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Negatively regulation of MAVS-mediated antiviral innate immune response by E3 ligase RNF5 in black carp

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ABSTRACT

Mitochondrial antiviral signaling protein (MAVS) is as an adaptor in RIG-I like receptor (RLR) signaling, which plays the key role in interferon (IFN) production during host antiviral innate immune activation. MAVS is fine tuned to avoid excess IFN production, which have been extensively studied in human and mammals. However, the regulation of MAVS in teleost still remains obscure. In this manuscript, we cloned ring finger protein 5 (bcRNF5) of black carp (*Mylopharyngodon piceus*) and characterized this teleost E3 ubiquitin ligase as a negative regulator of MAVS. The coding region of bcRNF5 consists of 615 nucleotides which encode 205 amino acids, containing two *trans*-membrane domain (TM) and a ring-finger domain (RING). The transcription regulation of bcRNF5 varies in host cells in response to stimulations of LPS, poly (I:C), grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV). bcRNF5 migrates around 22 KDa in immunoblot (IB) assay and distributes mainly in cytoplasm by immunofluorescent (IF) staining test. Moreover, bcRNF5 significantly inhibits bcMAVS-mediated IFN promoter transcription. In addition, both IF and co-immunoprecipitation assay showed that bcRNF5 interacts with bcMAVS. Furthermore, bcMAVS-mediated antiviral ability is distinctly impaired by bcRNF5. Taken together, these results conclude that bcRNF5, as a negative regulator of the MAVS-mediated IFN signaling, may play a key role in host protection upon virus infection in black carp.

1. Introduction

The innate immune response provides the first barrier of host defense against invading pathogens, such as bacteria, DNA and RNA viruses. Among them, the RNA viruses are the primary pathogens causing serious infectious and immunological diseases [1]. Upon infection, viral RNAs are recognized by pattern-recognition receptors (PRRs), including retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) [2], which both contain a DExD/Hbox RNA helicase domain that directly senses viral RNA [3]. After RNA recognition, the two caspase recruitment domains (CARDs) at the N termini of RIG-I and MDA5 initiate downstream signaling through interaction with the CARD domains of the adaptor protein mitochondrial antiviral signaling protein (MAVS; also called as Cardif, IPS-1, or VISA) [4,5], and then recruits IκB kinase ε (IKKε) and TANK-binding kinase 1 (TBK1) to activate interferon regulatory factor 3/7 (IRF3/7) or nuclear factor kappa B (NF-κB), which subsequently drives expression of interferon β (IFNβ) and other antiviral factors [6].

MAVS-mediated signaling is essential for host resistance to RNA viruses, but it is tightly regulated to prevent excessive immune response causing cell damage or death [7,8]. Previous studies have reported that several E3 ubiquitin ligases, such as SMAD specific E3 ubiquitin protein ligase 2 (SMURF2), can negatively regulate MAVS-mediated antiviral signaling pathway by promoting K48-linked poly-ubiquitination and proteasome mediated degradation of MAVS [9]. Moreover, emerging evidence has been showing that ring finger protein 125 (RNF125) is associated with MAVS and promotes K48-linked poly-ubiquitination and degradation of MAVS [10]. Although many enzymes have been found to play important roles in MAVS-mediated signaling pathways in mammals, the regulatory mechanisms of MAVS in teleost still need to be further investigated.

Ring finger protein 5 (RNF5), also known as G16 or RMA1, is an E3 ubiquitin ligase anchored to the endoplasmic reticulum (ER) [11]. Recent studies have shown that RNF5 contains a *trans*-membrane

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Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Primer information
bcRNF5		
bcRNF5-N-F	ACTGACGGTACCATGGAGGCCGCAGAGC	Flag-bcRNF5
bcRNF5-N-R	ACTGACGCGGCCGCTCACACGCTCAGCATC	
Q-PCR		
Q-EPC actin-F	AAGGAGAAGCTCTGCTATGTGGCT	*ex vivo* q-PCR
Q-EPC actin-R	AAGGTGGTCTCATGGATACCGCAA	
Q-EPC viperin-F	GCAAAGCGAGGGTTACGAC	
Q-EPC viperin-R	CTGCCATTACTAACGATGCTGAC	
Q-EPC Mx1-F	TGGAGGAACCTGCCTTAAATAC	
Q-EPC Mx1-R	GTCTTTGCTGTTGTCAGAAGATTAG	
Q-EPC ISG15-F	TGATGCAAATGAGACCGTAGAT	
Q-EPC ISG15-R	CAGTTGTCTGCCGTTGTAAATC	
Q-EPC Viperin-F	AGCGAGGCTTACGACTTCTG	
Q-EPC Viperin-R	GCACCAACTCTCCCAGAAAA	
Q-EPC IFN-F	ATGAAAACTCAAATGTGGACGTA	
Q-EPC IFN-R	GATAGTTTCCACCCATTTCCTTAA	
Q-SVCV-G-F	GATGACTGGGAGTTAGATGGC	
Q-SVCV-G-R	ATGAGGGATAATATCGGCTTG	
Q-SVCV-*M*-F	CGACCGCGCCAGTATTGATGGATAC	
Q-SVCV-*M*-R	ACAAGGCCGACCCGTCAACAGAG	
Q-SVCV-N-F	GGTGCGAGTAGAAGACATCCCCG	
Q-SVCV-N-R	GTAATTCCCATCATTGCCCCAGAC	
Q-SVCV-P-F	AACAGGTATCGACTATGGAAGAGC	
Q-SVCV-P-R	GATTCCTCTTCCCAATTGACTGTC	

domain (TM) in C-terminal and a ring-finger domain in N-terminal, which has multiple biological functions in mammals. In vertebrates, RNF5 regulates cell motility. One of the mechanism is that RNF5 could ubiquitinate paxillin, which resulting in altered paxillin localization and impaired cell motility [12]. Meanwhile, RNF5 has been reported as a novel regulator of breast cancer progression. For example, RNF5 could regulate the dynamic rearrangement of actin skeleton and promote the proliferation of breast cancer cells [13]. In addition, quite a few studies have shown that RNF5 regulates the host innate immune response through the ubiquitin-proteasome pathway. RNF5 interacts with stimulator of interferon response cGAMP interactor 1 (STING) and MAVS, and promotes their K48-ubiquitination and proteasomal degradation, subsequently inhibiting the production of IFNβ [14,15]. However, comparing to its mammalian counterpart, limited knowledge exists on the role of RNF5 in innate immune response in teleost.

In the present study, black carp RNF5 (bcRNF5) was cloned and characterized, and the negative role of bcRNF5 in bcMAVS-mediated antiviral signaling pathway was confirmed. Our data showed that bcRNF5, like its mammals, was expressed in the cytoplasm. Overexpression of bcRNF5 in SVCV-infected EPC cells observably dampened the mRNA expression levels of SVCV-related genes, IFN and ISGs in qRT-PCR assay. Moreover, bcRNF5 interacted with bcMAVS and negatively regulated MAVS/IFN signaling during SVCV infection. Thus, this study demonstrates that bcRNF5 is associated with MAVS and limits the ability of MAVS to induce type I interferon, suggesting an important yet novel regulatory factor of antiviral innate immune response for the first time in fish.

2. Materials and methods

2.1. Cells and plasmids

HEK293T cells and HeLa cells were kept in our laboratory [16], *epithelioma papulosum cyprinid* (EPC) cells and *ctenopharyngodon idella* kidney (CIK) cells were obtained from Dr. Pin Nie (Institute of Hydrobiology, CAS). *mylopharyngodon piceus* kidney (MPK) cells were obtained from Dr Tiansheng Chen (Jimei University, Xiamen, China) [17]. HEK293T and HeLa were cultured at 37 °C; EPC, CIK and MPK cells were cultured at 26 °C. All the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and were exposed in 5% CO_2.

pcDNA5/FRT/TO, pcDNA5/FRT/TO-Flag-bcMAVS, pRLTK, the two kinds of group I type I IFNs, including Luci-bcIFNa (for black carp IFNa promoter activity analysis) and LuciDrIFNφ1 (for zebrafish IFNφ1 promoter activity analysis) were kept in the lab [18]. The recombinant expression vectors pcDNA5/FRT/TO-Flag-bcRNF5 and pcDNA5/FRT/TO-HA-bcRNF5 were constructed by linking the open reading frame (ORF) of bcRNF5 to pcDNA5/FRT/TO fused with a HA tag or FLAG tag at the N-terminus. All the primer sequences were referenced in Table 1.

2.2. Virus production and titer detection

Spring viremia of carp virus (SVCV/strain: SVCV741) and grass carp reovirus (GCRV/strain: GCRV106) were kept in the lab and propagated in EPC cells or CIK cells separately at 26 °C in the presence of 2% fetal bovine serum. Viral titer were detected using EPC cells by viral plaque assay as previously described [19]. In brief, EPC cells were infected with 10-fold serial dilutions of viral supernatant, after incubation for 1 h at 26 °C, the supernatant was replaced with DMEM containing 2% fetal bovine serum (FBS) and 1% methylcellulose, plaques were counted on the third day after infection.

2.3. Sequence and phylogenetic analysis

The RNF5 homologous amino acid sequences of black carp and other species were aligned by MEGA-X software and analyzed by GeneDoc program, and the neighbor-joining technique with 1000 replications of bootstrap of MEGA-X program was used to construct the phylogenetic tree. In addition, the protein domain of bcRNF5 was predicted using the CDS sequence of black carp RNF5 as a reference, through NCBI Database CDD (Conserved Domain Database) and protein domain prediction website SIMPLE MODULAR ARCHITECTURE RESEARCH TOOL (SMART) (HTTP://SMART.EMBLHEIDELBERG.DE/). The amino acid sequences of RNF5 from all different species were searched at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/).

2.4. Lipopolysaccharides (LPS) and poly (I:C) treatment

MPK cells were treated with LPS or poly (I:C) stimulation for different time periods (2 h, 8 h, 12 h, 24 h and 48 h) after seeding MPK cells in 6-well plates (2×10^6 cells/well) for 24 h. Poly (I:C) was heated in a water bath at 55 °C for 5 min and cooled to room temperature before use, and the original medium of MPK cells was replaced with poly (I:C)-containing media at final concentrations of 5 μg/ml and 50 μg/ml. For LPS stimulation, MPK cells were directly added to the media at concentrations of 1 μg/ml and 50 μg/ml in the manner described above.

2.5. Quantitative real-time PCR

The relative mRNA level of SVCV-associated proteins (G, M, N, P), IFN and interferon stimulated genes (Viperin, ISG15, MX1) in SVCV-infected EPC cells were detected by quantitative real-time PCR (q-PCR) with the SYBR Green staining. The primer sequences were listed in Table 1. The operation procedure of qPCR is as follows:1 cycle of 95 °C/10 min, 40 cycles of 95 °C/15 s, 60 °C/1 min. The $2^{-\triangle\triangle}$CT method was used to calculate the relative expression levels of the target gene.

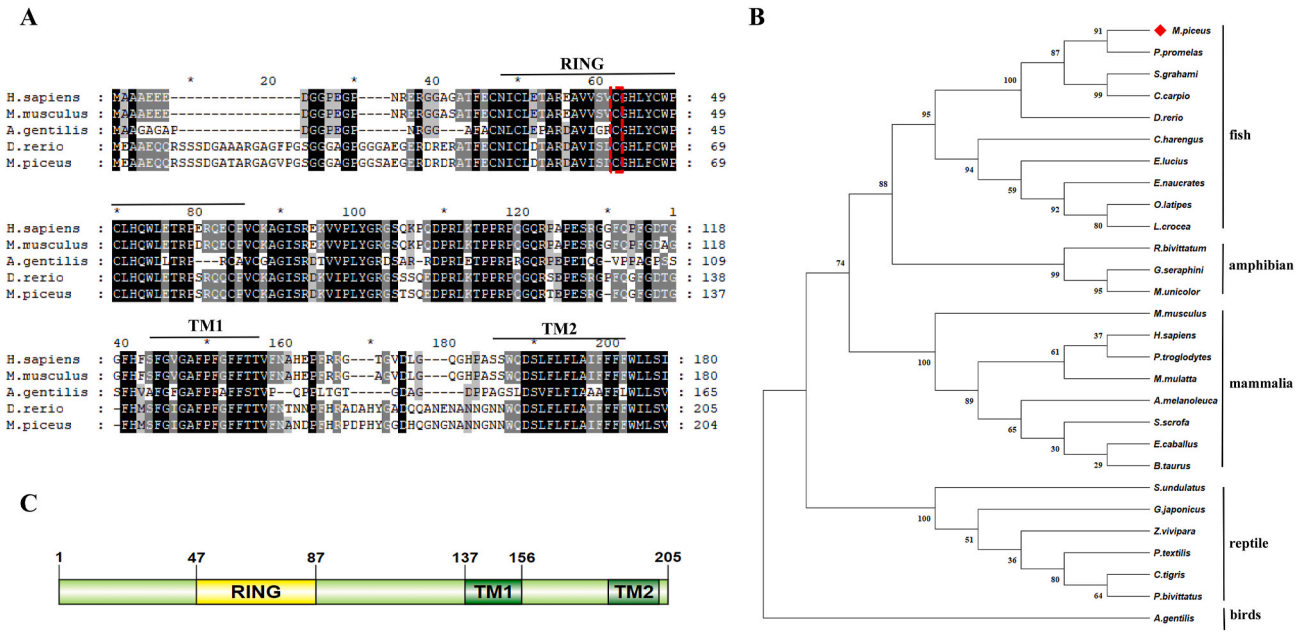

Fig. 1. Sequence analysis of bcRNF5.
(A) Multiple alignments of RNF5 from *H. sapiens* (CAG33496.1), *M. musculus* (NP_758488.1), *A. gentilis* (XP_049649052), *D. rerio* (NP_775365.1), *M. piceus* (OP771624). (B) By using MEGAX program, maximum likelihood phylogenetic tree was generated from vertebrate RNF5 of different species which include (GenBank accession number): *H. sapiens* (UQL51192.1), *M. musculus* (NP_062276.1), *Bos taurus* (NP_001098915.1), *D. rerio* (XP_003200552.1), *Pan troglodyte* (XP_001164301.1), *Sus scrofa* (NP_001116696.1), *Pimephales promelas* (XP_039534418.1), *Sinocyclocheilu grahami* (XP_016096383.1), *Cyprinus carpio* (XP_042601838.1), *Clupea harengus* (XP_012691514.1), *Clupea lucius* (XP_010898489.1), *Echeneis naucrates* (XP_029369492.1), *Oryzias latipes* (XP_004073806.1), *Larimichthys crocea* (XP_019119861.1), *Rhinatrema bivittatum* (XP_029440604.1), *Geotrypetes seraphini* (XP_033772209.1), *Microcaecilia unicolor* (XP_030053145.1), *Macaca mulatta* (NP_001192045.1), *Ailuropoda melanoleuca* (XP_002929683.1), *Equus caballus* (XP_001493502.1), *Sceloporus undulatus* (XP_042306811.1), *Gekko japonicus* (XP_015270904.1), *Zootoca vivipara* (XP_034962999.1), *Python bivittatus* (XP_007430366.1), *A. gentilis* (XP_049649052). The bar stands for scale length and the numbers on different nodes stand for bootstrap value. (C) structure domain of bcRNF5.

2.6. Immunoblotting

Immunoblotting was used to detect the expression of bcRNF5 in HEK293T cells and EPC cells. Either bcRNF5 or empty vector was transfected into HEK293T cells and EPC cells using polyethyleneimine (PEI) for 48 h, then transfected cells were harvested and lysed for immunoblot (IB) assay analysis as described previously [20]. In brief, the lysed cells were separated by 12% SDS-PAGE, and transferred to PVDF membranes. Then PVDF membranes of loading with the target protein were probed with mouse monoclonal anti-Flag-antibody. Finally, NBT/BCIP alkaline phosphatase substrate was used for visual analysis of target proteins.

2.7. Immunofluorescence microscopy

HeLa cells was co-transfected with bcRNF5 and bcMAVS or empty vectors by using LipoMax, approximately 24 h later, transfected cells were treated with 4% (v/v) histiocytes fixative, Triton X-100 (0.2% in PBS) and 10 %FBS as described previously [21], and then the cells were incubated with mouse monoclonal anti-Flag antibody at the ratio of 1:500 for 1 h, followed with alexa 488-conjugated secondary antibody at the ratio of 1:1000 for 1 h. After the above treatment, 6 μl DAPI was added to the microslide for nuclear staining, the slides were air-dried and photographed with laser scanning confocal microscope (Olympus FV1200, Japan).

2.8. Dual luciferase reporter assay

To investigate the effect of bcRNF5 on bcMAVS-mediated IFN promoter transcription, different doses of bcRNF5 (50 ng/100 ng/200 ng) was co-transfected into EPC cells in 24-well plates with bcMAVS, pRL-TK and Luci-bcIFNa (or Luci-DrIFNφ1) for 24 h, then cells were lysed with PLB and used for luciferase activities examination according to the instruction of the Dual-Luciferase Reporter Assay System kit (Promega, USA).

2.9. Co-immunoprecipitation

bcRNF5 was co-transfected with bcMAVS or empty vector into HEK293T cells in 10 cm plate, 48 h after transfection co-immunoprecipitation experiments were performed as previously described [22]. In short, the whole cell lysates were incubated with protein A/G agarose beads at 4 °C for 90 min. After precleaning and concentration, the anti-FLAG conjugated protein A/G agarose beads were added and incubated with the supernatant media at 4 °C for 4 h. The beads were heat-denatured in 5 × SDS-sample buffer after 5 times washes with 1% NP-40 and used for IB as above.

2.10. Statistics analysis

For the statistical analysis of the data of viral titer measurement, qPCR and luciferase reporter assay, all data were performed with one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or Dunnett's post-hoc test. Data are presented as the mean ± SEM. Data are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$.

3. Results

3.1. Sequence and phylogenetic analysis of bcRNF5

To investigate the role of RNF5 in teleost, we cloned the full-length cDNA of *bcRNF5* gene and constructed bcRNF5 expression plasmids with different labels. Sequence analysis results show that the coding

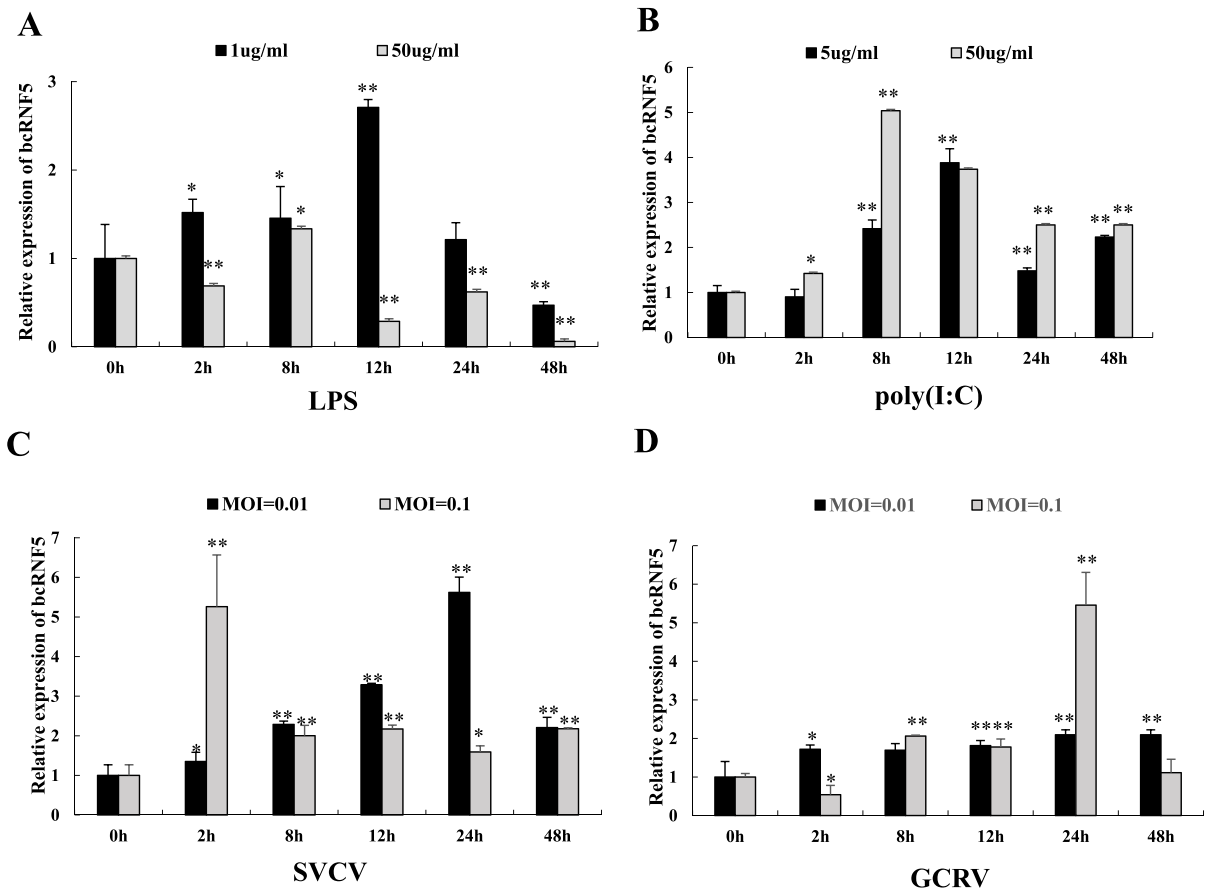

Fig. 2. mRNA Expression patterns of bcRNF5 in response to different stimuli.
The mRNA levels of bcRNF5 in MPK cells at different time points (2 h, 8 h, 12 h, 24 h, 48 h) after stimulated with LPS (1 μg/mL or 50 μg/mL) (A) or Poly (I:C) (5 μg/mL or 50 μg/mL) (B). Expression profile of bcRNF5 in MPK cells at different time points post SVCV (C) or GCRV (D) infection (2 h, 8 h, 12 h, 24 h, 48 h) with the indicated MOI (0.01 or 0.1).

sequence of bcRNF5 (NCBI accession number: OP771624) contains 615 nucleotides and encodes 205 amino acids which include two *trans*-membrane domain (TM) and a ring-finger domain (RING), similar to mammalian RNF5 (Fig. 1C). The predicted molecular weight of bcRNF5 is 22.2 kDa and the theoretical isoelectric point of the protein is 6.05 (calculated by EXPASy Compute PI/Mw).

To understand the conservation of RNF5 in vertebrate, the protein sequence of *Danio rerio, Homo sapiens, Mus musculus, Accipiter. gentilis and M. piceus* were aligned together using the ClustalW2 program and edited with GeneDoc software. The results revealed that bcRNF5 is a conserved protein among vertebrates, especially for its RING domain and TM domain (Fig. 1A). Phylogenetic analysis of RNF5 proteins from the selected species demonstrated that these RNF5 homologues could be divided into five groups, consisting of mammalia, aves, reptilia, amphibia and fish branches. Distinctly, the results showed that bcRNF5 and RNF5 of other fish species are clustered in the same monophyletic group (Fig. 1B).

3.2. *bcRNF5 expression ex vivo in response to different stimuli*

In order to explore the expression patterns of bcRNF5 in host innate immunity, MPK cells treated with LPS (1 μg/ml, 50 μg/ml) or poly (I:C) (5 μg/ml, 50 μg/ml) were used for qPCR to detect the mRNA level of bcRNF5. In the LPS treatment group, the transcription of *bcRNF5* gene was increased immediately after stimulation and reached the highest level at 12 h (1 μg/ml, 2.7 folds) (Fig. 2A), which indicated that bcRNF5 might participate in bacteria-triggered immune response. In poly (I:C) treatment group, bcRNF5 mRNA abundance began to increase at 2 h, reached the maximum at 8 h (50 μg/ml, 5.0 folds), and then showed a downward trend (Fig. 2B). When MPK cells were infected with SVCV, the mRNA level of *bcRNF5* gene in 0.1 MOI group was immediately increased at 2 h post infection and reached the highest level (5.3 folds), but when the MOI of SVCV was 0.01, the highest fold change appeared at 24 h post infection (5.6 folds) (Fig. 2C). Differently, right after GCRV infection, the mRNA level of *bcRNF5* gene decreased in high MOI group (MOI = 0.1) and increased again, then reached the peak at 24 h post infection (5.5 folds). However, in the low MOI group (MOI = 0.01), the mRNA level of *bcRNF5* gene increased immediately after infection and kept stable within 48 h post infection (Fig. 2D). Taken together, these data suggested that bcRNF5 was recruited in host innate immune response against GCRV and SVCV, but through different mechanisms.

3.3. *Protein expression and subcellular distribution of bcRNF5*

To verify the expression of the bcRNF5, bcRNF5 was over-expressed in HEK293T and EPC cells, respectively. Then bcRNF5 protein was detected by immunoblotting (IB) assay, in which the anti-Flag antibody was used to probe the exogenous bcRNF5. The specific band of around 22 kDa was detected in the whole cell lysates of HEK293T and EPC cells expressing bcRNF5 but not in that of the control cells, indicating that bcRNF5 was well expressed in both mammalian and fish systems (Fig. 3A&B). Then, to determine the subcellular distribution of bcRNF5, HeLa cells were transfected with bcRNF5 and used for immunofluorescence (IF) staining. In the results of IF assay, the red color representing bcRNF5 expression area surrounded the nuclei (blue color) tightly, indicating that bcRNF5 mainly distributed in the cytoplasm (Fig. 3C).

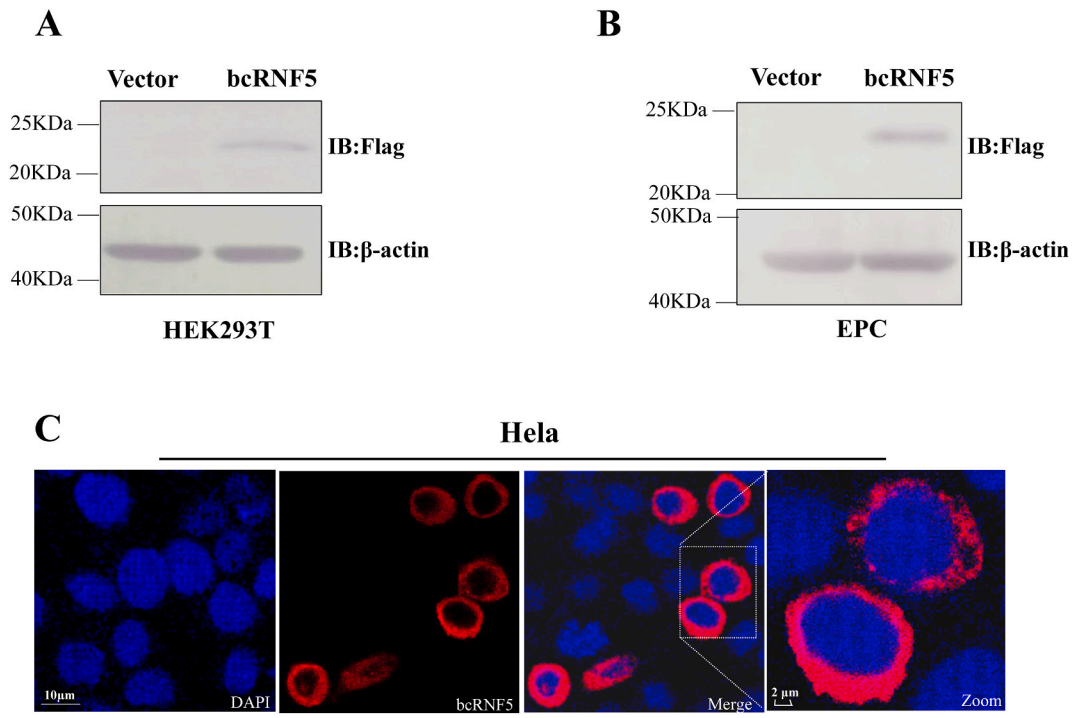

Fig. 3. Protein expression and subcellular distribution of bcRNF5.
HEK293T (A) cells or EPC (B) cells were transfected with bcRNF5 or empty vector (3 μg/well) by using PEI, and the expression of bcRNF5 was detected by immunoblotting (IB). (C) HeLa cells in 24-well plates were transfected with bcRNF5 by using LipoMAX, then the transfected cells were used for immunofluorescence staining. The bars stand for the scale of 2 μm or 10 μm. bcRNF5: pcDNA5/FRT/TO/Flag-RNF5, Vector: pcDNA5/FRT/TO.

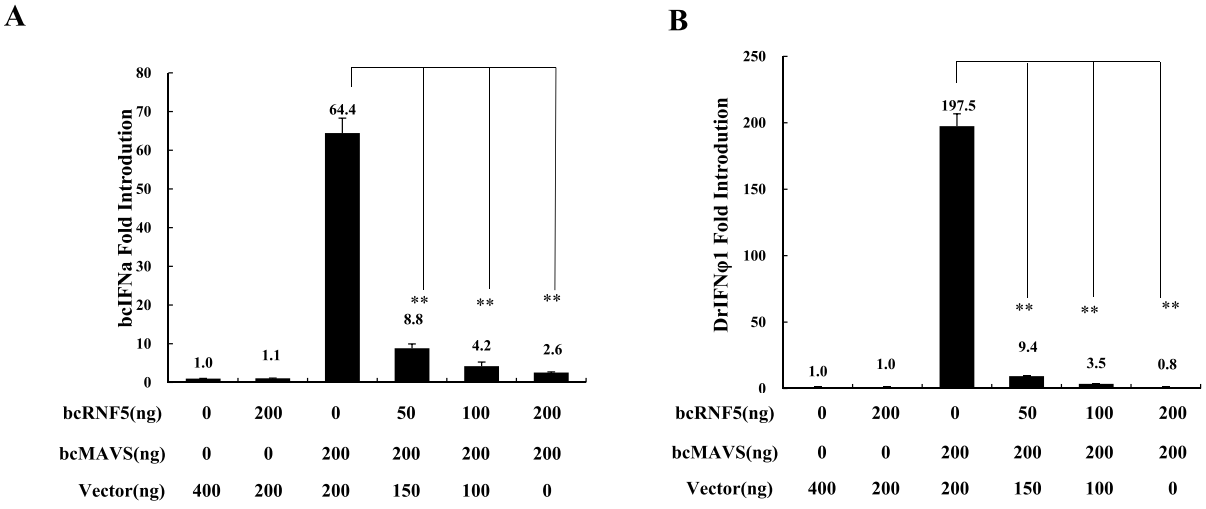

Fig. 4. bcRNF5 inhibits bcMAVS-mediated IFN promoter transcription.
The reporter plasmids pRL-TK, Luci-bcIFNa (A) or DrIFNφ1 (B) were co-transfected into EPC cells in 24-well plate with the indicated plasmids bcRNF5 and bcMAVS, respectively. The total amount of plasmids in each well were balanced by empty vectors. The transcription activities of IFN promoters were detected by the luciferase reporter assay 24 h after transfection. The numbers above the error bars stand for the average IFN fold induction. bcRNF5: pcDNA5/FRT/TO/Flag-bcRNF5, bcMAVS: pcDNA5/FRT/TO-Flag-bcMAVS; Vector: pcDNA5/FRT/TO. Data are representative of three independent experiments. *P < 0.05, **P < 0.01.

3.4. bcRNF5 inhibits bcMAVS-mediated interferon signaling

To further elucidate the role of bcRNF5 in IFN signaling pathway, EPC cells were co-transfected with plasmids expressing bcRNF5 and/or bcMAVS, then used for luciferase reporter assay. In the reporter assay, over-expressed bcMAVS fiercely activated bcIFNa and DrIFNφ1 promoters, however, over-expressed bcRNF5 had no induction effect on bcIFNa and DrIFNφ1 promoters. When co-expressed with bcMAVS, bcRNF5 significantly inhibited bcMAVS-mediated transcription of bcIFNa (Fig. 4A) and DrIFNφ1 (Fig. 4B) in a dose dependent manner. The results implied that RNF5 played a negatively regulatory role in MAVS/IFN cascade in the innate immunity of black carp.

3.5. bcRNF5 restrains bcMAVS-mediated antiviral activity

It has been reported that the antiviral activity of MAVS, as a critical

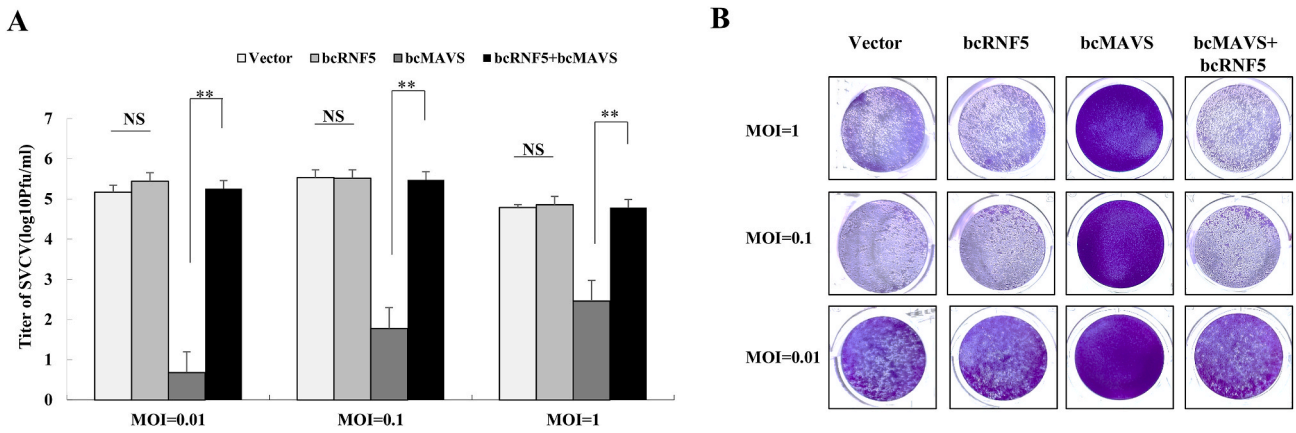

Fig. 5. bcRNF5 suppresses bcMAVS-mediated antiviral activity.
EPC cells in 24-well plate were transfected with bcRNF5 and/or bcMAVS respectively, and the transfected cells were infected with SVCV respectively. The supernatant was collected at 24 h post infection for virus titer assay (A), and the monolayer cells were used for crystal violet staining (B). bcMAVS: pcDNA5/FRT/TO/Flag-bcMAVS; bcRNF5: pcDNA5/FRT/TO/Flag-bcRNF5; Vector: pcDNA5/FRT/TO/Flag. Data are representative of three independent experiments. *P < 0.05, **P < 0.01.

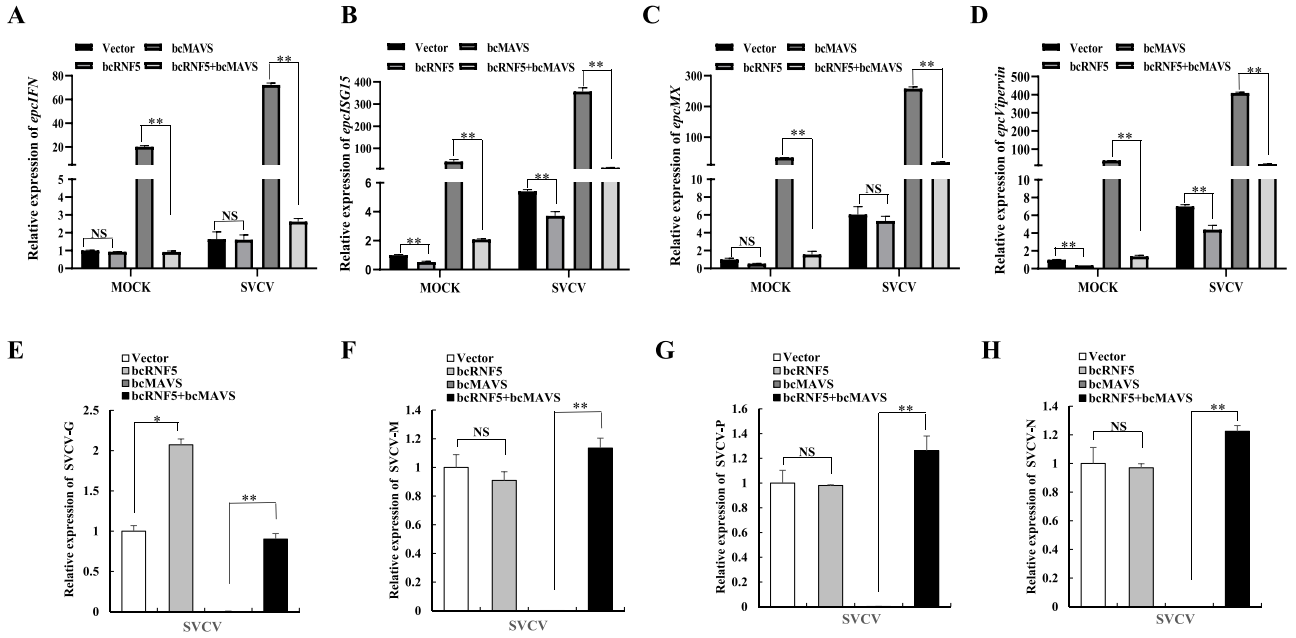

Fig. 6. The mRNA levels of SVCV encoded proteins and IFN/ISGs in EPC cells after SVCV infection.
EPC cells in 24-well plate were transfected with bcRNF5 and/or bcMAVS respectively, and the transfected cells were infected with SVCV (0.1 MOI) separately. The cells were harvested and used for RNA isolation at 24 h post infection. The relative mRNA level of *epcIFN* (A), *epcISG15* (B), *epcMX* (C), *epcViperin* (D) and SVCV-G (E), M (F), P (G), N (H) were examined by q-PCR. bcMAVS: pcDNA5/FRT/TO/Flag-bcMAVS; bcRNF5: pcDNA5/FRT/TO/Flag-bcRNF5; Vector: pcDNA5/FRT/TO/Flag. Data are representative of three independent experiments. *P < 0.05, **P < 0.01.

adaptor required for virus-triggered induction of type I IFNs, was significantly inhibited by the E3 ubiquitin ligase RNF5 in mammals [15]. Our previous study revealed that bcMAVS exhibited strong antiviral activity against SVCV and GCRV [20], which made us feel interested in the regulation of the antiviral activity of MAVS by RNF5 in black carp. To investigate the influence of RNF5 on MAVS-mediated antiviral signaling pathway in black carp, EPC cells were co-transfected with bcRNF5 and/or bcMAVS, and infected with SVCV virus with different MOI (MOI = 1/0.1/0.01). The plaque assay results showed that the viral titers in the supernatant of EPC cells co-transfected with bcRNF5 and bcMAVS were observably higher than those of EPC cells expressing bcMAVS alone (Fig. 5A). Moreover, the crystal violet staining results also showed that the mortality of EPC cells co-transfected with bcRNF5 and bcMAVS was fiercely higher than that of EPC cells expressing bcMAVS alone (Fig. 5B). Meanwhile, the qPCR data showed that the mRNA expression levels of SVCV encoded proteins G (Fig. 6E), M (Fig. 6F), P (Fig. 6G) and N (Fig. 6H) in EPC cells co-expressing bcRNF5 and bcMAVS were remarkably increased than that in EPC cells expressing bcMAVS alone. On the contrary, the mRNA expression of *epcIFN* (Fig. 6A), *epcISG15* (Fig. 6B), *epcMx1* (Fig. 6C) and *epcViperin* (Fig. 6D) in EPC cells co-transfected with bcRNF5 and bcMAVS were dramatically depressed. These results clearly demonstrated that bcRNF5 notably suppressed bcMAVS-mediated antiviral activity against SVCV.

3.6. The interaction between bcRNF5 and bcMAVS

To further investigate the mechanism behind the negative regulation of bcMAVS by bcRNF5, HEK293T cells were co-transfected with bcMAVS and bcRNF5 and co-immunoprecipitation (co-IP) assay was performed to examine the association between bcRNF5 and bcMAVS.

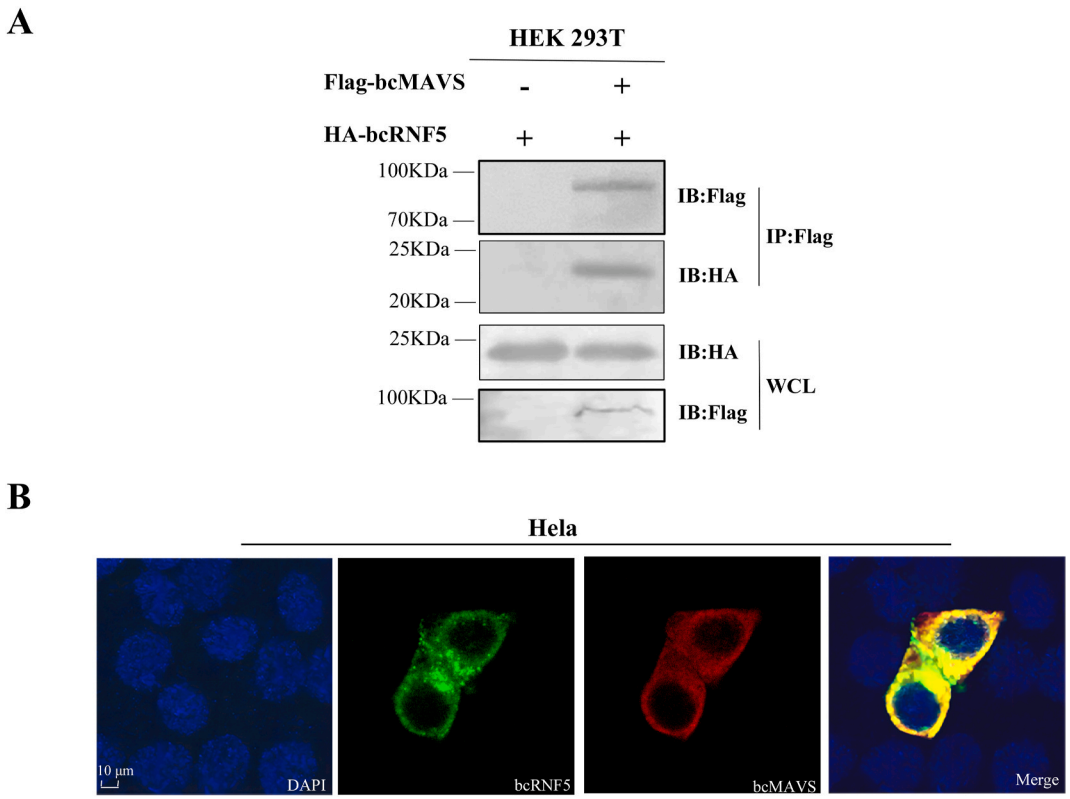

Fig. 7. The interaction between bcRNF5 and bcMAVS.
(A) HEK293T cells in 10 cm dish were co-transfected with bcMAVS and/or bcRNF5. The cells were harvested 48 h post transfection and used for co-immunoprecipitation assay. IP: immunoprecipitation; IB: immunoblot; WCL: whole cell lysate. (B) HeLa cells in 24-well plates were transfected with bcRNF5 and bcMAVS by using LipoMAX, and the transfected cells were used for immunofluorescence staining. The bars stand for the scale of 10 μm. bcMAVS: pcDNA5/FRT/TO/Flag-bcMAVS; bcRNF5: pcDNA5/FRT/TO/HA-bcRNF5; Vector: pcDNA5/FRT/TO.

The results showed that the specific band of 22 kDa representing bcRNF5 was detected in the Flag-bcMAVS-precipitated proteins, which demonstrated the interaction between these two molecules (Fig. 7A). Meanwhile, HeLa cells were co-transfected with bcRNF5 and bcMAVS and used for IF assay. The results showed that subcellular distribution of bcRNF5 (green color) largely overlapped with that of bcMAVS (red color) (Fig. 7B). Thus, these results demonstrated that bcRNF5 interacted with bcMAVS and dampened bcMAVS-mediated antiviral signlaing.

4. Discussion

MAVS is an essential adaptor protein, mediating IFN production in the RLR/IFN signaling pathway [5]. Excessive IFN production may lead to immunotoxicity or autoimmune diseases. Thereby, key factors in RLR signaling, such as MAVS, are strictly regulated to maintain intracellular homeostasis. In our previous study, several negative regulators, such as TUFM, NLK, RIPK1, and NLRX1, have been characterized to inhibit MAVS-mediated antiviral signaling during the antiviral innate immune activation in black carp through different mechanisms [23–26]. In mammals, growing post-translational modifications (PTMs) involved in MAVS regulation have been reported, such as phosphorylation, ubiquitination, and acetylation, etc [27]. However, the regulatory mechanisms of PTM of MAVS in teleost fishes remain obscure. Nemo-like kinase (NLK) can interact with MAVS and inhibit the MAVS-mediated IFN signaling pathway in black carp, implying that fish NLK might phosphorylate and degrade MAVS to limit IFN production like its mammalian counterpart [24]. In sea perch (*Lateolabrax japonicas*), E3 ubiquitin ligase ring finger protein 114 (RNF114) targets MAVS and TRAF3 for K27- and K48-linked ubiquitination, which promotes the proteasomal degradation of MAVS, and inhibits IFN production [28].

In this paper, bcRNF5 has been identified and characterized, which is aimed to elaborate the role of RNF5 in host antiviral innate immunity in teleost fish. Sequence analysis of bcRNF5 demonstrates that the amino acid sequences of RING domain, TM1 and TM2 in bcRNF5 possess highly similarities to those of mammals, birds and zebrafish, which hints the conserved function of RNF5 in vertebrates. In mammals, the RING domain is essential for the regulatory function of RNF5, especially, the 42nd cysteine (Cys^{42}) in the RING domain is critical for the E3 ubiquitin ligase activity of RNF5. RNF5-C42S, cysteine (C) mutated into serine (S) at site 42nd in the RING domain, lost the activity of E3 ubiquitin ligase, and failed to ubiquitinate and degrade MAVS to inhibit type I IFN production [29]. In this paper, the amino acid sequence alignment analysis of bcRNF5 showed that the 62nd cysteine in black carp and zebrafish is conserved, corresponding to the 42nd cysteine in vertebrates. Hence, it is speculated that Cys^{62} of bcRNF5 might be the active site of ubiquitin ligase, which play vital role in antiviral innate immunity.

RNF5, as an E3 ubiquitin ligase, was found to be a negative regulatory protein in the antiviral innate immunity in mammals. Numerous studies have shown that RNF5 plays different roles in response to diverse virus invasions. For instance, RNF5 was involved in Newcastle disease virus (NDV) V-mediated MAVS degradation, which restrained IFN signaling pathway [30]. Moreover, RNF5 could limit the type I IFN antiviral response in HSV keratitis by inhibiting STING/IRF3 signaling [31]. In addition, the recently research showed that RNF5 could mediate the ubiquitination of SARS-CoV-2 M at residue K15 to enhance the interaction of the viral envelope protein (E) with M, leading to the rapid release of virions [32]. However, the role of RNF5 in antiviral innate immunity in teleost fish has not been determined. In this paper, RNF5 has been cloned and identified in fish for the first time. Our results showed that RNF5 interacts with MAVS and significantly inhibits the MAVS-mediated antiviral activity against SVCV in black carp. Although

the mechanism behinds this still remains unknown, it is still could be speculated that bcRNF5 might ubiquitinate and degrade bcMAVS, thereby inhibiting bcMAVS-mediated IFN production, which needs to be further explored in the lab in the future.

CRediT authorship contribution statement

Jun Yan: Investigation, and . **Guoxia Qiao:** Investigation. **Enhui Wang:** Investigation. **Yuqing Peng:** Investigation. **Jiamin Yu:** Investigation. **Hui Wu:** Formal analysis. **Meiling Liu:** Validation. **Jiagang Tu:** Data curation, All persons who have made substantial contributions to the work reported in the manuscript are named in the manuscript and have given their written permission to be named. **Yongan Zhang:** Conceptualization. **Hao Feng:** Supervision, Conceptualization, Writing – review & editing.

Data availability

Data will be made available on request.

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