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RIOK3 Is an Adaptor Protein Required for IRF3-Mediated Antiviral Type I Interferon Production

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ABSTRACT

Detection of cytosolic nucleic acids by pattern recognition receptors leads to the induction of type I interferons (IFNs) and elicits the innate immune response. We report here the identification of RIOK3 as a novel adaptor protein that is essential for the cytosolic nucleic acid-induced type I IFN production and for the antiviral response to gammaherpesvirus through two independent kinome-wide RNA interference screens. RIOK3 knockdown blocks both cytosolic double-stranded B-form DNA and double-stranded RNA-induced IRF3 activation and IFN-β production. In contrast, the overexpression of RIOK3 activates IRF3 and induces IFN-β. RIOK3 functions downstream of TBK1 and upstream of IRF3 activation. Furthermore, RIOK3 physically interacts with both IRF3 and TBK1 and is necessary for the interaction between TBK1 and IRF3. In addition, global transcriptome analysis shows that the expression of many gene involved antiviral responses is dependent on RIOK3. Thus, knockdown of RIOK3 inhibits cellular antiviral responses against both DNA and RNA viruses (herpesvirus and influenza A virus). Our data suggest that RIOK3 plays a critical role in the antiviral type I IFN pathway by bridging TBK1 and IRF3.

IMPORTANCE

The innate immune response, such as the production of type I interferons, acts as the first line of defense, limiting infectious pathogens directly and shaping the adaptive immune response. In this study, we identified RIOK3 as a novel regulator of the antiviral type I interferon pathway. Specifically, we found that RIOK3 physically interacts with TBK1 and IRF3 and bridges the functions between TBK1 and IRF3 in the activation of type I interferon pathway. The identification of a cellular kinase that plays a role the type I interferon pathway adds another level of complexity in the regulation of innate immunity and will have implications for developing novel strategies to combat viral infection.

The innate immune response acts as the first line of defense, limiting infectious pathogens directly and shaping the adaptive immune response (1, 2). The production of type I interferons (IFNs), such as IFN-α and IFN-β, is one of the most immediate responses upon infection (1–4). These secreted IFNs then bind to the IFN receptors (IFNARs) on the cell surface and activate the JAK/STAT pathway, inducing the expression of a wide range of IFN-stimulated genes (ISGs), which collectively mediate the inhibition of pathogens (5–7).

Type I IFNs can be stimulated upon sensing certain highly conserved molecular components in bacteria or viruses, termed pathogen-associated molecular patterns (PAMPs), by several classes of germ line-encoded pattern recognition receptors (PRRs) (1, 8–10). Nucleic acids are among the most potent and broadly recognized PAMPs (11). Previous studies have led to the discovery of several different classes of PRRs that recognize nucleic acids (1, 10, 12, 13). One of the major classes is the membrane-associated Toll-like receptors (TLRs), the leucine-rich repeat domains of which can recognize specific types of nucleic acids, such as double-stranded RNA (dsRNA) detected by TLR3, single-stranded RNA by TLR7/8, and unmethylated CpG DNA by TLR9 (14). TLRs dimerize upon ligand stimulation and differentially recruit adaptor proteins MyD88 or TRIF. The activation of adaptor proteins initiates the downstream signaling cascade culminating in the activation of NF-κB, mitogen-activated protein kinase, and IFN regulatory factors (IRFs) (14). TLRs are only expressed in a subset of specialized cells, such as macrophages and dendritic cells, whereas almost all nucleated cells are able to recognize foreign nucleic acids and induce the production of type I IFNs (15). RIG-I-like receptors (RLRs), including two RNA helicase proteins (retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), represent a class of ubiquitously expressed PRRs (10, 16, 17). Both RIG-I and MDA5 contain caspase recruitment domains (CARDs) and DExD/H-box helicase domains, and they can respond to cytosolic dsRNA. Upon ligand recognition, RIG-I and MDA5 activate the adaptor protein mitochondrial antiviral signaling protein MAVS (also known as IPS-1, CARDIF,

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and VISA) via CARD domain interaction (18–21). MAVS then triggers the activation of TBK1/IKKε and NF-κB, which in turn induces the production of type I IFN (18–21). Recent studies have also identified a group of potential cytosolic DNA sensors, such as RNA polymerase III, DAI, IFI16, DDX41, and cGAS, which induce type I IFNs independent of TLR signaling (22–26). Similar to the cytosolic RNA sensing pathway, recognition of cytosolic DNA also leads to activation of TBK1 and IRF3 and production of type I IFNs. The recently identified adaptor protein stimulator of IFN genes (STING, also known as MITA, MPYS, ERIS, and TMEM173) plays a critical role in the signaling pathway upstream of TBK1 in the cytosolic DNA recognition pathway (27–30).

Although a variety of receptors present in the cytosol can induce type I IFNs, the majority converge at the level of TBK1/IKKε (3, 31). These kinases phosphorylate and activate the transcription factor IRF3, which resides in the cytoplasm in unstimulated cells (3, 31–34). Phosphorylation of IRF3 leads to its dimerization, nuclear translocation, and association with CREB binding protein (CBP)/p300 (35–37). Activated IRF3 then assembles into a transcriptional enhanceosome with the transcription factors NF-κB and activating transcription factor 2 (ATF-2)/c-Jun, all of which function cooperatively to drive IFN-α/β gene transcription (4, 36, 38).

Upstream of TBK1/IKKε, adaptor molecules integrate signals between different classes of PRRs and TBK1/IKKε kinase activity. To date, several distinct adapter molecules have been identified, including MyD88 and TRIF, Toll/interleukin-1 receptor (TIR) domain-containing adapter molecules that are recruited either directly or indirectly to TIR domains of TLRs; MAVS, a CARD domain-containing adaptor molecule that is localized to the mitochondrion and peroxisomes and relays signals from the RLRs; and STING, an endoplasmic reticulum-resident protein that transduces signals from the cytosolic DNA sensors (27, 39).

Despite the tremendous advances in understanding the type I IFN pathway, new cytosolic nucleic acid sensors and signaling components are still being identified. In the present study, we report the identification of RIOK3 as a novel regulator of type I IFN pathway and viral restriction factor. Knockdown of RIOK3 abolishes the induction of IFN-β production by either cytosolic double-stranded B-form DNA (BDNA) or dsRNA. Knockdown of RIOK3 inhibits the cellular antiviral responses against both DNA and RNA viruses. Epistasis experiments show that RIOK3 functions downstream of TBK1 and upstream of IRF3 phosphorylation. Ectopic expression of RIOK3 activates IRF3. Mechanistically, RIOK3 interacts with both TBK1 and IRF3 and is required for the interaction between TBK1 and IRF3. Collectively, our data suggested that RIOK3 plays a critical role in the antiviral type I IFN pathway by bridging TBK1 and IRF3.

MATERIALS AND METHODS

High-throughput screening. A 384-well plate-based assay was optimized to identify small interfering RNAs (siRNAs) that influence cellular response to cytosolic BDNA. A human kinome library comprised of 2068 synthetic siRNAs targeting 517 unique human genes in total was arrayed in 384-well plates such that each well contained two unique and identifiable siRNAs per gene (7 ng of siRNA per gene, per well). There were on average 2 wells per gene or 4 siRNAs per gene. Each plate also contained positive controls (RIG-I siRNAs), negative controls (GL2-luciferase siRNA), and scramble siRNA controls. The library was introduced into the HEK293T_ISRE luciferase reporter line by a high-throughput reverse transfection process. Poly(dA-dT) was added at 1 μg/ml to the wells 30 h after siRNA transfection. At 16 h after stimulation, Bright-Glo (Promega) was added in equal volumes to each well, and the luminescence associated with each sample was analyzed. The screen was run in duplicates and statistically analyzed.

Cells, virus, plasmids, and siRNAs. HEK293T, THP-1, Vero, and MDCK cells were obtained from the American Type Culture Collection and were maintained in Dulbecco modified Eagle medium or RPMI medium supplemented with 10% fetal bovine serum. Transient transfections in HEK293T and THP-1 cells were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Poly(dA-dT) and poly(I·C) high molecular weight was obtained from Invivogen. For stimulation of cells, poly(dA-dT) or poly(I·C) was mixed with Lipofectamine 2000 (Invitrogen) at a ratio of 1:1 (wt/vol) and then added to cells at a final concentration of 5 μg/ml. SeV was purchased from Charles River. For stimulation, cells were infected with 500 HAU of SeV/ml.

MHV-68-M3/FL virus was propagated in Vero cells, and the virus titer was measured by firefly luciferase assay. HEK293T cells were seeded in 80 μl of medium in each well of a 96-well plate 1 day prior to infection, and 40-μl portions of different dilutions of samples containing MHV-68-M3/FL virus were added to each well. The luciferase activities were determined by using the Bright-Glo luciferase assay system (Promega). Influenza A virus (A/WSN/33) was propagated by infecting MDCK cells, and the virus titer was measured by limiting dilution.

The ISRE and IFN-β firefly luciferase promoter reporter constructs and the PGK *Renilla* luciferase reporter construct were kind gift from Genhong Cheng and Lili Wu, respectively (University of California at Los Angeles). RIOK3 cDNA clone was purchased from DNASU plasmid repository and was subcloned into pCMV-FLAG vector by PCR amplification and standard recombinant DNA techniques using XhoI and NotI sites and following primers: RIOK3-F (5′-CCG CTC GAG CGG CCA TGG ACT ACA AAG ACG ATG ACG ACA AGA TGG ATC TGG TAG GAG TGG-3′) and RIOK3-R (5′-ATT TGC GGC CGC TTT ACT ATT CAT CAT ATA GTA GTG GTG GGT CTC C-3′). Expression constructs for human RIG-I, MAVS, TBK1, and IRF3 were constructed by PCR amplification using the following primers: RIG-I-F, 5′-ACC ACC GAG CAG CGA-3′; RIGI-R, 5′-CCC TCG AGG TAT GAC CAC CGA GCA GCG A-3′; MAVS-F, 5′-GAA GAT CTT CAT GCC GTT TGC TGA AGA CAA GAC-3′; MAVS-R, 5′-CCG CTC GAG CGC TAG TGCAGA CGC CGC CGG TA-3′; TBK1-F, 5′-GAA GAT CTT CAT GCA GAG CAC TTC TAA TCA TCT-3′; TBK1-R, 5′-GGG GTA CCC CCT AAA GAC AGT CAA CGT TGC GAA-3′; IRF3-F, 5′-GGA ATT CCA CCA TGG GAA CCC CAA AGC CAC G; and IRF3-R, 5′-CCG CTC GAG TCA GCT CTC CCC AGG GC-3′. The PCR products were then cloned into pCMV-HA vector using standard recombinant DNA techniques. The siRNAs used in the present study were purchased from Qiagen.

Reporter analysis. HEK293T cells were seeded at 5×10^4 cells per well in a 48-well plate 12 h prior to transfection. The cells in each well were cotransfected with 50 ng of luciferase reporter plasmid, together with 400 ng of expression plasmids or 10 pmol of various siRNA constructs using Lipofectamine 2000 (Invitrogen). As an internal control, 5 ng of pPGK-RL was transfected simultaneously. Cells were then stimulated with poly(dA-dT) or SeV or left untreated. At 20 h after stimulation, the luciferase activity was measured by the dual-luciferase reporter assay system (Promega).

Real-time PCR. For real-time PCR analysis, total RNA was extracted from cells using a PureLink RNA minikit (Invitrogen) and reverse transcribed into cDNA with qScript cDNA synthesis kit (Quantas). Reverse transcription-PCR was then performed with the following primers to quantify cellular transcripts: *Gapdh*, 5′-TGC ACC ACC AAC TGC TTA GC-3′ and 5′-GGC ATG GAC TGT GGT CAT GAG-3′; human *ifnb1*, 5′-GCT TGG ATT CCT ACA AAG AAG CA-3′ and 5′-ATA GAT GGT CAA TGC GGC GTC-3′; human *isg15*, 5′-ACT CAT CTT TGC CAG TAC AGG-3′ and 5′-CAG CTC TGA CAC CGA CAT G-3′; human *riok3*, 5′-TGT GGC ATG CTG GAA AGG TCT G-3′ and 5′-GCT TCC TTG ACT CCT CCT TTC TGG-3′; mouse *ifnb1*, 5′-CAG CTC CAA GAA AGG ACG

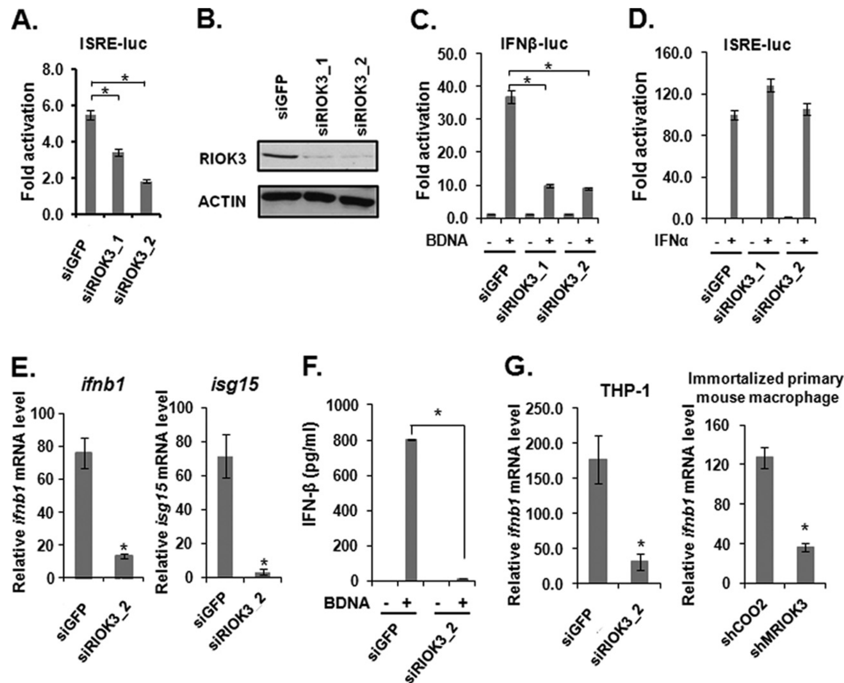

FIG 1 RIOK3 is required for IFN-β induction by BDNA. (A) Effect of RIOK3 RNAi on BDNA-induced ISRE promoter activation. HEK293T_ISRE cells were transfected with the indicated siRNAs and were left untreated or transfected with BDNA. The fold activation was calculated compared to the untreated cells. (B) Effect of RIOK3 RNAi on RIOK3 expression. HEK293T cells were transfected with the indicated siRNAs. Lysates were analyzed by immunoblotting using the indicated antibodies. (C and D) Effect of RIOK3 RNAi on BDNA-induced IFN-β promoter activation and IFN-α-induced ISRE promoter activation. HEK293T cells were cotransfected with the indicated siRNAs, PGK_Renilla-luciferase reporter and either IFN-β_luc (C) or ISRE_luc (D). Cells were left untreated or transfected with BDNA (C) or treated with 3×10^4 U of IFN-α/ml (D) for 20 h before luciferase assays were performed. The fold activation was calculated compared to the untreated cells. (E and F) Effect of RIOK3 RNAi on BDNA-induced *ifnb1* and *isg15* transcription and IFN-β expression. The relative *ifnb1* and *isg15* mRNA level was measured by quantitative PCR (QPCR) (E), and the concentration of IFN-β in the supernatant was measured by ELISA (F). (G) Effect of RIOK3 RNAi on BDNA-induced *ifnb1* expression in THP-1 cells and murine BMMs. The relative *ifnb1* mRNA level was measured by QPCR. *, $P < 0.05$ (Student *t* test). Error bars indicate the SD. All results are representative of three replicate experiments.

AAC-3′ and 5′-TGC TTT TTC AGC GGT AGA ATG TT-3′; and mouse *riok3*, 5′-ATC CAA CCA CGT TTT TAA TGC CT-3′ and 5′-TGC TTT TTC AGC GGT AGA ATG TT-3′.

ELISA. HEK293T cells were transfected with various siRNA constructs and stimulated with poly(dA-dT) or SeV. Supernatants were collected 12 h after stimulation. IFN-β enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer's instructions (PBL).

RNA-seq. HEK293T cells were transfected with various siRNA constructs and stimulated with poly(dA-dT) or SeV. Total RNA was extracted from cells, and RNA-seq was performed using an Illumina HiSeq 2000 sequencing system. Genes were filtered for differences in expression with the criterion of a *P* value of $<$0.05. Pathway analysis was done through the use of IPA (Ingenuity Systems).

Immunoblotting and immunoprecipitation. For immunoblotting, cells were directly lysed on the plate with a 1× protein sample buffer (0.625 M Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerin, 5% β-mercaptoethanol, 0.002% bromophenol blue), transferred to Eppendorf tubes and boiled for 10 min prior to SDS-PAGE. For all immunoprecipitation assays, HEK293T cells were washed once with phosphate-buffered saline (PBS) and then lysed on ice with lysis buffer (50 mM Tris [pH 7.5], 0.5% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 1 mM NaF. The cell lysates were then incubated with a specific antibody (2 μg of antibody per sample) or control IgG-conjugated Sepharose-protein G beads overnight at 4°C. The immunoprecipitates were washed three times with lysis buffer and then boiled and analyzed by Western blotting. The antibodies used here were as follows: rabbit anti-IRF3 (Santa Cruz, catalog no. sc-9082), rabbit anti-phospho-IRF3 (Epitomics, catalog no. s2562-1), rabbit anti-TBK1 (Cell Signaling, catalog no. s3013), rabbit anti-phospho-TBK1 (Cell Signaling, catalog no. s5483), mouse anti-FLAG (Sigma, catalog no. sF3165), HA (Sigma, catalog no. H3663), and V5 (Invitrogen, catalog no. sR960-25).

Statistical analysis. Statistical significance was determined using a two-tailed unpaired Student *t* test.

RESULTS

Screen for candidate molecules mediating cytosolic BDNA sensing. To systematically identify additional essential molecular components for cellular response to cytosolic BDNA, we conducted a kinome siRNA library screen. In this screen, we transfected individual siRNA targeting kinases into HEK293_ISRE cells harboring a stable luciferase reporter under the control of an IFN-sensitive response element (ISRE), which is responsive to transfected BDNA [poly(dA-dT)]. Subsequently transfected cells were stimulated with 1 μg of BDNA/ml to induce ISRE reporter activity. The activation of the ISRE reporter was measured by a dual-luciferase assay, and the fold induction was calculated in comparison to the untreated sample. Since HEK293T cells do not express TLR9 or other nucleic acid TLRs, it is likely that the observed activation is primarily a result of a cytoplasmic DNA recognition response pathway. Since it was reported that poly(dA-dT) sensing in HEK293T cells is mainly through RNA polymerase III and RIG-I, the screen was optimized with siRNAs against RIG-I. si-

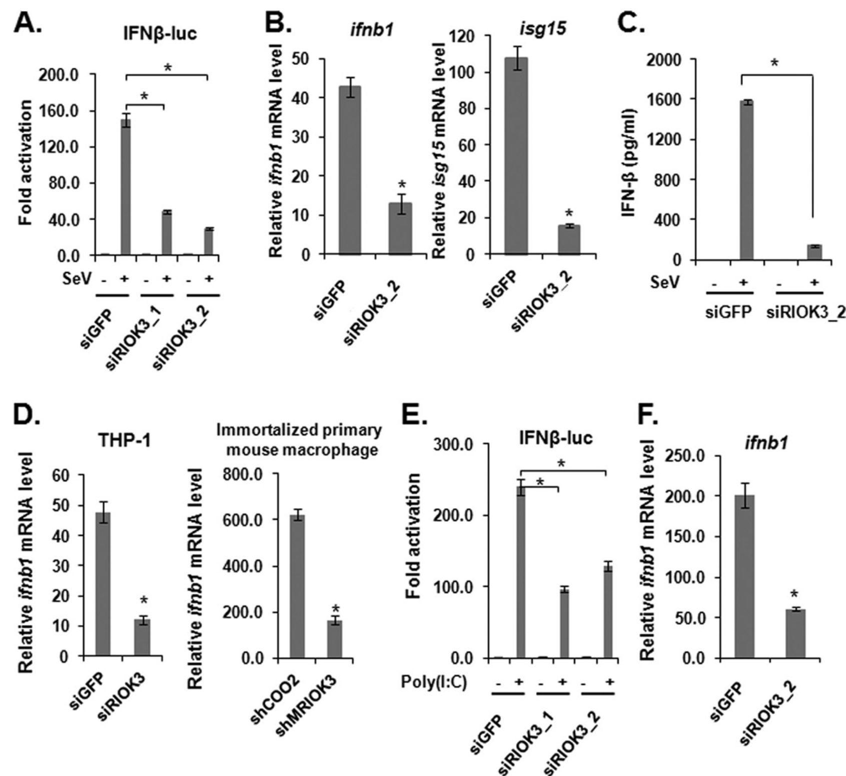

FIG 2 RIOK3 is required for IFN-β induction by SeV and poly(I·C). (A and E) Effect of RIOK3 RNAi on SeV (A)- or poly(I·C) (E)-induced IFN-β promoter activation. HEK293T cells were cotransfected with IFN-β_luc, PGK_Renilla-luciferase reporter, and the indicated siRNAs, and cells were left untreated or infected with 100 HAU of SeV (A)/ml or transfected with poly(I·C) (E) for 20 h before luciferase assays were performed. The fold activation was calculated compared to the untreated cells. (B and C) Effect of RIOK3 RNAi on SeV-induced *ifnb1* and *isg15* transcription and IFN-β protein expression. HEK293T cells were transfected with the indicated siRNAs and were left uninfected or infected with 100 HAU of SeV/ml 40 h later. The relative *ifnb1* and *isg15* mRNA levels were measured by QPCR (B), and the concentration of IFN-β in the supernatant was measured by ELISA (C). (D) Effect of RIOK3 RNAi on SeV-induced *ifnb1* expression in THP-1 cells and murine BMMs. The relative *ifnb1* mRNA level was measured by QPCR. (F) Effect of RIOK3 RNAi on poly(I·C)-induced *ifnb1* transcription. HEK293T cells were transfected with the indicated siRNAs and were left untreated or treated with poly(I·C) 24 h later. The relative *ifnb1* mRNA level was measured by QPCR. *, $P < 0.05$ (Student *t* test). Error bars indicate the SD. All results are representative of three replicate experiments.

RIG-I inhibited BDNA-induced ISRE promoter activity by >30-fold. The screen data were analyzed using redundant siRNA activity (40), and we were able to identify 85 cellular genes, for which at least two siRNAs reduced the ISRE promoter activity by 35% or more (~2 standard deviations [SD] from the mean of the negative controls).

We hypothesize that siRNAs that inhibit genes involved in the cytoplasmic response to DNA would, in turn, enhance replication of DNA viruses. We compared the results of this screen to a parallel kinome siRNA screen that was designed to identify genes that regulate murine gammaherpesvirus 68 (MHV-68) replication. Briefly, individual siRNA of a kinome-wide siRNA library was transfected into HEK293T cells, and the transfected cells were infected with MHV-68-M3/FL virus, which is replication competent and expresses luciferase as a reporter (41). After 2 days, viral supernatants were transferred to naive HEK293T cells, and luciferase expression was monitored after 20 h to gauge the effects of siRNAs on MHV-68 replication. Remarkably, we identified 23 genes that were found in the top 10% of both screens ($P = 1.5 \times 10^{-9}$). We further validated the role of these 23 genes in the innate response to cytoplasmic DNA.

RIOK3 was one of the top hits from both screens, and its siRNAs led to the significant inhibition of the BDNA-induced ISRE reporter activity. RIOK3 belongs to the RIO family, which is conserved from *Archaea* to humans (42). The mRNA expression profile from the GNF Tissue Atlas shows that the expression of RIOK3 is detected at low levels in various human tissues, but it is highly expressed in cells of lymphoid and myeloid lineage, which play major roles in immune surveillance (43). Although RIOK3 was previously shown to negatively regulate tumor necrosis factor alpha (TNF-α)-induced NF-κB activation and positively regulate hedgehogs signaling, its function in type I IFN pathway was never explored (44, 45).

In addition to the four siRNA constructs used in the initial screen, two more RIOK3 siRNA constructs that can knock down RIOK3 expression efficiently (Fig. 1B) were subsequently confirmed to block BDNA-induced ISRE promoter activity (Fig. 1A). (siRIOK3_2 was used for all of the experiments described below. Similar results were obtained with siRIOK3_1.) To examine whether RIOK3 is required for type I IFN production or signaling, we first performed the IFN-β promoter reporter assay as an indicator for IFN-β production. As shown in Fig. 1C, knockdown of RIOK3 significantly inhibited BDNA-induced IFN-β promoter activity. We next tested the role of RIOK3 in type I IFN signaling by assaying the induction of ISRE promoter activity in the presence of IFN-α treatment. The IFN-α-induced ISRE promoter ac-

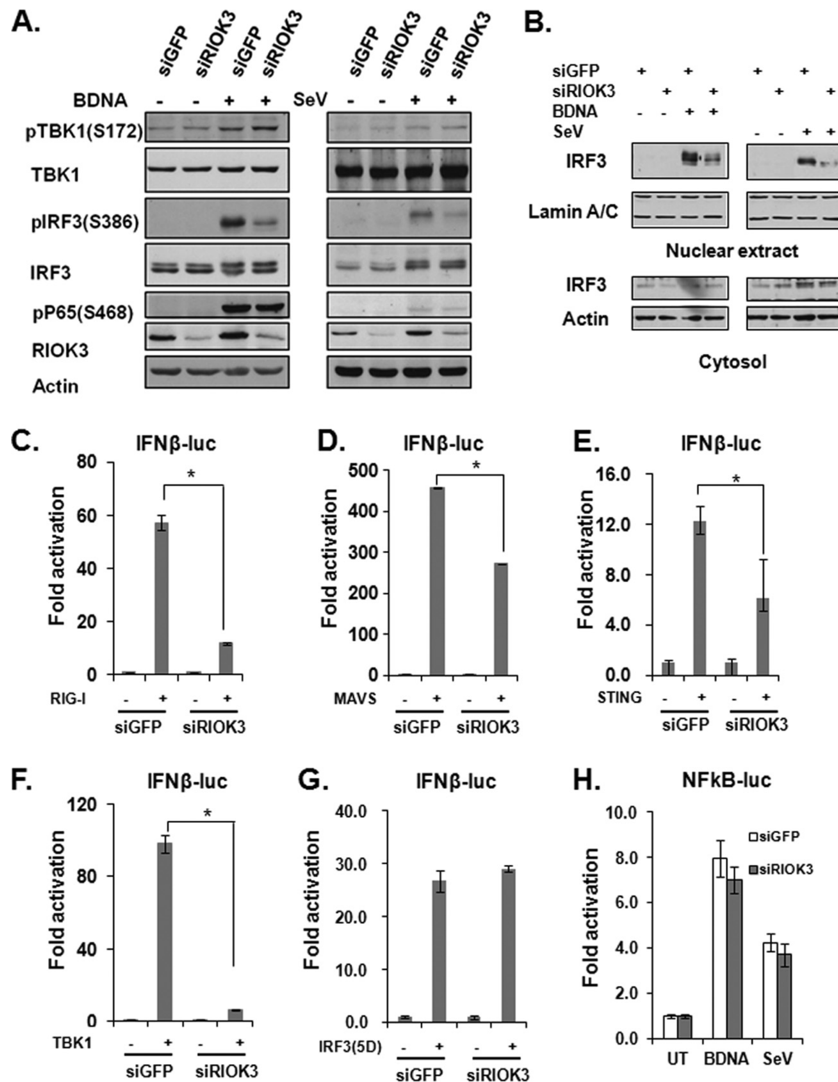

FIG 3 RIOK3 functions downstream of TBK1 and upstream of IRF3. (A) Effect of RIOK3 RNAi on BDNA (left)- or SeV (right)-induced TBK1, IRF3, and p65 phosphorylation. HEK293T cells were transfected with the indicated siRNAs and were left untreated or treated with BDNA or SeV 40 h later. Cell lysates were collected 4 h posttreatment, and immunoblotting was performed with the indicated antibodies. (B) Effect of RIOK3 RNAi on BDNA- and SeV-induced IRF3 nuclear translocation. HEK293T cells were transfected with the indicated siRNAs and were left untreated or treated with BDNA or SeV 40 h later. Nuclear extracts were prepared at 6 h posttreatment and analyzed by immunoblotting with the indicated antibodies. (C to G) HEK293T cells were cotransfected with IFN-β_luc and PGK_Renilla-luciferase reporter, together with the indicated cDNA plasmids and siRNAs. Luciferase assays were performed at 48 h posttransfection. (H) Effect of RIOK3 RNAi on BDNA- or SeV-induced NF-κB promoter activation. HEK293T cells were cotransfected with NF-κB_luc and PGK_Renilla-luciferase reporter, together with the indicated cDNA plasmids and siRNAs. At 24 h posttransfection, the cells were left untreated or infected with 100 HAU of SeV/ml or transfected with BDNA for 20 h before luciferase assays were performed. The fold activation was calculated compared to the untreated cells. *, $P < 0.05$ (Student *t* test). Error bars indicate the SD. All results are representative of three replicate experiments.

tivity was not affected by reduced RIOK3 expression achieved by siRNA (Fig. 1D). These results indicate that RIOK3 is required for the induction of type I IFN production but not for IFN downstream signaling. We next confirmed the reporter assay results by examining the endogenous gene expression. Knockdown of RIOK3 was sufficient to block the BDNA-induced transcription of the endogenous IFN-β1 (*ifnb1*) and the downstream ISG15 (*isg15*) by 5-fold and 24-fold, respectively (Fig. 1E), as well as the BDNA-induced production of IFN-β protein by 60-fold (Fig. 1F).

In addition to HEK293T cells, we also tested the role of RIOK3 in THP-1 cells and murine bone marrow-derived macrophages (BMMs). Similarly, knockdown of RIOK3 in THP-1 cells and BMMs also markedly attenuated the BDNA-induced transcription of *ifnb1* (Fig. 1G), further supporting the critical role of RIOK3 in the type I IFN pathway.

RIOK3 is required for cytosolic dsRNA induced type I IFN induction. Because RIOK3 is essential for type I IFN induction upon cytosolic DNA recognition, we then examined whether RIOK3 is also required for type I IFN induction by Sendai virus (SeV), which is an RNA virus of the *Paramyxoviridae* family and can be sensed through the RIG-I pathway (17). As shown in Fig. 2A, both RIOK3 siRNAs significantly reduced the activation of IFN-β promoter induced by SeV infection. Consistently, the induction of the *ifnb1* and *isg15* transcription (Fig. 2B), as well as

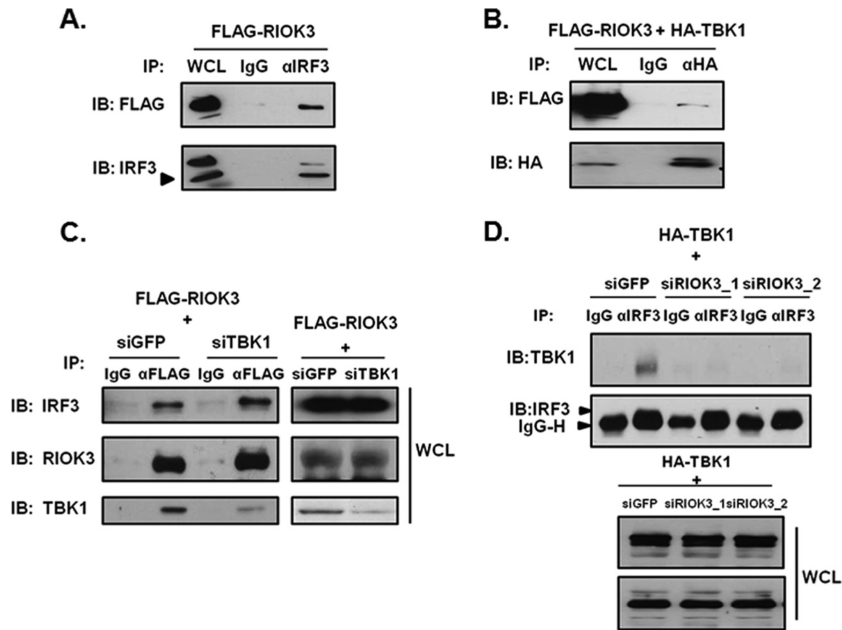

FIG 4 RIOK3 links TBK1 to IRF3. (A) RIOK3 interacts with IRF3. HEK293T cells were transfected with FLAG-RIOK3. Cell lysates were immunoprecipitated with the indicated antibodies. The arrow indicates the IRF3 band, and the upper band is the FLAG-tagged RIOK3 from the FLAG antibody blotting. (B) RIOK3 interacts with TBK1. HEK293T cells were transfected with FLAG-RIOK3 and HA-TBK1. Cell lysates were immunoprecipitated with the indicated antibodies. (C) TBK1 is not required for the interaction between RIOK3 and IRF3. HEK293T cells were transfected with FLAG-RIOK3 and the indicated siRNAs. Cell lysates were immunoprecipitated with the indicated antibodies. All results are representative of three replicate experiments. (D) RIOK3 is required for the interaction between TBK1 and IRF3. HEK293T cells were transfected with HA-TBK1 and the indicated siRNAs. Cell lysates were immunoprecipitated with the indicated antibodies. All results are representative of three replicate experiments.

IFN-β protein production (Fig. 2C) by SeV, was strongly attenuated in the absence of RIOK3. Similarly, knockdown of RIOK3 in THP-1 cells and BMMs also markedly attenuated the SeV-induced transcription of *ifnb1* (Fig. 2D).

In addition, we also tested whether RIOK3 plays a role in the long form poly(I·C)-mediated type I IFN production, which is largely governed by MDA5 (46). Knockdown of RIOK3 resulted in a reduction in the IFN-β promoter activity and *ifnb1* expression (Fig. 2E and F). Thus, we conclude that RIOK3 is crucial for both RIG-I- and MDA5-dependent signaling. Together with the data presented in Fig. 1, these results suggest that RIOK3 functions as a shared node of both intracellular dsDNA and dsRNA recognition pathways.

RIOK3 functions downstream of TBK1 and upstream of IRF3 phosphorylation. Since the induction of IFN-β production by BDNA and SeV is IRF3 dependent, we examined whether knockdown of RIOK3 affected IRF3 activation. Inactive IRF3 resides in the cytoplasm and becomes activated by phosphorylation at several serine and threonine residues. Activated IRF3 translocates into the nucleus and forms a complex with CBP to activate transcription of IFN-β (33, 35). Knockdown of RIOK3 significantly decreased BDNA-induced IRF3 phosphorylation and nuclear translocation (Fig. 3A and B), which are the hallmarks of IRF3 activation. Similarly, phosphorylation and nuclear translocation of IRF3 upon SeV infection were also largely reduced by knockdown of RIOK3 (Fig. 3A and B). These results indicate that RIOK3 functions upstream of IRF3 phosphorylation.

Previous studies have demonstrated that various proteins are directly or indirectly involved in IRF3 activation, including RIG-I, MAVS, STING, TBK1, IKKε, etc. Thus, we sought to determine the epistatic relationship between RIOK3 and these signaling molecules upstream of IRF3 activation. Consistent with current knowledge, the overexpression of RIG-I, MAVS, STING, or TBK1 could induce IFN-β promoter activity. We found that the ability of these proteins to induce the IFN-β promoter was impaired by the depletion of RIOK3 with RNA interference (RNAi) (Fig. 3C, D, E, and F), but the IFN-β promoter activity induced by the constitutively active form of IRF3 was not affected by siRIOK3 (Fig. 3G). TBK1 is one of the kinases responsible for IRF3 phosphorylation (3, 34), and TBK1 itself is activated by phosphorylation. Although RIOK3 is critical for IRF3 phosphorylation induced by BDNA or SeV, knockdown of RIOK3 had no effect on TBK1 phosphorylation at serine 172, an important phosphorylation event for TBK1 activation (Fig. 3A). Because BDNA transfection or SeV infection also activates NF-κB and RIOK3 was previously shown to negatively regulate TNF-α-induced NF-κB signaling (44), we determined whether knockdown of RIOK3 had any effect on NF-κB activation. As shown by reporter assay and Western blotting, knockdown of RIOK3 did not affect BDNA- or SeV-induced NF-κB activation (Fig. 3A and H). Collectively, the results indicate that RIOK3 functions downstream of TBK1 activation and upstream of IRF3 phosphorylation in the signaling pathway leading to IFN-β production.

RIOK3 physically interacts with IRF3 and bridges TBK1 to IRF3. As suggested by our data, RIOK3 functions downstream of TBK1 and upstream of IRF3. To further examine the mechanisms underlying the function of RIOK3, we sought to determine whether RIOK3 interacted with TBK1 and IRF3. RIOK3 was cotransfected with either TBK1 or IRF3 into HEK293T cells. The coimmunoprecipitation results showed that RIOK3 could inter-

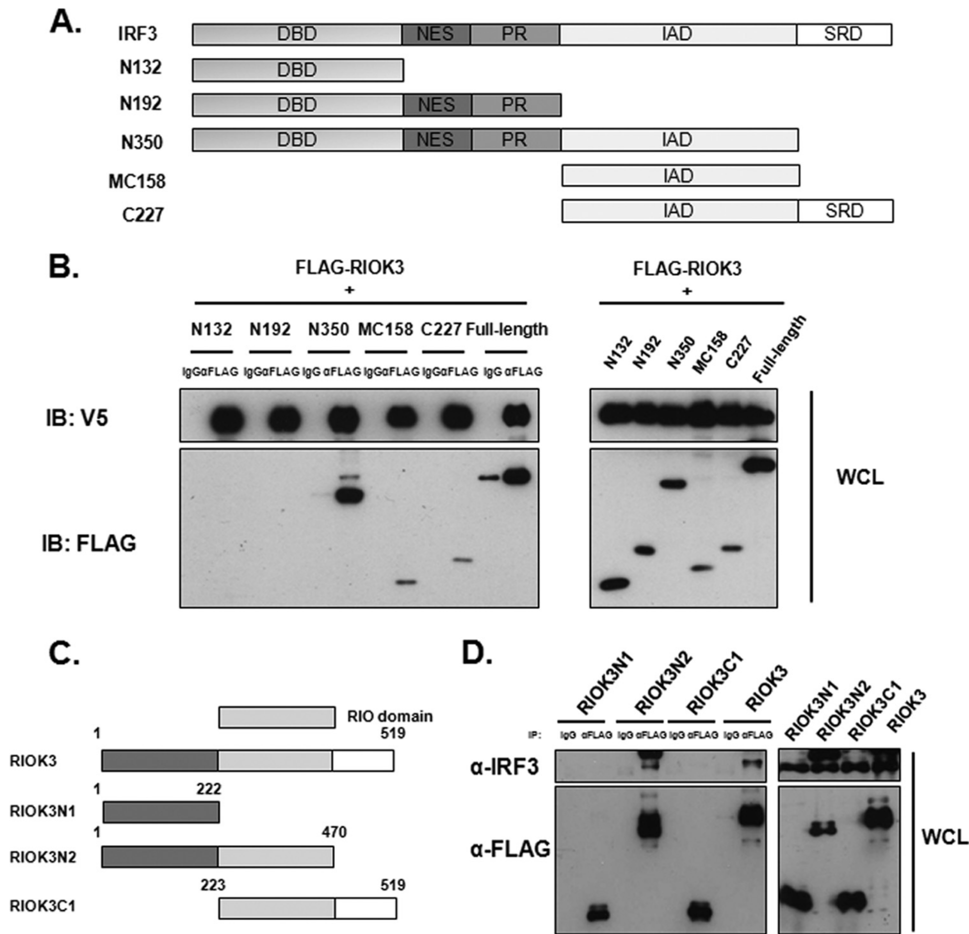

FIG 5 Map of the interaction domain on IRF3 and RIOK3. (A and C) Schematic illustration of IRF3 mutants (A) and RIOK3 mutants (C). (B and D) Domain map of the RIOK3-IRF3 interaction. HEK293T cells were transfected with the indicated plasmids, and immunoprecipitation and immunoblot analysis were performed with the indicated antibodies.

act strongly with endogenous IRF3 (Fig. 4A). To map the interaction domain on IRF3 and RIOK3, we constructed several IRF3 and RIOK3 truncation mutants (Fig. 5). It was observed that the IRF association domain of IRF3 is necessary and sufficient for its interaction with RIOK3 (Fig. 5B). Of the RIOK3 protein, the RIO domain is critical for its interaction with IRF3 (Fig. 5D).

In contrast, there is a low level of interaction between RIOK3 and TBK1 (Fig. 4B). Since TBK1 is responsible for IRF3 phosphorylation and activation (3, 34), we hypothesized that RIOK3 serves as an essential adaptor bridging TBK1 and IRF3. To determine whether RIOK3 is required for recruitment of TBK1 to IRF3, we examined the effect of RIOK3 knockdown on the association of TBK1 with IRF3. The coimmunoprecipitation experiments revealed that depletion of RIOK3 impaired the association of TBK1 with IRF3 (Fig. 4D). However, when we knock down TBK1 using siRNA, RIOK3 and IRF3 can still interact with each other (Fig. 4C), indicating that TBK1 is linked to IRF3 through RIOK3.

Kinase activity is essential for RIOK3 signaling. To further analyze the function of RIOK3, we constructed an RIOK3 expression plasmid. Ectopic expression of FLAG-tagged RIOK3 in HEK293T cells activated the IFN-β promoter activity in a dose-dependent manner (Fig. 6A). Consistently, overexpression of RIOK3 induced IRF3 phosphorylation in a dose-dependent manner (Fig. 6E), but not TBK1 phosphorylation. Furthermore, RIOK3 overexpression potentiated IFN-β promoter activity upon BDNA or SeV stimulation (Fig. 6B and C).

To determine whether the kinase activity of RIOK3 is required for its function in the type I IFN pathway, we constructed a RIOK3 kinase-dead mutant K290A, in which the lysine at position 290 critical for ATP binding was mutated to alanine (44) and tested whether the kinase activity of RIOK3 is required for activation of the IFN-β promoter activity. Although the kinase-dead mutant was still able to interact with both IRF3 and TBK1 (Fig. 6D), overexpression of the kinase-dead mutant did not activate the IFN-β promoter (Fig. 6A) or induce IRF3 phosphorylation (Fig. 6D). These data suggest that the ability of RIOK3 to induce IRF3 activation and IFN-β production depends on its kinase activity.

Global analysis of gene expression dependent on RIOK3. To assess RIOK3-dependent global gene expression changes in response to BDNA or SeV, we utilized RNA-seq analyses. We transfected HEK293T cells with control siRNA or siRIOK3 and then stimulated the cells with BDNA or SeV. The mRNA expression profiles were analyzed 10 h later by RNA-seq. We compared the fold changes in mRNA expression between BDNA-transfected cells and mock-transfected cells and between SeV-infected cells and mock-infected cells. Both BDNA and SeV can induce a wide

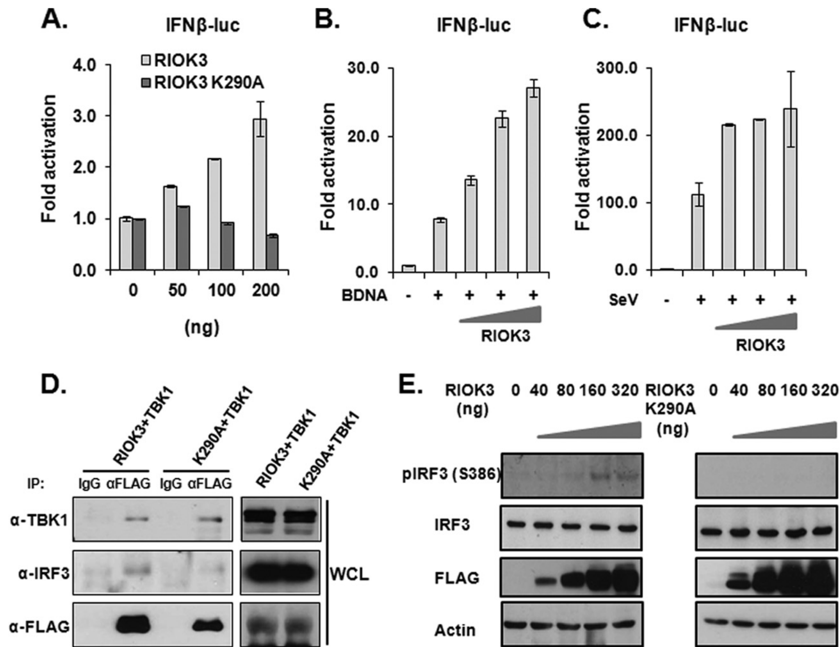

FIG 6 Overexpression of RIOK3 activates IRF3. (A) RIOK3 activates IFN-β promoter in a dose-dependent manner in HEK293T cells. HEK293T cells were cotransfected with IFN-β_luc and PGK_Renilla-luciferase reporter, together with increased amounts of RIOK3 or RIOK3 K290A. A luciferase assay was performed at 48 h posttransfection. The fold activation was calculated compared to the control vector. (B and C) Overexpression of RIOK3 boosts the BDNA and SeV-induced IFN-β promoter activity. HEK293T cells were cotransfected with IFN-β_luc and PGK_Renilla-luciferase reporter, as well as the indicated amount of RIOK3. At 24 h posttransfection, the cells were either left untreated or treated with BDNA (B) or SeV (C) for 20 h before luciferase assays were performed. The fold activation was calculated compared to the untreated control vector. (D) Kinase-dead RIOK3 interacts with TBK1 and IRF3. HEK293T cells were transfected with the indicated plasmids. Cell lysates were immunoprecipitated with the indicated antibodies. (F) Overexpression of RIOK3 results in IRF3 phosphorylation. HEK293T cells were transfected with the indicated amount of RIOK3 or RIOK3 K290A. After 24 h, cell lysates were collected and analyzed by immunoblotting with the indicated antibodies. All results are representative of three replicate experiments.

array of genes, in addition to type I IFN, in the cells transfected with control siRNA. The induction of many of these genes appeared to be RIOK3-dependent since knockdown of RIOK3 greatly reduced their induction by BDNA or SeV (see Tables S1 and S2 in the supplemental material).

Next, we performed pathway analysis of genes with significantly less induction in RIOK3 knockdown cells compared to the control cells after stimulation with BDNA or SeV through the use of IPA (Ingenuity Systems). This analysis showed that RIOK3 knockdown cells had significantly lower expression of genes encoding molecules functionally related to IRF activation by cytosolic PRRs and antiviral innate immunity than did cells transfected with control siRNA ($P < 0.01$; Fig. 7A and B). These data further confirmed the idea that loss of RIOK3 protein negatively regulated the transcriptional program of innate immune response to cytosolic nucleic acids.

Loss of RIOK3 affects host defense against DNA and RNA viruses. As indicated by the RNA-seq data, we next evaluated the importance of RIOK3 in mediating cellular antiviral response. To test this, two viruses were used: MHV-68, a DNA virus of the herpesvirus family, and influenza A virus (A/WSN/33), an RNA virus of the orthomyxovirus family. HEK293T cells were first transfected with control or RIOK3 siRNA and then infected with a reporter MHV-68 virus (MHV-68-M3/FL) (41) or influenza A virus at a multiplicity of infection (MOI) of 0.05. At 48 h postinfection, the virus titers in the supernatants were quantified by luciferase assay for MHV-68 and by limiting dilution for influenza A virus, respectively. Knockdown of RIOK3 led to an increase of both MHV-68-M3/FL and influenza A virus titer by 5-fold and 15-fold, respectively, compared to the control cells (Fig. 7C and D). These data indicated that the depletion of RIOK3 rendered cells more susceptible to viral replication, possibly due to impaired production of type I IFN. Thus, we conclude that RIOK3 plays a pivotal role in cellular antiviral response.

DISCUSSION

Both cytosolic foreign dsDNA and dsRNA can be recognized by different PRRs in the cytosol and induce the production of type I IFNs. Recent studies have identified and characterized two important adaptor molecules MAVS and STING downstream of PRRs (18–21, 27–30). The major converging point of the two cytosolic nucleic acids recognition pathways is TBK1/IKKε and IRF3, which can ultimately induce the robust expression of type I IFNs. Type I IFNs are then secreted, after which they bind to IFN receptors in an autocrine or paracrine fashion, and activate the JAK/STAT pathway to initiate the production of ISGs which are the potent effectors of antiviral innate immune responses. In the present study, we report the identification of a critical adaptor protein, RIOK3, in the signaling pathway leading to the type I IFN production induced by both cytosolic DNA and RNA. More specifically, RIOK3 is required for type I IFN production rather than downstream signaling. Knockdown of endogenous RIOK3 inhibited IRF3 activation induced by BDNA and SeV, therefore suppressing IRF3-mediated IFN-β production. Conversely, ectopic expression of RIOK3 resulted in enhanced activation of IRF3, therefore inducing the IFN-β promoter activity. In addition, the induction of the IFN-β promoter activity

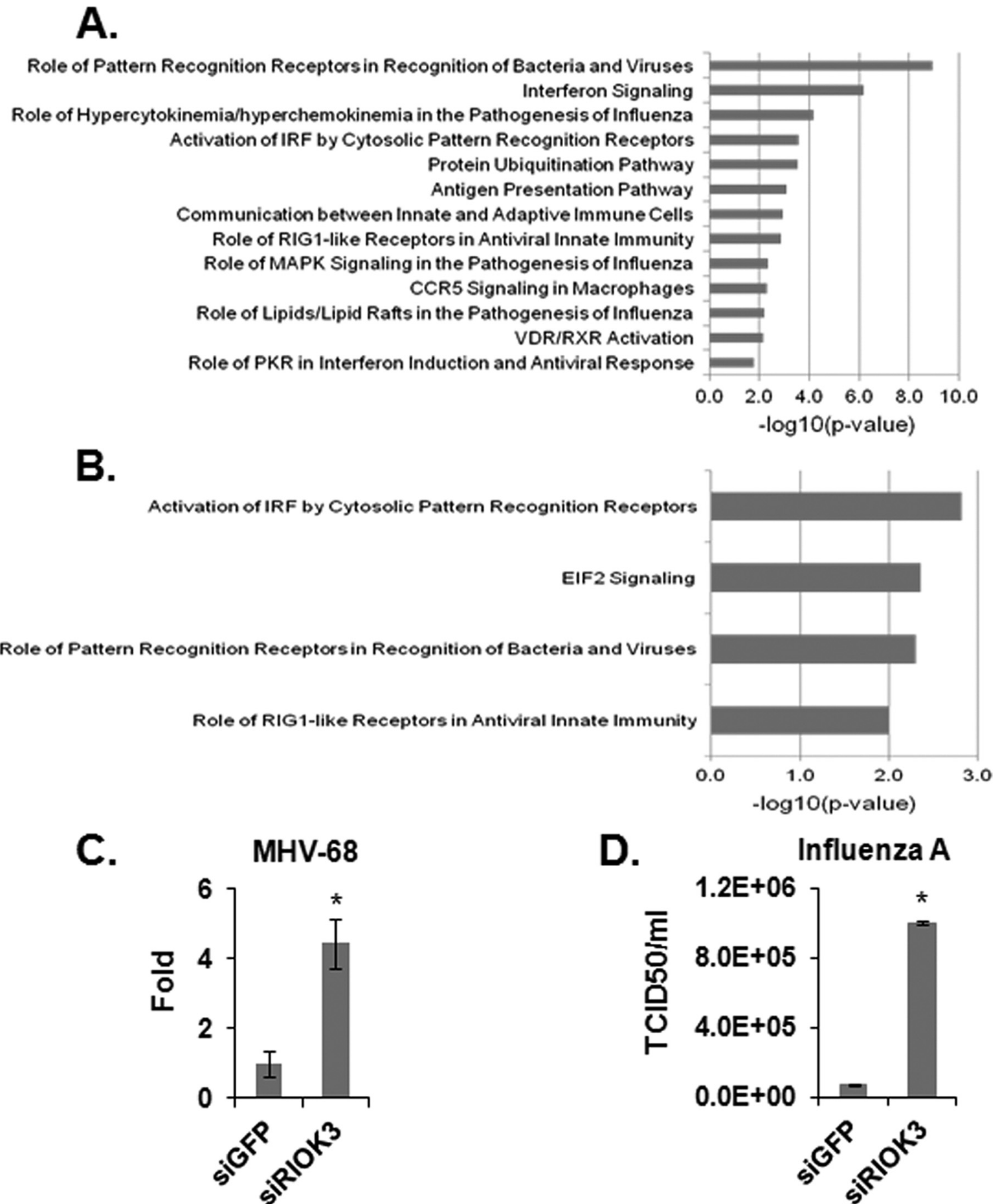

FIG 7 Loss of RIOK3 affects host defense against viruses. (A and B) IPA pathway analysis of differentially expressed genes between RIOK3 knockdown cells and control knockdown cells upon BDNA or SeV induction. (C) Effect of RIOK3 RNAi on MHV-68 replication. HEK293T cells were transfected with the indicated siRNAs and were infected with MHV-68-M3/FL luciferase reporter virus at an MOI of 0.05 for 48 h. The relative virus titer was measured by luciferase assay 24 h later. (D) Effect of RIOK3 RNAi on influenza virus A replication. HEK293T cells were transfected with the indicated siRNAs and were infected with influenza A virus at an MOI of 0.01 for 48 h. The virus titer was measured by limiting dilution. *, $P < 0.05$ (Student *t* test). Error bars indicate the SD. All results are representative of three replicate experiments.

with BDNA or SeV was further increased by RIOK3 overexpression. The significance of RIOK3 in the cellular antiviral response was implicated by the overall gene expression profiles and manifested by the enhanced replication of both MHV-68 and influenza A viruses upon knockdown of RIOK3.

Our data suggest that RIOK3 acts downstream of TBK1. First, knockdown of RIOK3 inhibited RIG-I-, MAVS-, STING-, and TBK1-mediated IFN-β activation but not IRF3(5D)-mediated IFN-β activation. Second, reduced RIOK3 expression blocked IRF3 phosphorylation but had little effect on TBK1 activation

indicated by phosphorylation at serine 172. Third, overexpression of RIOK3 activated only IRF3 but not NF-κB, whereas TBK1 activated both NF-κB and IRF3. Furthermore, coimmunoprecipitation experiments showed that RIOK3 interacted with both IRF3 and TBK1. Importantly, the interaction of IRF3 and TBK1 could be detected when TBK1 was overexpressed, and this interaction was disrupted by knockdown of RIOK3. Based on these results, we propose that RIOK3 acts as an adaptor protein essential for the recruitment of TBK1 to IRF3. This function makes RIOK3 distinct from the previously identified positive regulators of IRF3-mediated type I IFN production. Unlike TBK1, the other IRF3 kinase IKKε is only expressed in certain immune cells (47). It has been demonstrated that IKKε is also required for the amplification of type I IFNs such as IRF7 (47). Further studies on the interaction between RIOK3 and other components of the type I IFN pathway, such as IRF7 and IKKε, will provide more insight into the functions and mechanisms of RIOK3.

In addition to acting as an adaptor, our results also suggest that RIOK3 functions as a kinase during IRF3 activation. We showed that ectopic expression of RIOK3, but not the kinase-dead mutant, was able to induce IRF3 phosphorylation. RIOK3 belongs to the RIO family of atypical kinases, which is conserved from *Archaea* to humans (42). There are three RIO kinases: RIO1, RIO2, and RIO3. All organisms contain at least two RIO proteins, which are homologous to yeast RIO1 and RIO2. Previous studies using yeast have shown that RIO1 and RIO2 are important for ribosome biogenesis and cell cycle progression (42). In addition, the role of human RIO1 and RIO2 in ribosome biogenesis has been recently demonstrated (42). The yeast RIO1 and RIO2 are capable of autophosphorylation, and their kinase activity is essential for their reported functions. Unlike RIO1 and RIO2, RIO3 was only discovered in the multicellular eukaryotes, and the biological function of human RIOK3 remains largely unknown (48). RIOK3 is highly expressed in erythroid cells and is required for erythroblast enucleation (43, 49). The next most abundant RIOK3-expressing cells are of lymphoid and myeloid lineage, but the function of RIOK3 in these cell types has not been studied (43). There is evidence that RIOK3 can undergo autophosphorylation (44). Although the phosphorylation and activation of IRF3 is thought to be mediated by TBK1 and IKKε (33, 35, 50, 51), the exact phosphorylation sites and the contribution of each phosphorylated serine and threonine residue to IRF3 activation remains controversial (33, 35, 50, 51). Since RIOK3 is thought to possess serine/threonine kinase activity similar to other RIO family members, one hypothesis would be that IRF3 is a substrate for RIOK3. Although our *in vitro* kinase assay failed to demonstrate phosphorylation of IRF3 by RIOK3, such failure is difficult to interpret without a proper positive control. Alternatively, RIOK3 may indirectly activate another kinase through phosphorylation, which in turns acts on IRF3 or TBK1. Future studies to identify the interacting proteins and physiological substrates of RIOK3 will shed lights on the underlying molecular mechanisms by which RIOK3 facilitates TBK1-mediated activation of IRF3.

We have shown that the loss of RIOK3 expression renders cells more susceptible to MHV-68 and influenza A virus replication, thereby proposing that the RIOK3-dependent pathway is central to antiviral response against a wide variety of viruses. Ultimately, experiments using RIOK3-deficient mice are needed to address the *in vivo* significance of RIOK3 in antiviral immune responses. Nevertheless, the results presented here clearly support a critical role of RIOK3 in the signaling pathway of sensing cytosolic nucleic acids to IRF3 activation and type I IFN production. In addition, RIOK3 may add another level of complexity in the regulation of innate immunity. Therefore, further work to elucidate how RIOK3 functions will be useful for developing novel strategies to combat viral infection.

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