IFI16 is an innate immune sensor for intracellular DNA

Andrew G Bowie, Leonie Unterholzner, Sinead E Keating, Marcin Baran, Kristy Amanda Horan, Soren B Jensen, Søren Riis Paludan, Kate A Fitzgerald, Shruti Sharma, Cherilyn M. Sirois, et al.

IFI16 is an innate immune sensor for intracellular DNA

Leonie Unterholzner[1]*, Sinead E. Keating[1]*, Marcin Baran[1]†, Kristy A. Horan[2]†, Søren B. Jensen[2, 3], Shruti Sharma[3], Cherilyn M. Sirois[3], Tengchuan Jin[4], Eicke Latz[3, 5], T. Sam Xiao[4], Katherine A. Fitzgerald[3], Søren R. Paludan[2] & Andrew G. Bowie[1]

[1]School of Biochemistry and Immunology, Trinity College Dublin, Dublin 2, Ireland.[2]Department of Medical Microbiology and Immunology, Aarhus University, Aarhus, Denmark.[3]Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, Massachusetts, USA. [4]Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA. [5]Institute of Clinical Chemistry and Pharmacology, University of Bonn, Germany.

**These authors contributed equally to this work. †These authors contributed equally to this work.*

Correspondence should be addressed to A.G.B. (e-mail: agbowie@tcd.ie).

Abstract

The detection of intracellular microbial DNA is critical to an appropriate innate immune response, however current knowledge on how such DNA is sensed is limited. Here we identified IFI16, a PYHIN protein, as an intracellular DNA sensor mediating interferon-β (IFN-β) induction. IFI16 directly associated with IFN-β-inducing viral DNA motifs. STING, a critical mediator of IFN-β responses to DNA, was recruited to IFI16 after DNA stimulation. Reduction of expression of IFI16, or its murine ortholog p204, by RNA interference inhibited DNA- and herpes simplex virus (HSV)-1-induced gene induction and IRF3 and NF-κB activation. IFI16 (p204) is the first PYHIN protein shown to be involved in IFN-β induction. Thus, the PYHIN proteins IFI16 and AIM2 form a new family of innate DNA sensors we term AIM2-like receptors (ALRs).

INTRODUCTION

An effective immune response to viruses is dependent on the induction of host cytokines and type I interferons such as interferon-β (IFN-β)[http://www.signaling-gateway.org/molecule/query?afcsid=A001237][1]. This largely occurs in response to signaling by intracellular pattern recognition receptors that detect viral nucleic acids, such as viral RNA and DNA, viral replicative intermediates and viral transcription products[2].

The innate immune response to viral RNA has been well-characterized, in that the endosomal Toll-like receptors (TLRs) and cytoplasmic RIG-I-like receptors (RLRs) sense viral RNA leading to IFN-β induction via activation of downstream signaling pathways[2]. TLR3 senses double-stranded RNA (dsRNA) leading to the recruitment of the TLR adaptor protein TRIF (TICAM-1), which then triggers the activation of TANK-binding kinase 1(TBK1) [http://www.signaling-gateway.org/molecule/query?afcsid=A001597] and IKKβ, kinases that phosphorylate and activate IRF3 and NF-κB respectively[3,4]. Thereafter, IRF3 and NF-κB mediate an anti-viral gene induction program that includes production of IFN-β. TLR7 and TLR8 both bind viral single-stranded RNA (ssRNA) leading to recruitment of MyD88, and like TLR3 activate IKKβ and NF-κB, while in contrast to TLR3 they activate IKKα leading to IRF7 activation and induction of the type I interferon IFN-α[2]. Cytosolic RNA is detected by the RLRs RIG-I and Mda-5, which via the adaptor protein IPS-1 (also known as MAVS, Cardif or VISA) turn on a signaling pathway similar to TLR3, in that IFN-β induction occurs via TBK1-mediated activation of IRF3 (ref.[2]).

In contrast to the situation for viral RNA described above, the current knowledge of intracellular DNA sensors that mediate IFN-α, IFN-β and cytokine induction in response to DNA viruses, such as herpes viruses and poxviruses, is much more limited. TLR9 can detect some viral DNAs in endosomes in plasmacytoid dendritic cells (DCs), leading to IFN-α induction via MyD88 and IKKα[5]. However it has been known for some time that exogenous dsDNA introduced into the cytoplasm, such as would be the case during infection by a DNA virus, leads to a potent innate

immune response in multiple cell types, the hallmark of which is the induction of IFN-β (reviewed in [6]). An innate immune response has been observed in response to bacterial and viral DNA, self DNA from apoptotic cells and synthetic oligonucleotides such as poly(dA:dT) and a ds 45mer oligonucleotide termed IFN-stimulatory DNA (ISD). In all these cases, the induction of IFN-β relies on signaling though TBK1 and IRF3 (refs.[7],[8]). Apart from TBK1 and IRF3, a further critical downstream component of the response to intracellular DNA has recently been identified as STING (also called MITA, ERIS or TMEM173)[9-11]. STING was shown to be required for IFN-β induction by poly(dA:dT), ISD and herpes simplex virus-1 (HSV-1) in mouse embryonic fibroblasts (MEFs) and monocytes, and for the immune response to HSV-1 *in vivo*[12]. STING acts proximally to TBK1, and in the presence of intracellular DNA, relocalizes from the endoplasmic reticulum to cytoplasmic foci containing TBK1 (refs. [12,13]). Although cytoplasmic IFN-β responses to intracellular DNA have been shown to involve STING, TBK1 and IRF3, the identity of the initiating upstream DNA sensors that engage the STING-TBK1-IRF3 pathway has remained elusive.

Two such intracellular DNA sensor candidates leading to IFN-β induction have recently been identified, namely DAI (DNA-dependent activator of IRFs, also called ZBP1) and RNA polymerase III (RNA Pol III). DAI was discovered based on the ability of poly(dA:dT) to induce IFN-β when transfected into cells[14]. However, the role of DAI has been shown to be very cell-type specific, and DAI-deficient murine embryonic fibroblasts (MEFs) and monocytes responded normally to poly(dA:dT)[15,16]. RNA Pol III was also shown to respond to transfected poly(dA:dT), which it transcribed into an RNA ligand for RIG-I, leading to DNA-mediated IFN-β induction via the RIG-I pathway[17,18]. However, the ability of many exogenous DNAs, especially non-AT rich DNA, to induce IFN-β in multiple cell types is not accounted for by DAI and RNA Pol III, and their relevance in pathogen detection is still unclear.

Clearly then, at least one further intracellular sensor for viral DNA leading to IFN-β induction remains to be identified[6].

To search for additional cytoplasmic DNA sensors, we used an IFN-β-inducing vaccinia virus (VACV) DNA motif to affinity purify DNA-binding proteins from cytosolic extracts of human monocytes. This VACV motif, when transfected into cells induced IFN-β in a TLR- DAI- and RNA Pol III-independent, but STING- TBK1- and IRF3-dependent manner. Among the proteins identified that interacted with this DNA was IFI16, a member of the PYHIN protein family that contains a pyrin domain and two DNA binding HIN domains. IFI16 was shown to directly bind to the IFN-β–stimulating viral DNA, and to recruit STING upon stimulation of cells with transfected DNA. Small interfering RNA (siRNA) targeting IFI16, or its murine ortholog p204, inhibited DNA-, but not RNA-induced IRF3 and NF-κB activation, and IFN-β induction. Importantly, responses to a DNA virus, but not to an RNA virus, were also dependent on p204, in that HSV-1-, but not Sendai virus-stimulated transcription factor activation and gene induction were strongly impaired by p204 siRNA. Interestingly, AIM2 [http://www.signaling-gateway.org/molecule/query?afcsid=A004152], another PYHIN protein, has previously been shown to be a sensor for cytosolic DNA for the signaling pathway that activates caspase 1, leading to interleukin-1β release[19-22]. Thus we propose that the PHYIN proteins represent a new family of innate DNA sensors termed AIM2-like receptors (ALRs).

RESULTS

IFN-β induction in monocytes by viral DNA sequences

To investigate the cellular response to exogenous DNA we first compared the ability of different types of exogenous DNA to stimulate cytosolic DNA sensing pathways in human cells. Cells were transfected with poly(dA:dT), viral DNA from VACV, mammalian DNA or bacterial DNA, and induction of the *Ifnb* promoter was

measured. In HEK293 cells, which have previously been shown to have a functional RNA Pol III DNA sensing pathway[17, 18], only poly(dA:dT) stimulated the *Ifnb* promoter (**Fig. 1a**). In contrast, all dsDNAs tested induced IFN-β mRNA when transfected into in human monocytic THP-1 cells (**Fig. 1b**). This result suggested the existence of one or more DNA sensing pathways for non-AT rich dsDNA in THP-1 cells that were not present in HEK293 cells. To select a defined IFN-β–inducing viral dsDNA to screen for host DNA sensors in THP-1 cells, we noted a 70 bp long sequence, which is strongly conserved in different poxviral genomes such as VACV, in the inverted terminal repeat region and often repeated multiple times[23] (**Supplementary Fig. 1**). When transfected into cells, this dsDNA 70 bp motif (hereafter referred to as VACV 70mer) strongly induced IFN-β in THP-1 cells (**Fig. 1c** and **Supplementary Fig. 2a**) and in MEFs (**Fig. 1d**), but was only stimulatory as duplex DNA and not single-stranded (**Fig. 1c,d**). Furthermore, VACV 70mer also induced IFN-β in human peripheral blood mononuclear cells, immortalized mouse bone marrow-derived macrophages (BMDMs) and in mouse bone marrow-derived DCs (**Supplementary Fig. 2**), but not in HEK293 cells (**Fig. 1e**).

We next assessed whether the particular sequence of the VACV 70mer, and/or its length were critical to the IFN-β response. The response to VACV 70mer was independent of AT content, but strongly dependent on length, since in either human or murine monocytic cells changing the AT content from 67% to 10% did not affect the response, whereas even a reduction of 10 bp in length impaired IFN-β induction (**Fig. 1f, g**). Consistent with the cytosolic IFN-β response being length-dependent but sequence-independent, the previously described 45 bp ISD[7] gave a comparable IFN-β response to a 50 bp oligonucleotide derived from the VACV 70mer (**Fig. 1f,g**). Furthermore, an unrelated dsDNA 60mer oligonucleotide derived from the HSV-1 genome (herein referred to as HSV 60mer) also induced IFN-β (**Supplementary Fig. 3a**). A number of different oligonucleotides derived from the HSV-1 genome were

screened for IFN-β-inducing capacity (data not shown) and the HSV 60mer was selected as having the highest (see Methods for sequence).

IFNβ induction via a novel intracellular DNA sensor

We next investigated the role of known DNA sensors in the IFN-β response to the VACV 70mer and HSV 60mer. The response to VACV 70mer in BMDMs lacking the TLR signaling adaptors MyD88 or TRIF (TICAM-1) was not reduced compared to wild-type control cells (**Fig. 2a,c**) while TLR-induced IFN-β expression was strongly inhibited in the mutant cells (**Fig. 2b,d**). Analogously, the response to the HSV 60mer was unimpaired in macrophages lacking both MyD88 and TRIF (**Supplementary Fig. 3a**). BMDMs lacking DAI (ZBP1) also responded normally to VACV 70mer (**Fig. 2e**), and to poly(dA:dT) (**Fig. 2f**). The IFN-β response to poly(dA:dT) is known to involve transcription of the DNA into RNA RIG-I ligands[17,18]. Consistent with this notion, when RNA was extracted from poly(dA:dT)-treated murine macrophages, MEF or human THP-1 cells, and transfected into HEK293 cells, this RNA induced *Ifnb* promoter expression (**Fig. 2g** and **Supplementary Fig. 4a, b**). In contrast, *Ifnb* was not expressed in HEK293 cells transfected with RNA extracted from cells stimulated with VACV 70mer (**Fig. 2g** and **Supplementary Fig. 4a, b**), indicating that, unlike poly(dA:dT), the VACV 70mer was not transcribed into immune-stimulatory RNA . The HSV-1 60mer also induced IFN-β independently of RNA Pol III, since IFN-β induction was not prevented by the RNA Pol III inhibitor ML60812 (**Supplementary Fig. 3b**). Consistent with this result, there was no response to the HSV 60mer in HEK293 cells where the RNA Pol III pathway is operational (**Supplementary Fig. 3c**).

All cytosolic DNA sensing pathways inducing IFN-β described thus far, including those where no receptor has been identified such as for the ISD, are known to do so via activation of the transcription factor IRF3 by a complex containing TBK1

(ref. [6]). Consistent with such a requirement, the VACV 70mer response in MEFs was abolished in the absence of IRF3 (**Fig. 2h**), while in either MEFs or BMDMs lacking TBK1, the VACV 70mer response was strongly impaired (**Fig. 2i,j**). Hence IFN-β induction by the VACV 70mer is TLR-, DAI- and RNA Pol III-independent, yet dependent upon TBK1 and IRF3.

IFI16 is a candidate DNA sensor for viral DNA

Since the IFN-β response to VACV 70mer was not mediated by any of the known DNA-sensing pathways, we designed a screen to isolate novel cytoplasmic DNA sensors. Biotinylated ss or ds VACV 70mer was coupled to streptavidin beads to affinity purify DNA-binding proteins from cytosolic extracts of THP-1 cells. Proteins interacting with VACV 70mer were identified by mass spectrometry (see Methods). Among the proteins identified was IFI16, a member of the PYHIN protein family that contains a pyrin domain and two DNA binding HIN domains, termed HINa and HINb below (**Fig. 3a**). Interestingly, AIM2, another PYHIN protein, has been shown to be a receptor for cytosolic DNA that regulates caspase 1 activation, leading to interleukin-1β (IL-1β) release[19-22]. However, no PYHIN protein has yet been implicated in mediating an IFN-β response to DNA.

In THP-1 cells cytosolic IFI16 was detectable by immunoblot in a complex with immobilized ss or dsVACV 70mer (**Fig. 3b**), consistent with the previous finding that it HINa domain bound both ssDNA and dsDNA[24]. Given that the ssVACV 70mer was unable to induce IFN-β (**Fig. 1c**), we reasoned that it might bind IFI16 but fail to induce signaling to IFN-β. Consistent with this hypothesis, the ssVACV 70mer acted as an antagonist of dsVACV 70mer-induced IFN-β expression when co-transfected into THP-1 cells, while not affecting the response to poly(I:C) (**Fig. 3c**). Furthermore, poly(dA:dT)-induced IFN-β in HEK293 cells was also unaffected by transfection of the ssVACV 70mer (**Fig. 3c**). Previously IFI16 was shown to be mainly localized to

the nucleus when overexpressed in HEK293 cells[19], which was confirmed in this study (data not shown). However in THP-1 cells, endogenous IFI16 was isolated from the cytoplasm in association with VACV 70mer (**Fig. 3b**), and while predominantly nuclear, was clearly visible in the cytoplasm by immunofluorescence (**Fig. 3d**). Further, mitotracker co-staining indicated that IFI16 might partially localize to mitochondria (**Supplementary Fig. 5a**). Compellingly, when cells were stained with 4'6-diamidino-2-phenylindole (DAPI) to detect nucleic acid, VACV 70mer, but not poly(I:C), was found to co-localize with endogenous IFI16 in the cytoplasm (**Fig 3d**). Furthermore, fluorescein isothiocyanate (FITC)-labeled HSV 60mer also co-localized with endogenous cytoplasmic IFI16 (**Fig. 3e**). To determine whether IFI16 was capable of directly binding the viral DNAs *in vitro*, the HIN domains of IFI16 were expressed in *Escherichia coli* and purified (see Supplementary methods and **Supplementary Fig. 6**). An AlphaScreen was then used to determine *in vitro* binding between viral DNA and purified IFI16 HIN domains. Biotinylated dsVACV 70mer was found to bind cooperatively to HINb, and to the region of IFI16 containing both HINa and HINb (HINab) with higher affinity than to HINb alone, but not to bind HINa alone (**Fig. 3f**). Similar results were obtained for the HSV 60mer (**Supplementary Fig. 3d**). The co-localization of endogenous cytoplasmic IFI16 with IFN-β inducing viral DNA, and its direct and cooperative binding to this DNA *in vitro* suggested that IFI16 might be a DNA sensor mediating IFN-β induction.

STING is recruited to IFI16 upon DNA stimulation

We next assessed whether IFI16 could interact with any of the downstream signaling components known to be involved in IFN-β induction. Like IFI16 both cytosolic TBK1 and DDX3 (which has a role in IRF3 activation by TBK1[25]), were enriched in immobilized dsVACV 70mer complexes, compared to ssVACV 70mer complexes (**Fig. 4a**). STING is a recently identified signaling protein known to be required for TBK1-dependent IFN-β responses to viruses and DNA[12,13]. Transfection of VACV

70mer into cells caused a DNA-dependent association between endogenous STING and IFI16 in THP-1 cells (**Fig. 4b**). IFI16 from DNA-stimulated THP1 cells associated with immobilized Myc-tagged human STING and to a lesser extent mouse STING (**Fig. 4c**). STING was not only recruited to IFI16 after viral DNA stimulation, but was also required for IFN-β induction by VACV 70mer or HSV 60mer since in BMDMs lacking STING (TMEM173), IFN-β secretion from cells transfected with the viral DNAs was completely inhibited (**Fig. 4d,e**). ASC (apoptosis-associated speck like protein containing a CARD domain) is an adaptor recruited to AIM2 which mediates caspase 1 activation by viruses and poly(dA:dT)[19]. However ASC had no role in the IFN-β response mediated by exogenous DNA, since in BMDMs lacking ASC both VACV 70mer- and HSV 60mer-induced IFN-β expression was unimpaired, as was the poly(dA:dT) response (**Fig. 4f**). Thus STING and ASC likely have distinct roles in regulating PYHIN-mediated IFN-β induction and caspase 1 activation, respectively.

Gene induction and signaling by viral DNA requires IFI16

Further evidence for a role for IFI16 in mediating an IFN-β response to DNA stems from the correlation between expression of IFI16 and responsiveness of cells to VACV 70mer. Thus, PMA-treated THP-1 cells displayed enhanced IFI16 expression and increased VACV 70mer-induced IFN-β expression compared to untreated cells (**Fig. 5a**). Whereas in HEK293 cells that failed to respond to VACV 70mer (**Fig. 1e**), IFI16 expression was undetectable (**Fig. 5a** and **Supplementary Fig. 5b**). In THP-1 cells, IFI16 expression was inducible by both IFN-α and VACV 70mer (**Supplementary Fig. 5b,c**), consistent with an antiviral role for IFI16. To clarify the role of IFI16 in mediating IFN-β induction by the VACV 70mer we used transient transfection of short interfering RNA oligonucleotides (siRNAs) to knock down *IFI16* in THP-1s, which led to reduced expression of IFI16 protein compared to cells treated with control siRNA (**Fig. 5b**). Importantly, this treatment with siRNA targeting IFI16

led to an inhibition of IFN-β induction in response to VACV 70mer (**Fig. 5c**). The IFN-β response to HSV 60mer was also inhibited in human cells by siRNA targeting IFI16 (**Supplementary Fig. 7a**).

We next considered whether IFI16 might also have a role in DNA sensing in mouse cells. Upon examining the mouse PYHIN family, only one member was found to display the same domain structure as IFI16, namely p204, in that this PYHIN protein also contained one pyrin and two HIN domains (**Fig. 5d**). Also, a BLAST search with human IFI16 revealed p204 as the most similar mouse PYHIN protein (37% amino acid identity). Expression of p204 was detected in mouse RAW264.7 cells by immunoblot, and this protein increased upon transfection with VACV 70mer (**Fig. 5e**). Treatment of these cells with siRNA targeting p204 led to reduced p204 protein (**Fig. 5e**) and mRNA (**Fig. 5f**) abundance. Targeted p204 knockdown led to impaired VACV 70mer-mediated gene induction of *Ifnb*, *Ccl5* and *Tnf* (**Fig. 5f**). The requirement for p204 for transfected DNA-mediated gene induction was not restricted to RAW264.7 cells, since in MEFs, p204 siRNA strongly reduced p204 mRNA (**Fig. 5g**) and led to potent inhibition of VACV 70mer-mediated IFN-β, CCL5 and TNF expression (**Fig. 5g**). Suppression of p204 expression by siRNA in RAW264.7 cells also inhibited the IFN-β response to the HSV 60mer (**Fig. 5h**). Furthermore, in BMDMs, p204 siRNA inhibited both VACV 70mer- and HSV 60mer-stimulated IFN-β protein secretion from cells (**Fig. 5i**). Hence the IFI16 (p204) DNA sensing pathway is not restricted to one DNA sequence nor to one cell type, and is operational both in human and mouse cells.

A role for p204 as a sensor for DNA predicts that inhibition of p204 expression should affect DNA-mediated gene induction due to an effect upstream of transcription factor activation. Thus, we examined the effect of p204 siRNA on VACV 70mer-induced NF-κB and IRF3 activation. To measure transcription factor activation, the translocation of endogenous p65 (an NF-κB subunit) and IRF3 from the cytosol to the

nucleus was monitored by confocal microscopy upon DNA transfection of RAW264.7 cells, in the presence of either control or p204-targeting siRNA. Transfection of cells with VACV 70mer or poly(I:C) for 6 h led to an accumulation of both p65 and IRF3 in the nucleus in the presence of the control siRNA (**Fig. 6a,b**). Compellingly, both p65 and IRF3 translocation was prevented in the presence of p204 siRNA in cells transfected with DNA but not with RNA (**Fig. 6a,b**). This result confirmed that p204 has a role in gene induction upstream of transcription factor activation. Furthermore, it showed a role for p204 upstream of both the NF-κB and IRF3 pathways for DNA and not RNA, consistent with a role in direct DNA sensing.

IFI16 is required for HSV-1-induced gene expression

In order to extend these findings to a relevant DNA virus, we examined the role of various DNA sensing pathways in mediating gene induction and signaling responses to HSV-1 in monocytes[26]. The IFN-β response to live HSV-1 in BMDMs was largely TLR-independent, since in cells lacking both MyD88 and TRIF, only marginal inhibition of IFN-β mRNA induction was observed (**Fig. 7a**). The IFN-β response to HSV-1 in monocytes was also RNA Pol III-independent, in contrast to a previous study[17], we found no impairment of IFN-β induction by HSV-1 in the presence of the RNA Pol III inhibitor, although the response to poly(dA:dT) was inhibited (**Fig. 7b**). Furthermore, consistent with a lack of a role for RNA Pol III in sensing HSV-1, no IFN-β induction was detectable in HSV-1-infected HEK293 cells, cells in which the RNA Pol III DNA sensing pathway is operational (**Supplementary Fig. 3c**). IFN-β expression in response to HSV-1 was STING-dependent (**Fig. 4e**) as previously shown[12].

In order to examine the role of p204 in detecting HSV-1, cells were treated with p204 siRNA prior to infection with HSV-1. This led to a severe inhibition of HSV-1-induced IFN-β mRNA (**Fig. 7c**), which was in contrast to the minor role for TLR

detection of HSV-1 and the absence of a role for RNA Pol III in IFN-β induction by HSV-1. Furthermore, induction of the IRF3-dependent gene *Cxcl10*, and the NF-κB-dependent genes *Il6* and *Tnf* by HSV-1 infection of RAW264.7 cells were also impaired by p204 siRNA (**Fig. 7c**). In contrast, treatment of cells with p204 siRNA had no effect on induction of IFN-β mRNA in response to an RNA virus (Sendai) (**Fig. 7d**). p204 siRNA also reduced IFN-β protein secretion in response to HSV-1 but not Sendai virus (**Fig. 7e**). This result confirmed that knockdown of p204 expression did not lead to a global reduction in gene induction in response to viral infection. The IFN-β response to HSV-1 was also inhibited in human cells by siRNA targeting IFI16 (**Supplementary Fig. 7a**). Consistent with the effect of p204 siRNA on HSV-1-induced IRF3- and NF-κB-dependent genes (**Fig. 7c**), and similar to the case for the viral DNA oligonucleotides (**Fig. 6a**), p204 was required for HSV-1-induced translocation of NF-κB (p65) and IRF3 from the cytosol to the nucleus (**Fig. 7f,g**). In contrast, Sendai virus-stimulated transcription factor translocation was unaffected by p204 siRNA (**Fig. 7f,g**). Further, triggering the p204 pathway induced anti-HSV-1 activity in cells, since pre-treatment of cells with the HSV 60mer potently suppressed HSV-1 replication (**Fig. 7h**).

HSV-1 replicates in the nucleus. Therefore IFI16 and p204 could in principle detect HSV-1 DNA in that compartment, and certainly the nucleus contains high amounts of IFI16 (**Fig. 3d**). Using a specific labeled oligonucleotide that is complementary to HSV-1 DNA, viral DNA was detectable in the cytoplasm mislocated from the viral capsid (as stained by an antibody to the viral protein VP5) in RAW264.7 macrophages (**Supplementary Fig. 8**), BMDMs and THP-1 cells (not shown). Thus, IFI16 and p204 might detect the presence of HSV-1 DNA either in the cytoplasm and/or the nucleus. Together, these results demonstrate that human IFI16 and mouse p204 are PYHIN proteins that act as sensors for exogenous DNA, but not RNA, directly detecting the presence of viral DNA leading to transcription factor activation and gene induction via a STING-dependent pathway.

DISCUSSION

Currently there is much interest in defining the mechanisms whereby the innate immune response detects exogenous DNA, since this detection process is critical to understand how cells respond to DNA viruses and to immune-stimulatory bacterial and self-DNA[6]. Although IFI16 has previously been shown to have a role in regulating cell proliferation and differentiation[27], our data here identify human IFI16 and its mouse ortholog p204 as a critical sensor for exogenous dsDNA, especially with regard to IRF3- and NF-κB-dependent gene induction, and as essential for the IFN-β response to live HSV-1. Until now, the sensor for intracellular non-AT-rich dsDNA in MEFs and some monocyte-derived cells was undefined[6], although IRF3, TBK-1 and STING were known to be required[7,8,12]. Since the IFI16 (p204) pathway to IFN-β induction via STING, TBK-1 and IRF3 identified here operates in both MEFs and macrophages it may account for these responses.

IFI16 is the first example of a pyrin domain-containing protein sensing DNA to mediate IFN-β induction. We showed that the HIN domains of IFI16 bound the VACV 70mer and the HSV 60mer, but it remains to be determined which features of the dsDNA are critical for this interaction, and how dsDNA stimulates IFI16 to recruit STING. It is possible that DNA of a certain length in the cytosol causes oligomerization of IFI16 leading to signaling, consistent with the observation that the IFN-β response was strongly length-dependent.

Previously pyrin domains have been shown to mediate inflammasome activation. For example, AIM2 is another PYHIN family member which does sense DNA and poxviruses, but this interaction leads to activation of caspase 1, via the adaptor ASC, and not IFN-β induction[19]. In contrast, here IFI16 recruited STING, and required STING but not ASC, for DNA-mediated IFN-β induction. Further, previously it has been shown that IFI16 and ASC do not interact[19]. Interestingly,

within the PYHIN family, the pyrin domain of AIM2 is most like inflammasome-related pyrin domains, while the pyrin domains of the rest of the family are distinct from AIM2, and more like IFI16 (ref. [28]). It remains to be determined whether other PYHIN family members may also be capable of regulating STING-dependent pathways that may be independent of ASC.

Given that IFI16 senses DNA to induce IFN-β, and AIM2 senses DNA to activate caspase 1, we propose that the PYHIN proteins represent a new family of innate DNA sensors termed AIM2-like receptors (ALRs). Thus while exogenous RNA is sensed by both endosomal TLRs (TLR3, 7, 9) and intracellular RLRs[2], so exogenous DNA is sensed by both endosomal TLR9 and intracellular ALRs. Similar to the RLR family, the ALR family contains sensors for IFN-β induction (IFI16 compared to RIG-I and Mda5), inflammasome activators (AIM2 compared to RIG-I[29]) and negative regulators (p202, ref. [21], compared to LGP2, ref. [30]). Further, like the RLR family, the ALR family is subject to viral targeting: the poxviral protein M013 contains a pyrin domain and binds to ASC[31], which would inhibit the AIM2 response, while interestingly it has also been recently shown to inhibit poxviral-stimulated induction of NF-κB-dependent genes[32]. Also, the human cytomegalovirus protein pUL83, a known antagonist of IFN-inducible genes, has recently been shown to interact with IFI16 (ref. [33]). If IFI16 also has a role in sensing that DNA virus that could explain the ability of pUL83 to potently inhibit the IFN response.

It is possible that IFI16 also has a role in sensing bacterial DNA during infection with intracellular bacteria. Type I IFN induction in response to *Listeria* is independent of TLRs and RNA sensing and requires IRF3 (ref. [34]) and STING[12], while IFN-β induction in response to *Chlamydia* infection also involves a TLR- and RLR-independent but STING-dependent pathway[35]. Finally, DNA detection in the cytoplasm leading to IFN-β induction is thought to be one trigger for autoimmune conditions such as systemic lupus erythematosus (SLE), and it is interesting to note

that IFI16-specific antibodies are present and IFI16 expression is elevated in patients with SLE[36-38]. Thus detection of DNA by IFI16 or p204 may have a role not only in anti-viral innate immune responses but also in responses to bacterial pathogens and in autoimmunity.

ACKNOWLEDGEMENTS

We would like to thank C.Liu (New York University) for the anti-p204 antibody, S. Akira (Osaka University), T. Taniguchi (University of Tokyo), M. Kelliher (University of Massachusetts Medical School), T. Mak (University of Toronto) and G. Barber (University of Miami) for mice or femurs, D. Golenbock (University of Massachusetts Medical School) for immortalized BMDMs lacking MyD88 or TRIF and I. Julkunen (National Public Health Institute, Finland) for Sendai virus. This work was funded by Science Foundation Ireland grant 07/IN1/B934 (L.U., M.B., A.G.B.), the Irish Health Research Board (S.K., A.G.B.), the Danish Medical Research Council grant 09-072636 (S.R.P.), the Lundbeck Foundation grants R17-A1528 (S.R.P.) and R34-A3855 (K.A.H.), and by NIH grants AI083713 and AI067497 (to K.A.F). S.B.J. was supported by a PhD scholarship from the Faculty of Health Sciences, Aarhus. T.S.X. is supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, NIH, USA.

AUTHOR CONTRIBUTIONS

L.U. and S.E.K. did experiments, analyzed data and co-wrote the manuscript. M.B., K.A.H., S.B.J., S.S., C.M.S. and T.J. performed experiments and analyzed data. E.L. provided expertise. T.S.X., K.A.F. and S.R.P. supervised experiments and analyzed

data. A.G.B. designed and supervised the study, analyzed data and co-wrote the manuscript.

Figure 1 Induction of IFN-β by a VACV DNA motif. (**a**) HEK293T cells were transfected with nucleic acids (0.5 and 5 μg/ml) for 16 h and *ifnb* promoter activity was measured by reporter gene assay. (**b**) PMA-treated THP-1 cells were transfected with 1 μg/ml poly(dA:dT) or with 200 ng/ml DNA isolated from VACV, calf thymus or *Listeria* for 6 h and IFN-β mRNA was measured. (**c,d**) THP1 (**c**) or immortalized MEF (**d**) cells were transfected with 1 μg/ml (**c**) or 5 μg/ml (**d**) nucleic acids for 6 h and IFN-β mRNA was measured. (**e**) HEK293T cells were transfected with 5 μg/ml nucleic acids for 16 h, and *ifnb* promoter activity was measured. (**f,g**) THP-1 (**f**) or RAW264.7 (**g**) cells were transfected with different lengths (bp) of 1 μg/ml VACV 70mer-derived DNA, GC-rich 70mer (70(GC)) or interferon stimulatory DNA (ISD) for 6 h and IFN-β mRNA was measured. Data are from one experiment representative of two (Error bars, s.d.).

Figure 2 Induction of IFN-β by a VACV DNA motif is independent of known DNA sensing pathways. (**a-f,h-j**) Immortalized BMDMs or MEFs derived from mice lacking signaling components as indicated were transfected with 5 μg/ml VACV 70mer (**a**,**c**,**e**,**h-j**) or poly(dA:dT) (**f**) or stimulated with LPS (**b**) or poly(I:C) (**d**) for 6 h and IFN-β mRNA measured. Data are expressed as % of stimulation observed in cells from wild type mice and are from three independent experiments performed in triplicate (mean and s.e.m). (**g**) Immortalized BMDMs were transfected with nucleic acid for 6 h and IFN-β mRNA measured (left). 100 ng of the RNA extracted from BMDMs was transfected into HEK293T cells for 16 h, and *ifnb* promoter activity measured (right). Data are from one experiment representative of three (Error bars, s.d.).

Figure 3 IFI16 binds to immune stimulatory viral DNA. (**a**) Schematic representation of human PYHINs. Aa, amino acids. (**b**) Cytoplasmic extracts from PMA-treated THP-1 cells were incubated with biotinylated ss or dsVACV 70mer immobilized on streptavidin beads. Precipitated proteins were immunoblotted for IFI16. (**c**) Left, THP-1 cells were transfected with 1 μg/ml dsVACV 70mer or poly(I:C) in the absence or presence of 1 μg/ml ssVACV 70mer and IFN-β mRNA was measured after 6 h. Right, HEK293T cells were transfected with 50 ng/ml poly(dA:dT) alone, or together with 50 ng/ml ss or dsVACV 70mer and IFN-β promoter activity measured after 16 h. (**d**) PMA- and IFN-α-treated THP-1 cells grown on coverslips were transfected with 2.5 μg/ml VACV 70mer or poly(I:C) for 1 h. Cells were fixed and stained with anti-IFI16 antibody (red). DNA and poly(I:C) were visualized with DAPI (blue). (**e**) FITC-labeled HSV-1 60mer was transfected into PMA-treated THP-1 cells for 3 h. Cells were fixed and stained for IFI16 (red). DAPI-stained DNA is shown in blue (upper panel) and the HSV 60mer is shown in green (lower panel). Scale bar: 10 μm. (**f**) AlphaScreen assessment of IFI16 HIN domains binding dsVACV 70mer. Left, 30 nM DNA with increasing concentration of HIN domains or GB1 expression tag. Right, 30 nM protein domain with increasing concentrations of biotin-labeled VACV70mer. Data are from one experiment representative of three (mean and s.d. in **c**).

Figure 4 Role for STING in IFI16-mediated IFN-β induction. (**a**) Cytoplasmic extracts from PMA-treated THP-1 cells were incubated with biotinylated ss or dsVACV 70mer immobilized on streptavidin beads. Precipitated proteins were

immunoblotted for TBK1 and DDX3. (**b**) PMA- and IFN-α-pre-treated THP1 cells were transfected with 1 μg/ml VACV 70mer for 4 h. Resulting lysates were immunoprecipitated with a STING antibody and then immunoblotted for IFI16 and STING. (**c**) Myc-tagged human (hu) or mouse (mur) STING were overexpressed in HEK293 cells, immobilized on sepharose beads, and incubated with lysates from DNA-treated THP1 cells. Co-immunoprecipitated IFI16 was detected by immunoblotting. Ab, antibody heavy chain. (**d,e**) BMDMs lacking STING (from *Tmem173*$^{-/-}$ mice) were transfected with either VACV 70mer, HSV 60mer or infected with HSV-1 or Sendai virus for 18 h and IFN-β protein release measured. (**f**) Immortalized BMDMs from *Asc*$^{-/-}$ mice were transfected with DNA for 18 h and IFN-β protein secretion measured. Data are from one experiment representative of two (mean and s.d. in **d**, **e**, **f**).

Figure 5 IFI16 is required for DNA-mediated gene induction. (**a**) Left, IFI16 mRNA was measured in THP-1, PMA-treated THP-1, or HEK293 cells. Right, THP-1 or PMA-treated THP-1 cells were transfected with VACV 70mer for 6 h and IFN-β mRNA measured. (**b,c**) siRNA-treated THP-1 cells were transfected with 1 μg/ml VACV 70mer for 6 h, before detection of IFI16 protein expression by immunoblotting (**b**) or measurement of IFN-β mRNA (**c**). (**d**) Domain organization of human IFI16 compared to murine members of the PYHIN family, drawn to scale. Boxes represent conserved domains. aa, amino acids. p204 is indicated by an asterisk as most similar to human IFI16. (**e,f**) siRNA-treated RAW264.7 cells were transfected with 1 μg/ml VACV 70mer for 6 h and p204 protein expression was analyzed by immunoblotting

(**e**) or p204, IFN-β, CCL5 and TNF mRNA was measured (**f**). (**g**) siRNA-treated MEF cells were transfected with 1 μg/ml VACV 70mer for 6 h and p204, IFN-β, CCL5 and TNF mRNA was measured. (**h**) siRNA-treated RAW264.7 cells were treated with HSV 60mer DNA for 6 h before measurement of IFN-β mRNA. (**i**) BMDMs were electroporated with siRNA prior to transfection with oligomers for 18 h, and IFN-β protein release was measured. *P < 0.05 compared with control siRNA. Data are from one experiment representative of two or three (mean and s.d. in **a**,**c**,**f-i**).

Figure 6 IFI16 is required for VACV 70mer DNA-stimulated transcription factor activation. (**a**) siRNA-treated RAW264.7 cells grown on glass coverslips were mock-transfected or transfected with 2.5 μg/ml VACV 70mer or poly(I:C) for 6 h, fixed and stained for NF-κB p65 (red) and IRF3 (green). Nuclei were visualized by DAPI (blue). (**b**) Cells treated as described in **a** were qualitatively examined to assess staining of either p65 (left) or IRF3 (right) in the nucleus. Cells showing nuclear staining were counted and expressed as a percentage of total number of cells. At least 200 cells were counted per sample. Data shown are representative of four experiments.

Figure 7 IFI16 is required for the innate immune response to HSV-1. (**a**) BMDMs were infected with HSV-1 at an MOI of 10 for 6 h and IFN-β mRNA was measured. (**b**) RAW264.7 cells were pre-treated with ML60812 for 2 h prior to infection with HSV-1, or transfection with poly(dA:dT) for 6 h. (**c,d**)

siRNA-treated RAW264.7 cells were infected with HSV-1 or Sendai virus for 6 h and IFN-β (**c**,**d**), CXCL10 (**c**), IL-6 (**c**) and TNF (**c**) mRNA was measured. (**e**) siRNA-treated RAW264.7 cells were infected with HSV-1 or Sendai virus for 20 h and IFN-β protein expression determined. (**f**) siRNA-treated RAW264.7 cells grown on glass coverslips were mock-infected or infected with HSV-1 or Sendai virus for 6 h, fixed and stained for NF-κB p65 (red) and IRF3 (green). Nuclei were visualised by DAPI (blue). (**g**) Cells treated as described in **f** were qualitatively examined to assess staining of either p65 (left panel) or IRF3 (right panel) in the nucleus. Cells showing nuclear staining were counted and expressed as a percentage of total number of cells. At least 200 cells were counted per sample. (**h**) RAW264.7 cells transfected with HSV 60mer (2 μg/ml) were infected with HSV-1 at an MOI of 1 and culture supernatants were harvested at the indicated time points post infection for viral quantification by plaque assay. *$P < 0.001$ compared with control siRNA. Data are from one experiment representative of three (mean and s.d. in **a-e**,**h**).

METHODS

Mice, cells and viruses. *Myd88*$^{-/-}$ and *Ticam1*$^{-/-}$ mice were obtained from S. Akira, and were crossed to generate *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ mice. *Zbp1*$^{-/-}$ (DAI-deficient), *Irf3*$^{-/-}$, *Tnfrsf1a*$^{-/-}$ and *Tbk1*$^{-/-}$*Tnfrsf1a*$^{-/-}$ mice were from S. Akira, T. Taniguchi, M. Kelliher and T. Mak respectively. *Tmem173*$^{-/-}$ (STING-deficient) femurs were from G. Barber. Immortalized macrophage cell lines were generated from mouse femurs using J2 recombinant retrovirus carrying Mycl1 and v-raf/mil oncogenes[39] as described[40]. Immortalized MEFs were generated from *Irf3*$^{-/-}$ and *Tbk1*$^{-/-}$ mice. Mice were bred and maintained in the animal facilities of the University of Massachusetts Medical School and experiments were carried out in accordance with the guidelines set forth by the University of Massachusetts Medical School Department of Animal Medicine and the Institutional Animal Care and Use Committee. THP-1 cells were grown in RPMI containing 10% FBS, and were treated with 50 or 100 nM PMA and 1000 U/ml IFN-α for at least 16 h where indicated. All other cells were grown in DMEM containing 10% FBS. For viral infection, HSV-1 strain KOS, and Sendai virus strain Cantell (a gift from I. Julkunen) were used.

Antibodies. p204 antibody[41] was a gift from C. Liu. STING antibody[9] was a gift from G.Barber. Other antibodies were from the following sources: IFI16 (1G7: Santa Cruz) and p65 (F-6; Santa Cruz), β-actin (AC-74; Sigma), TBK1 (3013; Cell Signalling), DDX3 (A300-A74A; Bethel Laboratories), mouse IRF3 (51-3200; Zymed), VP5 (3B6; Virusys), myc (9E10; Sigma).

Nucleic acids and transfection. HEK293 cells were transfected using 4 μl/ml GeneJuice (Merck). All other cells were transfected using 1 μl/ml Lipofectamine2000 (Invitrogen). Poly(I:C), calf thymus DNA and poly(dA:dT) were from Sigma, and herring sperm DNA from Promega. *Listeria monocytogenes* DNA was a gift from S.

Corr. VACV DNA was purified from Western Reserve strain virus core particles by phenol-chloroform extraction. Oligonucleotides were synthesized by MWG Biotech, sequences are as follows: VACV 70mer, 5′-CCATCAGAAAGAGGTTTAATATTTTTGTGAGACCATCGAAGAGAGAAAGAGATAAAACTTTTTTACGACT-3′; GC-rich 70mer, 5′-CCGCCAGCCCGCGGGCTGGCGCCCCCACTCGGGCCGTCGGGGCCGCGCCTCCCCCGCGAGGCCGCCGGCG-3′; ISD, 5′-TACAGATCTACTAGTGATCTATGACTGATCTGTACATGATCTACA-3′; HSV-1 60mer, 5′-TAAGACACGATGCGATAAAATCTGTTTGTAAAATTTATTAAGGGTACAAATTGCCCTAGC-3′. The VACV 30mer, 50mer and 60mer sequences are identical to the 5′ portion of the VACV 70mer, but terminating at position 30, 50 and 60, respectively.

AlphaScreen. The AlphaScreen was set up as an association assay. Purified recombinant His-tagged IFI16 HIN domains were incubated at the indicated concentrations with biotinylated DNA and binding was measured as previously described[19].

RNA analysis. Human IFN-β mRNA was quantified by real-time PCR using the TaqMan gene expression assay Hs00277188_s1 and β-actin endogenous control VIC/MGB probe (Applied Biosystems). Other mRNAs were quantified using SYBR Green with the following primers: F-IFNβ(mouse), 5′-ATGGTGGTCCGAGCAGAGAT-3’; R-IFNβ(mouse), 5′-CCACCACTCATTCTGAGGCA-3′; F-CCL5(mouse), 5′-CTCACCATATGGCTCGGACA-3′; R-CCL5(mouse), 5′-ACAAACACGACTGCAAGATTG G-3′; F-TNF(mouse), 5′-TCCCCAAAGGGATGAGAAGTT-3′; R- TNF(mouse), 5′-

GTTTGCTACGACGTG GGCTAC-3′; F-β-actin(mouse), 5′-TCCAGCCTTCCTTCTTGG GT-3′; R-β-actin(mouse), 5′-GCACTGTGTTGGCATAGAGGT-3′; F-p204, 5′-TGGTCCCAAACAAGTGATGGTGC-3′; R-p204, 5′-TCAGTTTCAGTAGCCACGGTAGCA-3′; F-IFI16, 5′-CCGTTCATGACCAGCATAGG-3′; R-IFI16, 5′-TCAGTCTTGGTTTCAACGTGGT-3′. In Fig. 5h and 7, the following real-time PCR primers were used: F-TNFα, 5′-ATCGGCTGGCACCACTAGTT-3′; R-TNFα, 5′-GTAGCCCACGTCGTAGCAAAC-3′; F-IL6, 5′-AGAATTGCCATTGCACA-3′; R-IL6, 5′-CTCCCAACAGACCTGTCTATA-3′; β–actin-F, 5′-TAGCACCATGAAGATCAAGAT-3′; β–actin-R, 5′-CCGATCCACACAGAGTACTT-3′; IFNβ-F, 5′-CACGCTGCGTTCCTGCTGTG-3′, IFNβ-R, 5′-AGTCCGCCCTGTAGGTGAGGTT-3′; CXCL10-F, 5′-CGATGACGGGCCAGTGAGAATG-3′; CXCL10-R, 5′-TCAACACGTGGGCAGGATAGGCT-3′. mRNA expression was normalized to β-actin mRNA abundance.

ELISA. Cell culture supernatants were assayed for IFN-β by a custom ELISA, as previously described[42].

Luciferase assays. *Ifnb* promoter activation in HEK293 cells was measured as previously described[25].

Oligonucleotide pull-down and mass spectrometry. Cytoplasmic extracts were generated by disrupting PMA-treated THP1 cells in extraction buffer (10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, protease inhibitors) and pelleting nuclei by centrifugation at 1000 *g* for 10 min. The supernatant was further centrifuged at 15,700 *g* for 10min and pre-cleared with resin.

5 mg of total cytoplasmic protein was used for each nucleic acid affinity purification, together with 2 nmoles of ds or ss 5′ biotinylated VACV 70mer pre-coupled to 100 µl of Streptavidin UltraLink® Resin (Pierce; 50% slurry). Precipitated DNA-protein complexes were washed extensively in NP40-containing lysis buffer (0.5 % NP40, 100 mM NaCl, 5% glycerol, 0.5 mM EDTA and 50 mM HEPES pH7.5, 1 mM $NaVO_4$, protease inhibitors) and in 50 mM HEPES pH 7.5. Proteins were eluted by boiling in 1% SDS, resolved by SDS-PAGE, subjected to trypsin digestion and analyzed by liquid chromatography mass spectrometry.

Co-immunoprecipitation. PMA- and IFN-α-treated THP1s were either mock-transfected or transfected with 1 µg/ml VACV 70mer DNA for 4 h. Cells were lysed in NP40-containing lysis buffer (0.5% NP40, 100 mM NaCl, 10% glycerol, 1 mM EDTA and 50 mM HEPES pH7.5, 1 mM $NaVO_4$, protease inhibitors), pre-cleared and immunoprecipitated with STING antibody[9]. For co-immunoprecipitation with myc-STING, coding regions of human and mouse STING were amplified by PCR from full-length I.M.A.G.E. cDNA clones (IRATp970D0274D and IRAVp968F0688D, obtained from Imagenes) and cloned into the vector pCMV-myc (Clontech). STING constructs and empty vector were transfected into HEK293T cells using calcium phosphate. 24 h after transfection, cells were lysed in NP40-containing lysis buffer, and immunoprecipitated using immobilized myc antibody. Immunoprecipitates were washed in lysis buffer, and then incubated with pre-cleared lysates from PMA- and IFN-α-treated THP1 cells stimulated with 1 µg/ml 70mer. Immunoprecipitated proteins were washed in lysis buffer, eluted, resolved by SDS-PAGE and detected by immunoblotting.

RNA interference. siRNAs were chemically synthesised by Invitrogen (Stealth RNAi siRNAs) or by QIAGEN. Sequences were as follows: IFI16 siRNA, 5′-

GGUGCUGAACGCAACAGAAUCAUUU-3′ (Invitrogen); p204 siRNA 1, 5′-UUAGUUUACUGCCUGGUUCACACCU-3′ (Invitrogen); p204 siRNA 2, 5′-CGGAGAGGAAUAAAUUCAUTT-3′ (QIAGEN). p204 siRNA 2 was used in Fig. 5h, 5i and 7, together with the negative control siRNA, 5′-UUCUCCGAACGUGUCACGUTT-3′ (QIAGEN). In all other experiments, the Stealth RNAi negative control siRNA cat. no 12935-300 (Invitrogen) was used. 1 x 10^5 cells/well were seeded in 12-well plates and transfected with 12.5 pmol/ml (or 6.25 pmol/ml for THP-1 cells) siRNA using 1 µl/ml lipofectamine. RAW264.7 and THP1 cells were treated twice with siRNA on consecutive days, and grown for a further 48 h before stimulation. MEFs were treated once with siRNAs, and stimulated 48 h later.

HSV replication assay. RAW 264.7 cells were transfected with the HSV 60mer (2 µg/ml) 24 h prior to infected with HSV-1 (MOI 1). Supernatants were harvested 6, 24, and 48 h post infection, and virus was quantitated using standard plaque assay on Vero cells[26].

Confocal microscopy. Cells grown on glass coverslips were fixed in 4% paraformaldehyde, and permeabilized in 0.5% Triton X-100. Coverslips were pre-incubated in 5% BSA, 0.05% Tween20 in PBS or in 1%FCS in PBS and stained for 1-3 h with primary antibodies (1:100) at 25 °C, and for 1 h with Alexa488- and Alexa647-labeled secondary antibodies (1:500). Coverslips were mounted in MOWIOL 4-88 (Calbiochem) containing 1 µg/ml DAPI. Images were taken on an Olympus FV1000 scanning confocal microscope.

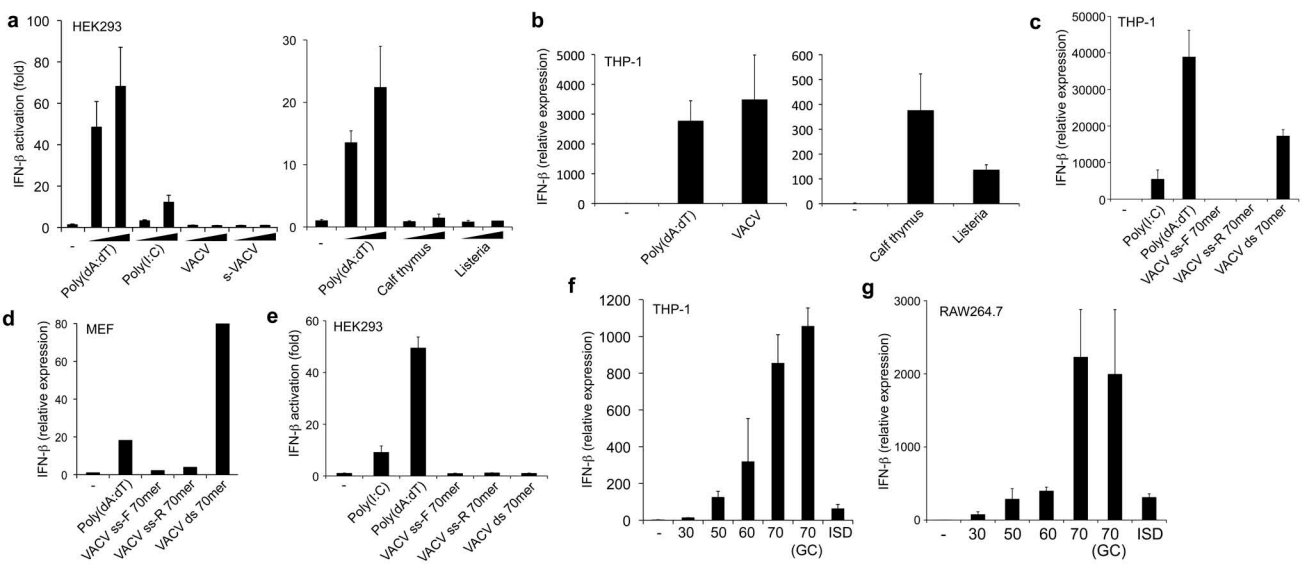
Statistics. Statistical significance was determined by Student's *t*-test.

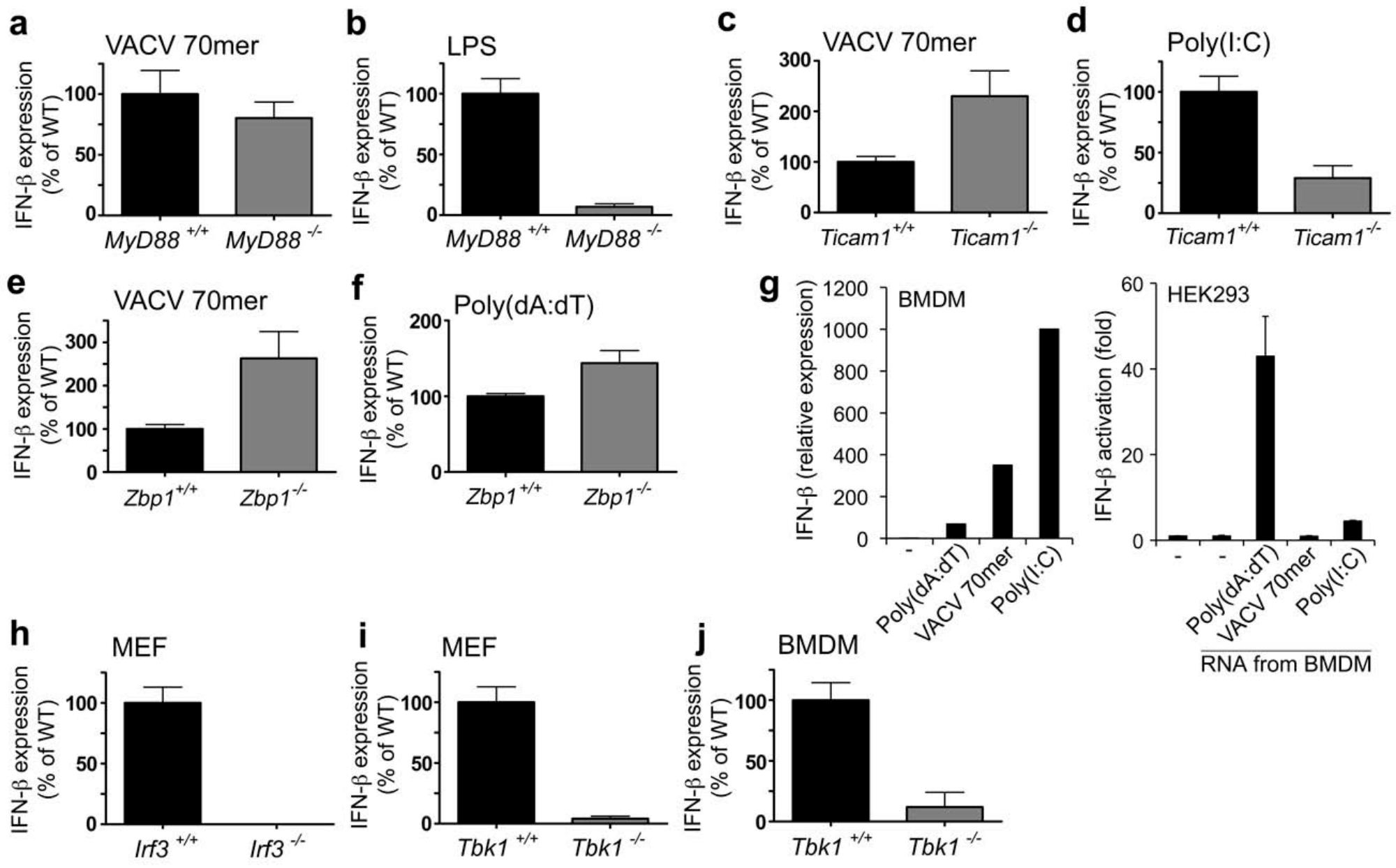
REFERENCES

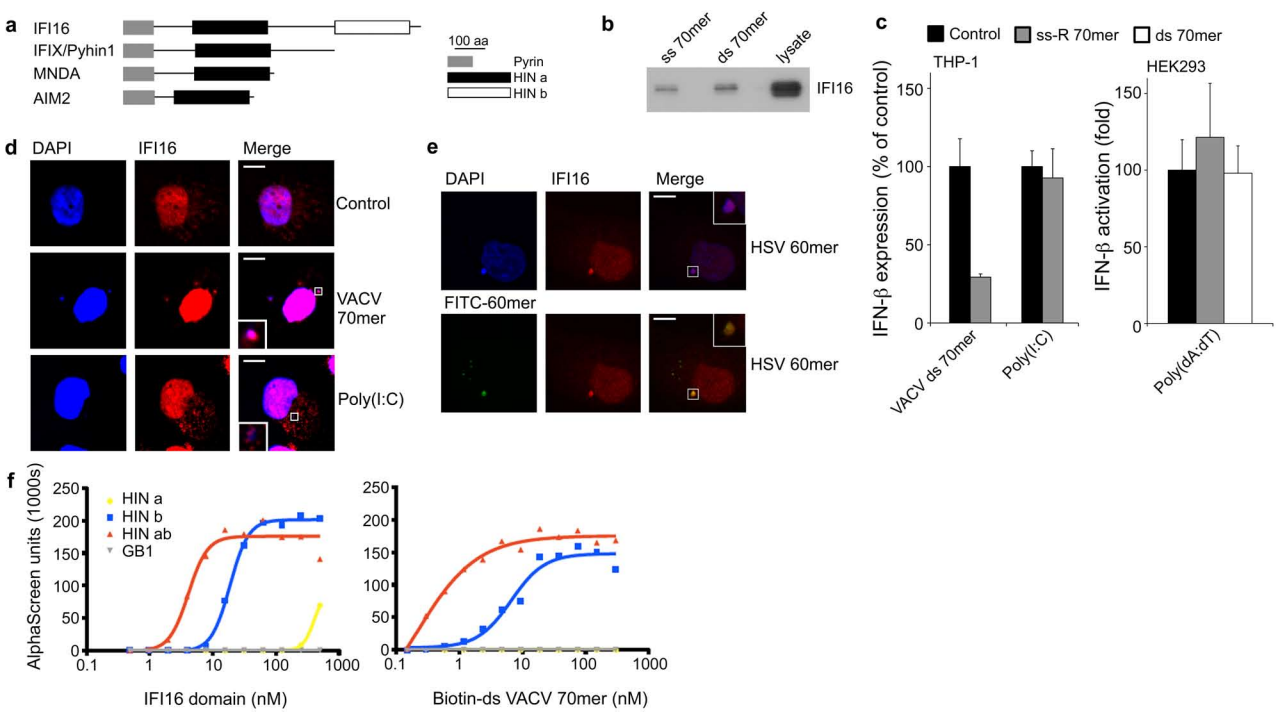
1. Medzhitov, R. Recognition of microorganisms and activation of the immune response. *Nature* **449**, 819–826 (2007).
2. Pichlmair, A. & Reis e Sousa, C. Innate recognition of viruses. *Immunity* **27**, 370–383 (2007).
3. Alexopoulou, L., Holt, A.C., Medzhitov, R. & Flavell, R.A. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature* **413**, 732–738 (2001).
4. O'Neill, L.A. & Bowie, A.G. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* **7**, 353–364 (2007).
5. Lund, J., Sato, A., Akira, S., Medzhitov, R. & Iwasaki, A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med* **198**, 513–520 (2003).
6. Hornung, V. & Latz, E. Intracellular DNA recognition. *Nat Rev Immunol* **10**, 123–130 (2010).
7. Stetson, D.B. & Medzhitov, R. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* **24**, 93–103 (2006).
8. Ishii, K.J. *et al.* A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol* **7**, 40–48 (2006).
9. Ishikawa, H. & Barber, G.N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**, 674–678 (2008).
10. Zhong, B. *et al.* The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* **29**, 538–550 (2008).
11. Sun, W. *et al.* ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. *Proc Natl Acad Sci USA* **106**, 8653–8658 (2009).
12. Ishikawa, H., Ma, Z. & Barber, G.N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788–792 (2009).
13. Saitoh, T. *et al.* Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. *Proc Natl Acad Sci USA* **106**, 20842–20846 (2009).
14. Takaoka, A. *et al.* DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* **448**, 501–505 (2007).
15. Lippmann, J. *et al.* IFNβ responses induced by intracellular bacteria or cytosolic DNA in different human cells do not require ZBP1 (DLM-1/DAI). *Cell Microbiol* **10**, 2579–2588 (2008).
16. Ishii, K.J. *et al.* TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* **451**, 725–729 (2008).
17. Chiu, Y.H., Macmillan, J.B. & Chen, Z.J. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* **138**, 576–591 (2009).
18. Ablasser, A. *et al.* RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat Immunol* **10**, 1065–1072 (2009).

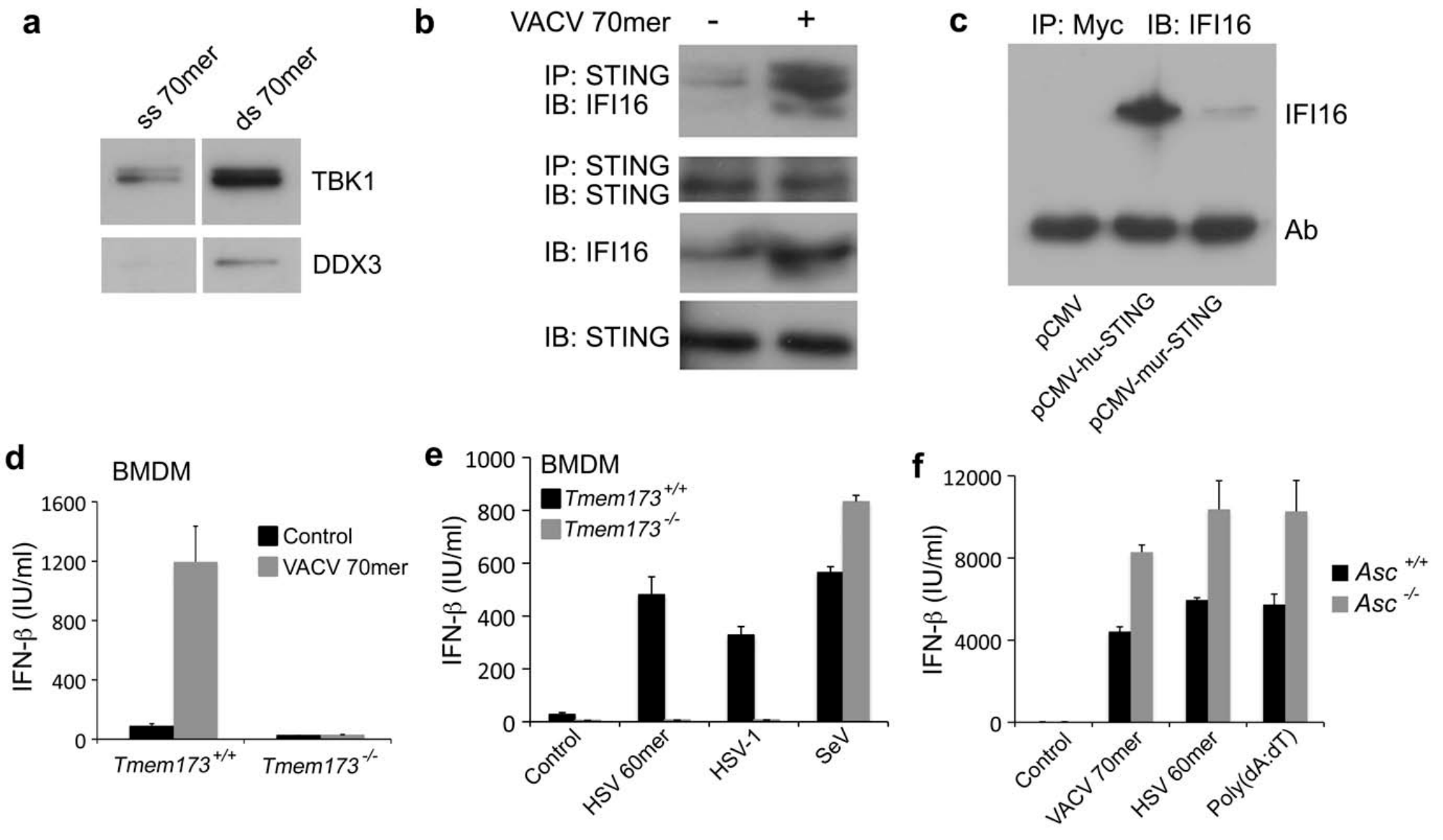
19. Hornung, V. *et al.* AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* **458**, 514–518 (2009).
20. Fernandes-Alnemri, T., Yu, J.W., Datta, P., Wu, J. & Alnemri, E.S. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* **458**, 509–513 (2009).
21. Roberts, T.L. *et al.* HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* **323**, 1057–1060 (2009).
22. Burckstummer, T. *et al.* An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat Immunol* **10**, 266–272 (2009).
23. Massung, R.F., Knight, J.C. & Esposito, J.J. Topography of variola smallpox virus inverted terminal repeats. *Virology* **211**, 350–355 (1995).
24. Yan, H. *et al.* RPA nucleic acid-binding properties of IFI16-HIN200. *Biochim Biophys Acta* **1784**, 1087–1097 (2008).
25. Schroder, M., Baran, M. & Bowie, A.G. Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKε-mediated IRF activation. *EMBO J* **27**, 2147–2157 (2008).
26. Rasmussen, S.B. *et al.* Type I interferon production during herpes simplex virus infection is controlled by cell-type-specific viral recognition through Toll-like receptor 9, the mitochondrial antiviral signaling protein pathway, and novel recognition systems. *J Virol* **81**, 13315–13324 (2007).
27. Luan, Y., Lengyel, P. & Liu, C.J. p204, a p200 family protein, as a multifunctional regulator of cell proliferation and differentiation. *Cytokine Growth Factor Rev* **19**, 357–369 (2008).
28. Liu, T., Rojas, A., Ye, Y. & Godzik, A. Homology modeling provides insights into the binding mode of the PAAD/DAPIN/pyrin domain, a fourth member of the CARD/DD/DED domain family. *Protein Sci* **12**, 1872–1881 (2003).
29. Poeck, H. *et al.* Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1β production. *Nat Immunol* **11**, 63–69 (2009).
30. Rothenfusser, S. *et al.* The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* **175**, 5260–5268 (2005).
31. Johnston, J.B. *et al.* A poxvirus-encoded pyrin domain protein interacts with ASC-1 to inhibit host inflammatory and apoptotic responses to infection. *Immunity* **23**, 587–598 (2005).
32. Rahman, M.M., Mohamed, M.R., Kim, M., Smallwood, S. & McFadden, G. Co-Regulation of NF-κB and Inflammasome-Mediated Inflammatory Responses by Myxoma Virus Pyrin Domain-Containing Protein M013. *PLoS Pathog* **5**, e1000635 (2009).
33. Cristea, I.M. *et al.* Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein. *J Virol* **84**, 7803–7814 (2010).
34. Monroe, K.M., McWhirter, S.M. & Vance, R.E. Induction of type I interferons by bacteria. *Cell Microbiol* **12**, 881–890 (2010).
35. Prantner, D., Darville, T. & Nagarajan, U.M. STING Is Critical for Induction of IFN-β during Chlamydia muridarum Infection. *J Immunol* **184**, 2551–2560 (2010).

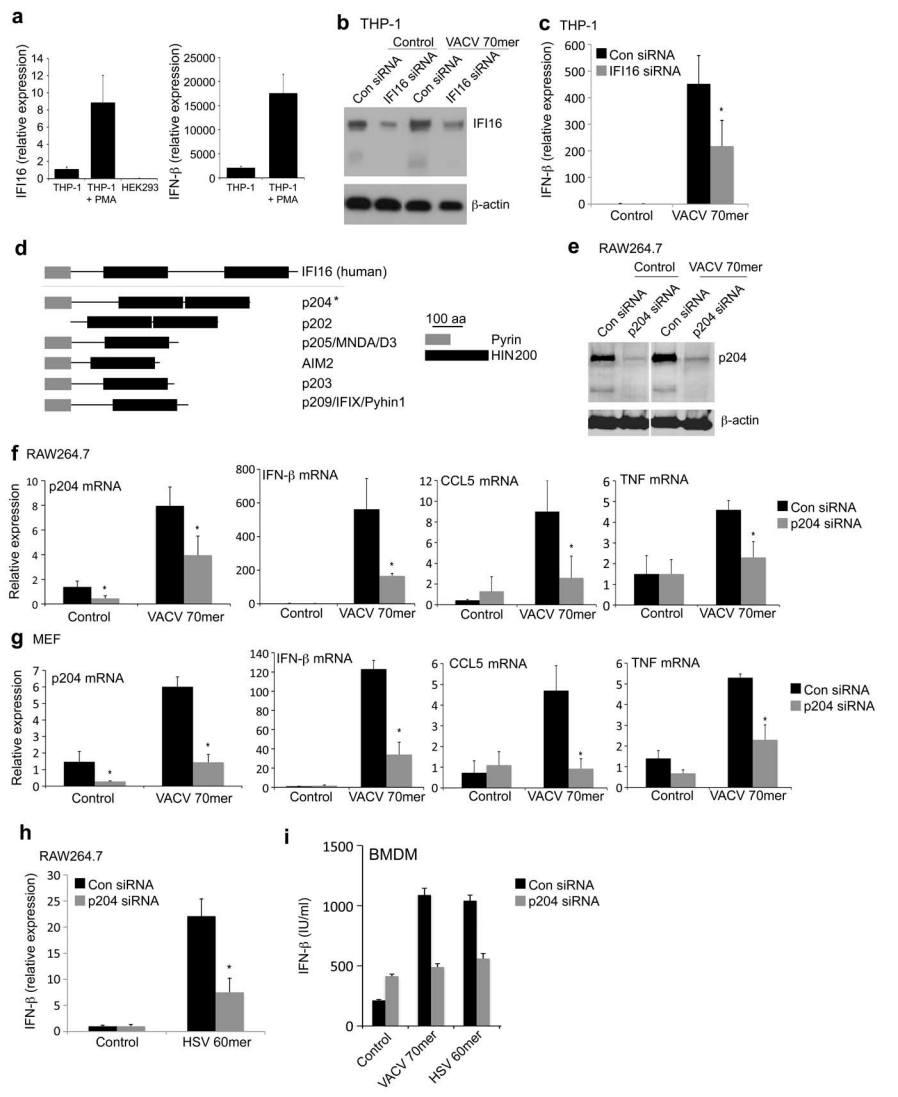
36. Mondini, M. *et al.* Role of the interferon-inducible gene IFI16 in the etiopathogenesis of systemic autoimmune disorders. *Ann N Y Acad Sci* **1110**, 47–56 (2007).
37. Choubey, D. & Panchanathan, R. Interferon-inducible Ifi200-family genes in systemic lupus erythematosus. *Immunology Letters* **119**, 32–41 (2008).
38. Kimkong, I., Avihingsanon, Y. & Hirankarn, N. Expression profile of HIN200 in leukocytes and renal biopsy of SLE patients by real-time RT-PCR. *Lupus* **18**, 1066–1072 (2009).
39. Roberson, S.M. & Walker, W.S. Immortalization of cloned mouse splenic macrophages with a retrovirus containing the v-raf/mil and v-myc oncogenes. *Cell Immunol* **116**, 341–351 (1988).
40. Hornung, V. *et al.* Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* **9**, 847–856 (2008).
41. Liu, C.J., Wang, H. & Lengyel, P. The interferon-inducible nucleolar p204 protein binds the ribosomal RNA-specific UBF1 transcription factor and inhibits ribosomal RNA transcription. *EMBO J* **18**, 2845–2854 (1999).
42. Roberts, Z.J. *et al.* The chemotherapeutic agent DMXAA potently and specifically activates the TBK1-IRF-3 signaling axis. *J Exp Med* **204**, 1559–1569 (2007).

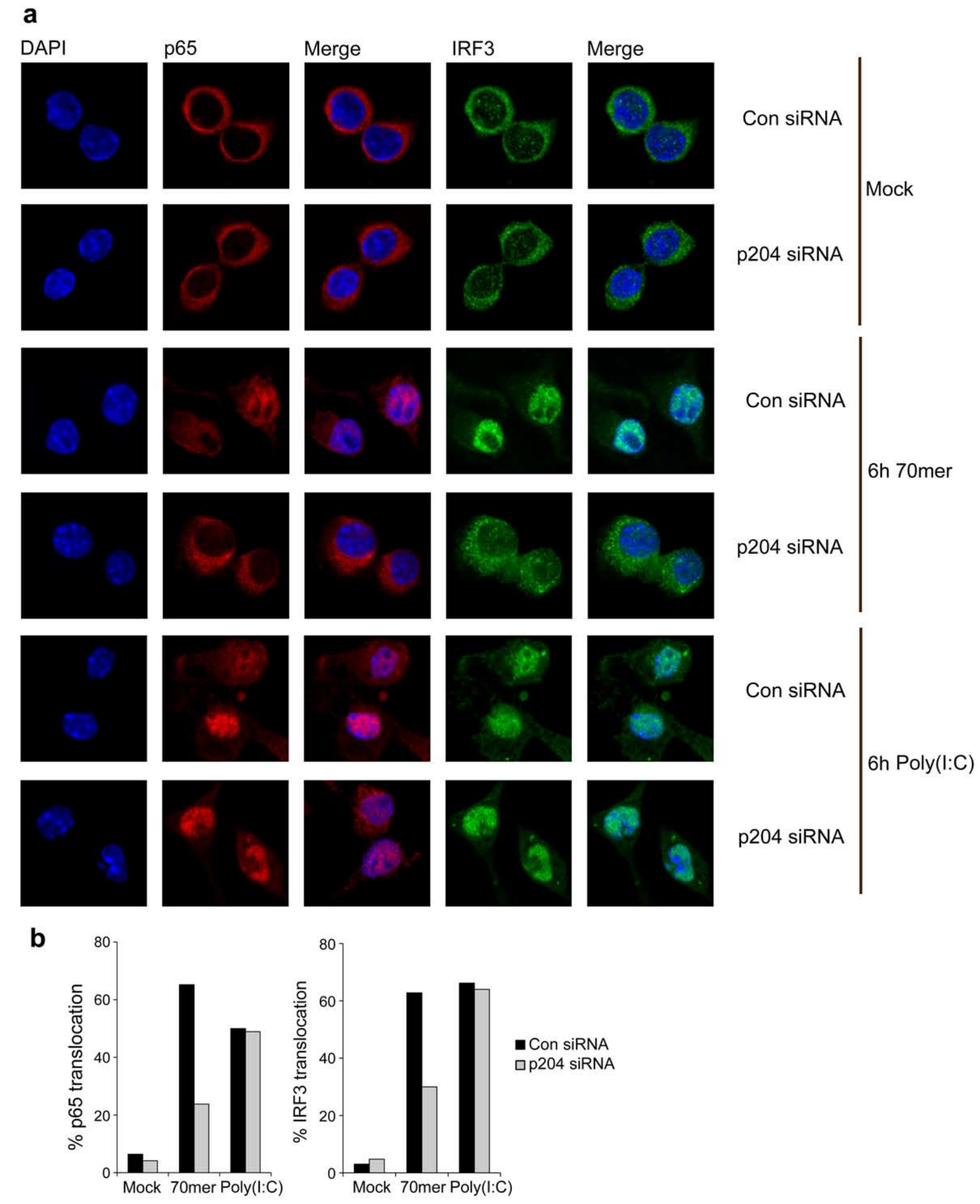

a
HEK293
IFN-β activation (fold)
100
80
60
40
20
0
30
20
10
0
-
Poly(dA:dT)
Poly(I:C)
VACV
s-VACV
Poly(dA:dT)
Calf thymus
Listeria
b
THP-1
IFN-β (relative expression)
5000
4000
3000
2000
1000
0
600
500
400
300
200
100
0
-
Poly(dA:dT)
VACV
Calf thymus
Listeria
c
THP-1
IFN-β (relative expression)
50000
40000
30000
20000
10000
0
-
Poly(I:C)
Poly(dA:dT)
VACV ss-F 70mer
VACV ss-R 70mer
VACV ds 70mer
d
MEF
IFN-β (relative expression)
80
60
40
20
0
-
Poly(dA:dT)
VACV ss-F 70mer
VACV ss-R 70mer
VACV ds 70mer
e
HEK293
IFN-β activation (fold)
60
40
20
0
-
Poly(I:C)
Poly(dA:dT)
VACV ss-F 70mer
VACV ss-R 70mer
VACV ds 70mer
f
THP-1
IFN-β (relative expression)
1200
1000
800
600
400
200
0
-
30
50
60
70
70
(GC)
ISD
g
RAW264.7
IFN-β (relative expression)
3000
2000
1000
0
-
30
50
60
70
70
(GC)
ISD

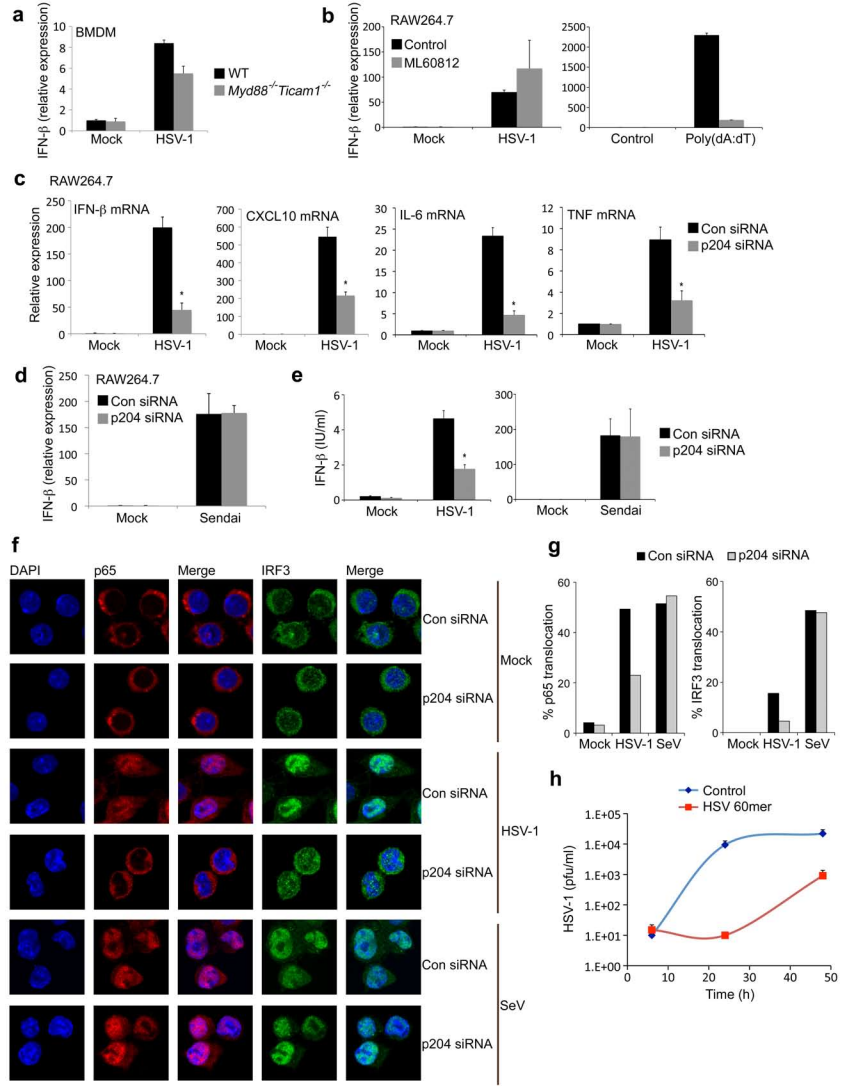

a
VACV 70mer
IFN-β expression (% of WT)
100
50
0
MyD88 +/+
MyD88 -/-
b
LPS
IFN-β expression (% of WT)
100
50
0
MyD88 +/+
MyD88 -/-
c
VACV 70mer
IFN-β expression (% of WT)
300
200
100
0
Ticam1+/+
Ticam1-/-
d
Poly(I:C)
IFN-β expression (% of WT)
100
50
0
Ticam1+/+
Ticam1-/-
e
VACV 70mer
IFN-β expression (% of WT)
300
200
100
0
Zbp1+/+
Zbp1-/-
f
Poly(dA:dT)
IFN-β expression (% of WT)
200
100
0
Zbp1+/+
Zbp1-/-
g
BMDM
IFN-β (relative expression)
1200
1000
800
600
400
200
0
-
Poly(dA:dT)
VACV 70mer
Poly(I:C)
HEK293
IFN-β activation (fold)
60
40
20
0
-
-
Poly(dA:dT)
VACV 70mer
Poly(I:C)
RNA from BMDM
h
MEF
IFN-β expression (% of WT)
100
50
0
Irf3 +/+
Irf3 -/-
i
MEF
IFN-β expression (% of WT)
100
50
0
Tbk1 +/+
Tbk1 -/-
j
BMDM
IFN-β expression (% of WT)
100
50
0
Tbk1 +/+
Tbk1 -/-


a
IFI16
IFIX/Pyhin1
MNDA
AIM2
100 aa
Pyrin
HIN a
HIN b
b
ss 70mer
ds 70mer
lysate
IFI16
c
Control
ss-R 70mer
ds 70mer
THP-1
HEK293
IFN-β expression (% of control)
IFN-β activation (fold)
150
100
50
0
VACV ds 70mer
Poly(I:C)
Poly(dA:dT)
d
DAPI
IFI16
Merge
Control
VACV 70mer
Poly(I:C)
e
DAPI
IFI16
Merge
HSV 60mer
FITC-60mer
HSV 60mer
f
AlphaScreen units (1000s)
HIN a
HIN b
HIN ab
GB1
250
200
150
100
50
0
0.1
1
10
100
1000
IFI16 domain (nM)
Biotin-ds VACV 70mer (nM)


a
ss 70mer
ds 70mer
TBK1
DDX3
b
VACV 70mer - +
IP: STING
IB: IFI16
IP: STING
IB: STING
IB: IFI16
IB: STING
c
IP: Myc IB: IFI16
IFI16
Ab
pCMV
pCMV-hu-STING
pCMV-mur-STING
d
BMDM
IFN-β (IU/ml)
1600
1200
800
400
0
Control
VACV 70mer
Tmem173+/+
Tmem173-/-
e
BMDM
IFN-β (IU/ml)
1000
800
600
400
200
0
Tmem173+/+
Tmem173-/-
Control
HSV 60mer
HSV-1
SeV
f
IFN-β (IU/ml)
12000
8000
4000
0
Asc+/+
Asc-/-
Control
VACV 70mer
HSV 60mer
Poly(dA:dT)


a
IFI16 (relative expression)
THP-1
THP-1 + PMA
HEK293
IFN-β (relative expression)
THP-1
THP-1 + PMA
b
THP-1
Control
VACV 70mer
Con siRNA
IFI16 siRNA
Con siRNA
IFI16 siRNA
IFI16
β-actin
c
THP-1
Con siRNA
IFI16 siRNA
IFN-β (relative expression)
Control
VACV 70mer
d
IFI16 (human)
p204*
p202
p205/MNDA/D3
AIM2
p203
p209/IFIX/Pyhin1
100 aa
Pyrin
HIN200
e
RAW264.7
Control
VACV 70mer
Con siRNA
p204 siRNA
Con siRNA
p204 siRNA
p204
β-actin
f
RAW264.7
p204 mRNA
IFN-β mRNA
CCL5 mRNA
TNF mRNA
Relative expression
Con siRNA
p204 siRNA
Control
VACV 70mer
g
MEF
p204 mRNA
IFN-β mRNA
CCL5 mRNA
TNF mRNA
Relative expression
Con siRNA
p204 siRNA
Control
VACV 70mer
h
RAW264.7
Con siRNA
p204 siRNA
IFN-β (relative expression)
Control
HSV 60mer
i
BMDM
Con siRNA
p204 siRNA
IFN-β (IU/ml)
Control
VACV 70mer
HSV 60mer


a
DAPI
p65
Merge
IRF3
Merge
Con siRNA
p204 siRNA
Mock
Con siRNA
p204 siRNA
6h 70mer
Con siRNA
p204 siRNA
6h Poly(I:C)
b
% p65 translocation
% IRF3 translocation
80
60
40
20
0
Mock
70mer
Poly(I:C)
Con siRNA
p204 siRNA


a
BMDM
IFN-β (relative expression)
WT
Myd88-/-Ticam1-/-
Mock
HSV-1
b
RAW264.7
Control
ML60812
IFN-β (relative expression)
Mock
HSV-1
Control
Poly(dA:dT)
c
RAW264.7
Relative expression
IFN-β mRNA
CXCL10 mRNA
IL-6 mRNA
TNF mRNA
Con siRNA
p204 siRNA
Mock
HSV-1
d
RAW264.7
Con siRNA
p204 siRNA
IFN-β (relative expression)
Mock
Sendai
e
IFN-β (IU/ml)
Con siRNA
p204 siRNA
Mock
HSV-1
Mock
Sendai
f
DAPI
p65
Merge
IRF3
Merge
Con siRNA
p204 siRNA
Mock
HSV-1
SeV
g
Con siRNA
p204 siRNA
% p65 translocation
% IRF3 translocation
Mock
HSV-1
SeV
h
Control
HSV 60mer
HSV-1 (pfu/ml)
Time (h)