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## Molecular mechanisms of innate immune inhibition by non-segmented negative-sense RNA viruses

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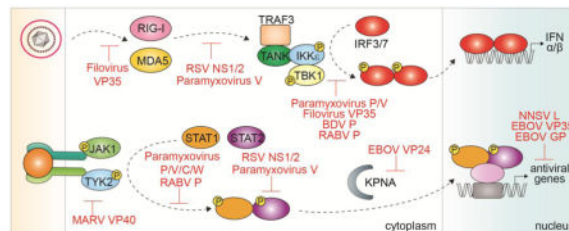
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### Abstract

The host innate immune system serves as the first line of defense against viral infections. Germline encoded pattern recognition receptors (PRRs) detect molecular patterns associated with pathogens and activate innate immune responses. Of particular relevance to viral infections are those PRRs that activate type I interferon (IFN) responses, which establish an antiviral state. The Order *Mononegavirales* is comprised of viruses that possess single-stranded, non-segmented, negative-sense (NNS) RNA genomes and are important human pathogens that consistently antagonize signaling related to IFN responses. NNS viruses (NNSVs) have limited encoding capacity compared to many DNA viruses. And as a likely consequence, most open reading frames (ORFs) encode multifunctional viral proteins that interact with host factors in order to evade host cell defenses while promoting viral replication. In this review, we will discuss the molecular mechanisms of innate immune evasion by select NNSVs. A greater understanding of these interactions will be critical in facilitating the development of effective therapeutics and viral countermeasures.

### Graphical Abstract



### Keywords

*Mononegavirales*; interferon antagonist; innate immune evasion; viral antagonism

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## Introduction

The onset of viral infections are first detected by the host innate immune system that stimulates the production of type I interferons (IFNs) and pro-inflammatory cytokines, which can limit multiple stages of the viral life cycle. Viruses can subvert host defenses by encoding for proteins that inhibit innate immune responses. Single-stranded, non-segmented, negative-sense RNA viruses (NNSVs) in the Order *Mononegavirales* include many efficient pathogens which encode multifunctional proteins. Versatile NNSV proteins have the ability to participate in multiple host-virus interactions in order to evade, antagonize, and disrupt host antiviral protein functions. A detailed understanding of host-virus relationships is crucial to the development of countermeasures to mitigate NNSV infections. Here we focus on the mechanistic basis of molecular interactions occurring between NNSV-encoded proteins and the host proteins of the IFN signaling cascade. This review is organized and presented in two major categories: 1) type I IFN induction due to viral infection and NNSV-mediated inhibition of IFN induction, and 2) type I IFN responses to viral infection and NNSV-mediated inhibition of IFN responses; this is followed by a brief discussion on NNSV-mediated modulation of the host ubiquitination system. While much work has been done in this area, NNSVs use few but multifunctional proteins to disrupt essentially every step in the IFN pathway. The key host-viral interactions described in this review will be important targets to develop novel countermeasures against NNSVs.

### Genome organization of NNSVs

NNSVs are divided into five viral families: *Bornaviridae*, *Filoviridae*, *Paramyxoviridae*, *Rhabdoviridae*, and *Nyamiviridae*, where the genomes range from 8 to 19 kilobases in size and contain 6 to 11 open reading frames (ORFs) within a single strand of RNA (Fig. 1). The proteins common to all NNSVs include nucleoprotein (N/NP), phosphoprotein (P), matrix protein (M), glycoprotein (G/GP), and the RNA-dependent RNA polymerase (RdRp) also known as large (L) protein. These proteins generally have conserved functions. For example, N/NP coats the viral RNA forming the nucleocapsid whereas G/GP permits attachment and fusion to the host cell. The M protein facilitates viral assembly and budding, and the L polymerase forms part of the viral replication complex along with the P protein [1, 2]. Bornaviruses and Nyamiviruses have an X protein that is involved in regulation of gene synthesis and immune inhibition [3–6]. Filoviruses and paramyxoviruses have more complex genomes that encode for several additional proteins. In filoviruses, VP35 and VP40 are the counterparts to the P and M proteins. In addition, filoviruses contain VP24 and VP30 proteins, which function as the minor matrix protein and to regulate replication/transcription, respectively. Some paramyxoviruses, including henipaviruses, rubulaviruses, pneumoviruses, and metapneumoviruses, also produce a fusion (F) protein and a small hydrophobic (SH) protein that are associated with the viral envelope to facilitate viral entry. The M2 protein found in pneumoviruses and metapneumoviruses are involved in regulation of replication/transcription. Finally, the non-structural proteins NS1 and NS2 are unique only to pneumoviruses that are not packaged as part of the virion, but are critical for inhibition of IFN induction and signaling [7, 8].

The limited RNA genome of NNSVs in some ways can restrict the genomic diversity and redundancy that is often observed in DNA viruses [9]. As a potential mechanism to overcome limited coding capacity of NNSVs, many NNSV encoded proteins perform multiple functions at distinct stages of the viral replication cycle. Recent studies are beginning to elucidate the molecular basis of the common and unique strategies employed by these viral proteins to evade host innate immune responses while promoting viral replication. In the following sections, we will provide an overview of the host type I interferon responses and discuss the different immune evasion strategies of NNSVs that target various points of the IFN  $\alpha/\beta$  signal transduction pathway (Fig. 2).

## Type I IFN induction during viral infection

The innate immune system is an evolutionarily conserved branch of the host response that serves as the first line of defense against invading pathogens. Detection of viral infection is mediated by pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs) absent in the host. Two major types of PRRs are RIG-I like receptors (RLRs) and Toll-like receptors (TLRs) [1, 10]. RLRs, including retinoic acid inducible gene I (RIG-I) and melanoma associated differentiation factor 5 (MDA5), are cytoplasmic proteins containing N-terminal caspase activation and recruitment domains (CARDs), a central DExD/H box helicase domain, and C-terminal RNA binding domains that recognize PAMPs from viral genomic material, including those generated due to viral replication within the host cells [11, 12]. RIG-I is activated by short double stranded RNA (dsRNA) with 5'-ppp or 5'-OH groups whereas MDA5 preferentially binds to longer lengths of dsRNA [11–17]. Signaling through CARD-CARD interaction between RLRs and the mitochondrial activator of viral signaling (MAVS, also known as IPS-1, VISA, or Cardif) located on the mitochondria [18, 19] (Fig. 2A) results in the activation of TANK binding kinase (TBK1) and inhibitor of nuclear factor kappa-B kinase subunit  $\epsilon$  (IKK $\epsilon$ ) through association with TANK (TRAF family member associated NF $\kappa$ B activator) and TRAF3 (TNF receptor associated factor 3). TBK1/IKK $\epsilon$  kinases phosphorylate the transcription factors interferon regulatory factors 3 and 7 (IRF3 and IRF7), which dimerize and translocate into the nucleus to activate the expression of type I IFNs [10].

TLRs are integral membrane glycoproteins that are present in cell, endosomal, and lysosomal membranes. TLR molecules bind to PAMPs through their extracellular domains, which contain leucine rich repeats. TLR2/4 molecules located in cell membranes bind to viral envelope proteins. TLR7/8 and TLR3 detects single stranded RNA (ssRNA) and dsRNA, respectively, in the endosomal compartments [17]. TLR3 activation followed by recruitment of the TIR domain containing adaptor-inducing IFN- $\beta$  (TRIF) or TLR7/8 activation and the recruitment of adaptor proteins myeloid differentiation factor 88 (MyD88) can also induce type I IFN production by activating IRF3 and nuclear factor kappa-B (NF $\kappa$ B) [17]. Signaling through MyD88 leads to the phosphorylation of inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta (IKK $\beta$ ). MyD88-mediated signaling ultimately leads to the activation of NF- $\kappa$ B that stimulate the expression of proinflammatory cytokines, which limit virus replication through a variety of mechanisms (Fig. 2A) [20, 21].

## NNSV-mediated inhibition of IFN induction

Type I IFN signaling functions to restrict viral infection and therefore, it is not surprising that viruses have developed strategies to circumvent or inhibit this system. Because PRRs provide immune surveillance to the cell and are the first to detect invasion by foreign particles, these receptors are highly targeted by viral proteins. However, as discussed in the examples below, NNSVs have developed mechanisms that target many critical steps in the IFN signaling cascade.

**Sequestration of viral RNA PAMPs**—One of best studied examples of PAMP sequestration by NNSVs is in filoviruses. Ebola virus (EBOV) and Marburg virus (MARV) VP35 proteins bind viral dsRNA to prevent their recognition by PRRs RIG-I and MDA5 [22, 23]. Structural studies of the C-terminal IFN inhibitory domain (IID) of EBOV VP35 in complex with an 8 bp *in vitro* transcribed RNA showed that a series of conserved basic residues facilitate binding of EBOV VP35 to the phosphodiester backbone of dsRNA (Fig. 3A and B) [23]. Mutational analysis of these residues, particularly Arg312, shows that these basic residues are important for dsRNA binding and IFN inhibition [22]. In fact, substitution of Lys319 and Arg322 with alanines renders a guinea pig adapted EBOV avirulent [24]. EBOV VP35 also caps the blunt ends of dsRNA through hydrophobic residues Phe235 and Phe239[22]. This dual interaction provides a mechanism that allows EBOV VP35 to efficiently sequester dsRNA from detection by and activation of RIG-I and MDA5. Similarly, MARV VP35 coats the dsRNA backbone. Although MARV VP35 is structurally homologous to EBOV VP35, with a backbone rmsd < 1.0 Å [23], MARV VP35 is unable to endcap dsRNA and preferentially binds longer dsRNA that are targeted by the RLR MDA5 [19, 23, 25] (Fig. 3C). These differences in MARV VP35 recognition of dsRNA motifs manifests in less efficient inhibition of RIG-I signaling compared to EBOV VP35 [26]. The impact of these interactions *in vivo* is likely to be more complex than indicated by the *in vitro* studies. However, the correspondence between *in vitro* studies that evaluate RNA sequestration with *in vivo* studies of corresponding mutant viral infections support the relevance of these mechanisms.

**Direct binding to RLR**—The V proteins of paramyxoviruses can bind to MDA5 and LGP2, but not RIG-I, in order to disrupt downstream signaling responsible for IFN induction [27–31]. The crystal structure of the parainfluenza virus 5 (PIV5) V protein in complex with MDA5 provides the molecular basis of how the V protein disrupts MDA5 function [27] (Fig. 4). The V protein C-terminal domain undergoes a large conformational change upon binding to MDA5 and refolds into a long  $\beta$ -hairpin stabilized by two zinc ions. This long hairpin displaces in MDA5 two  $\beta$ -strands  $\beta$ 1 and  $\beta$ 6 and the ATPase motif VI and is stabilized by two key interactions, a salt bridge between Glu174 of V and Arg803 of MDA5/LGP2, and a hydrophobic interaction between Trp179 of V and Gly805 of MDA5/LGP2. Consequently, V protein binding disrupts the ATPase activity of MDA5, which is required in the formation of a signaling platform with the CARD domains of MAVS [27, 32]. In RIG-I, Glu803 and Gly805 residues are substituted with a leucine and a glutamate, thus precluding its complex formation with V proteins [27, 31]. These structural observations suggest that specific inhibition of MDA5 and LGP2, but not RIG-I, is likely important for some paramyxoviruses in order to evade host innate immunity. While direct binding provides a highly effective

mechanism, modulation of post transcriptional modifications can also facilitate evasion of host immunity (See RLR dephosphorylation section).

The non-structural proteins NS1 and NS2 of respiratory syncytial virus (RSV) also appear to directly target the interaction between RIG-I and MAVS. Live cell imaging of a GFP expressing strain of RSV showed that the virus localized to the mitochondria, which is a signaling hub for IFN production [33]. Furthermore, immunofluorescence and electron microscopy show that transfected RSV NS1 colocalizes with MAVS, consistent with immunoprecipitation studies showing that MAVS binds to NS1 and prevents MAVS binding to RIG-I [33] (Fig. 5A). Similarly, RSV NS2 has been reported to disrupt RIG-I and MAVS binding through interaction with the N-terminal CARD domains of RIG-I (Fig. 5A). Coimmunoprecipitation studies show a loss of interaction between a truncated RIG-I containing only the CARD domains and MAVS in the presence of RSV NS2 proteins [34]. Direct targeting of the CARD domains potentially allows the virus to control the effector function of MAVS, which can limit signaling and antiviral responses.

**Suppression of RLR dephosphorylation**—Activation of RLRs requires binding of PAMPs and posttranslational modifications, such as dephosphorylation by protein phosphatase 1 (PP1) isoforms [35–37]. The V proteins from paramyxoviruses Nipah virus (NiV) and measles virus (MeV) can bind to PP1 $\alpha/\gamma$  to inhibit the dephosphorylation of MDA5 at Ser88 [38] (Fig. 5B). The C-terminal tail of MeV V protein contains a consensus PP1-binding motif within residues 288 to 291 consisting of amino acids Arg-Ile-Trp-Tyr-Thr. Deletion of this region results in enhanced dephosphorylation of MDA5 at Ser88 in human dendritic cells and negatively impacts viral propagation in human lung epithelial cells [38]. *In vitro* experiments further show that PP1 is capable of dephosphorylating MeV V proteins as MeV V is phosphorylated at several locations [39–42], suggesting that MeV V acts as a decoy substrate of PP1 to divert the dephosphorylation of MDA5 [38]. However, a consensus PP1 binding site is not present in NiV V protein and inhibition of phosphorylation by NiV and MeV V proteins is limited to MDA5 [38]. Biochemical and cell-biological studies provide important insights into regulatory mechanisms, but structural insights of this process are currently unexplored.

**Suppression of PACT induced RIG-I activation**—RIG-I can be activated by the cellular protein Protein Kinase R activator (PACT) [43–45], although the exact molecular mechanism is poorly understood. Some viral proteins like EBOV and MARV VP35 proteins target PACT in order to inhibit RIG-I signaling [26, 45] (Fig. 5C). PACT induces potent activation of RIG-I dependent IFN $\beta$  promoter activity, which is correlated with an increase in RIG-I ATPase activity. Expression of the EBOV VP35 C-terminal domain suppresses RIG-I ATPase activity as well as IFN $\beta$  promoter activity [26, 45]. Coimmunoprecipitation studies show that EBOV VP35 binds PACT and disrupts the interaction between PACT and RIG-I [45]. Moreover, EBOV VP35 residues critical for dsRNA binding, including Arg312, Arg322, and Phe239, are required for PACT binding. Interestingly, dsRNA binding does not appear to mediate the interaction between EBOV VP35 and PACT. Further studies are needed to elucidate upon the molecular mechanism of how PACT binding to VP35 regulates RIG-I activity as well as the role of PACT binding to VP35 on viral polymerase activity as

VP35 functions as a cofactor for the filoviral replication complex. [45–47]. The function of PACT in promoting translational inhibition through PKR activation requires additional studies in order to define the cellular role of its impact.

**Viral inhibition of transcription factors (IRF3/7)**—A large body of evidence suggests that several viral proteins, including Borna disease virus (BDV) and Rabies virus (RABV) P proteins, filoviral VP35 protein, and paramyxoviral V protein, block the phosphorylation of IRF3/7 by IKK $\epsilon$ /TBK1 kinases by acting as surrogate substrates, thereby preventing the translocation of IRF3/7 into the nucleus [39, 48–51] (Fig. 2A & Fig. 5D). These studies show that coexpression of the viral P/VP35/V proteins with IKK $\epsilon$ /TBK1 prevent phosphorylation of IRF3/7 in a dose-dependent manner. In addition, these viral proteins in turn are phosphorylated by IKK $\epsilon$ /TBK1. In the case of EBOV VP35, the binding site involves the kinase domain of IKK $\epsilon$  [50]. Incidentally, the kinase domain of TBK1 is highly homologous to that of IKK $\epsilon$ , which explains how VP35 can target both IKK $\epsilon$  and TBK1. However, it is currently unknown if these viral proteins act alone or in concert with other viral or cellular factors to block IKK $\epsilon$ /TBK1 activity.

EBOV VP35 has also been shown to interact with IRF7 and the SUMO E3 enzyme PIAS1 [52] through the N-terminus. VP35 binding potentially enhances the SUMOylation of IRF7 by forming a quaternary complex with IRF7, the E2 enzyme Ubc9, and PIAS1 [52]. This leads to the downregulation of IRF7 and inhibit IFN production.

## Type I IFN responses to viral infection

Type I IFNs,  $\alpha$  and  $\beta$ , are master regulators of antiviral responses [53, 54]. IFN $\alpha$  is predominantly produced by hematopoietic cells, including plasmacytoid dendritic cells, while IFN $\beta$  is more broadly expressed [55, 56]. IFN $\alpha$ / $\beta$  can act in an autocrine or paracrine fashion and bind to the IFN $\alpha$ / $\beta$  receptor (IFNAR) to activate the Janus kinase 1 (JAK1) and Tyrosine kinase 2 (Tyk2), which phosphorylate STAT1 and STAT2 (Fig. 2B). Phosphorylated STAT1 is recognized by a subset of the karyopherin  $\alpha$  (KPNA) family of nuclear transport proteins, the NPI-1 subfamily, which translocate STAT1-containing complexes to the nucleus [57, 58]. The phosphorylated STAT1/STAT2 heterodimer forms a ternary complex along with IRF9 in the nucleus that stimulates transcription of IFN stimulated genes (ISGs) through the IFN-stimulated gene response elements (ISREs). ISGs can inhibit different stages of viral infection including entry, replication, transcription, translation, assembly, and egress [59, 60]. As a result of the actions of ISG expression, an overall antiviral state is achieved within the infected and neighboring cells. Some specific functions of ISGs include inhibition of viral replication and transcription, degradation of viral nucleic acids, and modulation of lipid metabolism. Some well-studied ISGs include myxovirus resistance 1 (Mx1), IFN-inducible dsRNA-dependent protein kinase R (PKR), 2'-5'-oligoadenylate synthetase (OAS), interferon induced proteins with tetratricopeptide repeats (IFITs), apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC1), tripartite motif containing proteins (TRIM) molecules, and tetherin [20, 59–67].



## NNSV mediated inhibition of interferon response

In addition to the interferon induction signaling pathway, viral pathogens encode for proteins that target the IFN $\alpha$ / $\beta$  response signaling pathway at various points. In the examples described below, NNSVs have developed ways to inhibit JAK/STAT activity, interfere with the nuclear transport of transcription factor STAT1/STAT2, as well as inhibit the activity of IFN stimulated genes.

**Inhibition of phosphorylation of JAK/STAT pathway proteins**—IFN $\alpha$ / $\beta$  binding to IFNAR leads to the phosphorylation of JAK1 and TYK2. Although MARV and EBOV have similar genome organization, only MARV VP40 inhibits JAK1 dependent signaling pathways [68] (Fig. 2B). Expression of MARV VP40 inhibits the tyrosine phosphorylation of JAK1; TYK2, STAT1, and STAT2 in response to IFN $\gamma$ ; and IL6-mediated phosphorylation of STAT1 and STAT3 [68]. This is similar to a JAK1-deficient phenotype, suggesting that MARV VP40 targets JAK1, and not TYK2. Furthermore, MARV VP40 residues Ala57 and Ala165 appear to be important for inhibition of IFN signaling as mutation of these residues results in loss of Jak1 inhibition [68].

Paramyxoviruses, including NiV, Hendra virus (HeV), MeV, and Newcastle disease virus (NDV), also inhibit phosphorylation and activation of STAT1 in response to IFN stimulation (Fig. 2B). These viruses use the P ORF to encode V and W proteins through RNA editing by viral RdRp, and C proteins, which is a result of alternative AUG codon use, in addition to full-length P proteins. P, V, W, and C can target STAT1 to restrict its functions, albeit using independent mechanisms [69, 70]. Structural and biochemical studies of a complex of STAT1 N-terminal domain and the Sendai virus (SeV) C protein has revealed the molecular basis of one such strategy for STAT1 inhibition [70–72]. The C-terminal region (residues 99 to 204) of SeV C protein directly binds to the N-terminal domain of STAT1 (residues 1 to 126). The crystal structure of a STAT1 dimer bound to a dimer of C protein (Fig. 6A) shows how residues Met150 and Arg154 of SeV C protein stabilize this complex [70]. Arg154 makes electrostatic interactions with His58 of STAT1 (Fig. 6B), whereas Met150 inserts into a hydrophobic pocket within STAT1 (Fig. 6C). SeV C proteins carrying mutations at Arg154 and Met150 are unable to bind STAT1 and cannot inhibit IFN $\alpha$  signaling as efficiently as wild type C proteins. Further, structural comparisons of the STAT1-C complex with free STAT1 dimers revealed that C-binding induces conformational changes that allow STAT1 to form a tighter dimer [70]. STAT1 dimers bound to C are also stabilized by additional electrostatic interactions, including an inter-subunit interaction between residues Glu29 and Lys85 and an intra-subunit interaction between Lys85 and Asn82. Molecular models of parallel and antiparallel STAT1 dimers, bound to a monomer or dimer of SeV C protein respectively, were derived from the hetero-tetrameric structure of the STAT1 dimer and SeV C dimer. These models suggest that binding of two C proteins might restrict the STAT1 dimer in a parallel conformation that is not conducive to dephosphorylation at Tyr701. As a result, phosphorylated STAT1 could potentially accumulate in the cytoplasm, forming high molecular weight complexes [70–73].

While P/V/C mediated STAT1 binding retains the latter in the cytoplasm, W binds STAT1 and translocates into the nucleus. This is due to the presence of a classical nuclear

localization signal “KKAR” within the C-terminus of W, formed by the basic residues Lys439, Lys440, and Arg442, which allows it to bind to KPNA3 and KPNA4 [69]. In both cases, interaction of paramyxoviral P/V/C or W proteins leads to the sequestration of STAT1 away from the receptor kinase complexes and prevents phosphorylation of STAT1. Notably, the ability of P to inhibit STAT1 phosphorylation is much less pronounced compared to V and W, which may be due to the additional role of P as a cofactor for the viral polymerase complex. In addition, residues 114 to 140 in NiV P/V/W proteins are critical for STAT1 binding as mutation of these residues abolishes the ability of these proteins to bind to and inhibit STAT1 phosphorylation, and thereby IFN signaling [69].

**Inhibition of nuclear transport of STATs**—In contrast to MARV VP40, which inhibits JAK1, EBOV VP24 proteins block the nuclear translocation of phosphorylated STAT1 (pY-STAT1) complexes by targeting the STAT1 transporter KPNA [74–76]. Recent biochemical and structural studies have elucidated how EBOV VP24 affects STAT1 nuclear accumulation while maintaining other KPNA-mediated cargo delivery [76] (Fig. 2B, 7). All KPNA recognize cargo containing a classical nuclear localization signal (cNLS) through a major site on armadillo repeats (ARM) 2–4 and a minor site on ARM 6–8 [77–80]. However, pY-STAT1 is transported by the NPI-subfamily, including KPNA1, KPNA5, and KPNA6, which can recognize a relatively uncharacterized non-classical NLS (ncNLS) [58]. EBOV VP24 binds to KPNA with a significantly higher affinity than pY-STAT1, suggesting that EBOV VP24 competes with pY-STAT1 for binding to KPNA [76]. The crystal structure of EBOV VP24 in complex with the minimal binding region of KPNA5 encompassing ARM 7 to 10 shows a large surface area of interaction with a hydrophobic core and high shape complementarity [76]. The binding surface is formed by residues in KPNA that are conserved only among the NPI-subfamily and residues of EBOV VP24 that vary in the closely related MARV VP24 [76]. Use of the ncNLS allows STAT1 transport to occur independently of other nucleocytoplasmic trafficking of cargoes containing cNLSs. This may be important in EBOV pathogenesis by maintaining certain cellular functions that can facilitate viral replication.

The P protein encoded by RABV of the Rhabdoviridae family also targets the JAK/STAT pathway in order to block nuclear trafficking of STATs [49] (Fig. 2B). Like EBOV, RABV infection does not affect the phosphorylation of STAT1 or STAT2 in human epithelial cells, but greatly reduced the levels of activated STATs and IRF9 levels upon stimulation with IFN $\alpha$  and IFN $\gamma$  while increasing cytoplasmic accumulation of STAT1 and STAT2 [49]. Further studies suggest that the C-terminal domain of RABV P protein is critical for STAT1/2 binding, which may be mediated by RABV P residues W265 and M287 [81]. Mutation of Trp265 and Met287 greatly reduces RABV pathogenicity [81].

**Inhibition of IFN stimulated genes (ISGs)**—Type I IFN $\alpha/\beta$  signaling through the JAK/STAT pathway stimulates the production of more than 200 interferon stimulated genes (ISGs), which together modulate cellular function to limit viral propagation. One ISG product is the Protein Kinase R (PKR), which is activated upon binding to dsRNA or PKR activator (PACT) [82]. Activated PKR phosphorylates eukaryotic initiation factor 2 $\alpha$  (eIF2 $\alpha$ ) leading to the inhibition of mRNA synthesis that limits viral protein synthesis [82].



However, EBOV infection in human kidney cells coincides with the absence of PKR phosphorylation of eIF2 $\alpha$  [83]. Although the precise mechanism of EBOV mediated PKR inhibition is not known, the C-terminal IID domain of VP35 may be involved in the process [84]. Under basal conditions, transfection of plasmid DNA into mammalian cells activates PKR, which suppresses the expression of transfected DNA. In contrast, expression of VP35 is sufficient to relieve this suppression. Furthermore, residues Arg305, Lys309, and Arg312 within VP35 IID are important for PKR inhibition. However, inhibition of PKR may be independent of dsRNA binding since a mutated VP35 IID with a Arg312 to Ala substitution retains the ability to inhibit PKR even though it does not bind dsRNA [84]. The effect of VP35 and PACT interaction on the activity of PKR has not been studied. Nonetheless, the observation that phosphorylation status of eIF $\alpha$  can affect EBOV replication is suggestive that PKR may be involved [83].

The role of tetherin, a type II transmembrane protein with a phosphatidylinositol group at the C-terminus [85], has been well studied in viral egress in retroviruses, herpesviruses, arenaviruses, and filoviruses [86–90]. Activated tetherin forms a parallel homodimer that can insert one end into the cell membrane and the other into the viral membrane, thereby blocking progeny release [91]. Tetherins have been shown to block the release of EBOV VP40 mediated virus like particles (VLPs) [86, 92, 93], but this inhibition can be relieved by EBOV glycoprotein (GP) [92]. In contrast to HIV Vpu, filoviral GP are able to inhibit tetherin from multiple species and in a cell-specific manner without downregulating tetherin expression [67]. In-cell fluorescence resonance energy transfer (FRET) and coimmunoprecipitation assays show that tetherin associated with the GP2 transmembrane region of EBOV GP proteins, suggesting a unique mechanism of inhibition by GP2 [67]. However, it appears that GP does not target tetherin in a sequence-specific manner [94].

More recently, the IFN induced proteins with tetratricopeptide repeats or IFITs have been extensively studied [64, 65, 95]. Four classes of IFIT genes, IFIT1 (or ISG56), IFIT2 (or ISG54), IFIT3 (or ISG60, or IFIT4), and IFIT5 (or ISG58), can be induced by type I and type II IFNs [96]. IFIT1 and IFIT2 can also directly target the elongation factor eIF3E to perturb the formation of the translation preinitiation complex [97, 98]. IFITs recognize specific RNA motifs found in viral RNA but absent in host mRNA species. One example is the 5'-end cap that lacks 2'-O-methylation at the N7 position, a motif that is recognized by IFIT1 [99]. Binding of IFIT1 to the 5'-ends of viral mRNA precludes their interaction with host elongation factors like eIF4E thereby blocking viral translation [100, 101]. In order to avoid detection by IFITs, many viruses have devised a variety of mechanisms, including de novo synthesis of 2'-O methylated caps by West Nile Virus [102, 103], cap snatching from the host by influenza virus [104], hijacking the host capping machinery by HIV [105], or use of internal ribosome entry sites by picornaviruses [106]. NNSVs typically replicate in the cytoplasm where the host capping machinery is unavailable. To circumvent this hurdle, NNSVs possess a unique cap synthesis mechanism where the L polymerase contains guanylyl transferase activity that can transfer a GDP cap to the 5'-end of viral mRNAs [107]. The NNSV mRNA cap is usually methylated at two positions, G-N7 and 2'-O [10, 108]. Studies in the VSV L polymerase show that methylation at both G-N-7 and 2'O positions occur through a single S-adenosyl-L-methionine binding site within a catalytic

triad conserved among other 2' O methylases [10, 107]. This region was mapped to the C-terminal region of VSV L within residues Gly1670 to Gly1675 and Asp 1735 [10].

**Modulation of the host ubiquitin system**—Ubiquitination is required at several steps in IFN production and signaling and results in both recognition of viral pathogens as well as restriction of viral infection. For example, TRIM25 mediated ubiquitination of the CARD domains of RIG-I and MAVS are necessary for their activation and downstream signaling [32, 35, 109–111]. Further, activation of transcription factor NF- $\kappa$ B is dependent on the ubiquitination and proteasomal degradation of the inhibitor kinase I $\kappa$ B [112]. NNSVs encode for proteins that reprogram the ubiquitin system to facilitate degradation of host innate immune molecules that would otherwise restrict viral infection [113]. The V protein of paramyxoviruses, such as simian virus 5 (SV5), mumps virus, human parainfluenza virus 2 (PIV2), and PIV5, targets the damage-specific DNA binding protein 1 (DDB1)-Cullin4-Rbx1 E3 ligase complex to ubiquitinate and mediate proteasomal degradation of STATs [114–116] (Fig. 8A). Recent three-dimensional structures of DDB1 bound to the V protein from the SV5 reveals how the multifunctional V protein interacts with the E3 ligase complex [117–119] (Fig. 8B–D). DDB1 is composed of three  $\beta$ -propeller domains, BPA, BPB, and BPC. BPB binds to Cullin4-Rbx1 complex which links substrates to E2 ubiquitin conjugating enzyme, whereas the BPA and BPC module binds to a variety of substrate recognition adaptors, WD40 domain containing proteins [118]. SV5 V protein displaces the canonical substrate adaptors to bind to DDB1 at BPA-BPC and hijack the DDB1-Cul4-Rbx1 complex to reroute ubiquitination of host antiviral molecules [114–116]. SV5 V protein inserts its N-terminal  $\alpha$ -helix  $\alpha$ 1 into a pocket formed by the loop regions of BPA and BPC [117] (Fig. 8D). This interaction seems to be supported by the C-terminal region or core of V protein, where  $\beta$ 4 makes extensive hydrophobic interactions with BPC. The conserved Tyr127 on  $\alpha$ 2 of V protein also makes multiple van der Waals interactions with  $\alpha$ 1 and BPC that are important in stabilizing the V-DDB1 complex (Fig. 8D). The solvent exposed outer face of V protein is lined by relatively conserved hydrophobic and nonpolar residues along with Asn100 which are thought to be important in the ubiquitination of STAT proteins [117, 118]. SV5 V forms a complex with both STAT1 and STAT2 with the later providing species-specific contacts [115]. This is the basis for the inability of SV5 to ubiquitinate murine STATs. In fact, mutation of Asn100 can abolish the interaction between V and human STAT2 or even impart SV5 with the ability to antagonize murine STATs [117].

The NS1 and NS2 proteins from RSV are also thought to induce proteasomal degradation of many host immune molecules, including RIG-I, IRF3/7, and STAT2 [8, 120, 121]. Moreover, NS1 and NS2 seem to affect the cellular expression levels of different proteins in the IFN signaling pathway, and when expressed together, the two non-structural proteins produce the strongest effect [8]. It was further observed that the proteasomal inhibitor MG132 could partially relieve the NS1/2 mediated inhibition of host protein expression [8, 121]. However, the exact molecular mechanism underlying these observations have not been fully elucidated.

## Conclusions and future directions

While the genomes of NNSVs are limited in their coding capacity, it is becoming increasingly clear that NNSVs encode viral proteins that are capable of antagonizing or rewiring the function of many host genes in order to promote viral pathogenesis. The multifunctional nature of NNSV proteins combined with their high mutation rates allows these pathogens to target multiple cellular factors and adapt to specific hosts. Molecular biology tools, like the reverse genetics system, developed over the last two decades have been instrumental in our understanding of the basic functions of the NNSV genome. However, we are now beginning to appreciate the complex interplay that occurs between viral pathogens and the host innate immune system. A detailed molecular understanding of these host-virus interactions will likely be key to the development of effective antiviral therapies and management of viral outbreaks. Recent advances in functional genomics, along with transcriptomics and proteomics, are proving useful in the investigation of host-virus interactions in the context of specific tissue and cell types, closely related viral species, and a variety of cellular signals [122]. For example, several genome-wide and druggable genome screens using short interfering RNA (siRNA) and CRISPR/Cas are starting to uncover novel genes required for NNSV infections [123–126]. While these advances are identifying novel targets, mechanistic understanding of these key host-viral interactions and use of such information in countermeasure development has significantly lagged. These limitations are highlighted by the lack of well-defined interaction interfaces, which can not only provide insight into regulatory mechanisms, but inform on druggability as well as the potential to form escape mutants. Thus, studies aimed at providing molecular mechanisms will be critical to form the knowledge base to better understand NNSV immune evasion mechanisms and to develop countermeasures.

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## Abbreviations

<b>APOBEC1</b>	Apolipoprotein B mRNA–editing enzyme catalytic polypeptide
<b>ARM</b>	Armadillo
<b>ATPase</b>	Adenylpyrophosphatase
<b>BDV</b>	Borna disease virus
<b>BPA/B/C</b>	Beta-propeller A/B/C
<b>CARD</b>	Caspase activation and recruitment domain

<b>CAS</b>	CRISPR associated protein 9
<b>CRISPR</b>	Clustered regularly interspaced short palindromic repeats
<b>ds/ss/mRNA</b>	double-stranded/single-stranded/messenger ribonucleic acid
<b>DDB1</b>	Damage-specific DNA binding protein 1
<b>EBOV</b>	Ebola virus
<b>eIF2<math>\alpha</math></b>	Eukaryotic initiation factor 2 alpha
<b>F</b>	Fusion protein
<b>FRET</b>	Fluorescence resonance energy transfer
<b>G/GP</b>	Glycoprotein
<b>HIV</b>	Human immunodeficiency virus
<b>IFIT</b>	IFN induced proteins with tetratricopeptide repeat
<b>IFN</b>	Interferon
<b>IFNAR</b>	IFN alpha/beta receptor
<b>IID</b>	IFN inhibitory domain
<b>IKK<math>\beta</math></b>	Inhibitor of kappa light polypeptide gene enhancer in B-cells
<b>IKK<math>\epsilon</math></b>	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
<b>IPS</b>	IFN beta promoter stimulator protein 1
<b>IRF3/7/9</b>	IFN regulatory factor 3/7/9
<b>ISG</b>	IFN stimulated gene
<b>ISRE</b>	IFN stimulated gene response element
<b>JAK1</b>	Janus kinase 1
<b>KPNA</b>	Karyopherin alpha
<b>L</b>	Large polymerase
<b>LGP2</b>	Laboratory of genetics and physiology 2
<b>M</b>	Matrix protein
<b>MAPK</b>	Mitogen activated protein kinase
<b>MARV</b>	Marburg virus
<b>MDA5</b>	Melanoma differentiation associated protein 5

<b>MyD88</b>	Myeloid differentiation primary response gene 88
<b>MeV</b>	Measles virus
<b>Mx1</b>	Myxovirus resistance 1
<b>(n)cNLS</b>	(non) classical nuclear localization signal
<b>NDV</b>	Newcastle disease virus
<b>NFκB</b>	Nuclear factor kappa B
<b>NiV</b>	Nipah virus
<b>N/NP</b>	Nucleoprotein
<b>NNSV</b>	Non-segmented, negative-sense, single-stranded RNA virus
<b>NPI-1</b>	Nucleoprotein interacting protein 1
<b>NS1/2</b>	Non-structural protein 1/2
<b>OAS</b>	2'-5'-oligoadenylate synthetase
<b>OH</b>	Hydroxyl
<b>ORF</b>	Open reading frame
<b>P</b>	Phosphoprotein
<b>PACT</b>	PKR activator
<b>PAMP</b>	Pathogen associated molecular pattern
<b>PIAS1</b>	Protein inhibitor of activated STAT 1
<b>PKR</b>	Protein kinase R
<b>PP1</b>	Protein phosphatase 1
<b>PRR</b>	Pattern recognition receptor
<b>RABV</b>	Rabies virus
<b>Rbx1</b>	Really interesting new gene (RING) box protein 1
<b>RdRP</b>	RNA dependent RNA polymerase
<b>RIG-I</b>	Retinoic acid inducible gene I
<b>RLR</b>	RIG-I-like receptor
<b>RMSD</b>	Root mean square deviation
<b>SeV</b>	Sendai virus
<b>SH</b>	Small hydrophobic

<b>siRNA</b>	Small interfering RNA
<b>STAT1/2</b>	Signal transducer and activator of transcription 1
<b>SUMO</b>	Small ubiquitin-like modifier
<b>SV5</b>	Simian virus 5
<b>TANK</b>	TRAF family member associated NFκB activator
<b>TBK1</b>	TANK binding kinase 1
<b>TIR</b>	Toll-interleukin 1 receptor
<b>TLR</b>	Toll-like receptor
<b>TRAF3</b>	Tumor necrosis factor receptor associated factor 3
<b>TRIF</b>	TIR domain containing adaptor-inducing interferon-beta
<b>TRIM</b>	Tripartite motif containing proteins
<b>TYK2</b>	Tyrosine kinase 2
<b>VISA</b>	Virus-induced signaling adapter
<b>VP24/30/35/40</b>	Viral protein 24/30/35/40
<b>Vpu</b>	Viral protein unique
<b>VSV</b>	Vesicular stomatitis virus

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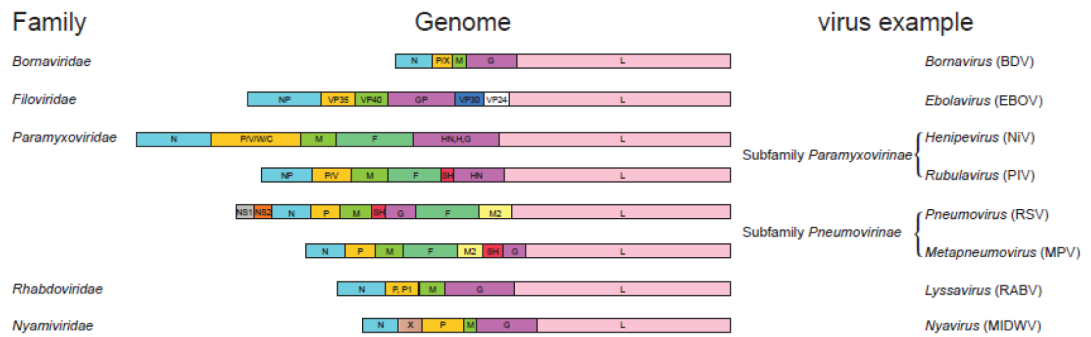


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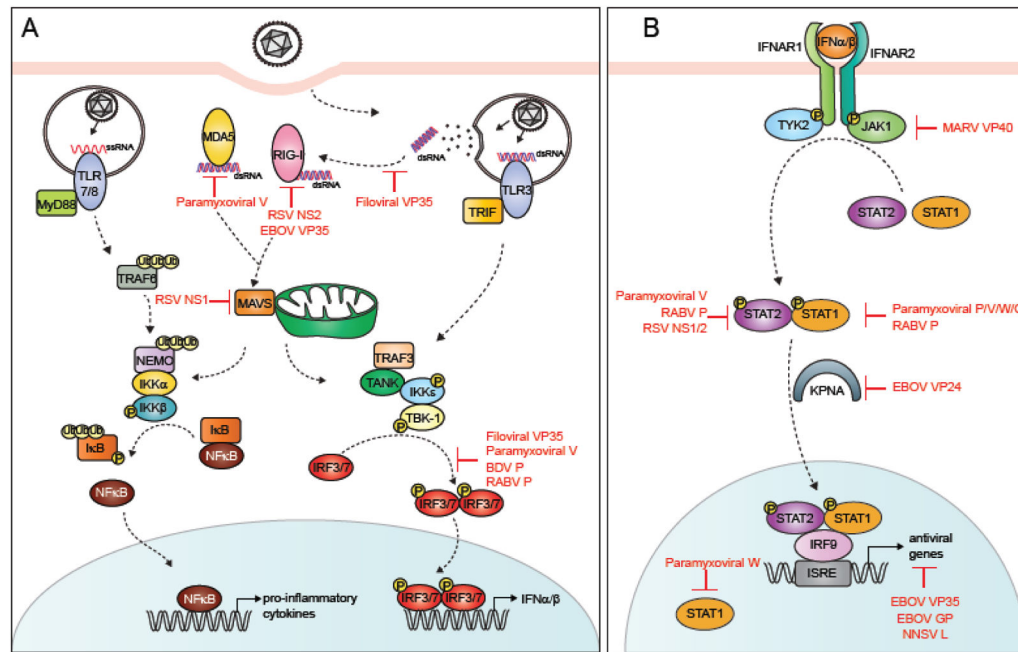
- Type I interferons are important for host innate immune responses.
- Non-segmented negative sense RNA viruses are important human pathogens.
- Recent studies are uncovering key host-virus interactions in immune evasion.
- These studies provide novel targets for development of countermeasures.



**Figure 1. Genome organization of NNSVs**

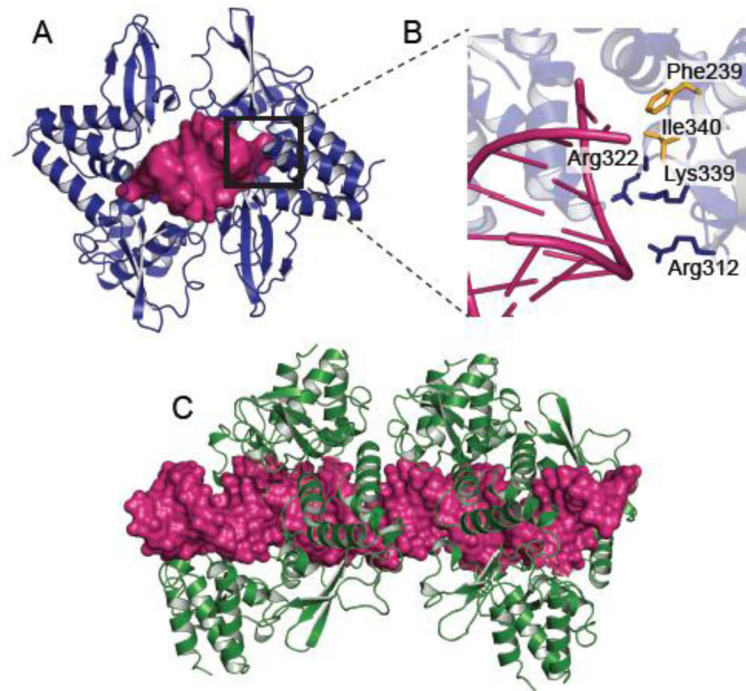
Shown are the different viral genomes included within the Order *Mononegavirales*.

Examples of genus and species (in parentheses) of viruses with the corresponding genome organization are listed on the right. Gene products common to all NNSVs are similarly colored, while genes unique to a specific family are different. Species abbreviations are as follows: Borna disease virus (BDV), Ebolavirus (EBOV), Nipah virus (NiV), Parainfluenza virus (PIV), respiratory syncytial virus (RSV), human metapneumovirus (MPV), rabies virus (RABV), and Midway virus (MIDWV).



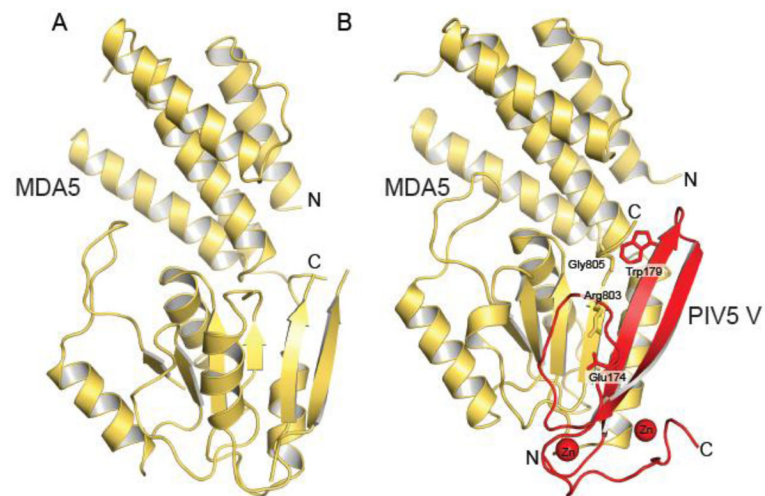
**Figure 2. Viral inhibitors of the type I IFN signaling pathway**

A simplified representation of the type I IFN signal transduction pathway. **A.** IFN induction. Viral PAMPs are detected by host PRRs, such as RLRs and TLRs, which lead to the production of type I IFNs and pro-inflammatory cytokines. **B.** IFN response. IFN $\alpha/\beta$  binding to IFNAR1/2 activates the Jak/STAT pathway leading to the expression of ISGs. Different steps of the pathway targeted by NNSV proteins are highlighted in red.



**Figure 3. PAMP sequestration by filoviral VP35 proteins**

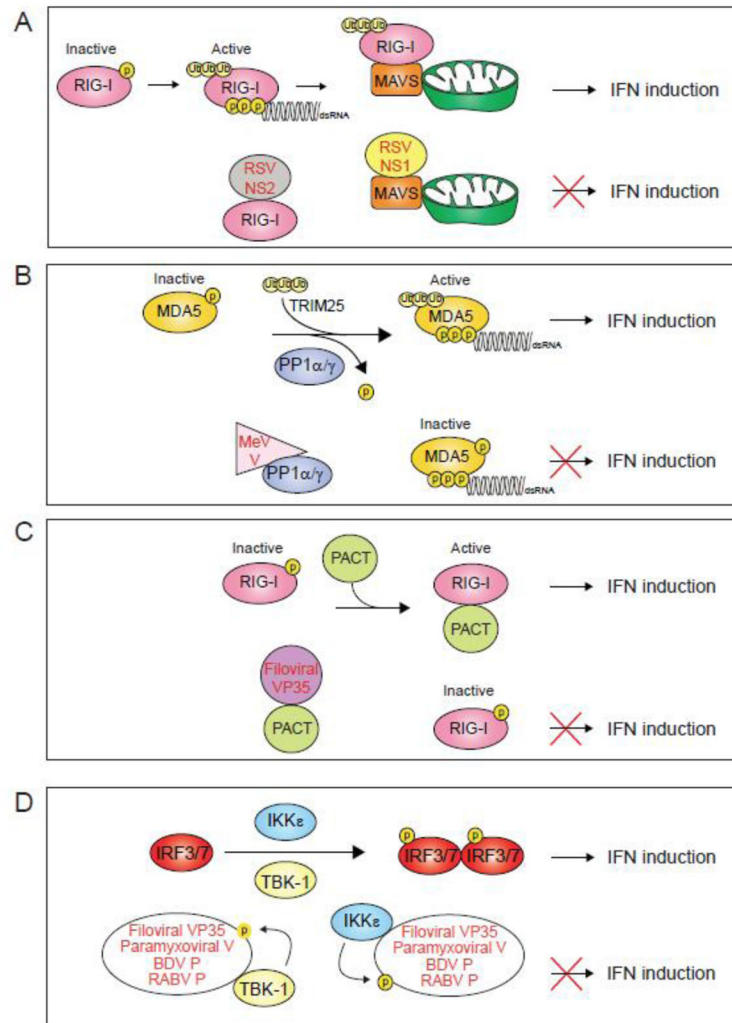
A. Crystal structure showing four molecules of Ebola virus VP35 (EBOV VP35) IID bound to an 8bp dsRNA (magenta) (PDB 3L25) [22]. The area marked with a black square is shown in greater detail in B. Residues in EBOV VP35 IID makes specific contacts to both the RNA phosphodiester backbone and the blunt ends. Residues colored blue make important protein-RNA backbone contacts while residues colored yellow contact the blunt ends. C. Crystal structure of Marburg virus VP35 (MARV VP35) IID bound to 18bp dsRNA (PDB 4GHL) showing that MARV VP35 IID does not end cap but coats the double stranded RNA backbone [23].



**Figure 4. Structural rearrangement of MDA5 upon binding to the PIV5 V protein**

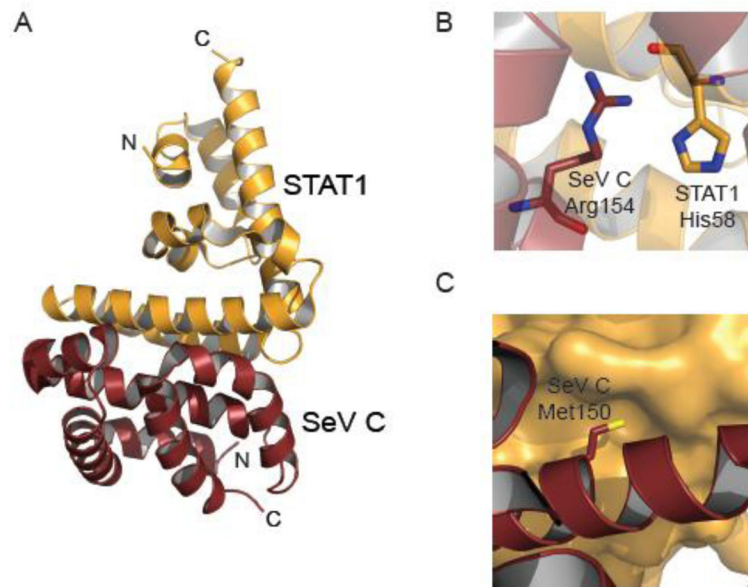
A. Crystal structure of the SF2 domain of human MDA5 (PDB 4GL2) [127]. B. Crystal structure of the SF2 domain of porcine MDA5 (yellow) bound to PIV5 V protein residues 168 to 219 (red) (PDB 4I1S) [27]. The key interactions that stabilize the complex are shown as sticks.





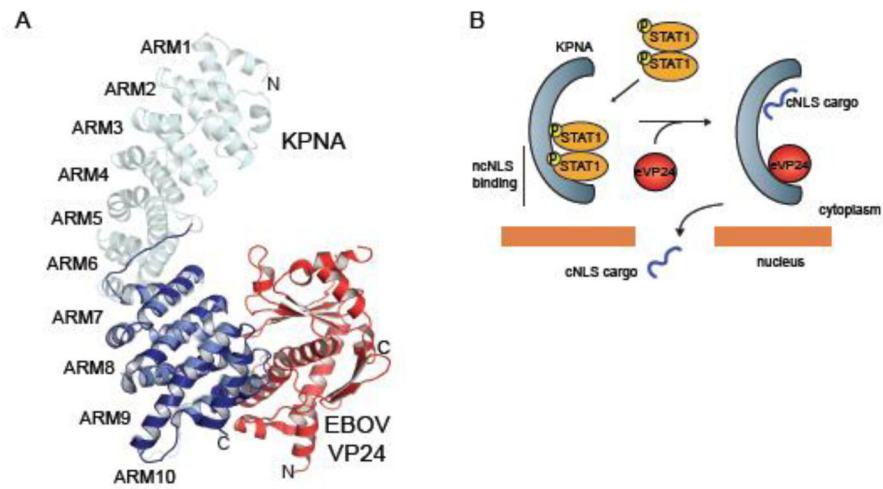
**Figure 5. Mechanisms of NNSV mediated inhibition of IFN induction**

A. The non-structural proteins NS1 and NS2 from respiratory syncytial virus (RSV) interact with MAVS and RIG-I, respectively, to disrupt RLR signaling. B. Measles virus (MeV) V protein binds to protein phosphatase 1  $\alpha/\gamma$  (PP1 $\alpha/\gamma$ ) and inhibits dephosphorylation and activation of RLRs. C. Ebolavirus VP35 (EBOV VP35) protein binds to PACT and inhibits PACT-induced activation of RIG-I. D. Filoviral VP35 proteins bind to kinases TBK1 and IKK $\epsilon$  and act as decoy substrates to inhibit the phosphorylation of IRF3/7. Paramyxoviral V protein and Bornavirus and Rhabdovirus P protein also inhibit IRF3/7 phosphorylation in a similar manner.



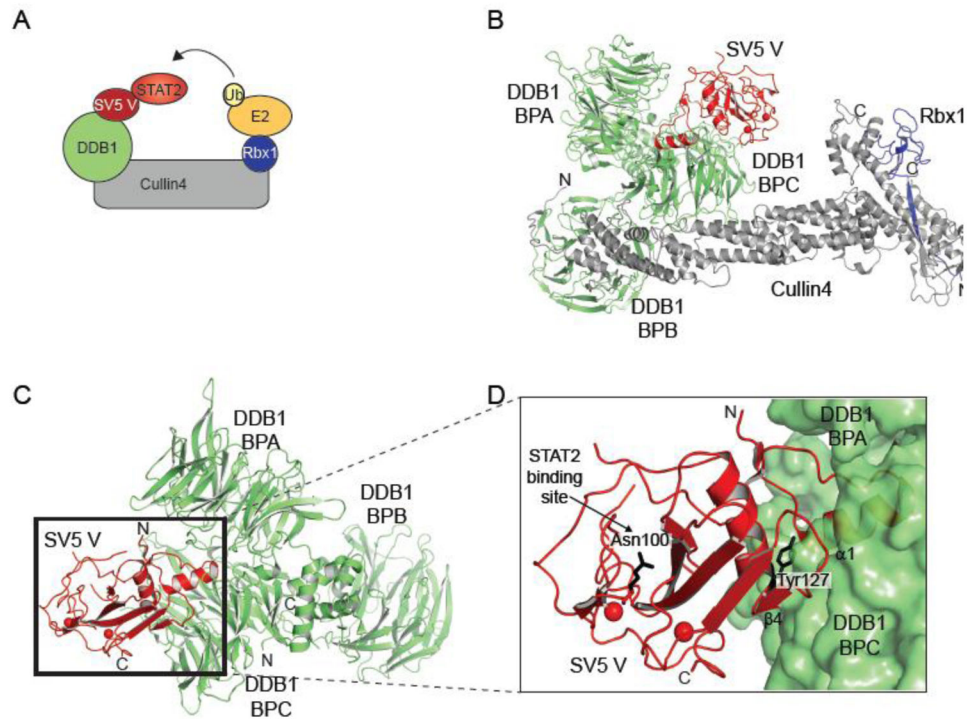
**Figure 6. SeV C protein targets STAT1**

A. Crystal structure of SeV C protein residues 99 to 204 bound to human STAT1 N-terminal domains (residues 1–126) (PDB 3WWT) [70]. B. Expanded view of a key electrostatic interaction between SeV C protein residue Arg154 and STAT1 residue His58, which highlights the role of charge complementarity at the host-viral interface. C. Expanded view of a hydrophobic pocket. SeV C residue Met150 inserts into the hydrophobic pocket within STAT1.



**Figure 7. Inhibition of the JAK/STAT pathway by EBOV VP24**

A. Crystal structure of EBOV VP24 (red) in complex with ARM 7–10 of KPNA5 (dark blue) (PDB 4U2X) [76]. ARM 7–10 is aligned to the structure of full length KPNA (PDB 1BK5) [77] as reference. B. Model showing how EBOV VP24 targets the non-classical nuclear localization signal (ncNLS) recognition of KPNA and blocks nuclear accumulation of pY-STAT1 while leaving classical NLS interactions intact.



**Figure 8. Viral modulation of host ubiquitination**  
[70]

A. Cartoon model depicting the mechanism of SV5 V protein mediated ubiquitination of STAT2. B. Crystal structure of the complex of SV5 V protein (red), DDB1 (green), Cullin4 (grey), and Rbx1 (Blue) (PDB 2HYE) [118]. BPB of DDB1 binds to the N-terminal domain of Cullin4, while Rbx1 binds to the C-terminal domain of Cullin4. C. Crystal structure of Simian virus 5 (SV5) V protein (red) and DDB1 (green) showing the three  $\beta$ -propellers BPA, BPB, and BPC (PDB 2B51) [117]. The SV5 V protein binds at a pocket within the BPA and BPC. D. Close up of the V binding pocket. N-terminal helix  $\alpha 1$  of V protein inserts into the hydrophobic pocket of DDB1. Tyr127 of V protein makes important inter-subunit interactions with BPC.