Interferon γ-inducible Protein (IFI) 16 Transcriptionally Regulates Type I Interferons and Other Interferon-stimulated Genes and Controls the Interferon Response to both DNA and RNA Viruses*

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The interferon γ-inducible protein 16 (IFI16) has recently been linked to the detection of nuclear and cytosolic DNA during infection with herpes simplex virus-1 and HIV. IFI16 binds dsDNA via HIN200 domains and activates stimulator of interferon genes (STING), leading to TANK (TRAF family member-associated NF-κB activator)-binding kinase-1 (TBK1)-dependent phosphorylation of interferon regulatory factor (IRF) 3 and transcription of type I interferons (IFNs) and related genes. To better understand the role of IFI16 in coordinating type I IFN gene regulation, we generated cell lines with stable knockdown of IFI16 and examined responses to DNA and RNA viruses as well as cyclic dinucleotides. As expected, stable knockdown of IFI16 led to a severely attenuated type I IFN response to DNA ligands and cytokines. In contrast, expression of the NF-κB-regulated cytokines IL-6 and IL-1β was unaffected in IFI16 knockdown cells, suggesting that the role of IFI16 in sensing these triggers was unique to the type I IFN pathway. Surprisingly, we also found that knockdown of IFI16 led to a severe attenuation of IFN-α and the IFN-stimulated gene retinoic acid-inducible gene I (RIG-I) in response to cyclic GMP-AMP, a second messenger produced by cyclic GMP-AMP synthase (cGAS) as well as RNA ligands and viruses. Analysis of IFI16 knockdown cells revealed compromised occupancy of RNA polymerase II on the IFN-α promoter in these cells, suggesting that transcription of IFN-stimulated genes is dependent on IFI16. These results indicate a broader role for IFI16 in the regulation of the type I IFN response to DNA and RNA viruses in antiviral immunity.

The innate immune system is crucial for regulation of early detection and clearance of invading pathogens. The innate detection of pathogens acts as a primer for the long term memory response governed by the adaptive immune system. Innate immunity is triggered by a panel of germ line-encoded pattern recognition receptors that sense foreign pathogens and trigger downstream signaling. This leads to the production of effector proteins such as type I interferons (IFNs), proinflammatory cytokines, and chemokines, which are important mediators of this response.

To date, several germ line-encoded pattern recognition receptors have been described. The Toll-like receptors (TLRs),2 which are present on both the cell surface and within endosomal compartments, are perhaps the most widely known and extensively studied. The TLRs recognize conserved pathogen associated molecular patterns, such as bacterial LPS, as well as danger-associated molecular patterns, including host cell DNA released from damaged cells during cellular stress. During viral infection, TLR3 and TLR7/8 recognize double-stranded RNA and single-stranded RNA, respectively. Additionally, in plasmacytid dendritic cells, TLR9 is responsible for the recognition of CpG DNA, leading to the production of type I IFNs (1, 2). The IFN response to nucleic acids is not exclusively mediated by TLRs, however. RNA and DNA that access or accumulate in the cytosol during viral and bacterial infection are also

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2 The abbreviations used are: TLR, Toll-like receptor; IFI16, interferon γ-inducible protein 16; STING, stimulator of interferon genes; TBK1, TANK-binding kinase-1; IRF, interferon regulatory factor; RIG-I, retinoic acid-inducible gene I; cGAMP, cyclic GMP-AMP; cGAS, cyclic GMP-AMP synthase; ISG, IFN-stimulated gene; ASC, apoptosis-associated specklike protein containing a caspase activation and recruitment domain; HSV, herpes simplex virus; ISRE, interferon-sensitive response element; CBP, cAMP-responsive element-binding protein (CREB)-binding protein; VACV, vaccinia virus; CDS, coding sequence; q-RT-PCR, quantitative RT-PCR; HPRRT, hypoxanthine-guanine phosphoribosyltransferase; RANTES, regulated on activation normal T cell expressed and secreted; PYHIN, pyrin and HIN domain containing; Pol, polymerase.
potent activators of innate immunity (3–5). RNA and DNA elicit TLR-independent responses, particularly those leading to robust induction of type I IFNs. This observation led to the discovery of several cytosolic nucleic acid sensors that sense RNA and DNA in the absence of TLRs and couple pathogen recognition to immune activation.

The DEX(D/H) box RNA helicases RIG-I and MDA5 detect 5′-ppp single-stranded RNA and long double-stranded RNA, respectively (6–9). A third member of this family, LGP2, has also been shown to cooperate with MDA5 (10). Growing evidence also supports the existence of multiple cytosolic and nuclear DNA-sensing receptors, which lead to activation of STING and TBK-1-dependent phosphorylation of IRF3 and transcription of type I IFN genes (11, 12). Through less well understood pathways, these sensors also elicit NF-κB activation and transcription of NF-κB-dependent genes (13).

We have been involved in implicating two members of the PYHIN (pyrin and HIN domain containing) protein family in the recognition of DNA. The first of these, absent in melanoma 2, which is highly conserved in mice and humans, binds DNA via a HIN200 domain and forms a multiprotein inflammusome complex with ASC and caspase-1. Activation of caspase-1 results in the cleavage of pro-IL-1β and pro-IL-18 to their mature, secreted forms (IL-1β and IL-18) as well as a novel cell suicide pathway called pyroptosis (3, 14–17). A second PYHIN protein, IFI16, as well as a murine PYHIN family member, IFI204 (also called p204), have also been shown to bind DNA via HIN200 domain(s). IFI16 was initially described as a transcriptional regulator (18). IFI16 contains a transcriptional regulatory domain and has been shown to bind to and transcriptionally repress p53 (18–20). It has also been shown to interact with SP-1-like factors to block human cytomegalovirus replication (21, 22) and in other studies to lead to epigenetic silencing of herpes simplex virus 1 (HSV-1) and transfected DNAs (23). In a series of biochemical and loss of function studies (24–26), IFI16 was shown to associate with viral or transfected DNA, activate STING, and coordinate IRF3 and NF-κB signaling in response to HSV-1 and transfected dsDNA (25). Although IFI16 is predominantly nuclear, the recognition of DNA by IFI16 was originally proposed to occur in the cytosol (25), but later IFI16 was also shown to recognize HSV-1 DNA in the infected cell nucleus (24, 26). Knockdown of IFI16 or IFI204 (in corresponding mouse cells) by RNAi leads to a decrease in IFN-β production in response to various synthetic DNA ligands and viruses. Li et al. (26) have shown that nuclear localization of IFI16 is essential for recognition of HSV-1 DNA in infected cells, and Orzalli et al. (24) showed that HSV-1 DNA must be delivered into the nucleus for recognition by IFI16. In contrast, IFI16 is localized in the cytoplasm of macrophages, and viral capsids are apparently degraded by a proteasomal process to release HSV-1 DNA to be recognized by the cytoplasmic IFI16 (27). Conradoy et al. (28) have shown that knockdown of IFI204 in corneal epithelium leads to susceptibility to HSV-1 infection. Other studies have shown that in endothelial cells IFI16 forms an inflammasome with ASC to produce IL-1β in response to human Kaposi sarcoma-associated herpesvirus (29). Most recently, IFI16 has been linked to inflammasome activation and pyroptotic death of bystander CD4 T cells during HIV infection (30–33).

Since the initial discovery of IFI16 and IFI204, compelling recent evidence from both human and mouse cells using RNAi, TALEN (transcription activator-like effector nuclease) knockdown approaches, and gene knock-outs has convincingly demonstrated the importance of a DNA-sensing enzyme called cyclic GMP-AMP synthase (cGAS) in the cytosolic response to dsDNA. Chen and co-workers (34–36) were the first to identify cGAS, which binds DNA in the presence of ATP and GTP, leading to the generation of a second messenger, cGAMP. cGAMP then binds to STING and leads to IRF3 activation. This is true for responses to viruses such as HSV-1 and HIV (33–36). Given the compelling insight into DNA sensing obtained from the studies of the cGAS-cGAMP pathway, further work is needed to fully elucidate the mechanism by which IFI16 contributes to the immune response to cytosolic dsDNA and DNA viruses.

In this study, we have furthered our understanding of the role that IFI16 plays in the induction of type I IFNs by examining responses to DNA as well as DNA viruses. Consistent with previous studies, we found a critical role for IFI16 in coordinating the induction of type I IFNs and IFN-stimulated genes in response to transfected DNA as well as DNA viruses. In contrast, we found that the induction of NF-κB-dependent genes such as IL-1 and IL-6 was independent of IFI16. Surprisingly, we also revealed that knockdown of IFI16 attenuates IFN/IFN-stimulated gene (ISG) responses to RNA viruses. The role of IFI16 in sensing RNA viruses was further characterized to demonstrate compromised IFN-α and ISG expression in response to synthetic ligands that engage the RIG-I pathway. Similar findings were made when bacterial or host cyclic dinucleotides were examined. These observations indicate broader roles for IFI16 in controlling antiviral immunity. Ectopic expression of IFI16 induced IFN-α reporter gene expression even in cells lacking STING. Moreover, we observed binding of IFI16 to the ISRE of the IFN-α promoter. Analysis of the IFN promoter revealed reduced occupancy of RNA polymerase II (Pol II) in cells with reduced expression of IFI16. Moreover, IRF3 failed to interact with cAMP-responsive element-binding protein (CREB) binding protein (CBP) in the absence of IFI16. Collectively, these studies describe a regulatory role for IFI16 in the transcriptional regulation of IFN-α gene expression and subsequent IFN-inducible gene expression in response to a broader array of IFN inducers than previously anticipated, expanding the function of IFI16 beyond strictly sensing microbial DNA.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—LPS and poly(dAdT) were obtained from Sigma-Aldrich. 5′-ppp RNA was from Invivogen (San Diego, CA). HSV 60-mer, VACV 70-mer, and immunostimulatory DNA oligonucleotides were synthesized as described (25). Cyclic-di-GMP was from Biolog (Hayward, CA). 2′3′-cGAMP was from Veit Hornung (University of Bonn, Germany). *Listeria monocytogenes* (clinical isolate 10403s) was from V. Boyartchuk (Norwegian University of Science and Technology (NTNU), Trondheim, Norway). HSV-1 (7134) was grown and titered on U2OS cells as described (23). Sendai virus (Cantrell strain) was pur-
chased from Charles River Laboratories (Wilmington, MA). The trypsin-independent human metapneumovirus isolate A1 (NL1\textsuperscript{001}) was from MedImmune (Gaithersburg, MD) and was propagated in Vero cells cultured in Iscove’s modified Dulbecco’s medium containing 4% BSA and trypsin (37, 38). Lipo- vectamine 2000 was from Invitrogen. Geneljuice was from Novagen (Madison, WI). Universal type I IFN was from PBL Interferon Source (Piscataway, NJ).

shRNA-mediated Silencing—Lentiviral shRNA sequences targeting IFI16 in the pLKO.1 TRC cloning vector were purchased from Sigma-Aldrich. The following IFI16-silencing sequences were from the MISSION TRC-Hs 1.0 (Human): TRCN0000019079 (CDS) and TRCN0000364735 (3’-UTR). The production of viral particles and transduction of target cells were conducted according to protocols available at the Broad Institute’s public RNAi consortium portal website. Lentiviral particles were produced in 293T HEK cells transfected with 3 μg of shRNA along with 4 μg of pSPAX and pMD2 for 48 h. Viral supernatant was collected, filtered, and then added to target THP-1 cells or U205 cells. THP-1 cells with shRNA knockdown were selected by puromycin 48 h later. Knockdown efficiency in stable knockdown cell lines was assessed by q-RT-PCR with the following primers: IFI16-F, 5’-CCC TTC ATG ACC AGA ATA GG-3’; IFI16-R, 5’-TCA GTC TTG GTT TCA ACG TGG T-3’.

ISRE Pulldown—Nuclear extracts were incubated with 40 μg/ml biontinated IFN-α4 ISRE consensus sequence and 20 or 40 μg/ml non-biontinated IFN-α4 ISRE consensus sequence for competition binding. IFN-α4 ISRE consensus sequence is as follows: 5’-biont-GAA TTT AGA AAA TGG AAA TTA GTA TGT TCA CTA TTT AA-3’; 3’-CTT AAA TCT TTT ACC TTT AAT CAT ACA AGT GAT AAA TT-5’. Streptavidin beads (50%, w/v) were obtained from Sigma.

Luciferase Assays—HEK 293T cells were transfected with a mixture containing 40 ng of the TK-Renilla luciferase reporter plus 40 ng of the IFN-β or IFN-α4 firefly luciferase reporter and either increasing amounts of pRGP-IFI16 plasmid or 80 ng of murine STING plasmid for 24 h. Cells were lysed with passive lysis buffer (Promega) and monitored for firefly or Renilla luciferase activity. Data are represented as -fold change over pcDNA3.1 empty vector control and relative to Renilla values.

IFI16 Addback—IFI16 coding sequence was cloned into the pRGP retroviral vector, and the production of viral particles and transduction of target cells were conducted according to the protocols referenced above. Addback cells were selected by puromycin.

Cell Culture, Stimulation, and ELISA—THP-1 cells were differentiated with 0.5 μM phorbol 12-myristate 13-acetate overnight prior to stimulation. For stimulations, poly(dADT) (1 μg/ml), immunostimulatory DNA sequence (3 μM), HSV 60-mer (3 μM), VACV 70-mer (3 μM), cyclic di-AMP (3 μM), 5’-ppp RNA (600 ng/ml), 2’,3’-cGAMP (3 μM) were transfected into the cells with Lipofectamine following the manufacturer’s instructions. Cells were infected with human metapneumovirus or HSV-1 at a multiplicity of infection of 10. Cells were infected with Sendai virus at 200 IU/ml. Cells were stimulated with pan-type I IFN at 1000 units/ml for 2, 8, or 24 h. For bacterial infection, cells were challenged with L. monocytogenes at a multiplicity of infection of 5 for 1 h. Infected cells were then washed twice, and medium containing gentamicin (100 μg/ml) was added to kill extracellular bacteria. Knockdown and control cells were challenged with stimulants or microbes for 6 (for RNA analysis and immunoblot analysis) or 12 h (for protein analysis by ELISA). Cytokine and IFN levels in culture supernatants were assayed for IL-1β and IL-6 (BD Biosciences) and IFN-α (Mabtech, Mariemont, OH) by sandwich ELISA.

NanoString and q-RT-PCR Experiments—Cells were treated for 6 h, and RNA was purified with a quick RNA miniprep kit (Zymo Research, Irvine, CA). RNA transcript counting was performed on total RNA hybridized to a custom gene expression CodeSet and analyzed on an nCounter digital analyzer. Counts were normalized to internal spiked-in and endogenous controls according to NanoString Technologies’ specifications. A pseudo count was added to all values such that the smallest value in the data set was equal to 1. cDNA was synthesized, and quantitative RT-PCR (q-RT-PCR) analysis was performed as described (39, 40). Primers used include: HPRT-F, 5’-ATC AGA CTT AAG AGC TAT TGT AAT GA-3’; HPRT-R, 5’-TGG CTT ATA TTA AAC ACT TCG TG-3’; IFN-α-F, 5’-CAC ACA GGC TTC CAG GCA TTC-3’; IFN-α-R, 5’-TCT TCA GCA CAA AGG ACT CAT CTG-3’; RIG-I-F, 5’-CTG GAC CCT ACC TAC ATC CTG-3’; RIG-I-R, 5’-GGC ATC CAA AAA GCC ACG G-3’; IFN-β-F, 5’-GTC TTC CCC AAA TTG CTC TC-3’; and IFN-β-R, 5’-ACA GGA GCT TCT GAC ACT GA-3’. Gene expression is shown as a ratio of gene copy number per 100 copies of HPRT ±S.D.

Immunoblotting—Cells were washed twice with PBS and lysed using a 1% Nonidet P-40 buffer. Immunoblotting was performed as described (41). Anti-murine IFI16 was from Abcam; anti-rabbit STAT-1, anti-rabbit phospho-STAT-1, anti-rabbit phospho-IRF3, anti-rabbit TBK1, and anti-rabbit USF2 were from Santa Cruz Biotechnology; anti-rabbit CBP and anti-murine IRF3 were from BD Biosciences; anti-rabbit phospho-TBK1 was from Cell Signaling Technology; anti-murine STING was a gift from Glen Barber (University of Miami Health System); and anti-rabbit cGAS was from Sigma.

Cell Viability Assay—Calcein AM staining was obtained from R&D Systems. Cells were treated as described above and cell viability was assayed according to the following protocol (Funakoshi Corp.). Cells were washed in PBS and incubated in calcein AM stain for 30 min at 37 °C. Calcein AM stain was then washed off with PBS, and cells were monitored for FITC fluorescence.

Chromatin Immunoprecipitation—8.0 × 10\textsuperscript{6} empty vector control and IFI16 knockdown THP-1 cells were stimulated with 200 hemagglutinating units of Sendai virus for 4 h, washed with PBS, and fixed with 1% formaldehyde for 5 min at room temperature. Formaldehyde fixation was stopped with the addition of 1.25 M glycine for 5 min at room temperature. The nuclear pellet was sonicated using a Bioruptor UCD-200 (Diagenode Inc., Sparta, NJ) to shear the DNA to obtain fragments ranging from 200 to 500 bp in size. 5 μg/immunoprecipitation sheared chromatin was immunoprecipitated with 2 μg of anti-RNA Pol II (Active Motif; Clone 4H18) or IgG1 isotype control (Imgenex) antibody overnight and 10 μl of magnetic beads for 1 h. Following reversal of the cross-linking and protein diges-
**RESULTS**

**DNA-induced Type I Interferon Production Is Dependent on IFI16**—The type I IFN response induced by DNA in the human THP-1 monocytic cell line or mouse RAW 264.7 cell line is dependent on IFI16 or its proposed mouse orthologue IFIG204 (25). siRNA studies have shown that transient knockdown of IFI16 in THP-1 cells leads to a decrease in IFN production and reduced IRF3 and NF-κB activation in response to HSV-1 as well as to DNA ligands (25). To conduct a more in-depth characterization of the DNA response through IFI16, we generated a stable IFI16 knockout in the human myeloid cell line THP-1 via lentiviral transduction of shRNA in a pLKO.1 vector. We also generated a stable IFI16 knockout in a second human cell line, the osteosarcoma cell line U2OS. Two different shRNA targeting vectors were used: one targeting the coding sequence of IFI16 (IFI16 shRNA CDS) and one targeting the 3′-UTR of IFI16 (IFI16 shRNA 3′-UTR). The empty pLKO.1 vector was used as a transduction control. In all IFI16 shRNA-transduced cell lines, we achieved at least 50% reduction in IFI16 mRNA and protein levels (Fig. 1).

We next determined whether the IFI16 knockout cells were hindered in their ability to produce type I IFN (α/β) in response to DNA ligands. THP-1 and U2OS IFI16 knockout cells were challenged with poly(dAdT) or HSV 60-mer (a double-stranded DNA sequence derived from the HSV-1 genome) or infected with HSV-1 7134 (a mutant strain of the virus that lacks the immunosuppressive ICP0 gene, a known inhibitor of IFN-β production) for 6 h, and then RNA was collected from the samples. IFN-α and IFN-β levels were measured by q-RT-PCR and normalized to TP53. Levels of IFN-β mRNA were decreased in IFI16 knockout THP-1 cells as compared with empty vector control cells in response to poly(dAdT), HSV 60-mer, and HSV-1 (Fig. 2, A, B, and C, respectively). We also monitored these responses in U2OS cells as an independent control knockdown cell line. Similar to THP-1 cells, levels of IFN-β mRNA were decreased in IFI16 knockout U2OS cells in response to poly(dAdT) and HSV 60-mer compared with empty vector controls (Fig. 2, D and E). Interestingly, levels of IFN-α were even more drastically reduced in U2OS IFI16 knockout cells as compared with the empty vector control cells in response to poly(dAdT) (Fig. 2F). This result is consistent with IFN-α production being downstream of IFN-β production and activation of IRF7 following DNA sensing. In contrast to THP-1 cells in which both DNA challenge and HSV-1 infection induced IFN-β mRNA, HSV-1 infection of U2OS cells did not induce detectable IFN-β mRNA in either control or knockdown cells, whereas DNA challenge did induce IFN-β in control U2OS cells. Similar observations were made using HSV-1 strains 7134 and d109 (a strain lacking all immediate early genes, including ICP0).3 These observations further support the idea that IFI16 functions at a level that is independent of DNA sensing during HSV-1 infection in this cell type and regulated IFN-α mRNA levels independently of IFN-β production.

**IFI16-dependent Interferon Production Occurs Independently of NF-κB**—In addition to reduced IFN-α mRNA levels, we also noticed a marked decrease in IFN-α protein levels measured by ELISA in response to poly(dAdT) in both THP-1 and U2OS IFI16 knockout cells compared with empty vector controls.

3 M. H. Orzalli, N. M. Broekema, and D. M. Knipe, manuscript in preparation.
IFI16 Transcriptionally Regulates IFN-α and RIG-I

Fig. 2. IFI16 knockdown cells display an abrogated type I IFN response to various DNA stimuli. IFI16 knockdown THP-1 cells were challenged with poly(dA:dT) (A), HSV 60-mer (B), or HSV-1 virus (C) for 6 h. Levels of IFN-β were measured by q-RT-PCR. IFI16 knockdown U2OS cells were challenged with poly(dA:dT) (D) or HSV 60-mer (E) for 6 h, and IFN-β levels were measured by q-RT-PCR. F, IFI16 knockdown U20S cells were challenged with poly(dA:dT), and IFN-α levels were measured by q-RT-PCR and normalized to HPRT. *, p < 0.05 assessed by two-tailed t test compared with empty vector control. Data are represented as mean ± S.E. (error bars). Data represent three biological replicates. KD, knockdown.

(Fig. 3, A and B). RANTES production was also decreased in U20S IFI16 knockdown cells in response to HSV 60-mer, VACV 70-mer (a double-stranded DNA sequence derived from the vaccinia virus genome), and poly(dA:dT) (Fig. 3C).

In contrast to the lower levels of type I IFNs, NF-κB-related cytokines IL-6 and IL-1β were unchanged or even increased in THP-1 IFI16 knockdown cells that were treated with poly(dA:dT), HSV 60-mer, or HSV-1 for 12 h (Fig. 3, D and E). An increase in proinflammatory cytokines may suggest a compensation for the lack of IFN production in these cells. We did not detect differences in phospho-IκBα protein expression in response to poly(dA:dT), HSV 60-mer, or HSV-1 between IFI16 knockdown and control cells (data not shown). Furthermore, we did not detect a significant difference in cell viability between the empty vector control and IFI16 knockdown cells under our experimental conditions. Poly(dA:dT) induces cell death (pyroptosis) via absent in melanoma 2 inflammasome activation (3, 41), and these events were independent of IFI16 (Fig. 3F).

To explore whether IFI16 is necessary and sufficient for the production of type I IFNs, we performed an addback experiment in which IFI16 was ectopically expressed in the knockdown cell lines. We transduced both CDS and 3′-UTR IFI16 knockdown cells with the pRGP-IFI16 retroviral vector to create a transient addback of IFI16. Although we did achieve expression of the IFI16 transgene in the CDS knockdown cells, the expression levels were modest (Fig. 4A). This was not surprising as the shRNA in this cell line could target the expression vector as well as the endogenous gene. However, we did achieve much higher expression of IFI16 transgene in the 3′-UTR
knockdown cell line (Fig. 4B). The IFI16 addback cells were then challenged with poly(dA:dT), HSV 60-mer, or VACV 70-mer, or immunostimulatory DNA sequence (25) (Fig. 4C) or infected with HSV-1 or Sendai virus (data not shown). Despite the modest IFI16 rescue in the CDS knockdown, we did see some restoration of IFN-α production in the addback cell line compared with the knockdown cells without IFI16 addback. The levels of IFN-α production were consistent with the amount of IFI16 expression in the addback cell line. We also saw almost a complete restoration of IFN-β production in the 3’-UTR addback cell line (Fig. 4D), also consistent with the amount of IFI16 expression in the addback cell line. These results suggest that IFI16 is necessary for the production of type I IFNs and that the phenotype that we observed was not due to off-target effects of the shRNA used to knock down IFI16.

IFI16 Knockdown Cells Display an Abrogated Type I Interferon Response to Various RNA Stimuli and cGAMP—IFI16 senses infection by HSV-1, and recent studies have shown that IFI16 senses infection by HIV-1 (23–33). Previous studies by Unterholzner et al. (25) have shown that siRNA knockdown of p204 in RAW cells or IFI16 in THP-1 cells did not have an effect on Sendai virus-induced type I IFN production. In contrast to these studies of transient IFI16 knockdown, we found that stable IFI16 knockdown cells display a decrease in type I IFN pro-

FIGURE 3. IFI16-dependent IFN production occurs independently of NF-κB. A, B, and C, IFI16 knockdown THP-1 and U2OS cells were challenged with poly(dA:dT), VACV 70-mer, or HSV 60-mer for 12 h. Levels of IFN-α (A and B) and RANTES (C) were measured by ELISA. D and E, IFI16 knockdown THP-1 cells were challenged with poly(dA:dT) or HSV 60-mer or infected with HSV-1 for 12 h. Levels of IL-6 (D) and IL-1β (E) were measured by ELISA. F, IFI16 knockdown cells were challenged with poly(dA:dT) or infected with HSV-1 for 6 h. Cells were stained with calcein for 1 h, and viability was determined by uptake of calcein stain and FITC fluorescence. *, p < 0.05 assessed by two-tailed t test compared with empty vector control. Data are represented as mean ± S.E. (error bars). Data represent three biological replicates. Bars without * are not significant. KD, knockdown.
duction in response to Sendai virus. Sendai virus-induced IFN-β levels were reduced in THP-1 cells expressing shRNA targeting either the CDS or 3'-UTR (Fig. 5A). This effect was not unique to THP-1 cells as we saw a similar defect when we measured both IFN-β and IFN-α in U2OS cells (Fig. 5, B and C). This inhibitory effect of IFI16 knockdown on IFN responses was not limited to Sendai virus signaling. We also saw defects in responses to L. monocytogenes, cyclic di-AMP (a second messenger that is secreted by some bacteria, including Listeria, and binds to and activates STING), 5’-ppp RNA (a synthetic RNA that signals through RIG-I), and human metapneumovirus (an RNA virus that signals through RIG-I) (Fig. 5, D, E, and F).

We also monitored the ability of IFI16 knockdown THP-1 cells to respond to cGAMP because cGAMP functions at a level downstream of DNA sensing by engaging STING directly. Empty vector control and IFI16 knockdown THP-1 cells were stimulated with 2’,3’-cGAMP, and supernatants were tested for IFN-α production by ELISA. Similar to the DNA and RNA ligands, IFI16 knockdown cells produced less IFN-α than the empty vector control cells in response to cGAMP (Fig. 5G). We also looked at protein levels of cGAS by Western blotting and found that cGAS protein levels were normal in the IFI16 knockdown cells as compared with the control cells even after stimulation with type I IFN and Sendai virus (Fig. 5H). We also detected a decrease in the response to encephalomyocarditis virus (EMCV), an RNA virus that signals through MDA5 (data not shown). In contrast to the IFN response, levels of IL-6 and IL-1β in response to Sendai virus were not affected by IFI16 knockdown (data not shown).

These results indicated that IFI16 not only plays a role in the detection of Sendai virus and signaling through RIG-I but also has a global effect on type I IFN production in response to a diverse panel of innate triggers. Furthermore, because cGAS/cGAMP signaling should bypass the need for IFI16 as a DNA sensor and directly activate STING, these results provide further evidence that although IFI16 can sense DNA leading to STING activation IFI16 also regulates ISG expression independently of its role in DNA recognition.

IFI16 Has a Global Effect on ISG Expression—Because IFI16 knockdown cells display a dramatic decrease in IFN production...
induced by multiple triggers and because we saw defects in response to both RNA and DNA stimulation, we next determined where IFI16 was acting in the type I IFN pathway. Western blot analysis demonstrated that expression levels of STING and TBK1 remained unchanged in IFI16 knockdown compared with control cells; however, there was a decrease in activation of TBK1 by DNA stimulants in IFI16 knockdown cells (Fig. 6A). Levels of phosphorylated TBK1 were lower in IFI16 knockdown THP-1 cells compared with empty vector control cells challenged with poly(dAdT) or HSV 60-mer (Fig. 6A). Similarly, levels of phosphorylated IRF3 were also decreased. These results are consistent with IFI16 acting as a DNA sensor controlling TBK1-dependent IRF3 activation and type I IFN induction.

We next wanted to determine what effect IFI16 had on the global expression of a panel of immune genes, including ISGs. We treated empty vector control and IFI16 knockdown cells with poly(dAdT) or infected them with Sendai virus for 6 h and collected RNA for multiplex gene expression analysis (nCounter, NanoString). NanoString uses fluorescently labeled probes that hybridize directly to target mRNA, allowing each individual mRNA to be counted in a highly sensitive manner (42). We found a decrease in the expression of many ISGs and an increase in NF-κB-related cytokine gene expression using this approach (Fig. 6B). Some immune genes such as NLRP3, MND, and MyD88 remained unchanged (data not shown). More interestingly, we saw a decrease in RIG-I mRNA expression both basally (Fig. 6A) and after treatment with LPS, poly(dAdT), and Sendai virus (Fig. 6D). The reduced expression of RIG-I in IFI16 knockdown cells may explain why there is a defect in Sendai virus signaling and further suggests a role for IFI16 in the transcriptional regulation of ISGs following challenge with RNA ligands or RNA virus infection.

IFI16 Acts at the Level of Chromatin to Regulate IFN-α and RIG-I

FIGURE 5. IFI16 knockdown cells display an abrogated type I IFN response to various RNA stimuli. A, B, and C, IFI16 stable knockdown THP-1 or U2OS cells were challenged with Sendai virus for 6 (for q-RT-PCR assay of IFN-β levels) or 12 h (for IFN-α levels measured by ELISA). THP-1 cells were stimulated with cyclic-di-AMP (c-di-AMP) or L. monocytogenes (Listeria) (D), 5’-ppp RNA (E), or human metapneumovirus (HMPV) (F) for 6 h, and IFN-β levels were determined by q-RT-PCR. G, THP-1 cells were stimulated with 2’,3’-cGAMP for 12 h, and IFN-α levels were measured by ELISA. H, empty vector (EV) and IFI16 knockdown (KD) cells were challenged with type I IFN or Sendai virus (SeV) for 2 h and monitored for cGAS expression by Western blotting. *, p < 0.05 assessed by two-tailed t test compared with empty vector control. Data are represented as mean ± S.E. (error bars). Data represent three biological replicates. Bars without * are not significant.
FIGURE 6. IFI16 has a global effect on ISG expression. A, IFI16 knockdown THP-1 cells were challenged with poly(dAdT) or HSV 60-mer for 6 h and monitored for protein expression by immunoblotting. B, IFI16 stable knockdown THP-1 cells were transfected with poly(dAdT) or infected with Sendai virus for 6 h, and RNA was collected. Graphs show selected genes from NanoString analysis. Basal (C) and stimulated (D) levels of RIG-I as determined by NanoString analysis. Data represent one experiment. KD, knockdown; pIRF3, phosphorylated IRF3; pTBK-1, phosphorylated TBK-1.
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also further up-regulated upon type I IFN signaling. To determine whether a decrease in type I IFN production was responsible for the lower expression levels of RIG-I in IFI16 knockdown THP-1 cells, we performed an IFN addback experiment. Cells were pretreated with pan-type I IFN for 2 h and then infected with Sendai virus for 2, 8, or 24 h. RNA was harvested from cells, and expression of RIG-I and IFN-α was monitored by q-RT-PCR. IFI16 knockdown cells primed with IFN were able to produce levels of RIG-I comparable with that of empty vector control cells (Fig. 7A). Because production of RIG-I was normal in the IFI16 knockdown cells after type I IFN stimulation, these results suggested that signaling through interferon α/β receptor (IFNAR) and STAT-1 was unaffected in these cells.

We next wanted to assess whether the impaired Sendai virus-induced IFN-α response in IFI16 knockdown cells was due to defective IFN production. We primed these cells with type I IFN prior to infection with Sendai virus to elevate RIG-I expression. IFN pretreatment prior to Sendai virus infection led to elevation of RIG-I levels, albeit at slightly delayed and lower levels than that seen in WT cells (Fig. 7A). However, despite the enhanced RIG-I levels, IFI16 knockdown cells were still completely unable to induce IFN-α mRNA (Fig. 7B). We also saw a similar effect when we monitored IRF7 (data not shown). This effect was specific because levels of IL-6 were either unchanged or increased following exogenous IFN and Sendai virus treatment of the IFI16 knockdown cells (data not shown). Taken together, these results suggested that IFI16 knockdown cells are capable of responding to exogenous IFN through the interferon α/β receptor/STAT-1 pathway to induce RIG-I expression. However, in response to Sendai virus infection, IFI16 knockdown cells are unable to drive IFN-α production, and as a consequence, these cells are unable to maintain high levels of RIG-I expression during infection. These effects resulted in a significant impairment of Sendai virus-dependent production of type I IFNs. These results led us to speculate that IFI16 may impact the transcription of IFN-α directly, perhaps acting in the nucleus to control gene transcription.

To more directly assess this potential role of IFI16 in controlling the transcription of IFN-α, we performed reporter assays in HEK 293T cells. We monitored the ability of IFI16 to induce expression of the IFN-β and IFN-α4 luciferase reporter genes. We transfected HEK 293T cells with increasing amounts of IFI16 plasmid and monitored luciferase activation after 24 h. Ectopic expression of IFI16 induced the IFN-β and IFN-α4 luciferase reporter by 8- and 4-fold over empty vector control plasmid, respectively (Fig. 7, C and D). These results suggested that IFI16 is capable of activating the promoter of type I IFNs. Because HEK 293T cells lack expression of STING, these results indicated that IFI16 has IFN-inducing capabilities that are beyond those reported previously that require STING.

We next monitored the ability of IFI16 to bind to the IFN-α4 ISRE consensus sequence. We infected THP-1 cells with Sendai virus for 6 h, incubated nuclear extracts with biotinylated IFN-α4 ISRE consensus sequence alone or biotinylated IFN-α4 ISRE consensus sequence with increasing amounts of non-biotinylated IFN-α4 ISRE consensus sequence for 1 h, and then pulled down the IFN-α4 ISRE consensus sequence with streptavidin beads. We then monitored IFI16 protein binding by Western blotting. IFI16 bound to the IFN-α4 ISRE consensus sequence after Sendai virus infection (Fig. 7E). Furthermore, we were able to compete off this binding with increasing amounts of non-biotinylated IFN-α4 ISRE consensus sequence (Fig. 7E). This provides evidence that IFI16 is capable of binding the IFN-α promoter. We next performed a chromatin immunoprecipitation assay in which we monitored RNA Pol II recruitment to the IFN-α promoter. Empty vector control and IFI16 knockdown THP-1 cells were infected with Sendai virus for 4 h, and cells were cross-linked with formaldehyde. Chromatin was immunoprecipitated with antibodies directed against RNA Pol II or IgG1 control. Primers for the region immediately upstream of the transcription start site of IFN-α (as well as IL-6) were used to detect Pol II recruitment to these regions via q-RT-PCR. Previous studies have shown that RNA Pol II is poised at some promoters and upon stimulation is released, leading to active transcription (43–46). We found that IFI16 knockdown cells have less basal RNA Pol II bound to the IFN-α promoter than the empty vector control cells (Fig. 7F). In contrast to the IFN-α promoter, RNA Pol II recruitment to the IL-6 promoter was higher in the IFI16 knockdown cells basally. IL-6 is a secondary response gene that is regulated following the recruitment of RNA Pol II. Consistent with this model, we also observed increased recruitment upon stimulation (Fig. 7F and data not shown), and this response was further enhanced in IFI16 knockdown cells. These results suggested that IFI16 contributes to RNA Pol II recruitment to the promoter of IFN-α basally.

IFN gene transcription is dependent on the nuclear translocation and DNA binding activity of IRF3 and IRF7. These factors form an enhanceosome, leading to the recruitment of CBP-p300. These events are essential for transcription of type I IFN genes. In IFI16 knockdown cells, we found that IRF3 and CBP failed to interact. Empty vector control cells and IFI16 knockdown cells were infected with Sendai virus for 6 h, and nuclear extracts were immunoprecipitated with CBP antibody. IRF3 protein levels were monitored by Western blotting. The IFI16 knockdown cells displayed decreased binding of IRF3 to CBP compared with control cells (Fig. 7G), providing further evidence that the transcriptional activation of type I IFNs is hindered in the IFI16 knockdown cells. A schematic model detailing our model of IFI16 and type I IFN gene regulation is shown (Fig. 7H).

DISCUSSION

IFI16 activates both inflammasome formation and IFN-β and ISG expression. Here we further expand our understanding of the role of IFI16 in innate immunity. We demonstrate a broader role for IFI16 in the transcriptional regulation of ISGs in response to multiple stimuli. In keeping with previous work (24–26), IFI16 knockdown led to impaired IRF3 activation, resulting in abrogated type I IFN production when cells were stimulated with either synthetic DNA or viral DNA. However, in contrast to previous work, we also saw a defect in type I IFN production in response to RNA ligands, including a defective IFN response to Sendai virus that was not seen in the original studies. Based on our results and previous IFI16 siRNA knock-
down studies, we suggest that there are multiple mechanisms by which IFI16 can induce type I IFN. IFI16 can act as an intracellular sensor and activate IRF3 to induce IFN in response to DNA. Importantly, our data also support a model whereby IFI16 also acts at the transcriptional level, regulating the transcription of IFN-α and ISGs, by facilitating Pol II placement at the promoter of the target gene, thus allowing for the production of genes that regulate type I IFN production. Lastly, type I

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**A.**

**B.**

**C.**

**D.**

**E.**

**F.**

**G.**

**H.**
IFNs can signal through the interferon α/β receptor, leading to the activation of STAT-1 and type I IFN production. IFI16 operates independently of this pathway.

The observed impact of IFI16 deficiency on the IFN response may be attributed to the fact that we used a stable knockdown of IFI16 whereas previous studies used only transient siRNA knockdown. Complete or chronic abrogation of IFI16 protein expression may be necessary to see the full effects of IFI16 deficiency on both DNA and RNA viral ligands. Consistent with these results, when IFI16 was added back to the knockdown cell lines, we saw levels of IFN-β restored toward normal production (comparable with empty vector control cells) when stimulated with various DNA and RNA ligands, suggesting that IFI16 is necessary for these responses.

In addition to a type I IFN response, viral nucleic acids trigger the production of NF-κB-dependent inflammatory cytokines. In contrast to a defect in type I IFN production, the NF-κB response was unchanged or even slightly elevated in IFI16 knockdown cells. We saw an increase in both IL-6 and IL-1β production in response to both DNA and RNA ligands. We did not see any changes in levels of phospho-IκBα postchallenge (data not shown), indicating that there is no defect in NF-κB signaling and that the impact of IFI16 deficiency on the antiviral response is specific to the type I IFN pathway. However, the increase in inflammatory cytokine production suggests that there may be a shift to a proinflammatory phenotype in IFI16 knockdown cells to compensate for a lack of a type I IFN response that is the primary defense when exposed to cytosolic nucleic acids.

In addition to a cytosolic or nuclear DNA sensing role for IFI16, we also saw a role for IFI16 in the transcriptional regulation of IFN-α gene expression as well. Both basal and stimulated levels of RIG-I, a major driver of the IFN response to RNA ligands, were decreased in IFI16 knockdown cells. We also saw a similar defect in other ISGs, including IRF7 and viperin (data not shown). We postulate that the lower levels of RIG-1 in IFI16 knockdown cells are due to the fact that RIG-I is itself an ISG, meaning that type I IFN production leads to the up-regulation of the RIG-I gene. When type I IFN was added to cells, expression levels of RIG-I returned to normal. In contrast, stimulation with Sendai virus did not restore expression of RIG-I in IFI16 knockdown cells but did enhance RIG-I levels in control cells.

To date, there have been conflicting studies in determining the role for IFI16 in innate immunity. Part of the perceived conflicts may be due to the varying cellular location of IFI16 in that IFI16 is nuclear or cytosolic in different cell types, and the function of this protein may differ by location. Several studies have shown a nuclear role for IFI16 during virus infection. IFI16 is capable of forming an inflammasome with ASC in the nucleus of epithelial cells in response to Kaposi sarcoma-associated herpesvirus (29) or in CD4 T cells in the case of bystander cells to HIV-infected T cells (31, 33). Other work shows that IFI16-dependent recognition of HSV-1 occurs in the nucleus of infected human foreskin fibroblasts and other cell types (24, 26). Other studies have shown that IFI16 is capable of sensing DNA in the cytoplasm. Acetylation of IFI16, which inhibits the nuclear localization of the protein, allows IFI16 to sense transfected DNA (26). Furthermore, it was shown that the HSV-1 capsid can be ubiquitinated and degraded by the proteasome in macrophages, leading to leaked DNA that is then recognized by IFI16 in the cytosol (27). In our current studies using THP-1 and U2OS cells, we noted that IFI16 is predominantly nuclear, and knockdown of IFI16 occurred in the nucleus (data not shown). The nuclear location of IFI16 along with the effect of IFI16 knockdown on IFN and ISG gene expression provides evidence for a regulatory role for the gene in addition to its role as a cellular DNA sensor.

We propose that IFI16 acts to position RNA Pol II in a complex with other transcription factors at the IFN-α promoter to regulate activation of transcription of type I IFN genes. Before IFI16 was implicated in innate sensing of DNA, much of the work focused on IFI16 as a transcriptional regulator in cancer cells. It is well known that IFI16 is capable of binding double-stranded DNA through its HIN200 domains (25, 27). Studies show that when IFI16 is fused to the GAL4 DNA binding domain and transfected into HeLa cells with GAL4-thymidine kinase-chloramphenicol acetyltransferase (tk-CAT), there was a dose-dependent decrease in chloramphenicol acetyltransferase activity, suggesting transcriptional repression. This activity is dependent on the HIN200 domain of IFI16 (18). Negative transcriptional regulation is also seen when IFI16 is transfected into HeLa cells with a reporter fused with the promoter of human CMV (18), and increased expression of transfected

**FIGURE 7. IFI16 acts at the level of chromatin to modulate IFN-α transcription.** A and B, pan-type IFI16 was added to cells 2 h before stimulation with Sendai virus (SV) for 2, 8, or 24 h. RIG-I (A) and IFN-α (B) expression was measured by q-RT-PCR. C and D, HEK 293T cells were transfected with a mixture containing 40 ng of TK-Renilla luciferase plus 40 ng of IFN-β or IFN-α4 firefly luciferase and increasing amounts of pRGP-IFI16 as indicated or 80 ng of STING. Luciferase values were monitored and normalized to Renilla values. Values are displayed as -fold change over empty vector control. Error bars represent ± S.E. (*, p < 0.05 assessed by two-tailed t test). Bars without * are not significant. KD, knockdown; RLU, relative luciferase units; IP, immunoprecipitation; IB, immunoblot; Bio, biotinylated; IFNaR, interferon α/β receptor; SEV, Sendai virus.

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We also saw a decrease in type I IFN production when IFI16 knockdown cells are stimulated with 2′,3′-cGAMP. This result provides further evidence that IFI16 plays a role in the regulation of IFN-α as cGAMP bypasses DNA sensing by IFI16 and directly activates STING.
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DNA is observed when IFI16 is depleted from cells (23). IFI16 was also shown to repress transcription of CMV DNA polymerase when bound in a complex with SP-1 and IR-1 promoter element (21, 22). The possible cross-talk between transcription regulation and viral nucleic acid sensing with IFI16 may prove to be an important addition to antiviral innate immunity whereby IFI16 not only recognizes an infection to trigger the production of type I IFNs but also promotes the transcription of important antiviral effectors.

There is also evidence that IFI16 plays a role in the cell cycle by negative regulation of p53. IFI16 was found to be bound directly to p53 (20). This interaction inhibits binding of p53 to the p21 promoter, leading to cell cycle arrest (19). Although we did not see an effect on cell death in the IFI16 knockdown cells, these studies provide further evidence that IFI16 is capable of acting in a regulatory manner. In our studies, we revealed a positive regulatory role for IFI16 on ISGs.

Further work is needed to determine the mechanism(s) by which IFI16 mediates the expression of IFN-α. ChIP analysis of IFI16 would be ideal; however, a suitable antibody for these studies is not yet available. We predict that IFI16 may bind directly to the promoter region of the IFN-α gene, possibly in complex with other transcription factors or co-regulators, thus regulating its expression. In addition, ChIP assays coupled to deep sequencing would provide further information on what other factors IFI16 may interact with that bind in that region. In the present study, we performed a ChIP assay in which RNA Pol II recruitment to the IFN-α promoter in both empty vector and IFI16 knockdown cells was assessed. Interestingly, we found that there was less Pol II recruitment to the IFN-α regulatory region in the IFI16 knockdown cells basally, consistent with the defect in IFN-α transcription. Previous studies have shown that serine 5 phosphorylated Pol II is bound and inactive on some promoters at a basal state. Upon stimulation, Pol II is phosphorylated on serine 2, leading to active transcription (43–46). IFI16 knockdown cells may recruit less Pol II to the IFN-α promoter basally, leading to a defect in IFN-α production and, therefore, a defect in basal levels of ISGs. Viral stimulation is not enough to overcome the absence of Pol II basally in these cells, thus leading to a continued defect in type I IFN production.

There are many conflicting results in the literature surrounding the cytosolic sensing of nucleic acids. Many sensors, including IFI16, DDX41, and CGAS, and even direct binding of DNA to STING have been implicated in the sensing of cytosolic DNA. More work needs to be done to clearly elucidate the contribution of IFI16 to these events and to determine whether these proteins are working together, playing redundant roles, or functioning in cell type-specific manners. This work offers a broader understanding of the role IFI16 plays in tight regulation of the IFN/ISG pathway.

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