Evasion of MDA5-Mediated Innate Immunity by Paramyxoviruses

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Evasion of MDA5-mediated Innate Immunity by Paramyxoviruses

A dissertation presented

by

Meredith Elizabeth Davis

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Evasion of MDA5-mediated Innate Immunity by Paramyxoviruses

Abstract

The innate immune sensor MDA5, a RIG-I-like receptor (RLR), is critical for the detection of viral nucleic acid, eliciting an antiviral immune response. Aberrant immune activation can be detrimental to the host; therefore, RLR activity is strictly regulated. In the uninfected cell, MDA5 is constitutively phosphorylated at S88, preventing antiviral signaling. Upon sensing of viral RNA, MDA5 is activated via dephosphorylation by the phosphatases PP1α/γ. Dephosphorylation of MDA5 allows interaction with the mitochondrial adaptor protein MAVS, inducing downstream signaling, leading to the production of antiviral cytokines including type-I interferons (IFNs). Many viruses have evolved sophisticated mechanisms to avoid detection by RLRs. Here, we present a novel evasion mechanism of paramyxoviruses to escape the MDA5-induced innate immune response: inhibition of its key regulators, PP1α and PP1γ. The V proteins of measles virus (MV) and multiple other paramyxoviruses interact with PP1α/γ, preventing MDA5 S88 dephosphorylation and subsequent innate immune signaling.

In Chapter 2, we identify a conventional PP1-binding motif in the unique C-terminal region of the MV V protein which mediates this interaction. Mutation of this motif abrogates PP1 binding and MDA5 antagonism without effecting other known activities of the V protein such as STAT inhibition. To determine the physiological relevance of the V-PP1 interaction for MDA5 antagonism, we generated a recombinant MV carrying a PP1-binding deficient V protein. This mutant virus no longer suppressed MDA5 dephosphorylation by PP1, resulting in increased expression of IFN and IFN-stimulated genes (ISGs) and impaired replication in lung epithelial and dendritic cells compared to the parental virus.
In Chapter 3, we expand our understanding of paramyxovirus antagonism of MDA5 by examining the virus-specificity of inhibition of S88 dephosphorylation. We found that the ability to interact with PP1 and inhibit MDA5 S88 dephosphorylation is shared by multiple viruses, including mumps (MuV), Nipah, and Hendra viruses. We mapped the PP1 interaction to a minimal binding region in the MuV-V C-terminal domain. This region contains a putative PP1-binding motif which is not conserved between viruses. The importance of this putative motif requires additional investigation. Together, our findings reveal PP1 antagonism as a novel immune evasion strategy of paramyxoviruses.
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To my parents, Dwight and Pamela Davis
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Chapter 1: Introduction-

RIG-I-like-Receptors, Their Regulation and Antagonism
Acknowledgements

Portions of Chapter 1 are adapted from the published work:


*Authors contributed equally to this manuscript
1.1. Pattern Recognition Receptors Detect Invading Pathogens

The immune system is divided into two arms, the innate and adaptive responses. The innate immune system is the first line of defense against invading pathogens. One component of the innate immune response are pattern recognition receptors (PRRs) which recognize conserved pathogen associated molecular patterns (PAMPs) and trigger signaling cascades resulting in anti-pathogen responses. For detection of virus-associated double stranded (ds) RNA, there are two main sets of PRRs: Toll-like receptors (TLRs) and RIG-I-like-receptors (RLRs). TLR3 and TLR7/8 are located on the cell surface and in endosomal compartments of cells where they are positioned to survey the extracellular milieu for viral infection. In contrast, the RLRs are ubiquitously expressed in the cytoplasm, and thus act as key sentinels to defend the host from intracellular pathogens. The RLRs recognize intracellular viral RNAs which accumulate during the course of infection and lead to the activation of the interferon (IFN) induction pathway. Upon detection of viral infection, both classes of PRRs initiate signaling cascades which result in the activation of transcription factors which induce the secretion of type-I and III IFNs and proinflammatory cytokines. Secreted IFN is then able to signal in an autocrine and paracrine manner to induce a large number of IFN stimulated genes (ISGs) which have direct antiviral effects as well as the ability to prime the adaptive immune response for viral clearance.

1.1.A. RIG-I-Like-Receptors Survey the Cytoplasm for Viral Nucleic Acid

While the existence of the TLRs has been known for decades, the RLRs were only more recently characterized. RIG-I was the first to be identified from a cDNA screen looking for factors which induce type-I IFN in response to dsRNA stimulation. This screen identified the RNA helicase retinoic acid-inducible gene-I (RIG-I) as a sensor of transfected dsRNA as well as New Castle disease virus (NDV). RIG-I was shown to act upstream of both IFN regulatory factor
3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), to activate two branches of the IFN-induction pathway.

Later in the same year, a separate screen identified melanoma differentiation-associated gene 5 (MDA5) as a related RNA helicase to detect viral RNA during infection. The V proteins of paramyxoviruses were known to inhibit the production of IFN-β; yet, their cellular target was unknown. A screen for interaction partners of the parainfluenza virus 5 (PIV5 previously called SV5) V protein by immunoprecipitation followed by mass spectrometry identified MDA5 as a novel factor whose activation leads to the production of type-I IFN. Further studies showed that RIG-I and MDA5 are both structurally and functionally related and they were classified together as the RIG-I-like receptors, or RLRs.

As a family, the RLRs share a common domain architecture consisting of two N-terminal caspase activation and recruitment domains (CARDs) which are necessary and sufficient for downstream signaling. They also contain an ATPase/helicase core domain and a C-terminal domain (CTD) both of which are involved in RNA binding. The helicase core consists of four major subdomains: Hel1, Hel2, a unique insertion domain (Hel2i), and a bridging domain (Br). Each of these subdomains contributes to optimal RNA binding. Hel2 and Hel1 interact with phosphates of the RNA backbone to then close around the RNA strand in a “C-clamp” fashion. After initial RNA binding, the Br domain interacts with Hel1 and the CTD to coordinate conformational changes necessary for tight interaction with the RNA. In the RNA-free state, the RIG-I Hel2i insertion interacts with CARD2, facilitating the auto-repressed, closed conformation. Upon RNA binding, Hel2i releases CARD2 and makes contacts with the CTD and dsRNA ligand. The MDA5 Hel2i insertion is shorter than that of RIG-I, and in the RNA-bound state, forms rigid contacts with the CTD, resulting in a more open conformation of the
CTD compared to RIG-I. This interaction partially accounts for the differences in RNA binding by the RLRs, with RIG-I “capping” the ends of RNA and MDA5 wrapping around the length of the dsRNA\(^8\). To date, there is no structure of the MDA5 helicase domain in the RNA-free state, leaving the role of Hel2i unknown; however, several studies suggest that MDA5 does not adopt a closed conformation\(^9,10\).

**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 IFN-\(\alpha/\gamma\) induction. In addition, all three RLR 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, peroxisomes, and MAM. (Adapted from Chiang JJ, Davis M.E, Gack MU. 2014\(^11\))

LGP2 (laboratory of genetics protein 2) is a third helicase-containing protein classified as a member of the RLR family. LGP2 shares the same helicase construction, but in contrast to RIG-I and MDA5, it lacks the CARD domains. For this reason, LGP2 is unable to directly signal for induction of IFN, but instead is thought to act as a cellular regulator of RIG-I and MDA5 function. The mechanisms that govern this regulation remain controversial as *in vitro* studies indicated LGP2 acts as a negative regulator of RLRs\(^12,13\), but *in vivo* studies implied a positive regulatory role\(^14,15\). Further studies now suggest that LGP2 may differentially regulate RIG-I and MDA5\(^16,17\), but the precise physiological role of LGP2 remains to be determined.
In addition to the common structure and function of the RLRs, it was found that they interact with a common downstream adaptor protein which acts as a scaffold for signal transduction. Four groups independently identified the mitochondrial antiviral-signaling protein (MAVS, also known as IPS-1, VISA, and Cardiff) as a mitochondria-associated protein essential for RLR signal transduction\textsuperscript{18-21}. MAVS is characterized by a C-terminal transmembrane domain containing a mitochondrial localization signal, a proline-rich domain (PRD), and a single N-terminal CARD domain. After detection of their ligands, both RIG-I and MDA5 oligomerize and translocate to the mitochondria where they interact with MAVS via homotypic CARD-CARD interactions to propagate antiviral signal transduction. Recent studies have revealed that the localization of MAVS, either on the mitochondria or on peroxisomes, dictates the type of immune response triggered by RLR activation\textsuperscript{22,23}. MAVS localized to the mitochondrial induces type-I IFN whereas MAVS localized to the peroxisomes induces type-III IFN.

The components of the RLR signaling cascade have been well defined (Figure 1.2). After activation, the RLR-MAVS interaction facilitates the formation of the ‘MAVS signalosome’ which includes tumor necrosis factor (TNF) receptor-associated death domain (TRADD), TRAF family member-associated NF-\(\kappa\)B activator (TANK), and the E3 ubiquitin ligases TNF receptor-associated factor (TRAF) 6 and 3 for NF-\(\kappa\)B and IRF activation, respectively\textsuperscript{24,25}. For NF-\(\kappa\)B activation, TRAF6 first recruits the TAK1/TAB1/2 complex. This complex of kinases then phosphorylates NF-\(\kappa\)B essential modulator (NEMO, also called IKK\(\gamma\)) activating it to serve as a scaffold for the additional IKKs: IKK\(\alpha\) and IKK\(\beta\). The IKK complex then recruits a complex consisting of I\(\kappa\)B (inhibitor of NF-\(\kappa\)B) and NF-\(\kappa\)B subunits, canonically the p65 and p50 subunits. IKK\(\beta\) phosphorylates I\(\kappa\)B which leads to its dissociation from p65/p50, allowing translocation to the nucleus to promote transcription of NF-\(\kappa\)B target genes. Similarly,
activation of IRF 3/7, the E3 ligase TRAF3 is recruited to MAVS\textsuperscript{26,27}. TRAF3 similarly activates NEMO which again forms a signaling complex, in this case consisting of TANK, IKK\textepsilon, and TBK1. After formation of this complex, TBK1 is activated to directly phosphorylate IRF3, leading to its dimerization and translocation to the nucleus to induce IRF3 target genes including type-I IFNs and proinflammatory cytokines.

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**Figure 1.2. RLR Signaling Cascade.** Schematic model of the signaling cascade induced by RLR activation, leading to the activation of NF-\kappaB, IRF3, and IRF7 for the induction of Type-I IFN and proinflammatory cytokines. The details of the signaling pathway are described in the text. (Adapted from Chiang JJ, Davis M.E, Gack MU. 2014\textsuperscript{13})
1.1.B. Distinct RNA Ligands of RLRs

The question of how RLRs distinguish viral RNA from host RNA has been an area of intense study since their discovery. Using synthetic RNAs such as poly(I:C) and in vitro transcribed RNAs, basic molecular features of these RNAs have been identified as RLR-activating PAMPs. Interestingly, despite their similarities in domain architecture, multiple studies have shown that RIG-I and MDA5 recognize discrete RNA species, possibly resulting in detection of distinct viral subsets. While there is still some debate over the physiological ligands for RLRs, there is a consensus that the key signature for RIG-I recognition is short dsRNA containing a 5’ tri-phosphate (5’ppp)\textsuperscript{28-31}. In contrast, studies of MDA5 have shown that it is activated upon binding to longer dsRNAs, as well as large aggregate RNA webs extracted from cells infected with encephalomyocarditis virus (EMCV), a picornavirus\textsuperscript{32}.

Recent structural studies have further defined the molecular mechanism by which these RNA species are recognized by RLRs. A basic groove in the CTD of RIG-I is required for binding to the negative charge of the 5’ppp present on RNA ligands\textsuperscript{28,33}. One study has indicated that this basic groove can accommodate 5’ di-phosphate (5’pp) ligands as well\textsuperscript{34}. This binding was further confirmed in two crystal structures which also show that the base-pairing that occurs in dsRNA stabilizes the association of the CTD with the RNA helix\textsuperscript{6,7}. In contrast, MDA5 binds to the RNA along its length and does not require a 5’ppp. Upon RNA binding, MDA5 appears to form large filaments dependent on the ATPase function of the helicase domain. These filaments wrap around the RNA and may serve to localize MAVS aggregates for downstream signaling\textsuperscript{9,35-37}.

Initial studies during virus infection of RIG-I or MDA5 knockout mice indicated that RIG-I and MDA5 recognize different subsets of viruses based on their distinct ligands. These
identified influenza virus, vesicular stomatitis virus (VSV), and arenaviruses as being solely detected by RIG-I\textsuperscript{13,38-42}. RIG-I was also thought to be the primary detector of paramyxoviruses, including Sendai virus (SeV), Newcastle Disease virus (NDV), and measles virus (MV)\textsuperscript{13,38,41-44}. MDA5 was found to be activated primarily in response to picornaviruses as well as RNA intermediates produced during vaccinia virus infection\textsuperscript{32,39,45}. Similar studies also revealed partially redundant roles of MDA5 and RIG-I in response to reovirus and flaviviruses such as dengue virus (DenV) and West Nile virus (WNV)\textsuperscript{39,41,46,47}.

These initial studies failed to consider the roles of viral antagonists of RLRs. Thus the contribution of either RIG-I or MDA5 to viral recognition was not immediately evident. One example of this was the apparent paradox of the antagonism of MDA5 by paramyxovirus V proteins. As mentioned, RIG-I was initially thought to be the sole RLR responsible for recognition of RNA species during paramyxovirus infection. However, \textit{in vivo} studies revealed that SeV infection of MDA5\textsuperscript{-/-} mice was more pathogenic and elicited much higher levels of proinflammatory cytokines compared to infection of wild type mice\textsuperscript{48}. Additional studies revealed that the contribution of MDA5 was previously not apparent due to the antagonistic functions of the V protein against MDA5: RIG-I knockdown cells infected with MV lacking the V protein induce higher levels of IFN\beta mRNA\textsuperscript{49}. It is likely that RIG-I and MDA5 cooperate to sense other infections. This has already been shown for certain viruses by examining the physiological ligands of MDA5 and RIG-I by IP and deep sequencing in infected cells. These studies have shown that each RLR recognizes distinct RNA PAMPs during infection\textsuperscript{50-52}. Further studies should explore this possibility, particularly where viral immune evasion mechanisms are known.
1.1.C. Cellular Regulation of RLRs

Activation of RLRs leads to the rapid expression of type-I IFNs and proinflammatory cytokines. Type-I IFNs act directly on cellular processes and also up-regulate a large set of ISGs. ISGs in turn have multiple antiviral functions, including the suppression of viral protein synthesis and the induction of apoptosis. Type-I IFNs also have great importance in the priming of the adaptive response which further mediates clearance of viral infection and development of a humoral response. A delicate balance in activation of innate immunity must be reached: an insufficient response may not completely clear the viral infection while an excessive response can be harmful to the host, resulting in cell/tissue damage and auto-immune disorders. To keep this balance, an elegant system of regulation of RLRs is in place.

Regulation of RIG-I

In the uninfected cell, RIG-I exists in a “closed,” auto-repressed conformation, preventing activation in the absence of virus infection (Figure 1.3). In this conformation, the CARDs are kept inaccessible for downstream signaling due to interactions between the Hel2i and CARD2 domains. In addition to this structural regulation, RIG-I is kept inactive through the phosphorylation of multiple residues. T770, S854, and S855 in the CTD are phosphorylated by casein kinase II (CK2) which promotes interactions necessary for auto-repression. Phosphorylation of S8 and T170 in the CARD domains by protein kinase C-α and β (PKCα/β) is also required to maintain RIG-I auto-repression in the absence of viral infection.
**Figure 1.3. Model of RIG-I activation and regulation.** (A) In uninfected cells, RIG-I is kept inactive via a closed conformation and inhibitory phosphorylation at specific residues in the CTD and CARDs by CKII and PKC-α/β, respectively. (B) Upon viral infection, RIG-I binds to its RNA ligand, inducing conformational changes and multimerization. Riplet mediates the K63-linked polyubiquitination of K788 in the CTD, which contributes to the release of RIG-I from its auto-repressed state. Dephosphorylation of the CTD is also required for RIG-I activation although the phosphatase responsible has not been identified. (C) Release of RIG-I from the auto-repressed conformation renders the CARDs accessible to the phosphatases PP1α/γ, which dephosphorylate S8 and T170. (D) Dephosphorylation of the CARDs allows interaction with TRIM25, which mediates K63-linked polyubiquitination of K172. (E) Ubiquitination of RIG-I facilitates its multimerization and the recruitment of the chaperone 14-3-3ε which translocates RIG-I to MAMs for interaction with MAVS. (F) Negative regulators of RIG-I signaling include the DUBs CYLD, USP3, and USP21, which remove K63-linked ubiquitin chains from RIG-I. LUBAC down-regulates RIG-I signaling by targeting TRIM25 for proteasomal degradation. USP15 counteracts the activity of LUBAC by removing K48-linked ubiquitin chains from TRIM25, stabilizing TRIM25 during viral infection. (Adapted from Chiang JJ, Davis M.E, Gack MU. 2014)

Both binding to viral RNA ligands and the modulation of post-translational modifications of RIG-I are required to exit the auto-repressed conformation for activation of signaling.

Structural studies have shown that both the CTD and the helicase domains are involved in binding to viral RNA. The CTD binds to the 5' end of the RNA. Historically, it was believed that a 5’-ppp was required for optimal RNA interaction with the CTD. However, recent evidence indicates that the basic groove in the CTD is also able to accommodate a 5’-diphosphate (5’-pp)
to induce similar levels of activation\textsuperscript{34}. After initial interaction of the CTD with the 5'-phosphate, the helicase domain binds to the RNA duplex\textsuperscript{6,7,62}. These interactions lead to an “end-capping” model of RIG-I binding to RNA.

Recognition of viral RNA induces dramatic conformational changes in RIG-I which result in the release of the CARD domains from interaction with the helicase and CTD, allowing the recruitment of additional regulatory factors. These additional factors facilitate a series of changes in post-translational modifications leading to the fully active form of RIG-I.

The E3 ubiquitin ligase Riplet (also RNF135/REUL) was characterized by multiple groups as a regulator of RIG-I through activating K63-linked ubiquitination\textsuperscript{63,64}. Among multiple residues that have been implicated for ubiquitination by Riplet, K788 appears to be the functionally important residue. Specifically, ubiquitination of K788 increases the ability of RIG-I to bind to its viral RNA ligand and also promotes the transition to the open conformation for further post-translational modifications and interaction with signaling partners\textsuperscript{63,65}.

The studies which identified the inhibitory phosphorylation marks in the RIG-I CARD (S8, T170) and CTD (T770, S854/855) showed that RNA binding is not sufficient for RIG-I activation\textsuperscript{56-58,66}. Therefore, following RNA binding, these phosphorylation marks must be removed. An siRNA screen of 257 human phosphatases identified protein phosphatase 1 (PP1) isoforms PP1\(\alpha\) and PP1\(\gamma\) as the phosphatases responsible for dephosphorylation of the RIG-I CARD domains\textsuperscript{66}. The PP1 family of phosphatases contains three catalytic isoforms, PP1\(\alpha\), PP1\(\beta\), and PP1\(\gamma\), that are involved in many cellular processes including cell cycle regulation, protein synthesis, and metabolism\textsuperscript{67}. This study demonstrated a role for PP1 in immune signaling
for the first time, showing that PP1α/γ are required for both RIG-I and MDA5 activation by dephosphorylating the CARD domains (RIG-I S8/T170 and MDA5 S88)⁶⁶.

Dephosphorylation of the RIG-I CARD domains induces further conformational changes within the CARD domain allowing the E3 ubiquitin ligase TRIM25 to bind and ubiquitinate RIG-I⁶⁸. TRIM25 is essential for RIG-I activation through the covalent attachment of ubiquitin to the residue K172 in the CARD domain. These K63-linked ubiquitin chains are required for interaction with MAVS as mutation of this residue prevented interaction⁶⁸.

Additional studies have indicated that Riplet ubiquitinates residues in the CARD domain (K154, K164, K172) as well as in the C-terminal region⁶⁴. In vivo studies confirmed the importance of the ligase in regulating RIG-I signaling: Riplet-deficient mice produced less IFN and were more susceptible to VSV infection than wild-type mice⁶⁹. Importantly, the K172 residue is not conserved between human and mouse RIG-I indicating that TRIM25 and Riplet may have species-specific roles in regulation of RIG-I⁷⁰.

Recently, there have been several reports showing a role for unanchored K63-linked polyubiquitin chains in the signaling activity of RIG-I and MDA5. Zeng, et al., first showed in a cell free system that in vitro generated K63-, but not K48- or linear, polyubiquitin chains were able to bind to RIG-I and facilitate its activation of IRF3⁷¹. This study also showed that RIG-I K172, the same residue covalently modified by TRIM25, was critical for the free ubiquitin binding of the CARD domains.

Structural studies have attempted to clarify the discrepancy of which K63-linked ubiquitination, covalently linked and/or unanchored chains, facilitates RIG-I activation. Initial studies showed that while the 2CARD truncation requires K63-linked ubiquitin, the full length
RIG-I structure could form oligomers and induce MAVS filament binding in the absence of ubiquitin. These ubiquitin-independent oligomers required ATP-dependent RNA binding of the helicase domain. Additional studies showed that non-covalent ubiquitin was able to induce RIG-I CARD tetramer formation and that in the crystal structure of the RIG-I CARD domains, ubiquitin chains are bound along the outside of the helical 2CARD tetramer, stabilizing interactions between distinct RIG-I 2CARD subunits. This study also sought to clarify the relationship between covalent and non-covalent ubiquitin chains for RIG-I activation. Several lines of evidence indicate that covalent K63-ubiquitination is important for RIG-I signaling. First, Peisley, et al., showed that K172 is not involved in interaction with unanchored chains, so the effect of the K172R mutation on signaling is due to the absence of covalent modification. Secondly, they showed that in the CARD-Ub crystal structure, the distance between residue K172 and the unanchored ubiquitin does not exclude conjugation of ubiquitin to this residue. Finally, in assays designed to observe the activation of MAVS by RIG-I, RIG-I CARDS conjugated to ubiquitin stabilized MAVS aggregates more than RIG-I bound to free ubiquitin. Together, these results show that RIG-I 2CARD acts both as a K63-linked ubiquitination target and also can bind to free ubiquitin. Both of these functions facilitate RIG-I CARD tetramerization which serves as a nucleation point for the formation of MAVS aggregates.

Recent studies have implicated roles for additional ubiquitin E3 ligases in regulating RIG-I signal transduction. Over-expression of TRIM4 led to increased IFN induction following infection with SeV. More detailed analysis indicated that TRIM4 interacted with RIG-I and led to the K63-linked ubiquitination of K154, K164, and K172 in the CARDS. However, the physiological role and contribution of K63-linked ubiquitination of RIG-I by TRIM4 to innate antiviral immunity has yet to be determined. Another regulatory mechanism of RIG-I activity
through K63-linked ubiquitination was recently discovered involving antiviral stress granules (avSG)\textsuperscript{76}. The E3 ligase MEX3C was shown to bind to viral RNA, resulting in its association with RIG-I inside avSGs. This study indicated that K48, K99, and K169 of RIG-I were ubiquitinated by MEX3C and that this ubiquitination increased type-I IFN induction. While this study strengthened the hypothesis that RIG-I’s subcellular localization may be important for viral RNA detection, the exact role of avSGs in innate immune signaling remains unclear.

In addition to the changes in post-translational modifications that RIG-I undergoes, RIG-I must be relocalized for signal activation. In the uninfected cell, RIG-I adopts a dispersed cytosolic localization to survey the intracellular space for invading pathogens; however, in order to signal, RIG-I must interact with MAVS which is located on the outer mitochondria and peroxisomal membranes\textsuperscript{22,23}. In order to traverse the cytoplasm, RIG-I requires the mitochondrial targeting chaperone protein 14-3-3\textsubscript{ε}\textsuperscript{77}. Following RIG-I-RNA binding and release from the auto-repressed conformation, 14-3-3\textsubscript{ε} forms a “translocon” complex consisting of RNA-bound RIG-I and TRIM25 which relocates to the mitochondrial associated membranes (MAM) to facilitate MAVS interaction and signaling. The interaction between RIG-I and 14-3-3\textsubscript{ε} was diminished by a K172R mutant RIG-I, indicating that only the ubiquitinated, active form of RIG-I can be translocated to MAVS.

A number of additional cellular factors have been shown to regulate RIG-I by modulating post-translational modifications, either by removing the activating K63-linked ubiquitination or by affecting RIG-I protein stability through K48-linked ubiquitination and proteasome-dependent degradation. As K63-linked ubiquitination of RIG-I is crucial for its signaling activity in response to virus infection, it is not surprising that several DUBs have been identified that counteract this modification. At least three different DUBs have been implicated in the inhibition
of RIG-I signaling through the removal of covalent K63-linked ubiquitin chains.

Cylindromatosis (CYLD) was the first DUB identified that led to RIG-I deubiquitination. In uninfected cells, CYLD was shown to keep RIG-I deubiquitinated, preventing any basal activation levels. This study further showed that, upon viral infection, CYLD was down-regulated, presumably allowing the full ubiquitination and activation of RIG-I. Notably, CYLD’s activity was not specific for RIG-I as TBK1 and IKKe were also targets of CYLD-mediated deubiquitination. Similarly, USP21 has been reported to negatively regulate RIG-I by removing K63-linked ubiquitination. USP21 was shown to interact with RIG-I both in uninfected cells and during VSV and SeV infection. This interaction led to RIG-I deubiquitination and a decrease in type-I IFN induction. More recently, USP3 has been shown to deubiquitinate RIG-I, leading to a decrease in IFN-β induction. Upon virus infection, USP3 interacted with RIG-I, likely acting as a negative feedback regulator. Interestingly, this study also showed a negative regulatory effect of USP3 on MDA5 activity; however, the precise mechanism by which USP3 affects MDA5’s signaling activity remains unclear, given the debatable role of K63-linked polyubiquitin in MDA5 activation.

RIG-I is also subject to proteasomal degradation following K48-linked ubiquitination. The E3 ligase RNF125 binds to and ubiquitinates RIG-I with K48-linked chains, leading to RIG-I degradation and decreased SeV-induced IFN induction, indicating a negative-feedback loop for regulation of RIG-I levels. The same study also showed that RNF125 interacted with and ubiquitinated MDA5 and MAVS, although to a lesser extent. This interaction led to a decrease in MDA5 and MAVS protein levels. This study identified RNF125 as a negative regulator of the RLR pathway by targeting RIG-I, MDA5, and MAVS for degradation.
Additionally, the linear ubiquitin assembly complex (LUBAC), composed of two E3 ligases, HOIL-1L and HOIP, has been shown to negatively regulate RIG-I signaling utilizing two distinct mechanisms\(^82\). First, LUBAC induced TRIM25 ubiquitination with K48-linked chains which led to proteasomal degradation. This ubiquitination was dependent on the RING-IBR-RING (RBR) domains of both HOIL-1L and HOIP. The second mechanism depends on the NZF (Npl4 zinc finger) domain of HOIL-1L which competes with TRIM25 for RIG-I binding, preventing TRIM25-mediated ubiquitination and activation of RIG-I\(^82\). USP15, identified as an interaction partner of TRIM25 by mass spectrometry, was recently shown to counteract the inhibitory effect of LUBAC\(^83\). Mechanistically, USP15 was found to bind to TRIM25 specifically during the later stages of viral infection, removing the LUBAC-induced K48-linked polyubiquitination of TRIM25 at its SPRY domain. This study indicated that USP15 specifically stabilizes the TRIM25 protein levels at later time points during infection, leading to sustained type-I IFN gene expression, facilitating virus clearance.

**Regulation of MDA5**

The regulatory mechanisms governing MDA5 activation remain relatively enigmatic; however, there is substantial evidence to suggest that the regulation of the two RLRs is quite distinct. Numerous studies have suggested that MDA5 does not adopt an auto-repressed conformation like RIG-I\(^9,84\). Rather, it seems that much of MDA5’s regulation comes from the way in which it binds to its RNA ligand. MDA5 monomers have a relatively low affinity for dsRNA, and interestingly, the CTD has an even lower affinity than the full length protein\(^36\). Structural studies have shown that protein-protein interactions between multiple MDA5 molecules are required for high-affinity RNA binding and MAVS activation. The current model is that the MDA5 helicase/CTD cooperatively binds to dsRNA in an ATP-dependent manner by
wrapping along its length forming a filamentous oligomer\textsuperscript{8,9,35,36,85}. The CARD domains are not involved in this filament formation, but instead are separated from the helicase by a flexible linker. At high concentrations, CARD-only constructs have been shown to organize into high molecular weight oligomers which adopt an elongated but heterogeneous conformation\textsuperscript{8}. Only the high molecular weight form of MDA5, not the CARD monomer, is capable of interacting with MAVS to facilitate signaling. In the cell, CARD oligomerization is likely facilitated by the formation of MDA5 helicase filaments along dsRNA, which increases the local concentration of the CARD domains to a level allowing oligomerization and activation of MAVS\textsuperscript{8}. It has been proposed that the innate propensity for MDA5 CARD oligomerization acts as an analogous platform to K63-linked ubiquitin-stabilized RIG-I CARD tetramers for formation of MAVS aggregates into signaling complexes.

One key commonality between the regulation of MDA5 and RIG-I is the inhibitory phosphorylation of specific residues in the CARD domains (Figure 1.4). As mentioned, RIG-I is phosphorylated at both S8 and T170 by PKCα/β\textsuperscript{57,58,66}. Likewise, MDA5 is constitutively phosphorylated at S88, keeping it inactive. Unlike RIG-I, the kinase responsible for phosphorylating MDA5 is unknown. Interestingly, upon binding to viral RNA, MDA5 is dephosphorylated by the phosphatases PP1 α/γ, the same phosphatases that remove RIG-I CARD phosphorylation. MDA5 harbors two PP1-binding motifs, one in the CARD domains and one in the helicase domain, which are both necessary for PP1 binding, leading to dephosphorylation and activation\textsuperscript{66}. S88 phosphorylation may prevent the self-association of CARD oligomers into their signaling complex, explaining the importance of this post-translational modification.
Figure 1.4. Model of MDA5 activation and regulation. (A) In uninfected cells, MDA5 is kept inactive by phosphorylation at residue S88 in CARD1. (B) Upon viral infection, MDA5 binds to dsRNA which recruits PP1α/γ to the CARDs to dephosphorylated S88. Cooperative RNA binding facilitates MDA5 multimerization. RAVER1 is thought to enhance the binding of MDA5 to its RNA ligand to enhance MDA5 signaling. In contrast, Arl5B binds to MDA5 and prevents interaction with RNA. The CTD also undergoes SUMOylation mediated by PIAS2β, which has been shown to enhance MDA5 signaling. (C) K63-linked ubiquitin chains associated with the MDA5 CARDs are thought to facilitate MDA5 multimerization and activation. Activated MDA5 then interacts with its downstream signaling partner, MAVS. (D) DAK is a negative regulator of MDA5 signaling; however, the precise mechanism of MDA5 inhibition by DAK is unknown. (Adapted from Chiang JJ, Davis M.E, Gack MU. 2014)

The roles of additional post-translational modifications for MDA5 activation remain controversial. The E3 ligase PIAS2β modifies the MDA5 CTD with the ubiquitin-like molecule SUMO. SUMOylation leads to increased MDA5 activation; however, the precise mechanism that governs this enhancement is unknown. The same group which identified unanchored ubiquitin binding by the RIG-I CARD domains saw a similar interaction with the MDA5 CARDs. MDA5 bound to K63-linked ubiquitin leading to its oligomerization and ability to activate IRF3 in vitro. However, subsequent studies investigating the oligomerization of the MDA5 CARD domains did not observe MDA5 activation by K63-polyubiquitin, leaving the role of K63-polyubiquitin in MDA5 activation ambiguous.
An additional study has shown that MDA5 is negatively regulated by the E3 ubiquitin ligase, TRIM13\textsuperscript{88}. In functional studies, expression of TRIM13 reduced MDA5-mediated signaling. Additionally, EMCV infection in TRIM13\textsuperscript{-/-} mice induced significantly higher levels of type-I IFN and promoted longer survival than in the wild-type mice\textsuperscript{88}. Although this study did not look specifically at ubiquitination of MDA5, TRIM13 is a functional ubiquitin ligase, so it would not be surprising if the mechanism of MDA5 regulation is through degradation following K48-linked ubiquitination.

Few cellular regulators of MDA5 have been described to-date. Ribonucleoprotein PTB-binding 1 (RAVER1) was identified through a mass spectrometry based screen as a positive regulator of MDA5\textsuperscript{89}. RAVER1 interacts with MDA5 upon viral infection and enhances MDA5 signaling by increasing interaction with poly(I:C). RIG-I was not found to interact with or be enhanced by RAVER1, possibly reflecting the different RNA-binding mechanisms of the RLRs. A yeast two-hybrid screen identified dihydroxyacetone kinase (DAK) as an interaction partner of MDA5 that inhibits MDA5 signaling\textsuperscript{90}. DAK did not inhibit interaction between over-expressed MDA5 and MAVS, suggesting that its effect is on RNA binding by MDA5. Interestingly, the kinase activity of DAK was not required for MDA5 inhibition\textsuperscript{90}, and over-expressed DAK does not phosphorylate MDA5 S88 (unpublished data). Another cellular protein was recently shown to interfere with MDA5-RNA interaction\textsuperscript{10}. ARF-like protein 5B (Arl5B) was shown to specifically inhibit MDA5 regulation in response to poly(I:C). Mechanistic studies showed that Arl5B interacts with the MDA5 CTD, preventing optimal RNA binding.
1.2. *Paramyxoviridae*: The viruses and their replication

RLRs function to protect the host against invading pathogens. Viruses rely on the host cell machinery for many aspects of their replication and must manipulate host proteins for their own functions. The host-pathogen interactions of the *Paramyxoviridae* family have been intensely studied over the years. In particular, the paramyxoviruses have evolved mechanisms to antagonize the IFN response. In order to fully understand paramyxovirus antagonism of RLRs, it is necessary to understand their replication cycle.

The order *Mononegavirales* encompasses five different virus families: *Paramyxoviridae, Rhabdoviridae, Filoviridae, Bornaviridae, and Nyamiviridae*. These viruses share common characteristics including a linear, non-segmented, single-stranded RNA genome of negative sense. The family *Paramyxoviridae* is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, based on morphological differences, genome construction, and differences in type and number of viral gene products. These subfamilies are further divided into genera based on sequence similarity and antigenic cross-reactivity. *Paramyxovirinae* includes the genera *Aquaparamyxovirus, Avulavirus, Ferlavirus, Henipavirus, Morbillivirus, Respirovirus*, and *Rubulavirus*. Many well known viruses with high medical and agricultural significance are classified in this subfamily, including MV, mumps virus (MuV), NDV, rinderpest virus (RPV), and the newly emerging Nipah (NiV) and Hendra viruses (HeV).

*Virus Attachment and Entry*

Paramyxoviruses are enveloped viruses which encode two membrane glycoproteins for infection of host cells: the tetrameric attachment protein hemagglutanin-neuraminidase (HN, sometimes H or G) and the trimeric fusion protein (F). HN binds to the cellular surface either
through a specific cellular receptor (*Morbillivirus* H, *Henipavirus* G) or through sialic acid-containing glycoproteins or glycolipids (*Respirovirus* and *Rubulavirus* HN)\(^{94-103}\).

Following initial cellular attachment, the F protein mediates fusion of the viral and cellular membranes at the neutral pH found at the cell surface. The F protein is synthesized as an inactive intermediate \(F_0\) protein which undergoes cleavage by host proteases to become the active pre-fusion \(F^{93,104,105}\). F is a class I fusion protein having stable pre- and post-fusion conformations. Following engagement of the attachment protein by cellular receptors, the fusion peptide of F inserts into the cellular membrane, inducing conformational changes to form a metastable fusion intermediate. Further conformational changes lead to the fusion of the membranes and the irreversible transition of F to the highly stable post-fusion form\(^{106}\). Late in infection when new particles are being assembled, F is expressed on the cell surface for incorporation into the budding particle. When this occurs, F can mediate cell-cell fusion to induce large syncytia formation, a hallmark of paramyxovirus induced cytopathic effect and potentially a mechanism of viral transfer between cells\(^{107,108}\).

**Gene Transcription**

Paramyxovirus genomes are single-stranded, negative-sense RNA and therefore must encode a viral RNA-dependent RNA polymerase (vRdRP) to complete all aspects of the virus replication cycle, including gene transcription and genome replication. The genome consists of 3’ and 5’ untranslated regions dubbed the leader and trailer, respectively. Between the leader and trailer, the genes encoding the viral proteins are in a conserved order (Figure 1.5). The genome is encapsidated by the nucleocapsid (N) protein, forming the RNA-nucleoprotein (RNP) complex. N covers exactly six nucleotides at a time, leading to the so called, “rule of six” requirement for paramyxovirus genomes to have lengths of an integer multiple of six nucleotides\(^{109}\).
Initially after infection, when N protein levels are limiting, the viral genome serves solely as a template for transcription of mRNAs for new protein synthesis. The vRdRP consists of the large (L), phospho- (P), and N proteins. The L protein contains all of the catalytic RdRP activities, including 5’ capping and 3’ polyadenylation, but requires the P protein for interaction with N in the RNP\textsuperscript{110-112}. Each gene is defined by certain nucleotide sequences to indicate the gene start (GS) and gene end (GE). Between each gene is a short stretch called the intergenic region (IG)\textsuperscript{113-115}. The polymerase generates mRNAs encoding each of the viral proteins by terminating at the GE, reading through the IG, and re-initiating at GS signals. Occasionally, the polymerase fails to re-initiate after termination, which results in a gradient of mRNAs produced from each gene with progressively fewer transcripts proportional to the distance from the 3’ end of the genome\textsuperscript{116-119} (Figure 1.5). Paramyxoviruses also exhibit expanded coding capacity through RNA editing and alternative ORF start sites within the P gene\textsuperscript{120} (discussed in further detail below).
**Genome Replication**

Viral mRNA transcription continues until the N protein is produced in sufficient amounts to encapsidate new viral genomes. At this point, it is presumed that the stoichiometry of the RdRP complex is altered and the polymerase transitions from production of mRNA to production of the positive sense antigenome by reading through all GE sequences\textsuperscript{121}. The antigenome serves only as a template for new genome synthesis and does not have any protein coding capacity. Following antigenome production, the polymerase initiates synthesis of the negative sense genome at the 3’ trailer of the antigenome\textsuperscript{122}. The trailer is immediately encapsidated by N, resulting in the synthesis of the full, encapsidated viral genome. Newly synthesized genomes can then either be incorporated into budding particles or serve as a template for further mRNA transcription or synthesis of new antigenomes.

**Virus Budding/Release**

Like many enveloped viruses, paramyxoviruses bud from the plasma membrane. The assembly of new virions occurs in multiple steps. First, the viral RNP is assembled in the cytosol following genome synthesis and encapsidation by N. P and L then interact with the N-RNA complex to form the complete RNP for packaging into the virion\textsuperscript{123}. The viral glycoproteins, HN and F, are trafficked to lipid rafts in the plasma membrane following synthesis\textsuperscript{124-127}. The matrix protein (M) serves as a scaffold to link the RNP complex with the cytoplasmic tails of the glycoproteins ensuring proper virion composition\textsuperscript{128-130}. The assembled virion buds out of the plasma membrane forming new particles. Some paramyxovirus HN proteins also possess neuraminidase function, acting to destroy potential receptors on the cells from which they bud, preventing re-infection.
**Paramyxovirus Accessory Proteins - The Poly-cistronic P Gene**

In addition to the proteins required for replication and structure, paramyxoviruses also encode accessory proteins for virus-host interaction. The gene encoding the P protein has an expanded coding capacity resulting in the synthesis of up to nine proteins from a single gene. The alternative initiation sites that encode the C proteins from overlapping reading frames were identified first\(^\text{131-137}\). Later, it was discovered that paramyxoviruses also employ pseudo-templated transcription, also called RNA editing\(^\text{120,138-140}\). RNA editing involves the addition of pseudo-templated G nucleotides at a specific site in the nascent mRNA causing frame shifts which result in the synthesis of multiple co-\textit{amino}-terminal proteins from a single gene. The number, type, and organization of gene products vary between paramyxovirus genera and species (Figure 1.6).

![Figure 1.6. Schematic of P/V/C Gene Products.](image)

The number and types of P/V/C gene products of different viruses are listed. The unedited transcript (+0) usually encodes the P protein, with the exception of the Rubulaviruses. The addition of pseudotemplated Gs results in a frame shift producing the V/W proteins. C proteins are expressed from alternate initiation sites. Genus names and representative species are indicated on the left. (Adapted from Audsley, MD and Moseley, GW 2013\(^\text{141}\))
The specific mechanism by which RNA editing occurs is through stuttering of the viral polymerase at a specific slippery sequence (e.g. 3’ UNN UUUUUU CCC). This stuttering causes the polymerase to backtrack and insert the untemplated nucleotide(s). More recent evidence suggests that the location of this slippery sequence within the N-encapsidated hexamer influences the site and frequency of RNA editing\(^ {109,142}\). The number and frequency of nucleotide insertion varies by species. Most paramyxoviruses encode the P protein as the faithful transcript and produce +1G (V protein) or +2G ORFs (D, W, or I proteins). Members of the Rubulavirus genus instead encode V as the unedited transcript with the P ORF in a +1G frame (Figure 1.6). This leads to proportionally higher amounts of V in infected cells and implies a larger dependency on the function of the V protein for these viruses\(^ {143}\). For the Henipaviruses, the frequency of RNA editing fluctuates throughout infection. Early during infection, the unedited P mRNA is transcribed in high amounts with very little V and W mRNA produced; however, later in infection, the V and W transcripts are found in higher amounts\(^ {144}\). The complete mechanism and regulation of RNA editing for paramyxoviruses remains an open field of investigation.

The P protein is the only P/V/C gene product that is essential for viral replication, serving as a link between the catalytic polymerase, L, and N for mRNA transcription and genome replication as discussed. The additional mRNAs from the P gene encode accessory proteins which affect viral pathogenesis and immune antagonism\(^ {145-147}\). The V protein C-terminal domain (CTD) contains a zinc-finger domain which coordinates two zinc atoms\(^ {148,149}\). The seven cysteines and one histidine that make up the zinc-fingers are highly conserved in the CTD of paramyxoviruses\(^ {148-150}\). The CTD and the zinc-fingers are important for the formation of complexes with cellular proteins; however the precise role of zinc binding is unclear\(^ {151,152}\).
Many viral proteins, including paramyxoviruses P/V/C gene products, have evolved diverse mechanisms to inhibit both the induction of IFNs and their signaling through the IFNAR pathway.

1.3. Viral Antagonism of RIG-I and MDA5

Activation of the innate immune response leads to the expression of IFNs, proinflammatory cytokines, and ISGs which lead to clearance of viral infections as well as the development of an adaptive immune response. To combat this, viruses have evolved mechanisms to evade the IFN response. As the initiators of a complex signaling cascade, RLRs present efficient viral targets to prevent innate immune activation. These evasion mechanisms can be grouped into four major categories: A) interference with multisite post-translational modifications of RLRs, B) inhibition of viral PAMP recognition, C) blocking of RLR’s enzymatic ATPase activity, and D) disruption of RLR interaction with MAVS.

1.3.A. Interfering with multisite post-translational modifications

RIG-I and MDA5 are tightly regulated through post-translational modification. One mechanism by which viruses can evade IFN production is preventing RLR activation through modulation of these modifications. Viral evasion mechanisms have been discovered that remove or prevent ubiquitination of RIG-I, either through viral protein interaction with E3 ubiquitin ligases or by encoding active viral DUBs. Interestingly, to date, no mechanisms have been described to affect RLR phosphorylation, a key modification of both RIG-I and MDA5. This is likely to change with further study as many viruses are known to encode kinases or to interfere with cellular kinases and phosphatases for other functions.
**Influenza NS1 interferes with RIG-I ubiquitination by inhibiting both TRIM25 and Riplet.**

The influenza A non-structural protein 1 (NS1) is a well described IFN antagonist. It was first shown to shield viral RNA from host detection; however, NS1 is now known to also antagonize RIG-I by antagonizing both TRIM25 and Riplet in a species-dependent manner (Figure 1.7). Initial studies showed that NS1 interacts with TRIM25 and prevents its association with and ubiquitination of RIG-I. NS1 binds to the coiled-coil domain of TRIM25 via its RBD and ED domains. These domains are important for other functions of NS1, including binding to RNA (RBD) and various host protein interactions (ED) including that with eIF-4G-I to modulate host translation. Interaction with NS1 prevents TRIM25 oligomerization which is necessary for its ubiquitination of RIG-I. A recombinant influenza virus containing a mutant NS1 that cannot interact with TRIM25 did not have an inhibitory effect on RIG-I signaling. The lack of conservation of K172 between human and mouse RIG-I prompted additional studies looking at NS1 antagonism of RIG-I in different species. This study showed that while human TRIM25 bound to NS1 proteins from viruses isolated from various species, Chicken TRIM25 bound preferentially to avian NS1 proteins. Mouse TRIM25 did not bind to any NS1 proteins. Further study showed that NS1 is still able to antagonize mouse RIG-I through inhibition of another E3 ubiquitin ligase, Riplet. These mechanisms of antagonism emphasize the importance of RIG-I activation through ubiquitination by TRIM25 and Riplet for innate immune activation.
**Figure 1.7. Viral inhibition of RIG-I through modulation of its K63-linked ubiquitination.** Schematic of viral proteins that modulate the K63-linked ubiquitination of RIG-I to suppress IFN induction. The NS1 proteins IAV interact with and inhibit TRIM25 and Riplet, blocking K63-linked ubiquitination of RIG-I at K172 in the CARDs and K788 in the CTD. The NS3/4A protease of HCV cleaves Riplet to suppress K63-linked ubiquitination of RIG-I at the CTD. KSHV, nairoviruses, arteriviruses, FMDV, and SARS-CoV encode DUBs that remove K63-linked ubiquitin from RIG-I CARDs and CTD. More details on viral inhibition of RIG-I ubiquitination are described in the text. (Adapted from Chiang JJ, Davis M.E, Gack MU. 2014)

**KSHV orf64 encodes a viral DUB to target RIG-I.**

Many herpesviruses encode viral DUBs that target cellular proteins for removal of ubiquitin moieties. One such viral DUB is encoded by Kaposi sarcoma-associated herpes virus (KSHV) orf64\(^{155}\). Orf64 is a component of the viral tegument and plays a structural role in virion assembly in addition to promoting lytic replication; however, it also has non-structural function based on its N-terminal DUB domain\(^{156,157}\). Inn, *et al*., demonstrated that orf64, but not a catalytically inactive mutant, was able to inhibit the production of type-I IFN. Further study revealed that it did so by reducing RIG-I 2CARD ubiquitination. This reduction was overcome by over-expression of TRIM25, indicating that orf64 specifically removes the ubiquitination essential for RIG-I activation\(^{156}\).
**Viral Papain-like Proteases possess DUB function with innate immune targets.**

Many RNA virus families share the characteristic that one or more of their genome ORFs is translated into a large poly-protein that must be cleaved into individual proteins required for the viral life cycle. This cleavage is facilitated by host and viral proteases including the viral papain-like proteases. In addition to their proteolytic function, multiple unrelated viruses have been shown to encode papain-like proteases which possess a DUB function. These include SARS-CoV papain-like protease (PLP), foot-and-mouth-disease virus (FMDV) leader proteinase (L\(^{pro}\)), and hepatitis E virus (HEV) papain-like cysteine protease (PCP).

Coronaviruses are enveloped, large RNA viruses that cause respiratory illnesses. During infection, the genome is translated into two polyproteins which are then processed by proteases, including PLP, into individual proteins. Structural and functional analysis revealed that the SARS-CoV PLP has a deubiquitinase activity that catalyzes the removal of both ubiquitin and ISG15 from substrates\(^{158-160}\). This function was shown to play a role in IFN antagonism; however, a catalytically dead mutant doesn’t completely lose activity\(^{161,162}\). Cell based deubiquitination assays identified RIG-I, TBK1, IRF3, and STING as targets of deubiquitination by SARS-CoV PLP for down-regulation of the IFN response\(^{163}\).

FMDV is the etiological agent responsible for a debilitating disease of cloven-hoofed animals which has serious economic impacts\(^{164}\). The FMDV L\(^{pro}\) is involved in shut off of host translation through cleavage of eIF-4G and has multiple roles in antagonism of IFN induction. Independent of its protease function, L\(^{pro}\) leads to decreases in IRF3, IRF7, and p65/RelA activation\(^{165-168}\). Like PLP, L\(^{pro}\) is a functional DUB that acts on several proteins involved in the IFN induction pathway, namely RIG-I, TBK1, TRAF3, and TRAF6. The deubiquitination of each of these proteins prevents activation and signaling to produce IFNs\(^{169}\).
The HEV ORF1 is translated into a poly-protein and then cleaved into individual proteins, including the PCP protease. This protease also has a DUB activity for ubiquitin, SUMO, and ISG15 removal. HEV PCP was shown to specifically deubiquitinate both RIG-I and TBK1, leading to inhibition of IFN induction in response to poly(I:C) stimulation. Together, these studies show that multiple viruses have evolved similar papain-like proteases with DUB function to broadly antagonize IFN induction through the deubiquitination of multiple targets, including RIG-I.

**Viral OTU-type DUBs target RIG-I for deubiquitination.**

Another class of viral DUBs is also encoded by two unrelated RNA viruses, the arteriviruses and the nairoviruses. The *Arterivirus* family are positive-sense, single-stranded RNA viruses that cause severe infections in livestock, including porcine respiratory and reproductive syndrome virus (PRRSV). *Nairoviruses* have tripartite, negative-stranded RNA genomes and include Crimean-Congo hemorrhagic fever virus (CCHFV), a tick-borne virus that causes sporadic hemorrhagic fever throughout the Middle East, Africa, Asia, and Southeastern Europe. Unlike viral PLPs which have a USP-like DUB structure, bioinformatics approaches revealed the existence of ovarian tumor (OTU) domain type DUBs in both the L protein of CCHFV and non-structural protein 2 (nsp2) of EAV and PRRSV. Over-expression of these viral DUBs, termed viral OTU proteins (vOTU), showed dual specificity for deconjugation of poly-ubiquitin chains and ISG15, leading to inhibition of both IFNβ and TNFα induction. Structural studies later determined that the dual-specificity of these vOTUs for ubiquitin and ISG15 derives from the angle of binding to the ubiquitin-like moiety. This orientation is rotated approximately 75 degrees in comparison to eukaryotic OTUs accommodating both ubiquitin and the larger ISG15. RIG-I was identified as one substrate for de-ubiquitination by vOTUs from
both arteriviruses and nairoviruses: wild-type vOTUs, but not catalytically dead mutants, inhibited RIG-I ubiquitination and downstream signaling\textsuperscript{176}.

1.3.B. Inhibition of RNA recognition and binding

Perhaps the most direct way to interfere with RLR-induced IFN production is to prevent initial recognition of viral PAMPs. There are three key mechanisms by which viruses protect their RNA from detection by RLRs: RNA sequestration, RNA processing, and sequestration of replication to membrane bound compartments.

\textit{Sequestration of viral RNAs prevents recognition by RLRs.}

Most RNA viruses encode RNA binding proteins (RBP) which ensure proper genome replication and delivery to newly formed virions. Some RBPs have the added function of preventing RNA recognition by RLRs. The nucleocapsid (N) protein of SARS-CoV has dsRNA binding properties and is the main component of the helical nucleocapsid present in the virion\textsuperscript{177-179}. In addition to being the major virion structural protein, the N protein has been shown to inhibit the IFN response induced by RLR-RNA ligands, but not by over-expression of the RLRs\textsuperscript{180,181}. This indicates an IFN antagonism mechanism linked to the RNA binding properties of N. This is further supported from structural studies of the HCoV-OC43 N protein in which an R106A mutant in the RBD led to decreased virus propagation due, at least in part, to an increased IFN response\textsuperscript{182}.

Vaccinia virus, a DNA virus, also encodes a dsRNA binding protein that is important for immune evasion\textsuperscript{183,184}. The E3L protein has an N-terminal Z-DNA-binding domain and a C-terminal dsRNA binding domain (RBD), both of which are important for antagonizing IRF3 activation and IFN\(\beta\) production\textsuperscript{185,186}. In fact, mutational studies showed that the dsRNA binding function is critical for immune evasion\textsuperscript{183}. This is particularly interesting for a DNA virus such as
vaccinia; however, there is evidence that dsRNA replication intermediates have the potential to activate RLR signaling\textsuperscript{187,188}.

Two of the best studied RBPs that inhibit IFN production are influenza A NS1 and filovirus VP35\textsuperscript{189-194}. Interestingly, these proteins have similar RBDs that bind to blunt ended 5’ppp dsRNA\textsuperscript{195-203}. Crystal structures of NS1 from IAV H5NI in complex with dsRNA reveal that binding of NS1 to the RNA forms a tube with a tunnel in the center to accommodate the RNA\textsuperscript{204}. Four crystal structures of the VP35-RNA interaction reveal virus species-specific differences in RNA binding. While the Ebola virus VP35 of Zaire and Reston both bind to short, blunt dsRNA, Marburg (MARV) VP35 shows preferential binding to longer dsRNA\textsuperscript{200,202,205,206}. Ebola VP35 monomers also form an “end-cap” on RNA ends whereas MARV VP35 primarily coats the RNA backbone\textsuperscript{207}. This binding difference is reflected in the differential abilities of the two virus species to inhibit RLRs: MARV VP35 can only inhibit RLR recognition of the backbone of dsRNA whereas Ebola VP35 can inhibit recognition of both the backbone and ends of dsRNAs\textsuperscript{206}.

\textit{RNA Processing removes PAMP signatures from viral RNAs.}

\textit{Arenaviruses} are classified into New World viruses and Old World Viruses, many of which are capable of causing hemorrhagic fever syndromes. The lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP), along with having various roles in viral replication and assembly, was the first identified IFN antagonist for arenaviruses\textsuperscript{208}. Later, the NPs of seven different Old World and New World arenaviruses were shown to inhibit type-I IFN induction\textsuperscript{209}. Several studies identified the C-terminal portion of NP as being critical for its IFN antagonistic function\textsuperscript{210,211,212,213}. Interestingly, in the related Old World arenavirus, Lassa virus (LASV), this region encodes a functional 3’-5’ exonuclease activity specific for dsRNA. Mutants of LASV
which lose this exonuclease activity also lose their inhibitory effect on IRF3 activation\textsuperscript{214}. Using knockout mice, Zhou et al. showed that NP antagonism of IFN is dependent on downstream signaling of MAVS. They further showed that NP binds to RIG-I and MDA5; however, mutation of key residues (D382A,G385A) abrogates IFN antagonism without altering this interaction\textsuperscript{215}. It has been proposed that the main mechanism by which the arenavirus NP evades the IFN response is to degrade its own dsRNA to prevent detection by PRRs. LCMV NP has also been shown to bind to IKK\(\varepsilon\) and thereby prevent IRF3 activation and virus induced NF-\(\kappa\)B activation\textsuperscript{212,213}. These activities are lost upon mutation of key residues of the putative exonuclease domain; however, the exact mechanism behind these downstream antagonistic functions is not known.

Members of the bunyavirus family, including Hantavirus, use a unique method of RNA priming which results in the removal of the 5’ppp PAMP\textsuperscript{42,216}. During RNA synthesis, the RdRP initiates at the first G residue in the anti-genome. After the nascent RNA is extended to a di-nucleotide, the template is realigned, resulting in a single 5’ppp G nucleotide overhang. Further copying from this genome results in the removal of this 5’ppp, ultimately resulting in blunt ended RNA which is not a strong inducer of IFN\textsuperscript{217}.

\textit{Sequestration of viral replication to discrete membrane compartments excludes RLRs.}

Some viruses conceal their PAMPs by directing replication and virion assembly to discrete membrane-bound structures in the cytoplasm. The SARS-CoV nsp4 protein acts as a membrane anchor to direct the assembly of double-membrane vesicles (DMVs) which exclude RLRs from the location of RNA synthesis\textsuperscript{218-221}. These structures have also been found in cells infected with the newly emerging MERS-CoV, indicating conservation of this evasion
mechanism among coronaviruses\textsuperscript{222}. Similar membranous structures are found in DenV and WNV infected cells. During infection, all DenV non-structural proteins, as well as dsRNA, can be found in these “convoluted membranes” (CM)\textsuperscript{223}. CM appear to be derived from the rough ER, localizing ribosomes near RNA synthesis for translation and providing a protected site for DenV replication and virion assembly\textsuperscript{223,224}.

1.3.C. Blocking ATP hydrolysis

The interferon evasion abilities of paramyxoviruses are dependent on one or more gene product from the P/V/C gene including the V, W, and C proteins. In particular, the V proteins of 13 different paramyxoviruses have been shown to inhibit MDA5 by binding to the helicase domain and inhibiting hydrolysis of ATP (Figure 1.8)\textsuperscript{4,225-228}. Recently, crystal structure analysis of porcine MDA5 and the parainfluenza virus 5 (PIV5) V protein indicated that inhibition of the ATPase activity is due to mutual structural unfolding\textsuperscript{229}. The antagonism of both IFN induction and signaling by paramyxovirus P/V/C gene products is discussed in greater detail below.

![Figure 1.8. Suppression of MDA5 activity through paramyxovirus ATPase activity. The V proteins of several paramyxoviruses, including PIV5, bind directly to the helicase domain of MDA5. This interaction induces conformational changes and suppresses the ATP hydrolysis activity of MDA5 and thereby its filament formation on viral dsRNA. (Adapted from Chiang JJ, Davis M.E, Gack MU. 2014)](https://example.com/fig18)

In addition to its function in shielding viral RNA from detection by RLRs, it has recently been described that Ebola VP35 has evolved a second mechanism to antagonize RIG-I signaling. PACT (for PKR activator) directly binds to RIG-I and acts as an activator of ATPase activity and
VP35 binds to PACT and prevents its association with RIG-I, thereby hindering RIG-I ATPase activity and subsequent IFN induction.

1.3.D. Interfering with RIG-I/MDA5-MAVS binding

The first step in the signaling cascade after RLR activation is CARD-CARD interaction between RIG-I/MDA5 and MAVS. This provides a central point for inhibition to block signaling mediated by both RLRs. Many viruses have evolved mechanisms to target this interaction through direct binding or degradation of one or more binding partners (Table 1.1).

Table 1.1. Viral inhibition of RIG-I and MDA5. Different viral antagonistic strategies are detailed, including information on the virus and the specific protein responsible for RLR antagonism. (Adapted from Chiang JJ, Davis M.E, Gack MU. 2014)

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Inhibition of RIG-I/MDA5 interaction with MAVS.

In addition to the deubiquitination of RIG-I by PLP and RNA encapsidation by N, SARS-CoV has evolved additional mechanisms to inhibit RLR-mediated IFN induction. The M protein
blocks RIG-I, MDA5, and MAVS induced IFNβ promoter activity by interacting with RIG-I, TBK1, IKKe, and TRAF3\textsuperscript{233}. The M protein is localized to membranes associated with the Golgi complex, so it is tempting to think that binding to RIG-I results in sequestration to these compartments, away from MAVS on the mitochondria. Two additional SARS-CoV proteins, ORF3b and ORF6, have also been shown to block the interaction between RIG-I and MAVS. Both proteins localize to the mitochondria where they likely bind to RIG-I or MAVS to inhibit IFN induction\textsuperscript{181,234}.

The Z protein of Arenaviruses has been implicated in many virus-host interactions including interacting with ribosomal protein P0, eIF4E, PML, and the proline-rich homeodomain protein\textsuperscript{235-237}. \textbf{Initially}, the Z protein of the New World arenaviruses Guanarito, Junin, Machupo, and Sabia, but not those of Old World LCMV or LASV, had been shown to have IFN antagonism activity\textsuperscript{238}. \textbf{A more recent study}, however, suggests that the difference in activity is reflected better in the pathogenicity of the virus rather than its origin\textsuperscript{239}. This study showed that the Z protein from all pathogenic arenaviruses, including LASV, inhibited IFN induction. Both studies showed that this inhibition is due to the direct interaction between Z and the CARD domains of both RIG-I and MDA5\textsuperscript{238,239}. This interaction prevents downstream CARD-CARD interactions between the RLRs and MAVS, thereby preventing the signaling cascade that results in IFN production.

Human metapneumovirus (hMPV) is a leading cause of respiratory infections, particularly in infants, the elderly, and immunocompromised individuals. Recombinant hMPV lacking the glycoprotein (G) attachment factor is attenuated \textit{in vivo}; however, only recently this attenuation has been attributed to loss of immune evasion mechanisms by the G protein. These studies showed that rhMPV-ΔG induced higher levels of IFN than did the intact rhMPV. This
was found to be due to an interaction between G and the RIG-I CARD domains which prevents RIG-I localization to the mitochondria and downstream signaling\textsuperscript{240,241}.

Like hMPV, respiratory syncytial virus (RSV) is a significant cause of debilitating respiratory infections, particularly in infants. The nonstructural proteins NS1 and NS2 of RSV are known to be antagonists of IFN induction, leading to inhibition or degradation of many proteins in the signaling cascade\textsuperscript{242-249}. Recent work has identified a large protein-organelle complex comprised of mitochondria, RSV NS1/NS2, and proteasome specific proteins, dubbed the NS degradasome (NSD). The mitochondrial association of the NSD is dependent on MAVS. When assembled, the NSD leads to the degradation of many IFN induction molecules including RIG-I, TRAF3, IKKε, IRF3, IRF7, and STAT2. Degradation of these innate signaling targets is due to the presence of both the proteasome and non-proteasomal proteases in the NSD\textsuperscript{250}.

**Cleavage of RLRs.**

Poliovirus induces cleavage of MDA5, but this effect is not due to either of its viral proteases, as recombinant viruses expressing catalytically dead proteases still induced MDA5 cleavage. Instead, degradation of MDA5 during poliovirus infection was dependent on both the proteasome and caspases. How poliovirus induces MDA5 cleavage by cellular factors is not known; however, mouse MDA5 is known to be cleaved during apoptosis\textsuperscript{251}. It is likely that poliovirus infection induces apoptosis which leads to caspase-cleavage of MDA5, resulting in decreased levels of IFN production\textsuperscript{252}.

Picornaviruses and flaviviruses are two families of viruses whose positive-sense RNA genome encodes a long poly-protein which undergoes post-translational processing into individual viral proteins. This processing is completed by both host and viral proteases. The viral proteases also have roles in antagonizing the host through cleavage of host proteins. Several
viruses have targeted these proteases to cleave MAVS. The NS3/4A proteases of hepatitis C virus (HCV) and the related flavivirus GB virus C (GBV-C) both directly cleave MAVS at C508, separating the CARD-containing cytoplasmic domain from the mitochondria, preventing downstream signaling\textsuperscript{253-255}. The unrelated picornavirus, hepatitis A virus (HAV) also encodes a protease which cleaves MAVS to down-regulate IFN production\textsuperscript{256,257}. However, unlike HCV where cleavage is via the mature protease, the mature HAV protease, 3C\textsuperscript{pro}, is unable to cleave MAVS. Instead the stable, catalytically active intermediate, 3ABC facilitates this cleavage due to the mitochondrial localization of a transmembrane domain in the 3A portion of the protein\textsuperscript{258}. It is interesting that these two disparate viruses have evolved such similar mechanisms to inhibit RLR signaling.

1.4. Paramyxovirus P/V/C Gene Products and IFN antagonism

The paramyxoviruses have also evolved diverse mechanisms for antagonizing the innate immune system. The majority of these mechanisms arise from functions of one or more of the P/V/C gene products. Here we detail what is currently known about the IFN antagonistic functions of the proteins encoded by the P/V/C gene.

1.4.A. IFN Signaling Antagonism

Secreted type-I IFNs bind to the extracellular IFN-\(\alpha/\beta\) receptor (IFNAR) to begin the IFN signaling cascade. The Janus family tyrosine kinases, JAK1 and TYK2 are associated with the cytoplasmic tail of IFNAR to facilitate signaling. Upon activation, JAK1 and TYK2 phosphorylate IFNAR tyrosines which creates a binding site for the transcription factors signal transducer and activator of transcription 1 (STAT1) and STAT2. STAT1 and STAT2 are also phosphorylated (STAT1 Y690, STAT2 Y701), leading to the formation of a heterodimeric
complex. STAT1/2 then form the IFN stimulatory gene factor 3 complex (ISGF3) through interactions with IRF9. ISGF3 is then fully activated to translocate to the nucleus where it promotes the expression of ISGs by activating promoters containing IFN stimulatory response elements (ISRE). Paramyxovirus proteins, including the P, V, C, and W proteins, antagonize IFN signal transduction to prevent the establishment of an antiviral state.

The PIV5 V protein was the first paramyxovirus protein identified as an IFN antagonist during infection. Expression of PIV5-V alone led to the rapid proteasomal degradation of STAT1. Further studies showed that this targeting of STAT proteins for degradation is a common feature of the Rubulaviruses; however, the precise mechanism varies depending on virus. Extensive research has shown both functionally and structurally that PIV5-V induces STAT1 degradation by recruiting an E3 ubiquitin ligase complex composed of DNA-damage response binding protein 1 (DDB1) and Cullin4A (Cul4A). This complex ubiquitinates STAT1 leading to its degradation. Interestingly, PIV5-V requires the expression of STAT2 for degradation of STAT1. These studies identified a single amino acid which is necessary for STAT2 binding by PIV5-V: N100 in the N-terminal sequence shared with the P protein. The interaction with DDB1, however, is mediated by the V protein CTD, meaning that only the V, and not the P protein, can target STAT1 for degradation. Subsequent studies revealed an intricate complex in which STAT2 serves as an adaptor to link PIV5-V to STAT1, while PIV5-V in turns acts as an adaptor to link DDB1 to STAT1. The related Rubulaviruses mumps (MuV) and parainfluenza virus 2 (PIV2) also target STATs for proteasomal degradation with differing specificity: PIV2-V targets STAT2, whereas MuV-V targets both STAT1 and STAT3. MuV-V has an additional mechanism to antagonize STAT signaling before DDB1-
mediated degradation occurs. The V protein is also able to prevent STAT activation by inhibiting their phosphorylation (STAT1 Y701 and STAT2 Y689) following IFN-β stimulation\textsuperscript{283,284}.

While most Rubulaviruses share the ability to degrade STAT proteins, there are notable exceptions. hPIV4 is completely incapable of antagonizing STAT signaling and none of the STAT binding sites are conserved in the hPIV4 V protein\textsuperscript{285}. The V protein of the recently identified Mapuera virus (MPRV), classified as a Rubulavirus due to structural and genetic similarities, does not target STAT proteins for degradation, but instead blocks both STAT1 and STAT2 action through protein-protein interactions, preventing their nuclear translocation\textsuperscript{286,287}.

The Avulavirus, NDV also inhibits STAT signaling through degradation of STAT1. Expression of the CTD of NDV-V was sufficient to reduce STAT1 protein levels in infected cells\textsuperscript{288,289}. The precise mechanism and cellular co-factors involved in degradation of STAT1 by NDV-V remains to be determined.

The Morbilliviruses, of which MV is the prototype, employ a different, non-degradative mechanism to antagonize the IFN signaling pathway. The MV P and V proteins interact with STAT1 dependent on a specific tyrosine, Y110, in their shared N-terminal region\textsuperscript{290,291}. The V protein has the additional ability to interact with and inhibit STAT2 through its unique CTD, specifically residues W240 and W250\textsuperscript{292-294}. These interactions inhibit IFN signaling by preventing STAT1/2 nuclear translocation. It has also been reported that MV-V can inhibit STAT1/2 phosphorylation by interacting with and inhibiting JAK1\textsuperscript{295,296}. The V protein binds to JAK1 via its N-terminal domain, but independent of STAT1 interaction as a Y110H mutant had no effect on JAK1 binding. Similar effects on STAT1/2 localization are caused by the V proteins of the related Morbilliviruses Rinderpest virus (RPV) and canine distemper virus (CDV)\textsuperscript{297,298}. 
The *Henipaviruses* also prevent STAT activation without inducing their proteasomal degradation. The P, V, and W proteins of both Hendra and Nipah virus inhibit STAT1 activation through interactions mediated by a single residue (G125) in their common N-terminal domain\(^{299-301}\). The effect of this interaction varies depending on which viral protein is bound to STAT1, primarily due to the subcellular localization of the viral protein. The P and V proteins adopt a cytosolic localization, whereas the W protein possesses a strong nuclear localization signal. The V protein antagonizes STAT signaling by sequestering STAT1/2 in high molecular weight complexes in the cytoplasm and preventing their phosphorylation\(^{302,303}\). In contrast, the W protein prevents nuclear STAT1/2 activity, presumably preventing binding to and activation of ISRE promoters\(^{300,304,305}\).

*Respiroviruses* are unique in that they encode multiple C proteins. hPIV1 encodes four C proteins and no V or W proteins\(^{306,307}\). SeV also encodes four C proteins in addition to its V protein. The hPIV1 C’ protein binds STAT1 and sequesters it in perinuclear complexes, preventing nuclear localization and full activation\(^{308}\). Likewise, the SeV C proteins (C, C’, Y1, and Y2) prevent STAT1 activation through direct interaction\(^{309,310}\). The precise mechanism of C protein antagonism of STAT signaling is unclear due to multiple and conflicting studies using different SeV strains and cell lines. Some suggest that the C protein prevents phosphorylation of STAT1 and STAT2,\(^{311,312}\) whereas others indicate C-mediated ubiquitination and degradation of STAT1\(^{310,313-315}\).

### 1.4.B. IFN Induction Antagonism

Aside from their antagonism of IFN signaling, the paramyxoviruses have also evolved mechanisms to inhibit the induction of IFN, also primarily through P/V/C gene products. These
antagonistic mechanisms act at varying stages in the IFN induction pathway, often with multiple targets to effectively abrogate production of IFN.

Several P/V/C gene products from different viruses, including PIV5-V\textsuperscript{316,317}, MV-V\textsuperscript{318,319}, and SeV-C\textsuperscript{320}, have been shown to have a regulatory role in polymerase function. It is thought that the V protein binds to the polymerase complex in place of the P protein, preventing efficient RNA synthesis. This inhibition of RNA synthesis prevents the over-production of viral RNA PAMPs, thereby preventing innate immune responses due to RLR and PKR detection.

The best-studied cellular target of paramyxovirus innate immune evasion is the RNA sensor MDA5\textsuperscript{4,225-227}. In fact, MDA5 was first identified in a screen to identify binding partners for PIV5-V\textsuperscript{4}. Subsequently, the V proteins from 13 different paramyxoviruses (PIV5, hPIV2, MuV, MenV, MPRV, MV, NiV, HeV, SeV, SalV, NDV, TioV, PorV) were shown to interact with and inhibit MDA5\textsuperscript{225}. The conserved cysteines, which make up the zinc coordinating loops of the V CTD, are critical for interaction with MDA5; however, the importance of individual cysteines varies depending on virus\textsuperscript{151}. In PIV5-V and NiV-V, the smaller zinc finger loop is sufficient, but for MV-V and MuV-V both loops are necessary for interaction.

The V protein interaction was mapped to the ligand-free helicase domain of MDA5 leading to speculation that the V protein blocks dsRNA detection by MDA5\textsuperscript{225,227}. Further studies showed for PIV5-V that individual MDA5 molecules may still be able to bind to dsRNA ligands but that cooperative binding to the dsRNA by multiple MDA5 monomers is inhibited. This finding was strengthened by structural studies showing that PIV5-V binding to MDA5 induces mutual unfolding of the proteins and inhibits the ATPase activity critical for cooperative RNA binding\textsuperscript{229}. Whether other paramyxovirus V proteins also induce this unfolding of MDA5 or if additional mechanisms are responsible for MDA5 inhibition remains to be determined.
While the V proteins bind to MDA5, they are unable to interact with RIG-I\textsuperscript{225,227}. However, V protein inhibition of RIG-I-mediated signaling may occur through an indirect mechanism\textsuperscript{321}. The PIV5 V protein interacts with the signaling-incompetent LGP2. Reports on the role of LGP2 in innate immune signaling have been ambiguous; therefore, it remains unclear exactly how LGP2 inhibits RIG-I signaling in complex with PIV5-V. Multiple studies have mapped the LGP2-PIV5-V binding site to a region highly conserved between MDA5 and LGP2, but not RIG-I, explaining why the V protein does not directly bind RIG-I\textsuperscript{228,229}.

In addition to targeting RNA sensors, some paramyxoviruses have evolved mechanisms to inhibit IFN induction farther downstream in the signaling cascade, providing the additional inhibition of TLR-mediated innate immune signaling. The V proteins of the *Rubulaviruses* PIV5, PIV2, and MuV prevent IRF3 phosphorylation and activation by acting as decoy substrates for phosphorylation by the kinases TBK1 and IKK\textsubscript{ε}\textsuperscript{322}. Similarly, MV-V acts as a decoy substrate for IKK\textalpha phosphorylation, inhibiting TLR7/9 signaling in dendritic cells\textsuperscript{323}. In both of these cases, the V proteins usurp the kinase activity of TBK1/IKK\textalpha or IKK\textsubscript{ε} for their own use, preventing IRF3 or IRF7 activation and induction of IFN. PIV2-V can also inhibit TLR7/9 signaling by binding to TRAF6 via the conserved V CTD to prevent the activation of IRF7 by TRAF6 mediated ubiquitination\textsuperscript{324}. The ability of paramyxovirus V proteins to antagonize IRF7 seems to be dependent on the presence of 2-3 conserved tryptophan residues\textsuperscript{325}.

Other viruses have evolved additional mechanisms to block downstream signaling as well. The V proteins of NDV and SeV directly interact with IRF3 in the cytoplasm to prevent its activation and nuclear import\textsuperscript{326}. *Henipaviruses*, utilizing the W proteins, are also able to block IRF3 activation\textsuperscript{304}. Nuclear localization of the W protein is required to inhibit IRF3 activation, possibly by preventing its phosphorylation or binding to ISRE promoters\textsuperscript{304}. The MV C protein
is another example of a nuclear-localized protein which inhibits IFN induction, although it does not directly target IRF3\textsuperscript{327}. The mechanism of MV-C inhibition of IFN induction is unknown.

Although the paramyxoviruses represent a large family of related viruses, individual viruses have evolved distinct characteristics, including mechanisms of innate immune antagonism. Our work began with a primary focus on MV and further characterizing the ability of MV-V to antagonize MDA5 by alternative mechanisms.

1.5. Measles Virus

Measles virus is one of the most contagious viruses that infect the human population. Clinical signs and symptoms begin approximately ten days following infection and include fever, cough, coryza, and conjunctivitis before onset of the characteristic rash\textsuperscript{328}. The macropapular rash begins on the face and spreads in a radial fashion to the extremities. The rash lasts 3-5 days and corresponds to the mounting of an immune response to viral infection. Once the rash is cleared, infectious virus is no longer present in the infected individual; however, viral RNA can be detected for many weeks. A small subset of measles infections lead to serious and often fatal neurological complications such as subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE)\textsuperscript{329}.

Prior to concerted vaccination efforts, measles was one of the most devastating human diseases, causing millions of deaths worldwide\textsuperscript{328}. Measles virus has only one serotype, no animal reservoir, and a highly effective vaccine, so it has been identified as an ideal target for eradication. The vaccination efforts that began in the 1960s have greatly decreased the number of measles cases and deaths in the past decades; however, recent declines in vaccination have led to the re-emergence of measles as a major public health concern. Measles also continues to be a
concern in developing countries. In particular, numbers of cases and outbreaks are projected to rise dramatically due to degradation of healthcare infrastructure in the wake of the 2014-2015 Ebola pandemic in West Africa.

1.5.A. Measles Virus Infection Cycle

It is well established that measles is spread via the respiratory route through aerosol droplets. Early models suggested that measles virus first infects the airway epithelium and then spreads into lymph nodes where monocytic cells are infected, leading to systemic spread of the infection. More recent evidence, including more complete characterization of cellular receptors and respiratory infection studies in macaques have revealed that infection likely occurs in the reverse manner, with sub-epithelial lymphocyte infection followed by infection of the airway epithelium from the basal surface.

At least three different cellular receptors have been identified to facilitate MV entry into cells. CD46 was the first receptor discovered for MV. CD46 is a ubiquitous membrane protein, expressed on all nucleated cells, and normally functions as a complement regulatory factor. There are four isoforms of CD46 which are expressed on different subsets of cells, but all isoforms share an N-terminal, extracellular domain consisting of 4 complement control protein repeats (CCP). The MV H protein interacts with CCP1 and CCP2 of each CD46 isoform for cellular attachment. While CD46 serves as an efficient receptor for vaccine and lab adapted strains of MV, it does not facilitate infection for wild type strains.

Additional studies identified CD150 (also SLAM, signaling lymphocyte activation molecule) as the primary receptor for infection by wild-type strains of MV. In contrast to CD46, CD150 is primarily expressed on lymphocytes and antigen presenting cells (APC) such
as dendritic cells (DCs) and macrophages. CD150 is also the receptor for the related Morbilliviruses CDV and RPV\textsuperscript{341}. CD150 consists of two glycosylated, immunoglobulin-like extracellular domains, the V and C2 domains. MV-H interacts with the V domain of CD150\textsuperscript{337}. Vaccine strains of MV can use either CD46 or CD150 as receptors, but seem to have a higher affinity for CD150\textsuperscript{342}. A recombinant MV (rMV) unable to use CD150 as a receptor was severely attenuated in the macaque model, underscoring the importance of this receptor \textit{in vivo}\textsuperscript{343}.

The preference of MV for CD150 receptor-usage does not explain the infection of airway epithelial cells. A third MV receptor has been identified which explains this discrepancy as well as other observations that were at odds with the traditional model for MV infection\textsuperscript{344-348}. Nectin-4, an adherens junction protein, was described by two independent groups to facilitate MV infection of epithelial cells\textsuperscript{349,350}. Nectin-4 is expressed solely on the basolateral surface of epithelial cells. It had been shown previously that airway epithelial cells cannot be infected from the apical surface which would be necessary if these cells were the initial target of MV infection. The binding site on MV-H which interacts with Nectin-4 partially overlaps with the sites for CD46 and CD150 interaction indicating that a single region of the glycoprotein is responsible for attaching to all three receptors\textsuperscript{351}.

The identification of CD150 and Nectin-4 as cellular receptors along with mechanistic studies of transmission has led to a revision in the model for MV infection \textit{in vivo}. Several animal models have been established, including transgenic mice\textsuperscript{352,353}, cotton rats\textsuperscript{354,355}, and ferrets\textsuperscript{356}; however, the macaque model (both rhesus and cynomologous) most closely replicates the disease progression seen in infected humans\textsuperscript{357-359}. Recent studies have looked specifically at the initial target cells of MV infection in the macaque model using an rMV engineered to express
EGFP as a marker. Rhesus macaques were infected via the aerosol route to closely recapitulate natural infection in humans. In these animals, the first cells to become EGFP positive were mononuclear cells in the alveoli followed by amplification in the bronchus-associated lymphoid tissue (BALT) and lymph nodes. Importantly, infection of respiratory tract epithelial cells was not observed until after systemic viremia. Studies in transgenic mice also showed evidence of early infection of lymphocytes.

Together, these studies suggest an alternative model for MV infection in the respiratory tract where sub-epithelial immune cells are the primary targets for infection via the CD150 receptor. Following amplification in the lymph nodes and wide-spread viremia, MV infects the airway epithelium through Nectin-4. MV then replicates to high titers in the epithelium to facilitate transmission via respiratory droplets.

1.5.B. Reverse Genetics System

The generation of recombinant negative-stranded RNA viruses lagged behind similar technology for positive-stranded RNA viruses. This is due to the inherent biology of the viruses: positive-stranded RNA genomes can be directly used by the cellular machinery as a template for transcription and translation. On the other hand, the cellular machinery has no way of utilizing negative-sense genomes. This means that positive-strand virus genomes or cDNA are infectious when transfected into cells, whereas negative-stranded viruses require encapsidation of the genome with the RNP consisting of the polymerase components.

Initial attempts to reconstitute negative-stranded viruses through reverse genetics systems involved assembling the RNP in vitro and introducing it into cells. This approach had some success for influenza; however, it was not feasible for members of Mononegavirales due to the
RNP structure. The ability to overcome this hurdle was first shown through the use of the hepatitis delta ribozyme to generate the proper genome length\(^ {366} \). Following this, Schnell et al., were the first to develop a full rescue system for a negative-stranded virus - Rabies\(^ {367} \). This system involved the introduction of antigenomic cDNA and plasmids encoding the vRdRP components under a T7 promoter and infection with a T7-polymerase-encoding vaccinia virus\(^ {367} \). Their approach differed from those previously attempted by influenza virologists because the viral nucleic acid was introduced to the cells in the form of the positive-sense antigenome. This relatively simple change alleviated a major antisense problem in virus recovery from transfection systems. When negative-sense genomic RNA is introduced into cells, the mRNAs produced by the transfected vRdRP can hybridize with the naked genomic RNA preventing further transcription and translation. By using the antigenome, new genomic RNA is first replicated and co-transcriptionally encapsidated, preventing hybridization with mRNA and drastically increasing viral yields from the *Rhabdovirus* recovery system.

This approach served as the basis for the development of reverse genetics systems for other negative-stranded viruses in the following years\(^ {368,369} \). The first recovery of measles virus occurred in 1995\(^ {370} \). This modified approach utilized HEK293 cells induced to stably express the T7 RNA polymerase and plasmids encoding MV N, P, and L under a T7 promoter, removing the need to infect with a helper virus carrying the T7 activity, and thereby eliminating the purification of progeny MV from MVA-T7 after recovery\(^ {370} \). The system was further improved by the addition of the ribozyme sequence from hepatitis delta virus to the antigenomic cDNA plasmid, leading to processing of the genome to the proper length in agreement with the paramyxovirus “rule of six”\(^ {371} \). This approach resulted in successful recovery of full-length measles viruses.
In recent years, the MV reverse genetics system has been further modified allowing the rescue of wild-type MV in cells expressing CD150 naturally or through stable transfection (Vero-hCD150)\textsuperscript{360,372-375}. Rather than using stable expression of the vRdRP components and T7 polymerase, the vRdRP plasmids can be transiently transfected following infection with a replication-deficient fowl pox expressing the T7 polymerase. Use of the replication-deficient fowl pox efficiently provides the T7 polymerase, but eliminates the virion purification step following recovery\textsuperscript{372}. The ability to make recombinant negative-stranded viruses has led to the discovery of many determinants of viral pathogenesis for these viruses as well as the introduction of reporter proteins, such as EGFP, to more efficiently track and titer the viruses\textsuperscript{360,374,375}.

The reverse genetics system for negative-strand RNA viruses has been used to study the roles of individual viral proteins during infection. This has been particularly useful in identifying the roles of the V proteins of paramyxoviruses. V-deleted viruses have shown the importance of the V protein as a pathogenicity factor for SeV\textsuperscript{376}, MuV\textsuperscript{377}, NDV\textsuperscript{288}, and MV\textsuperscript{49} and others. Prior to our work, no recombinant MV had been constructed to investigate the effects of a specific mutation in the V protein on MDA5 antagonism.

1.6 Summary

RLRs, including MDA5, are key sensors of viral infection. Through an intricate system of regulation including conformational changes and post-translational modification, RLRs activate the IFN response to induce an antiviral state in the infected organism. In order to combat recognition, viruses have evolved multiple mechanisms to antagonize the activation of RIG-I and MDA5. The V proteins of the paramyxovirus family have been described as antagonists of MDA5 activation; however, the characterization of the precise details for this antagonism has
been delayed. In the following chapters, we probe the mechanistic details of this antagonism. In Chapter 2, we show that the V protein of MV potently antagonizes dephosphorylation of MDA5, preventing its activation. In Chapter 3, we show that inhibition of dephosphorylation is a common evasion strategy among pathogenic paramyxoviruses, but that the precise details vary between viruses. Finally, in Chapter 4, we discuss the implications of these virus-specific activities and discuss remaining open questions in MDA5 activation.
Chapter 2:

Antagonism of the Phosphatase PP1 by the Measles Virus V Protein Is Required for Innate Immune Escape of MDA5
Chapter 2 is derived from the published work:


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2.1. Abstract

The cytosolic sensor MDA5 is crucial for the antiviral innate immune defense against various RNA viruses including measles virus; as such, many viruses have evolved strategies to antagonize MDA5’s antiviral activity. Here, we identify a novel mechanism of measles virus for escaping detection by MDA5, through targeting its key regulators, the phosphatases PP1α and PP1γ. The V protein of measles virus and the related paramyxovirus Nipah virus interacts with PP1α/γ, preventing PP1-mediated dephosphorylation of MDA5 and thereby its activation. We further identify that PP1 interaction with the measles V protein is mediated by a conserved PP1-binding motif in the C-terminal region of the V protein. A recombinant measles virus expressing a mutant V protein deficient in PP1 binding is unable to antagonize MDA5 and is growth-impaired due to its inability to suppress interferon induction. This identifies PP1 antagonism as a novel mechanism employed by paramyxoviruses for evading innate immune recognition.

2.2. Introduction

Pattern recognition receptors (PRRs) are critical components of the host’s innate immune sensing apparatus for detecting microbial pathogens. PRRs recognize conserved pathogen-associated molecular patterns (PAMPs) and then activate signaling cascades leading to the production of proinflammatory cytokines and type-I interferons (IFN-α/β), ultimately resulting in an antiviral state and activation of adaptive immune responses. Retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), the best characterized members of the RIG-I-like receptor (RLR) family, play an essential role in cytosolic detection of RNA viruses.
RIG-I is activated by 5’ triphosphate short dsRNA structures present in negative-strand RNA viruses as well as polyuridine/cytosine motifs in the positive-strand RNA of hepatitis C virus\(^{30,40,382}\). In contrast, MDA5 recognizes long dsRNA or RNA web structures produced during the replication cycle of picornaviruses\(^{39}\). Recent studies have also provided evidence that MDA5 acts in concert with RIG-I to respond to certain flaviviruses, reoviruses, and paramyxoviruses, such as measles virus (MV) and Sendai virus (SeV)\(^{41,48,49}\).

Despite their differences in ligand specificity, RIG-I and MDA5 share a common domain structure consisting of tandem caspase activation and recruitment domains (CARDs) at the N-terminus that are necessary and sufficient for signal transduction, as well as a helicase/ATPase domain and a C-terminal domain (CTD), both of which are important for RNA recognition\(^3,28\). Once activated, RIG-I and MDA5 form a complex with the mitochondrial-localized adaptor molecule MAVS/VISA/IPS-1/Cardif, resulting in downstream signaling to activate the transcription factors NF-κB, AP1 and IRF3/7, leading to IFN-α/β gene expression\(^{381}\).

Recent studies demonstrated that host cells are equipped with an elegant system for regulating RLR-induced signaling to avoid aberrant or premature immune activation\(^{381,383}\). Posttranslational modifications of the N-terminal CARDs as well as conformational changes induced by the CTD have been shown to play an important role in regulating RLR signaling activities\(^{55,68}\). Recently, we demonstrated that RIG-I and MDA5 are tightly controlled by an intricate balance of phosphorylation and dephosphorylation of their CARDs, and identified the phosphatase PP1 – specifically PP1\(\alpha\) and PP1\(\gamma\) isoforms – as key regulators of RIG-I and MDA5 activation\(^{57-59,66}\). In uninfected cells, RIG-I and MDA5 signaling is prevented by constitutive phosphorylation of specific serine/threonine residues located in the CARDs: serine-8 (S8) and threonine-170 (T170) in RIG-I and serine-88 (S88) in MDA5. Upon binding to RNA ligands,
RIG-I and MDA5 are dephosphorylated by PP1α/γ, allowing RLR interaction with MAVS and IFN-α/β induction\textsuperscript{66}.

Paramyxoviruses are enveloped, non-segmented, negative-strand RNA viruses comprising various human and animal pathogens including MV, mumps virus, parainfluenza virus 5 (PIV5), and the newly emerging Nipah (NiV) and Hendra viruses. To combat recognition and clearance by the immune system, these viruses have evolved sophisticated mechanisms to antagonize both IFN induction and IFN receptor signal transduction\textsuperscript{147,384}. This immunosuppression is particularly well-known for MV; in fact, many cases of mortality associated with MV infection are due to its potent inhibition of innate and adaptive immune responses\textsuperscript{385}.

The IFN-antagonistic activity of paramyxoviruses is due to one or more gene products of the paramyxovirus P/V/C gene, which encodes the essential phospho-(P) protein and – through alternative reading frames or RNA editing – the virulence factors C, V, and/or W proteins. Of the three, the V protein is the best characterized IFN antagonist. A major target of paramyxovirus V proteins is the immune sensor MDA5\textsuperscript{4,225,226,228}, however, the molecular mechanisms by which the V proteins inhibit MDA5 activity have only begun to be elucidated. It has been shown that the V proteins physically interact with the helicase domain of MDA5, inhibiting its ATPase activity and thereby MDA5 filament formation\textsuperscript{227}. Recently, co-crystal structure analysis of porcine MDA5 and the V protein of PIV5 provided evidence that the V protein inhibits the ATP hydrolysis activity of MDA5 through structural unfolding\textsuperscript{229}. Given the large diversity of IFN-antagonistic strategies employed by members of the paramyxovirus family, the mechanism of MDA5 inhibition by their V proteins is likely more complex than the present data suggests.
Here we identify a novel mechanism of MDA5 inhibition employed by MV and NiV in which their V proteins target PP1α and PP1γ, two essential activators of MDA5 signaling. PP1α/γ binding of the V protein prevents dephosphorylation of MDA5 and thereby its activation. Generation of a PP1 binding-deficient recombinant (r) MV demonstrates that PP1 antagonism by the V protein is an important mechanism for suppressing MDA5-mediated type-I IFN induction.

2.3. Materials and Methods

Plasmid Construction. FLAG-MDA5, GST-MDA5 2CARD, MAVS-CARD-PRD-FLAG, HA-PP1α, HA-PP1β, and HA-PP1γ were previously described\(^66,68\). pCR3-FLAG-PIV5-V and pCR3-FLAG-MV-V (Schwarz strain) were kindly provided by Karl-Klaus Conzelmann (LMU, Munich) and have been described\(^386\). pCAGGS-HA-NiV-V was provided by Chris Basler (Mount Sinai) and has been described\(^304\). FLAG- and V5-tagged PP1γ were subcloned into the pIRES-FLAG and pIRES-V5 vectors, respectively, between \(Mlu\) and \(Xba\)l. HA-tagged PIV5-V and MV-V genes were subcloned into the pCAGGS plasmid between \(Eco\)R\) and \(Xho\)l. FLAG-tagged NiV-V was subcloned into pEF-Bos vector containing an N-terminal FLAG tag between \(Nol\) and \(Sal\)l. GST-MV-V\(_N\) (aa 1-231), GST-MV-V\(_C\) (aa 232-299), GST-NiV-V\(_N\) (aa 1-407), and GST-NiV-V\(_C\) (aa 408-456) were constructed by subcloning into the pEBG vector between \(BamH\)I and \(Cla\)I. GST-MV-V\(_C\) and HA-MV-V AIAA mutants were generated through site-directed mutagenesis. GST-MV-V\(_C\) and HA-MV-V Δtail mutants were generated through subcloning residues 232-283 or 1-283 into the pEBG or pCAGGS vector, respectively. All constructs were sequenced to verify 100% agreement with the original sequence.

Cell Culture and Transfection. HEK293T, HeLa, 2fTGH, and immortalized MDA5-deficient MEF cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with
10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and 1% (w/v) penicillin-streptomycin (Pen-Strep; Gibco-BRL). Immortalized MDA5-deficient MEFs were described previously. A549-hCD150 and Vero-hCD150 were cultured in Advanced Modified Eagle’s Medium (MEM) supplemented with 10% (v/v) FBS and 2 mM GlutaMAX (Gibco-BRL). Stable expression of CD150 was maintained in A549-hCD150 and Vero-hCD150 using 500 µg/µL Zeocin (Invitrogen) and 400 µg/µL G418 (Sigma) respectively. Transient transfections were performed with calcium phosphate (Clontech) or Lipofectamine LTX and Plus Reagent (Invitrogen) according to the manufacturer’s instructions.

Immature monocyte-derived DCs were cultured as described before. In short, peripheral blood mononuclear cells, obtained from buffy coats of healthy donors, were isolated by a Lymphoprep (Axis-shield) gradient step and monocytes were subsequently isolated by a Percoll (Amersham biosciences) gradient step. Purified monocytes were differentiated into immature DCs in the presence of 500 U/ml interleukin-4 and 800 U/ml granulocyte-macrophage colony-stimulating factor (Schering-Plough, Brussels, Belgium). DCs were used for experiments at day 6-7.

**Viruses.** MV<sup>Ed</sup> and EMCV (strain EMC) were purchased from ATCC. DenV serotype 2 (strain 16681) was kindly provided by Lee Gehrke (Harvard/MIT). SeV (Cantell strain) was purchased from Charles River Laboratories.

**Generation of an rMV expressing EGFP and a truncated V protein.** rMV<sup>KS</sup> is based on a wild-type genotype B3 virus isolated from PBMC collected in 1997 from a severe measles case in Khartoum, Sudan. rMV<sup>KS</sup>-EGFP(3) was generated by insertion of EGFP as an additional transcription unit. The plasmid template for rMV<sup>KS</sup>-EGFP(3) was further modified to
generate rMV\textsuperscript{KS}EGFP(3)V\Delta\text{tail} by exchanging the \textit{AfeI}/Ascl fragment with one containing two in-frame stop codons in the sequence encoding the unique C-terminus of the V protein. Sequence changes to introduce the stop codons were designed to be silent in the overlapping P reading frame. The first stop codon terminates the V protein just before the RIWY motif. Plasmid and primer sequences are available on request. Recombinant viruses were recovered from fowlpox-T7-infected Vero-hCD150 cells transfected with the full-length plasmids along with plasmids expressing MV N, P and L. Virus stocks were grown in B-LCL and tested negative for contamination with mycoplasma species. Virus titers were determined by endpoint titration in Vero-hCD150 cells, and expressed in 50% tissue culture infectious dose (TCID\textsubscript{50}/ml).

**Reagents.** HMW-poly(I:C) complexed with LyoVec was purchased from Invivogen. The following phosphatase inhibitors were used to test MV-V phosphorylation: Calyculin A (25 nM) (Sigma), Cantharidic acid (100 nM, 500 nM, and 1 \( \mu \)M) (Abcam), Endothall (10 \( \mu \)M) (Millipore), and (-)-p-bromotetramisole oxalate (50 \( \mu \)g/mL) (Enzo Life Sciences). Silencing of endogenous MDA5 in A549-hCD150 cells was achieved by transduction of lentiviral particles expressing MDA5-specific shRNAs, or non-targeting control shRNAs (Santa Cruz Biotechnology) following the manufacturer’s instructions.

**GST Pull-Down Assay, Immunoprecipitation (IP), and Immunoblot Analysis.** HEK293T and A549-hCD150 cells were lysed in NP-40 buffer, followed by GST pull-down, IP and western blot analysis as previously described\textsuperscript{57,68}. For V-PP1 binding, Co-IPs were rigorously washed with NP40 lysis buffer containing 1 M NaCl.

**Antibodies.** For immunoblotting the following primary antibodies were used: anti-FLAG (M2; 1:2,000) (Sigma), anti-HA (1:2,000) (Clone HA-7; Sigma), anti-glutathione \( S \)-transferase (anti-
GST) (1:2,000) (Sigma), anti-measles nucleoprotein [3E1] (1:500) (Abcam), anti-PP1α (1:2,000) (Bethyl laboratories), anti-PP1γ (1:2,000) (Bethyl Laboratories), anti-MDA5 (1:1,000) (Enzo Life Sciences), anti-pan pSer antibody (Abcam), and anti-β-Actin (1:10,000) (Abcam). The phosho-specific pS88-MDA5, pS8-RIG-I, and pT170-RIG-I rabbit polyclonal antibodies have been previously described

**Quantitative Real-time PCR.** A549-hCD150 cells were infected with rMV^KS^EGFP(3) WT or VΔtail virus at an MOI of 0.05. 24 h post-infection, cells were harvested and total RNA was extracted with the E.Z.N.A. HP Total RNA Kit (Omega). RNA was used for qRT-PCR using SuperScript III Platinum One-Step Quantitative RT-PCR System with ROX kit (Invitrogen) with IFNβ, IFIT2, MxA, OAS1, MDA5, and GAPDH gene-specific primers (IDT) on a 7300 Real Time PCR System (Applied Biosystems).

**RIG-I and MDA5 Phosphorylation Analysis by Flow Cytometry.** For determining RIG-I and MDA5 phosphorylation, DCs were either left unstimulated, or pre-incubated with Raf-1 kinase inhibitor GW507427 (1 µM) (Sigma) for 2 h, and subsequently stimulated with poly(I:C)-LyoVec or infected with rMV^KS^EGFP(3) WT or VΔtail virus at the indicated MOI. Eight or 16 h after infection, cells were fixed with 4% (w/v) paraformaldehyde and permeabilized in 90% (v/v) methanol. Phosphorylation of endogenous RIG-I and MDA5 was assessed using pS8-RIG-I, pT170-RIG-I, and pS88-MDA5 antibodies. After incubation with PE-conjugated donkey anti-rabbit antibodies (Jackson), fluorescence was measured by flow cytometry.

**Luciferase Reporter Assay.** HEK293T cells were seeded into 12-well plates. 24 hours later, cells were transfected with 200 ng IFN-β luciferase and 300 ng β-gal-expressing pGK-β-gal as well as 500 ng FLAG-MDA5, 400 ng GST-MDA5 2CARD, and 500 ng V protein. At 48 h post-
transfection, whole cell lysates (WCLs) were prepared and subjected to a luciferase assay (Promega). Luciferase values were normalized to β-galactosidase to measure transfection efficiency.

**In vitro Dephosphorylation Assay.** MV-V-FLAG protein was purified from transfected HEK293T cells using anti-FLAG M2 agarose (Sigma). Immunoprecipitates were washed extensively with RIPA buffer, followed by washing twice with PBS. The *in vitro* MV-V protein dephosphorylation reaction was carried out at 30 °C for 1 h in phosphatase buffer (25 mM TRIS-HCl pH 7.5, 150 mM NaCl, 5 mM DTT) using 0.1 units of purified PP1α protein (Millipore). Immunoprecipitates were washed twice with NP40 buffer and the reaction was stopped by adding SDS-Laemmli buffer. Samples were then subjected to SDS-PAGE, followed by immunoblot analysis.

**Quantitative Real-time PCR- DCs.** RNA isolation from primary human DCs was performed by using the mRNA capture kit (Roche Diagnostic Systems). cDNA was synthesized with a reverse transcriptase kit (Promega). For real-time PCR analysis, PCR amplification was performed in the presence of SYBR green in a 7500 Fast Real-time PCR System (ABI). Transcription of the target gene was adjusted for *GAPDH* transcription with Nt = 2Ct(GAPDH)-Ct(target). Primers for IFN-β, MxA and ISG15 were kindly provided by C.L. Verweij, VUmc, Amsterdam.

**Microscopy.** Bright-field images of infected A549-CD150 cells were taken on a Nikon Eclipse Ti microscope.

**Confocal Microscopy.** For assessing PP1-V colocalization, HeLa cells, grown on cover slips, were transfected with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. At 36 h post-transfection, cells were stained with 500 µM MitoTracker-Alexa-fluor-
633 (Life Technologies) in DMEM for 30 min. Cells were fixed with 2% (w/v) paraformaldehyde for 10 min and permeabilized with 0.1% (v/v) Triton X-100, followed by blocking with 5% bovine serum in PBS for 1 h. Cell preparation and confocal microscopy analysis were performed as previously described (Gack et al., 2007). For immunostaining of FLAG-PP1\(\gamma\) and HA-MV-V, rabbit anti-FLAG (Sigma) and mouse anti-HA (clone HA-7, Sigma) antibodies were used, followed by incubation with donkey anti-rabbit Alexa-fluor-594 and donkey anti-mouse Alexa-fluor-488 (Life Technologies), respectively. Nuclei were stained with DAPI. Histogram profiles of confocal images were created using the RGB-Profiler function of ImageJ\textsuperscript{389}.

For determining the ability of MV-V WT and mutant proteins to prevent STAT2 nuclear translocation, 2fTGH cells were transfected with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. At 24 h post-transfection, cells were treated with 1000 U/ml IFN\(\alpha\)2 (PBL Biomedical Laboratories) for 30 min. Cells were fixed with 1% paraformaldehyde for 15 min and permeabilized in ice-cold 1:1 methanol/acetone for 10 min at -20 °C. Samples were washed with PBS and blocked with 5% bovine serum in PBS for 30 min. For immunostaining of HA-tagged MV-V proteins and endogenous STAT2, mouse anti-HA (Sigma) and rabbit anti-STAT2 (clone C-20, Santa Cruz) antibodies were used, followed by incubation with goat anti-mouse Alexa-fluor-594 and donkey anti-rabbit Alexa-fluor-488 (Life Technologies), respectively.

For assessing the nuclear translocation of endogenous IRF3, A549-hCD150 cells were infected with rMV\(^{KS}\)EGFP(3) WT or V\(\Delta\)tail (MOI 2), or left uninfected. At 18 h post-infection, cells were fixed and permeabilized as described for HeLa cells, followed by blocking with 10% (v/v) goat serum in PBS for 1 h. IRF3 was stained using a polyclonal rabbit anti-IRF3 (Santa
Cruz Biotechnology) antibody and a donkey anti-rabbit Alexa-fluor-594 antibody (Abcam). Nuclei were stained with DAPI. All laser scanning images were taken on an Olympus IX81 confocal microscope.

**Statistical Analysis.** Statistical analysis was performed by unpaired, two-tailed Student’s *t*-test.

**2.4. Results**

**The V protein of measles and Nipah virus suppresses MDA5 S88 dephosphorylation**

MDA5 signaling activity is tightly regulated by constitutive phosphorylation, keeping it inactive. Upon viral RNA sensing, MDA5 is activated via dephosphorylation by PP1, allowing innate immune signaling. We hypothesized that viruses may modulate this intricate balance in phosphorylation status to prevent MDA5 activation, and thus escape innate immune detection. To address this, we tested three RNA viruses, all known to be sensed by MDA5, for their effect on the S88 phosphorylation of FLAG-MDA5 by immunoblot (IB) using a phospho-(p)-S88-MDA5 specific antibody: encephalomyocarditis virus (EMCV), a picornavirus; dengue virus (DenV), a flavivirus; and MV, a paramyxovirus (Figure 2.1A). Infection with EMCV and DenV induced MDA5 S88 dephosphorylation, indicative of activation (Figure 2.1A). In striking contrast, the Edmonston (Ed) vaccine strain of MV (MV\textsuperscript{Ed}) did not induce any change in MDA5 S88 phosphorylation levels, even at a high MOI (Figure 2.1A), suggesting that MV\textsuperscript{Ed} modulates the MDA5 phosphorylation state. However, MV\textsuperscript{Ed} triggered RIG-I S8 dephosphorylation as efficiently as SeV, a virus known to induce RIG-I activation (Figure 2.1B). Similarly, while transfection of polyinosine-polycytidylic acid [poly(I:C)], a potent MDA5 agonist, efficiently triggered S88 dephosphorylation of endogenous MDA5 in primary human dendritic cells (DCs), infection with an enhanced green fluorescent protein (EGFP)-expressing rMV based on the wild-
type (WT) Khartoum-Sudan (KS) strain [rMVKS\textsuperscript{EGFP}(3)] did not change the MDA5 S88 phosphorylation compared to uninfected cells (Figure 2.1C). However, rMVKS infection efficiently induced RIG-I S8 and T170 dephosphorylation in DCs at this time point (Figure 2.1C). Collectively, these results suggest that MV, but not DenV and EMCV, blocks the dephosphorylation of the sensor MDA5.

**Figure 2.1. Measles virus inhibits MDA5 S88 dephosphorylation.** (A) S88 phosphorylation of FLAG-MDA5 in HEK293T infected with MV\textsuperscript{Ed} (MOI 1 or 3), or DenV (MOI 5) for 24 h, or with EMCV (MOI 0.5) for 3 h, assessed by IP with anti-FLAG, followed by immunoblot (IB) with anti-pS88-MDA5. Efficient MV infection was determined by IB with anti-MV nucleoprotein (MV-N). (B) S8 phosphorylation of FLAG-RIG-I in HEK293T, either left uninfected or infected with MV\textsuperscript{Ed} (MOI 3) for 8 or 24 h, or SeV (50 HAU/ml) for 24 h, assessed by IP with anti-FLAG, followed by IB with anti-pS8-RIG-I. Expression of MV-N was determined as in (A), two different blot exposures are shown. (C) Endogenous MDA5 S88 and RIG-I S8 or T170 phosphorylation in human DCs either unstimulated, treated with poly(I:C)-LyoVec for 3 h (upper panels), or infected with rMVKS\textsuperscript{EGFP}(3) WT for 16 h (lower panels), determined by flow cytometry using phospho-specific pS88-MDA5, pS8-RIG-I and pT170-RIG-I antibodies. Data are representative of three individual donors.

The V proteins of various paramyxoviruses, including MV, have been shown to interact with and inhibit MDA5\textsuperscript{4,225}, however, the molecular mechanism of this viral antagonism remains unclear. We therefore sought to determine whether the MDA5 phosphorylation-modulating activity of MV was caused by the V protein. To address this, we transfected increasing amounts of the MV V protein (MV-V) together with FLAG-MDA5 (Figure 2.2A). Over-expressed FLAG-MDA5, in contrast to endogenous MDA5, is not fully phosphorylated due to a low-level, constitutive binding to PP1\textsubscript{α/γ}\textsuperscript{66}, allowing examination of interference with the MDA5 S88 dephosphorylation by PP1. Indeed, MV-V strongly enhanced MDA5 S88 phosphorylation in a
dose-dependent manner, indicating interference with MDA5 dephosphorylation. The V protein of NiV also robustly enhanced MDA5 S88 phosphorylation, while that of PIV5 only marginally increased S88 phosphorylation levels (Figures 2.2B). Notably, MV-V and NiV-V did not affect RIG-I CARD phosphorylation, indicating that the V protein specifically modulates the phosphorylation state of MDA5, but not RIG-I (Figure 2.2D).

Figure 2.2. The paramyxovirus V protein inhibits MDA5 S88 dephosphorylation. (A) S88 phosphorylation of FLAG-MDA5 in HEK293T upon expression of increasing amounts of HA-MV-V protein, assessed as in (2.1 A). (B-D) IB analysis of FLAG-MDA5 S88 (B), FLAG-MDA5-R806L S88 (C), or FLAG-RIG-I S8 (D) phosphorylation upon expression of HA-tagged V proteins, determined by IP with anti-FLAG and IB using the indicated phospho-antibodies. (E and F) Interaction of GST-MDA5-2CARD (E) or GST-MDA5-2CARD-S88A (F) with MAVS-CARD-PRD-FLAG in the presence of HA-MV-V or HA-NiV-V, assessed by GST-pull down (GST-PD). (G and H) IFN-β luciferase activity in HEK293T cells transfected with GST-MDA5 2CARD (G) or FLAG-MDA5 (H) together with vector, MV-V, or NiV-V, normalized to constitutive pGK-β-gal. The results are expressed as means +/- s.d. (n=3). *P < 0.05; **P < 0.01.
Paramyxovirus V proteins have been reported to bind to the helicase domain of MDA5, leading to disruption of the ATP hydrolysis site\textsuperscript{229}. Residue R806 in MDA5 was shown to be critical for V-MDA5 interaction as mutation to leucine (R\textsubscript{806}L) abrogated or strongly reduced the binding of paramyxovirus V proteins to the helicase\textsuperscript{228,229}. To address whether modulation of MDA5 CARD phosphorylation and disruption of ATP hydrolysis are dependent or independent activities of the V protein, we determined the effect of the V protein on S88 phosphorylation of the MDA5 R\textsubscript{806}L mutant (Figure 2.2C). As with WT MDA5, ectopic expression of MV-V and NiV-V strongly increased S88 phosphorylation of MDA5 R\textsubscript{806}L, while PIV5-V had only a marginal effect (Figure 2.2C). These results indicate that the ability of MV-V and NiV-V to modulate MDA5 phosphorylation is independent of binding to the ATP-hydrolysis domain.

In uninfected cells, MDA5 S88 phosphorylation prevents MDA5 binding to the downstream adaptor MAVS; in contrast, viral RNA binding induces S88 dephosphorylation, allowing MAVS binding and leading to IFN gene expression\textsuperscript{66}. We therefore examined the effect of MV-V and NiV-V on the CARD-CARD interaction between MDA5 and MAVS (Figure 2.2E). In the absence of the V protein, GST-MDA5 2CARD efficiently interacted with the CARD-proline-rich domain of MAVS (MAVS-CARD-PRD); however, co-expressed MV-V or NiV-V potently diminished this interaction (Figure 2.2E). Crucially, the MV-V and NiV-V proteins did not affect the binding of MAVS-CARD-PRD to an MDA5 mutant in which the S88 residue was replaced with alanine (GST-MDA5 2CARD S\textsubscript{S88}A), reinforcing that the inhibition of MDA5-MAVS binding by the V proteins is a direct effect of their ability to enhance MDA5 S88 phosphorylation (Figure 2.2F). Consistent with their inhibition of MDA5-MAVS binding, MV-V and NiV-V markedly suppressed the IFN-\(\beta\) promoter activation induced by MDA5 2CARD or full-length MDA5 (Figures 2.2G and 2.2H). Collectively, these results indicate that the V
proteins of MV and NiV inhibit MDA5 S88 dephosphorylation, thereby suppressing MDA5-MAVS binding and downstream signaling.

**The V proteins of MV and NiV interact with the phosphatases PP1α/γ**

To elucidate the mechanism by which MV-V and NiV-V prevent MDA5 S88 dephosphorylation, we tested their potential interaction with PP1. Co-Immunoprecipitation (Co-IP) showed that MV-V and NiV-V bound to endogenous PP1α and PP1γ (Figures 2.3A and 2.3B). In contrast, PIV5-V did not interact with PP1α/γ (Figures 2.3A and 2.3C). Confocal microscopy showed that MV-V preferentially localized to the cytoplasm, with a minor fraction localized to the nucleus, whereas PP1γ was localized both in the nucleus and cytoplasm (Figure 2.3D). When both proteins were expressed together, however, PP1γ re-localized to the cytoplasm, where it extensively co-localized with MV-V at mitochondria-associated membranes, a subcellular compartment important for MDA5 signaling (Figures 2.3D and 2.3E). Furthermore, MV-V bound specifically to PP1α and PP1γ, which dephosphorylate MDA5, but not to PP1β which is not involved in innate immune signaling66 (Figure 2.3F). To further characterize the PP1-V interaction, we asked whether their binding is mediated by MDA5. To this end, we determined the binding of MV-V to PP1 in mouse embryonic fibroblasts (MEFs) derived from MDA5−/− mice (Figure 2.3G). MV-V bound efficiently to PP1 in MDA5−/− cells, suggesting that the binding between the V protein and PP1 is not mediated by MDA5 and thus likely to be a direct interaction. These results indicate that the V proteins of MV and NiV, but not that of PIV5, efficiently interact with the phosphatases PP1α and PP1γ.
Figure 2.3. The V proteins of MV and NiV bind to PP1α/γ. (A and B) Endogenous PP1α (A) or PP1γ (B) binding to the indicated HA-tagged V proteins in transfected HEK293T cells, determined by Co-IP. (C) HEK293T cells were transfected with V5-tagged PP1γ together with vector, HA-tagged MV-V, NiV-V, or PIV5-V protein. PP1γ binding was assessed by IP with anti-HA, followed by IB with anti-V5 antibody. (D) Confocal scanning laser images of HA-MV-V (green) and FLAG-PP1γ (red) in transfected HeLa cells. Nuclei were stained with DAPI (blue). Co-localization of MV-V and PP1γ was assessed by histogram profiles of merged images. (E) Confocal scanning laser images of HA-MV-V (green) and FLAG-PP1γ (red) in transfected HeLa cells. MAM were stained with MitoTracker (purple). (F) Co-IP of HA-tagged PP1α, PP1β, or PP1γ and FLAG-MV-V in transfected HEK293T cells. (G) Binding of FLAG-MV-V to HA-PP1γ in transfected MDA5−/− MEFs, assessed by IP with anti-FLAG and IB using anti-HA.

The V protein inhibits the PP1-MDA5 interaction and is a substrate for dephosphorylation by PP1

To address the mechanism by which the V protein interaction with PP1α/γ prevents MDA5 dephosphorylation, we asked whether the MV-V protein (i) inhibits PP1’s enzymatic activity, (ii) blocks the interaction of PP1 with MDA5, and (iii) whether it serves as a substrate
for PP1-mediated dephosphorylation. As neither measles infection nor MV-V expression suppressed RIG-I dephosphorylation by PP1α/γ (Figures 2.1B-C and 2.2D), it is unlikely that the V protein inhibits the enzymatic activity of PP1. To test whether the V protein competes with MDA5 for PP1 binding, we first compared the PP1α/γ binding of MDA5 and MV-V (Figure 2.4A). This showed that MV-V had a stronger association with PP1 than did MDA5. Next, we determined the binding of endogenous MDA5 to PP1 induced by poly(I:C) in the presence or absence of MV-V (Figure 2.4B). Poly(I:C) stimulation efficiently triggered PP1 binding to MDA5 in the absence of MV-V; in contrast, no MDA5-PP1 interaction was observed in poly(I:C)-stimulated cells expressing MV-V (Figure 2.4B). Finally, we examined endogenous PP1-MDA5 binding in poly(I:C)-stimulated or rMVKS-infected A549 lung epithelial cells stably expressing the WT MV entry receptor, CD150 (A549-hCD150) (Figure 2.4C). While poly(I:C) stimulation increased PP1 binding to MDA5, there was a near-complete loss of PP1-MDA5 interaction in rMVKS-infected cells (Figure 2.4C).
Figure 2.4. The V protein inhibits PP1 binding to MDA5 and serves as a PP1 substrate. (A) FLAG-MDA5 or FLAG-MV-V binding to HA-PP1α and HA-PP1γ in transfected HEK293T cells, determined by Co-IP. (B) Endogenous MDA5-PP1γ binding in Mock-treated or poly(I:C)-stimulated HEK293T cells, transfected with vector or FLAG-MV-V, assessed by IP with anti-MDA5, followed by IB with anti-PP1γ. (C) Endogenous MDA5-PP1α binding in Mock-treated, poly(I:C)-stimulated, or rMV-V6EGFP(3)-infected A549-hCD150 cells, determined by Co-IP. Efficient MV infection was determined by IB with anti-MV-N (two different blot exposures shown). (D) Phosphorylation of MV-V-FLAG in transfected HEK293T cells treated with the indicated inhibitors (or DMSO control) for 4 h, determined by IB using anti-pan-phospho-Ser antibody (anti-pSer). (E) IB analysis of MV-V-FLAG phosphorylation upon treatment with 100 nM, 500 nM, or 1 μM of Cantharidic acid for 4 h in transfected HEK293T cells. Arrow heads indicate phosphorylated forms of MV-V. (F) In vitro dephosphorylation of FLAG-MV-V by purified PP1α, assessed by IB analysis using anti-pSer.

Paramyxovirus P and V proteins are phosphorylated at multiple Ser/Thr sites. The kinases responsible for V protein phosphorylation have been identified, but the phosphatase(s) responsible for its dephosphorylation are unknown. We thus tested whether MV-V, upon binding to PP1, serves as a substrate for dephosphorylation by PP1. We first determined the phosphorylation of FLAG-MV-V after treatment with phosphatase inhibitors specific for PP1 and/or the related phosphatase PP2A (Figure 2.4D). This showed that specific inhibitors of both
PP1/PP2A (Calyculin A and Cantharidic acid), but not the PP2A-specific inhibitor Endothall, or an alkaline phosphatase inhibitor (Bromotetramisole), greatly enhanced the phosphorylation of MV-V, evident from a stronger signal in IB using a pan-phospho-Ser antibody, as well as a shift of the bands of MV-V, representing its phosphorylated forms (Figure 2.4D). In support of this, while treatment with Cantharidic acid at concentrations inhibiting only PP2A did not affect the phosphorylation state of MV-V, high concentrations of Cantharidic acid also blocking PP1 activity induced a shift in the multiple-band pattern of MV-V, indicating its enhanced phosphorylation (Figure 2.4E). Finally, purified PP1α efficiently dephosphorylated MV-V in an *in vitro* dephosphorylation assay, demonstrating direct enzymatic activity of PP1 towards the V protein (Figure 2.4F). Together, these results indicate that the MV-V protein blocks PP1 binding to MDA5 and serves as a substrate for PP1-mediated dephosphorylation, preventing MDA5 S88 dephosphorylation.

**A PP1 binding-deficient V protein is unable to inhibit MDA5 dephosphorylation but retains MDA5 binding and STAT inhibition activities**

We next sought to identify the site in the V protein that is necessary for PP1 binding. The paramyxovirus V protein is expressed through RNA editing of the P/V/C gene. As such, it shares the N-terminal sequence (VN) with the P and W proteins, but has a unique, cysteine-rich C-terminal domain (VC) which is responsible for many of its specific effector functions (Figure 2.5A). We thus determined the PP1-binding capacity of the P/V/W-shared VN and the unique VC of the MV-V and NiV-V proteins. Co-IP studies revealed that specifically the MV-VC and NiV-VC bound to PP1, while there was no interaction of PP1 with their VN domains (Figure 2.5B). Consistent with this binding mode, ectopic expression of MV-VC or NiV-VC fragments was
sufficient to enhance MDA5 S88 phosphorylation in a dose-dependent manner (Figures 2.5C and 2.5D).

Figure 2.5. The C-terminal domain of the V protein (Vc) of measles and Nipah virus is sufficient for PP1 binding and inhibition of MDA5 S88 dephosphorylation. (A) Domain structures of the paramyxovirus P and V proteins, and schematic representation of GST-fused truncation constructs of MV-V and NiV-V. Numbers indicate amino acids. (B) Binding of HA-PP1γ to GST-fused Vc or Vn of MV or NiV in transfected HEK293T cells, assessed by GST-PD and IB using anti-HA. (C and D) HEK293T cells were transfected with FLAG-MDA5 together with GST or 4 μg, 8 μg, or 12 μg of GST-fused MV-Vc (C) or NiV-Vc (D). WCLs were subjected to IP with anti-FLAG antibody, followed by IB with anti-pS88-MDA5 or anti-FLAG antibody. Expression of GST and GST-fused Vc were determined by IB with anti-GST antibody.

A hallmark of PP1-binding proteins is the presence of defined PP1-binding motifs. Indeed, sequence alignment of the Vc of several paramyxoviruses revealed that MV-V harbors a canonical PP1-binding motif, R/K-x(0,1)-V/I-x-F/W/Y (RIWY), in the extreme C-terminal portion of Vc (from now on referred to as C-terminal ‘tail’) (Figure 2.6A, upper panel). This PP1-binding motif was conserved among various vaccine and WT strains of MV. In contrast, no conventional PP1-binding motif was identified in the Vc of the other paramyxoviruses.

To determine the role of PP1 binding for MDA5 antagonism by the MV-V protein, key residues in the identified PP1-binding motif were mutated to alanine (MV-V AIAA). In addition,
a truncation mutant of MV-V was generated in which the C-terminal tail region (aa 284-299) containing the PP1 motif was deleted (MV-V Δtail) (Figure 2.6A, lower panel). These mutant V proteins were tested for their PP1-binding ability (Figure 2.6B). While MV-V WT efficiently interacted with PP1, the MV-V AIAA and Δtail mutants showed a reduced and near-complete loss of PP1 binding, respectively (Figure 2.6B). Consistent with its abolished PP1-binding ability, the Δtail V protein did not inhibit PP1-MDA5 binding, nor did it block MDA5 S88 dephosphorylation; in contrast MV-V WT robustly enhanced MDA5 phosphorylation, indicative of inhibition of MDA5 dephosphorylation, while the AIAA mutant only slightly enhanced MDA5 S88 phosphorylation (Figures 2.6C and 2.6D). Together, these results identify a classical PP1-binding motif in the C-terminal tail region of the MV-V protein, which is necessary for PP1 binding and suppression of MDA5 CARD dephosphorylation.
Figure 2.6. PP1 binding of the measles V protein is required for its inhibitory effect on MDA5 S88 dephosphorylation. (A) (Upper panel) Protein sequence alignment of the Vc of paramyxoviruses. Alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/). Asterisks (*) indicate a single, fully conserved residue. Colons (:) indicate conservation between groups of strongly similar properties, and periods (.) indicate conservation between groups of weakly similar properties. Conserved residues of the zinc-finger motif responsible for MDA5 interaction are highlighted in grey. (Lower panel) Protein sequences of the MV-Vc WT, AIAA and Δtail mutant. Identified PP1-binding motif in MV-Vc is indicated (red box). Numbers indicate amino acids. (B) Interaction of HA-PP1γ with GST-fused MV-Vc WT or mutant proteins in transfected HEK293T cells, determined by GST-PD. (C) S88 phosphorylation of FLAG-MDA5 in HEK293T expressing HA MV-V WT or mutant proteins, analyzed by IP. (D) Endogenous MDA5-PP1 binding in Mock-treated or poly(I:C)-stimulated HEK293T cells, transfected with vector or HA-tagged MV-V WT or Δtail, assessed by IP with anti-MDA5, followed by IB with anti-PP1α.

To further characterize the PP1 binding-defective MV-V mutant proteins, we first tested their ability to bind MDA5, an activity mediated by conserved histidine and cysteine residues in the Vc domain (Figure 2.6A, upper panel). In contrast to their defective PP1-binding ability, MV-V AIAA and Δtail mutants interacted with MDA5 as efficiently as MV-V WT (Figure 2.7A). Furthermore, MV-V is known to prevent the nuclear translocation of signal transducers and activators of transcription (STAT) proteins to suppress IFN receptor signal transduction. Therefore, we also tested the MV-V AIAA and Δtail mutant proteins for their ability to block STAT2
nuclear translocation. Ectopically expressed MV-V AIAA and Δtail proteins prevented STAT2 nuclear translocation as effectively as MV-V WT (Figure 2.7B). This demonstrates that a mutant V protein in which the PP1-binding motif is deleted, is unable to bind PP1 and inhibit PP1-mediated MDA5 dephosphorylation, but retains MDA5 binding and STAT inhibition.

**Figure 2.7.** Mutation or deletion of the PP1-binding motif in the measles V protein does not affect other known activities. (A) Binding of FLAG-MDA5 to HA-MV-V WT or mutant proteins, determined by Co-IP. (B) Confocal images of 2fTGH cells, that had been transfected with either HA-tagged MV-V WT, MV-V AIAA, or MV-V Δtail, were treated with IFNα for 30 min, or left untreated. Cells were then stained for endogenous STAT2 (green) or HA (MV-V, red). Nuclei were stained with DAPI (blue). Arrow heads indicate cells that show expression of MV-V.

**PP1 antagonism is required for inhibition of type-I IFN induction and optimal replication of MV in epithelial cells**

To assess the role of PP1 antagonism by the MV-V protein in innate immune escape of MDA5-mediated IFN induction, we sought to construct an rMV which expresses a mutant V protein deficient in PP1 binding. Based on our characterization of the MV-V Δtail mutant protein, we argued that an rMV expressing a Δtail V protein will reveal the contribution of PP1 antagonism to IFN suppression and virus replication. We therefore designed a cloning strategy that – by introducing two stop codons in the P/V/C gene – resulted in the deletion of the C-
terminal tail region (aa 288-299) of the MV-V protein while leaving P protein expression unaffected (Figure 2.8A). Using reverse genetics methodology as previously described\textsuperscript{360}, we then generated an EGFP-expressing rMV\textsuperscript{KS} harboring the MV-V\textsuperscript{Δtail} (rMV\textsuperscript{KS}EGFP(3)-V\textsuperscript{Δtail}, referred to as ‘V\textsuperscript{Δtail} virus’). We first examined the replication of the V\textsuperscript{Δtail} virus compared to the parental rMV\textsuperscript{KS}EGFP(3) WT virus (referred to as ‘WT virus’) in IFN-defective Vero cells (Figure 2.8B). Both viruses replicated with comparable efficiency, indicating that the V\textsuperscript{Δtail} virus does not have a general growth defect (Figure 2.8B). In striking contrast, the V\textsuperscript{Δtail} virus exhibited profoundly reduced replication capacity compared to WT virus in A549-hCD150 lung epithelial cells, with an intact IFN system: the titers of the V\textsuperscript{Δtail} virus were reduced by ~2 and ~3 logs compared to WT virus at 60 h.p.i. and 72 h.p.i., respectively. At later time points, the V\textsuperscript{Δtail} virus was not detectable, whereas the WT virus still replicated efficiently (Figure 2.8C). Depletion of endogenous MDA5 enhanced the replication of the V\textsuperscript{Δtail} virus to titers comparable to those of WT virus, indicating that the reduced replication capacity of the V\textsuperscript{Δtail} virus in A549-hCD150 cells is indeed due to its inability to antagonize MDA5 (Figures 2.8D and 2.8E). Consistent with its impaired replication, the V\textsuperscript{Δtail} virus had a greatly reduced ability to induce syncytium formation and cause cell death of A549-hCD150 cells; in contrast, cells infected with the WT virus formed large syncytia and died more rapidly (Figure 2.8F).
Figure 2.8. An rMV expressing a PP1-binding deficient mutant V protein shows impaired replication in human epithelial cells. (A) Schematic of rMV<sup>KS</sup>EGFP(3)V<sub>Δtail</sub> (lower inset) nucleotide sequence of mutated region with P and V protein translations showing uninterrupted P sequence and in-frame V stop codons (underlined in red). Mutated nucleotides are shown as lowercase letters. (B) Replication of rMV<sup>KS</sup>EGFP(3) WT and V<sub>Δtail</sub> virus in Vero-hCD150 cells infected at an MOI of 0.02. Viral titers were determined at 60 h.p.i. and are expressed as mean 50% tissue culture infectious dose (TCID<sub>50</sub>/ml) +/- s.d. (n=3). (C) A549-hCD150 cells were infected with WT or V<sub>Δtail</sub> virus (MOI 0.02). Virus titers in the supernatant were determined by endpoint titration in Vero-hCD150 cells, and expressed as TCID<sub>50</sub>/ml. (D) Viral titers of rMV<sup>KS</sup>EGFP(3) WT and VΔtail were determined 48 h.p.i. in A549-hCD150 cells, that had been transduced with lentiviral particles expressing MDA5-specific shRNA (sh.MDA5) or non-targeting control shRNA (sh.C). Data are expressed as mean TCID<sub>50</sub> +/- s.d. (n=2) and are representative of 2 independent experiments. (E) Confirmation of MDA5 knockdown from the experiment shown in (B) as determined by qRT-PCR normalized to gapdh mRNA levels. The results are expressed as means +/- s.d. (n=2). (F) Bright-field images of A549-hCD150 cells infected with WT or VΔtail virus at 48 h.p.i. and 60 h.p.i.

We argued that the growth defect of the VΔtail virus in A549-hCD150 cells is due to its diminished ability to suppress the IFN induction pathway activated by MDA5. To address this, we determined the ability of WT and VΔtail virus to induce the nuclear translocation of IRF3, a transcription factor required for type-I IFN gene expression. Infection with the VΔtail virus
efficiently triggered nuclear translocation of endogenous IRF3; whereas, cells infected with WT virus exhibited primarily cytoplasmic IRF3 localization, comparable to uninfected cells (Figure 2.9A). Consistently, infection with the V∆tail virus resulted in significantly higher mRNA levels of IFN-β and the interferon-inducible genes (ISGs), *Mx1*, *Oas1*, *isg15*, and *ifi16*, compared to infection with WT virus (Figure 2.9B). Taken together, these results show that an rMV harboring a mutant V protein that is deficient in PP1 binding efficiently induces IRF3-mediated type-I IFN induction in lung epithelial cells, and thus has impaired growth kinetics.

**Figure 2.9.** An rMV expressing a PP1-binding deficient mutant V protein shows diminished IFN antagonism in human epithelial cells (A) Confocal microscopy images of endogenous IRF3 (red) in rMV*K*EGFP(3) WT or V∆tail virus-infected A549-hCD150 cells (green) at 18 h.p.i. Nuclei were stained with DAPI (blue). Quantification of the percentage of cells with nuclear or cytoplasmic IRF3 (200 cells counted) (right) (B) A549-hCD150 cells were infected with WT or V∆tail virus (MOI 0.05). 24 h later, total RNA was extracted and transcript levels of IFNβ and ISGs were determined by quantitative real-time PCR. Transcript levels were normalized to GAPDH and are expressed as fold levels compared to mock-infected cells. Data are expressed as means +/- s.d. (n=3). **P<0.01.

The PP1 binding-deficient V∆tail virus is unable to suppress MDA5 S88 dephosphorylation and type-I IFN induction in primary human DCs

After MV infection of the lung, DCs are among the first cells that become infected. Thus we investigated whether MV, using its V protein, also targets PP1α/γ in DCs to evade detection by MDA5. We determined the ability of the V∆tail virus and WT virus to modulate
MDA5 S88 phosphorylation in primary human DCs at early (8 h.p.i.) and late (16 h.p.i.) time points during infection (Figure 2.10A, upper panel). In DCs infected with the WT virus, MDA5 remained phosphorylated at both time points, indicating that MV blocks MDA5 phosphorylation early and late during infection. In contrast, in VΔtail virus-infected cells MDA5 was in the dephosphorylated, active state at 16 h.p.i., indicating an inability to block MDA5 dephosphorylation (Figure 2.10A, upper right panel). Interestingly, at 8 h.p.i. MDA5 was phosphorylated in the VΔtail virus-infected cells. RIG-I was also kept in the S8/T170-phosphorylated, inactive state at 8 h.p.i. in WT and VΔtail virus-infected cells (Figure 2.10A, middle and lower left panels). In contrast to MDA5 suppression, the inhibition of RIG-I dephosphorylation was transient and not affected by the V protein, as RIG-I was fully dephosphorylated at 16 h.p.i. in both WT and VΔtail virus-infected cells (Figure 2.10A, middle and lower right panels). These results demonstrate that in DCs, MV uses V-dependent and V-independent mechanisms for preventing RLR activation: while the V-dependent inhibitory mechanism specifically targets MDA5, the V-independent mechanism blocks both RIG-I and MDA5 activation, specifically early during infection\(^{394}\). This early, V-independent inhibitory mechanism of MV is triggered via DC-specific DC-SIGN signaling and subsequent Raf-1 kinase activation, as treatment with the Raf-1 inhibitor GW5074 induced dephosphorylation of MDA5 and RIG-I at 8 h.p.i., but notably had no effect on both RLRs at 16 h.p.i. (Figure 2.10A). Our study combined with the results by Mesman et al. thus indicates that early during infection in DCs, MDA5 and RIG-I are inhibited through virus-induced DC-SIGN-Raf-1 signaling; later during infection, however, when viral protein expression occurs, the V protein of MV keeps MDA5, but not RIG-I, in the phosphorylated, repressed state.
Figure 2.10. The VΔtail rMV is defective in MDA5 suppression and IFN antagonism in primary human DCs.

(A) Primary human DCs were either DMSO-treated or pre-incubated with Raf-1 inhibitor GW5074 for 2 h, and then infected with WT or VΔtail virus (MOI 0.5). Phosphorylation of endogenous MDA5 S88 (upper panel), RIG-I S8 (middle panel), and RIG-I T170 (lower panel) was determined at 8 h.p.i. (left) and 16 h.p.i. (right) by flow cytometry using phospho-specific pS88-MDA5, pS8-RIG-I and pT170-RIG-I antibodies. Data are representative of three independent donors.

(B-D) DCs that had been either DMSO-treated or pre-incubated with GW5074 for 2 h, were infected with WT or VΔtail virus (MOI 0.5). IFN-β and ISG mRNA levels were determined by qRT-PCR at 24 h.p.i. Data are pooled from three (B and C) or two (D) independent donors and presented as means +/- s.d. *P<0.05; n.s., not statistically significant.

To determine the contribution of MV-V inhibition of MDA5 to the type-I IFN response in DCs, we compared IFN-β and ISG induction upon infection with WT virus or VΔtail virus (Figures 2.10B-2.10D). DCs infected with the VΔtail virus had markedly increased mRNA levels of Ifnb1, Mx1, and isg15 compared to cells infected with WT virus, specifically when DC-SIGN signaling was blocked using the Raf-1 inhibitor GW5074, but not upon DMSO treatment (Figures 2.10B-2.10D). Collectively, these results demonstrate that in DCs, MV uses V-
dependent and V-independent mechanisms to block RLR signaling: in addition to RLR inhibition through DC-SIGN-Raf-1 signaling, the V protein is important for MV to block MDA5 dephosphorylation-dependent activation and thus for optimal IFN antagonism. The V protein specifically targets MDA5, late during infection, while DC-SIGN signaling through Raf-1 blocks both RIG-I and MDA5, early during infection.

2.5. Discussion

The Paramyxoviridae family encompasses several clinically relevant human pathogens that pose a serious global health concern. MV, a member of the genus Morbillivirus, continuously causes high morbidity and mortality world-wide, the latter of which is caused by its ability to induce a generalized suppression of the immune system\(^{385}\). It is known that the immunosuppressive effect of MV is partly due to its ability to potently antagonize type-I IFN induction in various cell types including lung epithelial cells and immune cells. Similar to other paramyxoviruses, the IFN-antagonistic activity of MV is largely due to its non-structural V protein, a virulence factor that is dispensable for virus replication \textit{in vitro}\(^{395}\), but enhances pathogenicity \textit{in vivo}\(^{396,397}\). While it is well known that the V protein antagonizes various host innate immune proteins including MDA5 and STAT1/2, the precise mechanisms and physiological relevance of these virus-host interactions remain largely unknown. In this study, we show that the V protein of MV blocks PP1\(\alpha/\gamma\)-mediated dephosphorylation of MDA5, which keeps MDA5 in the phosphorylated, inactive state. Despite the fact that PP1\(\alpha/\gamma\) are responsible for both MDA5 and RIG-I dephosphorylation, MV-V specifically blocked MDA5, but not RIG-I, dephosphorylation. In addition, the V protein of NiV, of the Henipavirus genus, also robustly modulated the phosphorylation state MDA5 at S88. Maintained phosphorylation of MDA5 by MV-V and NiV-V subsequently blocked its interaction with MAVS, preventing downstream
signaling and IFN induction. In contrast, no appreciable enhancement of MDA5 phosphorylation was detected with PIV5-V, of the Rubulavirus genus. Our study thus identifies an important mechanism of MDA5 inhibition that is distinct from that previously described involving a direct interaction with and disruption of the MDA5 ATP hydrolysis domain. Given that paramyxoviruses are equipped with multiple mechanisms to manipulate IFN receptor signaling, it is not surprising that these viruses have also evolved multiple strategies to block MDA5-mediated IFN induction. Our study, combined with previous studies, indicates that whereas the V proteins of some paramyxoviruses, such as PIV5, block MDA5 by disrupting its ATPase activity, the V proteins of other paramyxoviruses, such as MV and NiV, suppress MDA5 activation through modulating the MDA5 phosphorylation state.

Mechanistically, our study revealed a physical interaction of the V protein of MV and NiV with the phosphatases PP1α/γ. In contrast, PIV5-V did not interact with PP1α/γ. Binding of MV-V to PP1 strongly inhibited the PP1-MDA5 interaction. MDA5-PP1 binding was also strongly impaired in MV-infected, but not poly(I:C)-stimulated, cells.

Since PP1 substrates or PP1-interacting proteins usually possess defined consensus motifs that mediate binding\textsuperscript{393}, we searched for known PP1-binding sequences in various paramyxovirus V proteins. The conventional PP1 sequence\textsuperscript{288RIWY\textsuperscript{291}} was identified in the very C-terminal “tail” region of the MV-V protein. The PP1-binding motif in MV-V was required for PP1 interaction and for MV-V’s inhibitory effect on MDA5 dephosphorylation. In contrast, no known PP1-binding motif was found in the V protein of PIV5 or NiV, the latter of which also efficiently interacted with PP1α/γ. It is thus tempting to speculate that the V protein of NiV, which evolved in bats, interacts with PP1α/γ utilizing an as yet unknown PP1-binding motif. Alternatively, it is possible that the binding of NiV-V may be mediated by MDA5 or
Another cellular protein. Further analyses will be required to define the molecular architecture of the NiV V-PP1 complex and the molecular mechanism of how NiV-V blocks MDA5 activation by PP1. Furthermore, future studies will be directed toward the precise details of how NiV and MV-V block the PP1-mediated activation of MDA5, but not RIG-I.

Several reports indicate that the V protein of paramyxoviruses is a multi-phosphorylated protein. Recent studies showed that the V proteins utilize cellular kinases for their own phosphorylation by mimicking a kinase substrate to antagonize innate immunity. For example, the V proteins of PIV5 and mumps virus were shown to compete with IRF3 for phosphorylation by TBK1/IKKε, thereby abrogating IRF3-induced gene expression. Since no phosphatase for V protein dephosphorylation has been identified, we asked whether the MV-V protein serves as a PP1 substrate. Experiments using chemical inhibitors of PP1 and an *in vitro* dephosphorylation assay indicated that PP1 dephosphorylates MV-V. It is conceivable that the targeting of cellular kinases (TBK1 and IKKs) or phosphatases (PP1α/γ), that play key roles in innate signal transduction pathways, is a common theme of the paramyxovirus V protein for innate immune suppression. However, more detailed studies are needed to determine whether MV-V indeed serves as a ‘decoy’ substrate of PP1α/γ, or whether V dephosphorylation by PP1 is a critical part of MV replication.

The V proteins of paramyxoviruses have been shown to suppress IFN induction and IFN receptor signaling through a number of different mechanisms; however, the physiological relevance and contribution of specific IFN-antagonistic functions of the V proteins to immune suppression remain largely undetermined. To determine the relevance of PP1 antagonism for innate immune evasion, we generated an rMV in which the PP1-binding motif in the V protein has been deleted. This VΔtail mutant virus was severely growth-impaired compared to the
parental virus in human lung epithelial cells which correlated with its ability to robustly trigger IRF3 activation and enhance IFN induction compared to the parental virus, indicating an important role of PP1 antagonism by the MV-V protein for inhibiting type-I IFN induction in epithelial cells. Infection studies in primates or hCD150-transgenic mice are needed to reveal the physiological role of PP1 antagonism in V-mediated innate immune suppression in vivo.

We extended our studies to primary human DCs, which are also key target cells of MV in vivo, to determine the contribution of PP1 antagonism by MV-V in immune cells. In contrast to the parental virus, the VΔtail mutant virus efficiently triggered MDA5 S88 dephosphorylation at 16 h.p.i., demonstrating that the PP1 binding-deficient VΔtail virus loses its ability to suppress MDA5 dephosphorylation and activation. Interestingly, MV also blocked RIG-I dephosphorylation specifically at early time points, but not at later time points during infection of DCs. At this early time point during infection MDA5 dephosphorylation was also blocked in a V protein-independent manner. This early V-independent inhibition of both RIG-I and MDA5 was caused by virus-induced PP1α/γ inhibition through activation of the DC-SIGN-Raf1 signaling cascade. This reveals that in DCs, MV has evolved two mechanisms – V-dependent and V-independent – for blocking the dephosphorylation-dependent activation of RLRs, indicating the importance of modulating this pathway. In the case of MDA5, specifically at later time points when viral gene expression occurs, this involves a direct interaction of the V protein with PP1α/γ, as presented here. In contrast, to block RIG-I and MDA5 dephosphorylation at early time points in infection, MV activates DC-SIGN signaling which triggers Raf-1 kinase activation, ultimately also resulting in PP1α/γ inhibition. Since MV inhibited both RIG-I and MDA5 at early time points but specifically blocks MDA5 activation at later time points, it is tempting to speculate that these receptors sense viral RNAs in DCs at different times during MV
infection: while both RIG-I and MDA5 may contribute to RNA detection at early time points, MDA5 may be the main sensor for detecting viral RNA species generated later during MV infection. A similar model of sequential RLR activation was recently proposed for West Nile virus infection\textsuperscript{50}. However, further studies are needed to determine the temporal contribution of RIG-I and MDA5 to viral RNA sensing during MV infection.

Taken together, these results demonstrate that PP1 antagonism is an important viral strategy for escaping RLR immune signaling and also emphasize the vital role of PP1\textsubscript{α} and PP1\textsubscript{γ} in innate immune activation.
Chapter 3:

Characterization of MDA5 evasion through PP1 inhibition by the paramyxovirus V proteins
Acknowledgements

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3.1. Abstract

The pattern recognition receptor, MDA5 recognizes viral dsRNA and initiates a signaling cascade resulting in the induction of type-I IFNs and the establishment of an antiviral state. MDA5 activation is intricately regulated by phosphorylation and dephosphorylation at S88 in the CARD domain. Our previous studies have shown that the V proteins of the paramyxoviruses measles and Nipah virus antagonize MDA5 by inhibiting S88 dephosphorylation by the phosphatase PP1α/γ. Here we investigate the virus-specificity of paramyxovirus V protein antagonism of MDA5 dephosphorylation. We find that the V proteins of additional, highly pathogenic paramyxoviruses, including mumps, Menangle, and Hendra virus, antagonize MDA5 dephosphorylation. We also identify a minimal binding region in the mumps V protein, MuV-V189, which encompasses a putative PP1 binding site. This study expands our knowledge of V protein antagonism of MDA5 and illustrates virus-specific differences in V protein functions.

3.2. Introduction

The innate immune system is the first line of defense against invading pathogens. PRRs detect infection through the recognition of conserved pathogen associated molecular patterns (PAMPs). The RLRs, RIG-I and MDA5 are ubiquitously expressed PRRs that survey the cytosol for pathogen-associated RNAs. The RLRs share a common domain architecture consisting of tandem N-terminal caspase activation and recruitment domains (CARDs), a central helicase domain, and a C-terminal domain (CTD). Each RLR senses a distinct type of RNA ligand, dependent on interactions between the CTD and helicase with the RNA. RIG-I senses primarily short double stranded (ds) RNAs with a 5′ triphosphate (5′ ppp). On the other hand, MDA5 has been shown to recognize longer dsRNAs and RNA aggregates. Upon detection of their
viral RNA ligands, both RIG-I and MDA5 bind to MAVS via CARD-CARD interactions which induces a signaling cascade ultimately resulting in the activation of the transcription factors IRF3/7 and NF-κB which induce type I IFNs and proinflammatory cytokines for establishment of an antiviral state\textsuperscript{379,381}.

Because aberrant IFN production can be harmful to the host, PRR signaling is tightly regulated. Both RIG-I and MDA5 are constitutively phosphorylated in the uninfected cell, keeping them inactive. RIG-I is phosphorylated at S8 and T170 in the first and second CARDs, respectively, by protein kinase C (PKC) α and β\textsuperscript{57,58}. MDA5 is phosphorylated at S88 in the first CARD by an as yet unidentified kinase. Binding to viral RNA triggers the dephosphorylation of both RIG-I and MDA5 by the phosphatases PP1α and PP1γ, allowing downstream signaling through MAVS\textsuperscript{66}. RIG-I further requires K63-linked ubiquitination of K172 by TRIM25 for interaction with MAVS and downstream signaling\textsuperscript{68}; however, the role of K63-linked ubiquitination of MDA5 remains controversial.

Viruses have evolved mechanisms to evade the innate immune response to promote their replication and transmission\textsuperscript{378,384}. The paramyxovirus V protein is a well known IFN antagonist which functions to target both JAK/STAT signaling and MDA5 activation. Previous studies have shown that the V proteins of 13 paramyxoviruses bind to and inhibit MDA5\textsuperscript{4,225,227}; however, there is some question as to the precise mechanism of this inhibition. Structural studies have shown that the V protein of parainfluenza virus 5 (PIV5) induces a conformational change upon binding to MDA5 that results in the inability of MDA5 to cooperatively bind to RNA\textsuperscript{229}. In contrast, our previous work showed that the measles (MV) and Nipah (NiV) virus V proteins counteract MDA5 dephosphorylation by interacting with the phosphatases PP1α/γ\textsuperscript{378}. For MV-V
this interaction was due to a canonical PP1 binding motif located in the very C-terminal tail of
the V protein. This binding site is not conserved among other V proteins and does not account
for the ability of NiV-V to bind to PP1.

The paramyxovirus family encompasses a large number of viruses of varying
pathogenicity. Here further investigate the ability of the V protein to modulate MDA5 S88
phosphorylation and determine the virus specificity of this function. We assembled a large panel
of paramyxovirus V proteins from multiple genera: Rubulaviruses, Morbilliviruses, and
Henipaviruses. The Rubulavirus panel consists of PIV5, human parainfluenza virus 2 (PIV2),
mumps virus (MuV), Menangle virus (MenV), and Mapuera virus (MPRV). PIV5 causes kennel
cough in dogs, but is not pathogenic in humans. PIV2 is a major cause of respiratory disease in
children and immunocompromised individuals. MuV is the etiologic agent of mumps, a severe
disease characterized by severe malaise and inflammation, particularly of the parotid glands,
with frequent complications of meningitis or encephalopathy. MenV and MPRV are both
viruses that have fruit bats as a natural host. These viruses have emerged in human populations
through zoonotic transmission. MenV was isolated following an outbreak of reproductive disease
at an Australian piggery and has been associated with influenza-like respiratory disease in at
least two humans. MPRV, on the other hand, was isolated from the Sturnira lilium species
of bat in Brazil. Its natural host range remains unknown, but it is not thought to be pathogenic
in humans. The Morbillivirus MV causes severe respiratory illness and the distinctive
macropapular rash characteristic of the disease. The Henipaviruses NiV and HeV are recently
emerged zoonotic viruses from pteropid fruit bats. NiV passes from bats to pigs to humans,
and has caused severe outbreaks of respiratory disease with encephalitic complications across
Southeast Asia. On the other hand, HeV has horses as an intermediate host and has been
associated with outbreaks in Australia. There is a licensed veterinary HeV vaccine for horses that is in widespread use in Australia\textsuperscript{403}.

In this study, we show that multiple paramyxovirus V proteins are able to modulate MDA5 S88 phosphorylation and interact with PP1. This ability is spread across genera, and seems to correlate with the pathogenicity of the viruses. We also were able to map the interaction of MuV V protein with PP1 to a minimal binding region in the unique $V_C$ of MuV-V. This minimal binding region was not sufficient for interaction with or antagonism of MDA5. Contained within this region is a putative, degenerate PP1-binding motif which is not conserved among the other V proteins. Further study will be necessary to show the importance of this putative motif in for function of the V protein, specifically for MDA5 antagonism.

3.3. Materials and Methods

**Plasmid Construction.** GST-MDA5 2CARD, HA-PP1$\alpha$, and HA-PP1$\gamma$ were previously described\textsuperscript{66,68}. HA-MDA5 was cloned into the pCDNA3.1 vector between $KpnI$ and $XhoI$. pCR3-FLAG-PIV5-V and pCR3-FLAG-MV-V (Schwarz strain) were kindly provided by Karl-Klaus Conzelmann (LMU, Munich) and have been described\textsuperscript{386}. pCAGGS-HA-NiV-V was provided by Chris Basler (Mount Sinai) and has been described\textsuperscript{304}. Myc-tagged PIV2-V, MenV-V, MPRV-V, and HeV-V constructs were kindly provided by Stephen Goodbourn (University of London) and were described previously\textsuperscript{225,287}. FLAG-tagged PIV2-V, MenV-V, MPRV-V, NiV-V, and HeV-V were subcloned into the pEF-Bos vector containing an N-terminal FLAG tag between $NotI$ and $SalI$. pEF-Bos-FLAG-MuV-V was obtained from AddGene. GST-MuV-V_N (aa 1-169), GST-MuV-V_C (aa 170-224), GST-MuV-V_{179} (aa 1-179), GST-MuV-V_{189} (aa 1-189), GST-MuV-V_{199} (aa 1-199), GST-MuV-V_{209} (aa 1-209), GST-MuV-V_{219} (aa 1-219), and GST-MuV-V (aa 1-224)
were constructed by subcloning into the pEBG vector between *KpnI* and *NotI*. All constructs were sequenced to verify 100% agreement with the original sequence.

**Cell Culture and Transfection.** HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and 1% (w/v) penicillin-streptomycin (Pen-Strep; Gibco-BRL). Transient transfections were performed with calcium phosphate (Clontech) according to the manufacturer’s instructions.

**GST Pull-Down Assay, Immunoprecipitation (IP), and Immunoblot Analysis.** HEK293T cells were lysed in NP-40 buffer, followed by GST pull-down, IP and western blot analysis as previously described57,68.

**Antibodies.** For immunoblotting the following primary antibodies were used: anti-FLAG (M2; 1:2,000) (Sigma), anti-HA (1:2,000) (Clone HA-7; Sigma), anti-glutathione S-transferase (anti-GST) (1:2,000) (Sigma). The phospho-specific pS88-MDA5 and pS8-RIG-I rabbit polyclonal antibodies have been previously described58,66.

### 3.4. Results

**The V proteins of multiple paramyxoviruses modulate MDA5 S88 phosphorylation.**

To test the virus-specificity of paramyxovirus V protein antagonism of MDA5 S88 dephosphorylation, we assembled an extended panel of V proteins from different species. We included V proteins of the *Rubulaviruses* PIV5, PIV2, MuV, MenV, and MPRV; and the *Henipaviruses* NiV and HeV. The MV V protein was included as a positive control from our previous study378. We tested this panel for the ability to modulate MDA5 S88 phosphorylation levels by over-expression in HEK293T cells by immunoblot (IB) following immunoprecipitation
(IP) of HA-MDA5 using our phospho-specific MDA5 antibody. As we had seen previously, both MV-V and NiV-V led to an enhancement of the phospho-S88 signal (Figure 3.1A). We also observed robust modulation of MDA5 S88 phosphorylation upon expression of the MuV-V, MenV-V, and HeV-V proteins. The PIV5-V, PIV2-V, and MPRV-V proteins had only a minimal effect on MDA5 S88 phosphorylation. These effects on phosphorylation were confirmed through over-expression of increasing amounts of the V proteins. As we had seen with both MV-V and NiV-V, the V proteins of MuV, HeV, and MenV enhance phosphorylation of MDA5 in a dose-dependent manner (Figure 3.1C-E). Importantly, PIV2-V had no effect on MDA5 S88 phosphorylation even at high concentrations (data not shown). These results show that the V proteins from multiple species of paramyxovirus have evolved the ability to antagonize MDA5 through modulation of S88 phosphorylation.

Figure 3.1. Paramyxovirus V proteins modulate MDA5 S88 Phosphorylation. (A) MDA5 S88 phosphorylation of HA-MDA5 in HEK293T upon expression of the indicated panel of FLAG-tagged paramyxovirus V proteins, assessed by IP with anti-HA antibody and IB with phospho-specific-MDA5-S88 rabbit polyclonal antibody. (B) RIG-I S8 phosphorylation of GST-fused RIG-I 2CARD in HEK293T upon expression of FLAG-tagged V proteins by GST PD followed by IB with phospho-specific-RIG-I-S8 antibody. (C-E) S88 phosphorylation assessed as in A upon expression of 4, 8, or 12µg of transfected FLAG-tagged MuV-V (C), MenV-V (D), or HeV-V (E).
The antagonism by MV-V and NiV-V is specific to MDA5 as neither protein had any effect on RIG-I S8 phosphorylation. To test if the other V proteins also have this specificity, we examined RIG-I S8 phosphorylation by IB in transfected HEK293T using a RIG-I phospho-S8-specific antibody. No effect was observed on RIG-I S8 phosphorylation upon over-expression of any of these V proteins, indicating that like MV-V and NiV-V, phosphorylation modulation is specific for MDA5 antagonism (Figure 3.1B).

**Multiple paramyxovirus V proteins interact with PP1α/γ**

Our previous study on MV antagonism of MDA5 showed that MV-V interacts with PP1α/γ to prevent MDA5 dephosphorylation. Therefore, we tested our expanded panel of V proteins for the ability to interact with PP1. Similar to our previous results on MV-V and NiV-V, all V proteins which modulated MDA5 S88 phosphorylation also interacted with both PP1α and PP1γ (Figure 3.2A, B). Surprisingly, the PIV2 V protein which had no observable effect on MDA5 S88 phosphorylation also interacted with over-expressed PP1α/γ. The MPRV V protein which had a modest to negligible effect on MDA5 S88 phosphorylation also showed minimal interaction with over-expressed PP1. More detailed binding studies with endogenous PP1 are needed to determine the physiological relevance of this interaction. However, these results suggest that interaction with PP1 and inhibition of MDA5 dephosphorylation is conserved among paramyxoviruses.
Figure 3.2. Paramyxovirus V proteins interact with PP1α/γ. Interaction of HA-PP1γ (A) or HA-PP1α (B) with the indicated FLAG-tagged paramyxovirus V proteins in HEK293T, assessed by FLAG IP followed by IP with anti HA antibody.

**Mumps virus V protein interacts with PP1 via its C-terminal domain**

When we investigated the PP1 binding ability of MV-V and NiV-V, we determined that MV-V has a canonical PP1 binding site located in the very C-terminal tail; however, there are no canonical PP1-binding sites located in the VC of NiV-V. Upon examining the sequence of the full length V proteins, we identified two putative PP1-binding sites in the MuV-VN. To test the functionality of these putative motifs, we first made GST-fused truncations of the MuV VN and VC (Figure 3.3A). We tested these truncation constructs for their ability to bind to PP1 through co-IP experiments. Surprisingly, even though the VN harbors two distinct putative PP1-binding sites, it was unable to interact with PP1 (Figure 3.3B). In contrast, the MuV-VC interacted with PP1 as efficiently as the full length V protein. To further map this interaction, we generated a series of mutants adding 10 amino acids each, beginning at the RNA editing site (Figure 3.3A). We tested these mutants for their ability to interact with PP1 by co-IP and found that a minimal binding region present in MuV-V189 was sufficient for interaction with PP1 (Figure 3.3C). Examination of MuV-V189 revealed a \(^{184}\text{RVFEW}^{188}\) motif that closely resembles a PP1-binding motif (R/K-(0,1)-V/I-x-F/W). This suggests that PP1 binding may be mediated by a previously unknown motif in the MuV-VC.
Figure 3.3. A Mumps V protein minimal binding region is sufficient for PP1 binding. (A) Schematic representation of truncation mutants of GST-fused MuV V protein. The VN is indicated in blue, VC in red. Red bars within the VC represent residues which constitute the conserved zinc fingers. Putative PP1-binding motif sequences are indicated. Summary of binding activities are displayed to the right of the diagram. (B) Interaction of HA-PP1γ with MuV-V terminal truncations in HEK293T by GST pull down (PD) followed by IB with anti-HA. (C) Interaction of HA-PP1γ with MuV-V truncation mutants as in (B). (D) Interaction of HA-MDA5 with GST-MuV-V truncation mutants by GST PD followed by IB with anti-HA.

The VC of all paramyxovirus V proteins contain seven cysteines and one histidine (represented by dark bars in Figure 3.3A) which participate in the formation of two zinc coordinating loops\(^{151}\). For MuV-V, all eight of these residues are required for MDA5 binding. We tested the ability of our truncation mutants to interact with MDA5. As expected, only the truncation possessing all the zinc finger residues, MuV-V\(_{219}\), was able to interact MDA5 (Figure 3.3D). This indicates that MuV-V is able to bind to PP1 independently of MDA5 as multiple truncations retained binding to PP1 but not to MDA5 (Figure 3.3C). Additional study will be needed to verify the importance of the putative PP1-binding motif and fully characterize the mechanism of MuV-V antagonism of MDA5 dephosphorylation.
3.5. Discussion

The V proteins of paramyxoviruses are well-defined innate immune antagonists. They have been particularly well characterized in their targeting of both IFN induction (through MDA5) and signaling (through STAT1/2). It is widely accepted that the V proteins have genus- and species-specific functions in antagonizing the JAK/STAT pathway. The Rubulaviruses recruit a ubiquitin E3 ligase complex consisting of DDB1 and Cul4 to target STATs for degradation\textsuperscript{274,275}. Morbilliviruses prevent STAT nuclear import\textsuperscript{294}, and the Henipaviruses sequester STAT1/2 in high molecular weight complexes in the cytosol\textsuperscript{302}. Despite these differences in activity, V protein antagonism of MDA5 has been generalized to a single mechanism. The V proteins of 13 paramyxoviruses interact with the MDA5 helicase domain. Early studies implied that V-MDA5 binding disrupts ATPase function and prevents cooperative binding of MDA5 to RNA\textsuperscript{225,227}. Structural studies have confirmed helicase inhibition as the primary mechanism for MDA5 antagonism by the PIV5 V protein\textsuperscript{229}. In Chapter 2, we identified a novel mechanism by which the V proteins of MV and NiV, but not PIV5, are able to antagonize MDA5 dephosphorylation through interaction with the phosphatases PP1\textgreek{a}/\textgreek{g}\textsuperscript{378}. In this chapter, we further examined the virus-specificity of this mechanism of antagonism and found major differences between the different V proteins.

The V proteins of the highly pathogenic Rubulaviruses, MuV and MenV, as well as MV and the Henipaviruses, NiV and HeV, were found to modulate MDA5 S88 phosphorylation levels. Mechanistically, this modulation could be thought to occur either through the inhibition of the phosphatases, PP1\textgreek{a}/\textgreek{g}, or through the recruitment of a kinase to keep MDA5 phosphorylated. Our studies of MV-V showed that the former, antagonism of PP1, governed this antagonism. Similarly, we found that all V proteins which had an effect on MDA5 S88
phosphorylation also interacted the both PP1α and PP1γ (Figure 3.4A). Interestingly, the PIV2 V protein which had no effect on MDA5 S88 phosphorylation even at high protein levels (data not shown), also interacted with over-expressed PP1. The physiological relevance and functional implications of this interaction are unknown. Our work on MV-V showed that the V protein was itself dephosphorylated by PP1. It is possible that the PIV2 V protein also requires dephosphorylation for its normal function and therefore interacts with PP1. Additionally, there are significant sequence differences between the VN of PIV2 and MuV which could account for varied effector functions despite binding to the same cellular factors.

Figure 3.4. Model of V protein inhibition of MDA5. (A) Schematic representation of the actions of the V proteins examined in this study. MV-V interacts with PP1 via a canonical PP1-binding motif, sequestering PP1 away from MDA5 and itself serving as a substrate for dephosphorylation. PIV5-V, PIV2-V, and MPRV-V have no effect on MDA5 S88 phosphorylation and therefore are thought to inhibit MDA5 activity solely through direct interaction with the helicase domain. The V proteins of MuV, MenV, NiV, and HeV all inhibit MDA5 S88 dephosphorylation and interact with PP1, although the precise molecular mechanism of this inhibition remains unknown. (B) Protein sequence alignment of the VC of paramyxoviruses. Alignment was performed using ClustalW2. Asterisks (*) indicate a single, fully conserved residue. Colon (:) indicate conservation between groups of strongly similar properties, and periods (.) indicate conservation between groups of weakly similar properties. Conserved residues of the zinc-finger motif responsible for MDA5 interaction are in grey. The confirmed PP1-binding site in MV-V is indicated in red text. The putative PP1-binding motifs in MuV-V and PIV2-V are highlighted by the red box.
Mumps is a common, but severe childhood illness that often has complications of encephalitis and meningitis. The widespread use of the MMR (measles, mumps, and rubella) vaccine has limited the number of cases since its introduction in the 1960s; however, multiple factors have led to a resurgence in cases in the last few years. First, there has been declined use of the MMR vaccine for social reasons. Additionally, it has recently come to light that the MuV segment of the MMR vaccine may be less effective than initially thought. The combination of these two factors has led to an increased number of cases and with it, increased interest in a more complete understanding of MuV biology. Thus, we further probed MuV-V antagonism of MDA5 by mapping the interaction between the V protein and PP1. We began by looking at the discreet domains of the V protein: the VN which shares its sequence with the P protein and the unique VC. PP1 binding to regulatory subunits and substrates can be defined by the presence of one or more of several defined PP1-binding motifs. Sequence analysis revealed two putative PP1-binding motifs in the MuV-V_N; however, we found that these motifs do not facilitate PP1 binding. In contrast, the V_C was necessary and sufficient to interact with PP1. Using terminal truncation mutants, we mapped this interaction to a minimal binding region which resembles a degenerative PP1-binding motif. The putative motif is not conserved between the other paramyxovirus V proteins; however, the analogous residues in PIV2-V also fit into the degenerative R/K-x(0,1)-V/I-x-F/W motif pattern (Figure 3.4B). We have not tested the importance of these residues for PIV2-V interaction with PP1, but it would be straightforward to pursue this after confirming the necessity of the motif for MuV-V.

MuV-V_{189} was not sufficient for interaction with MDA5. Importantly, this implies that MuV-V binding to PP1 is independent of MDA5. It is possible that by disrupting the zinc fingers of the V protein in our truncation mutants that the proper structure and function of the V protein
was also disrupted. This may also account for what appears to be more efficient binding of the truncations to PP1 when compared to the full length MuV-V or MuV-VC. The disruption of the zinc fingers may allow PP1 better access to the binding site without steric hindrance. We intend to address the importance of this putative PP1-binding site through mutational analysis in additional experiments. It will be most informative to mutate this motif in the context of the full length V protein to determine the potential of a PP1-binding defective mutant to antagonize MDA5.

Our studies have illustrated that some, but not all, paramyxovirus V proteins are able to antagonize MDA5 through inhibition of dephosphorylation by PP1 (Figure 3.4A). Multiple V proteins including those from MuV, MenV, MV, NiV, and HeV are able to interact with PP1; however, the motifs that govern this interaction are not conserved among these viruses. The MV-V PP1-binding motif is located in an extended C-terminal tail which is not shared with most V proteins (Figure 3.4B). Likewise, the putative PP1-binding motif of MuV-V is located in a stretch of lower sequence conservation between the other V proteins. Only the PIV2-V protein has a similar sequence at the location of the PP1-binding motif. The MuV V protein is unique among the Rubulaviruses in that in addition to recruiting a ubiquitin E3 ligase complex to degrade STAT2, it also targets the RACK1 kinase to prevent STAT1/2 phosphorylation. It is not surprising that this V protein has also evolved an additional mechanism to target MDA5. The MPRV V protein also has an extended C-terminal region; however, its importance is unknown. It is known the MPRV-V, unlike other Rubulaviruses, does not recruit an E3 ligase complex to degrade STAT but instead sequesters STAT1/2 in the cytoplasm \(^{287}\). It is possible that the extended C-terminal region is responsible for this altered function.
How multiple V proteins have evolved to target PP1 without a conserved binding site is a particularly interesting question. The specific mechanism by which the MuV V protein antagonizes MDA5 S88 dephosphorylation remains unclear. We showed previously that MV-V sequesters PP1 from MDA5, preventing dephosphorylation. Our current study indicates that a minimal binding region MuV-V$^{189}$ mutant is able to bind to PP1 but not to MDA5. Further study will be required to distinguish this precise mechanism.

Overall, our studies have added complexity to the known mechanisms of paramyxovirus V protein antagonism of MDA5. While some V proteins (PIV5-V, PIV2-V, and MPRV-V) seem to primarily act by inducing conformational changes in MDA5 abrogating the helicase function, other V proteins, particularly those from more highly pathogenic viruses (MV-V, MuV-V, NiV-V, and HeV-V) have evolved additional mechanisms to target MDA5 dephosphorylation. More work is needed to distinguish the individual molecular mechanisms for this inhibition, but it is becoming clear that these viruses have evolved species-specific mechanisms to target MDA5 for immune evasion.
Chapter 4:

Discussion
Our work has identified a novel mechanism of inhibition of MDA5 employed by the V proteins of a subset of paramyxovirus species. The V proteins of measles, mumps, Menangle, Nipah, and Hendra viruses target the phosphatases PP1α/γ to prevent MDA5 dephosphorylation and activation. Historically, the mechanism of V protein antagonism of MDA5 has been thought to be universal among paramyxovirus species. In this chapter, we discuss the issues of generalizing viral protein functions to such a large family of viruses. The discovery of PP1 inhibition as an evasion mechanism was only possible due to our understanding of MDA5 regulation through phosphorylation. Further knowledge of regulatory mechanisms will undoubtedly reveal additional targets for viral evasion. We discuss the open questions in RLR regulation and physiological activities which may provide insights into additional viral evasion mechanisms. We also address the recent evidence that mutations resulting in misregulation of RLR activity may induce autoimmune diseases.

4.1 Paradigm for Virus-Specific V protein Functions

The Paramyxovirinae subfamily encompasses a wide variety of viruses which infect a broad range of host species. The pathogenicity of these viruses varies depending on both virus and host species. For example, parainfluenza 5 (PIV5) causes minor disease in dogs, but no disease in humans while the closely related mumps virus (MuV) causes a highly pathogenic infection in humans\textsuperscript{399,404}. In contrast, the Henipaviruses Nipah and Hendra viruses are asymptomatic in their bat reservoirs but cause severe illness in humans following zoonotic transmission\textsuperscript{403}. Due to this varied host range, it is not surprising that these viruses would have established varied interactions with the host cellular proteins.
The paramyxovirus V proteins have evolved as potent IFN antagonists to increase virus replication by inhibiting the innate immune response. Most paramyxovirus V proteins have at least two cellular targets for evasion of the innate immune response - the JAK/STAT pathway for IFN signaling and MDA5 for IFN induction. Studies focused on many aspects of paramyxovirus biology, including the action of the V protein, have centered on the prototypic PIV5 and generalize its traits to the other members of the family. Subsequent studies have shown virus-specific differences within the family for inhibition of the JAK/STAT pathway; however, many studies still implied a shared mechanism for MDA5 antagonism.

Paramyxoviruses do share many commonalities; however, they have evolved independently in their host organisms and therefore have distinct characteristics as well. This is particularly clear from studies investigating the antagonism of IFN signaling by these viruses. Different gene products of the P/V/C gene have been shown to target the JAK/STAT pathway with the V protein playing a particularly important role. The V protein is produced through a mechanism of RNA editing of the P gene which results in the P and V protein sharing their N-terminal sequences with differing C-terminal sequences following the induced frame shift. The different domains of the V protein have different degrees of sequence conservation between viruses. The N-terminus (VN) of the V (and P protein) is important for antagonism of the JAK/STAT pathway and is not well conserved between viruses. In contrast, the VC, which targets MDA5, is highly conserved, even between genera. Most notably, seven cysteines and one histidine which form zinc binding loops are conserved between all paramyxovirus V proteins. The importance of zinc coordination remains unclear, but these residues have been shown to be important for V protein binding to MDA5. It seems likely that the differences in sequence conservation between the domains have led to the disparity in mechanistic studies on VN.
antagonism of IFN signaling compared to studies on $V_C$ antagonism of MDA5 - higher variability in the $V_N$ sequence more clearly indicates variability in function.

Although there is a high level of conservation in the $V_C$ sequence between paramyxoviruses, there are notable sequence differences as well. Despite the complete conservation of the zinc finger residues, not all paramyxovirus V proteins require all eight residues for MDA5 interaction. In particular, PIV5-V and NiV-V only require the second zinc finger made up of C2-4, whereas MV-V and MuV-V require both zinc fingers for MDA5 binding. Not only do the V proteins of different paramyxoviruses require distinct motifs for interaction with MDA5, they interact with different regions of MDA5. Initial studies looking at the binding of V to MDA5 mapped the interaction to a CARD-deleted construct. Further studies using truncations of the MDA5 helicase/CTD indicated that some, but not all, V proteins make separate contacts in distinct regions of the helicase domain. All V proteins tested interacted with a minimal region of MDA5 (residues 676-816) in the helicase domain; however, the Salem virus, MenV, and PIV5 V proteins interacted with 2-4 additional regions in the helicase/CTD domains. The intervening sequences between the H and C1 and C2 and C3 of the zinc coordinating loops are longer and show more variability than other regions of the $V_C$. These stretches of sequence show some conservation between viruses of the same genus, but generally not between genera. Our mapping studies of MuV-V indicated that the sequence between H and C1 is the minimal binding region for PP1 interaction. Importantly, there are significant differences in the sequence between MuV-V, which binds to PP1, and the PIV5-V which does not. There are also significant differences between the MuV-V minimal binding region and the analogous sequence in MV-V. In contrast, the MV-V protein has an extended $V_C$, which we have termed the “C-terminal tail.” This tail encompasses the motif required for PP1
binding, leading to inhibition of MDA5 dephosphorylation; however, the PP1 motif is only a small portion of this extended sequence. Further importance of this “tail” region is unknown. It may be involved in additional mechanisms of IFN antagonism as our MV-V-Δtail mutant had more profound effects on MDA5 inhibition than the MV-V-AIAA motif mutant. Overall, the differences in sequence and binding requirements across paramyxovirus V proteins, even within the larger conserved region of the V_C, warrant further research to define the distinct, virus-specific functions of the V proteins during infection. Of particular interest would be characterizing the interaction of PP1 with the additional V proteins which do not have defined PP1-binding motifs such as MenV-V, NiV-V, and HeV-V.

Many studies have focused on the function of the V proteins as being a determinant for pathogenicity of paramyxoviruses. These studies have shown that highly pathogenic viruses have more functionally-active V proteins. This has been shown for individual viruses, such as MuV and MV which have evolved additional mechanisms to antagonize IFN induction and signaling. MuV-V not only targets STAT1 for degradation through the recruitment of DDB1 and Cul4 like other Rubulaviruses, but it also prevents STAT1 phosphorylation through interaction with receptor activated kinase 1 (RACK1) to further antagonize IFN signaling. Likewise, the MV-V protein blocks not only MDA5-mediated induction of IFN, but can also block TLR- and NLR-responses. The MV-V protein interacts with IKKα, serving as a decoy substrate for phosphorylation at the expense of IRF7. Inhibition of IRF7 is cell-type specific, occurring in DCs, a prominent target cell of MV infection. The MV V protein has also been shown to bind to NLRP3 in Thp-1 cells and to relocalize it to the perinuclear region following inflammasome activation, suppressing pro-inflammatory cytokine production. It is not surprising that these
two highly pathogenic viruses have evolved the additional function for MDA5 inhibition by targeting PP1 with the V protein to more efficiently inhibit MDA5-mediated IFN induction.

In 2012, a new paramyxovirus was isolated from pteropid bats. Cedar virus (CedPV) shares many features with the Henipaviruses, and was thus classified as a novel member of this genus. Interestingly, CedPV does not have an editing site in the P gene and therefore does not produce a V protein. CedPV induces high levels of IFNβ following infection in cell culture, consistent with the lack of the V protein-mediated IFN antagonism. CedPV was also found to be non-pathogenic in small animal models. It is evident that the lack of V protein greatly contributes to the lack pathogenicity of this virus. It would be interesting to confirm this through generation of a recombinant CedPV engineered to express a V protein from a different paramyxovirus. This rCedPV should have the ability to antagonize IFN, and therefore increased pathogenicity.

Not only do highly pathogenic paramyxovirus species have expanded functions of the V protein, but strains within a single species have been shown to be more pathogenic based on V function. Studies looking at viruses harboring point mutations in the zinc fingers of the SeV V protein found that disruption of interaction with MDA5 correlated with lower pathogenicity in mice. Additional studies have looked at the mutations that occurred in MV as wild-type isolates were attenuated to produce the vaccine strains. Of particular interest are the mutations found in the V protein of the Edmonston-tag (MVEdtag) strain. MVEdtag is more severely attenuated than other strains in the same lineage. Several studies have shown that the MVEdtag-V protein is defective in inhibition of both IFN induction and signaling due to specific mutations. At least two of these mutations lead to the increased attenuation of MVEdtag compared to other MVEd lineage strains- Y110H and C272R. The substitution Y110H was
shown to cause a defect in STAT1 binding by the V protein\textsuperscript{295}. The R272C mutation disrupts the zinc-binding domain of the V protein important for MDA5 interaction\textsuperscript{151,412}. Interestingly, there is an additional mutation- Y291H. This mutation changes the PP1-binding motif identified in our work from RIWY to RIWH, presumably abrogating interaction with PP1\textsubscript{α/γ}.\textsuperscript{Takaki, et al.,} looked into the significance of these point mutations by making single residue reversion mutations and testing their IFN antagonistic capabilities. The R272C revertant restored binding to MDA5 and reduced IFN-β induction approximately 2-fold following transfection of MDA5 or poly(I:C). The single H291Y mutant did lead to a reduction in MDA5-mediated IFN-β induction; however, this reduction was not significant. Our studies on MV-V binding to PP1 showed that this binding takes place in the perinuclear region of the cell. This is a critical area for innate immune signaling. How the V protein targets only a small subset of the cellular PP1 is unclear. One potential mechanism is that the V protein first binds to MDA5 and then interact with PP1 as local PP1 levels increase for MDA5 dephosphorylation. If the V protein must first bind MDA5 to target PP1, it is possible that a double revertant mutant of MV\textsuperscript{Edtag-V} with both R272C and H291Y would lead to more efficient antagonism of IFN induction by the V protein as MDA5 would be targeted both through direct binding and through inhibition of PP1.

Our findings on the inhibition of MDA5 S88 dephosphorylation add to the paradigm of virus-specific V protein functions observed for JAK/STAT inhibition. We found that the V protein of multiple highly pathogenic paramyxoviruses can block MDA5 S88 dephosphorylation by binding to PP1\textsubscript{α/γ}, but the precise mechanisms for this function vary among viruses. While the MV-V protein has a canonical PP1-binding motif responsible for this interaction, this motif is not conserved between the other V proteins shown to bind to PP1. For all V proteins tested, PP1 binding was dependent on the V\textsubscript{C}, but, at least for MV-V and MuV-V, this interaction was
independent of interaction with MDA5. The MuV-V_c contains a putative, degenerate PP1-binding motif which is present in a minimal fragment that interacts with PP1. In contrast, neither of the Henipavirus V proteins nor the Menangle virus V protein which interact with PP1 show evidence of a PP1-binding motif. These three viruses evolved in pteropid bats, infecting humans through zoonosis. Little is known about the virus-host interaction in bats. It is possible that in the bat host, these paramyxovirus V proteins utilize previously uncharacterized PP1-binding motifs not evident from our human-centric studies. In the last few years, a great emphasis has been put on the large number of viruses present in asymptomatic bats. Interestingly, characterization studies of RLRs in the Pteropus alecto species of flying fox revealed that while there are differences in the phosphorylation sites in the RIG-I CARD domains, both S88 and the PP1-binding sites in MDA5 are conserved. Investigating the bat host factors involved in paramyxovirus replication will likely lead to new insights into paramyxovirus biology as well as virus-host interactions, potentially characterizing PP1 interaction and MDA5 antagonism.

Overall, our results on the varied ability of paramyxovirus V proteins to target PP1 for MDA5 antagonism add an additional level of complexity to a mechanism which is often oversimplified in the literature. The individual activities of paramyxovirus V proteins lead to variations in efficiency of IFN antagonism, and thus to the varied pathogenicity of individual virus species and strains.

4.2. Open Questions in MDA5 regulation

RIG-I-like receptors are critical for the innate immune response to virus infection. Despite the similar timing of their discovery, much more is known of the molecular details of RIG-I regulation compared to MDA5. There are two main mechanisms by which RLRs are
regulated: (1) control of RNA ligand recognition and binding and (2) regulation of CARD-CARD interaction between the RLRs and MAVS for downstream signaling. It is clear from numerous studies, that although there are parallels between RIG-I and MDA5 activation, there are also great differences. For example, while both RIG-I and MDA5 CARDs are kept inactive in the uninfected cell through constitutive phosphorylation of key residues in the CARD domains, the kinase responsible for MDA5 S88 phosphorylation remains unknown. The relevance of additional post-translational modifications for MDA5 activity also remains unclear. Our work on the paramyxovirus V protein antagonism of PP1 was predicated on the regulation of MDA5 through S88 phosphorylation. A more complete understanding of the regulation of MDA5 will inform future studies of viral evasion mechanisms. Here we will address some of the open questions in MDA5 regulation, focusing on helicase and CARD regulation, particularly through post-translational modification.

4.2.A. MDA5 Regulation through Helicase-RNA binding

The mechanisms by which the RLR helicase domains interact with RNA have been intensively studied in the past several years. These studies have revealed distinctions between RIG-I and MDA5 C-terminal interactions with RNA. The most obvious difference underlies the ligand specificity seen between these RLRs - the RIG-I CTD makes contacts with the 5’ppp or 5’pp of RNA with the helicase domain wrapping around like an end-cap28,34,40,72-74. In contrast, MDA5 does not require the phosphate signature of RNA and instead wraps around the length of longer dsRNA. Interestingly, MDA5 monomers have very low affinity for RNA, and require cooperative binding facilitated by contacts between the helicase domains of multiple MDA5 molecules8,9,35,36,85. This cooperative binding to RNA results in large filamentous complexes of MDA5-RNA. Filament formation is dependent on the ATPase activity of the MDA5 helicase.
domain: as MDA5 monomers hydrolyze ATP, they dissociate from RNA resulting in filament disassembly. Disassembly is favored at the filament ends, which leads to RNA length discrimination - shorter filaments will disassemble more quickly than longer filaments, preventing MDA5 activation upon binding to short dsRNA\textsuperscript{35,36}. Importantly, these studies were all completed in cell-free \textit{in vitro} systems. Further research will be needed to understand the physiological filament formation of MDA5 and whether additional factors are involved in this process.

Before the molecular details of MDA5 cooperative RNA binding were clarified, one study suggested that the MDA5 helicase/CTD is modified by SUMOylation\textsuperscript{86}. The exact residues of SUMOylation were not identified; however, modification potentiated IFN induction. It is possible that this SUMOylation facilitates MDA5 filament formation by stabilizing helicase contacts between MDA5 molecules. It is also possible that additional post-translational modifications occur in the MDA5 helicase/CTD that regulate its ability to interact with RNA, either through modulation of monomer interaction or filament formation.

The first defined mechanism by which paramyxovirus V proteins antagonize MDA5 signaling was linked to the direct interaction of the V proteins with the MDA5 helicase domain\textsuperscript{226,227}. This was hypothesized to disrupt the ATPase domain of MDA5. More recently, crystal structures have revealed that the interaction of the PIV5 V protein with MDA5 mediates mutual misfolding of the proteins, resulting in the inability of MDA5 to cooperatively bind to RNA, but leaving monomer MDA5-RNA interaction unaffected\textsuperscript{228,229}. Combined with our research, this illustrates the vast differences between V protein antagonism of MDA5, with some V proteins (PIV5-V) specifically targeting the MDA5 C-terminus and others (MuV-V, MV-V, NiV-V) also affecting MDA5 CARD activity.
4.2.B. MDA5 CARD Regulation through S88 Phosphorylation

**MDA5 dephosphorylation- what regulates the regulator?**

In contrast to the large number of genes encoding Ser/Thr kinases in the human genome (~400) there are very few of the catalytically opposed enzymes, Ser/Thr phosphatases (~40). This disparity in number is made up for in function by a vast number of regulatory subunits that complex with the phosphatases to form the active holoenzyme. This method of regulation and function has been particularly well described for phosphoprotein phosphatase 1 (PP1). PP1 phosphatases are a small family of catalytic isoenzymes which are ubiquitously expressed and control a vast number of cellular functions including entry into mitosis and protein translation, and a more recently defined role in innate immune signaling\(^\text{67,415,416}\). It would seem impossible for such a small number of enzymes to control so many processes; however, these catalytic subunits cannot function as monomers, and must form heterodimeric or heterotrimeric complexes to dephosphorylate substrates. PP1 regulatory subunits control substrate specificity either by directly bridging the substrate to the catalytic domain or by conformationally altering the substrate binding site on the catalytic subunit to facilitate binding\(^\text{67,415-420}\).

Both RIG-I and MDA5 are activated upon viral infection by the dephosphorylation of residues in the CARD by the isoenzyme phosphatases PP1\(\alpha/\gamma\); however, how this dephosphorylation is controlled by PP1 regulatory subunits is unknown\(^\text{66}\). It is interesting to consider the characteristics of the ideal PP1 regulatory subunit for RLR dephosphorylation. As the RLRs must be kept phosphorylated and inactive in the resting cell, the regulation of PP1-mediated dephosphorylation is of critical importance. The phosphatase must be recruited to MDA5 following virus infection and only dephosphorylate S88 following viral RNA binding. It is possible that the relocalization of PP1 to MDA5 is triggered to coincide with MDA5-RNA
binding by other aspects of virus infection. For example, certain PP1 regulatory subunits have been shown to be induced by PKR activation following sensing of foreign RNA. Having the active PP1 holoenzyme already recruited to MDA5 would minimize any delay in activation of MDA5 following RNA detection. Although MDA5 does not adopt an auto-repressed conformation as does RIG-I, it is still likely that rearrangement occurs following MDA5-RNA interaction to allow the binding of the PP1 holoenzyme to the MDA5 helicase domain for CARD dephosphorylation. This could prevent dephosphorylation prior to RNA binding even if the phosphatase was recruited prematurely. Additionally, it could be beneficial if the PP1 regulatory subunit was IFN-inducible leading to amplification of RLR signaling throughout viral infection.

If RIG-I and MDA5 share a regulatory subunit for PP1-mediated dephosphorylation, their differences in ligand specificity could explain their differences in activation kinetics. However, it is not necessary that a single regulatory subunit control PP1-mediated dephosphorylation of both RIG-I and MDA5. This difference could add another level of complexity to the innate immune response by these related PRRs. Distinct regulatory subunits may also explain how the paramyxovirus V proteins target PP1 to prevent MDA5 but not RIG-I activation despite its position as a common RLR regulator. We have speculated that this disparity could also be due to the binding of the V protein to MDA5, localizing it near the relevant PP1 holoenzymes when they are recruited for S88 dephosphorylation. While this is a possibility, it is also possible that an MDA5-specific PP1 regulatory subunit could be the target for the V protein. We have also shown that the MV V protein is itself dephosphorylated by PP1. Perhaps the same regulatory subunit that targets PP1 to MDA5 is required for interaction with the V protein. Importantly, the precise role of PP1-mediated dephosphorylation of the V protein remains unclear. We know that
the V protein association with and dephosphorylation by PP1 prevents MDA5 dephosphorylation, but whether dephosphorylated V has modulated activity is unknown.

Concurrent with our studies of the MV V protein targeting of PP1 for MDA5 antagonism, Mesman, et al. identified a V protein-independent mechanism for RLR antagonism via PP1 inhibition in dendritic cells\textsuperscript{394}. In our collaborative study, we saw that MV virion binding to DC-SIGN prevented both RIG-I and MDA5 dephosphorylation at early time points. This was due to activation of the Raf-1 signaling cascade leading to the phosphorylation of PP1 subunit inhibitor-1 (I-1) which then interacted with GADD34 preventing PP1-mediated dephosphorylation of the RLRs. This study identified GADD34-PP1 as the holoenzyme responsible for RLR dephosphorylation and activation. Previous studies had indicated that GADD34 may have a role in IFN induction, particularly in DCs and macrophages\textsuperscript{422-424}. It is feasible, given the differences in innate immune signaling between DCs and non-immune cells, that different regulatory mechanisms control these pathways in specific cell types. Further study will be necessary to determine if GADD34 acts as the RLR-specific PP1 regulatory subunit in other cells or if this is a DC-specific response.

**Kinase responsible for inhibitory phosphorylation of MDA5 S88.**

RIG-I S8 and T170 are constitutively phosphorylated by the kinases PKC\textalpha/\textbeta\textsuperscript{58}. In contrast, the kinase(s) responsible for the analogous phosphorylation of MDA5 S88 remains unknown. Unpublished data from our lab suggests that PKC\textalpha/\textbeta are not universal RLR regulators, as PKC over-expression had no effect on MDA5 S88 phosphorylation. The ideal kinase for MDA5-S88 phosphorylation would be constitutively expressed and active in the uninfected cell. Expression or activity of the kinase may be down-regulated following virus infection, decreasing the activation threshold for MDA5 signaling. Kinase prediction software
indicates that the motif encompassing S88 may be a consensus site for RSK, CAMK, and MAPK type kinases. These families include a vast number of kinases and prediction software is not always accurate so a screen would likely be the most efficient method to identify the kinase responsible for MDA5-S88 phosphorylation. This is a key open question in MDA5 regulation.

When we began our experiments looking at V protein antagonism of MDA5, we considered that the V proteins may recruit a kinase to keep MDA5 S88 phosphorylated rather than inhibiting MDA5 dephosphorylation by PP1. While this turned out not to be the case for MV, it is plausible that other viruses may recruit either physiological or viral kinases to phosphorylate MDA5, preventing its activation and downstream signaling.

4.2.C. Regulation of MDA5 CARD domain signaling

Following dephosphorylation of the RIG-I CARD domains, TRIM25 is recruited to ubiquitinate K172 in the first CARD domain with K63-linked polyubiquitin chains. This ubiquitination has been shown to be required for interaction with MAVS. Mechanistically, the ubiquitin chains wrap around the RIG-I CARD domains, stabilizing a tetrameric interaction which imprints a lock-washer interaction on MAVS CARD domains for oligomerization into signaling competent aggregates. The MDA5 CARD domains are also thought to oligomerize to form complexes that induce MAVS aggregation and signaling. In contrast to RIG-I activation, the MDA5 CARD domains have not been shown to be ubiquitinated. One study has indicated that, like RIG-I, MDA5 may bind to unanchored K63-linked ubiquitin chains; however, other groups did not see an activating role of K63-ubiquitination for MDA5. This represents yet another critical difference between RIG-I and MDA5 regulation. In vitro assays have indicated that the MDA5 CARD domains have a high intrinsic ability to form oligomers at high concentrations. This is in contrast to RIG-I CARD oligomerization which requires K63-linked
ubiquitination to form higher order complexes. It has been suggested that MDA5’s higher intrinsic oligomerization may negate the need for the stabilization induced by K63-linked ubiquitination. This is also reflected in the RNA binding properties of MDA5 versus RIG-I, where MDA5 forms much longer filaments along dsRNA, increasing the local concentration of MDA5 CARD domains, allowing their intrinsic oligomerization and activation of MAVS. An important caveat to these studies is that they were all done in a cell-free system. The formation of these long MDA5 filaments has not been seen in cells, so it is possible that the physiological mechanism of CARD oligomerization varies from what is seen in vitro. It has been shown in cells that TRIM25 does not catalyze K63-ubiquitination to MDA5 2CARD68. If the MDA5 2CARD is ubiquitinated, it is likely that a different E3 ligase is responsible. A more intriguing possibility is that MDA5 CARD is stabilized by ubiquitin-like (Ubl) modifiers. Recently, increasing evidence has shown the importance of these modifications in signaling cascades. Some of these Ubl modifiers are IFN inducible. The conjugation of such modifiers would be of particular interest as it would facilitate a positive feedback loop similar to the IFN-inducible state of TRIM25 for RIG-I activation. Alternatively, additional cellular factors may be required for optimal MDA5 oligomerization and signaling. Further systematic studies will be necessary to identify additional regulatory mechanisms for MDA5 CARD signaling activity.

The final step following RIG-I or MDA5 activation is the translocation of the activated RLR to MAVS on the mitochondria for downstream signaling. For RIG-I, the mitochondrial chaperone protein 14-3-3ε forms a “translocon complex” with ubiquitinated RIG-I and TRIM25 and relocalizes them to MAVS77. How activated MDA5 is targeted to MAVS remains an unanswered question. It is unknown if 14-3-3ε also completes this translocation or if additional
chaperone proteins are involved. The kinetics of an additional chaperone may partially explain the differences in kinetics between RIG-I and MDA5 activation.

In the last several years, great advancement has been made in our understanding of RLR regulation and signaling; however, much more needs to be done, in particular to clarify the regulatory mechanisms governing MDA5 activity.

4.3. Controversial Role of LGP2 in IFN Induction

One of the largest controversies in RLR regulation and function is the role of the third family member, LGP2. Unlike RIG-I and MDA5, LGP2 does not possess the N-terminal tandem CARD domains, rendering it incapable of direct signaling through MAVS. The lack of CARD domains has led to the assumption that LGP2 acts as a regulator of RIG-I and MDA5 function; however, the precise mechanism of this regulation remains unclear. Initial studies of LGP2 function identified it as an IFN-inducible, dominant-negative regulator of both RIG-I and MDA5 with the proposed mechanism of RNA sequestration\textsuperscript{12,13}. Multiple structural studies have suggested that the LGP2 CTD binds to the ends of blunt dsRNA independent of a 5’ppp\textsuperscript{425-428}. Additional studies implied that LGP2 functions by making inhibitory protein-protein interactions with RIG-I or MAVS. First, the LGP2 RD was thought to bind to RIG-I and inhibit activation similar to RIG-I auto-regulation\textsuperscript{55}. An additional study showed that LGP2 binds to MAVS, but not via the CARD domain. Instead, LGP2 interacted with the long MAVS cytoplasmic region, preventing association with the downstream kinase IKKe\textsuperscript{429}. LGP2\textsuperscript{−/−} mouse studies intended to clarify the physiological role of LGP2 only made the field more confusing by indicating that LGP2 has an inhibitory role in RIG-I signaling, but an activating role for MDA5 signaling. VSV infection in these mice was less lethal while EMCV infection was more lethal\textsuperscript{15}. Another study
generated new LGP2/- mice and found that they had a diminished response to several virus infections including VSV, SeV, Reovirus, and EMCV among others\textsuperscript{14}. Interestingly, this study saw no differences in IFN induction following IAV infection or poly(I:C) transfection, implying that there may be a stimulus-specific LGP2 response. This study also generated a transgenic mouse expressing LGP2 with a K30A mutation abrogating ATPase function. LGP2\textsuperscript{K30A/K30A} mice displayed IFN responses similar to LGP2/- mice, indicating that the positive regulatory role of LGP2 for both RLRs is dependent on ATPase function\textsuperscript{14}. Additional studies also showed LGP2 ATPase function to be essential for the synergistic activation of MDA5 signaling in response to EMCV infection or poly(I:C) transfection\textsuperscript{430}.

It has become clear in recent years that the intracellular concentrations of LGP2 have some bearing on its ability to regulate RLR signaling, especially the MDA5-mediated response. These studies show that at low concentrations, LGP2 acts as a positive regulator of MDA5. As LGP2 protein levels increase, either through its IFN-inducibility or in over-expression experiments, MDA5 signaling is inhibited\textsuperscript{16,431,432}. From these studies, it has been suggested that LGP2 regulates MDA5 recognition of RNA for multiple reasons: 1) MDA5 affinity for RNA is increased in the presence of LGP2\textsuperscript{16}, 2) MDA5 and LGP2 have both been shown to cooperatively bind to RNA\textsuperscript{425,427}, and 3) MDA5 and LGP2 only interact with each other when dsRNA ligand is present\textsuperscript{431}. In fact, one study identified a viral RNA ligand of MDA5 indirectly by purifying LGP2-RNA complexes from EMCV infected cells\textsuperscript{433}. When this RNA was subsequently transfected into cells, MDA5 was activated to induce IFN. More recently, Bruns, et al., put forth a structural model by which LGP2 regulates MDA5 function\textsuperscript{16}. MDA5 must cooperatively bind to dsRNA in order for the 2CARD domain to oligomerize and signal through MAVS. However, studies have shown that if MDA5 filaments become too long, signaling is
diminished. In their recent study, Bruns et al., show that LGP2 is intricately involved in the regulation of MDA5 filament length. In their model, LGP2 binds to RNA with a fast on-rate and caps the ends of MDA5-RNA filaments preventing further addition of MDA5 and keeping the filaments the optimal length for interaction with MAVS. The ATPase function of LGP2 is critical to this function; however, in contrast to MDA5 ATP hydrolysis which results in dissociation from RNA, ATP hydrolysis by LGP2 promotes RNA interaction. The limitation of MDA5-RNA filament length by LGP2 may explain why long MDA5 filaments have not been observed in infected cells. While this is a fascinating model, it remains to be seen if it has physiological relevance. If this model does hold true, it should be possible to visualize long MDA5 filaments in LGP2−/− or LGP2K30A/K30A cells.

One particularly intriguing aspect of LGP2 biology is that it is also targeted by the paramyxovirus V proteins. Early mapping studies of the interaction between the V proteins and MDA5 pointed out that the minimal binding region of MDA5 is very similar (57% identity and 78% similarity) to the LGP2 helicase. In fact, later studies implicated a single residue as important for V-MDA5 interaction, R806 in MDA5. This residue is also conserved in LGP2 (R455) and mediates interaction with MV-V. These studies showed that the V protein antagonizes the ATPase activity of LGP2. The precise role of this inhibition in LGP2 function is unknown; however, one study suggested that the PIV5 V protein blocked the interaction between MDA5 and LGP2 on RNA. This would be consistent with the model of LGP2 control of MDA5 filament length. It is possible that the V protein targets LGP2 to more completely inhibit MDA5 signaling. One additional study observed an LGP2-dependent inhibitory function of the V protein on RIG-I signaling. The authors suggest that the V protein
stabilizes the inhibitory interaction between RIG-I and the LGP2 RD\textsuperscript{321}; however, these results were not replicated in additional studies\textsuperscript{17} and their physiological relevance remains unclear.

The role of LGP2 in the RLR-mediated IFN response remains controversial. In particular, little effort has focused on how RIG-I might be inhibited or enhanced by LGP2. The evidence of LGP2’s role in RIG-I signaling has been inconsistent and deserves a concerted effort towards clarification. In contrast, it has become accepted that LGP2 stimulates MDA5 activity. While the model of LGP2 regulation of RNA-bound MDA5 filament length is intriguing, the importance of MDA5 filaments in general, and specifically in relation to LGP2, remains to be seen in a physiological setting. A more complete understanding of the role of LGP2 will also illuminate the role of V protein interaction with the LGP2 helicase.

4.4. Cooperation of RIG-I and MDA5 for Optimal IFN Induction

The majority of studies investigating the function of RLRs have relied on transient over-expression of RIG-I and MDA5 and/or the transfection of synthetic RNA agonists. This leaves the precise physiological roles of RLRs uncertain. Of particular interest is the relatively novel idea that RIG-I and MDA5 may cooperate for optimal IFN induction following virus infection. Early studies in knockout mice indicated that RIG-I and MDA5 recognize distinct subsets of viruses; however, growing evidence reveals that this is an overly simplistic view. Multiple studies have now shown that RIG-I and MDA5 cooperate, likely in a temporal manner, to activate IFN in response to a single viral.

Initial studies in RIG-I\textsuperscript{-/-} mice infected with WNV displayed a delayed, but not abrogated IFN response compared to wild type mice\textsuperscript{434}. Further study showed that MDA5 was activated later during WNV infection and was able to partially compensate for the lack of RIG-I\textsuperscript{47}. These
studies began to show that RIG-I and MDA5 act in a concerted manner to induce an optimal immune response. These findings were confirmed in knockout mice where single RIG-I or MDA5 knockout had an abnormal response to WNV infection. In contrast, double knockout mice had essentially no IFN response and were more susceptible to WNV infection. Furthermore, the mechanism behind this effect has been explained. As WNV infection progresses, multiple different PAMP RNAs are generated. RIG-I responds to RNA species present early in infection, dependent on the presence of 5’ppp. Certain sequence-specific regions of early replication intermediates were also shown to be recognized by RIG-I. In contrast, MDA5 recognizes longer RNA species that accumulate at later times during infection.

Similar to WNV, early studies indicated that both MDA5 and RIG-I are involved in sensing of paramyxoviruses. At first, the contribution of MDA5 was not evident due to the potent antagonism of MDA5 by the V protein. Careful study using V deleted viruses and MDA5−/− cells revealed the activation of MDA5 by paramyxovirus infection. Runge, et al., recently investigated the question of the physiological RLR ligands during MV infection. In order to purify RNA bound to the low affinity interactor MDA5, the authors used a novel protein-RNA cross-linking approach. Purified RNAs were then subjected to deep sequencing. This study identified distinct RNA species associated with RIG-I and MDA5. Both RLRs were able to recognize DI copy-back RNA products and showed preference for AU-rich regions. The amounts of RNA associated with RIG-I were much higher than those associated with MDA5, reflecting their intrinsic RNA binding affinities. RIG-I was found to bind to both negative-stranded genomic RNA and positive-stranded antigenomic and mRNA transcripts dependent on the presence of a 5’ppp. On the other hand, MDA5 was only found associated with positive-stranded mRNA transcripts derived from the L gene. This study confirms prior in vitro studies...
suggesting that different RNA species are recognized by individual RLRs and also corroborates that RIG-I and MDA5 cooperate for detection of a single virus. The preference of MDA5 for AU-rich regions of RNA was explained through functional studies. The ATPase function of MDA5 was reduced with RNA of high AU percentage. Because ATP-hydrolysis by MDA5 leads to its dissociation from RNA, the authors speculate that this impaired activity leads to higher affinity interaction with AU-rich RNA.

The RNA purification in this study was done at 24 hours following infection, a relatively late time point for MV infection. It would be interesting to look at a time-course of infected cells. The enrichment seen in RIG-I-associated RNA may reflect this late time point - RIG-I is activated early in infection by genomic and DI RNAs, potentially outpacing the RNA binding of MDA5 at later time points by additional RNA intermediates. This temporal activation was also indicated in our collaborative study looking at MV antagonism of RLRs in DCs. We saw that at early time points, both RIG-I and MDA5 were inhibited by DC-SIGN signaling. However, at later times during infection, this inhibition is released and only MDA5 remains inhibited through the actions of the V protein. This would correlate to the production of RIG-I-specific PAMP RNAs early in infection and MDA5-specific PAMPs later in infection.

These are the first full reports of RIG-I and MDA5 cooperating to sense viral infection, but it seems likely that this will hold true for additional virus infections as well. Of particular interest are those viruses already known to be sensed by both RIG-I and MDA5 including other flaviviruses and reoviruses. In fact, one recent study suggested a role for MDA5 in the response to hepatitis C virus (HCV). Like paramyxoviruses where the V protein potently inhibits MDA5, HCV encodes a protease, NS3A, which cleaves MAVS from the mitochondria, abrogating any RLR-mediated IFN production. Many studies investigating RLR detection of
HCV have shown that RIG-I plays a primary role by detecting both the 5’ppp end of the genome as well as the structure of the poly-U/UC stretch in the 3’ UTR\(^{382}\). To investigate a possible role for MDA5 in HCV infection, the authors generated Huh7.5 cells expressing a cleavage-resistant MAVS mutant\(^{52}\). Huh7.5 are defective in RIG-I activity; however, IFN-β was induced following HCV infection in these cells. These results were confirmed by transient knockdown of RIG-I or MDA5 in primary hepatocytes. Knockdown of each RLR resulted in an incomplete defect in IFN-β induction, indicating that RIG-I and MDA5 are both important for HCV detection. The HCV 3’ UTR poly-U/UC region has long been known to be a RIG-I specific PAMP; however, this study indicates that MDA5 may recognize an additional, uncharacterized HCV RNA PAMP\(^{52}\). The exact details and contributions of the two RLRs to HCV detection require further research.

While some virus-specific RNA PAMPs have been identified, the physiological ligands for RLRs are only beginning to be defined for specific viruses. Further studies should be focused on identifying these ligands and the individual and cooperative contributions of RIG-I and MDA5 to IFN induction in response to viral infection. Similar cross-linking and immunoprecipitation techniques could be used to identify the physiological ligands of RLRs during infection. Perhaps most intriguing would be the identification of the RNA intermediates that serve as RIG-I ligands during DNA virus infections such as HSV-1 and adenovirus.

4.5. Emerging Role for MDA5 in Autoimmune Disease

The importance of proper regulation of innate immune responses has become particularly evident in recent years with the discovery that aberrant immune activation can lead to autoimmune disorders. Single nucleotide polymorphisms (SNPs) in \textit{ifih1}, the gene encoding
MDA5, have been implicated in the pathogenesis of multiple autoimmune disorders including systemic lupus erythematosus (SLE), Aicardi-Goutières syndrome (AGS), and type I diabetes (T1D).

SLE is an autoimmune disease that results in chronic, multisystem inflammation including that of the skin, joints, and kidneys. Multiple factors have been linked to SLE, including both genetic and environmental factors. One key signature of SLE and other types of inflammatory disease is elevated basal IFN production. In fact, IFN therapy administered to treat other diseases can induce spontaneous development of SLE-like symptoms which abate after IFN therapy is terminated. Elevated IFN does not necessarily induce SLE progression and has been dubbed a “heritable risk factor,” as it is present in both affected and unaffected members of SLE families. Multiple causes for this elevated IFN have been found in individuals, including TLR7 polymorphisms and the production of auto-antibodies to nuclear factors such as dsDNA.

AGS is an inheritable encephalopathy due to inflammation in the brain. AGS patients typically display intellectual disabilities and dystonia. AGS is lethal in approximately 25% of patients by 17 years of age. Many genes linked to innate immune recognition and metabolism of nucleic acids have been associated with progression of AGS including TREXI, SAMHD1, ADARI, and multiple RNaseH genes. AGS shares many commonalities with SLE, including the association of elevated IFN production as a risk factor.

Recently, multiple studies have identified SNPs in ifih1 (MDA5) which are linked to SLE and/or AGS development through GWAS (genome wide association studies). These mutations led to hyperactivation of MDA5 signaling. The first SNP implicated in SLE results in
an A946T change in the helicase domain. This SNP had previously been associated with increased risk for type 1 diabetes (T1D) and the autoimmune hypothyroidism disorder Graves’ disease\textsuperscript{445,446}. Additional studies identified at least seven more SNPs in \textit{ifih1} in SLE and AGS patients, all within the helicase domain\textsuperscript{442}. Each of these mutations led to an increase in basal MDA5 activity. The authors speculate that this results from an increase in MDA5-RNA binding avidity, but the constitutive RNA ligand was not identified. It remains to be determined if this RNA ligand is derived from a persistent viral infection or misrecognition of host RNA.

An additional study identified a similar mutation in mice that leads to a lupus-like illness. \textit{Funabiki, et al.}, generated mice using ENU (N-ethyl-N-nitrosurea) mutagenesis. One subset of these mice developed a heritable condition with lupus-like nephritis. These mice harbored an \textit{ifih1} SNP resulting in a G821S mutation. Unlike the previously identified human SNPs, this mutation resulted in hyperactive MDA5 signaling even in the absence of ligand. Additionally, G821S MDA5 did not respond to viral infection, suggesting a complete dysregulation of MDA5 activity. The authors further examined this mutation as well as the previously described A946T. Atomic force microscopy indicated that each of these mutations resulted in a conformational change in comparison to wt MDA5. The authors speculate that this conformation is more suited to signaling but not RNA binding, resulting in the phenotype of constitutive MDA5 signaling in absence of ligand\textsuperscript{444}.

Just this year, an additional autoimmune disorder has been linked to a gain-of-function mutation in MDA5. Singleton-Merton syndrome, a disorder characterized by aortic calcification, osteopenia, and dental anomalies, had a high association with an R822Q mutation\textsuperscript{447}. This is directly adjacent to the G821S mutation generated in the mouse model of SLE and may therefore have a similar impact on MDA5 conformation and function.
Conversely, multiple SNPs in *ifih1* have been shown to convey resistance to certain autoimmune diseases. Two SNPs associated with protection from T1D were found to cause loss of function mutations in MDA5: an E627* truncation and I923V^{448,449}. The E627* truncation loses the ability to bind to dsRNA, and therefore is unable to signal. In contrast, the I923V mutation in the CTD is still able to bind to dsRNA but remains nonfunctional for unknown reasons^{449}. A similar effect of loss-of-function mutations in MDA5 was seen for psoriasis protection. Presence of these mutations (I923V or H460R) resulted in a decreased risk for developing psoriasis in patients^{450,451}.

The overarching question of how gain-of-function mutations can cause constitutive MDA5 activity is unclear. In addition to RNA binding, activation of MDA5 requires dephosphorylation of the CARD domains and subsequent interaction with MAVS. Some of the described mutations caused by the identified SNPs seem to increase overall RNA binding ability, which would not be at odds with the normal activation cascade of MDA5. However, Funabiki, *et al.*, indicate that the G821S and A946T mutations do not require RNA for their constitutive signaling. This raises the question of how the CARD domains are activated without a proper ligand signal. The authors also speculate that these mutations correlate with a conformational change. How this change in conformation would induce signaling is unclear. It would be interesting if this conformational change allows the constitutive binding of PP1, resulting in dephosphorylated MDA5 S88. Alternatively, this conformation could preclude kinase interaction and S88 may not be phosphorylated for inhibition. These are vital questions which require further investigation for a complete understanding of how these mutations affect MDA5 activity.

The association of certain viruses with autoimmune disorders can perhaps provide a link between the genetic and environmental inducers of these diseases. It is easy to imagine that
individuals with a gain-of-function SNP in MDA5 may mount an aberrant immune response to infection, leading to over-activation of the immune response. Specifically, EBV infection has been linked to the development of SLE. The primary mechanism for this seems to be molecular mimicry in antibody responses, as antibodies against the viral protein EBNA-1 cross-react against nuclear factors acting as auto-antibodies\textsuperscript{452,453}. Similarly, infection with certain picornaviruses (sensed by MDA5) has been correlated with development of diabetes\textsuperscript{454,455}. Precisely how enteroviruses may predispose individuals to development of T1D remains unclear; however, in some patients, direct infection and destruction of pancreatic $\beta$-cells has been observed\textsuperscript{454}. This is a particularly interesting link which is not fully explained by the association of MDA5 SNPs with T1D. The gain of function mutation may lead to an over-active immune response and destruction of $\beta$-cells instead of virus clearance. However, the role of loss-of-function SNPs in protecting individuals from T1D development is not explained in this situation. Further investigation is necessary to fully explain the link between viral infection, MDA5 SNPs, and autoimmune disorders.

4.6. Concluding Remarks

The co-evolution of virus and host has often been described as an arms race, where the host mounts an effective immune response and viruses evolve ways to antagonize these responses. Our data detail one small battle in this arms race, how certain paramyxoviruses target the regulatory phosphorylation of MDA5 to prevent effective activation of the IFN response. Specifically, the V proteins of measles, mumps, Nipah, Hendra, and Menangle viruses interact with the phosphatases PP1$\alpha/\gamma$ to block dephosphorylation and activation of MDA5. The precise mechanism of this inhibition has been determined for MV-V. The V protein interacts with PP1,
sequestering it away from MDA5 and also acts as a substrate for its dephosphorylation. The mechanisms relating to the other V proteins may vary, in particular because there is no conserved PP1-binding site. This represents an important step in deconvoluting the functions of the V proteins of specific paramyxoviruses, which have often been generalized to a single mechanism.

As our knowledge of the regulation of the innate immune system in general, and MDA5 in particular, grows, we discover new methods by which viruses target these systems for their replication needs. There are many open questions in MDA5 activity, including additional roles of post-translational modifications and regulatory proteins. The function of the less-well studied RLR, LGP2, also remains a mystery. More recently, evidence indicates roles for MDA5 not only in virus detection, but also reveals naturally arising mutations that may serve as markers for predisposition or protection from certain autoimmune disorders. A more complete understanding of these naturally occurring variants will also shed light on the typical functions of MDA5.
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Paramyxovirus evasion of innate immunity: Diverse strategies for common targets
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