Cellular nucleic acid–binding protein is essential for type I interferon–mediated immunity to RNA virus infection

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Type I interferons (IFNs) are innate immune cytokines required to establish cellular host defense. Precise control of IFN gene expression is crucial to maintaining immune homeostasis. Here, we demonstrated that cellular nucleic acid–binding protein (CNBP) was required for the production of type I IFNs in response to RNA virus infection. CNBP deficiency markedly impaired IFN production in macrophages and dendritic cells that were infected with a panel of RNA viruses or stimulated with synthetic double-stranded RNA. Furthermore, CNBP-deficient mice were more susceptible to influenza virus infection than were wild-type mice. Mechanistically, CNBP was phosphorylated and translocated to the nucleus, where it directly binds to the promoter of IFNb in response to RNA virus infection. Furthermore, CNBP controlled the recruitment of IFN regulatory factor (IRF) 3 and IRF7 to IFN promoters for the maximal induction of IFNb gene expression. These studies reveal a previously unrecognized role for CNBP as a transcriptional regulator of type I IFN genes engaged downstream of RNA virus–mediated innate immune signaling, which provides an additional layer of control for IRF3- and IRF7-dependent type I IFN gene expression and the antiviral innate immune response.

CNBP | type I interferon | transcriptional regulator | RNA virus | antiviral

Infections due to RNA viruses constitute a significant threat to public health (1, 2). In addition, RNA viruses are very prominent among the causes of emerging or re-emerging infectious diseases: the severe acute respiratory syndrome coronavirus (SARS), SARS-CoV-2, Middle East respiratory syndrome coronavirus, Ebola, and Zika virus are recent, high-profile examples (3, 4). Several RNA viruses, such as influenza virus, can infect hundreds of millions of people around the world, leading to millions of deaths every year and also have enormous potential to cause a pandemic.

Innate immune responses are the first line of host defenses against virus invasion (5). Upon infection, the conserved microbial components, called pathogen-associated molecular patterns, are sensed by host cells via cellular pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), NOD-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and DNA sensors (6–10). The genetic material of RNA viruses may be single-stranded or double-stranded (ds) RNA. The viral RNA replicase generates 5′-triphosphate RNA and/or dsRNA in ample amounts during replication and transcription of viral RNA genomes that can be recognized by different receptors. Among the PRRs, the transmembrane and endosomal TLR3, expressed mostly in immune cells, recognizes virus-derived dsRNA, whereas intracellular viral RNA is detected by RLRs, including RIG-I and MDA5, which are expressed in most cell types (11). RIG-I and MDA5 play crucial roles in the innate immune response to different types of RNA viruses. Although both RIG-I and MDA5 can respond to Sendai virus (SeV), Dengue virus, and coronaviruses, they also recognize specific types of viruses. Various studies have shown that RIG-I recognizes a wide variety of RNA viruses, such as SeV, hepatitis C virus, Newcastle disease virus, vesicular stomatitis virus (VSV), respiratory syncytial virus, and influenza virus (12–15), while MDA5 recognizes the Picornaviridae family, such as encephalomyocarditis virus (EMCV). Further studies and structural analysis of RIG-I and MDA5 showed that RIG-I preferably recognizes viral 5′-triphosphate dsRNA and short dsRNA, while MDA5 has a higher affinity for long dsRNA (16). Nucleic acid–sensing receptors recruit various adaptor proteins, such as MYD88, TRIF, MAVS (also known as IPS1, VISA, or CARDIF), or STING (also known as MITA or ERIS) to activate downstream signaling pathways and subsequently lead to the transcription of NF-κB–dependent and IRF3-IRF7–dependent genes, including type I interferons (IFNs), IFN-α and IFN-β (17, 18). The type I IFNs initiate an antiviral state in cells through the induction of a large number of multifunctional IFN-stimulated genes (ISGs)—the products of which are critical to control acute viral infections (19–24).

The regulation of type I interferon gene expression occurs at the transcriptional level and involves the combinatorial action of distinct classes of sequence-specific transcription factors including IRF3 to IRF7, NF-κB, and activator protein 1 (AP1) each of which are activated through upstream kinases activated in response to viral infection (25, 26). Among them, IRF3 and IRF7 are primary transcription factors that regulate type I IFN production by binding to the IFN-stimulated response element (18, 27–29). The intricacy of IRF3 activation and modulation is currently under intensive study. Several proteins have been implicated in these regulations, including TRIM21, Pin1, Cull-1, TAK1, cPKCs, PI3K-AKT, MEKK1, and ASK1 kinase (30–32). *Irf3*$^{-/-}$, *Irf7*$^{-/-}$, or *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$ mice are substantially more susceptible to viral infection than wild-type (WT) mice and do not induce type I IFN efficiently (27). While the importance of IRF3 and IRF7 in antiviral immunity is well established, *Irf3*$^{-/-}$ × *Irf7*$^{-/-}$

Significance

Infections due to RNA viruses pose a substantial threat to human health around the globe. A better understanding of how the immune system controls RNA virus infection is critical for developing new treatments and vaccines. Here, we demonstrated that CNBP was required to control RNA virus infection through regulating the production of type I IFNs. Overall, we discovered an important role for CNBP in regulating innate immune responses and defined the underlying mechanisms, which suggest that CNBP might be a therapeutic target in controlling viral infections and inflammatory diseases.

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macrophages and dendritic cells (DCs) still elicit IFN-β after viral infection, indicating that additional transcription factors or coactivators must also be used in cells to control IFN gene expression (25, 33).

Cellular nucleic acid–binding protein (CNBP), also called zinc-finger protein 9 (ZNF9), is a DNA- and RNA-binding protein with broad sequence specificity that is involved in diverse cellular functions, including transcription and translation (34). CNBP is linked to age-related sporadic inclusion body myositis (sIBM), an inflammatory muscle disease characterized by progressive muscle weakness and atrophy (35). Furthermore, a dominantly transmitted $(CCTG)_n$ expansion in intron 1 of the *CNBP* gene leads to myotonic dystrophy type 2 (DM2), a disease associated with a high frequency of autoantibodies and autoreactive T cells (36, 37). These observations suggest that CNBP may function in the immune system. Indeed, our previous study identified CNBP as a signaling molecule downstream of multiple PRRs that acts as a key regulator of interleukin-12 (IL-12) gene transcription and T helper type 1 (Th1) immunity. *Cnbp*$^{-/-}$ mice were more susceptible to acute toxoplasmosis associated with reduced production of IL-12β, as well as a reduced Th1 cell IFN-γ response essential to controlling parasite replication (38). However, the potent role and molecular mechanisms of CNBP on type I IFN induction and antiviral function are currently unknown.

Here, we demonstrated that CNBP was required to control RNA virus infection through regulating the production of type I IFNs. CNBP deficiency markedly impaired IFN production in macrophages and DCs following infection with a panel of RNA viruses as well as stimulation with a synthetic dsRNA, polyinosinic:polycytidylic acid (poly[I:C]). Furthermore, *Cnbp*$^{-/-}$ mice were more susceptible to influenza virus infection than WT mice. Mechanistically, CNBP is phosphorylated and translocated to the nucleus where it directly binds the type I IFN promoter in response to RNA virus infection. In addition, we found that CNBP controls the recruitment of IRF3 and IRF7 to IFN promoters for maximal induction of IFN gene expression. These studies, therefore, reveal a previously unrecognized role for CNBP as a transcriptional regulator of type I IFN genes engaged downstream of RNA virus–mediated innate immune signaling, providing insights into the role of CNBP in innate immunity and adding an understanding of IRF3- and IRF7-dependent type I IFN gene expression and the antiviral innate immune response.

Results

CNBP Is Required for poly(I:C)-Induced Type I IFN Signaling. Our previous study demonstrated that CNBP could be pulled down by stimulatory oligos in a mass spectrometry assay. To study the role of CNBP in innate immune signaling, we generated *Cnbp*$^{-/-}$ mice. However, IFN-β messenger RNA (mRNA) and protein levels were induced at similar levels between the WT and *Cnbp*$^{-/-}$ mouse macrophages after treatment with DNA viruses, including herpes simplex virus (HSV-1-ICP0–deficient mutant) and murine cytomegalovirus (mCMV), or stimulation with DNA ligands, including poly(dA:dT) (an immune-stimulatory dsDNA) and cyclic diGMP. All of these stimulants signal via the STING pathway, suggesting that CNBP is not a receptor for cytosolic dsDNA controlling the type I IFN response. This body of work identified CNBP as a regulator of IL12b gene transcription in the TLR signaling pathway (38).

To further broaden our investigation, we examined the role of CNBP in controlling other aspects of TLR responses, including the type I IFN response. We prepared bone marrow–derived macrophages (BMDMs) from WT and *Cnbp*$^{-/-}$ mice. Primary BMDMs were stimulated with Pam3CSK4 (TLR2 ligand), poly(I:C) (TLR3 ligand), lipopolysaccharide (LPS, a TLR4 ligand), R848 (TLR7/8 ligand), and CpG (TLR9 agonist). *Cnbp*$^{-/-}$ BMDMs showed significantly impaired IFN-β production compared to WT BMDMs when triggered by poly(I:C) (high molecular weight, HMW) or LPS. Notably, direct addition of HMW poly(I:C) to the cultures of primary *Cnbp*$^{-/-}$ BMDMs resulted in the highest fold decrease (Fig. 1 *A* and *B*), while production of tumor necrosis factor (TNF) was comparable between the genotypes (*SI Appendix*, Fig. S1 *A* and *B*).

Poly(I:C), a synthetic dsRNA, has been used extensively as an experimental mimic to trigger the host's response to virus infection. Extracellular poly(I:C) is endocytosed and transported to the endosomal lumen for presentation to TLR3, whereas cytosolic transfected HMW poly(I:C) (~1.5 to 8 kb) is directly recognized by cytosolic RLRs, including RIG-I and MDA5. We used HMW poly(I:C) to transfect BMDMs to trigger IFN signaling. As shown in Fig. 1*C*, *Cnbp*$^{-/-}$ BMDMs had lower expression of IFN-β mRNA and protein than WT BMDMs in response to transfected (i.e., cytosolic) HMW poly(I:C). In addition, IL-12p40 and IL-6 were also down-regulated in *Cnbp*$^{-/-}$ BMDMs (*SI Appendix*, Fig. S1 *C* and *D*). Furthermore, this effect was not restricted to BMDMs, as similar results were seen in bone marrow DCs (BMDC) (Fig. 1*D*) and peritoneal macrophages (PEC) (Fig. 1*E*). Additionally, *Cnbp*$^{-/-}$ macrophages treated with low molecular weight (LMW) poly(I:C) (~0.2 to 1 kb) also produced less IFN-β (*SI Appendix*, Fig. S1*E*), further confirming that CNBP was required for poly(I:C)-induced type I IFN signaling in vitro.

Next, we examined the in vivo relevance of CNBP deficiency in poly(I:C) signaling. Mice were injected intraperitoneally with poly(I:C), and serum concentrations of IFN-β were detected. As shown in Fig. 1*F*, serum concentrations of IFN-β, IL-12p40, and IL-6 were greatly reduced in *Cnbp*$^{-/-}$ mice. However, the production of IL1-β, TNF-α, and CCL5 were not changed in either group. Collectively, these data suggest that CNBP has a general role in dsRNA-triggered type I IFN signaling in distinct cell types and in animals.

CNBP Is Essential for Viral RNA-Triggered Type I IFN Signaling. The RLRs are cytosolic PRR proteins that recognize viral RNA species, including dsRNA and 5′-triphosphate RNA. In the case of RNA viruses, viral RNA replicase generates 5′-triphosphate RNA and/or dsRNA in ample amounts during replication and transcription of viral RNA genomes. To investigate the role of CNBP in IFN expression during RNA virus infection, we employed a panel of viruses representing diverse viral families, including SeV, VSV, influenza A virus (IAV), and EMCV. We infected primary BMDMs or BMDCs with these viruses and monitored IFN-β production. In all infections examined, *Cnbp*$^{-/-}$ BMDMs exhibited down-regulated IFN-β, IL12p40, and IL-6 (Fig. 2*A* and *SI Appendix*, Fig. S2 *A* and *B*), while production of IL1-β and TNF-α were comparable between the genotypes (*SI Appendix*, Fig. S2 *C* and *D*), and this phenomenon was also found in BMDCs (Fig. 2*B* and *SI Appendix*, Fig. S2*E*). Additionally, the qPCR data confirmed that *Cnbp*$^{-/-}$ BMDMs produced less IFN-β and IFN-α mRNA levels in response to RNA virus infection than WT BMDMs (Fig. 2 *C*–*F*). Similar trends were observed when we examined the expression of the ISGs, including *Cxcl9*, *Cxcl10*, and *Viperin* (Fig. 2 *C*–*F*). These data demonstrated that CNBP plays an important role in murine BMDMs and BMDCs in sensing RNA viruses.

CNBP Is Required to Control the Replication of Diverse RNA Viruses. Because CNBP is essential for the RNA virus–induced production of IFN, which is critical for antiviral immunity, we examined the function of CNBP in host defense against RNA virus infection. To determine the function of CNBP in RNA virus infection, primary mouse embryonic fibroblasts (MEFs), BMDMs, and BMDCs were infected with a panel of viruses representing diverse viral families. As shown in Fig. 3 *A* and *B*, the replication of VSV, Sindbis virus (SINV), and influenza virus (PR-8 and WSN strain) was dramatically increased in *Cnbp*$^{-/-}$ MEFs, BMDMs, and BMDCs. Consistently, *Cnbp*$^{-/-}$ BMDMs and BMDCs showed decreased expression of *Ifnb* mRNAs after infection with VSV, SINV, or

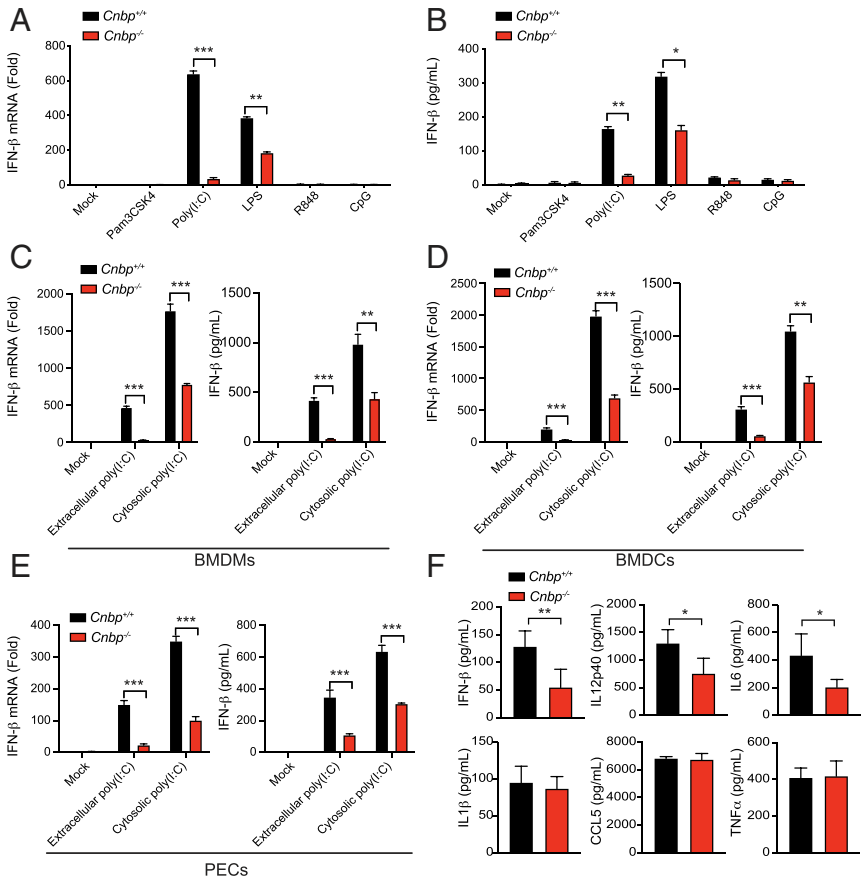

Fig. 1. CNBP is required for poly(I:C)-induced type I IFN signaling. (*A* and *B*) IFN-β mRNA and protein levels were measured by qRT-PCR (*A*) and ELISA (*B*) in $Cnbp^{+/+}$ and $Cnbp^{-/-}$ BMDMs treated with TLR ligands, including Pam3CSK4 (TLR2 ligand), poly(I:C) (TLR3 ligand), LPS (TLR4 ligand), R848 (TLR7/8 ligand), and CpG (TLR9 agonist). (*C*–*E*) qRT-PCR and ELISA analysis of IFN-β mRNA and protein level in $Cnbp^{+/+}$ and $Cnbp^{-/-}$ BMDMs (*C*), BMDCs (*D*), or PECs (*E*) stimulated with HMW poly(I:C) (extracellular) or transfected with HMW poly(I:C) (cytosolic). (*F*) $Cnbp^{+/+}$ and $Cnbp^{-/-}$ mice were given intraperitoneal injection of poly(I:C) (20 mg/kg body weight). Serum samples were collected at 6 h for ELISA of IFN-β, IL12p40, IL-6, IL-1β, TNF, and CCL5. Error bars represent SEM of triplicate biological replicates. All data are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

influenza virus (*SI Appendix*, Fig. S3 *A* and *B*). In addition, we generated polyclonal A549 CNBP knockout (CNBP pKO) cell lines using CRISPR-Cas9. We infected the CNBP pKO A549 cells with influenza virus and noted significantly elevated levels of intracellular viral RNA and decreased production of IFN-β compared to A549 control cells (*SI Appendix*, Fig. S3 *C* and *D*). Similar results were obtained using VSV-expressing GFP (VSV-GFP) or IAV (A/Puerto Rico/8, IAV-PR8)-expressing GFP (IAV-GFP) in MEFs, while the replication of a DNA virus, murine cytomegalovirus (mCMV)-expressing GFP, was not affected (*SI Appendix*, Fig. S3*E*). These data suggest that the up-regulation of IFN-β by CNBP is specific to RNA virus infection.

To further confirm that the increased viral replication is mainly due to IFN reduction, BMDMs were pretreated with recombinant (r)IFNb, rIFNa, or rIL12p40, then infected with RNA virus to detect virus replication. The data showed that administering exogenous rIFNb and rIFNa but not rIL12p40 decreased virus replication in KO BMDMs (Fig. 3 *C* and *D*). Conversely, the cells treated with neutralizing antibody anti-interferon-α/β receptor (anti-IFNAR), which blocks IFN-I signaling (*SI Appendix*, Fig. S3 *F* and *G*), showed increased viral replication, suggesting that increased viral replication is mainly due to IFN reduction and not IL12p40. CNBP, therefore, has a role in host defense against RNA virus infection through regulating the production of type I IFN.

CNBP Is Essential for the Innate Anti-RNA Virus Response In Vivo. To investigate the function of CNBP in antiviral immunity to RNA virus infection in vivo, we infected $Cnbp^{+/+}$ and $Cnbp^{-/-}$ mice with influenza virus IAV-PR8 (A/Puerto Rico/8/34, H1N1) through an intranasal route of infection and monitored both body weight changes and survival over time. $Cnbp^{-/-}$ mice lost significantly more body weight after infection with PR8 and had significantly increased mortality rates compared to WT mice (Fig. 4 *A* and *B*). Furthermore, the number of genomic copies of IAV in the lung was much higher in $Cnbp^{-/-}$ mice 2 d after viral infection (Fig. 4*C*). Consistently, we detected decreased IFN-β, IL12p40, and IL-6, but not IL-1β or TNF-α, compared with that of WT mice (Fig. 4 *D* and *E*). These results indicate that $Cnbp^{-/-}$ mice were significantly more susceptible to influenza virus–induced death and suggest that CNBP is important in host defense against RNA viruses in vivo.

Our previous study indicates that CNBP is broadly expressed in most tissues and cell types from humans and mice, albeit with different expression levels. Furthermore, we have demonstrated that IFN-β induction was impaired not only in BMDMs but also in PECs and BMDCs from $Cnbp^{-/-}$ mice; however, whether macrophages or DCs are important in vivo remains unclear. To define the cell-type–specific contributions of CNBP-dependent control of RNA virus infection in vivo, we generated mice deficient for *Cnbp* in specific cell types. *Cnbp*-floxed mice were generated and crossed to Vavi-Cre, LysM-Cre, or CD11c-Cre mice to delete *Cnbp* only in hematopoietic cells, myeloid cells, or DCs, respectively. These mice were infected with IAV-PR8. As shown in Fig. 4 *F* and *G*, Vavi-Cre

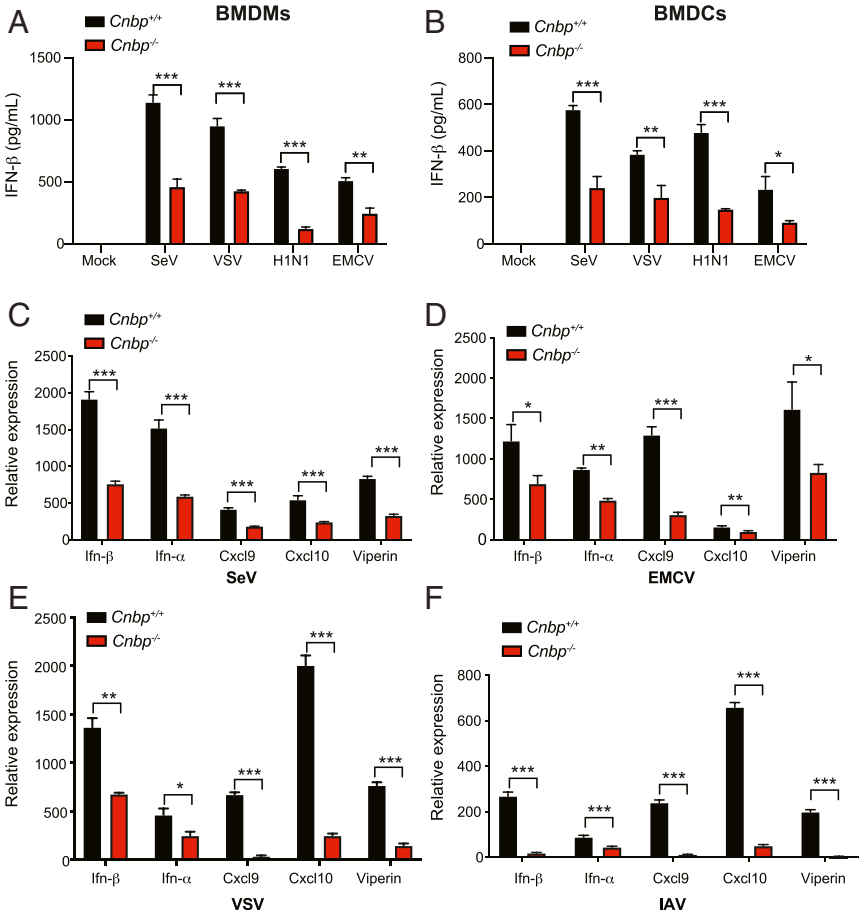

Fig. 2. CNBP is essential for viral RNA-triggered type I IFN signaling. (*A* and *B*) ELISA analysis of IFN-β protein level in $Cnbp^{+/+}$ and $Cnbp^{-/-}$ BMDMs (*A*) or BMDCs (*B*) infected with a panel of RNA viruses, including SeV, VSV, IAV (H1N1), and EMCV for 18 h. (*C–F*) qRT-PCR analysis of IFN-β, IFN-α, Cxcl9, Cxcl10, or Viperin mRNA in $Cnbp^{+/+}$ and $Cnbp^{-/-}$ BMDMs left unstimulated or infected with a panel of RNA viruses, including SeV (*C*), EMCV (*D*), VSV (*E*), or IAV (*F*). Error bars represent SEM of triplicate biological replicates. All data are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Cnbp conditional KO (cKO) mice had a 30% survival rate, LysM-Cre *Cnbp* cKO mice had a 40% survival rate, and CD11c-Cre Cnbp cKO mice had a 66.7% survival rate. Moreover, the replication of IAV (Fig. 4*H*) was significantly increased in organs from Vavi-Cre and LysM-Cre CNBP KO mice. However, all three cKO mice produced significantly lower levels of IFN-β than CNBPf/f mice in response to IAV-PR8 (Fig. 4 *I* and *J*). We also purified splenic DCs and exposed them to virus infection. WT and $Cnbp^{-/-}$ mice were previously treated with Flt3L to expand the DC pool. Isolated CD11c+ DCs were then infected ex vivo with IAV-PR8. As shown in *SI Appendix*, Fig. S4 *A–C*, the levels of IFN-β in virus-infected DCs were reduced while the replication of virus increased in the absence of CNBP. These results indicate CNBP functions to protect the host against RNA virus infection by promoting the production of type I IFN in vivo.

CNBP Is Activated by RNA Virus Infection and Binds to Type I IFN Promoters Directly. CNBP is a DNA- and RNA-binding protein with broad sequence specificity and is involved in diverse cellular functions, including transcription and translation. Our previous study, combined with another study, demonstrated that CNBP could be phosphorylated at conserved positions T173/177 after which it relocalizes from the cytosol to the nucleus in response to LPS (38, 39). However, whether CNBP is regulated in response to RNA virus infection had not been determined. Our results indicated that CNBP is phosphorylated and translocated into the nucleus after stimulation (Fig. 5 *A* and *B*), and the phosphorylation and translocation could be reduced by 5Z-7-Oxozeaenol, an inhibitor of TAK1 kinase (Fig. 5 *C* and *D*). Consistently, the phosphorylation and translocation of CNBP in TAK1 KO macrophage also decreased, suggesting that TAK1 functions to control CNBP in this setting (*SI Appendix*, Fig. S5 *A* and *B*). To test whether T173/177 positions are responsible for the phosphorylation, a pair of threonine codons at positions 173 and 177 in the mouse CNBP were mutated to alanine codons by site-directed mutagenesis, which reduced the phosphorylation of CNBP after treatment (Fig. 5*E*). We also found that CNBP expression was up-regulated at early time points after poly(I:C) stimulation or SeV infection, and the induction was independent of IFN-β (*SI Appendix*, Fig. S5 *C–E*). To further elucidate the molecular mechanisms of CNBP-dependent regulation of type I IFN, we used a luciferase reporter assay to examine whether ectopic expression of CNBP could enhance *Ifnb* expression. The result showed that *Ifnb* and *Ifna*-4 but not *Tnf* promoter activity could be increased by CNBP in a concentration-dependent manner (Fig. 5*F*). Furthermore, we found that the CNBP T173/177A mutant lost the ability to drive *Ifnβ* and *Ifnα*-4 promoter activity, suggesting that the phosphorylation of CNBP at these sites is necessary for type I IFN promoter activity (Fig. 5 *G* and *H*). In addition, chromatin immunoprecipitation (ChIP) analysis using an antibody to CNBP in primary BMDMs revealed very strong binding of CNBP to the *Ifnβ* promoter after poly(I:C) or SeV treatment (Fig. 5*I*). Collectively, these data indicate that phosphorylated CNBP translocates to the

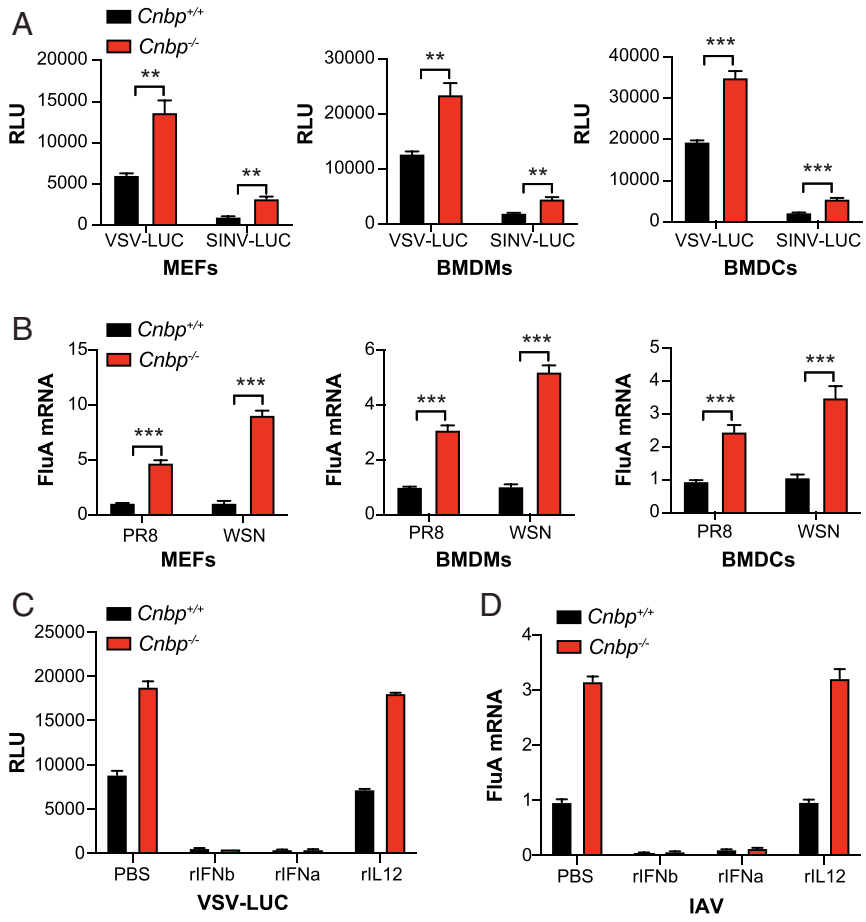

Fig. 3. CNBP is required to control the replication of diverse RNA viruses. (*A*) Matched *Cnbp*$^{+/+}$ and *Cnbp*$^{-/-}$ primary MEFs, BMDMs, or BMDCs were infected with VSV-LUC or SINV-LUC at an MOI of 0.1. VSV and SINV infections were monitored by luciferase activity assay at 18 h postinfection. (*B*) Matched *Cnbp*$^{+/+}$ and *Cnbp*$^{-/-}$ primary MEFs, BMDMs, or BMDCs were infected with influenza virus PR8 and WSN/33 strain. The replication was determined by qPCR at 24 h postinfection. (*C*) Luciferase activity analysis of VSV-LUC infection in *Cnbp*$^{+/+}$ and *Cnbp*$^{-/-}$ primary BMDMs pretreated with recombinant rIFNb, rIFNa, or rIL12p40. (*D*) qRT-PCR analysis of Flu A PR8 mRNA expression in *Cnbp*$^{+/+}$ and *Cnbp*$^{-/-}$ primary BMDMs pretreated with recombinant rIFNb, rIFNa, or rIL12p40. Error bars represent SEM of triplicate biological replicates. All data are representative of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$.

nucleus and directly binds to the IFN promoter in response to RNA virus infection.

CNBP Synergizes with IRF3/7 to Induce Type I IFN. To further explore how CNBP regulates the RNA virus–induced signaling pathways, we evaluated the activation of the TBK1-IRF3, NF-κB, and MAPK signaling pathways. The results showed that the phosphorylation of TBK1, IRF3, p65, ERK, and p38 was equivalent in *Cnbp*$^{+/+}$ and *Cnbp*$^{-/-}$ macrophages treated with poly(I:C) (*SI Appendix*, Fig. S6*A*). In addition, the translocation of IRF3 and p65 were unaffected (*SI Appendix*, Fig. S6*B*). These results indicate that CNBP is not involved in the phosphorylation and nuclear translocation of IRF3 and NF-κB.

Our previous study demonstrated that CNBP targets cRel to regulate IL-12b expression. To test whether CNBP regulates IFN through cRel, cRel-deficient BMDMs were generated for poly(I:C), SeV, or EMCV treatment. In contrast to what we have previously reported for IL-12b, IFNs and ISGs were unaffected in c-Rel KOs, suggesting that cRel was not involved in the CNBP mechanism examined here (*SI Appendix*, Fig. S6*C*).

The IFN regulatory factors (IRF), IRF3 and IRF7, are master transcription factors responsible for the induction of type I IFNs. Combinatorial interactions between distinct classes of sequence-specific transcription factors are important in regulating type I IFN expression. To study whether CNBP could synergize with IRF3 and IRF7, we cotransfected IRF3 or IRF7 with different amounts of CNBP into HEK293T cells and measured luciferase activity as a readout for IFN expression. The result demonstrated that overexpression of CNBP strongly synergized with IRF3 or IRF7 to activate the *Ifn-β* and *Ifn-α* promoter in a concentration-dependent manner (Fig. 6 *A* and *B*). We performed an endogenous coimmunoprecipitation experiment and found that CNBP associated with IRF3 after poly(I:C) treatment in BMDMs (Fig. 6*C*). In addition, ChIP–qPCR was performed in untreated, poly(I:C)-treated, or SeV-infected WT or *Cnbp*$^{-/-}$ BMDMs. The result showed that poly(I:C)- or SeV-induced recruitment of IRF3 to the *Ifnβ1* promoter or IRF7 to the *IFN-α4* promoter was significantly reduced in *Cnbp*$^{-/-}$ macrophages (Fig. 6 *D* and *E*). Furthermore, a comparable decrease in the mRNA level of IFN-β, Cxcl9, and Viperin in *Cnbp*$^{-/-}$ and *Irf3/7*$^{-/-}$ BMDMs after poly(I:C) stimulation was observed (Fig. 6*F*), while a less pronounced phenotype was observed after SeV virus treatment (*SI Appendix*, Fig. S6*D*). Taken together, these results demonstrate that CNBP synergizes with IRF3 and IRF7 to induce type I IFN gene expression.

Discussion

RNA viruses pose a significant threat to public health with enormous potential to impact society across the globe as highlighted by

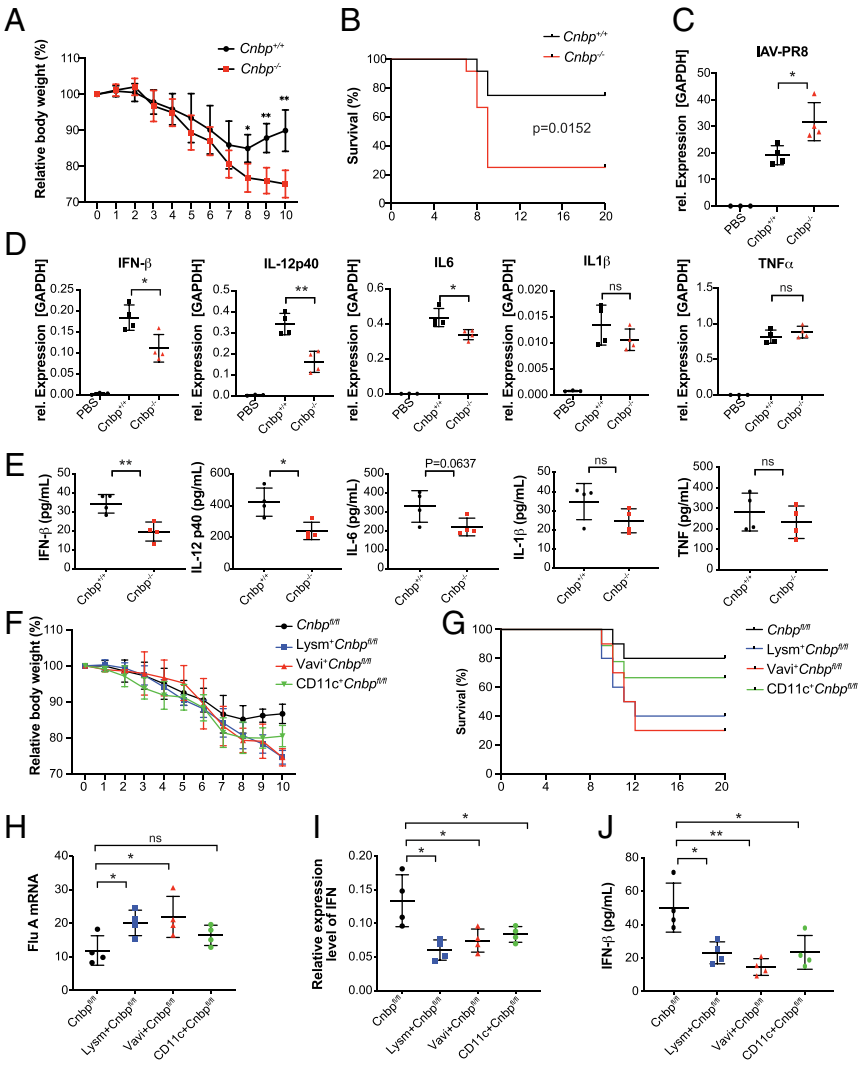

Fig. 4. CNBP is essential for the innate immune anti-RNA virus response in vivo. (*A* and *B*) WT and *Cnbp*$^{-/-}$ mice (ages 8 to 12 wk) were infected intranasally with a sublethal dose (50 PFUs [plaque-forming units]) of IAV-PR8 (H1N1), and morbidity, as a percentage of original weight (*A*) and survival (*B*), was assessed. Mice losing more than 30% of their initial body weight had to be euthanized and were recorded as dead ($n = 12$ per strain). Weight loss data are represented as mean values ± SEM. (*C*–*E*) WT and *Cnbp*$^{-/-}$ mice (ages 8 to 12 wk) were infected intranasally with a dose (1×10^5 PFUs) of IAV-PR8, on day 2 postinfection (pi), the lungs were collected for qRT-PCR analysis of virus mRNA expression (*C*) or cytokines mRNA expression (*D*), and the serum was collected for ELISA analysis of IFN-β, IL12p40, IL-6, IL-1β, and TNF (*E*). (*F* and *G*) *Cnbp*$^{f/f}$, Lysm^{+} *Cnbp*$^{f/f}$, Vavi^{+} *Cnbp*$^{f/f}$, or CD11c^{+} *Cnbp*$^{f/f}$ mice were infected intranasally with a sublethal dose (50 PFUs) of IAV-PR8, and morbidity, as a percentage of original weight (*F*) and survival (*G*), was assessed ($n = 8$ to 10 per strain). (*H* and *J*) *Cnbp*$^{f/f}$, Lysm^{+} *Cnbp*$^{f/f}$, Vavi^{+} *Cnbp*$^{f/f}$ or CD11c^{+} *Cnbp*$^{f/f}$ mice were infected intranasally with a dose (1×10^5 PFUs) of IAV-PR8. On day 2 pi, the lungs were collected for qRT-PCR analysis of virus mRNA expression (*H*) or IFN-β mRNA expression (*I*), and serum samples were collected for ELISA analysis of IFN-β (*J*). Each symbol represents an individual mouse; small horizontal lines indicate the mean. All data are representative of at least two to three independent experiments with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$; ns, not significant.

the COVID-19 pandemic (40). Type I IFNs play a central role in restricting RNA viruses by inducing a wide array of antiviral effectors and subsequent shaping of adaptive immunity (41). Therefore, understanding how type I IFNs are regulated is important. After infection with an RNA virus, the rapid induction of type I IFNs is regulated by extracellular and intracellular signals to activate both IRFs and NF-κB. CNBP has been implicated in the transcriptional control of some immune genes, including IL-12 and IL-6, suggesting that CNBP is important in the innate immune response (38, 39). In addition, CNBP-mediated diseases, including sIBM and DM2, were reported to be associated with a high frequency of autoimmune disease. However, the function of CNBP on virus infection and type I IFN regulation had not been studied.

In the present study, we demonstrated that CNBP was essential for the production of type I IFNs in response to RNA virus infection in vitro and in vivo. CNBP is constitutively expressed in most tissues and cell types from humans and mice, albeit with different expression levels, suggesting that CNBP might have a cell-type–specific function in response to viral infection. Our in vitro experiments showed that IFN-β induction was impaired not only in BMDMs but also in PECs and DCs. However, interestingly, CNBP-floxed mice crossed to Vavi-Cre, LysM-Cre, or CD11c-Cre mice showed different responses to IAV infection, suggesting that CNBP in hematopoietic cells and myeloid cells, but not DCs, plays important antiviral roles in vivo. Interestingly, although IAV replication was significantly increased in organs from Vavi-Cre and LysM-Cre but not CD11c-cre CNBPfl/fl mice, all three different cKO mice produced significantly lower levels of IFNb than CNBPfl/fl mice in response to IAV-PR8. Furthermore, isolated CNBP KO CD11c+ DCs showed decreased IFNb expression and

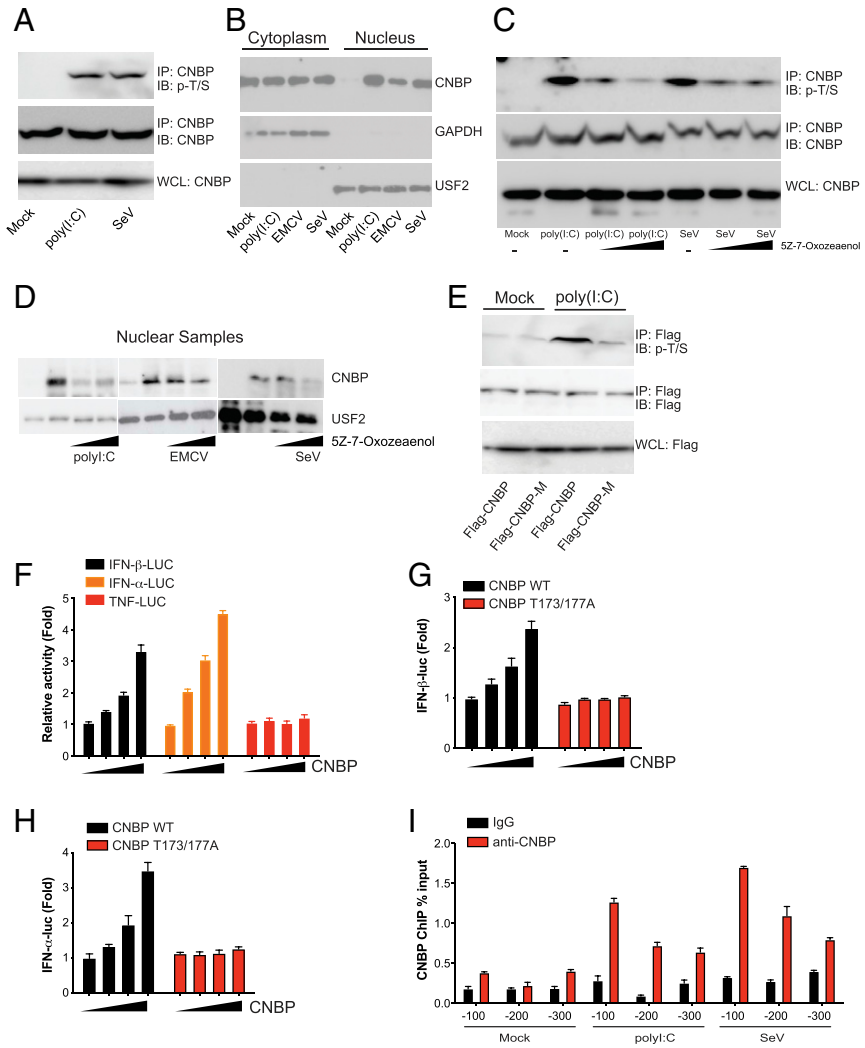

Fig. 5. CNBP is activated by RNA virus infection and binds to the type I interferon promoter. (*A*) BMDMs were untreated (mock) or treated with poly(I:C) or SeV as indicated. Endogenous CNBP protein was immunoprecipitated (IP) with anti-CNBP and immunoblotted (IB) with the indicated antibody. Phosphorylation of CNBP was determined by Western blot (anti–p-T/S). (*B*) The cytosolic and nuclear extracts were analyzed for CNBP by Western blotting in WT BMDMs treated with poly(I:C), SeV, or EMCV. (*C*) Phosphorylation of CNBP was determined by Western blot (anti–p-T/S) in BMDMs treated with increasing amounts of the TAK1 kinase inhibitor 5Z-7 Oxozeaenol in the presence or absence of poly(I:C) or SeV. (*D*) The nuclear extracts were analyzed for CNBP by Western blotting in BMDMs treated with increasing amounts of 5Z-7 Oxozeaenol in the presence or absence of poly(I:C), SeV, or EMCV. (*E*) Cells were transfected with Flag-CNBP or Flag-CNBPT173/177A for 36 h and then cell lysates were collected after treatment with poly(I:C) and IP with anti-Flag and IB with the indicated antibody anti–p-T/S. (*F*) Luciferase activity of IFN-β-Luc, IFN-α-Luc, or TNF-α-Luc in HEK293 cells after 36-h transfection with increasing amounts of plasmids encoding CNBP. (*G* and *H*) Luciferase activity of IFN-β-Luc (*G*) or IFN-α-Luc (*H*) in HEK293 cells after 36-h transfection with increasing amounts of plasmids encoding CNBP or CNBPT173/177A. (*I*) ChIP–qPCR of CNBP at the IFN-β promoter in BMDMs stimulated with poly(I:C) or SeV. Error bars represent SEM of triplicate biological replicates. All data are representative of three independent experiments.

increased virus replication during influenza virus infection suggesting that CNBP was important in various cell types, including macrophages and DCs, although CD11c-Cre Cnbp cKO mice showed less morbidity and mortality compared with Vavi-Cre and LysM-Cre Cnbp KO mice.

CNBP is a transcriptional regulator that up-regulates the expression of key innate immune genes, including IL-12, IL-6, c-Myc, and the macrophage colony-stimulating factor gene, whereas it down-regulates the expression of the early promoter enhancer of the John Cunningham virus, and the b-myosin heavy chain gene (34). In this study, we elucidated the mechanism of CNBP as a transcriptional regulator by demonstrating that CNBP directly binds the type I IFN promoter in response to multiple ligands. Posttranslational modification of IRF3 and IRF7, particularly by phosphorylation, is essential for efficient IFN-β transcription. Our result demonstrated that CNBP could be phosphorylated at T173/177 positions and induced to translocate to the nucleus to bind the IFN promoter after RNA viral infection as well as by stimulation with poly(I:C) in macrophages. Furthermore, a comparable decrease in the mRNA level of IFN-β, Cxcl9, and Viperin in Cnbp$^{-/-}$ and Irf3/7$^{-/-}$ BMDMs after poly(I:C) stimulation was observed suggesting that CNBP is similar to IRF3 and IRF7 in both its mode of activation and function.

The activity of both IRF3 and IRF7 is regulated by phosphorylation of several clustered serine residues within their regulatory domains. TANK-binding kinase 1 (TBK1) and the I-κB kinase-related IKK-ε were shown to exert this function (42, 43). The TAK1-JNK cascade was also reported to be required for IRF3 function through phosphorylating the N-terminal serine 173 residue of IRF3 instead of its canonical C-terminal segment (32). Our

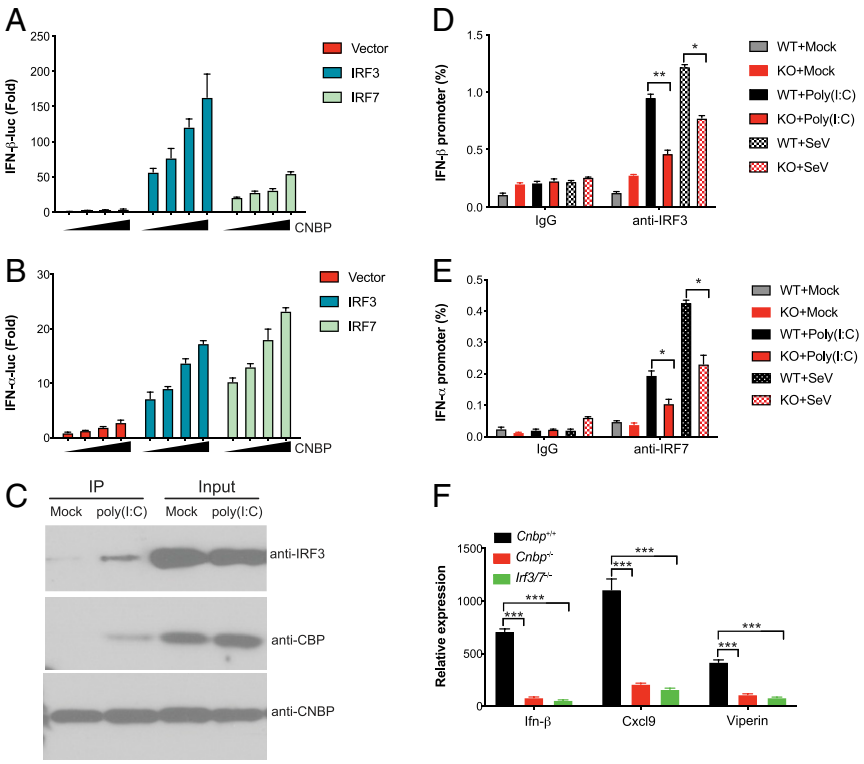

Fig. 6. CNBP synergizes with IRF3/7 to induce type I IFNs. (*A* and *B*) Luciferase activity of IFN-β-Luc (*A*) or IFN-α-Luc (*B*) in HEK293 cells after 36-h cotransfection of IRF3 or IRF7 with increasing amounts of CNBP. (*C*) Coimmunoprecipitation of CNBP and IRF3 or CREB-binding protein (CBP) in poly(I:C)-stimulated BMDMs. (*D*) ChIP–qPCR of IRF3 at the IFN-β promoter in *Cnbp*$^{+/+}$ and *Cnbp*$^{-/-}$ BMDMs stimulated with poly(I:C) or SeV. (*E*) ChIP–qPCR of IRF7 at the IFN-α promoter in *Cnbp*$^{+/+}$ and *Cnbp*$^{-/-}$ BMDMs stimulated with poly(I:C) or SeV. (*F*) qRT-PCR analysis of IFN-β, Cxcl9, and Viperin mRNA in *Cnbp*$^{-/-}$ or *Irf3/7*$^{-/-}$ BMDMs left unstimulated or stimulated with poly(I:C). Error bars represent SEM of triplicate biological replicates. All data are representative of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

experiments showed that cells treated with an inhibitor of TAK1 kinase (5Z-7-Oxozeaenol) had a dose-dependent decrease in CNBP nuclear translocation, suggesting that TAK1 kinase is essential for CNBP activation. The TAK1 signaling pathway leading to CNBP activation was parallel to that leading to IRF3/7 activation. Therefore, our studies reveal a layer of regulation in the induction of antiviral innate immune responses, which is induced via TAK1 and CNBP.

Both the IRF and NF-κB families of transcription factors are activated in response to virus infection, but the target genes that are ultimately induced through these signaling pathways are distinct. Our previous study demonstrated that CNBP could bind to cRel, which belongs to the NF-κB family, to regulate IL-12 expression (38). However, in this case, the CNBP-mediated promotion of IFN-β production is independent of cRel. Indeed, whereas the induction of proinflammatory cytokines requires NF-κB, type I IFN gene induction mainly relies on IRF activation. Our study demonstrated that CNBP is important for the recruitment of IRF3 and IRF7 to IFN promoters. CNBP and IRF3/7 synergized with each other to achieve maximal IFN production. However, how this synergy is fulfilled with respect to CNBP and IRF3 structure remains to be determined. In addition, although IRF3 and IRF7 have been assumed to be the predominant transcriptional regulators in canonical TLR and RLR signaling, the role of other IRF family members, such as IRF1, in innate immune responses remains a developing field (18, 44). There is clear evidence IRF1, which could be induced rapidly following virus infection or interferon stimulation, suppresses replication of a variety of RNA viruses in some cell types (45). Signaling pathways leading to IFN and/or ISG expression through IRF1 are interrelated and, to some extent, redundant with signaling via other IRF family members, raising questions as to whether IRF1 is essential for CNBP-mediated innate immune response to RNA viruses, which will be further studied in the future.

The IFN induction mediated by the TBK1–IRF3 immune axis is also required for the cGAS–STING pathway, which plays a critical role in mediating immune defense against dsDNA viruses, including HSV and mCMV (17). Interestingly, in this case, *Cnbp*$^{-/-}$ BMDMs produce normal IFN-β when infected with HSV or mCMV, suggesting that CNBP-mediated IFN-β production is not required for the cGAS–STING pathway. A growing list of kinases have been implicated in the regulation of the IRF3 response. For example, TAK1, cPKCs, PI3K-AKT, MEKK1, and ASK1 kinase have been reported to modulate IRF3 activity. IRF3 is known to be phosphorylated on multiple sites, with phosphorylation on Serines 386 and 396 by TBK1 being particularly critical (46). It is possible that specific kinase-dependent IRF3 phosphorylation at different sites may affect its functional activity, subcellular localization, and binding networks in transcriptional circuits during various virus infection conditions. Further research is required to address how the actions of these kinases are interrelated and to elucidate the molecular basis of their synergy or inhibition. Studies are also needed to determine how CNBP affects IRF3/7 activation and whether there is a difference of activation via CNBP between DNA virus and RNA virus infection.

In addition, viruses have developed various countermeasures to subvert IFN production and signaling to survive the innate antiviral response (21, 47). HSV-1 encodes several IFN antagonists, including ICP0, which inhibits IRF3 nuclear accumulation (48, 49). HSV-1 also encodes tegument protein VP16 that blocks

IRF3 recruitment to the coactivator CREB-binding protein (CBP) and, thus, abrogates IFN production (50, 51). Therefore, it is possible that the synergy between CNBP and IRF3/7 to achieve maximal IFN production could be a potential target for viruses to evade host IFN production and signaling.

In conclusion, our results describe a previously unknown role for CNBP in host defense against RNA viruses by regulating the production of type I IFNs. Exploiting or enhancing CNBP-mediated signaling may lead to the development of important targets for the treatment of infectious diseases. Improper CNBP function has been implicated in the pathology of various diseases, such as sIBM and DM2, which were reported to be associated with a high frequency of autoimmune disease, suggesting that CNBP may contribute to the pathogenesis of autoimmune diseases. Type I IFNs combined with other inflammatory cytokines are important modulators in the maintenance of immune homeostasis. Dysregulation of type I IFNs could result in autoimmune disease, inflammatory disease, and cancers. Therefore, further work should focus on clarifying the mechanisms by which CNBP bridges the type I IFN expression and pathogenesis of related diseases.

Materials and Methods

Generation of *Cnbp* KO Mice. *Cnbp*$^{-/-}$ mice were generated using embryonic stem (ES) cells obtained from the Knockout Mouse Repository (KOMP). ES cells (Cnbp$^{tm1a(KOMP)Wtsi}$) were generated by replacing the *Cnbp* genomic locus with a neomycin cassette under the control of a Pgk1 promoter. ES cells were injected into blastocytes to generate chimeric mice at the University of Massachusetts Medical School Transgenic Core. *Cnbp* heterozygous mice were obtained by gamete line transmission from mating the chimeric mice with WT C57BL/6 mice. *Cnbp* heterozygous mice were intercrossed to generate WT and KO alleles for experiments. For cKO mice, CNBP-floxed mice were generated and crossed to Vavi-Cre, Lysm-Cre, or CD11c-Cre mice to delete CNBP in hematopoietic cells, myeloid cells, or DCs only, respectively. *Irf3*$^{-/-}$x *Irf7*$^{-/-}$ mice were obtained from Dr. Tadatsugu Taniguchi (The University of Tokyo, Tokyo, Japan). *cRel*$^{-/-}$ mice were obtained from Dr. Stephen T. Smale (University of California, Los Angeles, CA) and Dr. Igor E. Brodsky (University of Pennsylvania, Philadelphia, PA). All mouse strains were bred and maintained under specific pathogen-free conditions in the animal facilities at the University of Massachusetts Medical School in accordance with the Institutional Animal Care and Use Committee.

Cell Culture and Stimulation. BMDMs, BMDCs, PECs, and Flt3 ligand-induced CD11c+ DCs were generated as previously described (38). MEF cells were isolated from WT or CNBP$^{-/-}$ embryos (day 13.5). A549 cell lines (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (volume[vol]/vol) fetal bovine serum (Invitrogen). For all experiments, cells were plated 1 d prior to stimulation. Cells were stimulated at the following concentrations (unless mentioned otherwise): LPS (100 ng/mL), HMW or LMW poly(I:C) (25 μg/mL). Transfection of BMDMs with HMW or LMW poly(I:C) was performed using lipofectamine 2000 (Invitrogen). BMDMs were infected with HSV (10 MOI [multiplicity of infection]), mCMV (10 MOI), IAV (5 MOI), SeV (Cantell strain purchased from Charles River Laboratories), and EMCV (5 MOI) for the indicated time points for mRNA and protein analysis.

RNA Extraction and RT-qPCR. Total RNA was extracted with the RNeasy RNA extraction kit (Qiagen) according to the manufacturer's instructions. Equal amounts of RNA were reverse transcribed using the iScript complementary DNA (cDNA) synthesis kit (Bio-Rad). Diluted cDNAs (1:100 final) were subjected to qPCR analysis using iQ SYBR Green Supermix reagent (Bio-Rad). Gene expression levels were normalized to *Gapdh* as housekeeping genes. Relative mRNA expressions were calculated by the change-in-cycling-threshold method as 2−ddC(t). The specificity of amplification was assessed for each sample by melting curve analysis. Primer sequences are provided in *SI Appendix*, Table S1.

Cytokine Measurements. Cell culture supernatants or serum were assayed for cytokine levels using commercially available sandwich ELISA kits: IFN-β (R&D Systems), CCL5 (BD Biosciences), IL12p40 (R&D Systems), IL6 (R&D Systems), IL-1β (R&D Systems), and TNF-α (eBioscience). All experiments for cytokine analysis by ELISA were performed in biological triplicates.

ChIP. ChIP experiments were performed essentially, as previously described (38). Briefly, 1×10^7 primary BMDMs were used to perform immunoprecipitation with mouse monoclonal anti-CNBP, anti-IRF3, or anti-IRF7. qPCR was performed on immunoprecipitated and input fractions from the immunoprecipitation.

Flow Cytometry. *Cnbp*$^{+/+}$ and *Cnbp*$^{-/-}$ primary MEFs were infected with IAV-PR8-GFP, VSV-GFP, or mCMV-GFP at an MOI of 0.05. Cells were collected by trypsinization and fixed in 2% paraformaldehyde. Flow cytometric analyses were performed on an LSRFortessa (BD Biosciences) and analyzed using FlowJo Software (Tree Star).

Luciferase Reporter Assay. 293T cells were seeded on 96-well plates (4×10^4 cells per well) and then transfected with 50 ng IFN-β1, IFN-α4, or TNF-luciferase reporter vector and 5 ng *Renilla*-luciferase reporter vector with increasing amounts of an expression vector for CNBP or plus 10 ng c-IRF3 or IRF7 expression vector. An empty control vector was added so that a total of 200 ng vector DNA was transfected into each well. Cells were collected 36 h after transfection, and luciferase activity was measured with a Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). Firefly luciferase activities were normalized to *Renilla*-luciferase activities. All reporter assays were repeated at least three times. Data shown were average values and SEM from one representative experiment.

Coimmunoprecipitation and Western Blot Analysis. Cells were lysed 36 to 48 h after transfection of expression plasmids using 50 mM Tris HCl, pH 8.0, 150 mM NaCl, and 1% Triton X-100 containing mixture. For immunoprecipitation, lysates were incubated with the appropriate antibodies for 2 h on ice, followed by precipitation with protein G Sepharose. Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. After blocking in phosphate buffered saline (PBS) containing 0.1% Tween-20 and 5% skim milk, the blots were probed with indicated antibodies. Western blot visualization was done with enhanced chemiluminescence.

Statistics. GraphPad Prism 5 software (GraphPad Software) was used for data analysis using a two-tailed unpaired *t* test. For mouse survival study, Kaplan–Meier survival curves were generated and analyzed for statistical significance. A P value of 0.05 was considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Data are shown as mean ± SD (unless otherwise indicated in the figure legend) of one representative experiment of at least three independent experiments showing similar results.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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