A Short Hairpin RNA Screen of Interferon-Stimulated Genes Identifies a Novel Negative Regulator of the Cellular Antiviral Response

Jianqing Li,[a] Steve C. Ding,[b] Hyelim Cho,[a] Brian C. Chung,[b] Michael Gale Jr.,[c] Sumit K. Chanda,[b] Michael S. Diamond[a,d,e]

Departments of Molecular Microbiology,[a] Medicine,[d] and Pathology and Immunology,[e] Washington University School of Medicine, St. Louis, Missouri, USA; Infectious and Inflammatory Disease Center, Sanford Burnham Medical Research Institute, La Jolla, California, USA[b]; Department of Immunology, University of Washington School of Medicine, Seattle, Washington, USA[c]

ABSTRACT **The type I interferon (IFN) signaling pathway restricts infection of many divergent families of RNA and DNA viruses by inducing hundreds of IFN-stimulated genes (ISGs), some of which have direct antiviral activity. We screened 813 short hairpin RNA (shRNA) constructs targeting 245 human ISGs using a flow cytometry approach to identify genes that modulated infection of West Nile virus (WNV) in IFN-β-treated human cells. Thirty ISGs with inhibitory effects against WNV were identified, including several novel genes that had antiviral activity against related and unrelated positive-strand RNA viruses. We also defined one ISG, activating signal cointegrator complex 3 (*ASCC3*), which functioned as a negative regulator of the host defense response. Silencing of *ASCC3* resulted in upregulation of multiple antiviral ISGs, which correlated with inhibition of infection of several positive-strand RNA viruses. Reciprocally, ectopic expression of human *ASCC3* or mouse *Ascc3* resulted in downregulation of ISGs and increased viral infection. Mechanism-of-action and RNA sequencing studies revealed that *ASCC3* functions to modulate ISG expression in an IRF-3- and IRF-7-dependent manner. Compared to prior ectopic ISG expression studies, our shRNA screen identified novel ISGs that restrict infection of WNV and other viruses and defined a new counterregulatory ISG, *ASCC3*, which tempers cell-intrinsic immunity.**

IMPORTANCE **West Nile virus (WNV) is a mosquito-transmitted virus that continues to pose a threat to public health. Innate immune responses, especially those downstream of type I interferon (IFN) signaling, are critical for controlling virus infection and spread. We performed a genetic screen using a gene silencing approach and identified 30 interferon-stimulated genes (ISGs) that contributed to the host antiviral response against WNV. As part of this screen, we also identified a novel negative regulatory protein, ASCC3, which dampens expression of ISGs, including those with antiviral or proinflammatory activity. In summary, our studies define a series of heretofore-uncharacterized ISGs with antiviral effects against multiple viruses or counterregulatory effects that temper IFN signaling and likely minimize immune-mediated pathology.**

Received 22 May 2013 **Accepted** 24 May 2013 **Published** 18 June 2013

Citation Li J, Ding SC, Cho H, Chung BC, Gale M, Jr, Chanda SK, Diamond MS. 2013. A short hairpin RNA screen of interferon-stimulated genes identifies a novel negative regulator of the cellular antiviral response. mBio 4(3):e00385-13. doi:10.1128/mBio.00385-13.

Editor Terence Dermody, Vanderbilt University School of Medicine

Address correspondence to Michael S. Diamond, diamond@borcim.wustl.edu.

Virus infection of mammalian cells induces several independent and interdependent signaling pathways to promote expression of genes that confer an antiviral state. RNA intermediates of virus replication are recognized by pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), which bind to adaptor molecules (e.g., MyD88, TRIF, and MAVS) that signal specific transcription factors (e.g., IRF-3, IRF-7, and NF-κB) to translocate into the nucleus and induce expression of type I interferon (IFN) and other genes encoding antiviral and immune regulatory activity (1, 2). Secreted type I IFN binds in an autocrine and paracrine manner to the IFN-α/β receptor (IFNAR) on the surface of cells and triggers a signaling cascade that induces hundreds of interferon-stimulated genes (ISGs) (3). The importance of this pathway is underscored by the vulnerability of *Ifnar*$^{-/-}$ mice to infection by many families of viruses (4–6).

Members of the *Flavivirus* genus are the most important arthropod-borne viruses causing disease in humans. This genus includes viruses (West Nile virus [WNV], Japanese encephalitis virus [JEV], yellow fever virus [YFV], and dengue virus [DENV]) that are endemic in several parts of the world and collectively cause hundreds of millions of infections each year (7). Flavivirus infection causes severe disease in humans, including hemorrhagic fever, shock syndrome, liver failure, and encephalitis. The enhanced spread of flaviviruses worldwide highlights a need for an improved understanding of mechanisms of immune control, as insight into the cell-intrinsic processes that restrict infection may facilitate novel strategies to limit disease (8).

Although type I IFN responses control the cell and tissue tropism of WNV and other flaviviruses (4), the molecules that restrict infection are not fully defined. In prior studies using deficient mice or cells, PKR and RNase L were identified as ISGs that contribute to IFN-mediated control of WNV infection (9, 10). More recent experiments in mice have suggested that IFIT1 and viperin restrict WNV infection *in vivo* with prominent effects in neurons of the central nervous system (11–13). IFITM genes also have been

reported to inhibit early entry steps in flavivirus infection (14–16), although these and other less well characterized ISGs (17–20) have not been extensively studied.

Here, we applied a genetic screen using a flow cytometry-based gene silencing approach to identify candidate ISGs that limit WNV infection. In contrast to prior screens that used ectopic expression to identify ISGs that were sufficient to confer an antiviral effect (18, 20, 21), we transduced a library of 813 lentivirus-encoded short hairpin RNAs (shRNAs) targeting 245 human ISGs in HeLa cells. We selected a gene silencing approach because it could (i) be performed using physiological concentrations of IFN-β, (ii) define the relative importance of any given ISG in the context of an intact IFN response, and (iii) identify ISGs that require a multicomponent complex to have antiviral function. Using this approach, we identified 30 genes that when silenced resulted in a 3- to 114-fold increase in WNV infection in the setting of exogenous IFN-β treatment. Novel ISGs with the greatest impact on WNV infection included *IFI6*, *IL13RA1*, *MAFK*, *SC4MOL*, and *PAK3*. We also identified one ISG, *ASCC3*, a putative DNA helicase, which negatively regulated cell-intrinsic antiviral responses. Mechanism-of-action studies revealed that ASCC3 functioned by dampening ISG expression through an interaction with the IRF-3 and IRF-7 pathway. Thus, type I IFN signaling promotes the expression of a large number of ISGs, several of which contribute to the antiviral response against WNV, with others having counterregulatory functions to minimize excessive immune activation.

RESULTS

An shRNA-based screen for defining ISGs with anti-WNV activity. To identify ISGs that inhibit replication of West Nile virus (WNV), we designed a lentivirus-based microRNA-adapted shRNA library containing 813 shRNA constructs against 245 different human ISGs (see Table S1 in the supplemental material) with, on average, four independent shRNAs per gene. ISGs were defined by our own and published microarray analyses as genes that were induced in cells at least 2-fold after treatment with IFN-α or IFN-β (22–26). We used our library to perform a screen in 96-well plates of human HeLa cells that were pretreated with inhibitory concentrations of IFN-β. The bicistronic pGIPZ lentiviral vector cotranscribes a microRNA carrying the shRNA and encoding green fluorescent protein (GFP), which marks transduced cells expressing the shRNA (Fig. 1A). HeLa cells were transduced transiently with lentiviruses carrying a single shRNA against a candidate ISG, treated with IFN-β for 6 h to induce ISG expression, and infected with WNV (multiplicity of infection [MOI] of 5). Viral infection was monitored by flow cytometry 48 h later for expression of viral envelope protein in GFP^+ transduced and GFP^- nontransduced cells (Fig. 1B and C) and normalized to wells transduced with a negative-control (scrambled) shRNA. shRNA constructs against ISGs with *Z* scores of $\geq$2 standard deviations from the mean were considered "hits" in the primary screen (Fig. 2A). Using this criterion, 80 shRNAs corresponding to 29 different ISGs were identified as putative antiviral molecules (see Table S2). For 26 of these candidate genes, transduction of at least two independent shRNAs targeting different mRNA segments resulted in increased WNV infection, suggesting that the observed phenotype was likely not due to off-target effects. The remaining three ISG (*IRF8*, *OAS1*, and *SAMHD1*) hits were retained for further study because they had low shRNA representation in the original library (see Table S2). Beyond these core ISG hits, we expanded the list for validation to include genes with multiple independent shRNAs that enhanced WNV infectivity greater than 2-fold by flow cytometry but yet fell short of achieving *Z* scores of >2. By relaxing the stringency of selection, we added another 33 ISGs corresponding to 81 shRNAs. The list of 62 (29 core plus 33 secondary) ISGs captured proteins involved in pattern recognition (*TLR3* and *MAVS*), IFN signaling (*STAT2*, *JAK2*, and *IRF9*), and known IFN effector functions against RNA viruses (*PKR*, *OAS1*, and *IFIT2*) (Fig. 2B). Expression of three shRNA constructs targeting one ISG, *ASCC3*, had opposing effects and resulted in inhibition of WNV infection, suggesting that it might be required for viral replication (27) or negatively regulate a host antiviral pathway.

Validation of the candidates from the primary screen. To validate hits from the primary screen, we optimized the efficiency of transduction of lentiviruses carrying shRNA (see Fig. S1A in the supplemental material) and measured a different virological endpoint; we assessed viral yield in the supernatant of WNV-infected HeLa cells at three time points (24, 48, and 72 h) (see Table S3). Silencing 47 of the 62 ISGs identified in the primary screen resulted in higher WNV titers in the supernatant for at least one time point relative to control shRNA (2- to 114-fold compared to control shRNA, $P < 0.05$); silencing 30 of these ISGs resulted in increased WNV infection relative to control shRNA (3- to 114-fold, $P < 0.05$) at all three time points (Fig. 2C). This subgroup of 30 ISGs included several genes with established innate immune recognition and signaling functions (*PKR*, *IRF3*, *IRF9*, *JAK2*, *STAT2*, *TLR3*, and *MAVS*). The efficiency of gene silencing of these ISGs was confirmed by quantitative reverse transcription-PCR (qRT-PCR) ($\geq$86% reduction compared to cells receiving nontargeting shRNA, $P < 0.05$) (Fig. 2D and data not shown), and importantly, no changes in cell proliferation or cytotoxicity were observed (see Fig. S1B and C). Silencing of several ISGs (*DDX24*, *IFI44L*, *IFI6*, *IFRD1*, *IL13RA1*, *MAFK*, *PAK3*, *SAMD9L*, and *SC4MOL*) not previously implicated in cell-intrinsic antiviral control of WNV resulted in increased infection at all time points (Fig. 3A to J). The specificity of silencing was validated, as no change in the expression of a reference antiviral gene (*PKR*) was observed in the transduced cells (see Fig. S1D). To determine whether the ISG hits were sufficient to restrict WNV infection, we ectopically expressed several with a C-terminal Flag tag in HeLa cells and measured WNV infectivity (Fig. 3K; see also Fig. S1E). Whereas *IFI6* and *SC4MOL* significantly inhibited WNV infection ($P < 0.01$), *DDX24*, *IFI44L*, *IFRD1*, *IL13RA1*, *MAFK*, *PAK3*, and *SAMD9L* did not show this effect. *IFITM3*, which was recently identified as an antiviral ISG against flaviviruses (14–16, 20) but was not picked up in the shRNA screen, also showed an inhibitory effect against WNV when expressed ectopically. In addition to WNV, DENV serotype 2 (DENV-2) and encephalomyocarditis virus (EMCV) infections also were enhanced after silencing several genes in the list, including *IFI6*, *MAFK*, *PAK3*, and *DDX24* (Fig. 3L and M). Among these validated hits, *IFI6* and *IFI44L* were suggested recently to inhibit infection of related *Flaviviridae* family members (YFV, DENV, and hepatitis C virus [HCV]) when expressed ectopically in cells in the absence of type I IFN signaling (18, 20).

***ASCC3* negatively regulates cell-intrinsic innate immunity.** While silencing of several different genes resulted in enhanced WNV infection, the opposing effect observed with *ASCC3* suggested that some ISGs might have counterregulatory functions to

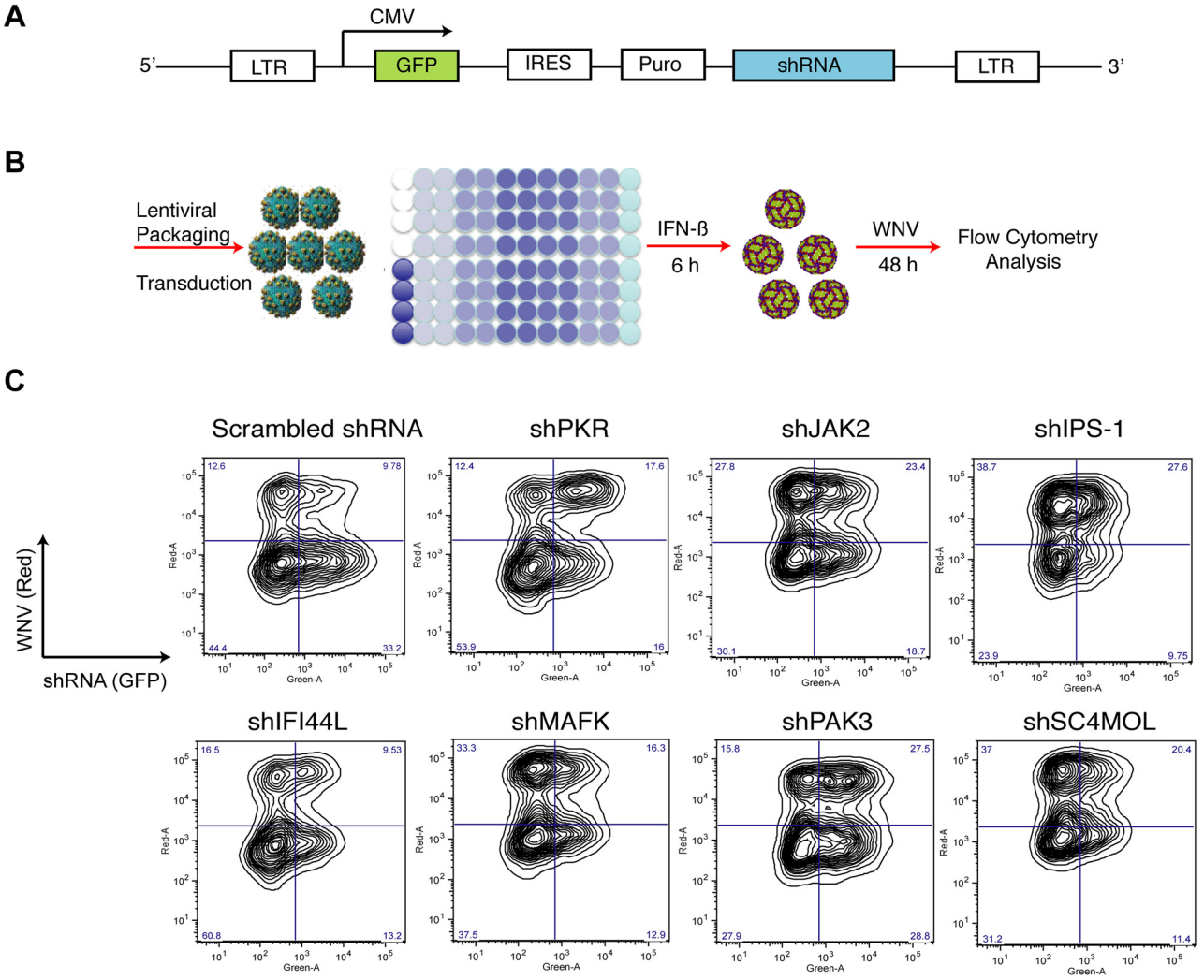

FIG 1 Lentivirus-based shRNA screen for ISGs that affect WNV infection. (A) Schematic of pGIPZ lentivirus vector expressing shRNA and GFP. LTR, long terminal repeat of HIV; CMV, immediate early promoter from human cytomegalovirus; IRES, internal ribosome entry site; Puro, puromycin selection marker. (B) Cartoon of the shRNA screen. Packaged and pseudotyped lentiviruses expressing 813 shRNAs were transduced into HeLa cells (list in Table S1 in the supplemental material). Forty-eight hours later, cells were treated with human IFN-β (10 IU/ml for 6 h) and then infected with WNV at an MOI of 5. Forty-eight hours later, cells were processed by flow cytometry. (C) Representative flow cytometry contour plots (from two independent screens) showing cells transduced with lentiviral vectors containing a scrambled shRNA control or shRNA specific against ISGs with top *Z* scores from the primary screen. The *x* axis reports the lentivirus transduction efficiency as represented by GFP expression, and the *y* axis shows infectivity of WNV, as judged by anti-envelope protein staining.

suppress host antiviral activity or could be required as cofactors supporting viral infection (27). Validation studies confirmed that silencing of *ASCC3* (82% reduction of mRNA levels, $P < 0.001$) (Fig. 4A) conferred an inhibitory effect on the yield on several positive-strand RNA viruses from *Flaviviridae* (WNV, 12-fold at 48 h, $P < 0.001$), *Togaviridae* (Chikungunya virus, 20-fold at 48 h, $P < 0.001$), and *Picornaviridae* (EMCV, 8-fold at 48 h, $P < 0.05$) families (Fig. 4B to D) without causing cytotoxicity (see Fig. S1C in the supplemental material).

Although *ASCC3* was reported as an ISG in transcriptional profiling studies (28), we validated this finding in primary cells. *ASCC3* expression was induced after treatment with exogenous IFN-β in murine macrophages (4.5-fold increase at 24 h, $P < 0.01$) and embryonic fibroblasts (murine embryonic fibroblasts [MEFs], 6.4-fold at 24 h, $P < 0.01$) but not in dendritic cells (see Fig. S2A in the supplemental material). Moreover, WNV infection of MEFs also induced expression of *ASCC3* (4-fold increase at 48 h, $P < 0.05$). In comparison, tumor necrosis factor alpha (TNF-α) treatment of MEFs did not induce *ASCC3* expression (see Fig. S2B to D). These data suggest that ASCC3 is regulated in a type I IFN-dependent and cell-type-specific manner.

To confirm the virological phenotype, we reciprocally and transiently expressed *ASCC3* with a C-terminal hemagglutinin (HA) epitope tag in HeLa cells (Fig. 4E) and assessed its impact on viral infection. WNV infection was enhanced (4-fold at 48 h, $P < 0.001$) in cells transfected with *ASCC3* compared to a control plasmid (Fig. 4F). Similar results were observed in human 293T cells (data not shown). Analogously, transduction of an shRNA targeting the mouse *Ascc3* ortholog in NIH 3T3

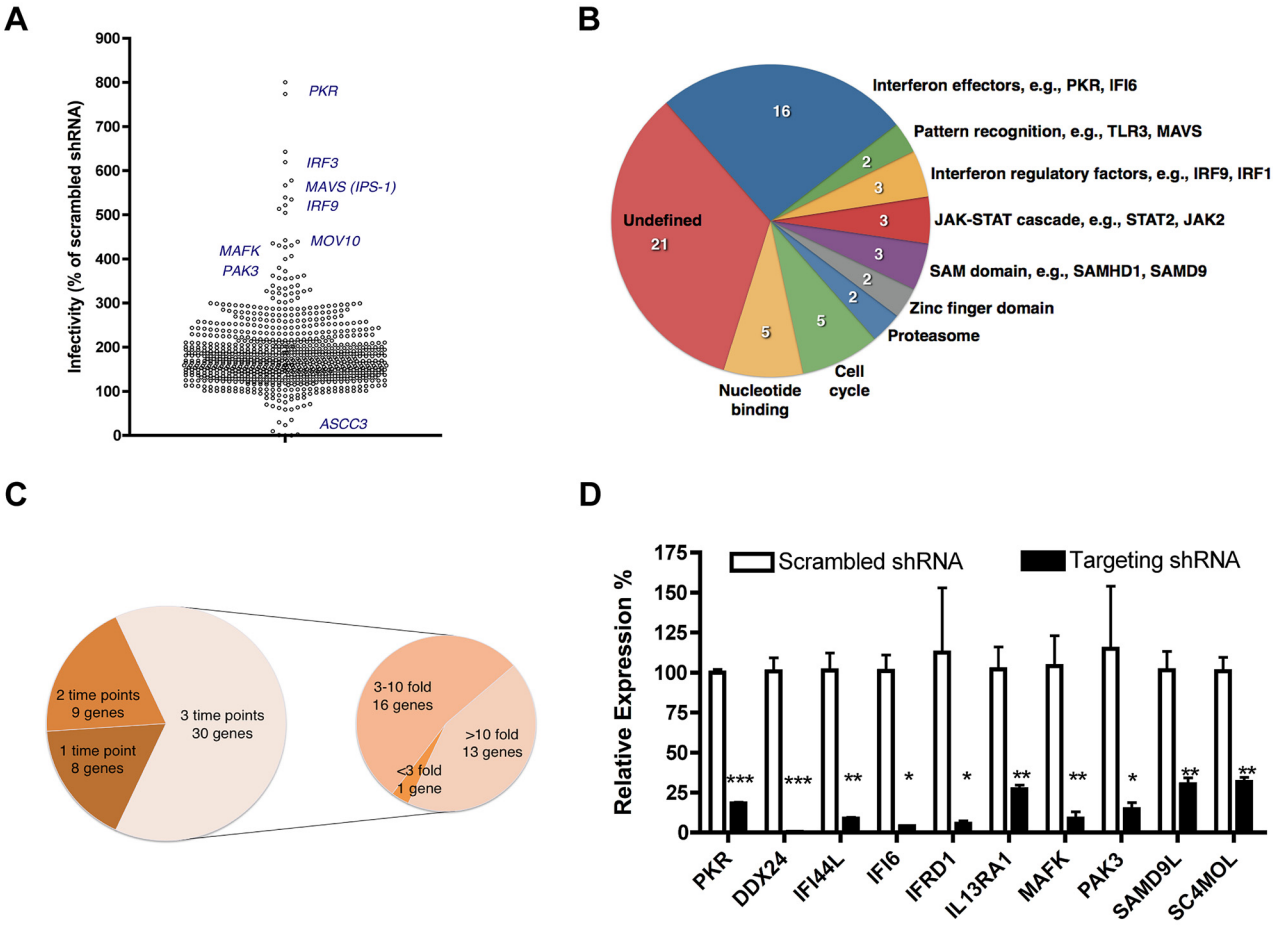

FIG 2 Validation of ISGs with antiviral potential. (A) Distribution of WNV infection in cells transduced with shRNA (normalized to the scrambled shRNA). Selected ISGs targeted by shRNA hits are labeled in blue. (B) Pie chart showing classification of top ISG hits, grouped by their known or putative functional categories (see Table S2 in the supplemental material). (C) Pie charts showing the breakdown of the 47 ISGs that were validated by viral yield assays. The chart on the left shows the number of ISGs in which silencing resulted in enhanced WNV production (relative to cells transduced with the scrambled shRNA) at different time points. The chart on the right shows the relative fold differences in viral yield associated with silencing of these ISGs (see also Table S3). (D) mRNA was harvested from cells transduced with the indicated shRNA or the scrambled shRNA control, and relative expression of the corresponding ISGs was determined by qRT-PCR (see also Fig. S1). The results are the averages of three independent experiments and are shown as means ± standard deviations. Statistical significance was determined by Student's *t* test (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

fibroblasts silenced its mRNA expression by 82% ($P < 0.05$) (Fig. 4G) and resulted in reduced WNV infection (up to 8-fold, $P < 0.01$) under basal conditions (Fig. 4H) or in the presence of IFN-β treatment (Fig. 4I).

***ASCC3* modulates cellular ISG expression.** As silencing of *ASCC3* resulted in an antiviral effect against three unrelated viruses, we speculated that it functioned to temper expression or activity of key host defense pathways. To evaluate this hypothesis, we sequenced mRNA (RNA-seq) from IFN-β-treated cells transduced with either scrambled shRNA or shRNA against *ASCC3* (see Table S4 in the supplemental material). Silencing of *ASCC3* resulted in the upregulation and downregulation of 199 and 42 genes, respectively, with *P* values of <0.05, indicating a significant impact on cellular gene expression. Notably, silencing of cellular *ASCC3* resulted in enhanced expression of RIG-I-like receptors (*DHX58*) and type I IFN-induced genes, including *IFI44*, *RSAD2*, and *IFIT2* (Fig. 5A). To validate the RNA-seq results, we measured by qRT-PCR basal or IFN-β-induced mRNA expression of 13 ISGs (*PKR*, *GBP1*, *IFI44*, *IFNA2*, *IFNB1*, *IRF1*, *ISG15*, *ISG20*, *MX1*, *OAS2*, *RNASE L*, *STAT1*, and *RSAD2*) that are induced through type I IFN-dependent or -independent (e.g., via IRF-3) signaling pathways (28, 29) (Fig. 5B and C). Silencing of *ASCC3* enhanced (2- to 31-fold, $P < 0.05$) expression of 7 ISGs at the basal level and 9 ISGs in the presence of IFN-β, suggesting that ASCC3 negatively regulates expression of selected ISGs.

***ASCC3* functions in an IRF-3- and IRF-7-dependent manner.** We hypothesized that ASCC3 might function to negatively regulate ISG expression through either NF-κB or IRF-3- and IRF-7-dependent transcriptional signals. However, ectopic expression of ASCC3 in *Ikk*$\beta^{-/-}$ fibroblasts showed enhanced WNV infection, suggesting that the integrity of the NF-κB activation pathway was not essential for ASCC3-dependent effects on infection (Fig. 6A). We also failed to observe substantive differences in the levels of IκBα or the p65 subunit of NF-κB in cells that were silenced for or that ectopically expressed ASCC3 (data not shown). Given this, we next assessed whether the functional effects of ASCC3 required IRF-3- and IRF-7-dependent signals. We silenced *Ascc3* expression in wild-type (WT) or *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ double-knockout

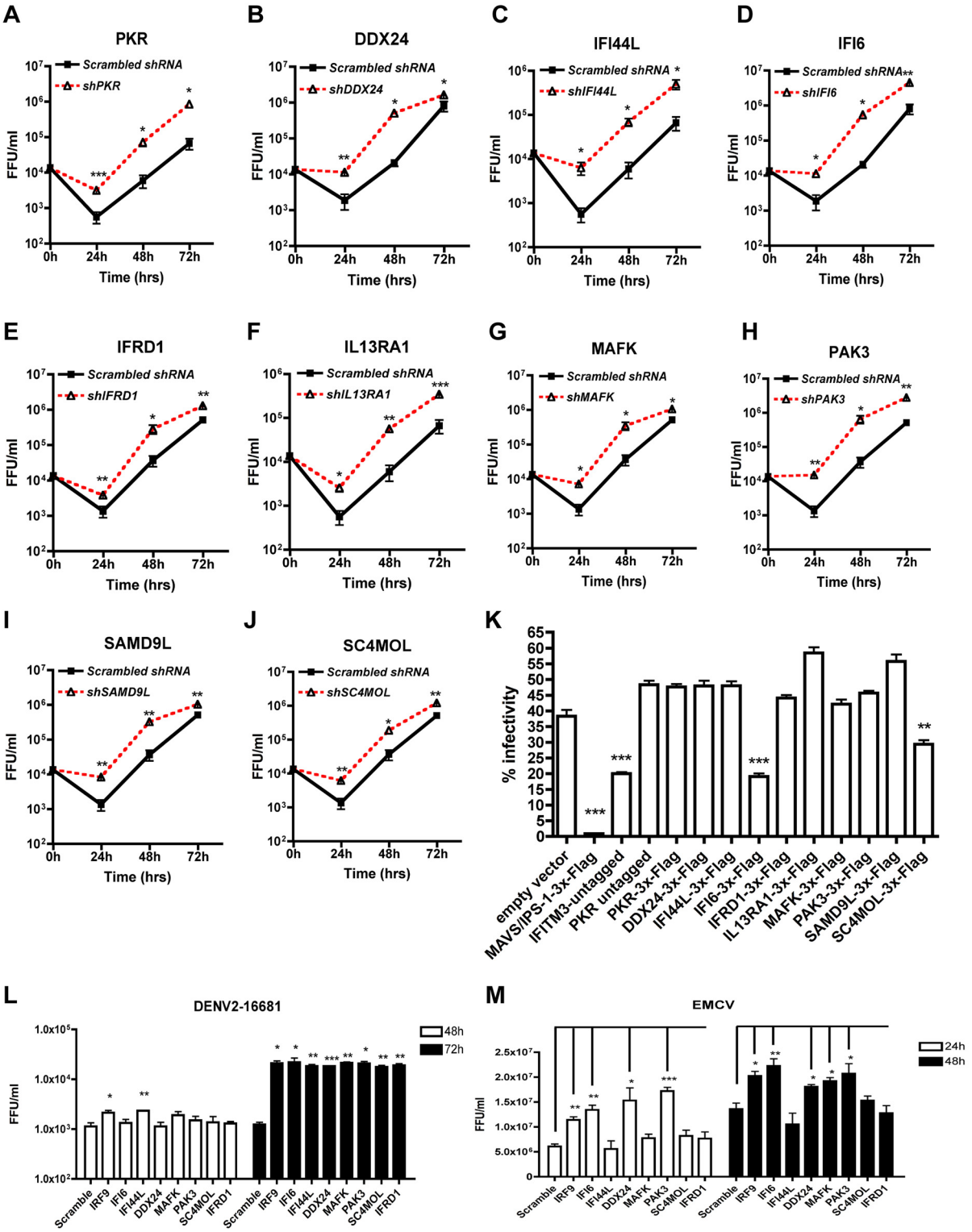

FIG 3 Antiviral genes against RNA viruses. (A to J) Multistep growth analysis of WNV infection on HeLa cells transduced with either scrambled shRNA or shRNA targeting the following top candidates: *PKR*, *DDX24*, *IFI44L*, *IFI6*, *IFRD1*, *IL13RA1*, *MAFK*, *PAK3*, *SAMD9L*, and *SC4MOL*. After transduction, cells were treated with 10 IU/ml of IFN-β for 6 h and then infected with WNV at an MOI of 0.05. The data are the averages of three independent experiments performed in triplicate with error bars indicating the standard deviations and asterisks marking values that are statistically different from the scrambled shRNA (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). (K) HeLa cells were transfected with ISGs tagged or untagged with 3× Flag. One day later, cells were infected with WNV at an MOI of 0.3. One day after this, viral infectivity was measured as the percentage of infected cells and is represented as mean ± standard deviation. Statistical significance was determined by Student's *t* test (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). (L and M) Titers of DENV-2 and EMCV grown from HeLa cells transduced with scrambled shRNA and shRNA targeting *IRF9*, *IFI6*, *IFI44L*, *DDX24*, *MAFK*, *PAK3*, *SC4MOL*, and *IFRD1*. Data are shown as means ± standard deviations. Statistical significance was determined by Student's *t* test (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

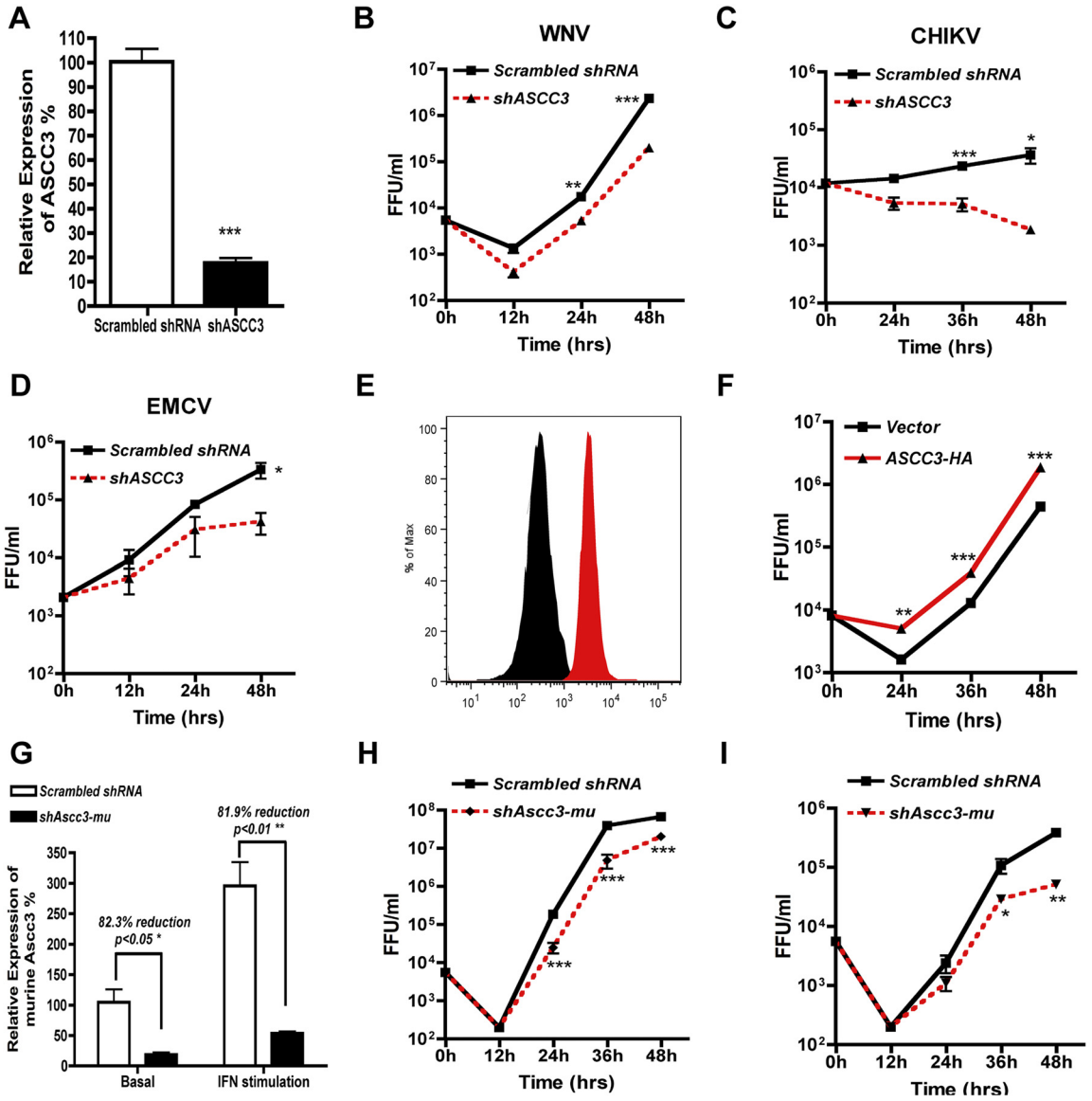

FIG 4 Silencing of *ASCC3* restricts viral replication. (A) Expression of *ASCC3* mRNA in HeLa cells expressing scrambled or *ASCC3* shRNA. The results are the averages of three independent experiments, and asterisks indicate differences that are statistically significant (***, $P < 0.001$). (B to D) Titers of WNV (B), Chikungunya virus (C), and EMCV (D) in the supernatants of HeLa cells transduced with either control or shRNA against *ASCC3* at the indicated time points. The results are the averages of three independent experiments performed in triplicate, and asterisks indicate differences from the scrambled shRNA that are statistically significant (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$). (E and F) HeLa cells were transfected with expression plasmids encoding GFP or human ASCC3 tagged at the C terminus with HA. (E) The transfection efficiency was measured by flow cytometry using an anti-HA tag antibody and an Alexa Fluor 647-conjugated secondary antibody. (F) Multistep growth analysis of WNV infection in GFP (vector) and human ASCC3-transfected cells. The results are the averages of three independent experiments, and asterisks indicate differences that are statistically significant (***, $P < 0.001$; **, $P < 0.01$). (G to I) NIH 3T3 cells were transduced with lentivirus carrying a control shRNA or shRNA against murine *Ascc3*. The mRNA levels of *Ascc3* were measured under basal or IFN-β-induced conditions by qRT-PCR and are expressed relative to those after transduction with scrambled shRNA (G). Multistep growth analysis of WNV infection was performed in the corresponding cells without (H) or with (I) IFN-β pretreatment (50 IU/ml for 6 h). The results are the averages of three independent experiments, and asterisks indicate differences that are statistically significant (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

(DKO) MEFs and measured WNV infection and levels of selected ISGs. *Ascc3* expression was decreased by up to 74% and 60% in WT and DKO MEFs, respectively ($P < 0.01$) (Fig. 6B). In untreated WT or DKO MEFs, we failed to observe an effect of Ascc3 silencing on WNV infection (see Fig. S3 in the supplemental material), possibly due to its lower level of expression in these cells. However, silencing of *Ascc3* in the context of IFN-β treatment resulted in decreased WNV infection at 72 h in WT MEFs (10.5-fold, $P < 0.001$), and this effect was not observed in DKO MEFs (Fig. 6C). Consistent with this, we observed increased ISG (*Ifi44*, *Irf1*, and *Rsad2*) expression in *Ascc3*-silenced WT but not *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ DKO MEFs (Fig. 6D to F). Collectively, our results suggest a model in which IFN-β induces expression of antiviral, proinflammatory, and counterregulatory ISGs, the last of which include proteins such as ASCC3. Expression of ASCC3 dampens the type I IFN-dependent signals likely by modulating activity of

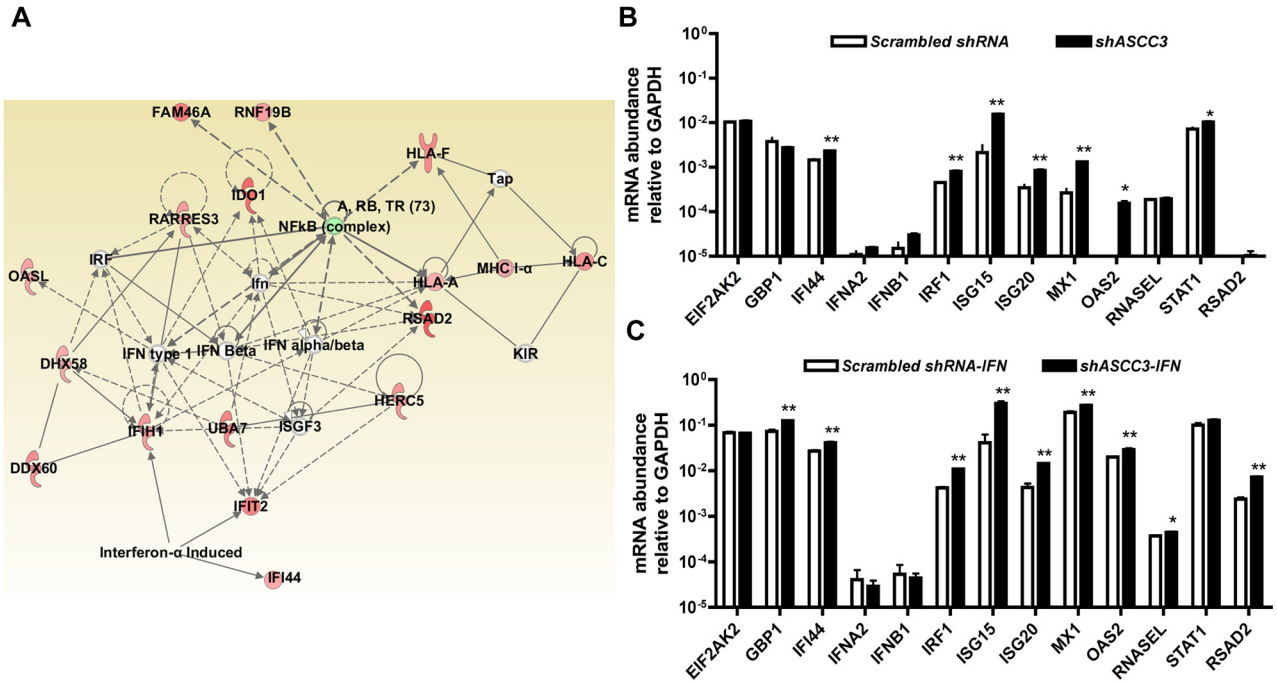

FIG 5 Silencing *ASCC3* expression upregulates cellular antiviral response. (A) HeLa cells were transduced with either scrambled shRNA or shRNA against *ASCC3*. Forty-eight hours later, cells were treated with 10 IU/ml of human IFN-β for 6 h. mRNAs were then harvested, purified, and sequenced. Ingenuity pathway analysis determined expression and interaction of ISGs and PRRs. Red and green colors indicate higher and lower gene expression levels, respectively, in *ASCC3*-silenced cells than in control cells. Fold differences in expression are listed in Table S4 in the supplemental material with a cutoff of a ≥2-fold change and an ANOVA *P* value with a Benjamini and Hochberg algorithm-corrected false discovery rate of <0.05. Shown are functional relationships that associate with antimicrobial pathway, inflammatory pathway, and posttranslational modification. (B and C) Gene expression profiles of 13 ISGs in HeLa cells that were transduced with an shRNA (scrambled or targeting ASCC3) under basal (B) or IFN-β treatment (50 IU/ml for 6 h) (C) conditions. mRNA abundance of ISGs was assayed using a commercial TaqMan array in 96-well plates and normalized to GAPDH. Statistical significance was determined by Student's *t* test (**, $P < 0.01$; *, $P < 0.05$).

IRF-3 and IRF-7 pathways and thus tempers the cellular host defense and inflammatory response.

DISCUSSION

In this report, we performed an shRNA screen and identified 30 candidate ISGs with antiviral activity against WNV. Our screening methodology relied on gene silencing through delivery of microRNA by lentiviruses. In comparison to prior ectopic gene expression screens (15, 18, 20, 21), our experiments were performed in the context of an intact IFN signaling pathway so that we could identify single ISGs that were required for an optimal host response against WNV. Subsequent validation studies with multiple independent shRNAs targeting an individual gene and virological growth curve analyses defined nine novel genes that strongly restricted WNV infection. Testing these candidates with related (DENV) and unrelated (EMCV) positive-strand RNA viruses revealed that several had antiviral activity against multiple viruses. Ectopic expression experiments demonstrated that a subset of ISGs (*IFI6* and *SC4MOL*) were sufficient for conferring antiviral effects against WNV in the absence of the exogenous type I IFN stimulation. We also identified a novel ISG (*ASCC3*), which had an opposing phenotype: silencing human *ASCC3* or mouse *Ascc3* decreased viral infection, and reciprocal ectopic expression enhanced viral infection. Mechanistic studies suggested that ASCC3 serves a counterregulatory function against cellular IFN-induced antiviral responses and acts in part by modulating signals of the IRF-3 and IRF-7 pathways.

Among the 30 ISGs identified in our screen with antiviral activity against WNV, seven genes are established components of innate immune recognition and signaling molecules (*PKR*, *IRF3*, *IRF9*, *JAK2*, *STAT2*, *TLR3*, and *MAVS*). Indeed, the mouse orthologs of several of these genes are required for control of WNV infection in mice or primary cells in the context of targeted gene deletion or silencing (9, 29–33). Four additional ISGs (*IFIT3*, *TRIM21*, *IFI6*, and *IFI44L*) have been reported to have inhibitory activity in the context of ectopic gene expression and infection by YFV, adenovirus, vesicular stomatitis virus, Sindbis virus, EMCV, or HCV (20, 21, 34–36). Among the known antiviral effects of these genes, TRIM21 has been suggested to sustain IRF-3 activation (34) whereas IFIT3 may sequester viral RNA displaying 5′-ppp (37) or inhibit translation initiation by binding subunits of eIF3 (38). While ectopic expression of IFI6 and SC4MOL inhibited viral infection, to date, no mechanism of action has been described for either gene. Beyond these, we identified a group of ISGs with heretofore-uncharacterized antiviral activity, including an RNA helicase (*DDX24*), a serine/threonine protein kinase (*PAK3*), a transcription factor (*MAFK*), an endoplasmic reticulum protein (*SC4MOL*), a tyrosine kinase-associated receptor (*IL13RA1*), an IFN-related development regulator (*IFRD1*), and a sterile alpha motif domain-containing protein (*SAMD9L*). Silencing expression of *DDX24*, *PAK3*, *MAFK*, and *IFI6* enhanced infection by a related flavivirus (DENV) and an unrelated picornavirus (EMCV), suggesting that their antiviral activities are more broad

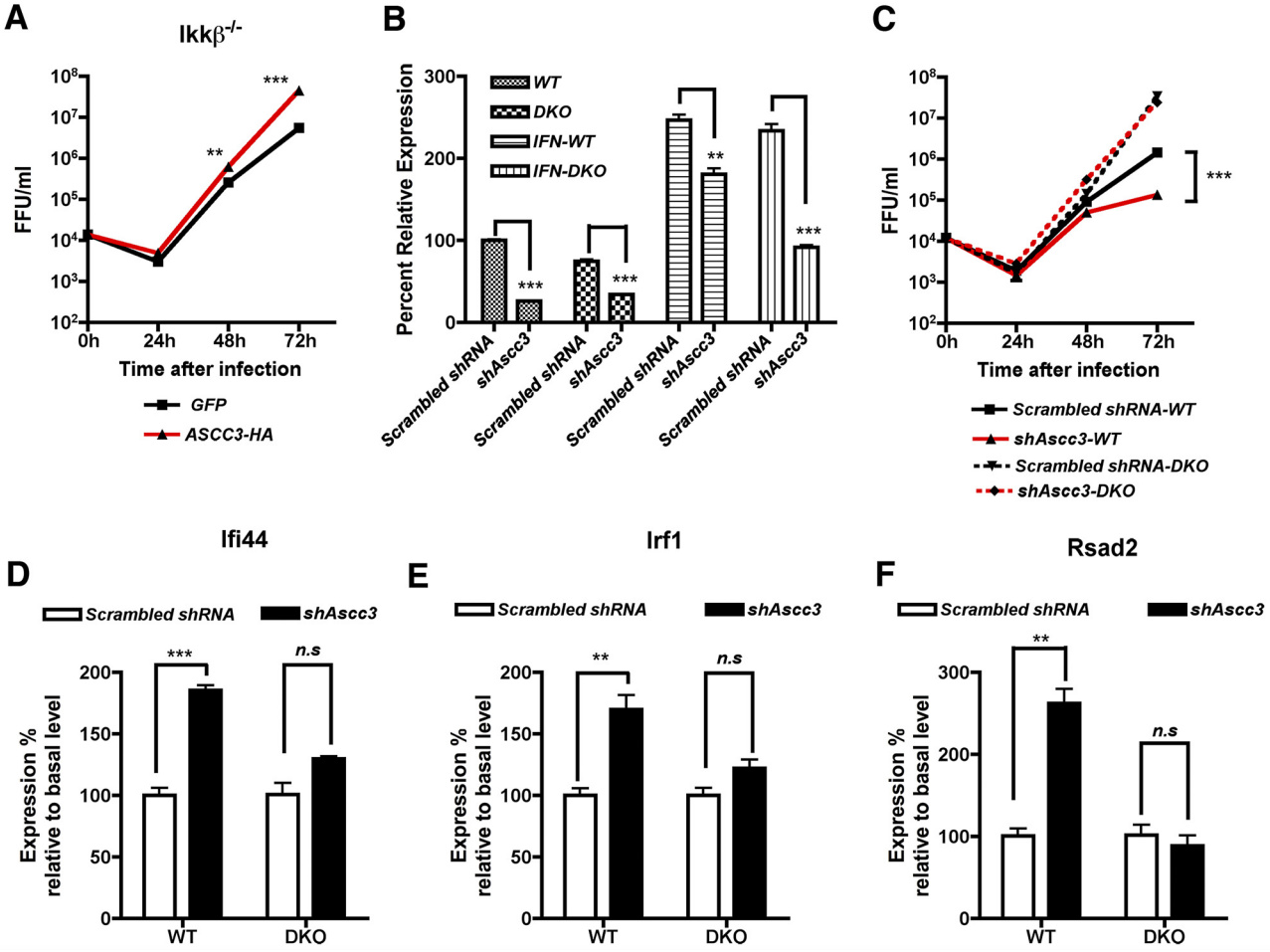

FIG 6 *ASCC3* functions through an IRF-3- and IRF-7-dependent pathway. (A) Primary *Ikkβ*$^{-/-}$ MEFs were transfected with pCAGGS-GFP or pCAGGS-ASCC3-HA and then infected with WNV at an MOI of 0.05. Viral titers were monitored through a focus-forming assay at indicated time points. The results are the averages of three independent experiments performed in duplicate, and asterisks indicate differences that are statistically significant (***, $P < 0.001$; **, $P < 0.01$). (B) Primary wild-type (WT) and *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ DKO MEFs were transduced with scrambled shRNA or shRNA against murine *Ascc3*. Cells were either untreated or treated with 10 IU/ml murine IFN-*β* for 6 h. Total RNA was harvested, and expression of *Ascc3* was determined by qRT-PCR. (C) Multistep growth analysis of WNV infection was performed on the corresponding cells after IFN-*β* treatment. (D to F) Expression of three ISGs was assayed in shRNA-transduced WT and *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ DKO MEFs at 72 h post-WNV infection. Relative expression levels of *Ifi44* (D), *Irf1* (E), and *Rsad2* (F) were normalized to scrambled shRNA-transduced cells. The results are the averages of three independent experiments performed in triplicate, and asterisks indicate differences from the scrambled shRNA control that are statistically significant (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., not significant).

spectrum in nature. By comparison, other ISGs (*IFI44L*, *SC4MOL*, and *IFRD1*) showed a more targeted restriction of flaviviruses in our experiments.

While systematic or candidate-based ectopic expression screens have identified ISGs with antiviral activities against *Flaviviridae* family members (15, 18, 20), we observed limited overlap in our shRNA-based screen, demonstrating the novelty of our approach. Beyond the differences in viruses (WNV, YFV, DENV, or HCV) and cell types (HeLa, 293T, Huh-7, and *STAT1*$^{-/-}$ fibroblasts) used during the primary screen, our shRNA-based strategy uniquely was performed in the presence of an active type I IFN response (associated with exogenous treatment) and thus is more likely to identify ISGs that are required, but not necessarily sufficient, for an optimal antiviral response (39, 40). Nonetheless, each approach identified both putative effector molecules and upstream signaling proteins that modulate the host response. For instance, genes in the RIG-I-like receptor (RLR) signaling pathway (ectopic screen, *RIG-I* [*DDX58*] and *MDA5* [*IFIH1*]; shRNA screen, *MAVS*) were identified. In comparison, some signaling proteins showed much greater antiviral activity when expressed ectopically, such as IRF-1. Expression of this transcription factor conferred broad-spectrum antiviral activity in multiple cell types, presumably by inducing other ISGs or antiviral genes (20). Most of our top candidates did not inhibit WNV infection when expressed ectopically in HeLa cells, which may explain why they were not captured in prior screens. These ISGs may require other IFN-stimulated factors to control viral infection. In addition to these targeted screens, two unbiased genome-wide small interfering RNA (siRNA) screens against HCV (39) and WNV (40) also identified ISGs with antiviral activity. A comparison of our hit list with the WNV study by Krishnan and colleagues (40) revealed only three matching genes (*ATCAY*, *LPGAT1*, and *SERPINB7*) that, when silenced, affected WNV infection. The disparity may reflect the absence of IFN stimulation in their system—our anti-

viral genes may not have been expressed at sufficient amounts at baseline to yield a phenotype. Overall, gene silencing and ectopic expression approaches likely identify different but overlapping sets of antiviral proteins because they screen for ISGs with necessary or sufficient activity, respectively. Ultimately, combining data from these two approaches will help identify ISGs with the greatest inhibitory activity against individual and multiple viruses and begin to suggest possible mechanisms of action.

Our experiments identified a function of *ASCC3*, to dampen the IFN-β-induced antiviral response by modulating IRF-3- and IRF-7-dependent transcriptional signals, suggesting a novel negative regulatory mechanism of the pathways. This counterregulatory mechanism was supported by the following data: (i) silencing of *ASCC3* resulted in increased expression of several ISGs (as defined by qRT-PCR and RNA-seq), which conferred antiviral activity against RNA viruses from different families; (ii) the virological and gene expression effects conferred by gene silencing of *Ascc3* were abolished in *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ DKO cells. These studies are most consistent with a model of ASCC3 acting as a negative regulator of the antiviral host response, which depends on the integrity of IRF-3 and IRF-7 transcriptional pathways. As ectopic expression of other ISGs (*ADAR*, *FAM46C*, *LY6E*, and *MCOLN2*) resulted in enhanced viral replication (20), additional negative regulatory pathways may exist.

The putative domain structure of ASCC3 suggests that it is comprised of two predicted superfamily II helicase regions (amino acids 440 to 1244 and 1327 to 2056). *In vitro* helicase activity assay experiments demonstrated that the region comprising amino acids 1301 to 2202 could unwind double-stranded DNA (41). Consistent with this function, preliminary cellular localization studies indicate that the full-length ASCC3 is present predominantly in the cytoplasm with a small fraction in the nucleus (J. Li and M. Diamond, unpublished results). These data suggest two possible models for ASCC3 function: (i) ASCC3 is induced after viral infection or type I IFN signaling, translocates into the nucleus, and either binds and unwinds DNA or recruits other transcriptional or epigenetic regulators (42); or (ii) ASCC3 binds other partner proteins in the cytoplasm or nucleus to modulate IRF-3- and IRF-7-dependent transcriptional activity. Studies are under way to address these unresolved mechanistic questions.

In summary, our shRNA-based screen identified a novel set of ISGs with antiviral activity against multiple RNA viruses. Future studies are planned with additional RNA (e.g., negative-strand viruses and retroviruses) and DNA viruses to determine the breadth of their inhibitory activities, their mechanisms of action, and their physiological role in restriction of pathogenesis in the context of targeted deletion of the murine orthologs. Our experiments also defined ASCC3 as an ISG with negative regulatory activity of IFN-dependent gene induction pathways; this finding provides an example of the delicate balance required for restriction of microbial infection and the need to mitigate host defense responses that could result in immune pathology or autoimmunity. Further investigation on the function of ASCC3 and possibly other newly discovered counterregulatory ISGs will clarify the host-pathogen interface and may provide novel avenues for pharmacological modulation to control excessive tissue damage mediated by the virus or host.

MATERIALS AND METHODS

Antibodies. The following antibodies were used for immunoblotting and immunofluorescence experiments: rabbit horseradish peroxidase (HRP)-conjugated anti-HA tag (Roche), mouse HRP-conjugated anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (Sigma), goat Alexa Fluor 488-anti-mouse IgG (Life Technologies), mouse anti-Flag (Sigma), and HRP-conjugated goat anti-rabbit or goat anti-mouse antibody (Jackson ImmunoResearch Laboratories).

Cells. Vero T144, NIH 3T3, HEK293T, and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific), 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH 7.3), and 10 mM nonessential amino acids (Cellgro) at 37°C and 5% CO_2. Parental WT and *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ DKO MEFs were prepared according to a previously published protocol (43, 44). Primary MEFs derived from *Ikk*$\beta^{-/-}$ mice were a generous gift of B. tenOever (Mount Sinai School of Medicine, New York, NY).

Cytokines. Human and murine IFN-β were purchased commercially (PBL InterferonSource) and used at concentrations of 10 IU/ml for viral growth curve experiments.

Viruses. The WNV-NY strain was isolated in New York in 2000 (45) and passaged once in C6/36 *Aedes albopictus* cells. DENV serotype 2 strain 16681 was propagated in C6/36 *Aedes albopictus* cells according to previously described protocols (46). EMCV strain K was grown in L929 cells, and Chikungunya virus (LR2006 OPY-1) was isolated from an outbreak in La Reunion (47), obtained from S. Higgs (Manhattan, KS), and passaged once in C6/36 *Aedes albopictus* cells.

Plasmids, oligonucleotides, and transfections. pCAGGS-*ASCC3*-HA was generated by subcloning *ASCC3* with a C-terminal HA tag downstream of the chicken β-actin promoter. ISGs appended with 3× Flag tags were cloned into pcDNA4.0, and transfections into HeLa cells were performed with FuGENE HD (Roche) according to the manufacturer's instructions. The primers used for cloning are listed in Table S5 in the supplemental material.

shRNA library and the ISG screen. The lentivirus-based shRNAmir library against the 245 human ISGs was custom generated and purchased commercially (Open Biosystems). The seed sequences for shRNA targeting each gene are listed in Table S1 in the supplemental material. The bicistronic vector coexpresses shRNA and GFP driven downstream of a cytomegalovirus (CMV) promoter. Individual shRNA constructs were packaged into lentiviral vectors in 96-well plates according to the manufacturer's instructions. HeLa cells were transduced with lentiviruses, and 48 h later, cells were treated with 10 IU/ml of IFN-β. Six hours later, cells were infected with WNV at an MOI of 5. After 48 h, cells were harvested, fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.1% (wt/vol) saponin, and incubated with an anti-WNV monoclonal antibody (MAb) (10 μg/ml of E16 [48]) and an Alexa Fluor 647-conjugated goat anti-mouse (1/500 dilution) secondary antibody. Cells were analyzed using a FACSArray flow cytometer (BD Biosciences). Viral infection was determined based on the percentage of WNV envelope protein-positive cells in shRNA-transduced (GFP$^+$) and untransduced (GFP$^-$) populations. The relative infectivity in each well was normalized to the wells containing a control scrambled shRNA sequence to obtain *Z* scores. Independent lentivirus stocks were used to validate the primary screen results in three independent replicates. Initial lead hits were defined as those *Z* scores greater than 2 or less than −2.

Viral growth kinetics. HeLa (human), MEF, or NIH 3T3 (mouse) cells transduced with shRNA-containing lentivirus in 24-well plates were treated with 10 IU/ml of human or murine IFN-β for 6 h before infection with WNV at an MOI of 0.05. Supernatants were harvested at specified times, and viral titer was determined by a focus-forming assay performed on Vero cells as previously described (49).

Infectivity assays by fluorescence imaging. HeLa cells in 96-well plates were transfected with individual ISGs tagged with 3× Flag. After 24 h, cells were infected with WNV at an MOI of 0.3 for another 24 h and

then fixed, permeabilized, and costained for WNV envelope protein (MAb E18) and the nucleus using 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Images were captured and processed using a Celigo cytometer (Cyntellect). Infected and uninfected populations were gated separately, and infectivity was measured as the percentage of infected cells from the total cell counts.

RNA-seq analysis. HeLa cells were transduced with scrambled shRNA or shRNA targeting ASCC3 and treated with 10 IU/ml of human IFN-β for 6 h. Total RNA was harvested using an RNeasy minikit (Qiagen) followed by mRNA extraction using a Dynal mRNA Direct kit. mRNA was then fragmented and reverse transcribed to double-stranded cDNA. Sequencing was performed in a single lane with Illumina HiSeq 2000 sequencing instrument with a 50-nucleotide read length. Short reads were aligned and assembled using the TopHat and Cufflinks package. Differential expression and interaction of genes were detected with Ingenuity Pathway Analysis.

Statistical analysis. Virological data sets were compared using an unpaired, two-tailed Student *t* test or analysis of variance (ANOVA) for multiple comparisons to determine statistical significance. Analysis of virological data was performed with Prism software (GraphPad Software). RNA-seq data were analyzed using an ANOVA with a Benjamini and Hochberg algorithm false discovery rate correction. Statistical significance was determined when P values were <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00385-13/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, TIF file, 2.5 MB.
Figure S2, TIF file, 0.2 MB.
Figure S3, TIF file, 0.5 MB.
Table S1, PDF file, 0.3 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.
Table S4, PDF file, 0.1 MB.
Table S5, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

NIH grants U54 AI081680 (Pacific Northwest Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research) (M.G., M.S.D., and S.K.C.), HHSN272200900041CU19 (M.S.D.), AI083019 (M.G. and M.S.D.), and R01 AI104972 (M.S.D. and S.K.C.) supported this work.

We thank Kevin Chen and David Wang (Washington University) for their assistance and advice with processing and analysis of the RNA-seq data.

J.L., S.C.D., and B.C.C. performed the experiments. J.L. and M.S.D. designed the experiments and wrote the initial draft of the manuscript. H.C., M.G., and S.K.C. contributed to the study design, analysis, and preparation of the manuscript.

The authors have no financial conflicts to disclose.

REFERENCES

1. **Colonna M.** 2007. TLR pathways and IFN-regulatory factors: to each its own. Eur. J. Immunol. **37:**306–309.
2. **Honda K, Taniguchi T.** 2006. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat. Rev. Immunol. **6:**644–658.
3. **Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD.** 1998. How cells respond to interferons. Annu. Rev. Biochem. **67:**227–264.
4. **Samuel MA, Diamond MS.** 2005. Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. J. Virol. **79:**13350–13361.
5. **Ryman KD, Klimstra WB, Nguyen KB, Biron CA, Johnston RE.** 2000. Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. J. Virol. **74:**3366–3378.
6. **Fiette L, Aubert C, Müller U, Huang S, Aguet M, Brahic M, Bureau JF.** 1995. Theiler's virus infection of 129Sv mice that lack the interferon alpha/beta or interferon gamma receptors. J. Exp. Med. **181:**2069–2076.
7. **Mackenzie JS, Gubler DJ, Petersen LR.** 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat. Med. **10:**S98–S109. doi: 10.1038/nm1144.
8. **Daffis S, Suthar MS, Gale M, Jr, Diamond MS.** 2009. Measure and countermeasure: type I IFN (IFN-alpha/beta) antiviral response against West Nile virus. J. Innate Immun. **1:**435–445.
9. **Samuel MA, Whitby K, Keller BC, Marri A, Barchet W, Williams BR, Silverman RH, Gale M, Diamond MS.** 2006. PKR and RNase L contribute to protection against lethal West Nile virus infection by controlling early viral spread in the periphery and replication in neurons. J. Virol. **80:**7009–7019.
10. **Scherbik SV, Paranjape JM, Stockman BM, Silverman RH, Brinton MA.** 2006. RNase L plays a role in the antiviral response to West Nile virus. J. Virol. **80:**2987–2999.
11. **Daffis S, Szretter KJ, Schriewer J, Li J, Youn S, Errett J, Lin TY, Schneller S, Zust R, Dong H, Thiel V, Sen GC, Fensterl V, Klimstra WB, Pierson TC, Buller RM, Gale M, Shi PY, Diamond MS.** 2010. 2′-O methylation of the viral mRNA cap evades host restriction by IFIT family members. Nature **468:**452–456.
12. **Szretter KJ, Brien JD, Thackray LB, Virgin HW, Cresswell P, Diamond MS.** 2011. The interferon-inducible gene viperin restricts West Nile virus pathogenesis. J. Virol. **85:**11557–11566.
13. **Szretter KJ, Daniels BP, Cho H, Gainey MD, Yokoyama WM, Gale M, Virgin HW, Klein RS, Sen GC, Diamond MS.** 2012. 2′-O methylation of the viral mRNA cap by West Nile virus evades Ifit1-dependent and -independent mechanisms of host restriction *in vivo*. PLoS Pathog. **8:**e1002698. doi: 10.1371/journal.ppat.1002698.
14. **Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ.** 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. Cell **139:**1243–1254.
15. **Jiang D, Weidner JM, Qing M, Pan XB, Guo H, Xu C, Zhang X, Birk A, Chang J, Shi PY, Block TM, Guo JT.** 2010. Identification of five interferon-induced cellular proteins that inhibit West Nile virus and dengue virus infections. J. Virol. **84:**8332–8341.
16. **Chan YK, Huang IC, Farzan M.** 2012. IFITM proteins restrict antibody-dependent enhancement of dengue virus infection. PLoS One **7:**e34508. doi: 10.1371/journal.pone.0034508.
17. **Taylor RT, Lubick KJ, Robertson SJ, Broughton JP, Bloom ME, Bresnahan WA, Best SM.** 2011. TRIM79α, an interferon-stimulated gene product, restricts tick-borne encephalitis virus replication by degrading the viral RNA polymerase. Cell Host Microbe **10:**185–196.
18. **Schoggins JW, Dorner M, Feulner M, Imanaka N, Murphy MY, Ploss A, Rice CM.** 2012. Dengue reporter viruses reveal viral dynamics in interferon receptor-deficient mice and sensitivity to interferon effectors *in vitro*. Proc. Natl. Acad. Sci. U. S. A. **109:**-14610–14615.
19. **Schoggins JW, Rice CM.** 2011. Interferon-stimulated genes and their antiviral effector functions. Curr. Opin. Virol. **1:**519–525.
20. **Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM.** 2011. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature **472:**481–485.
21. **Karki S, Li MM, Schoggins JW, Tian S, Rice CM, Macdonald MR.** 2012. Multiple interferon stimulated genes synergize with the zinc finger antiviral protein to mediate anti-alphavirus activity. PLoS One **7:**e37398. doi: 10.1371/journal.pone.0037398.
22. **Indraccolo S, Pfeffer U, Minuzzo S, Esposito G, Roni V, Mandruzzato S, Ferrari N, Anfosso L, Dell'Eva R, Noonan DM, Chieco-Bianchi L, Albini A, Amadori A.** 2007. Identification of genes selectively regulated by IFNs in endothelial cells. J. Immunol. **178:**1122–1135.
23. **Boonyaratanakornkit JB, Bartlett EJ, Amaro-Carambot E, Collins PL, Murphy BR, Schmidt AC.** 2009. The C proteins of human parainfluenza virus type 1 (HPIV1) control the transcription of a broad array of cellular genes that would otherwise respond to HPIV1 infection. J. Virol. **83:**1892–1910.
24. **Hayashi J, Stoyanova R, Seeger C.** 2005. The transcriptome of HCV replicon expressing cell lines in the presence of alpha interferon. Virology **335:**264–275.
25. **Fernald GH, Knott S, Pachner A, Caillier SJ, Narayan K, Oksenberg JR, Mousavi P, Baranzini SE.** 2007. Genome-wide network analysis reveals

the global properties of IFN-beta immediate transcriptional effects in humans. J. Immunol. **178:**5076–5085.
26. **Pfeffer LM, Kim JG, Pfeffer SR, Carrigan DJ, Baker DP, Wei L, Homayouni R.** 2004. Role of nuclear factor-kappaB in the antiviral action of interferon and interferon-regulated gene expression. J. Biol. Chem. **279:** 31304–31311.
27. **Seo JY, Yaneva R, Hinson ER, Cresswell P.** 2011. Human cytomegalovirus directly induces the antiviral protein viperin to enhance infectivity. Science **332:**1093–1097.
28. **Martinez FO, Gordon S, Locati M, Mantovani A.** 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J. Immunol. **177:**7303–7311.
29. **Bourne N, Scholle F, Silva MC, Rossi SL, Dewsbury N, Judy B, De Aguiar JB, Leon MA, Estes DM, Fayzulin R, Mason PW.** 2007. Early production of type I interferon during West Nile virus infection: role for lymphoid tissues in IRF3-independent interferon production. J. Virol. **81:**9100–9108.
30. **Daffis S, Samuel MA, Keller BC, Gale M, Jr, Diamond MS.** 2007. Cell-specific IRF-3 responses protect against West Nile virus infection by interferon-dependent and independent mechanisms. PLoS Pathog. **3:**e106. doi: 10.1371/journal.ppat.0030106.
31. **Daffis S, Samuel MA, Suthar MS, Gale M, Jr, Diamond MS.** 2008. Toll-like receptor 3 has a protective role against West Nile virus infection. J. Virol. **82:**10349–10358.
32. **Suthar MS, Ma DY, Thomas S, Lund JM, Zhang N, Daffis S, Rudensky AY, Bevan MJ, Clark EA, Kaja MK, Diamond MS, Gale M.** 2010. IPS-1 is essential for the control of West Nile virus infection and immunity. PLoS Pathog. **6:**e1000757. doi: 10.1371/journal.ppat.1000757.
33. **Pulit-Penaloza JA, Scherbik SV, Brinton MA.** 2012. Type 1 IFN-independent activation of a subset of interferon stimulated genes in West Nile virus Eg101-infected mouse cells. Virology **425:**82–94.
34. **Yang K, Shi HX, Liu XY, Shan YF, Wei B, Chen S, Wang C.** 2009. TRIM21 is essential to sustain IFN regulatory factor 3 activation during antiviral response. J. Immunol. **182:**3782–3792.
35. **Schmeisser H, Mejido J, Balinsky CA, Morrow AN, Clark CR, Zhao T, Zoon KC.** 2010. Identification of alpha interferon-induced genes associated with antiviral activity in Daudi cells and characterization of IFIT3 as a novel antiviral gene. J. Virol. **84:**10671–10680.
36. **Itsui Y, Sakamoto N, Kurosaki M, Kanazawa N, Tanabe Y, Koyama T, Takeda Y, Nakagawa M, Kakinuma S, Sekine Y, Maekawa S, Enomoto N, Watanabe M.** 2006. Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. J. Viral Hepat. **13:**690–700.
37. **Pichlmair A, Lassnig C, Eberle CA, Górna MW, Baumann CL, Burkard TR, Bürckstümmer T, Stefanovic A, Krieger S, Bennett KL, Rülicke T, Weber F, Colinge J, Müller M, Superti-Furga G.** 2011. IFIT1 is an antiviral protein that recognizes 5′-triphosphate RNA. Nat. Immunol. **12:**624–630.
38. **Fensterl V, Sen GC.** 2011. The ISG56/IFIT1 gene family. J. Interferon Cytokine Res. **31:**71–78.
39. **Fusco DN, Brisac C, John SP, Huang YW, Chin CR, Xie T, Zhao H, Zhang L, Chevalier S, Wambua D, Lin W, Peng L, Chung RT, Brass AL.** 2013. A genetic screen identifies interferon-alpha effector genes required to suppress hepatitis C virus replication. Gastroenterology **144:** 1438–1449.
40. **Krishnan MN, Ng A, Sukumaran B, Gilfoy FD, Uchil PD, Sultana H, Brass AL, Adametz R, Tsui M, Qian F, Montgomery RR, Lev S, Mason PW, Koski RA, Elledge SJ, Xavier RJ, Agaisse H, Fikrig E.** 2008. RNA interference screen for human genes associated with West Nile virus infection. Nature **455:**242–245.
41. **Dango S, Mosammaparast N, Sowa ME, Xiong LJ, Wu F, Park K, Rubin M, Gygi S, Harper JW, Shi Y.** 2011. DNA unwinding by ASCC3 helicase is coupled to ALKBH3-dependent DNA alkylation repair and cancer cell proliferation. Mol. Cell **44:**373–384.
42. **Jung DJ, Sung HS, Goo YW, Lee HM, Park OK, Jung SY, Lim J, Kim HJ, Lee SK, Kim TS, Lee JW, Lee YC.** 2002. Novel transcription coactivator complex containing activating signal cointegrator 1. Mol. Cell. Biol. **22:**5203–5211.
43. **Puel A, Reichenbach J, Bustamante J, Ku CL, Feinberg J, Döffinger R, Bonnet M, Filipe-Santos O, de Beaucoudrey L, Durandy A, Horneff G, Novelli F, Wahn V, Smahi A, Israel A, Niehues T, Casanova JL.** 2006. The NEMO mutation creating the most-upstream premature stop codon is hypomorphic because of a reinitiation of translation. Am. J. Hum. Genet. **78:**691–701.
44. **Daffis S, Suthar MS, Szretter KJ, Gale M, Jr, Diamond MS.** 2009. Induction of IFN-beta and the innate antiviral response in myeloid cells occurs through an IPS-1-dependent signal that does not require IRF-3 and IRF-7. PLoS Pathog. **5:**e1000607. doi: 10.1371/journal.ppat.1000607.
45. **Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD.** 2004. Genetic and phenotypic variation of West Nile virus in New York, 2002–2003. Am. J. Trop. Med. Hyg. **71:**493–500.
46. **Diamond MS, Edgil D, Roberts TG, Lu B, Harris E.** 2000. Infection of human cells by dengue virus is modulated by different cell types and viral strains. J. Virol. **74:**7814–7823.
47. **Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S.** 2007. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. **3:**e201. doi: 10.1371/journal.ppat.0030201.
48. **Oliphant T, Engle M, Nybakken GE, Doane C, Johnson S, Huang L, Gorlatov S, Mehlhop E, Marri A, Chung KM, Ebel GD, Kramer LD, Fremont DH, Diamond MS.** 2005. Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nat. Med. **11:**522–530.
49. **Fuchs A, Pinto AK, Schwaeble WJ, Diamond MS.** 2011. The lectin pathway of complement activation contributes to protection from West Nile virus infection. Virology **412:**101–109.