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RESEARCH ARTICLE

Krüppel-like factor 4 negatively regulates cellular antiviral immune response

Wei-Wei Luo, Huan Lian, Bo Zhong, Hong-Bing Shu and Shu Li

Viral infection triggers activation of the transcription factors NF-κB and IRF3, which collaborate to induce the expression of type I interferons (IFNs) and elicit innate antiviral response. In this report, we identified Krüppel-like factor 4 (KLF4) as a negative regulator of virus-triggered signaling. Overexpression of KLF4 inhibited virus-induced activation of ISRE and IFN-β promoter in various types of cells, while knockdown of KLF4 potentiated viral infection-triggered induction of *IFNB1* and downstream genes and attenuated viral replication. In addition, KLF4 was found to be localized in the cytosol and nucleus, and viral infection promoted the translocation of KLF4 from cytosol to nucleus. Upon virus infection, KLF4 was bound to the promoter of *IFNB* gene and inhibited the recruitment of IRF3 to the *IFNB* promoter. Our study thus suggests that KLF4 negatively regulates cellular antiviral response.
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Keywords: innate antiviral response; KLF4; promoter; translocation; type I interferons

INTRODUCTION

Germline-encoded pattern-recognition receptors (PRRs) detect the nucleic acid from invading viruses and initiate a series of cellular events that induce expression of type I interferons (IFNs).[1] Type I IFNs further induce transcriptional induction of a wide range of downstream antiviral genes, the products of which lead to inhibition of viral replication, apoptosis of virus-infected cells and cellular antiviral response.[1,2] Therefore, type I IFNs play a central role in innate antiviral immunity. It has been well documented that transcriptional induction of type I IFN genes requires the coordinated activation of multiple transcription factors and their subsequent assembly onto the enhancers of type I IFN genes. For example, the *IFNB1* gene promoter contains conserved enhancer elements recognized by NF-κB (κB site) and IRF3 (ISRE site, also known as PRDIII or IRFE).[1,3]

So far, at least two families of PRRs for recognition of viral RNAs have been well characterized.[4] One is membrane-bound Toll-like receptors (TLRs) such as TLR3 and TLR7/8. Engagement of TLR3 by double-stranded RNA triggers TRIF-mediated signaling pathways, while TLR7/8 recognizes ssRNA to activate MyD88-dependent signaling.[5,6] The other involves the cytosolic RIG-I-like receptors including RIG-I and MDA5.[7] The binding of the C-terminal RNA helicase domain of RIG-I to viral RNA results in its conformational change to form a prion-like structure of the N-terminal CARD domains, which recruits the mitochondrial adaptor VISA (also called MAVS, IPS-1, Cardif) and promote the CARD domain of VISA to form prion-like particles which trigger downstream signaling cascades.[8–13] A number of proteins have been implicated in mediating TRIF-, MyD88- or VISA-dependent signaling pathways, including TRAFs, MITA, IKKs, TRADD and RIP1.[14–18] Among these components, TRAF2 and TRAF6 facilitate K63-linked polyubiquitination of RIP and NEMO/IKKγ, respectively, which causes the activation of NF-κB. MITA and GSK3β are responsible for the recruitment and phosphorylation of TBK1, respectively, which leads to the phosphorylation and activation of IRF3 or IRF7.[19,20] These transcription factors enter nucleus to promote the transcription of type I IFN genes. However, uncontrolled induction of type I IFNs could cause severe side effects such as cytokine storm and even death. Host thus has developed various strategies to negatively regulate virus-triggered induction of type I IFNs by targeting distinct signaling components. For example, we have demonstrated that the E3 ubiquitin ligase RNF5 negatively regulates virus-triggered signaling by targeting MITA for ubiquitination and degradation, and the deubiquitinases OTUB1/2 inhibit TRAF3/6-mediated anti-viral signaling.[21–23] Whether and how other molecules are involved

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in regulating virus-triggered type I IFN induction is of great interest.

Krüppel-like factors (KLFs) belong to a family of transcriptional regulators consisting of seventeen members that contain highly conserved C-terminal DNA binding domains, which has been implicated in cell proliferation, differentiation and growth. The highly conserved C-terminal DNA binding domains consist of three C2H2 zinc fingers that bind to 'GC-box' or 'CACCC-box' motifs in the promoters and/or mediate protein–protein interactions. The N-terminal domains of KLFs contain much more variable activation or repression regions that allow KLFs to participate in different physiological processes.[24] In our previous study, we have identified KLF6 as a co-activator of NF-κB that is essential for NF-κB-mediated transcription of selected downstream genes after TNF-α- and IL-1β stimulation.[25] Whether and how other KLFs function in virus-triggered signaling is currently unknown.

In this study, we found that another KLF family member, KLF4, inhibited virus-triggered signaling. Overexpression of KLF4 inhibited virus-induced activation of IFN-β promoter in a dose-dependent manner. In contrast, knockdown of KLF4 potentiated virus-triggered transcription of *IFNB1* and downstream genes. In addition, KLF4 inhibited cellular antiviral response. Viral infection resulted in the translocation of KLF4 from cytosol to nucleus and KLF4 binding to *IFNB* promoter. Our findings thus demonstrate that KLF4 negatively regulates virus-triggered signaling.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant TNF-α (Peprotech: Rocky Hill, NJ, USA), recombinant IL-1β (Peprotech), mouse monoclonal antibodies against FLAG (Sigma: St, Louis, MO, USA), HA (Convance: Princeton, NJ, USA), β-tublin (Sigma), IRF3 (Santa Cruz Biotechnology: Santa Cruz, CA, USA), GFP (Santa Cruz Biotechnology), p65 (CST: Boston, MA, USA), p-P65(S536) (CST) and p-IκBα(S32/36) (CST) were purchased from the indicated manufacturers. SeV, GFP-vesicular stomatitis virus (VSV) were previously described.[15,26,27] Ectromelia virus (ECTV) is provided by Professor Hanzhong Wang in Wuhan Institute of Virology. Mouse anti-KLF4 was raised against recombinant human full-length KLF4.

Constructs

NF-κB, ISRE, the IFN-β promoter and IRF1 luciferase reporter plasmids, mammalian expression plasmids for HA- or Flag-tagged RIG-I, MDA5, VISA, MITA, TBK1, IKKε, IRF3 and IRF7 were previously described.[9,26,28] Mammalian expression plasmids for human HA-, Flag-KLF4 and its mutants were constructed by standard molecular biology techniques.

Transfection and reporter assays. The experiments were performed as previously described[29]

Briefly, HEK293 cells (1×10^5) were seeded on 24-well plates. One day later, the cells were transfected by calcium phosphate precipitation method. In each transfection, 0.01 μg of pRL-TK (Renilla luciferase) reporter plasmid was added as internal control. Eighteen hrs after transfection, luciferase assays were performed using a dual-specific luciferase assay kit (Promega: Madison, WI, USA). For RNAi experiments, luciferase assays were performed at least 24 h after transfection.

Immunoblot analysis

HEK293 cells were seeded on six-well plates or 10 cm dishes and transfected on the following day by calcium phosphate precipitation or lipofectin. Cells were selected by antibiotics and infected with virus for different time points. The cells were lysed in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were analyzed by standard immunoblot procedures.

Virus manipulation

Viral infection was performed when cells were 70% confluent. The culture medium was replaced by serum-free DMEM before GFP-VSV was added at various multiplicities of infection for 1 h. The cells were washed twice by PBS and fed with DMEM containing 2% FBS. GFP-VSV replication in HEK293 cells was visualized by fluorescence microscopy, and cells were harvested for flow cytometry and immunoblot analysis.

RNAi experiments

Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro RNAi plasmid (Oligoengine Inc.: Seattle, WA, USA). The following sequences were targeted for human KLF4 cDNA:

#1: CAGAATTGGACCCGGTGTA
#2: GGTCATCAGCGTCAGCAAA
#3: GGCAAAACCTACACAAAGA

Real-time PCR

Total RNA was isolated from cells and subjected to real-time PCR analysis to measure expression of mRNA. Gene-specific primer sequences were as follows:

IFNB1: TTGTTGAGAACCTCCTGGCT (forward),
TGACTATGGTCCAGGCACAG (reverse);
RANTES: GGCAGCCCTCGCTGTCATCC (forward),
GCAGCAGGGTGTGGTGTCCG (reverse);
ISG15: TCATCAGGTCAAGGATAGTC (forward),
CCACACTGTATTTGGTGTCTAGG (reverse);
GAPDH: GAGTCAACGGATTTGGTCGT (forward),
GACAAGCTTCCCGTTCTCAG (reverse).

Subcellular fractionation

HEK293 cells (2×10^7) infected with SeV or left uninfected for various time points were washed with PBS by three times. Subcellular fractionations were separated by Nucl-Cyto preparation kit (Applygen: Beijing, China). In brief, cells were lysed by douncing 25 times in 1 ml CER. The homogenates were centrifuged twice at 500*g* for 5 min to precipitate cytosol. The pellets were washed with NER and centrifuged twice at 4000*g* for 5 min to precipitate nucleus. The nucleus fraction was lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM

NaCl, 1 mM EDTA, and 1% Nonidet P-40, protease inhibitor mixture) for immunoblotting analysis.

Chromatin immunoprecipitation (ChIP) assays

Immunoprecipitation was performed with 2 μg of IRF3 antibody, 2 μg of Flag antibody, 5 μg of KLF4 antibody or control antibody, and the immune complexes were absorbed with protein A beads or protein G beads blocked with bovine serum albumin and salmon sperm DNA (Millipore: Bedford, MA, USA). Gene-specific primer sequences were as following: IFNB:

5′-CTGAAAGGGAGAAGTGAAAGTGG-3′ (forward),

5′-TCGAAAGGTTGCAGTTAGAATGTC-3′ (reverse).

Statistical analysis

Students *t*-test were performed this study. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Overexpression of KLF4 inhibits virus-induced signaling

Previously, we have screened KLFs that potentially regulate TNF-α- or IL-1β-triggered signaling and identified KLF6 as a co-activator of NF-κB in mediating TNF and IL-1 signaling.[25] Interestingly, we also noted that KLF4 substantially inhibited TNF-α- and IL-1β-induced activation of NF-κB. In addition, we found that KLF4 inhibited SeV-induced activation of NF-κB (Figure 1a). KLF4 is a tumor suppressor and is ubiquitously expressed in mammalian tissues. It is unclear whether KLF4 functions in cellular antiviral response. We thus made Flag- or HA-tagged expression plasmid encoding human KLF4 and examined its role in virus-triggered signaling.

As shown in Figure 1b, overexpression of Flag-KLF4 inhibited SeV-induced activation of IFN-β promoter and ISRE in a dose-dependent manner. In report assays, overexpression of

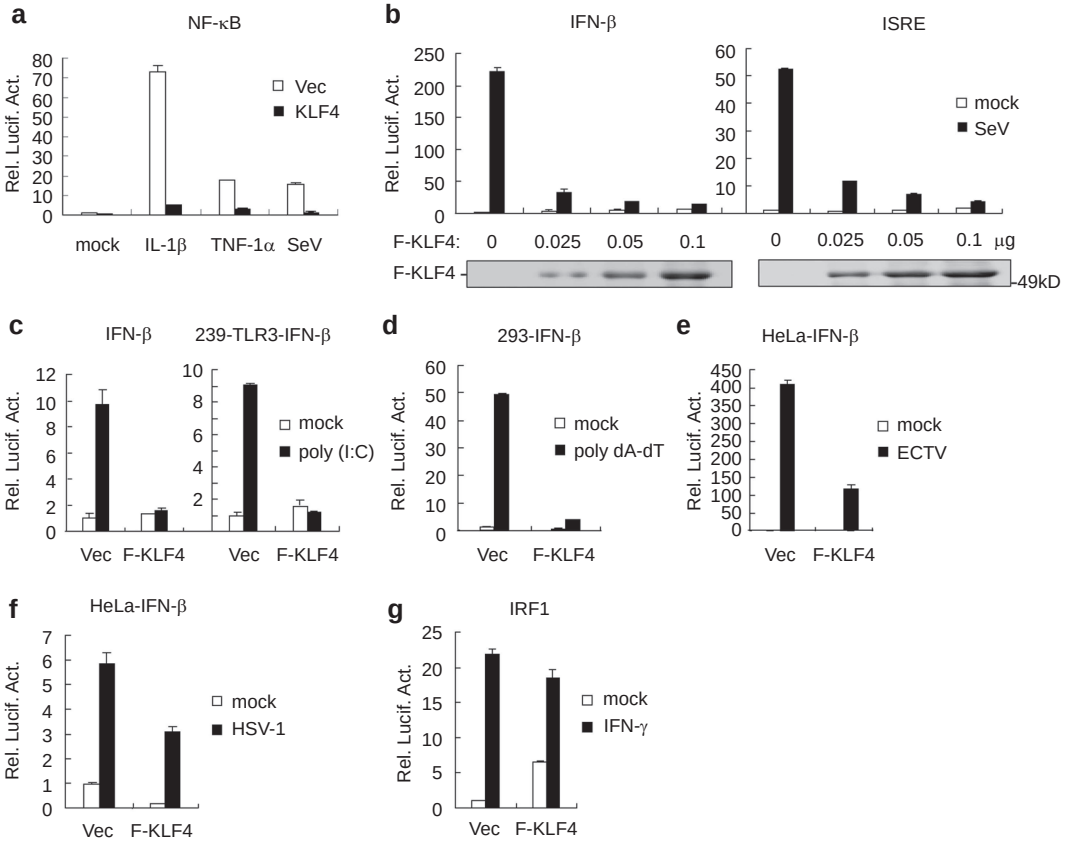

Figure 1 KLF4 inhibited virus-induced signaling. (**a**) KLF4 inhibited IL-1β-, TNF-α- and SeV-induced activation of NF-κB. HEK293 cells (1×10^5) were transfected with NF-κB reporter plasmid (0.05 μg) and Flag-KLF4 expression plasmids. Twenty hours after transfection, cells were left untreated or treated with IL-1β (10 ng/ml), TNF-α (20 ng/ml) or SeV for 12 hrs before luciferase assays were performed. (**b**) Overexpression of KLF4 inhibited SeV-induced activation of IFN-β promoter and ISRE in a dose-dependent manner. HEK293 cells (2×10^5) were transfected with the indicated reporter plasmid and an increased amount of KLF4 expression plasmids. Twenty hours after transfection, cells were left uninfected or infected with SeV for 12 h before luciferase assays and western blot were performed. (**c**) KLF4 inhibited poly(I:C)-triggered activation of the IFN-β promoter. HEK293 cells (1×10^5) or HEK293-TLR3 cells (1×10^5) were transfected with an IFN-β promoter reporter plasmid (0.05 μg) and the indicated expression plasmid (0.05 μg each) for 20 hrs. Cells were further transfected with poly(I:C) (1 μg) or add poly(I:C) (1 μg) in the media for 12 h before luciferase assays were performed. (**d**–**f**) KLF4 inhibited poly(dA:dT)-, ECTV- and HSV-1-induced activation of IFN-β promoter in the indicated cells. Reporter assay was performed similarly as B in except that different stimulators and cells were used. (**g**) KLF4 did not inhibit IFN-γ-induced IRF1 promoter activation. HEK293 cells (1×10^5) were transfected with IRF1 reporter plasmid (0.05 μg) and the indicated expression plasmids (0.05 μg). Twenty hours later cells were treated with IFN-γ (100 ng/ml) for 10 h before luciferase assays were performed. ECTV, Ectromelia virus; HSV, Herpes simplex virus; IFN, interferon; KLF, Krüppel-like factor 4; Rel. Lucif. Act, relative luciferase activity.

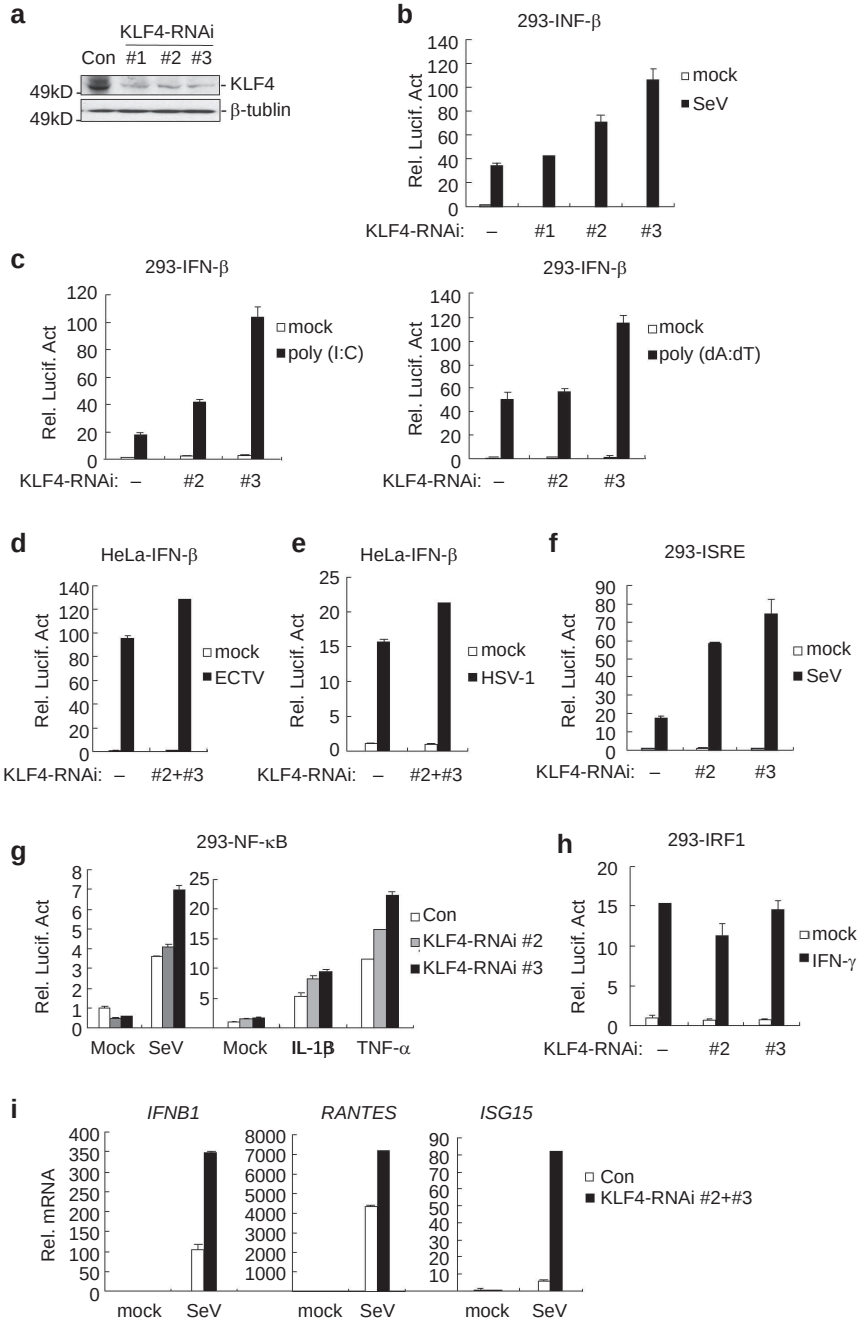

Figure 2 Effects of KLF4-RNAi plasmids on virus-induced signaling. (**a**) Effects of KLF4-RNAi plasmids on expression of their respective target proteins. HEK293 cells (2×10^5) were transfected with the indicated RNAi plasmids (2 μg each) for 36 hrs and cell lysates were analyzed by immunoblots with antibodies against KLF4 and b-Tublin respectively. (**b**) Effects of KLF4-RNAi on SeV-induced activation of the IFN-β promoter. HEK293 cells (1×10^5) were transfected with an IFN-β promoter reporter (0.05 μg) and the indicated RNAi plasmids (0.5 μg each) for 24 h, and then infected with SeV or left uninfected for 12 h before luciferase assays were performed. (**c**) Effects of KLF4-RNAi on poly(I:C)- and poly(dA:dT)-induced IFN-β activation in HEK293 cells. The reporter assays were performed similarly as in B except that different stimulations were used. (**d** and **e**) Effects of KLF4-RNAi on ETCV- and, HSV-1-induced IFN-β activation in HeLa cells. The reporter assays were performed similarly as in B except that different stimulations and cells were used. (**f** and **g**) Effects of KLF4-RNAi on SeV-induced ISRE, as well as IL-1β-, TNF-α- and SeV-induced NF-κB activation. The experiments were done similarly as in B except that ISRE or NF-κB reporter plasmid was used. (**h**) KLF4 did not inhibit IFN-γ-induced IRF1 promoter activation. HEK293 cells (1×10^5) were transfected with IRF1 reporter plasmid (0.05 μg) and the indicated expression plasmids (0.05 μg each). Twenty hours later, cells were treated with IFN-γ (100 ng/ml) for 10 hrs before luciferase assays were performed. (**i**) Effects of KLF4-RNAi on SeV-induced transcription of endogenous *IFNB1*, *RANTES* and *ISG 15* genes. HEK293 cells (2×10^5) were transfected with the indicated RNAi plasmids (2 μg each) for 36 h and then infected with SeV or left uninfected for 8 h before real-time PCRs were performed. ECTV, Ectromelia virus; HSV, Herpes simplex virus; IFN, interferon; KLF, Krüppel-like factor 4.

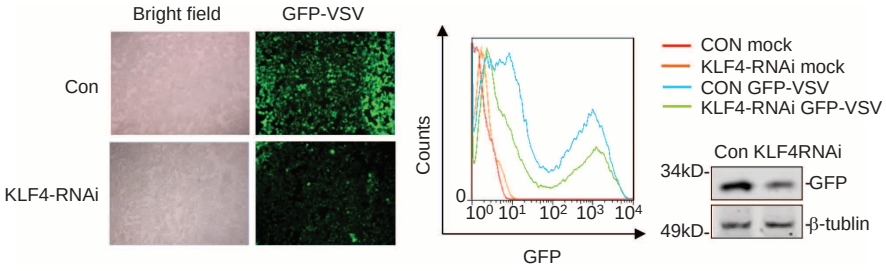

Figure 3 Knockdown of KLF4 inhibits VSV replication. HEK293 cells (1×10^5) were transfected with the control or KLF4-RNAi plasmid (1 μg each). The cells were infected with VSV-GFP (MOI=1) for 18 h followed by microscopy imaging (left panels) or with VSV-GFP (MOI=0.1) for 24 h followed by cell flow cytometry and immunobolt analysis (right panels), respectively. KLF, Krüppel-like factor 4; MOI, multiplicities of infection; VSV, vesicular stomatitis virus.

KLF4 potently inhibited poly(I:C)-triggered RIG-I-like receptor- or TLR3-mediated activation of the IFN-β promoter (Figure 1c) in HEK293 cells. In addition, poly(dA:dT)-, ECTV- or Herpes simplex virus 1 (HSV-1)-induced activation of IFN-β promoter was inhibited by overexpression of KLF4 in HeLa cells (Figure 1d–f). In similar experiments, KLF4 did not inhibit IFN-γ-induced activation of the IRF1 promoter (Figure 1g). These results suggest that KLF4 inhibits virus-triggered signaling.

Knockdown of KLF4 potentiates virus-induced expression of IFNB1

To determine the roles of endogenous KLF4, we made three RNAi constructs (pSuper-KLF4-RNAi #1–3) containing different sequences targeting the human *KLF4* mRNA. Immunoblot analysis indicated that these vectors could inhibit the expression of endogenous KLF4 (Figure 2a). Results from reporter assays showed that these three KLF4-RNAi vectors markedly enhanced SeV-, cytoplasmic poly(I:C)- or poly(dA:dT)-induced activation of the IFN-β promoter in HEK293 cells (Figure 2b and c). In addition, we found that knockdown of KLF4 potentiated ECTV- or HSV-1-induced activation of IFN-β promoter in HeLa cells (Figure 2d and e), indicating that KLF4 negatively regulates RNA or DNA virus-triggered activation of IFN-β promoter in multiple types of cells. We further found that knockdown of KLF4 potentiated SeV-triggered activation of ISRE and NF-κB, and IL-1β- or TNFα-induced activation of NF-κB in HEK293 cells in reporter assays (Figure 2f and g). In the similar experiments, knockdown of KLF4 had no effects on IFN-γ-triggered activation of IRF-1 promoter (Figure 2h). Consistent with these observations, results from real-time PCR experiments confirmed that knockdown of KLF4 potentiated SeV-induced transcription of *IFNB1*, *RANTES* and *ISG15* (Figure 2c). Taken together, these results suggest that KLF4 is a negative regulator of SeV-triggered signaling.

Because KLF4 inhibited virus-triggered signaling, we examined its role in cellular antiviral response. We found that the replication of GFP-tagged VSV was inhibited in KLF4-knockdown cells compared with control cells as monitored by GFP expression (Figure 3). These results indicate that KLF4 negatively regulates cellular antiviral response.

KLF4 regulates virus-triggered signaling at the level or downstream of IRF3

Various molecules have been reported to mediate SeV-triggered signaling, including RIG-I, MDA5, TRIF, VISA TBK1, IKKε, IRF3 and IRF7. To determine the molecular level at which KLF4 regulated virus-induced signaling, we performed reporter assays. As show in Figure 4a, overexpression of KLF4 inhibited ISRE activation induced by overexpression all of the above-mentioned molecules. On the contrary, KLF4-RNAi potentiated IRF3-mediated activation of ISRE (Figure 4b). However, in our transient transfection and coimmunoprecipitation assays, we did not observe the interactions between KLF4 and those signaling components (data not shown). These data

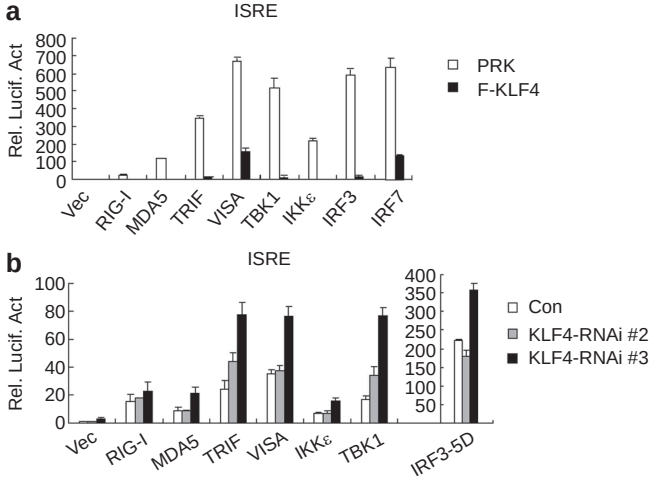

Figure 4 KLF4 regulates virus-triggered signaling at the level of or downstream of IRF3/7. (**a**) Overexpression of KLF4 inhibited activation of ISRE mediated by components of the virus-triggered signaling pathways. HEK293 cells (1×10^5) were transfected with the indicated expression plasmids (0.1 μg) together with ISRE-reporter plasmid (0.05 μg) and Flag-KLF4 expression plasmids for 20 h before reporter assays were performed. (**b**) The effects of KLF4-RNAi on activation of ISRE by components of the virus-triggered signaling pathways. HEK293 cells (1×10^5) were transfected with the indicated RNAi plasmids (0.5 μg). Twelve hours later cells were selected with puromycin for 24 h and further transfected with the indicated expression and reporter plasmids (0.1 μg each) for 20 h before reporter assays were performed. KLF, Krüppel-like factor 4.

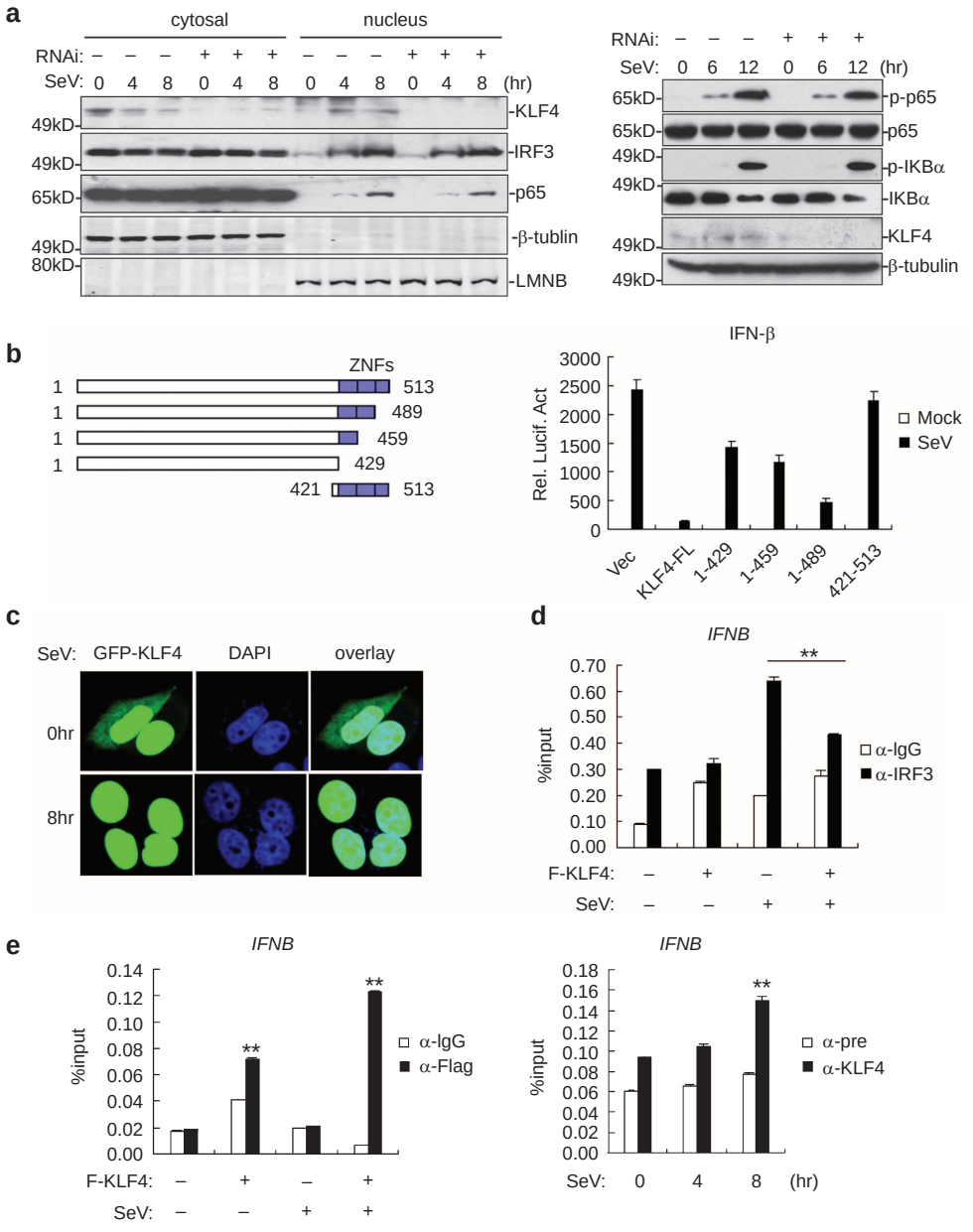

Figure 5 KLF4 bound to the *IFNB* promoter. (**a**) The effects of KLF4-RNAi on the virus-induce translocation of IRF3, p65 and phosphorylation of p65, IκBα. In the left panel, HEK293 cells (2×10^6) were transfected with the indicated RNAi plasmids (10 μg). Twelve hours later cells were selected with puromycin for 24 h and further infected with SeV for the indicated time points. Cell fractionations were performed and the fractions were analyzed by immunoblots with the indicated antibodies. In the right panel, HEK293 cells (2×10^5) were transfected with the indicated RNAi plasmids (5 μg). Twelve hours later, cells were selected with puromycin for 24 h and further infected with SeV for the indicated time points. Cell lysates were analyzed by immunoblots with the indicated antibodies. (**b**) The effect of KLF4 truncation mutants on virus-triggered signaling. Four truncation mutants were constructed as the left map. Report assay was performed as Figure 1b. (**c**) Confocal microscopy of the cellular localization of KLF4. HEK293 cells (1×105) were transfected with GFP-KLF4 expression plasmid. Twenty hours later, cells were left untreated or treated with SeV for 8 h. Transfected cells were stained with DAPI and observed by confocal microscopy. (**d**) Overexpressed KLF4 impaired DNA binding of IRF3 to promoter of *INFB*. HEK293 cells (2×10^6) were transfected with the indicated plasmids (5 μg) and 0.5 μg pSuper for puromycin selection. Tweenty hours later, cells were infected with SeV for 8 h or uninfected. ChIP assays were performed with the indicated antibodies. Binding of IRF3 to the *IFNB* promoter was determined by qPCR. (**e**) KLF4 was recruited to *IFNB* promoter after SeV infection. In the left panels, HEK293 cells (2×10^6) were transfected with the indicated plasmids (5 μg) and 0.5 μg pSuper for puromycin selection. Tweenty hours later, cells were infected with SeV for 8 h or uninfected. ChIP assays were performed with the indicated antibodies. Binding of KLF4 to the *IFNB* promoter was determined by qPCR. In the right panels, HEK293 cells (2×10^7) were infected with SeV for the Findicated time points. ChIP assays were performed with the indicated antibodies. Binding of KLF4 to the *IFNB* promoter was determined by qPCR. **P*<0.05, *n*=3; ***P*<0.01, *n*=3. Values are mean±s.d. for three experiments. ChIP, chromatin immunoprecipitation; KLF, Krüppel-like factor 4.

suggest that KLF4 functions at the level or downstream of IRF3/7 to inhibit virus-triggered signaling.

KLF4 binds to the promoter of *IFNB*

To determine the mechanisms by which KLF4 regulated virus-triggered type I IFN signaling, we examined the effects of KLF4-RNAi on virus-triggered phosphorylation of p65 and IκBα as well as the nuclear translocation of IRF3 or p65 that are hallmarks of activation of NF-κB and IRF3. However, knockdown of KLF4 neither affected the phosphorylation of p65 or IκBα nor potentiated the nuclear translocation of p65 or IRF3, indicating that KLF4 is not involved in the upstream signaling events after viral infection (Figure 5a).

It has been reported that the C-terminal domain of KLF4 contains DNA binding activity, while the N-terminal domain of KLF4 recruits cofactors for transcriptional repression or activation of target genes. In reporter assays, we found that both the N-terminal and C-terminal domains were required for optimal inhibition of virus-triggered activation of IFN-β promoter, indicating that the intact structure of KLF4 is important for its function (Figure 5b). In addition, we observed that KLF4 was located in the cytosol and nucleus, while SeV infection resulted in nuclear accumulation of KLF4 (Figure 5a and c), indicating that KLF4 may exerts the inhibitory function in the nucleus. Interestingly, we found that KLF4 was bound to the promoter of *IFNB* gene and this binding was promoted by SeV infection (Figure 5e). In addition, the binding of IRF3 to the promoter of *IFNB* gene was inhibited by overexpression of KLF4 (Figure 5d). These data suggest that KLF4 is a negative regulator of cellular antiviral response and functions on the promoter of *IFNB*.

DISCUSSION

The innate immune system is the first line of host defense against viral infection. After detection of invading viruses, host cells initiate a series of signaling events that lead to the expression of type I IFNs which play a crucial role in innate antiviral immunity. A set of molecules have been characterized as mediators for RNA virus-triggered signaling, including RIG-I, MDA5, VISA, MITA and TRAFs. However, uncontrolled induction of type I IFNs is harmful to the host. In the present study, we identified the transcription factor KLF4 as a negative regulator of innate cellular antiviral response.

In our study, we found that overexpression of KLF4 inhibited SeV-triggered activation of ISRE and IFN-β promoter in a dose-dependent manner, whereas knockdown of KLF4 potentiated virus-triggered induction of downstream genes including *IFNB1*, *RANTES* and *ISG 15* and inhibited replication of VSV. These results together demonstrate that KLF4 plays an inhibitory role in virus-triggered type I IFN signaling.

It has been demonstrated that KLF4 is a transcription factor that controls proliferation, differentiation, and apoptosis. KLF4 modulates the JAK–STAT3 pathway in cortical neural progenitors.[24,30] In our study, we found that KLF4 was located in both the cytosol and the nucleus. SeV infection resulted in the translocation of KLF4 from cytosol to nucleus. Overexpression of KLF4 inhibited virus-induced activation of ISRE and NF-κB, while knockdown of KLF4 had opposite effects. However, knockdown of KLF4 had no effect on virus-triggered phosphorylation of p65, IκBα or the nuclear translocation of IRF3 and p65. These data together indicate that KLF4 inhibits virus-triggered type I IFN signaling in the nucleus and functions at the level or downstream of IRF3. The C-terminal and N-terminal domains are responsible for the DNA binding and the recruitment of cofactors, respectively. Unexpectedly, neither the C-terminal nor the N-terminal domain alone inhibited SeV-induced activation of IFN-β promoter, indicating that the intact structure of KLF4 is necessary for its inhibitory function. We did not detect the interactions between KLF4 and RIG-I, MDA5, VISA, MITA, TRAFs, IRF3 or p65. Results from ChIP analysis indicated that KLF4 was bound to the promoter of *IFNB* gene and thus inhibited the binding of IRF3 to the *IFNB* promoter after viral infection. These data together suggest that KLF4 inhibits viral infection-triggered expression of *IFNB* by competitively binding to the *IFNB* promoter with IRF3. Further investigations need to be focused on characterizing the sequence target(s) of KLF4. Nonetheless, our study has demonstrated that KLF4 plays a previously uncharacterized role in virus-triggered signaling.

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1 Hiscott J. Convergence of the NF-kappaB and IRF pathways in the regulation of the innate antiviral response. *Cytokine Growth Factor Rev* 2007; **18**: 483–490.
2 Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783–801.
3 Honda K, Takaoka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity* 2006; **25**: 349–360.
4 Baccala R, Gonzalez-Quintial R, Lawson BR, Stern ME, Kono DH, Beutler B *et al*. Sensors of the innate immune system: their mode of action. *Nat Rev Rheumatol* 2009; **5**: 448–456.
5 Beutler B, Eidenschenk C, Crozat K, Imler JL, Takeuchi O, Hoffmann JA *et al*. Genetic analysis of resistance to viral infection. *Nat Rev Immunol* 2007; **7**: 753–766.
6 Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S *et al*. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci USA* 2004; **101**: 17264–17269.
7 Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M *et al*. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004; **5**: 730–737.
8 O'Neill LA, Bowie AG. Sensing and signaling in antiviral innate immunity. *Curr Biol* 2010; **20**: R328–R333.
9 Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* 2005; **19**: 727–740.
10 Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 2005; **122**: 669–682.

11 Meylan E,. Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R *et al*. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167–1172.
12 Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H *et al*. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 2005; **6**: 981–988.
13 Lei CQ, Zhang Y, Li M, Jiang LQ, Zhong B, Kim YH *et al*. ECSIT bridges RIG-I-like receptors to VISA in signaling events of innate antiviral responses. *J Innate Immun* 2014; doi:10.1159/000365971.
14 Ermolaeva MA, Michallet MC, Papadopoulou N, Utermöhlen O, Kranidioti K, Kollias G *et al*. Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses. *Nat Immunol* 2008; **9**: 1037–1046.
15 Zhong B, Yang Y, Li S, Wang YY, Li Y, Diao F *et al*. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 2008; **29**: 538–550.
16 Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 2008; **455**: 674–678.
17 Xu LG, Jin L, Zhang BC, Akerlund LJ, Shu HB, Cambier JC. VISA is required for B cell expression of TLR7. *J Immunol* 2012; **188**: 248–258.
18 Zhang L, Mo J, Swanson KV, Wen H, Petrucelli A, Gregory SM *et al*. NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING. *Immunity* 2014; **40**: 329–341.
19 Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT *et al*. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 2003; **4**: 491–496.
20 Lei CQ, Zhong B, Zhang Y, Zhang J, Wang S, Shu HB. Glycogen synthase kinase 3beta regulates IRF3 transcription factor-mediated antiviral response via activation of the kinase TBK1. *Immunity* 2010; **33**: 878–889.
21 Zhong B, Zhang L, Lei C, Li Y, Mao AP, Yang Y *et al*. The ubiquitin ligase RNF5 regulates antiviral responses by mediating degradation of the adaptor protein MITA. *Immunity* 2009; **30**: 397–407.
22 Li S, Zheng H, Mao AP, Zhong B, Li Y, Liu Y *et al*. Regulation of virus-triggered signaling by OTUB1- and OTUB2-mediated deubiquitination of TRAF3 and TRAF6. *J Biol Chem* 2010; **285**: 4291–4297.
23 Zhong B, Zhang Y, Tan B, Liu TT, Wang YY, Shu HB. The E3 ubiquitin ligase RNF5 targets virus-induced signaling adaptor for ubiquitination and degradation. *J Immunol* 2010; **184**: 6249–6255.
24 McConnell BB, Yang VW. Mammalian Kruppel-like factors in health and diseases. *Physiol Rev* 2010; **90**: 1337–1381.
25 Zhang Y, Lei CQ, Hu YH, Xia T, Li M, Zhong B *et al*. Kruppel-like factor 6 is a co-activator of NF-kappaB that mediates p65-dependent transcription of selected downstream genes. *J Biol Chem* 2014; **289**: 12876–12885.
26 Zhang J, Hu MM, Shu HB, Li S. Death-associated protein kinase 1 is an IRF3/7-interacting protein that is involved in the cellular antiviral immune response. *Cell Mol Immunol* 2014; **11**: 245–252.
27 Qin Y, Zhou MT, Hu MM, Hu YH, Zhang J, Guo L *et al*. RNF26 temporally regulates virus-triggered type I interferon induction by two distinct mechanisms. *PLoS Pathog* 2014; **10**: e1004358.
28 Diao F, Li S, Tian Y, Zhang M, Xu LG, Zhang Y *et al*. Negative regulation of MDA5- but not RIG-I-mediated innate antiviral signaling by the dihydroxyacetone kinase. *Proc Natl Acad Sci USA* 2007; **104**: 11706–11711.
29 Wang S, Li Y, Hu YH, Song R, Gao Y, Liu HY *et al*. STUB1 is essential for T-cell activation by ubiquitinating CARMA1. *Eur J Immunol* 2013; **43**: 1034–1041.
30 Qin S, Zou Y, Zhang CL. Cross-talk between KLF4 and STAT3 regulates axon regeneration. *Nat Commun* 2013; **4**: 2633.