Vimentin inhibits type I interferon production by disrupting the TBK1-IKKε-IRF3 axis

Highlights

- Vimentin expression is upregulated upon virus infection
- Vimentin negatively regulates type I interferon (IFN-I) production
- Vimentin inhibits IFN-I production by targeting TBK1 and IKKε
- Vimentin disrupts the TBK1-IKKε-IRF3 axis to inhibit IFN-I production

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In brief

Liu et al. identify vimentin as a negative regulator of type I interferon (IFN-I) production upon viral infection. Viral infection increases vimentin expression, and subsequently, the upregulated vimentin interacts with TBK1 and IKKε to disrupt the TBK1-IKKε-IRF3 axis, resulting in inhibition of IFN-I production.
Vimentin inhibits type I interferon production by disrupting the TBK1-IKKε-IRF3 axis

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SUMMARY

Cytoskeleton proteins have been reported to be involved in the host antiviral immune responses. However, how cytoskeletal proteins regulate host antiviral immune responses is not fully understood. Here we report that the cytoskeletal protein vimentin is a negative regulator of type I interferon (IFN-I) production upon viral infection. Ectopic expression of vimentin suppresses RNA- and DNA viruses-induced IFN-I production, whereas knockout of vimentin expression enhances IFN-I production. Viral infection increases vimentin expression and ultimately inhibits IFN-I production. Mechanistically, upregulated vimentin interacts with TBK1 and IKKε to disrupt the interactions of TBK1-IRF3 and IKKε-IRF3, resulting in inhibition of IRF3 phosphorylation and nuclear translocation. Furthermore, we generate vimentin knockout mice to confirm that deficiency of vimentin gene in mice suppressed encephalomyocarditis virus replication in vivo. Our findings demonstrate that vimentin plays an important role in regulating IFN-I production, revealing its antiviral function of the cytoskeletal protein vimentin.

INTRODUCTION

Cellular antiviral responses induced by type I interferons (IFN-Is) are considered the first line of host defense against viral infection (Randall and Goodbourn, 2008). The activation of the innate immune system depends on pattern recognition receptors (PRRs) and their corresponding downstream signaling pathways (Seth et al., 2006). The pathogen-associated molecular patterns of the invading viruses can be recognized by PRRs (Ivashik and Donlin, 2014). For example, viral DNA can be identified by cyclic GMP-AMP synthase (cGAS) (Sun et al., 2013), and viral RNA can be recognized by retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Kawasaki et al., 2011). Then, the adaptor proteins (such as STING for cGAS, MAVS/IP-1 for RIG-I) can be recruited (Liu et al., 2015), and the cascade signals are transmitted to the downstream TANK binding kinase 1 (TBK1)/I-kappa B kinase ε (IKKε) kinase complexes (Belgnaoui et al., 2011). The activated TBK1 and IKKε phosphorylate interferon regulation factor 3 (IRF3), leading to the dimerization and nuclear translocation of IRF3 (Fitzgerald et al., 2003; Reiss, 2009). Phosphorylated IRF3 binds to interferon-stimulated response elements (ISREs) and the promoters of the IFN-I genes, leading to the transcriptional induction of IFN-I (Garcia-Sastre and Biron, 2006).

Following the transcriptional activation and mRNA translation, IFN-I is secreted by the viral-infected cells and binds to the two cellular receptors, IFN-α receptor 1 (IFNAR1) and IFNAR2, which associate with tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1), respectively (Plataniás, 2005). Dimerization of the two receptors initiates the autophosphorylation of JAK1, leading to phosphorylation of the signal transducers and activators of transcription 1 (STAT1) and STAT2, which form a complex with IRF9, resulting in a well-characterized complex, IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus to bind to ISREs in the promoters of IFN-stimulated genes (ISGs) (Lukhele et al., 2019), and ultimately, it establishes the host antiviral status. Insufficient production of IFNs leads to decreased antiviral ability of the body, whereas excessive IFN production is associated with autoimmune or inflammatory diseases. Thus, precise control of IFN production is critical for efficient viral clearance without harmful immunopathology (Song et al., 2016).

Vimentin, a cytoskeletal protein that belongs to the type III intermediate filament protein family, is mainly expressed in cells of mesenchymal origin (Carey and Zehner, 1995). The vimentin monomer is an ∼54 kDa protein with a rod shape that is constituted by four alpha-helical segments joined by linkers and flanked by the disorganized head (N-terminal) and tail (C-terminal) domains (Herrmann and Aebi, 2016). Vimentin is present in the fibroblasts, endothelial cells, and immune cells. Vimentin filaments form a network that extends from the nuclear periphery toward the plasma membrane of cells (Schoumacher...
et al., 2010), which interacts with and modulates the function of other cytoskeletal components and acts as integrator of cellular mechanical processes, including cell migration, adhesion, and division (Battaglia et al., 2018; Duarte et al., 2019; Etienne-Manneville, 2018). Vimentin also works in maintaining the organelle position and homeostasis (Chernoivanenko et al., 2015; Styers et al., 2004), aggresome formation (Perez-Sala et al., 2015), and protection of the nucleus in situations of mechanical stress (Watabe and Nakaki, 2011). Furthermore, vimentin plays an essential role in cell signaling transduction by regulating the localization and activity of several MAP kinases, lipid metabolism, and even genome stability (Patteson et al., 2019).

Recently, vimentin was reported to play roles in many vital processes of the immune responses, and vimentin has been described as a ligand for some PRRs. For example, vimentin interacts with the nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and this interaction is required for subsequent NF-κB activation (Stevens et al., 2013). In addition, vimentin is required for assembly and activation of the NLRP3 inflammasome, which mediates the induction and release of proinflammatory cytokines associated with acute lung injury in bacterial and viral infections (Dos Santos et al., 2015). In turn, induction of IFN-I and IFN-III expression leads to increase expression of the vimentin gene (De Rivero Vaccari et al., 2012).

Our study demonstrated that vimentin is a negative regulator of innate antiviral immunity during RNA and DNA virus infection. Vimentin deficiency resulted in enhanced TBK1 activation, IRF3 phosphorylation, and IFN-I production through the activated cGAS-STING and RLRs signaling pathways. Consistently, vimentin−/− mice were more resistant to encephalomyocarditis virus (EMCV) infection than wild-type mice. Mechanistically, vimentin bound with TBK1 and IKKe to block the association between IRF3 and TBK1/IKKe, which inhibited IRF3 phosphorylation and nuclear translocation, resulting in a decrease in the IFN-I production.

RESULTS

Vimentin expression may depend on IFNAR1

To test whether vimentin expression could be upregulated by the IFN signaling, RAW264.7 cells were stimulated with poly(I:C), S′ppp-dsRNA (a RIG-I agonist) (Schmidt et al., 2009) or infected with Sendai virus (SeV), vesicular stomatitis virus (VSV), and encephalomyocarditis virus (EMCV) for 0, 4, 8, or 12 h; the results showed that the mRNA and protein levels of vimentin were significantly elevated in RAW264.7 compared with the untreated cells (Figure 1A). Consistent with these results, the mRNA and protein levels of vimentin in THP-1 cells were also increased when the cells were stimulated with poly(I:C) or infected with SeV, VSV, and EMCV (Figure S1A). Additionally, the mRNA and protein levels of vimentin were also upregulated in RAW264.7 and THP-1 cells, when the two cells were treated with cGAMP or ISD or infected with herpes simplex virus (HSV-1), pseudorabies virus (PRV), and adenovirus (ADV), respectively (Figures 1B and S1B).

Previous studies showed that IFN-I treatment could induce vimentin expression (Nedelec et al., 1995; Trivanovic et al., 2016). Consistently, we also noticed that IFN-α stimulation increased the mRNA and protein levels of vimentin in RAW264.7 cells (Figure 1C). To further confirm these results, we constructed a vimentin promoter reporter and assessed its activation induced by treatment with IFN-α, S′ppp-dsRNA, ISD, or infection with VSV and HSV-1. We found that both viral infection and IFN stimulation can promote the promoter activity of vimentin (Figures S2A–S2E).

Interestingly, the mRNA and protein levels of vimentin in the peritoneal macrophages isolated from wild-type mice were upregulated when the cells were infected with VSV or HSV-1 (Figures 1D and 1E) or transfected with poly(I:C) or poly(dA:dT) (Figures S1C and S1D). Additionally, mRNA and protein levels of vimentin in the peritoneal macrophages from mice-ifnar1−/− were not be affected upon infection with VSV and HSV-1 (Figures 1D and 1E) or stimulation with poly(I:C) and poly(dA:dT) (Figures S1C and S1D), suggesting that induction of vimentin expression may depend on IFNAR1. Consistent with these results, we noticed that the mRNA and protein levels of vimentin in the peritoneal macrophages from mice-ifnar1−/− could not be upregulated after stimulation with IFN-α (Figure 1F). Taken together, our findings indicate that vimentin expression could be upregulated in response to different stimuli, which may be dependent on IFNAR1, suggesting vimentin may play an important role in host antiviral responses.

Vimentin negatively regulates IRF3 activation and type I IFN production

To explore the function of vimentin in host antiviral responses, we generated stable vimentin-overexpressing THP-1 monocytes (THP-1-Vim) by lentivirus infection. The THP-1 cells and THP-1-Vim cells were infected with VSV or HSV-1 for 0, 4, 8, 12 h, and we found that the mRNA levels of Ifnb1 in THP-1-Vim cells were lower than that in THP-1 cells (Figures 2A and 2B), accompanied with enhanced virus replication (Figures 2C and 2D) and inhibition of phosphorylation of IRF3 and TBK1 (Figures 2E and 2F).

To further validate these observations, a vimentin gene knockout (KO) HeLa cell line (HeLa-vim−/−) was generated using CRISPR-Cas9 to examine the function of vimentin. We obtained 10 clones, and seven are vimentin−/− clones by western blotting analysis (Figure S3A). We randomly selected three vimentin−/− cell lines to infect with VSV or HSV-1, and the results from clone #2 (Figure S3B), clone #6 (Figure S3C), and clone #8 (Figure S3D) have no significant difference among the three different vimentin−/− clones. Therefore, we selected #2 cells for follow-up experiments. Consistent with the above results, both the mRNA level of Ifnb1 and the phosphorylation level of IRF3 were significantly increased in the HeLa-vim−/− cells infected with VSV or HSV-1 compared with the HeLa-vim−/− cells infected with VSV or HSV-1 (Figures 2G–2J). Furthermore, overexpressed vimentin in the HeLa-vim−/− cells inhibited IFN production (Figures S3B–S3G). To evaluate whether knockout of vimentin modulates the IFN-I signaling pathway by affecting cell viability, the apoptosis and cell viability of HeLa-vim+/+ and HeLa-vim−/− cells, and rescued cells were detected by flow cytometry using Annexin-V/PI staining or by WST-1 technology. The results showed that knockout of vimentin did not affect the apoptosis level and cell viability of these cells (Figures S3H and S3I). Of note, the results of an IFN sensitivity assay also showed that...
Vimentin inhibited IFN-I production induced by SeV infection in HEK293T cells (Figure 2K). Taken together, all these results indicated that vimentin negatively regulates IFN-I production.

Vimentin deficiency enhances the cellular antiviral responses in vitro

To dissect the function of vimentin in IFN-I production in vivo, we generated vimentin gene knockout mice (mice-vim−/−) by using a homologous recombination technique and validated them by genotyping and western blotting analysis (Figures S4A–S4C). We also validated the cell morphology and organelle positioning such as mitochondria, ER Golgi, and lysosomes in the peritoneal macrophages from mice-vim+/+ and mice-vim−/− by confocal experiments. We found that knockout of vimentin did not affect the morphology and localization of these intracellular organelles (Figures S4D). Spleens were isolated from mice-vim−/− and their wild-type littermates (mice-vim+/+), and total RNA was prepared for microarray analysis. As shown in Figures S4E and S4F, the expression of ISGs and PRRs of the spleen in the mice-vim+/+ was similar to mice-vim−/− without viral infection. Primary peritoneal macrophages from mice-vim−/− and mice-vim+/+ were infected with SeV, VSV, EMCV, and HSV-1 or treated with poly(I:C), cGAMP, ISD, poly(dA:dT), 5′ppp-dsRNA, and HT-DNA, respectively. Compared with peritoneal macrophages from the mice-vim+/+, peritoneal macrophages from mice-vim−/− showed higher mRNA expression of Ifnb1, Ifna4, Isg56, Tnf-α, Il-6, and Il-12 (Figures 3A–3F). To test whether the mRNA expression of Ifnb1 could be affected by vimentin, peritoneal macrophages isolated from mice-vim+/+ and mice-vim−/− were infected with SeV, VSV, EMCV, and HSV-1 or treated with poly(I:C), cGAMP, ISD, poly(dA:dT), 5′ppp-dsRNA, and HT-DNA, respectively. Compared with peritoneal macrophages from the mice-vim+/+, peritoneal macrophages from mice-vim−/− showed higher mRNA expression of Ifnb1 (Figures 3G and 3H) and Ifna4 (Figures 3I and 3J) at different time points. Furthermore, both the viral genomic copy number and viral titers in peritoneal macrophages from mice-vim−/− were significantly lower than that in the peritoneal macrophages from mice-vim+/+. To further verify the regulatory effect of vimentin in TLRs-mediated signaling pathway, primary bone-marrow-derived macrophages isolated from mice-vim−/− and
mice-\text{vim}^{-/-} were stimulated with the agonists of TLR2 (Pam3CSK4), TLR3 (poly I:C), TLR4 (LPS), TLR7 (R848), and TLR9 (CpG). Compared with primary bone-marrow-derived macrophages from the mice-\text{vim}^{+/+}, macrophages from mice-\text{vim}^{-/-} showed higher mRNA expression of \text{Ifnb1}, \text{Ifna4}, \text{Isg56}, \text{Tnf-a}, \text{Il-6}, and \text{Il-12} (Figures S5A–F). Collectively, these results indicate that knockout of vimentin expression enhanced the production of IFN-I, ISGs, and inflammatory cytokines, leading to the inhibition of viral replication.

Vimentin regulates host antiviral immune responses \textit{in vivo}

To further define the function of vimentin in inhibiting IFN-I production \textit{in vivo}, mice-\text{vim}^{-/-} and mice-\text{vim}^{+/+} were challenged with EMCV by intraperitoneal injections. As shown in Figure 4A, mice-\text{vim}^{-/-} were more resistant to EMCV infection than mice-\text{vim}^{+/+}. The mRNA level of \text{Ifnb1} in the heart and brain from mice-\text{vim}^{-/-} was significantly higher than that from mice-\text{vim}^{+/+} after infection with EMCV for 48 h (Figure 4B) and 72 h (Figure 4C). In agreement with these results, the EMCV genomic copy number in the heart and brain from the mice-\text{vim}^{-/-} was significantly lower than in mice-\text{vim}^{+/+} after infection with EMCV for 48 h (Figure 4D) and 72 h (Figure 4E).
The EMCV titers in the heart and brain from the mice-vim−/−/C0 were significantly lower than those in the mice-vim+/+ after infection with EMCV for 72 h (Figure 4F). Correspondingly, the protein levels of IFN-β in serum from the mice-vim−/−/C0 were also significantly increased (Figure 4G). In addition, we found that the brain and heart tissues obtained from EMCV-infected mice-vim−/−/C0 showed slight to mild histopathological changes. In contrast, the tissues obtained from EMCV-infected mice-vim+/+ show severe histopathological changes, and the brain and heart tissues from mice-vim+/+ infected with EMCV showed more inflammatory cell (mononuclear cells and lymphocytes) infiltration, whereas those from mice-vim−/−/C0 showed minor signs of inflammatory cell infiltration (Figure 4H).

Consistently, we also noticed that the mice-vim−/− infected with HSV-1 showed increased mRNA expression levels of Ifnb1 in liver and spleen compared with mice-vim+/+ (Figure 4I). In line with these results, the HSV-1 titers in liver and spleen were significantly lower in mice-vim−/− than that in mice-vim+/+ after infection with HSV-1 (Figure 4J). Based on these data, we concluded that vimentin deficiency enhanced host antiviral immune responses, which resulted in the inhibition of both RNA virus and DNA virus replication and the reduction of virus infection-mediated pathological injury.

**Vimentin targets TBK1 and IKKε**

To elucidate the underlying molecular mechanisms by which vimentin negatively regulates IFN-I production, we evaluated the effect of vimentin on IFN-β reporter activation induced by key molecules in the RLRs and cGAS-STING signaling pathways in HEK293T cells. As shown in Figures S7A–S7G, ectopically expressed vimentin significantly inhibited the IFN-β reporter activation induced by RIG-I, MDA5, MAVS, TBK1, IKKε, and cGAS+STING in a dose-dependent manner but not induced by IRF3-5D (a constitutively active IRF3 variant). In accordance with these results, the IFN-β promoter activation mediated by RIG-I, MDA5, MAVS, TBK1, IKKε, and cGAS+STING was significantly increased in HEK293T-vim−/− cells compared with that in HEK293T-vim+/+ cells, whereas the overexpression of vimentin in HEK293T-vim−/− cells restored the inhibition effect of vimentin on IFN-β promoter activation (Figure 5A). The mRNA levels of Ifnb1 induced by RIG-I, MDA5, MAVS, TBK1, IKKε, and cGAS+STING were also enhanced in HEK293T-vim−/− cells but not by IRF3-5D (Figure 5B). Taken together, these results indicate that vimentin might negatively regulate IFN-I production by targeting IRF3 or its binding partners, such as TBK1 and IKKε.

To further clarify the targets of vimentin, we examined the interaction between vimentin and RIG-I, MDA5, MAVS, TBK1,
IKKε, IRF3, cGAS, and STING. Co-immunoprecipitation (CoIP) results showed that vimentin interacted with TBK1 and IKKε but not others (Figure 5C). To identify the interaction between vimentin and TBK1 or IKKε, CoIP and reverse CoIP assays were performed. As shown in Figures 5D and 5E, vimentin co-immunoprecipitated with TBK1 or IKKε when the indicated proteins were co-expressed in HEK293T cells. In addition, we also found that endogenous vimentin interacted with endogenous TBK1 or IKKε in THP-1 cells following both VSV and HSV-1 infection (Figures 5F and 5G). Notably, the expression of vimentin was upregulated with increasing time of virus infection, and the interaction of vimentin-TBK1 or vimentin-IKKε was also increased. Immunofluorescence staining results also revealed that vimentin colocalized with TBK1 and IKKε in the cytoplasm (Figures 5H–5K). Taken together, all these data showed that vimentin targets the TBK1-IKKε-IRF3 axis.

N terminus domain of vimentin is required for its interaction with TBK1 and IKKε

Vimentin is comprised of 466 amino acids (aa) containing a head domain (1–95 aa), a central rod (coil) domain (96–407 aa), and a tail (C-terminal) domain (Perez-Sala et al., 2015). To identify which domain of vimentin is necessary for its interaction with TBK1 and IKKε, we first tested the interaction of GFP-Vimentin with Flag-TBK1 or Flag-IKKε. As shown in Figures S8A and S8B, GFP-Vimentin interacts with TBK1 or IKKε but not GFP. Subsequently, four plasmids expressing the GFP-fused truncated mutants of vimentin (GFP-Vimentin-N, GFP-Vimentin-Coil, GFP-Vimentin-C, and GFP-Vimentin-ΔN) were constructed (Figure S8C). Domain-mapping studies revealed that both TBK1 and IKKε interacted with GFP-Vimentin-WT and GFP-Vimentin-N but not GFP-Vimentin-Coil and GFP-Vimentin-C, suggesting that the N-terminal domain of vimentin is required for its interaction with both TBK1 and IKKε (Figures S6D and S8E).
To elucidate the functional domain by which vimentin negatively regulates IFN-I production, we assessed the effect of GFP-Vimentin-WT and its four truncated mutants on IFN-β promoter activation in HEK293T cells upon VSV or HSV-1 infection. As shown in Figures S8F and S8G, ectopically expressed GFP-Vimentin, GFP-Vimentin-N, and GFP-Vimentin-C, but not GFP-Vimentin-Coil, significantly inhibited the IFN-β promoter activation in a dose-dependent manner. Overall, our results demonstrated that vimentin interacted with TBK1 and IKKε through its N-terminal domain, while vimentin inhibited the IFN-β promoter activation by its N terminus and C terminus.

**Vimentin disrupts the interactions of TBK1-IRF3 and IKKε-IRF3**

IKKε and TBK1 are essential components of the IRF3 signaling pathway. The two proteins are structurally and enzymatically similar and share over 60% sequence identity. Both contain four domains: an N-terminal kinase domain (KD), a ubiquitin-like domain (ULD), and two coiled-coil (CC) domains (Fitzgerald et al., 2003). We constructed four deletion mutants of both TBK1 and IKKε to identify their binding domain to vimentin, respectively (Figures 6A and 6B). We found that the KD and KD+ULD mutants of TBK1 and IKKε interacted with vimentin, but not the CC and UDL+CC mutants (Figures 6A and 6B), suggesting that the KD regions of TBK1 and IKKε are indispensable for their interaction with vimentin.

It has been reported that TBK1, IKKε, and IRF3 form a complex upon virus infection, and then the activated TBK1 and IKKε phosphorylate IRF3 and promoted its nuclear translocation (Fitzgerald et al., 2003). The KD domain of TBK1 or IKKε is required for their interaction with IRF3 (Prins et al., 2009). Our results showed that vimentin interacted with the KD domain of TBK1 and IKKε. Therefore, we speculated that vimentin might directly inhibit the interactions of TBK1-IRF3 or IKKε-IRF3. As expected, overexpressed vimentin markedly
Figure 6. Vimentin disrupts the interaction of IRF3 and IKKε or TBK1
(A) Schematic of full-length TBK1 and its truncated mutants (top). CoIP analysis of the interaction of vimentin with TBK1 or its truncation mutants in HEK293T cells (below). *Non-specific bond.
(B) Schematic of full-length IKKε and its truncated mutants (top). CoIP analysis of the interaction of vimentin with IKKε or its truncation mutants in HEK293T cells (below).
(C and D) Overexpressed vimentin disrupts the interactions of TBK1 and IRF3 or TBK1-KD and IRF3. HEK293T cells were transfected with plasmids expressing Flag-IRF3 and HA-TBK1 or HA-TBK1-KD, together with a vector or a plasmid encoding HA-Vimentin. CoIP analysis of the interactions of TBK1-IRF3 and TBK1-KD-IRF3 in absence or presence of HA-Vimentin.
(E and F) Overexpressed vimentin disrupts the interactions of IKKε and IRF3 or IKKε-KD and IRF3. HEK293T cells were transfected with plasmids expressing Flag-IRF3 and HA-IKKε or HA-IKKε-KD, together with a vector or a plasmid encoding HA-Vimentin. CoIP analysis of the interactions of IKKε-IRF3 and IKKε-KD-IRF3 in absence or presence of HA-Vimentin.
(G) The peritoneal macrophages isolated from mice-vim+/+ and mice-vim−/− were infected with VSV or HSV-1 for 12 h, and CoIP was performed with an anti-IRF3 antibody. IgG was used as a negative control. The cell lysates and immunoprecipitates were analyzed by western blotting. Data are representative of three independent experiments with similar results (A–G).

Vimentin blocks IRF3 nuclear translocation
Previous reports showed that TBK1-IKKε-IRF3 complex formation is required for the phosphorylation and nuclear translocation of IRF3 upon viral infection, which leads to IFN-I production (Fitzgerald et al., 2003). To detect whether vimentin inhibits IRF3 phosphorylation and blocks IRF3 nuclear translocation, THP-1 and THP-1-Vim cells were infected by VSV and HSV-1. As shown in Figures 7A and 7B, the amount of IRF3 in the nucleus of THP-1-Vim cells was significantly reduced during VSV and HSV-1 infection, suggesting that the highly expressed vimentin in THP-1 cells can inhibit IRF3 nuclear translocation. Subsequently, peritoneal macrophages from mice-vim+/+ and mice-vim−/− were infected with VSV or HSV-1 for 0, 4, 8, 12 h, and the phosphorylation levels of IRF3 and TBK1 were detected. As shown in Figures 7C and 7D, the phosphorylation levels of IRF3 and TBK1 in the peritoneal macrophages from mice-vim−/− were higher than those from mice-vim+/+ infected with VSV or HSV-1, although the total protein levels of IRF3 and TBK1 were not affected. To further confirm these results, HeLa-vim+/+ cells and HeLa-vim−/− cells were infected with VSV and HSV-1, respectively. The nuclear-cytoplasmic separation results showed that nuclear translocation of IRF3 was significantly enhanced in HeLa-vim−/− cells compared with those in HeLa-vim+/+ cells during VSV and HSV-1 infection (Figures 7E and 7F). Consistent with these results, the confocal experiment results also displayed that vimentin deficiency promotes the nuclear translocation of IRF3 upon VSV and HSV-1 infection (Figures 7G and 7H). Taken together, our findings revealed that vimentin inhibits the nuclear translocation of IRF3 induced by VSV and HSV-1 infection.

DISCUSSION
Upon viral infection, the host PRRs sense viral nucleic acids to activate TBK1 and IKKε, resulting in the phosphorylation and
nuclear translocation of IRF3 to induce the production of IFN-I (Fitzgerald et al., 2003). Subsequently, the secreted IFN-I was recognized by IFNAR1 and IFNAR2 on the cell surface to activate the JAK-STAT pathway to induce transcription of hundreds of ISGs, including ISG15, ISG56, and Mx, to maintain host antiviral status (Schneider et al., 2014). In this study, we found that vimentin gene expression is upregulated upon viral infection, which is dependent on IFNAR1 expression. Overexpression of vimentin protein inhibited IFN-β production induced by both RNA viruses (such as SeV, VSV, and EMCV) and DNA viruses (such as HSV-1, PRV, and ADV). In contrast, vimentin gene deficiency enhanced IFN-I production and suppressed virus replication in vitro and in vivo. Consistently, we also observed that knockdown of vimentin gene significantly inhibited viral replication, due to the elevation of IFN-I mediated by vimentin gene knockdown. Mechanistically, vimentin disrupted the TBK1-IKKε-IRF3 axis, resulting in inhibition of the phosphorylation and nuclear translocation of IRF3.

Previous studies have demonstrated that vimentin plays various roles in many key processes involved in inflammatory responses, which are critical for viral clearance and recovery. For example, vimentin has been identified as a ligand for some PRRs. Vimentin interacts with the NOD2, and this interaction is required for subsequent NF-κB activation (Stevens et al., 2013). In addition, vimentin is required for assembly and activation of the NLRP3 inflammasome, which mediates the induction of pro-inflammatory cytokines associated with acute lung injury in bacterial and viral infections (Dos Santos et al., 2015). Extracellular receptors have been found to play essential roles in the innate immune response to viral infections (Ramos et al., 2011; Ramos and Fernandez-Sesma, 2012). It has been shown that extracellular vimentin is a ligand for the PRR Dectin-1 (Thiagarajan et al., 2013), an M2 macrophage marker (D’ Alessio et al., 2016; Nandakumar et al., 2017), and the interaction of vimentin and Dectin-1 triggers reactive oxygen species production. Extracellular vimentin also switches the cytokine profile...
of LPS-activated dendritic cells by decreasing the secretion of some pro-inflammatory cytokines (such as IL-6, IL-12) and increasing IL-10 secretion, an anti-inflammatory cytokine reducing Th1 differentiation of naive T cells (Yu et al., 2018). Importantly, vimentin has been identified as a metabolic controller of the activity of regulatory T cells, which are critical for immune homeostasis and the prevention of autoimmune and inflammatory disorders (Mcdonald-Hyman et al., 2018).

Previous studies reported that activation of the RIG-I by astroglialosis leads to increased expression of vimentin (De Rivero Vaccari et al., 2012). IFN-β treatment increases expression of vimentin at the mRNA and protein levels in differentiated embryonal carcinoma (PSMB) cells (Alldridge et al., 1989). In addition, the mRNA levels of vimentin gene were upregulated in human PTC cells when they are stimulated with inflammatory mediators, tumor necrosis factor alpha, and IFN-γ (Lv et al., 2015). In this study, we also noticed that IFN-α could upregulate the mRNA and protein levels of vimentin in the Raw264.7 and THP-1 cell lines. DNA and RNA virus infection also induced vimentin gene expression in mouse peritoneal macrophages, which is dependent on IFNAR1 expression. Interestingly, overexpressed vimentin inhibited IFN-1 production, indirectly promoting viral replication. It is well known that RIG-I depends on the feedforward loop of IFN/IFNAR signaling. Therefore, Ifnar−/− cells will show a reduced response to RIG-I ligands. RIG-I is extremely sensitive to IFN and PRR activation. Thus, it seems possible that poly(I:C) or viral infection still induces RIG-I production, resulting in vimentin expression. So, we could not rule out the possibility that vimentin cannot be induced by RIG-I in an IFN-independent manner.

Although hundreds of ISGs have been reported to play roles in inhibiting virus replication, until now, the functions of most ISGs have not been studied in detail. Accumulating evidence suggests that several ISGs regulate IFN-I and IFN-II signals to promote viral replication. For example, suppressor of cytokine signaling (SOCS)-1 is generally expressed at low levels in a physiological state. It is rapidly upregulated upon stimulation with viruses and works as a negative regulator of JAK/STAT signaling, thus creating a classic negative feedback loop. Therefore, SOCS-1 inhibits JAK-STAT signaling by binding to phosphorylated tyrosine residues on either the IFN receptors or the JAK proteins, resulting in inhibition of STAT activity (Hong and Carmichael, 2013). Both Usp18 and Isg15 genes are known to be strongly induced by IFN-I, genotoxic stress, and viral infection (Farrell et al., 1979). USP18 is arguably an ISG with the most important role in establishing and maintaining long-term homeostasis to IFN-I signaling. Usp18−/− mice are hypersensitive to IFN and more resistant to virus infection (Ritchie et al., 2004). Further study revealed that USP18 interacts with the intracellular domain of IFNAR2, which prevents its binding of JAK1, eventually inhibiting the IFN-I signaling (Malakhova et al., 2006). Isg15 is another of the most highly induced ISGs. Isg15 can be secreted from immune cells, thus taking on the role of a cytokine and stimulating the production of IFN-γ (Bogunovic et al., 2013). Furthermore, Isg15 plays a protective role for vaccinia virus infection. A previous study reported that wild-type and Isg15−/− mice are equally susceptible to VSV and lymphocytic choriomeningitis virus (Osiak et al., 2005). In contrast, mice-Isg15−/− are more susceptible to infection with influenza A and B, HSV-1, and Sindbis virus (Lenschow et al., 2007). Interestingly, USP18 is the first bona fide ISG15-specific protease. USP18 can remove ISG15 conjugates (de-ISGylation) through its isopeptidase activity, which can cause ISG15 to lose its function (Malakhov et al., 2002). In this study, our results revealed that upon stimulation with IFN-α, vimentin gene is rapidly induced to expression and functions as a negative regulator of IFN-I signaling, thus creating a classic negative feedback loop. Upon both RNA and DNA virus infection, the RIG-I/MDA5-MVAS and the cGAS-STING cascade signal converge at TBK1. It is well-known that activated TBK1 and IKKε recruit and activate IRF3. TBK1-IKKε-IRF3 complex formation is required for the phosphorylated and nuclear translocation of IRF3 upon viral infection, which leads to IFN-I production. In this study, we found IFN-α induced mRNA and protein expression of vimentin, and vimentin interacted with the KD domain of TBK1 and IKKε, which is required for TBK1 or IKKε to interact with IRF3 (Prins et al., 2009). Therefore, we revealed that the combination of vimentin with TBK1 or IKKε disrupted the TBK1-IKKε-IRF3 complex, resulting in inhibition of phosphorylation and nuclear translocation of IRF3 induced by RNA viruses and DNA viruses. Given that vimentin can broadly inhibit PRR signaling, we had thought that the IFN gene and pro-inflammatory genes would be upregulated in vimentin KO mice. However, we noticed that there is neither an IFN nor pro-inflammatory gene upregulated signature in the KO mice when they are not stimulated with poly(I:C) or cGAMP or infected with EMCV or HSV-1 (Figures S4E and S4F). We speculated that, in the absence of stimulation, the vimentin gene knockout mice maintained a resting state, where the innate immune signal pathway was not activated and cytokines levels are low. Only when this balance was altered by different stimuli or virus infection, vimentin then executes its function as an inhibitor.

In summary, we first proposed that vimentin expression could be upregulated in response to different stimuli, which may be dependent on IFNAR1. It works as a negative regulator of virus infection-induced IFN-β production in the RLRs- and the cGAS-STING signaling pathway. Our results uncovered a mechanism used by vimentin to negatively regulate host antiviral defense. These findings may be helpful to deeply understand the working mechanism employed by other cytoskeletal proteins to control innate immune responses.

Limitations of the study
Of note, the mRNA levels of most well-known ISGs are upregulated more than 2-fold upon IFN treatment. Our study found that the mRNA and protein levels of vimentin are upregulated 1- to 2-fold upon IFN stimulation. Therefore, whether vimentin can be defined as an ISG requires more detailed experiments to confirm. In this study, we found that depletion of vimentin does not affect cell viability, organelle localization, nor ISG and PRR expression. However, we noticed that knockout of vimentin affected all PRR-mediated IFN-I production we tested, including TLRs, RLRs, and cGAS. Whether knockout of vimentin affects other physiological functions needs to be further investigated.
STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111469.

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AUTHOR CONTRIBUTIONS

L.H., C.W., and H.L. conceived and coordinated the study; H.L. and G.Y. wrote the paper; L.H., C.W., X.L., M.X., Q.Z., L.Z., and K.Z. performed and analyzed the experiments. L.H., C.W., H.L., and G.Y. designed, modified, and wrote the paper; L.H., C.W., X.L., M.X., Q.Z., L.Z., and K.Z. performed and analyzed the experiments. L.H., C.W., and G.Y. designed, modified, and wrote the paper; L.H., C.W., X.L., M.X., Q.Z., L.Z., and K.Z. performed and analyzed the experiments. L.H., C.W., and H.L. conceived and coordinated the study; H.L. and G.Y. designed, modified, and wrote the paper; L.H., C.W., X.L., M.X., Q.Z., L.Z., and K.Z. performed and analyzed the experiments.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare regarding the content of this article.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-Flag-Rabbit    | SIGMA | Cat#F7425-2MG; RRID: AB-2572291 |
| anti-LaminB-Rabbit  | Solarbio | Cat# K106482P- |
| anti-HSV-1          | Abcam  | Cat# ab124764 |
| anti-IFNAR1-Rabbit  | Abcam  | Cat# ab124764 |
| anti-Flag-Mouse     | SIGMA | Cat# F1804-1MG |
| anti-HA-Rabbit      | SIGMA | Cat# SAB4300603 |
| anti-HA-Mouse       | SIGMA | Cat# HS658-2ML |
| anti-GFP-Rabbit     | Proteintech | Cat# 50430-2-AP |
| anti-Vimentin-Rabbit| Cell Signaling Technology | Cat# 57415 |
| anti-Vimentin-Mouse | Abcam  | Cat#ab20346 |
| anti-IRF3           | Cell Signaling Technology | Cat#4302; RRID: AB-1904036 |
| anti-IRF3 phosphorylated at S396 | Cell Signaling Technology | Cat#4947; RRID: AB-823547 |
| anti-TBK1           | Cell Signaling Technology | Cat#3504; RRID: AB-2255663 |
| anti-TBK1 phosphorylated at S172 | Cell Signaling Technology | Cat#5483 |
| anti-GAPDH          | Proteintech Group | Cat#60004-1-lg; RRID: AB-2107436 |
| Anti-IKKε           | RD systems  | Cat#MAB3199 |
| Alexa Fluor 488 goat anti-rabbit IgG(H + L) | Invitrogen | Cat#A11008 |
| Alexa Fluor 633 goat anti-rabbit IgG(H + L) | Invitrogen | Cat#A21071 |
| Alexa Fluor 633 goat anti-mouse IgG(H + L) | Invitrogen | Cat#A21052 |
| Alexa Fluor 594 goat anti-mouse IgG(H + L) | Invitrogen | Cat#A11032 |
| IRDye® 800CW Goat anti-rabbit IgG (H + L) | LI-COR | Cat#926-32211 |
| IRDye® 800CW Goat anti-mouse IgG (H + L) | LI-COR | Cat#926-32210 |
| **Bacterial and virus strains** |        |            |
| The Sendai/Cantell strain | ATCC | ATCC VR-907 |
| VSV-GFP             | Harbin Veterinary Research Institute | N/A |
| VSV-GFP             | Harbin Veterinary Research Institute | N/A |
| HSV-1-GFP           | Harbin Veterinary Research Institute | N/A |
| EMCV (HB10); GeneBank: JQ864080.1 | Isolated from Pig | N/A |
| ADV                 | Hanbio Biotechnology Co., Ltd. | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| anti-Flag (M2) beads | Sigma-Aldrich | Cat#M8823 |
| Protein agarose A/G | Santa Cruz Biotechnology | Cat#20397 |
| TRIzol reagent      | Invitrogen | Cat#1596026 |
| Poly(I:C)           | Sigma-Aldrich | Cat#P1530 |
| cGAMP               | Sigma-Aldrich | Cat#SML1232-1EA |
| Poly (dA:dT)        | Sigma-Aldrich | Cat#P0883-10UN |
| ISD                 | Invitrogen | Cat#trl-isdn |
| protease inhibitor Cocktail | Roche | Cat#4693132001 |
| Dulbecco’s Modified Eagle’s Medium (DMEM) | GIBCO | Cat#C11995500CP |
| RPMI 1640 medium    | GIBCO  | Cat#C11875500CP |
| fetal bovine serum (FBS) | GIBCO | Cat#10091-148 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Changjiang Weng (wengchangjiang@caas.cn).

Materials availability
Vim+/+ and Vim−/− mouse line and recombinant plasmids generated in this study are available from the Lead contact with a completed Materials Transfer Agreement. We are glad to share those materials if required.

Data and code availability
- Original data may be directed to and will be fulfilled by lead contact Changjiang Weng (wengchangjiang@caas.cn).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Vimentin−/− mice were generated using the homologous recombination technique (Cyagen, China). The primers used for mouse genotyping are listed in Table S3. All mice were generated and housed in specific pathogen-free (SPF) barrier facilities at the Harbin...
Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS) (Harbin, China). All animal experiments were performed according to animal protocols approved by the Subcommittee on Research Animal Care at the HVRI, and all female mice used were less than six months old.

**Cell lines and viruses**

HEK293T cells, HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), and THP-1, Peritoneal macrophages were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO2. HeLa-vim−/− and HEK293T-vim−/− cell lines were constructed using the CRISPR/Cas9 method. The Sendai/Cantell strain (SeV strain, product code VR-907), which is well documented to induce IFN production, was purchased from the American Type Culture Collection (ATCC) and was amplified in SPF eggs. The NDV LaSota strain, VSV and VSV-GFP were kindly provided by Prof. Zhigao Bu (HVRI, China). HSV-1 was kindly provided by Prof. Hongbin Shu (Wuhan University, China).

**METHOD DETAILS**

**Viral infection**

For viral replication assays, 2 × 10⁵ peritoneal macrophages were infected with SeV (1 MOI), VSV (0.1 MOI), EMCV (1 MOI), HSV-1 (10 MOI), PRV (5 MOI), or ADV (1 MOI) for 24 h. Viral replication was analyzed by qRT-PCR analysis. For mouse infection, six-to eight-week-old and sex-matched wild type and vimentin−/− transgenic littermates were intraperitoneally injected with EMCV (2 × 10⁵ PFU per mouse) or HSV-1 (2 × 10⁷ PFU per mouse). For survival experiments, the survival of animals was monitored every day after EMCV infection. The sera from EMCV-infected mice were collected for ELISA analysis at 48 h and 72 h post-infection. The heart and brain were collected for qPCR, EMCV titer, or histological analysis. The liver, spleen, lung of HSV-1-infected mice were collected for qPCR.

**Plasmids**

Plasmids expressing Flag-tagged RIG-I, MDA5, MAVS, TBK1, IKKε, and IRF3 have been previously described (Huang et al., 2020). The IFN-β reporter and TK-Renilla reporter were obtained from Prof. Hong Tang. To construct plasmids expressing HA-, Flag-, GFP-Vimentin, the cDNA corresponding to the human vimentin gene were amplified by standard reverse transcription-polymerase chain reaction (RT-PCR) using total RNA extracted from HEK293T cells and RAW264.7 cells as templates and then cloned into the pcAGGS-HA (pHA), pcAGGS-Flag (pFlag), pEGFP-C1 vector. All constructs were validated by DNA sequencing. The primers used in this study are available upon request.

**Generation of a HEK293T-vimentin and HeLa-vimentin knock-out cell lines**

As previously described, CRISPR/Cas9 genomic editing for gene deletion was used (Ran et al., 2013). HeLa-vim−/− and HEK293T-vim−/− cell lines were constructed using the CRISPR/Cas9 method. To create mammalian vimentin−/− cells, one CRISPR guide RNA (sgRNA) sequence targeting the vimentin locus in the genome was chosen based on the specificity scores (http://crispr.mit.edu/). The sgRNA sequence was used as follows: vimentin sgRNA, 5’-GCCGAGCTCGAGCAGCTCAA-3’.

**Luciferase reporter assay**

According to the manufacturer’s instructions, luciferase activities were measured with a Dual-Luciferase Reporter Assay System (Promega). Data are normalized for transfection efficiency by dividing Firefly luciferase activity with that of Renilla luciferase.

**RNA extraction and qPCR**

Total RNA was extracted using TRIzol reagent (Invitrogen, California, America), and reverse transcription was accomplished with the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). Real-time PCR was conducted using TB Green Premix Ex Taq II (TaKaRa, Tokyo, Japan) in a typical 20 μL PCR mixture that included 10 μL of TB Green Premix Ex Taq II, 1–5 μL of template cDNA, and 0.4 mM of each PCR primer. Cycling conditions were 95°C for 2 min, followed by 40 cycles at 95°C for 5 s, 60°C for 30 s, and samples were run on the Stratagene Mx3000P Real-Time PCR System (Stratagene, America). The data were normalized according to the level of β-actin expression in each sample. All experiments were performed at least in triplicate. The qPCR primers are listed in Table S1.

**ELISA**

According to the manufacturer’s instructions, the concentrations of IFN-β (PBL InterferonSource) in cell culture supernatants and sera were measured by ELISA kits.

**Co-immunoprecipitation and Western blot analysis**

Co-immunoprecipitation and Western blot analysis were performed as previously described (Li et al., 2015). In brief, HEK293T cells transfected with indicated plasmids for 24 h were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, and 10% glycerol) containing 1 mM PMSF and 1 x protease inhibitor Cocktail (Roche). Then the cell lysates were incubated with anti-Flag (M2) beads at 4°C overnight on a roller. The precipitated beads were washed five times with cell lysis buffer. For Western blot analysis, equal amounts of cell lysates and immunoprecipitates were resolved on a 10–12% sodium dodecyl
sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane (Millipore, Stafford, VA, USA). After incubation with primary and secondary antibodies, the membranes were visualized by the Odyssey two-color infrared fluorescence imaging system (LI-COR, America).

Confocal microscopy and co-localization analysis
The cells were transfected with the indicated plasmids and then fixed for 10 min in 4% paraformaldehyde in 1 × phosphate-buffered saline (PBS) pH 7.4. The fixed cells were permeabilized for 15 min with 0.3% Triton X-100 in 1 × PBS and then blocked in 1 × PBS with 10% BSA for 30 min. The cells were incubated with the appropriate primary antibodies and then stained with Alexa Fluor 594-labeled goat anti-rabbit immunoglobulin G and Alexa Fluor 488-labeled goat anti-mouse IgG. The subcellular co-localization of Vimentin, TBK1 or IKKε alone, or both, was visualized using a Zeiss LSM-880 laser scanning fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) under a 63× oil objective. Zeiss processing system software was used to determine the degree of Vimentin and TBK1 or IKKε co-localization. Ch3-T1 denotes the 633 nm channel, Ch2 GaAsP-T2 denotes the 488 nm channel, and Ch1-T3 denotes the 405 nm channel (DAPI).

Isolation of macrophages
Peritoneal macrophages were isolated from mice 4 days after injection of thioglycollate (BD Biosciences, Franklin, NJ, USA) and then cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO2.

Histopathology analysis
To assess histological changes in the brain and heart, wild-type and Vimentin−/− mice were infected with EMCV HB10 strain, and the brain and heart were fixed in 10% formalin neutral buffer solution overnight. The tissues were embedded in paraffin blocks and then sectioned at 4 μm thickness for staining with hematoxylin and eosin following standard procedures. The results were analyzed by light microscopy. Representative views of brain and heart sections are shown.

Generation of a THP-1-vim stable cell line
The human vimentin gene was cloned into the pLVX-ISRE-Puro vector (Clontech). The THP-1 cells were infected with the lentiviral particles expressing vimentin in the presence of 8 mg/mL polybrene. At 3 days post-infection, the cells were treated with 1 mg/mL puromycin for 2 weeks. Vimentin overexpression efficiency was assessed by Western blotting, and the cell line was named THP-1-Vim.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analysis was conducted using the unpaired Student’s t-test, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post-test. p values less than 0.05 were considered statistically significant. Sample sizes were chosen by standard methods to ensure adequate power, and no exclusion, randomization of weight or sex, or blinding was used for the animal studies.