PCBP2 maintains antiviral signaling homeostasis by regulating cGAS enzymatic activity via antagonizing its condensation

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Abstract

Cyclic GMP-AMP synthase (cGAS) plays a major role in detecting pathogenic DNA. It produces cyclic dinucleotide cGAMP, which subsequently binds to the adaptor protein STING and further triggers antiviral innate immune responses. However, the molecular mechanisms regulating cGAS enzyme activity remain largely unknown. Here, we characterize the cGAS-interacting protein Poly(rC)-binding protein 2 (PCBP2), which plays an important role in controlling cGAS enzyme activity, thereby mediating appropriate cGAS-STING signaling transduction. We found that PCBP2 overexpression reduced cGAS-STING antiviral signaling, whereas loss of PCBP2 significantly increased cGAS activity. Mechanistically, we showed that PCBP2 negatively regulated anti-DNA viral signaling by specifically interacting with cGAS but not other components. Moreover, PCBP2 inhibited cGAS enzyme activity by antagonizing cGAS condensation, thus ensuring the normal production of cGAMP and balancing cGAS-STING signal transduction. Collectively, our findings provide novel insight into mechanisms regulating cGAS-mediated antiviral innate immune signaling.

Introduction

The innate immune system serves as the first line of host defense against viral infection using a number of sensors known as pattern recognition receptors (PRRs). PRRs specifically recognize conserved microbial components termed pathogen-associated molecular patterns (PAMPs). Nucleic acids derived from viruses function as PAMPs and are detected by PRRs, consequently initiating the innate immune response and resulting in the production of type I interferons (IFNs) and proinflammatory cytokines. Because cGAS can detect both self and non-self DNA, its activity must be strictly regulated to maintain the balance in innate immune responses, subsequently preventing the development of autoimmune diseases. cGAS activity is regulated by multiple factors, including protein post-translational modifications, such as phosphorylation, glutamylation, sumoylation, acetylation, and ubiquitination. In addition to these post-translational modifications, cGAS function has been found to be negatively regulated by several other factors. For example, previous studies demonstrated that Caspase1/3 cleaves cGAS to inhibit cGAS-STING signaling, and Gasdermin D suppresses cGAS activity by disrupting K+ efflux. The autophagy protein Beclin-1 was found to inhibit cGAS enzymatic activity, and p62 is involved in the autophagic degradation of cGAS. Although previous studies have made significant progress in understanding the mechanisms regulating cGAS activity, how cGAS activity is tightly controlled to maintain immune homeostasis remains unclear.

Previous studies have indicated that cGAS can form dimers, undergo oligomerization after sensing cytosolic DNA, and form liquid droplets in vitro and in vivo. DNA-induced liquid phase condensation of cGAS promotes its enzymatic activity to increase cGAMP production and plays an important role in antiviral signaling. However, the mechanisms that dynamically regulate cGAS condensation to initiate an appropriate immune response to pathogens and prevent overreaction are poorly understood.

PCBP2 belongs to a class of proteins that bind to poly(C) stretches of both RNA and DNA, and it plays important roles in regulating mRNA stability and protein translation, and protein-protein interactions. A previous study demonstrated that PCBP2 is a negative modulator of innate immune responses against RNA virus infection by regulating MAVS stability via the HECT ubiquitin ligase AIP4. However, whether PCBP2 is involved in cGAS-STING signaling is still unclear.

To further explore the mechanism underlying the regulation of cGAS in the context of viral infection, we performed co-immunoprecipitation (Co-IP) experiments in combination with mass spectrometry assays and identified PCBP2 as a cGAS-interacting protein. We showed that PCBP2 overexpression significantly impaired cGAS-STING signaling, whereas PCBP2 deficiency remarkably enhanced the innate immune response induced by DNA stimulation or DNA virus infection in various cell lines. In addition, PCBP2 reduction in mouse embryonic fibroblasts (MEFs) apparently reduced HSV-1 virus replication. Mechanistically, we found that PCBP2 could interact with cGAS and negatively affect its enzymatic activity. Furthermore, we determined that PCBP2 evidently attenuated cGAS oligomerization and cGAS-DNA phase separation. Collectively, we revealed that PCBP2 negatively regulates cGAS-mediated innate immune responses against DNA virus infection by attenuating the enzyme activity of cGAS. Thus, our findings provide new insight into the mechanisms of innate immune responses against DNA virus infection that function to maintain immune homeostasis.

Results

Identification of PCBP2 as a cGAS-interacting factor

To better understand the molecular basis underlying the regulation of cGAS upon DNA virus infection, we aimed to identify proteins associated with cGAS. We infected human HEK293A cells with lentivirus expressing SFB-tagged cGAS or an empty vector for 48 h and then performed Co-IP experiments, followed by mass spectrometry analysis. Using this approach, we identified a number of proteins that were potentially associated with cGAS. Among these candidates, PCBP2 was of particular interest because it has been shown to regulate the MAVS-mediated antiviral response against RNA virus infection. To verify the association of PCBP2 with cGAS, we co-expressed PCBP2 and cGAS in HEK293T cells and performed additional Co-IP experiments. The results showed that PCBP2 was associated with cGAS (Fig. 1a). To determine whether the interaction between PCBP2 and cGAS is direct, we generated recombinant cGAS-His and GST-PCBP2 purified from Escherichia coli (E. Coli) and performed in vitro pull-down experiments. As shown in Fig. 1b and c, cGAS-His efficiently pulled down GST-PCBP2 but not the GST-GFP control, and GST-PCBP2 but not the GST-GFP control pulled down cGAS-His, suggesting that PCBP2 directly binds to cGAS. Next, to determine whether PCBP2 forms a complex with cGAS under physiological conditions, we used an anti-cGAS antibody to perform Co-IP assays in vivo.
THP-1 cells and found that PCBP2 was detected in the cGAS immuno-precipitants under normal physiological conditions. Interestingly, we also observed that HSV-1 infection enhanced cGAS-PCBP2 interaction (Fig. 1d), suggesting that endogenous cGAS and PCBP2 form a complex and that this interaction is regulated by viral infection in THP-1 cells. In addition, immunostaining assay results showed that PCBP2 was co-localized with cGAS following their co-transfection in HeLa cells (Fig. 1e). These data suggested that PCBP2 forms a complex with cGAS.

PCBP2 contains three heterogeneous nuclear ribonucleoprotein K-homology (KH) domains and a linker region. To map which domain is required for PCBP2 to associate with cGAS, we generated a series of deleted forms of PCBP2, in which the KH1, KH2, linker, or KH3 domain was deleted. As shown by the results of Co-IP assays, KH3 but not KH1, KH2, or the linker was important for PCBP2-cGAS interactions (Fig. 1f). Next, we aimed to identify the domain in cGAS that is required for its interaction with PCBP2. Because cGAS contains a less-conserved N-terminal disordered region and highly conserved C-terminal catalytic domains harboring NTase and Mab21 domains, we generated two deleted forms of cGAS for subsequent Co-IP experiments. As shown in Fig. 1g, C-terminal domain but not the N-terminal domain, were essential for the association between cGAS and PCBP2. These data suggested that the interaction between PCBP2 and cGAS is dependent on specific domains.

**PCBP2 inhibits cGAS-STING antiviral signaling**

Given that PCBP2 associates with cGAS and that this association is regulated by viral infection, we reasoned that PCBP2 might regulate cGAS-STING-mediated antiviral signaling by targeting cGAS. We first generated a cell-based luciferase reporter system to test the role of PCBP2 in the type-I IFN signaling induced by cGAS. In the control experiments, the co-expression of cGAS and STING activated the IFNβ promoter in HEK293T cells. In contrast, we found that PCBP2 overexpression significantly downregulated the activity of IFNβ induced by the co-expression of cGAS and STING, suggesting a role of PCBP2 in balancing cGAS-STING signaling activity (Fig. 2a). Of note, the stimulation of IFN-β requires the coordinated activation of both IRF3 and NF-κB transcription factors. To understand how PCBP2 regulates cGAS-STING signaling, we used an IFN-stimulated response element (ISRE) luciferase reporter that can be activated by IRF3 and an NF-κB luciferase reporter to perform cell-based luciferase assays. As shown in Fig. 2b and c, PCBP2 overexpression significantly reduced the activation of the ISRE reporter that was stimulated by the co-expression of cGAS and STING but had no effect on the NF-κB reporter. These results suggest that PCBP2 attenuated the cGAS-mediated induction of IFNβ activation by regulating IRF3 activation.

To verify our observation from reporter assays, we then performed quantitative reverse transcription PCR (qRT-PCR) analysis to measure the transcriptional levels of antiviral genes, such as *Ifnb1, Ifit1*, and *Cxcl10*, in human macrophage THP-1 cells with or without PCBP2 overexpression following the infection of herpes simplex virus type 1 (HSV-1), a double-stranded DNA virus. As shown in Fig. 2d–f, PCBP2 overexpression significantly reduced the mRNA levels of *Ifnb1, Ifit1*, and *Cxcl10* induced by HSV-1 infection. Given that the phosphorylation of TBK1 and IRF3 is a hallmark of activated antiviral signaling, we then tested whether PCBP2 overexpression affects the phosphorylation of these factors. As shown in Fig. 2g, we observed that PCBP2 overexpression in THP-1 cells significantly reduced the levels of phosphorylated TBK1 and IRF3 induced by Herring testis DNA (HT-DNA), which mimics viral DNA ligands that bind to cGAS. In addition, we tested whether PCBP2 regulates cGAS signaling in a mouse cell line and found that overexpressed PCBP2 significantly reduced the mRNA expression of *Ifnb1* and *Ifit1* but not *Il-6* in mouse fibroblast L929 cells (Supplementary Fig. 1a–c). Collectively, these findings suggest that PCBP2 overexpression antagonizes cGAS-mediated antiviral signaling.

The experiments described above indicated that the KH3 domain of PCBP2 is critical for its association with cGAS. We then examined whether the deletion of this domain affects the ability of PCBP2 to regulate cGAS signaling. As shown in Fig. 1h, luciferase reporter assays revealed that deletion of the KH3 domain of PCBP2 resulted in the loss of its ability to inhibit cGAS-STING activation, whereas other deletion mutants exhibited a similar inhibitory ability as full-length PCBP2. These results suggested that the KH3-mediated PCBP2-cGAS interaction is important for PCBP2 to antagonize cGAS-mediated antiviral signaling.

**Depletion of PCBP2 enhances cGAS-STING signaling**

Next, we tested whether the knockdown of endogenous PCBP2 affects cGAS-mediated antiviral signaling. For this purpose, we generated two lentiviral constructs expressing short hairpin RNAs (shRNAs) against different regions of human PCBP2 (shPCBP2-1 and shPCBP2-2). We infected THP-1 cells with a lentivirus carrying shPCBP2-1 or shPCBP2-2 and then performed western blotting. The results showed that shPCBP2-2 displayed a higher PCBP2 knockdown efficiency (Supplementary Fig. 2a), and thus it was selected for further experiments. As shown in Fig. 3a–c, PCBP2 knockdown significantly enhanced the mRNA level of *Ifnb1* and *Ifit1* induced by HSV-1 infection, whereas the mRNA expression of *Il-6*, a downstream target of NF-κB, was not significantly affected by PCBP2 knockdown compared with control cells (Fig. 3d). Consistently, the knockdown of PCBP2 in THP-1 cells further elevated the levels of phosphorylated TBK1 and IRF3, but not p65, stimulated by HT-DNA (Fig. 3e). To obtain more evidence supporting our conclusion, we studied the function of PCBP2 in mouse cells. We infected mouse macrophage Raw 264.7 cells with two shRNAs specifically targeting the coding region of mouse Pcbp2. As shown by qRT-PCR analysis, shPcbp2-2 exhibited a higher knockdown efficiency, and shPcbp2-2-mediated Pcbp2 knockdown significantly increased the production of *Ifnb1, Ifn4*, and *Ccl11* mRNA following HSV-1 virus infection compared with control cells (Supplementary Fig. 2b–e). We also observed similarly enhanced antiviral responses at different time points after HSV-1 infection (Fig. 3f–h). Collectively, these findings further support the notion that Pcbp2 acts as a negative regulator to balance cGAS signaling.

**Enhanced cGAS-STING signaling in Pcbp2-deficient cells**

To further substantiate the biological role of PCBP2 in cGAS-STING signal transduction, we used the CRISPR-Cas9 method to generate a Pcbp2 knockout L929 cell line and examined whether deletion of Pcbp2 affects cGAS-mediated antiviral signaling. Wild-type and Pcbp2-deficient cells from two different clones were transfected with HT-DNA, and the results of qRT-PCR assays showed that the transcriptional levels of *Ifnb1* and *Ifit1* stimulated by HT-DNA were remarkably increased in *Pcbp2*−/− cells compared with wild-type control cells (Fig. 4a, Supplementary Fig. 3a). Moreover, the levels of TBK1 and IRF3 phosphorylation induced by HT-DNA transfection were also enhanced in *Pcbp2*−/− cells compared with *Pcbp2*+/+ cells under the same experimental conditions (Fig. 4b). We also found that loss of Pcbp2 significantly increased the mRNA expression of *Ifnb1* following HSV-1 infection in a time-independent manner (Fig. 4c).
were co-transfected with PCBP2 or an empty vector into HEK293T cells, and then Co-IP assays were conducted to examine whether PCBP2 affected the self-

cGAS enzyme activity by regulating cGAS oligomerization. To test this hypothesis, we conducted the following experiments. First, HA-cGAS and Flag-cGAS

described above demonstrated that PCBP2 interacted with cGAS and inhibited its enzymatic activity. Thus, we hypothesized that PCBP2 likely modulated

PCBP2 inhibits cGAS enzymatic activity by antagonizing its condensation

PCBP2 attenuates the enzymatic activity of cGAS to balance cGAS signaling

Because cGAS is a synthase involved in the production of cGAMP, we reasoned that PCBP2 targets cGAS likely by negatively regulating its enzymatic activity,

PCBP2 modulates cGAS-STING signaling by specifically targeting cGAS

We next aimed to better understand the molecular mechanism by which PCBP2 antagonizes the cGAS-mediated antiviral response. To determine whether
cGAS is a specific target of PCBP2, we performed Co-IP experiments to examine if PCBP2 can interact with other known components in the cGAS-STING

PCBP2-decient cells and found that knockdown of cGAMP induced by HT-DNA transfection in THP-1 cells (Fig. 5h). In addition, we measured the levels of cGAMP in wild-type and

Our results described thus far support an intriguing model that PCBP2 negatively regulates the cGAS-STING signaling pathway likely by specifically targeting cGAS. To test our model, we next investigated whether PCBP2 overexpression affected the activation of components downstream from cGAS, including STING, TBK1, and IRF3. In contrast to its role in antagonizing the activation of cGAS, PCBP2 failed to influence the activation of STING, TBK1, or IRF3-5D (a constitutively active form of IRF3) (Fig. 5b–e). In particular, we found that overexpression of PCBP2 did not affect the levels of IRF3 and TBK1 phosphorylation induced by the ectopic expression of STING (Supplementary Fig. 4b). Collectively, these data suggest that PCBP2 is not a specific target of PCBP2, but rather that it functions upstream of STING to regulate cGAS-STING signaling.

PCBP2 Inhibits cGAS enzymatic activity by antagonizing its condensation

Next, we investigated how PCBP2 regulates the enzymatic activity of cGAS. Previous studies have shown that DNA binding induces cGAS conformational changes and that cGAS can form dimers and undergo oligomerization, which plays an important role in regulating its enzymatic activity. Our experiments described above demonstrated that PCBP2 interacted with cGAS and inhibited its enzymatic activity. Thus, we hypothesized that PCBP2 likely modulated cGAS enzyme activity by regulating cGAS oligomerization. To test this hypothesis, we conducted the following experiments. First, HA-cGAS and Flag-cGAS were co-transfected with PCBP2 or an empty vector into HEK293T cells, and then Co-IP assays were conducted to examine whether PCBP2 affected the self-

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association of cGAS. As shown in Fig. 6a, the self-association of cGAS was affected by PCBP2 overexpression. Second, we performed semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) assays to detect protein oligomerization. As shown in Fig. 6b, we found that overexpressed cGAS could form high molecular weight (HMW) polymers, whereas PCBP2 co-expression significantly reduced the levels of cGAS oligomerization in HEK293T cells. Conversely, PCBP2 knockout enhanced cGAS oligomerization induced by HT-DNA treatment in L929 cells (Fig. 6c). Similar results were obtained in PCBP2 knockout THP1 cells following HSV-1 infection (Supplementary Fig. 5a). In addition, we conducted an in vitro cGAS oligomerization assay and found that PCBP2 significantly attenuated cGAS oligomerization regardless of the presence or absence of HT-DNA treatments (Fig. 6d). Third, we conducted immunostaining experiments and found that PCBP2 overexpression significantly reduced the formation of cGAS granules in HEK293A cells (Fig. 6e). Conversely, cGAS formed substantially larger granules in L929 Pcbp2−/− cells than in control cells after stimulation of ISD, a 45 bp double-stranded interferon stimulatory DNA (Fig. 6f). Furthermore, larger cGAS granules were also observed in THP-1 PCBP2−/− cells compared with those in wild-type cells following ISD treatment (Supplementary Fig. 5b). Taken together, these data demonstrated that PCBP2 modulated cGAS oligomerization.

A previous study showed that cGAS could form liquid-like droplets after binding to DNA, which plays an important role in regulating cGAS activity. To test whether PCBP2 affects cGAS-DNA phase separation, we purified GFP-cGAS and GST-PCBP2 proteins and conducted in vitro protein phase separation analysis. As shown in Fig. 6g, GFP-cGAS formed larger droplets in the solution within 5 minutes after adding ISD. However, the addition of PCBP2 remarkably reduced the increased size of cGAS droplets induced by ISD treatment. We observed similar results when VACV70 (Fig. 6h) or HSV120 (120 bp dsDNA representing the genome of HSV-1) (Fig. 6i) was added. Collectively, these data demonstrate that PCBP2 modulates cGAS condensation.

Discussion

cGAS functions as a cytosolic sensor to detect the pathogenic DNA of viruses, bacteria, or damaged self-DNA. After binding to DNA, cGAS becomes activated and catalyzes the synthesis of cGAMP, which binds to STING to activate downstream signaling, subsequently inducing the production of IFNs and inflammatory cytokines. cGAS-cGAMP-STING-mediated signaling is tightly regulated to maintain innate immune system homeostasis and prevent the overproduction of IFNs, which can be detrimental to the host and even lead to the development of autoimmune diseases. Previous studies have reported several negative regulators of cGAS, such as AKT kinase, p62, Beclin1, Caspase 1, Caspase 3, Gasdermin D, and TTLL6/4. However, the processes that balance the activity of cGAS to avoid harmful overreaction remain largely unknown. In this study, we found that PCBP2 maintained proper cGAS signaling by targeting cGAS. Mechanistically, we found that PCBP2 attenuated the enzyme activity of cGAS to reduce cGAMP production by antagonizing cGAS condensation.

A previous study showed that PCBP2 mRNA and protein levels were induced by SeV infection. PCBP2 was mainly localized in the nuclei of untreated cells but could translocate to the cytosol, co-localize with MAVS in mitochondria upon SeV infection, and recruit the E3 ligase AIP4 to catalyze MAVS ubiquitination, thereby targeting it for degradation. We tested whether PCBP2 exhibited a similar function upon HSV-1 infection. We observed no apparent enhancement of PCBP2 mRNA, whereas cGAS and IFNB1 mRNA were significantly increased following HSV-1 infection in THP-1 cells (Supplementary Fig. 6a–c). However, immunostaining assays revealed that PCBP2 could translocate to the perinuclear region, where it showed a punctate distribution in HeLa cells after transfection with HT-DNA (Supplementary Fig. 6d). Similarly, we observed that PCBP2 translocated to the cytosol when cells were infected with HSV-1 virus or transfected with cGAS (Supplementary Fig. 6d and e). These results suggested that PCBP2 can translocate to the cytoplasm and target cGAS to inhibit antiviral signaling upon DNA virus infection. How PCBP2 translocates to the cytosol following virus infection is an interesting question that requires further study.

Oligomerization is important for cGAS to exert its enzyme activity. Upon binding to DNA, cGAS can undergo oligomerization and form liquid droplets in vitro and in vivo. Both DNA binding and self-protein-protein association of cGAS are important for the formation of the cGAS oligomeric complex; however, the mechanisms that dynamically regulate this complex to maintain an appropriate innate immune response are still unclear. In this study, we obtained extensive evidence supporting that PCBP2 inhibits cGAS oligomerization. First, we conducted Co-IP experiments and found that PCBP2 regulated cGAS self-association. Second, we conducted SDD-AGE assays and observed that PCBP2 overexpression reduced cGAS oligomerization in HEK293T cells, whereas PCBP2 deficiency enhanced cGAS oligomerization induced by HSV-1 infection and HT-DNA stimulation. We also demonstrated that PCBP2 significantly attenuated cGAS oligomerization in vitro. Third, we conducted immunostaining assays and found that PCBP2 overexpression reduced the size of cGAS granules. Conversely, cGAS formed substantially larger granules in PCBP2 knockout L929 and THP-1 cells compared with those in wild-type cells following ISD treatment. Forth, we performed in vitro phase separation and found that PCBP2 remarkably inhibited the DNA-induced liquid phase condensation of cGAS. Taken together, these data suggest that PCBP2 negatively regulates cGAS enzyme activity by modulating cGAS oligomerization to maintain the innate immune response. Importantly, our findings provide a novel understanding of the mechanism underlying the dynamic regulation of the cGAS oligomeric complex.

Methods

Ethics statements

All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols for animal studies were approved by the Committee on the Ethics of Animal Experiments of the Institute of Zoology, Chinese Academy of Sciences (Beijing, China) (approval number: IOZ15001).

Cell culture
HEK293T, HeLa, L929, and RAW264.7 cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. MEFs from wild-type and mutant mice were generated from 13.5-day-old embryos and cultured in complete DMEM containing 1 mM sodium pyruvate, 10 mM L-glutamine, 10 mM β-mercaptoethanol, and 1% nonessential amino acids. THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin, 1% streptomycin, 10 μM β-mercaptoethanol, and 5 mM HEPES.

**Plasmids**

Mammalian expression plasmids encoding Flag-tagged PCBP2, cGAS, STING, TBK1, IRF3, and IRF3-5D; hemagglutinin (HA)-tagged cGAS, STING, and PCBP2; SFB-cGAS, GFP-tagged cGAS and mCherry-tagged PCBP2; bacterial expression plasmids encoding histidine (His)-tagged cGAS and GFP-cGAS; and glutathione S-transferase (GST)-tagged PCBP2 were constructed using standard molecular biology methods. PCBP2 and cGAS mutants were generated by PCR using Pfu DNA polymerase. IFN-β, NF-κB, and ISRE-luciferase (Luc) reporter plasmids have been described previously. In our laboratory, the cGAS antibody was generated by immunizing mice or rabbits with purified human cGAS full-length from *E. coli*.

**DNA oligonucleotides.**

All DNA oligonucleotides were synthesized by Tsingke Biological Technology Company. For Cy3- or biotin-labeled ISD, VACV70 or HSV120, the sense strand was modified at the 5' end; the antisense strand was not modified. The sequences of oligonucleotides used in this study are as follows:

- **ISD45:**
  5'-TACAGATCTACTAGTGTACATGACTGATCTACATGATCTACA-3'.
- **VACV70:**
  5'-CCATCAGAAAGAGGTTTAATATTTTGTTGAGACCATGGAAGAGAAAGAGATAAAACTTTTTTACGACT-3'.
- **HSV120:**
  5'-AGAGGTTATATTTTGGCTTATCCTACTGTCGCCGTGACCAGACGTTTCTTGTTGGGATAGGCATGCCCAGAAAGCATATTGGGTTAACCCCTTTTTATTGTGGCGGGTTTTT3'.

**Luciferase reporter analysis and transfection**

HEK293T cells were transfected using the standard calcium phosphate transfection method or polyethyleneimine. A Renilla reporter plasmid and firefly luciferase reporter plasmids encoding IFNβ-Luc, NF-κB-Luc, or ISRE-Luc were co-transfected with the indicated expression plasmids. An empty control plasmid was used in the same experiment to ensure that the same amount of total DNA was transfected. Cells were lysed to measure luciferase activity. Firefly luciferase activity was normalized to Renilla activity. All reporter assays were repeated at least three times.

**Co-IP and immunoblotting analysis**

Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA) supplemented with a complete protease inhibitor cocktail (Roche). Clarified cell lysates were incubated with anti-Flag M2 agarose beads (Sigma Aldrich) for 4 h at 4°C. The immunoprecipitated complexes were washed with lysis buffer containing 300 mM NaCl three times and subjected to immunoblotting with the indicated antibodies. For endogenous IP, cell lysates were incubated with a cGAS or PCBP2 antibody overnight at 4°C, followed by further incubation with protein A/G beads (Pierce) for 2 h. Immunoblotting was carried out using standard procedures.

**Identification of cGAS-interacting proteins by mass spectrometry**

HEK293A cells were infected with lentivirus expressing SFB-tagged cGAS or an empty vector for 48 h and then lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 10% glycerol, 1 mM EDTA, 1 mM EGTA) containing a complete protease inhibitor cocktail, followed by centrifugation at 20,000 g for 10 min at 4°C. The supernatants were subjected to immunoprecipitation using S-protein agarose (Millipore). Immunoprecipitates were separated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. The entire lane was cut into 2-mm gel slices, digested with Trypsin, and subjected to LC-MS/MS analyses using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). The mass spectrometry data were analyzed using Thermo Proteome Discovery (version 2.3), and tandem mass spectra were searched against the UniProt-*Homo sapiens* database. Ranking of the identified proteins was based on the peptide abundance ratio of sample/control and reproducibility among the MS analyses of different samples.

**Expression and purification of recombinant proteins in *E. coli***

Constructs encoding human cGAS and GFP-tagged cGAS were cloned into the pET-28a vector carrying a C-terminal 6×His tag. GST-tagged full-length human PCBP2 and its mutant were inserted into a pGEX-4T-1 vector containing an N-terminal GST-tag. The plasmids were transformed into the BL21 *E. coli* strain. The fusion proteins were purified from the cell lysates using Ni-Sepharose beads (GE Healthcare) or Glutathione-Sepharose beads (GE Healthcare) in accordance with the manufacturer's protocols.
**Immunofluorescence staining**

HeLa, HEK293A, L929, or THP-1 cells were seeded on gelatin-coated glass coverslips and then transfected or infected as indicated. The cells were fixed with 4% paraformaldehyde for 15 min, permeabilized, and blocked with PBS containing 0.2% Triton-X-100 and 5% BSA for 25 min at room temperature. The cells were incubated with the primary antibody, followed by the secondary antibody. The cells were washed with PBST (PBS with 0.2% Tween 20) between each step. Images were acquired using a Zeiss LSM 710 META laser scanning confocal system.

**Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)**

Total RNA was isolated using TRIZOL reagent (Invitrogen). cDNA was generated using the SuperScript II First-Strand cDNA Synthesis kit (TianGen Biotech). qRT-PCR was conducted in duplicate using a SYBR Green Master Mix (CoWin Biosciences) on a Light Cycler 480® (Roche). Relative mRNA levels were normalized to GAPDH or actin mRNA levels in each sample. Relative expression changes were calculated by the $2^{ΔΔCt}$ method. Data are shown as the mRNA abundance relative to control groups. The primers used were listed in Supplemental table1.

**Lentivirus-mediated PCBP2 overexpression and shRNA knockdown**

Full-length cDNA encoding human PCBP2 was amplified and inserted into a pCDH-CMV-Puro vector. The lentivirus particles for PCBP2 overexpression were produced by co-transfecting a pCDH-CMV-Puro-PCBP2 construct into HEK293T cells with the packaging plasmids pMD2.G and pAS-MAX. To generate PCBP2-knockdown cells, we used pLKO.1-puro-based lentiviruses expressing specific short hairpin RNAs (shRNAs) against PCBP2. RAW264.7 cells or THP-1 cells were infected with lentiviruses expressing shRNAs against PCBP2 (shPCBP2) or control vector (pLKO), and the cells were selected with puromycin (2 μg/ml) to generate stable PCBP2 knockdown or control pLKO cells. The knockdown efficiency was determined by qRT-PCR or western blotting analysis. The shRNA sequences against human PCBP2 or mouse Pcbp2 are as follows:

Human PCBP2 shRNA-1#: 5’-ACCGGGGATTCACATCCATCTTGCTGAGCAATGATGGATTGTGGAATGCTTTTT-3’

Human PCBP2 shRNA-2#: 5’-ACCGGCCATGATCCTGTGTAGTTCTCGAGAACTACACAGATGATGGTTTTT-3’

Mouse Pcbp2 shRNA-1#: 5’-ACCGGCCATCCATAATCCTGCTGTTCTCGAGAACAGCAGGATTATGGATGGGTTTTT-3’

Mouse Pcbp2 shRNA-2#: 5’-ACCGGTCTGAGAAATTATCCTACTTCTCGAGAAAGTGATAATTCTCTCAGGATTTTT-3’

**CRISPR/Cas9-mediated PCBP2-knockout cell lines**

To generate PCBP2 knockout (PCBP2−/−) cells, lenti-CRISPRv2-sgPCBP2-Puro vectors were constructed in accordance with the method described by Sanjana et al40 and co-transfected with packaging plasmids into HEK293T cells. Two days after transfection, the viruses were harvested and used to infect THP-1 or L929 cells. The infected cells were selected with puromycin (2μg/ml) for at least 5 days. PCBP2-knockout cells were verified by immunoblotting. PCBP2 guide RNA sequences were as follows:

hPCBP2 sg-s: 5’-CACCCTAGGGTGACCGGGGGTCTAC-3’

hPCBP2 sg-as: 5’-AAACGTAGACCCCCGGTCACCCGT-3’

mPcbp2 sg-s: 5’-CACCCTAGGGTGACCGGGGGTCTAC-3’

mPcbp2 sg-as: 5’-AAACGTAGACCCCCGGTCACCCGT-3’

**CRISPR/Cas9-mediated PCBP2-knockout mice**

To generate Pcbp2 knockout mice, the CRISPR/Cas9-mediated gene deletion system was used. Cas9 mRNA and single-guide RNA targeting Pcbp2 sequences were co-injected into zygotes to obtain heterozygous mutants. Homozygous and heterozygous MEF cells were obtained from 13.5-day-old embryos by breeding heterozygous mutants. The sequences targeting Pcbp2 were (5’-3’): ATCTGGTTAAGATCCGGCG. The mutant was obtained with 8 bp deletion in the forth exon of Pcbp2, which prematurely terminate protein translation. Pcbp2 homozygous or heterozygous MEF cells were verified by immunoblotting analysis.

**cGAS enzyme activity assays and cGAMP quantification**

cGAS activity was analyzed as described previously 4,5. An in vitro cGAS reaction assay was conducted by mixing recombinant human cGAS protein with GST-PCBP2 or GST control in the presence or absence of HT-DNA (50 ng/μl) in a low-salt buffer (20 mM HEPES, pH 7.5, 5 mM MgCl2, 2 mM ATP, 2 mM GTP) and incubating the mixture at 37°C for 2 h. The reaction was terminated by heating at 95°C for 5 min to denature proteins, followed by centrifugation to
remove the denatured proteins. The supernatant was incubated with L929-ISRE cells that were permeabilized with digitonin solution (50 mM HEPES pH 7.0, 100 mM KCl, 3 mM MgCl₂, 85 mM sucrose, 0.1 mM DTT, 0.2% BSA, 1 mM ATP, 0.1 mM GTP, and 10 μg/ml digitonin) at 37°C for 30 min. The cells were cultured at 37°C for 12 h and then lysed for luciferase assays. Serial dilutions of cGAMP were used to generate the standard curve for quantifying cGAMP concentrations in the reactions.

A 2′-3′-cGAMP ELISA kit (Cayman Chemical) was used to measure cGAMP levels in HT-DNA transfected cells. Briefly, cells were transfected with HT-DNA using Lipofectamine 2000. Four hours after transfection, the cells were harvested and lysed in 100 µl of hypotonic buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 3 mM MgCl₂). Cell lysates were heated at 95°C for 5 min, followed by centrifugation to remove the denatured proteins. The heat-resistant supernatants were used to measure cGAMP abundance. The 2′-3′-cGAMP ELISA kit was used in accordance with the protocol of the manufacturer.

**Measurement of HSV-1 genomic DNA copy numbers**
Pcbp2⁺/⁺ and Pcbp2⁺/- MEFs were infected with HSV-1 (MOI=0.1) and incubated at 37°C in serum-free DMEM for 1 h. The cells were washed with warm PBS, cultured in complete DMEM for 18 h, and their genomic DNA was extracted. The HSV-1 genomic DNA copy numbers were determined by qRT-PCR using HSV-1-specific primers with the following sequence: 5’-TGGGACACATGCCTTCTTGG-3’; 5’-ACCCTTAGTCAGACTCTGTTACTTACCC-3’.

**SDD-AGE assay**
The cells were transfected as indicated and lysed in lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl, 150 mM NaCl, and 3 mM MgCl₂). The supernatants were separated by 1.5% SDD-AGE as previously described.

**In vitro phase separation assay**
Recombinant GFP-cGAS protein was mixed with GST or GST-PCBP2 and the indicated DNA in a glass-bottom cell culture dish. The mixtures were incubated in the buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mg/ml BSA at room temperature, and images were captured at the indicated times. Phase-separated droplets were imaged using an ANDOR CR-DFLY-505 confocal microscope equipped with a sCMOS Zyla 4.2 plus camera, and images were analyzed using Imaris software.

**Statistical analysis**
Results of all statistical analyses are shown as mean ± SD. Significant differences between samples under different experimental conditions were performed using two-tailed Student’s t-test. For all tests, P values < 0.05 were considered statistically significant.

**Declarations**

**Author contributions**
H.G., D.C., and Q.S. designed the experiments; H.G., J.Y, J.Z., P.Z., and L.L performed experiments; H.G., J.Y, J.Z., D.C., and Q.S. analyzed data; H.G., D.C., and Q.S. wrote the paper. All authors provided intellectual input, vetted and approved the final manuscript.

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**Competing interests**
The authors declare no competing interests.

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Identification of PCBP2 as a cGAS-interacting factor (a) HEK293T cells were co-transfected with Myc-tagged cGAS and Flag-tagged PCBP2 or an empty vector, and cell lysates were prepared and immunoprecipitated with anti-Flag beads, followed by immunoblot analysis. (b–c) Purified cGAS-His was incubated with GST-PCBP2 or GST-GFP and then pulled down with Ni-Sepharose beads (b) or glutathione-Sepharose beads (c). Western blotting was performed to detect the presence of His-tagged cGAS and GST-tagged PCBP2 proteins. (d) THP-1 cells were infected with HSV-1 or mock-infected for 12 h, and then cell lysates were prepared and immunoprecipitated with a rabbit anti-cGAS antibody or control IgG, followed by immunoblotting. (e) HeLa cells were transfected with GFP-cGAS or mCherry-PCBP2 individually or co-transfected with both plasmids. Twenty-four hours after transfection, the cells were fixed, stained with DAPI, and observed by confocal microscopy. Scale bars, 10 μm. (f) Schematic diagram of PCBP2 and its truncated mutants (upper panels). HEK293T cells were transfected with the indicated plasmids, and cell lysates were immunoprecipitated with anti-Flag beads, followed by immunoblotting (lower panels). (g) Schematic diagram of cGAS and its truncated mutants (upper panels). HEK293T cells were cotransfected PCBP2 with cGAS, its truncated mutants, or an empty vector, as indicated. The cell lysates were immunoprecipitated with anti-Flag beads, followed by immunoblotting with the indicated antibodies (lower panels).
Knockdown of PCBP2 increases cGAS-STING signaling (a–d) THP-1 cells were infected with lentiviruses-based shRNA targeting PCBP2 or an empty vector for 48 h and then left uninfected or infected with HSV-1 (MOI=5) for 6 h. The cells were harvested for qRT-PCR assays to measure the transcriptional levels of PCBP2 (a), IFNB1 (b), IFIT1 (c), and IL6 (d). (e) THP-1 cells were infected with a lentivirus-based shRNA targeting PCBP2 (shPCBP2-2) or an empty vector for 48 h, and then transfected with HT-DNA (2 μg/ml) for 6 h or mock-treated (Mock). The cells were lysed and followed by immunoblotting with the indicated antibodies. (f–h) RAW264.7 cells were infected with a lentivirus-based shRNA targeting Pcbp2 (shPcbp2-2) or an empty vector for 48 h and then infected with HSV-1 (MOI=5) for the indicated times. Transcriptional levels of Pcbp2 (f), Ifnb1 (g), and Ifna4 (h) were detected by qRT-PCR assays. The data shown in (a–d, f–h) are from one representative experiment of at least three independent experiments (mean ± SD of duplicate experiments). The two-tailed Student's t-test was used to analyze statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001; n.s. not significant versus the control groups.
PCBP2 deficiency augments cGAS-STING signaling. (a) Two different clones from L929 wild-type and Pcbp2-deficient cells were transfected with HT-DNA (2 μg/ml) for 6 h. The cells were harvested for qRT-PCR analysis to measure the transcriptional level of Ifnb1. (b) L929 Pcbp2+/+ and Pcbp2−/− cells were transfected with HT-DNA (2 μg/ml) for the indicated times, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. (c) L929 Pcbp2+/+ and Pcbp2−/− cells were infected with HSV-1 (MOI=5) for the indicated times and then harvested for the measurement of Ifnb1 mRNA levels by qRT-PCR. (d) L929 Pcbp2+/+ and Pcbp2−/− cells were first infected with a lentivirus expressing PCBP2 or an empty vector. After 48 h infection, cells were then transfected with or without HT-DNA (2 μg/ml) for 6 h, followed by qRT-PCR analysis. (e−f) THP-1 PCBP2+/+ and PCBP2−/− cells were infected with HSV-1 at an MOI of 5 for 6 h. The cells were harvested for qRT-PCR analysis to measure the mRNA levels of IFNB1 (e) and IFIT1 (f). (g) THP-1 PCBP2+/+ and PCBP2−/− cells were transfected with HT-DNA (2 μg/ml) for the indicated times, followed by immunoblotting with the indicated antibodies. (h−j) Pcbp2+/+ and Pcbp2−/− MEFs were infected with HSV-1 (MOI=5) for the indicated times and then lysed for the quantification of Ifnb1 (h), Ifit1 (i), and Cxcl10 (j) mRNA levels by qRT-PCR. (k) Pcbp2+/+ and Pcbp2+/- MEFs were infected with HSV-1 at an MOI of 5 for the indicated times and then lysed for immunoblotting with the indicated antibodies. (l) Pcbp2+/+ and Pcbp2−/− MEFs were infected with HSV-1 at an MOI of 0.1 for 18 h. The genomic DNA was extracted, and the relative HSV-1 genome copy numbers were measured using qRT-PCR. Data shown in (a, c−f, h−j, l) are from one representative experiment of at least three independent experiments (mean ± SD of duplicate experiments). Two-tailed Student’s t-test was used to analyze statistical significance. **P < 0.01; ***P < 0.001 versus the control groups.
PCBP2 specifically targets cGAS to antagonize its enzyme activity (a) THP-1 cells were transfected with or without HT-DNA (2 μg/ml), and cell lysates were prepared and immunoprecipitated with a PCBP2 antibody or control IgG, followed by immunoblotting with the indicated antibodies. (b–e) HEK293T cells were transfected with IFNβ-Luc and an expression vector encoding cGAS and STING (b), STING (c), TBK1 (d), or IRF3-5D (e) together with the increased amount of PCBP2 or an empty vector. Twenty-four hours after transfection, cells were lysed for luciferase reporter assays (upper panel) and immunoblotting assays (lower panels). (f) THP-1 PCBP2+/+ and PCBP2−/− cells were permeabilized with Perfringolysin O (PFO; 300 ng/ml) and then treated with or without cGAMP (0.2 μg/ml) for 4 h, followed by immunoblotting with the indicated antibodies. (g) THP-1 cells were infected with a lentivirus expressing PCBP2 or an empty vector for 48 h, followed by infection with HSV-1 for 9 h. Cytoplasmic cGAMP was extracted and quantified with a cGAMP ELISA kit. (h) THP-1 PCBP2-knockout and control cells were transfected with HT-DNA for 6 h and then harvested to measure the abundance of cGAMP using a cGAMP ELISA kit. (i) Pcbp+/+ and Pcbp−/− MEFs were transfected with HT-DNA for 6 h, and the cGAMP abundance in cytoplasmic extracts was measured with a cGAMP ELISA kit. (j) In vitro enzyme activity assays of cGAS were conducted by mixing recombinant human cGAS protein and GST, GST-PCBP2, or GST-PCBP2-ΔKH3 in the presence of HT-DNA (50 ng/μl) and incubating the mixture at 37°C for 2 h. The reaction was terminated by heating at 95°C for 5 min, and the denatured proteins were removed by centrifugation. The heat-resistant supernatants were used to determine the abundance of cGAMP using L929-ISRE cells. Data shown in (b–e, g–j) are from one representative experiment of at least three independent experiments (mean ± SD of duplicate experiments). Two-tailed Student's t-test was used to analyze statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001; n.s. not significant versus the control groups.
PCBP2 attenuates the oligomerization of cGAS (a) HEK293T cells were co-transfected with Flag-tagged cGAS and HA-tagged cGAS together with the increased amount of Myc-tagged PCBP2. Twenty-four hours after transfection, the cell lysates were prepared and immunoprecipitated with anti-Flag beads, followed by immunoblotting. (b) HEK293T cells were co-transfected with Flag-tagged cGAS in combination with the increased amount of Myc-tagged PCBP2. Twenty-four hours after transfection, the cell lysates were resolved with SDD-AGE (upper panel) or SDS-PAGE (lower panels), followed by immunoblot analysis with the indicated antibodies. (c) L929 Pcbp2+/+ and Pcbp2-/- cells were transfected with HT-DNA (2 μg/ml) for 6 h, and the cell lysates were resolved with SDD-AGE (upper panel) or SDS-PAGE (lower panels), followed by immunoblot analysis with the indicated antibodies. (d) Recombinant cGAS and PCBP2 proteins were incubated in cGAMP synthesis reaction buffer in the presence or absence of HT-DNA (2 μg/ml) at 37°C for 1.5 h and then resolved by SDD-AGE (upper panel) and SDS-PAGE (lower panels), followed by immunoblotting. (e) HEK293A cells were transfected with GFP-cGAS, together with mCherry-tagged PCBP2 or mCherry (a negative control). Twenty-four hours after transfection, the cells were fixed, stained with DAPI (blue), and observed by confocal microscopy. Scale bars, 10 μm. (f) L929 Pcbp2+/+ and Pcbp2-/- cells stably expressing mouse cGAS-GFP were transfected in the presence or absence of ISD (2 μg/ml) for 6 h. Cells were then fixed, stained with DAPI (blue), and observed by confocal microscopy. Scale bars, 10 μm. (g–i) Recombinant GFP-cGAS proteins were incubated with GST-PCBP2 or GST proteins in the presence of Cy3-ISD (g), Cy3-VACV70 (h), or HSV120 (i) for 5 min. Confocal images are representative of cGAS condensates of all fields. Scale bars, 10 μm.