Inhibition of Nuclear DNA Sensing by Herpes Simplex Virus 1

Dissertation Advisor: Dr. David Knipe Megan Horn Orzalli

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Abstract

The detection of immunostimulatory DNA is well documented to occur at several cellular sites, but there is limited evidence of nuclear innate DNA sensing. Prior to this study, the detection of herpesviral DNA was thought to be restricted to the cytosol so as to limit the sensing of host DNA in the nucleus. However, given the nuclear lifecycle of these viruses, we hypothesized that viral DNA could be sensed in the nucleus of infected cells. To test this hypothesis we examined the activation of interferon regulatory factor 3 (IRF-3) in response to herpes simplex virus 1 (HSV-1) infection of primary human foreskin fibroblasts (HFF). Using a mutant defective for expression of all viral genes, we observed that the release of viral DNA into the nucleus is necessary to activate IRF-3 signaling. Furthermore, we determined this response to be dependent on nuclear-localized interferon inducible protein 16 (IFI16) and the cytoplasmic stimulator of interferon genes (STING) adaptor protein.

The HSV-1 ICP0 E3 ubiquitin ligase has been observed to inhibit the induction of IRF-3 responsive genes in human fibroblasts; therefore, we investigated the effects of ICP0 on this pathway. We observed that nuclear ICP0 inhibits the induction of type I interferon by sequestering IRF-3 away from cellular

promoters and by promoting the degradation of IFI16 in a proteasome and RING-finger dependent manner.

IFI16 also plays a role in the restriction of HSV-1 replication by a STING-independent mechanism. We observed an IFI16-dependent reduction in immediate-early gene expression of ICP0-null mutant viruses in human fibroblasts. This response was not limited to viral DNA as the expression of transfected DNA was also inhibited by IFI16. Depletion of IFI16 did not affect the recruitment of PML to viral genomes, indicating this response does not involve the intrinsic resistance activity of ND10 domains.

Together, these studies show that nuclear IFI16 in normal human cells serves as both a sensor of viral DNA to initiate an innate signaling pathway and to activate an intrinsic resistance mechanism to foreign DNA. Furthermore we document a mechanism by which HSV-1 inhibits both these pathways through the expression of ICP0.

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Chapter One: Introduction

1.1 The *Herpesviridae* Family

The *Herpesviridae* family is comprised of a large number of double-stranded DNA viruses that were initially defined by structural similarities within the virion (Roizman et al., 2013). The linear genome of these viruses is contained within an icosahedral capsid, which is surrounded by an amorphous proteinaceous layer known as the tegument, and an envelope that contains viral glycoproteins. Along with the noted structural similarities, several biological activities are also shared amongst members of this virus family; including the expression of a number of enzymatic proteins involved in the virus lifecycle, the nuclear replication of the viral genome, the destruction of the host cell upon productive infection, and the ability of these viruses to establish latency in their natural host.

Within *Herpesviridae*, individual viruses are classified into three subfamilies (*Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae)* based on shared biological characteristics. Members of the *Alphaherpesvirinae* subfamily have a broad host range, replicate quickly, and establish latency in sensory ganglia of infected hosts. Within this subfamily, herpes simplex virus 1 and 2 (HSV-1, HSV-2) cause recurring oral and genital lesions, while varicella zoster virus is the causative agent of chicken pox and shingles.

In contrast to the alpha-herpesviruses, members of the *Betaherpesvirinae* subfamily are more restricted in their host range and replicate more slowly in cell culture. Furthermore, these viruses establish latency in cells of myeloid lineage. Infection with betaherpesviruses is often asymptomatic; however, these viruses can

cause severe disease in congenitally infected infants and immunocompromised individuals, including transplant recipients and individuals infected with human immunodeficiency virus (HIV). Human cytomegalovirus (HCMV), a major member of this subfamily, can cause hearing loss in symptomatic infants and is a major factor in post-transplant pathology.

The gammaherpesviruses are the most restrictive in their cellular tropism and mainly replicate in lymphoid cells. Unlike the alpha- and beta-herpesviruses, which undergo robust lytic replication *in vitro,* gammaherpesviruses generally default to latency in cell culture systems. For this reason, the latent lifecycle of gammaherpesviruses has been more thoroughly investigated than other *Herpesviridae* family members (Speck and Ganem, 2010). This family includes several significant human pathogens, including Epstein Barr virus (EBV), which is the causative agent of infectious mononucleosis.

1.2 HSV Epidemiology

In the United States approximately 57.7% and 17% of adults are seropositive for HSV-1 and HSV-2, respectively (Xu et al., 2006). A majority of HSV-1 seropositive individuals become infected before the age of 5, while the incidence of HSV-2 infection increases following the commencement of sexual activity. Both viruses can result in the formation of characteristic herpetic lesions at oral or genital mucosal surfaces. These lesions normally clear in the absence of intervention; however, in rare cases, HSV can cause severe morbidity and mortality in infected individuals. For instance, HSV infection

of the cornea, either during primary infection or upon reactivation of latent virus, can result in blindness due to the induction of a localized inflammatory response. Both HSV-1 and HSV-2 infection can result in herpes simplex encephalitis (HSE), where virus replication occurs in the brain, causing severe damage to the temporal lobe and, when untreated, a high incidence of death. In addition to being the causative agents in the diseases described above, evidence has emerged suggesting that HSV-2 infection increases the risk of human immunodeficiency virus (HIV) acquisition by 2- to 3-fold (Wald and Link, 2002). While the relationship between HSV-2 and HIV is not fully understood, disruption of the mucosal barrier during herpesvirus infection may recruit immune cells, increasing the localized pool of HIV-targeted cells.

1.3 HSV Pathogenesis

Herpes simplex viruses undergo a complicated biphasic lifecycle, which includes both lytic and latent stages. During an initial infection, the virus infects mucosal epithelium and undergoes a robust program of replication and production of progeny virions. Following virus release from infected epithelial cells, latency is established in enervating sensory ganglia. Under certain conditions the latent virus can reactivate, express lytic gene products and produce progeny virus. This reactivation event may be asymptomatic or replication competent virus may travel to the axonal termini inducing a secondary round of lytic replication at mucosal surfaces.

1.3.1 Viral Entry

HSV entry is mediated by a fusion event between the viral envelope and a host cell membrane. Initially, the virus attaches to target cells via interactions between the virion glycoproteins gB and/or gC and heparan sulfate, a glycosaminoglycan ubiquitously expressed on cell surfaces. This brings the viral gD glycoprotein in close proximity to its cognate cellular receptors, which triggers entry by the virion fusion machinery, gB and gH/gL. A variety of cellular proteins have been identified as receptors for gD binding, including herpesvirus entry mediator (HVEM) and nectin-1 [reviewed in (Spear, 2004)].

HSV can utilize two distinct entry pathways in a cell-type dependent manner. Initial studies in Vero and Hep2 cells suggested that HSV entry occurs by a pH-independent direct fusion event at the plasma membrane (Wittels and Spear, 1991). However, studies in additional cell lines, including HeLa and nectin-1 expressing CHO-cells, have identified an additional entry pathway utilizing endocytosis (Nicola et al., 2003). The molecular mechanisms that govern the entry pathway used have not been defined; however, differences in gD/receptor binding have been proposed (Karasneh and Shukla, 2011). In either case, both entry pathways can result in productive virus infection.

Following fusion with a cellular membrane, viral capsids are released into the cytoplasm and transported along microtubules to the nucleus where they accumulate at nuclear pore complexes (NPC) in an importin β-dependent manner (Ojala et al., 2000). Viral DNA is then rapidly injected into the nucleus, leaving empty viral capsids at the nuclear periphery. The viral tegument has been implicated in mediating the interaction

with NPC, because purified capsids lacking tegument are unable to bind to isolated nuclei *in vitro* (Ojala et al., 2000). While the exact viral proteins that mediate this interaction are unknown, inhibition of tegument VP1-2 cleavage, either through the use of the *ts*B7 temperature sensitive mutant (Batterson et al., 1983) or by chemical intervention (Jovasevic et al., 2008), causes the accumulation of electron dense capsids at the nuclear periphery, implicating this protein in the release of viral DNA.

1.3.2 Viral Gene Expression

Following the release of the linear viral genome into the nucleus, the DNA circularizes (Garber et al., 1993) and is rapidly chromatinized by host cell machinery (Cliffe and Knipe, 2008; Oh and Fraser, 2008). During lytic infection, histone association with HSV DNA is looser than the cellular chromatin (Lacasse and Schang, 2010) and is also less densely packed (Cliffe and Knipe, 2008). However, in the absence of viral gene expression HSV DNA is associated with increased levels of histone H3 as well as heterochromatic marks that are known to promote higher order chromatin structures indicative of transcriptional repression (Ferenczy and DeLuca, 2009). HSV proteins have been implicated in preventing this repression by modulating the chromatin structure during the early stages of infection (Knipe and Cliffe, 2008).

Lytic infection is characterized by a tightly regulated cascade of viral gene expression (Honess and Roizman, 1974) that is generally categorized into three classes: immediate-early, early, and late genes. The immediate-early genes are expressed in the absence of *de novo* viral protein synthesis, but expression is greatly

enhanced by the presence of the tegument-associated VP16 transactivator protein (Ace et al., 1989). The viral VP16 protein, in concert with the cellular proteins HCF-1 and Oct-1, binds to TATGARAT containing cis-regulatory elements in HSV immediate-early promoters to induce the expression of ICP0, -4, -22, -27, and -47 [reviewed in (Roizman et al., 2013)]. Members of the immediate-early class of proteins are involved in promoting the expression of the early-genes, which encode a range of proteins involved in viral DNA replication and nucleotide metabolism. The late genes are expressed upon viral DNA replication and consist of structural proteins and those involved in viral egress.

1.3.3 Viral Egress

Encapsidation of viral DNA leads to the formation of mature nucleocapsids, which must cross the nuclear membrane and transit through the cytoplasm in order to bud at the Golgi membranes. This process involves multiple envelopment and de-envelopment events as the capsid transits through the cell [reviewed in (Johnson and Baines, 2011)]. The first step in HSV nuclear egress involves the primary envelopment of nucleocapsids at the intranuclear membrane (INM). To gain access to the INM, viral proteins that make up the nuclear envelopment complex, including UL31 and UL34, mediate an extensive restructuring of the nuclear lamina (Reynolds et al., 2004), an underlying meshwork of cellular proteins that provide structure to the nuclear membrane. Enveloped virus particles in the inner nuclear space fuse with the outer nuclear membrane to release de-enveloped viral capsids into the cytoplasm. Here, the

capsids become associated with a majority of the tegument proteins found within extracellular virions. These tegument-coated viral capsids undergo a second round of envelopment when they bud into cytoplasmic organelles like the trans-Golgi network. The enveloped virus is then transported to the plasma membrane through the cellular exocytosis pathway and secreted into the extracellular space.

1.3.4 HSV Latent Infection

In contrast to the lytic cycle of HSV replication, far less is known about how herpes simplex viruses establish, maintain, and reactivate from latency. Following spread from the primary site of infection, HSV infects sensory neurons of the trigeminal or dorsal root ganglia. Retrograde transport of the viral nucleocapsid along axons leads to the deposition of the viral genome in cell bodies, where it is maintained as a circular episome in the nucleus. Unlike lytic HSV genomes, latent HSV DNA is associated with a regular array of nucleosomes and forms a classical laddering pattern following digestion with micrococcal nuclease (Deshmane and Fraser, 1989).

Latent infection is associated with a repression of viral gene expression, except for the latency-associated transcript (LAT), which can be detected at high levels in infected neurons. While LAT expression is not essential for the establishment of latency, it does negatively regulate lytic gene expression during this process (Chen et al., 1997; Garber et al., 1997). Furthermore, LAT -/- viruses have reduced levels of the H3K27me3 facultative heterochromatic modification on histones associated with HSV lytic gene promoters (Cliffe et al., 2009; Wang et al., 2005). Together, these results

indicate that LAT expression has a role in the repression of the viral genome during latency, although the exact mechanism(s) by which LAT acts remains elusive.

Periodic reactivation of latent HSV genomes occurs upon local injury, immunosuppression, or exposure to stimuli, such as ultra-violet (UV) light, hyperthermia, or emotional stress. Changes in chromatin structure are associated with reactivation (Neumann et al., 2007) and lytic gene expression is de-repressed resulting in the production of progeny virions, which travel in an anterograde fashion to the primary site of infection.

1.4 Infected Cell Protein 0

Infected cell protein 0 (ICP0) is encoded by the *α0* gene, which maps to the inverted repeats of the unique long (uL) component of the HSV genome; therefore, the virus contains two copies of the gene. It is one of the few viral gene products to require splicing during HSV infection, and the unspliced mRNA consists of three exons separated by two introns. The 2.7 kb spliced ICP0 mRNA encodes a 775 amino acid protein with a molecular weight of approximately 110 kDa.

ICP0 contains a nuclear localization signal in exon three and accumulates in the nucleus at early time points post-infection (Mullen et al., 1994). At late stages of infection, ICP0 translocates to the cytoplasm following viral DNA synthesis and/or late gene expression (Kawaguchi et al., 1997; Lopez et al., 2001). ICP0 has been reported to be a component of the viral tegument (Yao and Courtney, 1992) and has been

implicated in efficient trafficking of the viral capsids to the host nuclear periphery (Delboy and Nicola, 2011).

1.4.1 The E3 Ubiquitin Ligase Activity of ICP0

ICP0 contains a C_3HC_4 RING (really interesting new gene) finger domain, which exhibits E3 ubiquitin ligase activity *in vitro* and *in vivo*. Recombinant HSV-1 viruses with mutations in the RING finger domain of ICP0 functionally resemble ICP0-null viruses, indicating this domain is important for ICP0 function during infection (Everett, 1989; Lium and Silverstein, 1997). ICP0 has been shown to promote the degradation of a variety of cellular proteins [reviewed in (Boutell and Everett, 2012)], including USP7 (Canning et al., 2004), PML (Chelbi-Alix and de The, 1999), and RNF8 (Lilley et al., 2010). The diversity of these target proteins has indicated that ICP0 employs multiple mechanisms for substrate targeting.

One mechanism of ICP0-dependent substrate targeting occurs through a direct protein-protein interaction with the target protein. This is the case for the degradation of USP7, which is mediated by the C-terminus of ICP0. Mutation of one amino acid (K620) is sufficient to abolish the interaction with USP7 (Everett et al., 1999). Binding to USP7 enhances ICP0 stability by inhibiting autoubiquitination of the protein (Boutell et al., 2005).

Recently, ICP0 was shown to mimic a cellular fork-head associated (FHA) binding site through the phosphorylation of a conserved threonine (amino acid residue 67) within the N-terminus of the protein (Chaurushiya et al., 2012). This protein

modification allows ICP0 to interact with and target the RNF8 cellular E3 ubiquitin ligase for degradation during infection. Several cellular proteins, particularly those implicated in the DNA damage response contain FHA domains; however, while ICP0 can interact with additional members of this family (CKH2 and Nbs1), it does not appear to promote the degradation of these recently described interactors (Chaurushiya et al., 2012).

ICP0 has also been shown to promote the widespread loss of SUMO-conjugated proteins, including specific PML isoforms. This occurs in a SUMO-dependent manner, which has resulted in ICP0 being categorized as a SUMO-targeted ubiquitin ligase (STUbl). Several putative SUMO interaction motifs (SIMs) have been identified within the ICP0 sequence, and disruption of these sequences through mutational alteration inhibits the ability of transfected ICP0 to complement an ICP0-null virus (Boutell et al., 2011). At least one additional mechanism of ICP0-substrate targeting exists as ICP0 has been shown to promote the degradation of the PML isoform I independently of the mechanisms discussed above (Cuchet-Lourenco et al., 2012).

1.4.2 Effects on Gene Expression

ICP0 is one of the first proteins to be expressed during lytic infection. While not essential for virus replication, viral mutants that are defective for ICP0 are severely attenuated for growth, particularly at low multiplicity of infection (MOI). The defect in ICP0-null virus replication is cell-type dependent with the greatest defect observed in human fibroblasts (~1000 fold) and Vero cells (~100 fold). ICP0 mutant virus growth

can be complemented by the osteosarcoma U2OS cell line, which most likely lacks a component involved in the repression of HSV-1 genomes (Hancock et al., 2006).

ICP0 transactivates viral gene expression but does not mediate this effect by binding to viral DNA. Instead, ICP0 has been proposed to regulate viral chromatin structure in such a way as to enhance viral transcription. Consistent with this hypothesis, the expression of ICP0 promotes both histone removal and acetylation of viral DNA during lytic infection and the establishment of quiescence (Cliffe and Knipe, 2008; Ferenczy and DeLuca, 2009). Furthermore, the addition of ICP0 *in trans* results in a decrease in heterochromatin on quiescent genomes and a subsequent increase in viral promoter activity (Ferenczy and DeLuca, 2011).

The mechanism(s) by which ICP0 promotes these changes in chromatin structure have not been defined, though several possible mechanisms have been proposed. Nuclear domain 10 (ND10) associated proteins have been implicated in viral silencing (Boutell and Everett, 2012), and ICP0 disrupts ND10 accumulation at sites adjacent to viral DNA. However, disruption of ND10 by the simultaneous knockdown of PML, Sp100 and hDaxx only partially rescues the replication of ICP0-null viruses in human fibroblasts (Glass and Everett, 2012), indicating additional silencing mechanisms exist. Furthermore, a direct link between ND10 components and the observed chromatin effects on viral DNA have not been made.

1.5 The Innate Immune Response to Viral Infection

In an influential paper published in 1989, Charles Janeway hypothesized that activation of adaptive immunity is controlled by an initial innate response to a microbial infection (Janeway, 1989). He proposed that the host encodes pattern recognition receptors (PRRs), which recognize conserved pathogen associated molecular patterns (PAMPs) that are present or produced during bacterial or viral infection. Since the publication of this theory a large number of germline-encoded receptors have been identified, which recognize a wide variety of PAMPs. These receptors are classified according to their ligand specificity, localization, and evolutionary similarity, and include the Toll-like receptors (TLRs), the nucleotide binding and oligomerization domain-like receptors (NLRs), the RIG-I like receptors (RLRs), the AIM2-like receptors (ALRs), and a variety of cytosolic DNA sensors.

Upon sensing their cognate ligands these sensors trigger signaling cascades that induce the expression of antimicrobial factors, including pro-inflammatory cytokines and type I interferons (IFNα and IFNβ). The induction of pro-inflammatory cytokines generally relies on the activation of the nuclear factor κB (NF-κB) transcription factor, while expression of IFNα and IFNβ are induced by activation of NF-κB, interferon regulatory factors (IRFs), and additional transcription factors. Secreted pro-inflammatory cytokines and chemokines act upon resident myeloid cells (macrophage and dendritic cells), leading to their activation and migration to secondary lymphoid tissues where they present foreign peptide to T-cells. In contrast, secretion of type I interferons mediates a more localized response by acting directly on infected cells through the ubiquitously expressed type I interferon receptor (IFNAR). Signaling

through IFNAR activates the JAK-STAT signaling pathway [reviewed in (Stark and Darnell, 2012)] and induces the transcription of interferon-stimulated genes (ISGs) whose protein products mediate the antiviral state. It has since been shown that a subset of ISGs can be expressed independently of IFN, but signaling through this pathway enhances their expression substantially (Wathelet et al., 1992).

Interferon regulatory factor 3 (IRF-3) is a critical component of the innate immune response to virus infection and bridges the initial sensing of viral PAMPs to the production of type I IFNs and ISGs [reviewed in (Hiscott, 2007)]. This constitutively expressed transcription factor is localized exclusively in the cytoplasm in the absence of stimuli. However, signaling through PRRs leads to the activation of the serine-threonine kinases TANK binding kinase I (TBK1) and IKKε. These kinases have been shown to phosphorylate Ser/Thr residues in the C-terminus of IRF-3, leading to its homodimerization and nuclear translocation (Fitzgerald et al., 2003). Activated nuclear IRF-3 interacts with the co-activators CBP/p300 and undergoes a conformational change, which allows binding to interferon stimulated response elements (ISRE) in the promoter regions of ISGs (Lin et al., 1998; Wathelet et al., 1998; Weaver et al., 1998; Yoneyama et al., 1998). CBP/p300 promotes transcription of a variety of cellular genes through acetylation of histones, the relaxation of chromatin structure, and the recruitment of RNA polymerase II (Martinez-Balbas et al., 1998). Following transcriptional activation, IRF-3 is degraded by the ubiquitin proteasome pathway to down-regulate type I IFN production (Bibeau-Poirier et al., 2006; Lin et al., 1998).

1.5.1 Innate Sensors of HSV Infection

HSV infection activates innate signaling pathways by cellular recognition of three viral PAMPs: protein, RNA, or DNA. Within the membrane-bound TLR family, three of the eleven known members recognize HSV at different stages of infection. TLR2, which is localized to the plasma membrane, senses the viral gH/gL and gB glycoproteins upon virion binding to the cell surface (Leoni et al., 2012). In neonatal mice, TLR2 signaling in response to HSV infection of the brain produces an inflammatory response that leads to monocyte infiltration and lethal encephalitis (Kurt-Jones et al., 2004). However, TLR2 plays a protective role when mice are infected with HSV by intraperitoneal injection (Sorensen et al., 2008).

HSV is also sensed in endosomal compartments by TLR3 (Zhang et al., 2007) and TLR9 (Lund et al., 2003), which recognize dsRNA and unmethylated CpG rich DNA, respectively. Individuals with mutations in the TLR3 pathway show increased sensitivity to HSV-1 infection, indicating this pathway is important for the control of HSV (Casrouge et al., 2006; Lafaille et al., 2012; Zhang et al., 2007). The most potent producers of IFN in response to HSV-1 infection are activated plasmacytoid dendritic cells, conventional dendritic cells, and macrophages (Hochrein et al., 2004). Plasmacytoid dendritic cells, which are dedicated to antigen sampling in peripheral tissues, recognize infected epithelial cells through endocytosis. The internalized viral DNA activates TLR9 in endosomal compartments, leading to the induction of type I IFNs and other antiviral gene products (Krug et al., 2004).

Members of the RLR family of receptors sense viral RNA that accumulates in the cytoplasm of HSV infected cells. MDA5 has been shown to be important in the induction of an inflammatory response in response to HSV in primary human macrophages (Melchjorsen et al., 2010). It has also been reported that RIG-I is involved in sensing HSV-1, although indirectly through the RNA Pol III DNA sensor (Chiu et al., 2009). This sensor transcribes AT-rich DNA into 5' triphosphate-containing RNA, which is subsequently recognized by RIG-I. The importance of the RNA Pol III sensing pathway in HSV infection is unclear, as others have found it to be dispensable for the production of inflammatory cytokines during infection (Melchjorsen et al., 2010; Unterholzner et al., 2010).

Herpesviral DNA is a potent activator of a type I IFN and inflammatory cytokine response. Both HSV-1 and HCMV genomic DNA can induce IFNβ expression when transfected into mouse embryonic fibroblasts (Ishii et al., 2006). This DNA-dependent response to HSV-1 was later shown to be independent of TLR9, suggesting the existence of an additional DNA sensing mechanism (Rasmussen et al., 2007). Since these initial observations, several cytosolic DNA sensors have been described with varying importance in sensing HSV infection. While transient knockdown of the first identified cytosolic DNA sensor DNA-dependent activator of IRFs (DAI) was shown to be important in the response to HSV (Takaoka et al., 2007), DAI -/- mice were subsequently shown to respond normally to infection, suggesting the protein is dispensable or redundant in mice (Wang et al., 2008). In addition, several members of the DExD/H helicase family, including DHX9 (Kim et al., 2010), DHX36 (Kim et al.,

2010), and DDX41 (Zhang et al., 2011) have also been identified as cytosolic DNA sensors of HSV-1 infection in plasmacytoid dendritic cells and myeloid dendritic cells, respectively.

Recently, a member of the PYHIN (Pyrin and HIN200 domain-containing) family of proteins was described as a cytosolic DNA sensor. Interferon inducible protein 16 (IFI16) was identified as a protein that immunoprecipitated with biotinylated immunostimulatory vaccinia viral DNA (Unterholzner et al., 2010). Transient knockdown of the mouse ortholog p204 in RAW246.7 macrophages decreased the expression of type I IFNs in response to HSV-1 infection. Subsequent work in human corneal epithelial cells confirmed the involvement of IFI16 in the translocation of IRF-3 to the nucleus and the production of a chemokine, CXCL10, in response to HSV-1 infection (Conrady et al., 2012).

The multitude of DNA sensors that have been implicated in recognizing HSV-1 infection has complicated our understanding of this cellular process. Many different cell types have been used to investigate intracellular DNA sensing and it is likely that the components used for detection of foreign DNA are cell-type dependent. Interestingly, a majority of cytosolic DNA sensing pathways have been shown to be dependent on stimulator of interferon genes (STING), an endoplasmic reticulum (ER) resident adaptor protein that is involved in the activation of IRF-3 (Ishikawa and Barber, 2008; Ishikawa et al., 2009). Therefore, while the sensing components differ in a cell type-dependent manner, the functional response to HSV DNA appears to be the same.

1.5.2 HSV Evasion of Innate Immunity

The innate immune response is important for controlling HSV-1 replication, as demonstrated by the increased susceptibility of IFNAR deficient mice to HSV-1 infection (Leib et al., 1999). In addition, pre-treatment of cells with IFN inhibits HSV-1 immediate-early gene expression in a dose-dependent manner (Altinkilic and Brandner, 1988), suggesting that the induction of an antiviral state in neighboring cells can inhibit virus spread. The highly ubiquitous nature of this virus and its ability to spread to secondary tissues argues that HSV-1 employs means to evade the host immune response.

HSV-1 infection induces an IRF-3-dependent type I IFN and ISG response in human fibroblasts in the absence of viral gene expression (Everett et al., 2008b; Mossman et al., 2001; Preston et al., 2001). The induction of these effector molecules is potently inhibited by viral gene expression, suggesting that a viral protein inhibits this response. Like many large DNA viruses, HSV-1 encodes multiple proteins that have been implicated in host immune evasion. For instance, the ICP34.5 late gene product was recently reported to block type I IFN production by negatively regulating the phosphorylation of IRF-3 by TBK1 (Ma et al., 2012; Verpooten et al., 2009). However, infection with an ICP4 deletion mutant, which is restricted to IE gene expression (DeLuca et al., 1985), did not result in the induction of ISGs (Mossman et al., 2001), implicating an immediate-early protein in inhibiting IRF-3 signaling.

Using a panel of HSV-1 mutants, Eidson *et al* (2002) demonstrated that the ICP0 immediate-early protein could inhibit ISG induction during infection. A virus that expressed no viral gene products (*d*109) due to mutations within the five immediate-

early genes robustly induced ISG54 expression, while infection with the ICP0-expressing *d*106 virus inhibited this response (Eidson et al., 2002). Furthermore, the ICP0-dependent inhibition of ISG54 induction was, in part, dependent on a functional proteasome, indicating that ICP0 may promote the degradation of a cellular factor involved in the activation of IRF-3. While proteasome-dependent degradation of cellular proteins is a common immune evasion strategy adopted by viruses [reviewed in (McInerney and Karlsson Hedestam, 2009)], preliminary studies could not detect ICP0 RING finger-mediated degradation of any known IRF-3 pathway components (Lin et al., 2004).

Our lab has previously demonstrated that ICP0 expression promotes the increased turnover of Sendai virus (SeV) activated IRF-3 (Melroe et al., 2004). However, this phenotype does not fully explain ICP0's inhibitory activity because it occurs several hours following IRF-3 nuclear accumulation. We also observed an ICP0-dependent intranuclear relocalization of IRF-3 (Melroe et al., 2007), indicating that ICP0 may affect IRF-3 signaling through alternative mechanisms.

1.6 Intrinsic Resistance to Viral Infection

In addition to the activation of the innate immune response described above, viruses are also subject to a constitutively expressed resistance mechanism termed intrinsic immunity or intrinsic antiviral resistance (Bieniasz, 2004). Unlike innate immunity, which requires *de novo* protein synthesis to mediate its effects, intrinsic antiviral resistance involves constitutively expressed proteins that act directly on viruses

during infection. These innate and intrinsic responses are closely linked however, as many intrinsic mediators can be upregulated by type I interferon production. This response has been extensively investigated in the context of retroviral infection and several cellular proteins have been implicated in the restriction of these viruses [reviewed in (Bieniasz, 2004; Blanco-Melo et al., 2012)].

1.6.1 The Role of ND10 in Intrinsic Antiviral Resistance to HSV

The most well characterized intrinsic antiviral response to herpesvirus infection involves the action of promyelocytic leukemia protein nuclear bodies (PML NB), also known as nuclear domain 10 (ND10). These dynamic subnuclear domains are made up of a variety of cellular proteins, and have been implicated in diverse cellular processes, including gene expression, DNA damage, apoptosis, and aging (Lallemand-Breitenbach and de The, 2010). As suggested by the name, PML represents a major component of these domains and is important for the recruitment of the constitutive ND10 components Sp100 and hDaxx. The formation of ND10 requires the SUMOylation of PML because mutational alteration of the known PML SUMOylation sites results in a failure to recruit Sp100 and hDaxx to these domains (Ishov et al., 1999; Zhong et al., 2000). Furthermore, cells deficient in the lone SUMO E2 enzyme, ubc9, show dramatic defects in ND10 number and localization (Nacerddine et al., 2005).

During HSV-1 infection, ND10 components accumulate *de novo* in the nucleus at sites near incoming viral DNA (Everett and Murray, 2005; Everett et al., 2004b). This response is not observed in wild-type virus infection as expression of ICP0 rapidly

disrupts ND10 by promoting the degradation of PML and Sp100 (Chelbi-Alix and de The, 1999). The recruitment of these proteins to viral DNA is associated with the repression of ICP0-null virus replication in human fibroblasts as demonstrated by an increase in ICP0-null virus gene expression and replication upon individual knockdown of PML, Sp100, or hDaxx (Everett et al., 2008a; Lukashchuk and Everett, 2010). Simultaneous knockdown of these proteins indicated that ND10 components act cooperatively to repress herpesvirus infection, but are not responsible for the full defect in ICP0-null virus replication (Glass and Everett, 2012). Although it remains unclear how these proteins repress viral gene expression during infection, recent studies indicate they may be involved in the epigenetic repression of viral DNA (Kim et al., 2011; Shalginskikh et al., 2013).

Chapter Two: HSV-1 inhibits an antiviral response through the ICP0-dependent sequestration of IRF-3 from cellular promoters

Abstract

The interferon regulatory factor 3 (IRF-3) pathway is a critical component of cellular innate immunity and is important for the induction of type I interferons in response to virus infection. Herpes simplex virus 1 (HSV-1) activates IRF-3 signaling when viral protein synthesis is blocked, and viral gene expression inhibits this cellular response. We found that HSV-1 ICP0 induced the relocalization of active IRF-3 to nuclear foci and enhanced turnover of this protein. To study the mechanism of ICP0's effects on IRF-3 we measured IRF-3 responsive gene expression in the presence of transfected WT-ICP0 or a RING-finger mutant plasmid. Expression of ICP0 inhibited TBK1-activated endogenous IFNβ expression, indicating ICP0 is sufficient to inhibit IRF-3 signaling. Using a constitutively active IRF-3 construct, we found that both WT and RING finger mutant ICP0 inhibited the expression of IRF-3 responsive genes, suggesting that ICP0 inhibits IRF-3 independently of its E3 ubiquitin ligase activity. Infection with a HSV-1 RING finger mutant virus stabilized IRF-3 protein levels in cells co-infected with Sendai virus (SeV), but did not increase IRF-3 responsive gene expression, consistent with our transfection results. We also used chromatin immunoprecipitation to examine IRF-3 binding to endogenous promoters during co-infection with SeV and HSV-1 *d*106, which only expresses nuclear-retained ICP0, or co-infection with SeV and the HSV-1 *d*109 virus, which expresses no viral gene products. Infection with the *d*106 virus, but not *d*109, inhibited IRF-3 binding to the *IFN-β, ISG56,* and *ISG54* promoters at early times post-infection and prior to the degradation of IRF-3. Together, these results argue that ICP0-mediated degradation of IRF-3 is not involved in

the inhibitory activity of ICP0 and suggest that ICP0 inhibits this antiviral response by sequestering IRF-3 away from host chromatin.

Introduction

The cell-based innate immune response is a critical aspect of host immunity and provides a first line of defense against viral infection. A key outcome of this response is the production of pro-inflammatory cytokines, chemokines, and type I interferons (IFNs), which act to restrict virus replication at the site of infection and mobilize the adaptive immune response to promote viral clearance. Secreted type I IFNs act in an autocrine and paracrine manner to upregulate the expression of IFN-stimulated genes (ISGs), which are integral components of the antiviral response. However, expression of ISGs does not strictly rely on the type I IFN signaling pathway, as a subset of these proteins can be induced in the absence of *de novo* cellular gene expression (Bandyopadhyay et al., 1995; Mossman et al., 2001).

This aspect of innate immunity is controlled by a variety of germline-encoded pattern recognition receptors (PRRs) that recognize conserved microbial products and subsequently activate intracellular signaling cascades to induce IFN and ISG expression. In many cell types, the induction of these genes is dependent on interferon regulatory factor 3 (IRF-3), which is normally localized to the cytoplasm in an autoinhibited conformation (Lin et al., 1999). However, virus-induced phosphorylation of IRF-3 displaces the autoinhibitory domain (Lin et al., 1998), allowing protein dimerization, translocation to the nucleus, and association with the coactivator CBP/p300, which is recruited to the promoters of IRF-3 responsive genes (Lin et al., 1998; Weaver et al., 1998; Yoneyama et al., 1998).

The importance of IRF-3 in the control of viral replication is emphasized by the increased susceptibility of IRF-3 deficient mice to viral infections (Sato et al., 2000). Accordingly, this pathway has been identified as a major target of viral inhibition. Many viruses express immunomodulatory proteins that counteract the induction of antiviral genes by inhibiting the IRF-3 pathway, and viral proteins have been identified that target each of the stages of IRF-3 activation, including phosphorylation, dimerization, and association with CBP/p300 (Ren et al., 2011). In addition, several viral proteins inhibit the induction of IFN by promoting IRF-3 degradation (Bauhofer et al., 2007; Hilton et al., 2006; Yu and Hayward, 2010).

Herpes simplex virus 1 (HSV-1) is a ubiquitous human pathogen associated with a variety of maladies ranging from self-limiting herpetic lesions to ocular keratitis and herpes simplex encephalitis. The life cycle of this virus is biphasic and consists of an initial lytic cycle of replication in epithelial cells followed by the establishment of a life-long latent infection in enervating sensory neurons. The type I IFN response inhibits the replication of HSV-1 at the primary site of infection and spread to the transgeminal ganglia (TG), as demonstrated by an increase in peripheral and TG virus titers in $CD118^{-/-}$ mice infected via corneal scarification (Conrady et al., 2011). Furthermore, a direct correlation has been observed between virus replication in the periphery and the number of viral genomes present during latency (Thompson and Sawtell, 2000). Together these studies indicate that HSV-1 must overcome an antiviral response *in vivo* in order to replicate and then spread from the primary site of infection to secondary tissues.

The ability of HSV-1 to induce and inhibit the antiviral response has been investigated using cell culture models of lytic infection. HSV-1 is intrinsically capable of activating IRF-3 and ISG expression in certain cell types; however, this response is diminished upon viral gene expression, indicating that viral immune evasion mechanisms exist (Mossman et al., 2001; Preston et al., 2001). Several reports have implicated the viral immediate-early ICP0 protein in the inhibition of IRF-3 signaling, although its mechanism of action has remained unclear (Eidson et al., 2002; Lin et al., 2004; Melroe et al., 2004). We have previously examined the inhibition of Sendai virus (SeV) induced IFNβ expression in response to HSV-1 and observed both an ICP0-dependent nuclear relocalization and degradation of IRF-3 during infection (Melroe et al., 2004; Melroe et al., 2007). The relative importance of these two phenotypes in inhibiting IRF-3 activity has not been investigated.

In this study, we further characterized the reported ICP0-mediated inhibition of IRF-3 by transfection and infection experimental approaches. Our results indicate that HSV-1 infection controls IRF-3 responsive gene expression by sequestering IRF-3 rather than by promoting IRF-3 degradation.

Materials and Methods

Cell culture and Viruses. Human endometrial adenocarcinoma (HEC-1B), human osteosarcoma (U2OS), and African green monkey kidney epithelial (Vero) cell lines were obtained from American Type Culture Collection. Cells were grown in DMEM supplemented with 5% heat-inactivated fetal bovine serum and 5% heat-inactivated bovine calf serum, 2mM L- glutamine, streptomycin, and penicillin in a humidified 5% CO_2 atmosphere at 37^{o} C.

The SeV Cantel strain was obtained from Charles River laboratories (Wilmington, MA). HSV-1 wild-type KOS strain was propagated and titered on Vero cells. The ICP0-null (7134) and the ICP0-rescued (7134R) viruses were propagated and titered in parallel on U2OS cells (Cai and Schaffer, 1989). The ICP0-RING finger mutant virus (KOS.RFm) and its corresponding rescued virus (KOS.RFr) were constructed as previously described by homologous recombination into the ICP0-null 7134 virus (Cai and Schaffer, 1989). Briefly, infectious ICP0-null 7134 viral DNA was co-transfected into U2OS cells with a linearized plasmid containing ICP0 flanking regions as well as mutations within the ICP0 protein (pICP0 RFm). These cells were harvested at three days post-transfection, freeze-thawed, and dilutions were plated on U2OS cells to isolate plaques. An agarose overlay containing X-Gal was used to distinguish recombinant plaques that did not express beta-galactosidase. White plaques underwent three rounds of purification before viral DNA was harvested. The ICP0 RING finger domain was amplified, and mutations were confirmed by restriction endonuclease digestion and sequencing. The KOS.RFm infectious viral DNA and a WT ICP0

linearized plamid were used to construct the KOS.RFr virus in a similar manner. Virus stocks were grown and titered on U2OS cells.

Virus infections. Viruses were diluted in cold phosphate-buffered-saline (PBS) containing 0.1% glucose and 1% heat-inactivated BCS. Cells were infected at the stated MOI for 1h at 37° C, washed twice with PBS and overlaid with DMEM containing 1% heat inactivated BCS. Infected cells were incubated at 37° C for the indicated length of time.

Plasmids. The ICP0 expression vector (pICP0) contains 4.6 kb of DNA derived from the long terminal repeat of the HSV-1 genome cloned into the pUC18 vector (Thermo-Scientific). This was constructed through several subcloning steps. Briefly, a 6.5 kb SacI-PstI fragment was cloned from the pSG28 plasmid (Goldin et al., 1981) into pUC18 (pICP0 6.5) followed by subcloning of the 4.6 kb EcoRI-HpaI fragment of pICP0 6.5 into the EcoRI-HincII site of pUC18. The pICP0 RFm plasmid was constructed by sequential PCR mutagenesis of the XhoI-KpnI fragment of pICP0 to introduce C116G and C156A amino acid mutations. The predicted DNA sequence was confirmed and the fragment was introduced into pICP0. The Flag-TBK1 and pIRF-35D constructs were kindly provided by Dr. Katherine Fitzgerald (University of Massachusetts).

Transfection. HEC-1B cells were plated in 6-well plates to ensure 50% confluency on day of transfection. A total of 1μg of DNA was transfected into cells using Effectene

reagent (Qiagen) and media was changed at 6 hours post transfection (hpt). Cells were assayed at 36 hpt for RNA or protein expression.

Cellular RNA analysis by qRT-PCR. Total RNA was extracted from cell monolayers using Trizol reagent (Invitrogen) and 1μg of RNA was DNase-treated with the DNA-free kit (Ambion). 0.5ug of DNAse-free RNA was then reverse-transcribed using the iScript cDNA synthesis kit (Biorad) and quantified by real-time PCR (qPCR) using Power SYBR Green PCR master mix and a Prism 7300 sequence detection system (Applied Biosystems). PCR reactions were carried out in duplicate, and relative copy numbers were determined by comparison with standard curves. Mock reverse-transcribed samples were included as negative controls. Transcript levels were normalized to γ-actin and made relative to mock-infected samples.

Western blots. Cells were lysed in 1x Sample Loading Buffer (80mM Tris pH 6.8, 10.0% glycerol, 2.0% SDS, 0.01% bromophenol blue) and boiled at 100^{o} C prior to being resolved on NuPAGE® 4-12% Bis Tris Gels (Invitrogen). Proteins were transferred overnight to PVDF membranes and blocked with 5% milk in PBS. Membranes were probed with primary antibody at 4^{o} C, washed with PBS containing 0.05% Tween 20 and incubated in secondary antibody for 1h at room temperature. Membranes were washed three times to remove unbound antibody and were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

Indirect immunofluorescence. HSV-1 infected Vero cells grown on coverslips were fixed with 2% formaldehyde, permeabilized with 0.5% NP40, and blocked in 5% normal goat serum. Fixed cells were incubated with antibodies for 30 min at 37°C and washed twice with PBS containing 0.05% Tween 20 followed by one wash with PBS. An Alexa Fluor 594-conjugated secondary antibody was incubated with cells for 2h at 25°C. The coverslips were washed as above and mounted in ProLong Gold antifade reagent (Invitrogen). Images were acquired using an Axioplan 2 microscope (Zeiss) with a 63X objective and Hamamatsu CCD camera (Model C4742-95). Images were arranged in figures using Adobe Photoshop CS4 (Adobe Systems).

Isolation, amplification and digestion of viral DNA. Total cellular DNA was harvested using the Generation Capture Column Kit (Qiagen) at 4hpi. The RING finger domain of ICP0 was amplified using the High Fidelity polymerase reagent (Roche). The thermal cycling parameters were as follows: 1 cycle for 5 min at 37°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 49°C, and 90 sec at 72°C, followed by 1 cycle for 5 min at 72°C. The PCR product was digested with 10 units of Msc1 (New England Biolabs) diluted in appropriate buffer for 2h at 37^{o}C. 6x loading dye (30% v/v glycerol, 0.25% bromophenol blue) was added to digested DNA and resolved on a 2% agarose gel.

Chromatin Immunoprecipitation. Cells were incubated with 1% formaldehyde for 15 min to cross-link the chromatin at 5 hpi. Cells were washed twice with PBS and scraped into PBS containing 1x Complete protease inhibitor tablets (Roche Diagnostics). Cells

were resuspended in RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS) containing protease inhibitors and incubated on ice for 30 min. The samples were sonicated using a Biorupter 200 (Diagdnode) for eight cycles of 30s on 30s off for 5 min. This yielded DNA fragments averaging 500 bp in length. The samples were clarified by centrifugation at 13,000g at 4°C for 10 min. The clarified chromatin was diluted in ChIP dilution buffer (150 mM NaCl, 10 mM Na_2PO_4, 2 mM EDTA, 1.1% Triton, 0.1% SDS, 1 mM PMSF) pre-cleared with a salmon sperm DNA/protein A-agarose slurry for 1 h at 4°C. At this point, 1% of the total volume was removed and reserved as the input. Immunoprecipitation was carried out at 4°C overnight with 3 μg rabbit immunoglobulin G (IgG; Millipore) as the negative control or 3 μg anti-IRF-3 antibody (FL425, Santa Cruz Biotechnology).

Immunocomplexes were collected by incubation with a salmon sperm DNA/ protein A-agarose slurry for 1 h at 4°C with rotation. Beads were washed for 5 min at 4°C with rotation, twice with ChIP dilution buffer, twice with lithium chloride wash buffer (0.25 M LiCl, 1% NP-40, 0.7% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.1) and twice with Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The DNA-protein complexes were eluted by the addition of 200 μl of elution buffer (0.75% SDS, 0.1 M $NaHCO_3$) preheated to 65°C, rotating for 10 min at room temperature, incubating for 10 min at 65°C, and rotating for a final 10 min at room temperature. NaCl was added to a final concentration of 0.2 M to both the eluates and the inputs, and the samples were incubated at 65°C for 5 h in the presence of 1 μg RNase A (Ambion).

The samples were then digested with proteinase K (Qiagen), and the DNA was purified using a QIAquick PCR purification kit (Qiagen).

Antibodies. Antibodies used in Western blot experiments were mouse anti-ICP0 (1:1000, EastCoast Bio), mouse anti-FLAG (1:1000, abcam), mouse anti-GAPDH (G041, 1:5000, Applied Biological Materials), mouse anti-USP7 (C-2, 1:1000, Santa Cruz Biotechnology), and mouse anti-IRF3 (SL-12, 1:1000, Santa Cruz Biotechnology). Horse Radish Peroxidase-conjugated goat secondary antibodies were used at 1:10,000 (Santa Cruz Biotechnology).

For the indirect immunofluorescence study, anti-ICP0 primary antibody (1:150, EastCoast Bio) and goat anti-mouse Alexa 594 secondary antibody (1:500, Jackson ImmunoResearch) were used.

Primers.

Name	Use	Primer Sequences
IFNβ	qRT-PCR	5'-AAACTCATGAGCAGTCTGCA-3' 5'-AGGAGATCTTCAGTTTCGGAGG-3'
ISG56	qRT-PCR	5'-AAGGCAGGCTGTCCGCTTA-3' 5'-TCCTGTCCTTCATCCTGAAGCT-3'
ISG54	qRT-PCR	5'-ACGGTATGCTTGGAACGATTG-3' 5'-AACCCAGAGTGTGGCTGATG-3'
IL-6	qRT-PCR	5'-ACCGGGAACGAAAGAGAAGC-3' 5'-CTGGGAGTTCCAGGGCTAAG-3'
γ-actin	qRT-PCR	5'-CACCGCCGCATCCTCCTCTTC-3' 5'-GTGGTGCCGCCCGACAGC-3'
IFNβ promoter	qPCR	5'-ACTGAAAGGGAGAAGTGAAAGTGGA-3' 5'-TGCCAGAGCAAAGGCTTCGAAAGG-3'

ISG56 promoter	qPCR	5'-AGTTTCACTTTCCCCTTTCGC-3' 5'-ATCCTTACCTCATGGTTGCTG-3'
ISG54 promoter	qPCR	5'-AATGTCCCCAGACCCATCTTT-3' 5'-CCGGAGCTGAGTTGTGATCA-3'
ICP0 C116G	mutagenesis	5'-GACGAGGGCGACGTGGGAGCCGTGTGCACGGATG-3 5'-CATCCGTGCACACGGCTCCCACGTCGCCCTCGT-3'
ICP0 C156A	mutagenesis	5'-CGCAACACCTGCCCGCTGGCCAACGCCAAGCTGGTGTAC-3' 5'-GTACACCAGCTTGGCGTTGGCCAGCGGGCAGGTGTTGCG-3'
ICP0 RING Amp	PCR	5'-TTGCCCGTCCAGATAAAGTC-3' 5'-AGACCACCTTTGGTTGCAGA-3'

Results

HSV-1 ICP0 contributes to the inhibition of SeV induced IFNβ expression and is required for inhibition of ISG54. Previously, our lab reported the effect of HSV-1 on the induction of type I interferon (IFN) in Sendai virus (SeV) coinfected HEC-1B cells. These cells lack a functional type I IFN receptor (Wathelet et al., 1988) and allow the quantification of IFN and ISG expression without the confounding positive feedback loops observed with IFN induction. Using semi-quantitative RNAse protection assays, we observed that HSV-1 infection could inhibit the expression of *IFNβ* mRNA induced by SeV (Melroe et al., 2004). While a HSV-1 virus that only expressed ICP0 was sufficient to inhibit *IFNβ* production in response to SeV, ICP0-null viruses also retained their ability to inhibit *IFNβ* expression (Melroe et al., 2004), arguing that ICP0 was not necessary to inhibit the induction of *IFNβ*. In contrast, others have reported an increase in the expression of IRF-3 responsive genes during ICP0-null virus infection (Lin et al., 2004; Paladino et al., 2010). To reconcile these reports with our own results, we investigated HSV-1 mediated inhibition of SeV induced *IFNβ* expression using quantitative RT-PCR (qRT-PCR), which would allow us to identify subtle differences in cellular gene expression not detected in our original report.

To this end, HEC-1B cells were coinfected with SeV and either an ICP0-null virus (7134) or its rescue (7134R) at an MOI of 20. Total RNA was harvested from cells at 5 hours post infection (hpi) and *IFNβ, ISG56, and ISG54* mRNAs were measured by qRT-PCR. Infection with SeV alone robustly induced the expression of these genes, while wild-type HSV-1 significantly inhibited their induction (Figure 2.1, A-C), consistent with

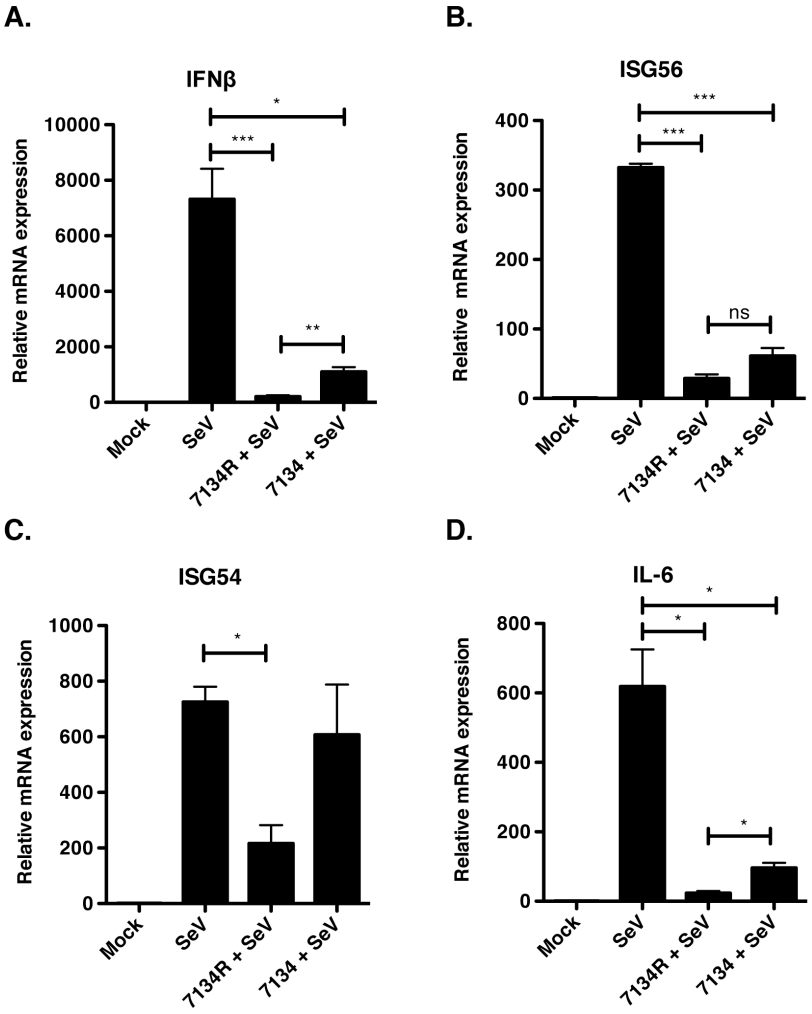

Figure 2.1: Deletion of ICP0 enhances antiviral gene expression. Total RNA was harvested at 5 hpi from HEC-1B cells infected with SeV alone or in conjunction with HSV-1 7134 or 7134R viruses. The harvested RNA was analyzed by qRT-PCR for the presence of (A) *IFNβ*, (B) *ISG56*, (C) *ISG54*, an (D) *IL-6* mRNA. Cellular RNA levels were normalized to *γ-actin* levels and made relative to mock-infected samples. Results are an average of four (*IFNβ* and *ISG56*) or three (*ISG54* and *IL-6*) independent experiments and error bars represent the standard error of means. *p≤.05, *** p≤.001 (Student's t test).

HSV-1 inhibiting SeV-induced innate immune signaling. In contrast to our previously published results, we observed an increase in *IFNβ* (5-fold) and *ISG56* (2-fold) expression in 7134-infected cells when compared to 7134R infection. While this increase was subtle, and did not account for the full inhibition observed, the change in gene expression was statistically significant (*IFNβ*) or reproducible (*ISG56*). In contrast, HSV-1 infection was unable to inhibit the induction of *ISG54* in the absence of ICP0 (Figure 2.1C). These results indicated that the requirement for ICP0 to inhibit SeV induced antiviral gene expression was dependent on the gene examined and confirmed that HSV-1 employs multiple mechanisms to inhibit *IFNβ* expression.

Expression of *IFNβ* is dependent on the activation of IRF-3; however, this protein alone is not sufficient to induce *IFNβ* expression. Instead, full activation of the *IFNβ* promoter relies on the synergistic activity of several transcription factors, including NF-κB, IRFs, and ATF-2/c-Jun (Wathelet et al., 1998). In contrast, IRF-3 is sufficient but not necessary to induce *ISG56* expression, while being both necessary and sufficient to induce *ISG54* (Andersen et al., 2008; Grandvaux et al., 2002). Interestingly, we observed a more robust inhibition of *IFNβ* expression (35-fold) than *ISG56* (11-fold) or *ISG54* (4-fold) in wild-type HSV-1-infected cells (Figure 2.1 A-C), suggesting that HSV-1 may affect the activity of transcription factors other than IRF-3. We therefore tested whether HSV-1 could inhibit SeV induced NF-κB signaling by examining the induction of an NF-κB responsive gene in our coinfection system. Interestingly, we observed a significant decrease in the induction of Interleukin-6 (IL-6) mRNA in HEC-1B cells coinfected with SeV and 7134R (Figure 2.1D). Infection with the ICP0-null 7134 virus

also inhibited the induction of *IL-6,* but to a lesser extent than 7134R. Together these results argue that in addition to its ability to inhibit IRF-3-dependent gene expression, HSV-1 inhibits SeV induction of NF-κB-dependent genes. Furthermore, our results indicate that ICP0 expression accounts for a portion of this inhibition of NF-κB signaling.

HSV-1 ICP0 is sufficient to inhibit IFNβ expression. The experiments above showed that ICP0 expression was necessary to inhibit a portion of *IFNβ* induction; however, it was unclear whether ICP0 was sufficient to inhibit this response. We previously reported that the *d*106 virus, which expresses ICP0 but no additional immediate-early genes, was capable of inhibiting IFNβ production (Eidson et al., 2002; Melroe et al., 2004); however, experiments with this virus do not rule out ICP0 acting in concert with incoming tegument proteins to mediate this response. To test whether ICP0 was sufficient to inhibit *IFNβ* expression in the absence of other viral genes, we examined the induction of *IFNβ* in the presence of ICP0 expressed from a transfected plasmid. HEC-1B cells were transfected with a FLAG-TBK1 construct and increasing doses of an ICP0 expression plasmid. Overexpression of FLAG-TBK1 induced the expression of *IFNβ* in comparison to empty vector-transfected cells (Mock), while ICP0 expression inhibited this induction in a dose-dependent manner (Figure 2.2A). This indicated that ICP0 was sufficient to inhibit *IFNβ* expression. Cell lysates from transfected cells were also examined for TBK1, ICP0 and IRF-3 protein levels. Interestingly, we observed an ICP0-dependent increase in the steady-state levels of FLAG-TBK1 (Figure 2.2B). This result was not unexpected, as ICP0 has been reported to be a promiscuous

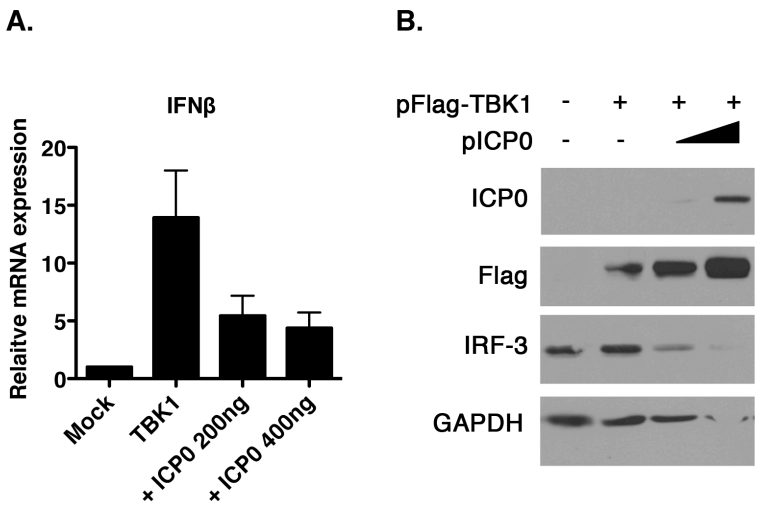

Figure 2.2: ICP0 expression is sufficient to inhibit *IFNβ* expression. HEC-1B cells were transfected with 500 ng pFLAG-TBK1 alone or co-transfected with either 200 or 400 ng of pICP0. The total amount of transfected DNA was brought up to 1000 ng with empty vector plasmid. Total RNA or whole cell lysates were harvested at 36 hours post transfection and analyzed by (A) qRT-PCR for endogenous *IFNβ* expression, or (B) western blot analysis using ICP0-, FLAG-, and IRF-3 specific antibodies. Cellular GAPDH levels were used as a recovery and loading control. Cellular RNA levels were normalized to *γ-actin* levels and made relative to empty vector transfected (mock) samples. qRT-PCR results are an average of two-independent experiments and error bars represent the standard error of means.

transactivator of HSV-1 and heterologous genes (Roizman et al., 2013), but suggested that our transfection results may underestimate the inhibitory activity of ICP0. We also observed an ICP0-dependent decrease in the levels of endogenous IRF-3, consistent with the loss of activated IRF-3 reported during SeV coinfection with either wild-type HSV-1 or the ICP0-expressing *d*106 virus (Melroe et al., 2004; Melroe et al., 2007). While this result suggested that ICP0 was also sufficient to promote IRF-3 degradation, we cannot rule out that the increased turnover of IRF-3 was not due to the observed increase in TBK1 expression, as the cellular response to downregulate IRF-3 activity involves proteasomal degradation of the protein (Lin et al., 1998).

Transfected ICP0 inhibits constitutively active IRF-3 in a RING finger–independent manner. ICP0 contains a C_3HC_4 zinc RING finger domain, which is responsible for the protein's E3 ubiquitin ligase activity (Boutell et al., 2002). Disruption of this domain through mutational alteration inhibits the ability of ICP0 to promote the ubiquitination and subsequent degradation of target proteins. Because expression of ICP0 induces the increased degradation of SeV activated IRF-3 during HSV-1 infection (Melroe et al., 2004), we asked whether the RING finger domain is necessary to inhibit IRF-3 activity. To answer this question, we generated a mutant ICP0 plasmid (pICP0 RFm) with two point mutations in the RING finger domain (C116G/C156A), which disrupts the catalytic activity of this protein (Lium and Silverstein, 1997; Vanni et al., 2012). Plasmids encoding wild-type or RING finger mutant ICP0 were then transfected into HEC-1B cells along with a constitutively active IRF-3 mutant (IRF-35D) and IRF-3

responsive gene expression was measured by qRT-PCR. Wild-type ICP0 inhibited IRF-35D-induced *IFNβ, ISG56* and *ISG54* expression in a dose-dependent manner (Figure 2.3A-C), recapitulating the inhibitory phenotype observed in our SeV coinfection model. Furthermore, of the three genes examined, ICP0 had the greatest effect on the expression of *IFNβ*, possibly due to the ability of ICP0 to inhibit both IRF-3 and NF-κB signaling. Although the extent of inhibition of these genes may appear modest (50-75% at highest ICP0 expression), western blot analysis of cell lysates from transfected cells revealed an ICP0-dependent increase in IRF-35D protein levels at lower levels of ICP0 (Figure 2.3D), similar to what we observed when co-transfecting TBK1 and ICP0 in Figure 2.2B. At the highest level of ICP0 expression we observed a decrease in IRF-35D levels, suggesting that ICP0 may overcome the transactivation of IRF-35D expression. These data indicated a possible underestimation of the inhibition provided by wild-type ICP0, consistent with the results reported in Figure 2.2.

Surprisingly, we also observed a decrease in the expression of *IFNβ, ISG56,* and *ISG54* in cells transfected with the RING finger mutant (Figure 2.3A-C), which did not transactivate IRF-35D or its own expression (Figure 2.3D), indicating that ICP0 can inhibit IRF-3 signaling though a RING finger-independent mechanism. However, due to the observed differences in both ICP0 and IRF-3 protein levels in these transfection studies, a direct comparison of the inhibition mediated by wild-type ICP0 and the RING finger mutant was not possible. Therefore, we cannot conclude what portion of ICP0 activity relies on a functional RING finger domain. Nevertheless, these results argue

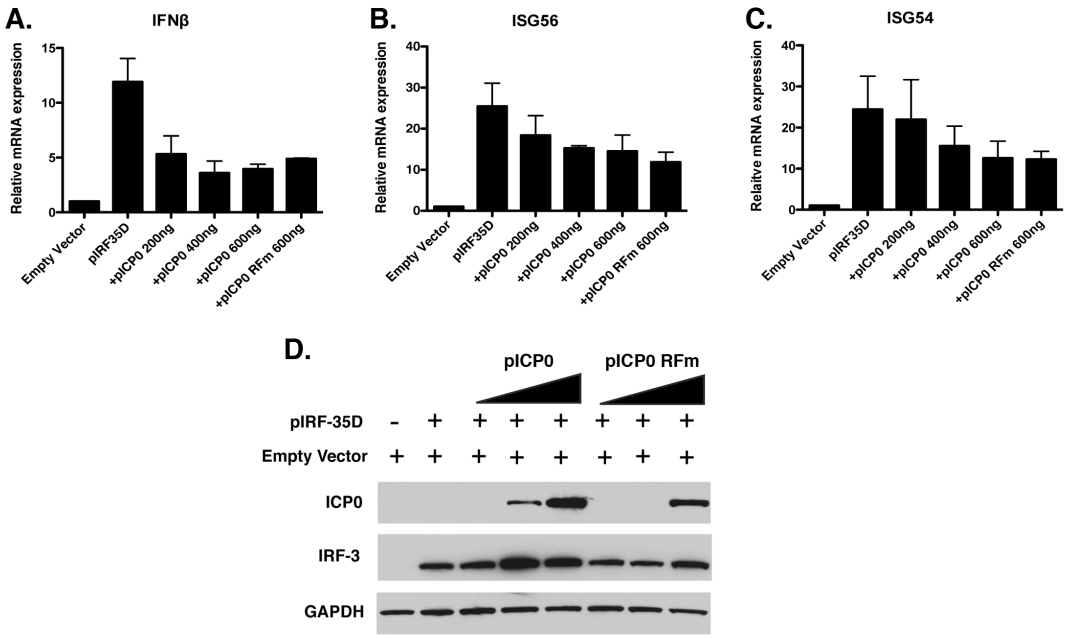

Figure 2.3: ICP0 inhibits IRF-3 independently of RING finger domain. HEC-1B cells were transfected with 400 ng pIRF35D alone or co-transfected with 200 to 600 ng of pICP0 or 600 ng pICP0 RFm. Total transfected DNA was brought up to 1000 ng with empty vector plasmid DNA. Total RNA or whole cell lysates were harvested at 36 hpt and analyzed by (A) qRT-PCR for endogenous *IFNβ, ISG56,* and *ISG54*, or (B) western blot analysis using ICP0- and IRF-3 specific antibodies. Cellular GAPDH proteins levels were used as a recovery and loading control. Cellular RNA levels were first normalized to *γ-actin* levels and then to empty vector (mock) samples.

that ICP0 is capable of inhibiting IRF-3 activity through a RING finger-independent mechanism.

Construction and validation of ICP0 RING finger mutant and rescued viruses. Due to the inherent difficulties in interpreting our ICP0 transfection studies, we next constructed an ICP0 RING finger mutant virus to examine the relative effect of the RING finger domain in inhibiting SeV activated IRF-3. Our RING finger mutant virus (KOS.RFm) was constructed by homologous recombination between infectious ICP0-null virus DNA and the pICP0 RFm plasmid, which contains complementary ICP0 flanking sequence. The successful recombination of this RING finger mutation introduced a novel Msc1 restriction site, which was confirmed by PCR amplification of the viral DNA and restriction endonuclease digestion (Figure 2.4A, Lane 5). Digestion of the corresponding rescue virus (KOS.RFr) DNA did not result in cleavage of the amplified DNA (Figure 2.4A, Lane 3), indicating the construction of a successful genotypic rescue.

We next examined these two viruses for known defects in ICP0 activity. ICP0 mutant viruses are restricted for growth in several cell lines, including Vero cells; therefore, we examined the replication of KOS.RFm and KOS.RFr in these cells. Consistent with a defect in ICP0 activity, we observed an approximate 3-log decrease in KOS.RFm viral yield in Vero cells compared to cells infected with the rescue virus or wild-type HSV-1 KOS strain (Figure 2.4B). Moreover, the KOS.RFm virus was unable to promote the degradation of USP7, a well-characterized direct target of ICP0-mediated

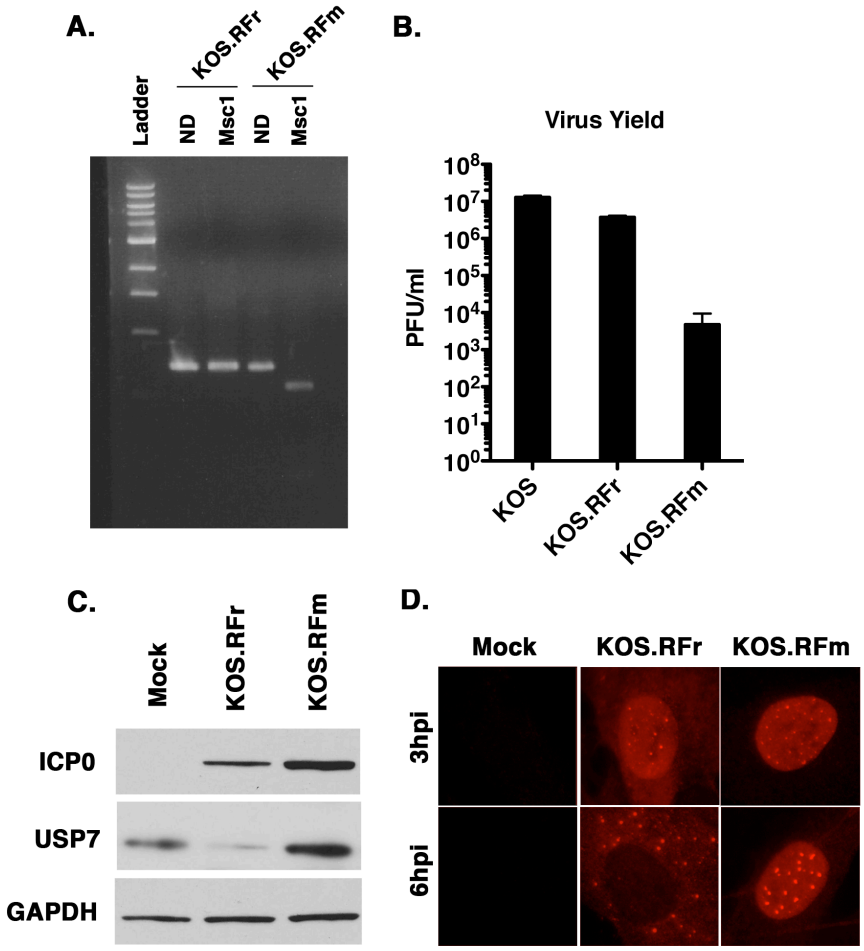
A.
Ladder
KOS.RFr
KOS.RFm
ND
Msc1
ND
Msc1
B.
Virus Yield
PFU/ml
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
KOS
KOS.RFr
KOS.RFm
C.
Mock
KOS.RFr
KOS.RFm
ICP0
USP7
GAPDH
D.
Mock
KOS.RFr
KOS.RFm
3hpi
6hpi

Figure 2.4

Figure 2.4 (Continued): An ICP0 RING finger mutant virus is defective for ICP0-dependent activities. (A) Total DNA from KOS.RFm and KOS.RFr infected cells were harvested at 4hpi and the RING finger domain of ICP0 was amplified by PCR, digested with Msc1, and visualized on a 2% agarose gel with 1kb ladder. (B) Vero cells were infected with wt-KOS, KOS.RFm or KOS.RFr viruses at an MOI of 0.1, harvested at 48hpi and viral yields were determined by plaque assay on U2OS cells. (C) Vero cells were mock-infected or infected with KOS.RFm or KOS.RFr at an MOI of 10 and whole cell lysates were harvested at 6 hpi and probed for ICP0, USP7 and GAPDH levels. (D) Vero cells grown on coverslips were infected with KOS.RFm or KOS.RFr at an MOI of 10. Samples were fixed at 3 and 6hpi, stained for ICP0, and visualized by indirect immunofluorescence

ubiquitination (Canning et al., 2004), while the rescued virus efficiently promoted the loss of this protein (Figure 2.4C). Finally, this mutation did not disrupt the ability of ICP0 to form nuclear foci at early time-points in infection (Figure 2.4D, 3hpi). However, ICP0 expressed from KOS.RFm did not accumulate in the cytoplasm at late time-points in infection consistent with the growth defect observed in Vero cells (Figure 2.4D, 6hpi) and indicating a delay in viral DNA replication. Together, these results argued that our KOS.RFm virus phenocopies previously described ICP0 RING finger mutant viruses (Canning et al., 2004; Everett, 1989; Lium and Silverstein, 1997), and that KOS.RFr successfully rescues the wild-type virus phenotype.

HSV-1 ICP0 promotes IRF-3 degradation in a RING finger-dependent manner. The IRF-3 signaling pathway is subject to a negative cellular feedback mechanism following activation, which promotes the degradation of IRF-3 and limits the expression of type I IFN as observed by immunoblot analysis following RNA virus infection (Lin et al., 1998). Previously, we have reported that ICP0 expression enhances the degradation of SeV activated IRF-3 (Melroe et al., 2004). To determine whether the observed degradation of IRF-3 was dependent on ICP0 E3 ubiquitin ligase activity, we examined the steady state levels of SeV-activated IRF-3 during infection with our ICP0 RING finger mutant virus.

HEC-1B cells were coinfected with SeV and either KOS.RFm or KOS.RFr at an MOI of 20. Cell lysates were harvested at 2, 4, 6, and 8 hpi and analyzed by western blot. Consistent with previously published results (Melroe et al., 2004), the KOS.RFr

virus enhanced the loss of SeV activated IRF-3 as early as 4 hpi (Figure 2.5, lane 6 vs lane 5). This phenotype was dependent on the RING finger activity of ICP0, as we did not observe an increase in IRF-3 degradation in KOS.RFm coinfected cells when compared to KOS.RFr coinfection or SeV infection alone (Figure 2.5, lane 6 vs lanes 5 and 7). Instead, we observed a stabilization of SeV-activated IRF-3 during coinfection with the ICP0 RING finger mutant virus, particularly at 6 and 8 hpi (Figure 2.5, lanes 10 and 13). Together, these results argued that HSV-1 enhances the degradation of SeV-activated IRF-3 in an ICP0 RING finger-dependent manner but that in the absence of a functional RING finger domain, ICP0 inhibits the normal cellular turnover of IRF-3.

HSV-1 ICP0 inhibits SeV-activated IRF-3 responsive gene expression independently of the RING finger domain. In Figure 2.1 we observed an ICP0-dependent inhibition of SeV-activated antiviral gene expression. To test whether this inhibition was dependent on the RING finger activity of ICP0, we examined the induction of IRF-3 responsive genes during co-infection with SeV and either KOS.RFm or KOS.RFr. At 5 hpi, RNA from co-infected cells was harvested and analyzed by qRT-PCR. We observed a significant reduction in SeV-induced *IFNβ, ISG56, ISG54,* and *IL-6* expression in KOS.RFr co-infected cells (Figure 2.6A-D), similar to our results with 7134R infection in Figure 2.1. Interestingly, we observed an increase in the expression of all four genes during KOS.RFm infection when compared to cells infected with KOS.RFr (Figure 2.5A-D), although the extent of this increase varied among the individual genes. The increases in antiviral gene expression were comparable to the

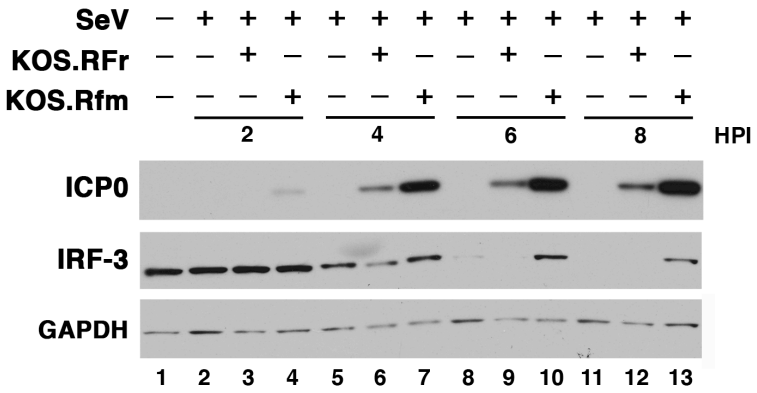

Figure 2.5: KOS.RFm inhibits the ICP0-dependent enhanced degradation of IRF-3. HEC-1B cells were mock infected, infected with SeV [100 hemagglutinating units (HAU)] alone or co-infected with KOS.RFr or KOS.RFm at an MOI of 20. Whole cell lysates were harvested at 2, 4, 6, and 8 hpi and probed for ICP0 and IRF-3 using specific antibodies. Cellular GAPDH protein levels were used as a recovery and loading control.

induction observed during ICP0-null virus infection (Figure 2.1), suggesting that the RING finger domain accounts for the ICP0-dependent effect on gene expression. However, this observation is in direct conflict with our transfection data that implicated a RING finger-independent mechanism of action (Figure 2.3).

One possible explanation to reconcile this difference may stem from the observed stabilization of IRF-3 during SeV coinfection with KOS.RFm (Figure 2.5). In other instances, decreasing the cellular turnover of IRF-3 through modifications like ISGylation enhances antiviral gene expression (Shi et al., 2010). Interestingly, we did not observe an increase in *ISG54* expression during KOS.RFm co-infection over that of SeV infection alone (Figure 2.6C), indicating that the RING finger mutant inhibited any potential IRF-3 activity associated with protein stabilization. In light of this observation, our results suggest that ICP0 can inhibit SeV-activated IRF-3 independently of the RING finger domain and IRF-3 degradation.

ICP0 expression inhibits IRF-3 occupancy on cellular promoters prior to promoting the degradation of IRF-3. Upon activation and accumulation in the nucleus, IRF-3 binds to specific DNA sequences in the promoter regions of cellular genes and recruits the CBP/p300 coactivator to promote their expression. During HSV-1 infection, ICP0 induces the intranuclear relocalization of SeV activated IRF-3 to ICP0 foci prior to the detectable degradation of IRF-3 (Melroe et al., 2007). Because the results of this study indicate that ICP0 inhibits IRF-3 independently of protein degradation, we hypothesized that ICP0 could inhibit the recruitment of IRF-3 to cellular

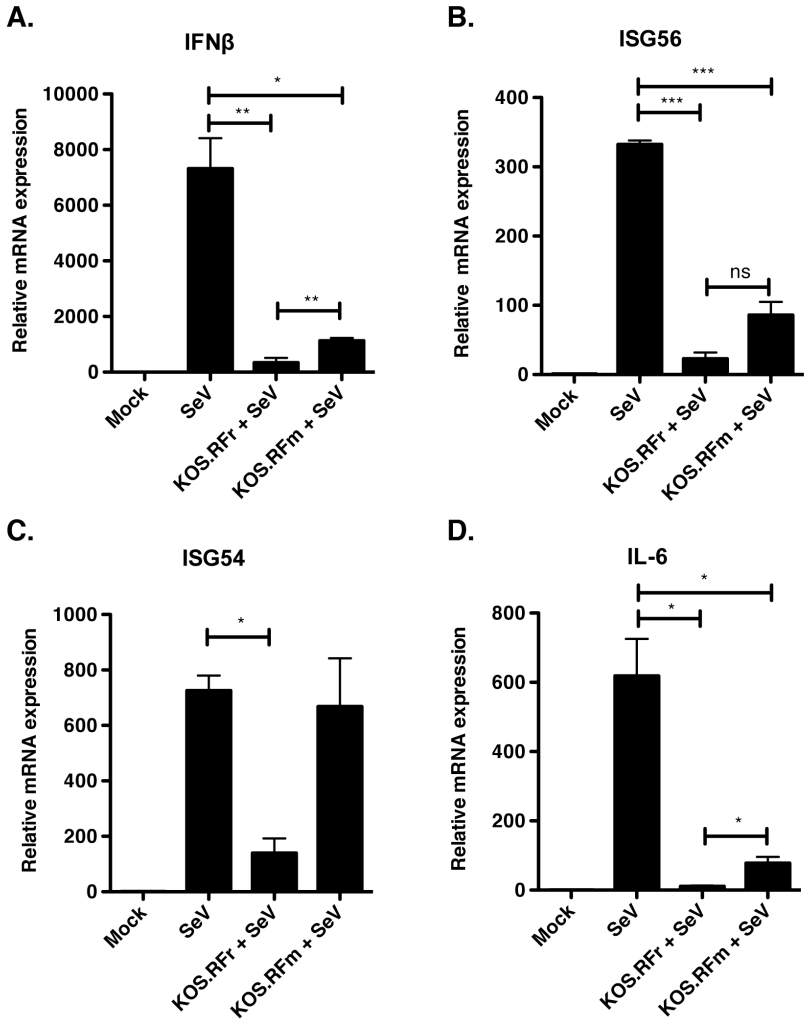

Figure 2.6: KOS.RFm inhibits the expression of antiviral genes. Total RNA was harvested at 5 hpi from HEC-1B cells infected with SeV alone or in conjunction with HSV-1 KOS.RFr or KOS.RFm viruses. The harvested RNA was analyzed by qRT-PCR for the presence of (A) *IFNβ*, (B) *ISG56*, (C) *ISG54*, an (D) *IL-6* mRNA. Cellular RNA levels were normalized to *γ-actin* levels and made relative to mock-infected samples. Results are an average of four (*IFNβ* and *ISG56*) or three (*ISG54* and *IL-6*) independent experiments and error bars represent the standard error of means. *p≤.05, ** p≤.01, ***p≤.001 (Student's t-test).

promoters. To test this hypothesis, we examined the ability of IRF-3 to associate with cellular DNA by chromatin immunoprecipitation (CHIP) during SeV co-infection with the HSV-1 *d*106 and *d*109 recombinant viruses. The *d*106 virus expresses ICP0, but not ICP4, -22, -27, or -47 (Samaniego et al., 1998). In contrast, the *d*109 virus does not express any viral immediate-early genes, including ICP0. The *d*106 virus was previously used to investigate ICP0-mediated inhibition of IRF-3 (Eidson et al., 2002), and it recapitulates the ICP0-dependent phenotypes observed during wild-type HSV-1 infection (Melroe et al., 2004; Melroe et al., 2007).

HEC-1B cells were infected with SeV alone or co-infected with either *d*106 or *d*109 at an MOI of 20. At 5 hpi, cells were fixed with formaldehyde and IRF-3-associated DNA was immunoprecipitated with an IRF-3 specific antibody and analyzed by qPCR using promoter specific primer sets. We observed a significant increase in IRF-3 association with the *IFNβ, ISG56, and ISG54* promoters in SeV-infected cells compared to mock-infected cells (Figure 2.7A-C), consistent with the induction of IRF-3 responsive gene expression at this time point (Figure 2.1 and 2.6). Co-infection with *d*106, but not the *d*109 virus, significantly inhibited the amount of IRF-3 bound to these genes, suggesting that ICP0 expression inhibits IRF-3 recruitment to cellular promoters.

Infection with the *d*106 virus has also been shown to increase the degradation of IRF-3, but this phenotype is delayed compared to wild-type infection (Melroe et al., 2004; Melroe et al., 2007). To test whether the decrease in IRF-3 occupancy on cellular promoters observed during *d*106 infection correlates with a decrease in IRF-3 protein levels, we examined the steady state levels of this protein in lysates from our CHIP

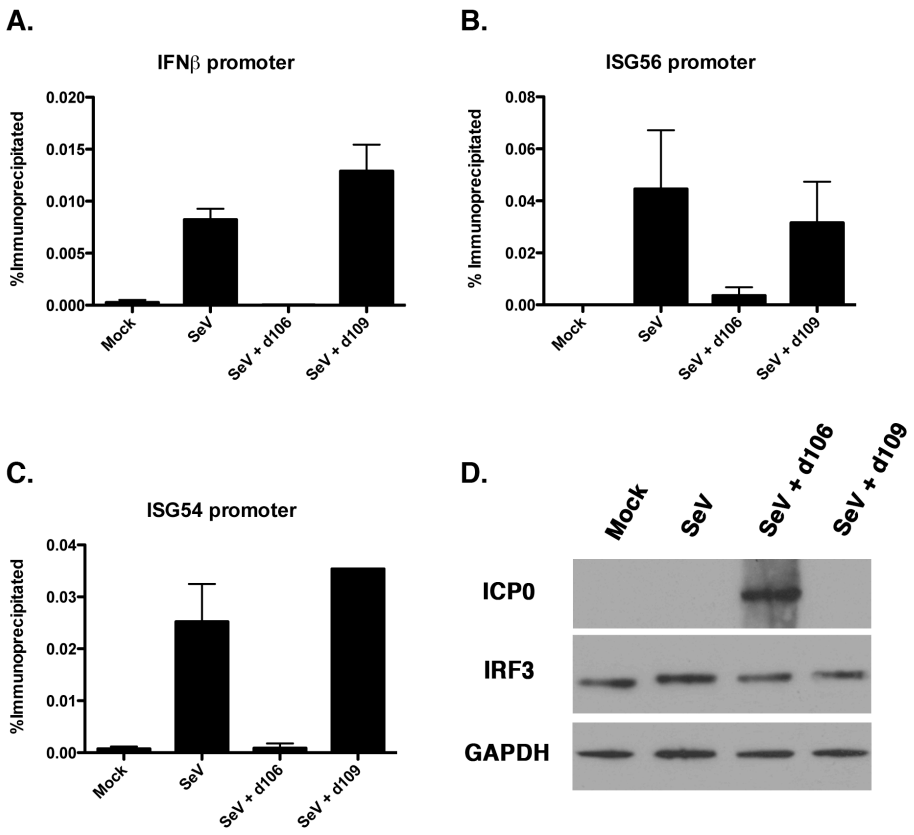

Figure 2.7: ICP0 expression inhibits IRF-3 occupancy on cellular promoters.

Chromatin immunoprecipitation assay measuring IRF-3 levels on cellular promoters. HEC-1B cells were mock infected, infected with SeV (100 HAU) alone or co-infected with *d*106 or *d*109 at an MOI of 20. Cells were fixed at 5 hpi and chromatin was prepared and subjected to immunoprecipitation using IRF-3 or control rabbit IgG-specific antibodies. Immunoprecipitated DNA was measured by qPCR using primers specific for the (A) *IFNβ*, (B) *ISG56*, and (C) *ISG54* gene promoters. Data are presented as a percent of total DNA immunoprecipitated and values are the average of three independent experiments. (D) Lysates from fixed samples were reverse cross-linked and probed for ICP0, IRF-3, and GAPDH levels by western blot.

assays. Infection with the *d*106 virus only slightly decreased the levels of IRF-3 compared to cells infected with SeV alone or with *d*109 virus (Figure 1.7D); however, not to the extent that would affect our CHIP analysis. Together these results indicate that the decrease in IRF-3 occupancy observed during *d*106 infection cannot be explained by ICP0-dependent turnover of IRF-3, and argued for an ICP0-dependent mechanism that relies on sequestration rather than degradation of IRF-3.

Discussion

In this study we further characterized the HSV-1-mediated inhibition of antiviral gene expression and the role of ICP0 in inhibiting this response. We confirmed that HSV-1 employs multiple mechanisms to inhibit *IFNβ* expression, but that inhibition of *ISG54* expression relies solely on ICP0. Prior to this study, we hypothesized that ICP0 could inhibit IRF-3 activity through two distinct mechanisms: either through degradation of IRF-3 or through an ability to sequester the protein away from host chromatin. Here we demonstrate that, although ICP0 promotes IRF-3 degradation in a RING finger-dependent manner, this response is not necessary to inhibit IRF-3 responsive gene expression. Instead, our results support a degradation-independent model that involves ICP0 sequestering IRF-3 from host chromatin.

Inhibition of NF-κB and IRF-3 responsive genes by HSV-1. HSV-1 infection inhibits both the expression of type I IFNs and ISGs to evade the host immune response. Initial attempts to identify the specific viral gene product(s) necessary to inhibit the induction of these genes were unsuccessful, as deletion of individual immediate-early genes had no effect on the production of either IFNβ or ISG56 (Melroe et al., 2004; Mossman et al., 2001). However, the expression of ICP0 during *d*106 infection was sufficient to inhibit *IFNβ* expression, suggesting the failure of these initial studies was due to the virus encoding multiple proteins that inhibit type I IFN production (Melroe et al., 2004). The study presented here confirms that ICP0 expression is mostly dispensable for the inhibition of both *IFNβ* and *ISG56* expression during HSV-1 infection, but was

unexpectedly necessary to inhibit *ISG54* expression. The differential requirement for ICP0 in the inhibition of these genes may be due to differences in the sufficiency of IRF-3 in their activation. It has become increasingly clear that the induction of antiviral genes can be regulated by many transcription factors, and while IRF-3 is required for *IFNβ* expression, it is not sufficient to induce its activation (Agalioti et al., 2000; Andersen et al., 2008). In contrast, IRF-3 is both necessary and sufficient to induce *ISG54* expression. Interestingly, while the *ISG56* gene is frequently used as a readout for IRF-3 activity, our results indicate that the requirements for its induction in response to SeV differ from that of *ISG54*. The exact nature of this difference remains unclear.

During the examination of additional virus-induced genes we observed that HSV-1 infection decreased the induction of *IL-6*, whose expression is dependent on NF-κB. Based on this observation, we hypothesize that a portion of the ICP0-independent inhibition of *IFNβ* expression observed during HSV-1 infection may involve the inhibition of NF-κB. HEC-1B cells do not induce an innate immune response to HSV-1 infection, even in the absence of viral gene expression (Melroe et al., 2004), indicating the targets of HSV-1 inhibition are within the signaling pathway that senses SeV infection in these cells. During infection, SeV RNA replication intermediates are sensed by cytosolic RIG-I or endosomal TLR7 in a cell type- dependent manner (Kato et al., 2005; Lund et al., 2004). HEC-1B cells express both RIG-I and TLR7; however, they do not respond to the TLR7 agonist Loxoribine (Aboussahoud et al., 2010), indicating that the response to SeV in these cells is mediated by RIG-I. Therefore, the targets of HSV-1 inhibition are within the RIG-I pathway in HEC-1B cells.

The viral gene(s) that inhibit the NF-κB response observed here are relatively unknown. Our results implicate ICP0 in inhibiting this response, but expression of this protein did not account for the full inhibition in *IL-6* mRNA production. Additional HSV-1 proteins have been reported to inhibit NF-κB activity. Deletion of the HSV-1 US11 protein was recently shown to slightly inhibit SeV-induced *IFNβ* expression at the step of RIG-I or MDA-5 activation (Xing et al., 2012). However, in our SeV co-infection model we have not observed a difference in the phosphorylation of IRF-3 during HSV-1 infection (Melroe et al., 2004), indicating that the effect on *IL-6* production occurs downstream of MAVS in the NF-κB arm of the RIG-I signaling cascade (Figure 2.8). Recently, we have identified the US3 tegument protein as a modulator of NF-κB signaling, albeit in response to TLR2 activation (Sen et al., 2013). Both the TLR2 and RIG-I pathways have considerable overlap in the signaling molecules used to activate NF-κB, including TRAF6, whose ubiqutination is inhibited by US3. Therefore, it is conceivable that US3 might be involved in inhibiting the RIG-I-dependent pro-inflammatory cytokine response through a similar mechanism.

ICP0 RING-finger dependent degradation of IRF-3. The ICP0 immediate-early protein is an E3 ubiquitin ligase, and many of its functions during infection are attributed to this activity (Roizman et al., 2013). We previously observed that ICP0 expression promoted the increased degradation of SeV activated IRF-3, indicating that the ICP0 RING finger domain might be involved in this phenotype. This hypothesis was supported in this study by the construction of an ICP0 RING finger mutant virus, which

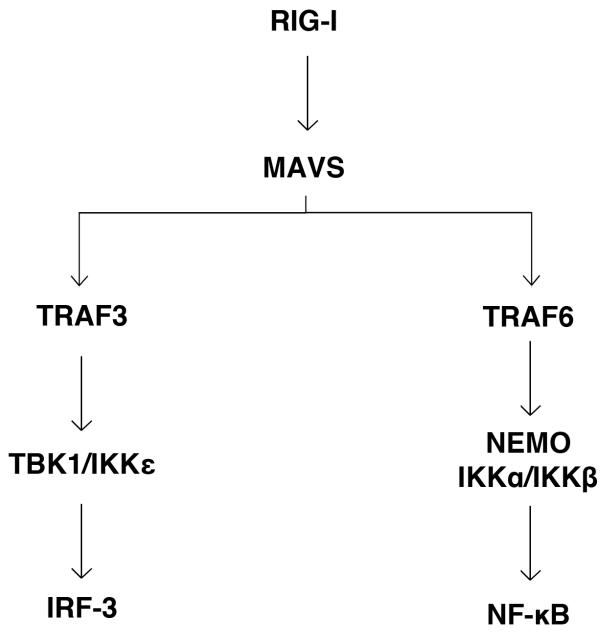

Figure 2.8: The RIG-I pathway. Viral double-stranded RNA is sensed by the RIG-I pattern recognition receptor. Activated RIG-I interacts with mitochondrial associated MAVS protein through homotypic interactions between caspase activation and recruitment domains (CARDs). MAVS activates the IRF-3 pathway through TRAF3 and subsequently TBK1/IKKε. The NF-κB pathway is also activated through MAVS via TRAF6 ubiquitination of NEMO and degradation of Iκβ.

was unable to promote IRF-3 degradation. Destabilization of IRF-3 through proteasomal degradation is a common immune evasion strategy employed by viruses (Viswanathan et al., 2010). Recently the varicella zoster virus ORF61 protein, an ortholog of HSV ICP0, was shown to degrade nuclear IRF-3 in a RING-finger dependent manner (Zhu et al., 2011), indicating this phenotype may be broadly active among alphaherpesvirus family members.

Interestingly, ICP0-dependent degradation of activated IRF-3 is not observed in human fibroblasts infected with HSV-1(Mossman and Smiley, 2002; Paladino et al., 2010), nor does it seems to be important for inhibiting IRF-3 activity in this study. This raises the possibility that the degradation of IRF-3 by ICP0 is only observed when activated by specific stimuli. In this study, we have stimulated IRF-3 through the RIG-I pathway; however, IRF-3 activation in response to HSV-1 infection occurs independently of RIG-I in fibroblasts (Paladino et al., 2006), although the MDA-5 RIG-I-like receptor has been implicated in sensing HSV-1 in other cell types (Melchjorsen et al., 2010). Furthermore, It has been noted that SeV, but not HSV-1, induces the hyperphosphorylation of IRF-3, which is associated with protein instability (Clement et al., 2008; Lin et al., 2004), indicating that while RNA and DNA viruses both activate IRF-3, distinct signaling pathways may modify IRF-3 differentially. We therefore hypothesize that the hyperphosphorylation of IRF-3 induced by SeV infection allows ICP0 to promote its degradation.

Mechanism of ICP0-mediated inhibition of IRF-3 responsive gene expression.

The results of this study indicate that ICP0-mediated degradation of IRF-3 is not necessary for the protein to inhibit IRF-3 responsive gene expression. Instead, our results support a model in which ICP0 sequesters IRF-3 away from cellular promoters. This may occur by ICP0 directly or indirectly interacting with IRF-3. In support of this hypothesis, we have previously observed that IRF-3 coimmunoprecipitates with ICP0 during infection, and that the two proteins colocalize by immunofluorescence (Melroe et al., 2007). This potential interaction is further supported by our observation that infection with the ICP0 RING finger mutant virus inhibits the cellular turnover of IRF-3. We speculate that ICP0 blocks cellular E3 ubiquitin ligases from degrading IRF-3 under these conditions, potentially by masking the binding site that is normally recognized by these proteins.

ICP0 interacts with cellular proteins through a variety of mechanisms, some of which depend on specific posttranslational modifications of either the target protein or ICP0 itself. For instance, ICP0 contains several SUMO interaction motifs, which allow ICP0 to interact with and degrade specific SUMOylated proteins (Boutell et al., 2011). Interestingly, IRF-3 has been shown to be SUMOylated upon activation with vesicular stomatitis virus (Kubota et al., 2008), which may provide an interaction surface for ICP0 to bind. Alternatively, ICP0 interacts with FHA (forkhead associated) domain-containing proteins by mimicking a cellular FHA phospho-binding site (Chaurushiya et al., 2012). While IRF-3 does not contain a FHA domain per se, the crystal structure of the IRF-3 IAD (IRF association domain) contains a basic face that structurally resembles the

CHK2 FHA domain (Qin et al., 2003). Further investigation of the ICP0 domains necessary for this association will elucidate the mechanism by which ICP0 sequesters IRF-3.

Chapter Two: Nuclear IFI16 Induction of IRF-3 Signaling During Herpesviral Infection and Degradation of IFI16 by the Viral ICP0 Protein

A version of this chapter has been published:

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Abstract

Innate sensing of microbial components is well documented to occur at many cellular sites, including at the cell surface, in the cytosol, and in intracellular vesicles, but there is limited evidence of nuclear innate signaling. In this study we have defined the mechanisms of interferon regulatory factor-3 (IRF-3) signaling in primary human foreskin fibroblasts (HFF) infected with herpes simplex virus 1 (HSV-1) in the absence of viral gene expression. We found that the interferon inducible protein 16 (IFI16) DNA sensor, which is required for induction of IRF-3 signaling in these cells, is nuclear, and its localization does not change detectably upon HSV-1 *d*109 infection and induction of IRF-3 signaling. Consistent with the IFI16 sensor being nuclear, conditions that block viral DNA release from incoming capsids inhibit IRF-3 signaling. An unknown factor must be exported from the nucleus to activate IRF-3 through cytoplasmic STING, which is required for IRF-3 activation and signaling. However, when the viral ICP0 protein is expressed in the nucleus, it causes the nuclear re-localization and degradation of IFI16, which inhibits IRF-3 signaling. Therefore, HSV-1 infection is sensed in HFF by nuclear IFI16 upon release of encapsidated viral DNA into the nucleus, and the viral nuclear ICP0 protein can inhibit the process by targeting IFI16 for degradation. Together these results define a pathway for nuclear innate sensing of HSV DNA by IFI16 in infected HFF and document the first mechanism by which a virus can block this nuclear innate response.

Introduction

Viral infection elicits a number of antiviral innate immune responses, including the secretion of type I interferons, interferons α and β (IFNα/β), which act in an autocrine or paracrine manner to induce the production of interferon-stimulated genes (ISG) whose protein products mediate the antiviral state (Stetson and Medzhitov, 2006). In addition to mediating a localized response, interferon bridges the innate and adaptive immune responses to promote immunological memory and clearance of viral infection (Iwasaki and Medzhitov, 2010).

The antiviral response is induced by a set of germ-line encoded pattern recognition receptors (PRRs) (Janeway, 1989) that recognize pathogen-specific moieties, which include viral genomic DNA and RNA species as well as RNA replication intermediates. These PRRs signal through distinct adaptor molecules but converge on the activation of TBK1 (Fitzgerald et al., 2003). This serine/threonine protein kinase phosphorylates the constitutively expressed interferon regulatory factor 3 (IRF-3) (McWhirter et al., 2004). Normally cytoplasmic, phosphorylated IRF-3 translocates to the nucleus where it promotes the transcription of ISRE responsive genes, including IFNβ (Wathelet et al., 1998).

The host cell is known to distinguish viral and self nucleic acids by sensing both chemical and compartmentalization differences between these molecules. For instance, the membrane bound toll-like receptors (TLRs) 3, and 7/8 detect RNA species that accumulate in endosomes (Kawai and Akira, 2009). In addition, resident phagocytic cells use these receptors to detect foreign pathogens during immune surveillance of

tissues (Schulz et al., 2005). Chemical differences between self and viral RNAs also exist and are detected by a class of cytosolic PRRs known as the RIG-I like receptors (RLRs). The eponymous member of this class, RIG-I, recognizes dsRNA that contains 5' triphosphates (Hornung et al., 2006), which are unique to RNA virus infection. MDA5, another member of this family, also binds viral dsRNA but potentiates signaling based on the length of the RNA species it detects (Kato et al., 2008).

While recognition of RNA virus infection is well understood, less is known about how DNA viruses are sensed by the host cell. The first DNA sensor identified was TLR9, which recognizes unmethylated CpG DNA in endosomal compartments and is particularly potent at detecting foreign DNA in plasmacytoid dendritic cells (Ahmad-Nejad et al., 2002; Latz et al., 2007). More recently, cytosolic PRRs have been identified which sense DNA virus infection in the cytoplasm and include DAI, Pol III, and the DEAD/H-box helicase DDX41 (Ablasser et al., 2009; Chiu et al., 2009; Takaoka et al., 2007; Zhang et al., 2011). These sensors are thought to distinguish cellular and viral DNA due to compartmentalization differences.

An interesting paradox in the DNA sensing field involves herpesviruses, which constitute a class of large double stranded DNA viruses that replicate in the nucleus of infected cells. While DNA from these viruses is a potent activator of IRF-3, it is unclear how they are sensed during infection. Currently, there is little evidence that nuclear DNA sensing can induce a type I interferon response to virus infection. Recently, IFI16, a member of the PYHIN family of proteins was implicated in the type I interferon response to herpes simplex virus I (HSV-1) (Unterholzner et al., 2010). While originally

reported to be a cytosolic DNA sensor (Unterholzner et al., 2010), IFI16 is localized in the nucleus of many cell types (Veeranki and Choubey, 2012), making it a potential candidate for sensing nuclear HSV-1 DNA.

The importance of the IRF-3 pathway in restricting virus replication has made it a target for virus-mediated inhibition. HSV-1 induces an IRF-3-dependent type I interferon/interferon stimulated gene (ISG) response in human fibroblasts in the absence of viral gene expression (Everett et al., 2008b; Mossman et al., 2001; Preston et al., 2001). This response is potently inhibited by viral gene expression, suggesting that a viral protein inhibits this response. Like many large DNA viruses, HSV-1 encodes multiple mechanisms for inhibiting interferon expression (Melroe et al., 2004). For instance, the late gene product ICP34.5 disrupts phosphorylation of IRF-3 by TBK1, while the US11 tegument protein inhibits RIG-I interaction with its downstream adaptor MAVS (Verpooten et al., 2009; Xing et al., 2012). In addition, the immediate-early ICP0 protein has long been known to inhibit IRF-3 signaling in human fibroblasts; however, the mechanism of its inhibition has not been determined (Eidson et al., 2002). Previously, we have shown that ICP0 causes the relocalization of Sendai virus (SeV) activated IRF-3 to nuclear foci (Melroe et al., 2007). This led us to hypothesize that ICP0 sequesters IRF-3 from cellular promoters to mediate inhibition of signaling. However, because SeV signals through RIG-I, it has remained unclear whether HSV-1 activated IRF-3 in a similar manner.

In this study, we investigated the initial activation of IRF-3 signaling in primary human fibroblasts in response to HSV-1. We observed that type I interferon expression

in response to replication-defective HSV-1 requires the accumulation of viral DNA in the nuclear compartment. Furthermore, we identified IFI16 as a nuclear sensor of HSV-1 infection. In addition, we describe an immune evasion strategy employed by ICP0, which inhibits IRF-3 signaling by promoting the degradation of IFI16 during infection.

Materials and Methods

Cell culture and viruses. Human foreskin fibroblasts (HFF) were grown in DMEM supplemented with 15% fetal bovine serum (FBS). RAW246.7 macrophage, U2OS, Vero, and FO6 cells were maintained in DMEM supplemented with 5% FBS, 5% bovine calf serum, and glutamine with appropriate selection media as needed.

The wild-type HSV-1 KOS strain virus was propagated and titered by plaque assay on Vero cells (Knipe and Spang, 1982). The *d*106 and *d*109 viruses were propagated on E11 and FO6 cells, respectively, and titered in parallel on FO6 cells (Samaniego et al., 1998). The ICP0-RING finger mutant virus (KOS.RFm) and its corresponding rescued virus (KOS.RFr) were propagated and titered on U2OS cells.

Infections. Virus was diluted in cold phosphate-buffered-saline (PBS) containing 0.1% glucose and 1% heat-inactivated BCS. Cells were infected at the stated MOI for 1 h at 37°C, washed twice with PBS and overlaid with DMEM containing 1% heat inactivated BCS. Infected cells were incubated at 37°C for the indicated length of time.

Drugs. Cells were treated with DMEM containing 5 ng/ml leptomycin B (Sigma-Aldrich) for 30 minutes prior to infection. For proteasome-inhibition studies, cells were treated with 1 μM MG132 (Sigma) or .01% DMSO. TPCK was used at a concentration of 5 μg/ml. Drugs were included throughout the adsorption period as well as in overlay medium.

Cytotoxicity assay. HFF were cultured in a 96-well plate overnight. The following day cells were treated with control or TPCK-containing media for 6 h. The viability of the cells was determined by assaying their metabolic capacity by the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Luminescence was measured by a Spectramax L microplate reader and values were normalized to control-treated cells.

Viral DNA analysis by qPCR. At 2 hours post-infection (hpi) total cellular DNA was harvested using a Qiagen Generation Capture Column Kit. DNA levels of specific sequences were determined by quantitative real-time PCR using the Power SYBR Green PCR master mix and a Prism 7300 sequence detection system (Applied Biosystems). PCR reactions were carried out in duplicate, and relative copy numbers were determined by comparison with standard curves. Viral DNA was normalized to cellular γ-actin (human) or GAPDH (mouse) levels.

Cellular RNA analysis by qPCR. Total RNA was extracted using the Qiagen RNeasy Kit and DNase treated using the DNA-free kit (Ambion). DNase-treated RNA was then reverse-transcribed and quantified by real-time PCR (qPCR) as above. Mock reverse-transcribed samples were included as negative controls. Experiments were conducted three times, and the values were averaged. Samples were normalized to either γ-actin mRNA or 18S rRNA. The Student t test was used to determine the statistical significance of differences between samples.

Nuclear cytoplasmic fractionation. Nuclear and cytoplasmic extracts were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). The purity of nuclear and cytoplasmic extracts was assessed by immunoblotting with anti-lamin A/C (cell signaling) and either anti-tubulin or anti-GAPDH antibodies (Abcam), respectively.

Western blots. Cells were lysed in NuPAGE® LDS Sample Buffer, and proteins were resolved on NuPAGE® 4-12% Bis Tris Gels (Invitrogen). Proteins were transferred to PVDF membranes, and western blots were developed using Luminate Forte Western HRP substrate (Millipore).

Indirect immunofluoresence. HSV-1 infected HFF grown on coverslips were fixed with 2% formaldehyde, permeabilized with 0.5% NP40, and blocked in 5% normal goat serum. Fixed cells were incubated with antibodies for 30 min at 37 °C and washed two times with PBS containing 0.05% Tween 20 followed by one wash with PBS. Alexa Fluor 488- and 594-conjugated secondary antibodies were incubated with cells for 2 hours at 25°C. The coverslips were washed as above and mounted in ProLong Gold antifade reagent (Invitrogen). Images were acquired using an Axioplan 2 microscope (Zeiss) with a 63X objective and Hamamatsu CCD camera (model C4742-95). Images were arranged in figures using Adobe Photoshop CS4 (Adobe Systems, Seattle, WA).

siRNA transfections. Double-stranded *IFI16*-specific, *STING*-specific and nontarget control siRNAs were purchased from Dharmacon. The pooled siRNA were transfected into HFF using the DarmaFECT 2 transfection reagent (Dharmacon) at a final siRNA concentration of 5 nM according to the manufacturer's instructions. At 3 days post-transfection cells were assayed for IFI16 or STING levels by immunoblotting and/or infected with HSV-1.

Flow cytometry. Infected HFF were trypsinized and washed once with ice-cold PBS containing 1 mM sodium orthovanadate. Cells were fixed for 20 minutes with 1.5% formaldeyde and incubated in permeabilization buffer (0.2% saponin, 0.5% bovine serum albumin, 1 mM sodium orthovanadate, 1 mM beta glycerophosphate, 50 mM sodium fluoride) for 15 minutes. Cells were incubated in permeabilization buffer with primary antibody for 1 hour at 25 °C, and then washed and stained with secondary goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (Jackson ImmunoResearch Labs, West Grove, PA) for 1 hour in permeabilization buffer and analyzed by flow cytometry.

Antibodies. Antibodies used in Western blot experiments were mouse anti-IFI16 (ab55328, 1:1000, Abcam), rabbit anti-phosphoTBK1 (Ser 172) (D52C2, 1:1000, Cell Signaling), mouse anti-GAPDH (G041, 1:5000, Applied Biological Materials), rabbit anti-TMEM173 (ab92650, 1:2000, Abcam), mouse anti-ICP0 (1:1000, EastCoast Bio), rabbit anti-Lamin A/C (1:2000, Cell Signaling), mouse anti-β-Tubulin (Clone JDR.3B8, 1:2000,

Sigma-Aldrich), rabbit anti-phosphoIRF3 (Ser396) (4D4G, 1:1000, Cell Signaling), and rabbit anti-p204 (1:500, (Liu et al., 1999). HRP-conjugated goat antibodies were used at 1:10,000 (Santa Cruz Bio-technology)

For the Indirect Immunofluorescence studies mouse anti-IFI16 (ab55328, 1:200, Abcam), rabbit anti-ICP0 (CLU7, 1:150,(Lium et al., 1996)), and mouse anti-IRF3 (SL-12, 1:150, Santa Cruz Bio-technology). Goat anti-mouse Alexa-488 (Jackson ImmunoResearch) and anti-rabbit Alexa 594 (Jackson ImmunoResearch) were used at 1:500 for secondary detection.

Primers.

Name	Use	Primer Sequence
hIFNβ	qRT-PCR	5'-AAACTCATGAGCAGTCTGCA-3' 5'-AGGAGATCTTCAGTTTCGGAGG-3'
hISG56	qRT-PCR	5'-AAGGCAGGCTGTCCGCTTA-3' 5'-TCCTGTCCTTCATCCTGAAGCT-3'
hISG54	qRT-PCR	5'-ACGGTATGCTTGGAACGATTG-3' 5'-AACCCAGAGTGTGGCTGATG-3'
hIL-6	qRT-PCR	5'-ACCGGGAACGAAAGAGAAGC-3' 5'-CTGGGAGTTCCAGGGCTAAG-3'
hγ-actin	qRT-PCR and qPCR	5'CACCGCCGCATCCTCCTCTTC-3' 5'-GTGGTGCCGCCCGACAGC-3'
h18s RNA	qRT-PCR	5'-GCATTCGTATTGCGCCGCTA-3' 5'-AGCTGCCCGGCGGGT-3'
mIFNβ	qRT-PCR	5'-AGCTCCAAGAAAGGACGAACAT-3' 5'-GCCCTGTAGGTGAGGTTGATCT-3'

mISG56	qRT-PCR	5'-TGGCCGTTTCCTACAGTT-3' 5'-TCCTCCAAGCAAAGGACTTC-3'
mISG54	qRT-PCR	5'-ATGAAGACGGTGCTGAATACTAGTGA-3' 5'-TGGTGAGGGCTTTCTTTTTCC-3'
mGAPDH	qRT-PCR	5'-CTGACGTGCCGCCTGGAGAAAC-3' 5'-CCCGGCATCGAAGGTGGAAGAGT-3'
ICP8	qPCR	5′-GCCCGGGCGCTGCTTGTTCTCC-3′ 5'-CGTCCGCCGTCGCAGCCGTATC-3′ (Cliffe and Knipe, 2008)

Results

Induction and inhibition of IRF-3 signaling in human fibroblasts. To investigate the mechanisms by which HSV infection induces IFNβ expression and how virus encoded ICP0 inhibits that process, we defined a system to investigate IRF-3 signaling in human foreskin fibroblasts (HFF) infected with the HSV-1 replication-defective *d*109 and *d*106 viruses. The *d*109 virus contains mutations in the five immediate-early genes *ICP0*, *ICP4*, *ICP22*, *ICP27*, and *ICP47*, effectively blocking all viral gene expression during infection (Samaniego et al., 1998). In contrast, the *d*106 virus expresses ICP0, but no additional immediate-early gene products. Consistent with previous results in human embryonic lung cells (Eidson et al., 2002), we found that HFF infected with *d*109 showed a MOI-dependent induction of the IRF-3 responsive gene *ISG54*, while *d*106 virus infection resulted in much lower levels of expression of this gene (Figure 3.1A). Because HSV-1 mutants that lack ICP0 have been shown to have differing particle to pfu ratios (Everett et al., 2004a), we also determined whether cells were exposed to the same amount of viral DNA during equal MOI infections with *d*109 and *d*106. This was particularly important in light of DNA sensing pathways playing a major role both in detecting HSV-1 and inducing IRF-3 signaling during infection (Conrady et al., 2012; Unterholzner et al., 2010). To test this possibility, we used real-time PCR (qPCR) to quantify the amount of viral DNA associated with cells during infection. Interestingly, infection with *d*109 led to 5-fold more viral DNA being associated with cells at 1 hour post-infection (hpi) (Figure 3.1B). When the amount of infectious virus was adjusted, we observed similar amounts of viral DNA in cells infected with the *d*109 and *d*106 viruses

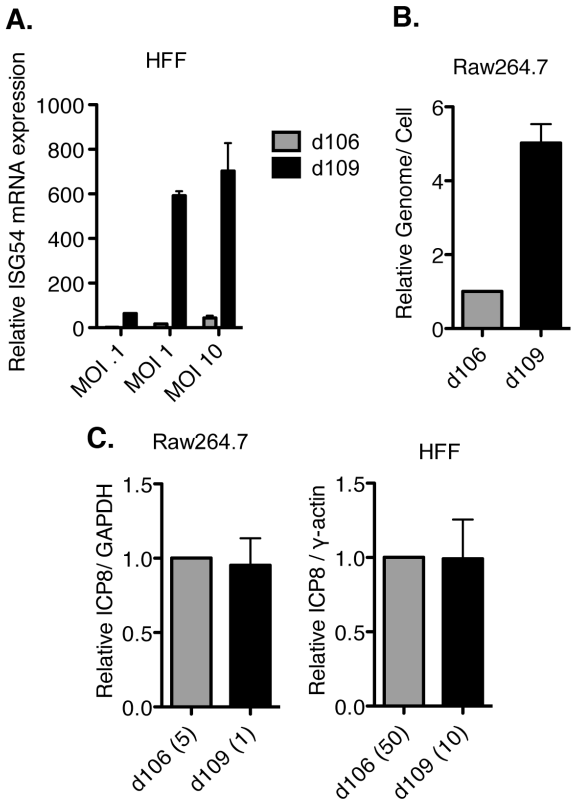

Figure 3.1: Establishment of an HSV-1 infection system to study IRF-3 signaling. (A) Induction of *ISG54*. HFF were infected with increasing MOIs of d106 or d109 virus and total cellular RNA was harvested at 6hpi. *ISG54* mRNA levels were normalized to γ-*actin* levels and further normalized to mock-infected samples. Mean values ± SEM are shown. (B) Viral genome numbers in infected cells. RAW264.7 cells were infected with d106 or d109 at an MOI of 1. Total cellular DNA was harvested at 2 hpi, and relative viral DNA levels were analyzed by qPCR using primers specific for the *ICP8* gene. Levels were normalized to cellular *GAPDH* gene levels. (C) *ICP8* gene levels from RAW264.7 *(Left)* or HFF *(Right)* infected with *d*106 at an MOI of 5 and 50, or *d*109 at an MOI of 1 and 10. Mean values ± SEM are shown (n=3).

(Figure 3.1C). These results suggested that *d*109 has a higher particle-to-pfu ratio than *d*106; therefore, further experiments were conducted with both equal-PFU and equal-genome infections.

To examine the kinetics of IRF-3 responsive gene expression in response to HSV-1 infection, we infected cells with *d*109 (MOI 10) or *d*106 (MOI 10 and 50), and harvested total RNA at 2, 4, and 6 hpi. The relative levels of *IFNβ, ISG56,* and *ISG54* transcripts were measured by qRT-PCR and normalized to *γ-actin* RNA levels. By 4hpi, the *d*109 virus induced measurable amounts of the responsive genes examined (Figure 3.2A-C). In contrast, we observed lower levels of expression of these genes in *d*106 virus-infected cells, confirming that ICP0 was sufficient to inhibit IRF-3 signaling. As a control, ICP0 expression had no effect on levels of expression of the NF-κB-dependent *IL-6* gene (Figure 3.2D). Similar results for the IRF-3 responsive genes were seen in infected RAW246.7 macrophage (Figure 3.3). These results argued that HSV-1 infection induces IRF-3 signaling early during infection and expression of ICP0 rapidly counteracts this pathway. Therefore, these results defined a system to study the mechanism of activation and inhibition of the IRF-3 pathway.

Conditions that block release of HSV DNA from capsids reduce IFNβ and ISG induction. The ability of *d*109 virus to induce IFNβ indicated that HSV-1 infection activates IFNβ prior to immediate-early gene expression. Therefore, to define the mechanism of induction of *IFNβ*, we assessed how *d*109 activates IRF-3 signaling in

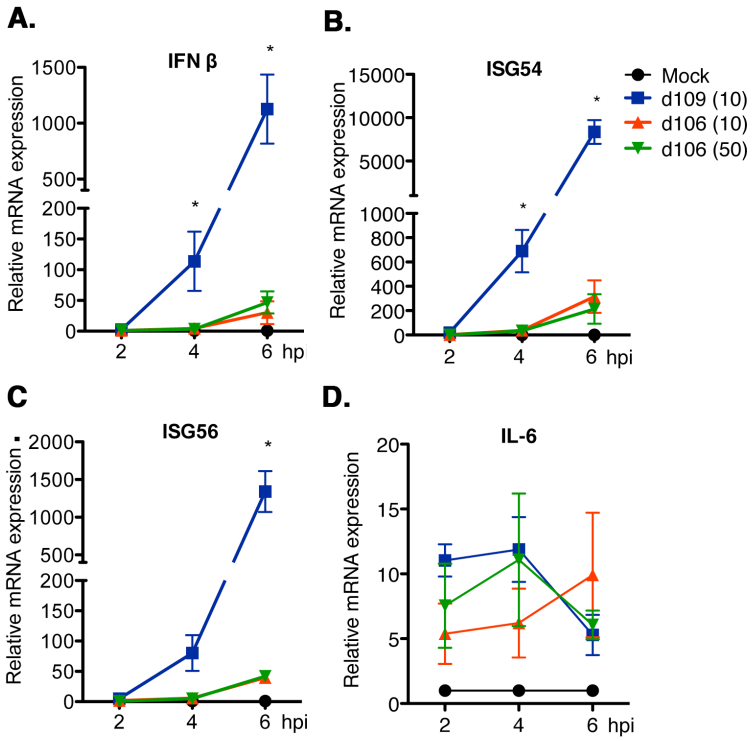

Figure 3.2: HSV-1 induces IRF-3 responsive genes in the absence of viral gene expression. RNA was harvested from HFF infected with *d*106 (MOI 10 or 50) or *d*109 (MOI 10) at 2, 4, and 6 hpi. RNA levels for (A) *IFNβ,* (B) *ISG54,* (C) *ISG56*, and (D) *IL-6* were determined by RT-qPCR. Cellular RNA levels were normalized to *γ-actin* levels and further normalized to control 6 hpi values. Mean values ± SEM are shown (n=4). *$p \leq 0.05$, compared with *d*106-infected cells (Student's *t*-test).

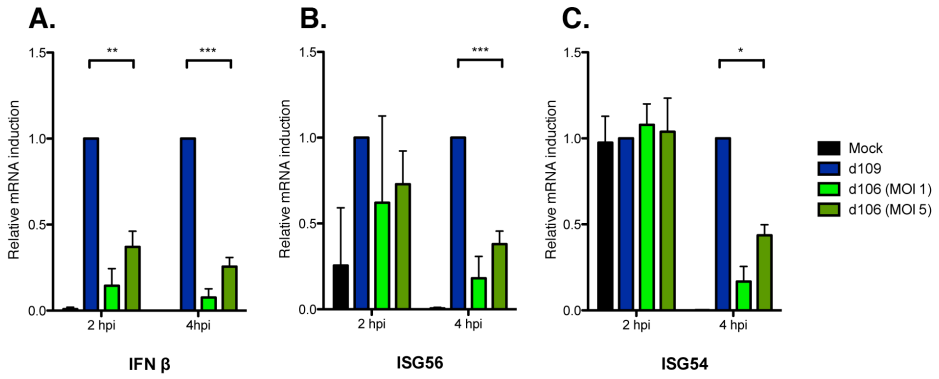

Figure 3.3: HSV-1 induces IRF-3 responsive genes in RAW264.7 cells the absence of viral gene expression. RNA was harvested from cells infected with *d*106 (MOI 1 or 5) or *d*109 (MOI 1) at 2 and 4hpi. RNA levels for (A) *IFNβ,* (B) *ISG56,* and (C) *ISG54* were determined qRT-PCR. Cellular RNA levels were normalized to *GAPDH* and further normalized to *d*109 values at their respective time points. Mean values ± SEM are shown (n=5). *p≤0.05, **p≤0.01,***p ≤0.001, compared with *d*109-infected cells (Student's *t*-test).

HFF. Upon entry, HSV-1 capsids rapidly translocate to the nuclear periphery where they dock at nuclear pores and subsequently release their viral DNA into the nucleus. To investigate whether accumulation of viral DNA in the nucleus was required for IRF-3-responsive gene expression, we examined *IFNβ* expression during infection in the presence of tosyl phenylalanyl chloromethyl ketone (TPCK). This serine/cysteine protease inhibitor inhibits cleavage of VP1-2 during infection and blocks viral DNA release into the nucleus (Jovasevic et al., 2008). HFF treated with TPCK or control medium were infected with *d*109 virus or treated with the TLR3 agonist polyinosinic:polycytidylic acid (poly I:C), total RNA was harvested at 6hpi and analyzed by qRT-PCR. TPCK treatment virtually eliminated the expression of *IFNβ* and *ISG54* in response to *d*109 virus infection compared to control cells (Figure 3.4 A and B) but had a less dramatic effect on the induction of *ISG54* in response to poly I:C (Figure 3.4C). These results argued for a specific effect of TPCK on HSV-induced signaling as opposed to poly I:C signaling. Although cell viability was not affected by TPCK treatment at the concentration used (Figure 3.4D, 5μM), TPCK could have additional effects on cellular processes, including the chymotrypsin-like activity of the proteasome. Therefore, we also treated cells with MG132 to determine the contribution of the proteasome in sensing of *d*109 infection. Interestingly, MG132 treatment had no effect on *ISG54* expression, although it did reduce *IFNβ* somewhat (Figure 3.4 A and B). This differential inhibition by MG132 may be explained by differences in transcription factor recruitment to the *IFNβ* and *ISG54* promoters. Although recruitment of IRF-3 is sufficient to activate the *ISG54* promoter, expression of *IFNβ* requires additional

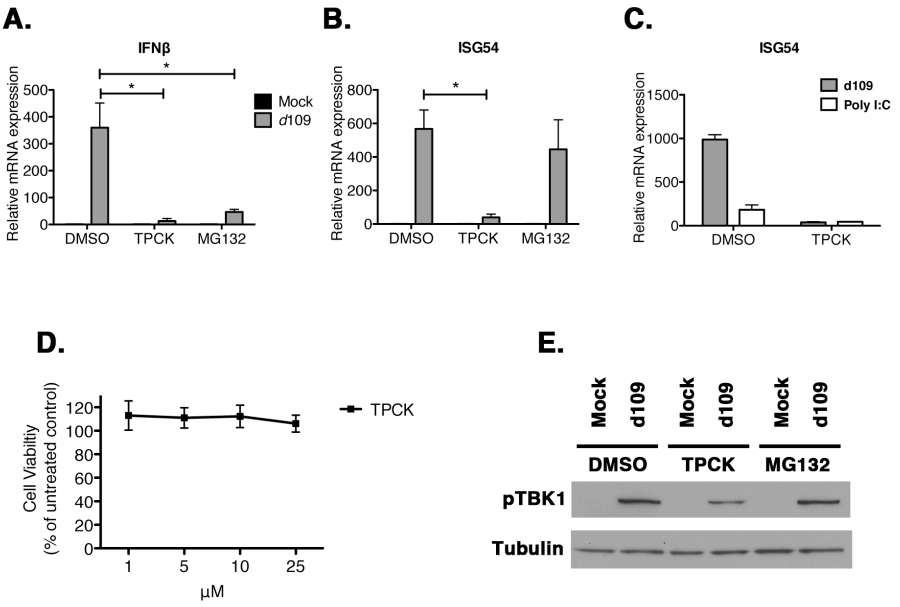

Figure 3.4: Induction of IRF-3 responsive genes requires viral DNA release from incoming capsids. HFF were pretreated with DMSO, TPCK, or MG132 for 30 minutes prior to and throughout infection with *d*109 at an MOI of 10. Cellular RNA was harvested at 6hpi and (A) *ISG54* and (B) *IFNβ* levels were determined by RT-qPCR. RNA levels were normalized to *18s rRNA* followed by normalization to corresponding mock treatment values. (C) HFF were pretreated with DMSO or TPCK for 30 minutes prior to and throughout infection with the *d*109 virus (MOI 10) or treatment with poly I:C (100 μg/ml). Cellular RNA was processed as above. (D) Cell viability assay using CellTiter-Glo Luminescent reagent. Cells were treated with increasing amounts of DMSO or TPCK and cell viability was analyzed at 6 hours post-treatment. (E) Western blot analysis of pTBK1 and Tubulin levels in drug-treated and *d*109-infected cells. Mean values ± SEM are shown (n=3). *$p \leq 0.05$, compared with DMSO-treated cells (Student's *t*-test).

transcription factors, including NF-κB whose activation is dependent on proteasomal degradation of the IκB inhibitor (Karin and Greten, 2005). Consistent with TPCK but not MG132 blocking IRF-3 responsive gene expression, TPCK reduced TBK1 phosphorylation at Ser-172 in *d*109-infected cells, whereas MG132 showed no effect (Figure 2.4E). Phosphorylation of this serine is essential for TBK1 kinase activity and thus the activation of IRF-3 (Kishore et al., 2002). In total, these results argued that the IRF-3 signaling response to HSV-1 infection requires the release of capsid-associated viral DNA into the nucleus and does not require proteasome activity.

IFI16 is necessary for HSV-1 induced IFNβ expression in HFF. The IFI16 DNA sensor and its mouse ortholog p204 have been shown to be necessary for IRF-3 responsive gene expression in response to HSV-1 infection in THP-1 monocytes, RAW264.7 macrophage, and corneal epithelial cells (Conrady et al., 2012; Unterholzner et al., 2010). To test the importance of IFI16 in HSV-1 activation of IRF-3 signaling in HFF, we examined *IFNβ* expression during infection in the absence of IFI16. HFF were transfected with control siRNA (siControl) or siRNA targeting IFI16 (siIFI16) for 3 days (d), followed by infection with *d*109 or SeV for 6 h. Western blot analysis of the resulting protein lysates showed robust knockdown of IFI16 in siIFI16-treated cells compared to cells treated with control siRNA (Figure 3.5A). When mRNA expression was examined in *d*109-infected cells by qRT-PCR, there was a marked reduction in *IFNβ* expression in siIFI16-treated cells compared to control cells (Figure 3.5C). In contrast, knockdown of IFI16 did not have a significant effect on SeV induced *IFNβ* expression

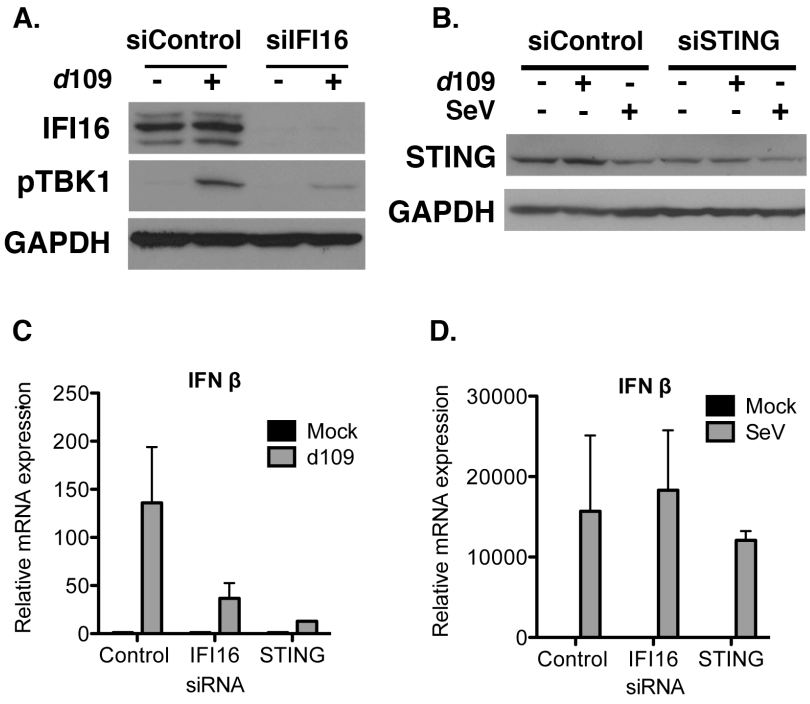

Figure 3.5: IFI16 and STING are required for HSV-1 induced IFNβ induction. HFF were treated with control siRNA (siControl) or siRNA targeting IFI16 (siIFI16) or STING (siSTING) for 3 d followed by infection with *d*109 (MOI 10) or SeV (100 HAU/8x10^5 cells). Cells were harvested for RNA and protein analysis at 6 hpi. (A and B) Western blot of IFI16, pTBK1, STING, and GAPDH levels in siRNA treated cells. (C and D) RNA samples were analyzed by RT-qPCR. *IFNβ* RNA levels were normalized to *18s rRNA* followed by normalization to corresponding mock values. Mean values ± SEM are shown (n=3).

(Figure 3.5D), consistent with previous findings (Unterholzner et al., 2010). Furthermore, phosphorylation of TBK1 at Ser-172 was diminished in *d*109-infected IFI16 knockdown cells relative to cells receiving control siRNA (Figure 3.5A). Together these results were consistent with IFI16 being necessary to activate the IRF-3 signaling cascade during HSV-1 infection.

STING, a cytoplasmic scaffolding protein, is essential for the IFNβ response to immunostimulatory DNA molecules and HSV-1 infection in mouse embryonic fibroblasts (Ishikawa et al., 2009). In addition, an immunostimulatory VACV 70mer has been shown to induce an association between STING and IFI16 in THP-1 cells by co-immunoprecipitation (Unterholzner et al., 2010). To determine whether STING was required for HSV-1 mediated IFNβ response in HFF, we examined *IFNβ* mRNA expression in HFF treated with STING siRNA. At 6 hpi, we observed a marked reduction in IFNβ expression in the absence of STING in *d*109-infected cells (Figure 3.5C). Treatment with STING siRNA had no significant effect on SeV induced IFNβ expression (Figure 3.5D). Interestingly, although STING protein expression was reduced only modestly upon treatment with STING siRNA (Figure 3.5B), its loss had a greater impact on IFNβ expression than IFI16 knockdown in response to *d*109 infection. In addition, we also observed a decrease in STING protein levels in SeV-infected siControl- and siSTING-treated HFF, consistent with the previously observed RNF5-mediated degradation of STING in response to SeV infection (Zhong et al., 2009). Together, these results argued that STING is important for the induction of *IFNβ* in response to HSV-1 infection and may play a role downstream of IFI16 in HFF.

IFI16 is nuclear in uninfected and *d*109-infected HFF cells. The subcellular localization of IFI16 can be either nuclear or cytoplasmic, depending on the cell type [reviewed in (Veeranki and Choubey, 2012)], and it has been reported to be nuclear in HFF (Cristea et al., 2010). We confirmed that IFI16 was nuclear in HFF by immunofluorescence (Figure 3.6A). Nuclear export of IFI16 has been reported during infection of endothelial cells with Kaposi sarcoma-associated herpesvirus (KSHV) and was associated with an induction of inflammasome and NF-κB signaling (Kerur et al., 2011). Interestingly, when we examined IFI16 localization in HFF during infection with *d*109, we did not observe a change in the localization of IFI16; i.e, IFI16 was nuclear in mock- and *d*109-infected cells (Figure 3.6A). Similar results were obtained using the 1G7 IFI16 monoclonal antibody (Santa Cruz) (data not shown), which showed nuclear export of IFI16 in a previous study (Kerur et al., 2011). To confirm this phenotype, we examined IFI16 localization by biochemical fractionation. Nuclear and cytoplasmic fractionation was performed on HFF infected with *d*106 or *d*109 viruses at 4 hpi. Consistent with our immunofluorescence results, IFI16 was completely nuclear in uninfected and *d*109-infected cells (Figure 3.6B).

STING was localized in the cytoplasm of uninfected cells as shown by fractionation, and it showed no change in localization following *d*109 infection (Figure 3.6B). Although in this experiment cells infected with a higher MOI of *d*106 showed a slight increase in nuclear-localized STING (Figure 3.6B), this was not observed in other experiments. The absence of IFI16 in the cytoplasm of *d*109-infected HFF suggested

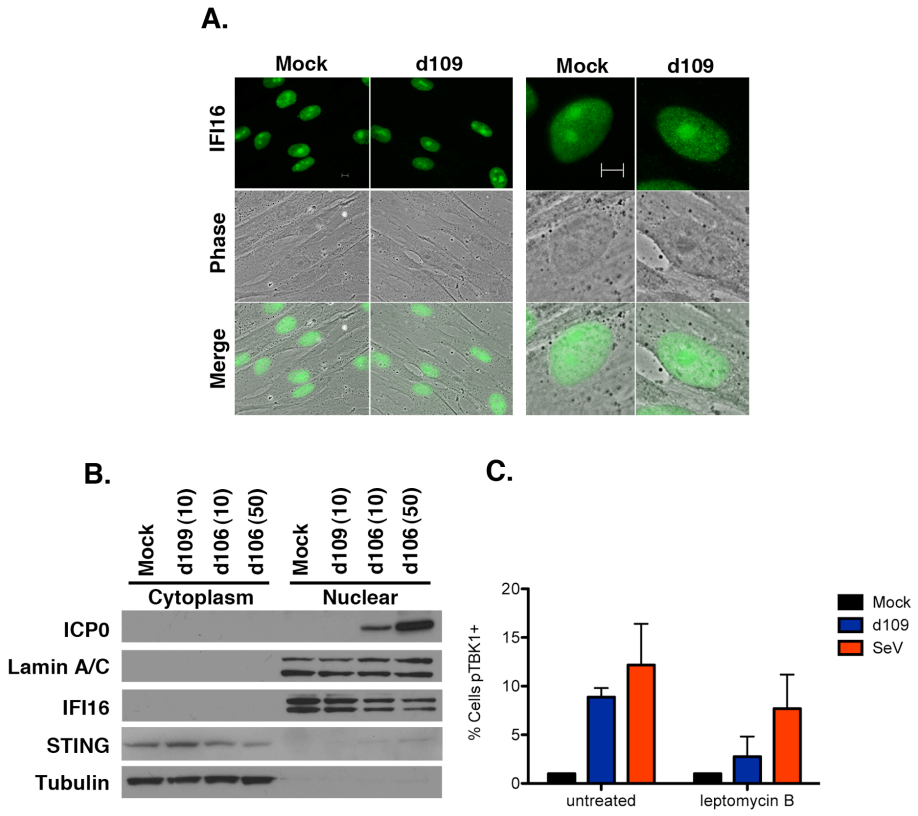

Figure 3.6: IFI16 is localized in the nucleus during HSV-1 infection. (A) Fibroblasts infected with *d*109 virus were fixed and stained with an antibody specific for IFI16 (shown in green) at 4hpi. The white bar represents 5μm. (B) Nuclear and cytoplasmic fractions were prepared from cells infected with *d*109 and *d*106, and analyzed by immunoblot for ICP0, IFI16, and STING localization. Tubulin and Lamin A/C represent the fractionation efficiency. (C) Cells were treated with leptomycin B for 30 minutes prior to and throughout infection with *d*109 or SeV for 8 h. Flow cytometry was then used to examine the phosphorylation status of TBK1. Mean values ± SEM are shown (n=4).

that IFI16 potentiates signaling from the nucleus to the cytoplasm without major changes in its distribution in response to HSV-1. To test whether nuclear export was required for activation of IRF-3 signaling during HSV-1 infection, we examined the phosphorylation status of TBK1 by flow cytometry when leptomycin B was used to inhibit nuclear export of proteins by the CRM1 pathway. HFF were treated with Leptomycin B for 30 min before to infection with HSV-1 *d*109 or, to stimulate through another sensor, infection with SeV. Infected cells were fixed and stained at 8 hpi with a specific antibody raised against phospho-TBK1 ser172. Treatment of HFF with Leptomycin B decreased the percentage of *d*109-infected cells that stained positive for TBK1 phosphorylation by ~70% (Figure 3.6C). In contrast, phospho-TBK1 staining was only slightly decreased in Leptomycin B-treated, SeV-infected cells compared to control-treated cells. These results suggested that signaling in response to HSV-1 infection requires CRM1-mediated export of a factor(s).

Nuclear ICP0 inhibits IRF-3 upstream and downstream of activation. The induction of *IFNβ* in response to HSV-1 infection is dependent on the IRF-3 transcription factor (Menachery and Leib, 2009; Menachery et al., 2010). The viral ICP0 protein has been implicated in inhibiting the IRF-3 pathway; however, the mechanism of inhibition has remained unclear (Eidson et al., 2002; Melroe et al., 2004). To determine the stage at which IRF-3 activation was inhibited by ICP0, we initially examined IRF-3 localization during HSV-1 infection by nuclear/cytoplasmic fractionation. Nuclear and cytoplasmic fractions were prepared from HFF infected with *d*106 or *d*109 at 4, 6, and 8 hpi. The

efficiency of fractionation was confirmed by the complete localization of GAPDH in the cytoplasmic and lamin A/C in the nuclear fractions (Figure 3.7). At 4hpi, equal amounts of activated IRF-3 (pIRF-3 S396) were seen in the nuclei of cells infected with equal-genome amounts of *d*109 (MOI=10) and *d*106 (MOI=50) (Figure 3.7A, lanes 14,16). Cells infected with an equal MOI of *d*106 (MOI=10) showed delayed IRF-3 accumulation, most likely due to decreased viral DNA being present during infection (Figure 3.7A, lane 15). Interestingly, by 6hpi pIRF-3 S396 had continued to accumulate in the nuclei of cells infected with *d*109, but was at very reduced levels in cells expressing ICP0 (*d*106). Additional experiments in RAW264.7 cells revealed a similar difference in *d*109 and *d*106 infected cells at 2 and 4hpi (Figure 2.7B). Because differences in *ISG54* expression were observed in HFF by 4hpi (Figure 2.2B), the results taken together suggest that ICP0 is capable of blocking IRF-3 activity at early times in the nucleus as well as upstream of IRF-3 activation at later stages of infection.

Previously, we have shown that ICP0 expression causes the relocalization of SeV activated IRF-3 in the nuclei of infected cells (Melroe et al., 2007). We therefore hypothesized that ICP0 sequesters IRF-3 from its normal nuclear activity. To determine whether this sequestration model applied to the situation at 4hpi in infected HFF, we examined IRF-3 localization by indirect immunofluorescence. At 6hpi, we fixed *d*109- and *d*106-infected HFF and stained them with antibodies specific for IRF-3 and ICP0. The IRF-3 antibody used in this experiment (SL-12) preferentially binds to activated IRF-3 (Melroe et al., 2004); therefore, we detected diffusely nuclear IRF-3 in *d*109-infected cells (Figure 3.8e). In contrast, cells infected with *d*106 showed a relocalization of IRF-3

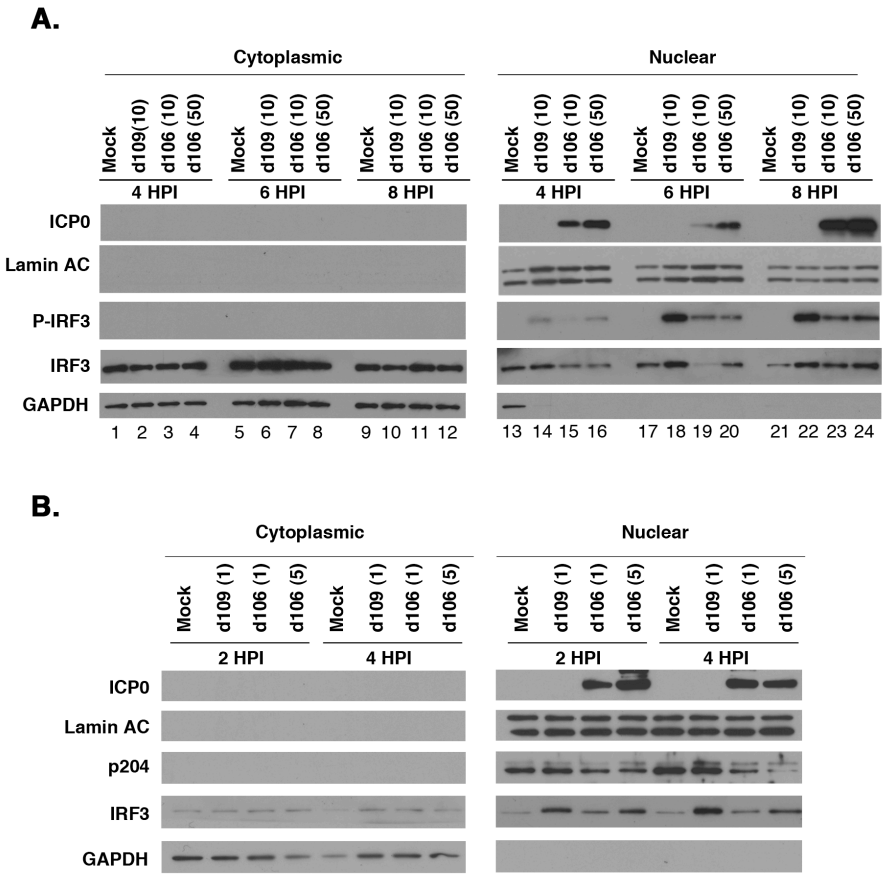

Figure 3.7: ICP0 inhibits nuclear accumulation of activated IRF-3. HFF (A) or RAW264.7 (B) cells were mock-infected or infected with the *d*109 or *d*106 viruses. Nuclear and cytoplasmic fractions were prepared at 2, 4, 6, and 8 hpi. Fractions were probed using antibodies specific for ICP0, IRF-3 and phospho-IRF-3 (Ser396). Fractionation efficiency was determined by localization of GAPDH (cytoplasm) and Lamin A/C (nucleus).

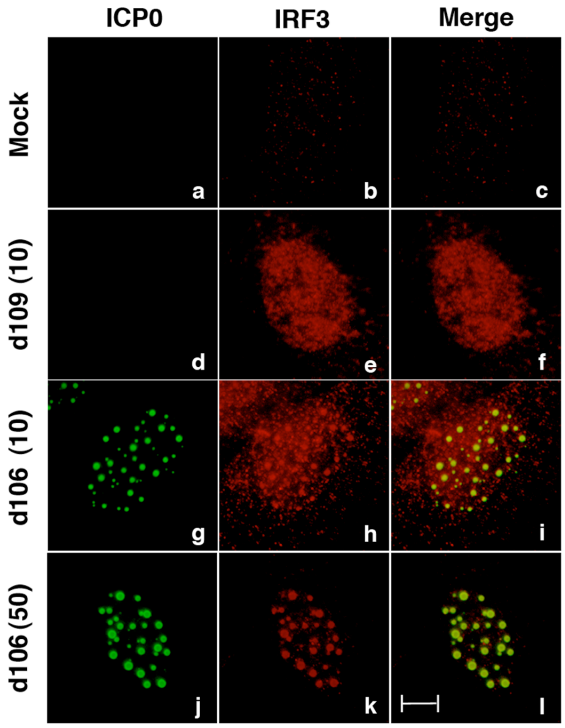

Figure 3.8: ICP0 sequesters nuclear localized IRF-3. HFF were infected with *d*109 or *d*106 viruses for 6 hpi. Samples were fixed and stained using antibodies specific for ICP0 (shown in green) and IRF-3 (shown in red). White bar represents 5μm.

to ICP0 foci (Figure 3.8, i and l). These results argued that nuclear ICP0 inhibits *IFNβ* expression early during infection by sequestering IRF-3 in the nucleus of infected HFF.

Effects of ICP0 expression on IFI16. In WT HSV-1 infection, ICP0 is localized to the nucleus initially but accumulates in the cytoplasm at late time points post-infection (Kawaguchi et al., 1997). This change in localization requires viral DNA replication and late gene expression (Lopez et al., 2001). Our nuclear fractionation and immunofluoresence studies argued that ICP0 expressed by *d*106 is mainly if not entirely nuclear (Figure 3.6 and 3.7), consistent with the replication-incompetent nature of the *d*106 virus. Given the nuclear localization of ICP0 and its apparent effect upstream of IRF-3 activation, we tested whether ICP0 expression affects nuclear IFI16. We infected HFF with *d*106 or *d*109, prepared protein lysates at 6 hpi, and examined them by Western blot analysis. Notably, cells infected with *d*106 at both an MOI of 10 and 50 (lanes 3 and 4) showed a marked reduction in the steady state levels of IFI16 compared to *d*109 virus- (lane 2) or mock-infected (lane 1) cells (Figure 3.9A). Interestingly, *d*106 infection in RAW264.7 cells also promoted the loss of p204, the murine IFI16 ortholog (Figure 3.7B, lanes 15 and 16). In addition, we examined IFI16 localization in *d*106- and *d*109-infected cells by immunofluorescence at 2, 4, and 6 hpi. At 2 hpi, IFI16 partially localized with nuclear ICP0 foci and subsequently was lost from cells by 4 and 6 hpi (Figure 3.9B). Together these results suggested that ICP0 expression during HSV-1 infection promotes the relocalization and subsequent degradation of IFI16.

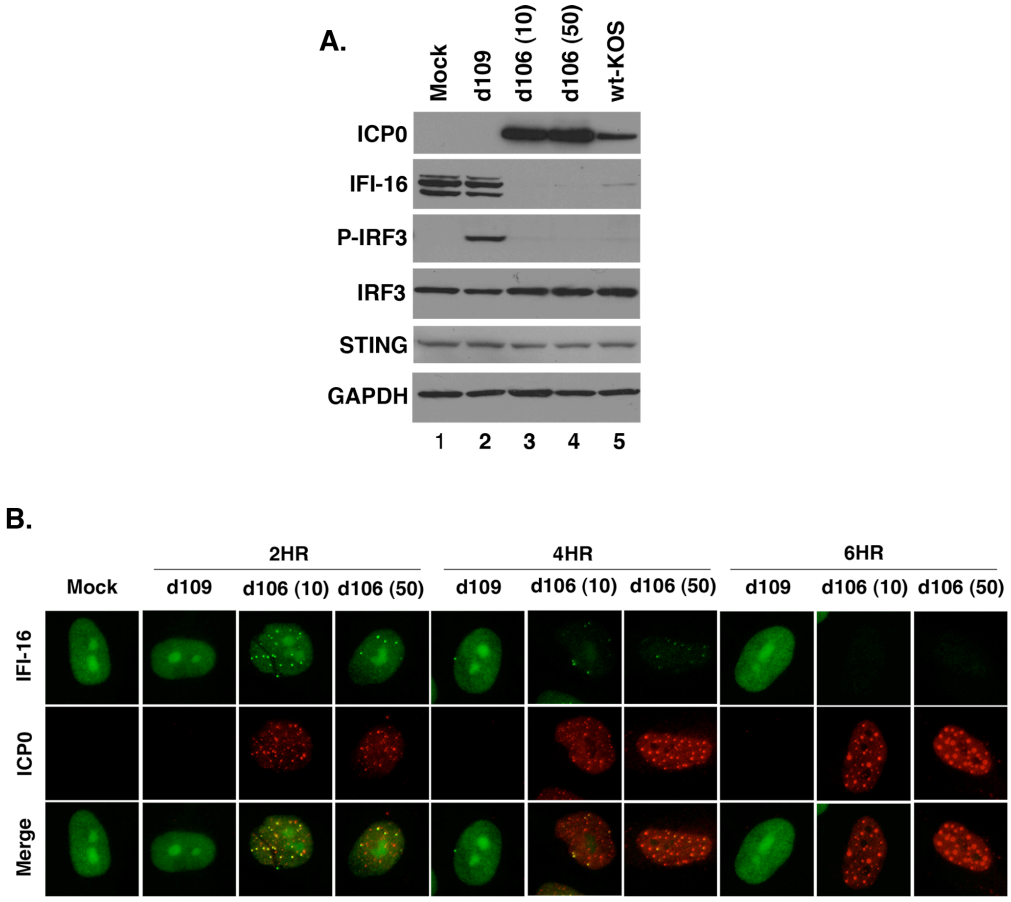

Figure 3.9: ICP0 expression promotes IFI16 relocalization and degradation. HFF were infected with *d*109, *d*106, or wt-KOS virus. (A) Whole cell lysates were harvested at 6 hpi and probed for ICP0, IFI16, IRF-3, phospho-IRF-3 (Ser396), STING, and GAPDH using specific antibodies. (B) Infected cells were fixed and stained at 2, 4, and 6 hpi for IFI16 (shown in green) and ICP0 (shown in red).

HSV-1 promotes degradation of IFI16 via an ICP0 RING finger-dependent mechanism. Previous studies have shown that ICP0 promotes the degradation of cellular proteins in a proteasome-dependent manner through the E3 ligase activity of its RING finger domain (Boutell et al., 2002). This domain coordinates the ubiquitination of specific target proteins and defines ICP0 as an E3 ubiquitin ligase. To test the involvement of the ubiquitin-proteasome pathway in the loss of IFI16, we examined IFI16 protein levels in the presence of the proteasome inhibitor MG132. We treated HFF with MG132 or DMSO for 30 minutes before infection with wt HSV-1 KOS strain, and drug treatment was continued throughout infection. At 8 hpi we harvested cell lysates and probed for IFI16 expression. Treatment with MG132 inhibited the loss of IFI16 observed during infection (Figure 3.10A). To test whether loss of IFI16 in our system was dependent on RING finger domain activity, we constructed an ICP0 RING finger mutant virus by mutational alteration of cysteine residues 116 and 156 to alanine and glycine, respectively. These mutations abolish ICP0 ligase activity by disrupting zinc coordination (Lium and Silverstein, 1997; Vanni et al., 2012). The RING finger mutant virus, KOS.RFm, was incapable of degrading USP7 (Boutell et al., 2005) as compared to the rescued virus, KOS.RFr, but KOS.RFm ICP0 localized normally in the nucleus of infected cells. We harvested cell lysates from KOS.RFm- or KOS.RFr-infected cells at 8 hpi and probed for ICP0 expression and IFI16 levels. We found that while KOS.RFm expressed similar levels of ICP0 as compared to KOS.RFr, the virus was unable to promote the degradation of IFI16 (Figure 3.10B). Based on these results

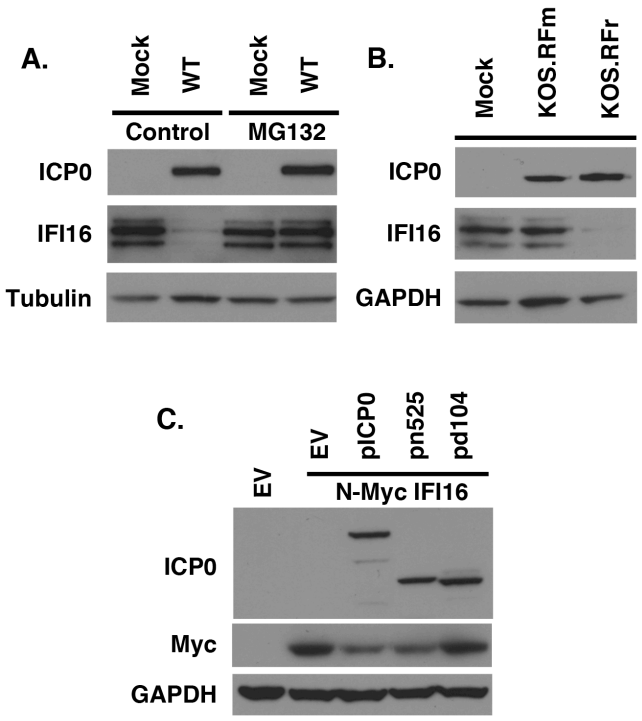

Figure 3.10: ICP0 promotes the degradation of IFI16 in a proteasome- and RING finger-dependent manner. (A) HFF were pretreated with DMSO or MG132 for 30 minutes prior to and throughout infection with wt-HSV (KOS) virus at an MOI of 10. Whole cell lysates were harvested at 8 hpi and ICP0. (B) Cells were infected with the ICP0 RING-finger mutant virus (KOS.RFm) and its Rescue (KOS.RFr). Whole cell lysates were harvested and analyzed as in (A). (C) HeLa cells were transfected with indicated plasmids for 24hpi and whole cell lysates were analyzed as in (A).

we concluded that ICP0 promotes the degradation of IFI16 in a proteasome- and RING finger domain-dependent manner.

We next examined whether additional domains of ICP0 are necessary for the observed degradation of IFI16. HeLa cells were co-transfected with a Myc-tagged IFI16 expression plasmid and either wt-ICP0 (pICP0), an ICP0 construct deleted for amino acids 1-104 (pd104) or an ICP0 nonsense mutant (pn525) that lacks amino acids 525-775 (Figure 3.10C). Both wt-ICP0 and pn525 promoted the degradation of IFI16, indicating that while ICP0 expression is sufficient to promote the loss of IFI16 the C-terminus of ICP0 is not necessary for this activity. Interestingly, the N-terminus of ICP0 appeared to be necessary for the degradation of IFI16, as we did not observe a loss of IFI16 in pd104-transfected cells.

Discussion

Sensing of microbial macromolecules by innate immune mechanisms has been demonstrated to take place at the cell plasma membrane, in internal vesicles, and in the cytoplasm, but there has been little clear evidence that innate sensing takes place in the cell nucleus. Furthermore, sensors of viral or bacterial DNA have been considered to be cytosolic in origin (Sharma and Fitzgerald, 2011). In this study, we demonstrate that HSV-1 DNA must be delivered to the cell nucleus for sensing by the nuclear IFI16 sensor and that, although IFI16 appears to remain in the nucleus after viral DNA recognition, signaling takes place by export of an unidentified molecule to activate IRF-3 through STING in the cytoplasm. The viral ICP0 protein in its nuclear form can re-localize IFI16 within the nucleus and promote its degradation, thereby blocking further signaling. The observations that viral DNA is delivered to the nucleus, that the sensor is nuclear, and that an inhibitor is nuclear strongly support the hypothesis that sensing of HSV-1 DNA occurs in the nucleus in human foreskin fibroblasts.

Nuclear Sensing of HSV-1 DNA. IFI16 has been defined as being involved in IFNβ and CXCL10 induction in HSV-infected THP-1 cells (Unterholzner et al., 2010) and epithelial cells (Conrady et al., 2012), respectively. Originally described as a cytosolic DNA sensor (Unterholzner et al., 2010), IFI16 was recently implicated in sensing Kaposi sarcoma-associated herpesvirus (KSHV) in the nucleus of infected cells (Kerur et al., 2011). The latter study found that IFI16 colocalized with nuclear KSHV genomes and was subsequently translocated to the cytoplasm where it induced inflammasome and

NF-κB signaling. We found that IFI16, which is nuclear in HFF, is involved in the induction of an IFN response to HSV-1 in this cell line, because knockdown of IFI16 decreased *IFNβ* expression during infection with the *d*109 virus. Endosomally localized TLR9 was the first sensor identified to recognize HSV-1 DNA (Lund et al., 2003); however, its expression is restricted to plasmacytoid dendritic cells and B cells in humans and thus is unlikely to be a sensor of HSV-1 in HFF. Indeed, Rasmussen et al previously reported that conventional dendritic cells, macrophage and mouse embryonic fibroblasts infected with HSV-1 could secrete IFNβ in a TLR9-independent but viral DNA-dependent manner (Rasmussen et al., 2007). Additional cytosolic sensors have been identified which recognize HSV-1 DNA; however, it has remained unclear how this viral DNA gains access to the cytosolic compartment during infection, given that the capsid likely protects viral DNA from cytosolic sensors during transport through the cytoplasm. Some have proposed that viral DNA may be made available to the cytosolic compartment by the degradation of viral capsid (Paludan et al., 2011). Our experiments revealed that chemical inhibition of the proteasome in HFF had no effect on *ISG54* expression in response to HSV-1. Instead, blocking the release of viral DNA into the nucleus of infected cells by TPCK treatment (Jovasevic et al., 2008) greatly diminished the cellular response to HSV-1 infection. These results are consistent with a need to deliver the viral DNA to the nucleus for IFNβ induction in HFF. Other cell types, however, may have means for freeing viral DNA in the cytoplasm or endosomes so that other receptors can initiate innate responses.

The sensing of viral DNA in the nucleus raises the question of how IFI16 distinguishes between nuclear viral and cellular DNA. The issue of specificity was one of the reasons that the compartmentalized cytosolic and endosomal sensing of DNA was attractive. IFI16 is known to bind to both single- and double-stranded DNA *in vitro* (Unterholzner et al., 2010); thus, it should be able to bind to cellular or viral DNA. IFI16 may bind preferentially to the under-chromatinized HSV DNA, DNA ends, or nicks and gaps in HSV DNA. HSV DNA is known to be rapidly chromatinized upon entry into the cell nucleus (Cliffe and Knipe, 2008; Oh and Fraser, 2008) although the histone association with HSV DNA is less densely packed (Cliffe and Knipe, 2008) and looser (Lacasse and Schang, 2010) than cellular chromatin. The altered chromatin structure may allow IFI16 binding and activation of the signaling pathway. IFI16 has been implicated in the DNA damage response by its interactions with BRCA1 and p53 (Aglipay et al., 2003; Johnstone et al., 2000). HSV infection is known to activate DNA damage response pathways (Lilley et al., 2005; Shirata et al., 2005; Wilkinson and Weller, 2004); therefore, the free ends of HSV DNA or nicks and gaps could provide DNA binding sites for IFI16 to activate the IRF-3 signaling pathway as well as potentially other signaling cascades. Currently there are conflicting reports as to whether DNA damage itself induces IFN-β expression. A recent study in primary human monocytes suggested that IFN-α and -γ, but not -β, were induced in response to the DNA damaging agent etopiside (Brzostek-Racine et al., 2011). However, additional reports have shown that treatment of young human diploid fibroblasts with bleomycin caused an increase in the expression of IFI16 (Duan et al., 2011), and irradiation of bone marrow-derived

mouse macrophages increased ISG expression in an IFNβ-dependent manner (Mboko et al., 2012). Interestingly, no detectable double-stranded break (DSB) response has been detected in UV-inactivated HSV-1 infected cells (Shirata et al., 2005), which would presumably mimic *d*109 infection in our system. However, current methods of detecting DSB may be insufficient to detect an initial DNA damage response from nonreplicating viral DNA. Therefore, further studies are needed to understand the relationship between IFI16 sensing of viral DNA and the DNA damage response.

As we were submitting this manuscript**,** Li et al reported results showing that IFI16 must be nuclear to sense HSV-1 infection (Li et al., 2012). In this study, the authors showed that a HEK293 cell line expressing IFI16 that lacked a functional nuclear localization signal was unable to induce *IFNβ* expression in response to HSV-1 compared to WT-IFI16 expressing cells. Our results are consistent with and complementary to their results and expand on the mechanisms of activation and inhibition of IFI16 sensing of HSV-1 infection.

Role for nuclear IFI16 in HSV-1 induced interferon expression. It is currently unclear how nuclear IFI16 induces the cytoplasmic IRF-3 signaling cascade in HFF. Previously, cytosolic IFI16 was shown to associate with STING upon activation with immunostimulatory DNA (Unterholzner et al., 2010). In our system, STING knockdown reduced the expression of IFN in response to HSV-1 infection, suggesting the involvement of this protein in the activation of IRF-3 signaling in HFF. However, we do not observe a measurable relocalization of IFI16 from the nucleus to the cytoplasm

during infection. Additional experiments using the CRM1 inhibitor leptomycin B revealed that a nuclear export event is required for the autophosphorylation of the TBK1 kinase, arguing that additional factors may translocate to the cytoplasm to initiate signaling upon IFI16 activation. The factor(s) that link nuclear IFI16 to cytosolic STING are currently unknown and represent a high priority for future studies.

Inhibition of IRF-3 signaling by nuclear ICP0. In this study we found that HSV ICP0 can inhibit the IRF-3 pathway through degradation of IFI16. HSV-1 infection has long been known to antagonize the IRF-3 signaling pathway, and the expression of ICP0 plays a major role in inhibiting this cellular response (Eidson et al., 2002; Melroe et al., 2004; Melroe et al., 2007). Our previous studies have shown that ICP0 can block RIG-I induced IRF-3 signaling in Sendai virus infected cells by sequestering nuclear IRF-3 and reducing its levels (Melroe et al., 2004; Melroe et al., 2007). In this study we have examined the mechanisms of ICP0 inhibition of IRF-3 activation in HSV-infected cells. In HFF and RAW246.7 macrophages, we observed that ICP0 inhibits the IRF-3 signaling pathway at two distinct steps. Nuclear/cytoplasmic fractionation and analysis of IFNβ and ISG expression revealed that ICP0 initially inhibits type I IFN expression at a stage after nuclear accumulation of phosphorylated IRF-3. This early inhibition is associated with a relocalization of IRF-3 to ICP0 nuclear foci and is consistent with the sequestration of IRF-3 in the SeV co-infection system. At later times after infection ICP0 inhibits the accumulation of IRF-3 in the nucleus coincident with the loss of IFI16.

ICP0 is an E3 ubiquitin ligase and promotes the degradation of cellular proteins to enhance virus replication and inhibit host innate responses (Boutell et al., 2002; Cai et al., 1993). Others have observed that the ability of ICP0 to inhibit IRF-3 signaling is at least partially dependent on functional proteasomes (Eidson et al., 2002; Paladino et al., 2010); however, no cellular target for degradation has been identified. In addition to sequestering IRF-3 in the nucleus, we observed that ICP0 promotes the degradation of the IFI16 DNA sensor in a proteasome- and RING finger-dependent manner. ICP0 is associated with the degradation of a number of cellular proteins, but only a limited number of these have been shown to directly interact with ICP0 and/or be directly ubiquitinated in a reaction involving ICP0 [reviewed in (Roizman et al., 2013)]. Therefore, it will be important to determine the mechanism by which ICP0 promotes degradation of IFI16. Most importantly, this study provides a mechanism by which a virus can inhibit nuclear IFI16 from activating IRF-3 signaling.

ICP0 may also inhibit IRF-3 signaling in the cytoplasm. At early times post-infection ICP0 is localized to the nucleus, whereas upon viral DNA replication the protein accumulates in the cytoplasm. A recent study showed that an ICP0 mutant protein without a NLS could inhibit signaling in human embryonic lung fibroblasts (Paladino et al., 2010). It is conceivable that ICP0 can affect IRF-3 activation in the cytoplasm when it accumulates there. Our results do not rule out additional effects of ICP0 in the cytoplasm of infected cells; however, our results argue strongly for the ability of nuclear localized ICP0 to inhibit IRF-3 signaling early during infection.

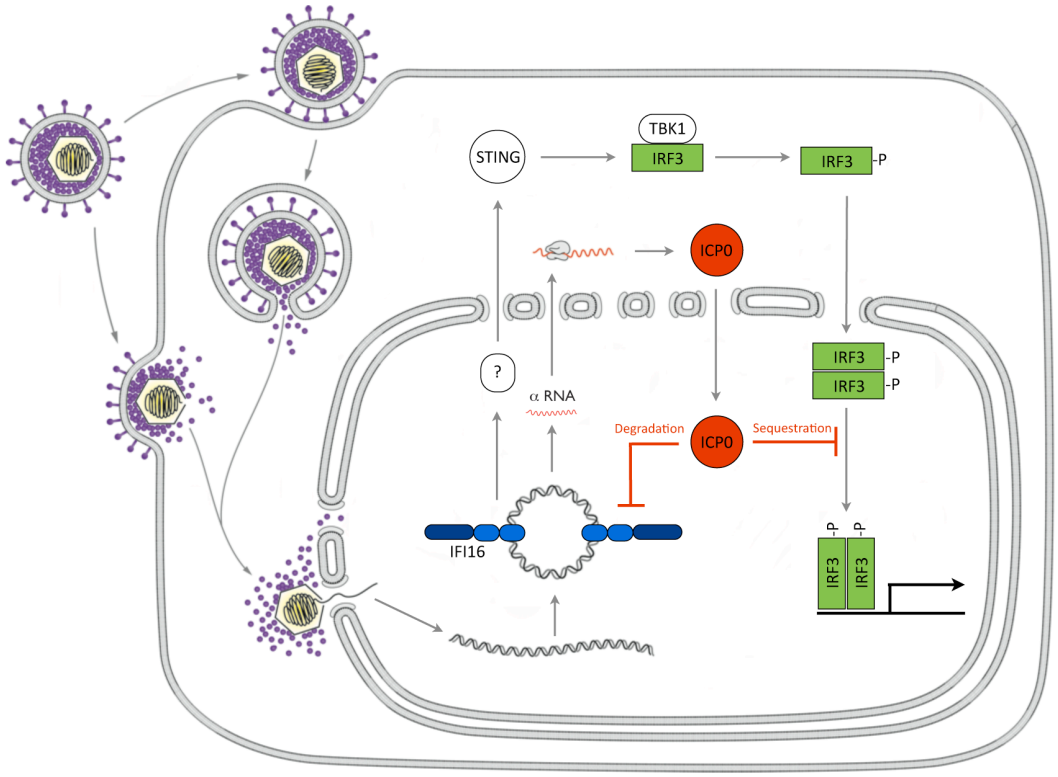

Figure 3.11: Model of nuclear HSV-1 DNA sensing and inhibition by ICP0. HSV-1 fusion at the plasma membrane or via endosomal compartments deposits viral capsids in the cytoplasm. Capsids traffic to nuclear pores where viral DNA is released into the nucleus. Nuclear IFI16 senses accumulating viral DNA inducing a nuclear-to-cytoplasmic signaling cascade activating IRF-3, which dimerizes and translocates to the nucleus. Immediate-early expression of ICP0 sequesters IRF-3 from cellular promoters and promotes degradation of IFI16 to inhibit IFNβ expression.

Based on the studies described here, we propose the following model for the activation and ICP0-mediated inhibition of type I interferon expression during HSV-1 infection (Figure 3.11). In HFF, HSV-1 infection is sensed initially by IFI16 upon the release of viral DNA into the nucleus. A nuclear-to-cytoplasmic signaling cascade is initiated that activates IRF-3 and induces its accumulation in the nucleus. ICP0 expressed at early times during infection sequesters this nuclear IRF-3 from cellular promoters and blocks type I IFN expression. In addition, ICP0 targets IFI16 for degradation, inhibiting additional signaling and activation of IRF-3. Determination of the mechanism of specific detection of viral DNA in the nucleus within the same cellular compartment as cellular DNA will likely shed light on basic cellular mechanisms for detection of "foreign" or altered DNA within the cell. HSV DNA activates DNA damage response pathways, and IFI16 has been implicated in the DNA damage response; thus, these results raise the possibility that the IFN response to viral DNA and the DNA damage response pathways share sensing or signaling components.

Chapter Four: Interferon inducible protein 16 acts as an intrinsic resistance factor to silence HSV-1 and transfected DNA gene expression

Abstract

The intrinsic cellular resistance to infection by large DNA viruses involves the silencing of viral DNA in the nucleus. During herpes simplex virus 1 (HSV-1) infection the ICP0 immediate-early protein counteracts this cellular response in part by disrupting ND10 bodies. However, ND10 components do not account for the total restriction observed in the absence of ICP0, suggesting that additional unidentified mechanisms contribute to silencing the viral genome. In this study we identify the nuclear IFI16 DNA sensor as an intrinsic factor involved in repression of foreign DNA. Knockdown of IFI16 enhanced the replication and immediate-early gene expression of an ICP0-null virus. This phenotype was independent of downstream STING signaling, and knockdown of IFI16 did not affect ND10 accumulation at viral genomes. Furthermore, overexpression of exogenous IFI16 in the permissive U2OS cell line restricted ICP0-null virus gene expression. The repressive activity of IFI16 was not limited to viral DNA as the expression of transfected DNA was also inhibited by IFI16. Together these results argue that in addition to its involvement as an innate pattern recognition receptor, IFI16 mediates an intrinsic immune response to foreign DNA by silencing its expression.

Introduction

Classically, the host mechanisms blocking viral infection have been divided into two distinct arms of host immunity: the innate and adaptive immune responses. However, a third aspect of host immunity has recently been described and termed intrinsic immunity or intrinsic antiviral resistance (Bieniasz, 2004). One major difference between these three host responses is the constitutive expression of resistance mechanisms. Both innate and intrinsic immunity act at the primary site of infection and mediate an initial cell-based immune response. However, while innate immunity requires *de novo* cellular gene expression to mediate its antiviral effects (e.g., induction of type I interferons), intrinsic resistance acts immediately to counteract viral infection through constitutively expressed proteins, known as intrinsic resistance factors. These two cell-based responses are closely linked, however, as many resistance factors are upregulated by type I interferons induced by the innate immune response.

The first intrinsic resistance factors to be characterized were identified as mediators of antiretroviral resistance. For instance, TRIM5α inhibits retroviral infection by modulating the capsid uncoating process (Wolf and Goff, 2008), while APOBEC3G is incorporated into newly synthesized retrovirus capsids and induces the hypermutation of reverse transcribing RNA (Bieniasz, 2004). In addition, tetherin/Bst-2 inhibits viral release by tethering viral particles to the cell surface (Perez-Caballero et al., 2009). The mechanisms employed by these retroviral restriction factors are closely linked to the lifecycle of the viruses they target, and as such are thought to have developed during a long-term co-evolution of these viruses with their respective hosts (Duggal and

Emerman, 2012). Interestingly, members of the *Herpesviridae* family, including herpes simplex viruses (HSV), have also co-evolved with their hosts, indicated by their high seroprevalence but modest pathogenicity. However, compared to antiretroviral intrinsic resistance, little is known about intrinsic resistance factors that target these large DNA viruses.

The most well characterized intrinsic immune response to herpesvirus infection involves the action of promyelocytic leukemia protein nuclear bodies (PML NB), also known as nuclear domain 10 (ND10) bodies. These dynamic subnuclear domains are made up of a variety of cellular proteins, and have been implicated in several cellular responses, including gene expression, DNA damage, apoptosis, and aging (Lallemand-Breitenbach and de The, 2010). During HSV-1 infection, ND10 components accumulate *de novo* in the nucleus at sites near incoming viral DNA, and this is associated with their ability to restrict viral gene expression (Everett and Murray, 2005). HSV-1 overcomes this restriction through expression of the viral ICP0 immediate-early protein, an E3 ubiquitin ligase that disrupts ND10 by promoting degradation of the associated PML and Sp100 proteins (Chelbi-Alix and de The, 1999; Everett et al., 1998; Muller and Dejean, 1999). The importance of counteracting this intrinsic response is documented by reports that ICP0-null viruses are significantly attenuated for viral replication, particularly in primary human fibroblasts (Everett et al., 2004a; Stow and Stow, 1986). However, depletion of ND10 by simultaneous knockdown of the three major ND10 components, PML, Sp100 and hDAXX does not completely rescue the replication of an ICP0-null

virus, indicating additional mechanisms are involved in the intrinsic resistance to HSV-1(Glass and Everett, 2012).

Recently it was reported that the interferon-inducible protein 16 (IFI16) DNA sensor restricts human cytomegalovirus (HCMV) replication in human fibroblasts (Gariano et al., 2012). While IFI16 is known to promote IRF-3 signaling in response to herpesvirus infection (Unterholzner et al., 2010), the reported IFI16-dependent restriction of HCMV was independent of IFNβ, indicating IFI16 may act as an intrinsic resistance factor in addition to categorization as an innate pattern recognition receptor. Furthermore, our recent identification of IFI16 as a target of ICP0-mediated degradation (Orzalli et al., 2012) prompted us to evaluate IFI16's activity as an intrinsic resistance factor to HSV infection. Here we report that IFI16 restricts HSV-1 replication in the absence of ICP0 and this phenotype is independent of STING and ND10-mediated intrinsic resistance. In addition, overexpression of a functional IFI16 in permissive U2OS cells confers resistance to an ICP0-null virus. Furthermore, we determined that IFI16 acts by silencing the expression of both viral and transfected DNA.

Materials and Methods

Cell culture and viruses. Human foreskin fibroblasts (HFF) and U2OS cells were obtained from American Type Culture Collection (ATCC). HFF were grown in DMEM supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2mM L- glutamine, streptomycin, and penicillin (15% DMEM). U2OS cells were grown in DMEM supplemented with 5% heat-inactivated FBS and 5% heat-inactivated bovine calf serum (BCS). The ICP0-null (7134) and rescued 7134R viruses were grown and titred in parallel on U2OS cells (Cai and Schaffer, 1989).

Virus infections. Virus was diluted in cold phosphate-buffered-saline (PBS) containing 0.1% glucose and 1% heat-inactivated BCS. Cells were infected at the stated MOI for 1 h at 37°C, washed twice with PBS and overlaid with DMEM containing 1% heat-inactivated BCS. Infected cells were incubated at 37°C for the indicated length of time.

siRNA transfections. Double-stranded *IFI16*-specific, *STING*-specific and nontarget control siRNAs were purchased from Dharmacon. The pooled siRNA were transfected into HFF using the DarmaFECT 2 transfection reagent (Dharmacon) at a final siRNA concentration of 5 nM according to the manufacturer's instructions. The siRNA containing media was replaced 24 hours post-transfection, and cells were assayed for IFI16 or STING levels by immunoblotting and/or infected with HSV-1 at 72 hours post-transfection (hpt).

Plasmids and DNA transfection. The N-Myc IFI16 plasmid and empty vector backbone were kindly provided by S. Conwell (Conwell and Knipe, unpublished data). U2OS cells were plated at a density to ensure 50% confluency on the day of transfection. Cells were transfected with 0.5 μg of empty vector or N-Myc IFI16 plasmid using the Effectene transfection reagent (Qiagen) and were infected with the indicated viruses at 48 hpt.

HFF were transfected with either 0.5 μg of an empty vector plasmid, pEGFP-C1 (Clonetech), or pEF1-GFP (provided by C. Cepko, Harvard Medical School) using the Lipofectamine LTX reagent (Invitrogen) at 48 hours post-siRNA treatment. Transfection media was replaced at 6 hpt with 15% DMEM and whole cell lysates were harvested and processed for flow cytometry or western blot at 36 hpt.

Cellular RNA analysis by qPCR. Total RNA was extracted using the Qiagen RNeasy Kit and DNase treated using the DNA-free kit (Ambion). Equal amounts of DNase-treated RNA was then reverse-transcribed and quantified by real-time PCR (qPCR) using the Power SYBR Green PCR master mix and a Prism 7300 sequence detection system (Applied Biosystems). PCR reactions were carried out in duplicate, and relative copy numbers were determined by comparison with standard curves. Mock reverse-transcribed samples were included as negative controls. Transcript levels were normalized to *18S rRNA* and made relative to mock-infected samples. Experiments were conducted three times, and the values were averaged.

Western blots. Cells were lysed in NuPAGE® LDS Sample Buffer, and proteins were resolved on NuPAGE® 4-12% Bis Tris Gels (Invitrogen). Proteins were transferred overnight to PVDF membranes and blocked with 5% milk in PBS. Membranes were probed with primary antibody at 4°C, washed with PBS containing 0.05% Tween 20 and incubated in secondary antibody for 1 h at room temperature. Western blots were developed using Luminate Forte Western HRP substrate (Millipore).

Indirect immunofluorescence. HSV-1 infected HFF grown on coverslips were fixed with 2% formaldehyde, permeabilized with 0.5% NP40, and blocked in 5% normal goat serum. Fixed cells were incubated with antibodies for 30 min at 37 °C and washed two times with PBS containing 0.05% Tween 20 followed by one wash with PBS. Alexa Fluor 488- and 594-conjugated secondary antibodies were incubated with cells for 2 h at 25°C. The coverslips were washed as above and mounted in ProLong Gold antifade reagent (Invitrogen). Images were acquired using an Axioplan 2 microscope (Zeiss) with a 63X objective and Hamamatsu CCD camera (model C4742-95). Images were arranged in figures using Adobe Photoshop CS4 (Adobe Systems, Seattle, WA).

Flow cytometry. Transfected HFF were trypsinized, pelleted and resuspended in 500 μl Accumax cell counting solution (Millipore). Cell suspensions were passed through a 40 μm filter to prevent clumping and stained with a 1:500 dilution of propidium iodine (PI). Fluorescence readings were collected for 20,000 cells. PI positive cells were gated out during analysis and GFP+ cells were defined on empty vector transfected

cells. Data analysis was performed using FlowJo (Version 9) and graphs were constructed using GraphPad Prism software.

Antibodies. Antibodies used in Western blot experiments were mouse anti-IFI16 (ab55328, 1:1000, Abcam), mouse anti-GAPDH (G041, 1:5000, Applied Biological Materials), rabbit anti-TMEM173 (ab92650, 1:2000, Abcam), mouse anti-ICP0 (1:1000, EastCoast Bio), mouse anti-β-Tubulin (Clone JDR.3B8, 1:2000, Sigma-Aldrich), mouse anti-IFIT2 (1:1000, ABCAM), and mouse anti-Myc (9E10, 1:2000, Santa Cruz Bio-technology). HRP-conjugated goat antibodies were used at 1:5,000-1:20,000 (Santa Cruz Bio-technology).

Antibodies used for indirect immunofluorescence studies were mouse anti-IFI16 (ab55328, 1:200, Abcam), rabbit anti-ICP8 (3-83, 1:500, (Knipe et al., 1987)), rabbit anti-PML (1:1000, Santa Cruz Bio-technology) and mouse anti-ICP4 (39S, 1:200, (Showalter et al., 1981). Goat anti-mouse Alexa-488 (Jackson ImmunoResearch) and anti-rabbit Alexa 594 (Jackson ImmunoResearch) were used at 1:500 for secondary detection.

Primers.

Name	Use	Primer Sequence
hIFNβ	qRT-PCR	5'-AAACTCATGAGCAGTCTGCA-3' 5'-AGGAGATCTTCAGTTTCGGAGG-3'
h18s RNA	qRT-PCR	5'-GCATTCGTATTGCGCCGCTA-3' 5'-AGCTGCCCGGCGGGT-3'
IFI16	qRT-PCR	5'-ACTGAGTACAACAAAGCCATTTGA-3' 5'-TTGTGACATTGTCCTGTCCCCAC-3'
STING	qRT-PCR	5'-CCTGAGCAGAACAACTGC-3' 5'-GGTCTTCAAGCTGCCCACAGT-3'

Results

Reduction of IFI16 enhances ICP0-null virus replication in normal human foreskin fibroblasts. To determine whether IFI16 plays a role in the reduced replication of ICP0-null viruses, we treated normal human foreskin fibroblasts (HFF) with siRNA to reduce IFI16 expression. HFF cells were transfected with siRNA specific for *IFI16* (siRNA IFI16) or nontargeting control siRNA (siRNA ctrl). We observed a significant decrease in the expression of IFI16 at both the mRNA and protein level at 72 hours post-transfection (hpt) (Figure 4.1A) and knockdown was robust through 120 hpt. (results not shown). siRNA-treated cells were subsequently infected with an ICP0-null virus (7134) or its corresponding rescue (7134R) at a low MOI (0.1), and virus yields were determined by plaque assay. At 48 hpi we observed a ~4 log defect in 7134 virus replication compared to 7134R in control-treated cells (Figure 4.1C), consistent with previously published reports (Everett and Murray, 2005). Interestingly, while we observed a minimal increase in 7134R virus yield in IFI16 siRNA-treated cells, replication of the 7134 virus was significantly increased (seven-fold) in the absence of IFI16. These results suggest that IFI16 restricts HSV-1 replication in the absence of ICP0 and that the IFI16 protein likely accounts for a portion of the attenuated ICP0-null phenotype seen in human fibroblasts.

Fibroblasts infected with ICP0-null viruses show enhanced expression of type I interferons and interferon-stimulated genes (ISGs) compared to wild-type viruses (Paladino et al., 2010) (Figure 4.1D). The induction of these antiviral genes are, at least

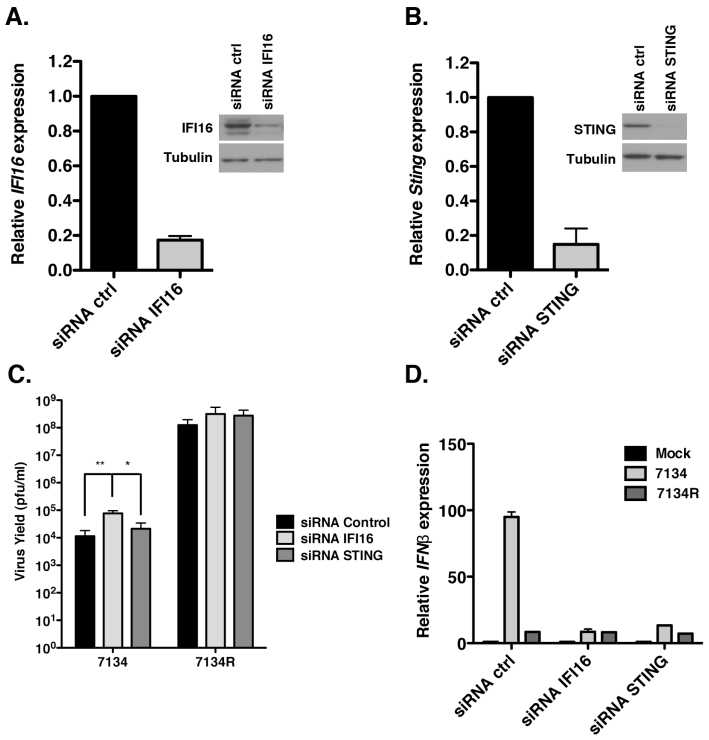

Figure 4.1: IFI16 negatively regulates the replication of an ICP0-null virus. (A) IFI16 and (B) STING transcript and protein levels were decreased following transfection of HFF with either nontarget control, *IFI16*-specific, or *STING*-specific siRNAs. (C) IFI16 knockdown resulted in an increase in ICP0-null virus replication relative to cells transfected with control siRNAs. siRNA-transfected cells were infected with HSV-1 ICP0-null (7134) or a rescue virus (7134R) at an MOI of 0.1, harvested at 48hpi, and virus yield was determined by plaque assay on U2OS cells. (D) IFI16 or STING knockdown decreased *IFNβ* transcript levels in response to ICP0-null virus infection. siRNA-transfected cells were infected with HSV-1 ICP0-null virus at an MOI of 10, and RNA was harvested at 6 hpi. Results are an average of three-(A and B), four-(C) or two-(D) independent experiments, and error bars represent the standard error of means.

in part, due to nuclear sensing of viral DNA by IFI16 (Orzalli et al., 2012). Therefore, it was possible that the increased replication of 7134 observed in our virus yield assay was due to the down regulation of IFI16-induced antiviral genes (Figure 4.1D). In a study by Everett et al (Everett et al., 2008b), a reduction in IRF-3 or STAT-1 protein levels enhanced wild-type and ICP0-null virus replication equally, suggesting that antiviral gene expression was not involved in restricting ICP0-null virus replication. However, in the aforementioned study, IRF-3 and STAT-1 protein levels were reduced by lentivirus vector expression of the parainfluenza protein 5 "V" protein or the NPro protein of bovine viral diarrhea virus, which downregulate these signaling pathways, respectively (Didcock et al., 1999; Hilton et al., 2006). In many cases, viral proteins are multifunctional and it is unclear whether these proteins could affect additional cellular pathways or HSV-1 replication. We therefore examined the involvement of these pathways in ICP0-null virus replication by examining virus yields in the absence of stimulator of interferon genes (STING). This protein is an adaptor in intracellular DNA sensing and decreased expression of STING greatly inhibits IRF-3 activation and type I interferon induction in response to HSV (Ishikawa and Barber, 2008; Ishikawa et al., 2009). In our system, knockdown of STING (Figure 4.1B) increased the replication of 7134 and 7134R by 1.8 and 2.2 fold (Figure 4.1D), respectively, confirming that signaling events downstream of STING, including the induction of type I interferons and ISGs, are not involved in the intrinsic resistance to ICP0-null viruses. Together these results indicated that IFI16 acts to restrict viral gene expression independently of STING and its role in IRF-3 signaling.

Knockdown of IFI16 enhances HSV-1 immediate-early gene expression. The experiments above showed that IFI16 can restrict ICP0-null virus replication and spread; however, it was unclear what stage in the viral lifecycle is inhibited by IFI16. To investigate the mechanism(s) of IFI16-mediated inhibition of viral replication we examined the expression of the viral ICP4 immediate-early gene during infection of siRNA-treated cells. HFF cells were transfected with IFI16 siRNA or non-target control siRNA, infected with 7134 or 7134R at an MOI of 10, and whole cell lysates were harvested at 6 or 24 hpi. Western blot analysis revealed an increase in the expression of ICP4 in 7134-infected IFI16 knockdown cells (Figure 4.2), compared to control-treated cells at both time points, consistent with the increase in viral replication observed in Figure 4.1C. Furthermore, we did not observe an increase in ICP4 expression during infection with 7134R, consistent with ICP0 overcoming IFI16-mediated inhibition by promoting the proteasomal degradation of IFI16. Together these results argue that IFI16 inhibits HSV-1 replication early during infection at the stage of immediate-early gene expression.

Knockdown of IFI16 enhances plasmid DNA expression. We next asked whether this repressive effect of IFI16 was also exerted on transfected DNA by testing whether IFI16 could restrict gene expression from plasmid DNA. HFF were treated with IFI16 or control siRNA and transfected with a GFP construct under the control of a CMV promoter (pCMV GFP) at 48 hours post-siRNA treatment. Whole cell lysates were harvested at 24 hpt and GFP protein levels were examined by western blot. We

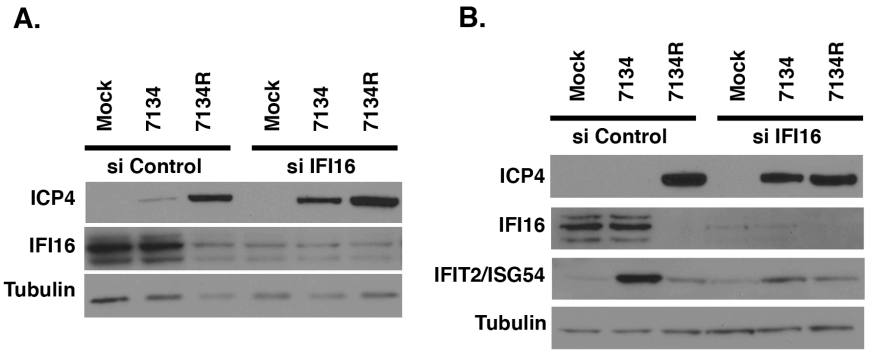

Figure 4.2: Reducing IFI16 protein levels increases the expression of a viral immediate-early protein. Immunoblots examining the levels of the HSV-1 ICP4 immediate early protein in HFF cells treated with IFI16 or non-targeting control siRNA. Treated cells were either mock-infected or infected with an ICP0-null (7134) or rescued virus (7134R) at an MOI of 10. Total whole cell lysates were harvested and probed at (A) 6 and (B) 24 hpi. The cellular tubulin gene was used as a recovery and loading control.

observed an increase in GFP expression in siIFI16 treated cells compared to control cells (Figure 4.3A). This increase was quantified by measuring GFP by flow cytometry and we observed a three-fold increase in GFP^+ cells in the absence of IFI16 (Figure 4.3B).

To determine whether this response was specific to the viral CMV promoter used to express the GFP plasmid above, we also examined GFP expression from a plasmid under the control of the endogenous elongation factor 1 (EF1) promoter. While the efficiency of transfection was lower in control cells transfected with pEF1 GFP compared to pCMV GFP transfected cells, we observed a similar increase in GFP^+ cells in the absence of IFI16 (Figure 4.3B). Together these results suggest that IFI16 inhibits foreign DNA expression regardless of whether the DNA is introduced to cells by infection or transfection and is not specific to DNA that contains viral promoter elements.

Overexpression of IFI16 in a permissive cell line reduces ICP0-null virus gene expression. HSV-1 mutants deficient in functional ICP0 are grown on the osteosarcoma U2OS cell line due to an intrinsic ability of these cells to complement ICP0-null viruses. Due to our observation that IFI16 restricts HSV-1 gene expression in HFF, we examined whether IFI16 is present and/or functional in U2OS cells. We observed that IFI16 could be detected by western blot in U2OS cells (Figure 4.4A, lane 1); however, in contrast to our observations in HFF, infection with 7134R virus at an MOI of 10 did not result in the degradation of IFI16 in these cells (Figure 4.4A, lane 3). Interestingly, we also observed an inability of ICP0 to degrade IFI16 in additional

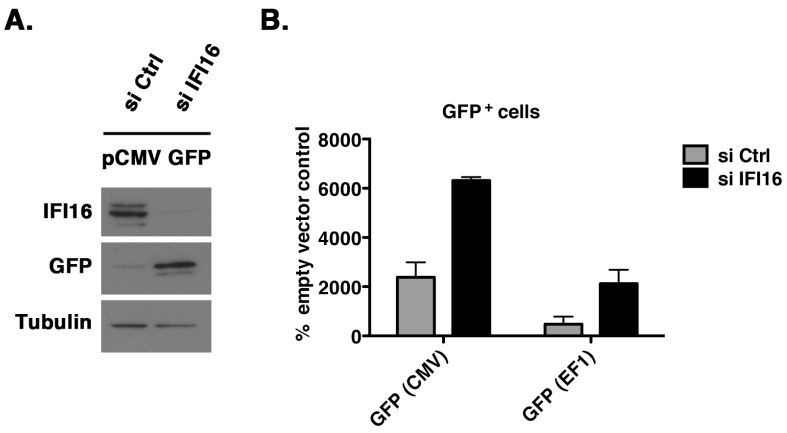

Figure 4.3: IFI16 restricts plasmid DNA expression in a promoter-independent manner. (A) Immunoblot examining GFP and IFI16 expression in pCMV GFP transfected HFF treated with non-targeting control or IFI16 siRNA. The cellular tubulin gene was used as a recovery and loading control. (B) Quantification of GFP^+ cells in the presence or absence of IFI16 by flow cytometry. HFF were transfected with an empty vector plasmid or pCMV GFP or pEF1 GFP at 48 hours post siRNA treatment. The results are represented as a % of the GFP signal from empty vector transfected cells and are an average of two-independent experiments.

cell lines, including HeLa and Hep2 cells (results not shown). Nevertheless, exogenous IFI16 introduced to U2OS cells by transfection was degraded during infection with the 7134R virus (Figure 4.4A, lane 6), suggesting that endogenous IFI16 in U2OS cells may be mutated or modified in some way that prevents ICP0 from promoting its degradation. The ability of ICP0 to target exogenous IFI16 in U2OS cells indicated the protein might be capable of restricting ICP0-null virus gene expression. In Figure 4.4A we observed no difference in the steady-state levels of ICP4 during infection with 7134 in the absence or presence of N-Myc IFI16 (lane 2 vs lane 5). However, this experiment was performed at an MOI of 10 to maximize our ability to detect ICP0-dependent degradation of IFI16. Defects in ICP0-null virus gene expression are more apparent at low MOI, so we examined the expression of ICP4 during infection at an MOI of 0.1. U2OS cells were transfected with an empty vector plasmid or N-Myc IFI16 for 48 h, infected with 7134 and 7134R, and whole cell lysates were harvested at 4 hpi. In contrast to our results at a high MOI of infection, expression of N-Myc IFI16 was associated with a decrease in ICP4 expression in low MOI 7134-infected cells compared to the empty vector control (Figure 4.4B, lane 2 vs lane 5). Consistent with its specificity for ICP0-null viruses, we did not observe a decrease ICP4 expression in the presence of N-Myc IFI16 during infection with 7134R (Figure 4.4B, lane 3 vs lane 6). Together these results provide further evidence of IFI16's activity as an intrinsic resistance factor to ICP0-null virus infection. Furthermore, our results suggest that endogenous IFI16 in U2OS cells is non-functional and accounts for at least a portion of this cell lines apparent permissivity to ICP0-null virus replication.

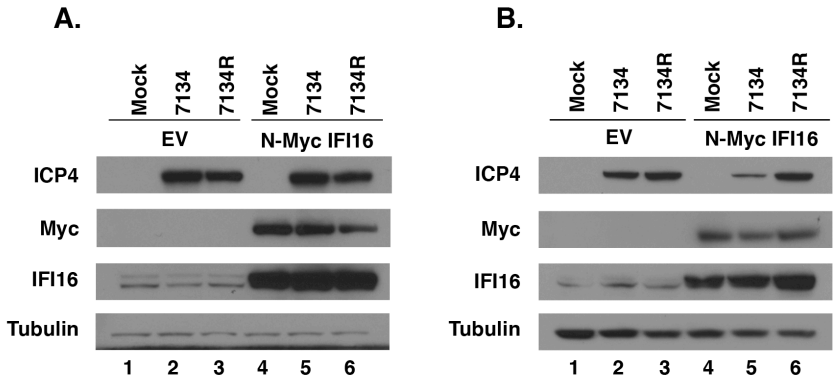

Figure 4.4: The effect of IFI16 overexpression on U2OS cell permissivity.

Expression of exogenous IFI16 resulted in the decreased expression the viral ICP4 immediate-early gene. U2OS cells were transfected with either an empty vector control or an N-terminally Myc-tagged IFI16 construct. At 48 hpt, cells were infected with an ICP0-null (7134) or rescued virus (7134R) at an MOI of (A) 10, or (B) 0.1. Whole cell lysates were harvested and subjected to Western blot analysis for ICP4, Myc, and IFI16 protein levels at 4 hpi. The cellular tubulin gene was used as a recovery and loading control.

IFI16 relocalizes to sites of HSV-1 viral DNA synthesis. In an earlier study we did not observe a detectable intranuclear relocalization of IFI16 during infection with a replication-defective HSV-1 recombinant virus (Orzalli et al., 2012). However, others have reported that endogenous IFI16 co-localizes with viral DNA during infection with KSHV, a gamma-herpesvirus, and IFI16 co-localizes with HSV-1 DNA when overexpressed in U2OS cells (Kerur et al., 2011; Li et al., 2012), consistent with the putative DNA binding activity of this protein (Unterholzner et al., 2010). It is possible that viral DNA synthesis enhances the relocalization of IFI16 due to an increased availability of the viral PAMP; therefore, we reinvestigated the localization of IFI16 during infection with replication competent HSV-1. HFF were infected with the 7134 or 7134R viruses at an MOI of 10, fixed, and processed for immunofluorescence at 3 and 6 hpi. Viral replication compartments, sites of viral DNA synthesis (de Bruyn Kops and Knipe, 1988), were visualized with an antibody specific for the viral ICP8 single-stranded DNA binding protein. In mock-infected HFF, IFI16 was nuclear and noticeably stained both the nucleoplasm and the nucleolus (Figure 4.5, panel a), as observed previously (Cristea et al., 2010; Orzalli et al., 2012). In 7134R-infected cells we observed the accumulation of IFI16 in nuclear foci and the subsequent loss of IFI16 (Figure 4.5, panels i and f), consistent with our previous study describing the ICP0-dependent loss of IFI16 during HSV-1 infection (Orzalli et al., 2012). This pattern of relocalization was not observed in 7134-infected cells. Instead, we initially observed a decrease in nucleolar IFI16 at 3 hpi (Figure 4.5, panel d). This loss did not appear to be due to degradation of the protein, as we did not observe a reduction in the steady state

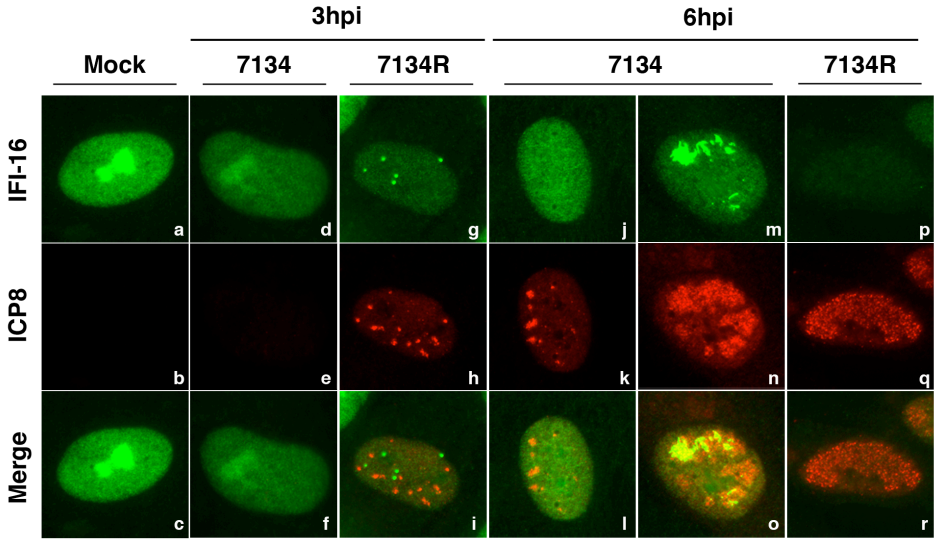

Figure 4.5: IFI16 relocalizes to replication compartments during HSV-1 infection. Immunofluorescence of HSV-1 infected HFF. HFF were infected with ICP0-null (7134) or rescued virus (7134R) at an MOI of 10 and fixed at 3 and 6 hpi. Cells were simultaneously stained with mouse anti-IFI16 (abcam) and rabbit anti-ICP8 antibodies followed by Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat-anti rabbit secondary antibodies.

levels of IFI16 by western blot (Figure 4.2). Instead, this initial redistribution is most likely a consequence of the viral disruption of cellular nucleoli (Calle et al., 2008; Greco et al., 2012). At 6 hpi, we observed two distinct populations of 7134 infected cells: (I) cells with small replication compartments and diffuse IFI16 staining (Figure 4.5, panel I) and (II), cells with large replication compartments and notable IFI16 accumulation within those compartments (Figure 4.5, panel e). These results indicate that in the absence of ICP0, IFI16 relocalizes to replication compartments as viral DNA synthesis progresses.

Knockdown of IFI16 does not affect PML recruitment to viral genomes. During the cellular response to HSV-1 infection, components of ND10 bodies accumulate at sites adjacent to viral DNA, which correlates with their involvement in the repression of viral replication (Everett et al., 2004b). This accumulation is observed during infection with ICP0-null viruses, as wild-type virus infection overcomes ND10-mediated repression by targeting components of these domains for degradation in an ICP0-dependent manner (Boutell and Everett, 2012). IFI16 has not been identified as an ND10 component, nor does it localize to nuclear foci that would be indicative of this domain in normal human foreskin fibroblasts (Figure 4.5) (Cristea et al., 2010; Orzalli et al., 2012). However, given the involvement of IFI16 in the restriction of viral gene expression, we asked whether the protein is involved in the recruitment of ND10 components to viral genomes. To answer this question, we utilized a technique previously developed for analyzing ND10 accumulation at sites of viral DNA entry into the nucleus. This assay involves imaging cells along the edge of a viral plaque, where incoming viral genomes

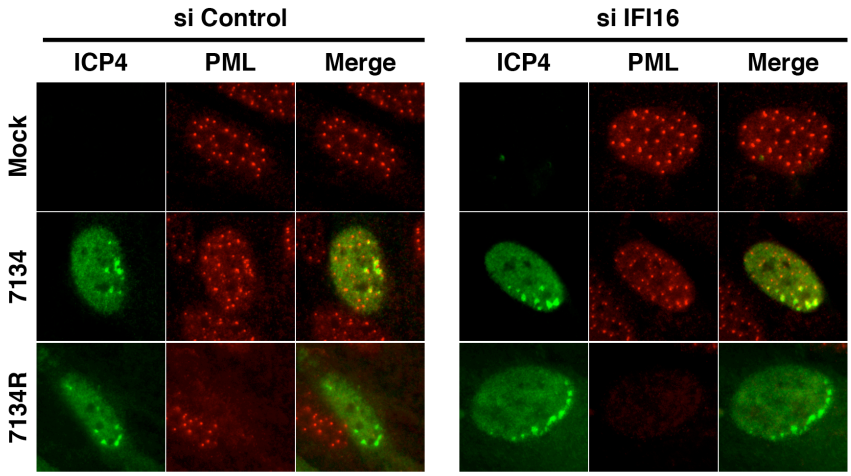

Figure 4.6. ND10 component PML is recruited to sites associated with viral genomes in the absence of IFI16. Immunofluorescence of non-targeting control or IFI16 siRNA transfected HFF infected with HSV-1. HFF were treated with indicated siRNA for 72 hours prior to infection with an ICP0-null (7134) or rescued virus (7134R) at an MOI of 1 or 0.001, respectively. Cells were fixed and simultaneously stained at 24 hpi with mouse anti-ICP4 and rabbit anti-PML antibodies followed by Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat-anti rabbit secondary antibodies.

accumulate asymmetrically in the cell nucleus (Everett and Murray, 2005; Silva et al., 2008; Taylor, 2002). HFF were treated with control siRNA or IFI16 siRNA to decrease IFI16 expression. Cells were then infected with 7134 (MOI 1) or 7134R (MOI 0.001) and fixed at 24 hours post-infection. Infection with 7134 at the higher MOI was necessary to observe plaque formation on HFF as ICP0-null viruses have up to a three-log defect in plaque forming efficiency on human fibroblasts (Everett et al., 2008b). Incoming viral genomes were visualized with an antibody specific for the viral ICP4 immediate-early protein, which has previously been shown to co-localize with viral DNA (Everett and Murray, 2005), and PML was used as a marker for ND10. We observed no difference in the accumulation of PML at ICP4 foci in 7134-infected IFI16-knockdown cells compared to control cells, and PML was degraded in 7134R-infected cells irrespective of knockdown (Figure 4.6). Furthermore, hDaxx was also recruited to genome complexes in the absence of IFI16 (results not shown), indicating this phenotype could be generalized to other ND10 components. Together these results argued that the IFI16-mediated restriction of an ICP0-null mutant is not due to a disruption in ND10 activity.

Discussion

During infection viruses are faced with a barrage of cellular intrinsic resistance and innate immune responses aimed to prevent their replication and spread. Herpesviruses, which are highly ubiquitous and can establish lifetime latent infections, robustly counteract these cellular responses through the expression of multiple immunomodulatory proteins. This complexity has hindered the investigation of the host response to these viruses, as gene knockdown approaches (e.g., siRNA) rarely provide robust phenotypic information when infecting with wild-type-viruses that modulate the pathways investigated. Therefore, to study the cellular responses to these large DNA viruses, one must use recombinant mutant viruses that do not express known immunomodulatory proteins. In the case of HSV-1, the viral ICP0 immediate-early protein has been shown to inhibit both intrinsic resistance and innate immune responses to viral infection (Boutell and Everett, 2012; Roizman et al., 2013). Previous studies using ICP0-null viruses have revealed that one major cellular response to DNA virus infection involves the silencing of incoming viral genomes, and while nuclear ND10 have been implicated in partially mediating this host response, additional unknown factors have yet to be identified.

In this study we established that the nuclear IFI16 DNA sensor is involved in the intrinsic cellular response to HSV-1 infection, as demonstrated by the increased expression and replication of an ICP0-null virus in the absence of IFI16. Our investigation revealed that IFI16 did not affect the recruitment of ND10 components to viral genomes, indicating the intrinsic activity of IFI16 is independent of a previously

described DNA silencing response. Interestingly, the complementation of the 7134 virus upon knockdown of IFI16 was similar to the remaining 10-fold inhibition observed in human fibroblasts when several ND10 components were simultaneously decreased by shRNA expression (Glass and Everett, 2012), suggesting that IFI16 may be the additional cellular repressor hypothesized in the aforementioned study. Future studies investigating the simultaneous knockdown of ND10 components and IFI16 will be necessary to determine whether additional intrinsic resistance factors modulate ICP0-null virus infection.

The extent to which ICP0-null viruses are restricted for replication is cell type-dependent. Human fibroblasts are among the most restrictive cells for ICP0-null virus replication, while ICP0 is dispensable for growth in U2OS cells. The cellular mechanisms that impart these differences have not been identified. Hancock *et al* (2006) reported that heterokaryon formation between U2OS cells and human fibroblasts imparted a dominant restrictive phenotype on U2OS cell nuclei, suggesting that U2OS cells lack a restrictive factor(s) that is functional in fibroblasts (Hancock et al., 2006). Others have examined the levels of known intrinsic resistance factors in U2OS cells (Lukashchuk and Everett, 2010), and it has been noted that these cells do not express ATRX, a component of ND10, however the addition of this protein failed to restrict HSV-1 growth (McFarlane and Preston, 2011). Surprisingly, U2OS cells expressed detectable IFI16 protein, which was inconsistent with the permissivity of these cells to ICP0-null virus infection. The ability of HSV-1 to promote the degradation of exogenous, but not endogenous, IFI16 indicates this protein might be dysfunctional in tumor cell

lines and is consistent with reports that transformed cells lack a functional DNA sensing mechanism (Iwasaki, 2012). However, the downstream factors involved in restricting viral gene expression appear to be functional, as exogenous IFI16 was able to restrict HSV-1 gene expression. We therefore speculate that the permissivity of cells to ICP0-null virus infection may, in part, rely on the functionality of IFI16 rather than whether the protein is expressed in a given cell-type. In addition, these results provide a basis for the further investigation of IFI16 activity in the absence of other restrictive phenotypes.

The inhibitory response mediated by IFI16 was not limited to DNA introduced by viral infection as we also observed an increase in the expression of transfected DNA in the absence of IFI16. It is well documented that exogenous plasmid DNA can be silenced upon transfection, and that this has been a significant drawback to the use of non-viral vectors in gene therapy (Al-Dosari and Gao, 2009; Jackson et al., 2006). Interestingly, cell lines that do not express IFI16 (293T cells) (Kwak et al., 2003) or have mutations that prevent the nuclear localization of IFI16 (PC3) (Xin et al., 2003), demonstrate enhanced mRNA expression of transfected DNA when compared to other cell lines (Karyala et al., 2010). While Karyala *et al* (2010) do not discuss the potential activity of IFI16 in their system, our results indicate IFI16 might play a role in inhibiting foreign DNA expression in non-transformed cells and warrants further investigation.

One critical question raised by this study involves the potential cellular components that act in concert with IFI16 to mediate its intrinsic activity. The ER-associated STING scaffolding protein has been shown to co-immunoprecipitate with IFI16 and is downstream of this nuclear protein in the activation of IRF-3 (Orzalli et al.,

2012; Unterholzner et al., 2010), but we found in our knockdown studies that STING is not involved in the intrinsic antiviral response to HSV-1 infection. As IFI16 is nuclear in HFF and inhibits viral gene expression in the nucleus, additional nuclear proteins may be involved in the observed phenotype. IFI16 is a member of the PHYIN (Pyrin and HIN) family of proteins, which includes AIM2, MNDA, and IFIX. Typically these proteins activate signaling cascades through homotypic interactions with other pyrin-containing proteins. For instance, upon binding cytosolic DNA, AIM2 interacts with ASC in a pyrin-dependent manner to initiate inflammasome signaling (Hornung et al., 2009). Interestingly, ASC has also been reported to co-localize with IFI16 in the nucleus during KSHV infection of endothelial cells (Kerur et al., 2011) and we have observed an intranuclear relocalization of IFI16 reminiscent of inflammasomes during ICP0-null virus infection. However, we have not determined whether this relocalization is necessary for IFI16 activity nor whether ASC co-localizes with IFI16 in human fibroblasts.

IFI16 has been shown to associate with other cellular proteins that lack pyrin domains, including BRAC1. Interestingly, BRAC1 has previously been shown to accumulate in HSV-1 replication compartments (Taylor and Knipe, 2004); however, this accumulation was observed during wild-type HSV-1 infection and we have observed no difference in BRCA1 recruitment in the presence or absence of ICP0 (results not shown), suggesting this protein is not involved in the silencing of viral DNA. The identification of additional IFI16-interacting proteins and their involvement in both the intrinsic and innate functions of IFI16 will further our understanding of IFI16 activity and is a high priority for future studies.

The mechanism(s) by which IFI16 decreases the expression of exogenously introduced DNA is unclear. In a study using HCMV, it was proposed that IFI16 sequesters the Sp1 transcription factor making it unavailable to promote viral gene expression (Gariano et al., 2012). However, several HCMV immediate-early promoters containing Sp1-binding sites were not enhanced by IFI16 knockdown and an additional study implicated IFI16 as positive regulator of CMV replication (Cristea et al., 2010), complicating this interpretation. In addition, we observed an IFI16-dependent repressive effect on a plasmid containing the EF1 alpha promoter, which does not contain an Sp1 binding site. Further insight into the activity of IFI16 may be garnered by work investigating HSV-1 ICP0's role in enhancing virus replication. ICP0 is a known transactivator of viral genes and its expression promotes both histone removal and acetylation of viral DNA during lytic infection (Cliffe and Knipe, 2008). Furthermore, Frenzcy *et al* (2009) reported that expression of ICP0 from a recombinant HSV-1 virus was sufficient to reduce the H3me3K9 heterochromatic mark on viral DNA when compared to a virus expressing no viral gene products (Ferenczy and DeLuca, 2009). Together, these data suggest ICP0 promotes a more "open" chromatin state, one that is associated with transcriptional activity. Based on these reports and our observations of both IFI16 restricting viral gene expression and ICP0 targeting IFI16 for degradation to promote virus replication, we hypothesize that IFI16 is involved in recruiting chromatin remodeling complexes and/or histones to foreign DNA.

In conclusion, the results presented in this study indicate that in addition to its involvement as an innate pattern recognition receptor, IFI16 plays a broader role in the sensing and silencing of foreign DNA as a mediator of intrinsic cellular resistance.

Chapter Five: Dissertation Perspective

Summary of Results.

The work presented in this dissertation was directed at understanding how HSV-1 is sensed by the cell upon infection, and how the viral ICP0 protein counteracts this response. Prior to this study, the induction of a type I interferon response to HSV-1 infection was thought to occur through cytosolic sensing of viral DNA. However, in light of the replication cycle of HSV-1, we hypothesized that viral DNA may also be sensed in the nucleus during infection. We tested this hypothesis by establishing an infection model in normal human foreskin fibroblasts where we could examine the requirements for IRF-3 activation in response to replication-defective HSV-1. The results from experiments using this model strongly argued that the release of viral DNA into the nucleus was required to activate a type I interferon response to infection. Furthermore, we identified the nuclear IFI16 DNA sensor as an important component of this response.

The identification of both the site of sensing and the components involved in this process allowed us to identify steps in the signaling cascade inhibited by the viral ICP0 protein. In chapter three we demonstrated that ICP0 has both an early and late effect on IFI16-dependent signaling. We observed that early during infection ICP0 promotes the intranuclear relocalization of IRF-3 and that this is associated with the inability of IRF-3 to bind to cellular promoters. Based on these results we hypothesize that ICP0 inhibits IRF-3 by sequestering the protein away from cellular promoters, although as discussed in chapter two, the mechanism of this sequestration is unknown. The inhibition of IRF-3 signaling by ICP0 later during infection appears to be due to the ICP0-dependent degradation of IFI16. Loss of IFI16 during infection inhibits activation

of downstream signaling and prevents accumulation of IRF-3 in the nucleus. Together these results argue for a dual mechanism of ICP0-mediated inhibition.

In chapter four of this dissertation, we further characterized the activity of IFI16 during HSV-1 infection and provided evidence of an IFI16-dependent intrinsic repressive response to foreign DNA. We demonstrated that IFI16 restricts the replication of an ICP0-null virus independently of STING, indicating that this response does not involve the antiviral activity of type I interferons. While knockdown of IFI16 in HFF enhanced ICP0-null virus immediate-early gene expression, the converse was true when IFI16 was overexpressed in the complementing U2OS cell line, indicating that IFI16-dependent repression occurs early during infection. The repressive activity of IFI16 was not limited to viral DNA as the expression of transfected DNA was also inhibited by IFI16, suggesting that IFI16 acts broadly to repress foreign DNA.

Discussion

Nuclear vs cytosolic innate sensing of HSV-1 DNA. The lifecycle of HSV-1 lends itself to being sensed by a variety of cell types during infection. While lytic replication occurs within the mucosal epithelium, innate immune cells that reside in the lamina propria continually sample this site for foreign pathogens. Current data suggests that the cellular components and compartments involved in sensing HSV-1 are cell type-dependent. For instance, dendritic cells, which are important interferon producers, sense HSV-1 in endosomal compartments in a TLR9-dependent manner (Lund et al., 2003). Cytosolic sensing of viral DNA has also been implicated in HSV-1 infection with the discovery of several putative cytosolic DNA sensors.

In this dissertation we present evidence of the existence of a nuclear sensing mechanism important for the activation of IRF-3 signaling in response to HSV-1. This response required the release of viral DNA into the nucleus and was, at least in part, dependent on the expression of the IFI16 DNA sensor. While exclusively nuclear in the normal human fibroblasts used in this study, IFI16 can be found at low levels in the cytosol of certain cell types, particularly those of myeloid lineage. IFI16-dependent cytosolic sensing of HSV-1 has been investigated in bone marrow-derived macrophage and PMA-differentiated THP-1 cells, both of which are highly restrictive for viral replication. While viral DNA is normally protected from cytosolic sensors by the viral capsid, Horen et al (2013) recently reported that in macrophages, viral DNA is made available to cytosolic sensors via proteasomal degradation of incoming viral capsids (Horan et al., 2013). This mechanism does not appear to be involved in sensing HSV-1

in human fibroblasts, as we did not observe an MG132-dependent effect on the activation of IRF-3 signaling. Furthermore, this response appears to be specific to immune cells as expression of a cytosolic restricted IFI16 in HEK293 cells was not sufficient to induce *IFNβ* expression in response to HSV-1(Li et al., 2012). These results indicate that non-immune cells potentially lack the components necessary to target viral capsids for degradation and the release of DNA into the cytosol.

The cell type specificity for cytosolic or nuclear sensing appears to correlate with the permissivity of these cells to HSV-1 infection. Myeloid cells are highly restrictive for viral gene expression and replication, while fibroblasts support robust productive replication. It is unclear why myeloid cells are restrictive for HSV-1 infection; however, cytosolic degradation of incoming capsids may inhibit efficient viral DNA accumulation in the nucleus thus preventing viral gene expression and replication. As such, the immune evasion strategies employed by HSV-1 that rely on viral gene expression may not be active in myeloid cells. This is consistent with reports that a type I interferon response is important for the control of HSV-1 infection *in vivo* (Conrady et al., 2011; Luker et al., 2003), even though permissive cells (e.g., epithelial cells and fibroblasts) robustly counteract the innate immune response. We therefore propose that the localization of IFI16-dependent sensing depends on both the compartment where IFI16 is expressed and the permissivity of these cells for viral replication. If this hypothesis proves correct, one would expect that the expression of the proteins required for promoting viral capsid ubiquitination and degradation would make cytosolic sensing of HSV-1 possible in

permissive cells. However, it is currently unclear how viral capsids are targeted for degradation in macrophages.

There appears to be at least one additional difference between IFI16-dependent cytosolic and nuclear sensing of HSV-1 DNA. Both the cytosolic and nuclear DNA sensing mechanisms require STING to mediate the activation of IRF-3 in response to HSV-1 infection. In addition, STING has also been shown to couple the cytosolic DNA sensing response to NF-κB activation (Ishikawa and Barber, 2008), and knockdown of IFI16 inhibited NF-κB signaling in HSV-1 infected macrophages (Unterholzner et al., 2010). However, in chapter two we observed that expression of an NF-κB-dependent gene was induced equally in both *d*109- and *d*106-infected HFF, suggesting this response was IFI16-independent. While it is still unclear how STING activates NF-κB signaling in response to DNA, our results indicate that this response is either not active in human fibroblasts, or alternatively, cytosolic and nuclear sensing of DNA differentially induce NF-κB signaling. This may indicate that cytosolic and nuclear IFI16 use different adaptor proteins to potentiate downstream signaling events.

Possible mechanisms for the differentiation of endogenous and foreign DNA in the nucleus. One of the most intriguing questions to have arisen from this study is how the cell differentiates between self and non-self DNA in the nucleus. One potential mechanism is informed by the recently described crystal structure of the IFI16 HINb domain in complex with DNA (Jin et al., 2012). Jin et al reported that IFI16 interacts with DNA in a non-sequence specific manner through electrostatic interactions with the

sugar-phosphate backbone. In addition, the DNA itself appears to act as a scaffold for IFI16 oligomerization. Cellular DNA is tightly associated with nucleosomes, which could preclude the DNA from acting as a signaling platform for IFI16, thus limiting the response to self-DNA. In contrast, while HSV DNA is known to be chromatinized rapidly upon entry into the nucleus (Cliffe and Knipe, 2008; Oh and Fraser, 2008), histone association with HSV DNA is less densely packed (Cliffe and Knipe, 2008) and looser (Lacasse and Schang, 2010) than cellular chromatin, and this reduced histone load may provide IFI16 access to viral DNA. Alternatively, HSV may be sensed prior to the chromatinization of its genome. While we have not directly shown that IFI16 binds to HSV DNA in our system, the former hypothesis is supported by our observation that IFI16 accumulates in replication compartments where newly synthesized DNA is known to be histone free (Maul et al., 1996; Oh and Fraser, 2008; Simpson-Holley et al., 2004).

This hypothesis can be further examined by directly testing whether IFI16 can bind DNA in the presence or absence of chromatin. As stated above, the IFI16 HIN (a and b) domains have previously been shown to bind DNA *in vitro*. Therefore, *in vitro* chromatinized DNA can be tested for IFI16 binding activity in a similar manner. If this hypothesis proves correct, we would expect the IFI16 HIN domains to bind *in vitro* chromatinized DNA less efficiently than naked DNA. The functionality of this hypothesis can also be tested in cell culture by examining the cellular innate immune response to virus infection in cells that do not express HIRA or asf1, the histone chaperone proteins important for the early chromatinization of HSV DNA (Oh et al., 2012; Placek et al., 2009).

An alternative, although not mutually exclusive, argument could be made for viral DNA being the second signal in a two-step mechanism of host cell sensing. Using HSV-1 VLPs that lack DNA, Holm et al (2012) recently reported that membrane perturbation induced a low level of IFNβ expression and enhanced the cellular response to subsequent stimuli. The authors hypothesized that membrane fusion could act as a "danger" signal to prime the cell for additional viral stimuli. The exact mechanism that links viral fusion to the production of type I interferons is unclear, although it appears to involve the activation of PI(3)K (Holm et al., 2012). Furthermore, others have proposed that PI(3)K may play a role in the phosphorylation of IRF-3 (Sarkar et al., 2004), although a potential mechanism was not explored in the referenced study. Based on these reports, it is possible that membrane perturbation primes IRF-3 in a way that enhances IFI16 signaling in response to DNA. However, it is currently unclear whether this priming response is necessary for the DNA-dependent response to HSV-1 infection.

Mechanisms of IFI16-dependent innate and intrinsic signaling. The results presented in chapters three and four of this dissertation argue that IFI16 is capable of acting as both an effector of the innate response and as an intrinsic resistance factor to foreign DNA. These IFI16-dependent responses appear to be separable; as the activation of type I interferon in human fibroblasts requires STING, while the intrinsic response acts independently of this adaptor molecule. However, it is currently unclear how IFI16 mediates either of these responses.

Our proposed model of innate nuclear HSV-1 DNA sensing requires the transduction of the sensing event to the cytoplasm to activate IRF-3 signaling. This is

supported by multiple observations, including the nuclear localization of IFI16, the involvement of cytoplasmic STING, and that blocking nuclear export results in decreased phosphorylation of TBK1 during infection. It has been proposed that a general feature of innate immune signaling is the ability to dissociate the site of PAMP recognition from that of signal transduction (Kagan, 2012). In many cases this appears to involve the transport of the sensor itself following ligand binding. While the intracellular localization of IFI16 can change through the acetylation of its nuclear localization signal (Li et al., 2012), we did not observe a measureable relocalization of IFI16 to the cytoplasm in *d*109-infected cells, suggesting that an additional protein may bridge these two compartments. However, we cannot rule out that a small portion of IFI16 translocates to the cytoplasm to initiate signal transduction.

While nuclear innate sensing of viral DNA is a novel finding of this study, the existence of an intrinsic repressive response to foreign nuclear DNA has been known for some time. The mechanisms underlying this response have remained unclear, although the repression has been observed for both viral and plasmid DNA. In this study we demonstrate that IFI16 is involved in the cellular silencing of exogenous DNA. However, we do not identify the mechanism by which IFI16 restricts gene expression.

IFI16 was first implicated as a repressor of transcription over a decade ago when IFI16 fused to a GAL4 DNA binding domain was shown to inhibit the expression of a GAL4 reporter construct (Johnstone et al., 1998). In a recent study using HCMV, it was proposed that IFI16 sequesters the Sp1 transcription factor making it unavailable to promote viral gene expression (Gariano et al., 2012). The co-immunoprecipitation of

IFI16 with Sp1 required the HIN-B domain of IFI16. However, this domain was dispensable for the repression observed by Johnstone et al (1998). In addition, in chapter four we observed an IFI16-dependent repression of the EF1α promoter, which does not contain an Sp1 binding site. Therefore, sequestration of Sp1 cannot account for several reported IFI16 repressive activities.

It has been hypothesized that changes in chromatin structure may play a role in the silencing of exogenous DNA. This is supported by observations that transcriptional repression of viral and plasmid DNA occurs concomitantly with an increase in histone associated-heterochromatic marks (Knipe and Cliffe, 2008; Riu et al., 2007). During HSV-1 infection the ICP0 protein counteracts this repression and promotes viral gene expression (Knipe and Cliffe, 2008). This appears to be partly due to the disruption of ND10 bodies (Boutell and Everett, 2012), which are sites of intrinsic antiviral resistance. However, results from our study indicate that ICP0-dependent degradation of IFI16 also plays a role in the derepression of viral DNA. Based on these observations, we hypothesize that IFI16 may silence viral and plasmid DNA by promoting their association with heterochromatin.

The factors that mediate both the innate and intrinsic effects of IFI16 have not been defined. The use of proteomics to identify potential IFI16 interactors may provide additional information on the activities of IFI16 observed in this study. Proteins identified by this approach can then be tested for their activity in both IRF-3 signaling and the repression of foreign DNA.

Potential for additional innate nuclear sensing mechanisms. As stated in the introduction, several putative DNA sensors have been identified as activators of type I interferon in response to viral DNA although none have been universally accepted by the field (Burdette and Vance, 2013). This is mainly due to the modest response seen when individual putative DNA sensors are silenced by siRNA. However, it has been hypothesized that this effect may be due to functional redundancy within the DNA sensing pathway. This has been observed recently in THP-1 cells where cytosolic sensing of HSV-1 DNA was shown to be dependent on both IFI16 or ddx41 (Horan et al., 2013).

In this study, we observed that knockdown of STING, although less efficient than IFI16 knockdown, resulted in a greater inhibition in the expression of *IFNβ* in response to HSV-1 infection. This could indicate that an additional nuclear sensing mechanism exists in these cells. Currently the AIM2-like family of receptors (ALR), of which IFI16 is a member, is composed of four human and thirteen mouse proteins. Expression of the individual ALR proteins in 293T cells expressing STING induced varying type I interferon responses (Brunette et al., 2012), indicating some functional redundancy within the family. While human AIM2 is cytoplasmic, and thus unlikely to be involved in the nuclear sensing of HSV-1, both IFIX and MNDA are localized to the nucleus, making them potential nuclear PRR candidates. Examining whether individual or simultaneous knockdown of the human ALR family members affects the IRF-3 response to HSV-1 infection would test this hypothesis.

Recently, Wu *et al* (2013) made the intriguing observation that both transfected DNA and HSV-1 infection induces the production of cyclic GMP-AMP (cGAMP), which binds to STING and promotes IRF-3 signaling (Wu et al., 2012). Knockdown of the cGAMP synthase responsible for the production of this second messenger resulted in a decreased antiviral response to a ΔICP34.5 HSV-1 virus in both mouse L929 cells and human THP1 cells (Sun et al., 2012). While expression of both STING and cGAMP synthase in HEK293T cells was sufficient to activate IRF-3 in response to transfected DNA, this sufficiency was not examined during HSV-1 infection. It is possible that IFI16 and cGAMP synthase work in concert to activate IRF-3 or are involved in distinct signaling pathways that both utilize STING as an adaptor molecule. The HSV-1 virus used by Wu, Sun, and colleagues (2012) expresses ICP0, and would thus potently inhibit the IFI16/p204-dependent response to viral infection; therefore, it is likely that IFI16 and cGAMP synthase sensing is separable. Furthermore, others have demonstrated that STING recognition of cyclic dinucleotides and the production of IFNβ in the response to transfected viral DNA (VV70mer) can be decoupled in bone marrow derived macrophage (Burdette et al., 2011), suggesting that multiple mechanisms of sensing exist within a single cell type.

How does ICP0 promote the degradation of IFI16? ICP0-null HSV-1 mutants are subject to an enhanced type I interferon response and repression through intrinsic resistance mechanisms. Prior to this study, ICP0 was known to counteract both responses; however, the mechanism of action had not been thoroughly defined. In this

dissertation we demonstrate that ICP0 overcomes these two DNA-dependent responses by promoting the degradation of nuclear IFI16.

Several questions remain unanswered with regards to this aspect of our study, including how ICP0 targets IFI16 for degradation. As stated in the Introduction, ICP0 promotes the degradation of cellular proteins through diverse means, including direct protein interactions, phosphospecific interactions, and SUMO-dependent and –independent mechanisms. Alternatively, it has been proposed that some ICP0-dependent degradation events may be downstream effects of ICP0 targeting another cellular protein. We believe that it is unlikely that the loss of IFI16 observed in this study is a secondary consequence of another degradation event due to the observed relocalization of IFI16 to ICP0 foci during infection. This phenotype is similar to that seen with several direct ICP0 targets, including the C-terminal domain of PML.I and RNF8 (Cuchet-Lourenco et al., 2012; Lilley et al., 2010). However, formal testing of whether IFI16 is a direct or indirect interactor of ICP0 will help strengthen this argument.

Currently, IFI16 has not been reported to possesses any of the substrate motifs that ICP0 is known to interact with when targeting proteins for degradation. In chapter three we attempted to elucidate the ICP0 domains necessary for this event by testing a panel of ICP0 mutants for their ability to degrade IFI16. Surprisingly, the N-terminus of ICP0 (amino acid residues 1-105) was important for this activity. Interestingly, over a decade ago, a potential role for the N-terminal domain of ICP0 in the transactivation of immediate-early gene expression was reported (Lium et al., 1998). Deletion of the first 105 amino acids of ICP0 decreased immediate-early gene expression and replication of

the mutant virus by approximately 10-fold. The FHA binding activity of ICP0 has been mapped to this region; however, we do not believe that this activity of ICP0 is necessary to degrade IFI16 because a mutant lacking this binding site showed very little growth defect in HFF (Chaurushiya et al., 2012). Additional mutational analysis of this region will potentially reveal a novel mechanism of ICP0 substrate specificity.

Implications for replication-defective vaccine design. Replication-defective HSV vaccines are currently being explored as potential vaccine vector candidates. The HSV-1 *d*106 recombinant virus used in this study has shown good immunogenicity in a rhesus macaque model of SIV infection (Kaur et al., 2007). However, the protective capacity of this vaccine vector to SIV challenge was limited for unknown reasons. While the correlates of protection for vaccine design are not well understood, eliciting a stronger immune response may increase the efficacy of this vaccine vector.

One potential approach to increasing the efficacy of virus-based vectors is to delete (or modify) immunomodulatory genes that are encoded by the vector. It is clear from this study and work by others (Eidson et al., 2002) that expression of ICP0 from the *d*106 virus blunts the cellular innate immune response to infection. However, expression of ICP0 also prevents silencing of viral chromatin and subsequently enhances the expression of the transgene to which an immune response is being elicited. Therefore, while deletion of ICP0 would enhance the innate immune response to the *d*106 virus, it would also likely decrease transgene-specific immunogenicity of this vector.

Prior to this study it was unclear whether the transactivating capability and immunomodulatory activity of ICP0 could be decoupled. While we demonstrated that both of these ICP0-dependent responses rely, in part, on the degradation of IFI16, we also observed that ICP0 inhibits the innate immune response by sequestering IRF-3. This response was independent of the RING-finger activity of ICP0, which is required for the protein's transactivating potential. By understanding how ICP0 sequesters IRF-3 in a RING finger-independent manner it may be possible to enhance the innate immune response to the *d*106 vector without decreasing transgene expression.

Dissertation References

Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K.A., and Hornung, V. (2009). RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nature immunology *10*, 1065-1072.

Aboussahoud, W., Aflatoonian, R., Bruce, C., Elliott, S., Ward, J., Newton, S., Hombach-Klonisch, S., Klonisch, T., and Fazeli, A. (2010). Expression and function of Toll-like receptors in human endometrial epithelial cell lines. Journal of reproductive immunology *84*, 41-51.

Ace, C.I., McKee, T.A., Ryan, J.M., Cameron, J.M., and Preston, C.M. (1989). Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. Journal of virology *63*, 2260-2269.

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000). Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. Cell *103*, 667-678.

Aglipay, J.A., Lee, S.W., Okada, S., Fujiuchi, N., Ohtsuka, T., Kwak, J.C., Wang, Y., Johnstone, R.W., Deng, C., Qin, J.*, et al.* (2003). A member of the Pyrin family, IFI16, is a novel BRCA1-associated protein involved in the p53-mediated apoptosis pathway. Oncogene *22*, 8931-8938.

Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R.M., and Wagner, H. (2002). Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. European journal of immunology *32*, 1958-1968.

Al-Dosari, M.S., and Gao, X. (2009). Nonviral gene delivery: principle, limitations, and recent progress. The AAPS journal *11*, 671-681.

Altinkilic, B., and Brandner, G. (1988). Interferon inhibits herpes simplex virus-specific translation: a reinvestigation. The Journal of general virology *69 (Pt 12)*, 3107-3112.

Andersen, J., VanScoy, S., Cheng, T.F., Gomez, D., and Reich, N.C. (2008). IRF-3-dependent and augmented target genes during viral infection. Genes and immunity *9*, 168-175.

Bandyopadhyay, S.K., Leonard, G.T., Jr., Bandyopadhyay, T., Stark, G.R., and Sen, G.C. (1995). Transcriptional induction by double-stranded RNA is mediated by

interferon-stimulated response elements without activation of interferon-stimulated gene factor 3. The Journal of biological chemistry *270*, 19624-19629.

Batterson, W., Furlong, D., and Roizman, B. (1983). Molecular genetics of herpes simplex virus. VIII. further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. Journal of virology *45*, 397-407.

Bauhofer, O., Summerfield, A., Sakoda, Y., Tratschin, J.D., Hofmann, M.A., and Ruggli, N. (2007). Classical swine fever virus Npro interacts with interferon regulatory factor 3 and induces its proteasomal degradation. Journal of virology *81*, 3087-3096.

Bibeau-Poirier, A., Gravel, S.P., Clement, J.F., Rolland, S., Rodier, G., Coulombe, P., Hiscott, J., Grandvaux, N., Meloche, S., and Servant, M.J. (2006). Involvement of the IkappaB kinase (IKK)-related kinases tank-binding kinase 1/IKKi and cullin-based ubiquitin ligases in IFN regulatory factor-3 degradation. J Immunol *177*, 5059-5067.

Bieniasz, P.D. (2004). Intrinsic immunity: a front-line defense against viral attack. Nature immunology *5*, 1109-1115.

Blanco-Melo, D., Venkatesh, S., and Bieniasz, P.D. (2012). Intrinsic cellular defenses against human immunodeficiency viruses. Immunity *37*, 399-411.

Boutell, C., Canning, M., Orr, A., and Everett, R.D. (2005). Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7. Journal of virology *79*, 12342-12354.

Boutell, C., Cuchet-Lourenco, D., Vanni, E., Orr, A., Glass, M., McFarlane, S., and Everett, R.D. (2011). A viral ubiquitin ligase has substrate preferential SUMO targeted ubiquitin ligase activity that counteracts intrinsic antiviral defence. PLoS pathogens *7*, e1002245.

Boutell, C., and Everett, R.D. (2012). Regulation of alphaherpesvirus infections by the ICP0 family of proteins. The Journal of general virology.

Boutell, C., Sadis, S., and Everett, R.D. (2002). Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. Journal of virology *76*, 841-850.

Brunette, R.L., Young, J.M., Whitley, D.G., Brodsky, I.E., Malik, H.S., and Stetson, D.B. (2012). Extensive evolutionary and functional diversity among mammalian AIM2-like receptors. The Journal of experimental medicine *209*, 1969-1983.

Brzostek-Racine, S., Gordon, C., Van Scoy, S., and Reich, N.C. (2011). The DNA damage response induces IFN. J Immunol *187*, 5336-5345.

Burdette, D.L., Monroe, K.M., Sotelo-Troha, K., Iwig, J.S., Eckert, B., Hyodo, M., Hayakawa, Y., and Vance, R.E. (2011). STING is a direct innate immune sensor of cyclic di-GMP. Nature *478*, 515-518.

Burdette, D.L., and Vance, R.E. (2013). STING and the innate immune response to nucleic acids in the cytosol. Nature immunology *14*, 19-26.

Cai, W., Astor, T.L., Liptak, L.M., Cho, C., Coen, D.M., and Schaffer, P.A. (1993). The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. Journal of virology *67*, 7501-7512.

Cai, W.Z., and Schaffer, P.A. (1989). Herpes simplex virus type 1 ICP0 plays a critical role in the de novo synthesis of infectious virus following transfection of viral DNA. Journal of virology *63*, 4579-4589.

Calle, A., Ugrinova, I., Epstein, A.L., Bouvet, P., Diaz, J.J., and Greco, A. (2008). Nucleolin is required for an efficient herpes simplex virus type 1 infection. Journal of virology *82*, 4762-4773.

Canning, M., Boutell, C., Parkinson, J., and Everett, R.D. (2004). A RING finger ubiquitin ligase is protected from autocatalyzed ubiquitination and degradation by binding to ubiquitin-specific protease USP7. The Journal of biological chemistry *279*, 38160-38168.

Casrouge, A., Zhang, S.Y., Eidenschenk, C., Jouanguy, E., Puel, A., Yang, K., Alcais, A., Picard, C., Mahfoufi, N., Nicolas, N.*, et al.* (2006). Herpes simplex virus encephalitis in human UNC-93B deficiency. Science *314*, 308-312.

Chaurushiya, M.S., Lilley, C.E., Aslanian, A., Meisenhelder, J., Scott, D.C., Landry, S., Ticau, S., Boutell, C., Yates, J.R., 3rd, Schulman, B.A.*, et al.* (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*, 79-90.

Chelbi-Alix, M.K., and de The, H. (1999). Herpes virus induced proteasome-dependent degradation of the nuclear bodies-associated PML and Sp100 proteins. Oncogene *18*, 935-941.

Chen, S.H., Kramer, M.F., Schaffer, P.A., and Coen, D.M. (1997). A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus. Journal of virology *71*, 5878-5884.

Chiu, Y.H., Macmillan, J.B., and Chen, Z.J. (2009). RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell *138*, 576-591.

Clement, J.F., Bibeau-Poirier, A., Gravel, S.P., Grandvaux, N., Bonneil, E., Thibault, P., Meloche, S., and Servant, M.J. (2008). Phosphorylation of IRF-3 on Ser 339 generates a hyperactive form of IRF-3 through regulation of dimerization and CBP association. Journal of virology *82*, 3984-3996.

Cliffe, A.R., Garber, D.A., and Knipe, D.M. (2009). Transcription of the herpes simplex virus latency-associated transcript promotes the formation of facultative heterochromatin on lytic promoters. Journal of virology *83*, 8182-8190.

Cliffe, A.R., and Knipe, D.M. (2008). Herpes simplex virus ICP0 promotes both histone removal and acetylation on viral DNA during lytic infection. Journal of virology *82*, 12030-12038.

Conrady, C.D., Jones, H., Zheng, M., and Carr, D.J. (2011). A Functional Type I Interferon Pathway Drives Resistance to Cornea Herpes Simplex Virus Type 1 Infection by Recruitment of Leukocytes. Journal of biomedical research *25*, 111-119.

Conrady, C.D., Zheng, M., Fitzgerald, K.A., Liu, C., and Carr, D.J. (2012). Resistance to HSV-1 infection in the epithelium resides with the novel innate sensor, IFI-16. Mucosal immunology *5*, 173-183.

Cristea, I.M., Moorman, N.J., Terhune, S.S., Cuevas, C.D., O'Keefe, E.S., Rout, M.P., Chait, B.T., and Shenk, T. (2010). Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein. Journal of virology *84*, 7803-7814.

Cuchet-Lourenco, D., Vanni, E., Glass, M., Orr, A., and Everett, R.D. (2012). Herpes simplex virus 1 ubiquitin ligase ICP0 interacts with PML isoform I and induces its SUMO-independent degradation. Journal of virology *86*, 11209-11222.

de Bruyn Kops, A., and Knipe, D.M. (1988). Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. Cell *55*, 857-868.

Delboy, M.G., and Nicola, A.V. (2011). A pre-immediate-early role for tegument ICP0 in the proteasome-dependent entry of herpes simplex virus. Journal of virology *85*, 5910-5918.

DeLuca, N.A., McCarthy, A.M., and Schaffer, P.A. (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. Journal of virology *56*, 558-570.

Deshmane, S.L., and Fraser, N.W. (1989). During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. Journal of virology *63*, 943-947.

Didcock, L., Young, D.F., Goodbourn, S., and Randall, R.E. (1999). The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. Journal of virology *73*, 9928-9933.

Duan, X., Ponomareva, L., Veeranki, S., Panchanathan, R., Dickerson, E., and Choubey, D. (2011). Differential roles for the interferon-inducible IFI16 and AIM2 innate immune sensors for cytosolic DNA in cellular senescence of human fibroblasts. Molecular cancer research : MCR *9*, 589-602.

Duggal, N.K., and Emerman, M. (2012). Evolutionary conflicts between viruses and restriction factors shape immunity. Nature reviews Immunology *12*, 687-695.

Eidson, K.M., Hobbs, W.E., Manning, B.J., Carlson, P., and DeLuca, N.A. (2002). Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. Journal of virology *76*, 2180-2191.

Everett, R.D. (1989). Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. The Journal of general virology *70 (Pt 5)*, 1185-1202.

Everett, R.D., Boutell, C., and Orr, A. (2004a). Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0. Journal of virology *78*, 1763-1774.

Everett, R.D., Freemont, P., Saitoh, H., Dasso, M., Orr, A., Kathoria, M., and Parkinson, J. (1998). The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. Journal of virology *72*, 6581-6591.

Everett, R.D., Meredith, M., and Orr, A. (1999). The ability of herpes simplex virus type 1 immediate-early protein Vmw110 to bind to a ubiquitin-specific protease contributes to its roles in the activation of gene expression and stimulation of virus replication. Journal of virology *73*, 417-426.

Everett, R.D., and Murray, J. (2005). ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection. Journal of virology *79*, 5078-5089.

Everett, R.D., Parada, C., Gripon, P., Sirma, H., and Orr, A. (2008a). Replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and Sp100. Journal of virology *82*, 2661-2672.

Everett, R.D., Sourvinos, G., Leiper, C., Clements, J.B., and Orr, A. (2004b). 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*, 1903-1917.

Everett, R.D., Young, D.F., Randall, R.E., and Orr, A. (2008b). STAT-1- and IRF-3-dependent pathways are not essential for repression of ICP0-null mutant herpes simplex virus type 1 in human fibroblasts. Journal of virology *82*, 8871-8881.

Ferenczy, M.W., and DeLuca, N.A. (2009). Epigenetic modulation of gene expression from quiescent herpes simplex virus genomes. Journal of virology *83*, 8514-8524.

Ferenczy, M.W., and DeLuca, N.A. (2011). Reversal of heterochromatic silencing of quiescent herpes simplex virus type 1 by ICP0. Journal of virology *85*, 3424-3435.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., Coyle, A.J., Liao, S.M., and Maniatis, T. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nature immunology *4*, 491-496.

Garber, D.A., Beverley, S.M., and Coen, D.M. (1993). Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. Virology *197*, 459-462.

Garber, D.A., Schaffer, P.A., and Knipe, D.M. (1997). A LAT-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. Journal of virology *71*, 5885-5893.

Gariano, G.R., Dell'oste, V., Bronzini, M., Gatti, D., Luganini, A., De Andrea, M., Gribaudo, G., Gariglio, M., and Landolfo, S. (2012). The Intracellular DNA Sensor IFI16 Gene Acts as Restriction Factor for Human Cytomegalovirus Replication. PLoS pathogens *8*, e1002498.

Glass, M., and Everett, R.D. (2012). Components of PML Nuclear Bodies (ND10) act cooperatively to repress herpesvirus infection. Journal of virology.

Goldin, A.L., Sandri-Goldin, R.M., Levine, M., and Glorioso, J.C. (1981). Cloning of herpes simplex virus type 1 sequences representing the whole genome. Journal of virology *38*, 50-58.

Grandvaux, N., Servant, M.J., tenOever, B., Sen, G.C., Balachandran, S., Barber, G.N., Lin, R., and Hiscott, J. (2002). Transcriptional profiling of interferon regulatory factor 3 target genes: direct involvement in the regulation of interferon-stimulated genes. Journal of virology *76*, 5532-5539.

Greco, A., Arata, L., Soler, E., Gaume, X., Coute, Y., Hacot, S., Calle, A., Monier, K., Epstein, A.L., Sanchez, J.C.*, et al.* (2012). Nucleolin Interacts with US11 Protein of Herpes Simplex Virus 1 and Is Involved in Its Trafficking. Journal of virology *86*, 1449-1457.

Hancock, M.H., Corcoran, J.A., and Smiley, J.R. (2006). Herpes simplex virus regulatory proteins VP16 and ICP0 counteract an innate intranuclear barrier to viral gene expression. Virology *352*, 237-252.

Hilton, L., Moganeradj, K., Zhang, G., Chen, Y.H., Randall, R.E., McCauley, J.W., and Goodbourn, S. (2006). The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. Journal of virology *80*, 11723-11732.

Hiscott, J. (2007). Triggering the innate antiviral response through IRF-3 activation. The Journal of biological chemistry *282*, 15325-15329.

Hochrein, H., Schlatter, B., O'Keeffe, M., Wagner, C., Schmitz, F., Schiemann, M., Bauer, S., Suter, M., and Wagner, H. (2004). Herpes simplex virus type-1 induces IFN-alpha production via Toll-like receptor 9-dependent and -independent pathways. Proceedings of the National Academy of Sciences of the United States of America *101*, 11416-11421.

Holm, C.K., Jensen, S.B., Jakobsen, M.R., Cheshenko, N., Horan, K.A., Moeller, H.B., Gonzalez-Dosal, R., Rasmussen, S.B., Christensen, M.H., Yarovinsky, T.O.*, et al.* (2012). Virus-cell fusion as a trigger of innate immunity dependent on the adaptor STING. Nature immunology *13*, 737-743.

Honess, R.W., and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. Journal of virology *14*, 8-19.

Horan, K.A., Hansen, K., Jakobsen, M.R., Holm, C.K., Soby, S., Unterholzner, L., Thompson, M., West, J.A., Iversen, M.B., Rasmussen, S.B.*, et al.* (2013). Proteasomal Degradation of Herpes Simplex Virus Capsids in Macrophages Releases DNA to the Cytosol for Recognition by DNA Sensors. J Immunol.

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D.R., Latz, E., and Fitzgerald, K.A. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature *458*, 514-518.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K.K., Schlee, M.*, et al.* (2006). 5'-Triphosphate RNA is the ligand for RIG-I. Science *314*, 994-997.

Ishii, K.J., Coban, C., Kato, H., Takahashi, K., Torii, Y., Takeshita, F., Ludwig, H., Sutter, G., Suzuki, K., Hemmi, H.*, et al.* (2006). A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nature immunology *7*, 40-48.

Ishikawa, H., and Barber, G.N. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature *455*, 674-678.

Ishikawa, H., Ma, Z., and Barber, G.N. (2009). STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature *461*, 788-792.

Ishov, A.M., Sotnikov, A.G., Negorev, D., Vladimirova, O.V., Neff, N., Kamitani, T., Yeh, E.T., Strauss, J.F., 3rd, and Maul, G.G. (1999). PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. The Journal of cell biology *147*, 221-234.

Iwasaki, A. (2012). A virological view of innate immune recognition. Annual review of microbiology *66*, 177-196.

Iwasaki, A., and Medzhitov, R. (2010). Regulation of adaptive immunity by the innate immune system. Science *327*, 291-295.

Jackson, D.A., Juranek, S., and Lipps, H.J. (2006). Designing nonviral vectors for efficient gene transfer and long-term gene expression. Molecular therapy : the journal of the American Society of Gene Therapy *14*, 613-626.

Janeway, C.A., Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harbor symposia on quantitative biology *54 Pt 1*, 1-13.

Jin, T., Perry, A., Jiang, J., Smith, P., Curry, J.A., Unterholzner, L., Jiang, Z., Horvath, G., Rathinam, V.A., Johnstone, R.W.*, et al.* (2012). Structures of the HIN domain:DNA complexes reveal ligand binding and activation mechanisms of the AIM2 inflammasome and IFI16 receptor. Immunity *36*, 561-571.

Johnson, D.C., and Baines, J.D. (2011). Herpesviruses remodel host membranes for virus egress. Nature reviews Microbiology *9*, 382-394.

Johnstone, R.W., Kerry, J.A., and Trapani, J.A. (1998). The human interferon-inducible protein, IFI 16, is a repressor of transcription. The Journal of biological chemistry *273*, 17172-17177.

Johnstone, R.W., Wei, W., Greenway, A., and Trapani, J.A. (2000). Functional interaction between p53 and the interferon-inducible nucleoprotein IFI 16. Oncogene *19*, 6033-6042.

Jovasevic, V., Liang, L., and Roizman, B. (2008). Proteolytic cleavage of VP1-2 is required for release of herpes simplex virus 1 DNA into the nucleus. Journal of virology *82*, 3311-3319.

Kagan, J.C. (2012). Signaling organelles of the innate immune system. Cell *151*, 1168-1178.

Karasneh, G.A., and Shukla, D. (2011). Herpes simplex virus infects most cell types in vitro: clues to its success. Virology journal *8*, 481.

Karin, M., and Greten, F.R. (2005). NF-kappaB: linking inflammation and immunity to cancer development and progression. Nature reviews Immunology *5*, 749-759.

Karyala, P., Namsa, N.D., and Chilakalapudi, D.R. (2010). Translational up-regulation and high-level protein expression from plasmid vectors by mTOR activation via different pathways in PC3 and 293T cells. PloS one *5*, e14408.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., Tsujimura, T., Takeda, K., Fujita, T., Takeuchi, O*., et al.* (2005). Cell type-specific involvement of RIG-I in antiviral response. Immunity *23*, 19-28.

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T.S., Fujita, T., and Akira, S. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. The Journal of experimental medicine *205*, 1601-1610.

Kaur, A., Sanford, H.B., Garry, D., Lang, S., Klumpp, S.A., Watanabe, D., Bronson, R.T., Lifson, J.D., Rosati, M., Pavlakis, G.N*., et al.* (2007). Ability of herpes simplex virus vectors to boost immune responses to DNA vectors and to protect against challenge by simian immunodeficiency virus. Virology *357*, 199-214.

Kawaguchi, Y., Bruni, R., and Roizman, B. (1997). Interaction of herpes simplex virus 1 alpha regulatory protein ICP0 with elongation factor 1delta: ICP0 affects translational machinery. Journal of virology *71*, 1019-1024.

Kawai, T., and Akira, S. (2009). The roles of TLRs, RLRs and NLRs in pathogen recognition. International immunology *21*, 317-337.

Kerur, N., Veettil, M.V., Sharma-Walia, N., Bottero, V., Sadagopan, S., Otageri, P., and Chandran, B. (2011). IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. Cell host & microbe *9*, 363-375.

Kim, T., Pazhoor, S., Bao, M., Zhang, Z., Hanabuchi, S., Facchinetti, V., Bover, L., Plumas, J., Chaperot, L., Qin, J.*, et al.* (2010). Aspartate-glutamate-alanine-histidine box motif (DEAH)/RNA helicase A helicases sense microbial DNA in human plasmacytoid dendritic cells. Proceedings of the National Academy of Sciences of the United States of America *107*, 15181-15186.

Kim, Y.E., Lee, J.H., Kim, E.T., Shin, H.J., Gu, S.Y., Seol, H.S., Ling, P.D., Lee, C.H., and Ahn, J.H. (2011). Human cytomegalovirus infection causes degradation of Sp100 proteins that suppress viral gene expression. Journal of virology *85*, 11928-11937.

Kishore, N., Huynh, Q.K., Mathialagan, S., Hall, T., Rouw, S., Creely, D., Lange, G., Caroll, J., Reitz, B., Donnelly, A.*, et al.* (2002). IKK-i and TBK-1 are enzymatically distinct from the homologous enzyme IKK-2: comparative analysis of recombinant human IKK-i, TBK-1, and IKK-2. The Journal of biological chemistry *277*, 13840-13847.

Knipe, D.M., and Cliffe, A. (2008). Chromatin control of herpes simplex virus lytic and latent infection. Nature reviews Microbiology *6*, 211-221.

Knipe, D.M., Senechek, D., Rice, S.A., and Smith, J.L. (1987). Stages in the nuclear association of the herpes simplex virus transcriptional activator protein ICP4. Journal of virology *61*, 276-284.

Knipe, D.M., and Spang, A.E. (1982). Definition of a series of stages in the association of two herpesviral proteins with the cell nucleus. Journal of virology *43*, 314-324.

Krug, A., Luker, G.D., Barchet, W., Leib, D.A., Akira, S., and Colonna, M. (2004). Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. Blood *103*, 1433-1437.

Kubota, T., Matsuoka, M., Chang, T.H., Tailor, P., Sasaki, T., Tashiro, M., Kato, A., and Ozato, K. (2008). Virus infection triggers SUMOylation of IRF3 and IRF7, leading to the negative regulation of type I interferon gene expression. The Journal of biological chemistry *283*, 25660-25670.

Kurt-Jones, E.a., Chan, M., Zhou, S., Wang, J., Reed, G., Bronson, R., Arnold, M.M., Knipe, D.M., and Finberg, R.W. (2004). Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. In Proceedings of the National Academy of Sciences of the United States of America, pp. 1315-1320.

Kwak, J.C., Ongusaha, P.P., Ouchi, T., and Lee, S.W. (2003). IFI16 as a negative regulator in the regulation of p53 and p21(Waf1). The Journal of biological chemistry *278*, 40899-40904.

Lacasse, J.J., and Schang, L.M. (2010). During lytic infections, herpes simplex virus type 1 DNA is in complexes with the properties of unstable nucleosomes. Journal of virology *84*, 1920-1933.

Lafaille, F.G., Pessach, I.M., Zhang, S.Y., Ciancanelli, M.J., Herman, M., Abhyankar, A., Ying, S.W., Keros, S., Goldstein, P.A., Mostoslavsky, G.*, et al.* (2012). Impaired intrinsic immunity to HSV-1 in human iPSC-derived TLR3-deficient CNS cells. Nature.

Lallemand-Breitenbach, V., and de The, H. (2010). PML nuclear bodies. Cold Spring Harbor perspectives in biology *2*, a000661.

Latz, E., Verma, A., Visintin, A., Gong, M., Sirois, C.M., Klein, D.C., Monks, B.G., McKnight, C.J., Lamphier, M.S., Duprex, W.P.*, et al.* (2007). Ligand-induced conformational changes allosterically activate Toll-like receptor 9. Nature immunology *8*, 772-779.

Leib, D.A., Harrison, T.E., Laslo, K.M., Machalek, M.A., Moorman, N.J., and Virgin, H.W. (1999). Interferons regulate the phenotype of wild-type and mutant herpes simplex viruses in vivo. The Journal of experimental medicine *189*, 663-672.

Leoni, V., Gianni, T., Salvioli, S., and Campadelli-Fiume, G. (2012). Herpes simplex virus glycoproteins gH/gL and gB bind Toll-like receptor 2, and soluble gH/gL is sufficient to activate NF-kappaB. Journal of virology *86*, 6555-6562.

Li, T., Diner, B.A., Chen, J., and Cristea, I.M. (2012). Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16. Proceedings of the National Academy of Sciences of the United States of America *109*, 10558-10563.

Lilley, C.E., Carson, C.T., Muotri, A.R., Gage, F.H., and Weitzman, M.D. (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*, 5844-5849.

Lilley, C.E., Chaurushiya, M.S., Boutell, C., Landry, S., Suh, J., Panier, S., Everett, R.D., Stewart, G.S., Durocher, D., and Weitzman, M.D. (2010). A viral E3 ligase targets RNF8 and RNF168 to control histone ubiquitination and DNA damage responses. The EMBO journal *29*, 943-955.

Lin, R., Heylbroeck, C., Pitha, P.M., and Hiscott, J. (1998). Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. Molecular and cellular biology *18*, 2986-2996.

Lin, R., Mamane, Y., and Hiscott, J. (1999). Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. Molecular and cellular biology *19*, 2465-2474.

Lin, R., Noyce, R.S., Collins, S.E., Everett, R.D., and Mossman, K.L. (2004). The herpes simplex virus ICP0 RING finger domain inhibits IRF3- and IRF7-mediated activation of interferon-stimulated genes. Journal of virology *78*, 1675-1684.

Liu, C.J., Wang, H., and Lengyel, P. (1999). The interferon-inducible nucleolar p204 protein binds the ribosomal RNA-specific UBF1 transcription factor and inhibits ribosomal RNA transcription. The EMBO journal *18*, 2845-2854.

Lium, E.K., Panagiotidis, C.A., Wen, X., and Silverstein, S. (1996). Repression of the alpha0 gene by ICP4 during a productive herpes simplex virus infection. Journal of virology *70*, 3488-3496.

Lium, E.K., Panagiotidis, C.A., Wen, X., and Silverstein, S.J. (1998). The NH2 terminus of the herpes simplex virus type 1 regulatory protein ICP0 contains a promoter-specific transcription activation domain. Journal of virology *72*, 7785-7795.

Lium, E.K., and Silverstein, S. (1997). Mutational analysis of the herpes simplex virus type 1 ICP0 C3HC4 zinc ring finger reveals a requirement for ICP0 in the expression of the essential alpha27 gene. Journal of virology *71*, 8602-8614.

Lopez, P., Van Sant, C., and Roizman, B. (2001). Requirements for the nuclear-cytoplasmic translocation of infected-cell protein 0 of herpes simplex virus 1. Journal of virology *75*, 3832-3840.

Lukashchuk, V., and Everett, R.D. (2010). Regulation of ICP0-null mutant herpes simplex virus type 1 infection by ND10 components ATRX and hDaxx. Journal of virology *84*, 4026-4040.

Luker, G.D., Prior, J.L., Song, J., Pica, C.M., and Leib, D.A. (2003). Bioluminescence imaging reveals systemic dissemination of herpes simplex virus type 1 in the absence of interferon receptors. Journal of virology *77*, 11082-11093.

Lund, J., Sato, A., Akira, S., Medzhitov, R., and Iwasaki, A. (2003). Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. The Journal of experimental medicine *198*, 513-520.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Iwasaki, A., and Flavell, R.A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proceedings of the National Academy of Sciences of the United States of America *101*, 5598-5603.

Ma, Y., Jin, H., Valyi-Nagy, T., Cao, Y., Yan, Z., and He, B. (2012). Inhibition of TANK binding kinase 1 by herpes simplex virus 1 facilitates productive infection. Journal of virology *86*, 2188-2196.

Martinez-Balbas, M.A., Bannister, A.J., Martin, K., Haus-Seuffert, P., Meisterernst, M., and Kouzarides, T. (1998). The acetyltransferase activity of CBP stimulates transcription. The EMBO journal *17*, 2886-2893.

Maul, G.G., Ishov, A.M., and Everett, R.D. (1996). Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. Virology *217*, 67-75.

Mboko, W.P., Mounce, B.C., Wood, B.M., Kulinski, J.M., Corbett, J.A., and Tarakanova, V.L. (2012). Coordinate regulation of DNA damage and type I interferon responses imposes an antiviral state that attenuates mouse gammaherpesvirus type 68 replication in primary macrophages. Journal of virology *86*, 6899-6912.

McFarlane, S., and Preston, C.M. (2011). Human cytomegalovirus immediate early gene expression in the osteosarcoma line U2OS is repressed by the cell protein ATRX. Virus research *157*, 47-53.

McInerney, G.M., and Karlsson Hedestam, G.B. (2009). Direct cleavage, proteasomal degradation and sequestration: three mechanisms of viral subversion of type I interferon responses. Journal of innate immunity *1*, 599-606.

McWhirter, S.M., Fitzgerald, K.A., Rosains, J., Rowe, D.C., Golenbock, D.T., and Maniatis, T. (2004). IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. Proceedings of the National Academy of Sciences of the United States of America *101*, 233-238.

Melchjorsen, J., Rintahaka, J., Soby, S., Horan, K.A., Poltajainen, A., Ostergaard, L., Paludan, S.R., and Matikainen, S. (2010). Early innate recognition of herpes simplex

virus in human primary macrophages is mediated via the MDA5/MAVS-dependent and MDA5/MAVS/RNA polymerase III-independent pathways. Journal of virology *84*, 11350-11358.

Melroe, G.T., DeLuca, N.A., and Knipe, D.M. (2004). Herpes simplex virus 1 has multiple mechanisms for blocking virus-induced interferon production. Journal of virology *78*, 8411-8420.

Melroe, G.T., Silva, L., Schaffer, P.A., and Knipe, D.M. (2007). Recruitment of activated IRF-3 and CBP/p300 to herpes simplex virus ICP0 nuclear foci: Potential role in blocking IFN-beta induction. Virology *360*, 305-321.

Menachery, V.D., and Leib, D.A. (2009). Control of herpes simplex virus replication is mediated through an interferon regulatory factor 3-dependent pathway. Journal of virology *83*, 12399-12406.

Menachery, V.D., Pasieka, T.J., and Leib, D.A. (2010). Interferon regulatory factor 3-dependent pathways are critical for control of herpes simplex virus type 1 central nervous system infection. Journal of virology *84*, 9685-9694.

Mossman, K.L., Macgregor, P.F., Rozmus, J.J., Goryachev, A.B., Edwards, A.M., and Smiley, J.R. (2001). Herpes simplex virus triggers and then disarms a host antiviral response. Journal of virology *75*, 750-758.

Mossman, K.L., and Smiley, J.R. (2002). Herpes simplex virus ICP0 and ICP34.5 counteract distinct interferon-induced barriers to virus replication. Journal of virology *76*, 1995-1998.

Mullen, M.A., Ciufo, D.M., and Hayward, G.S. (1994). Mapping of intracellular localization domains and evidence for colocalization interactions between the IE110 and IE175 nuclear transactivator proteins of herpes simplex virus. Journal of virology *68*, 3250-3266.

Muller, S., and Dejean, A. (1999). Viral immediate-early proteins abrogate the modification by SUMO-1 of PML and Sp100 proteins, correlating with nuclear body disruption. Journal of virology *73*, 5137-5143.

Nacerddine, K., Lehembre, F., Bhaumik, M., Artus, J., Cohen-Tannoudji, M., Babinet, C., Pandolfi, P.P., and Dejean, A. (2005). The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. Developmental cell *9*, 769-779.

Neumann, D.M., Bhattacharjee, P.S., Giordani, N.V., Bloom, D.C., and Hill, J.M. (2007). In vivo changes in the patterns of chromatin structure associated with the latent herpes simplex virus type 1 genome in mouse trigeminal ganglia can be detected at early times after butyrate treatment. Journal of virology *81*, 13248-13253.

Nicola, A.V., McEvoy, A.M., and Straus, S.E. (2003). Roles for endocytosis and low pH in herpes simplex virus entry into HeLa and Chinese hamster ovary cells. Journal of virology *77*, 5324-5332.

Oh, J., and Fraser, N.W. (2008). Temporal association of the herpes simplex virus genome with histone proteins during a lytic infection. Journal of virology *82*, 3530-3537.

Oh, J., Ruskoski, N., and Fraser, N.W. (2012). Chromatin assembly on herpes simplex virus 1 DNA early during a lytic infection is Asf1a dependent. Journal of virology *86*, 12313-12321.

Ojala, P.M., Sodeik, B., Ebersold, M.W., Kutay, U., and Helenius, A. (2000). Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. Molecular and cellular biology *20*, 4922-4931.

Orzalli, M.H., Deluca, N.A., and Knipe, D.M. (2012). Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein. Proceedings of the National Academy of Sciences of the United States of America *109*, E3008-3017.

Paladino, P., Collins, S.E., and Mossman, K.L. (2010). Cellular localization of the herpes simplex virus ICP0 protein dictates its ability to block IRF3-mediated innate immune responses. PloS one *5*, e10428.

Paladino, P., Cummings, D.T., Noyce, R.S., and Mossman, K.L. (2006). The IFN-independent response to virus particle entry provides a first line of antiviral defense that is independent of TLRs and retinoic acid-inducible gene I. J Immunol *177*, 8008-8016.

Paludan, S.R., Bowie, A.G., Horan, K.A., and Fitzgerald, K.A. (2011). Recognition of herpesviruses by the innate immune system. Nature reviews Immunology *11*, 143-154.

Perez-Caballero, D., Zang, T., Ebrahimi, A., McNatt, M.W., Gregory, D.A., Johnson, M.C., and Bieniasz, P.D. (2009). Tetherin inhibits HIV-1 release by directly tethering virions to cells. Cell *139*, 499-511.

Placek, B.J., Huang, J., Kent, J.R., Dorsey, J., Rice, L., Fraser, N.W., and Berger, S.L. (2009). The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. Journal of virology *83*, 1416-1421.

Preston, C.M., Harman, A.N., and Nicholl, M.J. (2001). Activation of interferon response factor-3 in human cells infected with herpes simplex virus type 1 or human cytomegalovirus. Journal of virology *75*, 8909-8916.

Qin, B.Y., Liu, C., Lam, S.S., Srinath, H., Delston, R., Correia, J.J., Derynck, R., and Lin, K. (2003). Crystal structure of IRF-3 reveals mechanism of autoinhibition and virus-induced phosphoactivation. Nature structural biology *10*, 913-921.

Rasmussen, S.B., Sorensen, L.N., Malmgaard, L., Ank, N., Baines, J.D., Chen, Z.J., and Paludan, S.R. (2007). Type I interferon production during herpes simplex virus infection is controlled by cell-type-specific viral recognition through Toll-like receptor 9, the mitochondrial antiviral signaling protein pathway, and novel recognition systems. Journal of virology *81*, 13315-13324.

Reynolds, A.E., Liang, L., and Baines, J.D. (2004). Conformational changes in the nuclear lamina induced by herpes simplex virus type 1 require genes U(L)31 and U(L)34. Journal of virology *78*, 5564-5575.

Riu, E., Chen, Z.Y., Xu, H., He, C.Y., and Kay, M.A. (2007). Histone modifications are associated with the persistence or silencing of vector-mediated transgene expression in vivo. Molecular therapy : the journal of the American Society of Gene Therapy *15*, 1348-1355.

Roizman, B., Knipe, D.M., and Whitley, R.J. (2013). Fields Virology, 6th edn (Lippincott Williams & Wilkins).

Samaniego, L.A., Neiderhiser, L., and DeLuca, N.A. (1998). Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. Journal of virology *72*, 3307-3320.

Sarkar, S.N., Peters, K.L., Elco, C.P., Sakamoto, S., Pal, S., and Sen, G.C. (2004). Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nature structural & molecular biology *11*, 1060-1067.

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N.*, et al.* (2000). Distinct and essential roles of

transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity *13*, 539-548.

Schulz, O., Diebold, S.S., Chen, M., Naslund, T.I., Nolte, M.A., Alexopoulou, L., Azuma, Y.T., Flavell, R.A., Liljestrom, P., and Reis e Sousa, C. (2005). Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature *433*, 887-892.

Sen, J., Liu, X., Roller, R., and Knipe, D.M. (2013). Herpes simplex virus US3 tegument protein inhibits Toll-like receptor 2 signaling at or before TRAF6 ubiquitination. Virology *439*, 65-73.

Shalginskikh, N., Poleshko, A., Skalka, A.M., and Katz, R.A. (2013). Retroviral DNA methylation and epigenetic repression are mediated by the antiviral host protein daxx. Journal of virology *87*, 2137-2150.

Sharma, S., and Fitzgerald, K.A. (2011). Innate immune sensing of DNA. PLoS pathogens *7*, e1001310.

Shi, H.X., Yang, K., Liu, X., Liu, X.Y., Wei, B., Shan, Y.F., Zhu, L.H., and Wang, C. (2010). Positive regulation of interferon regulatory factor 3 activation by Herc5 via ISG15 modification. Molecular and cellular biology *30*, 2424-2436.

Shirata, N., Kudoh, A., Daikoku, T., Tatsumi, Y., Fujita, M., Kiyono, T., Sugaya, Y., Isomura, H., Ishizaki, K., and Tsurumi, T. (2005). Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. The Journal of biological chemistry *280*, 30336-30341.

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

Silva, L., Cliffe, A., Chang, L., and Knipe, D.M. (2008). Role for A-type lamins in herpesviral DNA targeting and heterochromatin modulation. PLoS pathogens *4*, e1000071.

Simpson-Holley, M., Baines, J., Roller, R., and Knipe, D.M. (2004). Herpes simplex virus 1 U(L)31 and U(L)34 gene products promote the late maturation of viral replication compartments to the nuclear periphery. Journal of virology *78*, 5591-5600.

Sorensen, L.N., Reinert, L.S., Malmgaard, L., Bartholdy, C., Thomsen, A.R., and Paludan, S.R. (2008). TLR2 and TLR9 synergistically control herpes simplex virus infection in the brain. J Immunol *181*, 8604-8612.

Spear, P.G. (2004). Herpes simplex virus: receptors and ligands for cell entry. Cellular microbiology *6*, 401-410.

Speck, S.H., and Ganem, D. (2010). Viral latency and its regulation: lessons from the gamma-herpesviruses. Cell host & microbe *8*, 100-115.

Stark, G.R., and Darnell, J.E., Jr. (2012). The JAK-STAT pathway at twenty. Immunity *36*, 503-514.

Stetson, D.B., and Medzhitov, R. (2006). Type I interferons in host defense. Immunity *25*, 373-381.

Stow, N.D., and Stow, E.C. (1986). Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. The Journal of general virology *67 (Pt 12)*, 2571-2585.

Sun, L., Wu, J., Du, F., Chen, X., and Chen, Z.J. (2012). Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. Science.

Takaoka, A., Wang, Z., Choi, M.K., Yanai, H., Negishi, H., Ban, T., Lu, Y., Miyagishi, M., Kodama, T., Honda, K.*, et al.* (2007). DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature *448*, 501-505.

Taylor, T.J. (2002). Intranuclear localization of the herpes simplex virus ICP8 protein (Harvard University).

Taylor, T.J., and Knipe, D.M. (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*, 5856-5866.

Thompson, R.L., and Sawtell, N.M. (2000). Replication of herpes simplex virus type 1 within trigeminal ganglia is required for high frequency but not high viral genome copy number latency. Journal of virology *74*, 965-974.

Unterholzner, L., Keating, S.E., Baran, M., Horan, K.A., Jensen, S.B., Sharma, S., Sirois, C.M., Jin, T., Latz, E., Xiao, T.S.*, et al.* (2010). IFI16 is an innate immune sensor for intracellular DNA. Nature immunology *11*, 997-1004.

Vanni, E., Gatherer, D., Tong, L., Everett, R.D., and Boutell, C. (2012). Functional Characterization of Residues Required for the Herpes Simplex Virus 1 E3 Ubiquitin Ligase ICP0 To Interact with the Cellular E2 Ubiquitin-Conjugating Enzyme UBE2D1 (UbcH5a). Journal of virology *86*, 6323-6333.

Veeranki, S., and Choubey, D. (2012). Interferon-inducible p200-family protein IFI16, an innate immune sensor for cytosolic and nuclear double-stranded DNA: regulation of subcellular localization. Molecular immunology *49*, 567-571.

Verpooten, D., Ma, Y., Hou, S., Yan, Z., and He, B. (2009). Control of TANK-binding kinase 1-mediated signaling by the gamma(1)34.5 protein of herpes simplex virus 1. The Journal of biological chemistry *284*, 1097-1105.

Viswanathan, K., Fruh, K., and DeFilippis, V. (2010). Viral hijacking of the host ubiquitin system to evade interferon responses. Current opinion in microbiology *13*, 517-523.

Wald, A., and Link, K. (2002). Risk of human immunodeficiency virus infection in herpes simplex virus type 2-seropositive persons: a meta-analysis. The Journal of infectious diseases *185*, 45-52.

Wang, Q.Y., Zhou, C., Johnson, K.E., Colgrove, R.C., Coen, D.M., and Knipe, D.M. (2005). Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. Proceedings of the National Academy of Sciences of the United States of America *102*, 16055-16059.

Wang, Z., Choi, M.K., Ban, T., Yanai, H., Negishi, H., Lu, Y., Tamura, T., Takaoka, A., Nishikura, K., and Taniguchi, T. (2008). Regulation of innate immune responses by DAI (DLM-1/ZBP1) and other DNA-sensing molecules. Proceedings of the National Academy of Sciences of the United States of America *105*, 5477-5482.

Wathelet, M.G., Berr, P.M., and Huez, G.A. (1992). Regulation of gene expression by cytokines and virus in human cells lacking the type-I interferon locus. European journal of biochemistry / FEBS *206*, 901-910.

Wathelet, M.G., Clauss, I.M., Content, J., and Huez, G.A. (1988). Regulation of two interferon-inducible human genes by interferon, poly(rI).poly(rC) and viruses. European journal of biochemistry / FEBS *174*, 323-329.

Wathelet, M.G., Lin, C.H., Parekh, B.S., Ronco, L.V., Howley, P.M., and Maniatis, T. (1998). Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. Molecular cell *1*, 507-518.

Weaver, B.K., Kumar, K.P., and Reich, N.C. (1998). Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAF1. Molecular and cellular biology *18*, 1359-1368.

Wilkinson, D.E., and Weller, S.K. (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*, 4783-4796.

Wittels, M., and Spear, P.G. (1991). Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. Virus research *18*, 271-290.

Wolf, D., and Goff, S.P. (2008). Host restriction factors blocking retroviral replication. Annual review of genetics *42*, 143-163.

Wu, J., Sun, L., Chen, X., Du, F., Shi, H., Chen, C., and Chen, Z.J. (2012). Cyclic GMP-AMP Is an Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA. Science.

Xin, H., Curry, J., Johnstone, R.W., Nickoloff, B.J., and Choubey, D. (2003). Role of IFI 16, a member of the interferon-inducible p200-protein family, in prostate epithelial cellular senescence. Oncogene *22*, 4831-4840.

Xing, J., Wang, S., Lin, R., Mossman, K.L., and Zheng, C. (2012). Herpes simplex virus 1 tegument protein US11 downmodulates the RLR signaling pathway via direct interaction with RIG-I and MDA-5. Journal of virology *86*, 3528-3540.

Xu, F., Sternberg, M.R., Kottiri, B.J., McQuillan, G.M., Lee, F.K., Nahmias, A.J., Berman, S.M., and Markowitz, L.E. (2006). Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. JAMA : the journal of the American Medical Association *296*, 964-973.

Yao, F., and Courtney, R.J. (1992). Association of ICP0 but not ICP27 with purified virions of herpes simplex virus type 1. Journal of virology *66*, 2709-2716.

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E., and Fujita, T. (1998). Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. The EMBO journal *17*, 1087-1095.

Yu, Y., and Hayward, G.S. (2010). The ubiquitin E3 ligase RAUL negatively regulates type i interferon through ubiquitination of the transcription factors IRF7 and IRF3. Immunity *33*, 863-877.

Zhang, S.Y., Jouanguy, E., Ugolini, S., Smahi, A., Elain, G., Romero, P., Segal, D., Sancho-Shimizu, V., Lorenzo, L., Puel, A.*, et al.* (2007). TLR3 deficiency in patients with herpes simplex encephalitis. Science *317*, 1522-1527.

Zhang, Z., Yuan, B., Bao, M., Lu, N., Kim, T., and Liu, Y.J. (2011). The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. Nature immunology *12*, 959-965.

Zhong, B., Zhang, L., Lei, C., Li, Y., Mao, A.P., Yang, Y., Wang, Y.Y., Zhang, X.L., and Shu, H.B. (2009). The ubiquitin ligase RNF5 regulates antiviral responses by mediating degradation of the adaptor protein MITA. Immunity *30*, 397-407.

Zhong, S., Muller, S., Ronchetti, S., Freemont, P.S., Dejean, A., and Pandolfi, P.P. (2000). Role of SUMO-1-modified PML in nuclear body formation. Blood *95*, 2748-2752.

Zhu, H., Zheng, C., Xing, J., Wang, S., Li, S., Lin, R., and Mossman, K.L. (2011). Varicella-zoster virus immediate-early protein ORF61 abrogates the IRF3-mediated innate immune response through degradation of activated IRF3. Journal of virology *85*, 11079-11089.