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Cutting Edge: Ku70 Is a Novel Cytosolic DNA Sensor That Induces Type III Rather Than Type I IFN

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Cytosolic foreign DNA is detected by pattern recognition receptors and mainly induces type I IFN production. We found that transfection of different types of DNA into various untreated cells induces type III IFN (IFN-λ1) rather than type I IFN, indicating the presence of uncharacterized DNA sensor(s). A pull-down assay using cytosolic proteins identified that Ku70 and Ku80 are the DNA-binding proteins. The knockdown studies and the reporter assay revealed that Ku70 is a novel DNA sensor inducing the IFN-lambda1 activation. The functional analysis of *IFNL1* promoter revealed that positive-regulatory domain I and IFN-stimulated response element sites are predominantly involved in the DNA-mediated *IFNL1* activation. A pull-down assay using nuclear proteins demonstrated that the IFN-λ1 induction is associated with the activation of IFN regulatory factor-1 and -7. Thus, to our knowledge, we show for the first time that Ku70 mediates type III IFN induction by DNA. *The Journal of Immunology*, **2011, 186: 4541–4545.**

In the activation of innate immune responses triggered by infection with bacterial or viral pathogens, microbe-specific molecular patterns in the pathogens are detected by pattern-recognition receptors (PRR). This ligand–receptor interaction triggers the activation of the innate immune system (1–3). Foreign DNA is recognized by membrane-bound PRR and cytoplasmic PRR. Recent studies demonstrate that DNA-dependent activator of IFN-regulatory factor (DAI) (4), absence in melanoma 2 (AIM-2) (5), leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) (6), RNA polymerase III (7), and IFN-γ–inducible protein 16 (IFI16) (8) are cytoplasmic PRRs and induce production of type I IFN or IL-1β. In this study, we report that Ku70, a component of a heterodimeric Ku protein, which is required for a variety of nuclear processes, including nonhomologous end-joining DNA repair, V(D)J recombination, and telomerase maintenance (9, 10), also functions as a cytosolic PRR recognizing DNA and induces the production of IFN-λ1 (a member of type III IFN) (11, 12) rather than type I IFN. The induction is mediated via the activation of IFN regulatory factor (IRF)-1 and IRF-7.

Materials and Methods

Cell culture, mice, and HIV replication assay

Human embryonic kidney cell line 293 (HEK293), HEK293T, human rhabdomyosarcoma cell line (RD), and HeLa cells were obtained from American Type Culture Collection. Monocyte-derived macrophages (MDMs) and dendritic cells (DCs) were prepared as previously described (13, 14). HIV replication assay was performed as previously described (13). Female wild-type (WT) C57/B6.129 mice were provided by the National Cancer Institute (NCI)-Frederick. Ku70-deficient mice on a C57/B6.129 background (15) were provided by Dr. Andre Nussenzweig (NCI, Bethesda, MD). All experiments with mice were performed in compliance with the principles and procedures outlined in the National Institutes of Health *Guide for the Care and Use of Animals* and were approved by NCI-Frederick Animal Care and Use Committee.

Preparation of plasmid DNA and genomic DNA

All plasmids were purified using the Endofree Plasmid Maxi kit (Qiagen). Genomic DNA was extracted from HEK293 cells using QIAamp DNA mini kit (Qiagen).

Transfection

HEK293 cells (100×10^3 cells in 3 ml/well of 6-well plates) were transfected with 1 μg DNA or 5 nM siRNA using TransIT-293 (Mirus Bio) or RNAiMAX (Invitrogen), according to the manufacturer's instructions. The

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Abbreviations used in this article: AIM-2, absence in melanoma 2; DAI, DNA-dependent activator of IFN-regulatory factor; DC, dendritic cell; HA, hemagglutinin; HEK293, human embryonic kidney cell line 293; IFI16, IFN-γ–inducible protein 16; IRF, IFN regulatory factor; ISRE, IFN-stimulated response element; LRRFIP1, leucine-rich repeat flightless-interacting protein 1; MDM, monocyte-derived macrophage; NCI, National Cancer Institute; PRDI, positive-regulatory domain I; PRR, pattern-recognition receptor; qRT-PCR, quantitative real-time RT-PCR; RD, human rhabdomyosarcoma cell line; si-Ctrl, siRNA-control; si-Ku70, siRNA-Ku70; si-Ku80, siRNA-Ku80; siRNA, small interfering RNA; WT, wild-type.

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small interfering RNA (siRNA)-transfected HEK293 cells were cultured for 48 h followed by DNA transfection for 24 h. Primary monocytes were transfected with siRNA using a Nucleofactor Transfection kit (Lonza) and then differentiated into MDM as described earlier. DNA transfection into MDM and mouse spleen cells was performed using the Nucleofactor Transfection kit. All siRNAs were obtained from Ambion (Supplemental Table I).

Quantitative real-time RT-PCR

The quantitative real-time RT-PCR (qRT-PCR) was performed as previously described (13). All probes were obtained from Applied Biosystems (Supplemental Table II).

Microarray analysis

Gene expression profiles of DNA-transfected cells were analyzed using the Affymetrix Chip, as previously described (13).

Preparation of cytosolic fraction and nuclear extract

The cytosolic and nuclear proteins were extracted from HEK293 cells using a Nuclear Extraction kit (Active Motif).

Pull-down assay

A pull-down assay was performed using DNA or oligonucleotide-conjugated agarose beads as previously described (16).

Mass spectrometry analysis

Cytosolic proteins bound to beads were analyzed by mass spectrometer (LTQ XP; Thermo Finnigan) as previously described (17).

Western blot

Western blot analysis was performed as previously described (13), using anti-Ku70, anti-DAI, and anti–AIM-2 Abs (Abcam); anti-Ku80, anti–IRF-1, anti–NF-κB p65, and anti–NF-κB p50 Abs (Cell Signaling Technology); or anti–β-actin, anti–IRF-3, and anti–IRF-7 Abs (Santa Cruz Biotechnology).

Reporter assay

Luciferase activity was measured using the Dual-Glo luciferase reporter assay system (Promega) and normalized against *Renilla* luciferase activity following the manufacturer's protocol.

Statistics

All results are representative of at least three independent experiments. All values are expressed as the mean and SD of individual samples. Samples were analyzed using the Student *t* test.

Results and Discussion

Transfection of various types of DNA induces activation of IFNL1 in different human cells

We have previously reported that IL-27 inhibits replication of HIV-1 and hepatitis C virus (13, 18). In studies designed to better understand the role of IL-27 in host defense, we constructed an expression vector encoding the human IL-27 gene (pCMV9.IL27) and transiently transfected it into HEK293 cells. As controls, mock and a noncoding empty plasmid (pCMV9) were used. On 3 d after transfection, the culture supernatants were collected and then studied to determine their ability to inhibit HIV-1 replication in MDM. Surprisingly, anti-HIV activity was seen in both culture supernatants from pCMV9- and pCMV9.IL27-transfected cells (Fig. 1*A*). In contrast, culture supernatants from mock cells had no significant impact on the antiviral activity, indicating that the transfection of noncoding pCMV9 triggers the induction of anti-HIV mediators in culture supernatants.

To identify the nature of the anti-HIV mediators associated with the empty vector transfection, we compared patterns of gene expression between untreated and pCMV9-transfected HEK293 cells, using DNA microarray analysis. DNA transfection upregulated 496 genes and downregulated 147 genes >2-fold compared with the untreated HEK293 control. An annotation analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics tool (19) illustrated that the transfection led to an upregulation of genes associated with viral infection and immune responses including some known anti-HIV proteins, IFN-λ1 (20) and RANTES (21) (Supplemental Table III). A qRT-PCR assay confirmed that pCMV9 transfection induced high levels of *IFNL1* and *RANTES* mRNA with lower levels of *IFNA, IFNB*, and *IFNL2/3* mRNA (Fig. 1*B*). Quantitation of cytokine concentration using ELISA indicated that pCMV9 transfection significantly produced both IFN-λ1 and RANTES; however, the induction of IFN-β was below the level of detection (<25 pg/ml) (Fig. 1*C*).

To characterize the DNA-mediated *IFNL1* activation, cell-type specificity in the gene activation was analyzed. The activation of *IFNL1* and *RANTES* was detected not only in HEK293, but in RD, HeLa, MDMs, and DCs (Fig. 1*D*). It has been reported that DNA transfection induces RANTES (4); however, the induction of IFN-λ1 has not been reported

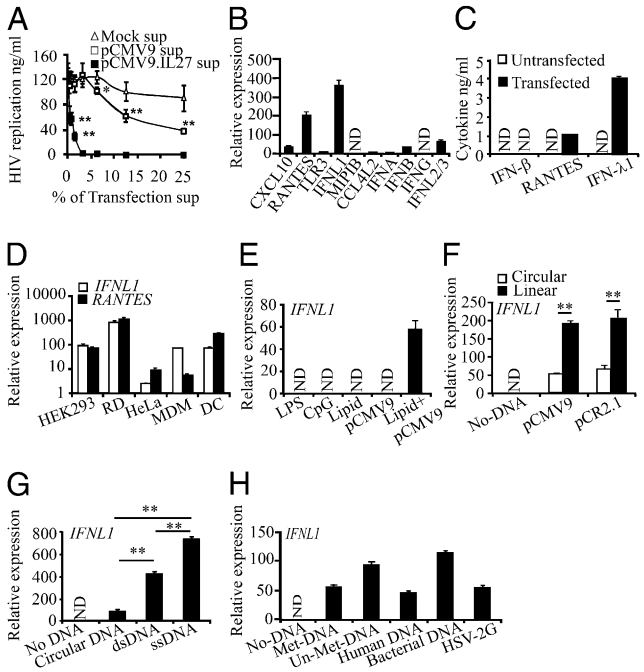

FIGURE 1. Various types of DNA induce *IFNL1* in different human cells. *A*, HIV-infected MDMs were cultured with various concentrations (% v/v) of culture supernatants from pCMV9.IL27- or empty pCMV9-transfected HEK293 cells. HIV replication was determined using a p24 Ag capture assay. *B*, Gene expression in mock- or pCMV9-transfected HEK293 cells was confirmed by qRT-PCR. *C*, Cytokine concentrations in the transfection supernatants were determined via ELISA. *D–H*, HEK293, HeLa, RD cells, MDMs, or DCs were transfected with pCMV9 (*D*); HEK293 cells were treated with 100 ng/ml LPS, 1 μM CpG oligodinucleotides (InvivoGen), 3 μl transfection lipid, 1 μg pCMV9, or a combination of the lipid and pCMV9 (*E*); HEK293 cells were treated with 1 μg supercoil (circular) or linearized plasmids (pCMV9 and pCR2.1) (*F*); circular pCR2.1 (circular DNA), linearized pCR2.1 (dsDNA), single-stranded pCR2.1 DNA (ssDNA) (*G*); linearized pCR2.1 (Met-DNA), PCR-amplified full-length pCR2.1 (Un-Met DNA), human DNA or bacterial DNA for 24 h, or infected with HSV-2G (multiplicity of infection = 5) for 18 h (*H*); then gene expression was analyzed by qRT-PCR. Gene expression is presented as relative expression units compared with mocked transfection after normalization to *GAPDH*. Data are mean ± SD. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$. ND, not detected.

yet. Thus, we mainly focused on the activation of *IFNL1*. To evaluate whether endogenous TLR4 or TLR9 is involved in *IFNL1* activation, we treated HEK293 cells with LPS (TLR4 ligand) or CpG motif oligodinucleotides (TLR9 ligand); then we analyzed the gene activation. As a positive control, MDMs were treated with LPS or CpG for 6 or 24 h. Even though LPS and CpG induced *IFNB* mRNA within 24 h in MDM (data not shown), neither reagent had any impact on the activation of *IFNL1* in HEK293 cells (Fig. 1*E*). Expression of *TLR7*, *DAI*, *AIM-2*, and *LRRFIP1* mRNA was not detected after 38-cycle qRT-PCR. Western blot illustrated that neither DAI nor AIM-2 was detected (data not shown), and transfection of siRNA RNA polymerase III (POLR3F) suppressed the expression of *POLR3F* mRNA by 50%; the siRNA, however, had no impact on *IFNL1* activation (Supplemental Fig. 1). Thus, we concluded that none of those DNA sensors is involved in the activation of *IFNL1* in HEK293 cells. To characterize the DNA-mediated *IFNL1* activation, we assessed DNA length, dose, incubation time, sequence, and structure dependency. The *IFNL1* mRNA was induced in a size-dependent (>500 bp) and dose-dependent (>250 ng/ml) DNA (Supplemental Fig. 2*A*, 2*B*). The kinetic experiment illustrated that the gene activation could be detected within 6 h of transfection, and that activation persisted for >48 h (Supplemental Fig. 2*C*). *IFNL1* mRNA was induced by both supercoil or linearized forms of pCMV9 (6.4 kbp) and pCR2.1 (3.9 kbp) plasmids, and the activation was significantly enhanced by the linearized plasmids (Fig. 1*F*). In addition, transfection of ss-pCR2.1, PCR-amplified pCR2.1 (Un-Met-DNA), fragmented human genomic DNA (~500 bp), bacterial DNA, and infection of DNA virus (HSV-2G) (Fig. 1*G*, 1*H*) also induced *IFNL1* activation. Taken together, these data indicated that an uncharacterized DNA sensor recognizes DNA without any restriction in structure or sequence, and induces activation of *IFNL1*. The sensor may preferentially recognize long linearized DNA. Because DNA fragment of human DNA induced *IFNL1* gene activation, apoptotic cells may also induce the gene activation.

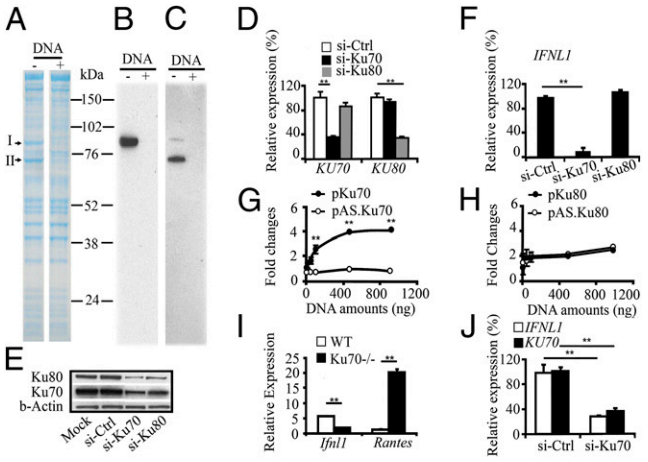

FIGURE 2. Ku70 is a cytosolic DNA sensor positively regulating *IFNL1* activation. *A*, Cytosol proteins from untreated HEK293 cells were incubated with DNA-conjugated beads in the absence or presence of DNA competitor (Supplemental Fig. 3). Proteins bound to the beads were separated on SDS-PAGE under reducing conditions, followed by Coomassie blue staining. *B* and *C*, Western blot analysis using anti-Ku70 (*B*) or anti-Ku80 (*C*) Ab demonstrated intended proteins. Because of a cross-reactivity in the anti-Ku70 Ab, it detected Ku70, as well as Ku80. *D–E*, HEK293 cells were transfected with si-Ctrl, si-Ku70, or si-Ku80, and the expression level of *KU70* or *KU80* mRNA (*D*) and protein (*E*) was analyzed by qRT-PCR and Western blot, respectively. The expression level of mRNA was compared with that in the cells transfected with si-Ctrl. Relative amounts of Ku70 and Ku80 protein levels were densitometrically analyzed using the National Institutes of Health image, and normalized against β-actin. *F*, HEK293 cells were transfected with si-Ctrl, si-Ku70, or si-Ku80 followed by DNA transfection. Expression levels of *IFNL1*, *KU70*, and *KU80* mRNA were determined by qRT-PCR. The level of mRNA was compared with that in the cells transfected with si-Ctrl. *G* and *H*, HEK293T cells were cotransfected with 100 ng full-length *IFNL1*-luciferase reporter plasmid and 10 ng *Renilla* luciferase plasmid with pKu70, FLAG-tagged anti-sense Ku70 (pAS.Ku70), pKu80, or HA-tagged Ku80 (pAS.Ku80) for 24 h, then stimulated for 18 h by transfection with 500 ng pCR2.1. *I*, Spleen cells from WT or knockout $Ku70^{-/-}$ mice were transfected with linearized pCR2.1 using Nucleofactor Transfection kit; then expression level of mRNA was analyzed by qRT-PCR. *J*, si-Ctrl or si-Ku70–transfected HEK293 cells were infected with HSV-2G; then gene expression was analyzed. Data are shown as the mean ± SD ($n = 3$). $^{**}p < 0.01$.

Ku70 is a novel cytosolic DNA sensor and positively regulates the activation of IFNL1

To identify the potential cytoplasmic DNA sensor present in HEK293 cells, we performed a proteomic approach using immobilized DNA beads and mass spectrometric analysis. Cytosolic fractions from untreated HEK293 cells were incubated with the DNA beads conjugated with PCR-amplified, full-length pCR2.1; then proteins bound on the beads were separated on SDS-PAGE, followed by Coomassie blue staining (Supplemental Fig. 3). To determine DNA-specific binding proteins on the gel, 10-fold excess amounts of pCR2.1 (DNA competitor) were mixed with the cytosol fraction before incubation with the beads. The addition of the competitor DNA reproducibly led to the disappearance of protein bands at molecular mass 80 (band I) and 70 kDa (band II) (Fig. 2*A*). The two bands were analyzed by mass spectrometer. Database searching revealed that band I is Ku80 and band II is Ku70 (Supplemental Fig. 4). Both Ku80 and Ku70 were confirmed

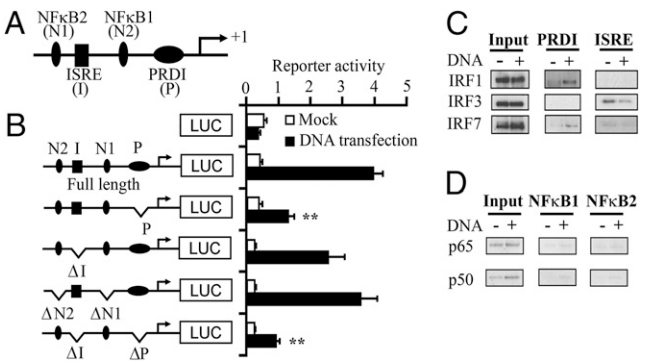

FIGURE 3. PRDI and ISRE elements of the *IFNL1* promoter are important for the DNA-mediated *IFNL1* activation, and *IFNL1* activation is associated with the activation of IRF-1 and IRF-7. *A* and *B*, Schematic representation of the *IFNL1* promoter region and different mutant constructs on the *IFNL1* promoter region. This diagram does not indicate the exact position of the elements. HEK293T cells were transfected with a series of variants of IFN-λ1–luciferase reporter and pTK-Renilla for 24 h, and then stimulated with transfection of pCR2.1; the luciferase activities were normalized with *Renilla* activities, and data are presented as fold inductions from promoter activity from basal promoter activation without pCR2.1 transfection. Data are shown as the mean ± SD. $n = 3$. $^{**}p < 0.01$. *C* and *D*, Nuclear extracts from mock- or DNA-transfected HEK293 cells were allowed to bind to oligonucleotides (PRDI or ISRE or NF-κB elements from the *IFNL1* promoter) conjugated to beads. Proteins bound to the beads were separated on SDS-PAGE, followed by Western blot analysis with specific Abs.

by Western blot (Fig. 2*B*, 2*C*). To determine the roles of Ku70 and Ku80 in the activation of *IFNL1*, we applied siRNA-Ku70 (si-Ku70) or siRNA-Ku80 (si-Ku80) in HEK293 and MDMs. Transfection with si-Ku70 into HEK293 cells led to a 70% reduction in *KU70* mRNA and no change in *KU80* mRNA compared with siRNA-control (si-Ctrl)–transfected cells, whereas transfection with si-Ku80 led to a 75% decrease in *KU80* mRNA and no change in *KU70* mRNA (Fig. 2*D*). Western blot using cytosol fraction from the siRNA-transfected cells resulted in si-Ku70 decreasing Ku70 protein by 54% (Fig. 2*E*). Consistent with other reports illustrating that each subunit of the Ku protein stabilizes the other (22), the si-Ku70 transfections also decreased the protein level of Ku80 by 65% (Fig. 2*E*). In MDMs, si-Ku70 transfection, but not si-Ku80, downregulated the expression of *KU70* mRNA by 40% compared with si-Ctrl–transfected cell (Supplemental Fig. 5*A*). The transfection of si-Ku70, but not si-Ku80, significantly decreased the DNA-mediated *IFNL1* activation in HEK293 cells and MDMs (Fig. 2*F*, Supplemental Fig. 5*B*). HSV-2G– and the ssDNA-mediated *IFNL1* activation was also significantly suppressed by si-Ku70 (Fig. 2*J*, Supplemental Fig. 6), indicating that only Ku70 is the positive regulator of *IFNL1* activation. FLAG-tagged Ku70 (pKu70) or hemagglutinin (HA)-tagged Ku80 (pKu80) expression vectors were transfected and *IFNL1* promoter activation was analyzed using a reporter assay, to further delineate the roles of Ku70 and Ku80. As a control, expression vector encoding FLAG-tagged anti-sense Ku70 (pAS.Ku70) or HA-tagged Ku80 (pAS.Ku80) was applied. Transfection of pKu70 and pKu80 overexpressed Ku70 and Ku80 proteins, respectively (Supplemental Fig. 7). Overexpression of Ku70, but not Ku80, increased *IFNL1* promoter activity in a dose-dependent manner (Fig. 2*G*, 2*H*). To precisely determine the role of Ku70, DNA was transfected into $Ku70^{-/-}$ mice spleen cells. DNA transfection induced transcripts of *Ifnl1* in WT but not in $Ku70^{-/-}$, whereas the transfection enhanced the expression of *Rantes* (Fig. 2*I*). Taken together, Ku70 is a novel cytosolic DNA sensor protein and positively regulates *IFNL1* activation by invaded cytosolic DNA. Because si-Ku70 inhibited the expression of *RATNES* mRNA in HEK293 cells (Supplemental Fig. 8), Ku70 may differentially regulate RANTES gene in between human and mouse.

IRF-1 and IRF-7 are associated with DNA-mediated IFN-λ1 activation

To investigate mechanism of the DNA-mediated *IFNL1* activation, we constructed mutants on the *IFNL1* promoter region lacking positive-regulatory domain I (PRDI), IFN-stimulated response element (ISRE), NF-κB1, NF-κB2, or different combinations (Fig. 3*A*, 3*B*). The construct lacking PRDI or ISRE domain predominantly reduced the Ku70-mediated *IFNL1* promoter activation, whereas the construct lacking NF-κB sites were able to induce the Ku70-mediated *IFNL1* promoter activation (Fig. 3*B*), indicating that the PRDI and ISRE binding sites play key roles in the DNA-mediated *IFNL1* activation. The pull-down assay using oligonucleotide (PRDI, ISRE, or NF-κB element)-conjugated beads with nuclear extract from mock- or pCR2.1-transfected cells, followed by Western blotting, demonstrated that DNA transfection significantly induced the binding activity of IRF-1 to the PRDI element, and a subtle increase of the IRF-7 binding activity to the same element rather than IRF-3 (Fig. 3*C*). Analysis of the activation profile of NF-κB indicated that DNA transfection increased only p65 and p50 binding activity (Supplemental Fig. 9). The pull-down assay illustrated that p65 and p50 bound to the NF-κB1 and NF-κB2 sites are at a low but detectable level (Fig. 3*D*). Taken together, these results indicated that both PRDI and ISRE sites are involved in the DNA-mediated *IFNL1* activation. IRF-1 and IRF-7 play key roles in DNA-dependent *IFNL1* activation.

It is known that the signaling pathway and biological activity of IFN-λ1 are the same as those of IFN-β; however, antiproliferative activity by IFN-λ1 is lower than that by IFN-β. Therefore, the selective induction of IFN-λ1 appears to be less cytotoxicity (23). The Ku70-mediated *IFNL1* activation required a longer size of DNA (>500 bp DNA). Ku protein bounds to multiple sites along linear DNA on dsDNA or ssDNA (24, 25); thus, unlike IFI16 (8), binding of multiple molecules of Ku70 on DNA may need to induce the gene activation. As previously reported (9), Ku70 and Ku80 protein expressed in cytosol fraction of all cell types tested (Supplemental Fig. 10*A*). A comparative analysis demonstrated no correlation between Ku70 expression and *IFNL1* activation (Supplemental Fig. 10*B*), indicating that although Ku70 plays a key role to activate *IFNL1* via activated IRF-1 and IRF-7, some other factor(s) may be involved in the activation of IRFs (Supplemental Fig. 11). Further study needs to precisely determine the mechanism by which Ku70 induces IFN-λ1 induction sensing dsDNA and ssDNA, and the physiological relevance in the selective induction of IFN-λ1 as innate immune response.

In summary, this study has demonstrated a role for Ku70 protein in the innate immune responses to foreign DNA through induction of *IFNL1* activation. The finding that an endogenously expressed cytosolic protein can immediately trigger IFN-λ1, but not IFN-β, production in response to exogenous DNA describes a new pathway of host defense to viral infection and DNA vaccination.

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Disclosures

The authors have no financial conflicts of interest.

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