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NLRX1 of black carp suppresses MAVS-mediated antiviral signaling through its NACHT domain

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ABSTRACT

NOD-like receptor (NLR) family member X1 (NLRX1) of human localizes on mitochondria and serves as a negative regulator of antiviral signaling. However, the function of NLRX1 in teleost fish still remains elusive. To explore its role in the innate immunity of teleost fish, NLRX1 homologue has been cloned and characterized from black carp (*Mylopharyngodon piceus*). Black carp NLRX1 (bcNLRX1) consists of 1008 amino acids, which includes a N-terminal mitochondrial targeting sequence, a central NACHT domain and a C-terminal leucine-rich repeat (LRR) domain. bcNLRX1 was identified as a cytosolic protein locating on mitochondria through immunofluorescence (IF) staining. The overlapped subcellular distribution of bcNLRX1 and black carp MAVS (bcMAVS) was detected in IF staining, and the direct interaction between these two molecules *in vitro* was identified through co-immunoprecipitation assay. When co-expressed with bcMAVS, bcNLRX1 fiercely reduced bcMAVS-mediated IFN induction in reporter assay. Accordingly, the antiviral activity of bcMAVS against both grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV) was forcefully repressed by bcNLRX1 in plaque assay. Mutagenic analyses further revealed that the NACHT domain of bcNLRX1 was essential for it to interact with bcMAVS and to suppress bcMAVS-mediated antiviral signaling. Taken together, our data support the conclusion that bcNLRX1 negatively regulates bcMAVS-mediated antiviral signaling through its NACHT domain during host innate immune activation.

1. Introduction

Innate immunity of vertebrates is the first line to defense against endogenous antigens and invasion of exogenous pathogens. Vertebrates typically use a range of pattern recognition receptors (PRRs) to initiate innate immune responses against pathogens, which are currently reported by researchers: Toll-like receptors (TLRs), C-type lectins receptors (CLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs) and OAS-like receptors (Parvatiyar et al., 2013; Broz and Monack, 2013; Jeannin et al., 2008; Kato et al., 2008; Takeuchi and Akira, 2010). The pathogen-associated molecular patterns (PAMPs) recognized by the above PRRs mainly include viral DNA and RNA, lipopolysaccharide (LPS), bacterial nucleic acid, flagellin, peptidoglycan, and lipoteichoic acid, etc. (Keating et al., 2011; Akira and Hemmi, 2003; Chen et al., 2016). After recognizing the components, PRRs sequentially activate downstream signaling pathways, resulting to the production of type-I interferon (e.g. IFNα/β) and many other inflammatory cytokines (e.g. TNFα, IL-1 and IL-18) (Barbalat et al., 2011).

NLR members contain three domains: a central NACHT domain, which is common to all NLRs, a C-terminal leucine-rich repeat (LRR) and a variable N-terminal interaction domain. Therefore, NLR family is divided the into five different subfamilies based on the N-terminal domain: NLRA、NLRB、NLRC、NLRP and NLRX (Motta et al., 2015). NLRs are not only involved in immune recognition, but also widely involved in the regulation of immune responses in mammal, especially the regulation of inflammatory responses (Moore et al., 2008). While some NLRs, such as NOD1, NOD2 and NLRP3, serve as the PRRs in response to various PAMPs, others, including NLRX1, NLRC3, NLRC5 and NLRP12, act as the negative regulators of innate immune activation (Schneider et al., 2012; Xia et al., 2011; Benko et al., 2010; Allen et al., 2012; Feng et al., 2017). NLRX1, also called NOD5/NOD9/CLR11.3, was found in 2003 to affect innate immunity to viruses and bacterial infection (Castaño-Rodríguez et al., 2014). In mammals, NLRX1 is located on the mitochondrial outer membrane, interacting with the CARD domain of mitochondrial antiviral signaling protein (MAVS) via its LRR domain. When NLRX1 was silenced, the induction of IFNβ by Sendai virus (SeV) or Sindbis virus (SINV) could be significantly enhanced (Lei

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Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Amplicon length (nt) and primer information
CDS		
bcNLRX1-F	ATGTGGAGATTTGGAAGACCATC	3021bp
bcNLRX1-R	TCATTTTGTACCGGTTCCTGTTC	complete bcNLRX1 CDS cloning
Expression construct		
HA-bcNLRX1-F	ACTGACGGATCCGCCACCATGTGGAGATTTGG	
HA-bcNLRX1-R	ACTGACGCGGCCGCTCATTTTGTACCGGTTCC	FRT-To-HA-bcNLRX1
HA-NACHT-F	ACTGACGGTACCGCCACCCTAAATGTTTTGCTA	FRT-To-HA-NACHT
HA-NACHT-R	ACTGACCTCGAGTCAATTCTGTTGCAAGAG	FRT-To-HA-LRR
HA-LRR-F	ACTGACGGTACCGCCACCAGCTTCCACCTGAAT	
HA-LRR-R	ACTGACCTCGAGTCAGGGATGTTCACGACA	
q-PCR		
bc-Q-actin-F	TGGGCACCGCTGCTTCCT	
bc-Q-actin-R	TGTCCGTCAGGCAGCTCAT	*In vitro* q-PCR
bc-QNLRX1-F1	CAAGGCACAAGGACTAAATG	
bc-QNLRX1-R1	CTACAAGGTCTCGAAGGGAT	*In vitro* q-PCR

et al., 2012; Moore et al., 2008). Recent evidence indicated that NLRX1 recruited PCBP2 to MAVS and induced the K48-linked polyubiquitination and degradation of MAVS, leading to the negative regulation of IFN signaling pathway and promoting HCV infection (Qin et al., 2017). Nevertheless, not all studies showed that NLRX1 acted as a negative regulator in innate immune responses against virus. Wild type and NLRX1-deficient mice exhibited unaltered antiviral and inflammatory gene expression following intranasal challenge with influenza A virus or intraperitoneal injection of poly (I:C) (Soares et al., 2013).

Teleost fish are an independent organism from the early developmental stages compared with human and mammals. Teleost fish possess both innate and adaptive immune system; however, they depend more on the innate immune system to defend themselves against the disadvantage environment and pathogen invasion (Rombout et al., 2005). Although the innate immunity of teleost fish has been extensively studied in recent years, the detailed role of teleost NLRX1 during viral infection still remains largely indefinite. Hitherto, NLRX1 homologues have been cloned and characterized in several species such as grass carp (*Ctenopharyngodon idella*), rainbow trout (*Oncorhynchus mykiss*), miiuy croaker (*Miichthys miiuy*), channel catfish (*Ictalurus punctatus*) and goldfish (Chu et al., 2018; Álvarez et al., 2017; Li et al., 2015; Sha et al., 2009; Xie et al., 2013). In NLRX1 deficient CIK cell, gene expression levels of IRF3, IRF7, and IFN-I were all significantly heightened after poly (I:C) stimulation, which suggested that NLRX1 was a negative regulator of IFN in innate immune responses, and NLRX1 functioned as an attenuator in innate immune by interacting with TRAF6 pathways in grass carp (Chu et al., 2018).

Black carp (*Mylopharyngodon piceus*) is one of the "Four Domesticated Fish" in China's freshwater aquaculture over a thousand years. This economically important species is susceptible to bulk of pathogenic microorganisms both in aquaculture and natural condition, including grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV) (Jiang et al., 2017). However, the innate immune system of black carp remains largely unknown. In our previous study, MAVS, the major adaptor and antiviral protein of RLR signaling pathway, has been cloned and characterized from black carp (bcMAVS) (Zhou et al., 2015). To illuminate the role of NLRX1 in the antiviral innate immune response in black carp, NLRX1 homologue of black carp (bcNLRX1) has been cloned and characterized in this paper. Our data speculated clearly that, bcNLRX1 functioned as a negative regulator in virus-triggered innate immune response, in which bcNLRX1 restrained RLR/MAVS/IFN signaling by attenuating the antiviral activity of bcMAVS against invading SVCV or GCRV. Our study has unlocked a key mechanism of fine tuning of innate immunity by which bcNLRX1 directly interacts with bcMAVS through its NACHT domain, which is reported in teleost fish for the first time.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, *Epithelioma papulosum cyprini* (EPC), *Ctenopharyngodon idella* kidney (CIK) and *Mylopharyngodon piceus* kidney (MPK) cells were kept in the lab (Zhou et al., 2015). HEK293T cells were cultured at 37 °C with 5% CO_2; EPC, CIK and MPK cells were cultured at 26 °C with 5% CO_2. All cell lines were maintained in DMEM supplement with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Transfection was done as previously described, calcium phosphate was used for 293T transfection, Lipomax (SUDGEN, China) was used for the transfection of EPC and MPK cells (Wang et al., 2018).

pcDNA5/FRT/TO (Invitrogen, USA), EGFP-bcMAVS, pRL-TK, Luci-eIFN (for fathead minnow IFN promoter activity analysis) and Luci-DrIFNφ3 (for zebrafish IFNφ3 promoter activity analysis) were kept in the lab (Huang et al., 2015). The recombinant expression vector pcDNA5/FRT/TO-HA-bcNLRX1 and pcDNA5/FRT/TO-bcNLRX1-HA were constructed by cloning the open reading frame (ORF) of bcNLRX1 fused with a HA or Flag tag at its N-terminus/C-terminus into pcDNA5/FRT/TO, respectively. The truncations of bcNLRX1 (NACHT/LRR) were generated by cloning NACHT domain and LRR domain of bcNLRX1 into pcDNA5/FRT/TO separately.

2.2. Cloning the cDNA of bcNLRX1

Primers (Table 1) were designed to amplify the cDNA of bcNLRX1 based on the data of transcriptome analysis of MPK cells. Total RNA from the spleen of black carp was isolated by RNAiso Plus (TaKaRa) and the first-strand cDNA were synthesized by using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) (Li et al., 2019). The coding sequence (CDS) of bcNLRX1 was cloned at the first attempt by using the primers. The cDNA of bcNLRX1 was cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titration

SVCV (strain: SVCV741) and GCRV (strain: GCRV106) were kept in the lab and propagated in EPC or CIK separately at 26 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque assay on EPC cells as previously described (Zhou et al., 2015). Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 26 °C. The supernatant was replaced with DMEM containing 2% FBS and 0.75% methylcellulose (Sigma, USA) after incubation. Plaques were counted at day 3 post-infection.

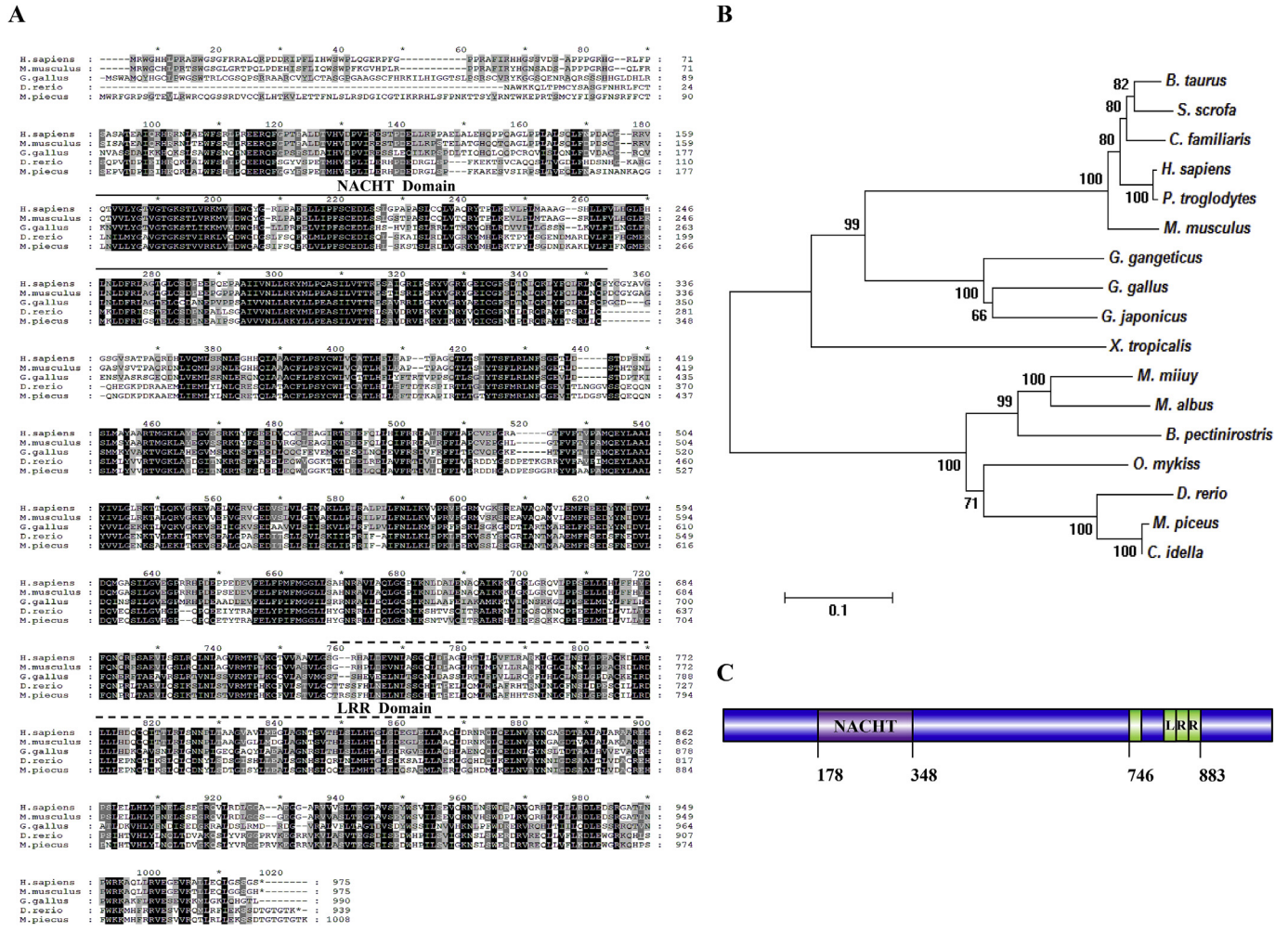

Fig. 1. Sequence analysis of bcNLRX1

(A): Comparisons of bcNLRX1 with other vertebrate NLRX1 by using MEGA 7.0 program and GeneDoc program. (B): Phylogenetic tree of vertebrate NLRX1. The amino acid sequence of bcNLRX1 was aligned with those of NLRX1 proteins from different species by using MEGA 7.0 software, which included (GenBank accession number): *H. sapiens* (AAI10891.1), *M. musculus* (NP_001157215.1), *B. taurus* (AAI51288.1), *G. gallus* (XP_015153689.1), *D. rerio* (XP_021322089.1), *P. Troglodyte* (XP_016777626.2), *S. Scrofa*(ADK79105.1), *O. Mykiss*(APD13818.1), *M. Albus*(XP_020474741.1), *B. Pectinirostris*(XP_020783434.1), *M. Miiuy*(ALJ32259.1), *C. Idella* (AXK69168.1) The bar stands for scale length and the numbers on different nodes stand for bootstrap value. (C): Diagrams the functional domains of bcNLRX1.

2.4. Quantitative real-time PCR

The relative bcNLRX1 mRNA level in MPK cells was determined by quantitative real-time PCR (q-PCR). The primers for bcNLRX1 and β-actin (as internal control) were listed in Table 1. The q-PCR program was: 1 cycle of 95 °C/10min, 40 cycles of 95 °C/15s, 60 °C/1min, followed by dissociation curve analysis (60 °C-95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the 7500 Real-Time PCR System and exported into a Microsoft Excel spreadsheet for subsequent data analysis where the relative expression ratios of target gene in treated groups versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method.

2.5. Luciferase reporter assay

EPC cells in 24-well plate (3×10^5 cells/well) were co-transfected with pRL-TK (25 ng), Luci-eIFN (or Luci-DrIFNφ3) (200 ng), pcDNA5/FRT/TO-HA-bcNLRX1 and/or pcDNA5/FRT/TO-HA-bcMAVS. For each transfection, the total amount of plasmid DNA (425 ng/well) was balanced with the empty vector. The cells were harvested and lysed on ice by renilla luciferase lysis buffer (Promega, USA) at 24 h post-transfection. The centrifuged supernatant was used to measure firefly luciferase and renilla luciferase activities according to the instruction of the manufacturer (Promega, USA) as described previously (Xiao et al., 2017).

2.6. Immunoblotting

EPC cells in 6-well plate (2×10^6 cells/well) were transfected with plasmid expressing HA-bcNLRX1, bcNLRX1-HA, HA-bcNLRX1 truncations (NACHT/LRR) or the empty vector separately. The transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described (Xiao et al., 2017). Briefly, the whole cell lysates were isolated by 10% SDS-PAGE and transferred to PVDF membrane. The transferred membranes were probed with mouse monoclonal anti-HA antibody (1:1000; Sigma, USA), which was followed by the incubation with goat-anti-mouse IgG (1:30000; Sigma, USA). The target proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma, USA).

2.7. Immunofluorescence microscopy

EPC cells and MPK cells in 24-well plate (3×10^5 cells/well) were transfected with plasmid expressing HA-bcNLRX1, Flag-bcMAVS or the empty vector separately. The transfected cells were fixed with 4% (v/v)

Table 2
Comparison of bcNLRX1 with other vertebrate NLRX1 (%).

Species	Full-length sequence	
	Similarity	Identity
Mylopharyngodon piceus	100	100
Homo sapiens	58.5	42.2
Rattus norvegicus	59.3	41.9
Bos taurus	59.3	41.9
Gallus gallus	62.1	43.5
Danio rerio	93.0	87.5
Pan troglodytes	58.0	41.7
Sus scrofa	58.7	42.0
Oncorhynchus mykiss	82.1	71.3
Monopterus albus	76.5	64.1
Boleophthalmus pectinirostris	75.2	63.0
Miichthys miiuy	75.2	64.2
Ctenopharyngodon idella	98.9	98.9

The proteins IDs of NLRX1 in the table are the same as those in Fig. 1B.

paraformaldehyde at 24 h post-transfection. The fixed cells were permeabilized with Triton X-100 (0.2% in PBS) and used for immune-fluorescent staining as previously described (Xiao et al., 2017). Mouse monoclonal anti-HA antibody (Sigma, USA) was probed at the ratio of 1:300; Alexa 594-conjugated secondary antibody (Invitrogen, USA) was probed at the ratio of 1:200 and Alexa 488-conjugated secondary antibody (Invitrogen, USA) was probed at the ratio of 1:800; DAPI was used for nucleus staining.

2.8. Statistics analysis

For the statistics analysis of the data of q-PCR, luciferase reporter assay and viral titer measurement, all data were obtained from three independent experiments with each performed in triplicate. Error bars represent the standard error of the mean (+SEM) of three independent experiments. Asterisk (*) stands for $p < 0.05$ and asterisk (**) stands for $p < 0.01$. The data were analyzed by two-tailed Student's t-test.

3. Results

3.1. Molecular cloning and sequence analysis of bcNLRX1

To explore the role of bcNLRX1 in black carp, the cDNA of NLRX1 orthologue was cloned from the spleen of black carp (NCBI accession number: MK424479). The open reading frame (ORF) of bcNLRX1 consists of 3021 nucleotides and the predicted bcNLRX1 protein contains 1008 amino acids (Supplementary Fig. 1). Initial sequence analysis of bcNLRX1 cDNA (predicted by SMART) predicts that bcNLRX1 contains two function domains, including a NACHT domain in the N terminus (178-348aa) and a C-terminal leucine-rich repeat (LRR) domain (746-883aa), which exist in most vertebrates NLRX1 homologues and vary from one species to another (Fig. 1A and C). The calculated molecular weight of bcNLRX1 is 110 KDa and the calculated isoelectric point is 8.59.

To gain insight into NLRX1 evolution, amino acid sequence of bcNLRX1 was subjected to multiple alignments with those of NLRX1 proteins from different species. bcNLRX1 shared high protein sequence similarity with cyprinid fish NLRX1, such as grass carp (98.9%) and

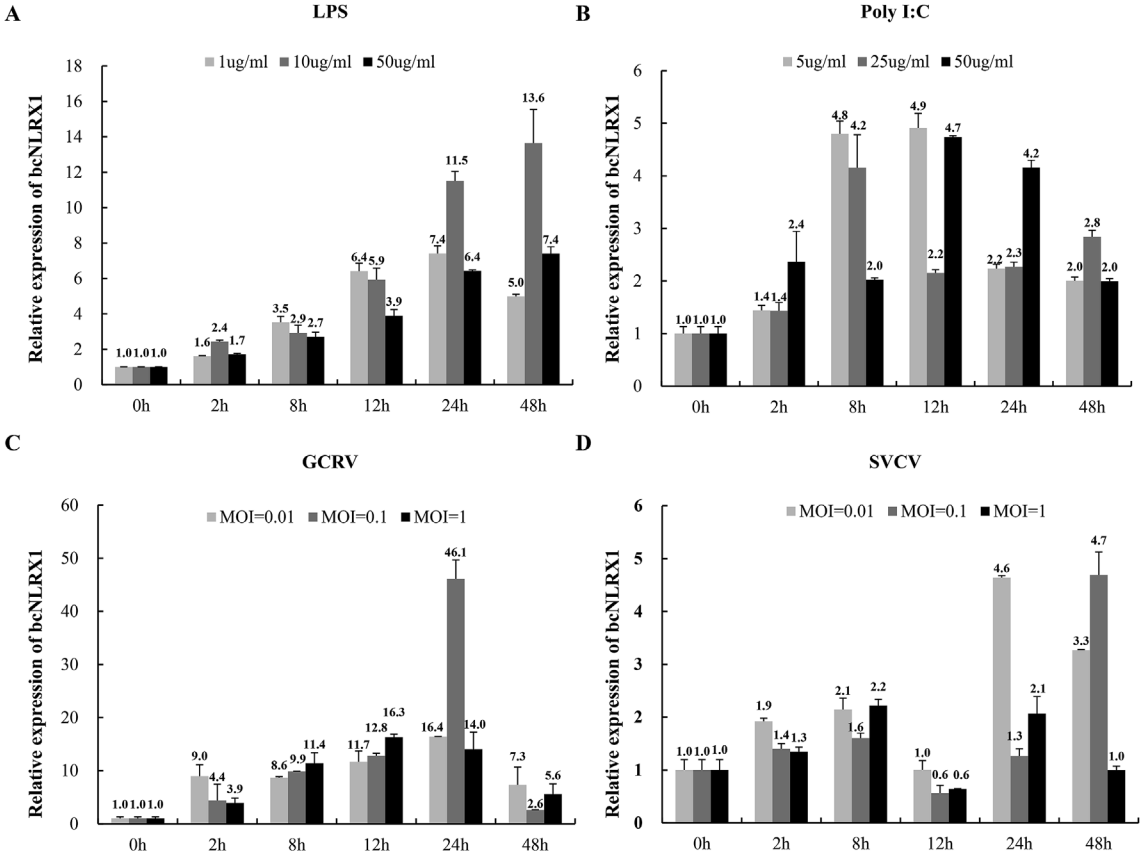

Fig. 2. Expression of bcNLRX1 in response to different stimuli
MPK cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before stimulation. The cells were treated with LPS or poly (I:C) at indicated dose (A&B), or infected with SVCV or GCRV at indicated MOI separately (C&D), and then harvested for q-PCR independently at the indicated time point post stimulation. The number above the error bar represents the average bcNLRX1 mRNA level. Error bar represents the standard error of the mean (+SEM) of three independent experiments. The relative expression of bcNLRX1 was normalized to the expression of β-actin.

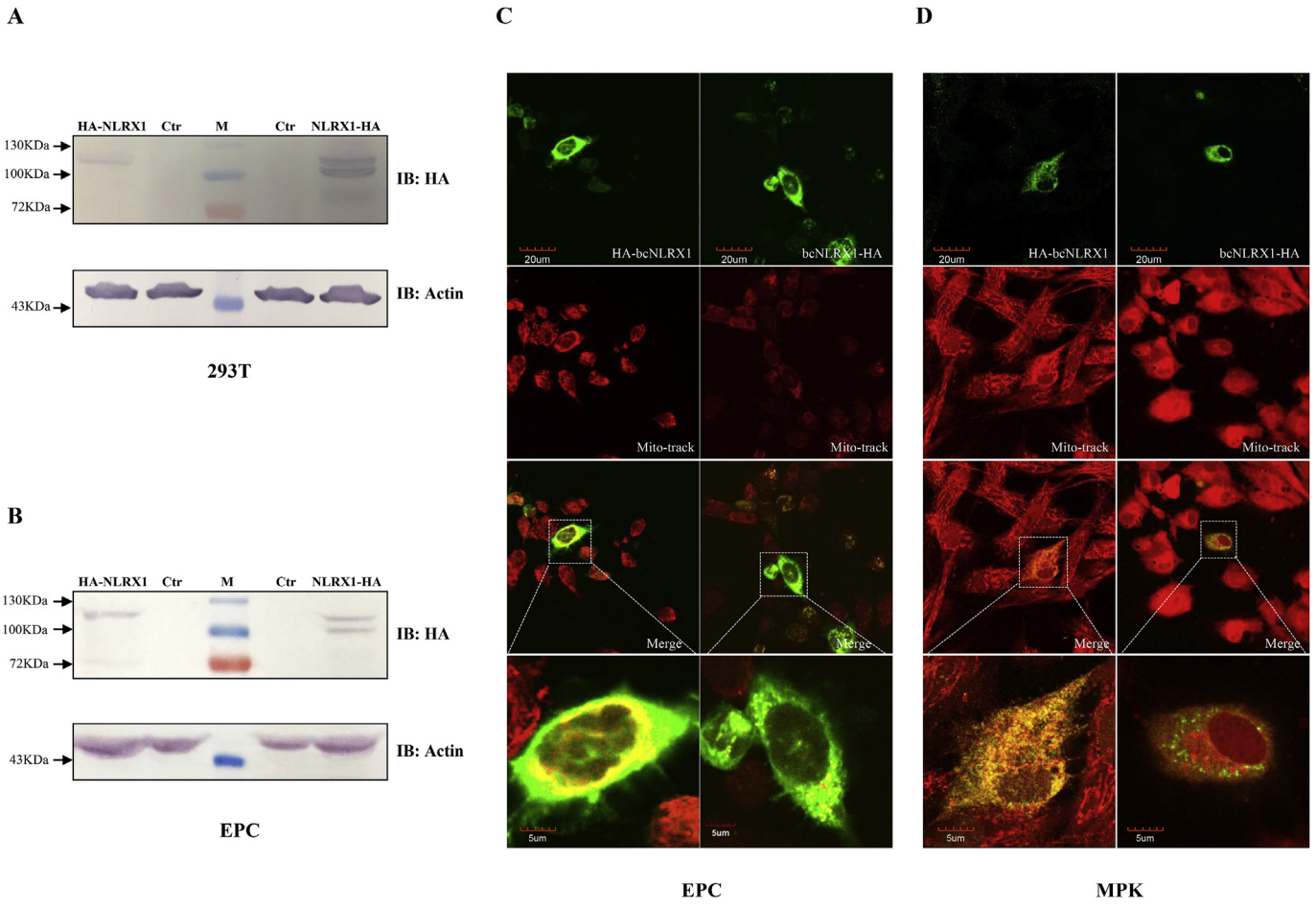

Fig. 3. Protein expression and subcellular distribution of bcNLRX1
(A)& (B): Immunoblot assay of bcNLRX1 in EPC cells and HEK293T cells. Ctr: the control cells transfected with empty vector, HA-NLRX1: pcDNA5-FRT- TO-HA-bcNLRX1; NLRX1-HA: pcDNA5-FRT-TO-bcNLRX1-HA. IB: immunoblot. (C)& (D): Immunofluorescence staining of bcNLRX1 in EPC and MPK cells. HA-NLRX1: pcDNA5-FRT-TO-HA-bcNLRX1; NLRX1-HA: pcDNA5-FRT-TO -bcNLRX1-HA. The bars stand for the scale of 20
mm and 5
mm accordingly.

zebra fish (87.5%) (Table 2). Phylogenetic analysis of NLRX1 proteins from the selected species demonstrated that these NLRX1 homologues could be divided into five groups, consisting of mammalia, aves, reptilia, amphibia and fish branches. Distinctly, bcNLRX1 was clustered with grass carp NLRX1 tightly, which correlated with the close genetic relationship of these two species (Fig. 1B).

3.2. bcNLRX1 expression in vitro in response to different stimuli

To examine the transcription of bcNLRX1 during innate immune activation, MPK cells were treated with poly (I:C) or LPS at different concentrations, or infected with SVCV or GCRV at different MOIs. MPK cells were harvested at indicated time points post stimulation and the mRNA level of bcNLRX1 was examined by qPCR. bcNLRX1 mRNA level in MPK cells was increased right after LPS treatment. The maximum relative mRNA level of bcNLRX1 (10 μg/ml dosage, 48 h point) in the LPS stimulated MPK cells within 48 post stimulation was up to 13.6-fold of that of control cells (Fig. 2A). In poly (I:C) treated MPK cells, the transcription of bcNLRX1 was increased right after stimulation and the maximum relative mRNA level of bcNLRX1 (25 μg/ml dosage, 12 h point) was up to 4.9-fold of that of control cells (Fig. 2B). By contrast, the increase rate of bcNLRX1 mRNA in LPS treated group was higher than that of poly (I:C) treated group. In GCRV infected MPK cells, bcNLRX1 mRNA was increased right after infection at all MOIs and the maximum relative mRNA level of bcNLRX1 (0.1 MOI, 24 h point) was up to 46-fold of that of control cells (Fig. 2C). bcNLRX1 transcription in SVCV infected MPK cells was increased right after infection and varied at different time points in different MOI, and the maximum relative mRNA level of bcNLRX1 (0.1 MOI, 48 h point) was up to 4.7-fold of that of control cells (Fig. 2D). In contrast to SVCV, GCRV infection triggered higher transcription level of bcNLRX1 mRNA within the first 48 h. The q-PCR data suggested that bcNLRX1 was involved in host innate immune response initiated by both GCRV and SVCV, however, might be recruited into different mechanisms.

3.3. Protein expression and subcellular distribution of bcNLRX1

EPC cells or HEK293T cells were transfected with plasmids expressing bcNLRX1 and used for immunoblotting (IB) assay to investigate the protein expression of bcNLRX1, in which mouse anti-HA antibody was used to detect the exogenous bcNLRX1. A specific band of ~110 KDa was detected in the whole cell lysate of both EPC cells and HEK293T cells transfected with HA-bcNLRX1or bcNLRX1-HA but not in the empty vector-transfected cells, which matched the predicted molecular weight of this fish protein (Fig. 3A and B). However, an additional smaller band (~100 KDa) was found in the lane of NLRX1-HA, which implied that this protein was degraded from its N terminal.

To determine the subcellular location of bcNLRX1, EPC and MPK cells were transfected with plasmids expressing HA-bcNLRX1 or bcNLRX1-HA separately and used for immunofluorescence staining

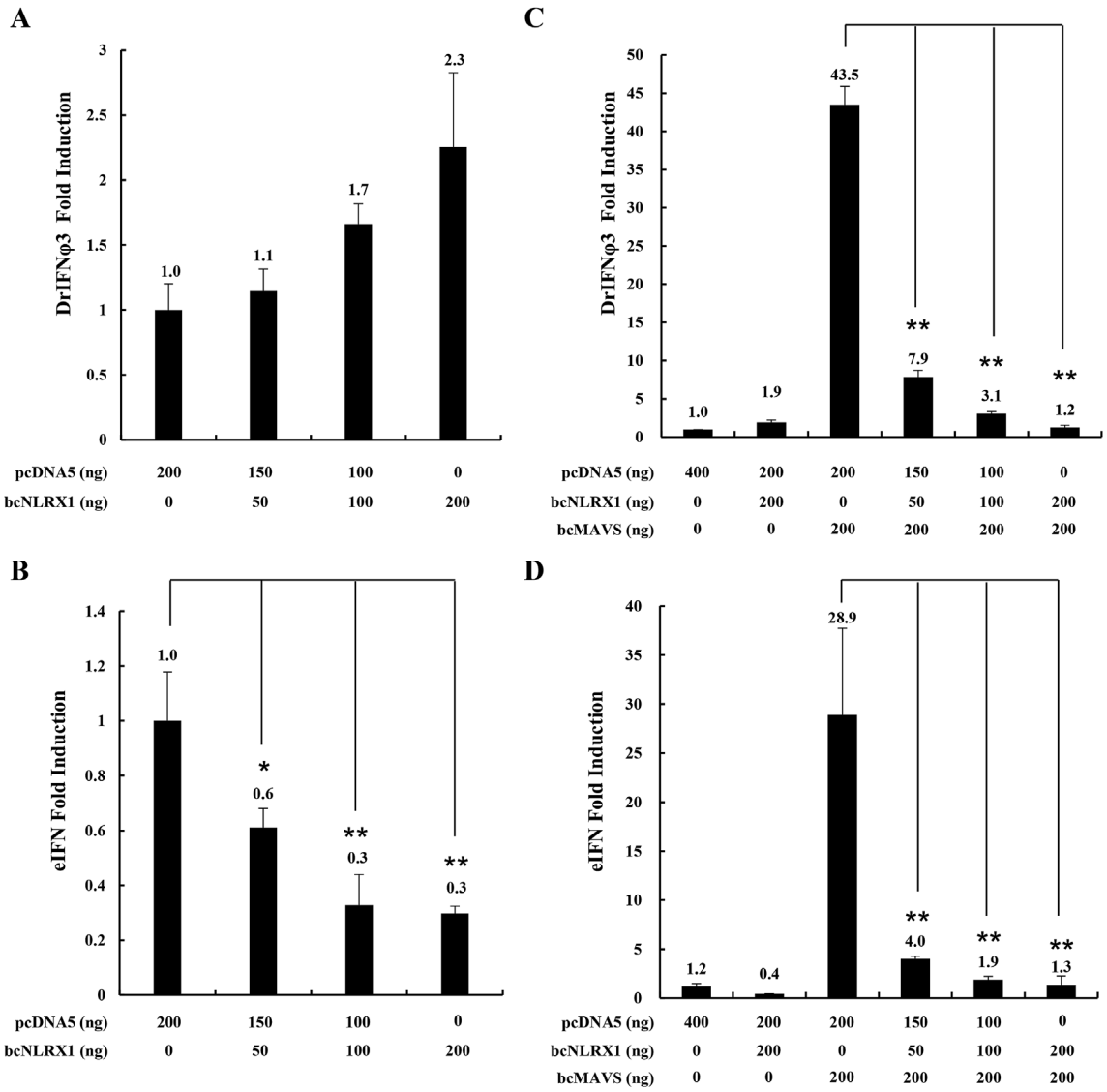

Fig. 4. IFN-inducing activity of RLR signaling members was down-regulated by bcNLRX1.
(A)& (B): EPC cells were transfected with bcNLRX1 and applied to reporter assay. For each transfection, the total amount of DNA was balanced with the empty vector. p
cDNA5: pcDNA5/FRT/TO-HA; NLRX1: pcDNA5/FRT/TO-HA-bcNLRX1; MAVS: pcDNA5/FRT/TO-HA-bcMAVS. (C)& (D): EPC cells were co-transfected with bcNLRX1 and bcMAVS and applied to reporter assay. For each transfection, the total amount of DNA was balanced with the empty vector. The numbers above the error bars stand for average induced IFN folds.

(IF). The IF data in both EPC and MPK cells showed clearly that bcNLRX1-expressing region (green) overlapped with Mito-track stained region (red), which demonstrated that bcNLRX1 was mainly distributed on mitochondria. Especially, brilliant green dots representing bcNLRX1 were widely scattered in the mitochondria region, which implied that the aggregation of bcNLRX1 molecules or the interaction of bcNLRX1 with other molecules (Fig. 3C and D).

3.4. The role of bcNLRX1 in IFN signaling

To investigate the effect of bcNLRX1 on IFN signaling, EPC cells were transfected with plasmid expressing bcNLRX1 and used for dual luciferase reporter assay. Overexpression of bcNLRX1 in EPC cells had little effect on the transcription of DrIFNφ3 (Fig. 4A). However, exogenous bcNLRX1 in EPC cells down regulated eIFN transcription in a dose dependent manner and the lowest eIFN transcription level in bcNLRX1-expressing cells was only 30% of that of control cells (Fig. 4B).

The previous study demonstrated that bcMAVS-mediated IFN signaling and enhanced the antiviral activity of EPC cells (Xiao et al., 2017). To study the role of bcNLRX1 in MAVS/IFN signaling, EPC cells were co-transfected with bcNLRX1 and bcMAVS, and applied to reporter assay. In EPC cells, while co-expressing bcMAVS and bcNLRX1, the induced transcription of both DrIFNφ3 and eIFN by bcMAVS was fiercely reduced by bcNLRX1 and the highest reduction was more than 95% of that of EPC cells expressing bcMAVS alone. What is more, bcNLRX1 suppressed bcMAVS-induced IFN transcription in a dose-dependent manner in the reporter assay (Fig. 4C and D). All the data demonstrated that bcNLRX1 was a suppressor of MAVS/IFN signaling of black carp and implied that this fish NLRX1 homologue most analogously functioned on mitochondria as its mammalian counterpart (Moore et al., 2008).

3.5. The interaction between bcNLRX1 and bcMAVS

To further identify the relationship between bcNLRX1 and bcMAVS,

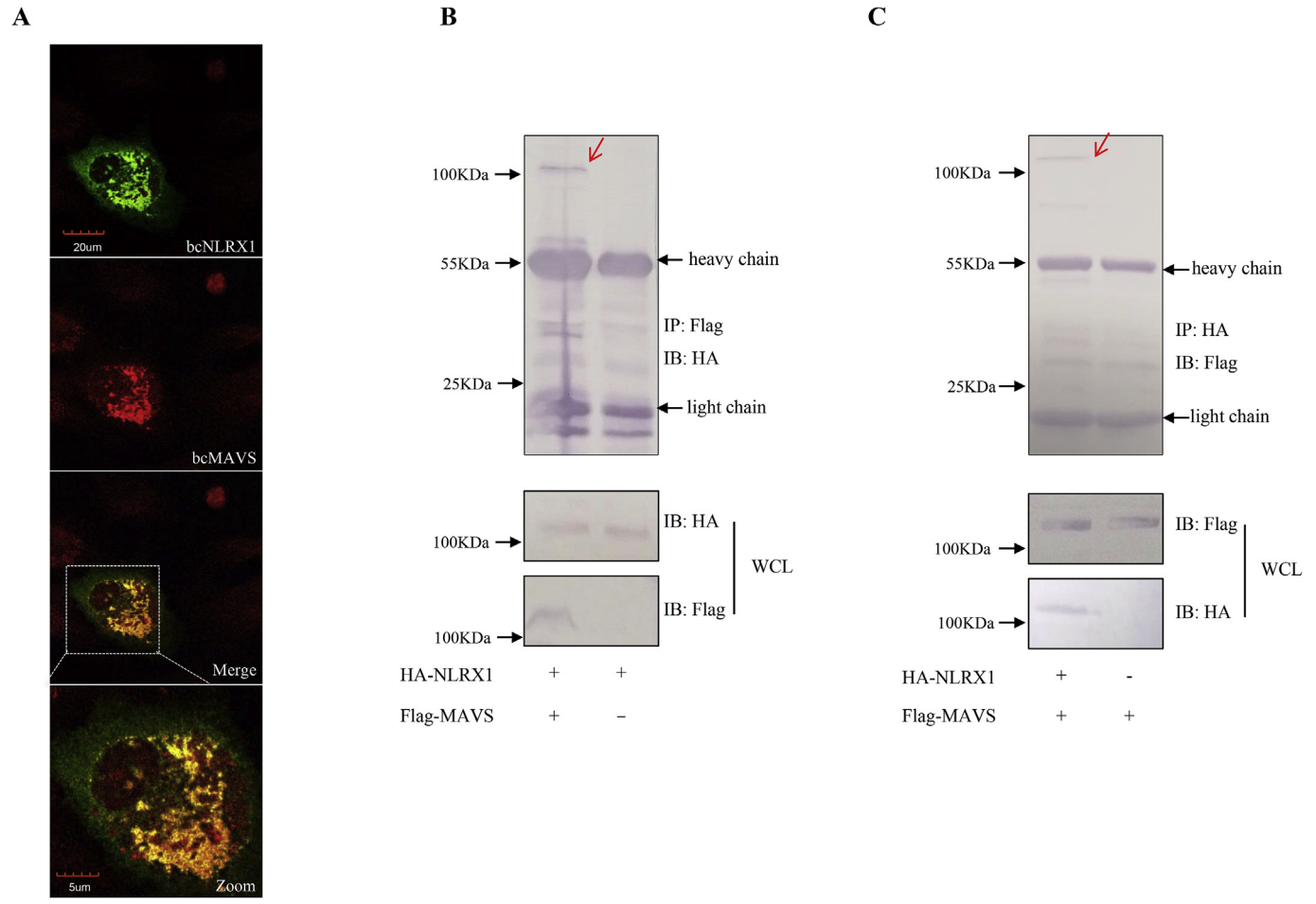

Fig. 5. The association between bcNLRX1 and bcMAVS

(A): Immunofluorescence staining of MPK cells co-transfected with plasmids expressing bcNLRX1 and bcMAVS. bcMAVS: pcDNA5/FRT/TO-Flag-bcMAVS; bcNLRX1: pcDNA5/FRT/TO-HA–bcNLRX1; the bars stand for the scale of 20 μm and 5 μm separately. (B): Co-IP in EPC cells between HA-bcNLRX1 and Flag-bcMAVS. IB:immunoblot; IP: immunoprecipitation; WCL: whole cell lysate; HA-bcNLRX1: pcDNA5/FRT/TO-HA-bcNLRX1; Flag-bcMAVS: pcDNA5/FRT/TO-Flag-bcMAVS.

immunofluorescence (IF) staining and co-IP assay were used to characterize the interaction between bcNLRX1 and bcMAVS. The IF data of MPK cells showed clearly that bcNLRX1-expressing region (green) overlapped well with bcMAVS-expressing region (red), which suggested the same subcellular distribution of these two molecules (Fig. 5A). In the co-IP assay of EPC cells, the specific band of HA-bcNLRX1 (~110 KDa) was detected in the proteins precipitated by Flag-bcMAVS (Fig. 5B, the red arrow indicated). On the other side, the specific band of Flag-bcMAVS (~120 KDa) was detected in the proteins precipitated by HA-bcNLRX1 (Fig. 5C, the red arrow indicated). At the same time, similar co-IP data was obtained in HEK293T cells (Supplementary Fig. 2). Therefore, the co-IP assay of bcNLRX1 and bcMAVS in both EPC and HEK293T cells sufficiently identified the direct interaction between these two fish proteins.

3.6. Down-regulated bcMAVS-mediated antiviral signaling by bcNLRX1

Our previous study identified that bcMAVS possessed strong antiviral activities during host innate immune response initiated by SVCV and GCRV (Xiao et al., 2017). To characterize the role of bcNLRX1 in bcMAVS-mediated antiviral signaling, EPC cells were co-transfected with bcMAVS and/or bcNLRX1 and then subjected to SVCV (MOI = 0.1, MOI = 0.01) or GCRV (MOI = 1, MOI = 0.1) infection separately. In SVCV group, the viral titer of EPC cells expressing bcNLRX1 was almost the same to the control group and the exogenous bcMAVS enhanced the antiviral activity of EPC cells. However, the viral titer of EPC cells expressing bcMAVS alone was obviously lower than the cells expressing both bcNLRX1 and bcMAVS, which demonstrated that bcMAVS-mediated antiviral activity in EPC cells against SVCV was restrained by bcNLRX1 (Fig. 6A). Similar to the data of SVCV group, GCRV titer of EPC cells expressing bcMAVS alone was obviously lower than the cells expressing both bcNLRX1 and bcMAVS, which demonstrated that bcMAVS-mediated antiviral activity in EPC cells against GCRV was suppressed by bcNLRX1 (Fig. 6B). Thus, our data demonstrated clearly that bcNLRX1 down regulated bcMAVS-mediated antiviral activity during host innate immune activation.

3.7. NACHT instead of LRR of bcNLRX1 was crucial for its suppression of IFN induction ability of bcMAVS

To test the roles of bcNLRX1 domains (NACHT and LRR) in its negative regulation of bcMAVS, the construct expressing NACHT or LRR was generated and the IB data of EPC cells demonstrated the successful expression of truncations of NACHT and LRR (Fig. 7E). The subsequent report assay demonstrated that both NACHT and LRR showed little effect on the activation of eIFN and DrIFNφ3 when they were expressed in EPC cells alone (Fig. 7A and B). When co-expressing with bcMAVS, NACHT significantly suppressed bcMAVS-induced activation of eIFN and DrIFNφ3 like bcNLRX1, nevertheless, LRR showed no effect on bcMAVS-mediated activation of eIFN or DrIFNφ3 (Fig. 7C and D). Co-IP data showed that NACHT was detected in the precipitated proteins by bcMAVS (Fig. 7F, the red arrow indicated, ~19 KDa), which suggested

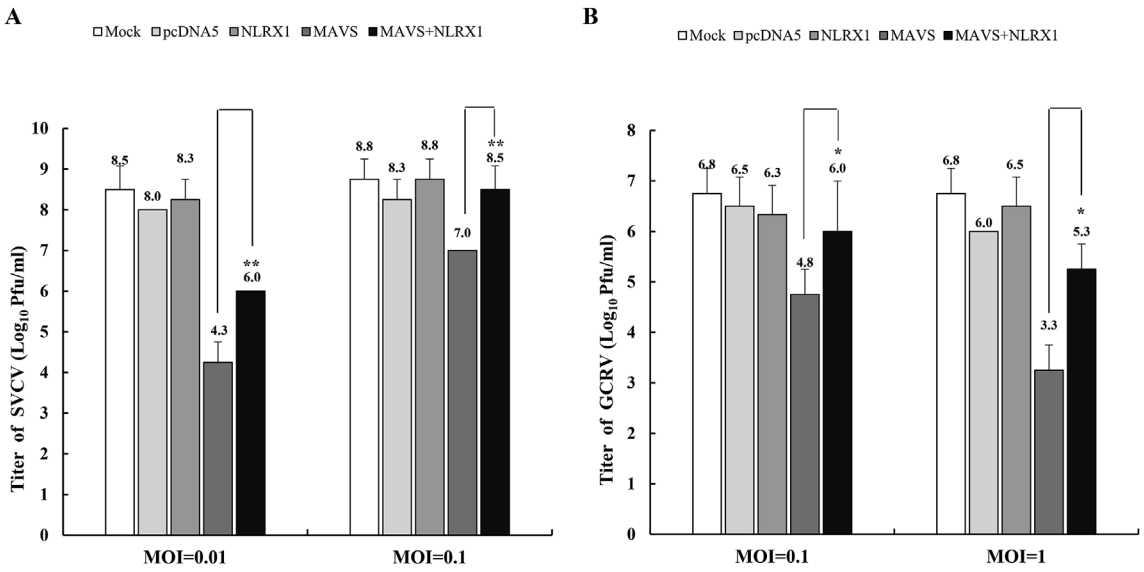

Fig. 6. Down-regulated antiviral ability of bcMAVS against GCRV and SVCV by bcNLRX1
(A): EPC cells in 24-well plate were transfected with bcMAVS and/or bcNLRX1. The transfected cells were infected with SVCV (MOI = 0.01 or MOI = 0.1) at 24 h post-transfection and the virus titers in the supernatant media were determined by plaque assay at 48 h post-infection. (B): EPC cells in 24-well plate were co-transfected with bcNLRX1 and/or bcMAVS. The transfected cells were infected with GCRV (MOI = 0.1 or MOI = 1) at 24 h post-transfection and the virus titers in the supernatant media were determined by plaque assay at 48 h post-infection. The numbers above the error bars stand for average virus titer and error bars denote mean with standard deviation and data represent three independent experiments. NLRX1: pcDNA5/FRT/TO-HA-bcNLRX1; MAVS: pcDNA5/FRT/TO-Flag-bcMAVS.; Mock: cells without transfection; pcDNA5: cells transfected with the empty vector.

the direct association between NACHT and bcMAVS. Nevertheless, the Co-IP between LRR and bcMAVS showed no interaction between these two proteins (data not shown). The above data clarified that NACHT domain of bcNLRX1 was crucial for its interaction with bcMAVS and for its down-regulation of bcMAVS-mediated antiviral signaling, which was incompletely similar to that of human MAVS and NLRX1 (Qin et al., 2017; Moore et al., 2008; Singh et al., 2018).

4. Discussion

The innate immune system, an evolutionarily conserved mechanism, is the first line of host defense against pathogens, which collaborates with the adaptive immune system to help vertebrates to survive from the disadvantage environments (Zeng et al., 2010). Activation of innate immunity is essential for host cells to restrict the spread of invading viruses and other pathogens. However, attenuation or termination of signaling is also necessary for preventing immune-mediated tissue damage and spontaneous autoimmunity, such as the interaction among TBK1, IKKε and SIKE (Wu and Chen, 2014). In the previous study, we reported that downstream molecule bcSIKE down regulated the IFN-inducing activity of bcMDA5, bcMAVS, bcTBK1, bcIRF3 and bcIRF7, which are key components of black carp RLR signaling (Li et al., 2019). As an upstream molecule of RLR signaling, NLRX1 is another example, which has been characterized as a physiological suppressor of MAVS that keeps MAVS and PCBP2 sequestered as inactive complexes to prevent unintended activation by either RLRs (Qin et al., 2017). To date, only several studies have been reported about the function of NLRX1, and the function of NLRX1 in teleost are not well confirmed. In this study, bcNLRX1 has been identified and characterized, which is aimed to explore the role of NLRX1 in host antiviral innate immune response.

NLRX1 was originally characterized as a negative regulator of MAVS-mediated antiviral signaling, through the inhibition of the virus-induced RLH (RIG-like helicase)-MAVS interaction (Moore et al., 2008). The initial report showed that human NLRX1 mutants containing NACHT domain was sufficient to associate with MAVS, and LRR domain acted as a negative regulator of MAVS-mediated signaling (Moore et al., 2008). Howbeit the recent research indicated that the NOD domain of NLRX1, was essential for NLRX1 to trigger MAVS degradation and the subsequent suppression of the activation of IFN production pathway during virus infection (Qin et al., 2017). In our study, bcNLRX1 down regulated the IFN-inducing activity of bcMAVS, which was a key component of black carp RLR signaling. Mechanistically, IF and co-IP assay identified the direct association between bcNLRX1 and bcMAVS; and plaque assay further confirmed the inhibition of bcMAVS-mediated antiviral activity by bcNLRX1. What's more, our study unlocked a key mechanism of innate immunity in teleost fish for the first time by which bcNLRX1 restrained MAVS/IFN signaling through its NACHT domain. The data generated in this paper was dissimilar to that of its mammalian counterpart.

The luciferase reporter assay in EPC cells (Fig. 4A) showed that the exogenous bcNLRX1 (200 ng in one well of 24-well plate)-induced DrIFNφ3 expression was only 2.3-fold of that of the control ($P > 0.05$, without obvious difference). Thus, it was speculated that over-expression of bcNLRX1 in EPC cells had little effect on the induction of DrIFNφ3. As to the decreased eIFN fold induction by bcNLRX1 (Fig. 4B), it could be explained by that the endogenous MAVS of EPC cells (EPC MAVS) induced the transcription of eIFN, which might be inhibited by bcNLRX1. However, it was possible that “the faintly increased transcription of DrIFNφ3 (2.3-fold)” was not induced by EPC MAVS, which might explain why exogenous bcNLRX1 did not obviously decrease the transcription of DrIFNφ3 in reporter assay.

NLRX1 (−/−) mice exhibited increased expression of antiviral signaling molecules after influenza virus infection, which included IFN-β, STAT2, OAS1, and IL-6 (Soares et al., 2013). In mice, NLRX1 interacts with TRAF6 and inhibits NF-κB activation (Allen et al., 2011). After LPS stimulation, human NLRX1 is rapidly ubiquitinated, disassociates from TRAF6, and then binds to the IKK complex, which leads to inhibition of IKKα and IKKβ phosphorylation and NF-κB activation (Xia et al., 2011). Our Q-PCR data showed that the mRNA level of bcNLRX1 was increased right after LPS stimulation. The results obtained from our study and previous research lead us to deeply explore the role of bcNLRX1 in inhibition of NF-κB activation. What's more, NLRX1-deficient cells generate an amplified STING-dependent host response to

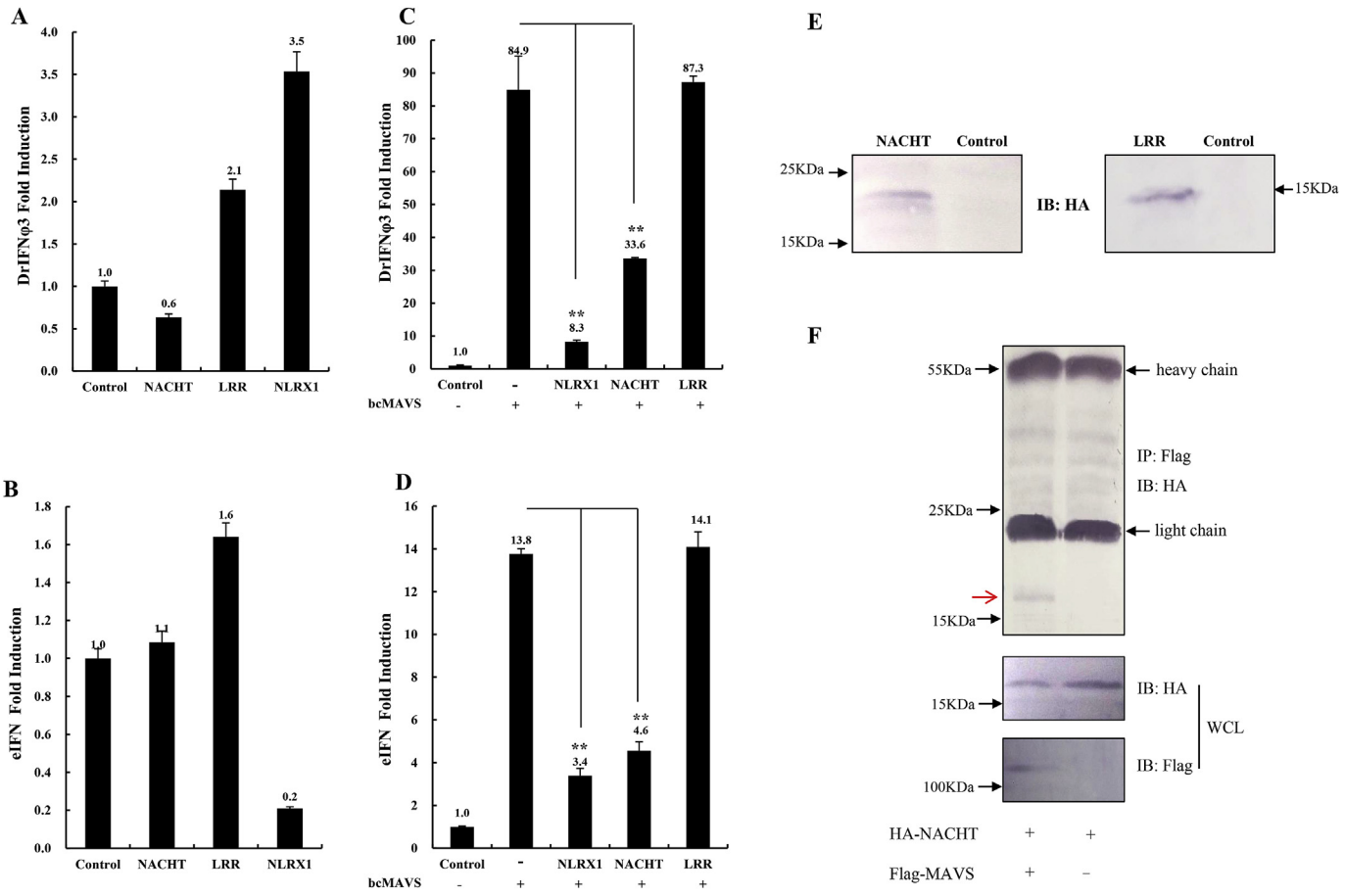

Fig. 7. NACHT of bcNLRX1 interacted with bcMAVS and suppressed it mediated IFN production

(A)&(B): EPC cells were transfected with plasmids expressing bcNLRX1, bcNACHT or bcLRR, and applied to reporter assay. (C)&(D): EPC cells were co-transfected with plasmids expressing bcNACHT, bcLRR and/or bcMAVS, and applied to reporter assay. NLRX1: pcDNA5/FRT/TO-HA-bcNLRX1; NACHT: pcDNA5/FRT/TO-HA-bcNACHT; LRR: pcDNA5/FRT/TO-HA-bcLRR; bcMAVS: pcDNA5/FRT/TO-HA-bcMAVS. For each transfection, the total amount of DNA was balanced with the empty vector. The numbers above the error bars stand for average induced IFN folds. (E): Immunoblot assay of bcNACHT and bcLRR in EPC cells. Mock: cells transfected with empty vector, NACHT: pcDNA5/FRT/TO-HA-bcNACHT; LRR: pcDNA5/FRT/TO-HA-bcLRR, IB: immunoblot. (F): Co-IP in EPC cells between HA-NACHT and Flag-bcMAVS. IB: immunoblot; IP: immunoprecipitation; WCL: whole cell lysate; HA-NACHT: pcDNA5/FRT/TO-HA-bcNACHT; Flag-MAVS: pcDNA5/FRT/TO-Flag-MAVS.

cytosolic DNA, c-di-GMP, cGAMP, HIV-1, and DNA viruses. Evidence supports that NLRX1 functions as an inhibitor of early innate immune responses against both RNA viruses and DNA viruses (Guo et al., 2016). Although innate immune is essential for host antiviral infection, a rabid immune response is detrimental to the host when left unchecked. The identification of the physiological suppressor of bcMAVS—bcNLRX1, may help us to get better understanding of the mechanism how host cells keep homeostasis when facing pathogens invading in teleost fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2019.03.001.

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