Activation and Antagonism of RIG-I-Mediated Innate Immune Signaling by Herpes Simplex Virus 1

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Activation and Antagonism of RIG-I-Mediated Innate Immune Signaling by

Herpes Simplex Virus 1

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Activation and Antagonism of RIG-I-Mediated Innate Immune Signaling by

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ABSTRACT

The cytosolic innate immune receptor retinoic acid-inducible gene-I (RIG-I) recognizes double-stranded RNA (dsRNA) that emerges during viral infection and initiates an antiviral response comprised of interferon (IFN) and proinflammatory cytokine induction. Although RIG-I plays a role in the immune response to DNA viruses such as herpes simplex virus 1 (HSV-1), little is known about the nature of RNA ligands that activate RIG-I during DNA virus infection, and moreover, how these viruses antagonize RIG-I signaling.

To identify physiological RNA ligands of RIG-I during HSV-1 infection, we performed RNA-Seq on RIG-I-bound RNA from HSV-1-infected cells and found that the 5S rRNA pseudogene RNA5SP141 was highly enriched with RIG-I specifically in infected cells and elicited a robust RIG-I-dependent antiviral response. In uninfected cells, RNA5SP141 is found primarily in the nucleus, but upon HSV-1 infection, its localization becomes predominantly cytoplasmic. Furthermore, HSV-1-induced shutoff of host protein expression results in the downregulation of proteins that normally interact with and shield RNA5SP141 from RIG-I detection. Depletion of endogenous RNA5SP141 strongly dampened the antiviral response to HSV-1 and the related Epstein-Barr virus, as well as influenza A virus, an RNA virus. Taken together, our data show that viral infection can lead to deshielding of endogenous RNAs that activate innate immunity.
The innate immune system is tightly regulated by a variety of regulatory mechanisms that prevent aberrant signaling. However, these pathways can be exploited by viruses to suppress antiviral signaling during infection. Here we show that the serine/threonine kinase US3 of HSV-1 suppresses RIG-I-mediated signaling by phosphorylating a regulatory residue in the RIG-I signaling domain. A recombinant HSV-1 encoding catalytically-inactive US3 was unable to phosphorylate RIG-I and elicited higher levels of IFNs and proinflammatory cytokines compared to wild-type (WT) HSV-1.

In summary, these studies have uncovered both a novel host mechanism for detecting DNA virus infection by sensing ‘exposed’ endogenous RNAs and a viral mechanism that co-opts a host regulatory pathway to suppress antiviral signaling. Our findings provide insight into the molecular details governing innate immunity and illustrate the complex interconnectedness of virus and host.
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ABBREVIATIONS

5’-ppp: 5’ triphosphate
AGS: Aicardi-Goutières syndrome
CARD: caspase activation and recruitment domain
cGAMP: cyclic GMP-AMP
cGAS: cyclic GMP-AMP synthase
CTD: C-terminal domain
DI: defective interfering
PKC: protein kinase C
dsDNA: double-stranded DNA
dsRNA: double-stranded RNA
DV: dengue virus
EBER: Epstein-Barr virus-encoded RNA
EBV: Epstein-Barr virus
ER: endoplasmic reticulum
HCMV: human cytomegalovirus
HEK: human embryonic kidney cells
Hel2i: RIG-I helicase 2 insertion domain
HHV: human herpesvirus
HMW poly(I:C): high molecular weight polycytidylic acid
hpi: hours post-infection
HSV: herpes simplex virus
HVEM: herpes entry mediator
IAV: influenza A virus
IB: immunoblot
ICP0: infected cell protein 0
IF116: interferon-inducible protein 16
IFN: interferon
IRF3: interferon regulatory factor 3
ISG: interferon-stimulated gene
ISRE: interferon-sensitive response element
KO: knockout
KSHV: Kaposi’s sarcoma-associated herpesvirus
LAT: latency-associated transcript
LGP2: laboratory of genetics and physiology 2
LNA: locked nucleic acid
MAVS: mitochondrial antiviral signaling protein
MDA5: melanoma differentiation-associated protein 5
MEF: mouse embryonic fibroblast
miRNA: micro RNA
mRNA: messenger RNA
MRPL18: mitochondrial ribosomal protein L18
mtDNA: mitochondrial DNA
NaB: sodium butyrate
NHLF: normal human lung fibroblast
NS: viral non-structural protein
OAS: 2-5-oligoadenylate synthase
PAMP: pathogen-associated molecular pattern
PKR: RNA-dependent protein kinase
Pol: RNA polymerase
PP1: phosphoprotein phosphatase 1
PRD: proline-rich domain
PRR: pattern recognition receptor
qRT-PCR: quantitative reverse transcription PCR
RD: regulatory domain
RIG-I: retinoic acid-inducible gene-I
RNA5SP141: 5S ribosomal RNA pseudogene 141
RNase L: ribonuclease L
RNA-Seq: next-generation RNA sequencing
RNU1-1: small nuclear RNA 1
RPL5: ribosomal protein L5
rRNA: ribosomal RNA
SeV: Sendai virus
siRNA: small interfering RNA
SLE: systemic lupus erythematosus
SMS: Singleton-Merton syndrome
snRNPs: small nuclear ribonucleoprotein particles
ssDNA: single-stranded DNA
ssRNA: single-stranded RNA
STING: stimulator of interferon genes
TLR: Toll-like receptor
TNF: tumor necrosis factor
TRIM25: tripartite motif protein 25
tRNA: transfer RNA
TST: thiosulphate sulphurtransferase
UL: herpesvirus genome unique long region
US: herpesvirus genome unique short region
vhs: virion host shutoff protein
VZV: varicella zoster virus
WCL: whole cell lysate
Parts of this chapter were adapted from previously published work:

RIG-I-LIKE RECEPTORS

Discovery of RIG-I-like receptors

The mammalian innate immune system uses a repertoire of germline-encoded pattern-recognition receptors (PRRs) to recognize microbes based on a defined set of pathogen-associated molecular patterns (PAMPs). Upon pathogen detection, PRRs activate signaling pathways that activate an antimicrobial defense program, characterized by type-I and -III interferons (IFNs) and other proinflammatory cytokines, which are secreted and act in both an autocrine and paracrine manner, alerting neighboring cells of infection and inducing the expression of hundreds of IFN-stimulated genes (ISGs). These ISGs encode antiviral restriction factors, key molecules in innate immune signaling pathways, chemokines, and cytokines, which together lead to the establishment of an antiviral state in both infected and non-infected cells and stimulate adaptive immunity.

PRRs are distinguished by the PAMPs they recognize and the subcellular compartments in which they are found. Two of the most well-characterized PRR families that have been described to detect viral RNA are the Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs) [reviewed in (Takeuchi and Akira, 2010)]. TLR3 and TLR7/8 are found primarily in the membrane of endosomes, where they are positioned to detect the double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) of incoming virions, respectively. While it has long been recognized that viral dsRNA also accumulates intracellularly during viral replication, the PRRs that detect these cytosolic RNA replication products were not discovered until relatively recently. In 2004, Yoneyama et al. identified the RNA helicase retinoic acid-inducible gene-I (RIG-I) as a sensor of viral RNA using a cDNA library screen of molecules that potently enhanced type-I IFN production in response to dsRNA transfection (Yoneyama et al., 2004). This study also demonstrated that
RIG-I functions upstream of the transcription factors essential for activating type-I IFN and proinflammatory cytokine promoters: IFN regulatory factor 3 (IRF3) and NF-κB. In the same year, Andrejeva et al. discovered a second cytosolic receptor of viral RNA, melanoma differentiation-associated protein 5 (MDA5) (Andrejeva et al., 2004). Based on the observation that paramyxovirus V proteins effectively block the production of IFN-β in response to intracellular dsRNA, Andrejeva et al. sought to identify the cellular protein targeted by the V protein in hopes of discovering key molecules involved in the TLR-independent IFN induction pathway. Mass spectrometry analysis of a protein that co-immunoprecipitated with the V protein of parainfluenza virus 5 (PIV5), identified MDA5 as a cytosolic viral RNA sensor required for antiviral IFN induction. In 2005, a third RLR family member, laboratory of genetics and physiology 2 (LGP2), was described to play a role in negative regulation of RIG-I signaling (Rothenfusser et al., 2005).

RLRs are essential for innate immune detection of RNA virus infection in nearly all cell types, including fibroblasts, epithelial cells, and conventional dendritic cells (Kato et al., 2005b). Since the discovery of RLRs, immense progress has been made toward understanding the molecular details of how these receptors sense viral infection and coordinate an effective antiviral defense program. Recent studies have shown that the signaling activities of RLRs are delicately controlled through a multi-step regulatory program to avoid excessive and uncoordinated cytokine production. Furthermore, viruses have developed a myriad of mechanisms to manipulate and dampen the immune response initiated by RLRs.

**RLR structures and signaling**

The RLRs belong to the DExD/H-box RNA helicase family and possess two RNA binding modules, a helicase core and a C-terminal domain (CTD), which are linked through a
bridging/pincer domain (Figure 1.1). The RLR helicase core is comprised of two helicase domains, Hel1 and Hel2 with a unique insertion domain (Hel2i) in Hel2. Together these domains work in concert to surround and make tight interactions with RNA ligands and possess ATPase activity (Kowalinski et al., 2011; Luo et al., 2011). In addition, RIG-I and MDA5 have two N-terminal caspase activation and recruitment domains (CARDs) that mediate interactions with downstream signaling partners, thereby inducing IFN production [reviewed in (Belgnaoui et al., 2011)]. LGP2 is composed of a helicase domain and a CTD but lacks the N-terminal CARDs and is therefore incapable of direct signaling. LGP2 has been shown to play a regulatory role in type-I IFN signaling in several contexts, including RLR signaling, T cell regulation, response to cytosolic dsRNA, and response to ionizing radiation in cancer cells [reviewed in (Bruns and Horvath, 2015)]. As a regulator of RLR signaling, LGP2 is thought to inhibit RIG-I activity and promote MDA5 activity, although some disparities have been observed, and the mechanisms behind this regulation remain a subject of debate.

**Figure 1.1.** Schematic representation of RLR and MAVS domain structures. RIG-I and MDA5, but not LGP2, possess tandem caspase activation and recruitment domains (CARDs), a signaling module allowing for MAVS binding and type-I IFN induction. All three RLR family members have a helicase core consisting of two helicase domains (Hel1 and Hel2), a helicase insertion domain within Hel2 (Hel2i) with ATPase activity, a bridging domain (Br), and a C-terminal domain (CTD). Both the helicase and the CTD have RNA binding abilities. MAVS is comprised of a single CARD, a proline-rich domain (PRD), and a transmembrane (TM) domain that anchors it to mitochondria and peroxisomes.
Soon after the identification of RIG-I and MDA5, their common downstream adaptor, the mitochondrial antiviral signaling protein (MAVS) (also known as VISA, IPS-1 or Cardif), was identified by four independent groups (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). MAVS is localized to the outer mitochondrial membrane (OMM) and is composed of a single CARD at the N-terminus, a central proline-rich domain (PRD), and a C-terminal transmembrane domain (TM) that contains a mitochondrial localization signal. Once activated, RIG-I and MDA5 bind MAVS through homotypic CARD-CARD interactions (Figure 1.2). In addition to residing in the OMM, a subset of MAVS can be found in peroxisomal membranes and mitochondrial-associated membranes (MAM), which connect the endoplasmic reticulum (ER) to mitochondria (Dixit et al., 2010; Horner et al., 2011). Peroxisomal MAVS signaling occurs rapidly upon viral detection and leads to type-III IFN production and expression of a subset of ISGs, while mitochondrial MAVS signals later during infection, triggering type-I IFN production and ISG expression (Odendall et al., 2014). To summarize, the current view is that peroxisomal MAVS is responsible for the establishment of a type-III IFN antiviral state early in infection while mitochondrial MAVS facilitates a sustained type-I IFN-dependent immune response. MAVS at the MAM is proposed to organize an ‘innate immune synapse’ for RLR-MAVS signaling by coordinating MAVS localization in peroxisomal membranes and mitochondria.
Figure 1.2. Schematic model of the signal transduction pathway induced by RLRs. RLR recognition of viral RNA activates a signaling cascade that leads to expression of type-I IFN and proinflammatory cytokines. Details of the model are described in the text.
Activation of MAVS facilitates the assembly of many proteins that induce downstream signaling (Figure 1.2). This ‘MAVS signalosome’ includes the TNF receptor-associated factor (TRAF) 2, 3, and 6, as well as tumor necrosis factor receptor-associated death domain (TRADD), TRAF family member-associated NF-κB activator (TANK), Fas-associated death domain (FADD), and receptor interacting protein 1 (RIP1) (West et al., 2011). Additionally, the mitochondria- and ER-associated protein stimulator of interferon genes (STING/MITA), a critical molecule in the sensing pathway of cytoplasmic viral DNA (Ishikawa et al., 2009), was reported to interact with the MAVS signalosome, promoting RIG-I signal transduction (Ishikawa and Barber, 2008; Zhong et al., 2008). Recent studies have shown that MAVS forms large prion-like aggregates, which represent the signaling-active forms of MAVS (Hou et al., 2011). MAVS signaling ultimately results in the activation of TANK-binding kinase 1 (TBK1) and IκB kinase-ε (IKK-ε) as well as the IKK-α/β/γ complex, resulting in IRF3/7 and NF-κB activation (Belgnaoui et al., 2011; Takeuchi and Akira, 2010). IRF3/7 and NF-κB translocate from the cytoplasm to the nucleus, where they – together with the activating transcription factor 2 (ATF2)/c-Jun – induce the transcriptional activation of genes encoding IFN-α/β, proinflammatory cytokines, and many other antiviral proteins, including RIG-I and MDA5 themselves, creating a positive feedback loop that amplifies the RLR response.

**RLR ligands**

The specific requirements for a given dsRNA to act as an immunostimulatory RIG-I ligand remains an area of intense study [reviewed in (Schlee and Hartmann, 2016)]. Numerous attempts to characterize the properties of RIG-I ligands using synthetic RNAs have been made, examining a variety of parameters including length, RNA duplexing, sequence specificity, tolerance for 5’-
and 3’-end overhangs, and 5’-end phosphorylation. Structures of the RNA-bound RIG-I CTD provide particularly strong evidence that the two critical features of a RIG-I ligand are a 5’-triphosphate (5’-ppp) moiety and base-pairing at the 5’ end (Lu et al., 2010; Wang et al., 2010). The length requirement for the 5’ base-paired stretch has been reported to be at least 10-19 bp (Schlee et al., 2009; Schmidt et al., 2009). Cellular concentrations of RIG-I may affect the ability of different lengths of dsRNA to trigger immune signaling; shorter duplexes require higher concentrations of RIG-I for recognition. Together, these characteristics are required for stabilizing the RNA helix for interactions with the RIG-I CTD and helicase domains. It has also been demonstrated that blunt-ended 5’-ppp dsRNAs are optimal for RIG-I activation, and nucleotide overhangs at the 5’ and 3’ ends of these RNAs substantially reduce their immunostimulatory ability.

The presence of 5’-ppp RNAs in the cytoplasm is thought to serve as a robust and broad means by which RIG-I can distinguish between self- and non-self-RNA. While newly-transcribed cellular RNA in the nucleus have a 5’-ppp, the 5’ ends of most mature RNAs are modified before they are exported to the cytoplasm. RNA polymerase I (Pol I) and Pol II transcripts are 5’-monophosphorylated, and Pol II transcripts receive a 7-methylguanosine cap. In contrast, viral genomic RNA and replication intermediates can contain 5’-ppp. The best-described viral RIG-I PAMPs are negative-sense ssRNA virus genomes, which possess a 5’-ppp and form blunt ended dsRNA panhandle structures from duplexing of the complementary viral genome 5’ and 3’ ends (Schlee et al., 2009). Interestingly, the uninfected host cell cytoplasm is not completely devoid of 5’-ppp RNA. For example, signal recognition particle RNA 7SL is a 5’-ppp-containing cellular Pol III transcript that is abundant in the cytoplasm but is not immunostimulatory, and it was speculated that 7SL RNA association with a protein shields it from detection by RIG-I (Hornung
et al., 2006; Plumet et al., 2007). A recent study demonstrated that breast cancer cells stimulate the release of exosomes from stromal fibroblasts that contain non-protein-bound 7SL RNA, which are taken up by breast cancer cells (Nabet et al., 2017). The unshielded 7SL RNAs are detected by and activate RIG-I signaling, thereby promoting inflammation and cancer progression.

Furthermore, there is increasing evidence that specific sequence motifs in the RNA ligand, such as poly-U/UC, are needed for optimal RIG-I activation (Saito et al., 2008; Uzri and Gehrke, 2009). It has also been shown that the introduction of certain modified RNA nucleotides into RIG-I ligands can reduce their immunostimulatory activity by disrupting early steps in RIG-I activation (Durbin et al., 2016). Structural studies have further defined the molecular determinants of RNA recognition by RIG-I. A basic groove in the CTD of RIG-I has been shown to bind the negative charge of the 5’-ppp group (Cui et al., 2008; Takahasi et al., 2008), while the adjacent short dsRNA stretches stabilize the association of the CTD with the RNA (Kowalinski et al., 2011; Luo et al., 2011). In addition to RIG-I’s function as a sensor of RNA viruses, it has also been proposed that RIG-I detects various DNA viruses by sensing small viral RNA species produced during replication (Ablasser et al., 2009; Chiu et al., 2009; Minamitani et al., 2011). Specifically, RIG-I was shown to bind the Epstein-Barr virus (EBV)-encoded RNAs (EBERs), small 5’ppp-containing untranslated RNAs synthesized by RNA Pol III, as well as adenovirus-associated RNA (VA).

A study demonstrating that an arenavirus genomic RNA can bind RIG-I but does not activate RIG-I signaling suggests that the ability of an RNA molecule to simply bind RIG-I does not necessarily mean it is immunostimulatory (Marq et al., 2010). In this case, the researchers speculated that a single unpaired nucleotide at the 5’ end of the arenavirus RNA may impede RIG-I translocation along the dsRNA and prevent the conformational changes required for RIG-I activation. Another study has demonstrated that the bottom (complementary) strand of an RNA
duplex is important for RIG-I ATPase activity but not RNA binding (Anchisi et al., 2015). The implications of these findings for an RNA-IP experiment such as the one performed in Chapter Two of this dissertation is that, although this experiment may identify many RNA species that transiently interact with RIG-I during viral infection, a *bona fide* RIG-I PAMP must also activate RIG-I ATPase activity and downstream antiviral signaling.

Despite the apparent requirement for 5’-ppp and duplexing, RIG-I ligands that do not strictly adhere to these specifications have also been identified. For example, HCV genome-derived RNase-L cleavage products have been shown to possess RIG-I PAMP activity (Malathi et al., 2007; Malathi et al., 2010). In this case, RIG-I recognition appears to depend on a 3’-monophosphate. In addition, while the immunostimulatory HCV RNA is predicted to contain duplexed stretches, its 5’ and 3’ ends are single-stranded. The ability of RIG-I to recognize viral dsRNA containing 5’-diphosphates has also been demonstrated (Goubau et al., 2014). These characteristics do not precisely adhere to the standard model of a RIG-I ligand and highlight the fact that the exact determinants of RNA recognition by RIG-I are not yet fully understood.

Compared with those of RIG-I, the molecular features of viral PAMPs that trigger MDA5 activation are even less well-understood. MDA5 does not require a 5’-ppp moiety for RNA recognition, and instead, is activated upon binding to longer dsRNAs or web-like RNA aggregates which are found, for instance, in cells infected with the picornavirus, encephalomyocarditis virus (EMCV) (Kato et al., 2008; Pichlmair et al., 2009). More recent studies have described cooperative binding and multimerization of MDA5 on viral RNA as a mechanism by which MDA5 discriminates between dsRNA species of different lengths (Feng et al., 2012; Peisley et al., 2012; Peisley et al., 2011). The ability of MDA5 to bind to the RNA helix along its length, forming large filaments, is dependent on the ATPase function of its helicase domain. Structural studies showed
that not only the helicase but also the CTD of MDA5 coordinates the formation of MDA5 filaments and subsequent interaction with MAVS (Berke et al., 2012). In addition to a length-dependent mechanism of RNA recognition, it has been proposed that MDA5 can discriminate host and viral RNA based on the 2′-O-methylation statuses of their 5′cap structures (Zust et al., 2011). Recent studies also suggest that LGP2 facilitates the interaction between MDA5 and its dsRNA ligands and enhances MDA5 signaling by promoting rapid assembly of short filaments with high immunostimulatory activity (Bruns et al., 2014; Bruns et al., 2013).

Initial infection studies performed in RIG-I or MDA5 knockout (KO) mice indicated that the two sensors recognize different subsets of viruses. Viruses found to be solely detected by RIG-I were influenza A virus (IAV), vesicular stomatitis virus (VSV), arenaviruses, and multiple paramyxoviruses, including Sendai virus (SeV), Newcastle disease virus (NDV), and measles virus (MV). In contrast, MDA5 was shown to be activated primarily in response to picornaviruses as well as RNA intermediates produced during vaccinia virus infection (Gitlin et al., 2006; Kato et al., 2006). Furthermore, detailed studies in cells deficient in either RIG-I or MDA5 revealed partially redundant roles of these sensors in the detection of reoviruses and flaviviruses such as dengue virus (DV) and West Nile virus (WNV) (Loo and Gale, 2011). While these studies greatly expanded our knowledge about the roles of RIG-I and MDA5 in antiviral immunity to distinct viral pathogens, they often ignored the fact that many viruses encode potent RLR antagonists that could obscure the contribution of RIG-I or MDA5 to virus recognition. For example, initial infection studies suggested that RIG-I, but not MDA5, is the primary sensor for the detection of RNA species during paramyxovirus infection; however, it has been demonstrated that the V proteins of many paramyxoviruses specifically inhibit MDA5 activation. Indeed, detailed in vivo studies showed that SeV infection of MDA5 KO mice was highly pathogenic and elicited much
lower levels of pro-inflammatory cytokines compared to infection of wild-type mice (Gitlin et al., 2010). Additional studies strengthened that indeed MDA5’s contribution to sensing of paramyxovirus infection was not apparent in previous infection studies due to the antagonistic function of the V protein; when RIG-I knockdown cells were infected with a recombinant MV lacking the V protein, high IFN-β induction was observed, which was triggered by MDA5 (Ikegame et al., 2010). In support of this, Runge et al. identified both distinct and common RNA species bound by RIG-I and MDA5 in MV-infected cells by using a novel protein-RNA cross-linking approach followed by next generation sequencing (RNA-Seq) (Runge et al., 2014). Both RLRs could recognize defective interfering (DI) copy-back RNA products and showed preference for AU-rich regions from the L gene. RIG-I also bound to both negative-stranded genomic RNA and positive-stranded mRNA transcripts, likely dependent on the presence of 5’-ppp. MDA5 on the other hand, was only found associated with positive-stranded mRNA transcripts as well as potentially dsRNA replication intermediates (Runge et al., 2014). A similar RNA pull-down and deep sequencing approach had previously defined shorter 5’ppp containing RNA segments of the IAV genome as well as DI genomes of both IAV and SeV as physiological ligands for RIG-I in infected cells (Baum et al., 2010). More recently, it has been shown that the nucleoprotein-encapsidated viral genomic RNA of incoming virions can also trigger RIG-I activation (Weber et al., 2013).

Additionally, recent studies have implied that RIG-I and MDA5 may respond to viral infection in a temporally distinct manner. WNV-infected RIG-I KO mice could produce IFN, but in a delayed manner compared to wild-type mice. Indeed, MDA5 was able to compensate for the lack of RIG-I, but was not activated as quickly in response to WNV (Fredericksen et al., 2008). This effect has been shown to be due to the recognition of two distinct RNA species that are present
in WNV infection: RIG-I responded to early RNA replication intermediates in a 5’ppp-dependent manner, whereas MDA5 responded to viral RNA species that accumulated as the infection progressed (Errett et al., 2013). It is thus tempting to speculate that in the context of many viral infections, during which a variety of viral RNA species are likely produced, RIG-I and MDA5 (and other PRRs) act in concert to sense viral PAMPs in a temporally distinct manner. Further studies will be required to identify these physiological PAMPs and the relative contributions of RIG-I, MDA5, and other PRRs to IFN induction in response to viral infection.

**RLR regulation by post-translational modifications**

A recent series of structural and biochemical studies has demonstrated a multi-step model of RIG-I activation that is subject to several intricate regulatory mechanisms. Control of RIG-I activation is primarily mediated by conformational changes and host enzyme-induced post-translational modifications (PTMs) (Figure 1.3). During the initial characterization of RIG-I as a viral sensor, Yoneyama et al. observed that the overexpressed CARDs of RIG-I were constitutively active and robustly induced signaling without viral RNA stimulation (Yoneyama et al., 2004); in contrast, full-length RIG-I had a low basal signaling activity in the absence of viral PAMPs, providing the first hint that RIG-I is maintained in an inactive, signaling-repressed state in uninfected cells. Subsequent studies showed that the deletion of the CTD of RIG-I (and also LGP2) leads to its constitutive activation, while overexpressed CTD alone had a dominant-negative effect on RIG-I-induced signal transduction (Saito et al., 2007). This suggested a model in which the CTD functions as a regulatory/repressor domain (RD) by keeping RIG-I in an inactive conformation in which the CARDs are masked and thus unable to signal. The model for RIG-I autorepression was further refined by crystal structure data that demonstrated distinct
Figure 1.3. Dynamics of post-translational modifications during RIG-I activation. (A) In uninfected cells, RIG-I inactivation is maintained via two mechanisms: a closed conformation mediated by the helicase domain, as well as through phosphorylation at the S8 and T170 residues in the CARDs mediated by protein kinases (PKC)-α/β. (B) Upon viral infection, RIG-I binds to its RNA ligand, which facilitates RIG-I conformational changes. Release of RIG-I from the autorepressed conformation renders the CARDs accessible to the phosphatases PP1-α/γ, which dephosphorylate S8 and T170. (C) Dephosphorylation of the CARDs allows for interaction with the E3 ligase TRIM25, which mediates K63-linked polyubiquitination of K172. (D) Ubiquitination of RIG-I facilitates its multimerization and translocation to mitochondria-associated membranes where RIG-I interacts with MAVS.
conformational states for RIG-I in the absence of PAMPs and upon activation through viral RNA binding (Kowalinski et al., 2011). These findings indicated that in uninfected cells, interactions between the helicase core – in particular Hel2i – and the CARD2 of RIG-I inhibit MAVS binding and downstream signaling mediated by the CARDs.

In addition to conformational auto-repression of RIG-I, multi-site serine/threonine (S/T) phosphorylation has been shown to play an important role in preventing aberrant RIG-I signaling in uninfected cells (Gack, 2014). RIG-I is constitutively phosphorylated at the CARDs and the CTD in uninfected cells, and undergoes rapid dephosphorylation upon viral infection, indicating that a balance of phosphorylation and dephosphorylation regulates RIG-I signal transducing activity. Phosphorylation of the residues T770 and S854/S855 in the RIG-I CTD by casein kinase II (CK2) was shown to be required for maintaining the autorepressed conformation (Sun et al., 2010). Phosphorylation of S8 and T170 in the RIG-I CARDs induced by protein kinase C-α (PKC-α) or PKC-β inhibits CARD-dependent downstream signaling by preventing the interaction of RIG-I with MAVS (Gack et al., 2010; Maharaj et al., 2012; Nistal-Villan et al., 2010). In contrast, dephosphorylation of both S8 and T170 allowed efficient MAVS binding, possibly caused by a distinct conformation of the tandem CARD triggered by dephosphorylation of these residues.

Extensive research has been performed to determine the molecular details of how RIG-I is released from its autorepressed state in response to viral infection. The current view is that RIG-I activation requires (i) Binding of 5′-ppp-containing dsRNA to the CTD and helicase domain, and (ii) a series of dephosphorylation and K63-linked ubiquitination events both in the CTD and the CARDs. As described above, the RIG-I CTD and helicase domain bind to the 5′-ppp moiety and duplex RNA, respectively (Kohlway et al., 2013; Kowalinski et al., 2011; Lu et al., 2010; Luo et al., 2011; Wang et al., 2010). Thermodynamic studies further supported the model that the CARDs contribute to
dsRNA binding specificity by controlling RNA access to the helicase domain (Vela et al., 2012). Upon infection, viral RNA binding is believed to induce conformational changes that release the CARDs, rendering them accessible to downstream interaction partners.

Studies addressing the role of inhibitory phosphorylation marks in RIG-I demonstrated that binding of viral RNA is insufficient for RIG-I activation, but that dephosphorylation of the RIG-I CARDs (at S8 and T170) and CTD (at T770, S854/855) is required for RIG-I signaling (Gack et al., 2010; Maharaj et al., 2012; Nistal-Villan et al., 2010; Sun et al., 2010). A screen identified two isoforms of the S/T phosphoprotein phosphatase 1 (PP1), PP1-α and PP1-γ, as essential regulators of RIG-I (and MDA5) signaling (Wies et al., 2013). The PP1 subfamily is a group of S/T phosphatases that in mammals consists of the isoforms PP1-α, PP1-β, and PP1-γ. PP1 phosphatases are involved in many cellular processes, including cell cycle regulation, metabolism, protein synthesis, and muscle function [reviewed in (Cohen, 2002)]. This study demonstrated that PP1-α/γ dephosphorylate specific CARD residues (S8 and T170 in RIG-I; S88 in MDA5), thereby allowing MAVS binding and downstream signaling. The dephosphorylation of specific sites in the RIG-I CTD is also thought to be required for RIG-I activation (Sun et al., 2010), but the responsible phosphatase(s) has not been identified.

In addition to regulatory phosphorylation sites, RIG-I signaling is tightly regulated by K63-linked ubiquitin chains which, in contrast to K48-linked ubiquitination, do not target proteins for proteasomal degradation but rather modulate their activity. In recent years, a growing number of substrates targeted for K63-linked ubiquitination have been described that are involved in diverse processes, ranging from DNA repair, protein transport, translation and innate immune signaling [reviewed in (Weissman, 2001)]. Using mass spectrometry analysis, Gack et al. identified that the RIG-I CARDs, purified from human cells, undergo covalent K63-linked ubiquitination at six
lysine residues (K99, K169, K172, K181, K190 and K193). Mutational analysis identified that K63-linked polyubiquitin attached to K172 in RIG-I is necessary for RIG-I’s ability to bind MAVS and to induce type-I IFN induction (Gack et al., 2007). Mass spectrometry analysis of RIG-I CARD-interacting proteins further identified the ubiquitin E3 ligase tripartite motif protein 25 (TRIM25) as being responsible for catalyzing the covalent K63-linked ubiquitination of the RIG-I CARDs at K172 (Gack et al., 2008). In addition to covalent K63-linked polyubiquitination, the RIG-I CARDs have been shown to bind short, unanchored K63-linked polyubiquitin chains in vitro (Zeng et al., 2010). TRIM25, together with the E2 ubiquitin-conjugating enzyme Ubc5 or Ubc13, were responsible for the synthesis of free polyubiquitin chains, which upon binding to the CARDs facilitated RIG-I multimerization and engagement with MAVS (Jiang et al., 2012).

Recently, crystal structure of the tetrameric 2CARD of human RIG-I revealed that both covalent and non-covalent K63-linked ubiquitin chains act synergistically in promoting RIG-I 2CARD oligomer assembly and signaling ability (Peisley et al., 2014). Furthermore, it has been unclear how RIG-I, upon its ubiquitination, engages MAVS to induce antiviral signal transduction. Ubiquitinated, activated RIG-I as well as TRIM25 were recently reported to form a complex with the mitochondrial targeting chaperone protein 14-3-3ε. This ‘translocon’ complex triggered the redistribution of RIG-I from the cytosol to the membranes where MAVS is found (Liu et al., 2012).

Compared with those of RIG-I, the mechanisms of MDA5 activation are not as well-characterized, but what is known suggests that some of the regulatory steps leading to MDA5 activation may be quite different from those of RIG-I. Unlike RIG-I, MDA5 is not kept inactive by an autorepressed conformation (Berke and Modis, 2012), suggesting that PTMs may play an even more important role in controlling MDA5 signaling activity. One commonality of RIG-I and MDA5 activation is the removal of repressive phosphorylation marks in the CARDs (Wies et al.,
2013). Mass spectrometry and mutational analysis of purified human MDA5 2CARD revealed that phosphorylation MDA5 S88 modulates MDA5 signaling (Wies et al., 2013). In infected cells, recruitment of PP1-α/γ to endogenous MDA5 induced dephosphorylation of S88, promoting MDA5 binding to MAVS and type-I IFN induction. These results demonstrated that phosphorylation/dephosphorylation of specific S/T residues in the N-terminal CARDs regulates MDA5 and RIG-I signaling abilities and identified the phosphatases PP1-α/γ as common upstream activators of both sensors. While it is now well-established that K63-linked ubiquitination is essential for RIG-I signaling, the requirement for K63-linked ubiquitin chains in MDA5 activation has been a subject of debate. No covalent K63-linked ubiquitination of MDA5 CARDs purified from human cells has been detected (Gack et al., 2007). Furthermore, while a study suggested that unanchored K63-ubiquitin chains promote MDA5 oligomerization and filament formation in vitro (Jiang et al., 2012); others have not observed a requirement for unanchored K63-linked ubiquitin chains in MDA5 activation (Wu et al., 2013).
HERPES SIMPLEX VIRUS 1

The *Herpesviridae* family of enveloped, double-stranded DNA viruses, comprises more than 200 members, including nine herpesviruses that have been identified in humans to date: herpes simplex virus (HSV) 1 and 2, varicella zoster virus (VZV), human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6) A and B, human herpesvirus 7 (HHV-7), Epstein-Barr virus (EBV), and Kaposi’s sarcoma-associated herpesvirus (KSHV). *Herpesviridae* family members are further divided into three subfamilies: (1) *Alphaherpesviruses* (including HSV-1, HSV-2, and VZV), which are characterized by rapid replication and latency in neurons, (2) *Betaherpesviruses* (including HCMV, HHV-6A/B, and HHV-7), which are characterized by latency in leukocytes, and (3) *Gammaherpesviruses* (including EBV and KSHV), which are characterized by latency in lymphocytes.

HSV-1 epidemiology and disease

The alphaherpesvirus HSV-1 causes oral and genital herpes in humans. Recent estimates by the World Health Organization state that HSV-1 infection affects 3.7 billion individuals under age 50, or 67% of the population (WHO, 2017). Initial infection occurs by contact through broken skin or mucus membranes, and is either asymptomatic or characterized by lesions near the area of contact. After the first phase of infection, the virus travels from the initial site of infection up the axons of sensory ganglia neurons and establishes latency in the neuronal cell body. Periodically, the virus is reactivated and carried by anterograde axonal transport to cells near the sites of original infection where it can manifest as lesions (Knipe and Howley, 2013). Reactivation events have been associated with triggers such as stress, immunosuppression, and UV radiation, but the exact
mechanisms behind this process remain unclear. Although symptoms of infection are usually mild and transient, HSV-1 infection and reactivation can in rare cases lead to a number of severe neurological illnesses, especially in immunocompromised individuals and neonates. HSV-1 invasion of the central nervous system (CNS) can cause fatal HSV encephalitis, meningitis, myelitis, and radiculitis. Infection of the eye can cause ocular lesions and keratoconjunctivitis that over time may lead to corneal scarring and blindness [reviewed in (Remeijer et al., 2004)]. Reactivation of latent HSV-1 in facial and vestibular nerves is associated with the neurological disorders Bell’s palsy, which is characterized by facial paralysis, and vestibular neuritis, which is characterized by acute, prolonged peripheral vertigo (Royal and Vargas, 2014). Additionally, individuals infected with HSV-1 have been shown to have an increased susceptibility to infection with human immunodeficiency virus (HIV) 1 (Heng et al., 1994).

The nucleoside analogue acyclovir is the most commonly used drug for treating both HSV-1 and HSV-2 infections. When incorporated into a nascent DNA chain, acyclovir terminates DNA synthesis and inactivates viral DNA polymerase activity, thereby preventing viral DNA synthesis and viral replication (Kimberlin and Whitley, 2007). While this drug is moderately efficacious and has a good safety profile, acyclovir resistance is a substantial problem, especially in patients that have undergone long-term treatment with the drug. Other nucleotide and nucleoside analogue drugs, penciclovir and valaciclovir, respectively, function in similar ways and are used to supplement or complement acyclovir treatment. In recent years, there has been a renewed interest in the development of antivirals to treat herpesviruses, and several candidate compounds targeting various stages of the viral lifecycle have emerged [reviewed in (Price and Prichard, 2011)]. Despite these promising advances, there are currently no treatments capable of eliminating latent HSV reservoirs, and the need for an efficacious vaccine also remains.
There are currently no vaccines against either HSV-1 or HSV-2, and the bulk of vaccine development efforts have focused on HSV-2. However, as HSV-1 is implicated in many serious neurological diseases and is also increasingly the cause of genital herpes (Ryder et al., 2009), there is a need for a vaccine that specifically targets HSV-1, or an HSV-2 vaccine that elicits cross-reactive immunity. Promisingly, several HSV-2 vaccine candidates have been shown to provide partial protection against HSV-1. A variety of HSV-2 vaccine strategies have been tested in clinical trials for either prophylactic or therapeutic use, including HSV-2 glycoprotein D (gD2) and B (gB2) subunit vaccines, single cycle gH deletion virus, HSV-2 peptides, plasmid DNA vaccines, replication defective viruses, and live attenuated viruses (Awasthi and Friedman, 2014). However, none of these methods have proven successful to date. A deeper understanding of the viral lifecycle and the virus’s interactions with host immunity may uncover novel drug targets or more effective means of viral attenuation.

**HSV-1 life cycle**

The HSV-1 virion consists of an outer envelope studded with surface glycoproteins enclosing a tegument layer that surrounds an icosahedral capsid containing the dsDNA viral genome core. The approximately 152 kbp HSV-1 genome is composed of two unique regions: the unique long (UL) and unique short (US) regions, which are separated by stretches of inverted repeat sequences. At least 84 transcriptional units in the UL and US regions encode proteins. HSV-1 also expresses several ncRNAs, most of which arise from an 8.3-9 kb latency-associated transcript (LAT) encoded by an inverted repeat region flanking the UL region. Sixteen microRNAs and two LAT-encoded small ncRNAs have been identified to date (Knipe and Howley, 2013).
During HSV-1 infection, the viral glycoproteins gB and gC mediate the attachment of virions to cells via glycosaminoglycans (GAGs) at the cell surface. Next, the viral glycoprotein gD interacts with its cellular receptors, which include nectins, herpesvirus entry mediator (HVEM), and heparan sulfate. These receptors are expressed on many cell types, including lymphocytes, fibroblasts, epithelial cells, and neurons, which may explain the broad tropism of HSV-1 [reviewed in (Karasneh and Shukla, 2011)]. This interaction triggers fusion of the viral envelope and plasma membrane, a process that can occur either at the cell surface or after uptake of the virus by endocytosis. Upon membrane fusion, viral tegument proteins and capsid are released into the cytosol. One of these tegument proteins, the ribonuclease UL41, also known as virion host shutoff (vhs) protein, degrades cellular mRNA, which functions both to reduce the expression of host antiviral proteins and to redirect cellular resources to the translation of viral proteins. After release from the envelope, the viral capsid, along with a few inner tegument proteins that remain associated, is transported along microtubules to nuclear pores at the outer nuclear membrane. The linear viral DNA genome is then extruded from the capsid through the nuclear pore complex (NPC) into the nucleoplasm, where it rapidly circularizes and becomes associated with histones.

Once in the nucleus, HSV-1 transcription is mediated by the host RNA polymerase II (Pol II). HSV-1 mRNA transcripts are processed by cellular machinery, and the resulting capped and polyadenylated viral transcripts exported into the cytoplasm are essentially indistinguishable from cellular transcripts. The processes of HSV-1 transcription can be described as a cascade, in which viral proteins expressed at early time points transactivate the transcription of later viral genes, resulting in several temporally-distinct waves of protein expression, broadly delineated as the immediate-early, early, and late phases. The six immediate-early genes are expressed first and
initiate viral replication in the absence of de novo viral protein synthesis. After the immediate-early proteins are translated in the cytoplasm, they return to the nucleus and transactivate expression of early genes, by either serving as transcription factors or relieving cellular repression mechanisms. The early genes are primarily involved in forming and regulating the viral DNA replication complex and include the major viral single-stranded DNA (ssDNA) binding protein, ICP8. Finally, structural proteins, including capsid, tegument, and envelope proteins, are expressed during the late phase of HSV-1 transcription. Capsid proteins are assembled into procapsids in the nucleus, into which replicated viral genomes are packaged. The newly-formed nucleocapsids exit through the nuclear membrane and pass through the cytosol and plasma membrane, acquiring viral tegument proteins and glycoproteins, and host-derived envelope along the way, to become a fully mature virion upon release into the extracellular space. The details of nuclear egress remain an intense area of debate, and the current prevailing model describes nucleocapsid exit via a process of envelopment and deenvelopment at the outer nuclear membrane [reviewed in (Mettenleiter et al., 2013)]. HSV-1 progeny virions released from infected cells go on to infect neighboring cells and can also enter sensory neurons that innervate the region near the site of infection. Virions travel up axons by retrograde transport to the neuronal cell body and establish latency in the nucleus. Latently-infected neurons are characterized by minimal levels of lytic gene expression and an abundance of viral LAT transcripts. During instances of reactivation, viral replication resumes, and the resulting virions are carried by anterograde transport along axons and establish productive infection in nearby cells.
Innate immune sensing of HSV-1

The stages of the HSV-1 lifecycle occur within specific subcellular compartments, and PRRs residing in these compartments are uniquely poised to detect the protein and nucleic acid PAMPs generated there and activate antiviral signaling.

Viral glycoproteins gB and gH/gL on incoming virions are detected by TLR2 – in complex with coreceptors TLR1 or TLR6 – at the plasma membrane (Cai et al., 2013; Leoni et al., 2012). Interestingly, the requirement for TLR2 varies depending on the route of infection (Sorensen et al., 2008), and not all strains of HSV-1 activate TLR2 signaling (Sato et al., 2006). HSV-1 genomic DNA is a potent activator of several types of PRRs, but the relative contribution of these PRRs, along with the timing and subcellular location of their activation, remains a topic of intense debate and study. In addition, the exact nature and source of viral DNA detected by PRRs also remains unclear. Two of the most well-characterized DNA-sensing PRRs, interferon inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS), detect viral DNA in the nucleus and cytoplasm, respectively, and induce a type-I IFN response. IFI16 is a PYHIN family protein that contains a pyrin domain and two DNA-binding HIN domains, which directly bind viral DNA (Unterholzner et al., 2010). IFI16 has been shown to detect HSV-1 derived DNA released into the nucleus and is antagonized by the viral protein ICP0 (Orzalli et al., 2012). Although IFI16 is predominantly nuclear, cytoplasmic IFI16 has also been observed in certain cell types and may also contribute to cytoplasmic sensing of viral DNA [reviewed in (Veeranki and Choubey, 2012)]. cGAS is a DNA sensor localized to the cytoplasm that, upon binding its DNA ligand, generates a second messenger, cyclic GMP-AMP (cGAMP), which then activates the downstream signaling molecule STING, which leads to the induction of the type-I IFN response (Sun et al., 2013; Wu et al., 2013). cGAS plays an important role in sensing HSV-1 infection and has been shown to be essential for
immune defense against HSV-1 infection in mice (Li et al., 2013). Additionally, viral DNA found in endosomes has been shown to activate the endosomal PRR TLR9 (Sorensen et al., 2008), although TLR9-mediated signaling is not required for the innate immune response to HSV-1 in some cell types (Hochrein et al., 2004).

Although mRNA transcripts of HSV-1 are highly similar to cellular mRNA and therefore presumed to be ‘invisible’ to cellular sensors, it has been proposed that other RNA species generated during HSV-1 infection can trigger innate immune signaling. Accumulation of dsRNA in HSV-1-infected cells has been observed, although it is unclear whether these transcripts are of viral or cellular origin (Weber et al., 2006). The endosomal dsRNA sensor TLR3 (Ma and He, 2014) and cytoplasmic dsRNA sensor MDA5 (Melchjorsen et al., 2010) have been shown to contribute to the antiviral response to HSV-1 in cell-type dependent manner, but in both cases, the sources of dsRNA during infection were not identified. In 2009, two different groups, Chiu et al. and Ablasser et al. reported that HSV-1 (along with EBV and adenovirus) may be sensed by the RLR pathway through RNA Pol III (Ablasser et al., 2009; Chiu et al., 2009). They showed that Pol III-mediated transcription of the synthetic dsDNA molecule poly(dA-dT) generates a 5’-ppp-containing RNA, which can be recognized by RIG-I. Pol III was shown to play a role in generating EBV-encoded small RNAs (EBERs), which are abundant during latent EBV infection and induce a RIG-I-dependent IFN response (Samanta et al., 2006). Another proposed mechanism for innate immune activation by dsRNA during HSV-1 infection involves the endolysosomal dsRNA transporter protein SIDT2, which has been shown to facilitate the release of imported viral dsRNA from endosomes into the cytosol, where they activate IFN induction in a MAVS-dependent manner (Nguyen et al., 2017). This phenomenon was observed in uninfected bystander cells, suggesting that the dsRNA does not arise from viral replication within the cell but rather is taken in from the
extracellular space. Although the exact origins of extracellular viral dsRNAs is unknown, it was speculated that they may be released from ruptured infected cells.

**HSV-1 antagonism of innate immunity**

The IFN response is one of the host’s major antiviral defense mechanisms, and its critical role in controlling HSV-1 infection is underscored by the numerous viral mechanisms that inhibit the pathway at multiple levels. For example, the viral E3 ligase ICP0 mediates the degradation of several IFN signaling proteins, including the PRR IFI16, adaptor proteins MyD88 and Mal, and the transcription complex protein p50 [reviewed in (Lanfranca et al., 2014)]. The viral S/T kinase US3 is also known to counteract IFN signaling by mediating inhibitory phosphorylation of a variety of targets, including the PRR TLR3, adaptor protein TRAF6, transcription complex subunit p65, and transcription factor IRF3 [reviewed in (Su et al., 2016)]. IRF3, a key transcription factor for type-I IFN expression, is an especially favored target; anti-IRF3 activities have been ascribed to seven herpesviral proteins to date [reviewed in (Kumari et al., 2015)].

In addition to inhibiting IFN signal transduction, HSV-1 also directly antagonize ISGs – the effector molecules of the IFN pathway. The viral mechanism of host translational shutoff is mediated by the viral proteins vhs (also known as UL41) and ICP27 via their ability to promote degradation of mRNAs in the cytoplasm and to inhibit pre-mRNA splicing. Host shutoff has a general effect on the suppression of ISG expression, and vhs, along with other viral proteins, has also been shown to downregulate specific key ISGs such as RNA-dependent protein kinase (PKR), 2-5-oligoadenylate synthase (OAS), components of nuclear domain 10 (ND10), and the viral restriction factors viperin, tetherin, and zinc finger antiviral protein (ZAP) [reviewed in (Su et al., 2016)].
Chapter 2: Viral unmasking of cellular 5S rRNA pseudogene transcripts induces RIG-I mediated immunity

This chapter was adapted from:


Attributions:
J.J.C. and M.U.G. conceived the study. J.J.C. performed and analyzed all experiments with the exception of the following: K.M.J.S. analyzed RNA-Seq data (Figure 2.3). K.M.J.S and M.vG. performed the endogenous RNA5SP141 immunoprecipitation experiment (Figure 2.4B, C). C.L. and K.P.H. performed the in vitro ATP hydrolysis experiment (Figure 2.7C). M.vG. performed an EBV reactivation experiment (Figure 2.14A, B). T.H. and N.O. constructed HSV-1mut. J.J.C. and M.U.G. interpreted data and wrote the text.
ABSTRACT

The innate immune sensor retinoic acid-inducible gene-I (RIG-I) detects double-stranded RNA derived from RNA viruses. Although RIG-I is also known to play a role in the antiviral response to DNA viruses, physiological RNA species recognized by RIG-I during DNA virus infection have remained unknown. Using next-generation RNA sequencing (RNA-Seq), we found that host-derived RNAs, most prominently 5S ribosomal RNA pseudogene 141 (RNA5SP141), bind to RIG-I during herpes simplex virus 1 (HSV-1) infection. HSV-1 infection induced the relocalization of RNA5SP141 from the nucleus to the cytoplasm, and virus-induced shutoff of host protein synthesis downregulated 5S rRNA-interacting proteins, thereby allowing RNA5SP141 to bind RIG-I and induce type I interferon. Depletion of endogenous RNA5SP141 strongly dampened the antiviral response to HSV-1 and other viruses, including Epstein-Barr virus (EBV) and influenza A virus (IAV). Taken together, our data show that viral infection can lead to the exposure of endogenous RNAs that activate innate immunity.

INTRODUCTION

The mammalian innate immune system surveils infected cells for the presence of microbes using a defined repertoire of pattern-recognition receptors (PRRs) that, upon sensing, induce the expression of type I interferons (IFNs) and other antiviral genes (Goubau et al., 2013; Takeuchi and Akira, 2010). Among the PRRs, RIG-I (encoded by DDX58) is a key sensor for detection of RNA virus infection that recognizes virus-specific double-stranded RNA (dsRNA) in the host cell cytoplasm and initiates signaling cascades leading to the activation of antiviral defense pathways. The C-terminal domain of RIG-I recognizes and binds the 5’ end of the RNA ligand, which is
followed by RNA binding to the RIG-I central helicase domain. RNA binding triggers a conformational change in RIG-I that allows for the release of its N-terminal caspase activation and recruitment domains (CARDs) and binding to mitochondrial antiviral-signaling protein (MAVS; also called IPS-1, VISA or Cardif) (Loo and Gale, 2011; Wu and Chen, 2014).

Two well-characterized features of RIG-I ligands are a 5’-triphosphate (5’-ppp) moiety and adjacent base-paired stretches of at least 10-19 bp (Hornung et al., 2006; Pichlmair et al., 2006; Schlee et al., 2009), as found in the genomes or replication intermediates of several RNA viruses including IAV and paramyxoviruses (Baum et al., 2010; Rehwinkel et al., 2010; Runge et al., 2014). Furthermore, viral RNAs bearing 5′-diphosphates as well as poly-U/UC sequences as found in the RNA of hepatitis C virus are also recognized by RIG-I (Ablasser et al., 2009; Goubau et al., 2014), indicating that RIG-I is capable of recognizing a diverse group of viral RNA species.

In addition to its role in the detection of RNA viruses, evidence is accumulating that RIG-I also plays a critical role in mounting an innate immune response to several DNA viruses, including herpesviruses (e.g., EBV and HSV-1) and adenoviruses (Ablasser et al., 2009; Chan and Gack, 2016; Chiu et al., 2009). For the detection of EBV-derived DNA, RNA polymerase III (Pol III) has been shown to convert poly(dA:dT) into short 5’-ppp-containing RNAs that are recognizable by RIG-I, suggesting that Pol III acts upstream of RIG-I by producing RIG-I ligands through conversion of dsDNA (Ablasser et al., 2009; Chiu et al., 2009). However, physiological ligands recognized by RIG-I during DNA virus infection have not been identified.

Here we show that HSV-1 infection leads to the nuclear-to-cytoplasmic relocalization and exposure of cellular 5S ribosomal RNA pseudogene 141 (RNA5SP141) transcripts, allowing their recognition by RIG-I and activation of innate immunity. RNA5SP141 is critical for antiviral cytokine induction in response to HSV-1 and EBV, as well as the RNA virus IAV that replicates
in the nucleus. Our study provides evidence that RIG-I-mediated immunity can be triggered by viral perturbation of the life cycle of host-derived RNAs.
RESULTS

RIG-I and intracellular DNA sensors play temporally-distinct roles in DNA virus sensing

Although recent evidence indicates that RIG-I plays an important role in the antiviral defense to DNA viruses, its contribution relative to other key intracellular sensors of DNA virus infection, in particular cyclic GMP-AMP synthase (Sun et al., 2012) (cGAS) and IFN-inducible protein 16 (Unterholzner et al., 2010) (IFI16), has not been determined. We hypothesized that RIG-I and DNA sensors may play temporally-distinct roles in response to DNA virus infection, possibly due to the emergence of specific pathogen-associated molecular patterns (PAMPs) recognized by these sensors at different times during infection. To test this hypothesis, we individually depleted endogenous RIG-I, cGAS, and IFI16 using small interfering RNA (siRNA) in primary normal human lung fibroblasts (NHLFs), followed by infection with HSV-1, a DNA virus of the Herpesviridae family. Knockdown of endogenous RIG-I had no significant effect on IFNB1 and TNF mRNA amounts induced by HSV-1 at early time points after infection [6 hours post-infection (hpi)], but substantially reduced cytokine expression at a late time point (16 hpi) (Figure 2.1A). In contrast, silencing of cGAS impaired cytokine induction at both time points, while knockdown of IFI16 reduced TNF induction at 6 hpi but not 16 hpi. To corroborate the role of RIG-I in the detection of HSV-1, we assessed HSV-1-mediated upregulation of selected cytokines, chemokines and IFN-stimulated genes (ISGs) in Rig-I-deficient mouse embryonic fibroblasts (Rig-I −/− MEFs) (Figure 2.1B). Compared to wild-type MEFs (Rig-I +/+), induction of antiviral and proinflammatory genes in Rig-I −/− MEFs was severely attenuated, particularly at late times post-infection. Together, these results indicated that cGAS, IFI16 and RIG-I contribute to
HSV-1 sensing in a temporally-distinct manner, with RIG-I acting specifically at later time points during infection when potential immunostimulatory RNAs may emerge.

**Figure 2.1. RIG-I plays a role in the antiviral response to HSV-1.** (A) Left two panels: qRT-PCR analysis of *IFNB1* and *TNF* mRNA in primary NHLF cells transfected with either non-targeting control siRNA (si.Ctrl) or RIG-I, cGAS-, or IFI16-specific siRNAs (si.RIG-I, si.cGAS, si.IFI16) for 30 h and then infected with HSV-1 (MOI 0.1) for the indicated times. Results were normalized to 18S rRNA, and fold induction is shown relative to mock-infected control cells. Right three panels: Knockdown efficiency of endogenous RIG-I (*DDX58*), cGAS (*MB21D1*) and IFI16 was confirmed by qRT-PCR. (B) qRT-PCR analysis of *IFNB1*, *IFIH1*, *OASL1*, *RSAD2*, *IFIT2*, and *CCL5* mRNA in Rig-I+/+ and Rig-I−/− MEFs infected with HSV-1 (MOI 1 each) for the indicated time points. Results were normalized and shown as in (A). Data represent mean and s.d. of n = 3 biological replicates, and are representative of at least two independent experiments. *P <0.05, **P <0.01, ***P <0.001 (unpaired t-test); n.s., statistically not significant; n.d., not detected.
Host-encoded 5S rRNA pseudogene transcripts bind to RIG-I during HSV-1 infection

To identify the physiological RNA species that are recognized by RIG-I during HSV-1 infection, we purified the RNAs that co-immunoprecipitated with FLAG-tagged RIG-I in transfected human embryonic kidney (HEK) 293T cells that had been infected with a recombinant HSV-1 (HSV-1mut) containing a mutation in the viral serine/threonine protein kinase US3 that abolishes its catalytic activity, as the viral kinase is known to antagonize type-I IFN responses (Peri et al., 2008) (Figure 2.2A). As controls, RNA species bound to FLAG-RIG-I in uninfected cells and RNA bound to FLAG-GFP from both HSV-1mut-infected and uninfected cells were also purified. The RIG-I-bound RNA, but not the GFP-bound RNA from HSV-1mut-infected cells, induced robust activation of an IFN-sensitive response element (ISRE)-driven promoter, albeit less efficiently than RNA co-immunoprecipitated with FLAG-RIG-I from HEK 293T cells infected with Sendai virus (SeV), which is an RNA virus that potently activates RIG-I and served as positive control (Figure 2.2B, C).
Figure 2.2. Purification of RNA by RIG-I co-immunoprecipitation during HSV-1 infection. (A) Schematic representation of the experimental setup for isolation and identification of RNAs from FLAG-RIG-I or FLAG-GFP precipitates. RNAseq, next-generation RNA sequencing. (B) ISRE-luciferase reporter activity in HEK 293T cells transfected for 18 h with 7 μl RNA from FLAG-RIG-I- or FLAG-GFP-precipitates from uninfected (Mock) or HSV-1_mut-infected cells, isolated as described in (A). RNA from FLAG-RIG-I- or FLAG-GFP-precipitates from HEK 293T cells infected with SeV (50 HAU/mL) served as a positive control. Luciferase activity is presented as fold induction relative to the values for FLAG-GFP-precipitates from uninfected cells, set as 1. (C) Equal pulldown (PD) of FLAG-RIG-I and FLAG-GFP from the experiment shown in (A) and (B) was confirmed by immunoblot (IB) with an anti-FLAG antibody. Data are representative of two independent experiments. (mean and s.d. of n = 3 biological replicates in B). **P<0.01 (unpaired t-test). n.s. statistically not significant.
In contrast, the immunostimulatory activity of RIG-I-bound RNAs extracted from uninfected cells was minimal, indicating the emergence of RIG-I ligands was induced by HSV-1 infection. Next, RIG-I-bound RNA and total RNA extracted from uninfected and HSV-1\textsubscript{mut}-infected cells were analyzed by RNA-Seq, and the resulting sequences were mapped to both the HSV-1 and human genomes. The analysis revealed that several human transcripts were highly enriched in the RIG-I-bound fraction from infected cells; in contrast, the enrichment of viral sequences was low (Figure 2.3A), suggesting that the RIG-I-stimulatory RNA species that emerge during HSV-1 infection are host- rather than virus-derived. The cellular transcripts that were most abundant in the RIG-I fraction were predominantly non-coding RNAs from different subclasses as well as some coding RNAs (Figure 2.3B, C). Among the top ten RNAs that bound to RIG-I specifically in HSV-1\textsubscript{mut}-infected cells were three 5S ribosomal RNA pseudogene transcripts (RNA5SP), of which 5S rRNA pseudogene 141 (RNA5SP141) was the most substantially enriched RNA in the RIG-I-bound fraction (Figure 2.3C). qRT-PCR analysis using primers designed to target unique regions in RNA5SP141 that are absent in the parental 5S rRNA confirmed that RNA5SP141 was highly enriched in RIG-I precipitates specifically from HSV-1\textsubscript{mut}-infected, but not uninfected cells or GFP precipitates from either condition (Figure 2.4A). RNA5SP141 was also highly enriched in RIG-I precipitates from cells infected with wild-type HSV-1 (HSV-1\textsubscript{WT}) (Figure 2.4A). Importantly, endogenous RNA5SP141 purified from total cellular RNA using specific locked nucleic acid (LNA) antisense oligos detectably triggered \textit{IFNB1} mRNA induction when transfected into NHLFs (Figure 2.4B, C) when compared to RNA purified with ‘scrambled’ LNA antisense oligos, or purified U1 RNA, which served as an additional control, further substantiating a role for RNA5SP141 in innate immune activation.
Figure 2.3. Endogenous non-coding RNAs co-immunoprecipitate with RIG-I during HSV-1 infection. (A) Relative enrichment of HSV-1-derived or human-derived transcripts in FLAG-RIG-I precipitates from HSV-1mut-infected cells, determined by RNAseq. Relative enrichment (log_2) of transcripts was calculated by comparing the abundance of transcripts in FLAG-RIG-I precipitates to FLAG-GFP precipitates. Data are from two independent experiments. (B) Relative enrichment (log_{10}) of human transcripts in FLAG-RIG-I precipitates from HSV-1mut-infected cells. Labeled genes represent the 9 most highly enriched non-coding transcripts. rRNA pseudogene (red); long non-coding RNA (lncRNA, green); small nucleolar RNA (snoRNA, yellow); small Cajal body-specific RNAs (scaRNA, purple). (C) Relative enrichment of the 10 most highly-enriched human transcripts (both non-coding and coding) in FLAG-RIG-I precipitates from HSV-1mut-infected cells, as compared to uninfected cells. Data are representative of two independent experiments.
Figure 2.4. Endogenous non-coding RNAs co-immunoprecipitate with RIG-I during HSV-1 infection. (A) Quantitative RT-PCR (qRT-PCR) analysis of RNA5SP141 transcripts from RNA isolated as described in (Figure 2.2A) from HEK 293T cells transfected with FLAG-RIG-I or FLAG-GFP and infected with HSV-1\textsubscript{WT} or HSV-1\textsubscript{mut} (both MOI 1), or left uninfected (Mock). (B) qRT-PCR analysis of *IFNB1* mRNA in primary NHLF cells transfected for 20 h with RNA purified by streptavidin pull-down from total RNA using 3′-biotinylated locked nucleic acid (LNA) oligos. Oligos designed to capture a nonsense scramble sequence (Scramble) and a highly abundant small nuclear RNA RNU1-1 (U1) that is not known to be immunostimulatory served as negative controls. (C) Representative enrichment of RNU1-1 and RNA5SP141 by pulldown as described in (B). Data are representative of two independent experiments (mean and s.d. of n = 3 technical replicates in A and C, n = 3 biological replicates in B). **P<0.01 (unpaired *t*-test). n.s. statistically not significant.
RNA5SP141 is a direct ligand of RIG-I

We next sought to examine if RNA5SP141 is a direct and specific ligand of RIG-I. Since very little is known about the expression and function of RNA5SP transcripts, we inspected their parent gene, 5S rRNA, for insight into how the transcribed pseudogenes may serve as RIG-I agonists. 5S rRNA is a 120 nucleotide Pol III transcript with a highly stable secondary structure composed of helices and hairpin loops organized in a three-helix junction (Szymanski et al., 2003). Structure prediction suggests that, like the parental 5S rRNA, RNA5SP141 has extensive base-pairing throughout and particularly at the 5’ end (Figure 2.5).

**Figure 2.5. Predicted secondary structure of RNA5SP141.** RNA5SP141 structure was modeled using the Vienna RNAfold web server. MFE, minimum free energy. 5’-ppp, 5’-triphosphate.
We hypothesized that these double-stranded regions, along with the 5’-ppp moiety that is characteristic of Pol III transcripts, may provide the molecular basis for RNA5SP141 recognition by RIG-I and stimulation of innate immunity. Indeed, \textit{in vitro}-transcribed RNA5SP141 robustly upregulated \textit{IFNB1} mRNA in primary NHLF cells, and also induced the expression of the proinflammatory cytokine \textit{TNF} and the ISGs \textit{ISG15} and \textit{DDX58} (Figure 2.6A, B). The cytokine and ISG induction by RNA5SP141 was comparable to, or even higher than, the induction triggered by a 58-nucleotide rabies virus leader (RABV\textsubscript{Le}) \textit{in vitro} transcript, which was previously shown to activate RIG-I (Cui et al., 2008; Hornung et al., 2006). Phosphatase treatment of RNA5SP141 to remove the 5’-ppp group, a well-defined feature of RIG-I (but not MDA5) ligands (Hornung et al., 2006; Pichlmair et al., 2006), abrogated its ability to stimulate ISRE-promoter activation (Figure 2.6C). \textit{In vitro} transcribed RNA5SP141 and RABV\textsubscript{Le} also strongly activated antiviral cytokine responses in HEK 293T cells, while a 59-nucleotide RNA derived from an internal rabies virus sequence [previously termed ‘RV2024-2080’ (Saito et al., 2008) and hereafter termed ‘RABV\textsubscript{INT}’], which served as negative control, was not immunostimulatory (Figure 2.6D). In agreement with these observations, RNA5SP141 transfection of HEK 293T cells induced the dimerization of interferon regulatory factor 3 (IRF3), a key transcription factor downstream of RIG-I and also other PRRs [reviewed in (Hiscott, 2007)], to levels comparable to those after SeV infection or RABV\textsubscript{Le} transfection (Figure 2.6E). Depletion of RIG-I, but not MDA5, using specific siRNAs severely impaired the ability of RNA5SP141 to elicit an IFN-β response, indicating that RNA5SP141 specifically activates RIG-I (Figure 2.6F). Silencing of MDA5, however, reduced the IFN-β response triggered by transfection of high molecular weight (HMW) polyinosine-polycytidylic acid [poly(I:C)], an MDA5 agonist which served as control. In a complementary approach and to rule out the possibility that potential dsRNA byproducts generated during \textit{in vitro}
Figure 2.6. In vitro-transcribed RNA5SP141 activates RIG-I signaling. (A) Virtual gel image of in vitro transcribed RNA5SP141, or in vitro transcribed RNA corresponding to the rabies virus leader sequence (RABV <sub>Le</sub>; positive control) or an internal rabies virus sequence (RABV <sub>INT</sub>; negative control), analyzed by Agilent 2100 Bioanalyzer. (B) qRT-PCR analysis of IFNB1 transcripts in primary NHLF cells transfected for 18 h with the indicated amounts of in vitro transcribed RNA5SP141 or RABV <sub>Le</sub> (positive control). (C) ISRE-luciferase activity in HEK 293T cells transfected for 18 h with 500 fmol in vitro transcribed RNA5SP141 or RABV <sub>Le</sub>, which had been pre-treated with calf alkaline phosphate (CIP), or left untreated. (D) qRT-PCR analysis of the indicated cytokine and ISG transcripts (vertical axes) in HEK 293T cells transfected for 16 h with 200 fmol or 1 pmol of the indicated in vitro transcribed RNAs. (E) Native PAGE and SDS-PAGE of lysates from HEK 293T cells transfected with 125 pmol of the indicated in vitro transcribed or infected with 50 HAU/mL SeV for 16 h, or left untreated (Mock). Endogenous IRF3 and actin were detected by IB with anti-IRF3 and anti-actin antibody, respectively. (F) Left panel: qRT-PCR analysis of IFNB1 mRNA in primary NHLF cells transfected with siRNA targeting RIG-I (si.RIG-I), MDA5 (si.MDA5), or non-targeting control siRNA (si.Ctrl), followed 30 h later by transfection for 16 h with 1 pmol of the indicated in vitro-transcribed RNAs, or treatment with 0.05 μg/mL HMW-poly(I:C) which served as a control. Knockdown efficiency of endogenous RIG-I (DDX58) and MDA5 (IFIH1) was confirmed by qRT-PCR (right panels). Data are representative of two (E) or three (B, C, D, F) independent experiments (mean and s.d. of n = 2 biological replicates in D, n = 3 biological replicates in B, C and F).
transcription triggered RIG-I activation, we also generated a DNA construct encoding the RNA5SP141 sequence under the Pol III promoter U6 and tested its immunostimulatory activity (Figure 2.7A). U6-expressed RNA5SP141 triggered robust ISRE-promoter activation, and this immunostimulatory activity was abrogated when endogenous RIG-I was depleted, while it was unchanged upon knockdown of MDA5. Silencing of MDA5 did, however, reduce ISRE-promoter activation triggered by transfection of the MDA5 agonist HMW poly(I:C). Furthermore, transfection of a DNA construct encoding U6-expressed RABV<sub>INT</sub> served as an additional control in this assay to confirm that DNA transfection itself did not induce activation of the ISRE-promoter in HEK 293T cells, which are known to be defective in cGAS-STING signaling (Sun et al., 2012). Similarly, U6-expressed RNA5SP141, but not RABV<sub>INT</sub>, robustly induced IFNB1 transcripts in primary NHLFs, and silencing endogenous RIG-I, but not MDA5, blunted IFNB1 induction triggered by U6-expressed RNA5SP141 (Figure 2.7B), further supporting that RNA5SP141 elicits cytokine responses via RIG-I. Finally, to confirm that RNA5SP141 directly interacts with and activates RIG-I, we performed an ATP hydrolysis assay using in vitro-transcribed RNA5SP141 and purified RIG-I protein. Upon binding, RIG-I agonists activate the ATPase activity of the central SF2 helicase domain of RIG-I (Civril et al., 2011), and we found that RNA5SP141 did indeed efficiently activate the RIG-I ATPase activity at levels comparable to RABV<sub>Le</sub> RNA (Figure 2.7C). Collectively, these findings demonstrate that RNA5SP141 is a direct ligand of RIG-I.
Figure 2.7. Pol III promoter-expressed RNA5SP141 activates RIG-I signaling. (A) ISRE-luciferase reporter activity (left panel) in HEK 293T cells transfected for 30 h with non-targeting control siRNA (si.Ctrl) or siRNAs targeting RIG-I or MDA5 (si.RIG-I or si.MDA5), and subsequently mock-transfected or transfected with 500 ng DNA construct encoding U6 promoter-expressed RNA5SP141 or RABV\textsubscript{INT} for 18 h. Treatment with 1 \( \mu \)g/mL HMW-poly(I:C) served as a control. Knockdown efficiency of endogenous RIG-I (\textit{DDX58}) and MDA5 (\textit{IFIH1}) was confirmed by qRT-PCR (right panels). (B) qRT-PCR analysis of \textit{IFNB1} mRNA (left panel) in NHLF cells that were transfected with the indicated siRNAs and U6 promoter-expressed DNA constructs as described in (A). (C) Quantification of hydrolyzed [\( \gamma \)-\textsuperscript{32}P]ATP by RIG-I incubated for the indicated times with 250 nM of \textit{in vitro}-transcribed RNA5SP141. Incubation of RIG-I with \textit{in vitro} transcribed RABV\textsubscript{La}, or no RNA, served as positive and negative controls, respectively. Free phosphate was separated from unhydrolyzed ATP by thin layer chromatography, and the percentage of hydrolyzed ATP in each sample was calculated. Data are representative of two independent experiments (mean and s.d. of \( n = 3 \) biological replicates in A and B, \( n = 3 \) technical replicates in C).
**HSV-1 infection induces nuclear-to-cytoplasmic relocalization of RNA5SP141**

To define the mechanism by which RNA5SP141 transcripts are recognized by RIG-I specifically in HSV-1-infected cells but not uninfected cells, we first considered the possibility that HSV-1 infection leads to changes in RNA5SP141 gene expression, thereby facilitating its sensing by RIG-I. While HSV-1 is known for its ability to downregulate most host mRNAs, a phenomenon termed virus-induced host shutoff, other cellular RNAs are not downregulated (e.g., tRNAs and rRNAs), or are instead upregulated (e.g., proinflammatory cytokine transcripts) [reviewed in (Glaunsinger and Ganem, 2006)]. Both RNAseq and qRT-PCR analysis showed that RNA5SP141 is upregulated ~2- to 3-fold in HSV-1-infected cells compared to uninfected cells; in contrast most host RNAs were downregulated during HSV-1 infection, consistent with previous reports [reviewed in (Glaunsinger and Ganem, 2006)] (Figure 2.8A, B). However, as RNA5SP141 was highly enriched in the RIG-I-bound fraction from HSV-1-infected cells (Figure 2.3C and 2.4A), we considered the possibility that additional changes in the distribution or availability of RNA5SP141 must occur during HSV-1 infection that enable RNA5SP141 detection by RIG-I.

![Figure 2.8. RNA5SP141 levels in HSV-1 infected cells. (A) Relative expression of RNA5SP141 in HEK 293T cells infected with HSV-1WT (MOI 1) for 16 h, as compared to uninfected cells, determined by RNAseq analysis. Red boundaries represent ± 2-fold change in gene expression. (B) qRT-PCR analysis of RNA5SP141 transcripts in primary NHLF cells infected with HSV-1WT (MOI 1) for the indicated times. Data represent mean and s.d. of n = 3 biological replicates, and are representative of two independent experiments.](image-url)
While little information exists about the life cycle of RNA5SP transcripts, it has been established that the parental 5S rRNA is transcribed in the nucleoplasm and then either imported into the nucleolus for ribosome biogenesis, or exported to the cytoplasm and mitochondria [reviewed in (Ciganda and Williams, 2011)]. We hypothesized that RNA5SP141 may follow a similar pattern of transcription and transport that, when perturbed by HSV-1, allows sensing of RNA5SP141 by RIG-I in the cytoplasm. As HSV-1 replication and egress from the cell nucleus are known to remodel and disrupt nuclear architecture [reviewed in (Johnson and Baines, 2011)], we tested whether HSV-1 infection triggers nuclear-to-cytoplasmic relocalization of RNA5SP141. To this end, we performed a fractionation assay of uninfected or HSV-1-infected HEK 293T cells and compared the abundance of RNA5SP141 in the cytoplasmic and nuclear fractions. We observed that, while RNA5SP141 was predominately nuclear in uninfected cells, it was predominantly cytoplasmic in HSV-1-infected cells (Figure 2.9A). As compared to RNA5SP141, the cytoplasmic levels of RNU2-1, MALAT1, and NEAT1, which are well-defined nuclear RNAs (West et al., 2014), were only marginally increased following HSV-1 infection. Furthermore, RNA5SP141 localization remained predominantly nuclear in cells infected with SeV (Figure 2.9B), suggesting that the induction of RNA5SP141 relocalization may be specific for infection with herpesviruses, or perhaps more generally, viruses that replicate in the nucleus. Collectively, these data showed that HSV-1 infection leads to increased cytoplasmic localization of RNA5SP141.
Figure 2.9. RNA5SP141 is relocalized from the nucleus to the cytoplasm during HSV-1 infection but not SeV infection. (A) Left four panels: Relative abundance of RNA5SP141 transcripts in the cytoplasmic and nuclear fractions of HEK 293T cells that had been infected with HSV-1<sub>WT</sub> (MOI 1) for 16 h, or left uninfected (Mock), determined by cytoplasmic-nuclear fractionation assay and qRT-PCR. Analysis of the relative abundance of RNU2-1, MALAT1, and NEAT1 RNA (all nuclear RNAs) served as controls. Right panel: IB analysis of Lamin A/C and β-Tubulin confirmed the purity of the nuclear and cytoplasmic fraction, respectively. IB analysis of whole cell lysates (WCL) with anti-HSV-1 infected cell protein 8 (ICP8) and anti-actin served as infection and loading controls, respectively. (B) Left panel: Relative abundance of RNA5SP141 transcripts in the cytoplasmic and nuclear fractions of HEK 293T cells that had been infected with SeV (50 HAU/mL) for 16 h or left uninfected (Mock), determined by fractionation assay and qRT-PCR analysis as described in (A). Right panel: IB analysis of cytoplasmic and nuclear fractions as in (A). IB analysis of WCL with anti-SeV confirmed SeV infection. Data are representative of two independent experiments.
**HSV-1-induced depletion of 5S rRNA-binding proteins allows for RNA5SP141 sensing by RIG-I**

When 5S rRNA is present in the cytoplasm, it is normally tightly associated with proteins that facilitate its subcellular transport, increase its stability, or mediate its function as part of the large ribosomal subunit [reviewed in (Ciganda and Williams, 2011)]. At least three major cytoplasmic 5S rRNA-binding proteins have been identified: (1.) ribosomal protein L5 (RPL5), which associates with 5S rRNA to form 5S ribonucleoprotein particles [reviewed in (Ciganda and Williams, 2011)]; (2.) mitochondrial ribosomal protein L18 (MRPL18) and (3.) thiosulphate-sulphur transferase (TST; also called rhodanese), both of which facilitate the import of cytoplasmic 5S rRNA into mitochondria [reviewed in (Niazi et al., 2013)]. We hypothesized that, as with parental 5S rRNA, RNA5SP141 is associated with these proteins when present in the cytoplasm. Under normal conditions the 5S rRNA-interacting proteins may ‘shield’ it from the immune surveillance apparatus, whereas HSV-1-mediated shutoff of their synthesis may liberate RNA5SP141 for recognition by RIG-I. To address this hypothesis, we tested whether (1.) RNA5SP141, like its parental 5S rRNA, also complexes with 5S rRNA-binding proteins; (2.) HSV-1 downregulates the expression of 5S rRNA-binding proteins; and (3.) downregulation of these proteins leads to increased binding of RNA5SP141 by RIG-I and thereby IFN induction. Co-immunoprecipitation showed that RNA5SP141, like the parental 5S rRNA, bound to RPL5, MRPL18, and TST, although with different binding affinities (Figure 2.10A). The mRNA levels of the 5S rRNA-binding proteins were significantly downregulated in HSV-1-infected cells compared to noninfected cells (Figure 2.10B, C), and the corresponding protein levels of MRPL18 and TST were also reduced over the course of HSV-1\textsubscript{WT} infection compared to uninfected cells.
Figure 2.10. 5S rRNA interaction proteins are downregulated during HSV-1 infection.

(A) Binding of biotinylated in vitro-transcribed RNA5SP141 to FLAG-tagged RPL5, MRPL18, and TST in transiently transfected HEK 293T cells, assessed by streptavidin pulldown (Strep-PD) and IB with anti-FLAG antibody. Whole cell lysates (WCLs) were probed by IB with anti-FLAG and anti-actin antibodies. Biotinylated 5S rRNA and a scrambled random RNA (Scrambled) were included as positive and negative controls, respectively.

(B) Relative mRNA expression of RPL5, MRPL18, and TST in HEK 293T cells infected with HSV-1 (MOI 1) for 16 h as compared to uninfected cells, determined by RNAseq. Red boundaries represent ±2-fold change in gene expression.

(C) qRT-PCR analysis of RPL5, MRPL18, and TST mRNA in HEK 293T cells infected with HSV-1 (MOI 1) and harvested at the indicated times. Data are representative of two (A, B) or three (C) independent experiments (mean and s.d. of n = 2 biological replicates in C).
In contrast, MRPL18 and TST protein levels were not reduced in cells infected with a recombinant HSV-1 with a deletion in the gene encoding the virion host shutoff (vhs) protein (HSV-1Δvhs), an mRNA-specific RNase that plays a major role in HSV-1-induced host translational shutoff (Kwong and Frenkel, 1987) (Figure 2.11A, left). Notably, despite downregulation of RPL5 transcripts upon HSV-1WT infection, we observed that RPL5 protein levels were not reduced by HSV-1WT or HSV-1Δvhs infection, which is consistent with previous observations that ribosomal protein levels are known to persist during HSV-1 infection despite viral shutoff mechanisms (Simonin et al., 1997). RNA5SP141 bound to RIG-I efficiently during infection with HSV-1WT, but not HSV-1Δvhs (Figure 2.11B), suggesting that virus-induced downregulation of MRPL18 and/or TST proteins leads to the unshielding of RNA5SP141 transcripts, thereby allowing recognition by RIG-I and activation of innate immunity. In support of this, IFNB1 transcripts induced by HSV-1Δvhs were lower than those induced by HSV-1WT infection (Figure 2.11C). However, the phenomenon of higher cytokine induction by infection with HSV-1WT, as compared to HSV-1Δvhs, was not generally applicable, as the induction of the proinflammatory cytokines IL6 and IL8, which are known to be direct targets of vhs-mediated degradation (Smiley, 2004), was repressed in HSV-1WT-infected cells infection (Figure 2.11D). To further test the role of 5S rRNA-interaction proteins in RNA5SP141-induced innate signaling, we used siRNAs to individually deplete endogenous RPL5, MRPL18, and TST, thereby mimicking the effects of HSV-1 infection (Figure 2.11E). Depletion of MRPL18 or TST markedly enhanced IFNB1 mRNA levels triggered by RNA5SP141 transfection but did not affect the response in mock-transfected cells. In contrast, depletion of RPL5 had no significant effect on RNA5SP141-induced IFNB1 mRNA expression. Inversely, ectopic expression of MRPL18 or TST, but not RPL5, dampened the IFN-β transcriptional activity triggered by RNA5SP141 in a dose-dependent
Figure 2.11. HSV-1-induced downregulation of 5S rRNA-interacting proteins leads to activation of RIG-I signaling. (A) IB analysis of endogenous RPL5, MRPL18, and TST proteins in the WCLs of HEK 293T cells infected with HSV-1 WT or HSV-1 Δvhs (both MOI 10) for the indicated times. IB analysis of HSV-1 ICP8 and cellular actin served as infection and loading controls, respectively. (B) HEK 293T cells were transfected with FLAG-RIG-I or FLAG-GFP. 24 h later, cells were infected with HSV-1 WT or HSV-1 Δvhs (both MOI 10) for 16 h, or left uninfected (Mock). RNA bound to FLAG-RIG-I or FLAG-GFP was precipitated from cell lysates using anti-FLAG PD as described in Figure 2.2A, followed by qRT-PCR analysis to assess bound RNA5SP141 transcripts. (C) qRT-PCR analysis of IFNB1 mRNA in NHLF cells infected with HSV-1 WT or HSV-1 Δvhs (both MOI 1) for 16 h and 24 h, or left uninfected (Mock). (D) qRT-PCR analysis of IL6 and IL8 transcripts in primary NHLF cells that were mock-infected or infected with HSV-1 Δvhs or HSV-1 WT (MOI 10 each) for 16 h. (E) qRT-PCR analysis of IFNB1 transcripts in HEK 293T cells transfected with the indicated siRNAs for 30 h and then transfected with either no RNA (Mock) or 1 pmol of in vitro-transcribed RNA5SP141 for 16 h. Values were normalized to GAPDH. (F) IFN-β luciferase activity in HEK 293T cells transfected for 30 h with the indicated amounts of plasmids expressing FLAG-tagged RPL5, MRPL18, or TST and subsequently transfected with 1 pmol of RNA5SP141 for 16 h to stimulate RIG-I signaling. Expression of FLAG-tagged proteins was confirmed in the WCL by IB with anti-FLAG. Data are representative of two (A-D) or three (E, F) independent experiments (mean and s.d. of n = 3 technical replicates in B, n = 2 biological replicates in C and F, n = 3 biological replicates in D and E). *P < 0.05, **P<0.01, ***P<0.001 (unpaired t-test). n.s., statistically not significant.
manner (Figure 2.11F), suggesting that the interaction of RNA5SP141 with MRPL18 and TST prevents RIG-I-dependent cytokine induction. Taken together, these findings support a model in which RNA5SP141 transcripts are mislocalized to the cytoplasm during HSV-1 infection, and virus-mediated downregulation of MLRP18 and TST allows RIG-I access to these host-encoded transcripts, resulting in activation of innate immunity.

**RNA5SP141 is required for mediating an antiviral cytokine response to HSV-1 and EBV infection**

To determine the relevance of endogenous RNA5SP141 in eliciting an antiviral response to HSV-1 infection, we performed siRNA knockdown of RNA5SP141 in NHLF cells and analyzed IFNB1 and TNF transcripts in response to HSV-1 infection (Figure 2.12A, B). As compared to cells transfected with non-targeting control siRNA, the levels of HSV-1-triggered IFNB1 and TNF mRNA in RNA5SP141-depleted cells were profoundly reduced and similar to levels in RIG-I-depleted cells. Importantly, depletion of RNA5SP141 had no effect on IFNB1 mRNA triggered by transfection of RABV1c or HMW-poly(I:C) (Figure 2.12A), ruling out the possibility that silencing RNA5SP141 has a general inhibitory effect on IFNB1 induction. In support of this finding, silencing of RNA5SP141, unlike knockdown of RIG-I, had no effect on IFNB1 and TNF transcripts or secreted IFNB1 or CCL5 protein triggered by infection with SeV (Figure 2.12C, D), a paramyxovirus that replicates in the cytoplasm and for which it has been well established that viral RNAs, in particular RNAs from defective interfering (DI) particles, trigger RIG-I activation (Baum et al., 2010; Strahle et al., 2006).
Figure 2.12. RNA5SP141 is essential for the induction of an antiviral cytokine response to HSV-1. (A) qRT-PCR analysis of IFNB1 mRNA in NHLF cells transfected with either non-targeting control siRNA (si.Ctrl), or siRNAs targeting RIG-I (si.RIG-I) or RNA5SP141 (si.5SP141) for 72 h and then transfected with 1 pmol in vitro-transcribed RABV Le or treated with 0.05 μg/mL HMW-poly(I:C) (controls), or infected with HSV-1 (MOI 0.1) for 16 h. (B) Left panel: qRT-PCR analysis of TNF mRNA in primary NHLF cells transfected with the indicated siRNAs as in (A) followed by infection with HSV-1 (MOI 0.1) for 16 h. Right two panels: Knockdown efficiency of endogenous RIG-I (DDX58) and RNA5SP141 (5SP141) was confirmed by qRT-PCR. (C) Left two panels: qRT-PCR analysis of IFNB1 and TNF mRNA in HEK 293T cells transfected with the indicated siRNAs as in (A) followed by infection with SeV (50 HAU/mL) for 16 h. Right two panels: Knockdown efficiency of endogenous RIG-I (DDX58) and RNA5SP141 (5SP141) was confirmed by qRT-PCR analysis. (D) ELISA of IFN-β (left panel) and CCL5 (right panel) in the supernatants of primary NHLF cells that were transfected with the indicated siRNAs as in (A) followed by infection with SeV (50 HAU/mL) for 24 h. Data are representative of two independent experiments (mean and s.d. of n = 3 biological replicates in A-C, n = 2 biological replicates in D. *P < 0.05, **P<0.01, ***P<0.001 (unpaired t-test). n.s., statistically not significant.
We next used two different LNA gapmers, each with a different targeting sequence than the siRNA used in our experiments, to deplete endogenous RNA5SP141. As a control, we also used two gapmers to deplete endogenous RIG-I (Figure 2.13A). Consistent with our siRNA results, gapmer-mediated depletion of either RNA5SP141 or RIG-I strongly reduced antiviral cytokine induction in response to HSV-1 (Figure 2.13B). In contrast, gapmer-mediated knockdown of RNA5SP141 had no effect on SeV-induced IFNB1 mRNA, while RIG-I depletion abolished IFNB1 induction by SeV as expected (Figure 2.13C). Gapmer knockdown of RNA5SP141 or RIG-I had no effect on IFNB1 induction in response to treatment with the MDA5 agonist HMW poly(I:C) (Figure 2.13D).
Figure 2.13. Gapmer depletion of RNA5SP141 dampens antiviral cytokine response to HSV-1. (A) Representative knockdown efficiency of RIG-I (DDX58) (left panel) and RNA5SP141 (right panel) in NHLF cells achieved by transfection of two different gapmers targeting RIG-I (Gap RIG-I_1 and Gap RIG-I_2) or RNA5SP141 (Gap 5SP141_1 and Gap 5SP141_2), assessed by qRT-PCR at 72 h post-transfection. Non-targeting control gapmer (Gap NT) served as control. (B) qRT-PCR analysis of IFNB1, CCL5, and TNF mRNA in NHLF cells transfected with either non-targeting control GapmeR (Gap NT), or two different GapmeRs targeting RIG-I (Gap RIG-I_1 and Gap RIG-I_2) or RNA5SP141 (Gap 5SP141_1 and Gap 5SP141_2) for 72 h and then infected with HSV-1 (MOI 0.1) for 16 h (C) qRT-PCR analysis of IFNB1 and IFIT1 mRNA in NHLF cells transfected as in (B) and then infected with SeV (25 HAU/mL) for 16 h. (D) qRT-PCR analysis of IFNB1 mRNA in NHLF cells transfected as in (B) and then treated with HMW poly(I:C) for 16 h. Data are representative of two (A, D) or three (B, C) independent experiments (mean and s.d. of n = 3 technical replicates in A, n = 3 biological replicates in B, C, D).
We next asked whether RNA5SP141 also serves as a RIG-I ligand during infection by other DNA viruses. EBV is a member of the *Gammaherpesvirinae* subfamily that also replicates in the nucleus and exhibits a strong host shutoff phenotype during lytic infection (Glaunsinger and Ganem, 2006). Using gastric adenocarcinoma cells harboring latent EBV episomes (AGS-EBV), in which EBV lytic replication can be induced by treatment with sodium butyrate (NaB), we found that EBV reactivation led to strong upregulation of *TNF*, *IL6*, and *IL8* transcripts, but not *IFNB1* mRNA (Figure 2.14A, B). Consistent with previous reports indicating that RIG-I plays a role in EBV detection (Ablasser et al., 2009; Chiu et al., 2009), knockdown of endogenous RIG-I markedly reduced *TNF* induction upon EBV reactivation in AGS-EBV cells, while depletion of endogenous MDA5 only had a very moderate effect (Figure 2.14C). Furthermore, siRNA-mediated depletion of RNA5SP141 resulted in profound reduction of *TNF* induction in response to EBV reactivation, similarly to RIG-I knockdown (Figure 2.14C), suggesting that RIG-I activation by EBV is predominantly mediated by RNA5SP141 in these cells. Consistent with this observation, silencing of RIG-I or RNA5SP141 strongly reduced *IL6* and *IL8* mRNA as well as IL8 protein secretion upon EBV reactivation (Figure 2.14D, E). Moreover, we observed that the 5S rRNA-interacting proteins MRPL18 and TST, but not RPL5, were downregulated upon EBV reactivation (Figure 2.14F), suggesting that recognition of RNA5SP141 by RIG-I is triggered by EBV-mediated unshielding of RNA5SP141.
Figure 2.14. RNA5SP141 contributes to the antiviral cytokine response to EBV.
(A) *IFNB1*, *TNF*, *IL6*, and *IL8* transcripts in AGS-EBV cells after treatment with 2.5 mM sodium butyrate (NaB) for the indicated times to induce EBV reactivation, assessed by qRT-PCR analysis. (B) Efficient EBV reactivation was confirmed by determining the transcript levels of EBV early gene *BMRF1* (EA-D) by qRT-PCR analysis. (C) qRT-PCR analysis of *TNF* mRNA in AGS-EBV cells transfected with the indicated siRNAs for 72 h, followed by treatment with 2.5 mM NaB for 24 h. (D) qRT-PCR analysis of *IL6* and *IL8* mRNA (left two panels) in AGS-EBV cells transfected with the indicated siRNAs for 72 h, followed by treatment with 2.5 mM NaB for the indicated times. EBV reactivation was confirmed by qRT-PCR analysis of EBV early gene *BMRF1* mRNA (right panel). (E) ELISA of IL-8 in the supernatants of AGS-EBV cells that were transfected with the indicated siRNAs for 72 h, followed by treatment with 2.5 mM NaB for 24 h. (F) Abundance of endogenous MRPL18, TST, and RPL5 proteins in the WCLs of AGS-EBV cells treated with 2.5 mM NaB for the indicated times, determined by IB. IB analysis of EBV early antigen-restricted p85 (Ea-R p85) and cellular actin served as infection and loading controls, respectively. Data are representative of two (A, B, D, E, F) or three (C) independent experiments (mean and s.d. of n = 3 technical replicates in A and B, n = 3 biological replicates in C and D, n = 2 biological replicates in E). *P < 0.05, **P<0.01, ***P<0.001 (unpaired t-test).
Unlike most RNA viruses, IAV and other members of the family *Orthomyxoviridae* replicate in the host cell nucleus. RIG-I is the major sensor of IAV infection in mammalian cells, and recognizes IAV genomes and RNA derived from DI particles (Baum et al., 2010; Rehwinkel et al., 2010). We, therefore, asked whether RNA5SP141 also contributes to induction of antiviral immunity in the case of IAV infection. siRNA-mediated knockdown of RNA5SP141 markedly reduced the amount of *IFNB1* and *CCL5* transcripts induced by IAV in both NHLF and HEK 293T cells (Figure 2.15A, B). siRNA-mediated silencing of RNA5SP141 also reduced the secretion of IFN-β and CCL5 proteins by NHLFs upon IAV infection (Figure 2.15C). Depletion of RNA5SP141 similarly decreased *IFNB1* induction in response to IAVΔNS1, a mutant virus that lacks NS1 and is therefore unable to efficiently antagonize innate signaling (Ayllon and Garcia-Sastre, 2015; Gack et al., 2009) (Figure 2.15D).

Depletion of RNA5SP141 using gapmers also led to strongly reduced *IFNB1* and *IFIT1* transcripts in response to IAV, although less efficiently than depletion of RIG-I (Figure 2.15E, F). Consistent with our model that virus-induced downregulation of 5S rRNA-binding proteins mediates unmasking of RNA5SP141 and allows for RIG-I activation, we also observed reduced levels of TST and MRPL18, as well as RPL5, in IAV-infected cells as compared to uninfected cells (Figure 2.15G). These data indicate that, during IAV infection, both virus-derived RNAs (Baum et al., 2010; Rehwinkel et al., 2010) and host-derived RNA5SP141 contribute to efficient detection of IAV by RIG-I. As IAV-induced nuclear rearrangements and virus-induced host shutoff effects differ substantially from those of herpesviruses, the precise molecular mechanisms that allow for RNA5SP141 sensing by RIG-I during IAV infection will require further study.
Figure 2.15. RNA5SP141 contributes to the antiviral cytokine response to IAV. (A) qRT-PCR analysis of IFNB1 (left) and CCL5 (right) mRNA in NHLF cells transfected with either non-targeting control siRNA (si.Ctrl), or siRNAs targeting RIG-I (si.RIG-I) or RNA5SP141 (si.5SP141) for 72 h and then infected with IAV (MOI 0.01) for 16 h. Cells transfected with 1 pmol RABVLe or treated with 0.05 μg/mL HMW-poly(I:C) for 16 h served as controls. (B) qRT-PCR analysis of IFNB1 and CCL5 transcripts in HEK 293T cells transfected as in (A) followed by infection with IAV PR8 (MOI 0.1) for 16 h. (C) ELISA analysis of IFN-β (left panel) and CCL5 (right panel) in the supernatants of NHLF cells that were transfected as in (A) and subsequently infected with IAV (MOI 0.1) for 24 h. (D) qRT-PCR analysis of IFNB1 mRNA in HEK 293T cells transfected with siRNAs as in (A) and then infected with IAVΔNS1 (MOI 0.1) for 16 h. (E) qRT-PCR analysis of IFNB1 mRNA in NHLF cells transfected with either non-targeting control gapmers (Gap NT), or gapmers targeting RIG-I (Gap RIG-I_1 and Gap RIG-I_2) or RNA5SP141 (Gap 5SP141_1 and Gap 5SP141_2) for 72 h and then infected with IAV (MOI 0.05) and harvested at 6, 12, and 24 hpi. (F) qRT-PCR analysis of IFIT1 mRNA in NHLF transfected with gapmers as in (E) for 72 h and then infected with IAV (MOI 0.1) and harvested at 16 hpi. (G) IB analysis of endogenous MRPL18, TST, and RPL5 protein abundance in the WCLs of NHLF cells infected with IAV (MOI 5) for the indicated times. IB analysis of IAV non-structural protein 1 (NS1) and cellular actin served as infection and loading controls, respectively. Data are representative of two (A, B, C, E-G) or three (D) independent experiments (mean and s.d. of n = 3 biological replicates in A, B, D and F, n = 2 biological replicates in C and E). *P < 0.05, **P<0.01, ***P<0.001 (unpaired t-test).
DISCUSSION

Intracellular innate immune sensors are known to function by recognizing specific molecular signatures that are unique to viral pathogens. The presence of 5’-triphosphate-RNAs in the cytoplasm is believed to serve as a means by which RIG-I discriminates between ‘self’ and ‘non-self’ RNA. In contrast to viral 5’-triphosphate-containing RNAs, most mature host RNAs are modified (e.g., tRNAs which bear 5’-monophosphates) or capped (e.g., mRNAs) after their export from the nucleus to the cytoplasm. However, several small RNAs bearing a 5’-triphosphate group (e.g. Pol III transcripts) are also present in the host cell cytoplasm, but it is unknown whether these endogenous RNA species can activate RIG-I. This study provides evidence that innate immunity can be triggered by signals that follow pathogen assault and originate from the host itself. Furthermore, our findings that RNA5SP141, a Pol III transcript, activates RIG-I during HSV-1 and EBV infection confirm previous reports showing that the Pol-III-RIG-I axis is important for activation of the innate immune response to DNA viruses (Ablasser et al., 2009; Chiu et al., 2009). Our work proposes a model in which the recognition of immunostimulatory cellular 5S rRNA pseudogene transcripts is normally prevented through association with 5S rRNA-interacting proteins (Figure 2.16, left). However, alterations in nuclear envelope integrity and specific shutoff of host protein synthesis caused by viral infection allow for recognition of these pseudogene transcripts, thereby triggering antiviral immunity (Figure 2.16, right). While virus-induced shutoff of host protein synthesis is believed to promote virus replication by reducing the competition between viral and host mRNAs for nuclear export and the cellular translational machinery, our data suggest that this strategy also makes the virus vulnerable to detection by the innate immune system. RNA5SP141 also seems to be critical for initiation of RIG-I-mediated cytokine responses.
Figure 2.16. Model of RNA5SP141 sensing by RIG-I during HSV-1 infection. In a normal, uninfected cell (left), RNA5SP141 is primarily localized to the nucleus. RNA5SP141 found in the cytoplasm is associated with the RNA binding proteins (RBPs) MRPL18 or TST, and does not interact with RIG-I. In an HSV-1-infected cell (right), RNA5SP141 is predominantly localized to the cytoplasm. The viral nuclease vhs degrades mRNA to downregulate levels of RBPs. The unshielded cytoplasmic RNA5SP141 binds to and activates RIG-I to induce a type-I IFN and proinflammatory cytokine response.
to other viruses, such as EBV, a DNA virus related to HSV-1, as well as IAV, which is unique amongst RNA viruses as it replicates in the nucleus. Based on our model, viruses that both inhibit host protein synthesis and disrupt nuclear integrity may be more likely to inadvertently expose host-derived immunostimulatory RNAs, but future studies are needed to determine the physiological relevance of RNA5SP141 in eliciting innate immunity to other viruses. We have observed that RNA5SP141 is important for the RIG-I-mediated immune response to DNA viruses and at least one RNA virus (IAV) that replicates in the nucleus. However, the full range and characteristics of viruses that trigger RNA5SP141-mediated activation of RIG-I remain to be determined.

Additionally, although our study revealed a prominent role for RNA5SP141 in activating RIG-I-mediated immunity, it is possible that other 5S rRNA pseudogene transcripts may also contribute to innate immune activation. Several hundred putative 5S rRNA pseudogenes with highly similar sequences have been identified in humans to-date, and based on our RNA-Seq data, a number of these pseudogene sequences are transcribed (data not shown). It is possible that depletion of RNA5SP141 using siRNA and antisense oligos may lead to the depletion of other expressed 5S rRNA pseudogenes. However, we have performed depletion experiments using two different knockdown approaches and targeting different regions of RNA5SP141 that are unique from the parental 5S rRNA sequence. Both approaches have demonstrated that RNA5SP141 is critical to the type-I IFN and proinflammatory cytokine response to several viruses. However, generating RNA5SP141 knockout cells will provide more unequivocal evidence of this specificity. Other endogenous RNA ligands of RIG-I that have recently been described include RNase L cleavage products (Malathi et al., 2007) and exosomal RNA shed from stromal cells (Nabet et al.,
2017). Taken together, these studies and ours further strengthen the concept of proinflammatory signal activation through host-derived RNAs.

Interestingly, while in vitro experiments have demonstrated that blunt-ended 5’-triphosphate dsRNAs activate RIG-I most strongly [reviewed in (Schlee and Hartmann, 2016)], structure predictions suggest that RNA5SP141, which has the characteristic RIG-I agonist features 5’-triphosphate moiety and dsRNA stretches, is not blunt-ended, similar to endogenous RNase L cleavage products (Malathi et al., 2007) and exosomal 7SL RNA (Nabet et al., 2017). This suggests that endogenous RIG-I ligands do not strictly adhere to the criteria of ‘classical’ RIG-I ligands and extends the diverse group of RNA species that RIG-I can apparently recognize.

A recent series of studies linked aberrant innate immune sensing of intracellular RNA or DNA to proinflammatory or autoimmune disorders, such as Aicardi-Goutières Syndrome, Singleton-Merten syndrome and systemic lupus erythematosus. Conceptually, these disorders arise from single-nucleotide polymorphisms in innate sensor or signaling molecules, or enzymes that degrade viral nucleic acids (e.g., TREX1), thereby leading to accumulation of stimulatory RNA or DNA ligands [reviewed in (Barrat et al., 2016)]. While our study reveals that the recognition of cellular non-coding RNAs by RIG-I is part of the antiviral defense program, it is conceivable that this mechanism of ‘self’ recognition could also be implicated in uncontrolled immune homeostasis, leading to harmful consequences for the host organism. It remains to be seen whether other sources of cellular disturbance, such as autoimmune-dependent changes in the host milieu, can also trigger the exposure of RNA5SP141 through downregulation of its cognate RNA-binding proteins and/or disturbance of nuclear integrity. Conversely, small inhibitors designed to disrupt the interaction of RNA5SP141 and MRPL18 or TST may be useful for stimulating antiviral innate immunity.
MATERIALS AND METHODS

Cell culture and viruses

HEK 293T (ATCC), Vero (ATCC), NHLF (Clonetics), RIG-I WT and knockout MEFs (Gack et al., 2007), as well as HEK 293T ISRE-luciferase reporter cells (Shapira et al., 2009) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM GlutaMAX (Gibco), and 1% (v/v) penicillin-streptomycin (Gibco) under standard tissue culture conditions. AGS-EBV cells (Marquitz et al., 2012) (generously provided by Nancy Raab-Traub, UNC-Chapel Hill) were cultured in Ham’s F12 medium supplemented with 10% (v/v) FBS, 2 mM GlutaMAX (Gibco), 1% (v/v) penicillin-streptomycin, and 500 µg/mL G418 (Gibco). Reactivation of EBV in AGS-EBV cells was induced by treatment with 2.5 mM sodium butyrate (NaB) (Sigma) dissolved in sterile water.

A US3 K220A HSV-1 mutant (HSV-1mut) was generated using a bacterial artificial chromosome (BAC) clone of HSV-1 strain (F) (pYEbac102; kindly provided by Yasushi Kawaguchi, University of Tokyo, Japan). To facilitate detection of infected cells, pYEbac102 was modified by insertion of the egfp (enhanced green fluorescent protein) gene into mini-F vector sequences, resulting in the pYEbac102-G construct that was used for further manipulation. Expression of EGFP was driven by the human cytomegalovirus (HCMV) immediate-early (IE) promoter. En passant mutagenesis (Tischer et al., 2010) was performed to introduce a mutation at amino acid residue 220 of the US3 protein kinase where the original lysine (K) was substituted with alanine (A), leading to the BAC pYEbac102-K220A-G construct. The following primers were used to amplify the PCR product that allowed for mutagenesis to occur:

(forward: 5’-TGACAGCAGCCACCCAGATTACCCCAACGGGTAATCGTG GCG GCGGGGTGGTACACGAGCAGC ACTAGGGATAACAGGGTAATCGAT-3’; reverse: 5’-
GCAGTCGCGCCTCGTGCTCGTGTACCACCCGCGC
CACGATTACCCGTTGGGGGTCCAGTGTTACAACCAATTACC-3’; (nucleotides in italics and bold represent the desired mutation). After confirmation of the constructs by restriction fragment length polymorphism (RFLP) and DNA sequencing (LGC Genomics, Germany), 1 µg each of purified BAC DNA was transfected into Vero cells at 90% confluency using polyethylenimine (PEI; Polysciences) according to procedures described elsewhere (Boussif et al., 1995). At 72 h post-transfection, when visible fluorescent plaques appeared, the respective virus mutant was reconstituted and harvested by 3 cycles of freeze-thaw.

The mutant HSV-1Δvhs (R2621) and its revertant virus (R2626) were generously provided by Bernard Roizman (University of Chicago) and have been described previously (Poon and Roizman, 1997). Sendai virus (SeV, Cantell strain) was purchased from Charles River Laboratories (Wilmington, MA). Influenza A/Puerto Rico/8/1934 and ΔNS1 recombinant virus (Mibayashi et al., 2007) were generously provided by A. Garcia-Sastre (Mount Sinai).

**DNA constructs and transfections**

FLAG-RIG-I and FLAG-GFP, cloned into the pCMV5-FRT-TO vector, have been previously described (Runge et al., 2014). pCMV6-Entry plasmids encoding Myc-DDK-tagged human RPL5, MRPL18, and TST were purchased from OriGene. The DNA construct encoding U6 promoter-expressed RNA5SP141 was generated by PCR using the following forward (F) and reverse (R) primers: F1 primer: 5’-TGGAAAGGACGAAACACCGTCTACGGCCATACCACCCTGAACGCGCCCGATCTCGTC-3’; F2 primer: 5’-CGCGCCCGATCTCGTCTGATCTCGGAAGCTAAGCAGGGTTGGGCCTGGTTAGTACTTGGATGGGAAGATACATCCAAAAACACGATGACTCACATG-3’; R primer: CATGTGAGTCATCGTGTTTTTGGATG. The DNA
construct encoding U6 promoter-expressed RABV<sub>INT</sub> was generated using the following sense (S) and antisense (A) primers: S primer: 5’- TGGAAAGGACGAAACACCGCCAGGATGGCGGCTCAAACTGCTTCTGGCCCTCCAGCCTTTGAATGGTCGGCCGCC-3’; A primer: 5’-GGCGGCCGACCATTCAAGGGCTGGAGGGCCAGAAGCAGTTTGAGCCGCCATCCTGGCGGTGTTTCGTCCCTTCCA-3’.

Transient transfection of cells was performed using calcium phosphate (Clontech), linear polyethylenimine (PEI [Polysciences, Inc.] at 1 mg/mL in 10 mM Tris pH 6.8), Lipofectamine and Plus reagent or Lipofectamine 2000 (both Life Technologies), according to the manufacturer’s instructions.

**Antibodies and other reagents**

The following antibodies were used for immunoblot analysis: anti-FLAG (1:2000, M2, Sigma), anti-Lamin A/C (1:2000, E-1, Santa Cruz), anti-Tubulin (1:2000, 2144, Cell Signaling), anti-β-Actin (1:5000, A1978, Sigma), anti-ICP8 (1:10,000, kindly provided by David Knipe (Harvard University)), anti-RPL5 (1:1000, GTX101821, GeneTex), anti-MRPL18 (1:500, ab67844, Abcam), anti-TST (1:200, H-75, Santa Cruz), anti-IRF3 (1:500, FL-425, Santa Cruz), anti-RIG-I (1:2000, Alme-1, Adipogen), anti-Sendai virus (1:1000, PD029, MBL), and anti-EBV Ea-R p85 (1:500, 6G7, Santa Cruz). Goat anti-mouse- or goat anti-rabbit-Horseradish Peroxidase (HRP) secondary antibodies (both 1:2000) were purchased from Cell Signaling (cat #7076S and #7074S, respectively).

Anti-FLAG (DYKDDDDK) magnetic beads for RNA co-IP were purchased from Origene or Clontech. Protease inhibitor cocktail used for RIG-I-RNA co-IP was purchased from Sigma (cat
ELISA kits for IFN-β (PBL Assay Science #), IL-8 (R&D Systems #D8000C), and CCL5 (Thermo Scientific #EHRNTS) were purchased and assays were performed according to the manufacturer’s instructions.

LyoVec-conjugated high molecular weight (HMW) poly(I:C) was purchased from Invivogen (cat #tlrl-piclv) and prepared according to the manufacturer’s instructions.

**Large-scale co-immunoprecipitation of RNA-bound RIG-I from infected cells**

HEK 293T cells (~ 2.5 x 10^6 cells per 100 mm dish, 1 dish per sample) were transfected with FLAG-RIG-I or FLAG-GFP (10 μg DNA per dish) using polyethylenimine. 24 h later, cells were infected with either HSV-1mut (MOI 300) or SeV (50 HAU/mL), or left uninfected. Cells were harvested 18 h later and lysed in Nonidet P-40 (NP-40) lysis buffer (50 mM HEPES pH 7.5, 150 mM KCl, 1 mM Na_3VO_4, 0.5% (v/v) NP-40, 0.5 mM Dithiothreitol (DTT, Sigma, cat # 10197777001), supplemented with protease inhibitor [Sigma]) for 30 min at 4°C. The lysates were cleared by centrifugation at 13,000 rpm for 20 min. To co-immunoprecipitate RNA-bound FLAG-RIG-I and FLAG-GFP, cleared lysates were mixed with a 50% slurry of anti-FLAG-conjugated magnetic beads (Origene or Clontech), and the binding reaction was incubated for 4 h at 4°C. Precipitates were washed three times with NP-40 lysis buffer and two times with high-salt wash buffer (50 mM HEPES, pH 7.5, 300 mM KCl, 1 mM Na_3VO_4, 0.5% (v/v) NP-40, 0.5 mM DTT, supplemented with protease inhibitor [Sigma]), followed by incubation with proteinase K (New England Biolabs) at 55°C for 1 h. The RNA bound to FLAG-RIG-I or FLAG-GFP was extracted using phenol/chloroform/isoamylalcohol (Amresco).
RNAseq analysis

RNA purified from FLAG-RIG-I or FLAG-GFP precipitates from mock-infected or HSV-1\textsubscript{mut}-infected HEK 293T cells, as well as their respective total RNA input samples were converted to a DNA library for sequencing, both performed at the Harvard Biopolymers Facility (Boston, MA). Up to 1000 ng total RNA was used in the Wafergen (formerly IntegenX) PrepX RNA-Seq Library Kit (cat #400039) and placed into the Wafergen Apollo, and run using a standard protocol. PCR was performed on samples using indexed primers according to Wafergen instructions and then cleaned using the Wafergen Apollo. The resulting samples were run on an Agilent 2200 Tape Station on a D1000 High Sensitivity Tape with ladder provided to assess the integrity and overall concentration of DNA. DNA libraries were run in a qPCR assay with SYBR green KAPA SYBR FAST Universal 2X qPCR Master Mix (Kapa Biosystems, cat #KK4602) and primers to the P5 and P7 regions of the adapters. Diluted PhiX was used for the standard curve to determine concentration. Libraries passing quality control were subjected to Illumina HiSeq 2500 sequencing (Harvard Biopolymers Facility). A local Galaxy server was used to process the RNAseq data (Afgan et al., 2016). Raw sequence reads were quality trimmed using TRIM Galore! [Cutadapt (Martin, 2011) and FastQC wrapper (Andrews, 2010)] and aligned to the human genome using Tophat2 (Kim et al., 2013) with settings that allow for zero mismatches in the final sequence alignment. Htseq-count was used to calculate the induction of human transcripts, and the obtained results were normalized using total count normalization (Dillies et al., 2013). Furthermore, the RIG-I-precipitate and GFP-precipitate counts were normalized to their respective input RNA. RIG-I-specific transcript enrichment was calculated by dividing the normalized RIG-I-precipitate counts through the normalized GFP-precipitate counts using R scripts. The data from two
biologically independent experiments were multiplied, and visualized in R (Team, 2013) using the ggplot2 package (Wickham, 2009).

**Immunoblot analysis**

Infected or transfected cells were lysed in NP-40 lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1% (v/v) NP-40, supplemented with protease inhibitor cocktail [Sigma]), followed by centrifugation at 13,000 rpm for 20 min to remove insoluble material. Cleared lysates were resolved on 7%, 10%, or 12% Bis-Tris SDS-PAGE gels, and subsequently transferred to a Polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a Trans-Blot SD (Bio-Rad). Membranes were blocked with 5% (w/v) non-fat dry milk (NFDM) in PBS for 1 h, and then probed with primary antibody in antibody dilution buffer (PBS supplemented with 0.05% (v/v) Tween-20 and 5% (w/v) NFDM) at the indicated dilutions (as described above) for either 1 h at RT, or at 4 °C overnight. Membranes were probed with goat anti-mouse- or goat anti-rabbit-HRP antibodies (Cell Signaling cat #7076S and #7074S, respectively) in antibody dilution buffer for 1 h at RT. Proteins were visualized using an enhanced chemiluminescence reagent (Pierce) and detected by Fujifilm luminescent image analyzer LAS-4000 or Amersham Imager 600.

**Native PAGE**

HEK 293T cells, seeded into 6-well plates (~ 3 x 10^5 cells per well), were transfected with 125 pmol of RABV_{Le} or RNA5SP141 in vitro transcripts, or infected with SeV (50 HAU/mL). 16 h later, cells were lysed by three cycles of freeze-thaw in NP-40 lysis buffer. Native PAGE was performed as previously described (Gack et al., 2008).
Luciferase reporter assays

HEK 293T cells were seeded into 12-well plates (~ 1 x 10^5 cells per well). The next day, cells were transfected with 200 ng IFN-β luciferase construct (Lin et al., 2000) and 300 ng β-galactosidase-expressing pGK-β-gal (Hatzivassiliou et al., 1997), along with 5 - 125 ng of the indicated constructs encoding 5S rRNA-interaction proteins, or empty vector, using Lipofectamine and Plus reagent (Life Technologies), according to the manufacturer’s instructions. 24 h later, cells were transfected with either no RNA or 10 pmol of in vitro-transcribed RNA5SP141 using Lipofectamine 2000 (Life Technologies), according to the manufacturer’s instructions. 24 h later, cells were harvested and lysates were subjected to luciferase and β-galactosidase activity assays (both Promega). Luciferase and β-gal activity was measured using a BioTek Synergy Microplate Reader in 96-well plates using 10 μl and 25 μl of cell lysates, respectively. Luciferase activity was normalized to β-galactosidase values, and luciferase induction was calculated relative to mock-transfected samples, set as 1.

For ISRE-luciferase assays, HEK 293T ISRE-luciferase reporter cells were seeded into 12-well plates (~ 1 x 10^5 cells per well) and transfected the next day with either 7 μl of RNA retrieved from RIG-I- or GFP-precipitates, or 200 fmol of CIP-treated or untreated in vitro-transcribed RABV_{Le} or RNA5SP141, using Lipofectamine 2000 (Invitrogen). 16 h later, cells were harvested and assayed for luciferase activity as described above. Fold induction of luciferase activity was calculated relative to mock-transfected samples, set as 1.

siRNA-mediated knockdown

Transient knockdown of endogenous RIG-I, cGAS, IFI16, RPL5, MRPL18, and TST in HEK 293T and NHLF cells, seeded in 12-well plates (~ 1 x 10^5 cells per well), was achieved by
transfection of gene-specific siGenome SMARTpool siRNAs (Dharmacon) with RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. As a control, non-targeting siRNA (Dharmacon, D-001206-14-20) was transfected. A final concentration of 150 nM per well was used. The following siRNAs were used: siRNAs targeting RIG-I (siGENOME SMARTpool M-012511-01-0010), cGAS (siGENOME SMARTpool M-015607-01-0005), IFI16 (siGENOME SMARTpool M-020004-01-0010), RPL5 (siGENOME SMARTpool M-013611-01-0005), MRPL18 (siGENOME SMARTpool M-017251-00-0005), and TST (siGENOME SMARTpool M-010120-00-0005) (all Dharmacon). At 48 h posttransfection, knockdown efficiency was confirmed by analyzing the transcript levels of the individual genes by qRT-PCR using specific primers (from IDT), or by determining their protein abundance by immunoblot analysis. Transient knockdown of endogenous RNA5SP141 in HEK 293T, NHLF, and AGS-EBV cells was achieved by RNAiMAX transfection of custom-made siRNA with the sense sequence 5'-UGGGAGAAAUACAUCCAAAUU-3' (Dharmacon). 72 h post-transfection, knockdown efficiency was confirmed by analyzing the transcript levels of RNA5SP141 by qRT-PCR as described below.

**In vitro RNA transcription**

DNA templates for *in vitro* transcription of scrambled, 5S rRNA, and RNA5SP141 RNA were generated by PCR with the primers listed in Table 2.1 using KOD Hot Start Polymerase (EMD Millipore). DNA templates for *in vitro* transcription for control rabies virus leader sequence (RABV<sub>Le</sub>) and rabies virus internal sequence (RABV<sub>INT</sub>) were generated by annealing sense and antisense oligonucleotides as previously described (Saito et al., 2008). RNA was synthesized using the MEGAshortscript *in vitro* transcription kit (Ambion) and purified using the MEGAclean
transcription reaction purification kit (Ambion). RNA integrity was evaluated with the Agilent 2100 Bioanalyzer System using the Small RNA Analysis Kit (Agilent).

**Table 2.1.** Forward (F) and reverse (R) DNA oligonucleotides used to construct in vitro transcription templates of RNA5SP141, parental 5S rRNA, and ‘scrambled’ RNA.

<table>
<thead>
<tr>
<th>Name</th>
<th>5’-3’ Sequence</th>
</tr>
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<tbody>
<tr>
<td>T7 promoter F</td>
<td>TAATACGACTCATATAG</td>
</tr>
<tr>
<td>T7 scrambled F1</td>
<td>TAATACGACTCATATAGGACCTCTACTTTAGATGTTGACCGCATGTAGCAGCTAGCGATAAGCATGACGCGCTTTCAAG</td>
</tr>
<tr>
<td>T7 scrambled F2</td>
<td>GACGCGCTTTCAAGGTCGCGAGTATGTGAAACAAAGGCTCCGGCACAGGACACTATATACCTGCTTTGATAT</td>
</tr>
<tr>
<td>T7 scrambled R</td>
<td>CATGTGAGTCATGTGTTTTTGGATG</td>
</tr>
<tr>
<td>T7 5SP141 F1</td>
<td>TAATACGACTCATATAGGCTACGGCCATACCACCCTGAACGCGCCCGATCTCGTCTGATCTCGGAAG</td>
</tr>
<tr>
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<tr>
<td>T7 5S rRNA R</td>
<td>AAAGCCTACAGCAGCGGTA</td>
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**Phosphatase treatment of RNA**

Calf intestinal alkaline phosphatase (CIP, New England Biolabs) was used according to the manufacturer’s instructions to treat RABV<sub>Le</sub> and RNA5SP141 in vitro transcripts for 3 h at 37 °C. The RNA was purified using the MEGAclear kit (Ambion) and run on a 15% TBE-Urea gel to validate RNA integrity. RNAs were subsequently tested for their immunostimulatory activity by ISRE-luciferase assays as described above.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was purified from cells using an RNA extraction kit (Omega Bio-tek) as per the manufacturer’s instructions. RNA quality was assessed using NanoDrop 2000. Equal amounts
of RNA (25 - 500 ng) were used for a one-step qRT-PCR reaction using the SuperScript III Platinum One-Step qRT-PCR kit with ROX (Life Technologies) with commercially available FAM reporter dye primers (IDT) for the indicated genes. Gene expression levels were normalized to 18S (in experiments with HSV-1) or GAPDH (for all other experiments). Fold induction of each target gene relative to mock-infected or mock-treated cells was calculated using the Comparative CT Method (ΔΔCT Method).

For EBV BMRF1 transcript analysis, custom FAM reporter dye primers based on previously-described primer sequences (Dillon et al., 2013) were used (forward: CAACACCGCACTGGAGAG, reverse: GCCTGCTTCACTTTCTTGG, probe: AGGAAAAGGACATCGTGAGGC) (IDT).

For RNA5SP141 transcript analysis, total RNA including small RNAs (<200 nt) were purified from cells using the miVana miRNA isolation kit (Life Technologies). Reverse transcription (RT) and qPCR were performed using the following TaqMan MicroRNA Assays which include a target-specific RT primer and a target-specific FAM reporter dye primer: RNA5SP141 (ThermoFisher cat #CSN1ESE) and let-7a (ThermoFisher cat #4440887). Equal amounts of RNA (10 - 100 ng) were reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) with the target-specific RT primers. cDNA was then pre-amplified using the TaqMan PreAmp Master mix using the target-specific RT primers. qRT-PCR analysis was performed with the TaqMan Fast Advanced Master Mix (Applied Biosystems) using the target-specific TaqMan FAM reporter dye primers. Fold induction of RNA5SP141 relative to mock-infected or mock-treated cells was calculated using the ΔΔCT method, normalizing to let-7a, a miRNA that is maintained at stable levels during HSV-1 infection and serves as an internal
control for miRNA levels during infection (Cui et al., 2006). All qRT-PCR assays were performed using a 7500 FAST RT-PCR System (Applied Biosystems).

**RIG-I ATPase hydrolysis assay**

The RIG-I ATPase assay was performed as previously described (Lassig et al., 2015). Briefly, 250 nM of *in vitro*–transcribed RNA were incubated with 100 nM of RIG-I protein purified from insect cells (as previously described (Cui et al., 2008)) and 3 mM unlabeled ATP, including trace amounts of [γ-32P]ATP (Hartmann Analytik, Germany) in EMSA buffer (5 mM MgCl₂, 50 mM KCl, 50 mM HEPES, 1 mM TCEP, 0.1 mg/mL BSA, pH 7) for 0, 0.5, 1, 2, or 3 hours at 37 °C. Free phosphate was separated from unhydrolyzed ATP by thin layer chromatography in TLC running buffer (1 M formic acid, 0.5 M LiCl) on polyethyleneimine cellulose TLC plates (Sigma-Aldrich, Germany). [γ-32P]Pi and [γ-32P]ATP were detected using a phosphor-imaging system (GE Healthcare, Germany), quantified using ImageJ, and the percentage of hydrolyzed ATP for every time point was calculated. The average value of three replicates was calculated and average hydrolyzed ATP was plotted against time.

**Cytoplasmic-nuclear fractionation assay**

HEK 293T cells were seeded into 6-well plates (~3 x 10⁵ cells per well). The next day, cells were infected with HSV-1 (MOI 10) or SeV (50 HA units/ml) for 18 h, or left uninfected. Cells were fractionated using the Ambion PARIS fractionation kit according to the manufacturer’s instructions. Briefly, cell pellets were lysed in cell fractionation buffer on ice for 15 min and subjected to low-speed centrifugation to pellet nuclei. The supernatant (cytoplasmic fraction) was removed, and nuclear pellets were lysed in cell disruption buffer. Lysates from each fraction were
divided into two equal samples and used for protein and RNA purification. Fractionated protein lysates were subjected to immunoblot analysis to assess the levels of the nuclear protein lamin A/C and the cytoplasmic protein β-tubulin. Fractionated RNA samples were subjected to qRT-PCR analysis to assess the levels of nuclear RNA markers (MALAT1, NEAT1, and RNU2-1) and RNA5SP141 transcripts in each fraction.

**RNA pulldown assays**

For purification of endogenous RNA5SP141 from total cellular RNA, 3’-biotinylated LNA oligos (Exiqon) with the following sequences were used: Scramble: AGTTCCGTCGTCGACTAGGA/3BioTEG/; U1: TACCACAAATTATGCAGTCGA/3BioTEG/; and RNA5SP141: ATGTGAGTCATCGTGTTT/3BioTEG/. For each sample, 50 μg of phenol-chloroform purified total cell RNA was incubated with 375 pmol LNA oligos for 30 minutes at room temperature. Next, MyONE Streptavidin C1 magnetic beads (ThermoFisher cat # 65002) were added to the samples and incubated for 30 minutes at 25 °C. Beads were washed using buffers from the Dynabeads mRNA DIRECT Kit (Life Technologies cat #61011) according to the manufacturer’s instructions, and RNA was eluted from the beads by incubation at 65 °C for 2 min.

For pull-down of 5S rRNA-interacting proteins, biotinylated scramble, 5S rRNA, and RNA5SP141 RNA (Supplementary Fig. 3b) were *in vitro* transcribed using a Biotin-16-UTP RNA labeling mix (Roche), and RNA was purified using the MEGAclear transcription reaction purification kit (Ambion). RNA binding of 5S rRNA-interacting proteins was assessed by streptavidin pulldown (PD) as previously described (Maharaj et al., 2012). Briefly, one to four 10-cm dishes of HEK 293T cells per sample were each transfected with 15 μg of FLAG-RPL5, FLAG-
MRPL18, or FLAG-TST. Two days after transfection, cells were lysed in NP-40 lysis buffer supplemented with protease inhibitor (Sigma) and cleared by centrifugation at 13,000 rpm for 20 min. Cell lysates were incubated for 1 h at 22 °C with no RNA or 1 μg of scramble, 5S rRNA or RNA5SP141 biotinylated RNA. Samples were incubated with a 50% slurry of streptavidin agarose (Pierce) for 2 h at 4 °C and washed extensively with NP-40 lysis buffer. WCLs and PD samples were subjected to immunoblot analysis to test for expression and binding of FLAG-tagged RPL5, MRPL18 and TST, respectively.

**RNA secondary structure prediction**

Prediction of RNA5SP141 secondary structure was performed using the Vienna RNAfold web server (Lorenz et al., 2011) using standard settings.

**LNA gapmer-mediated knockdown**

Transient knockdown of endogenous RIG-I and RNA5SP141 in HEK 293T and NHLF cells, seeded in 24-well plates (~ 5 x 10⁴ cells per well), was achieved by transfection of gene-specific LNA gapmers (Exiqon) with Lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer's instructions at a final concentration of 50 nM LNA gapmer per well. Non-targeting scrambled antisense LNA gapmer (Exiqon, 300610) was transfected as a negative control. Custom-made LNA gapmers with the following sequences were used: RIG-I_1: CAAATGCGCAGAGGTC, RIG-I_2: TCGGTTGGGATAATTC, RNA5SP141_1: CATGTCGATGCATCGTG, RNA5SP141_2: TTTGGATGTATTCTC. At 72 h posttransfection, knockdown efficiency was confirmed by analyzing the transcript levels of the individual genes by qRT-PCR using specific primers (IDT).
Statistical analysis

Unpaired two-tailed Student’s $t$ tests were used to compare differences between two unpaired experimental groups in all cases. A p-value of <0.05 was considered statistically significant.
Chapter 3: HSV-1 antagonizes innate immune signaling through US3-mediated phosphorylation of RIG-I

Attributions:
Jessica J. Chiang (J.J.C.) and Michaela U. Gack (M.U.G.) conceived the study. J.J.C. performed and analyzed all experiments. Teng Huang and Nikolaus Osterrieder constructed HSV-1_{K220A}. J.J.C. and M.U.G. interpreted data.
ABSTRACT

Recent studies have demonstrated that the signaling activity of the cytosolic pathogen sensor retinoic acid-inducible gene-I (RIG-I) is modulated by a variety of post-translational modifications (PTMs) that fine-tune the type-I interferon (IFN) response to viral infection. Whereas K63-linked ubiquitination by the E3 ligase TRIM25 activates RIG-I, phosphorylation of RIG-I at S8 and T170, located in its N-terminal caspase activation and recruitment domains, represses RIG-I signal transduction by preventing the TRIM25-RIG-I interaction and subsequent TRIM25-mediated RIG-I ubiquitination. Several viruses, with both RNA and DNA genomes, have evolved to suppress RIG-I signaling by manipulating its K63-polyubiquitin-dependent activation. However, it has been unknown whether viruses also promote the inhibitory phosphorylation of RIG-I to escape antiviral immunity. Here, we show that the serine/threonine (S/T) kinase US3 of herpes simplex virus-1 (HSV-1) binds to RIG-I and phosphorylates RIG-I at S8. US3-mediated phosphorylation suppressed RIG-I-mediated IFN-β induction, and a recombinant HSV-1 encoding a catalytically-inactive mutant US3 protein (K220A) was unable to phosphorylate RIG-I at S8. The US3 mutant virus elicited higher levels of type-I IFNs, IFN-inducible genes (ISGs), and proinflammatory cytokines in a RIG-I-dependent manner. The viral kinase-mediated RIG-I evasion mechanism appears to be conserved among alphaherpesviruses. Collectively, our study reveals a novel immune evasion mechanism for alphaherpesviruses in which US3 kinases mediate the phosphorylation of RIG-I to maintain RIG-I in its signaling-repressed state.
INTRODUCTION

The RIG-I-like receptors (RLRs) are a family of cytoplasmic pattern recognition receptors (PRRs) essential for innate immune detection of viral infection in nearly all cell types. RIG-I and MDA5 are composed of three domains: N-terminal tandem caspase activation and recruitment domains (CARDs), a central DExD/H box RNA helicase domain, and a C-terminal domain. The CARDs mediate RIG-I and MDA5 interactions with their common downstream signaling partner, mitochondrial antiviral signaling protein (MAVS), and the helicase and C-terminal domains are important for the recognition of RNA ligands and RIG-I autorepression. RLR activation upon PAMP recognition triggers a signaling cascade that leads to the recruitment and activation of several and additional effector proteins, resulting in the phosphorylation, dimerization, and activation of the transcription factors interferon regulatory factor (IRF) 3 and IRF7, which translocate from the cytoplasm to the nucleus, where they mediate transcriptional activation of type-I IFN and other proinflammatory cytokine genes [reviewed in (Schneider et al., 2014)]. Type-I IFNs can act directly on cellular processes and upregulate a large set of ISGs that carry out a variety of antiviral functions, including the suppression of viral protein synthesis and the induction of apoptosis.

Since the RLR-mediated antiviral response is characterized by high levels of cytokine production and the activation of a diverse set of genes that control essential cell processes, dysfunctions in this process can be severely detrimental to the host. Many points of regulation exist along the RLR signaling pathway to maintain a balance between viral clearance and host preservation. Post-translational modification of the RIG-I and MDA5 CARDs are a key mechanism by which RLR activity is regulated. A series of PTMs have been shown to be essential
for RIG-I regulation. In uninfected cells, RIG-I and MDA5 are maintained in an inactive state by phosphorylation at S8/T170 (Gack et al., 2010; Nistal-Villan et al., 2010) and S88 (Wies et al., 2013), respectively. In the case of RIG-I, both S8 and T170 were bioinformatically identified as potential phosphorylation sites for the S/T kinases PKA and PKC. However, detailed biochemical analysis revealed that PKC-α/β are the primary kinases that phosphorylate the regulatory residues of RIG-I (Maharaj et al., 2012). The kinase(s) that phosphorylates MDA5 S88 has not yet been identified. Upon viral infection, activation requires the removal of these inhibitory phosphorylation marks by the protein phosphatases PP1α/γ (Wies et al., 2013). In the case of RIG-I, dephosphorylation is followed by K63-linked (non-proteasome-targeting) polyubiquitination of K172 by the E3 ligase tripartite motif-containing protein 25 (TRIM25) (Gack et al., 2007). Polyubiquitination of RIG-I facilitates its multimerization and translocation to mitochondria-associated membranes and peroxisomes where RIG-I interacts with MAVS (Dixit et al., 2010; Seth et al., 2005). These regulatory steps have been demonstrated to be essential for RLR activation and serve as targets for viral antagonism. Much emphasis has been placed on elucidating mechanisms by which RNA viruses antagonize RLRs, but little is known about the RLR evasion mechanisms employed DNA viruses such as HSV-1.

In this study, we identify an innate immune evasion strategy employed by HSV-1 in which the viral S/T kinase US3 antagonizes RLR signaling by phosphorylating the regulatory residue S8 of RIG-I.
RESULTS

HSV-1 S/T kinase US3 interacts with RIG-I and mediates phosphorylation of RIG-I regulatory residue S8

In uninfected cells, RIG-I and MDA5 are maintained in an inactive state by phosphorylation of specific residues in their CARDs. Upon detection of viral infection, dephosphorylation of these inhibitory marks by a cellular phosphatase is required for downstream signaling. We hypothesized that virally-encoded kinases may counteract this early step in RLR activation by mediating phosphorylation of regulatory residues in RIG-I and MDA5, thereby maintaining the sensors in a signaling-inactive state. To address this hypothesis, we assessed the effect of HSV-1 S/T kinase US3 on phosphorylation of RIG-I and MDA5 regulatory residues.

To determine the effect of HSV-1 US3 on phosphorylation at regulatory residues in RIG-I and MDA5, we coexpressed US3 with either RIG-I or MDA5 CARDs tagged with GST (GST-RIG-I/MDA5 2CARD) and analyzed the lysates by immunoblot (IB) using phospho-specific antibodies against RIG-I pS8, RIG-I pT170, and MDA5 pS88. In the presence of US3, phosphorylation of GST-RIG-I 2CARD at S8 was strongly enhanced, while phosphorylation at T170 remained unchanged (Figure 3.1A). In keeping with the model that RIG-I phosphorylation precludes the K63-linked ubiquitination of RIG-I, we observed that the enhanced phosphorylation of GST-RIG-I 2CARD in the presence of ectopically expressed US3 corresponded with a near-complete loss of GST RIG-I 2CARD ubiquitination. The phosphorylation levels of GST-MDA5 2CARD at S88 remained unchanged when coexpressed with US3 and were similar to the phosphorylation levels of GST-MDA5 2CARD co-transfected with empty vector (Figure 3.1B). Examination of the S8 phosphorylation sequence in RIG-I revealed that it is similar to the
Figure 3.1. HSV-1 US3 phosphorylates RIG-I S8. HEK 293T cells were co-transfected with (A) GST-RIG-I 2CARD or (B) GST-MDA5 2CARD together with empty vector or FLAG-US3. Whole cell lysates (WCLs) harvested 48 hours post-transfection (hpt) were subjected to pull-down with glutathione-sepharose beads (GST-PD), followed by immunoblot (IB) with the indicated antibodies. (C) Top: consensus sequences for alphaherpesvirus kinase US3 and cellular protein kinases (PK) C and PKA. For US3, n is ≥2, X can be R, A, V, P, or S; Y can be any amino acid except an acidic residue. For PKC, X can be any residue. For PKA, Φ can be any hydrophobic residue. Bottom: regulatory phosphorylation sites in the RIG-I and MDA5 CARDs. (D) HEK 293T cells were co-transfected with full-length FLAG-RIG-I together with empty vector or V5-US3. WCLs harvested 48 hpt were subjected to pull-down with M2 antibody-conjugated-sepharose beads (FLAG-PD), followed by IB with the indicated antibodies. (E) Normal human lung fibroblasts (NHLFs) were transfected with V5-US3. At 24 hpt, cells were treated with 1,000 U/ml IFN-β for 24 h. Cells were treated with calyculin A and WCLs were subjected to immunoprecipitation (IP) with anti-RIG-I antibody, followed by IB with the indicated antibodies. (F) HEK 293T cells were cotransfected with GST-RIG-I 2CARD and either empty vector, V5-US3 WT or the catalytically-inactive mutant V5-US3 K220M. At 48 hpt, cells were lysed and subjected to GST-PD, followed by IB with the indicated antibodies. Data are representative of two independent experiments.
consensus phosphorylation sequence described for HSV-1 US3, while the RIG-I T170 and MDA5 S88 sequences do not fit the motif (Figure 3.1C). In uninfected cells, RIG-I assumes a closed, autoinhibited conformation that limits exposure of the CARDs. Since the cellular kinases that maintain constitutive phosphorylation of RIG-I act on this inactive form of RIG-I, we next tested whether US3 can phosphorylate full-length RIG-I. Coexpression of US3 with FLAG-tagged full-length RIG-I (FLAG-RIG-I) resulted in a robust enhancement of FLAG-RIG-I S8 phosphorylation as compared to FLAG-RIG-I cotransfected with empty vector (Figure 3.1D), suggesting that US3 is similarly able to access the phosphosite on the autoinhibited RIG-I. RIG-I is an ISG, and therefore normally present at relatively low levels in uninfected cells. To test the ability of US3 to phosphorylate endogenous RIG-I, we overexpressed US3 in primary normal human lung fibroblast (NHLF) cells and treated the cells with IFN-β to upregulate endogenous RIG-I expression. In this more physiologically relevant context, we again observed a strong enhancement of RIG-I S8 phosphorylation in the presence of US3 as compared to co-transfection of empty vector (Figure 3.1E). We next sought to determine whether the kinase activity of US3 is required for the observed enhancement of RIG-I S8 phosphorylation. Mutation of the HSV-1 US3 K220 residue (both K220A and K220M have been described) renders the kinase catalytically inactive (Kato et al., 2005a; Mou et al., 2007). We introduced the K220M mutation into HSV-1 US3 and coexpressed GST-RIG-I 2CARD with either WT US3 or kinase-dead mutant US3 K220M. This showed that that US3 WT robustly enhanced GST-RIG-I 2CARD S8 phosphorylation as compared to empty vector cotransfection, while US3 K220M did not affect the level of GST-RIG-I 2CARD S8 phosphorylation, demonstrating that US3 kinase activity is required for the observed enhancement of RIG-I phosphorylation (Figure 3.1F). To determine whether RIG-I and US3 interact in the context of viral infection, we infected HEK 293T cells with wild-type HSV-1 (HSV-1WT) and
observed by IB that US3 co-immunoprecipitated with endogenous RIG-I (Figure 3.2A). This interaction was also supported by immunofluorescence microscopy in which overexpressed RIG-I was observed to colocalize with US3 during HSV-1 infection (Figure 3.2B). Together, these data suggest that US3 interacts with and directly phosphorylates RIG-I during HSV-1 infection.

**Figure 3.2. US3 interacts with RIG-I during HSV-1 infection.** (A) HEK 293T cells were infected with HSV-1WT at a multiplicity of infection (MOI) of 1. At 20 hours post-infection (hpi), cells were lysed and subjected to co-IP with anti-RIG-I antibody, followed by IB with the indicated antibodies. (B) HeLa cells were transfected with FLAG-RIG-I. At 48 hpt, cells were re-seeded on to coverslips and infected with HSV-1WT (MOI 1). At 20 hpi, cells were fixed and stained with the indicated antibodies and analyzed by confocal microscopy. Images and spectral profiles were processed using ImageJ software. Data are representative of two independent experiments.
US3 inhibits the activation of RIG-I and its interaction with the downstream signaling adaptor MAVS

Dephosphorylation of RIG-I S8 is a prerequisite for its interaction with the E3 ubiquitin ligase TRIM25, which subsequently mediates the K63-linked ubiquitination of RIG-I that is required for full RIG-I activation. The interaction between RIG-I and TRIM25 can be readily observed in virus-infected cells. Since US3 counteracts RIG-I dephosphorylation at S8, we asked whether the presence of US3 inhibits the interaction of RIG-I and TRIM25 induced by SeV infection. To test this, we transfected HEK 239T cells with either empty vector or V5-tagged US3, and assessed the levels of endogenous RIG-I-TRIM25 binding in SeV or mock infected cells by co-IP and IB. SeV infection efficiently triggered RIG-I binding to TRIM25, and their interaction in infected cells was reduced when US3 was present (Figure 3.3A). The interaction between TRIM25 and RIG-I during viral infection leads to the TRIM25-mediated K63-linked ubiquitination of RIG-I, which is required for downstream RIG-I signaling. To test whether US3 inhibits the K63-linked ubiquitination of RIG-I during viral infection, we transfected HEK 293T cells with US3 or empty vector along with an HA-tagged ubiquitin protein HA-K63Ub. In HA-K63Ub all lysines except K63 are mutated to arginines, which means HA-K63Ub can only form ubiquitin chains containing K63 linkages. The cells were subsequently infected with SeV or left uninfected (mock), and K63-linked ubiquitination of endogenous RIG-I was evaluated by IB. The presence of US3 during SeV infection resulted in a strong reduction of K63-linked ubiquitination of RIG-I (Figure 3.3B). After undergoing K63-linked ubiquitination, RIG-I is primed for translocation to the mitochondria where it interacts with its signaling partner, MAVS. The two CARD domains of RIG-I interact with MAVS via its single CARD domain. Therefore, we tested the effect of exogenous expression of V5-tagged US3 on the binding of FLAG-tagged MAVS
CARD-proline-rich domain (CARD-PRD) and GST-RIG-I 2CARD. In the absence of V5-US3, GST-RIG-I 2CARD and MAVS-CARD-PRD was readily observed. In the presence of V5-US3, this binding was strongly reduced, indicating that US3 inhibits the RIG-I-MAVS interaction (Figure 3.3C). Collectively, these data show that phosphorylation of RIG-I S8 by US3 inhibits the K63-polyubiquitin-mediated activation of RIG-I and its interaction with TRIM25 and MAVS.

Figure 3.3. HSV-1 US3 inhibits the K63-linked ubiquitination of RIG-I and its binding to TRIM25 and MAVS. (A) HEK 293T cells were transfected with V5-US3 as indicated. At 24 hpt, cells were infected with 50 HA units/mL of SeV. At 24 hpi, cells were lysed and subjected to co-IP with an anti-TRIM25 antibody, followed by IB with the indicated antibodies. (B) HEK293T cells were transfected with V5-US3 and HA-K63 Ub as indicated. At 24 hpt, cells were infected with 50 HA units/mL of SeV. At 24 hpi, cells were lysed and subjected to co-IP with anti-RIG-I antibody, followed by IB with the indicated antibodies. (C) HEK 293T cells were co-transfected with GST-RIG-I 2CARD, FLAG-MAVS CARD-proline rich domain (PRD), and empty vector or V5-US3 as indicated. At 48 hpt, WCLs were subjected to FLAG-PD followed by IB with the indicated antibodies. Data are representative of two independent experiments.
Inhibition of RIG-I-mediated IFN-β induction by HSV-1 US3 is dependent on S8 in RIG-I

To assess the effect of US3 on the RIG-I-induced type-I IFN response, we coexpressed titrating amounts of HSV-1 US3 with an IFN-β luciferase reporter gene. Upon infection with SeV, which is a strong and specific activator of RIG-I, IFN-β luciferase reporter activity was suppressed by exogenously expressed US3 in a dose-dependent manner (Figure 3.4A). Notably, very low amounts of US3 (5 - 50ng) had a potent inhibitory effect on IFN-β induction triggered by SeV infection. To determine whether US3-mediated inhibition of the IFN-β is due specifically to the ability of US3 to phosphorylate S8 in RIG-I, we coexpressed US3 with either GST-RIG-I 2CARD WT or two phospho-null mutant constructs (GST-RIG-I 2CARD T170A or S8A) along with an IFN-β luciferase reporter gene. Expression of the GST-RIG-I 2CARD WT induced a robust IFN-β response, which was suppressed by US3 in a dose-dependent manner (Figure 3.4B, left). Expression of GST-RIG-I 2CARD T170A, in which T170 is replaced with an alanine and is thereby unable to be phosphorylated, induced a robust IFN-β response, which was also suppressed by US3 in a dose-dependent manner (Figure 3.4B, middle). Expression of GST-RIG-I 2CARD S8A, in which S8 is replaced with an alanine, also induced a robust IFN-β response. In contrast to GST-RIG-I 2CARD WT and T170A, the IFN-β response induced by GST-RIG-I 2CARD S8A was unaffected by the presence of US3 even at high concentrations (Figure 3.4B, right). Taken together, these data suggest that the inhibitory effect of US3 on RIG-I signaling is due to US3-mediated phosphorylation of one specific residue in RIG-I, S8.
Figure 3.4. Inhibition of RIG-I-mediated IFN-β induction by HSV-1 US3 is dependent on S8 in RIG-I. (A) HEK 293T cells were co-transfected with IFN-β luciferase reporter construct (IFN-β-luc) and a β-galactosidase plasmid (pGK-β-gal) as a transfection control, along with increasing amounts of FLAG-US3 as indicated. 48 hpt, cells were infected with 5 HA units/mL of SeV for 16 h. IFN-β promoter activity was measured by luciferase assay. (B) HEK 293T cells were co-transfected with IFN-β-luc and pGK-β-gal, and increasing amounts of FLAG-US3 as indicated, along with 1 ng of the indicated GST-RIG-I 2CARD construct (WT, T170A, or S8A). IFN-β promoter activity was measured by luciferase assay at 48 hpt. Data are representative of three independent experiments (mean and SEM of n = 3 biological replicates in A and B).
US3 inhibits RIG-I activation and innate immune response during HSV-1 infection

Having established that ectopic expression of the HSV-1 US3 protein phosphorylates RIG-I S8 and thereby inhibits RIG-I-mediated IFN-β induction, we next sought to characterize the role of US3 in RIG-I phosphorylation and signal inhibition in the context of authentic HSV-1 infection. Since the kinase activity of US3 is required for its ability to mediate RIG-I S8 phosphorylation, we generated a recombinant HSV-1 (F strain) encoding the kinase-dead US3 K220A mutant protein (HSV-1K220A), using a bacterial artificial chromosome (BAC) system as previously described (Tischer et al., 2010). In agreement with other groups that have assessed recombinant HSV-1 variants lacking US3 kinase activity, we found that HSV-1K220A exhibited slightly reduced replication kinetics compared to WT HSV-1 but otherwise grew normally. The current model of RIG-I activation during infection includes an initial desphosphorylation step in which RIG-I S8 and T170 are rapidly dephosphorylated by the cellular phosphatases PP1α/γ, a modification that is required for RIG-I downstream signaling (Wies et al., 2013). To evaluate the effect of US3 on RIG-I S8 phosphorylation during HSV-1 infection, we assessed the phosphorylation state of FLAG-RIG-I in cells infected with either HSV-1WT or HSV-1K220A (Figure 3.5A). RIG-I purified from cells infected with HSV-1WT was found to have a robust level of S8 phosphorylation, similar to RIG-I isolated from uninfected cells. In contrast, RIG-I purified from cells infected with HSV-1K220A had reduced S8 phosphorylation, suggesting that RIG-I had undergone robust dephosphorylation at the S8 residue. We next asked whether the inability of HSV-1K220A to antagonize RIG-I activation corresponds to higher levels of IFN-β induction in response to HSV-1K220A infection compared to HSV-1WT infection. To test this, we infected both immortalized cell lines (HEK 293, HEK 293T, and human hepatoma Huh7) and primary human cells (human foreskin fibroblasts HFF-1 and NHLF) with HSV-1WT or HSV-1K220A. In all cell types tested, we
Figure 3.5. US3 counteracts RIG-I S8 dephosphorylation and the RIG-I-mediated innate immune response during HSV-1 infection. (A) HEK 293T cells were transfected with FLAG-RIG-I as indicated. At 48 hpt, cells were left uninfected (mock), or infected with HSV-1 WT or HSV-1 K220A (MOI 0.1). At 18 hpi, cells were treated with calyculin A (Cal A). WCLs were subjected to FLAG-PD, followed by IB with the indicated antibodies. (B) qRT-PCR analysis of IFNB1 mRNA in the indicated cell types infected with HSV-1 WT or HSV-1 K220A (each MOI 1) for 16 h. (C) qRT-PCR analysis of the indicated mRNAs in primary NHLFs infected as described in (B). (D) Left: qRT-PCR analysis of IFNB1 mRNA in NHLF cells transfected with either non-targeting control siRNA (si.C) or siRNA targeting RIG-I (si.RIG-I) for 48 h and then infected with HSV-1 WT or HSV-1 K220A (MOI 1) for 16 h. Right: Knockdown efficiency of endogenous RIG-I (DDX58) was confirmed by qRT-PCR. Data are representative of two (A, D) or three (B, C) independent experiments (mean and s.d. of n = 3 biological replicates in B-D). *P < 0.05, **P<0.01, ***P<0.001 (unpaired t-test). n.s., statistically not significant.
detected significantly higher levels of IFNB1 induction in HSV-1\textsubscript{K220A}-infected cells compared to HSV-1\textsubscript{WT}-infected cells at 16 hpi (Figure 3.5B). In addition to upregulating IFNB1, HSV-1\textsubscript{K220A} infection also elicited significantly higher levels of ISGs (ISG15, Mx1, and OAS1) and the proinflammatory cytokine TNF compared to HSV-1\textsubscript{WT} infection (Figure 3.5C). To test whether HSV-1\textsubscript{K220A} triggers IFN induction in a RIG-I-dependent manner, we depleted endogenous RIG-I by using RIG-I-specific siRNA and measured IFNB1 transcripts in response to infection with either HSV-1\textsubscript{K220A} or HSV-1\textsubscript{WT}. In NHLF cells transfected with non-targeting control siRNA (si.C), infection with HSV-1\textsubscript{K220A} induced significantly higher levels of IFNB1 than infection with HSV-1\textsubscript{WT}, as previously observed (Figure 3.5D). Importantly, in cells transfected with siRNA targeting RIG-I, HSV-1\textsubscript{K220A} did not induce significantly higher levels of IFNB1 mRNA compared to HSV-1\textsubscript{WT}, suggesting that the enhanced immunogenicity of HSV-1\textsubscript{K220A} is due to its inability to antagonize RIG-I.

**Ability to phosphorylate RIG-I S8 is conserved amongst alphaherpesvirus US3 homologs**

Our finding that HSV-1 US3 antagonizes RIG-I through direct phosphorylation of the sensor at S8 led us to ask whether US3 homologs of other alphaherpesviruses, and even more broadly, unrelated herpesvirus-encoded kinases, are also capable of phosphorylating RIG-I-like receptor (RLR) regulatory residues. Herpesviruses encode two main families of proteins kinases: US3 kinases (McGeoch and Davison, 1986), and conserved herpesvirus-encoded protein kinases (CHPKs) (Gershburg and Pagano, 2008) (Table 3.1). The US3 kinases are conserved only amongst the alphaherpesviruses, while CHPKs are conserved amongst all herpesviruses. We tested the ability of several herpesviral kinases to phosphorylate the regulatory residues RIG-I S8 and MDA5 S88. IB with our phospho-specific RIG-I pS8 antibody showed that titrating amounts of HSV-1 US3, HSV-2 US3, and VZV ORF66 induced higher levels of S8 phosphorylation of GST-RIG-I.
2CARD as compared to empty vector co-transfection (Figure 3.6A). Other herpesviral kinases did not affect GST-RIG-I 2CARD S8 phosphorylation. These preliminary results suggest that the ability of US3 homologs to phosphorylate RIG-I S8 is likely conserved amongst the alphaherpesviruses, although additional studies are required to confirm this observation in virus-infected cells.

Table 3.1. **Herpesvirus-encoded serine/threonine kinases tested in this study.** Herpes simplex virus (HSV), varicella zoster virus (VZV), human cytomegalovirus (HCMV), murine herpesvirus 68 (MHV68), Epstein-Barr virus (EBV), Kaposi sarcoma-associated herpesvirus (KSHV), Conserved herpesvirus-encoded protein kinases (CHPK).

<table>
<thead>
<tr>
<th>Herpesvirus subfamily</th>
<th>Virus</th>
<th>US3 kinases</th>
<th>CHPKs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alphaherpesvirus</strong></td>
<td>HSV-1</td>
<td>US3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HSV-2</td>
<td>US3</td>
<td>UL13</td>
</tr>
<tr>
<td></td>
<td>VZV</td>
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<td>ORF66</td>
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<tr>
<td><strong>Betaherpesvirus</strong></td>
<td>HCMV</td>
<td></td>
<td>UL96</td>
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<tr>
<td><strong>Gammaherpesvirus</strong></td>
<td>MHV68</td>
<td></td>
<td>ORF36</td>
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<td></td>
<td>EBV</td>
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<td>BGLF4</td>
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<td></td>
<td>KSHV</td>
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<td>ORF36</td>
</tr>
</tbody>
</table>
Figure 3.6. Alphaherpesvirus US3 kinases enhance RIG-I S8 phosphorylation. HEK 293T cells were co-transfected with GST-RIG-I 2CARD along with titrating amounts of the indicated herpesviral kinase constructs. Whole cell lysates (WCLs) harvested 48 hpt were subjected to pull-down with glutathione-sepharose beads (GST-PD), followed by immunoblot (IB) with the indicated antibodies. Data are representative of two independent experiments.
DISCUSSION

RLRs are highly regulated by PTMs, which are mediated by a specific complement of cellular proteins. Modifications in the RLR CARDs have been demonstrated to follow a prescribed sequence of events. In the case of RIG-I, two critical early steps in activation are dephosphorylation of specific inhibitory residues, followed by K63-linked ubiquitination. Recent studies have uncovered a variety of mechanisms utilized by viruses to subvert these regulatory steps to evade the innate immune response [reviewed in (Chan and Gack, 2016)]. The K63-linked ubiquitination step of RIG-I activation is targeted by the NS1 protein of influenza A virus (Gack et al., 2009), which inhibits K63-linked ubiquitination of RIG-I. Several virally-encoded deubiquitinating enzymes (DUBs), which remove K63-linked ubiquitination from RIG-I, have also been identified (Clementz et al., 2010; Inn et al., 2011; van Kasteren et al., 2013; Wang et al., 2011). The dephosphorylation step of RLR activation is also manipulated by viruses for innate immune evasion. Two recent studies demonstrated that the V protein of measles virus prevents removal of inhibitory phosphorylation marks in RIG-I and/or MDA5 by antagonizing the cellular phosphatase PP1, thereby inhibiting downstream RLR activation and signaling (Davis et al., 2014; Mesman et al., 2014). These studies highlight the importance of phosphorylation-dependent inhibition of RLRs and led us to ask whether viruses also employ mechanisms that, rather than prevent RLR dephosphorylation, instead mimic cellular kinases and promote phosphorylation of RLRs at inhibitory sites. We speculated that one such mechanism could be a virally-encoded kinase that directly phosphorylates RLR at regulatory residues.

In this study, we found that the HSV-1-encoded S/T kinase US3 potently enhanced phosphorylation of RIG-I at S8 and had no observed effect on the phosphorylation levels of RIG-
I T170 or MDA5 S88. It has previously been shown that the phosphatases PP1α/γ regulate the phosphorylation state of all three regulatory residues (Wies et al., 2013). The phosphatase inhibitor Calyculin A (CalA) has potent activity against PP1 and is used in our experiments to maintain the phosphorylation state of RIG-I or MDA5 for detection by immunoblot. We observed that the phosphorylation state of RIG-I T170 and MDA5 S88 remained unchanged in the presence or absence of US3 despite CalA treatment. These observations support the idea that US3 phosphorylation of RIG-I S8 is somewhat specific. Examination of the RIG-I S8 phosphorylation sequence revealed that it fits the consensus phosphorylation motif described for US3.

We also showed that US3 phosphorylation of RIG-I corresponds to the inhibition of subsequent downstream RIG-I activation steps, including TRIM25 binding, K63-linked ubiquitination, and MAVS binding. While two inhibitory phosphorylation marks have been described for RIG-I, removal of both appears to be necessary for full RIG-I activation (Gack et al., 2010; Maharaj et al., 2012; Wies et al., 2013). Therefore, it is not surprising that US3 phosphorylation of a single residue would result in effective RIG-I inhibition. Our finding that US3 suppresses WT RIG-I 2CARD-induced signaling but not S8A RIG-I 2CARD-mediated signaling suggests that the ability of US3 to inhibit the RIG-I-induced IFN-β response is largely dependent on its ability to phosphorylate S8 in RIG-I. We also demonstrated that US3 kinase activity is essential for mediating RIG-I S8 phosphorylation and that US3 and RIG-I interact during HSV-1 infection. Collectively, these findings suggest that US3 binds and directly phosphorylates RIG-I S8 during viral infection. Given that US3 performs a number of functions in infected cells, it is tempting to speculate that a specific subcellular population or isoform of US3 may be responsible for RIG-I antagonism. US3 is categorized as a ‘leaky-late’ protein and is found in both the nucleus and cytoplasm during HSV-1 infection as well as in the tegument of mature
virions. Interestingly, US3 was found to cofractionate with mitochondria in infected cells, although a mitochondrial targeting sequence in HSV-1 US3 has not yet been identified (Poon et al., 2006). Future studies will be required to determine the precise kinetics of US3 phosphorylation of RIG-I during infection and whether RIG-I phosphorylation is mediated by tegument US3 or de novo-expressed US3 (or both). In HSV-1 and pseudorabies virus, US3 contains an alternative start site, which in the case of HSV-1 gives rise to a minor truncated US3 (US3.5) that lacks the first 76 N-terminal residues. Further studies are necessary to determine whether the localization and/or isoform of US3 play a role in its ability to inhibit IFN signaling.

It is important to note that US3 is a promiscuous kinase, and while many putative substrates have been identified, not all may be of equal importance for viral pathogenesis. Therefore, we also sought to demonstrate the functional relevance of RIG-I antagonism by US3 in the context of viral infection. RIG-I isolated from cells infected with HSV-1WT was found to have similar levels of S8 phosphorylation as RIG-I isolated from uninfected cells, suggesting that HSV-1WT can counteract the dephosphorylation of S8. In contrast, RIG-I isolated from cells infected with HSV-1K220A was found to have undergone robust S8 dephosphorylation. These findings suggest that HSV-1WT can effectively counteract RIG-I dephosphorylation by encoding an active US3 kinase that facilitates the phosphorylation of RIG-I S8. On the other hand, HSV-1K220A, which encodes a catalytically-inactive US3 kinase, loses the ability to maintain RIG-I S8 phosphorylation, thereby allowing the dephosphorylation and activation of RIG-I to proceed normally. US3 has previously been implicated in type-I IFN response suppression; infection with HSV-1 encoding a kinase-dead US3 was found to induce higher levels of IFN-β than infection with WT virus (Peri et al., 2008).

We found that, in RIG-I depleted primary fibroblasts, HSV-1K220A did not induce significantly higher levels of IFNB1 compared to cells transfected with non-targeting control
siRNA, indicating that the enhanced immunogenicity of HSV-1<sub>K220A</sub> is RIG-I-dependent. Our data suggest that HSV-1<sub>K220A</sub> is unable to promote inhibitory phosphorylation of RIG-I S8 and thereby loses its ability to suppress IFN induction.

HSV-1 US3 is a multifunctional protein that has been shown to play a role in a wide range of viral processes, including nuclear egress of viral capsids and virus-mediated inhibition of apoptosis. Although the observation that siRNA depletion of RIG-I abrogates the enhanced type-I IFN response elicited by the HSV-1 US3 mutant virus suggests that US3 acts on the pathway by regulating RIG-I signaling, it remains a possibility that US3 also targets other components of the RLR pathway in addition to RIG-I. Indeed, it has been suggested that US3-mediated hyperphosphorylation of the translation factors IRF3 and p65/RelA also dampens antiviral cytokine responses (Wang et al., 2014; Wang et al., 2013). In addition to acting directly on RLR pathway signaling molecules, other activities of US3, such as the modulation of nuclear structure via phosphorylation of nuclear lamin proteins (Mou et al., 2007) may also contribute to the observed phenotype of enhanced type-I IFN induction during infection with a US3 mutant virus.

Collectively, our data point to a model in which US3 exerts its major inhibitory activity on the type-I IFN response at the RIG-I level by counteracting RIG-I dephosphorylation. Our findings provide an example of how viral proteins can take advantage of preexisting cellular regulatory mechanisms to antagonize the antiviral response. US3 mimics the activity of cellular kinases PKC-α/β to mediate phosphorylation-dependent repression of RIG-I signaling. This mechanism may be employed by viruses beyond the Herpesviridae family. All large DNA virus families, including Poxviridae and Baculoviridae, encode conserved S/T kinases. The possibility that other viral kinases can also suppress the innate immune response by promoting inhibitory phosphorylation of signaling molecules remains to be explored.
MATERIALS AND METHODS

Cell culture and viruses

HEK 293T (ATCC), Vero (ATCC), NHLF (Clonetics), HEK 293 (ATCC), HeLa (ATCC), HFF-1 (ATCC), and HEK 293T ISRE-luciferase reporter cells (Shapira et al., 2009) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM GlutaMAX (Gibco), and 1% (v/v) penicillin-streptomycin (Gibco) under standard tissue culture conditions.

A US3 K220A HSV-1 mutant (HSV-1mut) was generated using a bacterial artificial chromosome (BAC) clone of HSV-1 strain (F) (pYEbac102; kindly provided by Yasushi Kawaguchi, University of Tokyo, Japan). To facilitate detection of infected cells, pYEbac102 was modified by insertion of the egfp (enhanced green fluorescent protein) gene into mini-F vector sequences, resulting in the pYEbac102-G construct that was used for further manipulation. Expression of EGFP was driven by the human cytomegalovirus (HCMV) immediate-early (IE) promoter. En passant mutagenesis (Tischer et al., 2010) was performed to introduce a mutation at amino acid residue 220 of the US3 protein kinase where the original lysine (K) was substituted with alanine (A), leading to the BAC pYEbac102-K220A-G construct. The following primers were used to amplify the PCR product that allowed for mutagenesis to occur:

(forward: 5’-TGACAGCAGCCACCCAGATTACCCCCCAACGGGTAATCGTG GCG
GCGGGGTGTTACACGAGCACTAGGGATAACAGGGTAATCGAT-3’; reverse: 5’-GCAGTCGCACCTCGTGCTCGTGCTCATCGTGTACCACCCCGC CGC
CAGATTACCCCGTTGGGGTGCTTAAACCCCAATTTAACC-3’; (nucleotides in italics and bold represent the desired mutation). After confirmation of the constructs by restriction fragment length polymorphism (RFLP) and DNA sequencing (LGC Genomics, Germany), 1 μg
each of purified BAC DNA was transfected into Vero cells at 90% confluency using polyethylenimine (PEI; Polysciences) according to procedures described elsewhere (Boussif et al., 1995). At 72 h post-transfection, when visible fluorescent plaques appeared, the respective virus mutant was reconstituted and harvested by 3 cycles of freeze-thaw.

**DNA constructs and transfections**

Plasmids encoding GST-RIG-I-2CARD, GST-MDA5-2CARD, GST-RIG-I-2CARD S8A, GST-RIG-I-2CARD T170A, FLAG-RIG-I, and FLAG-MAVS-CARD-PRD have been previously described (Gack et al., 2010; Gack et al., 2007; Wies et al., 2013). Plasmid encoding US3<sub>HSV-1</sub> (FLAG-US3, V5-US3) was a gift from David Knipe (Harvard University) (Sen et al., 2013). Plasmids encoding US3<sub>HSV-2</sub> (pSG5-US3) and UL13<sub>HSV-2</sub> (pSG5-UL13) were gifts from Lynda Morrison (Saint Louis University) (Geiss et al., 2004). Plasmid encoding ORF66<sub>VZV</sub> (pGK2-HA66) was a gift from Paul Kinchington (University of Pittsburgh) (Kinchington et al., 2000). Plasmid encoding UL97<sub>HCMV</sub> (pHA-UL97) was a gift from Donald Coen (Harvard University) (Kamil and Coen, 2007). Plasmid encoding ORF36<sub>MHV68</sub> was a gift from Pinghui Feng (University of Southern California). Plasmid encoding BGLF4<sub>EBV</sub> (pcDNA-BGLF4–FLAG) was a gift from Manfred Marschall (Friedrich-Alexander Universität Erlangen-Nürnberg) (Marschall et al., 2002). Plasmid encoding ORF36<sub>KSHV</sub>-myc (pcDNA4-ORF36-myc/His) was a gift from Frank Neipel (Friedrich-Alexander Universität Erlangen-Nürnberg) (Sander et al., 2008). Plasmid encoding V5-US3<sub>K220M</sub> was generated by site-directed mutagenesis using primers containing the desired mutation.
Transient transfection of cells was performed using calcium phosphate (Clontech), Lipofectamine and Plus reagent or Lipofectamine 2000 (both Life Technologies), according to the manufacturer’s instructions.

**Antibodies and other reagents**

The following antibodies were used for immunoblot analysis: anti-FLAG (1:2000, M2, Sigma), anti-β-Actin (1:5000, A1978, Sigma), anti-ICP8 and anti-US3 [(1:10,000 and 1:1000, respectively, generous gifts from David Knipe (Harvard University)], anti-RIG-I (1:2000, Alme-1, Adipogen), anti-TRIM25 (1:1000, 2/EFP, BD Biosciences), anti-GST (1:2000, GST-2, Sigma), anti-ubiquitin (1:500, P4D1, Santa Cruz), anti-V5 (1:2000, R96025, Invitrogen), anti-myc (1:2000, 9E10, Covance), phospho-specific pS8-RIG-I, pT170-RIG-I and pS88-MDA5 antibodies have been previously described (Gack et al., 2010; Maharaj et al., 2012). Goat anti-mouse- or goat anti-rabbit-Horseradish Peroxidase (HRP) secondary antibodies (both 1:2000) were purchased from Cell Signaling (cat #7076S and #7074S, respectively).

**Confocal immunofluorescence microscopy**

HeLa cells were grown on coverslips and transfected with FLAG-RIG-I. 24 hours post-transfection, cells were infected with HSV-1 (MOI 1). 20 hpi, cells were fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.1% (v/v) TritonX-100 (Sigma). Immunostaining of FLAG-RIG-I and HSV-1 US3 was performed using anti-FLAG and anti-US3 antibodies (see above), followed by incubation with anti-mouse Alexa-fluor-594 (Invitrogen) and anti-rabbit Alexa-fluor-488 (Invitrogen), respectively. Coverslips were mounted using VECTASHIELD mounting media with DAPI (Vector Laboratories). Laser scanning images
were acquired using an Olympus IX81 confocal microscope (Leica Microsystems) as previously described and analyzed using ImageJ.

Co-immunoprecipitation, pull-downs, and immunoblot analysis

HEK 293T cells were lysed in NP-40 buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% v/v NP-40, protease inhibitor cocktail [Sigma] and Ser-Thr phosphatase inhibitor cocktail [Sigma]), followed by centrifugation at 13,000 rcf for 20 min. GST pull-down was performed using gluathione-conjugated Sepharose beads (Amersham Biosciences). FLAG pull-down was performed using anti-FLAG-conjugated sepharose beads (Sigma). RIG-I and TRIM25 co-immunoprecipitation (co-IP) was performed using anti-RIG-I antibody (Alme-1, Adipogen) and anti-TRIM25 antibody (2/EFP, BD Biosciences), respectively, followed by incubation with protein A/G agarose (Pierce).

Luciferase reporter assays

HEK 293T cells were seeded into 12-well plates and transfected with 200 ng IFN-β luciferase construct (Lin et al., 2000) and 300 ng β-galactosidase-expressing pGK-β-gal (Hatzivassiliou et al., 1997), using Lipofectamine and Plus reagent (Life Technologies) according to the manufacturer’s instructions. The following effectors were used: 1 ng of plasmid encoding GST-RIG-I 2CARD or infection with SeV (5 HA units/mL) 24 h post-transfection. 48 h after transfection or 16 hpi, cells were harvested and lysates were subjected to luciferase and β-galactosidase activity assays (both Promega). Luciferase and β-gal activity was measured using a BioTek Synergy Microplate Reader in 96-well plates using 10 μl and 25 μl of cell lysates,
respectively. Luciferase activity was normalized to β-galactosidase values, and luciferase induction was calculated relative to mock-transfected samples, set as 1.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was purified from cells using an RNA extraction kit (Omega Bio-tek) following the manufacturer’s instructions. RNA quality was assessed using NanoDrop 2000. Equal amounts of RNA (25 - 500 ng) were used for a one-step qRT-PCR reaction using the SuperScript III Platinum One-Step qRT-PCR kit with ROX (Life Technologies) with commercially available FAM reporter dye primers (IDT) for the indicated genes. Gene expression levels were normalized to GAPDH. Fold induction of each target gene relative to mock-infected or mock-treated cells was calculated using the Comparative CT Method (ΔΔCT Method).

**siRNA-mediated knockdown**

Transient knockdown of endogenous RIG-I in NHLF cells, seeded in 12-well plates, was achieved by transfection of siRNAs targeting RIG-I (siGENOME SMARTpool M-012511-01-0010, Dharmacon) using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's instructions. As a control, non-targeting siRNA (Dharmacon, D-001206-14-20) was transfected. A final concentration of 150 nM per well was used. At 48 h post transfection, knockdown efficiency was confirmed by analyzing the transcript levels of the individual genes by qRT-PCR using specific primers (IDT).
Chapter 4: Dissertation Perspectives
SUMMARY OF RESULTS

In this dissertation, we have elucidated a mechanism by which HSV-1 activates innate immune signaling via the innate immune sensor RIG-I and how the virus in turn counteracts RIG-I signaling.

In Chapter 2, we described how HSV-1 infection leads to the exposure of endogenous pseudogene RNA5SP141 to innate immune sensor RIG-I. Specifically, we demonstrated that:

- The RIG-I mediated antiviral response to HSV-1 infection is temporally distinct from those induced by other innate immune receptors that detect HSV-1 infection.
- RNA-IP and deep sequencing of RIG-I ligands during HSV-1 infection shows enrichment of several noncoding RNAs (ncRNAs), including 5S rRNA pseudogenes.
- 5S rRNA pseudogene RNA5SP141 is a bona fide RIG-I ligand.
- The ability of RNA5SP141 to serve as a RIG-I ligand during viral infection is dependent on its cytoplasmic localization and the degradation of 5S rRNA binding proteins by the viral protein vhs.
- Depletion of RNA5SP141 by siRNA and gapmers inhibits the cytokine response to HSV-1 but does not affect the IFN response to SeV and MDA5 agonist HMW poly(I:C).
- Depletion of RNA5SP141 by siRNA and gapmers inhibits the cytokine response to EBV, and IAV.
In Chapter 3, we described how the HSV-1 tegument protein US3 antagonizes type-I IFN induction by mediating the inhibitory phosphorylation of a RIG-I regulatory residue. Specifically, we demonstrated that:

- US3 inhibits RIG-I interaction with the E3 ligase TRIM25 and downstream signaling adaptor MAVS.
- Inhibition of RIG-I-mediated IFN-β induction by US3 is RIG-I S8-dependent.
- A recombinant HSV-1 encoding a catalytically-inactive US3 mutant protein is unable to counteract RIG-I S8 dephosphorylation during infection and elicits an enhanced antiviral cytokine response.

Taken together, our findings suggest a model in which viral infection both activates innate immune sensing by revealing host immunostimulatory nucleic acids while also repressing the same signaling pathway (Figure 4.1). It remains to be elucidated precisely how these processes fit into the overall viral lifecycle and whether they represent evolutionary adaptations that favor viral fitness or host survival.
Figure 4.1. Model of RIG-I activation and antagonism during HSV-1 infection.

In a normal, uninfected cell (left), (A) RIG-I is maintained in an inactive state by constitutive phosphorylation of the regulatory residue S8. (B) RNA5SP141 is primarily localized to the nucleus, and RNA5SP141 in the cytoplasm is associated with RNA binding proteins (RBPs). During HSV-1 infection (right), (C) RNA5SP141 localization becomes predominantly cytoplasmic, and the viral virion host shutoff protein (vhs) mediates the downregulation of RBPs, which increases the availability of unshielded RNA5SP141. (D) RNA5SP141 binding triggers RIG-I activation, leading to RIG-I S8 dephosphorylation by the cellular phosphatases PP1α/γ. RIG-I signaling induces an antiviral type-I IFN and proinflammatory cytokine response. (E) The viral kinase US3 is able to antagonize RIG-I activation by mediating RIG-I S8 phosphorylation, thereby maintaining RIG-I repression.
DISCUSSION

Endogenous immunostimulatory nucleic acids

Our observation that endogenous RIG-I ligands are exposed during HSV-1 infection (Chapter 2) adds to the growing number of examples of immunostimulatory ‘self’ nucleic acids. The advancement of technologies such as RNA-Seq and microscopy in recent years have helped bring to light the mechanisms by which endogenous nucleic acids set off innate immune signaling. Although the mechanisms described to date involve a diverse set of ligands and sensors, a common theme is often observed: in healthy cells, potentially immunostimulatory endogenous nucleic acids are shielded from immune sensors by membrane-bound compartmentalization and/or association with cellular proteins. Innate immune signaling is triggered when the physical separation between ligand and sensor is lost. In some cases, this activation can be further exacerbated if cellular proteins that normally edit or clear improperly localized nucleic acids do not function correctly. In the case of RNA5SP141, containment within the nucleus appears to play a major role in preventing aberrant RIG-I signaling in the absence of infection, and other membrane-bound compartments such as the mitochondria and autophagosome are also critical for preventing autoactivation of PRRs.

Soon after the discovery of cGAS, endogenous mitochondrial DNA (mtDNA) was found to be a potent activator of the cGAS-mediated IFN response that can act as a switch between proinflammatory or immunologically silent cell death (Rongvaux et al., 2014; White et al., 2014). In normal cells, mtDNA are sequestered from cGAS sensing within the mitochondria. During apoptosis, the proteins Bax and Bak form a channel in the mitochondrial membrane that allows the escape of mtDNA into the cytoplasm, and in the absence of specific caspases, the cytosolic
mtDNA activates IFN induction via cGAS. Other sources of cellular disturbance have also been shown to lead to mtDNA activation of innate immune signaling. For example, the loss of transcription factor A, mitochondrial (TFAM) was found to result in aberrant mtDNA packaging, which allowed for mtDNA escape into the cytoplasm and detection by cGAS (West et al., 2015). In the same study, mtDNA escape and immunostimulatory activity was also observed during HSV-1 infection, due to mitochondrial damage mediated by the viral protein UL12. A recent study demonstrated that the dengue virus (DV) nonstructural (NS) protein NS2B targets cGAS for degradation to avoid cGAS activation by cytoplasmic mtDNA during DV infection (Aguirre et al., 2017). Taken together, these studies suggest that the release of mtDNA into the cytoplasm may occur during infection by many different viruses and serve as an endogenous signal of cellular damage. Other studies have shown that mtDNA can escape autophagosomes and activate innate immune signaling via TLR9 in endolysosomes (Oka et al., 2012). In addition to mtDNA, recent studies have revealed that DNA in the cytoplasm can also originate from the nucleus (Dou et al., 2017). In cells exhibiting signs of malignant transformation, chromatin fragments were observed to bleb out from the nucleus and activate cGAS. The resulting cGAS-mediated cytokine response was shown to contribute to the senescence-associated secretory phenotype (SASP), which serves to arrest the growth of potentially cancerous cells.

Another source of endogenous immunostimulatory nucleic acids is the 2-5A pathway, which generates small cellular RNAs that amplify the antiviral response by activating RLR signaling during infection [reviewed in (Silverman, 2007)]. The 2-5A pathway is triggered by IFN-mediated upregulation of 2-5A synthetase (OAS), which converts ATP into 2,5-linked oligoadenylate, a second messenger that subsequently activates RNase L to cleave cytoplasmic RNA. RNase L-mediated cleavage of viral RNAs in the cytoplasm serves as a general
antiviral mechanism to reduce viral replication, but RNAse L activation was also found to upregulate IFN in the absence of viral infection, suggesting that self-RNA products of RNAse L cleavage also contribute to the antiviral response (Malathi et al., 2007). In infected cells, RNAse L-cleaved RNAs containing 3’-phosphoryl groups were shown to enhance RLR-mediated signaling, but whether specific cellular RNAs are targeted for RNAse L cleavage remains unknown.

HSV-1 is an ancient DNA virus that is well-equipped for evasion of sensing and signaling by PRRs. HSV-1 mRNA products are largely indistinguishable from host mRNA, and these viruses encode a large arsenal of proteins that antagonize immune signaling pathways, often with multiple layers of redundancy. Utilizing ‘self’ RNA ligands like RNA5SP141 to set off RLR signaling may be an endogenous solution for responding to viral infection, in which inappropriate exposure of endogenous RNAs acts as indirect evidence of obligatory steps of the viral lifecycle such as nuclear egress and viral shutoff of host protein synthesis. In a similar manner as mtDNA and RNA generated by the 2-5A system, RNA5SP141-mediated signaling does not rely on viral nucleic acids, which can be masked or sequestered by the virus, but rather utilizes the immunostimulatory potential of the cell’s own RNA. Although potentially useful in certain contexts, endogenous nucleic acids that can trigger PRRs also pose the risk of aberrant signaling in the absence of infection. PRR activation by ‘self’ nucleic acids are often implicated in diseases involving inappropriate IFN signaling, also known as ‘interferonopathies.’

**Endogenous PRR ligands and interferonopathies**

Early evidence that autoimmune disease could be caused by innate immune receptor activation by nucleic acid autoantigens was provided by the observation that U1 small nuclear
ribonucleoprotein particles (snRNPs) from apoptotic cells could activate TLR7 and TLR8 signaling in plasmacytoid dendritic cells (pDCs) and monocytes, respectively (Vollmer et al., 2005). Autoimmunity to U1 snRNP had previously been associated with the autoimmune disease systemic lupus erythematosus (SLE), but the discovery that the RNA component of U1 snRNP alone could actively trigger IFN-α and TNF production demonstrated that in addition to playing a passive role in eliciting autoantibodies, U1 snRNP could also directly stimulate innate immune signaling via TLRs (Kattah et al., 2010).

Another example of endogenous activation of TLR-associated autoimmunity is the sensing of aggregated self-DNA and aggregated self-RNA by TLR9 and TLR7/8, respectively (Ganguly et al., 2009; Lande et al., 2007). Overexpression of the antimicrobial peptide LL37, which is associated with psoriasis, was shown to nucleate the aggregation of endogenous DNA and RNA and deliver the resulting complexes to endosomal compartments where they activated TLR signaling. In this mechanism, the high concentration and abnormal localization of endogenous nucleic acids confers their ability to trigger immune signaling.

Aicardi-Goutières syndrome (AGS) is an autoimmune disorder that is characterized by sustained type-I IFN activity. AGS has been linked to mutations in cellular proteins such as RNA editing enzyme ADAR1 and exonucleases TREX1, RNAse HII, and SAMHD1 [reviewed in (Crow and Manel, 2015)]. Activation of MDA5 and cGAS has been implicated in AGS, but the endogenous PAMP that triggers PRR signaling remains unclear. One popular hypothesis is that deficiencies in RNA editing and exonuclease activities fail to edit or remove intracellular nucleic acids generated by transcriptionally active endogenous retroelements, thereby allowing them to serve as PAMPs for PRRs and activate IFN responses in the absence of infection [reviewed in (Volkman and Stetson, 2014).]
Mutations in RIG-I and MDA5 have been shown to result in Singleton-Merton syndrome (SMS), an autoimmune disorder characterized by high levels of IFN (Jang et al., 2015; Rutsch et al., 2015), but whether RLR signaling in SMS is dependent on an endogenous dsRNA trigger is unknown. Further study is needed to determine whether RNA5SP141 and/or other endogenous ncRNA ligands of RIG-I contribute to autoimmune disorders with type-I IFN signatures. Additionally, although our results suggest that RNA5SP141 is shielded from innate immune recognition by binding with cellular proteins, it remains to be seen whether there are cellular proteins that edit or degrade RNA5SP141 and related immunostimulatory RNAs as a safeguard against autoimmunity.

**Pseudogenes and other ncRNAs in innate immunity**

Pseudogenes are generally defined as stretches of DNA arising from mutation and/or gene duplication that encode formerly-functional (parental) genes that are no longer expressed due to loss of promoter and enhancer elements. Although pseudogenes were traditionally thought of as being transcriptionally silent, recent advances in RNA sequencing have revealed that many pseudogenes are in fact expressed, and some even encode functional proteins [reviewed in (Milligan and Lipovich, 2014)]. Very little is known about the functions of transcribed pseudogenes, but some instances of pseudogene transcript-mediated regulation of signaling and gene functions have been described. For example, the long noncoding RNA (IncRNA) pseudogene Lethe was found to act as a negative regulator of proinflammatory cytokine expression by binding and inhibiting the activity of NF-κB subunit RelA (Rapicavoli et al., 2013). Expression of Lethe itself is upregulated by TNF signaling, and it is proposed to act as a braking mechanism for the inflammatory response. Other pseudogenes have been shown to modulate levels of parent gene
expression. Antisense pseudogenes can hybridize directly to the mRNA of the parent gene or be processed into siRNAs that silence the parent gene. Sense pseudogenes can affect levels of parent mRNA by acting as miRNA sponges. For example, the phosphatase and tensin homolog pseudogene (PTENP1) elevates the mRNA levels of its parent gene, PTEN, by acting as a decoy target for miRNA binding (Poliseno et al., 2010).

When preparing the cDNA library for RNA-Seq analysis of RNA bound to RIG-I during HSV-1 infection (Chapter 2), we attempted to capture the widest possible range of RNA species by choosing not to perform the standard-practice ribosomal reduction on the RNA pool before cDNA library construction and by choosing a sequencing read length of 50 nt in order to include small RNAs. Using this method, we found that a number of small 5S rRNA pseudogene transcripts, and most prominently RNA5SP141, were highly enriched in the RIG-I-pulldown fraction exclusively in HSV-1-infected cells. This experiment revealed that many 5S rRNA-like sequences previously designated as ‘pseudogenes’ have the ability to be expressed, and further study is required to determine the biological relevance of these transcripts. Like other pseudogenes, 5S rRNA pseudogenes may play a role in regulating the levels of 5S rRNA. Our observation that RNA5SP141 interacts with proteins that typically bind to its parent 5S rRNA suggests that RNA5SP141 and other 5S rRNA pseudogenes could also potentially affect ribosomal assembly and cellular translation.

**Determinants of RNA5SP141 immunostimulatory activity**

The findings described in Chapter 2 suggest that the ability of RNA5SP141 to serve as a RIG-I ligand is dependent on its relocalization from the nucleus to cytoplasm during infection, but many questions about this process remain unanswered. For example, which stage of the viral life
cycle specifically leads to relocalization, and what is the mechanism behind this relocalization? Based on our observation that RIG-I activation occurs late in infection (Figure 2.1A), we hypothesize that RNA5SP141 relocalization may correspond with viral nuclear egress or the onset of virus-induced apoptosis. Future experiments may help clarify this point. For example, time course experiments could be performed to assess the localization of RNA5SP141 over the course of HSV-1 infection, for example, by qRT-PCR of nuclear and cytoplasmic cellular fractions, or by fluorescence in situ hybridization microscopy.

Our observation that depletion of RNA5SP141 dampens the IFN response to several viruses (HSV-1, EBV, and IAV) suggests that host protein translation shutoff and mislocalization of nuclear RNAs may serve as somewhat generic ‘danger signals,’ of viral infection, since they are characteristic of infection by a diverse range of viruses. Shutoff of host translation is a feature of infection by many viruses, and it acts both to direct cellular resources towards production of viral proteins and to prevent the translation of host defense proteins. Although the mechanisms employed are highly divergent, examples of viral manipulation of host translation can be found in almost all virus families [reviewed in (Walsh and Mohr, 2011)]. We observed by immunoblot that 5S rRNA interaction protein levels are reduced during infection with both EBV and IAV (Figure 2.14F, 2.15G), and future experiments testing the ability of other viruses to downregulate these proteins may help identify other viruses that trigger RNA5SP141-mediated immune signaling.

Although it remains to be determined whether the localization of RNA5SP141 or other nuclear RNAs is altered during infection EBV and IAV infection, both viruses are known to alter nuclear structure, which may be a prerequisite for RNA5SP141 localization to the cytoplasm. EBV infection has been observed induce disassembly of the nuclear lamina (Lee et al., 2008), and IAV has been shown to trigger enlargement of nuclear pore complexes (Muhlbaier et al., 2015). In
general, perturbations of the nucleus are thought to facilitate or arise from the process of viral egress, but viruses that do not replicate within the nucleus have also been observed to manipulate nuclear function. For example, the ssRNA picornavirus, poliovirus, has been shown to alter nuclear pore structures and increase the permeability of the nuclear envelope to facilitate nucleocytoplasmic exchange of proteins (Belov et al., 2004). In this study, poliovirus-infected cells were unable to retain nuclear proteins, and bidirectional movement of proteins was also observed. It’s tempting to speculate that this type of nuclear disruption may also facilitate the efflux of immunostimulatory nucleic acids to the cytoplasm. Similar to the phenomenon of viral shutoff of host protein expression, virus-induced alterations of the nuclear envelope and nuclear pore complex viruses are also a hallmark of infection for many viruses [reviewed in (Le Sage and Mouland, 2013; Mettenleiter, 2016)].

Aside from viral infection, disruption of the nuclear membrane can be observed in other contexts such as cancer [reviewed in (Chow et al., 2012)] and aging (D'Angelo et al., 2009). Interestingly, Pol I- and Pol III-transcribed ncRNAs, including 5S rRNA, are upregulated in oncogenically transformed cells. RNA5SP141 or similar pseudogenes may be similarly induced and serve as a danger signal for innate immune ‘sensing’ of cancer [reviewed in (Fuertes et al., 2013)]. In aging cells, the exposure of endogenous RNA ligands may contribute to sterile inflammation and immunosenescence. In general, performing RNA-Seq experiments to compare the ncRNA composition of the cytoplasmic and nuclear fractions of healthy vs. diseased cells may reveal novel endogenous immunostimulatory RNA species.
Implications for therapeutics and vaccine design

Vertebrate immunity is divided into two arms: the innate immune system and the adaptive immune system, which work synergistically to mount an effective and sustained defense against pathogens. While the adaptive immune system takes days to produce a custom antibody response, the innate immune system initiates an immediate response that is more nonspecific in comparison and mounted in the first minutes and hours after pathogen exposure. As a safeguard against inappropriate immune responses, full activation of both adaptive and innate responses requires multiple signaling inputs that indicate the origin and pathogenicity of a detected substance. A vaccine that delivers inactivated virus or viral protein may elicit antibodies but does not stimulate as robust of an innate immune response compared to a live virus, since crucial components of the innate immune system are activated by signals that are only generated during viral replication and other parts of the viral life cycle. The use of adjuvants attempts to overcome this limitation by introducing substances, such as metal salts or oil emulsions, that promote additional immune activation, usually via an unknown mechanism.

The discovery that PRRs such as TLRs and RLRs function as key regulators of antiviral cytokine signaling prompted the idea that PRR agonists could be useful as vaccine adjuvants [reviewed in (McKee and Marrack, 2017)]. Benefits of PRR agonists compared to traditional methods are that the mechanistic basis of activation is well-understood, and the elicited immune response is broad and well-defined. A variety of RIG-I agonists have been shown to act as effective virus vaccine adjuvants, including synthetic 5’-triphosphate dsRNA (Kulkarni et al., 2014) and a Sendai virus-derived RNA (Martinez-Gil et al., 2013), among others. Our observation that RNA5SP141 is a very potent RIG-I agonist that triggers high levels of type-I IFN and other cytokines (Chapter 2) suggests that RNA5SP141 may be effectively used as a vaccine adjuvant.
Some features of RNA5SP141 may provide particular advantages for its use in this application. For example, rRNAs (along with tRNAs) are highly stable compared to other forms of RNA, which may facilitate more sustained RIG-I signaling. Additionally, an endogenous RNA such as RNA5SP141 may be better tolerated by the cell compared to a virus-derived or synthetic sequence.

Many of the most effective vaccines to date have been live attenuated vaccines (LAVs), which are replicating viruses that are theoretically capable of ‘naturally’ eliciting full immune activation without the aid of adjuvants. Our observation (described in Chapter 3) that a single point mutation in the HSV-1 US3 kinase, K220A, abolishes the ability of US3 to antagonize RIG-I activation suggests that this mutation could be used to attenuate HSV-1 (or HSV-2) in the context of a vaccine. The K220A mutation, combined with mutations in other key viral proteins, could potentially generate a safe and effective LAV that is unable to antagonize innate immune responses.
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