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ABSTRACT

In a genome-wide siRNA screen, we recently identified the interferon (IFN) inducible protein 35 (IFI35, also known as IFP35) as a factor required for VSV infection. Studies reported here were conducted to further understand the role and requirement of IFI35 in VSV infection. Consistent with the siRNA screening data, we found that depletion of IFI35 led to reduced VSV replication at the level of viral gene expression. Although no direct interaction of IFI35 with the viral replication machinery was observed, we found that IFI35 negatively regulated the host innate immune response and rescued poly(I:C)-induced inhibition of VSV replication. Promoter-driven reporter gene assays demonstrated that IFI35 overexpression suppressed the activation of IFNβ and ISG56 promoters, whereas its depletion had opposite effect. Further investigation revealed that IFI35 specifically interacted with RIG-I and negatively regulated its activation through mechanisms that include: (i) suppression of dephosphorylation (activation) of RIG-I and (ii) proteasome-mediated degradation of RIG-I via K48-linked ubiquitination. Overall, the results presented here suggest a novel role for IFI35 in negative regulation of RIG-I mediated antiviral signaling, which will have implications for diseases associated with excessive immune signaling.

IMPORTANCE

Mammalian cells employ a variety of mechanisms including production of interferons (IFNs) to counteract invading pathogens. In this study, we identified a novel role for a cellular protein, the IFN inducible protein 35 (IFP35/IFI35), in negatively regulating the host IFN response during vesicular stomatitis virus (VSV) infection. Specifically, we found that IFI35 inhibited activation of the RNA sensor, the retinoic acid inducible gene I (RIG-I), leading to inhibition of IFN production and thus resulting in better replication of VSV. The identification of a cellular factor that attenuates IFN response will have implications towards understanding of inflammatory diseases in humans such as Systemic Lupus Erythromatosus and Psoriasis that have been found to be associated with defects in the regulation of host IFN production.

INTRODUCTION

Negative strand RNA viruses employ diverse strategies to counter the host innate immune response (1). Vesicular stomatitis virus (VSV), a prototypic member of the *Rhabdoviridae* family with a non-segmented negative-strand RNA genome, replicates exclusively in the cytoplasm of the host cells. Among the five proteins encoded by VSV, the nucleocapsid protein (N) and the matrix protein (M) are crucial to evade as well as impair cellular antiviral responses (2). VSV N protein binds to newly synthesized viral genomic RNA during replication, which prevents the formation of dsRNA intermediates and thereby helps avoid the recognition by the viral RNA sensors to mount innate immune responses (1). On the other hand, VSV M causes a global inhibition of host gene expression by abrogating the nucleo-cytoplasmic export of host mRNAs (3-6). This leads to down-regulation of the overall antiviral response during VSV infection. Although VSV is highly efficient in invading a wide range of cell types, its growth is attenuated in cells with a pre-existing antiviral state (1). This suggests that VSV lacks inherent mechanism(s) to counteract an active innate immune response immediately after entry into the cells. In this context, VSV may need to depend on host cell factors to counter the antiviral response to allow efficient replication.

Our recent genome-wide screen for host factors identified the interferon inducible gene IFI35 (also known as IFP35) as a factor required for VSV infection (7). It was intriguing to find the requirement for an interferon stimulated gene (ISG) in virus infection, since majority of ISGs are known to exert antiviral functions to facilitate clearance of virus infection. IFI35 is a 35 kDa protein first identified by screening cDNA libraries of HeLa cells stimulated with IFN-γ (8). It contains an atypical leucine zipper

69 domain that lacks the basic region essential for DNA binding but can homo- and hetero-
70 dimerize with its binding partners through the N-myc interacting domains (NIDs) (9, 10).
71 It also interacts with another ISG called N-myc interacting protein (Nmi) to form a 200-
72 400 kDa high molecular mass complex (HMMC) in response to IFN-α treatment (10).
73 The interaction of IFI35 with Nmi prevents IFI35 from proteasomal degradation (11).
74 However, the functional consequence of the HMMC formation and/or the proteasomal
75 degradation of IFI35 in the context of antiviral signaling are not yet understood.

76 In contrast to the classical role of ISGs in antagonizing virus infections, several
77 ISGs are known to play regulatory functions to control excessive antiviral signaling.
78 Recently, ISGs such as ISG56, Optineurin, gC1qR, LGP2 and SARM have been shown
79 to negatively regulate the antiviral response by interfering with interactions between
80 signaling components (12-16) whereas another group of ISGs including ISG15, A20,
81 RNF125, TRIAD3a, PCBP2 and Ro52 induce degradation of signaling factors via the
82 ubiquitin-proteasome system (17-22). Importantly, the physiological relevance of
83 negative regulators is highlighted by the fact that many inflammatory diseases in
84 humans such as Systemic Lupus Erythromatosus and Psoriasis have been found to be
85 associated with defects in the regulation of host IFN production (23). The observations
86 that IFI35, an ISG, is required for VSV infection and that it does not interact directly with
87 any of the viral components led us to consider the possibility that IFI35 may support
88 VSV infection by functioning as a negative regulator of innate antiviral response in
89 infected cells.

90 RIG-I is a well-known pathogen recognition receptor (PRR) for negative
91 stranded viruses such as VSV, Sendai virus, Influenza and some positive-stranded

92 viruses like hepatitis C and dengue virus (24). RIG-I recognizes short dsRNA and
93 ssRNA with 5□ triphosphate ends through a conserved DExD/H RNA helicase domain.
94 The RNA helicase domain is essential for viral RNA recognition and activation whereas
95 the CARD domains are essential for downstream signaling (25). Many recent studies
96 have focused on understanding of the regulatory mechanisms of RIG-I activation
97 mediated through post-translational modifications such as phosphorylation and
98 ubiquitination (26, 27), This is particularly important since activation of PRRs, in general,
99 is crucial to prevent excessive signaling during virus infection and also after the infection
100 is cleared. Studies have shown that dephosphorylation (of Ser8 and Thr170 residues) of
101 RIG-I by the phosphatases PP1α/γ (28) and subsequent K63-linked ubiquitination are
102 two major modifications that promote RIG-I activation (29-31). On the other hand, K48-
103 linked ubiquitination and ISG15 conjugation (ISGylation) are the prominent mechanisms
104 to down-regulate RIG-I activation (17, 19, 28). Thus, the array of regulatory
105 mechanisms employed by the host cells underscore the importance of fine tuning of the
106 PRR response during virus infection.

107 In this report, we have investigated the supportive role of IFI35 in VSV infection.
108 Our studies reveal that IFI35 functions as a negative regulator of the host antiviral
109 response. Specifically, we have found that IFI35 negatively regulates the RIG-I
110 mediated antiviral signaling pathway. Our data suggest that IFI35 suppresses RIG-I
111 activation and also mediates its proteasomal degradation through ubiquitination. Since
112 RIG-I is the primary sensor during VSV infection (32), its down-regulation may help the
113 virus to replicate better. Overall, the key findings of this study uncover a new role for
114 IFI35 in negatively regulating virus triggered RIG-I antiviral signaling.

MATERIALS AND METHODS

Cells and reagents. HeLa and HEK293 cells were obtained from ATCC and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and the antibiotics (PKS): penicillin (100 units/ml), kanamycin (20 units/ml) and streptomycin (20 units/ml). Baby hamster kidney (BHK-21) cells were maintained in MEM supplemented with 5% FBS and 1X PKS. We generated HEK293 cells stably expressing a 3xFlag tagged IFI35 (3xF-IFI35) by transfection of cells with a plasmid encoding IFI35 tagged with 3xFlag sequence at its amino-terminus and continuously passaging the cells in DMEM supplemented with G418 (1mg/ml). A similar stable cell line (EV cells) transfected with an empty vector was also generated and used in control experiments. Following isolation, the stable cells were maintained in complete media containing G418 (250 µg/ml). G418 was purchased from Invitrogen, MG132, poly(I:C) and calyculin A were purchased from Sigma Aldrich.

Viruses, infection and titration. Sendai virus (SeV, Cantell strain) was obtained from Charles River Laboratories. Stocks of wt VSV and VSV-eGFP were prepared as described earlier (33, 34). VSVΔG virus and VSV nucleocapsids (NCs or RNPs) were prepared as described previously (35). VSV titers were determined by standard plaque assay on HeLa and BHK-21 cells. SeV infections were performed using 50 haemagglutinin units per ml (HA units/ml) for 16 h. VSV infections were performed at an MOI of 1 or 0.1 plaque forming unit (PFU) per cell, unless otherwise indicated.

Antibodies. VSV anti-M (23H12) antibody and anti-phospho RIG-I (Ser8) antibody were generously provided by Dr. D. Lyles (Wake Forest School of Medicine) and Dr. M. U. Gack (Harvard Medical School), respectively. Anti-ISG56 (rabbit polyclonal) antibody

has been described before (36). Mouse monoclonal antibody against IFI35 from Santa Cruz (Sc-100769), which detects a closely migrating doublet of endogenous IFI35 and a single band in plasmid-transfected cells, was used. IRF-3 (Sc-33641) and actin (Sc-47778) antibodies were from Santa Cruz Biotechnology Inc.; anti-phospho IRF-3 (Ser-396) antibody (# 4947) was from Cell Signaling Technology Inc.; anti-RIG-I (ab-65588) antibody was from Abcam; anti-HA (mouse Mab, HA.11 clone) antibody from Covance; anti-Flag (mouse Mab, Clone M2) antibody, HRP-conjugated goat anti-mouse (A4416) and goat anti-rabbit (A6154) antibodies were from Sigma Aldrich. Alexa-594 conjugated goat anti-mouse IgG (A11032), Alexa-488 donkey anti-mouse IgG (A21202) and Alexa-594 goat anti-rabbit (A11037) antibodies were from Invitrogen.

Plasmid constructs. The cDNA encoding the full length ORF of IFI35 (Cat # SC320222) was obtained from Origene and cloned into pcDNA 3.1(+) Neo vector (Invitrogen) at the KpnI and NotI sites to obtain the plasmid pcDNA-IFI35. An N-terminal HA-tagged IFI35 (HA-IFI35) was constructed by cloning the IFI35 ORF into a modified pHyg vector (Clontech). An N-terminal 3xFlag-IFI35 was also constructed by inserting the IFI35 ORF from the pcDNA-IFI35 into a modified pcDNA-3xFlag Neo vector. All the constructs were sequence verified and all the primers used are described in Table 1. 5x-NF-κB luciferase (37), IFNβ-luciferase and ISG56-luciferase (38), myc-TBK-1 (39), pEFBos-RIG-I and pEFBos-N-RIG-I (37) constructs have been described previously. Myc-MAVS was a kind gift from Dr. L. Zhang (University of Nebraska-Lincoln). N-terminal tagged GFP-IRF-3 plasmid was a generous gift from Dr. M. U. Gack. pRK5-HA-Ubiquitin-K48 (17605) was purchased from Addgene (40).

siRNA-mediated silencing. For depletion of endogenous IFI35, we used a combination of two ORF targeting siRNA duplexes from Qiagen (Hs_IFI35_1 # SI00445760 and Hs_IFI35_2 # SI00445767). Non-targeting (NT) siRNA (Qiagen # 1027281) was used as negative control. All siRNAs were used at a final concentration of 15nM unless otherwise indicated. siRNA transfections were performed following the protocol described previously (7).

Promoter-driven reporter gene assays. HEK293 cells were used for IFI35 knockdown using siRNAs as mentioned above and 3xFlag-IFI35 stable cells were used in IFI35 overexpression experiments. Both these cells were transfected with 0.5 µg of IFNβ-luc, NF-κB-luc or ISG56-luc plasmids along with 50 ng of pRL-TK plasmid (transfection control) for 32 h. Subsequently, cells were infected with 50 HA units of SeV for 16 h before harvesting. Cell lysates were subjected to luciferase assay using a Dual-Luciferase assay kit (Promega). Luciferase activity was measured using $20/20^{n}$ Luminometer (Turner Biosystems). Luciferase activity was expressed as the relative fold induction (n-fold) over the level of activity in the NT siRNA transfected or stable EV cells after normalization to the *Renilla* luciferase activity.

Quantitative real time PCR (qRT-PCR). Total cellular RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. First strand cDNA was synthesized with 2 µg of total RNA using M-MLV reverse transcriptase (Invitrogen) according to manufacturer's protocol. Oligo-dT primers were used for synthesis of all cDNAs except for VSV anti-genome, in which VSV2955R primer was used. All primers were used at a final concentration of 200 nM. The cDNA was diluted 2 fold and 200 ng of cDNA was used for each qRT-PCR reaction. In each reaction, iTaq Universal SYBR

183 green supermix (Bio-Rad) was used according to the manufacturer's protocol and
184 carried out using Step One Plus Real time PCR system (Applied Biosystems) following
185 the standard protocol provided by the manufacturer. Relative fold changes were
186 automatically calculated by the Step One Plus Real time PCR system software (Applied
187 Biosystems) following the ΔΔCT method. All primer sequences are listed in Table 1.
188 **Plasmid transfections and western blotting.** Transfection of plasmids was performed
189 using Lipofectamine 2000 (Invitrogen) as per manufacturer's instructions. Usually, 4-6 h
190 after transfection, fresh growth media was added and incubated for 44-48 h.
191 Transfection of viral RNPs was performed as described above but incubated for 6-8 h or
192 until eGFP expression is observed by fluorescent microscopy. Western blotting and
193 quantification of bands were performed as described before (35). Concentrations of
194 primary antibodies used are as follows unless otherwise indicated: anti-VSV M (1:2000),
195 actin (1:10,000), ISG56 (1:10,000), IFI35 (1:2000), IRF-3 (1:2000), p-IRF-3 (1:1000),
196 RIG-I (1:1000), p-RIG-I (1:1000), Flag Mab (1:2000) and HA Mab (1:2000). The
197 corresponding secondary antibodies were used at 1:2000 to 1:5000 dilutions.
198 **Immunofluorescence (IF) microscopy.** Stable EV cells or 3xF-IFI35 cells were
199 transfected with IRF-3-GFP plasmid and subsequently infected with 50 HA units/ml SeV
200 for 16 h and fixed with 4% paraformaldehyde. IRF-3 was visualized from the fused GFP
201 fluorescence whereas 3xFlag-IFI35 was immunostained with anti-Flag Mab (1:600) and
202 the secondary antibody Alexa-594 goat anti-mouse (1:1000). In other experiments,
203 HEK293 cells were mock-infected or infected with SeV, fixed and stained with
204 antibodies for endogenous RIG-I (1:200) and IFI35 (1:200). The secondary antibodies
205 used were Alexa-594 goat anti-rabbit (1:400) and Alexa-488 donkey anti-mouse (1:400),

206 respectively. Nuclei were stained with 10 ng/ml 4',6-diamidino-2-phenylindole (DAPI)
207 (Invitrogen). All images were acquired at 60X magnification using an Olympus
208 FV500/IX81 inverted laser scanning confocal microscope.

209 **Co-immunoprecipitation (co-IP) assay.** 6-well plates containing 50-60% confluent
210 HEK293 cells were used for co-IP experiments. For the interaction studies, cells were
211 transfected with 1 µg of EV, HA-IFI35 or Flag-RIG-I plasmids for 30 h. Then, one set of
212 cells was infected with SeV (50HA units/ml) or left uninfected for another 16 h. After 46
213 h of transfection/infection, cells were lysed in lysis buffer as described before (35). For
214 ubiquitination experiments, 0.5 µg of Flag-RIG-I and HA-Ubiquitin-K48 plasmids along
215 with increasing amounts IFI35 plasmid (0.5, 1 and 2 µg) were transfected for 36 h and
216 then treated with MG132 or its vehicle (DMSO) for another 12 h. Antibodies used for
217 immunoprecipitation are: anti-Flag Mab (1:125) and anti-HA Mab (1:250). Following
218 overnight incubation with the antibodies, 4 mg of protein A-Sepharose beads (GE
219 Healthcare Bioscience AB) washed 3 times in the lysis buffer were added to each
220 sample and incubated for 6-8 h. Beads with bound immune complexes were thoroughly
221 washed with lysis buffer, 30-40 µl 2X SDS-polyacrylamide gel electrophoresis (PAGE)
222 sample preparation buffer was added and the proteins were resolved in 10% SDS-
223 polyacrylamide gel. Subsequently, immunoblotting was carried out as described in the
224 western blotting section.

225 **Statistical analysis.** Statistical significance between groups was determined by two-
226 tailed paired Student's t test. $p < 0.05$ was considered statistically significant.

RESULTS

Depletion of IFI35 inhibits VSV replication

Our recent genome-wide RNAi screen identified IFI35 as a factor required for VSV infection (7). Since IFI35 is an ISG, it was intriguing to observe its requirement in the virus infection. To understand the involvement of IFI35 in VSV infection, we initially reconfirmed the high-throughput screen results using siRNA duplexes targeting the open reading frame of IFI35 mRNA and examined the levels of IFI35 protein as well as the extent of virus replication. With increasing amounts of siRNA, we observed 8-10 fold reduction in the overall levels of IFI35 protein (Fig. 1A). Although IFI35 depletion appeared to have reached its peak with 5 nM siRNA, use of lower (1 nM or less) concentrations resulted in IFI35 deletion in a dose-dependent manner (data not shown). A 4-6 fold reduction in VSV M protein levels (measure of virus replication) was observed in cells depleted of IFI35 (Fig. 1A). Depletion of IFI35 also reduced VSV progeny production significantly (Fig. 1B). These data reconfirmed our genome-wide screening results and suggested that IFI35 is required for VSV replication and growth.

To identify the step(s) of VSV infection cycle where IFI35 might be involved, we determined the levels of viral M protein expression in cells depleted of IFI35 and transfected with viral nucleocapsids, infected with VSVΔG virus or infected with VSV under single-cycle (MOI=3) or multi-cycle (MOI=0.1) infection conditions as described previously (7, 35), and found that IFI35 primarily affected virus replication at the levels of viral gene expression (data not shown). This observation was further confirmed by examining the level of viral P mRNA (measure of transcription) and viral antigenome (measure of replication) by quantitative real time PCR. Results showed a 4-5 fold

reduction in P mRNA (Fig. 1C) and anti-genome (Fig. 1D) in cells depleted of IFI35 as compared to the levels in NT siRNA-treated control cells. Since the observed reduction in genome replication could be due to inhibition of transcription, we examined VSV replication independent of transcription. This was performed by measuring replication of VSV defective interfering (DI) particle genomes in HEK293 cells (41) constitutively expressing the replication proteins of the virus (N, PeGFP, and L). We observed that DI particle RNA replication in the cells depleted of IFI35 was significantly (4-5 fold) reduced (Fig. 1E). Importantly, the levels of the viral N, PeGFP, and L proteins were not affected (data not shown), indicating that IFI35 depletion was responsible for the observed reduction of VSV DI RNA replication. Collectively, these data suggest a role for IFI35 in VSV infection at the level of viral RNA transcription and replication.

IFI35 rescues VSV infection by negatively regulating the host antiviral response

Since our results demonstrated that IFI35 depletion led to reduced VSV RNA transcription and replication, we investigated if IFI35 co-localizes or interacts with the VSV transcription and replication machinery. We did not observe any co-localization or specific interaction of IFI35 with the viral replication proteins N, P, and L (data not shown), suggesting that IFI35 is not directly involved in VSV replication but may be indirectly involved in modulating host cell pathways required for viral replication. IFI35 is an ISG and is induced in response to IFNα/γ treatment (8, 10). In contrast to the classical role of ISGs in combating virus infection, many ISGs are known to regulate the antiviral response through negative feedback mechanisms (23, 42). We considered the possibility that IFI35 is a negative regulator of antiviral response and thereby it may support VSV replication. Thus, we first examined the IFNβ mRNA production in HEK293

cells stably expressing 3xFlag-tagged IFI35 (3xF-IFI35) by real time PCR. Stable cells (EV) transfected with an empty vector were used as control. Because VSV is known to efficiently block host gene expression, we used Sendai virus (SeV) infection, which more potently activates a similar antiviral pathway as VSV (1). In comparison to EV cells, we observed 4-fold reduction in IFNβ mRNA levels in the 3xF-IFI35 stable cells infected with SeV (Fig. 2A). Additionally, we observed that when HEK293 cells were depleted of IFI35 using siRNAs, IFNβ mRNA levels were increased significantly (Fig. 2B). The effect of IFI35 on IFNβ mRNA production was similar whether SeV infection or poly(I:C) transfection was used (data not shown). To further investigate if IFI35 affects the production of ISGs, we examined the levels of endogenous ISG56 in EV or 3xF-IFI35 cells infected with SeV. Our results show that the ISG56 protein levels were significantly reduced in 3xF-IFI35 cells compared to the EV cells (Fig. 2C). Collectively, the results suggest that IFI35 down-regulates the host antiviral response.

The observation that depletion of IFI35 potentiated the production of IFNβ and also resulted in reduced VSV infection prompted us to examine if overexpression of IFI35 could rescue VSV infection from suppression induced by the host antiviral response. Indeed, VSV growth was enhanced (~10 fold) in poly(I:C) transfected 3xF-IFI35 stable cells when compared to the EV cells (Fig. 2D). Overall, these results indicate that IFI35 negatively regulates the host antiviral response and supports efficient replication of VSV.

IFI35 attenuates IFNβ activation

To further probe into the IFN signaling pathway that is negatively regulated by IFI35, we examined the effect of IFI35 depletion and overexpression on the induction of

296 various promoters including IFNβ, NF-κB and ISG56, all which are prominently activated
297 in response to most virus infections (43). First, we tested the effect of IFI35 depletion on
298 IFNβ promoter induction. To this end, HEK293 cells treated with NT or IFI35 siRNA
299 were transfected with a plasmid (IFNβ-Luc) encoding firefly luciferase under an IFNβ
300 promoter. Subsequently, these cells were infected with SeV to induce the IFNβ
301 promoter. Under these conditions, we observed a 4-fold increase in the luciferase
302 activity in IFI35 depleted cells compared to control (Fig. 3A). In SeV-infected 3xF-IFI35
303 stable cells, significant (5-fold) reduction in luciferase activity was observed compared
304 to Sev-infected EV cells (Fig. 3B). These data suggest that IFI35 down-regulates the
305 IFNβ promoter induction, providing further support for our earlier observation on
306 reduced IFNβ mRNA levels (Fig. 2A and 2B). Next, we examined the effect of IFI35 on
307 induction of NF-κB promoter. We found only a 2-fold increase or decrease in NF-κB
308 dependent promoter activity when IFI35 was depleted or overexpressed, respectively, in
309 SeV-infected cells (Fig. 3C and 3D), suggesting that NF-κB pathway may not play a
310 major role in IFI35-mediated down-regulation of IFNβ activation. Since we observed that
311 IFI35 overexpression suppressed ISG56 protein induction (Fig. 2C), we then examined
312 if this suppression was due to down-regulation of ISG56 promoter activity. We found
313 about 5-fold enhancement in ISG56 promoter induction in cells depleted of IFI35 (Fig.
314 3E) and a similar reduction (6-fold) in reporter activity was observed in cells
315 overexpressing IFI35 as compared to the control (EV) cells (Fig. 3F). Taken together,
316 these results suggest that IFI35 down-regulates IFNβ activation pathway.
317
318

IFI35 down-regulates IRF-3 and IRF-7 activation

Down-regulation of IFNβ induction by IFI35 could occur either by direct interaction of IFI35 with the promoter elements or by interfering with activation of upstream signaling molecules. Since IFI35 lacks DNA binding activity (9) and therefore may not be directly interacting with the promoter elements, we investigated whether IFI35 suppresses activation of signaling factors such as NF-κB, IRF-3 and IRF-7 that function upstream of IFNβ production. We focused our investigation on IRF-3 and IRF-7 activation as IFI35 showed relatively lower effect on NF-κB promoter activation (Fig. 3C and 3D). We first examined if IRF-3 phosphorylation and nuclear translocation, both of which are hallmarks of IRF-3 activation (44), are affected by IFI35 depletion or overexpression. Depletion of IFI35 in HEK293 cells infected with SeV resulted in increased IRF-3 phosphorylation compared to NT siRNA treated cells infected with SeV (Fig. 4A). Furthermore, IRF-3 phosphorylation was strongly suppressed in 3xF-IFI35 stable cells when infected with SeV (Fig. 4B). As phosphorylation of IRF-3 leads to its dimerization and nuclear translocation (45), we examined if IFI35 inhibits nuclear translocation of IRF-3. To this end, HEK293 cells stably expressing 3xF-IFI35 or control (EV) cells were transfected with a N-terminal GFP tagged IRF-3 construct (GFP-IRF-3). Infection of EV cells with SeV led to translocation of IRF-3-GFP from the cytoplasm into the nucleus whereas the nuclear translocation was abolished in 3xF-IFI35 stable cells (Fig. 4C). These results indicate that IFI35 inhibits the activation of IRF-3, which likely accounts for the down-regulation of IFNβ promoter induction.

Since IRF-7 in most cells other than plasmacytoid dendritic cells is induced upon virus infection and activated through positive feedback from IFN-α/β signaling (46), we

examined if IFI35 negatively regulates IRF-7 induction in the HEK293 cells used in our studies here. Our results show that in SeV-infected cells, IRF-7 mRNA levels were up-regulated when IFI35 was depleted (Fig. 4D) and reduced when IFI35 was overexpressed (Fig. 4E). Taken together, these results suggest that IFI35 negatively regulates activation of IRF-3 and IRF-7. Thus, the down-regulation of IFNβ promoter is likely due to the combined effect of suppression IRF-3 and IRF-7 activation.

IFI35 negatively regulates RIG-I pathway activation

To investigate if IFI35 down-regulates the antiviral signaling pathway upstream of IRF-3 and IRF-7, we examined the activation of IFNβ promoter by the signaling components including TBK1, MAVS/IPS-1 and RIG-I, all of which are known to be activated during VSV or SeV infection (43). In 3xF-IFI35 stable cells, we observed significant reduction (3-5 fold) in IFNβ promoter activation induced by RIG-I and the constitutively active form of RIG-I (N-RIG-I). But the activation was not significantly altered by the downstream signaling molecules, MAVS and TBK1 (Fig. 5A). In consistent with this data, we also found significant enhancement in IFNβ promoter activation by RIG-I and N-RIG-I in HEK293 cells depleted of IFI35 (Fig. 5B), whereas no significant effect was observed with MAVS and TBK1 expression in these cells (Fig. 5B). These data suggest that IFI35 negatively regulates the host antiviral pathway by exerting its effect at the level of RIG-I.

To understand how IFI35 regulates the activity of RIG-I, we examined the phosphorylation status of RIG-I. Dephosphorylation of Ser8 is a necessary prerequisite step in RIG-I activation process (30). In SeV-infected HEK293 cells depleted of IFI35, we observed significantly reduced phosphorylated form of RIG-I by using Ser8

365 phospho-specific RIG-I antibody (Fig. 5C). The total RIG-I levels were also slightly
366 increased. In contrast, the level of RIG-I phosphorylation was partially restored in 3xF-
367 IFI35 stable cells infected with SeV and the total RIG-I was also reduced, indicating that
368 IFI35 overexpression led to suppression of RIG-I activation (Fig. 5D). Since we
369 observed changes in total RIG-I levels by depletion or overexpression of IFI35, we
370 examined if IFI35 affected RIG-I induction at the level of transcription. Results showed
371 that RIG-I mRNA was up-regulated in cells depleted of IFI35 (Fig. 5E), whereas in 3XF-
372 IFI35 stable cells, it was reduced (Fig. 5F). Since RIG-I is an ISG, the reduced levels of
373 RIG-I mRNA may be due to overall down-regulation of IFNβ activation. These results
374 indicate that IFI35 down-regulates the activation of RIG-I, leading to overall suppression
375 of the antiviral response.

376 **IFI35 interacts with and promotes RIG-I degradation via K48-linked ubiquitination**

377 To further understand the mechanism by which IFI35 negatively regulates RIG-I
378 activation, we examined if IFI35 and RIG-I co-localize and/or interact in cells. We
379 observed enhanced co-localization of endogenous IFI35 and RIG-I in SeV-infected cells
380 compared to mock-infected cells (Fig. 6A). To determine if the observed co-localization
381 was due to physical interaction between IFI35 and RIG-I, we performed co-IP assay in
382 HEK293 cells. Cells were co-transfected with plasmids encoding Flag-RIG-I and HA-
383 IFI35, and subsequently were mock-infected or infected with SeV. The cell extracts
384 were subjected to co-IP with Flag (Fig. 6B, left panel) or HA (Fig. 6B, right panel)
385 antibody and immunoblotted with HA or Flag antibody, respectively. Results show that
386 RIG-I and IFI35 interact with each other in transfected cells in presence or absence of

SeV infection (Fig. 6B). Overall, both the co-localization and interaction studies suggest that IFI35 specifically interacts with RIG-I.

Since the negative regulators such as RNF125 and Siglec-G induce proteasomal degradation of RIG-I (19, 47), we examined if IFI35 promotes degradation of RIG-I. Indeed, when HEK293 cells were transfected with Flag-RIG-I along with increasing amounts of IFI35, we observed a dose dependent reduction in the levels of Flag-RIG-I but this effect was reversed when cells were treated with a proteasome inhibitor MG132 (Fig. 6C). This suggests that IFI35 promotes degradation of RIG-I through the proteasome machinery. It is well known that proteins degraded through proteasome pathway are conjugated to ubiquitin chains via lysine48 (K48) linkage (48). Thus, we examined if IFI35 promotes K48-linked ubiquitination of RIG-I. As shown in Fig. 6D, a dose dependent increase in K48-linked ubiquitination of RIG-I was observed with increasing amounts of IFI35. The levels of free HA-Ub-K48 in whole cell lysates decreased, indicating that the expressed HA-Ub-K48 was being conjugated to RIG-I. Concomitant reduction of RIG-I levels was observed in the whole cell lysates (Fig. 6D). Further, we observed reduction in K48-linked ubiquitination of RIG-I when IFI35 was depleted using siRNAs (Fig. 6E). These data suggest that IFI35 interacts with RIG-I and negatively regulates its activation by promoting K48-linked ubiquitination and degradation by proteasome pathway.

406 **DISCUSSION**

407 In the present study, we have uncovered a new role for IFI35 as a negative
408 regulator of the host antiviral response during VSV infection. Consistent with our recent
409 high-throughput genome-wide siRNA screening study (7) revealing a requirement for
410 IFI35 in VSV infection, we found that depletion of IFI35 led to significant reduction in
411 VSV replication. Conversely, overexpression of IFI35 could rescue VSV growth from
412 poly(I:C)-induced antiviral response. The observation that IFI35 did not interact with any
413 of the viral components suggested that IFI35 likely exerted its effect by influencing
414 cellular pathways. The results presented here indicate that IFI35 down-regulates the
415 host antiviral response mediated by RIG-I. IFI35 appears to down-regulate RIG-I activity
416 by at least two modes: (i) by keeping the RIG-I in its phosphorylated (inactive) form and
417 (ii) by mediating proteasomal degradation of RIG-I though ubiquitination. Whether the
418 requirement of IFI35 is specific to VSV infection is not known at this time, although, we
419 have observed a moderate requirement of IFI35 during lymphocytic choriomeningitis
420 virus infection (7).

421 Current understanding of the role of IFI35 in virus infections is limiting. IFI35 is an
422 ISG that is induced in response to treatment of cells with IFN-α/γ (8, 10). We have found
423 that it is also induced by transfection of cells with poly(I:C) or infection with SeV (data
424 not shown). The only study reported so far suggests that IFI35 interacts with the bovine
425 foamy virus (BFV) trans-activator protein Tas and that this interaction blocks the ability
426 of Tas to activate viral gene transcription, resulting in inhibition of BFV replication (49).
427 So, in this context, IFI35 exhibits an antiviral role and suppresses the virus replication.
428 Since infection of HeLa or HEK293 cells (which are of nonhematopoietic origin) by

429 foamy viruses do not induce type I IFN response (50, 51), it is possible that the negative
430 regulatory role of IFI35 in innate immune signaling was not observed readily in these
431 studies. However, our data clearly demonstrates the supportive role of IFI35 in VSV
432 infection through negative regulation of innate immune signaling. As viruses differ
433 significantly in their ability to induce or suppress the host innate antiviral responses,
434 additional studies will be needed to evaluate the role and requirement of IFI35 during
435 infection with diverse families of viruses.

436 Although host innate antiviral response is of paramount significance for
437 controlling viral infections, unregulated or excessive response may be detrimental to the
438 host. Therefore, identification of host cell factors and understanding the mechanisms
439 that negatively regulate these processes are critical to prevent damage to the infected
440 and neighboring uninfected cells. In this regard, the role of ISGs that function as
441 negative regulators of antiviral signaling is being increasingly appreciated. Previous
442 studies have shown that several ISGs, such as ISG56, Optineurin, RNF125 and A20
443 down-regulate the antiviral response through diverse mechanisms (12, 13, 19, 52) that
444 include interference with interactions between various signaling components (12-16) or
445 degradation of signaling factors via the ubiquitin-proteasome pathway. Our studies
446 presented here show that IFI35 negatively regulates RIG-I mediated antiviral pathway
447 through multiple mechanisms. Previous studies have shown that activation of RIG-I is
448 controlled through ubiquitination or deubiquitination of the CARD domains (26). K48-
449 linked ubiquitination of RIG-I is primarily promoted by the E3 ubiquitin ligases RNF125
450 (19) and the recently identified c-Cbl (47), which results in degradation RIG-I through
451 the proteasome machinery. On the other hand, removal of K63-linked ubiquitin chains is

452 mediated by the tumor suppressor CYLD which leads to inactivation of RIG-I (53). The
453 results presented here show that IFI35 down-regulates the host antiviral response by
454 promoting proteasomal degradation of RIG-I. IFI35 interacts with RIG-I and promotes its
455 degradation through the proteasome pathway via K48-linked ubiquitination (Fig. 6). We
456 did not observe any significant changes in K63-linked ubiquitination of RIG-I under
457 conditions of IFI35 overexpression (data not shown), suggesting that negative
458 regulation of RIG-I mediated by IFI35 occurs primarily through K48-linked ubiquitination
459 and proteasomal degradation and may not involve deubiquitination. At this time, it is not
460 known whether IFI35 directly mediates the K-48 linked ubiquitination or facilitates the
461 recruitment of an E3 ubiquitin ligase. Since IFI35 does not contain any typical E3
462 ubiquitin ligase domains such as HECT, RING or F-box motifs (54), the latter possibility
463 of recruiting an E3 ubiquitin ligase seems more likely. It will be interesting to examine if
464 the K48-linked ubiquitination of RIG-I in the IFI35-RIG-I complex is mediated by known
465 E3 ubiquitin ligases of RIG-I such as RNF125 and c-Cbl or by a yet unidentified E3
466 ubiquitin ligase (Fig. 7).

467 Another mechanism of RIG-I inactivation by IFI35 appears to be through
468 suppressing the dephosphorylation of RIG-I. Previously, it was shown that RIG-I is
469 maintained in an inactive state by phosphorylation mediated by PKC-α/β (55). Initial
470 step of RIG-I activation involves dephosphorylation of the CARD domains mediated by
471 the phosphatases PP1α/γ (28). We observed that IFI35 overexpression led to
472 enhancement of RIG-I phosphorylation (Fig. 5D), suggesting that IFI35 also regulates
473 the activation of RIG-I through direct involvement in the phosphorylation event or
474 through an indirect mechanism. Interestingly, our preliminary bioinformatics analysis

475 has uncovered several potential phosphorylation sites on IFI35 which are predicted to
476 be phosphorylated by the protein kinase C (PKC) family of kinases (data not shown).
477 Although the role of phosphorylation/dephosphorylation in modulating IFI35 function is
478 not clear at this time, it is possible that phosphorylation of both IFI35 and RIG-I by a
479 common family of kinases may represent an intricate mechanism for IFI35-mediated
480 negative regulation of RIG-I antiviral response.

481 Recently, it was reported that Nmi negatively regulates the host antiviral signaling
482 by promoting degradation of IRF-7 (56). Since Nmi is known to interact with IFI35 and
483 enhance its stability (11), it is possible that both proteins may act in a synergistic or
484 additive fashion to negatively regulate a common antiviral signaling pathway, albeit
485 through different targets. Since the formation of a high molecular mass IFI35-Nmi
486 complex has been shown to be an IFN-α stimulated event (10), it will be interesting to
487 investigate if the interaction of Nmi and IFI35 plays any role in augmenting the negative
488 regulatory effects during virus infections.

489 IFI35 belongs to a group of atypical helix-loop-helix (HLH) proteins such as Id,
490 which lack the basic DNA binding region and thus function in a dominant negative
491 fashion to negatively regulate function of other b-HLH group of transcription factors (57,
492 58). In this study, we have uncovered a novel role of IFI35 in negatively regulating the
493 host antiviral response by targeting the RIG-I signaling pathway. Mechanistically, we
494 have demonstrated that IFI35 down-regulates RIG-I activation by promoting its
495 proteasomal degradation as well as keeping it in its phosphorylated (inactive) form.
496 Further studies are required to understand the mechanistic details of this process and
497 also identify other factors involved in the IFI35 mediated negative regulatory loop. In

498 conclusion, identification of IFI35 introduces a new player to the existing group of
499 molecules that regulate RIG-I antiviral signaling pathway and also highlights the
500 importance of negative regulation of cellular antiviral responses.

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694 **FIGURE LEGENDS**

695 **Figure 1.** Depletion of IFI35 inhibits VSV replication**.** (A) HeLa cells were transfected
696 with 15 nM of NT siRNA or increasing amounts (5, 10 and 15 nM) of a combination of
697 two different siRNAs targeting IFI35. At 60 h post-transfection, cells were infected with 1
698 MOI VSV for 4 h. Levels of IFI35 and VSV M were determined by immunoblotting with
699 specific antibodies. Actin served as the loading control. (B) HeLa cells were transfected
700 with 15 nM of NT or IFI35 siRNA for 60 h and infected with 0.1 MOI VSV for 18 h.
701 Supernatant was harvested and virus titer was determined by plaque assay and
702 expressed as pfu/ml. Average titers for NT and siIFI35 were $3.9x10^4$ and $2.7x10^5$ pfu/ml,
703 respectively. (C and D) IFI35 depletion inhibits VSV infection at the level of virus
704 transcription and replication. HeLa cells were transfected with 15 nM of NT or IFI35
705 targeting siRNA for 60 h. Subsequently, the cells were infected with 0.1 MOI VSV for 12
706 h. VSV P mRNA (C) and VSV antigenomic RNA (D) levels in IFI35 depleted HeLa cells
707 was determined by qRT-PCR. Values were normalized to the internal control β-actin
708 and expressed as relative change over the NT sample, which was set at 100. (E) IFI35
709 knockdown inhibits VSV DI RNA replication. NPeGFPL stable cells were transfected
710 with 15 nM NT or IFI35 targeting siRNA for 60 h. Then, the cells were infected with DI
711 particles for 14 h and the RNA replication products (both genomic and antigenomic)
712 were quantitated by semiquantitative RT-PCR as described previously (41). IFI35 and
713 RPL32 (internal control) mRNA levels also were examined. Data presented in B-D
714 panels are from three independent experiments and error bars represent mean ± SD of
715 duplicates. *$p < 0.05$; **$p < 0.01$.

716 **Figure 2.** IFI35 rescues VSV infection by negatively regulating host antiviral response.
717 (A-B) IFI35 down-regulates IFNβ mRNA synthesis. EV and 3xF-IFI35 stable cells (A) or
718 HEK293 cells transfected with NT or IFI35 siRNAs (B) were infected with SeV for 16 h.
719 Total RNA was isolated and IFNβ mRNA was quantified by qRT-PCR. Values were
720 normalized to the internal control GAPDH and expressed as relative fold change over
721 the mock-infected NT or EV samples, which were set at 1. Error bars represent mean ±
722 SD of duplicate reactions from two independent experiments. ** $p < 0.01$. (C) IFI35
723 overexpression suppresses ISG56 induction. EV and 3xF-IFI35 stable cells were
724 infected with SeV for 16 h. Cell lysates were used for immunoblotting using ISG56, Flag
725 M2 (detects 3xF-IFI35) and actin antibodies. (D) IFI35 overexpression rescues poly(I:C)
726 induced suppression of VSV infection. EV and 3xF-IFI35 cells were transfected with 2
727 μg poly(I:C) for 16 h and infected with 0.1MOI VSV for 18 h. The supernatants were
728 harvested and virus titers were determined by plaque assay on BHK-21 cells. Virus
729 titers are expressed as $\log_{10}$ pfu/ml. Error bars represent mean ± SD from three
730 independent experiments. ** $p < 0.01$.
731 **Figure 3.** IFI35 attenuates IFNβ activation and signaling. (A-B) Effect of IFI35 on IFNβ
732 promoter activation. (A) HEK293 cells were transfected with 15nM NT or IFI35 targeting
733 siRNAs for a total of 76 h. After first 24 h, cells were transfected with 500 ng of IFNβ
734 luciferase reporter plasmid (IFNβ-Luc) along with 50 ng of pRL-TK plasmid and were
735 incubated for another 36 h. For the final 16 h prior to harvesting, cells were either mock-
736 infected or infected with SeV. Cell lysates were used for dual luciferase assay. (B) EV
737 and 3xF-IFI35 stable cells were transfected with 500 ng of IFNβ luciferase reporter
738 plasmid along with 50 ng of pRL-TK plasmid for 32 h. Subsequently, cells were infected

739 with SeV for 16 h and cell lysates were used for dual luciferase assay. (C-D) Effect of
740 IFI35 on NF-κB promoter activation. Experimental conditions are similar to those in
741 panels A-B except 500 ng of NF-κB luciferase construct (NF-κB-Luc) was transfected in
742 place of IFNβ-Luc. (E-F) Effect of IFI35 on ISG56 promoter activation. Experimental
743 conditions are similar to those in panels A-B except 500 ng of ISG56 luciferase
744 construct (ISG56-Luc) was transfected in place of IFNβ-Luc construct. Values
745 represented in all the promoter reporter assays are normalized to NT or EV control cells
746 and expressed as relative fold change over the mock-infected NT or EV samples, which
747 were set at 1. Error bars represent mean ± SD from three independent experiments. * p
748 < 0.05; ** p < 0.01.
749 **Figure 4.** IFI35 down-regulates activation of IRF-3 and IRF-7. (A-B) IFI35 down-
750 regulates IRF-3 phosphorylation. (A) HEK293 cells were transfected with 15 nM NT or
751 IFI35 siRNAs for 60 h and infected with SeV for another 16 h. Cell lysates were
752 analyzed by immunoblotting using the indicated antibodies. (B) EV and 3xF-IFI35 stable
753 cells were infected with SeV for 16 h and cell lysates were analyzed immunoblotting
754 using the indicated antibodies. (C) IFI35 overexpression inhibits nuclear translocation of
755 IRF-3. EV and 3xF-IFI35 stable cells were grown on coverslips and transfected with 1
756 μg GFP-IRF-3 plasmid for 32 h and then infected with SeV for 16 h. 3xFlag-IFI35 was
757 immunostained using anti-Flag antibody. Nuclei were stained with DAPI. (D-E) IFI35
758 down-regulates IRF-7 induction. (D) HEK293 cells transfected with 15 nM of NT or IFI35
759 siRNAs for 60 h and infected with SeV for another 16 h. Total RNA was isolated and
760 subjected to qRT-PCR using IRF-7 specific primers. (E) IRF-7 mRNA was quantified as
761 described in D from EV and 3XF-IFI35 stable cells infected with SeV for 16 h. Values

762 were normalized to the internal control GAPDH and expressed as relative fold change
763 over the mock-infected EV or NT samples, which were set at 1. Error bars represent
764 mean ± SD of duplicate reactions from two independent experiments. * $p < 0.05$.
765 **Figure 5.** IFI35 negatively regulates RIG-I pathway activation. (A) EV and 3xF-IFI35
766 stable cells were transfected with the plasmids encoding RIG-I pathways components
767 (0.5μg each) MAVS, TBK1, RIG-I and N-RIG-I along with 0.25 μg IFNβ-Luc and 0.025
768 μg pRL-TK plasmid for 48 h. The cells were lysed and used for dual luciferase assays.
769 (B) HEK293 cells were transfected with 15 nM of NT or IFI35 siRNAs for 24 h and then
770 transfected with the plasmids as described in A for another 48 h. Cells were lysed and
771 used for dual luciferase assays. Luciferase values were normalized to the EV or NT
772 control cells and expressed as relative fold change over the mock-infected EV or NT
773 samples, which were set at 1. Error bars represent mean ± SD of two independent
774 experiments performed in duplicates. * $p < 0.05$; ** $p < 0.01$; ns; not significant. (C-D)
775 IFI35 negatively regulates RIG-I activation. (C) HEK293 cells were transfected with 15
776 nM NT or IFI35 siRNAs for 60 h and infected with SeV for another 16 h. The cells were
777 treated with 100 nM calyculin A for 30 min before harvesting. Cell lysates were analyzed
778 by immunoblotting using the indicated antibodies. Actin served as loading control. (D)
779 EV and 3xF-IFI35 stable cells infected with SeV for 16 h and processed as described in
780 C. (E-F) Knockdown or overexpression of IFI35 enhances or suppresses RIG-I
781 transcription, respectively. (E) HEK293 cells were transfected with 15 nM NT or IFI35
782 siRNAs for 60 h and infected with SeV for another 16 h. Total RNA was isolated and
783 used for quantification of RIG-I mRNA levels by qRT-PCR. (F) EV or 3xF-IFI35 stable
784 cells were infected with SeV for 16 h and processed as in E. Values were normalized to

785 the internal control GAPDH and expressed as relative fold change over the mock-
786 infected EV or NT samples set at 1. ** $p < 0.01$.

787 **Figure 6.** IFI35 interacts with and promotes degradation of RIG-I via K48-linked
788 ubiquitination. (A) SeV infection enhances co-localization of IFI35 and RIG-I. HEK293
789 cells were grown on coverslips and mock-infected or infected with SeV for 16 h. The
790 cells were fixed and immunostained with the indicated antibodies. Nuclei were stained
791 with DAPI and images were collected at 60X magnification. (B) IFI35 interacts with RIG-
792 I in transfected cells. HEK293 cells were transfected with 0.5 µg of the indicated
793 plasmids for 32 h. One set of cells were mock-infected while the other set was infected
794 with SeV for another 16 h. Co-IP and immunobloting was performed with the indicated
795 antibodies. Expression of proteins from the transfected plasmids was analyzed in the
796 whole cell lysates (WCL) using the indicated antibodies. (C) IFI35 promotes
797 proteasomal degradation of RIG-I. Sets of HEK293 cells were transfected with 0.5 µg of
798 Flag-RIG-I along with increasing amounts of IFI35 (0.5 and 1 µg) for 36 h. One set of
799 cells was treated with 10 µM MG132 and the other set with DMSO for 12 h. Cell lysates
800 were analyzed by immunoblotting with the indicated antibodies. (D) IFI35
801 overexpression enhances K48-linked ubiquitination of RIG-I. Sets of HEK293 cells were
802 transfected with Flag-RIG-I and HA-Ub-K48 constructs (0.5 µg each) along with
803 increasing amounts of plasmid expressing IFI35 (0.5, 1 and 2 µg) for 36 h. One set of
804 cells was treated with MG132 (10 µM) for 12 h and subsequently lysed and used for co-
805 IP and immunoblotting with the indicated antibodies. Another set of cells was treated
806 with DMSO for 12 h and the whole cell lysates were analyzed by immunoblotting with
807 the indicated antibodies. (E) IFI35 knockdown reduces K48-linked ubiquitination of RIG-

I. Sets of HEK293 cells were transfected with NT siRNA or increasing amounts of IFI35 siRNA (5, 10 and 20 nM) for 24 h. The cells were transfected with Flag-RIG-I and HA-Ubiquitin-K48 constructs (0.5 µg each) for another 36 h. Cells were subsequently processed as described in D.

Figure 7. A proposed model depicting the role of IFI35 in negative regulation of RIG-I signaling. For the sake of simplicity, we have only shown the ubiquitin-mediated degradation pathway. Viruses like VSV and SeV are recognized by RIG-I, which signals through downstream factors (IRF-3/IRF7) leading to production of type I IFNs (IFN-α/β). IFN-α/β in turn induce the synthesis of ISGs including IFI35 which interacts with RIG-I and inhibits its activation by promoting K48-linked ubiquitination and proteasomal degradation. The identity of the associated E3 ubiquitin ligase is unknown at this time. The negative feedback loop mediated by IFI35 leads to down-regulation of the host antiviral response.

821 **Table 1. Primers used in this study**

822

Primer name	Primer sequence (5' to 3')	Used for
IFI35 KpnI-F	ATATATGGTACCGCCGCCACCATGTCAGCCCCACTGGATG	Amplification and cloning of IFI35 in pcDNA vector
IFI35 NotI-R	ATATATGCGGCCGCCTAGCCTGACTCAGAGGTG	
HAIFI35 KpnI-F	ATATATGGTACCGCCGCCACCATGTACCCATACGATGTTCCAGATTACGCTTCAGCCCCACTGGATGCC	Amplification and cloning of HA tagged IFI35
IFI35 NotI-R	ATATATGCGGCCGCCTAGCCTGACTCAGAGGTG	
VSV P-F	GTGACGGACGAATGTCTCATAA	Amplification of VSV P mRNA
VSV P-R	TTTGACTCTCGCCTGATTGTAC	
VSV 2795-F	GTGACGGACGAATGTCTCATAA	Amplification of VSV antigenomic RNA
VSV2955-R	TGATGAATGGATTGGGATAACA	
Actin-F	CAAGTACTCCGTGTGTGGAT	Amplification of Actin mRNA
Actin-R	CATACTCCTGCTTGCTGAT	
IFN-β-F	ATGACCAACAAGTGTCTCCTCC	Amplification of IFN-β mRNA
IFN--β-R	GGAATCCAAGCAAGTTGTAGCTC	
IRF7-F	CCCACGCTATACCATCTACCT	Amplification of IRF7 mRNA
IRF7-R	GATGTCGTCATAGAGGCTGTTG	
RIG-I-F	CTGGACCCTACCTACATCCTG	Amplification of RIG-I mRNA
RIG-I-R	GGCATCCAAAAAGCCACGG	
GAPDH-F	GCAAATTCCATGGCACCGT	Amplification of GAPDH mRNA
GAPDH-R	TCGCCCCACTTGATTTTGG	
VSV DI	ACGAAGACCACAAAACCAGATAAAAA	Amplification of DI RNA
RPL32-F	GCCAGATCTTATGCCCCAAC	Amplification of RPL32 mRNA
RPL32-R	CGTGCACATGAGCTGCCTAC	
IFI35-F	AACAAAAGGAGCACACGATCA	Amplification of IFI35 mRNA
IFI35-R	CTCCGTTCCTAGTCTTGCCAA	

823 Underlined sequences indicate the restriction sites