nature immunology

IFI16 is an innate immune sensor for intracellular DNA

Leonie Unterholzner[1,6], Sinead E Keating[1,6], 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]

The detection of intracellular microbial DNA is critical to appropriate innate immune responses; however, knowledge of how such DNA is sensed is limited. Here we identify IFI16, a PYHIN protein, as an intracellular DNA sensor that mediates the induction of interferon-β (IFN-β). 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. Lowering the expression of IFI16 or its mouse ortholog p204 by RNA-mediated interference inhibited gene induction and activation of the transcription factors IRF3 and NF-κB induced by DNA and herpes simplex virus type 1 (HSV-1). IFI16 (p204) is the first PYHIN protein to our knowledge shown to be involved in IFN-β induction. Thus, the PYHIN proteins IFI16 and AIM2 form a new family of innate DNA sensors we call 'AIM2-like receptors' (ALRs).

An effective immune response to viruses is dependent on the induction of host cytokines and type I interferons such as interferon-β (IFN-β (A001237))[1]. This occurs mainly 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 RNA helicase RIG-I–like receptors (RLRs) sense viral RNA, which leads to the induction of IFN-β through activation of downstream signaling pathways[2]. TLR3 senses double-stranded RNA (dsRNA), which leads to recruitment of the TLR adaptor protein TRIF (also known as TICAM-1); this then triggers activation of TBK1 (A001597) and IKKβ, kinases that phosphorylate and activate the transcription factors IRF3 and NF-κB respectively[3,4]. Thereafter, IRF3 and NF-κB mediate an antiviral gene-induction program that includes the production of IFN-β. TLR7 and TLR8 both bind viral single-stranded RNA, which leads to recruitment of the adaptor MyD88, and like TLR3, they activate IKKβ and NF-κB, whereas in contrast to TLR3, they activate IKKα, which leads to IRF7 activation and induction of the type I interferon IFN-α[2]. Cytosolic RNA is detected by the RLRs RIG-I and Mda5, which via the adaptor protein IPS-1 (also known as MAVS, Cardif or VISA) turn on a signaling pathway similar to that of TLR3 in that IFN-β induction occurs via TBK1-mediated IRF3 activation[2].

In contrast to the situation for viral RNA described above, knowledge of the intracellular DNA sensors that mediate the induction of IFN-α, IFN-β and cytokines 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, which leads to IFN-α induction via MyD88 and IKKα[5]. However, it is known that exogenous dsDNA introduced into the cytoplasm, as would happen during infection by a DNA virus, leads to a potent innate immune response in many cell types, the hallmark of which is the induction of IFN-β[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 double-stranded 45–residue oligonucleotide called interferon-stimulatory DNA (ISD). In all these cases, the induction of IFN-β relies on signaling though TBK1 and IRF3 (refs. 7,8). In addition to TBK1 and IRF3, a further critical downstream component of the response to intracellular DNA has been identified as STING (also called MITA, ERIS or TMEM173)[9–11]. STING is required for IFN-β induction by poly(dA:dT), ISD and herpes simplex virus type 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, it relocalizes from the endoplasmic reticulum to cytoplasmic foci containing TBK1 (refs. 12,13). Although cytoplasmic IFN-β responses to intracellular DNA 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 been identified: DAI (DNA-dependent activator of IRFs; also called ZBP1) and RNA polymerase III. DAI was discovered on the basis of the ability of poly(dA:dT) to induce IFN-β when transfected into cells[14]. However, the role of DAI is very cell type specific, and DAI-deficient MEFs and monocytes responded

[1]School of Biochemistry and Immunology, Trinity College Dublin, Dublin, 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, Maryland, USA. [5]Institute of Clinical Chemistry and Pharmacology, University of Bonn, Bonn, Germany. [6]These authors contributed equally to this work. Correspondence should be addressed to A.G.B. (agbowie@tcd.ie).

Received 6 July; accepted 9 August; published online 3 October 2010; doi:10.1038/ni.1932

normally to poly(dA:dT)[15,16]. RNA polymerase III also responds to transfected poly(dA:dT), which it transcribes 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 many cell types is not accounted for by DAI and RNA polymerase 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. When transfected into cells, this VACV motif induced IFN-β in a manner independent of TLRs, DAI and RNA polymerase III but dependent on STING, TBK1 and IRF3. 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 directly bound to the IFN-β-stimulating viral DNA and recruited STING after stimulation of cells with transfected DNA. Small interfering RNA (siRNA) targeting IFI16 or its mouse ortholog p204 inhibited DNA-induced but not RNA-induced activation of IRF3 and NF-κB, as well as IFN-β induction. Notably, responses to a DNA virus, but not to an RNA virus, were also dependent on p204, in that transcription factor activation and gene induction stimulated by HSV-1, but not those stimulated by Sendai virus, were impaired by p204-specific siRNA. Notably, AIM2 (A004152), another PYHIN protein, is a sensor for cytosolic DNA for the signaling pathway that activates caspase-1, leading to the release of interleukin 1β[19–22]. Thus, we propose that the PHYIN proteins represent a new family of innate DNA sensors we call '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 various types of exogenous DNA to stimulate cytosolic DNA-sensing pathways in human cells. We transfected cells with poly(dA:dT), viral DNA from VACV, mammalian DNA or bacterial DNA and measured induction of the promoter of the gene encoding IFN-β (*IFNB*). In HEK293 human embryonic kidney cells, which have been shown to have a functional RNA polymerase 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 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 sequence 70 base pairs in length that was conserved in various poxviral genomes such as VACV (VACV 70mer); it was located in the inverted terminal repeat region and was often repeated multiple times[23] (**Supplementary Fig. 1**). When transfected into cells, this dsDNA 70–base pair motif (dsVACV 70mer) induced IFN-β in THP-1 cells (**Fig. 1c** and **Supplementary Fig. 2a**) and in MEFs (**Fig. 1d**), but it was stimulatory only as duplex DNA and not in the single-stranded form (ssVACV 70mer; **Fig. 1c**,**d**). Furthermore, dsVACV 70mer also induced IFN-β in human peripheral blood mononuclear cells, immortalized mouse bone marrow–derived macrophages (BMDMs) and mouse bone marrow–derived dendritic cells (**Supplementary Fig. 2**), but not in HEK293 cells (**Fig. 1e**).

We next assessed whether the sequence of dsVACV 70mer and/or its length were critical to the IFN-β response. The response to dsVACV 70mer was independent of AT content but was dependent

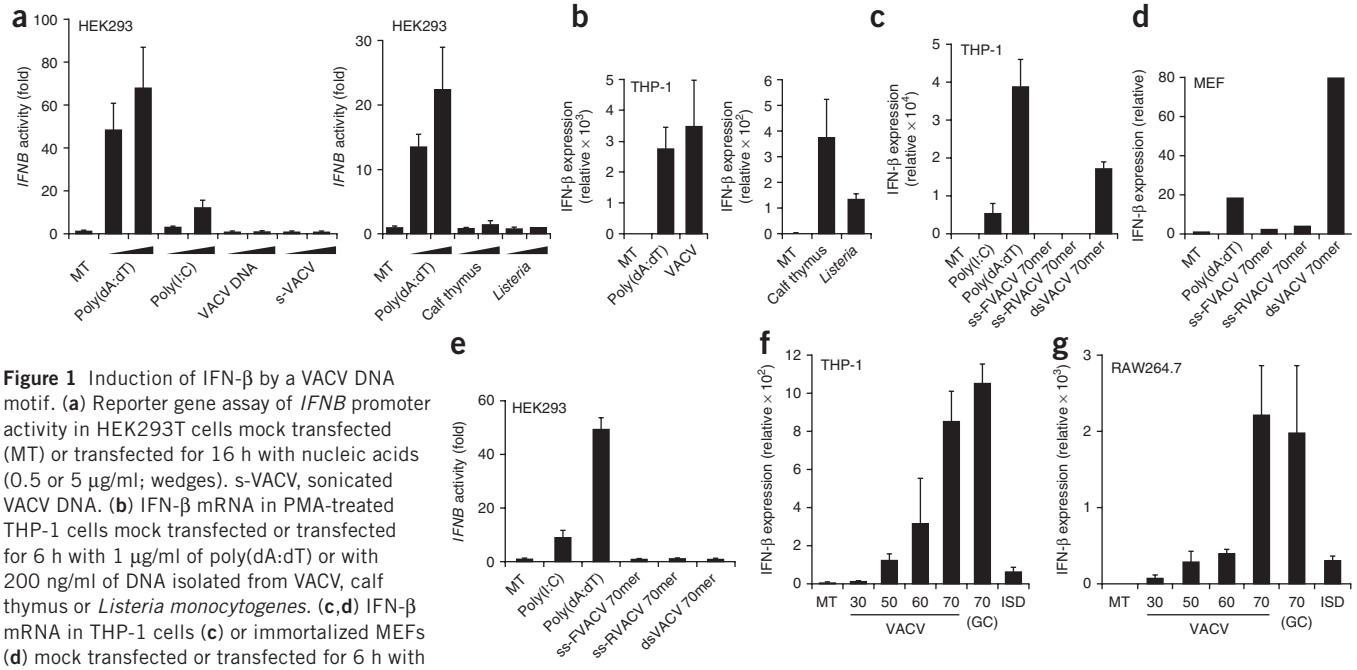

Figure 1 Induction of IFN-β by a VACV DNA motif. (**a**) Reporter gene assay of *IFNB* promoter activity in HEK293T cells mock transfected (MT) or transfected for 16 h with nucleic acids (0.5 or 5 μg/ml; wedges). s-VACV, sonicated VACV DNA. (**b**) IFN-β mRNA in PMA-treated THP-1 cells mock transfected or transfected for 6 h with 1 μg/ml of poly(dA:dT) or with 200 ng/ml of DNA isolated from VACV, calf thymus or *Listeria monocytogenes*. (**c**,**d**) IFN-β mRNA in THP-1 cells (**c**) or immortalized MEFs (**d**) mock transfected or transfected for 6 h with nucleic acids at a concentration of 1 μg/ml (**c**) or 5 μg/ml (**d**). ss-FVACV 70mer, forward strand only of dsVACV 70mer; ss-RVACV 70mer, reverse strand only of dsVACV 70mer. (**e**) *IFNB* promoter activity in HEK293T cells mock transfected or transfected for 16 h with nucleic acids at a concentration of 5 μg/ml. (**f**,**g**) IFN-β mRNA in THP-1 cells (**f**) or RAW264.7 cells (**g**) mock transfected or transfected for 6 h with DNA oligonucleotides or sequences of various length (horizontal axis; in base pairs) derived from VACV 70mer (sequences, **Supplementary Methods**), GC-rich VACV 70mer (70 (GC)) or ISD, each at a concentration of 1 μg/ml. Results for mRNA and promoter activity are presented relative to those of mock-transfected cells. Data are from one experiment representative of two (error bars, s.d.).

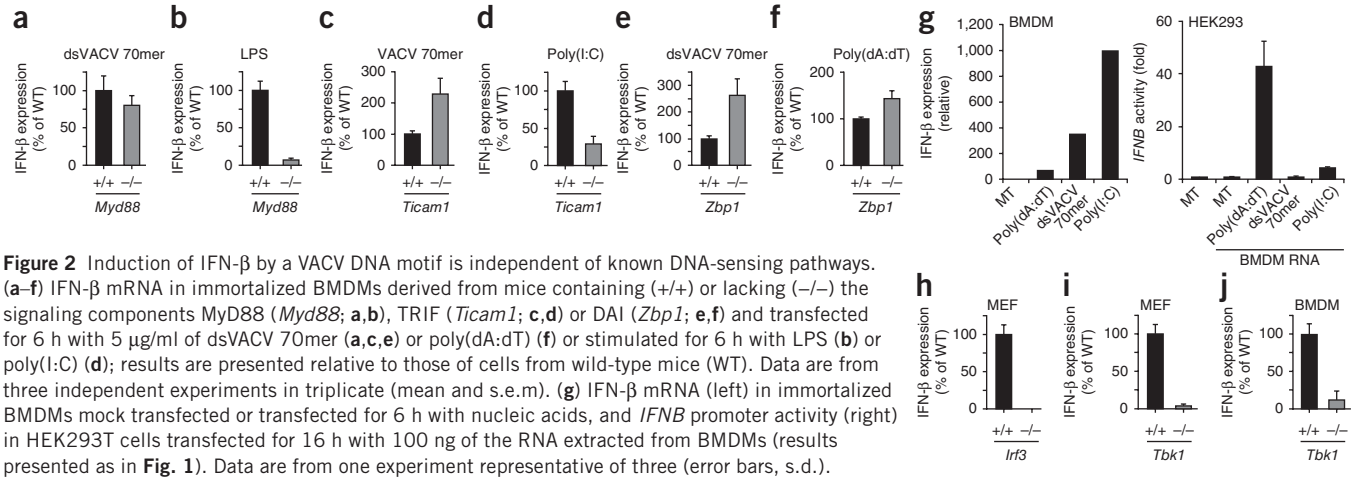

Figure 2 Induction of IFN-β by a VACV DNA motif is independent of known DNA-sensing pathways. (**a**–**f**) IFN-β mRNA in immortalized BMDMs derived from mice containing (+/+) or lacking (−/−) the signaling components MyD88 (*Myd88*; **a**,**b**), TRIF (*Ticam1*; **c**,**d**) or DAI (*Zbp1*; **e**,**f**) and transfected for 6 h with 5 μg/ml of dsVACV 70mer (**a**,**c**,**e**) or poly(dA:dT) (**f**) or stimulated for 6 h with LPS (**b**) or poly(I:C) (**d**); results are presented relative to those of cells from wild-type mice (WT). Data are from three independent experiments in triplicate (mean and s.e.m). (**g**) IFN-β mRNA (left) in immortalized BMDMs mock transfected or transfected for 6 h with nucleic acids, and *IFNB* promoter activity (right) in HEK293T cells transfected for 16 h with 100 ng of the RNA extracted from BMDMs (results presented as in **Fig. 1**). Data are from one experiment representative of three (error bars, s.d.). (**h**–**j**) IFN-β mRNA in immortalized BMDMs or MEFs derived from mice lacking the signaling component IRF3 (*Irf3*; **h**) or TBK1 (*Tbk1*; **i**,**j**) and transfected for 6 h with dsVACV 70mer (5 μg/ml); results are presented as in **a**–**f**. Data are from three independent experiments in triplicate (mean and s.e.m).

on length, as in either human or mouse monocytic cells, changing the AT content from 67% to 10% did not affect the response, whereas a decrease of even 10 base pairs in length impaired IFN-β induction (**Fig. 1f**,**g**). Consistent with the finding that the cytosolic IFN-β response was length dependent but sequence independent, the previously described 45–base pair ISD[7] gave a similar IFN-β response to a 50–base pair oligonucleotide derived from dsVACV 70mer (**Fig. 1f**,**g**). Furthermore, an unrelated 60–base pair dsDNA oligonucleotide derived from the HSV-1 genome (HSV 60mer) also induced IFN-β (**Supplementary Fig. 3a**). We screened many different oligonucleotides derived from the HSV-1 genome for IFN-β–inducing capacity and selected HSV 60mer as having the greatest capacity (data not shown).

IFN-β induction via a previously unknown intracellular DNA sensor

We next investigated the role of known DNA sensors in the IFN-β response to dsVACV 70mer and HSV 60mer (**Fig. 2**). The response to dsVACV 70mer in BMDMs lacking the TLR signaling adaptors MyD88 or TRIF (TICAM-1) was not lower than that in wild-type control cells (**Fig. 2a**,**c**), whereas TLR-induced IFN-β expression was inhibited in the mutant cells (**Fig. 2b**,**d**). Analogously, the response to HSV 60mer was unimpaired in macrophages lacking both MyD88 and TRIF (**Supplementary Fig. 3a**). BMDMs lacking DAI (ZBP1) also responded normally to dsVACV 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 that, when we extracted RNA from poly(dA:dT)-treated mouse macrophages, MEFs or human THP-1 cells and transfected it into HEK293 cells, we found that this RNA induced expression of the *IFNB* promoter (**Fig. 2g** and **Supplementary Fig. 4a**,**b**). In contrast, *IFNB* was not expressed in HEK293 cells transfected with RNA extracted from cells stimulated with dsVACV 70mer (**Fig. 2g** and **Supplementary Fig. 4a**,**b**), which indicated that unlike poly(dA:dT), dsVACV 70mer was not transcribed into immune-stimulatory RNA. HSV 60mer also induced IFN-β independently of RNA polymerase III, as IFN-β induction was not prevented by the RNA polymerase III inhibitor ML60812 (**Supplementary Fig. 3b**). Consistent with that result, there was no response to HSV 60mer in HEK293 cells in which the RNA polymerase III pathway was operational (**Supplementary Fig. 3c**).

All cytosolic DNA-sensing pathways that induce IFN-β described thus far, including those for which 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 response to dsVACV 70mer in MEFs was abolished in the absence of IRF3 (**Fig. 2h**), whereas in either MEFs or BMDMs lacking TBK1, the response to VACV 70mer was strongly impaired (**Fig. 2i**,**j**). Hence, IFN-β induction by dsVACV 70mer was independent of TLRs, DAI and RNA polymerase III but was dependent on TBK1 and IRF3.

IFI16 is a candidate DNA sensor for viral DNA

As the IFN-β response to dsVACV 70mer was not mediated by any of the known DNA-sensing pathways, we designed a screen to isolate previously unknown cytoplasmic DNA sensors. We coupled biotinylated ssVACV 70mer or dsVACV 70mer to streptavidin beads to affinity-purify DNA-binding proteins from cytosolic extracts of THP-1 cells. We used mass spectrometry to identify proteins that interacted with ssVACV 70mer or dsVACV 70mer. Among the proteins identified was IFI16, a member of the PYHIN protein family that contains a pyrin domain and two DNA-binding HIN domains, called 'HINa' and 'HINb' here (**Fig. 3a**). Notably, AIM2, another PYHIN protein, has been shown to be a receptor for cytosolic DNA that regulates activation of caspase-1, which leads to the release of interleukin 1β[19–22]. However, no PYHIN protein has yet been linked to the mediation of an IFN-β response to DNA.

In THP-1 cells, cytosolic IFI16 was detectable by immunoblot analysis in a complex with immobilized ssVACV 70mer or dsVACV 70mer (**Fig. 3b**), consistent with the published finding that its HINa domain binds both ssDNA and dsDNA[24]. Given that 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 our hypothesis, ssVACV 70mer acted as an antagonist of dsVACV 70mer–induced IFN-β expression when transfected together with dsVACV 70mer into THP-1 cells, while not affecting the response to poly(I:C) (**Fig. 3c**). Furthermore, induction of the *IFNB* promoter by poly(dA:dT) in HEK293 cells was also unaffected by the transfection of ssVACV 70mer (**Fig. 3c**). IFI16 has been shown to be localized mainly to the nucleus when overexpressed in HEK293 cells[19], a result we confirmed (data not shown). However in THP-1 cells, endogenous IFI16 was isolated from the cytoplasm in association with ssVACV 70mer or dsVACV 70mer (**Fig. 3b**), and although it was predominantly nuclear, it was distinctly visible in the cytoplasm

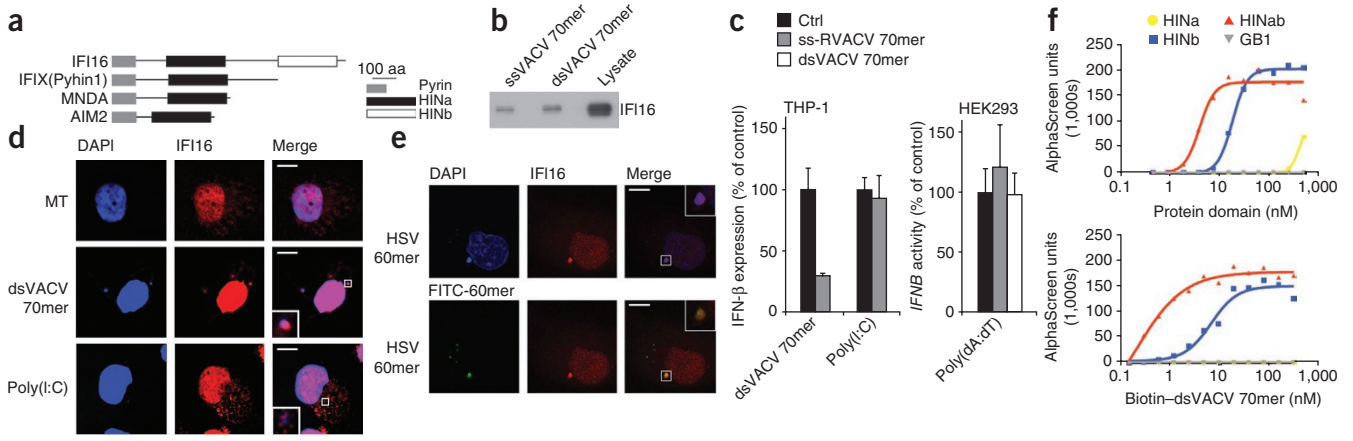

Figure 3 IFI16 binds to immunostimulatory viral DNA. (**a**) Human PYHIN proteins (left), including the pyrin, HINa and HINb domains (scale bar (right), size in amino acids (aa)). (**b**) Immunoblot analysis of IFI16 among proteins precipitated from cytoplasmic extracts of PMA-treated THP-1 cells incubated with biotinylated ssVACV 70mer or dsVACV 70mer immobilized on streptavidin beads. (**c**) IFN-β mRNA (left) in THP-1 cells transfected for 6 h with 1 μg/ml of dsVACV 70mer or poly(I:C) alone (control (Ctrl)) or in the presence of 1 μg/ml of the reverse strand only of dsVACV 70mer (ss-RVACV 70mer), and *IFNB* promoter activity (right) in HEK293T cells transfected for 16 h with 50 ng/ml of poly(dA:dT) alone (Ctrl) or together with 50 ng/ml of the reverse strand only of dsVACV 70mer (ss-RVACV 70mer) or dsVACV 70mer (results presented relative to control). (**d**) Microscopy of PMA- and IFN-α-treated THP-1 cells grown on coverslips and mock transfected or transfected for 1 h with 2.5 μg/ml of dsVACV 70mer or poly(I:C), then fixed and stained with antibody to IFI16 (red) and with the DNA-intercalating dye DAPI (blue), for visualization of DNA and poly(I:C). (**e**) Microscopy of PMA-treated THP-1 cells transfected for 3 h with fluorescein isothiocyanate–labeled HSV 60mer (FITC-60mer; green), then fixed and stained for IFI16 (red) or with DAPI (blue; DNA). Inset (**d**,**e**), magnification of area outlined in main image. Scale bar (**d**,**e**), 10 μm. (**f**) AlphaScreen assessment of the binding of dsVACV 70mer to the IFI16 domains HINa, HINb or HINab (both HINa and HINb) or a GB1 expression tag: top, 30 nM DNA with increasing concentrations of HIN or GB1; bottom, 30 nM HIN or GB1 with increasing concentrations of biotin-labeled dsVACV 70mer. Data are from one experiment representative of three (mean and s.d. in **c**).

by immunofluorescence (**Fig. 3d**). Further, MitoTracker costaining indicated that IFI16 might partially localize to mitochondria (**Supplementary Fig. 5a**). Notably, in cells stained with the DNA-intercalating dye DAPI for the detection of nucleic acid, dsVACV 70mer localized together with endogenous IFI16 in the cytoplasm, but poly(I:C) did not (**Fig. 3d**). Furthermore, fluorescein isothiocyanate–labeled HSV 60mer also localized together with endogenous cytoplasmic IFI16 (**Fig. 3e**). To determine whether IFI16 was able to directly bind the viral DNAs *in vitro*, we expressed the HIN domains of IFI16 in *Escherichia coli* and purified them (**Supplementary Methods** and **Supplementary Fig. 6**). We then used an AlphaScreen to assess *in vitro* binding between viral DNA and purified IFI16 HIN domains. Biotinylated dsVACV 70mer bound cooperatively to HINb or to the region of IFI16 containing both HINa and HINb (HINab), as indicated by the sigmoidal binding curve for both (**Fig. 3f**). The affinity of dsVACV 70mer was higher for HINab than for HINb alone, whereas dsVACV 70mer did not bind HINa alone (**Fig. 3f**). We obtained similar results for HSV 60mer (**Supplementary Fig. 3d**). The colocalization 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 that mediates IFN-β induction.

STING interacts with IFI16

We next assessed whether IFI16 interacted with any of the downstream signaling components known to be involved in IFN-β induction. As with IFI16, immobilized dsVACV 70mer complexes were enriched for both cytosolic TBK1 and DDX3 (which has a role in the activation of IRF3 by TBK1; ref. 25) compared with their presence in ssVACV 70mer complexes (**Fig. 4a**). STING (TMEM173) is a signaling protein

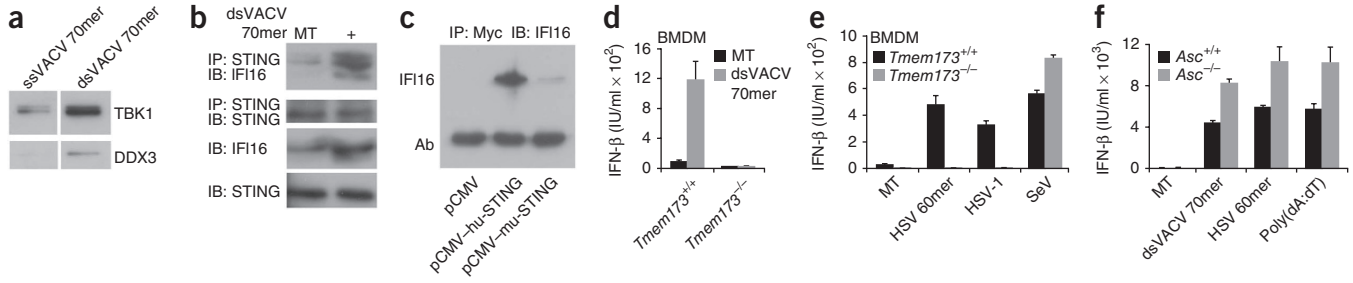

Figure 4 Role for STING in IFI16-mediated IFN-β induction. (**a**) Immunoblot analysis of TBK1 and DDX3 among proteins precipitated from cytoplasmic extracts of PMA-treated THP-1 cells incubated with biotinylated ssVACV 70mer or dsVACV 70mer immobilized on streptavidin beads. (**b**) Immunoassay of THP-1 cells pretreated with PMA and IFN-α and mock transfected or transfected for 4 h with 1 μg/ml of dsVACV 70mer (+); lysates were immunoprecipitated (IP) with antibody to STING and then analyzed by immunoblot (IB) for IFI16 and STING. (**c**) Immunoassay of the association of IFI16, from lysates of DNA-treated THP-1 cells, with Myc-tagged human (hu) or mouse (mu) STING overexpressed in HEK293 cells and immobilized on sepharose beads; coimmunoprecipitated IFI16 was detected by immunoblot analysis. pCMV, vector; Ab, antibody heavy chain. (**d**,**e**) Release of IFN-β protein from BMDMs containing (*Tmem173*$^{+/+}$) or lacking (*Tmem173*$^{-/-}$) STING, mock transfected or transfected for 18 h with either dsVACV 70mer or HSV 60mer or mock transfected or infected for 18 h with HSV-1 or Sendai virus (SeV). (**f**) Secretion of IFN-β protein from immortalized *Asc*$^{-/-}$ BMDMs mock transfected or transfected for 18 h with DNA. Data are from one experiment representative of two (mean and s.d. in **d**–**f**).

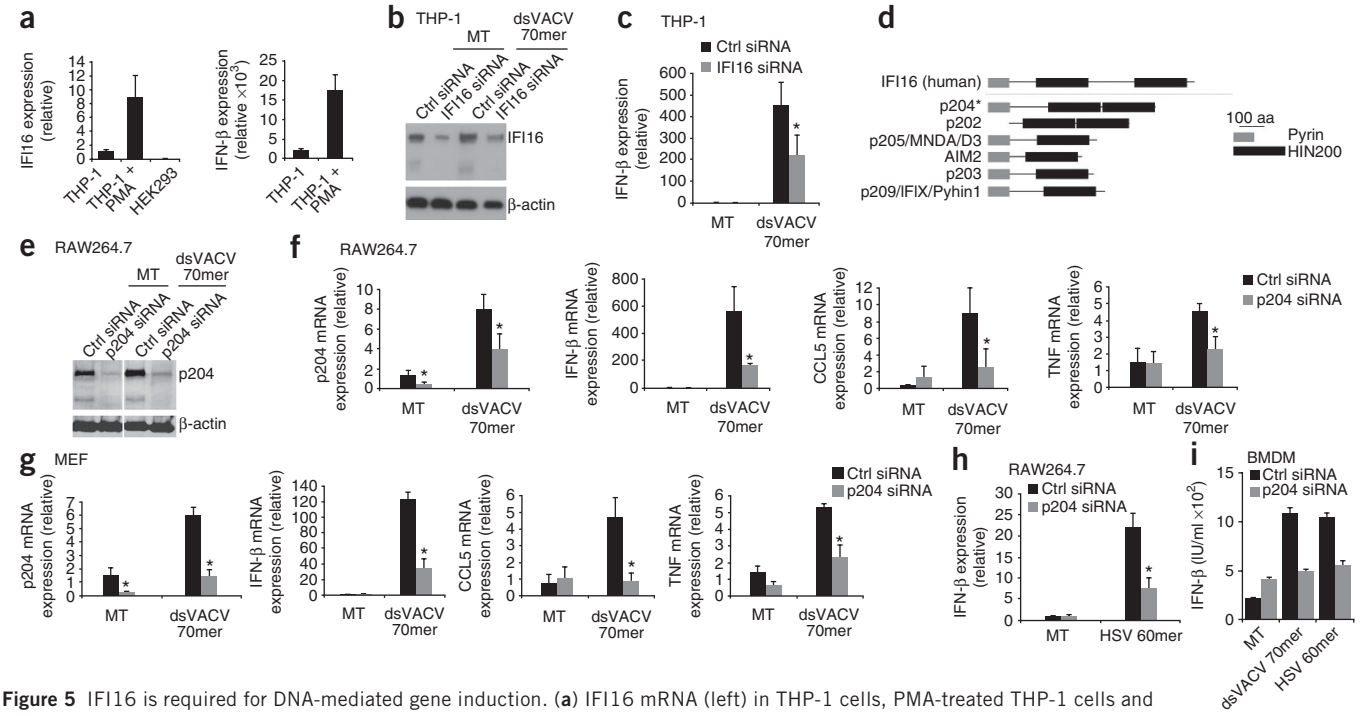

Figure 5 IFI16 is required for DNA-mediated gene induction. (**a**) IFI16 mRNA (left) in THP-1 cells, PMA-treated THP-1 cells and HEK293 cells, and IFN-β mRNA (right) in untreated or PMA-treated THP-1 cells transfected for 6 h with dsVACV 70mer (results presented as in **Fig. 1**). (**b**,**c**) Immunoblot analysis of IFI16 protein (**b**) and real-time PCR analysis of IFN-β mRNA (**c**) in THP-1 cells treated with control (Ctrl) or IFI16-specific siRNA (key) and mock transfected or transfected for 6 h with 1 μg/ml of dsVACV 70mer. (**d**) Domain organization (left) of human IFI16 (top) and members of the mouse PYHIN family (below), drawn to scale (right). Boxes indicate conserved domains. *, mouse PYHIN protein most similar to human IFI16 (p204). (**e**,**f**) Immunoblot analysis of p204 (**e**) and real-time PCR analysis of p204, IFN-β, CCL5 and TNF mRNA (**f**) in RAW264.7 cells treated with control (Ctrl) or p204-specific siRNA (key) and mock transfected or transfected for 6 h with 1 μg/ml of dsVACV 70mer. (**g**) Analysis of p204, IFN-β, CCL5 and TNF mRNA in siRNA-treated MEFs mock transfected or transfected for 6 h with 1 μg/ml of dsVACV 70mer. (**h**) IFN-β mRNA in siRNA-treated RAW264.7 cells mock transfected or transfected for 6 h with HSV 60mer DNA. (**i**) Release of IFN-β protein from BMDMs electroporated with siRNA and then mock transfected or transfected with oligomers for 18 h; results are presented relative to those of mock-transfected cells not treated with siRNA. $*P < 0.05$, compared with control siRNA (Student's *t*-test). Data are from one experiment representative of two or three (mean and s.d. in **a**,**c**,**f**–**i**).

known to be required for TBK1-dependent IFN-β responses to viruses and DNA[12,13]. Transfection of dsVACV 70mer into cells caused a DNA-dependent association between endogenous STING and IFI16 in THP-1 cells (**Fig. 4b**). IFI16 from DNA-stimulated THP-1 cells associated with immobilized Myc-tagged human STING and, to a lesser extent, mouse STING (**Fig. 4c**). STING not only was recruited to IFI16 after viral DNA stimulation but was also required for IFN-β induction by dsVACV 70mer or HSV 60mer, as in BMDMs lacking STING, IFN-β secretion from cells transfected with the viral DNAs was completely inhibited (**Fig. 4d**,**e**). ASC is an adaptor recruited to AIM2 that 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, as in BMDMs lacking ASC, IFN-β expression induced by either dsVACV 70mer or HSV 60mer was unimpaired, as was the poly(dA:dT) response (**Fig. 4f**). Thus, STING and ASC probably have distinct roles in regulating PYHIN-mediated induction of IFN-β and activation of caspase-1, 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 stemmed from the correlation between expression of IFI16 and responsiveness of cells to VACV 70mer. Thus, THP-1 cells treated with the phorbol ester PMA had higher expression of IFI16 and dsVACV 70mer-induced IFN-β than did untreated cells (**Fig. 5a**), whereas in HEK293 cells that failed to respond to dsVACV 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 dsVACV 70mer (**Supplementary Fig. 5b**,**c**), consistent with an antiviral role for IFI16. To clarify the role of IFI16 in mediating IFN-β induction by dsVACV 70mer, we used transient transfection of small interfering RNA (siRNA) to knock down *IFI16* in THP-1 cells, which led to lower expression of IFI16 protein than that in cells treated with control siRNA (**Fig. 5b**). Notably, this treatment with siRNA targeting IFI16 led to inhibition of IFN-β induction in response to dsVACV 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 also has a role in DNA sensing in mouse cells. After examining the mouse PYHIN family, we found that only one member, p204, had the same domain structure as IFI16 in that p204 also contained one pyrin domain and two HIN domains (**Fig. 5d**). Also, a BLAST search showed that p204 was the mouse PYHIN protein most similar to human IFI16 (37% amino acid identity). We detected expression of p204 in RAW264.7 mouse macrophages by immunoblot analysis, and this protein increased after transfection with dsVACV 70mer (**Fig. 5e**). Treatment of these cells with siRNA targeting p204 led to lower abundance of p204 protein (**Fig. 5e**) and p204 mRNA (**Fig. 5f**). Targeted knockdown of p204 led to impaired dsVACV 70mer–mediated induction of mRNA for IFN-β, the chemokine CCL5 and tumor necrosis factor (TNF; **Fig. 5f**). The requirement for p204 in transfected DNA–mediated gene induction was not restricted to RAW264.7 cells, as in MEFs, p204-specific siRNA resulted in much less p204

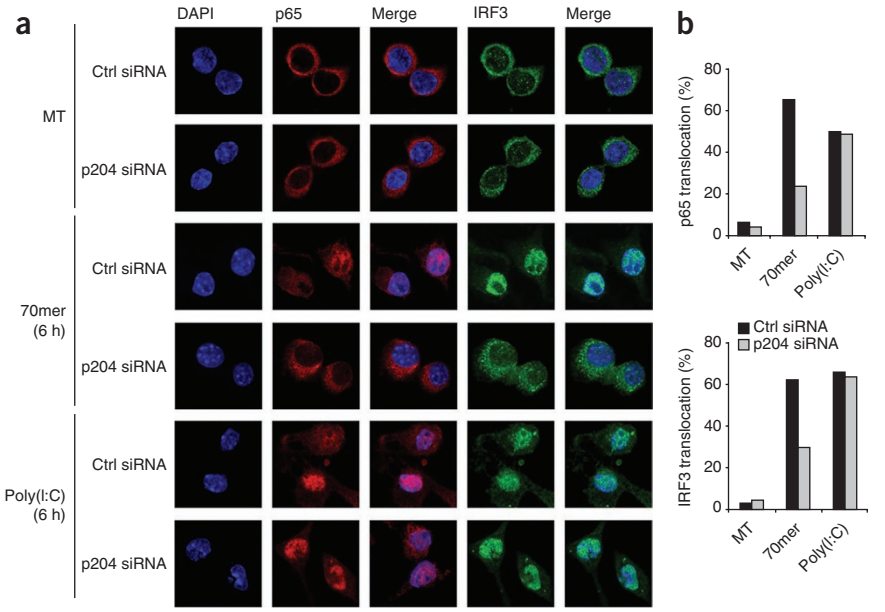

Figure 6 IFI16 is required for VACV 70mer DNA-stimulated transcription factor activation. (**a**) Confocal microscopy of siRNA-treated RAW264.7 cells grown on glass coverslips and mock transfected or transfected for 6 h with 2.5 μg/ml of dsVACV 70mer or poly(I:C), then fixed and stained for NF-κB p65 (red) and IRF3 (green); nuclei were visualized by DAPI (blue). Original magnification, ×60. (**b**) Staining of p65 (top) or IRF3 (bottom) in the nucleus of cells treated as in **a**; cells with nuclear staining are presented as a percentage of total cells (n = 200 cells per sample). Data are representative of four experiments.

mRNA (**Fig. 5g**) and led to potent inhibition of dsVACV 70mer–mediated expression of IFN-β, CCL5 and TNF (**Fig. 5g**). Suppression of p204 expression by siRNA in RAW264.7 cells also inhibited the IFN-β response to HSV 60mer (**Fig. 5h**). Furthermore, in BMDMs, p204-specific siRNA inhibited the secretion of IFN-β protein from cells stimulated by either dsVACV 70mer or HSV 60mer (**Fig. 5i**). Hence the IFI16 (p204) DNA-sensing pathway is not restricted to one DNA sequence or to one cell type and is operational both in human and mouse cells.

A role for p204 as a sensor for DNA indicated that inhibition of p204 expression should affect DNA-mediated gene induction because of an effect upstream of transcription factor activation. Thus, we examined the effect of p204-specific siRNA on the activation of NF-κB and IRF3 induced by dsVACV 70mer. To measure transcription factor activation, we monitored the translocation of endogenous p65 (an NF-κB subunit) and IRF3 from the cytosol to the nucleus by confocal microscopy after transfecting DNA or RNA into RAW264.7 cells in the presence of either control or p204-targeting siRNA. Transfection of cells with dsVACV 70mer or poly(I:C) for 6 h led to accumulation of both p65 and IRF3 in the nucleus in the presence of the control siRNA (**Fig. 6**). Notably, translocation of both p65 and IRF3 was prevented in the presence of p204-specific siRNA in cells transfected with DNA but not in those transfected with RNA (**Fig. 6**). This result confirmed that p204 has a role in gene induction upstream of transcription factor activation. Furthermore, it demonstrated a role for p204 upstream of both the NF-κB pathway and the IRF3 pathway for DNA but not RNA, consistent with a role in direct DNA sensing.

IFI16 is required for HSV-1-induced gene expression

To extend the findings reported above 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, as we observed only marginal inhibition of IFN-β mRNA induction in cells lacking both MyD88 and TRIF (**Fig. 7a**). The IFN-β response to HSV-1 in monocytes was also independent of RNA polymerase III, in contrast to a published study[17], as we found no impairment in IFN-β induction by HSV-1 in the presence of RNA polymerase III inhibitor, although the response to poly(dA:dT) was inhibited (**Fig. 7b**). Furthermore, consistent with a lack of a role for RNA polymerase III in sensing HSV-1, no IFN-β induction was detectable in HSV-1-infected HEK293 cells, which are cells in which the RNA polymerase III DNA-sensing pathway is operational (**Supplementary Fig. 3c**). IFN-β expression in response to HSV-1 was STING dependent (**Fig. 4e**), as shown before[12].

To examine the role of p204 in detecting HSV-1, we treated cells with p204-specific siRNA before infecting them with HSV-1. This led to a considerable inhibition of HSV-1-induced IFN-β mRNA (**Fig. 7c**), in contrast to the minor role for TLR detection of HSV-1 and the absence of a role for RNA polymerase III in IFN-β induction by HSV-1. Furthermore, induction of the IRF3-dependent gene *Cxcl10* (encoding the chemokine CXCL10) and the NF-κB-dependent genes *Il6* (encoding interleukin 6) and *Tnf* by HSV-1 infection of RAW264.7 cells was also impaired by p204-specific siRNA (**Fig. 7c**). In contrast, treatment of cells with p204-specific siRNA had no effect on the induction of IFN-β mRNA in response to an RNA virus (Sendai virus; **Fig. 7d**). Treatment with p204-specific siRNA also resulted in less secretion of IFN-β protein in response to HSV-1 but not in response to Sendai virus (**Fig. 7e**). This result confirmed that knockdown of p204 expression did not lead to a global decrease 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-specific siRNA on HSV-1-induced IRF3- and NF-κB-dependent genes (**Fig. 7c**), and similar to the results obtained with 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 translocation of transcription factors was unaffected by p204-specific siRNA (**Fig. 7f,g**). Furthermore, triggering the p204 pathway induced anti-HSV-1 activity in cells, as pretreatment of cells with 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 large amounts of IFI16 (**Fig. 3d**). Using a specific labeled oligonucleotide complementary to HSV-1 DNA, we detected viral DNA 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 (data not shown). Thus, IFI16 and p204 might detect the 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, which directly detect the presence of viral DNA, leading to the activation of transcription factors and gene induction via a STING-dependent pathway.

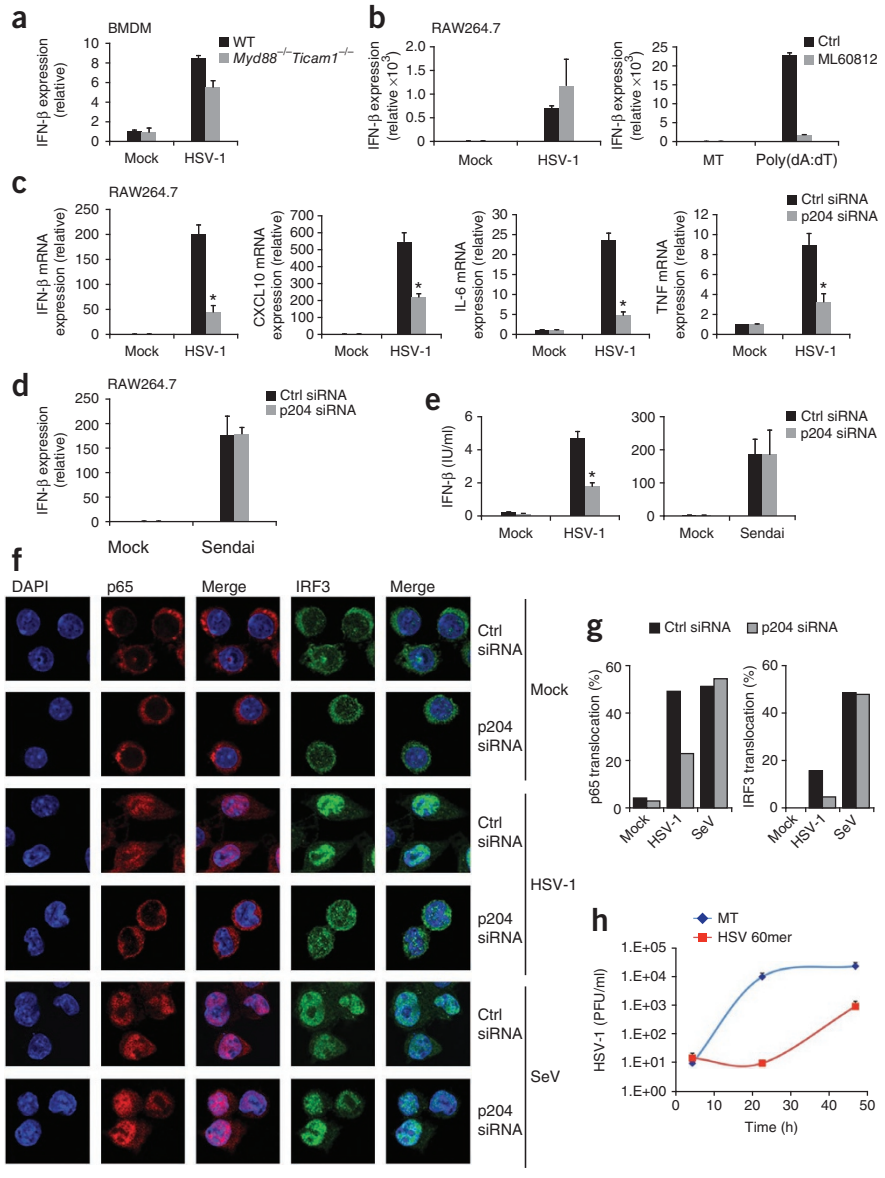

Figure 7 IFI16 is required for the innate immune response to HSV-1. (**a**) IFN-β mRNA in wild-type (WT) or *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ BMDMs mock infected or infected for 6 h with HSV-1 (multiplicity of infection, 10). (**b**) IFN-β mRNA in RAW264.7 cells given no pretreatment (Ctrl) or pretreated for 2 h with ML60812, then mock infected or infected for 6 h with HSV-1, or mock transfected or transfected for 6 h with poly(dA:dT). (**c**,**d**) IFN-β mRNA (**c**,**d**), CXCL10 mRNA (**c**), interleukin 6 (IL-6) mRNA (**c**) and TNF mRNA (**c**) in siRNA-treated RAW264.7 cells mock infected or infected for 6 h with HSV-1 (**c**) or Sendai virus (**d**). (**e**) IFN-β protein expression in siRNA-treated RAW264.7 cells mock infected or infected for 20 h with HSV-1 or Sendai virus. (**f**) Confocal microscopy of siRNA-treated RAW264.7 cells grown on glass coverslips and mock infected or infected for 6 h with HSV-1 or Sendai virus, then fixed and stained for NF-κB p65 (red) and IRF3 (green); nuclei were visualized by DAPI staining (blue). Original magnification, ×60. (**g**) Staining of p65 (left) or IRF3 (right) in the nucleus of cells treated as in **f**; cells with nuclear staining are presented as a percentage of total cells ($n = 200$ cells per sample). (**h**) Plaque assay of virus in culture supernatants of RAW264.7 cells mock transfected or transfected with HSV 60mer (2 μg/ml) and infected with HSV-1 (multiplicity of infection, 1). PFU, plaque-forming units. Results for mRNA (**a**–**d**) are presented relative to those of mock-transfected cells. *$P < 0.001$, compared with control siRNA (Student's *t*-test). Data are from one experiment representative of three (mean and s.d. in **a**–**e**,**h**).

DISCUSSION

At present there is much interest in defining the mechanisms whereby the innate immune response detects exogenous DNA, as this detection process is critical to understanding how cells respond to DNA viruses and to immune-stimulatory bacterial and self DNA[6]. Although IFI16 has a role in regulating cell proliferation and differentiation[27], our data here have identified human IFI16 and its mouse ortholog p204 as critical sensors for exogenous dsDNA, especially for IRF3- and NF-κB-dependent gene induction, and as being 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]. As 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 to our knowledge of a pyrin domain–containing protein sensing DNA to mediate IFN-β induction. We showed that the HIN domains of IFI16 bound dsVACV 70mer and 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.

Pyrin domains have been shown before to mediate inflammasome activation. For example, AIM2 is another PYHIN family member that 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. Furthermore, IFI16 and ASC do not interact[19]. Notably, in the PYHIN family, the pyrin domain of AIM2 is most like inflammasome-related pyrin domains, whereas the pyrin domains of the rest of the family are distinct from that of AIM2 and more like that of IFI16 (ref. 28). It remains to be determined whether other PYHIN family members are also able to regulate 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 we call 'AIM2-like receptors' (ALRs). Thus, whereas exogenous RNA is sensed by both endosomal TLRs (TLR3, TLR7 and TLR9) 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; analogous to RIG-I and Mda5), inflammasome activators (AIM2; analogous to RIG-I (ref. 29)) and negative regulators (p202 (ref. 21); analogous to LGP2 (ref. 30)). Furthermore, like the RLR family, the ALR family is subject

to viral targeting: the poxvirus protein M013 contains a pyrin domain and binds to ASC[31], which would inhibit the AIM2 response, although it has also been shown to inhibit poxvirus-stimulated induction of NF-κB-dependent genes[32]. Also, the human cytomegalovirus protein pUL83, a known antagonist of interferon-inducible genes, can 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 interferon response.

It is possible that IFI16 also has a role in sensing bacterial DNA during infection with intracellular bacteria. The induction of type I interferon in response to *Listeria* is independent of TLRs and RNA sensing and requires IRF3 (ref. 34) and STING[12], whereas 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, and IFI16-specific antibodies are present and IFI16 expression is higher in patients with systemic lupus erythematosus[36–38]. Thus detection of DNA by IFI16 or p204 may have a role not only in antiviral innate immune responses but also in responses to bacterial pathogens and in autoimmunity.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A001237, A001597 and A004152.

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank C. Liu (New York University) for antibody to p204; S. Akira (Osaka University) for *Myd88*$^{-/-}$, *Ticam1*$^{-/-}$ and *Zbp1*$^{-/-}$ mice; T. Taniguchi (University of Tokyo) for *Irf3*$^{-/-}$ mice; M. Kelliher (University of Massachusetts Medical School) for *Tnfrsf1a*$^{-/-}$ mice; T. Mak (University of Toronto) for *Tbk1*$^{-/-}$*Tnfrsf1a*$^{-/-}$ mice; G. Barber (University of Miami) for *Tmem173*$^{-/-}$ femurs; D. Golenbock (University of Massachusetts Medical School) for immortalized BMDMs lacking MyD88 or TRIF; I. Julkunen (National Public Health Institute, Finland) for Sendai virus; and S. Corr (Trinity College Dublin) for *Listeria monocytogenes* DNA. Supported by Science Foundation Ireland (07/IN1/B934 to L.U., S.E.K., M.B. and A.G.B.), the Irish Health Research Board (S.E.K. and A.G.B.), the Danish Medical Research Council (09-072636 to S.R.P.), the Lundbeck Foundation (R17-A1528 to S.R.P. and R34-A3855 to K.A.H.), the US National Institutes of Health (AI083713 and AI067497 to K.A.F.; Division of Intramural Research, National Institute of Allergy and Infectious Diseases to T.S.X.) and the Faculty of Health Sciences, Aarhus (S.B.J.).

AUTHOR CONTRIBUTIONS

L.U. and S.E.K. did experiments, analyzed data and cowrote the manuscript; M.B., K.A.H., S.B.J., S.S., C.M.S. and T.J. did experiments and analyzed data; E.L. provided expertise; T.S.X., K.A.F. and S.R.P. supervised experiments and analyzed data; and A.G.B. designed and supervised the study, analyzed data and cowrote the manuscript. M.B. and K.A.H. contributed equally to this work.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/natureimmunology/.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/.

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. NY Acad. Sci.* **1110**, 47–56 (2007).
37. Choubey, D. & Panchanathan, R. Interferon-inducible Ifi200-family genes in systemic lupus erythematosus. *Immunol. Lett.* **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).

ONLINE METHODS

Mice, cells and viruses. *Myd88*$^{-/-}$ and *Ticam1*$^{-/-}$ mice from S. Akira 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 with 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 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 medium containing 10% (vol/vol) FBS and were treated for at least 16 h (where indicated) with 50 or 100 nM PMA and 1,000 U/ml of IFN-α. All other cells were grown in DMEM containing 10% (vol/vol) FBS. For viral infection, HSV-1, KOS strain, and Sendai virus, Cantell strain (a gift from I. Julkunen), were used.

Antibodies. Antibody to p204 (anti-p204)[41] was a gift from C. Liu; anti-STING[9] was a gift from G. Barber; and other antibodies were as follows: anti-IFI16 (1G7; Santa Cruz), anti-p65 (F-6; Santa Cruz), anti-β-actin (AC-74; Sigma), anti-TBK1 (3013; Cell Signaling), anti-DDX3 (A300-A74A; Bethel Laboratories), anti–mouse IRF3 (51-3200; Zymed), anti-VP5 (3B6; Virusys) and anti-Myc (9E10; Sigma).

Nucleic acids and transfection. HEK293 cells were transfected with GeneJuice (4 μl/ml; Merck). All other cells were transfected with Lipofectamine2000 (1 μl/ml; Invitrogen). Poly(I:C), calf thymus DNA and poly(dA:dT) were from Sigma, and herring sperm DNA was from Promega. *Listeria monocytogenes* DNA was a gift from S. Corr. VACV DNA was purified from core particles of Western Reserve strain virus by phenol-chloroform extraction. Oligonucleotides were synthesized by MWG Biotech (sequences, **Supplementary Methods**).

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

RNA analysis. Human IFN-β mRNA was quantified by real-time PCR with the TaqMan gene expression assay Hs00277188_s1 and a β-actin endogenous control VIC-MGB probe (6-carboxyrhodamine–minor groove binder; Applied Biosystems). Other mRNAs were quantified with SYBR Green (primers, **Supplementary Methods**). The abundance of mRNA was normalized to that of β-actin mRNA.

Enzyme-linked immunosorbent assay. Cell culture supernatants were assayed for IFN-β by a custom enzyme-linked immunosorbent assay as described[42].

Luciferase assays. *IFNB* promoter activation in HEK293 cells was measured by luciferase assay as described[25].

Oligonucleotide precipitation and mass spectrometry. Cytoplasmic extracts were generated by disruption of PMA-treated THP-1 cells in extraction buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol and protease inhibitors) and pelleting of nuclei by centrifugation at 1,000*g* for 10 min. Supernatants were further centrifuged at 15,700*g* for 10 min and precleared with resin. Each nucleic acid affinity purification included 5 mg total cytoplasmic protein, together with 2 nmol 5′-biotinylated VACV 70mer (double- or single-stranded) precoupled to 100 μl Streptavidin UltraLink Resin (50% slurry; Pierce). Precipitated DNA-protein complexes were washed extensively in lysis buffer (0.5% (vol/vol) Nonidet P-40, 100 mM NaCl, 5% (vol/vol) glycerol, 0.5 mM EDTA, 50 mM HEPES, pH 7.5, 1 mM $NaVO_4$ and protease inhibitors) and in 50 mM HEPES, pH 7.5. Proteins were eluted by boiling of samples in 1% SDS and were resolved by SDS-PAGE, digested with trypsin and analyzed by liquid chromatography mass spectrometry.

Coimmunoprecipitation. PMA- and IFN-α-treated THP-1 cells were mock transfected or transfected for 4 h with 1 μg/ml of VACV 70mer. Cells were lysed in lysis buffer (0.5% (vol/vol) Nonidet P-40, 100 mM NaCl, 10% (vol/vol) glycerol, 1 mM EDTA, 50 mM HEPES, pH 7.5, 1 mM $NaVO_4$ and protease inhibitors), then were precleared and were immunoprecipitated with anti-STING[9]. For coimmunoprecipitation with Myc-tagged 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; imaGenes) and were cloned into the vector pCMV-myc (Clontech). STING constructs and empty vector were transfected into HEK293T cells by the calcium-phosphate method. At 24 h after transfection, cells were lysed in Nonidet P-40–containing lysis buffer and proteins were immunoprecipitated with immobilized anti-Myc. Immunoprecipitates were washed in lysis buffer and then were incubated with precleared lysates of PMA- and IFN-α-treated THP-1 cells stimulated with 1 μg/ml VACV 70mer. Immunoprecipitated proteins were washed in lysis buffer, eluted, resolved by SDS-PAGE and detected by immunoblot analysis.

RNA-mediated interference. The siRNAs were chemically synthesised by Invitrogen (Stealth RNAi siRNAs) or by Qiagen (sequences, **Supplementary Methods**). Cells were seeded in 12-well plates at a density of 1×10^5 cells per well and were transfected with siRNA at a concentration of 12.5 pmol/ml (or 6.25 pmol/ml for THP-1 cells) with Lipofectamine (1 μl/ml). RAW264.7 and THP-1 cells were treated twice with siRNA on consecutive days and were grown for a further 48 h before stimulation. MEFs were treated once with siRNA and stimulated 48 h later.

HSV replication assay. RAW 264.7 cells were transfected with HSV 60mer (2 μg/ml) 24 h before being infected with HSV-1 (multiplicity of infection, 1). Supernatants were collected 6, 24 and 48 h after infection and virus was quantified by standard plaque assay on Vero African green monkey kidney cells[26].

Confocal microscopy. Cells grown on glass coverslips were fixed in 4% (wt/vol) paraformaldehyde and were made permeable in 0.5% (vol/vol) Triton X-100. Coverslips were preincubated in 5% (wt/vol) BSA and 0.05% (vol/vol) Tween-20 in PBS or in 1% (vol/vol) FCS in PBS and were stained for 1–3 h at 25 °C with primary antibodies (1:100 dilution) and for 1 h with Alexa Fluor 488– and Alexa Fluor 647–labeled secondary antibodies (1:500 dilution). Coverslips were mounted in Mowiol 4-88 (Calbiochem) containing DAPI (4,6-diamidino-2-phenylindole; 1 μg/ml). Images were obtained with an Olympus FV1000 scanning confocal microscope.

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

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).