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SENP2 negatively regulates RIG-I/MDA5 mediated innate immunity in black carp

Yixia Chen [a,1], Jun Li [b,1], Jiaxin Fu [a], Lili Xiao [a], Jixiang Chu [a], Wei Qin [a], Jun Xiao [a,c,*], Hao Feng [a,c,**]

[a] State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, China
[b] Key Laboratory of Hunan Province for Study and Utilization of Ethnic Medicinal Plant Resources, College of Biological and Food Engineering, Huaihua University, Huaihua, 418008, China
[c] Institute of Interdisciplinary Studies, Hunan Normal University, Changsha, 410081, China

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ABSTRACT

Mammalian SUMO specific peptidase 2 (SENP2) plays vital roles in a variety of biological procedures including the immune response. However, the effects of teleost SENP2 are still mostly unexplored. In this study, the SENP2 of black carp (*Mylopharyngodon piceus*) was cloned and characterized. The open reading frame of black carp SENP2 (bcSENP2) consists of 1800 nucleotides, which encode 600 amino acids. The reporter assay results showed that over-expression of bcSENP2 alone had a weak effect on interferon (IFN) promoter transcription activity, whereas it significantly reduced bcMDA5/bcRIG-I mediated IFN promoter transcription activity. The interaction between bcSENP2 and bcMDA5 or bcRIG-I was detected by immunoprecipitation experiments. The plaque assay and qPCR results indicated that bcMDA5 or bcRIG-I mediated antiviral capacity was attenuated by bcSENP2, while knockdown of bcSENP2 led to enhanced antiviral resistance to SVCV in host cells. In addition, the expression level of bcMDA5/bcRIG-I protein was attenuated by co-expressed bcSENP2 and MG132 treatment rescued this attenuating effect. All of these data support the conclusion that bcSENP2 inhibits bcMDA5/bcRIG-I mediated antiviral signaling by enhancing ubiquitin-proteasome mediated degradation of bcMDA5/bcRIG-I in black carp.

1. Introduction

The vertebrates depend on adaptive immunity (also name specific immunity) and innate immunity (also name nonspecific immunity) to resist invasion of pathogenic microbes. Due to the undeveloped adaptive immunity, fish rely more on innate immunity to combat pathogenic microorganisms [1]. The innate immunity protects the host through recognizing conserved pathogen associated molecular patterns (PAMPs) by germline-encoded pattern-recognition receptors (PRRs) [2,3], which mainly include retinoic acid inducible gene-I-like receptors (RLRs), Toll-like receptors (TLRs), NOD-like receptors (NODs) and C-type Lectin receptors (CLRs) [4,5]. Among all the above PRRs, RLRs are the primary cytoplasmic receptors to recognize viral RNA and prevent virus replication in cells. RLRs are comprised of three elements, RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [6,7], all of which have a central helicase structural domain and a putative carboxy-terminal domain (CTD) for the detection of immunostimulatory RNAs [8]. Both RIG-I and MDA5 possess two amino-terminal Caspase recruitment domains (CARDs) to mediate signaling downstream, while LGP2 lacks CARDs, therefore is commonly considered to regulate RIG-I and MDA5 [9]. In the resting state, RIG-I and MDA5 are self-repressed by phosphorylating in their individual CARDs [10,11]. Upon detecting the invading RNA viruses, RIG-I or MDA5 was activated which then triggered a series of downstream signaling promotes the generation of type I interferon (IFN) and pro-inflammatory cytokines [12,13], ultimately leading to the antiviral

* Corresponding author. State key laboratory of developmental biology of freshwater fish, college of life science, Hunan Normal University, Changsha, 410000, China.
** Corresponding author. State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, China.
E-mail addresses: xiaojun@hunnu.edu.cn (J. Xiao), fenghao@hunnu.edu.cn (H. Feng).
[1] These authors contributed equally to this paper.

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immune response.

IFN are key cytokines in host antiviral response. However, over synthesis of IFN might lead to autoimmune diseases [14]. RLRs play a significant role in inducing IFN production. As the membership of RLRs, the activity of RIG-I and MDA5 are strictly regulated by oligomerization, ubiquitination, phosphorylation and sumoylation [7]. Oligomerization of RIG-I can impact its own activity, whereas oligomerization of MDA5 enhances the binding of the CARDs to downstream MAVS thereby strengthening signal transduction [10,15]. Ubiquitination of RIG-I and MDA5 is important for maintenance of self-stability. RNF125, TRIM40 and G3BP1 mediate K48-linked ubiquitination and induce degradation of RIG-I or MDA5 [16,17]. HSPBP1 promotes cellular RLR-mediated antiviral responses by inhibiting K48-linked ubiquitination of RIG-I [18]. TRIM38 enhances K63-linked ubiquitination of RIG-I and MDA5, whereas USP3 is a negative regulator of the ubiquitination of RIG-I and MDA5 [19]. RIG-I and MDA5 activities are regulated by a dynamic balance of phosphorylation and dephosphorylation. In uninfected cells, phosphorylation at the CARDs of RIG-I and MDA5 prevents IFN production prematurely preceding viral infection. In the presence of pathogen invasion, RIG-I and MDA5 are rapidly dephosphorylated, to initiate downstream cascade reactions [20]. In addition, RIG-I and MDA5 have been found to be regulated by sumoylation modification [21].

Sumoylation targets proteins with isoforms of small ubiquitin-like regulatory factors (SUMOs) that play important roles in a variety of biological processes [22,23]. Characterization of the covalent binding of SUMO proteins to the ε-amino group of lysine residues of intracellular target proteins during ATP-dependent processes [24]. The reverse sumoylation process named "desumoylation" has a dynamically reversible reaction, and this dynamically reversible process is reversed by a family of Sentrin/SUMO-specific proteases (SENPs). SUMO specific peptidase 2 (SENP2) is one of the de-sumoylation enzymes, which can de-sumoylate proteins through breaking the covalent bond between SUMO protein and the target protein [25,26]. In mammals, SENP2 has been reported to play vital roles in embryonic development. Knocking down SENP2 in mouse resulted in the defective development of heart, in which the expressions of Gata4 and Gata6 required for heart development were significantly reduced [27,28]. SENP2 is also engaged in regulating the developmental process of cancer cells. SENP2 can reduce stemness and improve sensitivity to multi-targeted agents in hepatocellular carcinoma by inhibiting the AKT/GSK3b/CTNNB1 pathway [29]. SENP2 can also act as tumor suppressor by inhibiting bladder cancer cell migration and invasion [30]. Recently, SENP2 has been found to exert a negative role in the innate immunity. Several studies have shown that SENP2 regulates the activities of p53 by SENP2-Mdm2-p53 pathway [31,32]. Over-expression of SENP2 promotes the de-sumoylation of IRF3 and K48-linked ubiquitination, which regulates antiviral innate immunity and provides an idea regarding the connection between the ubiquitin and SUMO pathway [33]. In fish, SENP2 has been identified in Yellow Catfish (*Pelteobagrus fulvidraco*), which shows higher mRNA expression in seminal, ovarian, and gill tissues than in kidney and spleen tissues. However, the function of SENP2 in teleost is largely unknown.

In this paper, bcSENP2 was cloned and characterized. The dual-luciferase report assay revealed that bcSENP2 played a negative regulatory role in the RLR signaling pathway. Knocking down of bcSENP2 improved the antiviral ability of host cells. The further results indicated that bcSENP2 interacted with bcRIG-I and bcMDA5 and inhibited the antiviral capacity of bcRIG-I and bcMDA5 by promoting their protein degradation. Therefore, the results in this study provide new insights into the regulation of RIG-I and MDA5 in innate immunity in teleost.

2. Material and methods

2.1. Cells, virus and plasmids

HEK293T cells, HeLa cells, EPC cells and *M. piceus* kidney cells (MPK cells) were preserved in the laboratory. The above cells were cultured in the CO_2 incubator with DMEM supplemented and 10 % fetal bovine serum (FBS), at 26 °C for EPC cells and MPK cells and at 37 °C for HEK293T cells and HeLa cells. The open reading frame (ORF) of bcSENP2 was cloned from the total RNA of the black carp kidney and inserted into pcDNA5/FRT/TO with a Flag tag or a Myc tag at the C-terminus separately. The shRNAs designed from the website (http://rnaidesigner.thermofisher. com/rn aiexpress) targeting bcSENP2 were inserted into PLKO.1. The other plasmids used in this study were kept in the lab. The constructed plasmids were verified by sequencing, and the primers, which included the information on the restricted enzyme sites, were listed in Table 1. SVCV (strain: SVCV741) was maintained in the lab, propagated in EPC cells at the presence of 2 % FBS.

2.2. Analysis of sequence and phylogeny

The molecular weight (Mw) of bcSENP2 was analyzed by the online EXPASy software (https://web.expasy.org/compute_pi/). The MEGAX program was used for multiple sequence comparison to explore the similarities of SENP2 among black carp and other species, and then the outcome was modified through GeneDoc program. The SENP2 protein sequences of multiple species were inserted into the same fasta format file and aligned by MEGAX to obtain the phylogenetic tree. Protein three-dimensional structure of bcSENP2 and human SENP2 was predicted by the online SWISS-MODEL software.

2.3. Quantitative real-time PCR (qPCR)

To test the influence of over-expressed bcSENP2 on bcRIG-I or bcMDA5 -mediated antiviral signaling, EPC cells in 6-well plate were transfected with bcSENP2, bcRIG-I or bcSENP2 and bcRIG-I. The gene expression levels of RIG-I/MDA5 downstream immune factors (IFN, Mx1, Viperin and ISG15), and the mRNA expression levels of virus genes (SVCV-G, M, N, P) were detected by qPCR 24 h post SVCV infection. To test the influence of knocking-down bcSENP2 on black carp antiviral signaling, the mRNA expression levels of host antiviral genes such as bcIFN, bcViperin, bcMx1, bcPKR and virus genes (SVCV-G, M, N and P), were detected by qPCR 24 h post SVCV infection. The SYBR Green detection system (Invitrogen) and QuantStudio 5 Real-Time PCR Systems (Thermo Fisher, USA) were utilized for qPCR, and the program was same as used in previous paper [34]. The qPCR data were analyzed using the $2^{-\Delta\Delta}$CT relative quantification method, and the fold change represented relative expression of the target gene. Table 1 lists the primers for qRT-PCR.

2.4. Reporter assay

To investigate the effect of bcSENP2 on the RLR signaling pathway mediated transcription activities of the IFN promoter, the reporter plasmids pRL-TK (as internal control reporter, with stable expression of renilla luciferases) and Luci-bcIFNa, Luci-DrIFNφ1 or Luci-epcIFN (as experimental reporter, with the expression of firefly luciferases regulated by the indicated IFN promoter) were co-transfected into EPC cells with the expressing vector of bcSENP2, bcMDA5, bcRIG-I, bcMAVS, bcIRF3, bcIRF7, respectively. The luciferase reporter assay allows us to study the regulatory control of a gene of interest. The presence of luminescent signal means that luciferase is transcribed, and therefore the regulatory region is active. For dosage dependent assays, the Luci-bcIFNa and pRL-TK were transfected with the bcSENP2-expressing vector of the different doses like 0 ng, 50 ng, 100 ng or 200 ng per well into EPC cells, and 100 ng of bcMDA5 or bcRIG-I per well, respectively. All reporter experiments were performed by harvesting cells for dual luciferase reporter experiments 24 h after the transfection. The methods were previously outlined in Ref. [34]. Simply stated, subsequent experiments were performed after lysing the cells with 100

Table 1
Primers in this paper.

Primer name	Sequence (5′-3′)	Amplicon length (nt) and primer information
bcSENP2-F	ATGTATGAATGGATAGTTGACGGATTAT	PMD-18T
bcSENP2-R	CTGCAGCAGCTTTTGGTGAGGATC	
bcSENP2-Flag-F	ACTGACGGTACCGCCACCATGTATGAATGGATAGTTGACGG	Expression construct
bcSENP2-Flag-R	ACTGACCTCGAGCTGCAGCAGCTTTTGGTTG	
Flag-bcSENP2-F	ACTGACGGTACCATGTATGAATGGATAGTTGACGG	
Flag-bcSENP2-R	ACTGACCTCGAGCTACTGCAGCAGCTTTTGGT	
bcSENP2-sh1-F	CCGGAGATGGCGTCTGAGAAATATTCTCGAGAATATTTCTCAGACGCCATCTTTTTTG	ShRNA oligos
bcSENP2-sh1-R	AATTCAAAAAAGATGGCGTCTGAGAAATATTCTCGAGAATATTTCTCAGACGCCATCT	
shbcSENP2-2-F	CCGGGAAGCCTCCAATCACAAATTTCTCGAGAAATTTGTGATTGGAGGCTTCTTTTTG	
shbcSENP2-2-R	AATTCAAAAAGAAGCCTCCAATCACAAATTTCTCGAGAAATTTGTGATTGGAGGCTTC	
bcSENP2-sh3-F	CCGGGGTGTGTTCGTATGCAAATATCTCGAGATATTTGCATACGAACACACCTTTTTG	
bcSENP2-sh3-R	AATTCAAAAAGGTGTGTTCGTATGCAAATATCTCGAGATATTTGCATACGAACACACC	
epc-actin-F	AAGGAGAAGCTCTGCTATGTGGCT	*in vitro* q-PCR
epc-actin-R	AAGGTGGTCTCATGGATACCGCAA	
epc-IFN-F	ATGAAAACTCAAATGTGGACGTA	
epc-IFN-R	GATAGTTTCCACCCATTTCCTTAA	
epc-MX1-F	TGGAGGAACCTGCCTTAAATAC	
epc-MX1-R	GTCTTTGCTGTTGTCAGAAGATTAG	
epc-ISG15-F	TGATGCAAATGAGACCGTAGAT	
epc-ISG15-R	CAGTTGTCTGCCGTTGTAAATC	
epc-Viperin-F	GCAAAGCGAGGGTTACGAC	
epc-Viperin-R	CTGCCATTACTAACGATGCTGAC	
bc-actin-F	TGGGCACCGCTGCTTCCT	
bc-actin-R	TGTCCGTCAGGCAGCTCAT	
bc-SENP2-F	GGACCCTTGCTACCATCCAG	
bc-SENP2-R	TTGAGCAAGAGCAGCACTCA	
bc-IFNb-F	GACCACGTTTCCATATCTTT	
bc-IFNb-R	CATTTTTTCTTCATCCCACT	
bc-MX1-F	TGAGCGTAGGCATTAGCAC	
bc-MX1-R	CCTGGAGCAGCAGATAGCG	
bc-PKR-F	GAGCGGACTAAAAGGACAGG	
bc-PKR-R	AAAATATATGAGACCCAGGG	
bc-Viperin-F	CCAAAGAGCAGAAAGAGGGACC	
bc-Viperin-R	TCAATAGGCAAGACGAACGAGG	
SVCV-G-F	GATGACTGGGAGTTAGATGGC	
SVCV-G-R	ATGAGGGATAATATCGGCTTG	
SVCV-M-F	CGACCGCGCCAGTATTGATGGATAC	
SVCV-M-R	ACAAGGCCGACCCGTCAACAGAG	
SVCV-N-F	GGTGCGAGTAGAAGACATCCCCG	
SVCV-N-R	GTAATTCCCATCATTGCCCCAGAC	
SVCV-P-F	AACAGGTATCGACTATGGAAGAGC	
SVCV-P-R	GATTCCTCTTCCCAATTGACTGTC	

μl passive lysis buffer (PLB, Promega, USA) each well. Lysates of whole cells placed on a shaker and cultured for 15 min were used for luciferase reporter assays. The luminescence values of the firefly luciferase and renilla luciferase were determined according to the Due-Luciferase Reporter Assay System kit (Promega, USA) as described previously [34]. Typically, the "experimental" reporter was correlated with the effect of the expression of tested genes (such as bcSENP2, bcMDA5 etc.), while the activity of the co-transfected "control" reporter provided an internal control that serves as the baseline response. The firefly luciferase activity were normalized with renilla luciferase activity among samples. Each group performed three experimental replications.

2.5. RNA extraction

Total mRNA extraction for each sample was performed using the HiPure Total RNA Kit from Magen. The PrimeScript™ RT Reagent Kit (Takara, Code No. RR047A) was used to synthesis the first strand cDNA.

2.6. Immunoblotting

To validate the *in vitro* expression of bcSENP2 vectors, bcSENP2 or the empty vector was transfected into HEK293T (4×10^6 per well) or EPC cells (2×10^6 per well) in 6-well plates, separately. The expression of bcSENP2 was detected by immunoblotting (IB). To detect the knockdown efficiency of the bcSENP2, HEK293T in 6-well plates were transfected with the plasmids expressing bcSENP2 and three different shbcSENP2 or scramble shRNAs, respectively, and bcSENP2 expression levels were detected by immunoblot (IB) assay. Briefly, the harvested whole cells were twice rinsed with PBS and resuspended in 1 × SDS loading buffer and boiled at 95–100 °C for 10 min. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes, detained with 5 % milk and incubated with mouse monoclonal anti-Flag antibody (1:5000, Abmart) at 4 °C for overnight. The membrane were cleaned with tris buffered saline containing 1 ‰ tween (TBST) for 4 times and then probed with anti-mouse second antibody (1:30000, Sigma). Following these, the membrane washed by TBST and visualized using AP substrate solution (NBT/BCIP).

2.7. Immunofluorescence

To confirm the subcellular distribution of bcSENP2 in cells, HeLa cells in 24-well plates were transfected with bcSENP2 and used for immunofluorescence. 24 h post transfection, the cells were treated with 4 % (v/v) of the fixative paraformaldehyde, permeabilized through 0.2 % Triton X-100, cleaned in PBS, and incubated with the 1: 500 diluted primary antibody(mouse monoclonal anti-Flag, Abmart). Subsequently, the cells were infiltrated with the secondary antibody which was a 488-conjugated anti-mouse antibody (Sigma) at a dilution of 1:1000. Prior to sealing the coverslips, the cells were subjected to DAPI (Sigma). Photographs of the samples were obtained using a laser scanning confocal microscope.

Table 2
Identity and Similarity of SENP2 in different species (%).

Species	GenBank accession number	Full-length sequence	
		Identity (%)	**Similarity (%)**
Pongo pygmaeus	XP_054339543.1	34.6	51.2
Hylobates moloch	XP_032027720.2	34.6	51.2
Homo sapiens	Pan troglodytes	34.6	51.2
Pan troglodytes	XP_516925.4	34.6	51.2
Mus musculus	NP_083733.1	35.1	51.0
Caretta caretta	XP_048719705.1	34.3	50.5
Chelonia mydas	XP_037764418.1	34.6	50.1
Dermochelys coriacea	XP_038272904.1	34.7	50.0
Gallus gallus	XP_040535733.1	39.2	57.6
Phasianus colchicus	XP_031452399.1	39.3	55.1
Centrocercus urophasianus	XP_042676960.1	39.0	58.3
Lagopus muta	XP_048811352.1	53.7	72.7
Lagopus leucura	XP_042734410.1	38.5	57.0
Bombina bombina	XP_053568827.1	51.1	70.0
Stegostoma fasciatum	XP_048389964.1	33.8	51.6
Rhincodon typus	XP_020366377.1	34.6	50.6
Chiloscyllium plagiosum	XP_043549328.1	34.7	52.2
Scyliorhinus canicula	XP_038642620.1	34.8	51.5
Danio rerio	XP_684283.2	85.1	91.1
Labeo rohita	XP_050968638.1	90.6	94.5
Cyprinus carpio	XP_042613815.1	90.5	93.5
Rhinichthys klamathensis goyatoka	XP_056122878.1	93.8	96.2
Pimephales promelas	XP_039545362.1	93.2	96.1
Megalobrama amblycephala	XP_048056648.1	96.8	97.8
Ctenopharyngodon idella	XP_051752162.1	99.3	99.5

2.8. Co-immunoprecipitation (Co-IP)

HEK293T cells were co-transfected with plasmids expressing bcSENP2 with or without bcRIG-I, or bcMDA5 with or without bcSENP2 in 10 cm dishes. At 48 h post-transfection, the cells were harvested and added with 10 μl protease inhibitor in 1 ml of 1 % NP40. The whole cell lysate (WCL) was subjected to ultrasonication on ice and subsequently subjected to centrifugation at 12,000 rpm for a period of 5 min at the temperature of 4 °C to eliminate any residual debris. Thereafter, the resulting supernatant was aspirated and transferred to a fresh 1.5 ml tube, where it was incubated at 4 °C for 90 min with protein A/G beads on a rotor to facilitate the removal of any non-specific binding proteins that may have been attached to the agarose beads. The WCL was subjected to centrifugation at 5000 rpm for a duration of 2 min at 4 °C, with the objective of discarding the mentioned beads. Subsequently, the anti-Flag-conjugated beads were introduced into the resulting solution and incubated overnight at 4 °C. Following 5 times washes with 1 % NP40 buffer, the anti-Flag-conjugated beads were boiled. The proteins that have been eluted were then employed in IB as previously described.

2.9. Crystal violet staining

EPC cells were transfected with the indicated plasmids. 24 h post-transfection, each group was infected with SVCV at 0.01, 0.1 or 1 MOI for 2 h. Then, the cells were washed thrice by DMEM and replaced with media containing 2 % FBS and 0.75 % methylcellulose. At 72 h post-infection, the cells were fix with 10 % methanol and stained with crystal violet solution for photograph [34].

2.10. Virus infection and titer

Virus titration was performed in EPC cells following the method in Ref. [34]. In summary, the cells were treated with serial 10-fold dilution of virus. Following 2 h innovation period, the viral supernatant was replaced with DMEM medium supplemented with 2 % FBS and 0.75 % methylcellulose. Plaques were enumerated under a microscope 72 h post-infection. To exploring the effect of shbcSENP2 in host cells, the MPK cells (3×10^5 per well) were plated in 12-well plates and transfected with plasmids expressing shbcSENP2 or control plasmids. 24 h post transfection, the cells were infected with SVCV and the virus titer assays were conducted as mentioned above. All tests were carried out in triplicate.

3. Result

3.1. Cloning and sequence analysis of bcSENP2

The sequencing results showed that bcSENP2 was composed of 1800 nucleotides, which encoded 600 amino acids, containing a ULP1 domain (Fig. 1A). The multiple sequence alignment among *Homo sapiens, Gallus gallu, Chelonia mydas,* Danio rerio and Mylopharyngodon piceus indicated that bcSENP2 was poorly conserved, except for the function domain ULP1 (Fig. 1A). The results of computational predictions indicate that the complete length of the bcSENP2 is distinguished from its mammalian homologue by structural dissimilarities, whereas the structural domain of the ULP1 exhibits structural similarities to that of mammals (Fig. 1B). The genome information (unpublished data) showed that bcSENP2 consisted of 17 exons and 16 introns and located on chromosome 6 of black carp (Fig. 1C). The phylogenetic analysis showed bcSENP2 had a high degree of similarity to several carp species, with the closest affinity to *Ctenopharyngodon idellus*, followed by *Megalobrama amblycephala* (Fig. 1D).

3.2. The protein expression of bcSENP2 and its subcellular distribution

The predicted molecular weight of bcSENP2 was 68 kD using the website (EXPASy). The IB assays demonstrated the presence of a specific band with an approximate molecular weight of 70 kD in cells that had been transfected with bcSENP2, but not in the control cells. This finding indicated that the expression vectors could be correctly expressed in both mammalian and fish cells (Fig. 2A and B). To ascertain the cellular localization of bcSENP2, the plasmids encoding bcSENP2 were introduced into HeLa cells by transfection, and immunofluorescence staining was conducted 24 h post-transfection. The fluorescent microscopy data showed that bcSENP2 was mainly distributed in the nucleus, in particular nuclear membrane (Fig. 2C).

3.3. bcSENP2 inhibits bcMDA5 and bcRIG-I mediated IFN production

The reporter assay results showed that over-expression of bcSENP2 alone in EPC cells had little effect on transcription activity of IFN promoter (Fig. 3A–C). However, bcSENP2 fiercely inhibited key factors in the RLR signaling pathway such as bcMDA5, bcRIG-I, bcMAVS and bcIRF7 (Fig. 3A–C). In mammals, the desumoylation of MDA5 and RIG-I by SENP2 has been shown to regulate the efficient innate immunity to RNA viruses and their timely termination [21]. So we investigated the regulation role of bcSENP2 on MDA5 and RIG-I under virus infection. The resulus showed that the tendency of bcSENP2 inhibitory effect on cMDA5 and bcRIG-I remained consistant before and after SVCV infection (Fig. 3D). In addition, further study showed the bcMDA5 and bcRIG-I mediated bcIFNa promoter transcription activities were depressed by bcSENP2 in a dose-dependent manner (Fig. 3E and F). Together, the above results confirmed that bcSENP2 inhibited bcMDA5 and bcRIG-I mediated IFN production.

3.4. Over-expression of bcSENP2 suppresses bcRIG-I/bcMDA5 mediated antiviral activity

The results of the plaque assay demonstrated that co-expression of bcSENP2 with bcRIG-I or bcMDA5 reduced the viral titer in supernatant compared to the control that only transfected with bcRIG-I or bcMDA5, which suggested that the inhibition of bcMDA5 and bcRIG-I on SVCV

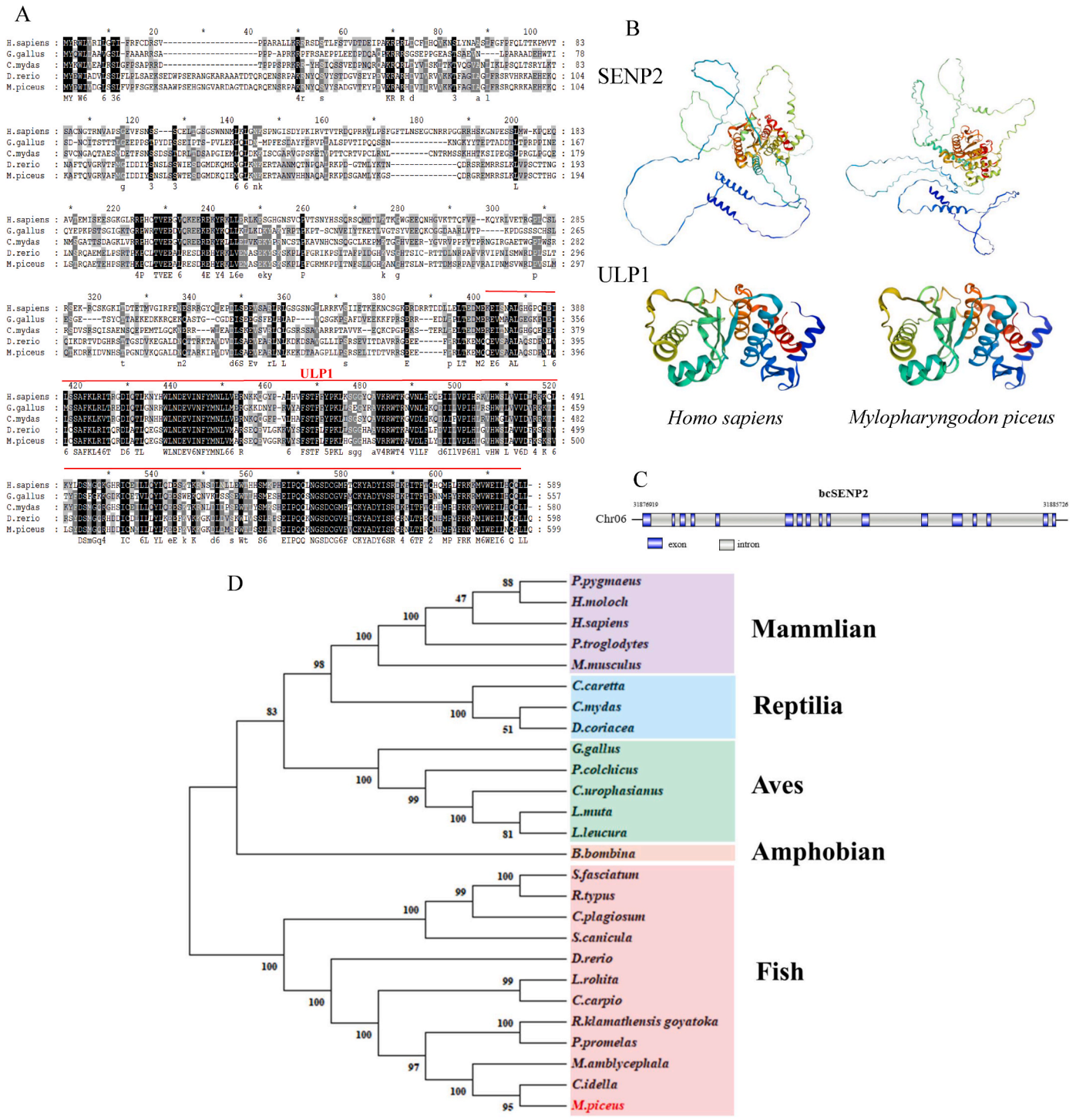

Fig. 1. Sequence alignment, gene location and structure prediction of bcSENP2 (A) Multisequence alignment of SENP2 from *Mylopharyngodon piceus* (PQ492338), *Homo sapiens* (NP_067640.2), *Gallus gallus* (XP_040535733.1), *Chelonia mydas* (XP_037764418.1), and *Danio rerio* (XP_684283.2). (B) Prediction of the three-dimensional structures of the entire protein and ULP1 domain between bcSENP2 and human SENP2. (C) Location and the composition of bcSENP2 in black carp genome. The blue sections represented exons, and the gray sections represented introns. (D) Phylogenetic tree was generated from vertebrate SENP2 of different species by using MEGAX program. The species information were showed in Table 2.

replication were reduced by bcSENP2 (Fig. 4A, B, E, F). The mRNA levels of SVCV-G, M, N and P in the cells that co-transfected with bcSENP2 and bcRIG-I or bcMDA5 were significantly higher than in the cells transfected with bcRIG-I/bcMDA5, bcSENP2 or empty vector, respectively (Fig. 4C–G). In addition, the mRNA levels of bcIFN, bcMx1, bcISG15, bcViperin in the cells over-expressing bcSENP2 and bcRIG-I or bcMDA5 were much lower than those of the control (Fig. 4D–H). These results revealed that bcSENP2 played a negative role in the IFN signaling during the antiviral innate immune response.

3.5. *Interfering bcSENP2 expression reinforces antiviral ability in MPK cells*

The IB results indicated that the over-expressed bcSENP2 in

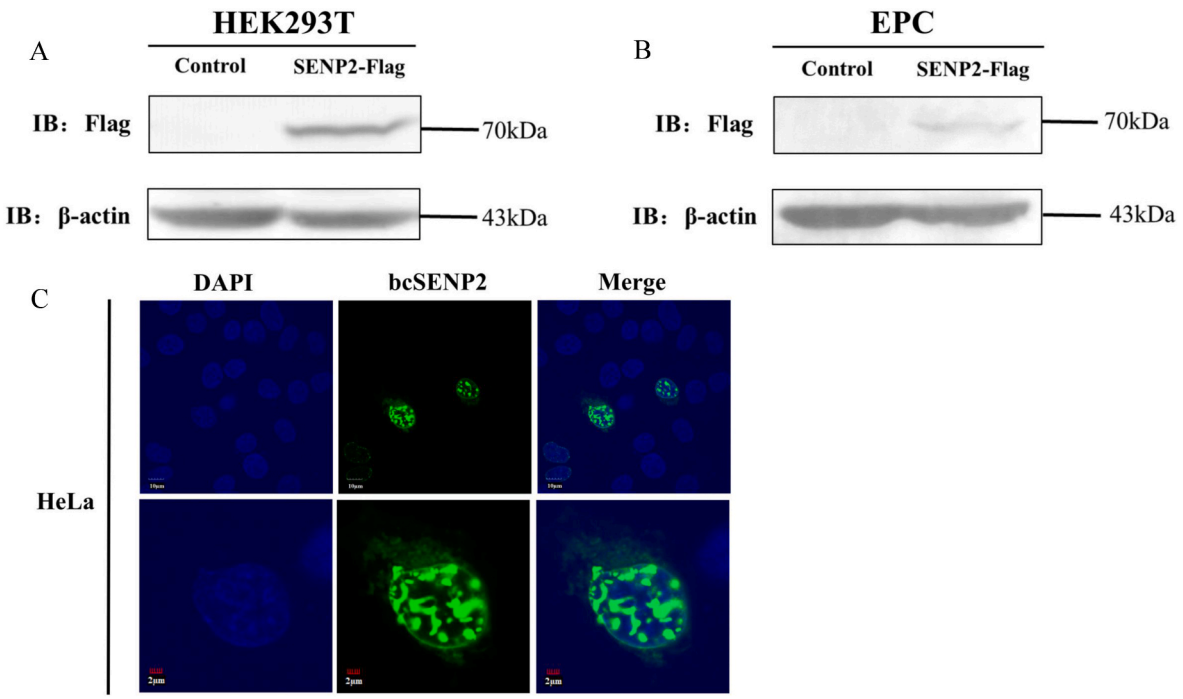

Fig. 2. The protein expression of bcSENP2 (A) HEK293T cells in 6-well plate were transfected with bcSENP2, or the empty vector (3 μg), and detected by IB. (B) EPC cells in 6-well plate were transfected with bcSENP2, or the empty vector (3 μg), and detected by IB. (C) HeLa cells in 24-well plate were transfected with bcSENP2 (500 ng) and used for immunofluorescence staining. The scales in the pictures stand for 10 μm (upper panel) or 2 μm (lower panel) separately.

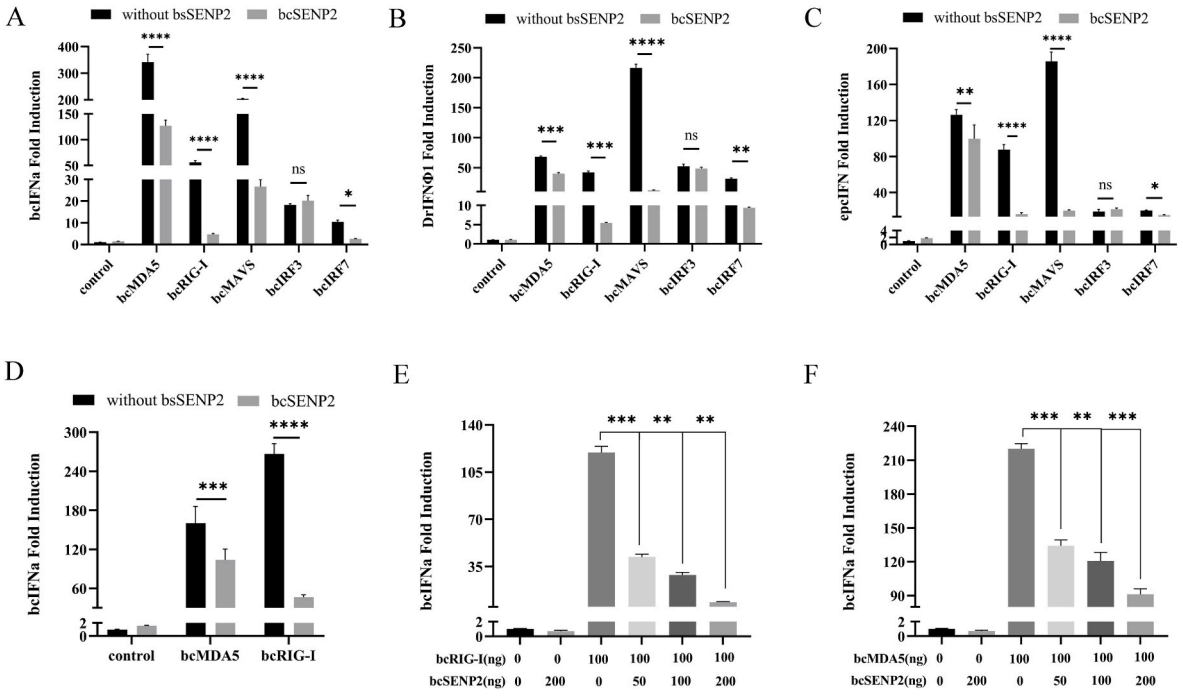

Fig. 3. bcSENP2 inhibits MDA5 and RIG-I mediated interferon production (A–C) The bcMDA5/bcRIG-I/bcMAVS/bcIRF3/bcIRF7 (100 ng/well) and the reporter plasmids pRL-TK (50 ng/well), Luci-bcIFNa (A)/Luci-DrIFNφ1(B)/Luci-epcIFN (C) (200 ng/well) were co-transfected in EPC cells with or without bcSENP2 (100 ng/well), respectively. 24 h post transfection, the cells were harvested for reporter assay. (D) EPC cells were transfected with the reporter plasmids pRL-TK (50 ng/well), Luci-bcIFNa (200 ng/well), bcMDA5/bcRIG-I (100 ng/well) with or without bcSENP2 (100 ng/well), respectively. After 24 h, the cells were infected with SVCV (MOI = 0.1) and harvested for reporter assay 6 h post infection. (E–F) EPC cells in 24-well plate were transfected with the reporter plasmids pRL-TK (50 ng/well), Luci-bcIFNa (200 ng/well),bcRIG-I (E)/bcMDA5 (F) (100 ng/well) and different dosage of bcSENP2, respectively. 24 h post infection, the cells were harvested for reporter assay.

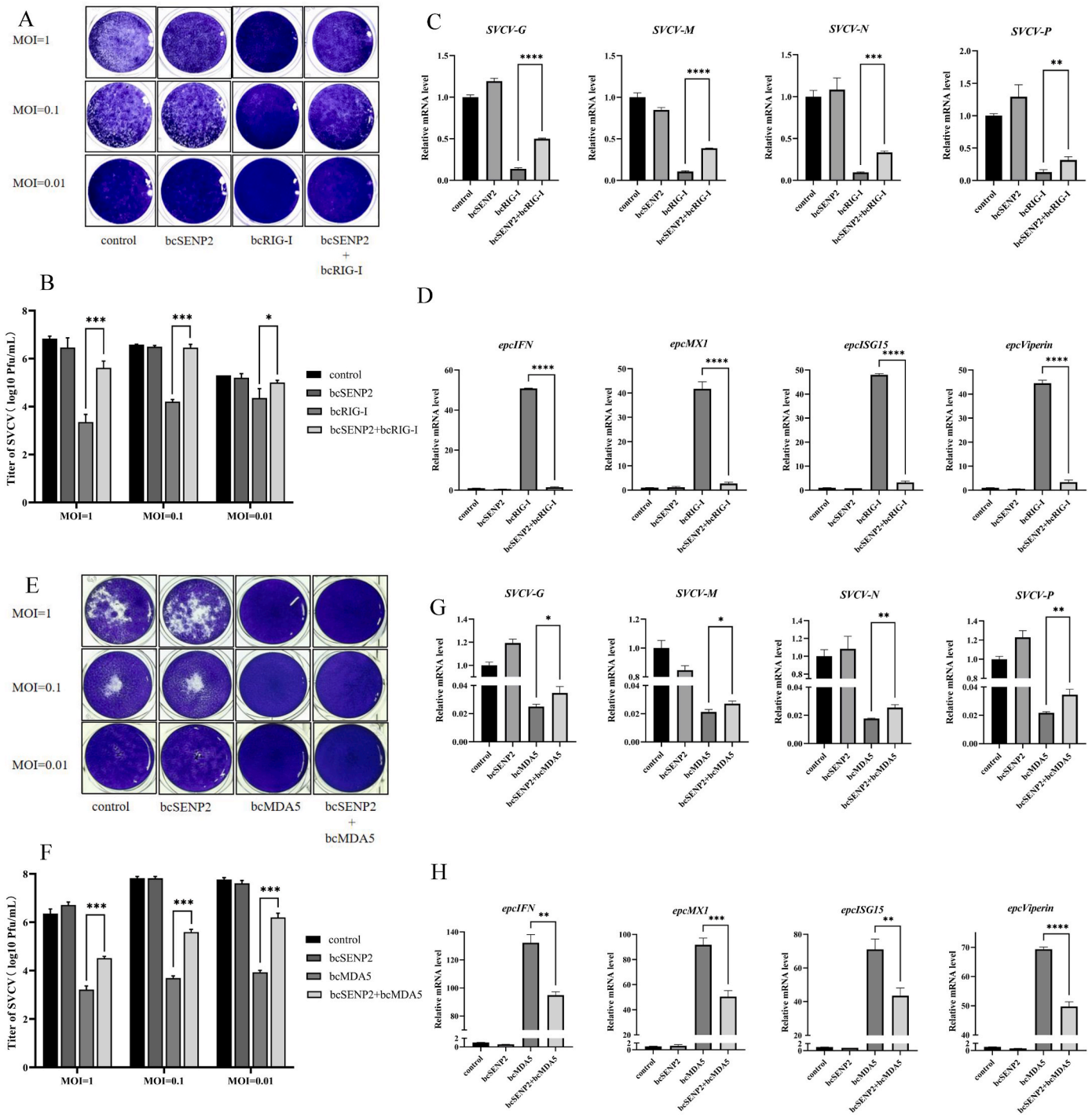

Fig. 4. Over-expression of bcSENP2 suppress bcRIG-I/bcMDA5 mediated antiviral activity (A–B) EPC cells in 24-well plates were transfected with empty vector, bcSENP2, bcRIG-I or co-transfected with bcSENP2 and bcRIG-I. The cells were infected SVCV (MOI = 0.01, 0.1, 1). The cells were used for crystal violet staining (A) and the supernatant were used for plaque assay (B). (C–D) EPC cells in 6-well plates were transfected with empty vector, bcSENP2, bcRIG-I or co-transfected with bcSENP2 and bcRIG-I. 24 h post transfection, the cells were infected with SVCV (MOI = 0.1) and collected for RNA extraction 24 h post infection. The mRNA level of SVCV proteins (C) and immune factors of the IFN signaling pathway (D) were examined by qPCR. (E–H) The same experiments as in A-D, only replaced bcRIG-I with bcMDA5.

HEK293T cells could be reduced by shbcSENP2-1 and shbcSENP2-3 for 36 % and 89 %, respectively (Fig. 5A). Consequently, shbcSENP2-3 was introduced into MPK cells to knockdown the endogenous bcSENP2. The qPCR results showed that over-expressing shbcSENP2-3 in host cells significantly decreased the mRNA expression of endogenous bcSENP2 (Fig. 5B), thus leading to an increase in the mRNA expression levels of bcIFN, bcMX1, bcPKR, and bcViperin (Fig. 5D). MPK cells transfected with shbcSENP2-3 or scramble shRNA were infected with SVCV at the MOI of 0.1, and the plaque assay results showed lower viral titers in bcSENP2 knockdown MPK cells than in controls (Fig. 5C). In addition, knocking down of bcSENP2 remarkably decreased the expression of SVCV-G, M, N and P in MPK cells infected with SVCV (Fig. 5E). All these results indicated that bcSENP2 knockdown enhanced IFN signaling.

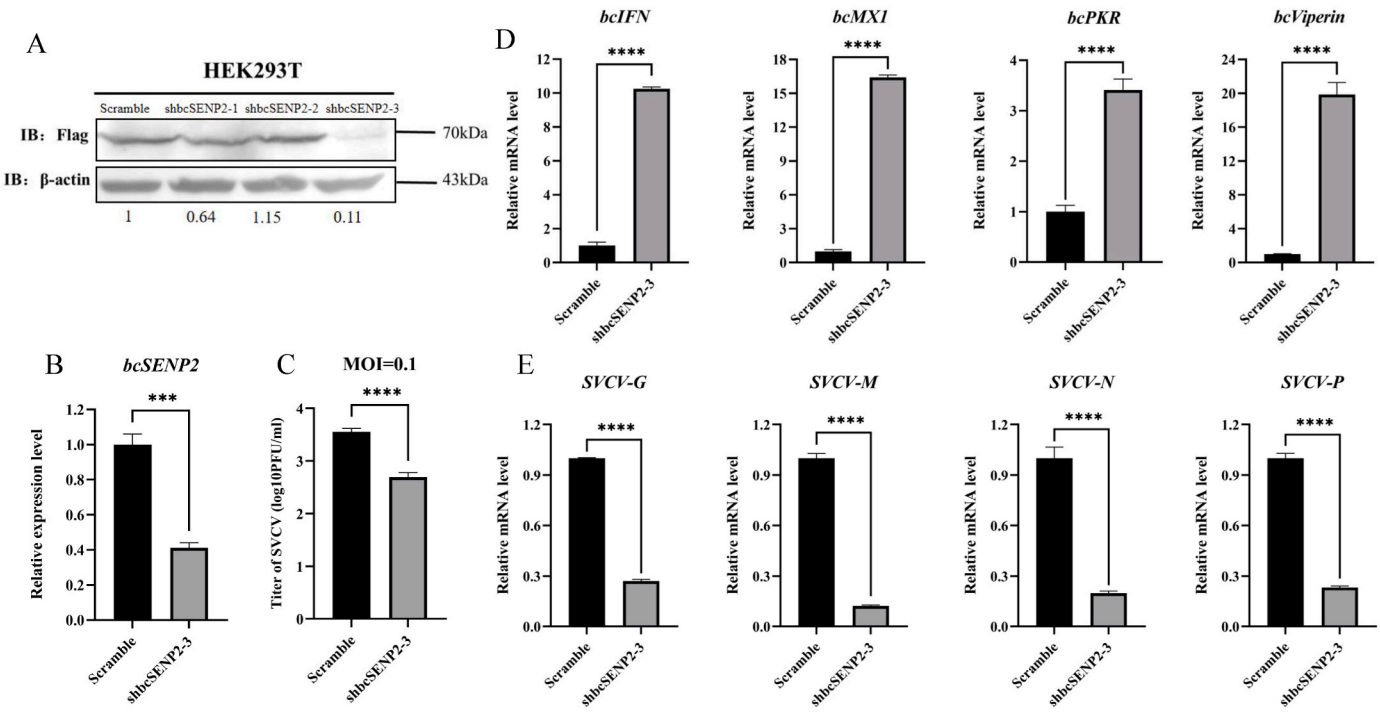

Fig. 5. Interfering bcSENP2 expression reinforces antiviral ability in MPK cells (A) HEK293T cells in 6-well plates were transfected with bcSENP2 (1 μg) and scramble (control vector, 2 μg)/shbcSENP2-1 (2 μg)/shbcSENP2-2 (2 μg)/shbcSENP 2–3 (2 μg), respectively. After 48 h the cells were harvested to test protein expression by IB assay. (B) MPK cells in 6-well plates were transfected with bcSENP2 (1 μg) and scramble or shbcSENP2-3 (2 μg), respectively. After 24 h, the cells were harvested to for RNA extraction and the mRNA expression of endogenous bcSENP2 were examined by qPCR. (C–E) MPK cells in 6-well plates were transfected with bcSENP2 (1 μg) and scramble (control vector) or shbcSENP2-3 (2 μg), respectively. 24 h post transfection, the cells were infected with SVCV (MOI = 0.1). The supernatants were used for virus titer assays (C) and the cells were collected for RNA extraction 24 h post infection. The mRNA level of downstream factors of the IFN signaling pathway (D) and SVCV proteins (E) were examined by qPCR.

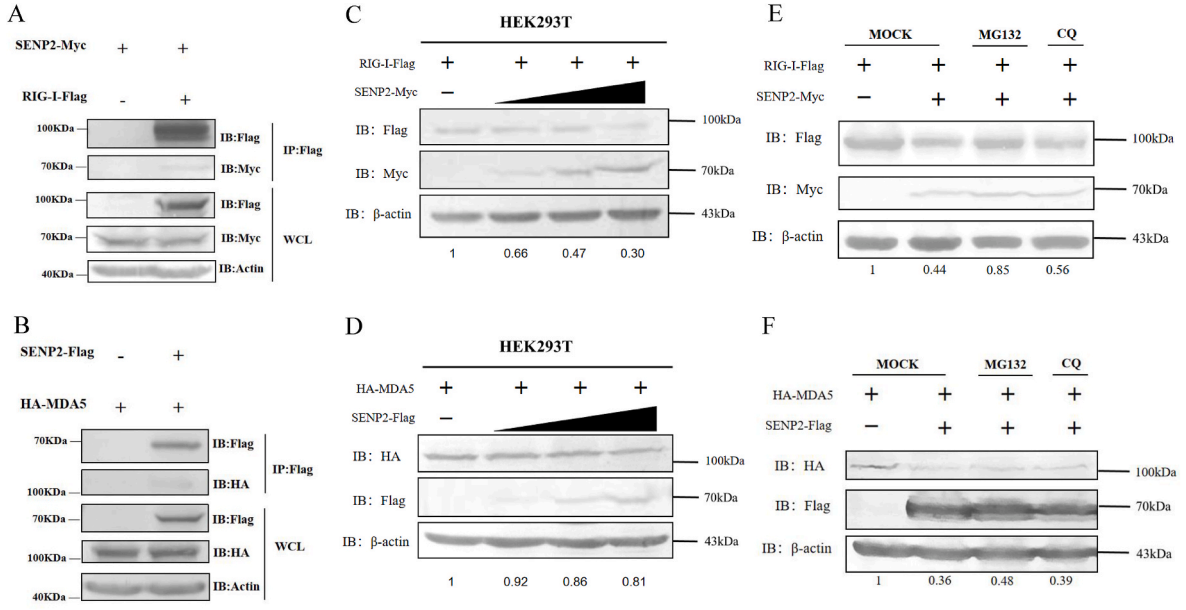

Fig. 6. bcSENP2 interacts with bcRIG-I and bcMDA5 (A) HEK293T cells in 10 cm dish were co-transfected with bcSENP2 and with or without bcRIG-I. The cells were harvested at 48 h post transfection and used for immunoprecipitation (IP). (B) HEK293T cells in 10 cm dish were co-transfected with bcMDA5 and with or without bcSENP2. The cells were harvested 48 h post transfection and used for IP. (C–D) HEK293T cells in 6 cm dish were transfected with bcRIG-I (C) or bcMDA5 (D) and with different dosage of bcSENP2. The cells were harvested 48 h post transfection and used for immunoblotting (IB). (E–F) HEK293T cells in 6 cm dish were transfected with bcRIG-I (E) or bcMDA5 (F) and with or without bcSENP2. After 36 h of transfection, 10 μl of DMSO or MG132 of 20 μM or CQ of 10 μM were added, and the cells were collected 12 h post drug treatment and used for IB.

3.6. bcSENP2 interacts with bcRIG-I and bcMDA5

The co-IP results showed that bcSENP2 could be pulled down by bcMDA5 and bcRIG-I, which indicated bcSENP2 interacted with bcMDA5 and bcRIG-I (Fig. 6A and B). To further reveal the regulation mechanism of bcSENP2 on bcRIG-I and bcMDA5 mediated antiviral signaling, the influence of bcSENP2 on the protein expression levels of bcRIG-I and bcMDA5 were tested. The IB results showed that bcSENP2 reduced the expression of bcRIG-I at the protein level by dose-dependent and impaired the protein expression of bcMDA5 (Fig. 6C and D), which indicated that bcSENP2 interacted with bcRIG-I and bcMDA5 and inhibited the antiviral signaling mediated by bcRIG-I and bcMDA5 by promoting their protein degradation. To explore the protein degradation pathway by which bcSENP2 reduced bcRIG-I/bcMDA5, we treated cells co-expressing bcSENP2 and bcRIG-I/bcMDA5 with the MG132 or chloroquine diphosphate (chloroquine diphosphate, CQ). The results of IB detection and relative gray value analysis showed that there were more obvious reversion effect of bcRIG-I after MG132 treatment, while there was little reversion effect after CQ treatment (Fig. 6E). Under MG132 treatment, bcSENP2 also had a reversion effect on the degradation of bcMDA5 (Fig. 6F). Therefore, these results suggested that bcSENP2 mediated the protein degradation of bcRIG-I/bcMDA5 mainly through the proteasome pathway.

4. Discussion

IFN is an essential mediator of innate immunity,and their expression must be strictly regulated to prevent organismal damage [29,30]. IFN protect immunity against infection or cancer by stimulating cellular cytotoxicity, but may also lead to immunologically mediated lesions [31,32]. RLRs are critical for the recognition of viral RNAs which the RIG-I and MDA5-mediated IFN signaling pathways are extremely vital in the defense against pathogenic microorganisms, and thus the regulation of RIG-I and MDA5 has been extensively studied in recent years [35]. Recently study showed that sumoylation of RIG-I enhanced the expression of downstream factors by increasing its K63-linked ubiquitination modification, which promotes the increase of IFN [36]. Sumoylation of MDA5 enhanced the expression of IFN during viral infections, thus resisting the viral invasion [37]. In this study, we found that bcMDA5 or bcRIG-I mediated antiviral capacity was attenuated by bcSENP2, while knockdown of bcSENP2 led to enhanced antiviral resistance to SVCV in host cells. In addition, bcSENP2 inhibited bcMDA5/bcRIG-I mediated antiviral signaling by enhancing ubiquitin-proteasome mediated degradation of bcMDA5/bcRIG-I in black carp.

By sequence and 3D structures analysis, we found that the functional structural domain ULP1 in SENP2 was relatively conserved among different species (Fig. 1). The three-dimensional structure predictions of the ULP1 functional structural domain between black carp and human are very similar, suggesting that bcSENP2, as a de-sumoization enzyme, the functions performed by this gene during evolution should be comparable (Fig. 1). In previous studies, ULP1 has been shown to support cell growth rates to regulate cellular processes, and it can cleave down SUMO proteins *in vitro*, which plays a critically important role [38]. It has been demonstrated that a catalytically inert mutant in the ULP1 domain of SENP2, SENP2 - C548A, exhibits a markedly diminished capacity to inhibit virus-induced activation of the IFN-b promoter [33]. This finding suggests that this structural domain exerts a pivotal function within the antiviral signaling pathway. The ULP1 structural domain of bcSENP2 exhibited great conservation in this study, which suggests that it may serve a functional structural role in the negative regulation of the RLR antiviral signaling pathway. RIG-I and MDA5 play extremely important roles in resisting viral invasion [39]. In this paper, bcSENP2 acted as a negative regulator of the RLR signaling pathway and targeted bcRIG-I and bcMDA5, which attenuated the antiviral ability of both (Figs. 3 and 4). In mammals, SENP2 has been reported to bind to IRF3 and modulate its ubiquitination thereby regulating negatively the cellular antiviral response [33]. But in the present study, SENP2 does not modulate IRF3 in fish, which might result from the variation in the immune systems of mammals and fish (Fig. 3A-C). For example, in mammals KAT8 undergoes acetylation by binding directly to IRF3, inhibiting the recruitment of IRF3 to the promoter of the IFN-I gene and decreasing the transcriptional activity of IRF3; in fish KAT8 prevents the activity of IRF3 by acetylating it, leading to the low-affinity interactions of the ISRE response element with IRF3, which in turn inhibits the nucleic acid-induced innate immune response [40,41]. Furthermore, OTUD6B interacts with IRF3 and attenuates TRAF6-mediated K63-linked ubiquitination of the IRF3 and attenuates the binding of TBK1 to IRF3, leading to impaired phosphorylation in zebrafish, thereby inhibiting cellular antiviral responses. Nevertheless, in mammals OTUD6B acts as a positive regulator of IRF3 and promotes innate antiviral immune signaling in type I IFN [42,43].

It has been shown that TRIM38-mediated dynamic sumoylation of RIG-I and MDA5 in mammals maintains their stability by opposing the K48-linked ubiquitination and degradation of RIG-I and MDA5. While SENP2 negatively regulates RIG-I and MDA5 by inhibiting the TRIM38 mediated sumoylation of RIG-I and MDA5 and promoting their ubiquitin-proteasome mediated protein degradation [21]. In this paper, bcSENP2 can negatively regulate bcRIG-I and bcMDA5 mediated IFN signaling. In the meanwhile, co-expression of bcSENP2 resulted in reduced protein levels of bcRIG-I and bcMDA5 and MG132 treatment rescued this attenuating effect (Fig. 6). Therefore, it is probably that bcSENP2 can inhibit sumoylation of bcRIG-I and bcMDA5 and promotes their protein degradation, thereby negatively regulating bcRIG-I and bcMDA5 like its mammalian counterpart, but the exact mechanism needs further investigation.

In this study, we demonstrated that direct binding of bcSENP2 to bcRIG-I/bcMDA5 is required for negative regulation of both, but in mammals, SENP2 regulates RIG-I and MDA5 indirectly, which is difference between the two. Furthermore, all these data support the conclusion that bcSENP2 inhibits bcRIG-I/bcMDA5 mediated antiviral innate immune. In conclusion, this is the first study showing that SENP2 negatively regulates the RLR signaling pathway by targeting RIG-I and MDA5 and promoting their proteasome degradation in fish. Nevertheless, the specific mechanism of bcSENP2 on bcRIG-I or bcMDA5 needs to be further investigated to reveal its significance in the vertebrate innate immune system.

CRediT authorship contribution statement

Yixia Chen: Investigation, Writing – original draft. **Jun Li:** Investigation, Validation. **Jiaxin Fu:** Investigation, Visualization. **Lili Xiao:** Methodology, Visualization. **Jixiang Chu:** Investigation. **Wei Qin:** Investigation. **Jun Xiao:** Conceptualization, Writing – review & editing. **Hao Feng:** Writing – review & editing.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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Data availability

The data that has been used is confidential.

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