



Nuclear interferon-stimulated gene product maintains heterochromatin on the herpes simplex viral genome to limit lytic infection

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Interferons (IFN) are expressed in and secreted from cells in response to virus infection, and they induce the expression of a variety of genes called interferon-stimulated genes (ISGs) in infected and surrounding cells to block viral infection and limit spread. The mechanisms of action of a number of cytoplasmic ISGs have been well defined, but little is known about the mechanism of action of nuclear ISGs. Constitutive levels of nuclear interferon-inducible protein 16 (IFI16) serve to induce innate signaling and epigenetic silencing of herpes simplex virus (HSV), but only when the HSV infected cell protein 0 (ICP0) E3 ligase, which promotes IFI16 degradation, is inactivated. In this study, we found that following IFN induction, the pool of IFI16 within the infected cell remains high and can restrict wild-type viral gene expression and replication due to both the induced levels of IFI16 and the IFI16-mediated repression of ICP0 levels. Restriction of viral gene expression is achieved by IFI16 promoting the maintenance of heterochromatin on the viral genome, which silences it epigenetically. These results indicate that a nuclear ISG can restrict gene expression and replication of a nuclear DNA virus by maintaining or preventing the removal of repressive heterochromatin associated with the viral genome.

interferon | herpes simplex virus | ICP0 | lytic infection | HSV

Mammalian cells respond to microbial infection as a result of cellular pathogen recognition receptors recognizing microbial molecules that bear pathogen-associated molecular patterns and inducing innate immune signaling pathways that resist microbial infection (1). During infection, one of the major induced innate immune responses consists of the expression and secretion of interferon (IFN) family cytokines, named for their ability to interfere with viral replication (1). IFNs act in an autocrine or paracrine manner, binding to receptors and inducing the expression of IFN-stimulated genes (ISGs) whose products can act as effectors for inhibition of microbes. The mechanisms of action of a number of the cytoplasmic ISG proteins, including the IFN-inducible proteins with tetratricopeptide repeats (IFITs), protein kinase R (PKR), and Mx family proteins, have been defined (1, 2), but much less is known about ISGs acting within the nucleus, particularly during DNA virus infections.

Nuclear factors such as promyelocytic leukemia protein (PML), interferon-inducible protein 16 (IFI16), and Sp100 are expressed at constitutive levels and can restrict infection by certain DNA viruses (3–11). However, their expression can be further stimulated by IFN cytokines (12–15). There is evidence that PML can restrict wild-type (WT) HSV-1 (herpes simplex virus 1) replication when stimulated by IFN (16) and that both PML and Sp100 can limit WT viral gene expression in IFN-stimulated cells (16, 17), but the specific mechanisms by which these cellular gene products function as ISGs to mediate IFN control within the nucleus have yet to be defined.

HSV-1 is a prototype of a nuclear-replicating DNA virus and a member of the *Herpesviridae* family of large double-stranded DNA (dsDNA) viruses, as well as a widespread human pathogen, with a majority of the world's population estimated to be infected with HSV-1 (18, 19). Although often asymptomatic, HSV-1 infection can result in clinically severe disease, such as ocular keratitis and encephalitis, particularly in immunocompromised individuals (20). Individuals with defective IFN induction or signaling response pathways are more susceptible to both severe HSV infection and to more frequently recurring disease (21–25). IFN signaling is a critical component of the front-line, innate immune response to HSV infection and secretion of IFN cytokines by both immune and nonimmune cells can contribute to host immune control at both lytic and latent stages of infection (26–33).

Significance

Upon viral infection, cells produce and secrete proteins called interferons (IFNs) that bind to receptors on infected or neighboring cells to induce the expression of genes whose products act to interfere with viral replication. The mechanisms of action of certain of the IFN-induced proteins functioning in the cytoplasm have been defined, but little is known about the mechanisms of restriction of IFN-inducible proteins that act within the nucleus. In this study, we showed that a cellular nuclear protein, when induced by IFN, can reduce wild-type herpes simplex virus infection through effects on viral chromatin. This study defines the impact of this nuclear IFN-stimulated gene and provides opportunities for future antiviral strategies relating to epigenetic silencing within the nucleus.

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HSV-1 genomes enter the nucleus as naked DNA but are rapidly associated with cellular histones. During lytic infection, HSV genes are transcribed in a sequential progression from immediate-early (IE) to early (E) to late (L) gene products that enables viral DNA synthesis and the production of new infectious viral particles to occur (34, 35). The transcriptional cascade is accomplished in part by the actions of viral and cellular proteins that counter cellular epigenetic silencing. For example, virion protein 16 organizes a complex of cellular proteins that binds to IE gene promoters, converts heterochromatin to euchromatin, and allows access of transcriptional activators (36–38). The infected cell protein 0 (ICP0) protein is an E3 ubiquitin ligase (39, 40) that promotes the degradation of several host restriction factors including PML, IFI16, and Sp100 (41–43), which allows for the conversion of

heterochromatin to euchromatin on the E and L genes (44, 45). Therefore, while the host cell silences the incoming HSV-1 genome with heterochromatin, viral proteins can modulate the levels of the deposited heterochromatin and promote euchromatin formation to allow lytic gene transcription.

Host-cell defenses against HSV infection include an array of proteins that function to restrict virus replication and limit spread (34). In particular, IFI16 has been shown to associate with viral DNA rapidly following nuclear entry (46) and bind at sites across the viral genome (3, 47–49). We and others identified previously a role for IFI16 in the intrinsic restriction of an ICP0-negative HSV mutant virus (3, 50, 51). IFI16 is a predominantly nuclear protein that preferentially binds non-nucleosomal DNA (52). During WT virus infection, IFI16 is targeted by the IE protein

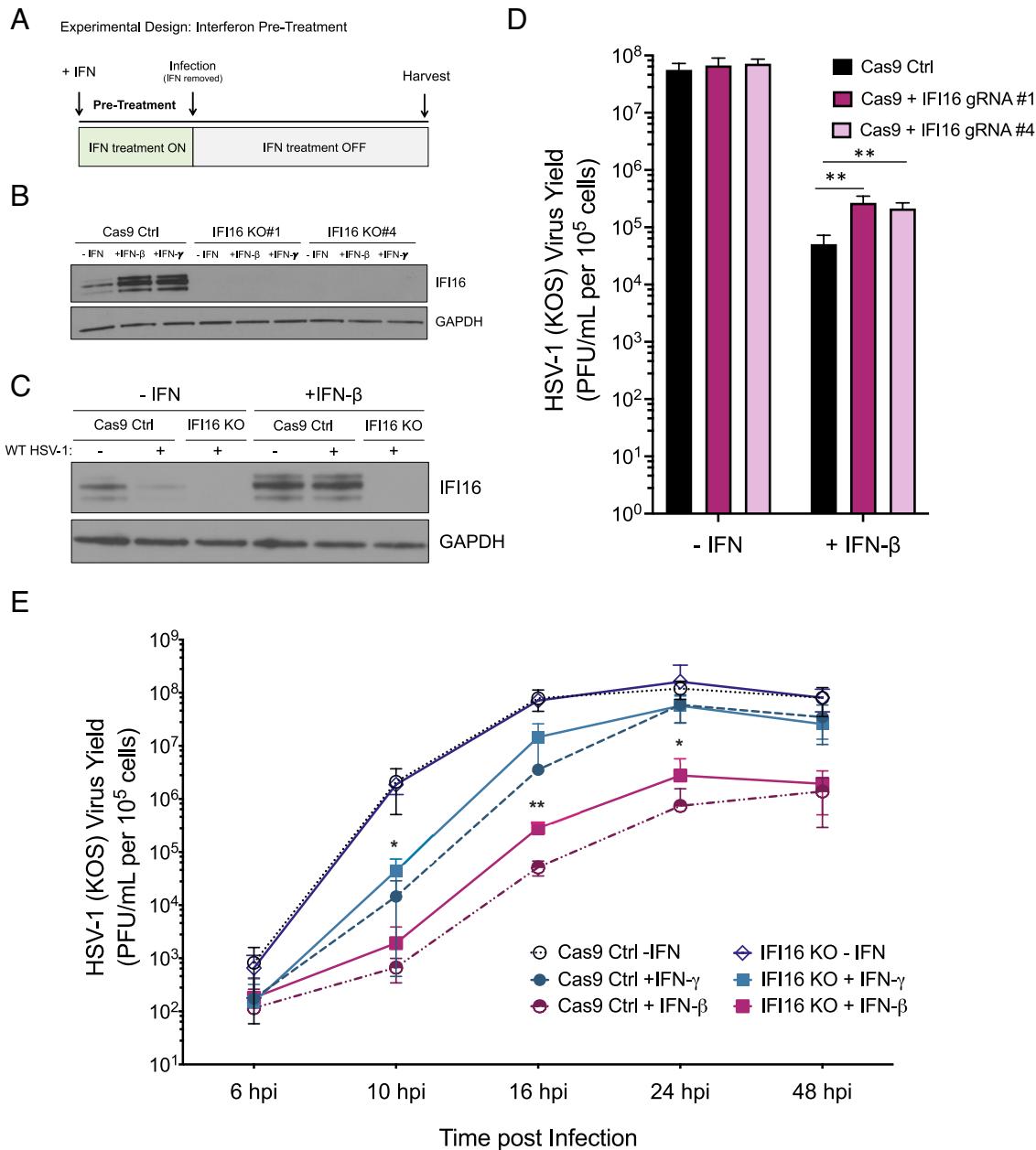


Fig. 1. IFI16 reduced replication of WT HSV-1 following IFN pretreatment. (A) IFN pretreatment was performed 20 h prior to infection as diagrammed. (B) Immunoblot analysis of IFI16 protein levels in pretreated Cas9-transduced control and IFI16 knockout cells ($n = 3$). (C) Immunoblot analysis of IFI16 expression following mock infection or infection of the control and IFI16 knockout fibroblasts with WT HSV-1 KOS (MOI 1, 10 hpi). (D) Viral yields at 16 hpi with the HSV-1 KOS strain (MOI 1) were determined by plaque assay following IFN- β pretreatment (1,000 IU/mL) of the IFI16 knockout and control fibroblasts ($n = 3$). (E) A time course was conducted following IFN- γ or IFN- β treatment to assess viral yield following HSV-1 (KOS, MOI 1) infection. Statistical significance was determined from at least three biological replicates by two-way ANOVA followed by Tukey's multiple comparison test. Mean \pm SD (error bars), * $P < 0.05$, ** $P < 0.01$.

ICP0 for degradation. Consequently, IFI16 at basal levels does not significantly impact viral IE or E gene expression or affect viral yield during WT HSV infection (3, 41, 50). However, in the absence of ICP0 expression, IFI16 promotes the association of restrictive heterochromatin (H3K9me3) with viral IE and E gene promoters, and IFI16 depletion results in increased IE and E gene transcription and expression as well as increased viral replication and yield (3, 50, 53). In addition, ectopic expression of IFI16 can also restrict WT HSV-1 gene expression and replication (48).

IFN-mediated restriction during HSV lytic infection has been linked previously to the suppression of IE gene expression within

the nucleus (27, 28, 54). IFI16 has an established ability to restrict HSV-1 gene expression following nuclear entry. While there is a constitutive basal level of IFI16 expression, IFI16 is also an IFN-inducible gene and its expression can be elevated by type I and type II IFN cytokines (12, 13). IFI16 could thus potentially contribute to IFN-mediated restriction of HSV in addition to its established role in intrinsic restriction. As we showed previously that IFN-induced IFI16 could play a role in HSV-2 replication (32), we hypothesized that IFN-stimulated levels of IFI16 may be sufficient to overcome ICP0-mediated degradation in HSV-1 infected cells and restrict WT viral gene expression and replication. We were particularly interested in whether IFN-mediated

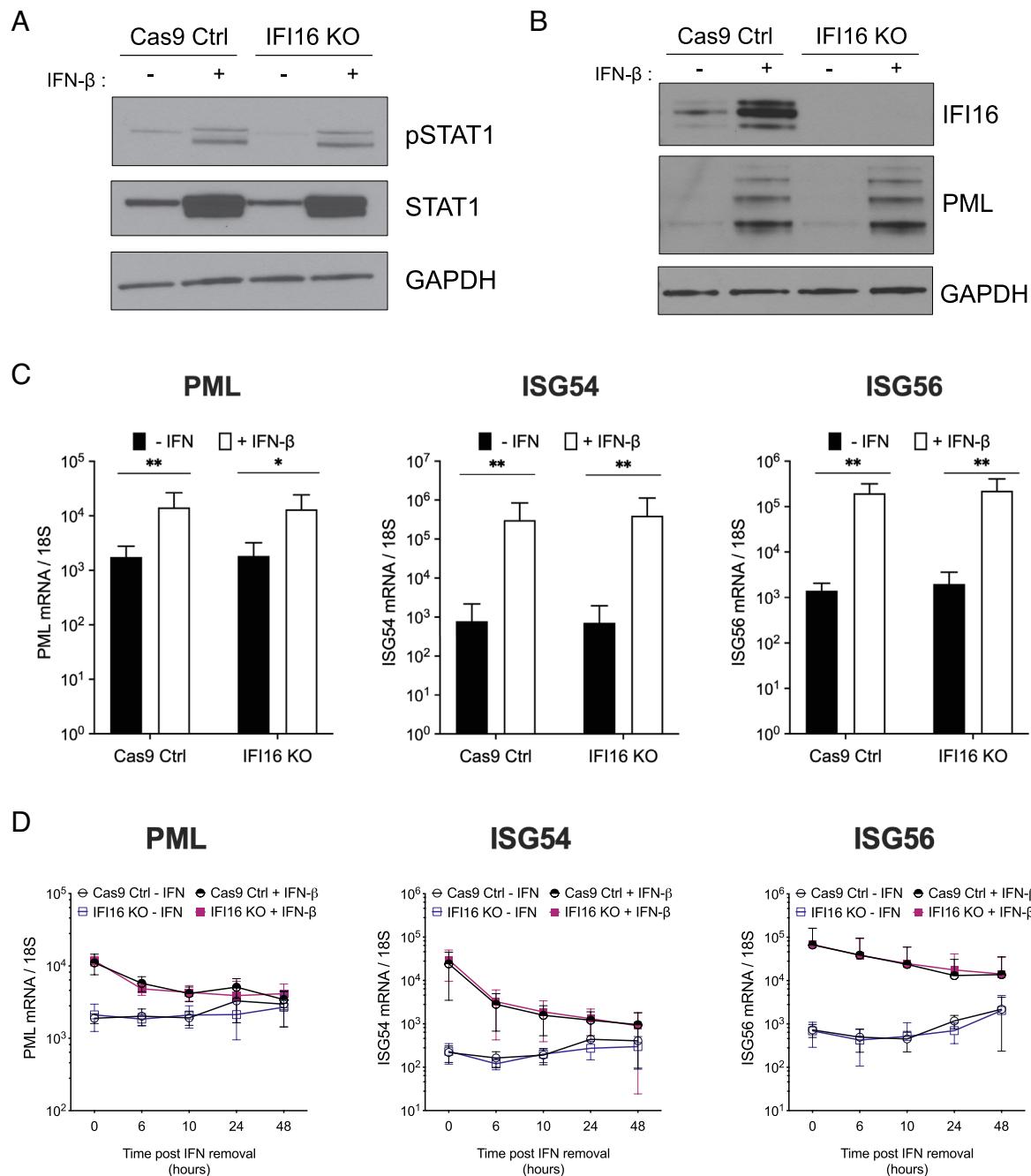


Fig. 2. IFI16 knockout and control fibroblasts responded comparably to IFN- β stimulation. Cas9-transduced cells were treated for 20 h with IFN- β (1,000 IU/mL) or left untreated (-IFN). (A and B) Immunoblot analyses of (A) STAT1 and pSTAT1 (Tyr 701) protein levels, and (B) IFI16 and PML protein levels in pretreated Cas9-transduced cells ($n = 3$). (C) qRT-PCR analyses of IFI16 and interferon-stimulated gene (ISG) transcript levels in pretreated Cas9-transduced control and IFI16 knockout HFFs. (D) qRT-PCR analysis of ISG transcript levels following IFN- β treatment, with RNA isolated from whole-cell lysate harvested at indicated time points post IFN removal ($n = 3$). Statistical significance in C was determined by two-way ANOVA followed by Tukey's multiple comparison test from four biological replicates. Mean \pm SD (error bars), * $P < 0.05$, ** $P < 0.01$.

restriction of viral replication could in part be due to epigenetic repression of viral gene expression during lytic infection.

Results

IFI16 Contributes to IFN-Mediated Restriction of WT HSV-1 Replication

As discussed above, ICP0 targets IFI16 for degradation; therefore, constitutive levels of IFI16 restrict ICP0-null strains of HSV-1 but not ICP0-positive viruses. To test whether IFN-stimulated levels of IFI16 could overcome ICP0 degradation and restrict WT HSV-1 infection and contribute to IFN-induced host-cell defenses, we first established conditions for induction of IFI16 in human foreskin fibroblasts (HFFs) using an IFN pretreatment approach in which cells were treated with IFN for 20 h prior to infection (Fig. 1A). We observed elevated levels of IFI16 protein expression in fibroblasts following treatment with either type I (IFN- β) or type II (IFN- γ) family cytokines (Fig. 1B and *SI Appendix*, Fig. S1 A–C), with IFN treatment increasing IFI16 levels in the nucleus (*SI Appendix*, Fig. S1D). IFI16 protein expression was not detected in either of two IFI16-knockout cell lines, either with or without IFN stimulation. Furthermore, in IFN-treated cells, IFI16 expression remained elevated following infection with WT HSV-1 (Fig. 1C), indicating that IFN-stimulated levels of IFI16 can persist regardless of targeting by ICP0.

To define the role of IFI16 in IFN-mediated inhibition of WT virus infection, we compared viral yields following infection with the HSV-1 KOS strain in the IFN-pretreated IFI16 knockout cell lines to yields in the pretreated Cas9-transduced control cell line. We observed that following IFN treatment, HSV-1 WT virus replication increased ~5-fold in the IFI16 knockout cell lines relative to the control cells (Fig. 1D). No IFI16 restriction of WT HSV-1 was observed in the absence of IFN treatment (Fig. 1D), consistent with previously published results (3, 50). Furthermore, the observed effect on virus yield was IFN dose-dependent, with the extent of both IFN-mediated restriction and IFI16-dependent restriction correlating with the levels of induced IFI16 protein expression (*SI Appendix*, Fig. S2). The ~10-fold increase in HSV-1 yield following siRNA-mediated depletion of IFI16 in WT HFFs relative to control cells provided further confirmation that the observed effect was due to IFI16 (*SI Appendix*, Fig. S3).

We observed increased replication of WT HSV-1 in the IFI16 knockout cells following IFN- γ treatment as well as following IFN- β treatment (Fig. 1E). However, the effect of IFN- γ on WT virus replication in fibroblasts was relatively modest compared to that of IFN- β . Accordingly, combined treatment with IFN- β and IFN- γ only slightly increased HSV-1 restriction and had no significant effect on IFI16 restriction compared to IFN- β treatment alone (*SI Appendix*, Fig. S4C).

We observed previously that IFN- γ -stimulated levels of IFI16 contributed to control of HSV-2 in epithelia (32). Consistent with previous results, we observed increased HSV-2 replication following IFN- β stimulation in the IFI16 knockout cells relative to control cells (*SI Appendix*, Fig. S5).

IFI16 Knockout and Control Fibroblasts Respond Comparably to IFN Treatment

IFI16 can affect innate signaling as well as epigenetic silencing through its role as a nuclear DNA sensor (3, 41, 55). IFI16 has been shown to promote IFN- β production upon sensing of viral DNA released into the nucleus, and it can thus potentially influence host-cell IFN signaling or response pathways. To test whether initial IFN signaling was impaired in the absence of IFI16 expression, we first compared the extent of STAT1 induction and phosphorylation following IFN treatment

in the Cas9-transduced control cells and the IFI16 knockout cells. STAT1 was up-regulated and phosphorylated following IFN- β treatment (Fig. 2A) as well as following IFN- γ treatment (*SI Appendix*, Fig. S6A). We observed that the levels of STAT1 and phosphorylated STAT1 expression were equivalent in the Cas9-transduced control cells and the IFI16 knockout fibroblasts (Fig. 2A). Thus, initial signaling through the respective cell-surface IFN receptors was not impaired in the IFI16 knockout cells.

While IFI16 accounted for a portion of the IFN-mediated restriction of WT HSV-1, IFN-stimulated levels of IFI16 alone were insufficient to drive full restriction. Thus other ISGs, in addition to IFI16, are likely to act collectively to inhibit WT virus replication. We tested whether ISG induction was comparable in IFI16 knockout vs. control cells by measuring PML protein levels following IFN- β treatment (Fig. 2B) and IFN- γ treatment (*SI Appendix*, Fig. S6B) in the IFI16 knockout cells and the Cas9-transduced control cells. Like IFI16, PML is an ISG and has been previously implicated in both intrinsic restriction and IFN-mediated restriction of HSV-1 (16, 56). We observed equivalent PML induction in the two cell lines by immunoblot and observed equivalent induction of *PML* as well as *ISG54/IFIT2* and *ISG56/IFIT1* gene transcripts using qRT-PCR analysis (Fig. 2C and *SI Appendix*, Fig. S6C).

Given that the observed restriction by IFI16 occurred several hours after IFN treatment, we further compared the transcript levels of *PML*, *ISG54*, and *ISG56* in uninfected IFI16 knockout and control cells at the indicated times following removal of the IFN pretreatment (Fig. 2D and *SI Appendix*, Fig. S6D). We observed equivalent elevation in transcript levels at all times measured post IFN removal. Together, these results indicated that neither type I

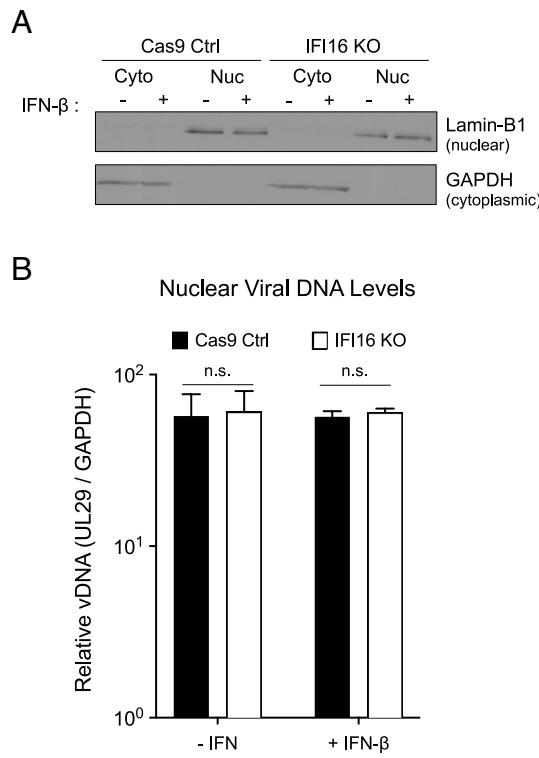


Fig. 3. Nuclear entry of WT virus is unaltered by IFN treatment or IFI16 levels. (A) Purity of cytoplasmic (Cyto) and nuclear (Nuc) fractions were determined by probing for Lamin-B1 and GAPDH ($n = 3$). (B) Levels of viral DNA in the nucleus following IFN- β pretreatment and infection of Cas9-control and IFI16-knockout cells with HSV-1 KOS (MOI 1, 2 hpi). Statistical significance was determined from three biological replicates by two-way ANOVA followed by Tukey's multiple comparison test. Mean \pm SD (error bars), * $P < 0.05$, ** $P < 0.01$.

nor type II IFN signaling was significantly compromised in the absence of IFI16.

Nuclear Levels of Input WT Viral DNA Are Unaltered by IFN Treatment or IFI16 Expression Level in Fibroblasts. The ISG MxB had been shown to restrict HSV nuclear entry in A549 cells and THP-1-derived macrophages (57–59); thus, IFN treatment of fibroblasts or the level of IFI16 expression may alter nuclear levels of input viral DNA. To test this, we measured nuclear levels of input viral DNA by isolating total DNA from infected cell nuclei at 2 h post infection (hpi) following IFN pretreatment and assessing the relative viral genome copy numbers by qPCR in the control and IFI16-knockout fibroblasts. The purity of the isolated nuclear fraction was confirmed by immunoblot (Fig. 3A). We observed that neither IFN treatment nor IFI16 expression level altered viral DNA levels in the nucleus at this stage of infection (Fig. 3B and *SI Appendix*, Fig. S7A). We confirmed that this assay was quantitative by showing that nuclear viral DNA increased proportionately to the multiplicity of infection (MOI) (*SI Appendix*, Fig. S7B). Thus, these results indicated that in fibroblasts both IFN-mediated restriction and IFI16-dependent restriction were occurring at a stage of viral infection following nuclear entry. Because our findings differed from reports in

earlier papers that MxB can limit infection in IFN-treated cells by restricting nuclear entry (57–59), we tested whether MxB was expressed in HFF cells. As shown in *SI Appendix*, Fig. S7C, MxB is induced upon IFN- β treatment of HFFs; thus, IFN-mediated restriction and/or ISG restriction of nuclear entry may differ depending on cell type or cell line.

IFN-Stimulated Levels of IFI16 Restrict WT HSV-1 Gene Expression.

We hypothesized that the IFN-mediated restriction attributable to IFI16 may be due to its capacity to bind viral DNA in the nucleus and regulate viral gene expression (3, 5, 48, 60). To test this, we measured the levels of viral gene transcripts and proteins produced in IFN-treated control and IFI16-knockout cells following WT HSV-1 infection. For WT virus, as observed previously, IFI16 did not affect viral transcript levels in the absence of IFN treatment (Fig. 4A). However, following IFN pretreatment, IE and E gene transcript levels were approximately fivefold higher in IFI16-knockout cells relative to control cells (Fig. 4A). Loss of IFI16 resulted in an ~14-fold increase in *ICP4* gene transcript levels at 2 hpi with IFN- β treatment, relative to control cells (Fig. 4B). We observed a similar trend for IFN- γ treatment, with an ~10-fold increase in *ICP4* transcript levels in IFI16 knockout cells relative to control cells (*SI Appendix*, Fig. S8). At constitutive levels of expression, IFI16 is known to associate rapidly

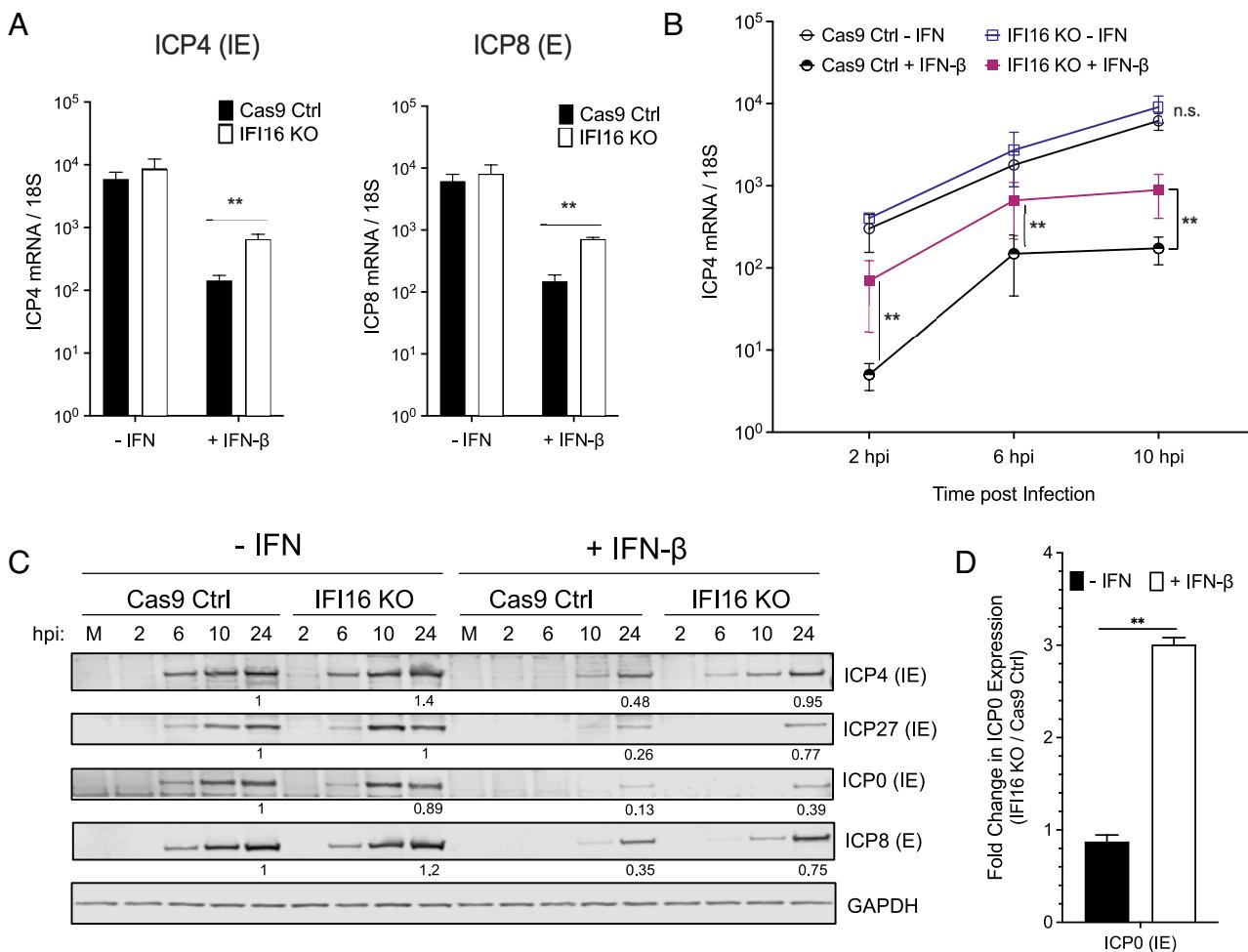


Fig. 4. IFN-stimulated levels of IFI16 restricted WT HSV-1 gene expression. (A) qRT-PCR analysis of viral transcript levels in IFI16 knockout and Cas9-control fibroblasts infected with HSV-1 KOS (MOI 1, 10 hpi) following IFN- β pretreatment. (B) Time-course analysis of IE gene *ICP4* transcript levels in HSV-1-infected (KOS, MOI 1) Cas9-control and IFI16-knockout fibroblasts following either no treatment or IFN- β pretreatment prior to infection. (C) Immunoblot analysis of viral protein expression in IFI16 knockout and Cas9-control fibroblasts treated with IFN- β prior to infection with HSV-1 KOS (MOI 1; n = 3). Relative HSV-1 protein levels are shown for 24 hpi. (D) Fold increase in *ICP0* expression in the IFI16 knockout cells relative to control cells, in the absence of IFN treatment or following IFN- β treatment. *ICP0* signal normalized to GAPDH prior to analysis. Statistical significance in A and B was determined from three biological replicates by two-way ANOVA followed by Tukey's multiple comparison test and in C from three biological replicates by the unpaired t test. Mean \pm SD (error bars), *P < 0.05, **P < 0.01.

with incoming viral DNA entering the nucleus. These results were consistent with IFN-stimulated levels of IFI16 operating similarly, with its contribution to IFN-mediated restriction also functioning at the onset of nuclear entry. Consistent with the yield results above, IFN- γ treatment, and IFN- γ -stimulated levels of IFI16, had only modest effects overall on virus transcript levels as infection progressed (*SI Appendix*, Fig. S8). Changes to viral protein expression in the absence of IFI16 reflected the observed effects on transcript levels (Fig. 4C). Notably, IFN treatment reduced ICP0 protein levels; thus, the total effect of IFN was to elevate IFI16 and reduce ICP0 levels. IFI16 also contributed to this IFN-mediated suppression of ICP0, with an ~threefold increase in ICP0 expression observed in the IFI16 knockout fibroblasts relative to control cells (Fig. 4D). Overall, these results were consistent with IFI16 contributing to IFN-mediated restriction through effects on transcription.

Viral DNA Synthesis is Reduced by IFN-Stimulated Levels of IFI16. We hypothesized that reduced gene expression could lead to reduced replication of viral DNA, and we therefore isolated total cell DNA and measured viral DNA levels by qPCR. We observed equivalent viral DNA levels at 2 hpi across all treatment conditions (Fig. 5 and *SI Appendix*, Fig. S9). Differences in viral DNA resulting from IFN treatment were not apparent until 6 hpi, with total viral DNA levels decreased >20-fold following IFN- β treatment (Fig. 5) and ~11-fold following IFN- γ treatment (*SI Appendix*, Fig. S9). At 10 hpi, we observed an ~sixfold increase in viral DNA in the IFN- β pretreated IFI16 knockout cells relative to the control cells (Fig. 5). Consistent with the relatively modest effect of IFN- γ -stimulated IFI16 on virus yield observed above, there was only a modest (~twofold) increase in viral DNA levels in the IFI16-knockout cells relative to control cells following IFN- γ treatment (*SI Appendix*, Fig. S9). From these results, we concluded that in addition to, or as a consequence of, the observed suppression of viral gene expression, IFN-stimulated levels of IFI16 led to reduced levels of viral DNA synthesis.

IFN-Stimulated Levels of IFI16 Stabilized Virus-Associated Heterochromatin. IFN-stimulated levels of IFI16 did not affect

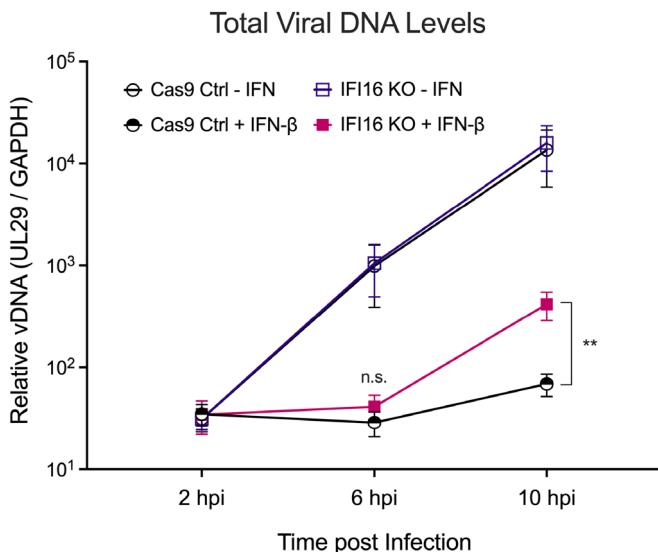


Fig. 5. Viral DNA synthesis is reduced by IFN-stimulated IFI16. qPCR time-course analysis of total viral DNA levels isolated from Cas9-control cells or IFI16-knockout HFFs at the indicated times post infection with HSV-1 KOS, following either no IFN treatment or IFN- β pretreatment. Statistical significance was determined from four biological replicates by two-way ANOVA followed by Tukey's multiple comparison test. Mean \pm SD (error bars), * P < 0.05, ** P < 0.01.

nuclear entry of WT viral DNA but still reduced HSV-1 gene transcript levels at the onset of infection. Thus, we hypothesized that IFI16 may influence the loading and composition of chromatin associating with incoming WT viral DNA to limit transcription and subsequent DNA replication. We therefore used ChIP-qPCR analysis to measure the levels of histone H3 levels and the H3K9me3 heterochromatin mark associated with viral gene promoters at 2 hpi and 6 hpi. IFI16 had been linked previously to increased association of the heterochromatin mark H3K9me3 with ICP0-null mutant viral DNA (3, 53); thus, we investigated this mark to determine the effects of IFN-induced IFI16 on epigenetic silencing during WT HSV-1 infection. We observed that levels of input viral DNA associated with histone H3 were not significantly altered at 2 hpi by IFN pretreatment (Fig. 6). However, at 6 hpi we observed elevated levels of histone H3 on the *ICP4* and *ICP27* gene promoters in both the Cas9-transduced control cells and the IFI16-knockout cells following IFN treatment, relative to untreated cells (Fig. 6 and *SI Appendix*, Fig. S10). Similarly, H3K9me3 levels were equal with or without IFN treatment at 2 hpi, but we observed significantly reduced levels of H3K9me3 by 6 hpi only in the absence of IFN. We observed a similar increase in total H3 and H3K9me3 levels in WT HFFs following IFN pretreatment and HSV-1 infection (*SI Appendix*, Fig. S11A). Comparing total histone H3 levels and H3K9me3 levels in the Cas9-transduced control cells with the IFI16-knockout cells, we again observed no change at 2 hpi, with or without IFN pretreatment. At 6 hpi, however, following IFN treatment, we observed a significant decrease in the association of both histone H3 and H3K9me3 in the IFI16 knockout cells relative to the control cell line on both the *ICP4* and *ICP27* viral gene promoters (Fig. 6A and *SI Appendix*, Fig. S10). In the absence of IFN stimulation, IFI16 had no effect on the composition of viral chromatin, consistent with the gene transcription and replication data described above.

The levels of total histone H3 and H3K9me3 histone marks associated with the *GAPDH* pseudogene were unchanged by either IFN treatment or IFI16 expression levels (Fig. 6B), and thus, no impact was observed on the cellular *GAPDH* pseudogene locus. Consistent with the results described above for total viral DNA levels, IFN treatment did not affect the levels of viral DNA in 2 hpi input samples but resulted in reduced levels of viral DNA at 6 hpi (Fig. 6C). Furthermore, as observed above, within each IFN treatment group, the relative viral genome levels in chromatin samples at 2 hpi and 6 hpi did not differ between IFI16-knockout and control HFFs. Thus, the observed differences in viral chromatin did not result from differences in total viral DNA in the chromatin preparations. Furthermore, the equivalent levels of vDNA associated with histone H3 at 2 hpi confirm the cell fractionation data showing equivalent amounts of nuclear input vDNA with or without IFN treatment.

Overall, these results indicated that the total chromatin and levels of heterochromatin associating with viral DNA were maintained over time by IFN-stimulated levels of IFI16.

Discussion

IFN cytokines induce a variety of IFN-stimulated genes or ISGs that act to block viral replication. Mechanisms of action for several cytoplasmic ISGs such as the IFITs, PKR, and Mx proteins have been elucidated (1), but little is known about the mechanism of action of the nuclear ISGs. The nuclear ISG IFI16 can restrict DNA virus infection, but IFI16 is usually degraded by the HSV-1 IE protein ICP0, so it does not normally impact WT infection. In this study, we found that the higher levels of IFI16 protein

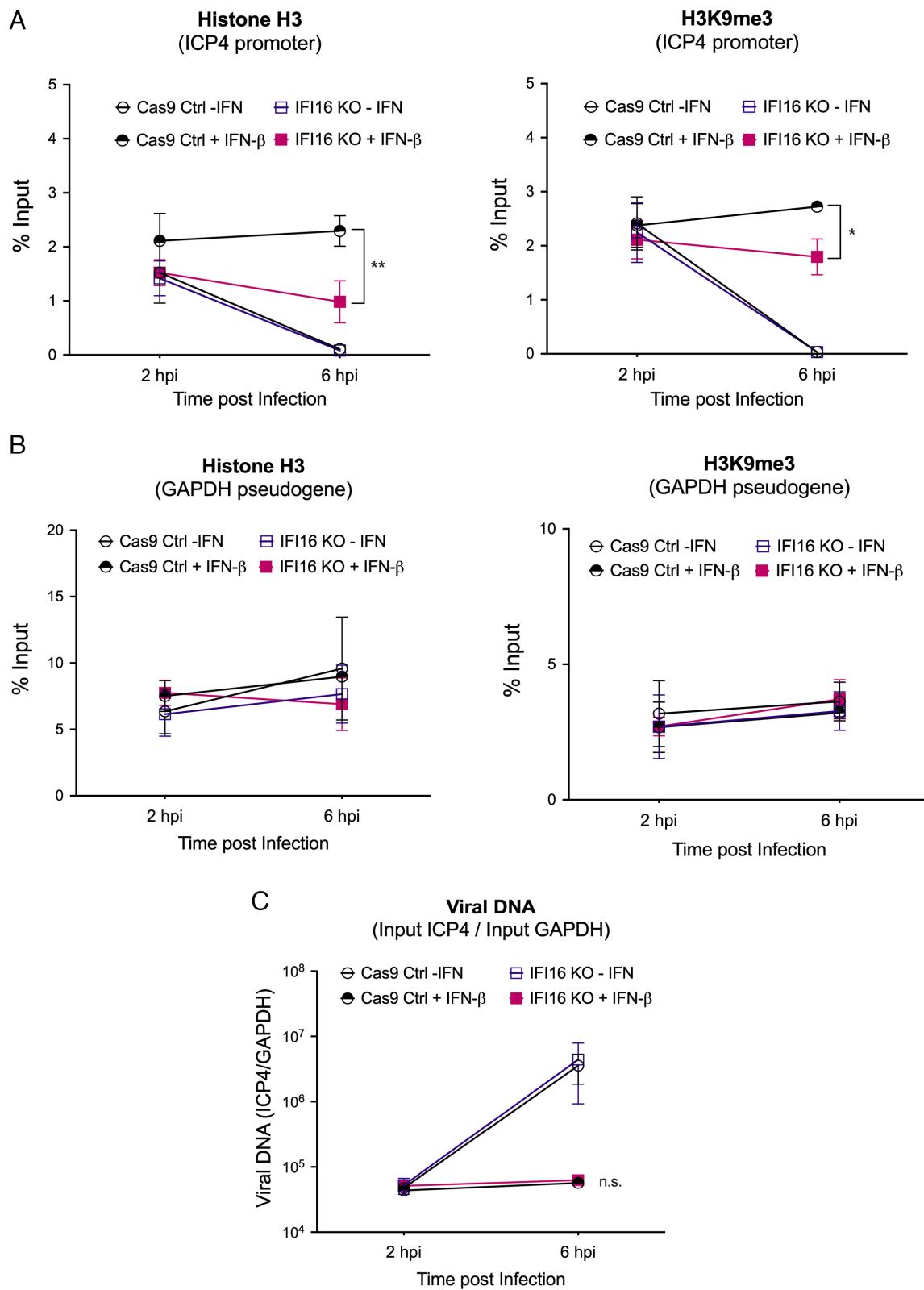


Fig. 6. IFN-stimulated levels of IFI16 promote chromatin maintenance and stabilize heterochromatin at viral gene promoters. ChIP-qPCR analysis of Cas9 control cells and IFI16 knockout HFFs pretreated with IFN- β and infected with HSV-1 KOS (MOI 1), using antibodies specific to histone H3 (Left) and H3K9me3 (Right) and primers specific to (A) the virus IE gene *ICP4* and (B) the *GAPDH* pseudogene (negative control). (C) Viral genome levels determined following qPCR analysis of ChIP input samples. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison test. Mean \pm SD (error bars), * P < 0.05, ** P < 0.01.

stimulated by IFN treatment can withstand ICP0-mediated degradation and promote the maintenance of heterochromatin on the viral genome, resulting in reduced viral transcription starting

with IE genes and reduced viral DNA replication (Fig. 7). These results define the broad mechanisms by which nuclear IFI16 can act as an ISG to restrict WT viral replication.

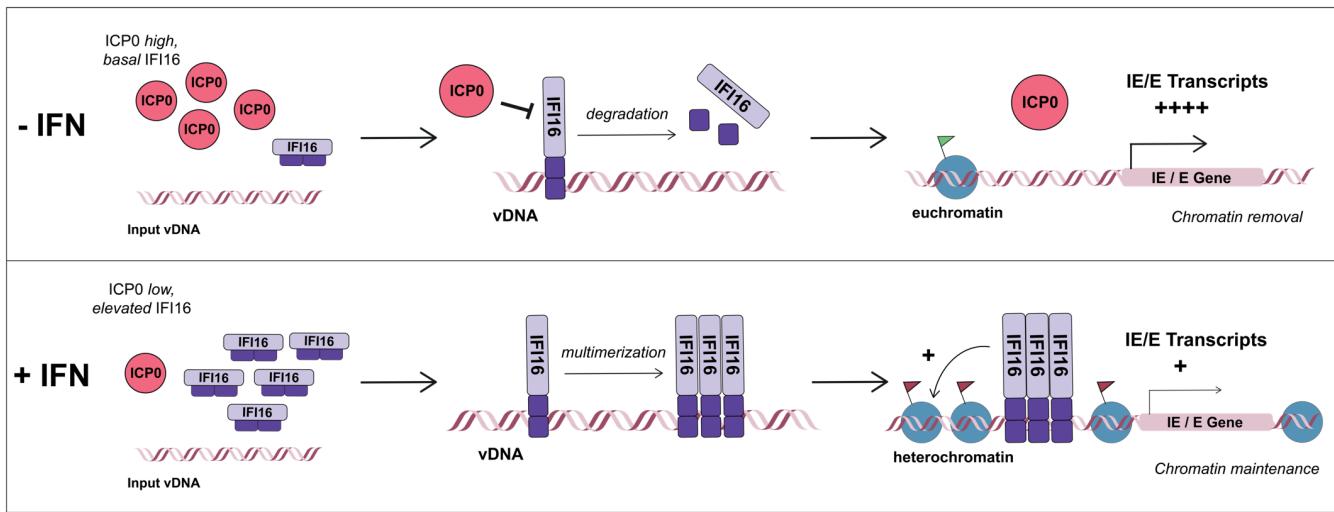


Fig. 7. IFN-stimulated levels of IFI16 provide competitive edge to host-cell defenses in the race against ICP0 and WT virus via effects on HSV-1 chromatin. In the absence of IFN treatment ("−IFN"): constitutively expressed IFI16 is targeted by the HSV-encoded E3 ubiquitin ligase ICP0 for degradation early in infection and does not restrict WT virus gene transcription. ICP0 promotes chromatin removal and euchromatin deposition to increase HSV-1 genome accessibility and facilitate ongoing WT virus gene transcription and replication. Following IFN stimulation ("+IFN"): elevated, IFN-stimulated levels of IFI16 combine with suppressed ICP0 expression to enable IFI16 multimers to persist on viral DNA. IFI16, through interactions with as-yet-unidentified host-cell chromatin-modifying proteins, facilitates the retention of nucleosomes and restrictive heterochromatin on viral DNA to inhibit WT virus transcription and replication. IFN-stimulated levels of IFI16 can thus restrict WT virus replication by promoting the maintenance of restrictive chromatin and inhibiting viral gene expression, including that of ICP0, at the onset of nuclear infection to ultimately favor host-cell suppression of infecting virus.

ICP0 vs. IFI16. It is likely that a major part of the mechanism by which IFN-induced IFI16 can resist ICP0-mediated degradation is by mass action, with the increased levels of IFI16 preventing full degradation by ICP0. Additionally, by decreasing the amount of ICP0 expressed, IFI16 further limits its own degradation. In cells infected in the absence of IFN treatment, HSV-1 ICP0 promotes the degradation of constitutive, basal levels of IFI16 to counteract its restriction and favor continued HSV-1 replication (Fig. 7). In contrast, in IFN-treated cells, enhanced levels of IFI16 can reduce expression of ICP0 and other viral gene products to limit ICP0 degradation and instead facilitate host-cell suppression of the infecting virus. Because additional inhibitors of ICP0 or viral gene expression in general may also be induced by IFN, it would be informative to conduct a screen to identify other cellular proteins besides IFI16 that are involved in the inhibition of ICP0 or WT HSV-1 in IFN-treated human cells.

Overall, the race between IFI16 and ICP0 has divergent outcomes in infections occurring without or with IFN treatment, with these different outcomes relating to decreased or increased retention of heterochromatin on viral DNA and resulting effects on viral gene expression. Key remaining questions include how IFI16—directly or indirectly—promotes the maintenance of heterochromatin on viral DNA and how the race between IFI16 and ICP0/WT HSV-1 may ultimately operate *in vivo*.

Mechanism of Heterochromatin Maintenance on Viral DNA. IFI16 is known to bind dsDNA and translocate along the DNA allowing multimerization and cooperative tight binding (52). IFI16 can restrict expression from genes delivered into cells without chromatin, such as incoming HSV-1 genomes during infection or genes delivered on plasmids (3). IFI16 binds tightly to unchromatinized or underchromatinized DNA and promotes its epigenetic silencing. Aspects of how this epigenetic silencing of incoming HSV-1 DNA is mediated, and the specific role of IFI16 in this process, remain puzzling. For example, IFI16 localizes to input HSV-1 DNA by 15 min post infection (46), and while we observed effects of IFN-induced IFI16 on gene expression by 2 hpi in this study, effects on chromatin were not observed by 2 hpi on input genomes but instead at later times such as 6 hpi. Thus,

there may be a direct effect of IFI16 binding to HSV-1 DNA on viral DNA accessibility to RNA polymerase II at 2 hpi and an indirect effect of IFI16 binding to viral DNA at later times such as 6 hpi through recruitment of chromatin-modifying enzymes that maintain heterochromatin. Further work is therefore needed to both characterize IFI16 restriction at distinct stages of infection and to define the kinetics and determinants of this restriction more precisely following nuclear entry, including in IFN-treated cells.

IFI16 has no known intrinsic chromatin-modifying ability, but it has been shown to interact with a number of cellular transcriptional and chromatin-modifying proteins in HSV-infected cells, including the methyl-H3K9-binding chromodomain protein M-phase phosphoprotein 8 and the heterochromatin-associated macroH2A histone variants (61). However, no interactions have been defined that could fully explain the epigenetic effects of IFI16 during HSV-1 infection. IFN-stimulated levels of IFI16, and IFN treatment generally, may stabilize viral heterochromatin by inhibiting the removal of initially deposited chromatin and/or by promoting heterochromatin formation on viral DNA, either via increasing de novo chromatin deposition or via changes in histone modifications. IFI16 has been shown to bind HSV DNA at sites across the viral genome (48, 49). Thus, increased binding following IFN stimulation may prevent chromatin removal, potentially by directly reducing viral genome accessibility, or may facilitate heterochromatin formation by enabling restrictive chromatin-modifying cellular proteins to be recruited to viral DNA. IFI16 has been reported to interact with the H3K9 methyltransferases SUV39H1 (Suppressor of variegation 3-9 Homolog 1) and G9a-like protein to regulate KSHV chromatin and with the KRAB-associated protein 1 during EBV infection (62, 63). These proteins may be key to any IFI16-mediated addition or maintenance of heterochromatin-associated H3K9me3 marks on chromatin during HSV infection as well, although depletion of SUV39H1 slightly reduced, rather than increased, HSV-1 ICP0-mutant virus replication (64). Given the relevance of both IFN restriction within the nucleus and IFI16 restriction to the broader herpesvirus family of viruses, IFI16-mediated epigenetic silencing as well as epigenetic silencing in general may be important components of innate immune control across a range of viral pathogens. Further work is thus needed to precisely define how

IFI16 may mediate changes to virus-associated chromatin and to fully understand how resulting changes in viral chromatin may—either indirectly or directly—influence HSV-1 transcription and replication.

In Vivo Role for IFI16 during Human infections. Several lines of evidence indicate that IFI16 may play a role in control of HSV infections in humans. First, specific combinations of genetic polymorphisms in the *IFI16* gene are associated with resistance to genital herpes infection (65). Second, the *IFI16* gene shows high positive selection in primates (66), indicating that IFI16 is involved in an ongoing evolutionary conflict between the human host and likely an infectious agent. Third, IFI16 has been shown to be highly expressed in the tissue around a genital herpes recurrent lesion (32).

Our cell-culture studies implicate IFN-stimulated levels of IFI16 in control of WT HSV infection. Although *in vitro* IFN pretreatment may not reflect *in vivo* infection conditions in humans, the paracrine effects of IFN are part of the classical description of how IFNs function, i.e., on the surrounding cells to protect against further infection. Our recent study (32) has shown a potentially similar effect of IFI16 in human genital HSV-2 lesions. This study found that genital HSV-2 lesion tissue contains acutely infected tissue areas within the lesion in which viral antigens are present but IFI16 is absent, consistent with its degradation by HSV-2-encoded ICP0. The cells in the tissue around the acute lesion, meanwhile, contained elevated levels of nuclear IFI16, consistent with IFI16 induction by the IFN- γ produced by T cells also present in the lesion. IFN- γ secretion was linked to the induction of IFI16 and other ISGs in tissues surrounding active and healed lesions, suggesting that IFI16-mediated restriction is relevant to immune control of HSV following IFN stimulation *in vivo* as well as *in vitro* (32).

Our current study reveals how IFN-induced IFI16 acts to restrict WT HSV-1, which is consistent with the previously observed role for IFI16 in controlling HSV-2 clinical reactivation in genital lesions. The convergence of these clinical and molecular-virology studies demonstrates the relevance of this nuclear ISG, and host IFN defenses more generally, to effective immune control of HSVs during infection in humans and strongly validates the need for further studies of the molecular mechanisms by which IFI16, and other nuclear ISGs, control virus infection.

Materials and Methods

Cell Culture. HFFs (CRL-1635) and Vero cells (CCL-81) were obtained from the American Type Culture Collection (ATCC). HFFs were maintained in Dulbecco's modified Eagle medium (DMEM; Corning) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco) and penicillin-streptomycin (Pen-Strep; Gibco). Vero cells were maintained in DMEM supplemented with 5% heat-inactivated bovine calf serum (BCS; Gibco), 5% heat-inactivated FBS, and Pen-Strep. Cas9 control cell lines and IFI16 knockout cells were generated in HFFs as described previously using the lentiCRISPR v2 lentiviral transfer plasmid (IFI16 gRNA sequences: GTTCGAGGTGATGCTGGTT and TTGATGGAAGAAAGTCCG) (50, 67) and maintained under puromycin selection (1 μ g/mL). All cell lines were grown at 37 °C with 5% CO₂.

Viruses and Infections. WT HSV-1 strain KOS (68, 69) stock preparations and virus yield experiment titrations were performed on Vero cells. Virus stock titers and yield titers were determined by plaque assay following standard procedures (70). Infections were conducted at the indicated MOI for 1 h in a 37 °C shaking incubator. Cell monolayers were incubated with virus diluted in phosphate-buffered saline avidin-biotin complex (PBS-ABC) supplemented with 1% heat-inactivated BCS and 0.1% glucose. Following inoculum removal, cells were maintained in DMEM supplemented with 1% heat-inactivated BCS and Pen-Strep at 37 °C until the indicated time of harvest.

IFN treatment. HFFs were either left untreated (−IFN; media change only) or pretreated with IFN- β (1,000 IU/mL; R&D Systems) or IFN- γ (1,000 IU/mL; R&D Systems) for 20 h prior to infection in DMEM containing 10% FBS and Pen-Strep. Control and IFN-pretreated cells were washed once with PBS-ABC (PBS with MgCl₂ and CaCl₂) following media removal, prior to infection. Infections and subsequent incubations were conducted without the addition of IFN.

Nucleic Acid Isolation and Quantification of Total Cellular DNA and RNA by qPCR. Viral RNA and DNA were quantified as described previously (67). Briefly, total cellular DNA and RNA were isolated from fibroblasts using the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's instructions. Purified RNA was quantified with a NanoDrop spectrophotometer (Thermo Scientific) and treated with DNase (DNA-free, Ambion) before equal amounts of DNase-treated RNA were reverse transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Relative DNA and cDNA levels were determined from samples run in duplicate using the specific primers (Integrated DNA Technologies) listed in Table 1 with the FAST SYBR green master mix and the 7500 Fast real-time PCR system (Applied Biosystems). A standard curve was used for quantification and was generated from 10-fold serial dilutions of either DNA or cDNA prepared from HFFs infected with WT virus.

Immunoblotting. Immunoblots were conducted as described previously (64). Briefly, whole-cell lysates were collected using 1× NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen) containing 5% 2-mercaptoethanol and a protease and phosphatase inhibitor cocktail (Halt; Thermo Scientific). Proteins from the harvested lysate were separated using 4 to 12% NuPAGE Bis-Tris polyacrylamide gels (Invitrogen) and transferred to nitrocellulose (Bio-Rad) or polyvinylidene difluoride (Bio-Rad) membranes. Membranes were blocked in either Odyssey blocking buffer (LI-COR) or a solution of 5% (w/v) nonfat milk in PBS containing 0.1% Tween 20 (PBS-T). Membranes were incubated overnight with primary antibody and washed with PBS-T prior to secondary antibody incubation at room temperature. The primary and secondary antibodies used are listed in Table 2. Horseradish peroxidase signal was detected by X-ray film (HyBlot CL; Denville) and Immobilon Classico or Forte Western HRP substrates (Millipore). Fluorescent-labeled antibody signal was detected using the Odyssey Clx Infrared Imaging System (LI-COR). Immunoblot quantification was performed using ImageJ.

Cell Fractionation. Nuclear and Cyto fractions were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific), following established protocols (75). Cells were pretreated with IFN for 24 h prior to infection and infected with the WT HSV-1 KOS strain at an MOI of 1 before harvesting at 2 hpi for analysis. Proteins extracted from the nuclear and Cyto fractions were subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and immunoblotting. Viral DNA was extracted from the nuclear fraction and purified using the DNeasy Blood & Tissue kit (Qiagen), with subsequent quantification with the 7500 FAST Real-Time PCR system (Applied Biosystems) using a similar approach to that described above for total cellular DNA.

ChIP Assays. ChIP assays were performed as described previously (75, 76), with minor modifications. Briefly, HFFs at a density of 2×10^6 cells per 100-mm dish were infected with WT HSV-1 at an MOI of 1 following 20 h of IFN treatment. At the indicated times post infection, cell monolayers were fixed with 1% formaldehyde (Sigma) for 10 min at 37 °C prior to quenching with 125 mM cold glycine for 3 min. Cells were washed 3× with cold PBS-ABC containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and harvested by scraping into PBS-ABC supplemented with protease inhibitor cocktail (cComplete, Roche), pelleted by centrifugation at 1,500 rpm for 5 min, and stored at -80 °C.

Cells were subsequently thawed on ice and lysed in cold lysis buffer I [50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1× Roche cComplete protease inhibitor cocktail] for 10 min with rotation. Samples were spun at 1,000 × g for 10 min at 4 °C, and pellets were resuspended in cold lysis buffer II [10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 0.5 mM egta-zinc acid, and 1× Roche cComplete protease inhibitor cocktail] prior to incubation with rotation for 10 min at 4 °C and collection by centrifugation.

Pelleted nuclei were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1, and 1× Roche cComplete protease inhibitor cocktail) and transferred to 1.5 mL TPX (polymethylpentene) tubes (Diagenode) prior to sonication.

Table 1. Primer sequences for qRT-PCR and qPCR

Target	Sequence (5' -> 3')	Reference
qRT-PCR (cDNA)		
18S forward	GCCGCTAGAGGTGAAATTCTTG	(46)
18S reverse	CTTCGCTCTGGTCCGTCTT	(46)
ICP4 forward	CGGTGATGAAGGAGCTGCTGTTGC	(71)
ICP4 reverse	CTGATCACGCCGCTGCTGTACA	(71)
ICP27 forward	AGACGCCCTCGTCCGACGGA	(71)
ICP27 reverse	GAGGCGCGACCACACACTGT	(71)
ICP8 forward	CATCAGCTGCTCCACCTCGCG	(71)
ICP8 reverse	GCAGTACGTGGACCAGGCGGT	(71)
IFI16 forward	ACTGAGTACAACAAAGCCATTGA	(3)
IFI16 reverse	TTGTGACATTGTCCTGTCACAC	(3)
PML forward	GGAC CCTATTGACGTTGACC	(50)
PML reverse	TTGATGGAGAAGGCGTACAC	(50)
ISG54 forward	ACGGTATGCTTGGAACGATTG	(41)
ISG54 reverse	AACCCAGAGTGTGGCTGATG	(41)
ISG56 forward	AAGGCAGGCTGTCGCTTA	(41)
ISG56 reverse	TCCTGTCCTTCATCCTGAAGCT	(41)
qPCR (DNA)		
GAPDH forward	CAGGCGCCAATACGACCAAATC	(50)
GAPDH reverse	TTCGACAGTCAGCCGATCTTCTT	(50)
ICP8/UL29 forward	GAGACCGGGGTTGGGAATGAATC	(72)
ICP8/UL29 reverse	CCCCGGGGTTGTCTGTGAAGG	(72)
ICP4/RS1 forward	TAGCATGCGAACCGAACGC	(44)
ICP4/RS1 reverse	CGCATGGCATCTCATTACCG	(44)
ICP27/UL54 forward	ACCCAGCCAGCGTATCCACC	(72)
ICP27/UL54 reverse	ACACCATAAGTACGTGGCATGT	(72)

Chromatin was sheared using a Bioruptor Standard sonication system (UCD-200, Diagenode) in 30-s pulses for 40 min at the maximum power setting, with sufficient fragmentation (<1 kbp) verified by size in agarose gels.

Sheared chromatin was diluted in ChIP dilution buffer (150 mM NaCl, 10 mM Na₂HPO₄, 2 mM EDTA, 1.1% Triton X-100, 0.01% SDS, and 1× Roche cComplete protease inhibitor cocktail). Equal amounts of chromatin (20 µg) were incubated overnight with 2.5 µg of histone-specific antibody or rabbit IgG, with 10% of each sample reserved to determine input. Specific antibodies used in ChIP assays are listed in Table 2. Following the overnight incubation, samples were incubated with 20 µL of protein A magnetic beads (MagnaChIP, Millipore) for 3 h at 4 °C. Magnetic beads were subsequently separated and washed 3× with cold low-salt, high-SDS wash buffer (150 mM NaCl, 10 mM Na₂HPO₄, 2 mM EDTA, 1.1% Triton X-100, 0.1% SDS, and 1 mM PMSF), 3× with cold lithium chloride wash

Table 2. Antibodies for immunoblot and chromatin immunoprecipitation (ChIP) experiments

Antibody target	Source	Identifier
Immunoblot		
GAPDH	Abcam	ab8245
ICP0	East Coast Bio	H1A027
ICP4 (58S)	(73)	
ICP27	Abcam	ab31631
ICP8 (3-83)	(74)	
IFI16	Abcam	ab55328
PML	Bethyl	A301-167A
STAT1	Cell signaling	9176S
pSTAT1 (Y701)	Cell signaling	9167S
Lamin-B1	Abcam	ab16048
Mouse IgG, HRP-conjugated (secondary antibody)	Cell signaling	7076S
Rabbit IgG, HRP-conjugated (secondary antibody)	Cell signaling	7074S
Mouse IgG, IRDye 680RD (secondary antibody)	LICOR	926-68070
Rabbit IgG, IRDye 800CW (secondary antibody)	LICOR	926-32211
ChIP		
Histone H3 (ChIP Grade)	Abcam	ab1791
H3K9me3 (ChIP Grade)	Abcam	ab8898
Normal Rabbit IgG, rabbit polyclonal	Millipore	12-370

buffer [50 mM HEPES (pH 7.5), 500 mM lithium chloride, 1 mM EDTA, 1% NP-40, 0.7% sodium deoxycholate, and 1 mM PMSF], and 1× with Tris-EDTA [10 mM Tris-HCl (pH 8.1) and 1 mM EDTA]. DNA was eluted from beads using two consecutive 10-min incubations at 65 °C in 100 µL of elution buffer (1% SDS and 100 mM sodium bicarbonate). Samples were incubated at 65 °C overnight with NaCl added to a concentration of 200 mM for cross-link reversal, and, following RNase (Ambion) and proteinase K (Roche) treatment, eluted DNA was purified using the QIAquick PCR purification kit (Qiagen). Purified DNA was analyzed by qPCR as described above, using primers listed in Table 1.

Statistical Analyses. Data analyses were performed using GraphPad Prism version 9.0, with means across multiple groups analyzed by ANOVA and relevant post hoc tests and means between two groups analyzed by the unpaired *t* test. Statistical significance is indicated when relevant, with **P* < 0.05; ***P* < 0.01; and ns = not significant. Where indicated, the sample size (*n*) denotes the number of independent, biological replicates performed.

Data, Materials, and Software Availability. Primary data for the results of this study are available in the Harvard Dataverse Repository (<https://doi.org/10.7910/DVN/DKNKA>) (77). Cell lines and materials used in this study are available from the corresponding author upon reasonable request.

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