Guanylate-binding protein 1 restricts avian coronavirus infectious bronchitis virus-infected HD11 cells

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ABSTRACT The Infectious Bronchitis Virus (**IBV**), a coronavirus, is a key avian pathogen that causes acute and highly infectious viral respiratory diseases. IBV is an enveloped, positive-sense RNA virus, and the host factors that restrict infection and replication of the virus remain poorly understood. Guanylate-binding protein 1 (**GBP1**), an interferon-gamma (**IFN-γ**)-inducible guanosine triphosphatase (**GTPase**), is a major player in host immunity and provides defense against viral replication. However, the role of chicken GBP1 (**chGBP1**) in the IBV-life cycle is not well understood. Therefore, this study aimed to reveal the potential role of IFN-γ-induced chGBP1 in mediating host anti-IBV infection responses. We identified the host restriction factor, chGBP1, in IBV-infected chicken macrophages HD11 cell lines. We showed that chGBP1 was upregulated by treatment with both IFN-γ and IBV in HD11 cells. chGBP1 inhibited IBV replication in a dose-dependent manner and enhanced IFN-γ anti-IBV activity. Importantly, the GTPase domain of chGBP1 played a pivotal role in its anti-IBV activity. Furthermore, chGBP1 interacts with IBV Nucleocapsids protein to degrade IBV-N protein through the autophagy pathway. Taken together, our results demonstrate a critical role of chGBP1 in anti-IBV in macrophages HD11 cells.

Key words: infectious bronchitis virus (**IBV**), interferon-gamma, guanylate-binding protein 1, GTPase activity, nucleocapsids protein

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INTRODUCTION

The infectious bronchitis virus (**IBV**) is the primary cause of the acute infection disease known as avian infectious bronchitis, which affects the respiratory and reproductive systems (Cook et al., 2012). At present, infectious bronchitis in chickens exists in almost all countries in the world and presents a local epidemic trend. IBV belongs to the gammacoronavirus Coronaviridae (Wille and Holmes, 2020). IBV is a single-stranded RNA virus with an enveloped, positive-sense RNA genome without a segment. The IBV virion structure is similar to that of other coronaviruses, which consists of an envelope and a nucleocapsid (Li et al., 2021). The envelope membrane contains spike (**S**), envelope (**E**), and membrane (**M**) proteins. The nucleocapsid (**N**) protein is to assemble with genomic RNA into the viral RNA-protein **(vRNP)** complex (Lu et al., 2021). The whole IBV genome length is about 27.6 kb, and the genome composition is 5′UTR–ORF1–S–ORF3–E–M–ORF5–N-3′UTR. Translation of ORF1 produces two large polypeptides, pp1a and pp1ab, which are cleaved by papain-like protease and 3C-like protease to produce nonstructural proteins (Jackwood et al., 2012; Snijder et al., 2016; Yu et al., 2017).

Upon infection with the virus, the host immune response is activated, inducing expression of restrict factors to inhibit the virus. The innate immune system plays a crucial role in limiting viral replication at the initial stage of the infection (Thaiss et al., 2016). Host restrict factors play an indispensable role in IBV infection. IBV induces type I, II, and III IFN production following infection of chicken trachea and kidney tissues (Yang et al., 2018). Moreover, macrophages play an important role in a host's innate and acquired immune responses to IBV infection. After IBV infection of the chicken macrophage HD11 cell lines and chicken peripheral blood mononuclear cell-derived macrophages, *MHCII*, Fc receptor, *TLR3*,

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IFN-α, CCL4, MIF, IL-1β, IL-6, and *iNOS* were significantly upregulated (Sun et al., 2021). Whole-genome gene expression microarrays have shown that *MX1, C1S, IRF7, TLR3, C1R, CCLi7, ISG12-2,* and *IFITM3* are all strongly upregulated in response to IBV infection (Smith et al., 2015). However, expression patterns of other factors differed in response to different IBV strains and in different cell types (Chhabra et al., 2018; Zhu et al., 2020). Guanylate-binding proteins (**GBPs**), which are IFN-inducible guanosine triphosphatases (**GTPases**), are a conserved superfamily that includes human, murine, and so on (Praefcke, 2018; Tretina et al., 2019). The GBP1 structure includes a globular N-terminal Large GTPase (**LG**) domain, followed by a helical domain, which is further subdivided into a middle domain and a C-terminal α12/13-domain. The LG domain is involved in GTPase activity and confers the main biochemical functions of GBPs (Prakash et al., 2000; Cui et al., 2021). GBP1 has been reported to restrict several viral infections in vitro, via its GTPase activity (Honkala et al., 2019). Early studies showed that GBP1 mediated antiviral effects against vesicular stomatitis virus (**VSV**) and encephalitis myocarditis virus (**EMCV**). Compared with control cells, GBP1 overexpression attenuated the cytopathic effects of VSV and EMCV and produced fewer viral progeny (Anderson et al., 1999). GBP1 also suppressed the replication of hepatitis C virus (**HCV**), classical swine fever virus (**CSFV**), and Kaposi's sarcoma-associated herpesvirus (**KSHV**) (Itsui et al., 2006; Li et al., 2016; Zou et al., 2017).

However, viruses use various mechanisms to resist the antiviral effects of hosts. For instance, the HCV viral replicase nonstructural (**NS**) protein 5B and CSFV NS5A both interact with the GTPase domain of GBP1 to block its GTPase activity, thus antagonizing its antiviral effects (Itsui et al., 2006; Li et al., 2016). GBP1 also inhibits KSHV through its GTPase activity and disturbs the natural cytoskeletal structure by interfering with actin filament formation. Additionally, KSHV encodes the E3 ligase RTA, which interacts with GBP1 and mediates its degradation (Zou et al., 2017). Yet, to date, the function of chicken GBP1 in the IBV life cycle has not been elucidated.

In this study, we aimed to investigated chGBP1 inhibits its IBV replication in chicken macrophages and elucidate the mechanism of chGBP1 exert's antiviral response. We analyzed the expression of chIFN-γ after IBV infection. More importantly, chGBP1, as the interferon-stimulated gene produced by chIFN-γ activation, mediates antiviral effects. Our data show that the IBV infection activates chIFN-γ production, which in turn increases downstream chGBP1 expression via signal cascades. We found that chGBP1 inhibits IBV replication through N-terminal GTPase activity and C-terminal. In addition, we also found that chGBP1 interacts with IBV nucleocapsid **(N)** protein**,** which degrades N protein via the autophagy pathway. Our findings demonstrate the regulation and function of IFN-γ-induced chGBP1 in IBV-infected macrophages, reveal the potential role of chGBP1 in mediating host anti-coronavirus infection responses.

MATERIALS AND METHODS

Cells, Viruses and Antibodies

The chicken macrophages cell lines (**HD11**) and embryo fibroblast cell lines (**DF-1**) were cultured in Dulbecco's modified Eagle's medium (**DMEM**, Cellmax, Beijing, China) supplemented with 10% fetal bovine serum (**FBS**, Cellmax, Beijing, China) and 100 IU/mL penicillin–streptomycin solution (Cellmax, Beijing, China). All cells were cultured in an incubator at 37°C and 5% CO_2.

The IBV Beaudette strain (GenBank: DQ001339) was kindly gifted by Prof. Ding-Xiang Liu, South China Agricultural University. The IBV M41 strain and Qx strain was stored in our laboratory.

Mouse monoclonal antibodies specific for Flag, hemagglutinin (**HA**), β-actin, HRP-conjugated Affinipure Goat Anti-Mouse IgG(H+L) and HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) were purchased from Proteintech (Proteintech, Wuhan, China). Rabbit polyclonal antibodies specific for chGBP1 were generated in our laboratory.

Plasmid Construction

Full-length chicken *IFIT5* (NM_001320422.2), *DDX17* (XM_416260.8), *ATF3* (XM_046939659.1), *OASL* (NM_001397447.1), *MX1* (NM_204609.2), *IRF1* (KC250010.1), *IFITM10* (XM_046942295.1), *RAB25* (XM_040690945.2), and *IFI6* (NM_001001296.6) was amplified from HD11 cells and cloned into pcDNA3.1-Flag. Full-length chicken *GBP1* (NM_204652.2) (Prakash et al., 2000) was amplified from HD11 cells and cloned into pcDNA3.1-Flag to generate pcDNA3.1-Flag-chGBP1. The gene sequences of *chGBP1* N-terminal and C-terminal domains were cloned into pcDNA3.1-Flag to generate pcDNA3.1-Flag-chGBP1$_{1\text{-}306}$ and pcDNA3.1-Flag-chGBP1$_{307\text{-}576}$. The mutated *chGBP1* GTPase domains *chGBP1* R48A and *chGBP1* K52A were cloned into pcDNA3.1-Flag. The IBV *N* sequence was amplified from cDNA extracted from the IBV M41 strain and Beaudette strain, then cloned into pcDNA3.1-HA empty vectors. *chGBP1* was fused to GFP and cloned into a pEGFP-C2. IBV-*N* was fused to mcherry and cloned into a pcDNA3.1-mcherry. The primers used for the construction of each plasmid are listed in Table 1.

Cell Transfection

The cells were plated in 6-well plates (5×10^5 cell/mL) for 12 h. Then, a Lipofectamine 8000 (Beyotime Biotech, Beijing, China) was used with the indicated amount of expression construct, according to the manufacturer's instructions. In the same experiment, empty control plasmids were added to ensure that each transfection received equal amounts of total DNA. After transfection, cells were collected and transfection efficiency was measured by western blotting.

Table 1. Primers used in this study.

Primers	Forward (5'−3')	Reverse (5'−3')
chIFIT5	CTCTAGAATGAGTACCATTTCCAAG	CGGATCCGCTTGAGAGGGAAAGTCG
chDDX17	CGAATTCATGAGGGGCTTCGGGGA	CGGATCCTTTGCGTGAGGGTGGAGG
chATF3	CTCTAGAATGCCGTTTAAGATTAA	CGGATCCACCTTGTAATGTTCCTTC
chOASL	GGGTACCATGGAGCTGGGCGTGAGG	CTCTAGAGGAGGGCACGCAGCGTCT
chMX1	GGGTACCATGAACAATCCACGGT	CTCTAGACAGAGACTTAAAGTCTACCAG
chIRF1	GGGTACCATGCCCGTCTCAAGGATG	CTCTAGACAAGCTGCAGGAGATGG
chIFITM10	CGAATTCATGACAACAATGATAACAAA	CTCTAGAGGTAATCGGTGAGGGGGTA
chRAB25	GGGTACCATGAGCAGCGCCGAGGAG	CTCTAGAGATGGCCACGCAGCACGG
chIFI6	GGGTACCATGTCTGACCAGAACGTCC	CTCTAGAGCGCCTTCCTCCTTTGCCA
chGBP1	CGGAATTCATGGACACTCCGGTGCT	CCTCGAGTCAGAGTACAGTGCACTTGG
$chGBP1_{1\text{-}306}$	GGAATTCATGGACACTCCGGTG	CGGATCCGCAGGGCACAGAGC
$chGBP1_{307\text{-}576}$	GGAATTCATGGTGGAGAGTGCAGTGA	CGGATCCGAGTACAGTGCACTTGGGT
IBV-N	CGGATCCATGGCAAGCGGTAAGGCA	CCCTCGAGTCAAAGTTCATTCTCTCCT
$chGBP1_{GFP}$	CGGAATTCATGGACACTCCGGTGCT	CGGATCCTCAGAGTACAGTGCACTTGG
$IBV\text{-}N_{mcherry}$	CGGATCCATGGCAAGCGGTAAGGCA	CCCTCGAGTCAAAGTTCATTCTCTCCT
qchIFN-γ	ACACTGACAAGTCAAAGCCGCACA	AGTCGTTCATCGGGACCTTGGC
qchGBP1	AAGTCCTTCCTGATGAACC	CTTGGTCTCCGCATACAC
qIBV-N	GAAGAAAACCAGTCCCAGA	TTACCAGCAACCCACAC
GAPDH	CATCACAGCCACACAGAAG	GGTCAGGTCAACAACAGAGA

In order to measure the different dose of chGBP1 for anti-IBV replication, HD11 cells was transfected with pcDNA3.1-Flag-chGBP1 300 ng, 500 ng, or 1,000 ng for 24 h.

In order to detect the effect of chGBP1 on the stability of IBV-N protein. HD11 cells were co-transfected pcDNA3.1-Flag-chGBP1 1,000 ng with either pcDNA3.1-HA-IBV_{Beau}-N 1,000 ng, or pcDNA3.1-HA-IBV_{M41}-N 1,000 ng for 24 h. Further, to explore the effect of different doses of chGBP1 on the level of IBV-N protein. HD11 cells were co-transfected with different concentrations of pcDNA3.1-Flag-chGBP1 1,000 ng or 2,000 ng and pcDNA3.1-HA-N 1,000 ng for 24 h.

Antiviral Genes Screening Assay

The cells were plated in 6-well plates (5×10^5 cell/mL) for 12 h. HD11 cells were transfected with plasmids pcDNA3.1-Flag-chIFIT5 (1,000 ng), pcDNA3.1-Flag-chDDX17 (1,000 ng), pcDNA3.1-Flag-chATF3 (1,000 ng), pcDNA3.1-Flag-chOASL (1,000 ng), pcDNA3.1-Flag-chMX1 (1,000 ng), pcDNA3.1-Flag-chGBP1 (1,000 ng), pcDNA3.1-Flag-chIRF1 (1,000 ng), pcDNA3.1-Flag-chIFITM10 (1,000 ng), pcDNA3.1-Flag-chRAB25 (1,000 ng) or pcDNA3.1-Flag-chIFI6 (1,000 ng) for 24 h. Then, infected with MOI = 5 of IBV Beaudette strain and harvested cells after 24 h, qRT-PCR detect IBV N gene mRNA.

Enzyme-Linked Immunosorbent Assay (ELISA)

In order to measure the expression of chIFN-γ after IBV infection, HD11 cells was infected with IBV Beaudette strain at MOI = 5. Following viral infection, the cell culture supernatants were collected at 0 h, 6 h, 12 h, 24 h, 36 h, and 48 h. Using chicken IFN-γ ELISA kits (MEIMIAN Industrial, Jiangsu, China), as according to the manufacturer's instructions. For significance analysis of the values obtained by ELISA, the 0 h value was used as the comparative value.

50% Tissue Culture Infective Dose ($TCID_{50}$)

IBV virus titer was quantified using a $TCID_{50}$ assay. Briefly, 10-fold dilutions of IBV were inoculated into HD11 cells grown in a 96-well tissue culture plate at 1,000 cells/well. The plate was incubated at 37°C for 5 d, followed by observation of the cytopathic effect in each well under light microscopy. $TCID_{50}$ was calculated using the Reed−Muench method (Reed, and Muench, 1938).

RNA Interference Assay

To investigate the function of chGBP1, we silenced its expression through siRNA. siRNA-1# and siRNA-2# target nucleotide sequences of the N-terminal GTPase domain of chGBP1, and siRNA-3# target nucleotide sequences of the C-terminal domain of chGBP1. All siRNAs used in this study were designed and synthesized by Sangon Biotech (Sangon Biotech, Shanghai, China) target *chGBP1* coding region. The siRNA sequences used in this study are listed in Table 2.

To analyze the effect of chGBP1 siRNA. HD11 cells (5×10^5 cell/mL) were seeded in 6-well plates at 50% confluency. Cells were transfected with siRNA-ctrl, siRNA-1#, siRNA-2#, or siRNA-3# (100 pmol/well) using a Lipofectamine 8000 (Beyotime Biotech, Beijing, China) and stimulated with chIFN-γ (100 ng/mL) for 6 h. The treated cells were incubated for 48 h. Then, cells were harvested and detected by western blotting or quantitative real-time polymerase chain reaction (qRT-PCR) assay.

GTPase Enzyme-Linked Inorganic Phosphate Assay (ELIPA)

To determine the chGBP1 GTPase activity and the effect of mutants on GTPase activity, HD11 cells

Table 2. siRNA sequence used in this study.

siRNA	Forward (5'−3')	Reverse (5'−3')
siRNA-ctrl	UUCUCCGAACGU GUCACGUTT	ACGUGACACGU UCGGAGAATT
siRNA-1#	CCUGGUGUACAAC AGCAUUTT	AAUGCUGUUGUACA CCAGGTT
siRNA-2#	CCAACUUUGUCA GCAUCUUTT	AAGAUGCUGACAA AGUUGGTT
siRNA-3#	GCACAUGGCUGG AGGAGCATT	UGCUCCUCCAGCC AUGUGCTT

(5×10^5 cell/mL) were seeded in 6-well plates and cultured for 12 h; transfected with pcDNA3.1-Flag-chGBP1, pcDNA3.1-Flag-chGBP1$_{R49A}$, pcDNA3.1-Flag-chGBP1$_{K52A}$, pcDNA3.1-Flag and harvested at 36 h. An ATPase/GTPase enzyme-linked inorganic phosphate assay (**ELIPA**) kit (catalog no. BK051/BK052, Cytoskeleton, Denver, CO) was used to measure the amount of inorganic phosphate generated (absorbance at 360 nm) according to the manufacturer's instructions.

Confocal Fluorescence Microscopy

The cells were seeded on glass coverslips in 35-mm cell culture dishes and cultured overnight. Thereafter, the HD11 cells were co-transfected with pEGFP-C2-chGBP1 and pcDNA3.1-mcherry-N. After 24 h, the cells were washed three times with cold phosphate buffered saline (**PBS**) and fixed with 4% paraformaldehyde for 15 min at room temperature. Subsequently, the cells were incubated with DAPI at 37°C for 10 min and washed with cold PBS. Finally, images were captured using a laser scanning confocal microscope (Zeiss, Jena, Germany).

Co-Immunoprecipitation Assays

HD11 cells cultured in 6-well plates were co-transfected with the following plasmids: pcDNA3.1-Flag-chGBP1, pcDNA3.1-Flag-chGBP1$_{1\text{-}306}$, pcDNA3.1-HA-chGBP1$_{307\text{-}576}$, and pcDNA3.1-HA-N using Lipofectamine 8000 (Beyotime). After transfected 24 h, the cells were harvested and lysed by RIPA lysis buffer (500 μL/well) containing 1 mM PMSF (Beyotime). After incubation on ice for 30 min, the cell lysates were centrifuged at 13,000 g for 30 min. Approximately 25% of the supernatant was subjected to input assays, and the remainder was used for a Co-immunoprecipitation (**Co-IP**) assay with an anti-Flag agarose affinity gel (Beyotime) according to the manufacturer's instructions. Briefly, 50 μL of the agarose affinity gel was centrifuged for 30 s at 4°C to remove glycerol and was washed with cold TBS. The cell lysate was added to the equilibrated resin and gently rocked on a rotating platform at 4°C overnight. The resin was washed with cold TBS, and the protein samples were evaluated by western blotting.

Protein Extraction and Western Blot

Cultured cells in 6-well plates post transected were harvested and lysed by RIPA lysis buffer (500 μL/well) containing 1 mM PMSF (Beyotime). After incubation on ice for 30 min, the cell lysates were centrifuged at 13,000 g for 30 min, and collected the supernatant. Protein concentration was quantified using the BCA protein concentration assay kit (Beyotime). Finally, 30μg protein was taken for Western blot detection.

Cultured cells were lysed in RIPA lysis buffer for 30 min on ice. The SDS-PAGE sample loading buffer was added, and the samples were heated for 5 min at 95° C. The samples were subjected to 10% SDS-PAGE at 120 V for 100 min. The protein was transferred to a PVDF membrane (pore size, 0.22 μM; Beyotime Biotech, Beijing, China) for 1.5 h at 200 mA. Afterwards, the membrane was blocked with a blocking buffer containing 1% BSA for 1 h at room temperature. It was then incubated overnight at 4°C with a primary antibody (1:1000 dilution). The membrane was then washed three times and subsequently incubated with a secondary antibody (1:2000 dilution) for 1 h at room temperature. After three washes, protein bands were detected using BeyoECL Moon chemiluminescent system (Beyotime).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA from cultured cells were isolated by RNAiso Plus reagent (Takara, 9109) according to the manufacturer's instruction. The RNA (1 μg) was further processed using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher, K1621) to produce cDNA in accordance with the manufacturer's instructions. qRT-PCR was performed using a Bio-Rad system (Hercules, CA). ChamQ SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China) and gene-specific primers were used in a 20-μL volume. The samples were heated to 95°C for 10 min, followed by 40 cycles of PCR involving 10 s at 95°C, 20 s at 60°C and 15 s at 72°C. The primers name of qchIFN-γ was used to detect chIFN-γ mRNA level. The primers name of qchGBP1 was used to detect chGBP1 mRNA level (Table 1). The primer name of qIBV-N was used to detect IBV replication level (Table 1). The glyceraldehyde-3-phosphate dehydrogenase gene (**GAPDH**) was used as a housekeeping gene to normalize (relative) gene expression using the $2^{-\Delta\Delta CT}$ formula. The qRT-PCR primers used in this study are listed in Table 1.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (Mann–Whitney test, one-way and two-way ANOVAs test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). Statistical significance was set at $P < 0.05$.

RESULTS

Screening of Antiviral Genes Against IBV Replication in HD11 Cells

To screen the antiviral genes with anti-IBV activity in chicken macrophages, we constructed ten chicken genes expression plasmids with Flag-tag. Notably, overexpression *chIFIT5, chDDX17, chATF3, chOASL, chMX1, chGBP1, chRAB25* and *chIFI6* significantly reduced IBV mRNA level (Figure 1). Interestingly, chGBP1 (Chicken Guanylate-Binding Protein 1), an interferon induced interferon-stimulated gene, showed the highest inhibitory efficiency on IBV replication.

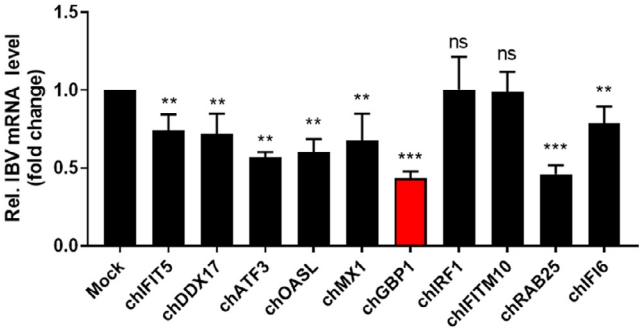

Figure 1. Screening of antiviral genes against IBV replication in HD11 cells. HD11 cells were transfected with plasmids pcDNA3.1-Flag-chIFIT5, pcDNA3.1-Flag-chDDX17, pcDNA3.1-Flag-chATF3, pcDNA3.1-Flag-chOASL, pcDNA3.1-Flag-chMX1, pcDNA3.1-Flag-chGBP1, pcDNA3.1-Flag-chIRF1, pcDNA3.1-Flag-chIFITM10, pcDNA3.1-Flag-chRAB25 or pcDNA3.1-Flag-chIFI6 for 24 h. Then infected with IBV at MOI = 5 and harvested cells after 24 h, qRT-PCR detect IBV N gene mRNA.

IBV or chIFN-γ Induced Upregulation of chGBP1 Expression

GBP1 is one of the IFN-γ inducible ISGs. Upon investigation, we found that IBV infection significantly induced *chGBP1* expression in HD11 cells (Figure 2A). Furthermore, HD11 cells treated with chIFN-γ (100 ng/mL) showed similar results to those obtained with IBV infection (Figure 2B). Additionally, western blotting showed that IBV infection could stimulate chGBP1 protein expression, similar to the changes at transcription levels (Figure 2C and D). These results indicate that both chIFN-γ and IBV infection can stimulate chGBP1 expression in HD11 cells.

IBV Induced Chicken IFN-γ Production in Macrophages HD11 Cells

Given the significant role of type II IFN in controlling virus infections, and to verify IBV-induced type II IFN production, we verified chIFN-γ expression in IBV-infected chicken macrophages HD11 cells. chIFN-γ expression levels were significantly upregulated (Figure 3A) after IBV infection of HD11 cells. Moreover, ELISA detection of chIFN-γ at protein level showed similar results (Figure 3B). Thus, we confirmed that chIFN-γ is expressed in response to IBV infection of chicken macrophages HD11 cells.

Knockdown chGBP1 Promoted IBV Replication

To clarify the function of chGBP1 further, we knockdown chGBP1 expression in HD11 cells. The results showed that siRNA-2# efficiently downregulated chGBP1 at both the mRNA and protein levels (Figure 4A and B). Consistently, siRNA knockdown cells were infected with

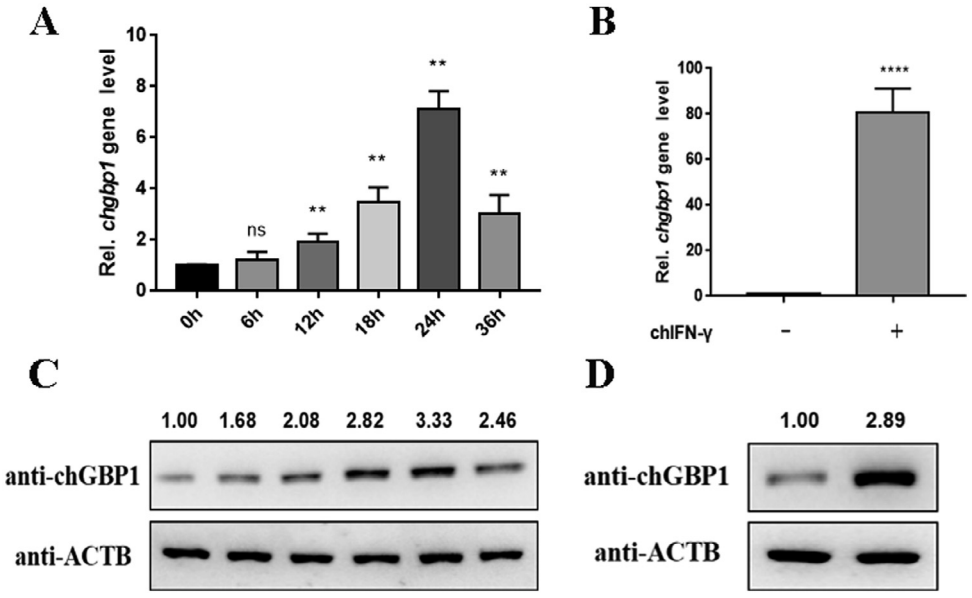

Figure 2. IBV or chIFN-γ induce chGBP1 expression in HD11 cells. (A) HD11 cells infected with IBV at MOI = 5 were harvested after 0 h, 6 h, 12 h, 18 h, 24 h, and 36 h, qRT-PCR detect chGBP1 mRNA. (B) HD11 cells treated with chIFN-γ (100 ng/mL) were harvested after 6 h, qRT-PCR detect chGBP1 mRNA. (C-D) All the protein level measure using anti-chGBP1 antibodies. β-actin was used as a loading control. Data were presented as means ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

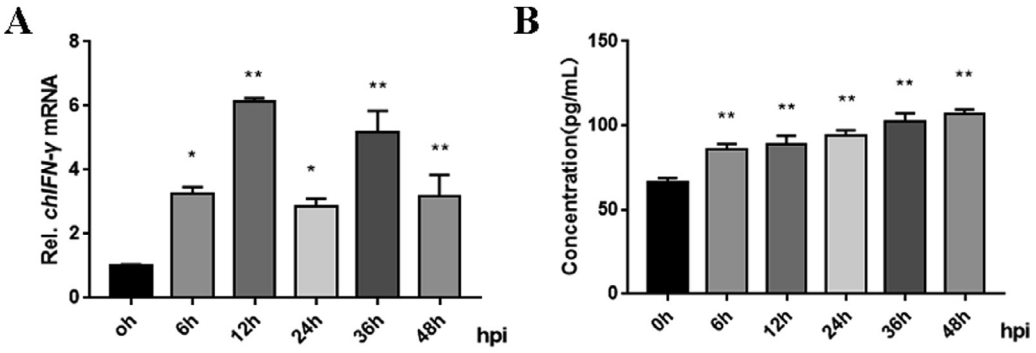

Figure 3. IBV infection induced chIFN-γ production in macrophages HD11 cells. HD11 cells were infected with IBV at MOI = 5 and then harvested cells and cell culture supernatants after 0 h, 6 h, 12 h, 24 h, 36 h, and 48 h. (A) chIFN-γ mRNA levels production was analysised by qRT-PCR. (B) chIFN-γ protein levels was detected by ELISA. Data were presented as means ± SD. $^{**}P < 0.01$.

IBV and used qRT-PCR targeting the IBV-*N* gene to detect the virus replication. The replication level and viral titers of IBV were increased in chGBP1 knockdown cells (Figure 4C and D). Thus, chGBP1 is involved in the host antiviral response to restrict IBV infection.

chGBP1 Inhibited IBV Replication and Enhanced IFN-γ-mediated Inhibition of IBV Replication

In order to investigated whether chGBP1 was involved in the response of macrophages to IBV infection by overexpressing chGBP1 in HD11 cells. We found that, compared with the empty vector, overexpressed chGBP1 inhibited viral replication significantly more (Figure 5A). $TCID_{50}$ was significantly reduced by chGBP1 (Figure 5B). Also, chGBP1 repressed IBV replication and reduced virus titers in DF-1 cells (Figure 5C and D) and chicken embryo fibroblasts CEF cells (Figure 5E and F). Next, we overexpressed chGBP1 at different doses in HD11 cells (Figure 5G) and simultaneously used qRT-PCR targeting the IBV *N* gene to detect the virus. chGBP1 inhibited IBV replication in a dose-dependent manner (Figure 5H).

To verify whether chGBP1 was necessary for the effect of chIFN-γ against IBV-infection in HD11 cells, we treated HD11 cells with chIFN-γ in vitro. Treatment with chIFN-γ effectively restricted IBV infection, while chGBP1 enhanced the chIFN-γ antiviral effect in

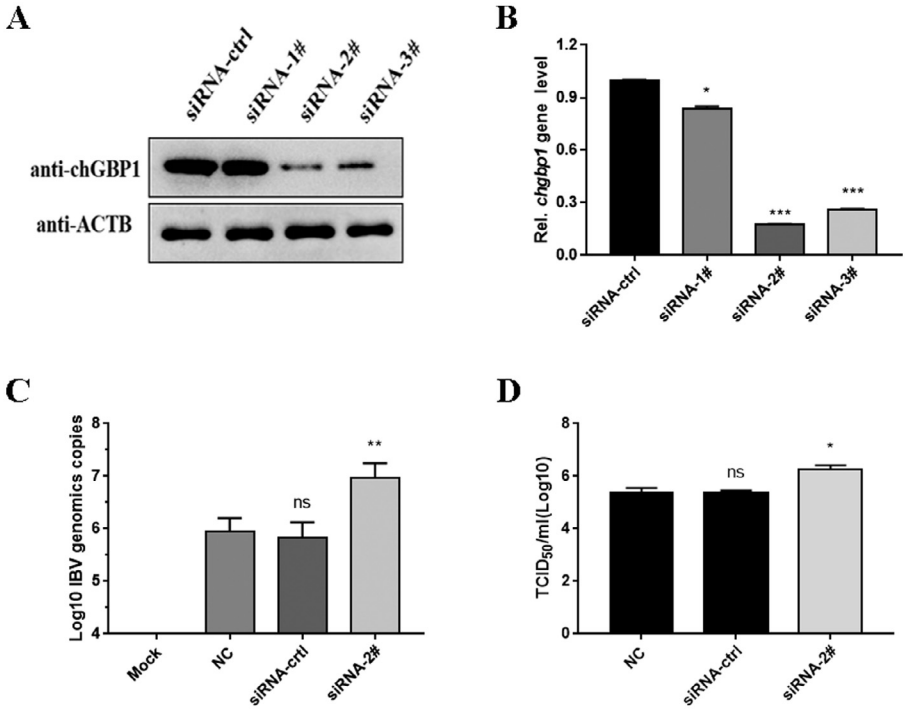

Figure 4. The loss of chGBP1 promotes IBV replication. HD11 cells were transfected with negative control siRNA (siRNA-ctrl) or siRNA against chGBP1 with chIFN-γ (100 ng/mL) treatment 6 h. (A) chGBP1 protein expression was analyzied by Western blotting with anti-chGBP1and anti-β-actin antibodies. (B) The mRNA of chGBP1 was quantified by qRT-PCR using GAPDH as the reference gene. (C) HD11 cells were transfected with negative control siRNA (siRNA-ctrl), siRNA-2# or NC (It doesn't contain any siRNA) for 48 h and then infected with IBV at MOI = 5, harvested cells after 36 h. Virus RNA levels were quantified by qRT-PCR. (D) Virus titers in the supernatants were analyzed by a $TCID_{50}$ assay. Data were presented as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, ns = not significant.

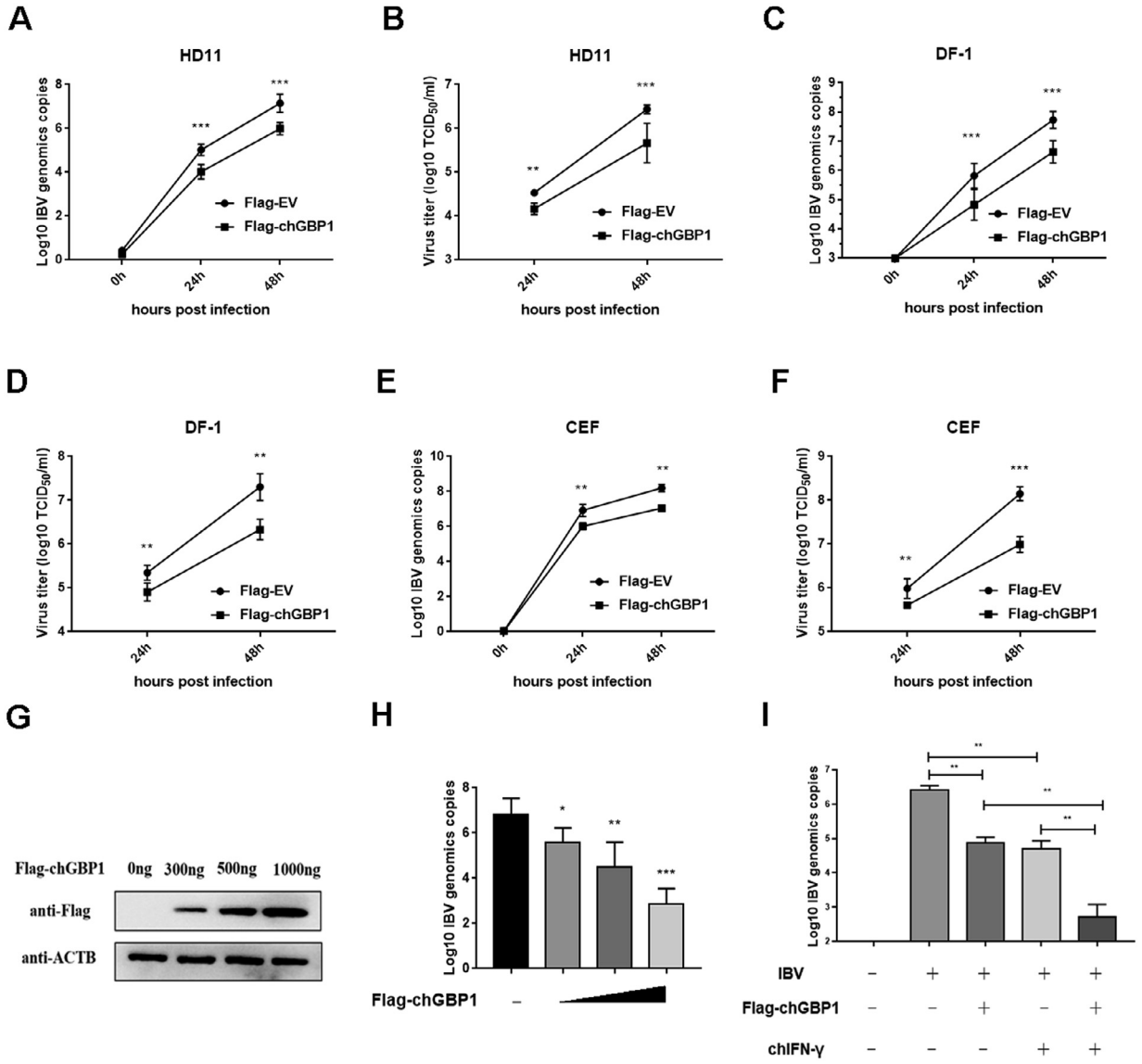

Figure 5. Overexpression chGBP1 inhibit IBV replication. (A) HD11 cells were transfected with pcDNA3.1-Flag-chGBP1 (1,000 ng) or pcDNA3.1-Flag (1,000 ng) for 24 h and then infected with IBV at MOI = 5 were harvested at different points in time, qRT-PCR detect IBV N gene mRNA. (B) Virus titers in the supernatants were analyzed by a $TCID_{50}$ assay. (C) DF-1 cells were transfected with pcDNA3.1-Flag-chGBP1 (1,000 ng) or pcDNA3.1-Flag (1,000 ng) for 24 h, then infected with IBV at MOI = 5, and harvested at different points in time. (D) Virus titers in the supernatants were analyzed by a $TCID_{50}$ assay. (E) CEF cells were transfected with pcDNA3.1-Flag-chGBP1 (1,000 ng) or pcDNA3.1-Flag (1,000 ng) for 24 h, then infected with IBV at MOI = 1, and harvested at different points in time. (F) Virus titers in the supernatants were analyzed by a $TCID_{50}$ assay. (G) HD11 cells were transfected with pcDNA3.1-Flag-chGBP1 (300 ng, 500 ng, and 1,000 ng) or pcDNA3.1-Flag (1,000 ng) for 24 h, western blot measured protein. β-actin was used as a loading control. (H) HD11 cells transfected with pcDNA3.1-Flag-chGBP1 (300 ng, 500 ng, and 1,000 ng) or pcDNA3.1-Flag (1,000 ng) for 24 h and then infected with IBV at MOI = 5 were harvested after 36 h, qRT-PCR detect IBV-*N* gene mRNA. (I) HD11 cells were transfected with pcDNA3.1-Flag-chGBP1 (1,000 ng) or pcDNA3.1-Flag (1,000 ng) for 24 h and then incubated with chIFN-γ (1,000 ng/ml) for 12 h. Subsequently, HD11 cells infected IBV at MOI = 5 were harvested after 36 h, qRT-PCR detect IBV-*N* gene mRNA. Data were presented as means ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

macrophages (Figure 5I). Thus, chGBP1 is an important effector of chIFN-γ against IBV replication in HD11 cells.

N-terminal GTPase Domain of chGBP1 was Essential for Anti-IBV Effects

The GBP1 protein has two structural domains, namely the N-terminal GTPase domain and the C-terminal helical domain (Figure 6A). Therefore, western blot confirmed that the chGBP1 full length, N-terminal ($chGBP1_{1\text{-}306}$) and C-terminal ($chGBP1_{307\text{-}576}$) were expressed in HD11 cells (Figure 6B). After IBV infection of HD11 cells, the chGBP1 GTPase domain significantly reduced viral replication (Figure 6C). Taken together, our data indicated that the N-terminal GTPase domain of chGBP1 was essential for restricting IBV replication.

R49 and K52 were Critical for chGBP1-Mediated Anti-IBV

To further study the biological function of chGBP1, we constructed two mutant of chGBP1(R49A) and

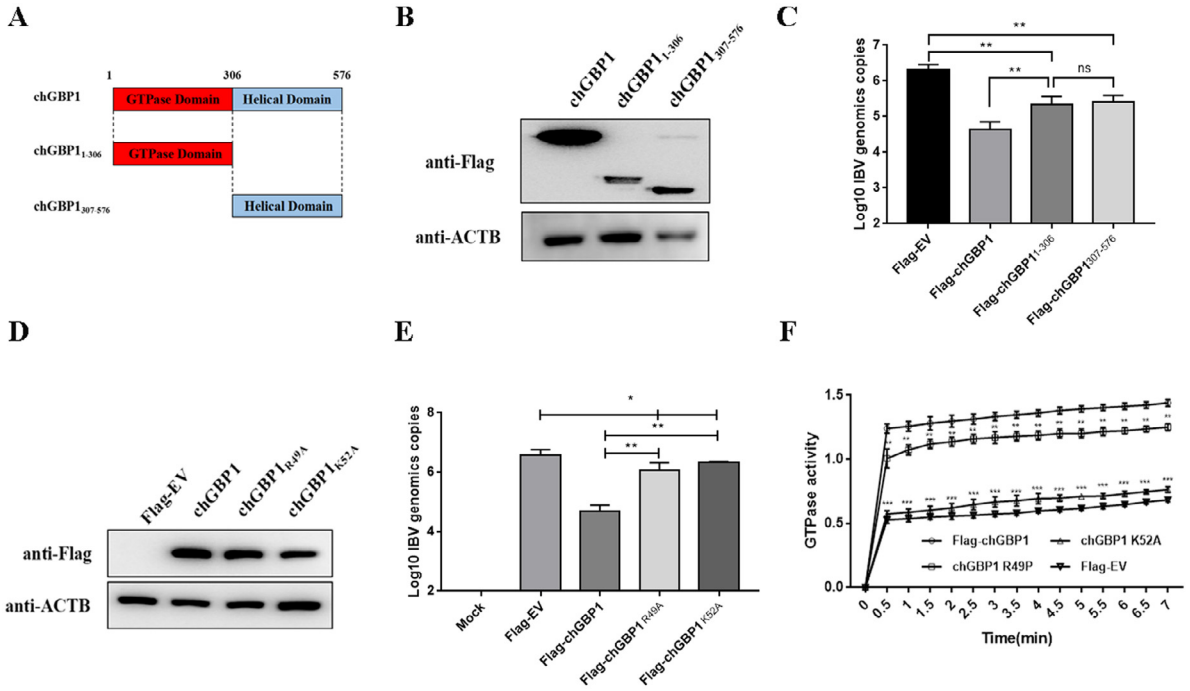

Figure 6. The critical regions of chGBP1 to repress IBV replication. (A) According the structure of chGBP1, construction N-terminal GTPase domain and C-terminal helical domain expression plasmid. (B) HD11 cells were transfected with pcDNA3.1-Flag-chGBP1$_{1\text{-}306}$, pcDNA3.1-Flag-chBGP1$_{307\text{-}576}$ or pcDNA3.1-Flag-chGBP1, the protein measured by western blot with anti-Flag antibodies. (C) HD11 cells were transfected with pcDNA3.1-Flag-chGBP1, pcDNA3.1-Flag-chGBP1$_{1\text{-}306}$, pcDNA3.1-Flag-chBGP1$_{307\text{-}576}$ or pcDNA3.1-Flag and then infected with IBV at MOI = 5 for 36 h. Virus RNA levels were quantified by qRT-PCR. (D) HD11 cells were transfected with pcDNA3.1-Flag-chGBP1, pcDNA3.1-Flag-chGBP1$_{R49P}$, pcDNA3.1-Flag-chGBP1$_{K52A}$, or pcDNA3.1-Flag for 24 h and then infected with IBV at MOI = 5 for 36 h. The protein measured by western blot with anti-Flag antibodies. (E) Virus RNA levels were quantified by qRT-PCR. (F) HD11 cells were transfected with pcDNA3.1-Flag-chGBP1, pcDNA3.1-Flag-chGBP1$_{R49P}$, pcDNA3.1-Flag-chGBP1$_{K52A}$, or pcDNA3.1-Flag for 36 h. The GTPase activity was measured using the ELIPA kit at 360 nm absorbance. Data were presented as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, ns = not significant.

chGBP1(K52A). Western blot confirmed that the chGBP1 wild type, chGBP1(R49A) and chGBP1 (K52A) were expressed in HD11 cells (Figure 6D). We found that wild-type chGBP1 effectively reduced the level of IBV mRNA compared with the empty vector, and that both chGBP1 R49A and chGBP1 K52A mutants partially restored the level of viral replication (Figure 6E). This indicated that both R49 and K52 are critical for controlling viral replication.

To analyze the enzymatic functions of the mutant chGBP1 R49A and chGBP1 K52A proteins, their GTPase activity was examined using ELIPA. GTPase activity was significantly higher in chGBP1-expressing cells than in empty vector-transfected cells. After HD11 cells were transfected with chGBP1 K52A and chGBP1 R49A constructs, the ELIPA test proved that the two mutants had reduced GTPase activity, as compared with cells with wild-type chGBP1 (Figure 6F). These results suggest that the GTPase activity of chGBP1 is critical for inhibiting IBV replication, and that R49/K52 of chGBP1 are essential for anti-IBV activity.

chGBP1 Protein Degraded IBV N Protein Through Autophagy Pathway

We used co-immunoprecipitation (**Co-IP**) to analyze chGBP1- and IBV-interacting proteins. IBV nucleocapsid protein was identified as a chGBP1-interacting protein in IBV-infected HD11 cells. Subsequently, the interaction between chGBP1 and IBV-N protein was confirmed using Co-IP. The results showed that chGBP1 C-terminal domain interacted with IBV-N protein (Figure 7A). Furthermore, confocal microscopy indicated that IBV-N could co-localize with chGBP1 in the cytoplasm of HD11 cells (Figure 7B). Next, we examined the effects of chGBP1 protein on IBV-N protein stability. Overexpression chGBP1 reduced IBV-N protein levels, both IBV Beaudette strain (Figure 7C) and M41 strain (Figure 7D). We also found that the protein level of IBV-N were reduced by chGBP1 protein in dose-dependent manner (Figure 7E). In addition, treatment HD11 cells with an autophagy inhibitor (3-MA and Baf.A1) restored the IBV N protein levels reduced by chGBP1, but not proteasome inhibitor (MG-132), which included IBV Beaudette strain N protein (Figure 7F) and M41 strain N protein (Figure 7G). These results indicated that the chGBP1 interacts with the IBV N protein to reduce its protein levels, inhibiting IBV replication in HD11 cells.

DISCUSSION

Herein, we investigated the regulation and function of IFN-γ-induced chGBP1 in IBV-infected macrophages.

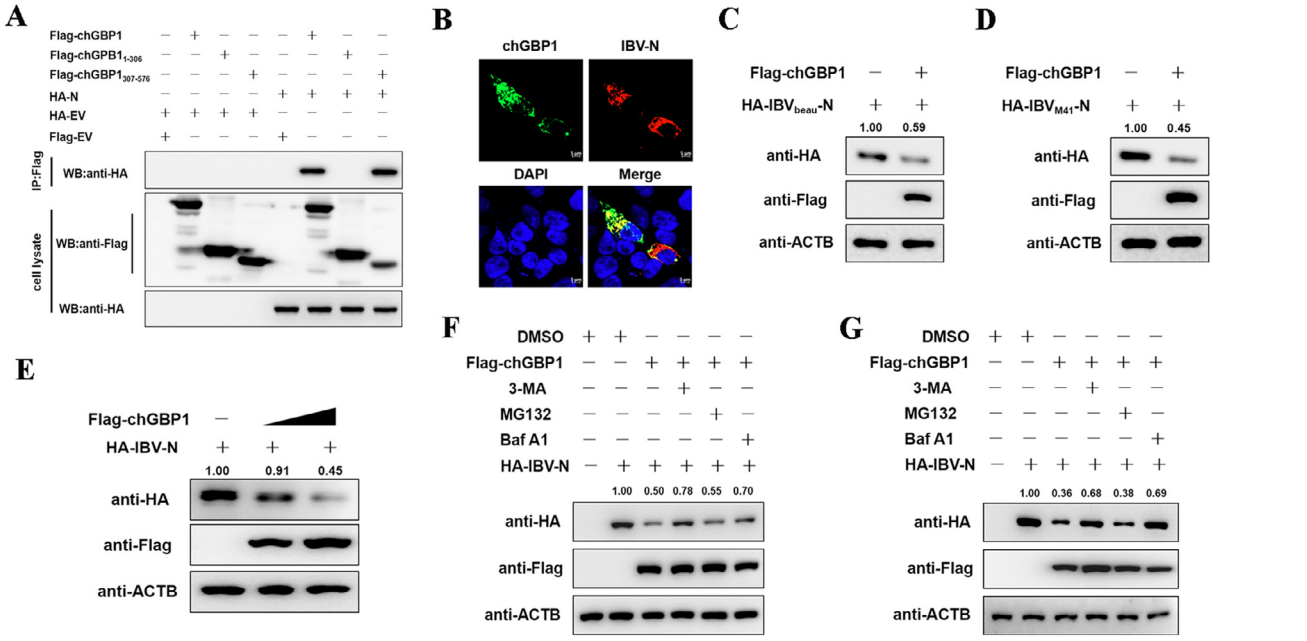

Figure 7. chGBP1 reduced IBV-N protein levels to block IBV replication. (A) HD11 cells were co-transfected with pcDNA3.1-Flag-GBP1, pcDNA3.1-Flag-GBP1$_{1\text{-}306}$, pcDNA3.1-Flag-GBP1$_{307\text{-}576}$ with either pcDNA3.1-HA-N or pcDNA3.1-HA. The cell lysate was harvested. Coimmunoprecipitation (**Co-IP**) was performed using an anti-Flag antibody (MAb) (1:100). The precipitated proteins were analyzed by Western blotting using anti-HA antibodies. (B) HD11 cells were transfected with GFP-chGBP1 or mcherry-N. At 30 h post transfected, co-localization was performed using Confocal fluorescence microscopy. (C-D) HD11 cells were co-transfected pcDNA3.1-Flag-chGBP1 (1,000 ng) with either pcDNA3.1-HA-IBV$_{Beau}$-N (1,000 ng), or pcDNA3.1-HA-IBV$_{M41}$-N (1,000 ng). The proteins were analyzed by Western blotting. (E) HD11 cells were co-transfected with different concentrations of pcDNA3.1-Flag-chGBP1 (1,000 ng or 2,000 ng) and pcDNA3.1-HA-N (1,000 ng). At 30 h post transfected, the proteins were analyzed by Western blotting. (F-G) HD11 cells were co-transfected pcDNA3.1-Flag-chGBP1 (1000ng) with either pcDNA3.1-HA-IBV$_{Beau}$-N (1000ng), or pcDNA3.1-HA-IBV$_{M41}$-N (1000ng). After 24 h, the cells were treated with dimethyl sulfoxide (DMSO), 3-MA (0.5 mg/mL), MG-132 (20 μM), and Baf.A1 (100 nM) for 6 h. Protein expression was measured through western blotting.

We showed that chGBP1 was upregulated by both IFN-γ and IBV in HD11 cells. Meanwhile, chGBP1 inhibited IBV replication in a dose-dependent manner and enhanced IFN-γ anti-IBV activity. We found that the chGBP1 N-terminal GTPase domain was crucial for its anti-IBV activity, with amino acids R49 and K52 in chGBP1 playing critical roles in its GTPase activity and anti-IBV effects. More than that, we found that chGBP1 interacts with IBV N protein to destroy the stability of protein via the autophagy pathway.

IFN-induced proteins, such as GBP1, are required for host-mediated immune responses to pathogen invasion (Samuel, 2001). Initially, the antiviral effect of GBP1 against VSV and EMCV was shown (Anderson et al., 1999). Subsequently, GBP1 was reported to inhibit RNA viruses such as Flaviviridae viruses, including HCV (Itsui et al., 2009) and CSFV (Li et al., 2016), in vitro, and more recently, the effect of GBP1 against the DNA virus KSHV was reported (Zou et al., 2017). We screened for antiviral cytokines and found that GBP1 significantly inhibited IBV replication. GBPs were identified because they are strongly induced by type II IFN (**IFN-γ**) (Cheng et al., 1983). IFN-γ is a type II IFN and enhances the specific immune response by activating T cells and macrophages (Boehm et al., 1997). We then examined chGBP1 transcription levels at different times of IBV infection and found that chGBP1 was upregulated at both the gene and protein levels after virus infection, as compared with that in the control group. What's more, the same results were obtained when HD11 cells were stimulated with exogenous chIFN-γ. Chicken macrophages HD11 cells are susceptible to the IBV Beaudette strain, and infected macrophages produce a high load of virions (Han et al., 2017). We found that chIFN-γ production increased significantly after IBV infection in chicken macrophages, which may be related to host resistance to IBV infection. Further, we demonstrated by gain- and loss-of-function that chicken GBP1 inhibits IBV replication in HD11 cells in a dose-dependent manner.

IFNs are complex mixtures of bioactive molecules with complex functions within the innate immune system and are divided into types I, II, and III IFN (Pestka et al., 2004). IFNs have a range of antiviral effects on viral infection and replication, especially in the suppression of coronaviruses, including SARS, MERS, and SARS-CoV-2 (Mesev et al., 2019; Park and Iwasaki, 2020). We further demonstrated that IFN-γ significantly inhibited IBV replication. Moreover, chGBP1 enhanced the antiviral effect of IFN-γ in vitro. Similarly, silencing of GBP1 abolishes the antiviral effect of IFN-γ on HEV (Glitscher et al., 2021).

Also, GBP1 inhibits Classical Swine Fever Virus (**CSFV**) and Porcine Reproductive and Respiratory Syndrome Virus (**PRRSV**) via GTPase activity (Li et al., 2016; Duan et al., 2022). We showed that the GTPase domain of chGBP1 was critical for inhibiting IBV replication. The GTPase domain is a key region for GBP1 to play an antiviral function. In the conserved

structure of GBP1 GTPase in humans and mice, an R48 mutation weakens GTPase activity, while a K51 mutation causes a loss of function (Kravets et al., 2012; Yu et al., 2020). Compared with human/mouse amino acid sequences, these sites are at positions 49 and 52, respectively, in chicken GBP1. We found that R49A and K52A mutations attenuated the anti-IBV effect of chGBP1, suggesting that the GTPase activity may be required for this effect.

In addition, the researchers also found that GBP1 exhibited antiviral activity against viruses independent of GTPase activity. GBP1 anti-VSV was independent of its GTPase activity and isoprenylation (Gu et al., 2021b). GBP1 competitively binding to the VSV-N substituting for the VSV-P decreased RNA synthesis, repress the VSV genome transcription (Gu et al., 2021b; Zhang et al., 2021). Interestingly, our results suggest chGBP1 C-terminal represses IBV replication observably, independent of GTPase activity. chGBP1 reduced the IBV nucleocapsid (**N**) protein level by interacting with IBV-N protein to inhibit IBV. Coronavirus nucleocapsid protein, whose main function is to package the viral genome RNA molecule into a ribonucleoprotein (**RNP**) complex (Jayaram et al., 2006; Carlson et al., 2020). Ribonucleocapsid packaging is a fundamental part of viral self-assembly (Chang et al., 2014). Interestingly, our results showed that chGBP1 through the autophagy pathway degrades IBV-N protein. This may be a new strategy of GBP1 to exert antiviral activity. In HEV infection, GBP1 anti-HEV mainly through the autophagosomal pathway, independent of the GTPase-activity (Glitscher et al., 2021). GBP1 to form GBP1 homodimers targets the viral capsid protein to the lysosomal compartment, leading to inactivation of the viral particle(Glitscher et al., 2021). Chinese tree shrew GBP1 (**tGBP1**) interacted with tSTING, sequestosome 1, and microtubule associated protein 1 L chain 3, forming a complex which promotes autophagy in response to HSV-1 infection (Gu et al., 2021a). Thus, the mechanism of chGBP1 through the autophagy pathway to degrade IBV-N protein is worth further study.

In conclusion, we demonstrated that coronavirus IBV infection activates an chIFN-γ-induced host restriction factor, chGBP1, whose both C-terminal and N-terminal GTPase domain effectively inhibits IBV replication in chicken macrophages HD11 cells. This study provides insight into the host factor chGBP1 anti-IBV infection and will facilitate an understanding of viral pathogenesis and promote development of new antiviral strategies.

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DISCLOSURES

The authors declare that they have no competing interests.

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