TRIM27 in HSV-1 Infection: A Story of Loss and Death

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TRIM27 in HSV-1 Infection: A Story of Loss and Death

A dissertation presented
by
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to
The Division of Medical Sciences

in partial fulfillment of the requirements
for the degree of
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TRIM27 in HSV-1 Infection: A Story of Loss and Death

Abstract
During viral infection, the host subjects the virus to an array of protective mechanisms. Viruses have evolved evasion strategies to thwart these defenses, and must simultaneously regulate multiple cellular pathways and resources to achieve successful replication. To orchestrate such complex regulation, viruses, including herpes simplex virus 1 (HSV-1), rely on multifunctional proteins such as the E3 ubiquitin ligase ICP0. This protein counteracts various host defenses by targeting cellular factors for degradation. We undertook a proteomic screen to identify binding partners of ICP0, and identified the Tripartite Motif 27 (TRIM27) protein, a cellular transcriptional repressor, as a novel interacting protein of ICP0. This interaction resulted in rapid loss of TRIM27 during HSV-1 infection. However, replication of an ICP0-null mutant virus required TRIM27, suggesting a complex interaction between TRIM27 and viral infection.

To further characterize regulation of TRIM27 by HSV-1, we evaluated whether infection affected TRIM27 levels independently of ICP0. Infection with an ICP0-null virus resulted in TRIM27 protein loss, but at a greatly reduced rate. TRIM27 protein exhibited a short half-life in uninfected cells, indicating that viral regulation of transcript levels could affect protein levels during infection. HSV-1 reduced TRIM27 transcripts through the virion host shutoff (VHS) function and a global inhibition of host
transcription. The compound regulation of TRIM27 levels during infection demonstrated the redundant mechanisms by which HSV-1 regulates the cellular proteome.

Because degradation targets of ICP0 often function to restrict viral infection, we hypothesized that TRIM27 could contribute to an antiviral pathway. Based on the involvement of TRIM27 in programmed cell death resulting from Tumor Necrosis Factor (TNF) signaling, we evaluated the role of TRIM27 in cells treated with TNF. TRIM27 was required for TNF-dependent programmed necrosis, or necroptosis, in mouse cells. HSV-1 infection of these cells induced TNF-dependent necroptosis, reducing viral yield. This pathway required mouse TRIM27, and cells expressing human TRIM27 in place of mouse TRIM27 did not exhibit necroptosis. The differing capacities of mouse and human TRIM27 to support necroptosis during HSV-1 infection suggested that TRIM27 contributes to species-specific restriction of HSV-1 and to the selective pressure driving viral evasion of this protective host response.
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Chapter One: Introduction
1.1 Classification and Properties of Herpesviruses

1.1.1 The Family Herpesviridae

The family *Herpesviridae*, of the order *Herpesvirales*, spans the 200 herpesviruses that claim mammals, birds and reptiles as their hosts. Although these viruses represent a diverse set of pathogens, they share distinct structural features and exhibit characteristic biological properties. The herpes virion consists of an encapsidated, double-stranded DNA genome, surrounded by a proteinaceous tegument and enveloped in an outer lipid bilayer. One hallmark of herpesviruses is their ability to undergo both lytic and latent infection. Lytic infection results in death of the host cell, while latency allows the virus to persist in its host organism. Another defining feature of herpesviruses is their nuclear replication. These shared properties arise from a set of 41 core genes, which facilitate the nucleotide metabolism, DNA replication, gene regulation, virion assembly, and virion structure that are common to this set of pathogens (Roizman, 2013).

The family *Herpesviridae* is divided into three subfamilies based on host range, tissue tropism, and infection kinetics (Roizman et al., 1981). The *Alphaherpesvirinae* subfamily includes the human pathogens herpes simplex virus (HSV)-1, HSV-2, and varicella zoster virus (VZV), and features a large host range and rapid cell death following a short replication cycle. Viruses of the *Betaherpesvirinae* subfamily are characterized by a more limited host range with slower kinetics of infection, and include human cytomegalovirus (HCMV). The *Gammaherpesvirinae* subfamily is typically restricted to infecting lymphocytes of a single host, and includes both Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV).
1.1.2 Herpes Simplex Viruses

Herpes simplex virus (HSV) infection results from direct exposure of mucosal surfaces to HSV-1 or HSV-2. These two viruses are highly related, with 83% similarity in their genomes (Dolan et al., 1998). Estimates suggest that 54% of Americans are seropositive for HSV-1 and 16% are seropositive for HSV-2 (Bradley et al., 2014). Primary infection occurs in epithelial cells of the oral mucosa (HSV-1) or the genital mucosa (HSV-2). While HSV-1 is typically associated with orolabial lesions, it is becoming an increasingly frequent cause of genital lesions as well (Xu et al., 2006). Following the initial lytic stage of infection, virus spreads to the sensory ganglia, where it establishes latent infection and persists in the host. Periodic reactivation of productive infection leads to recurrent lesions. Although these benign lesions are the most common symptom associated with HSV-1, under rare circumstances infection can cause serious conditions including blindness (Binder, 1977) and fatal encephalitis (Olson et al., 1967).

1.1.3 Virion Structure and Entry

The HSV-1 virion is approximately 200nm in diameter. Each virion contains a single copy of the double-stranded DNA genome, packaged in an icosahedral capsid. An unstructured but somewhat ordered layer of 18 proteins, termed the tegument, lies between the capsid and the lipid envelope of the virus (Roizman, 2013).

The viral envelope is decorated by at least 13 glycoproteins, (including gB, gC, gD, gH, and gL). These proteins interact with various molecules on the cellular membrane to facilitate entry of the virus into the cell, either by fusion with the plasma
membrane or by endocytosis [reviewed in (Krummenacher et al., 2013)]. The virion first docks on the cell surface through interactions of gB and gC with cellular glycosaminoglycans. Any of three cellular receptors can then be bound by gD, including nectin, herpesvirus entry mediator (HVEM), and 3-O-sulfated heparan sulfate. Receptor binding induces fusion of the viral envelope with the cellular membrane, mediated by the gH/gL heterodimer and gB.

Membrane fusion releases the viral capsid and tegument proteins into the cytoplasm of the host cell. The capsid is transported to the nuclear membrane, where the viral genome is released into the nucleus through a nuclear pore (Batterson and Roizman, 1983; Ojala et al., 2000). Tegument proteins include the virion host shutoff function (VHS), which is an endonuclease that degrades polysome-bound mRNAs of both cellular and viral genes (Kwong and Frenkel, 1987), and VP16, the transcriptional activator that initiates viral gene expression (Campbell et al., 1984; McKnight et al., 1987). After VP16 is released into the cytoplasm following viral entry, it interacts with the cellular transcription factor HCF, and both proteins translocate into the nucleus (La Boissiere et al., 1999). In the nucleus, VP16 interacts with other cellular transcription factors to recruit cellular RNA polymerase II to viral promoters to activate transcription of viral genes (Herrera and Triezenberg, 2004).

1.1.4 Lytic Infection

During lytic, or productive, infection, the virus diverts cellular resources and macromolecular synthesis machinery to produce viral proteins and replicate its genome, leading to virion assembly and egress. The virus exerts control over the cellular
environment throughout infection, while evading the many protective mechanisms of the host. For successful productive infection, the virus must orchestrate a complex array of functions. The gene products responsible for these functions are expressed in a cascade, consisting of immediate-early (IE) genes, early (E) genes, and late (L) genes (Honess and Roizman, 1974). The tegument protein VP16 brought in with the virion recruits cellular transcription factors to IE promoters to initiate this cascade by transactivating expression of the IE genes (Campbell et al., 1984).

The IE genes encode two proteins that are essential during infection, ICP4 and ICP27, and four proteins that may increase the efficiency of infection but are not absolutely required (ICP0, ICP22, ICP47, and U₅1.5). ICP4 is a DNA-binding protein that functions as the major regulator of viral transcription (Michael and Roizman, 1989), with both activating and repressive effects (Faber and Wilcox, 1986; Smith et al., 1993). ICP27 is a multifunctional regulatory protein that inhibits pre-mRNA splicing to reduce host protein synthesis (Hardy and Sandri-Goldin, 1994) and is also essential in activating expression of viral genes (Rice and Knipe, 1988; Uprichard and Knipe, 1996).

The products of the IE genes, particularly ICP4, ICP27, and ICP0, are required for the activation of the early (E) and late (L) gene classes. The E and L genes are distinguished by the dependence of L gene expression on viral DNA synthesis. Many proteins encoded by the E gene class are directly involved in viral DNA replication and nucleotide metabolism, while L proteins include the virion components, tegument proteins, and mediators of assembly and egress. Viral capsids are assembled in the nucleus (Gibson and Roizman, 1972). Once they are loaded with a single copy of the newly synthesized viral genome, they must be transported out of the cell, passing
through the nuclear membrane, the cytoplasm, and the plasma membrane *en route*. While multiple models of virion egress exist, the dominant model suggests that capsids are first enveloped and then de-enveloped as they traverse the two layers of the nuclear membrane (Roizman, 2013). Capsids and their associated tegument proteins then bud into cytoplasmic vesicles derived from the *trans*-Golgi network, and are subsequently transported to the plasma membrane where they are released from the cell.

1.1.5 *Latent Infection*  
Primary infection of epithelial cells results in cell lysis and the release of viral progeny. The latent phase of infection occurs in sensory neurons of the dorsal root ganglia, and allows the virus to persist in its host. Following entry at the axonal termini, virions reach the cell body by retrograde transport (Bearer et al., 2000). Once in the nucleus, the viral genome is bound by nucleosomes (Deshmane and Fraser, 1989) and maintained as an episome in the latently infected cell. The primary gene product during this phase is the latency-associated transcript (LAT) (Stevens et al., 1987), which is thought to contribute to the silencing of the lytic gene products by promoting the assembly of heterochromatin marks on the viral genome (Cliffe et al., 2009; Wang et al., 2005). In response to various stimuli such as stress, fever, or ultraviolet light, the virus reactivates from its latent state, entering a productive phase which releases viral progeny from the axonal termini to induce recurrent, lytic infection in the mucosal epithelium.
1.2 HSV-1 ICP0

1.2.1 Structural Elements of ICP0

The immediate early protein ICP0 of HSV-1 is a multifunctional molecule. A major biochemical function of ICP0 is its E3 ubiquitin ligase activity, which is mediated by its zinc-binding, really interesting new gene (RING)-finger domain and promotes proteasome-dependent degradation of various proteins. Ubiquitination by ICP0 requires one of two E2 enzymes, UBE2D1 or UBE2E1 (Boutell et al., 2002). ICP0 is also capable of autoubiquitination (Canning et al., 2004), leading to its destabilization. Interaction of ICP0 with the deubiquitinase USP7 protects ICP0 from autoubiquitination, while resulting in ubiquitination and loss of USP7 during infection (Boutell et al., 2005). ICP0 contains a nuclear localization signal (Everett, 1988), which targets it to the nucleus early in infection. As infection proceeds and viral DNA synthesis occurs, ICP0 is shuttled to the cytoplasm (Lopez et al., 2001). At the C-terminus, ICP0 encodes a sequence implicated in dimerization and multimerization (Ciufo et al., 1994).

The DNA sequence of ICP0 exhibits 98% identity among six HSV-1 strains (Watson et al., 2012). While HSV-1 and HSV-2 ICP0 exhibit only 62% identity (McGeoch et al., 1991), the RING finger domain, nuclear localization signal, and multimerization sequence are the most highly conserved regions (Halford et al., 2010). Viruses expressing orthologues of ICP0 include bovine herpesvirus 1 (BICP0), equine herpesvirus 1 (Eg63), pseudorabies virus (EP0), and VZV (Vg61) (Reviewed in Parkinson and Everett, 2000)]. Although these orthologues exhibit high sequence variability, the RING domain remains conserved (Parkinson and Everett, 2000). The beta and gamma herpesviruses also express proteins that share functions with ICP0.
For example, HCMV IE1 and EBV BZLF1 promote degradation of PML, and some of the other functions of ICP0 are carried out by additional viral proteins (Salsman et al., 2008).

1.2.2 ICP0 Function During Infection

HSV-1 ICP0 is required for efficient infection (Sacks and Schaffer, 1987). While the expression of viral genes and production of viral progeny are generally impaired in the absence of ICP0, the fitness of an ICP0-null virus depends on the multiplicity of infection (MOI) and the cell type. For example, an ICP0-null virus behaves like wild-type virus in U2OS cells, but in Vero cells, gene expression is reduced during infection with an ICP0-null virus at low MOI (Yao and Schaffer, 1995).

The reduced gene expression observed with ICP0-null mutant virus infection is partly a direct effect of deleting ICP0, as this protein normally transactivates expression of all three classes of viral genes (Cai and Schaffer, 1992). ICP0 is a promiscuous transactivator of gene expression, and even promotes transcription from exogenous DNA transfected into the cell (Quinlan and Knipe, 1985). The mechanism underlying ICP0’s transactivation function is unclear, as it does not bind DNA (Everett et al., 1991). Another open question is whether the ubiquitination and transactivation activities of ICP0 are separable, as the RING-finger domain appears to be required for both, and mutations disrupting one activity also disrupt the other (Boutell et al., 2002).

ICP0’s ability to activate genes introduced by infection and transfection may arise from its function in counteract the cell’s defensive silencing mechanisms. ICP0 appears to affect the abundance and acetylation state of histones on viral and transfected DNA,
thereby facilitating transcription in a template-independent manner (Cliffe and Knipe, 2008). It also disrupts the REST/CoREST/HDAC1/LSD1 complex by binding to CoREST, which leads to increased acetylation as HDAC1 is dissociated from the complex (Ferenczy et al., 2011). Transcriptional repression may be relieved through the degradation of RNF8 and RNF168 by ICP0, resulting in reduced histone ubiquitination (Lilley et al., 2010). Additional factors that have been implicated in ICP0’s suppression of silencing include the histone acetyltransferases CLOCK (Kalamvoki and Roizman, 2010) and PCAF (Li et al., 2009). In addition to allowing ICP0 to counteract repressive chromatin during lytic infection, these mechanisms may contribute to the ability of ICP0 to promote reactivation from quiescent or latent infection (Ferenczy and DeLuca, 2009).

1.2.3 Intrinsic Resistance and ND10s

Intrinsic resistance refers to the ability of constitutively expressed cellular proteins to restrict viral infection. In the HSV-1 infected cell, many proteins conferring intrinsic resistance are associated with nuclear domain 10 (ND10) structures. These dynamic compartments contain over 160 proteins, and have been hypothesized to contribute to a wide variety of cellular functions. While their exact role is unclear, ND10s appear to function as nuclear depots or as nuclear scaffolds for post-translational modification [Reviewed in (Van Damme et al., 2010)]. Shortly after viral infection, ND10s assemble near incoming viral genomes (Everett et al., 2004). If ICP0 is present, it disrupts ND10 bodies (Maul et al., 1993) by degrading PML (Everett et al., 1998) and SP100 (Chelbi-Alix and de The, 1999). If ICP0 is absent, ND10s restrict
infection through the actions of PML and SP100 (Everett et al., 2008a) as well as two other ND10 proteins, DAXX and ATRX (Lukashchuk and Everett, 2010). Depletion of any of these four proteins can increase the efficiency of infection with an ICP0-null virus.

While multiple mechanisms may contribute to the accumulation and release of ND10 proteins, the recruitment of many of these components is regulated by SUMOylation. This post-translational modification is also a mechanism by which ICP0 can target ND10 proteins for degradation. ICP0 can bind SUMO directly, and can mediate degradation of all SUMOylated proteins (Boutell et al., 2011). However, ICP0 also degrades ND10 proteins independently of its interaction with SUMO. For example, even though ICP0 can target PML through its SUMO modification, it can also bind and degrade specific PML isoforms in a SUMO-independent manner (Cuchet-Lourenco et al., 2012).

### 1.2.4 Degradation Targets

In addition to USP7, PML, and SP100, ICP0 targets a range of proteins for degradation. ICP0’s ability to target the DNA sensor IFI16 for degradation blocks innate immune signaling (Orzalli et al., 2012) and contributes to reduced heterochromatin on viral lytic genes (Orzalli et al., 2013). Degradation of the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) by ICP0 inhibits the nonhomologous end-joining arm of DNA repair (Parkinson et al., 1999), and degradation of two other DNA damage proteins, RNF8 and RNF168, prevents recruitment of downstream DNA repair effectors, in addition to potentially counteracting transcriptional repression (Chaurushiya et al., 2012; Lilley et al., 2010). ICP0 targets RNF8 through its forkhead-associated (FHA)
domain and can interact with other FHA-containing proteins such as CHEK2, NBN, and FOXK2, although the outcome of ICP0’s interaction with these other proteins has not been evaluated (Chaurushiya et al., 2012). ICP0 disrupts centromeres during infection by targeting multiple centromeric proteins for degradation, including CENPA, CENPB, CENPC, CENPH, CENPI, and CENPN (reviewed in (Boutell and Everett, 2013). In addition to degrading cellular proteins, ICP0 can also regulate levels of viral proteins by targeting them for degradation, shown in the case of UL46 (Lin et al., 2013).

1.3 TRIM27

1.3.1 Structural Elements of TRIM27

TRIM27, also known as RET finger protein (RFP), is a member of the tripartite motif (TRIM) family. This family of over 70 proteins share three conserved, N-terminal domains: a RING-finger, one or two B-Boxes, and a region of coiled-coils. Many TRIM proteins have been shown to exhibit antiviral activity [reviewed in (Rajsbaum et al., 2014)], either by an involvement in innate immune signaling or in intrinsic resistance. The ND10 protein PML, for example, is a TRIM protein (TRIM19).

TRIM27 features a single B-Box between its RING-finger and coiled-coil region. Its C-terminus contains a PRYSPRY domain, an element shared by many TRIM proteins and thought to determine protein-protein interactions (Grutter et al., 2006). Although TRIM27 localization can vary by cell type (Tezel et al., 1999), it is predominantly nuclear and can localize to ND10 bodies (Cao et al., 1998). Not all TRIM27 is associated with ND10s, and not all ND10 bodies contain TRIM27.
Recruitment of TRIM27 to ND10 bodies may be mediated by its interaction with PML, which results in the formation of heterooligomers (Cao et al., 1998), or through SUMOylation of TRIM27 by PIAS family members (Matsuura et al., 2005).

1.3.2 TRIM27 Function

TRIM27 has been previously characterized as a transcriptional repressor involved in chromatin regulation (Shimono et al., 2003). TRIM27 functions as an E3 ubiquitin ligase (Gillot et al., 2009) and is capable of conjugating various types of polyubiquitin chains to different substrates. Ubiquitination by TRIM27 modulates the activity of a given substrate rather than inducing its proteasomal degradation, with the exception of NOD2 which is degraded by TRIM27 (Zurek et al., 2012). Ubiquitination by TRIM27 reduces the activity of PI3KC2β in T cells (Cai et al., 2011), inhibits PTEN to activate AKT1 (Lee et al., 2013), modifies WASH1 of the WASH regulatory complex to facilitate actin polymerization (Hao et al., 2013), and upregulates USP7-dependent deubiquitination of RIP1 in tumor necrosis factor (TNF)-dependent apoptosis (Zaman et al., 2013).

1.4 Programmed Cell Death in HSV-1 Infection

1.4.1 Apoptosis

The canonical pathway for programmed cell death, apoptosis, serves important functions during development of the organism and in response to cell stress. This mode of controlled cell suicide is characterized by certain morphological changes in the cell,
including membrane blebbing, chromatin condensation, and nuclear fragmentation. Two major pathways result in the induction of apoptosis, the extrinsic and the intrinsic pathways [reviewed in (Nguyen and Blaho, 2007)]. The extrinsic pathway is initiated by the engagement of death receptors at the cell surface, leading to activation of proteases known as caspases. Ligand binding to the death receptor results in the formation of large protein complexes of adaptor molecules. These adapters activate caspase-8, which then cleaves and activates effector caspases. Effector caspases go on to cleave cellular proteins, resulting in the characteristic morphology of apoptosis.

The intrinsic pathway occurs in response to DNA damage, heat shock, or oxidative stress. In response to these stimuli, the balance of pro-apoptotic and anti-apoptotic Bcl-2 proteins in the outer mitochondrial membrane shifts to favor pro-apoptotic factors, resulting in permeabilization of the membrane. Cytochrome c is then released into the cytoplasm, where it activates the initiator caspase, caspase-9. Caspase-9 then cleaves effector caspases, and apoptosis proceeds in the same fashion as in the case of the extrinsic pathway.

1.4.2 Apoptosis in HSV-1 Infection

HSV-1 triggers apoptosis at early stages of infection in certain types of human cells (Galvan and Roizman, 1998), but evades this protective cellular response as infection proceeds. Viruses deficient in IE genes such as ICP4 and ICP27 exhibit increased apoptosis, likely due to deficient E and L gene expression (Roizman, 2013). Of the E proteins, ICP6 blocks caspase-8 activation (Dufour et al., 2011) and U53 blocks the intrinsic pathway and prevents activation of caspase-3 (Munger and
Roizman, 2001). Although the underlying mechanism is not known, the L proteins gD and gJ appear to prevent apoptosis [[reviewed in (Nguyen and Blaho, 2007)]].

Additionally, the latency-associated transcript (LAT) may block apoptosis by inhibiting caspase-8 during infection (Henderson et al., 2002).

1.4.3 Necroptosis

Necroptosis is programmed, necrotic cell death. This type of death, characterized by cell swelling and early permeabilization of the plasma membrane relative to apoptosis, only occurs in certain types of cells. While multiple proteins are involved in the induction of necroptosis, levels of RIP3 expression are correlated with the tendency of a given cell type to support death through this pathway (He et al., 2009). In cells that do support necroptosis, the typical pathway proceeds downstream of TNF receptor engagement (Holler et al., 2000). Other receptors can also induce this pathway, including TLR3 and TLR4 (He et al., 2011) as well as interferon receptors (Thapa et al., 2013). Binding of TNF to its receptor induces Complex I formation, promoting NF-κB activation and survival signaling. Interfering with Complex I or blocking survival signaling promotes Complex II, which leads to apoptosis when caspase-8 is active. If caspase-8 is inhibited, RIP1 and RIP3 interact through their RIP homotypic interaction motifs (RHIMs) to form the necosome. RIP1 deubiquitination by the CYLD deubiquitinase (DUB) also promotes necroome activation by acting on RIP1 (Moquin et al., 2013). Phosphorylation events within the necosome activate RIP3 and result in MLKL phosphorylation, oligomerization, and translocation to the plasma membrane (Wang et al., 2014a), eventually leading to cell death by necroptosis.
1.4.4 Necroptosis in Viral Infection

Many viruses have evolved strategies to avoid triggering apoptosis in the host cell, including inhibition of caspases. This action, however, primes the infected cell for necroptosis. The RIP1-RIP3 necrosome is induced by vaccinia virus (VV) in mice, and results in TNF-dependent restriction of viral infection (Chan et al., 2003; Cho et al., 2009). A non-canonical necrosome is induced during murine cytomegalovirus (MCMV) infection, formed by RIP3 and DAI, a cellular RHIM-containing protein (Upton et al., 2012). MCMV encodes a RHIM-containing protein, vIRA, that blocks the induction of necroptosis by binding RIP3 (Upton et al., 2010).

HSV-1 also encodes a RHIM protein, ICP6, which binds RIP3 and prevents formation of the necrosome in human cells (Guo et al., 2015). In mouse cells, however, this interaction appears to directly activate TNF-independent necroptosis, resulting in restriction of viral replication (Huang et al., 2015; Wang et al., 2014b). These species-specific mechanisms dictating the induction and evasion of necroptosis suggest that this pathway may contribute to the selective pressure driving the evolution of host-pathogen dynamics.
Chapter Two: Identification of TRIM27 as a Novel Degradation Target of Herpes Simplex Virus 1 ICP0

A version of this chapter has been published:


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Author contributions: S.E.C. designed research, performed research, analyzed data, and wrote the paper, with guidance from D.M.K. Mass spectrometry and data analysis were performed in the lab of J.W.H. with the assistance of A.E.W.
Abstract

The herpes simplex virus 1 immediate early protein ICP0 performs many functions during infection, including transactivation of viral gene expression, suppression of innate immune responses, and modification as well as eviction of histones from viral chromatin. Although these functions of ICP0 have been characterized, the detailed mechanisms underlying ICP0’s complex role during infection warrant further investigation. We thus undertook an unbiased proteomic approach to identifying viral and cellular proteins that interact with ICP0 in the infected cell. Cellular candidates resulting from our analysis included the ubiquitin-specific protease USP7, the transcriptional repressor TRIM27, DNA repair proteins NBN and MRE11A, regulators of apoptosis including BIRC6, and the proteasome. We also identified two HSV-1 early proteins involved in nucleotide metabolism, UL39 and UL50, as novel candidate interacting proteins of ICP0. Because TRIM27 was the most statistically significant candidate interactor, further experiments investigated the relationship between TRIM27 and ICP0. We observed rapid, ICP0-dependent loss of TRIM27 during HSV-1 infection. TRIM27 protein levels were restored by disrupting the RING domain of ICP0 or by inhibiting the proteasome, arguing that TRIM27 is a novel degradation target of ICP0. A mutant ICP0 lacking E3 ligase activity interacted with endogenous TRIM27 during infection as demonstrated by reciprocal coimmunoprecipitation and supported by immunofluorescence data. Surprisingly, ICP0-null mutant virus yields decreased upon TRIM27 depletion, arguing that TRIM27 has a positive effect on infection despite being targeted for degradation. These results
illustrate a complex interaction between TRIM27 and viral infection with potential positive or negative effects of TRIM27 on HSV under different infection conditions.
Introduction

During lytic infection, a virus must hijack the synthesis machinery of its host cell to manufacture its own components. The virus redirects cellular metabolism, chromatin regulation, transcription factors, and translation machinery, all while attempting to avoid detection by host immune defenses. Even viruses with a relatively large coding capacity, such as the DNA virus herpes simplex virus 1 (HSV-1), must express multifunctional proteins to exert such widespread control over cellular processes. One example of this strategy is the viral ICP0 E3 ubiquitin ligase, an immediate early protein that modulates multiple cellular pathways simultaneously by targeting various cellular proteins for degradation.

HSV-1 ICP0 is required for efficient infection (Sacks and Schaffer, 1987). While the fitness of an ICP0-null virus depends on the multiplicity of infection (MOI) and the cell type, the expression of viral genes and production of viral progeny are generally impaired in the absence of ICP0. Reduced gene expression is in part a direct effect of deleting ICP0, as this protein normally transactivates expression of all three classes of viral genes (Cai and Schaffer, 1992). Other functions associated with ICP0 such as its dissociation of nuclear domain 10 (ND10) bodies (Everett and Maul, 1994), downmodulation of innate immunity (Melroe et al., 2007; Orzalli et al., 2012), modification and eviction of histones (Cliffe and Knipe, 2008), and counteraction of the DNA damage response (Lilley et al., 2010) likely contribute to impaired gene expression in its absence.

The mechanism behind certain functions of ICP0 has been linked directly to its E3 ubiquitin ligase activity. This activity is mediated by the really interesting new gene
(RING)-finger domain of ICP0 and promotes proteasomal degradation of various cellular proteins. For example, dissociation of ND10s is caused by loss of two of its constituents, PML and Sp100 (Chelbi-Alix and de The, 1999), as well as ICP0’s ability to generally target all SUMOylated proteins for degradation (Boutell et al., 2011). Degradation of the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) by ICP0 inhibits the nonhomologous end-joining arm of DNA repair (Parkinson et al., 1999), and degradation of two other DNA damage proteins, RNF8 and RNF168, prevents recruitment of downstream DNA repair effectors (Lilley et al., 2010). ICP0’s ability to target the DNA sensor IFI16 for degradation blocks innate immune signaling (Orzalli et al., 2012) and contributes to reduced heterochromatin on viral lytic genes (Orzalli et al., 2013).

While ICP0 has been reported to degrade many cellular proteins, only a subset of these have been shown to coimmunoprecipitate (co-IP) with ICP0 in infected cells. One example is that of ubiquitin-specific protease 7 (USP7) (Everett et al., 1999), which binds ICP0 and protects it from autoubiquitination, while itself being ubiquitinated by ICP0 and targeted to the proteasome. ICP0 has also been shown to bind the forkhead-associated (FHA) domain of proteins such as RNF8 (Lilley et al., 2010). ICP0’s recognition of RNF8 through its FHA domain results in loss of this protein during infection, but the outcome of ICP0’s interaction with other FHA domain-containing proteins such as CHEK2, NBN, and FOXK2 has not been evaluated (Chaurushiya et al., 2012). ICP0 can also interact with SUMO1 (Boutell et al., 2011), and SUMOylation can facilitate ICP0’s interaction with various substrates. For example, ICP0 can target SUMOylated PML through this modification, but ICP0 can also bind specific PML
isoforms in a SUMO-independent manner (Cuchet-Lourenco et al., 2012). Additional proteins shown to co-IP with ICP0 in infected cells include the E3 ubiquitin ligase SIAH1 (Nagel et al., 2011) as well as p65 and p50 of NF-κB (Zhang et al., 2013).

To further define the mechanisms by which ICP0 alters its cellular environment during infection, we performed proteomic analysis to identify additional proteins that interact with ICP0 in infected cells. We examined complexes containing an enzymatically inactive RING finger mutant variant of ICP0 in addition to the wild-type protein. We then evaluated the relationship between ICP0 and the most statistically significant candidate from our screen, the tripartite motif (TRIM) protein TRIM27, also known as RET finger protein (RFP). This protein has been previously characterized as a transcriptional repressor involved in chromatin regulation (Shimono et al., 2003). Although TRIM27 localization can vary by cell type (Tezel et al., 1999), it is predominantly nuclear and can localize to ND10s (Cao et al., 1998). TRIM27 functions as an E3 ubiquitin ligase (Gillot et al., 2009) and is capable of conjugating various types of polyubiquitin chains to different substrates. Ubiquitination by TRIM27 modulates the activity of a given substrate rather than inducing its proteasomal degradation with the exception of NOD2 which it degrades (Zurek et al., 2012). Ubiquitination by TRIM27 reduces the activity of PI3KC2β in T cells (Cai et al., 2011), inhibits PTEN to activate AKT1 (Lee et al., 2013), modifies WASH1 of the WASH regulatory complex to facilitate actin polymerization (Hao et al., 2013), and upregulates USP7-dependent deubiquitination of RIP1 in tumor necrosis factor (TNF)-dependent apoptosis (Zaman et al., 2013). We report here that TRIM27 is a novel degradation target of ICP0 during HSV-1 infection, and we characterize its role in the infected cell.
Materials and Methods

Cell culture. Human foreskin fibroblast (HFF) cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). HEK293T, HeLa, U2OS, and Vero cells were maintained in DMEM supplemented with 5% FBS and 5% heat-inactivated bovine calf serum (BCS). Viral inocula were prepared by diluting the virus to the specified MOI in phosphate buffered saline (PBS) supplemented with 0.1% glucose and 1% BCS. The cells were then overlaid with the inoculum for 1 hour at 37°C with shaking. Infected-cell monolayers were maintained at 37°C in DMEM containing 1% BCS. For determination of viral yield, infected cells and supernatant were harvested by scraping the infected-cell monolayer into an equal volume of sterile nonfat milk solution. Following two cycles of freezing and thawing, viral yields were determined by plaque assay on the indicated cell type.

Viruses and Plasmids. The wild-type HSV-1 KOS strain virus (Schaffer et al., 1973) was propagated in Vero cells, and the titers were determined on Vero cells (Knipe and Spang, 1982). The ICP0 mutant viruses and their respective rescued strains (7134 and 7134R (Cai and Schaffer, 1989) and KOS.RFm and KOS.RFr (Orzalli et al., 2012)) were propagated in U2OS cells, and the titers of the viral stocks were determined on U2OS cells. To construct the HA-ICP0 and HA-RFm viruses, molecular cloning was used to append an N-terminal hemagglutinin (HA) tag with an alanine linker to the wild-type or RING finger mutant (RFm) ICP0 protein expressed from a pUC18 vector plasmid, after which the DNA sequence of each tag was verified. These plasmids were then used to
construct full-length viruses by homologous recombination, introducing each of the HA-tagged ICP0 constructs into viral DNA derived from the 7134 ICP0-null virus (Cai and Schaffer, 1989). Following linearization, the HA-ICP0 or HA-RFm plasmids were transfected into U2OS cells with 7134 viral DNA using Effectene (Qiagen). Cell lysates were harvested at 72 hours after transfection, and dilutions of the lysate were plated onto Vero cells to isolate white plaques of recombinant virus using an agarose overlay containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Following three rounds of plaque purification, the HA tag and the RING domain regions of each virus were confirmed by sequencing. Stocks of HA-ICP0 and HA-RFm viruses were then grown in U2OS cells, and the titers were determined on U2OS cells.

**Immunoblotting.** Cells were harvested in NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Life Technologies), and proteins were resolved on NuPAGE 4 to 12% Bis Tris gels (Life Technologies) before transfer to polyvinylidene difluoride (PVDF) or nitrocellulose membranes. The membranes were then blocked for 1 h in PBS with 0.1% Tween 20 (PBST) containing 5% nonfat dry milk or in Odyssey blocking buffer (LI-COR) before overnight incubation with primary antibodies at 4°C. Primary antibodies included mouse monoclonal USP7 (catalog no. sc-137008; Santa Cruz Biotechnology) (1:100), mouse monoclonal ICP0 (EastCoast Bio) (1:2,000), ICP27 (Abcam) (1:2,000), mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam) (1:8,000), and rabbit polyclonal TRIM27 (IBL) (1:100). PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz) at a dilution of 1:5,000 (mouse) or 1:10,000 (rabbit). HRP signal was detected using Super
Signal West Pico chemiluminescent substrate (Pierce) or Luminata Forte Western HRP substrate (Millipore). Nitrocellulose membranes were incubated with a 1:15,000 dilution of mouse or rabbit secondary antibodies labeled with near infrared fluorescent dye for detection with an Odyssey CLx imaging system and analysis using ImageStudio software (LI-COR).

**Proteomic screen.** Approximately $4 \times 10^7$ 293T cells were infected with 7134R, HA-ICP0, or HA-RFm virus at an MOI of 10. At 4 h postinfection (hpi), the infected-cell monolayers were harvested by scraping. The cells were then washed twice with cold PBS before centrifugation and freezing of the cell pellets. After the cell pellets were thawed on ice, the cells were then lysed in 4 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Nonidet P-40, Complete EDTA-free protease inhibitor cocktail [Roche], 10 mM NaF, 1 mM beta glycerol phosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 nM okadaic acid). Protein purification, mass spectrometry (MS), and analysis of spectra were performed essentially as described previously (Sowa et al., 2009). Proteins were immunoprecipitated with anti-HA agarose beads (Sigma), eluted using HA peptide, and digested with trypsin. Samples were analyzed on an LTQ Velos (Thermo) mass spectrometer, and the spectra were searched using the Sequest algorithm and a composite database containing both human and HSV-1 open reading frames in their forward and reverse orientations. Correct spectral matches were distinguished from random matches using linear discriminant analysis, limiting the peptide false discovery rate to 1.99% (Huttlin et al., 2010). **Comparative Proteomic Analysis Software Suite (CompPASS)** analysis was
subsequently performed as described previously to identify high-confidence interacting proteins (HCIPs) from the technical duplicate runs corresponding to each sample (Sowa et al., 2009). The number of HSV-1 peptides reported here reflects the total number of peptides identified in duplicate mass spectrometry runs.

**Coimmunoprecipitation.** Approximately $6 \times 10^7$ HeLa cells were harvested after 4 h of mock infection or infection with HA-RFm virus at an MOI of 10. The cells were then lysed as described above under the same conditions as for the proteomic samples. Cell lysates were incubated with 2 µg of ICP0 or TRIM27 immunoglobulin G (IgG) and species-matched control IgG (normal mouse IgG [Millipore] or normal rabbit IgG [Millipore]) overnight at 4°C. Antibody-bound complexes were then precipitated using protein A/G Plus-agarose beads (Santa Cruz) according to the manufacturer's instructions. After the complexes were washed with lysis buffer, they were eluted from the beads and detected by Western blotting as described above. Clean-Blot immunoprecipitation (IP) detection reagent (Thermo) was used to detect TRIM27 primary antibody to minimize signal from the antibody heavy chain.
Results

Construction and validation of HA-ICP0 and HA-RFm viruses. To identify additional viral and cellular proteins that interact with ICP0, we performed affinity purification to isolate ICP0-containing complexes from infected-cell lysates, followed by liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). To facilitate immunoprecipitation (IP) of ICP0-associated proteins in infected cells, we inserted a hemagglutinin (HA) epitope tag at the 5’ end of the ICP0 open reading frame. The HA-ICP0 coding sequence was then introduced into the HSV-1 genome by homologous recombination as described in Materials and Methods. In parallel, HA-ICP0 encoding a modified RING finger domain with the amino acid substitutions C116G and C156A was also introduced into the viral genome (HA-RFm, Figure 2.1A). These mutations disrupt the ability of ICP0’s RING finger domain to coordinate zinc, abrogating its E3 ubiquitin ligase activity (Lium and Silverstein, 1997). The two resulting viruses containing HA-tagged wild-type and HA-tagged RFm ICP0 were named HA-ICP0 and HA-RFm, respectively. The use of infected-cell lysates for the IP ensured the expression of all viral genes and the recapitulation of any changes in cellular protein levels induced by infection. To evaluate the effect of the HA tag on ICP0 function, we compared the viral progeny yields of the HA-ICP0 and HA-RFm viruses to those of the ICP0-null (7134) and rescued (7134R) viruses following infection at a low multiplicity of infection (MOI) of 0.1, conditions in which ICP0-deficient viruses exhibit a growth defect. We observed that the HA-ICP0 virus grew similarly to the virus expressing wild-type ICP0, and that the HA-RFm virus grew similarly to an ICP0-null virus in 293T cells (Figure 2.1B). The HA tag did not compromise the ability of ICP0 to induce degradation
Figure 2.1. Construction and characterization of HSV-1 recombinants. (A) Structure of the ICP0 variants used herein, indicating the HA tag (black), the disrupted RING finger domain (gray), and the length of the n680 and n720 nonsense mutant proteins. (B) Virus yields as determined by plaque assay on U2OS cells. HEK293T cells were infected with 7134 (ICP0-null), 7134R (rescued), HA-ICP0 (expressing HA-tagged ICP0) or HA-RFm (expressing HA-tagged RING mutant ICP0) viruses at an MOI of 0.1 and harvested at 24 hpi. Values represent the means plus standard errors of the means (SEM) (error bars) (n = 2). (C) Levels of USP7, ICP0, ICP27, and GAPDH as determined by Western blotting. HEK293T cells were infected with the indicated viruses at an MOI of 10; lysates were harvested at 4 hpi.
of a known target of ICP0’s E3 ubiquitin ligase activity, ubiquitin-specific protease 7 (USP7) (Figure 2.1C). Compared to the levels of USP7 observed in uninfected cells or those infected with an ICP0-null or HA-RFm virus, the HA-ICP0 virus reduced levels of USP7 to the same extent as seen following infection with the rescued virus. The levels of ICP0 appeared comparable in the HA-ICP0 and rescued viruses, but were elevated following infection with the HA-RFm virus. Because ICP0 has previously been shown to be capable of autoubiquitination (Canning et al., 2004), the increase in ICP0 protein levels observed during HA-RFm infection was likely due to an inability of the catalytically inactive ICP0 to mediate its own degradation.

Identification of viral and cellular interacting proteins of HSV-1 ICP0. We used the HA-ICP0 and HA-RFm viruses to express tagged ICP0 in the context of infection for our IPs, allowing for the identification of both viral and cellular interacting proteins of ICP0 by mass spectrometry. We then implemented the Comparative Proteomic Analysis Software Suite (CompPASS) (Sowa et al., 2009) to analyze candidate cellular interacting proteins of ICP0. This method was designed for the comparison of various baits prepared in parallel and has been validated for nonreciprocal data sets such as ours. Its algorithm draws on duplicate mass spectrometry runs for each sample to account for the reproducibility of potential interactions. CompPASS also determines the uniqueness of a given interaction using a database of IPs performed under the same conditions, in this case 77 IPs of HA-tagged proteins expressed in 293T cells, prepared using the same protocol. CompPASS incorporates these factors into the calculation of a normalized weighted D (NWD) score reflecting the abundance, reproducibility, and uniqueness of a given interaction.
Proteins with NWD scores of >1 are considered likely bona fide interactors referred to as high-confidence interacting proteins (HCIPs). Because the CompPASS database was generated using uninfected 293T cells, this analysis was possible only for the cellular candidate interacting proteins of ICP0, as the uniqueness of viral proteins could not be assessed using this method.

To prepare the IP samples, we infected 293T cells with each virus at an MOI of 10 and collected cells at 4 hours post-infection (hpi). We chose to harvest our cell lysates at 4 hpi because our primary interest was in identifying cellular proteins that mediate known functions of ICP0 in the nucleus. This timing allowed for maximal expression of the ICP0 bait protein before the accumulation of ICP0 in the cytoplasm (Lopez et al., 2001). Following lysis and IP using HA antibody-coupled resin, we subjected the affinity-purified proteins to duplicate runs of LC-MS/MS and searched the resulting MS/MS spectra using Sequest to identify both cellular and viral interacting proteins. To evaluate the specificity of the HA IP, we also infected cells with 7134R and analyzed them in parallel. Our analysis revealed that the abundance of HA-RFm bait peptides was 44% greater than the abundance of HA-ICP0 bait peptides (Table 2.1). The greater number of HA-RFm peptides may correspond to the increased levels of HA-RFm that we observed by western blotting (Figure 2.1C). Our IP of HA-ICP0 and HA-RFm brought down complexes containing 13 and 11 HSV-1 proteins, respectively, identifying a total of 16 different candidate HSV-1 binding partners. Peptides from these proteins were absent from the 7134R sample controlling for nonspecific IP of viral proteins. We next analyzed the cellular interacting proteins using the CompPASS platform to determine the HCIPs of the two ICP0 baits, taking into account the
abundance, reproducibility, and uniqueness of these peptides as compared to the database of comparable IPs with 77 different HA-tagged baits in 293T cells. Including both HA-ICP0 and HA-RFm, this analysis resulted in the identification of 39 different HCIPs (Table 2.2). Nine HCIPs were shared between the two baits. Two of these, USP7 (Everett et al., 1999) and NBS1 (gene symbol NBN; gene symbols from the HUGO Gene Nomenclature Committee [www.genenames.org]) (Chaurushiya et al., 2012), had previously been shown to bind ICP0. The most unique, abundant, and reproducible candidate interacting protein of both ICP0 variants as measured by NWD score was the TRIM27 transcriptional repressor. Our subsequent analysis thus assessed the role of TRIM27 during HSV-1 infection and the interaction between TRIM27 and ICP0.

**ICP0 decreases TRIM27 protein levels during HSV-1 infection.** Due to ICP0’s established role as a viral E3 ubiquitin ligase, we hypothesized that ICP0 may degrade TRIM27 during infection. We thus examined the levels of TRIM27 during infection in the presence or absence of ICP0 using an ICP0-null mutant virus, 7134, and its corresponding rescued virus, 7134R. HeLa cells were mock infected or infected with the 7134 ICP0-null mutant virus or the 7134R rescued virus at an MOI of 10 and levels of TRIM27 were monitored over time (Figure 2.2A). We observed a slow but reproducible loss of TRIM27 protein in cells infected with the 7134 ICP0-null virus (Figure 2.2A, lanes 2, 4, 6, and 8). In comparison, infection with the 7134R rescued virus induced rapid loss of TRIM27, starting as early as 2 hpi and continuing through 6 hpi (Figure 2.2A, lanes 3, 5, 7, and 9). To determine whether ICP0 was sufficient for this observed
Table 2.1. Total HSV-1 peptides identified via MS following IP of HA-ICP0 and HA-RFm complexes in duplicate runs.

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aRR1, ribonucleotide reductase large subunit; TK, thymidine kinase; NUC, nuclease; RR2, ribonucleotide reductase small subunit; MCP, major capsid protein; PPS, polymerase processivity subunit; OBP, origin-binding protein; DNA Pol, DNA polymerase.
b—, no peptides found.
Table 2.2. HCIPs from HA-ICP0 and HA-RFm CompPASS analysis.

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\( ^a \) Gene symbols from the HUGO Gene Nomenclature Committee (www.genenames.org).

\( ^b \) –, protein not identified as HCIP.

\( ^c \) NWD, normalized weighted D score.

\( ^d \) TSC, total spectral counts averaged across duplicate runs.
Figure 2.2: ICP0 is necessary and sufficient for loss of TRIM27. (A) Western blot analysis of ICP0, TRIM27, and GAPDH in HeLa cells infected with the indicated viruses at a MOI of 10. (B) Western blot analysis of ICP0, TRIM27, and GAPDH in cell lysates of HeLa cells 24 hours following transfection of 1 ug pUC19, ICP0, or RFm plasmids using Effectene (Qiagen). (C) Representative Western blot as in panel A performed on cell lysates collected at 4 hpi. Italicized numbers reflect the background-adjusted fluorescence signals of the ICP0 or TRIM27 bands normalized to the GAPDH loading control, measured by the Odyssey CLx imaging system and relative to the highest value for each protein (n.d., not detected).
loss, HeLa cells were transfected with a vector control (pUC19), a plasmid expressing ICP0 (pICP0), or a plasmid expressing the RING finger mutant (pRFm). We collected lysates 24 hours after transfection and assessed TRIM27 protein levels by western blotting (Figure 2.2B). Compared to the TRIM27 levels observed in the vector-only or pRFm transfections, less TRIM27 protein was visible following transfection of pICP0. These experiments demonstrated that ICP0 is both necessary and sufficient to reduce protein levels of TRIM27.

To evaluate the domain(s) of ICP0 required for degradation of TRIM27, we infected cells with viruses expressing various nonsense mutant forms of ICP0 (Figure 2.1A) (Cai and Schaffer, 1989). HSV-1 expressing the N-terminal 680 residues of ICP0 (n680) did not reduce TRIM27 levels more than infection with an ICP0-null virus (Figure 2.2C, lanes 2 and 3). In contrast, HSV-1 expressing the N-terminal 720 residues (n720) of ICP0 induced a reduction in TRIM27 levels equivalent to the rescued virus (Figure 2.2C, lanes 4 and 5). Residues 680 to 720 have been previously implicated in ICP0 localization to ND10s during infection (Everett and Maul, 1994) as well as in binding to CoREST (Gu and Roizman, 2007). Residues 682 to 685 also contain a putative SUMO interaction motif (SIM), SLS-7 (Boutell et al., 2011). Because TRIM27 is SUMOylated (Matsuura et al., 2005), we considered whether ICP0’s putative SIM in the region could mediate the interaction of these two proteins. To examine this possibility, we used predictive software to determine the most likely SUMOylation site of TRIM27 (Ren et al., 2009) and constructed a myc-tagged TRIM27 expression vector with the predicted SUMOylated lysine, K157, mutated to arginine. Loss of myc-TRIM27 and myc-TRIM27 K157R appeared comparable (Supplemental Figure 1). Because the TRIM27
antibody detects the C-terminus of TRIM27, loss of the N-terminally myc-tagged protein confirms that TRIM27 is degraded, rather than truncated, during infection.

**HSV-1 ICP0 induces proteasomal degradation of TRIM27.** We next tested whether the RING finger domain of ICP0 was required for the observed reduction in TRIM27 protein levels during infection. We infected HeLa cells with the KOS.RFm mutant or the KOS.RFr rescued virus (Orzalli et al., 2012) and evaluated TRIM27 protein levels over time. While KOS.RFr induced TRIM27 loss, TRIM27 was more stable in the cells infected with the KOS.RFm virus (Figure 2.3A, lanes 2, 4, 6, and 8). The major reduction of TRIM27 thus required an intact ICP0 RING finger domain, suggesting that TRIM27 is a novel target of ICP0’s E3 ubiquitin ligase activity. To determine whether proteasome activity was required for TRIM27 loss, we infected HeLa cells in the presence of the proteasome inhibitor MG132 (Figure 2.3B, lane 3). This treatment largely rescued TRIM27 levels during wild-type infection, indicating that the loss was due to proteasomal degradation. These results together argued that ICP0’s E3 ubiquitin ligase activity induces proteasomal degradation of TRIM27 during infection.

To determine the relative localization of TRIM27 and ICP0, we used indirect immunofluorescence (IF) to examine cells that were mock infected or infected with the KOS.RFm or KOS.RFr virus. Cells were fixed at 2 or 4 hpi and processed for IF. We found that in mock-infected cells, TRIM27 exhibited punctate nuclear staining with occasional prominent foci (Figure 2.4a). TRIM27 was lost as early as 2 hpi in the presence of functional ICP0 (Figure 2.4c and e). In cells infected with the KOS.RFm virus, the diffuse TRIM27 relocalized into numerous foci colocalizing with ICP0 (Figure 2.4l and n). These observations suggested that TRIM27 and ICP0 may interact in the
Figure 2.3: ICP0 RING finger activity and cellular proteasome functionality are required for the observed reduction in TRIM27 levels during infection. (A) Western blot analysis of ICP0, TRIM27, and GAPDH in HeLa cells infected with the indicated viruses at an MOI of 10. (B) Western blot analysis as in panel A performed on mock- or KOS-infected HeLa cells at an MOI of 10 and collected at 4 hpi. Cells were pretreated for 30 minutes with 1 µM MG132 (Sigma) (+) or the dimethyl sulfoxide (DMSO) vehicle (-) as indicated. Treatment was maintained in the inoculum and the overlay.
Figure 2.4: TRIM27 colocalizes with ICP0 following infection with the KOS.RFm virus. HeLa cells on coverslips were infected with KOS.RFm or KOS.RFr viruses at an MOI of 10. At 2 and 4 hours after infection, the cells were fixed in 2% formaldehyde for 10 minutes, permeabilized with 0.5% Nonidet P-40 for 10 minutes, and blocked overnight at 4°C with 0.32% human IgG (catalog no. 1215581, Baxter). Coverslips were then exposed to primary antibodies targeting ICP0 (1:500) or TRIM27 (1:100) for 1 hour at ambient temperature. After the coverslips were washed, they were exposed to Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) Antibody (Life Technologies, 1:500) and Alexa Fluor® 594 F(ab')2 Fragment of Goat Anti-Rabbit IgG (Life Technologies, 1:250) for 1 hour at ambient temperature before mounting with ProLong Gold Antifade (Life Technologies). Confocal images were obtained with a point scanning microscope, the Olympus FluoView 1000 microscope, using a 60× PlanApo objective with a numerical aperture of 1.42. Bar, 10 µm.
nucleus early during infection, and that TRIM27 loss occurred in the presence of functional ICP0 due to its E3 ubiquitin ligase activity.

**TRIM27 interacts with ICP0 during infection.** To validate TRIM27 as an ICP0-associated cellular protein, we performed immunoprecipitation (IP) studies in HeLa cells. In the HeLa lysates prepared from cells infected with the HA-RFm virus, we detected TRIM27 after IP with an ICP0 antibody (Figure 2.5A, lane 6). When the same infected cell lysate was subjected to IP with a TRIM27 antibody, we detected ICP0 in the IP (Figure 2.5B, lane 6). The interaction was dependent on the presence of the specific antibodies, as control co-IPs using species-specific immunoglobulin G (IgG) control antibodies did not pull down ICP0 or TRIM27 (Figures 2.5A and B, lanes 3 and 4). TRIM27 was not detected following IP with the ICP0 antibody in the absence of infection (Figure 2.5A, lane 5). These results indicated that endogenous TRIM27 interacts with HA-RFm ICP0 during infection, either directly or as part of a larger complex.

**Effect of TRIM27 knockdown on viral yields.** To evaluate the role of TRIM27 during infection, we depleted TRIM27 using an RNA interference approach. We transfected HFF cells with pooled small interfering RNAs (siRNAs), either with four nontargeting siRNAs or with four siRNAs designed to target TRIM27. At 3 days posttransfection (dpt), TRIM27 protein levels were reduced below the limit of detection by immunoblotting (Figure 2.6A). The transfected cells were then infected with ICP0-null or rescued virus to determine whether depletion of endogenous TRIM27 affected viral replication. Surprisingly, in HFF cells the yield of an ICP0-null virus decreased significantly at late times postinfection following TRIM27 depletion (Figure 2.6B),
Figure 2.5: Coimmunoprecipitation of ICP0 and TRIM27. (A and B) Extracts from mock- or HA-RFm-infected HeLa cells (MOI of 10, 4 hpi) were subjected to IP with antibodies specific for either ICP0 (A), endogenous TRIM27 (B), or species-specific control IgG. Samples were then analyzed for the presence of ICP0 and TRIM27 by Western blotting (WB). Whole cell lysate (WCL) samples contain 2.5% of the input for each IP. A longer exposure was required to detect TRIM27 in the WCL, compared to the shorter exposure used to detect TRIM27 in the IP samples.
Figure 2.6: Effect of TRIM27 knockdown on yields of an ICP0-null or rescued virus in HFF cells. (A) Western blot analysis indicating TRIM27 and GAPDH levels following transfection of HFF cells with pooled nontargeting siRNA (control), pooled TRIM27 siRNA, or individual TRIM27 siRNAs (TRIM27 siRNAs 5 to 8). Pooled non-targeting control siRNAs (catalog no. D-001810-10, Dharmacon) and TRIM27-targeting siRNAs (catalog no. L-006552-00-0005, Dharmacon) were transfected with DharmaFECT 2 reagent (catalog no. T-2002-01, Dharmacon) according to the manufacturer’s instructions. The cells were transfected twice for 72 hours. (B) Viral yields from siRNA-transfected HFF cells infected with 7134 or 7134R virus at a MOI of 0.1 were collected over time. Values indicate means ± SEM (error bars) (n = 2). Significance was determined using a two-sided Student’s t test for independent samples (*, P < 0.001) (C) Yields from infected HFF cells following siRNA transfection as in panel B (infected at a MOI of 0.1, at 48 hpi). Values indicate means plus SEM (n = 2). (D) TRIM27 mRNA levels following siRNA transfection normalized to 18S mRNA levels, relative to nontargeting siRNA transfection. Values indicate mean plus SEM (n = 2). (E) Fold reduction in 7134 yield from panel C, TRIM27 siRNA transfection relative to non-targeting siRNA transfection.
arguing that TRIM27 can exert a positive effect on viral infection. In comparison, no change in replication was observed in HeLa cells despite efficient knockdown of TRIM27 (Supplemental Figure 2A and B). To ensure that the reduction in 7134 yield observed in HFFs was not an off-target effect of the siRNA pool, we assessed viral yields following transfection of these cells with the four individual siRNAs targeting TRIM27 (Figure 2.6C). The reduction in 7134 yield (Figure 2.6E) correlated with the knockdown efficiency of individual TRIM27 siRNAs (Figure 2.6D). Thus, while ICP0 clearly degrades TRIM27 during infection, an ICP0-null virus replicates more efficiently in the presence of endogenous TRIM27.
Discussion

We describe here a proteomic screen performed in infected cells to identify viral and cellular interacting proteins of wild-type and catalytically inactive ICP0. Previous screens of ICP0 using affinity purification followed by mass spectrometry have employed a transfection approach (Boutell and Everett, 2013), which may not fully recapitulate the proper abundance, localization, and viral regulation of ICP0. The construction of full-length viruses expressing tagged ICP0 allowed us to evaluate potential interacting proteins of ICP0 in the context of infection, maintaining proper regulation of the ICP0 bait protein. Additionally, evaluating ICP0-associated proteins during infection ensured exposure of the ICP0 bait to the most relevant pool of cellular proteins, preserving any viral perturbations to the cellular environment that might affect binding of ICP0, such as changes in the abundance, localization, post-translational modification, or binding partners of cellular proteins.

Evaluating proteins that interact with ICP0 in the context of infection also facilitated the unbiased identification of viral interactors, which has not been possible with previous approaches. ICP0 has been shown to interact with ICP4 (Yao and Schaffer, 1994), VP22 (Maringer et al., 2012), and U₇46 (Lin et al., 2013), and all three of these proteins were identified as precipitating with both HA-ICP0 and HA-RFm in our analysis. The viral proteins appearing in the greatest abundance in our results, the large subunit of the U₇39 ribonucleotide reductase and the U₇50 dUTPase, are novel candidate interacting proteins of ICP0. Because these two early proteins are both involved in nucleotide metabolism, it is possible that ICP0 may regulate this process.
Interestingly, dUTPase peptides were only present in the HA-ICP0 sample, indicating that this interaction requires an intact RING finger domain.

The use of infected 293T cell lysates in the preparation of our IP samples allowed us to analyze our cellular mass spectrometry results using the CompPASS platform, a particularly successful method for identifying bona fide interactors from parallel nonreciprocal datasets based on the uniqueness and abundance of particular interacting proteins as well as on the reproducibility of the interactions. This analysis yielded 39 total HCIPs of ICP0 (Figure 2.7), including the two previously characterized interactors USP7 and NBN. These two proteins were identified as HCIPs with both of our ICP0 baits. USP7 has previously been shown to bind ICP0 directly at amino acid residues 616 to 629 (Everett et al., 1999), whereas NBN interacts with ICP0 at its TELF motif at residues 67 to 70 (Chaurushiya et al., 2012). While the role of ICP0’s association with NBN during infection has not been evaluated, identification of the DNA repair protein MRE11A as an HCIP suggests that ICP0 may target the FHA domain of NBN to regulate the MRN complex, of which NBN and MRE11A are both members. The previously characterized targets of ICP0 involved in double-strand break repair, RNF8 and RNF168 (Lilley et al., 2010), were absent in our IPs. Considering that RNF8 can bind to and ubiquitinate NBN (Lu et al., 2012), further investigation is required to determine whether ICP0 exerts independent effects on these various DNA repair proteins.

A comparison of the CompPASS results for the HA-ICP0 and HA-RFm baits reveals key differences in the cellular interactors of ICP0 presence and absence of an intact, functional RING finger domain. Of the 39 HCIPs, only nine proteins were
Figure 2.7: Interaction map of HCIPs from HA-ICP0 and HA-RFm CompPASS analysis. Each node represents a likely bona fide interacting protein of ICP0 or high-confidence interacting protein (HCIP) resulting from our CompPASS analysis (NWD > 1). Proteins identified following IP of HA-ICP0 only are depicted in black, proteins identified following IP of HA-RFm only are depicted in white, and proteins identified following IP of both ICP0 variants independently are depicted in gray.
common to both ICP0 baits and thus independent of the integrity of ICP0’s RING domain. Twelve proteins were specific to the HA-ICP0 bait, including nine proteasomal proteins. The presence of proteasome subunits exclusively in the HA-ICP0 IPs likely reflects the bait protein in the process of being degraded due to autoubiquitination, in that disruption of the E3 ligase activity of ICP0 would inhibit this process. While it is possible that disrupting the RING domain of ICP0 could prevent another E3 ubiquitin ligase from recognizing ICP0 as a substrate, this scenario seems unlikely. The one such ubiquitin ligase shown to target ICP0 for degradation, SIAH-1, was able to ubiquitinate RING-deficient ICP0 when both proteins were overexpressed (Nagel et al., 2011). The total absence of proteasome subunits in our HA-RFm results suggests that ubiquitination by cellular ligases such as SIAH-1 plays a relatively minor role in regulating levels of ICP0 by proteasomal degradation when compared to the effect of what we presume is autoubiquitination of HA-ICP0.

We observed 17 HCIPs specific to the HA-RFm IP. Some of these proteins may have been absent from the wild-type screen due to lower expression of the HA-ICP0 bait, as few peptides of these interactors were observed in the HA-RFm samples. While TRAF2, BIRC2, and AIMP2 fall into this category of low abundance HCIPs, previously described interactions among these three proteins and their involvement in regulating apoptosis may suggest a functional role for ICP0 in this pathway. An additional regulator of apoptosis, BIRC6, exhibited relatively high abundance in the HA-RFm despite its total absence in the HA-ICP0 IP. The presence of this HCIP exclusively in the HA-RFm sample may suggest that this protein could be a novel degradation target of ICP0. The other protein found abundantly, and exclusively, with the HA-RFm bait is
the stress response protein STIP1. This protein’s role in the heat shock response raises the question of whether this interaction occurs only with HA-RFm due to recruitment of chaperones and stress response proteins that may not occur with properly folded, wild-type ICP0. We conclude that while subtractive proteomics can provide useful information under certain circumstances [as reviewed in (Kirkpatrick et al., 2005)], a comparison of peptide abundance between our two baits is not sufficient to indicate whether a given candidate may be targeted for degradation in the presence of a functional RING domain. Even in the straightforward example of ICP0’s known substrate USP7, we did not observe an increase in USP7 peptides in the HA-RFm sample as compared to HA-ICP0 after normalizing for the number of ICP0 bait peptides.

We describe here our analysis of the most statistically significant hit from our screen, the transcriptional repressor TRIM27. We find that TRIM27 is rapidly targeted for degradation by ICP0 during HSV-1 infection, and that this loss occurs in a RING- and proteasome-dependent manner. Due to TRIM27’s previously characterized role in chromatin regulation (Shimono et al., 2003) and its association with ND10s (Cao et al., 1998), we initially hypothesized that TRIM27 might play a role in intrinsic resistance to HSV-1. Surprisingly, though, we found no evidence to support a role for TRIM27 in restricting virus in the presence or absence of ICP0. We note also that depletion of PML did not affect levels of TRIM27 (Figure 3.2A), and depletion of TRIM27 did not affect PML levels or the recruitment of ICP0 to ND10s (Figure 3.2A, Supplemental Figure 3).

While ICP0’s rapid targeting of TRIM27 for degradation during infection suggested that TRIM27 would restrict an ICP0-null virus, we observed instead that
depletion of TRIM27 prevented replication in the absence of ICP0. A paradoxical dependence of HSV-1 on a protein that is lost during infection has been observed in the case of another target of ICP0’s E3 ubiquitin ligase activity, USP7. While ICP0 degrades USP7 during infection, viral gene expression appears accelerated when degradation is blocked (Kalamvoki et al., 2012). TRIM27 appeared to exert a positive effect on viral replication in low MOI infections at 36 to 48 hpi, a time at which the virus is spreading from cell to cell. Therefore, TRIM27 could affect viral spread or innate responses that impact viral replication at late times postinfection. Degradation of TRIM27 in high-MOI infections but decreased viral replication in the absence of TRIM27 may indicate different roles for TRIM27 under different infection conditions.

In this study we have focused on ICP0-dependent phenotypes involving TRIM27, which require not only the use of low MOI infection but also the use of restrictive cell types such as HFFs. These conditions limit the replication of an ICP0-null virus to the extent that effects on early infection cannot be assessed. However, TRIM27 depletion did not affect viral yield in more permissive cell types such as HeLa cells (Supplemental Figure 2). These results argue that TRIM27 may be required only under conditions that show a strong dependence of efficient viral infection on ICP0. This could possibly be due to the involvement of TRIM27 in a pathway such as innate immune signaling that is intact in restrictive cell types such as HFFs but not functional in more permissive lines. Future experiments will require careful experimental design to isolate specific functions of TRIM27 during infection and to investigate the context-dependent nature of the viral phenotype observed with TRIM27 depletion.
Chapter Three: HSV-1 Infection Reduces TRIM27 Protein Levels By Multiple Mechanisms
Abstract

Herpes simplex virus 1 (HSV-1) infection regulates levels of the TRIM27 E3 ubiquitin ligase through multiple mechanisms. We previously reported that the HSV-1 immediate-early (IE) ICP0 protein, a viral E3 ubiquitin ligase, interacted with and degraded TRIM27. In this study we found that another target of ICP0, USP7, affected TRIM27 stability but was not required for TRIM27 degradation. TRIM27 transcript levels declined rapidly after infection, consistent with the gradual loss of protein at late times after infection with an ICP0-null virus. While the IE ICP27 protein was not required for the reduction in transcript levels, the virion host shutoff (VHS) protein contributed significantly. The rate of VHS-independent TRIM27 transcript loss was equivalent to the rate of TRIM27 transcript decay in uninfected cells, indicating that global inhibition of host gene transcription could account for the residual TRIM27 loss. The compound regulation of TRIM27 protein and transcript levels during infection demonstrates the redundant mechanisms by which HSV-1 regulates the cellular proteome.
Introduction

During productive infection, the incoming virus hijacks the host cell’s macromolecular synthesis machinery to manufacture its own components. While the virus is redirecting cellular processes to replicate its own genome, synthesize viral proteins, and produce viral progeny, it must also evade host protective mechanisms including intrinsic immunity and programmed cell death. The large coding capacity of the herpes simplex virus 1 (HSV-1) genome allows it to draw on at least 84 gene products during infection, with multiple proteins often perturbing the same or overlapping cellular pathways. In some cases, this overlap constitutes functional redundancy, while in other cases, viral proteins appear to have opposing effects on a given pathway.

The HSV-1 ICP0 IE protein is an E3 ubiquitin ligase capable of disrupting a diverse panel of cellular pathways. This multifunctional protein is required for efficient infection, in part due to its ability to transactivate viral gene expression (Cai and Schaffer, 1992; Sacks and Schaffer, 1987). ICP0 also counteracts multiple cellular defense mechanisms by targeting various cellular proteins for degradation. Examples include its disruption of nuclear domain 10 (ND10) bodies through PML and Sp100 degradation (Chelbi-Alix and de The, 1999; Everett and Maul, 1994), inhibition of the DNA damage response by RNF8 and RNF168 degradation (Lilley et al., 2010), and evasion of innate immune signaling through degradation of IFI16 (Melroe et al., 2007; Orzalli et al., 2012). We recently used a proteomic approach to identify novel cellular targets of ICP0 and identified a cellular E3 ubiquitin ligase, TRIM27, as both a binding partner and a degradation target of ICP0 in the infected cell (Conwell et al., 2015).
TRIM27 has previously been reported to localize to ND10s and to function as a transcriptional repressor (Cao et al., 1998; Shimono et al., 2003), suggesting that TRIM27 could contribute to intrinsic resistance. Despite the rapid loss of TRIM27 induced by ICP0 during infection, replication of an ICP0-null virus was reduced in the absence of TRIM27. This surprising result led us to speculate that TRIM27 could play a complex role during infection, requiring multiple viral proteins to tailor its regulation to suit various cellular environments or stages of the viral life cycle.

To examine the contribution(s) of other viral proteins to the regulation of TRIM27 levels during infection, we characterized the ICP0-dependent and -independent loss of TRIM27 and evaluated the relationship of TRIM27 to other proteins regulated by ICP0. We measured the half-life of TRIM27 protein in uninfected cells to be much shorter than the average cellular protein (Schwanhausser et al., 2011), so we examined TRIM27 transcripts over the course of infection to determine whether transcript loss could contribute to the apparent ICP0-independent reduction in protein. We observed a rapid decline in TRIM27 transcripts, in part due to active degradation by the virion host shutoff function (VHS) (Read and Frenkel, 1983). Although splicing of TRIM27 could be inhibited by ICP27 (Hardy and Sandri-Goldin, 1994), infection with an ICP27 gene deletion mutant did not affect loss of TRIM27 transcripts. The remaining loss of transcripts observed following infection with a catalytic mutant of VHS was consistent with the global decrease in host transcription during infection (Preston and Newton, 1976). These studies evaluated TRIM27 regulation in the context of the widespread effects of HSV-1 infection on the cellular proteome. Our emphasis on evaluating all potential viral effects on cellular protein levels arose from discrepancies between our
observations of ICP0-dependent loss of IFI16 (Orzalli et al., 2012) and a conflicting report claiming that ICP0 does not regulate IFI16 stability during infection (Cuchet-Lourenco et al., 2013). The results described here seek to clarify potential effects of infection on the cellular proteome, using the example of TRIM27.
Materials and Methods

Cell culture. Human foreskin fibroblast (HFF) cells (ATCC, SCRC-1041) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). HeLa, U2OS, Vero, and FO6 cells (Samaniego et al., 1998) were maintained in DMEM supplemented with 5% FBS and 5% heat-inactivated bovine calf serum (BCS). Viral inocula were prepared by diluting the virus to the specified MOI in phosphate buffered saline (PBS) supplemented with 0.1% glucose and 1% BCS. The cells were then overlaid with the inoculum for 1 hour at 37°C with shaking. Infected-cell monolayers were maintained at 37°C in DMEM containing 1% BCS.

Viruses. The wild-type HSV-1 KOS strain virus (Schaffer et al., 1973), the HSV-1 KOS vhs1 catalytic U41 mutant virus (Read and Frenkel, 1983), the HSV-1 KOS NHB U41 nonsense mutant virus, and the HSV-1 KOS U41 rescued virus NHBR (Strelow and Leib, 1995) were all propagated in Vero cells, and titers of the stocks of these viruses were determined on Vero cells (Knipe and Spang, 1982). The ICP0 mutant viruses and their respective rescued strains, HSV-1 KOS 7134 and 7134R (Cai and Schaffer, 1989), HSV-1 strain F R7910 and R7911 (Van Sant et al., 1999)), as well as the USP7-binding mutant virus HSV-2 strain F R6702 (Kalamvoki et al., 2012) were propagated in U2OS cells, and the titers of the virus stocks were determined on U2OS cells. The HSV-1 KOS d27-1 ICP27-null virus was propagated and titrated in V27 cells (Rice and Knipe, 1990). The HSV-1 KOS d109 virus, which lacks immediate-early gene expression, was propagated and titrated in FO6 cells (Samaniego et al., 1998).
Transfections and Compound Treatments. HFF cells were transfected with pooled non-targeting control small interfering RNA (siRNA) (Dharmacon, catalog no. D-001810-10), USP7-targeting siRNA (Dharmacon, catalog no. L-006097-00-0005), PML-targeting siRNA (Dharmacon, catalog no. L-006547-00-0005), TRIM27-targeting siRNA (Dharmacon, catalog no. L-006552-00-0005), or DAXX-targeting siRNA (Dharmacon, catalog no. L-004420-00-0005) using DharmaFECT 2 reagent (Dharmacon, catalog no. T-2002-01) according to the manufacturer’s instructions. Cells were transfected for 72 hours prior to infection or treatment. Protein stability was examined by treating cells with 100 µg/ml cycloheximide (Sigma-Aldrich) or an equivalent volume of sterile water. RNA stability was examined by treating cells with 2.5 µg/ml actinomycin D (Life Technologies) or an equivalent volume of dimethyl sulfoxide (DMSO). Actinomycin D was maintained in the viral inoculum as well as in the post-infection medium. Inhibition of the proteasome was achieved by treating cells with 1 µM PS-341 (BioVision) or an equivalent volume of DMSO. PS-341 was maintained in the medium for a 30-minute pre-treatment period, in the inoculum for the duration of the infection, and in the post-infection medium.

Immunoblotting. Cells were harvested in NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Life Technologies), and proteins were resolved in NuPAGE 4-12% Bis Tris gels (Life Technologies) before transfer to polyvinylidene difluoride (PVDF) or nitrocellulose membranes. The membranes were then blocked for 1 hour in PBS with 0.1% Tween 20 (PBST) containing 5% nonfat dry milk or in Odyssey blocking buffer (LI-COR) before overnight incubation with primary antibodies at 4°C. Primary antibodies
included anti-Daxx mouse monoclonal (catalog no. ab130198; Abcam) (1:2000), anti-USP7 mouse monoclonal (catalog no. sc-137008; Santa Cruz Biotechnology) (1:100), anti-ICP0 mouse monoclonal (EastCoast Bio) (1:2,000), anti-ICP4 mouse monoclonal (EastCoast Bio) (1:5000), anti-ICP27 mouse monoclonal (Abcam) (1:2,000), anti-GAPDH mouse monoclonal (Abcam) (1:8,000), anti-TRIM27 rabbit polyclonal (IBL) (1:100), and anti-PML rabbit polyclonal (Bethyl Labs) (1:2000). PVDF membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz) at a dilution of 1:5,000 (mouse) or 1:10,000 (rabbit). HRP signal was detected using Super Signal West Pico chemiluminescent substrate (Pierce) or Luminata Forte Western HRP substrate (Millipore). As a second method of immunoblot development, nitrocellulose membranes were incubated with a 1:15,000 dilution of mouse or rabbit secondary antibodies labeled with near infrared fluorescent dye for detection with an Odyssey CLx imaging system and analysis using ImageStudio software (LI-COR).

**RNA Analysis by qPCR.** Total cDNA was prepared from HFF cells as described previously (Orzalli et al., 2013). Relative amounts of TRIM27 and 18S rRNA RT products were determined by quantitative PCR (qPCR) using Fast SYBR Green Master Mix and the StepOne Plus Real-Time PCR System (Applied Biosystems). Each qPCR reaction was performed in a final volume of 25 µl, containing 100 nM of two primers targeting either TRIM27 (5’-CAGGCACGAGCTGAACTCT [forward], 5’-AGCTGCTCAAACTCCAAAC-3’ [reverse] (Kwon et al., 2009)) or 18S rRNA (5’-GCATTCGTATTGCGCCGCTA-3’ [forward], 5’-AGCTGCCCAGCGGT-3’ [reverse]). For each sample, qPCR reactions were run in duplicate, and negative controls that had
not been subjected to RT were included in parallel. Standard curves were used to
determine relative copy numbers for each target sequence, and TRIM27 RNA values
were then normalized to the corresponding 18S rRNA values.
Results

**Loss of TRIM27 protein during HSV-1 infection.** To examine the relative contributions of ICP0 and other viral proteins to TRIM27 protein loss during infection, we infected HFF and HeLa cells with either the 7134 ICP0- virus or the 7134R ICP0+ virus and measured TRIM27 protein levels over time. Loss of TRIM27 in ICP0-infected cells proceeded more rapidly in HFFs than in HeLa cells (**Figure 3.1A and B**, lanes 5 to 8, **Figure 3.1C**). This result was unexpected, because IE gene expression is generally more restricted in HFFs compared to gene expression in HeLa cells (Chen and Silverstein, 1992; Everett et al., 2008b). Viral gene expression was indeed restricted in HFFs compared to the expression in HeLa cells, because the level of ICP0 plateaued in HeLa cells after 4 hours, but continued to increase over the course of infection in HFFs. Although our initial focus was on the kinetics TRIM27 loss in ICP0+ virus-infected cells, we also observed reduction of TRIM27 in cells infected with the ICP0- virus (**Figure 3.1A and B**, lanes 1 to 4, **Figure 3.1C**). This effect was moderate compared to the ICP0-dependent loss, with 70% of the TRIM27 protein remaining in HeLas after 8 hours of infection and 80% remaining in HFFs.

**Relationship of TRIM27 to USP7, PML, and DAXX.** We observed previously that replication of an ICP0-null virus was reduced in the absence of TRIM27, despite degradation of TRIM27 by ICP0 during infection. This surprising result led us to consider that the stability of TRIM27 and proteins that restrict replication of an ICP0-null virus, such as PML or DAXX, may be interdependent (Chee et al., 2003; Lukashchuk and Everett, 2010). In knockdown experiments targeting PML or DAXX in HFFs, we did not observe any changes in TRIM27 levels, nor did knockdown of TRIM27 affect levels
Figure 3.1: Effect of HSV-1 infection on TRIM27 protein levels. (A and B) Immunoblot analysis of ICP0, ICP27, TRIM27, and GAPDH in HFF cells (A) or HeLa cells (B) infected with the indicated viruses at a MOI of 10. (C) Quantification of immunoblots in panels A and B. Relative background-adjusted fluorescence intensity of TRIM27 normalized to the GAPDH loading control, measured by the Odyssey CLx imaging system and normalized to the value at the time of infection.
of PML or DAXX (Figure 3.2A and B). We also examined the relationship between TRIM27 and USP7, another binding partner and degradation target of ICP0 (Boutell et al., 2005; Everett et al., 1999). USP7 exhibits a similar phenotype to TRIM27 in that it is degraded by ICP0, but viral gene expression appears accelerated when this degradation is blocked (Kalamvoki et al., 2012). Although we did not observe an effect of TRIM27 depletion on USP7 levels, we did observe that TRIM27 protein levels were reduced following USP7 depletion (Figure 3.2C, lanes 2 and 3). We observed that TRIM27 degradation still occurred in USP7-depleted cells during infection (Figure 3.2D, lanes 2 and 4), arguing that degradation of TRIM27 by ICP0 is independent of USP7.

To further examine any potential relationship between the degradation of USP7 and TRIM27 by ICP0, we obtained a mutant virus expressing ICP0 unable to bind USP7, R6702, and compared it to its parental strain, F, and an ICP0-null mutant F strain virus (Kalamvoki et al., 2012). R6702 did not appear to degrade USP7 (Figure 3.2E), and the lower levels of ICP0 suggested that USP7 was not able to bind ICP0 and protect it from autoubiquitination (Canning et al., 2004). However, R6702 also seemed deficient in PML degradation, in contrast to published results. We were thus unable to determine the effect of disrupting the interaction between USP7 and ICP0 on TRIM27 loss using these viruses.

We next measured the effect of USP7 depletion on TRIM27 protein half-life by transfecting HFF cells with nontargeting or USP7 siRNAs and then treating with cycloheximide (CHX). USP7 depletion decreased the half-life of TRIM27 protein more than two-fold, from 5.4 hours to 2.3 hours (Figure 3.3A and B). The change in rate constant reflecting this decrease in stability was significant ($P < 0.0001$). In uninfected
Figure 3.2: Relationship of TRIM27 protein levels to PML, DAXX, and USP7. (A, B, and C) Immunoblot analysis indicating PML, TRIM27, GAPDH, DAXX, and USP7 levels following transfection of HFF cells with nontargeting (nt), TRIM27, PML, DAXX, or USP7 siRNA for 72 hours. (D) Immunoblot analysis indicating USP7, ICP0, TRIM27, and GAPDH levels following transfection with nt or USP7 siRNA as in panel C and mock infection or infection with HSV-1 (7134R) at a MOI of 10 for 4 hours. Values indicate the fluorescence intensity of TRIM27 normalized to GAPDH, measured by the Odyssey CLx imaging system and normalized to the uninfected sample transfected with control siRNA. (E) Infection of HFF with F strain viruses R6702 (USP7-binding mutant of ICP0), R7910 (ICP0-null), and R7911 (rescued) compared to the KOS strain 7134 (ICP0-null) and 7134R (rescued) at a MOI of 10. Whole cell lysates were collected 6 hours postinfection and levels of USP7, PML, ICP0, TRIM27, and GAPDH were assessed by Western blot.
Figure 3.3: Effect of USP7 on TRIM27 half-life in uninfected cells. (A) Immunoblot analysis measuring USP7, TRIM27 and GAPDH levels in HFF cells with or without transfection of USP7 siRNA. Cells were treated with 100 µg/ml cycloheximide (CHX) at 72 hours posttransfection and whole cell lysate samples were collected over time as indicated. (B) Quantification of immunoblots in panel A. Relative background-adjusted fluorescence intensity of TRIM27 normalized to the GAPDH loading control, measured by the Odyssey CLx imaging system and normalized to the value in HFFs without knockdown at the time of treatment. Half-life values were calculated using a one-phase decay equation (GraphPad Prism). The rate constant modeling the best-fit curves of the decay was determined to be significantly different using the extra sum-of-squares F test (*, P < 0.0001). (C) Immunoblot analysis indicating TRIM27 and GAPDH levels following CHX treatment of HeLa cells. (D) As in panel B, calculation of the TRIM27 protein half-life in uninfected HeLa cells. Blots are representative of three independent experiments.
HeLa cells, we calculated the half-life of TRIM27 to be 3.1 hours (Figure 3.3C and D). The reduced TRIM27 half-life in HeLa cells as compared to HFF cells was consistent with the greater ICP0-independent loss of TRIM27 observed in HeLas compared to HFFs. (Figure 3.1). These results indicate that although PML and DAXX do not regulate TRIM27 protein levels, loss of USP7 by ICP0 could destabilize TRIM27 and contribute to the reduction in TRIM27 protein we observe following infection with an ICP0⁺ virus.

**Loss of TRIM27 mRNA during infection.** To evaluate whether the observed loss of TRIM27 protein could be due to effects of viral infection on TRIM27 mRNA, we measured the levels of TRIM27 transcripts over the course of wild-type KOS infection in HFFs using RT-qPCR and observed a 95% reduction (Figure 3.4A). Because HSV-1 reduces host mRNA levels in multiple ways, we sought to characterize the source(s) of this reduction. To evaluate the contribution of ICP27 and VHS to reduced TRIM27 mRNA, we compared the levels of TRIM27 transcripts following infection with wild-type virus to the levels observed following infection with an ICP27 gene deletion mutant, d27-1 (Rice and Knipe, 1990), or a catalytic mutant of VHS, vhs1 (Read and Frenkel, 1983). While TRIM27 mRNA levels were reduced similarly during wild-type and ICP27-null virus infection (Figure 3.4A), the levels decreased to a lesser extent in cells infected with the vhs1 mutant virus (P < 0.01). While these results indicate that VHS degrades TRIM27 transcripts, a part of the TRIM27 transcript loss appeared to be independent of VHS, possibly due to the global reduction in host gene transcription during infection. The viral proteins required for shutoff of host transcription can vary from gene to gene, but generally involve ICP4 and ICP27 (Spencer et al., 1997). Because we do not see
Figure 3.4: Effect of VHS and ICP27 on TRIM27 transcript loss in HSV-1–infected cells. (A) TRIM27 mRNA levels from HFF cells were normalized to 18S rRNA and relative to the start of infection. Samples were collected over time following infection with wild-type (KOS), ICP27-null (d27-1), or VHS mutant (vhs1) virus at a MOI of 10. Values indicate means ± SEM (error bars) (n = 3). The significance of the difference in TRIM27 transcript levels during vhs1 and KOS infections was determined by two-way repeated measures ANOVA (*, P < 0.01). (B) TRIM27 mRNA levels from HFF cells, normalized to 18S rRNA and relative to the start of infection. Samples were collected over time following mock infection or infection with a virus expressing no immediate early genes (d109) at a MOI of 10. Values indicate means ± SEM (error bars) (n = 3). TRIM27 transcript levels following d109 infection differed significantly from levels in mock infected cells at 1 and 2 hpi, as determined by a Bonferroni post test following two-way repeated measures ANOVA (*, P < 0.05). (C) HFF cell lysates were collected at the start of the experiment (t = 0) or 8 hours after infection as in (A), but adding a 30-minute pretreatment with DMSO or 1 µM PS-341. Whole cell lysates collected in sample buffer were subjected to Western blotting to determine levels of ICP4, ICP0, TRIM27, and GAPDH.
rescued levels of TRIM27 transcripts during infection with an ICP27-null virus (Figure 3.4A), we speculate that ICP4 may contribute to this residual loss.

To further investigate the role of tegument VHS in actively degrading TRIM27 transcripts, we examined the mRNA levels following infection with d109, a virus incapable of de novo viral gene expression due to disruption of the immediate-early genes (Samaniego et al., 1998). Transcript levels were reduced significantly at 1 and 2 hpi following d109 infection compared to mock-infected cells (P < 0.05) (Figure 3.4B). Decay of TRIM27 transcripts was accelerated following d109 infection, supporting the role of an incoming virion component in actively destabilizing TRIM27 mRNA immediately following infection.

To evaluate the role of VHS in ICP0-independent TRIM27 protein loss during infection, HFF cells were infected with the vhs1 mutant virus in the presence of the proteasome inhibitor PS-341 to prevent degradation by ICP0’s E3 ubiquitin ligase activity. Although PS-341 would be expected to increase steady-state levels of TRIM27 protein by preventing normal protein turnover, we evaluated whether TRIM27 protein accumulation was diminished through transcript degradation by VHS. In the presence of PS-341, TRIM27 protein accumulated to a lesser extent in cells infected with wild-type virus compared to mock-infected cells at 8 hpi (Figure 3.4C). The level of TRIM27 protein was equivalent in cells infected with wild-type and vhs1 mutant virus in the presence of PS-341. Consistent with our observation that TRIM27 is still substantially reduced during infection with the vhs1 mutant (Figure 3.4A), these results suggested that, at the protein level, VHS-dependent loss of TRIM27 mRNA was masked by the global block in host-gene transcription during infection.
**VHS affects the stability of TRIM27 mRNA.** To further define the effect of VHS on TRIM27 mRNA, we measured the half-life of TRIM27 transcripts following infection with wild-type virus or vhs1 mutant virus. Treatment with actinomycin D at the time of infection blocked further cellular gene transcription and blocked expression of HSV-1 genes, isolating our analysis to the effect of incoming virions on TRIM27 transcript stability. While levels of TRIM27 mRNA in vhs1-infected cells remained unchanged compared to mock-infection, transcripts were significantly lower ($P < 0.05$) in wild-type infected cells (Figure 3.5A). The half-life of TRIM27 mRNA was reduced from 2.5 hours in mock- or vhs1-infected cells to 1.8 hours in the presence of functional VHS ($P < 0.05$) (Figure 3.5B). These results indicated that VHS released into the cell with the tegument actively degraded TRIM27 transcripts, and that the vhs1 mutation fully blocked this effect. We also evaluated the effect of a VHS-null virus and its corresponding rescued virus, NHB and NHBR (Strelow and Leib, 1995), on transcript levels following actinomycin D treatment (Figure 3.5C). The difference in TRIM27 mRNA levels following NHB and NHBR infection was significant at 2 hours ($P < 0.05$), but the VHS deletion did not fully restore transcript levels. While the source of the differing effects of vhs1 and NHB infection on TRIM27 mRNA remained unclear, we concluded that tegument VHS actively degrades TRIM27 transcripts following entry of the virus into the cell.
Figure 3.5: Effect of VHS on TRIM27 transcript levels following HSV-1 infection in the absence of de novo viral gene expression. (A) TRIM27 transcript levels from HFF cells treated with actinomycin D at the time of infection, normalized to 18S rRNA and to untreated, uninfected cells. Samples were collected over time following mock infection or infection with wild-type (WT) or VHS mutant (vhs1) virus at a MOI of 10 and concurrent treatment with 2.5 µg/ml actinomycin D or DMSO vehicle. Values indicate means ± SEM (error bars) (n = 4). The significance of the difference in TRIM27 transcript levels following vhs1 and KOS infections was determined by two-way repeated measures ANOVA (*, P < 0.05). (B) Half-life of TRIM27 mRNA following actinomycin D treatment. Values were calculated using a one-phase decay equation (GraphPad Prism). The significance of the difference in the best-fit curves following vhs1 and KOS infection was determined by the extra sum-of-squares F test (*, P < 0.05). (C) As in panel A, TRIM27 mRNA levels relative to 18S rRNA following actinomycin D treatment. Cells were mock infected or infected with a VHS-null virus (NHB) or its corresponding rescued virus (NHBR). Values indicate means ± SEM (error bars) (n = 2). The significance of the difference in TRIM27 transcript levels during NHB and NHBR infection vhs1 and KOS infections was determined by a Bonferroni post test following two-way repeated measures ANOVA (*, P < 0.05).
Discussion

Regulation of TRIM27 Levels During HSV-1 Infection. The experiments described here examine the multiple mechanisms by which HSV-1 infection perturbs the cellular proteome, using TRIM27 as an example. In addition to the ICP0-dependent loss of TRIM27 protein that we previously reported, infection with an ICP0-null mutant virus also results in loss of TRIM27 protein, but at a greatly reduced rate. The kinetics of ICP0-independent loss of TRIM27 are consistent with viral regulation of TRIM27 transcript levels during infection, manifest as an ICP0-independent decrease in steady-state levels of TRIM27 protein at late times. After confirming that transcript levels rapidly decline after infection, we considered the known mechanisms by which HSV-1 reduces cellular transcripts. These mechanisms include VHS degradation of host mRNAs (Read and Frenkel, 1983), ICP27 inhibition of host splicing (Hardy and Sandri-Goldin, 1994; Rice and Knipe, 1990), and the global decrease in cellular transcription induced by ICP4 and ICP27 (Preston and Newton, 1976; Spencer et al., 1997). Although TRIM27 transcripts are spliced, infection with an ICP27-null virus does not alter loss of TRIM27 transcripts. Existing transcripts are actively degraded by VHS to a significant extent. The kinetics of the residual VHS-independent decline in transcripts are consistent with TRIM27 transcript decay as HSV hijacks the transcriptional machinery of the infected cell.

Multiple Viral Effects on Protein Levels. Our results demonstrate the complex effects of HSV-1 infection on the host cell, emphasizing the importance of considering all potential consequences of viral infection. Viral effects on transcript levels, such as transcript degradation by VHS, are particularly critical in the study of proteins such as
TRIM27, which exhibits a short half-life compared to the average cellular protein (Schwanhausser et al., 2011). Although the primary effect of infection on TRIM27 is degradation by ICP0, infection also reduces TRIM27 transcripts and results in an apparent ICP0-independent loss when the existing protein turns over. The reduction in transcript levels is due to active degradation by VHS and also to transcript decay as the transcriptional machinery is co-opted by the virus. A similar scenario in which a target of ICP0’s E3 ubiquitin ligase activity can also exhibit loss through other means during infection occurs in the case of IFI16 (Orzalli et al., 2012). Our report of IFI16 degradation by ICP0 has been challenged due to the observation of ICP0-independent effects on IFI16 protein levels during infection (Cuchet-Lourenc et al., 2013). To compensate for the delayed kinetics of ICP0-null infection, Cuchet-Lourenc et al. increased the MOI of their ICP0-null virus anywhere from 2- to 25-fold above the wild-type MOI in their experiments. While this strategy could equalize the progression of infection across the two viruses, introduction of more VHS into the cell could increase degradation of cellular transcripts. Discrepancies in the progression of infection constitute a valid concern when comparing mutant viruses, but the effects of compensatory strategies must also be taken into account. In our infections with the VHS mutant virus, viral gene expression equals or exceeds that of the wild-type virus (Supplemental Figure 4), consistent with the ability of VHS to degrade both viral and cellular transcripts (Kwong and Frenkel, 1987).

Specificity of Viral Effects on Cellular Protein Levels. These studies allude to the issue of specificity as it relates to viral perturbation of the host cell environment. Distinguishing targeted effects of viral proteins from nonspecific effects of infection is
conceptually useful in examining the significance of virus-host interactions. In the case of TRIM27, although it is a degradation target and binding partner of ICP0, the outcome of this interaction is unclear. Because TRIM27 is SUMOylated (Matsuura et al., 2005), ICP0’s SUMO-targeted ubiquitin ligase activity could result in its loss (Boutell et al., 2011). TRIM27 could also be specifically targeted through a SUMO-independent mechanism, or, as in the case of PML, by both SUMO-dependent and -independent mechanisms (Cuchet-Lourenco et al., 2012).

The ICP0-independent loss of TRIM27 likely reflects the fate of many cellular proteins, determined in large part by inherent protein and transcript stability. Aside from the biochemical specificity of VHS, which is similar to RNase A, VHS targets two distinct populations of mRNA: actively translated transcripts in polyribosomes and those containing AU-rich elements (Esclatine et al., 2004; Strom and Frenkel, 1987; Taddeo and Roizman, 2006). Neither TRIM27 nor IFI16 contain AU-rich elements (Halees et al., 2008), suggesting that they are targeted due to their active translation. While degradation of these transcripts may be nonspecific, this does not undermine the significance of their degradation by VHS. Loss of an interferon-stimulated gene such as IFI16 may account for the previously described ability of VHS to blunt the interferon response (Chee and Roizman, 2004). TRIM27, unlike many other TRIM proteins, is not induced by interferon (Iwata et al., 1999). Whether targeted or nonspecific, the potential for VHS to reduce transcripts of ICP0’s degradation targets represents a redundant strategy for regulating the cellular environment that may be required under specific conditions of infection.
Direct and Indirect Effects of ICP0. Distinguishing the direct and indirect effects of ICP0 is a complex undertaking. Because ICP0 increases the efficiency of infection, effects absent during infection with an ICP0-null virus could be falsely attributed to direct activities of ICP0. For example, ICP0 was also initially implicated in promoting the assembly of EIF4F complexes, later shown to result from the dependence of ICP6 expression on ICP0 (Walsh and Mohr, 2004, 2006). Indirect effects of ICP0 on cellular proteins can result from degradation of known targets, or complex interactions can arise from relationships among degradation targets. For example, USP7 depletion increases PML levels, although ICP0 degrades both proteins (Sarkari et al., 2011). Evaluating the relationship of TRIM27 to other targets, we find that its stability is reduced in the absence of USP7. Stabilization of an E3 ubiquitin ligase by USP7 is reminiscent of the relationship between ICP0 and USP7. USP7 stabilizes ICP0 protein during infection by protecting it from autoubiquitination (Canning et al., 2004). TRIM27 is also capable of autoubiquitination (Napolitano et al., 2011), raising the possibility that USP7 may protect TRIM27 in a similar manner. In support of this argument, TRIM27 and USP7 have recently been reported to interact (Zaman et al., 2013).

Role of TRIM27 in HSV-1 Infection. In addition to using TRIM27 as an example of a protein regulated by multiple mechanisms during infection, we undertook these studies to shed light on the role of TRIM27 in the infected cell. Our previous observation that TRIM27 is both degraded by ICP0 and also required for efficient infection of an ICP0-null virus suggests that TRIM27 may have more than one activity during HSV infection, or that an indirect effect of TRIM27 or ICP0 could account for this
counterintuitive finding. Similar observations have been made in the case of USP7, in that this protein is degraded by ICP0 but viral gene expression appears accelerated when this degradation is blocked (Kalamvoki et al., 2012). While the significance of the various mechanisms by which HSV regulates TRIM27 remains unclear, the redundant effects of infection on this protein highlight the complexity of viral effects in the infected cell and emphasize the need for careful experimental design and interpretation.
Chapter Four: TNF-Dependent Necroptosis Restricts Herpes Simplex Virus 1 in Mouse Embryonic Fibroblasts and Requires TRIM27
Abstract

The host cell subjects herpes simplex virus 1 (HSV-1) to an array of protective mechanisms that must be overcome for efficient infection. Programmed necrosis, or necroptosis, serves as a backup mechanism to restrict viruses capable of blocking apoptosis. We observed TNF-dependent necroptosis in mouse embryonic fibroblasts (MEFs) infected with HSV-1 and found that induction of this pathway mediated the ability of TNF to restrict viral growth. Necroptosis required the E3 ubiquitin ligase TRIM27, as TRIM27−/− MEFs were resistant to both chemical-induced and HSV-induced necroptosis. Expression of human TRIM27 restored sensitivity of TRIM27−/− MEFs to necroptosis induced by chemicals, but not by HSV-1. The differing capacities of mouse and human TRIM27 to support necroptosis during HSV-1 infection suggest that TRIM27 contributes to species-specific restriction of HSV-1 and to the selective pressure driving viral evasion of this protective host response.
Introduction

Viral infection triggers an array of cellular defense mechanisms and viral strategies of evading these defenses. Such reciprocal countermeasures are evident in the case of necroptosis, or programmed cell death by necrosis. The classic pathway for programmed cell death, apoptosis, protects the host against viral infection through the activation of caspases, proteolytic cleavage events, cell death, and clearance of infected cells. To circumvent this protective measure, many viruses have evolved strategies to block caspase activation. Caspase inhibition, however, primes the infected cell for an alternate modality of programmed cell death known as necroptosis. The canonical stimulus of necroptosis is TNF receptor engagement (Holler et al., 2000), but other receptors can also initiate this pathway, including TLR3 and TLR4 (He et al., 2011) as well as interferon receptors (Thapa et al., 2013). Following TNF receptor engagement, Complex I promotes NF-κB activation and survival signaling (Figure 4.1A). Interfering with Complex I or blocking survival signaling promotes Complex II formation and programmed cell death. Complex II leads to apoptosis when caspase-8 is active (Figure 4.1B). If caspase-8 is inhibited, RIP1 and RIP3 interact through their RIP homotypic interaction motifs (RHIMs) to form the necrosome (Figure 4.1C). Phosphorylation of RIP3 by RIP1, which can be blocked by the RIP1 inhibitor necrostatin-1 (Degterev et al., 2008), leads to phosphorylation of MLKL by RIP3. MLKL then oligomerizes and translocates to the plasma membrane (Wang et al., 2014a), where it forms disruptive pores that result in cell death by necroptosis.

The ubiquitination status of RIP1 affects multiple checkpoints in the induction of necroptosis. Ubiquitinated RIP1 in Complex I supports NF-κB activation and
Figure 4.1: Model of TNF-induced necroptosis, highlighting the potential role of TRIM27 and the action of compounds used to promote or inhibit necroptosis. (A) TNF receptor engagement recruits Complex I, comprised of TRADD, RIP1, cIAP1, cIAP2, TRAF2, and TRAF5. Ubiquitinated RIP1 allows for docking of the TAK1-TAB2-TAB3 complex and activation of NF-κB signaling, promoting cell survival. (B) TNF binding also induces formation of Complex II, comprised of TRADD, FADD, RIP1, RIP3, and caspase-8. Complex II is promoted in the absence of survival signaling, as in cycloheximide treatment, and when RIP1 is deubiquitinated. RIP1 deubiquitination occurs through CYLD, when TRIM27 activates USP7 by nondegradative ubiquitination. Active caspase-8 cleaves RIP1 and RIP3, inducing apoptotic cell death. (C) If caspases are inhibited, as in the case of Z-VAD-FMK treatment, RIP1 and RIP3 form the necrosome, which phosphorylates MLKL to induce necroptosis. Inhibition of RIP1 activity by necrostatin-1 prevents the induction of necroptosis.
downstream survival signaling. Inhibition of RIP1 ubiquitination blocks this pathway and triggers programmed cell death. Formation of the necosome requires RIP1 deubiquitination, and the CYLD deubiquitinase (DUB) has been shown to promote necroptosis by activating RIP1 (Moquin et al., 2013). Another DUB, USP7, has been implicated in regulating TNF-dependent apoptosis through RIP1 deubiquitination (Zaman et al., 2013). The E3 ubiquitin ligase TRIM27 forms a complex with USP7 and RIP1, where ubiquitination of USP7 by TRIM27 facilitates deubiquitination of RIP1 (Figure 4.1, B and C). Because RIP1 deubiquitination sensitizes cells to both apoptosis and necroptosis, the TRIM27-USP7-RIP1 complex has the potential to contribute to trigger both modalities of programmed cell death.

The protective role of necroptosis during viral infection has been demonstrated in a number of contexts. The RIP1-RIP3 necrosome is induced by vaccinia virus (VV) in mice and contributes to the TNF-dependent control of viral infection (Chan et al., 2003; Cho et al., 2009). Murine cytomegalovirus (MCMV) infection induces a non-canonical, TNF-independent necrosome formed by RIP3 and DAI, a cellular RHIM-containing protein (Upton et al., 2012). MCMV counteracts necroptosis by binding RIP3 with a RHIM-containing protein of its own, the viral inhibitor of RIP activation (vIRA) (Upton et al., 2010). An antiviral role of necroptosis during herpes simplex virus 1 (HSV-1) infection has recently been described, with important species-specific differences between human and mouse experimental systems. The HSV-1 RHIM-containing protein ICP6 exhibits RHIM-RHIM binding to RIP3, much like vIRA, and also to RIP1. In mouse cells, these interactions directly activate TNF-independent necroptosis to restrict viral replication (Huang et al., 2015; Wang et al., 2014b). Human cells, in contrast,
exhibit TNF-dependent necroptosis that is blocked by RHIM-RHIM interactions of ICP6 with RIP1 and RIP3, and thus only restricts viral replication when the ICP6 RHIM sequence is mutated (Guo et al., 2015).

In previous studies, we observed degradation of TRIM27 by the HSV-1 ICP0 E3 ubiquitin ligase during infection (Conwell et al., 2015). Considering the potential role of the TRIM27-USP7-RIP1 complex in the induction of necroptosis, we examined the role of TRIM27 during infection in mouse embryonic fibroblasts (MEFs). We observed TNF-dependent necroptosis resulting in restriction of HSV-1, indicating that infection can induce necroptosis by multiple means. Necroptosis was not observed in TRIM27 knockout MEFs. Stable expression of human TRIM27 restored the ability of the knockout MEFs to support necroptosis in response to chemical stimuli, but not in response to HSV-1 infection. We thus report that HSV-1 induces TNF-dependent necroptosis in MEFs, and that differences in human and mouse TRIM27 may contribute to the ability of HSV to evade necroptosis in a species-specific context.
Materials and Methods

Cell culture and viral infections. Mouse embryonic fibroblasts were obtained from Zaman et al. (2013). These cells were isolated from TRIM27+/+ or TRIM27−/− C57BL/6 embryos, and the wild-type (WT) and knockout (KO) cell lines were generated by spontaneous immortalization. Human TRIM27 was stably introduced into the KO MEFs using a retroviral vector followed by puromycin selection (hTRIM27). MEFs were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). U2OS cells were maintained in DMEM supplemented with 5% FBS and 5% heat-inactivated bovine calf serum (BCS). The ICP0-null virus (7134) and its corresponding rescued strain (7134R) (Cai and Schaffer, 1989) were propagated in U2OS cells, and the titers of the viral stocks were determined on U2OS cells. Viral inocula were prepared by diluting the virus to the specified MOI in phosphate buffered saline (PBS) supplemented with 0.1% glucose and 1% BCS. The cells were then overlaid with the inoculum for 1 hour at 37°C with shaking. Infected-cell monolayers were maintained at 37°C in DMEM containing 1% BCS. For determination of viral yield, infected cells and supernatant were harvested by scraping the infected-cell monolayer into an equal volume of sterile nonfat milk solution. Following two cycles of freezing and thawing, viral yields were determined by plaque assay on U2OS cells.

Immunoblotting. Cells were harvested in NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Life Technologies), and proteins were resolved on NuPAGE 4 to 12% Bis Tris gels (Life Technologies) before transfer to nitrocellulose membrane. The
membranes then blocked for 1 hour in Odyssey blocking buffer (LI-COR) before overnight incubation with primary antibodies at 4°C. Primary antibodies included mouse monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam) (1:8,000) and rabbit polyclonal TRIM27 (IBL) (1:100). Membranes were then incubated with a 1:15,000 dilution of mouse or rabbit secondary antibodies labeled with near infrared fluorescent dye for detection with an Odyssey CLx imaging system and analysis using ImageStudio software (LI-COR).

**Indirect Immunofluorescence.** Cells were plated on cover slips and fixed 24 hours later in 2% formaldehyde for 10 minutes, permeabilized with 0.5% Nonidet P-40 for 10 minutes, and blocked overnight at 4°C with 0.32% human IgG (catalog no. 1215581, Baxter). Cells on the coverslips were incubated with rabbit polyclonal TRIM27 antibody (1:100) for 1 hour at ambient temperature. After the coverslips were washed, they were incubated with Alexa Fluor® 594 F(ab')2 Fragment of Goat Anti-Rabbit IgG (Life Technologies, 1:250) and 4',6-diamidino-2-phenylindole (DAPI) (Thermo) for 1 hour at ambient temperature before mounting with ProLong Gold Antifade (Life Technologies). 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 CS5 (Adobe Systems).

**Compound treatments.** All treatments were maintained in the pre-treatment medium, viral inoculum, and post-infection medium, where relevant. Control cells were treated with equal volumes of vehicle at the time of each addition. Treatments included dilution
of cycloheximide (Sigma-Aldrich) in sterile water to 10 µg/ml, Z-VAD-FMK (Santa Cruz) in dimethyl sulfoxide (DMSO) to 10 µM, necrostatin-1 (Santa Cruz) in DMSO to 10 µM, and mouse TNF (Millipore) in PBS to 10 ng/ml.

**Cell viability assays.** Sixteen hours before the beginning of each experiment, MEFs were seeded into 96 well plates with 2 X 10^4 cells per well. Following treatment and infection as indicated, cell viability was assessed with Cell Titer Glo reagent (Promega) using a SpectraMax L plate reader (Molecular Devices) to measure chemiluminescent signal. All treatments were performed in duplicate wells.
Results

Characterization of TRIM27 knockout MEFs. We obtained the MEFs described by Zaman et al. (2013), including TRIM27\textsuperscript{+/+} (WT), TRIM27\textsuperscript{−/−} (KO), and TRIM27\textsuperscript{−/−} stably expressing human TRIM27 (hTRIM27). The hTRIM27 MEFs expressed nearly twice as much TRIM27 as compared to the wild-type MEFs, as detected by a polyclonal antibody raised against a C-terminal TRIM27 peptide shared by the human and mouse sequences (Figure 4.2A). The localization of human and mouse TRIM27 was similar, with both WT and hTRIM27 cells exhibiting nuclear punctate staining with occasional, prominent foci (Figure 4.2B). This pattern was consistent with previous observations that TRIM27 localizes to nuclear domain 10 (ND10) structures (Cao et al., 1998). A comparison of the mouse and human TRIM27 protein sequences revealed that these proteins differ in only 6 residues (Figure 4.2C), making TRIM27 one of the most conserved TRIM proteins between these two species. The similarity of mouse and human TRIM27 is notable, considering that TRIM27 is one of 34 TRIM proteins classified as rapidly evolving and often species-specific in mammals (Sardiello et al., 2008).

TRIM27 is required for induction of necroptosis in MEFs. To evaluate the role of TRIM27 in necroptosis, we treated the WT, KO, and hTRIM27 MEFs with TNF (T) to induce formation of Complex I, and cycloheximide (C) to block protein synthesis and thereby inhibit survival signaling downstream of Complex I (Figure 4.1A). We also tested the effect of the pan-caspase inhibitor Z-VAD-FMK (Z) which promotes necroptosis by inhibiting caspase-8 in Complex II, and the RIP1 inhibitor necrostatin-1
Figure 4.2: Characterization of mouse embryonic fibroblasts (MEFs). (A) Immunoblot analysis of TRIM27 and GAPDH levels in MEFs, including wild-type (WT), TRIM27 knockout (KO), and TRIM27 knockout expressing human TRIM27 (hTRIM27). Italicized numbers reflect the background-adjusted fluorescence signals of the TRIM27 bands normalized to the GAPDH loading control, measured by the Odyssey CLx imaging system and relative to WT (n.d., not detected). (B) TRIM27 localization in WT, KO, and hTRIM27 MEFs. Cells were processed for indirect immunofluorescence 24 hours after plating on coverslips. Staining indicates primary antibody detection of mouse or human TRIM27 (green), or cell nuclei as visualized by 4',6-diamidino-2-phenylindole (DAPI) (blue). Bar, 10 µm. (C) Alignment of the reference protein sequences of mouse and human TRIM27, highlighting the 6 differing residues (yellow), the RING domain (blue), the B-Box (green), and the two coiled-coil domains (red).
(N) which blocks formation of the necrosome (Figure 4.1, B and C). TNF addition was preceded by cycloheximide treatment by 1 hour, and by Z/N treatment by 30 minutes. We measured cell viability 6 hours after TNF addition. In WT MEFs, TCZ treatment decreased cell viability by 50% (Figure 4.3). KO MEFs were resistant to this induction of cell death (Figure 4.3), indicating that TRIM27 is required for the TNF-dependent death induced by Z-VAD-FMK. Stable expression of hTRIM27 in the KO cells restored sensitivity of the cells to TCZ treatment. The reduction in viability following TCZ treatment of WT and hTRIM27 MEFs was fully blocked by necrostatin-1 (TCZN; Figure 4.3), confirming the involvement of necroptosis. Surprisingly, hTRIM27 MEFs also exhibited reduced survival with TC treatment in the absence of Z-VAD-FMK (TC; Figure 4.3), differing significantly from both WT and KO MEFs. This death was largely inhibited by TCN treatment, suggesting that hTRIM27 MEFs tend toward necroptosis rather than apoptosis even when caspases are active. No toxicity of the individual compounds was observed in the absence of TC treatment or in conjunction with T or C only (Supplemental Figure 5).

**TRIM27 is required for HSV-induced necroptosis in MEFs.** After demonstrating the involvement of TRIM27 in necroptosis in MEFs, we evaluated whether it contributed to necroptosis induced by HSV-1 infection. Following a 30-minute treatment with DMSO, Z-VAD-FMK, and/or necrostatin-1, we infected WT, KO, and hTRIM27 MEFs with the ICP0⁻ (7134) or ICP0⁺ (7134R) virus at an MOI of 10. Four hours later, we treated with TNF or PBS, and 6 hours after TNF addition (10 hpi total) we measured cell viability. WT MEFs exhibited a significant amount of death following infection with both viruses in the presence of T and TZ treatment (Figure 4.4, B and C).
Figure 4.3: Role of TRIM27 in the induction of necroptosis in MEFs. TRIM27<sup>+</sup> MEFs (WT), TRIM27<sup>−/−</sup> MEFs (KO), or TRIM27<sup>−/−</sup> MEFs stably expressing human TRIM27 (hTRIM27) were treated with the indicated compounds or equivalent amounts of DMSO, PBS, or sterile water. MEFs were treated with cycloheximide (C, 10 µg/ml) 1 hour before treatment with mouse TNF (T, 10 ng/ml), and necrostatin-1 (N, 10 µM) and/or Z-VAD-FMK (Z, µM) was added 30 minutes before TNF. Six hours after TNF addition, cell viability was determined by measuring ATP levels. Values represent the mean viability relative to vehicle-treated cells, averaged across two separate experiments with technical duplicates. Error bars indicate standard errors of the means (SEM), and significance was determined using a two-way ANOVA (**, P < 0.01; ***, P < 0.001).
Figure 4.4: Role of TRIM27 in HSV-induced necroptosis in MEFs. TRIM27<sup>−/−</sup> MEFs (WT), TRIM27<sup>+/−</sup> MEFs (KO), or TRIM27<sup>−/−</sup> MEFs stably expressing human TRIM27 (hTRIM27) were mock-infected (A), infected with ICP0<sup>−</sup> virus (B), or infected with ICP0<sup>+</sup> virus (C). MEFs were pre-treated with DMSO, necrostatin-1 (N, 10 µM) and/or Z-VAD-FMK (Z, µM) for 30 minutes. Four hours after infection, TNF (T, 10 ng/ml) or an equivalent amount of PBS was added. Six hours after TNF addition, cell viability was determined by ATP assay. Values represent the mean viability relative to mock-infected, vehicle-treated cells, averaged across two separate experiments with technical duplicates. Error bars indicate standard errors of the means (SEM), and significance was determined using a two-way ANOVA (***, P < 0.001).
The induction of death in the presence of T and TZ required HSV infection, as the viability of mock-infected cells remained unchanged by these treatments (Figure 4.4A). The reduction in WT viability during infection was significantly greater with the ICP0\(^+\) virus compared to the ICP0\(^-\) virus (Figure 4.4, B and C). HSV-induced death in the presence of T and TZ treatment was not observed in KO or hTRIM27 MEFs. In both infected and uninfected hTRIM27 MEFs, a small, but reproducible, reduction in viability resulted from TZ treatment. Because TZN treatment resulted in the same reduction, this effect appeared to be independent of necroptosis.

**TRIM27 is required for TNF-dependent restriction of HSV replication.** To study further the TRIM27-dependent effects on cell viability observed in the presence of TNF, we assessed the effect of TNF treatment on viral growth in the WT, KO, and hTRIM27 MEFs. We tested both ICP0\(^-\) (7134) and ICP0\(^+\) (7134R) viruses at low (0.1) and high (10) MOI. In WT MEFs, both viruses exhibited reduced replication in the presence of TNF (Figure 4.5, A and B). Restriction of viral replication by TNF was greater following infection at the low MOI. The TNF-dependent reduction in viral yield was similar for the two viruses at both multiplicities, suggesting that ICP0 activity does not counteract the effects of TNF treatment. No TNF-dependent changes in yield were observed in the KO or hTRIM27 MEFs. TNF treatment thus reduced viral replication through a mechanism requiring mouse TRIM27, but the human TRIM27 protein did not restore sensitivity to TNF-dependent restriction of viral replication in the KO MEFs.

**TNF restricts viral growth by inducing necroptosis in a TRIM27-dependent manner.** To evaluate the mechanism(s) by which TRIM27 mediated TNF-dependent restriction of viral replication, we infected WT, KO, and hTRIM27 MEFs with HSV-1
Figure 4.5: Effect of TRIM27 and TNF treatment on yields of ICP0⁻ or ICP0⁺ HSV-1. (A) Viral yields from TRIM27⁺/⁺ MEFs (WT), TRIM27⁻/⁻ MEFs (KO), or TRIM27⁻/⁻ MEFs stably expressing human TRIM27 (hTRIM27). MEFs were infected with ICP0⁻ (7134) or ICP0⁺ (7134R) virus at a MOI of 0.1 and cells were treated with TNF or PBS vehicle at 4 hpi. Infected samples were harvested at 24 hpi. Values indicate means plus SEM (error bars) (n = 2), and significance was determined using a two-way ANOVA (*, P < 0.05). (B) As in (A), but infections were performed at a MOI of 10 and samples were harvested at 16 hpi.
after promoting or inhibiting necroptosis with Z-VAD-FMK (Z) or necrostatin-1 (N). We pre-treated the MEFs for 30 minutes prior to infection, and added TNF (T) at 4 hpi. The reduction in viral growth observed in WT cells following TNF treatment was greater when cells were sensitized to necroptosis with Z-VAD-FMK (Figure 4.6). Restriction of viral replication from T and TZ treatment was blocked completely by necrostatin-1, demonstrating that the effect of TNF treatment resulted from the induction of necroptosis during infection. This induction required TRIM27, because viral yields remained unaffected by compound treatment in the KO MEFs. The hTRIM27 MEFs behaved similarly to the KO MEFs, although TZ and TZN treatment resulted in a minor reduction in viability, consistent with our cell viability data (Figure 4.3). Overall, these results indicate that mouse TRIM27 restricts viral growth in response to TNF by promoting necroptosis, and that this restriction is enhanced when caspases are inhibited.
Figure 4.6: Effect of TRIM27 on HSV-1 yields in MEFs sensitized to necroptosis. Viral yields from TRIM27+/+ MEFs (WT), TRIM27−/− MEFs (KO), or TRIM27−/− MEFs stably expressing human TRIM27 (hTRIM27). MEFs were treated with the indicated compounds or equivalent amounts of DMSO, PBS, or sterile water. Prior to infection with HSV-1 (7134R) at an MOI of 10, MEFs were pre-treated with DMSO, necrostatin-1 (N, 10 µM) and/or Z-VAD-FMK (Z, µM) for 30 minutes. Four hours after infection, TNF (T, 10 ng/ml) or an equivalent amount of PBS was added. Infected samples were harvested at 16 hpi. Values indicate means plus SEM (error bars) (n = 2), and significance was determined using a two-way ANOVA (*, P < 0.05).
Discussion

Deubiquitination of RIP1. We report here that HSV-1 infection induces TNF-dependent necroptosis and that the necroptosis pathway requires TRIM27 in both infected and uninfected MEFs. We propose that TRIM27’s ability to promote RIP1 deubiquitination by USP7, previously shown to facilitate TNF-dependent apoptosis (Zaman et al., 2013), is also necessary for the induction of necroptosis. TRIM27, USP7, and RIP1 interact in a complex, where TRIM27 modifies USP7 by nondegradative ubiquitination and USP7 removes ubiquitin chains from RIP1. TRIM27 thus regulates the ubiquitination status of RIP1, much like the CYLD DUB which promotes necroptosis through RIP1 deubiquitination (Moquin et al., 2013). The actions of USP7 and CYLD on RIP1 do not appear to be redundant. Differences in the specificity of these proteins for particular ubiquitin linkages or lengths of ubiquitin chains may account for the apparent role of multiple DUBs in promoting necroptosis through RIP1.

TNF-Dependent and -Independent Necroptosis. Previous studies of HSV-infected mouse cells describe TNF-independent necroptosis resulting from RHIM-RHIM interactions of ICP6 with RIP1 and/or RIP3 (Huang et al., 2015; Wang et al., 2014b). Because TRIM27 deficiency does not affect HSV-induced necroptosis in the absence of TNF, our results describe a second, TNF-dependent mechanism of induction. These two modalities of necroptosis could work in concert during infection to protect the host. TNF-independent necroptosis appears at late stages of infection (Huang et al., 2015; Wang et al., 2014b), and blocking this pathway delays the normal onset of HSV-induced cell death. Treatment of infected MEFs with TNF rapidly promotes premature cell death, and blocking this with necrostatin-1 or TRIM27 deficiency restores yields to the
levels of untreated cells. TNF contributes to the immune response to HSV-1 infection in mice, restricting viral replication and increasing life expectancy in infected animals (Nguyen et al., 1994; Sergerie et al., 2007). If TNF receptor engagement occurs and survival is blocked in an infected cell, either by dysregulation of NF-κB signaling (Amici et al., 2006) or by a global reduction in host transcription (Preston and Newton, 1976), programmed cell death ensues. Caspase-8 is inhibited by ICP6 during lytic infection (Dufour et al., 2011), and may be inhibited by the latency-associated transcript (LAT) during latent infection (Henderson et al., 2002). As HSV infection proceeds, ICP6 may promote TNF-dependent necroptosis by inhibiting caspase-8, while directly facilitating necrosome formation independently of TNF by binding RIP1 and RIP3.

**Role of ICP0 in Necroptosis.** Our initial interest in TRIM27’s role in necroptosis arose from our previous findings that ICP0 binds to and promotes degradation of TRIM27 during HSV-1 infection (Conwell et al., 2015). While we hypothesized that degradation of TRIM27 and/or USP7 by ICP0 during HSV-1 infection could regulate the survival of infected cells in the presence of TNF receptor engagement, we did not observe a significant role for ICP0 under the conditions tested here. ICP0-dependent effects may require specific conditions of infection, with cell type, MOI, time of TNF treatment, and time of assay presenting opportunities for optimization. We also observe an ICP0-independent reduction in TRIM27 protein levels over the course of infection due to transcript loss (**Figures 3.1, 3.4A, and 3.5**), resulting from degradation by the virion host shutoff (VHS) endonuclease (Read and Frenkel, 1983) as well as the shutoff of host transcription (Preston and Newton, 1976). ICP0-independent loss could obscure the contribution of TRIM27 protein degradation in protecting infected cells from TNF-
dependent necroptosis. In human cells, where ICP6 disrupts the necroptosome, infection with an ICP0-null virus may enhance necroptosis due to the dependence of ICP6 expression on transactivation by ICP0 (Desai et al., 1993).

**Host-Specific Responses.** Differences between the potential of mouse and human TRIM27 to support necroptosis may underlie species-specific aspects of this pathway. Human TRIM27 appears to support necroptosome formation even in the absence of caspase inhibitors, while mouse TRIM27 requires treatment with Z-VAD-FMK to sensitize uninfected cells to necroptosis. Additionally, while human TRIM27 can functionally replace the mouse protein in compound-induced necroptosis, it does not support necroptosis during HSV infection. Although we have not excluded the possibility that differences in expression levels of exogenous human TRIM27 and endogenous mouse TRIM27 may contribute to the abilities of the WT and hTRIM27 MEFs to support necroptosis, the levels of TRIM27 in these cells does not vary more than 2-fold. We thus hypothesize that the difference we observe is due to sequence variation between the two proteins. Human and mouse TRIM27 differ in only six amino acid residues, with two in the RING finger domain, one in the B-Box domain, and two in the coiled-coil region. These sequence differences could alter enzymatic activity or binding affinity in the TRIM27-USP7-RIP1 complex, leading to altered susceptibility to caspase-8 cleavage or ICP6 binding. In mouse cells, ICP6 binds RIP1 and RIP3, but does not disrupt the interaction of RIP3 with RIP1, MLKL, or other RIP3 molecules (Huang et al., 2015). In human cells, the interaction of ICP6 with RIP1 and RIP3 inhibits formation of the RIP1-RIP3 necroptosome (Guo et al., 2015). It is tempting to speculate that expression of human TRIM27 in MEFs could facilitate necroptosome disruption by
ICP6, explaining the ability of human TRIM27 to replace mouse TRIM27 in compound-induced death but not during HSV-1 infection. Whether the species-specific differences arise during induction or evasion of necroptosis, our results raise the likelihood that this pathway is shaped by selective pressure resulting from host-pathogen dynamics.
Chapter Five: Discussion and Future Directions
HSV-1 ICP0 and TRIM27

These studies investigate the effects of HSV-1 infection on the cellular E3 ubiquitin ligase TRIM27, and evaluate the significance of these effects by exploring potential roles of TRIM27 in the infected cell. This line of research was prompted by a proteomic screen identifying novel interacting proteins of ICP0, in which TRIM27 was our most statistically significant hit. The previously described involvement of TRIM27 in chromatin regulation and transcriptional repression (Shimono et al., 2003), as well as its association with PML at ND10s (Cao et al., 1998), led us to hypothesize that ICP0 might bind and degrade TRIM27 to relieve restriction of the virus during infection.

Our hypothesis that TRIM27 restricts HSV-1 was supported by our findings that endogenous TRIM27 interacted, directly or indirectly, with the RING finger mutant of ICP0 in infected cells, and that ICP0 induced rapid proteasomal degradation of TRIM27 at ND10s. We sought to confirm a restrictive role for TRIM27 through siRNA knockdown experiments, depleting cells of TRIM27 and examining changes in viral gene expression and yields. Knockdown of intrinsic resistance proteins often has no effect on wild-type infection, but allows ICP0-null infection to proceed more efficiently, diminishing the apparent defect of an ICP0-null virus (Chee et al., 2003; Lukashchuk and Everett, 2010; Orzalli et al., 2013). The effect of TRIM27 knockdown on infection, however, did not follow this paradigm. Instead of increasing the efficiency of ICP0-null virus infection, TRIM27 depletion blocked replication of an ICP0-null virus.

Our finding that a degradation target of ICP0 could exert a positive effect on HSV-1 infection resembled results from studies investigating USP7 (Kalamvoki et al., 2012). The positive role of USP7 in infection appears to result from its stabilization of
ICP0. We considered the possibility that TRIM27 depletion might negatively regulate a factor that promotes infection, such as USP7, or positively regulate a protein involved in restriction, such as PML, DAXX, or IFI16. However, knockdown of TRIM27 did not affect the levels of these proteins or their transcripts in uninfected cells. We also examined whether knockdown of these proteins affected TRIM27 protein levels to see if loss of TRIM27 could be a secondary effect resulting from ICP0’s degradation of another protein. While the stability of TRIM27 was reduced by knockdown of one other target of ICP0, USP7, this protein was not required for degradation of TRIM27 by ICP0.

Because the primary functions of ICP0, TRIM27, and USP7 all involve regulating proteins by altering their ubiquitination status, it is possible that these proteins affect each other’s activity through shared interactions or by competing for resources in the infected cell. TRIM27 and ICP0 can execute their E3 ubiquitin ligase function through two of the same E2 ubiquitin-conjugating enzymes, UBE2D1 and UBE2E1 (Napolitano et al., 2011; Vanni et al., 2012). ICP0 may target TRIM27 through its interaction with one or both of these E2 enzymes, or ICP0 and TRIM27 could affect each other’s activity by competing for E2 proteins. Another potential contributing factor to these dynamics is USP7, which binds UBE2E1 (Sarkari et al., 2013). The USP7-UBE2E1 interaction results in decreased UBE2E1-mediated ubiquitination, despite the stabilization of UBE2E1 by USP7. While investigating the effects of E2 enzyme regulation would not account for TRIM27’s role in ICP0-null infection, examining the effect of depleting or overexpressing UBE2D1 and UBE2E1 on the activity of ICP0 could reveal mechanisms by which TRIM27, ICP0, and USP7 affect each other’s stability and activity.
Examining whether purified ICP0 and TRIM27 interact in vitro would indicate whether these proteins interact directly or indirectly, and, if the interaction is direct, allow us to identify the residues of ICP0 and TRIM27 mediating this interaction. The purified proteins could also be used for in vitro ubiquitination experiments, which have previously been used to confirm the ubiquitination of USP7 and SUMO by ICP0 (Boutell et al., 2005; Boutell et al., 2011). Identification of the residues required for the ICP0-TRIM27 interaction and the ubiquitination site(s) of TRIM27 would facilitate further investigation of the relationship between these two proteins. Residues 680-720 of ICP0 are important for TRIM27 degradation, but this region of ICP0 is implicated in many functions of ICP0. Mapping the critical residues on both ICP0 and TRIM27 could allow us to separate the role of the ICP0-TRIM27 interaction from the role of TRIM27 loss during infection. The use of binding mutants would also reveal the relationship of TRIM27 loss to other functions attributed to residues 680-720 of ICP0, including retention of ICP0 in ND10s (Gu et al., 2013), binding of ICP0 and CoREST (Gu and Roizman, 2009), and potential interaction with SUMOylated proteins through a putative SUMO Interaction Motif (SIM) (Boutell et al., 2011). Identifying the site(s) of TRIM27 SUMOylation (Matsuura et al., 2005) would allow us to determine whether ICP0 binds and degrades TRIM27 in a SUMO-dependent manner.

**Role of TRIM27 During HSV-1 Infection**

To follow up on our finding that ICP0 degrades TRIM27 despite this protein’s ability to promote infection in the absence of ICP0, we explored potential functions of TRIM27 in the infected cell. We hypothesized that TRIM27 could play multiple roles,
thereby exerting both positive or negative effects on the virus. Different functional roles of TRIM27 could be limited to particular conditions of infection, affected by parameters such as MOI and cell type. Alternatively, spatially distinct populations of TRIM27 could serve different functions in the infected cell. The role of TRIM27 could change as infection proceeds, with TRIM27 required immediately following infection and then lost shortly thereafter. While these possibilities are difficult to explore through siRNA knockdown, the construction of ICP0 and TRIM27 binding mutants, or degradation-resistant TRIM27, would be useful tools in investigating the parameters that dictate the overall effect of TRIM27 on viral infection.

The function(s) of TRIM27 during HSV-1 may be context-dependent, a trait that has been observed with other degradation targets of ICP0, such as PML. Wild-type MEFs and PML knockout MEFs are similar in their restriction of an ICP0-null virus (Chee et al., 2003). However, upon interferon treatment, the restrictive effect of PML is potentiated, and replication is reduced to a greater extent in the wild-type than in the PML knockout MEFs. In our TRIM27 knockdown experiments, we observe an effect of TRIM27 in HFF cells, but not in HeLa cells. This difference leads us to speculate that a pathway intact in HFFs, but not in HeLa cells, could be required for TRIM27-dependent effects on infection. TRIM27 has been implicated in the inhibition of innate immune signaling (Zha et al., 2006), raising the question of whether knockdown of TRIM27 restricts ICP0-null viral replication by enhancing the innate immune response. However, we do not observe an effect of TRIM27 knockdown on innate immune signaling in HFFs (Supplemental Figure 6A). We also did not observe an effect of TRIM27 knockdown on TLR-2 signaling as tested in H2-14 cells (Kurt-Jones et al.,
2002) (Supplemental Figure 6B) or TLR-3 signaling as tested in Hec-1B cells (Supplemental Figure 6C).

In light of the role of TRIM27 in promoting RIP1 deubiquitination (Zaman et al., 2013), we hypothesized that ICP0’s degradation of TRIM27 is a viral strategy to prevent cell death. Necroptosis only occurs in certain types of cells, and is generally correlated with the level of RIP3 expression (He et al., 2009). When we began examining the role of TRIM27 and HSV-1 in necroptosis, MEFs offered a more relevant experimental system for our studies than the established human cell lines used for studying necroptosis, such as HT-29 colon carcinoma cells and Jurkat immortalized lymphocytes. It is now clear that necroptosis occurs in epithelial cells including keratinocytes (Dannappel et al., 2014). The use of siRNA knockdown or CRISPR knockout approaches to alter TRIM27 levels in keratinocytes would allow us to translate our results on HSV-1-induced necroptosis in MEFs to the context of human cells.

Necroptosis in HSV-1 Infection

While inducing TNF-dependent necroptosis in cell culture can involve a cocktail of treatments, it is probable that cells encounter these stimuli in the context of the infected organism. HSV-1 infection in mice induces TNF expression, which restricts viral replication and increases the life expectancy of the infected animal (Nguyen et al., 1994; Sergerie et al., 2007). TNF is also produced in human macrophage cells in response to HSV-1 infection (Paludan and Mogensen, 2001). If TNF binds to the receptor of an infected cell, downstream survival signaling is likely blocked by dysregulation of NF-κB signaling (Amici et al., 2006) or inhibition of host transcription
(Preston and Newton, 1976), leading to programmed cell death through Complex II. In this complex, caspase-8 could be inhibited by ICP6 (Dufour et al., 2011), and formation of the necroosome would result in death of the infected cell by necroptosis. The progression of HSV-1 infection in TRIM27−/− mice needs to be examined to establish the role of this protein in limiting viral infection by promoting necroptosis. Based on studies performed in RIP3−/− mice (Huang et al., 2015; Wang et al., 2014b), we would expect TRIM27−/− mice to exhibit increased viral spread and reduced survival compared to TRIM27+/− mice, following infection with HSV-1 expressing ICP6 with a mutant RHIM domain. Evaluating wild-type HSV-1 infection in TRIM27−/− mice would clarify the significance of the TNF-dependent necroptosis we observe in relation to the ICP6-RIP3 necroosome described in previous studies (Huang et al., 2015; Wang et al., 2014b). It is possible that TRIM27 would not affect the progression of wild-type infection, because the ICP6-RIP3 necroosome seems to form downstream of RIP1. While RIP3−/− and MLKL−/− MEFs are completely spared from HSV-induced necroptosis that leads to death of wild-type MEFs, RIP1−/− MEFs exhibit an intermediate phenotype (Huang et al., 2015). Necroptosis thus appears to proceed through both RIP1-dependent and -independent means, at least in MEFs. The ICP6-RIP3 necroosome should form properly in TRIM27−/− mice, but TNF-dependent necroptosis involving the TRIM27-USP7-RIP1 complex would not occur. While RIP1−/− mice die shortly after birth, knock-in mice expressing catalytically inactive RIP1 are viable (Polykratis et al., 2014). We would expect such mice to exhibit a similar progression of HSV-1 infection relative to TRIM27−/− mice, exhibiting greater pathogenicity than infection of wild-type mice, but not as great as infection of RIP3−/− mice. Infection of TRIM27−/− mice with an ICP6-null
virus could yield insights into TNF-dependent apoptosis in the infected animal. Without ICP6, active caspase-8 would likely result in apoptotic death, which could also be regulated by the TRIM27-USP7-RIP1 complex. Examining an ICP6-null virus in TRIM27−/− mice could reveal important differences regarding TNF-dependent apoptosis and TNF-dependent necroptosis in the extent of their induction and in their contribution to limiting viral spread.

HSV-1 infection of TRIM27−/− mice would also reveal whether TRIM27 is involved in neurovirulence and/or latency. It is possible that the primary function of TRIM27 is relevant only in an infected animal, and that cultured cells undergoing lytic infection are an insufficient experimental system for examining the significance of TRIM27’s degradation by ICP0. The observation that LAT inhibits caspase-8 suggests that HSV-1 may need to suppress cell death pathways during latent infection (Henderson et al., 2002), and reveals a potential mechanism for the induction of TNF-dependent necroptosis in infected neurons. In a mouse model of Parkinson’s disease, TRIM27 deficiency protected neurons by blocking apoptosis (Liu et al., 2014b). If this protection is mediated by an increase in RIP1 ubiquitination in the absence of TRIM27, this same mechanism could prevent necroptosis when caspases are inhibited by HSV-1. Neurons have been shown to undergo necroptosis in response to TNF (Liu et al., 2014a), and TNF-deficient mice are more susceptible to HSV-induced encephalitis and death (Nguyen et al., 1994; Sergerie et al., 2007). These observations support a model in which uncontrolled viral infection in neurons could lead to fatal encephalitis if TNF-dependent necroptosis is completely blocked. While inducing death of the host organism through fatal encephalitis would inhibit transmission of the virus, perhaps
unimpeded necroptosis also prevents transmission by resulting in complete control of the virus by the host. HSV-1 may have evolved strategies to modulate cell death pathways in the infected organism to avoid either of these extreme outcomes. One caveat of performing such studies in mice is that adaptive strategies of the virus may not translate across different species. An alternative approach would be to search for a link between mutant *TRIM27* alleles in patients and altered HSV-1 pathogenesis, a method which has proven successful in demonstrating a linke between TLR3 deficiency and HSV-induced encephalitis (Zhang et al., 2007).

**TRIM27 and USP7**

While our results suggest that the TRIM27-USP7-RIP1 complex promotes TNF-dependent necroptosis during HSV-1 infection, this hypothesis needs to be examined directly. The use of biochemical methods to detect interactions among these proteins and examine their ubiquitination states is required to demonstrate that the TRIM27-USP7-RIP1 complex which mediates TNF-dependent apoptosis (Zaman et al., 2013) also promotes TNF-dependent necroptosis. If this complex is required for necroptosis during HSV-1 infection, the role of ICP0’s degradation of USP7 and TRIM27 in regulating this pathway must be evaluated. Although we do not observe ICP0-dependent effects on necroptosis in these experiments, our results do not exclude the possibility that ICP0 could affect this pathway under different conditions of infection.

Examining the localization of the components of the TRIM27-USP7-RIP1 complex could yield insights into its regulation and the potential for ICP0 to affect its formation. Although our initial studies focused on functions of TRIM27 in the nucleus,
the events that result in necroptosis generally take place in the cytoplasm. We observe that TRIM27 promotes necroptosis in MEFs even though it appears predominantly nuclear. In HFFs, we observe that TRIM27 is primarily nuclear, but we can detect cytoplasmic TRIM27 upon fractionation (Supplemental Figure 7). The cytoplasmic fraction of TRIM27 is lost later during infection than the nuclear fraction, and this loss coincides with the accumulation of ICP0 in the cytoplasm. If MEFs behave similarly, delaying TNF treatment relative to the time of infection could allow ICP0 more time to access TRIM27 and/or USP7 in the cytoplasm and induce their degradation before necroptosis is triggered. Examining the location and kinetics of TRIM27-USP7-RIP1 complex formation could also help clarify the relative contributions of ICP6-dependent and TNF-dependent necroptosis to the restriction of HSV-1.

**Deubiquitination of RIP1**

RIP1 ubiquitination is a key factor in determining the outcome of TNF receptor engagement (O'Donnell et al., 2007). Depletion of the cIAP E3 ligases responsible for RIP1 ubiquitination blocks the ability of Complex I to activate survival signaling (Bertrand et al., 2008). While two DUBs, A20 and CYLD, have been implicated in promoting necroptosis through RIP1 deubiquitination, depletion of A20 does not reduce cell survival (Moquin et al., 2013). CYLD⁻/⁻ MEFs exhibit reduced necroptosis, but the degree of protection varies across different means of induction. CYLD is critical for necroptosis in cells treated with TNF and Z-VAD-FMK, dispensable in cells treated with TNF and cycloheximide, and of intermediate significance when cells are treated with TNF, cycloheximide, and Z-VAD-FMK (Moquin et al., 2013). Because RIP1
ubiquitination is required for signaling downstream of Complex I, and RIP1 deubiquitination promotes necroptosis, the original model of TNF signaling proposed that the same deubiquitination event resulted in both disruption of the Complex I and formation of the necrosome. However, ubiquitination of soluble RIP1 is increased in CYLD−/− MEFs (Moquin et al., 2013). The role of CYLD in RIP1 deubiquitination appears to take place in the insoluble compartment of the necrosome rather than in Complex I, and results in activation of the kinase activity of RIP1 rather than in its dissociation from the TNF receptor complex.

We propose that RIP1 is deubiquitinated independently by CYLD and by the TRIM27-USP7 complex, and that the two deubiquitination events may regulate RIP1 in different ways. Given the role of CYLD in RIP1 deubiquitination in the necrosome, USP7 may act on RIP1 outside of the necrosome, possibly in Complex I. Differential specificities of CYLD and USP7 for particular ubiquitin linkages may account for sequential RIP1 deubiquitination events, each serving a different function in promoting necroptosis. While both USP7 and CYLD are relatively promiscuous DUBs, USP7 exhibits more activity relative to CYLD in cleaving all di-ubiquitin linkages except K27 and linear chains (Ritorto et al., 2014). However, the catalytic domain of CYLD features longer ubiquitin binding loops which render it capable of endocleavage (Komander et al., 2008). This allows CYLD to cleave long ubiquitin chains, whereas the primary activity of USP7 is limited to mono- and di-ubiquitin (Schaefer and Morgan, 2011). K63-linked ubiquitination at lysine 377 has been shown to be the primary modification allowing RIP1 to promote survival signaling, but mutation of lysine 377 does not completely prevent this function (Li et al., 2006). It would be interesting to evaluate the
roles of CYLD and the TRIM27-USP7 complex in RIP1 deubiquitination by examining CYLD<sup>−/−</sup> and TRIM27<sup>−/−</sup> MEFs side-by-side. Such studies could reveal regulation of RIP1 by different ubiquitin chains, and possibly shed light on the role of RIP1 deubiquitination in the context of Complex I.

**TRIM27 and Species-Specific Restriction**

Many proteins in the TRIM family mediate antiviral responses through their involvement in innate immune signaling or intrinsic resistance [reviewed in (Rajsbaum et al., 2014)], including PML (TRIM19) which plays a role in restricting HSV-1 infection and is counteracted by ICP0. Restriction of viruses by TRIM proteins is often associated with species-specific responses, most notably in the case of TRIM5α which restricts retroviral infection in primates. The majority of TRIM proteins exhibit increased expression in response to interferon (Rajsbaum et al., 2008). While TRIM27 is among the members of this family that are not stimulated by such treatment, it is classified as rapidly evolving and associated with species-specific functions in mammals (Sardiello et al., 2008).

Given the differences that we observe in the ability of mouse and human TRIM27 to support necroptosis, we propose that this protein may be involved in species-specific restriction of HSV-1 infection. Human TRIM27 supports necroosome formation even in the absence of caspase inhibitors, while mouse TRIM27 requires the additional stimulus of Z-VAD-FMK. We find that human TRIM27 can functionally replace the mouse protein only in compound-induced necroptosis, not in necroptosis induced by HSV-1 infection. These results are compelling in light of recent observations regarding the role of HSV-1
ICP6 in the induction of necroptosis in human and mouse cells. In human cells, ICP6 binds to RIP3 and prevents necrosome formation (Guo et al., 2015). In mouse cells, ICP6 interacts with RIP3 to form a noncanonical necrosome which restricts viral spread (Huang et al., 2015; Wang et al., 2014b). These differences in the induction of necroptosis and the suppression of this protective host response by HSV-1 suggest that proteins involved in necroptosis are subject to selective pressure, with only the natural host evolving mechanisms to counteract evasion strategies of the virus.

The difference that we observe in the ability of human and mouse TRIM27 to support necroptosis is unexpected, considering that these proteins differ in only six amino acid residues. All of these differences are found in the N-terminal TRIM domains: two in the RING, one in the B-Box, and two in the coiled-coil region. These changes may alter substrate specificity or catalytic activity in the RING domain, or could affect the ability of TRIM27 to form multimers through its coiled-coils. The function of the B-Box is not yet clear, although its structure is highly similar to that of the RING-finger (Massiah et al., 2006). It has been proposed that B-Box domains may be capable of E3 ubiquitin ligase activity. This hypothesis would be interesting to evaluate in the case of TRIM27, as the RING domain is dispensable for its ability to ubiquitinate USP7 in the TRIM27-USP7-RIP1 complex (Zaman et al., 2013). The observation that RING-deleted TRIM27 retains E3 ubiquitin ligase functionality in vitro also suggests that its catalytic activity may be mediated by the B-Box (Napolitano et al., 2011). Thus, studies to determine the mechanism underlying species-specific differences of TRIM27 in the necroptosis pathway may also reveal a novel function of the B-Box, a common motif with evolutionary origins in prokaryotes (Burroughs et al., 2011).
Appendix
Supplemental Figure 1: Effect of infection on exogenous myc-TRIM27 and myc-TRIM27 K157R mutant protein. HeLa cells were transfected with vector plasmid or plasmid encoding myc-TRIM27 or myc-TRIM27 K157R as described in Figure 2.2. At 24 hours after transfection, cells were mock-infected or infected with wild-type virus at an MOI of 10, and collected 4 hours later. Western blotting was performed as described in the Materials and Methods in Chapter 2, with the addition of the mouse anti-Myc antibody (9E10; 1:2,000; Santa Cruz Biotechnology).
Supplemental Figure 2: Effect of TRIM27 knockdown on yields of an ICP0-null or rescued virus in HeLa cells. (A) Western blot analysis indicating TRIM27 and GAPDH levels following transfection of HeLa cells with pooled non-targeting control siRNAs (catalog no. D-001810-10, Dharmacon) or TRIM27-targeting siRNAs (catalog no. L-006552-00-0005, Dharmacon) using HiPerFect (Qiagen). The cells were transfected twice for 72 hours. (B) Viral yields from siRNA-transfected HeLa cells infected with 7134 or 7134R virus at a MOI of 0.1 were collected at 24 hpi. Values indicate means ± SEM (error bars) (n = 2).
Supplemental Figure 3: Localization of ICP0 to ND10 following TRIM27 depletion. Pooled non-targeting control siRNAs (catalog no. D-001810-10, Dharmacon) (panels a, b, and c) and TRIM27-targeting siRNAs (catalog no. L-006552-00-0005, Dharmacon) (panel d) were transfected into HFF cells using DharmaFECT 2 reagent (catalog no. T-2002-01, Dharmacon) according to the manufacturer’s instructions. The cells were transfected twice for 72 hours before infection with the RFm or RFr viruses at an moi of 10, and plated onto coverslips 24 hours before the start of infection. At 4 hours after infection, cells were processed for immunofluorescence as described in Figure 2.4, with the addition of polyclonal goat PML antibody (catalog no. sc-9862, Santa Cruz) (1:50) followed by Alexa Fluor® 594 Chicken Anti-Goat IgG (H+L) Antibody (Life Technologies, 1:250), Alexa Fluor® 488 Chicken Anti-Mouse IgG (H+L) Antibody (Life Technologies, 1:500), and Alexa Fluor®647 F(ab')2 Fragment of Goat Anti-Rabbit IgG (Life Technologies, 1:250). Confocal images were obtained with a point scanning microscope, the Olympus FluoView 1000 microscope, using a 60× PlanApo objective with a numerical aperture of 1.42.
Supplemental Figure 4: Levels of ICP4 and ICP0 mRNA during infection with ICP27 and VHS mutant viruses. ICP4 (A) and ICP0 (B) mRNA levels from the HFF cells infected in Figure 3.4 were normalized to 18S rRNA, with values expressed relative to 2-hour infection with KOS. As in Figure 3.4, samples were collected over time following infection with wild-type (KOS), ICP27-null (d27-1), or VHS mutant (vhs1) virus at a MOI of 10. Values indicate means ± SEM (error bars) (n = 2).
Supplemental Figure 5: Toxicity of treatments used to induce necroptosis. TRIM27<sup>+/−</sup> MEFs (WT), TRIM27<sup>−/−</sup> MEFs (KO), or TRIM27<sup>−/−</sup> MEFs stably expressing human TRIM27 (hTRIM27) were treated with the indicated compounds or equivalent amounts of DMSO, PBS, or sterile water. MEFs were treated with cycloheximide (C, 10 µg/ml) 1 hour before treatment with mouse TNF (T, 10 ng/ml), and necrostatin-1 (N, 10 µM) and/or Z-VAD-FMK (Z, µM) were added 30 minutes before TNF. Six hours after TNF addition, cell viability was determined by measuring ATP levels. Values represent the mean viability relative to vehicle-treated cells, averaged across technical duplicates.
Supplemental Figure 6: Effect of TRIM27 depletion on innate immune activation in HFF, H2-14, and HEC-1B cells. All values represent relative RNA levels as described in Chapter 3, Materials and Methods, normalized to mock-treated samples. (A) Pooled non-targeting control siRNAs (catalog no. D-001810-10, Dharmacon) and TRIM27-targeting siRNAs (catalog no. L-006552-00-0005, Dharmacon) were transfected into HFF cells using DharmaFECT 2 reagent (catalog no. T-2002-01, Dharmacon) according to the manufacturer’s instructions. The cells were transfected twice for 72 hours before infection with 100 HA units/8×10⁵ cells of Sendai virus, and were collected at 6 hpi. (B) H2-14 cells were transfected with nt and TRIM27 siRNA pools for 72 hours using HiPerFect (Qiagen). Cells were treated with Zymosan at the concentrations indicated and were collected 6 hours later. (C) HEC-1B cells were transfected with nt and TRIM27 siRNA pools for 72 hours using HiPerfect (Qiagen). Cells were treated with 1 µg/ml polyinosinic:polycytidylic acid and were collected 6 hours later. Primers included ISG54 (5′–GCGTCGTCAGGTCGT–3′ [forward], 5′–CGCGGAGACGGAGA–3′ [reverse]), IL-8 (5′–ATGACTTCCAAGGCTGCGGCT–3′ [forward] and 5′–TTCTCAGCCCCTGCTTACAAACTTCTC–3′ [reverse]), and γ-actin (5′– CACCGCCGCATCCTCTCTCTTC–3′ [forward] and 5′–GTGGTGCACGCCCCACGC–3′ [reverse]).
Supplemental Figure 7: Degradation of nuclear and cytoplasmic fractions of TRIM27 in infected HFF cells. Western blot analysis of ICP0, TRIM27, and GAPDH in mock- or KOS-infected HFF cells, infected at an MOI of 10 and collected at the indicated time points. Fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) according to the manufacturer’s instructions, resuspending the two fractions in equal volumes.


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