Journal of Virology,* **85**(15): 7474-7482.

Roizman B, Whitley RJ. 2013. An inquiry into the molecular basis of HSV latency and reactivation. *Annual Review of Microbiology,* **67**: 355-374.

Salsman J, Jagannathan M, Paladino P, Chan PK, Dellaire G, Raught B, Frappier L. 2012. Proteomic profiling of the human cytomegalovirus ul35 gene products reveals a role for UL35 in the DNA repair response. *Journal of Virology,* **86**(2): 806-820.

Sedlackova L, Perkins KD, Lengyel J, Strain AK, Van Santen VL, Rice SA. 2008. Herpes simplex virus type 1 ICP27 regulates expression of a variant, secreted form of glycoprotein C by an intron retention mechanism. *Journal of Virology,* **82**(15): 7443-7455.

Shen GH, Wang KZ, Wang S, Cai MS, Li ML, Zheng CF. 2014. Herpes simplex virus 1 counteracts viperin via its virion host shutoff protein UL41. *Journal of Virology,* **88**(20): 12163-12166.

Showalter SD, Zweig M, Hampar B. 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. *Infection and Immunity,* **34**(3): 684-692.

Shukla S, Kavak E, Gregory M, Imashimizu M, Shutinoski B, Kashlev M, Oberdoerffer P, Sandberg R, Oberdoerffer S. 2011. CTCF-promoted RNA polymerase II pausing links DNA methylation to splicing. *Nature,* **479**(7371): 74-79.

Silva L, Cliffe A, Chang L, Knipe DM. 2008. Role for A-type lamins in herpesviral DNA targeting and heterochromatin modulation. *PLoS Pathogens,* **4**(5): e1000071.

Smith S, Reuven N, Mohni KN, Schumacher AJ, Weller SK. 2014. Structure of the herpes simplex virus 1 genome: manipulation of nicks and gaps can abrogate infectivity and alter the cellular DNA damage response. *Journal of Virology,* **88**(17): 10146-10156.

Song B, Liu JJ, Yeh KC, Knipe DM. 2000. Herpes simplex virus infection blocks events in the G1 phase of the cell cycle. *Virology,* **267**(2): 326-334.

Taddeo B, Zhang WR, Roizman B. 2006. The UL41 protein of herpes simplex virus 1 degrades RNA by endonucleolytic cleavage in absence of other cellular or viral proteins. *Proceedings of the National Academy of Sciences of the United States of America,* **103**(8): 2827-2832.

Taddeo B, Zhang WR, Roizman B. 2013. The herpes simplex virus host shutoff RNase degrades cellular and viral mRNAs made before infection but not viral mRNA made after infection. *Journal of Virology,* **87**(8): 4516-4522.

Taylor TJ, Knipe DM. 2004. Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. *Journal of Virology,* **78**(11): 5856-5866.

Teif VB, Beshnova DA, Vainshtein Y, Marth C, Mallm JP, Höfer T, Rippe K. 2014. Nucleosome repositioning links DNA (de)methylation and differential CTCF binding during stem cell development. *Genome Research,* **24**(8): 1285-1295.

Volcy K, Fraser NW. 2013. DNA damage promotes herpes simplex virus-1 protein expression in a neuroblastoma cell line. *Journal of Neurovirology,* **19**(1): 57-64.

Wang S, Long J, Zheng CF. 2012. The potential link between PML NBs and ICP0 in regulating lytic and latent infection of HSV-1. *Protein & Cell,* **3**(5): 372-382.

Wang XJ, Patenode C, Roizman B. 2011. US3 protein kinase of HSV-1 cycles between the cytoplasm and nucleus and interacts with programmed cell death protein 4 (PDCD4) to block apoptosis. *Proceedings of the National Academy of Sciences of the United States of America,* **108**(35): 14632-14636.

Wang Y, Yang Y, Wu SF, Pan S, Zhou CD, Ma YJ, Ru YX, Dong SX, He B, Zhang CZ, Cao YJ. 2014. p32 is a novel target for viral protein ICP34.5 of herpes simplex virus type 1 and facilitates viral nuclear egress. *Journal of Biological Chemistry,* **289**(52): 35795-35805.

Weitzman MD, Carson CT, Schwartz RA, Lilley CE. 2004. Interactions of viruses with the cellular DNA repair machinery. *DNA Repair,* **3**(8-9): 1165-1173.

Weitzman MD, Weitzman JB. 2014. What's the damage? The impact of pathogens on pathways that maintain host genome integrity. *Cell Host & Microbe,* **15**(3): 283-294.

Wilkinson DE, Weller SK. 2004. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. *Journal of Virology,* **78**(9): 4783-4796.

Wilkinson DE, Weller SK. 2006. Herpes simplex virus type I disrupts the ATR-dependent DNA-damage response during lytic infection. *Journal of Cell Science,* **119**(Pt 13): 2695-2703.

Wysocka J, Herr W. 2003. The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. *Trends in Biochemical Sciences,* **28**(6): 294-304.

Yun MH, Hiom K. 2009. CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. *Nature,* **459**(7245): 460-463.

Zaborowska J, Baumli S, Laitem C, O'reilly D, Thomas PH, O'Hare P, Murphy S. 2014. Herpes Simplex Virus 1 (HSV-1) ICP22 protein directly interacts with cyclin-dependent kinase (CDK)9 to inhibit RNA polymerase II transcription elongation. *PloS One,* **9**(9): e107654.

Zhou Q, Li TD, Price DH. 2012. RNA polymerase II elongation control. *Annual Review of Biochemistry,* **81**: 119-143.

Zhou YH, Liang YB, Pang W, Qin WH, Yao ZH, Chen X, Zhang CY, Zheng YT. 2014. Diverse forms of HIV-1 among Burmese long-distance truck drivers imply their contribution to HIV-1 cross-border transmission. *BMC Infectious Diseases,* **14**: 463.