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Black carp RNF5 inhibits STING/IFN signaling through promoting K48-linked ubiquitination and degradation of STING

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ABSTRACT

Ubiquitination is one of the important post-translational modifications (PTMs) of proteins that plays a vital role in regulating substrate degradation to ensure cellular homeostasis. Ring finger protein 5 (RNF5) is an essential E3 ubiquitin ligase for inhibiting STING-mediated interferon (IFN) signaling in mammals. Nevertheless, the function of RNF5 in STING/IFN pathway remains obscure in teleost. Here, we reported that over-expression of black carp RNF5 (bcRNF5) inhibited STING-mediated transcription activity of bcIFNa, DrIFNφ1, NF-κB and ISRE promoters and antiviral activity against SVCV. Moreover, knockdown of bcRNF5 increased the expression of host genes, including bcIFNa, bcIFNb, bcILβ, bcMX1 and bcViperin, and also enhanced the antiviral capability of host cells. Immunofluorescence (IF) and Co-immunoprecipitation (Co-IP) assay confirmed that bcRNF5 was mainly localized in the cytoplasm and interacted with bcSTING. The expression level of bcSTING protein was attenuated by co-expressed bcRNF5 and MG132 treatment rescued this attenuating effect, suggesting that bcRNF5-mediated bcSTING degradation was dependent on the proteasome pathway. Subsequent, Co-IP and immunoblot (IB) experiments identified that bcRNF5 triggered the K48-linked but not K63-linked ubiquitination of bcSTING. Altogether, above results conclude that RNF5 suppresses STING/IFN signaling by enhancing K48-linked ubiquitination and protease degradation of STING in black carp.

1. Introduction

Innate immunity is the primary line of host defense against pathogens invasion, in which host cells employ a repertoire of germline encoded pattern recognition receptors (PRRs) to recognize the conserved pathogen-associated molecular patterns (PAMPs) of pathogens (Jeannin et al., 2008; Rehwinkel and Gack, 2020). PAMPs consist of a series of biomacromolecules introduced by invading pathogens, such as viral nucleic acids and proteins (Janeway and Medzhitov, 2002; Akira et al., 2006; Li and Wu, 2021), among them, the identification of pathogenic nucleic acid is a common strategy for host to detect invading pathogens. After detecting the components of invading pathogens, type I IFN and interleukin-1 (IL-1)-mediated proinflammatory responses are initiated to resolve the threats (Mayer-Barber et al., 2014; Mayer-Barber and Yan, 2017; Chen and Holtzman, 2022).

Stimulator of interferon genes (STING), as an important regulator for pathogenic DNA/RNA mediated antiviral or antimicrobial responses, plays a pivotal role in innate immunity against pathogens in vertebrates (Ishikawa and Barber, 2008; Sun et al., 2009; Couillin and Riteau, 2021). STING consists of two parts: the transmembrane regions (TM) at N-terminal, which anchors STING on the endoplasmic reticulum (ER), and mitochondria and mitochondrial associated membrane (MAM) (Ishikawa et al., 2009; Jin et al., 2011), and the C-terminal spherical domain, which faces the cytoplasm and is involved in STING-mediated signaling pathway (Ishikawa et al., 2009). Although STING exerts key roles in immune responses, aberrant activation of STING causes

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autoinflammatory and autoimmune diseases. Therefore, STING was tightly regulated by many kinds of post translational modification (PTM) in human and mammals, including phosphorylation and ubiquitination (Hopfner and Hornung, 2020). In addition, STING was also regulated by some viral proteins (Ran et al., 2014). Among them, ubiquitination of STING has become a core regulatory mechanism for positively and negatively controlling its antiviral signaling (Bhoj and Chen, 2009). For example, STING could be activated by MUL1 (mitochondrial E3 ubiquitin protein ligase 1) triggering K63-linked ubiquitination at K224 (Ni et al., 2017). In contrast, TRIM29 was associated with STING and catalyzed its K48-linked ubiquitination and degradation, thus limiting STING-mediated antiviral responses (Li et al., 2018).

In teleost fish, STING homologues have been identified in several species. For instance, grass carp (*Ctenopharyngodon idella*) (Feng et al., 2014), zebrafish (*Danio rerio*) (Biacchesi et al., 2012), gold fish (*Carassius auratus*) (Sun et al., 2011), grouper (*Epinephelus coioides*) (Huang et al., 2015), common carp (*Cyprinus carpio*) (Cao et al., 2016) and black carp (*Mylopharyngodon piceus*) (Lu et al., 2017). The above reports have indicated that STING played important role in antiviral signaling pathway in fish. For example, gold fish STING could restrain viral replication capacity through activating IRF3/7-dependent IFN signaling. Zebrafish STING contributed to RIG-I mediated IFN production, and the fish cells over-expressing STING could induce the expression of IFN/ISG. Grass carp STING has been shown to participate in innate immunity through the TBK1-IRF3/IRF7 cascade. However, the regulation mechanism of STING has not been well identified in teleost.

RNF5 is anchored to the outer membrane of ER as an E3 ubiquitin ligase (Kyushiki et al., 1997; Matsuda et al., 2001; Adir et al., 2021). Initial studies have found that human RNF5, as a new cell movement regulator, could inhibit cell movement by targeting ubiquitination of cytoskeletal protein paxillin (Didier et al., 2003). Subsequently, it was found that RNF5 is not only related to the assembly of muscle attachments and muscle degenerative diseases, but also to the regulation of cancers such as breast cancer and glioma (Didier et al., 2003; Delaunay et al., 2008). Besides, previous reports found that RNF5 could inhibit host antiviral responses in mammals through catalyzing K48-linked ubiquitination and degradation of MAVS and STING (Zhong et al., 2009, 2010). However, it remains unknown whether RNF5 regulates the STING/IFN signaling pathway through ubiquitination modification in teleost fish.

In our previous research, bcSTING has been reported to have antiviral effects in host cells against SVCV/GCRV (Lu et al., 2017). In this study, we found that bcRNF5 promoted the polyubiquitination and degradation of bcSTING through the proteasome pathway, thus inhibiting bcSTING-mediated antiviral immune responses. Meanwhile, over-expression and knockdown of bcRNF5 also indicated that bcRNF5 had an inhibitory effect in IFN signaling pathway. These data reveal that bcRNF5 is a negative regulatory factor in the STING/IFN signaling, and provide a reference for further research on the regulatory mechanism in STING mediated signaling.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, HeLa and *epithelioma papulosum cyprinid* (EPC) cells were kept in the lab. *Mylopharyngodon piceus fin* (MPF) cells were gained from Dr. Tiansheng Chen (College of Aquatic Sciences, Jimei University) (Xue et al., 2018). These cells were cultured according to the reference (Yan et al., 2023).

The pcDNA5/FRT/TO, pcDNA5/FRT/TO-Flag-bcSTING, pcDNA5/FRT/TO- HA-Ub, pcDNA5/FRT/TO-HA-Ub-K48O/K63O (only one lysine remains at position 48/63), pRL-TK, two type I IFN promoter vectors including Luci-bcIFNa (for analyzing the activity of black carp IFNa promoter) and Luci-DrIFNφ1 (for analyzing the activity of zebrafish IFNφ1 promoter), and Luci–NF–κB (for analyzing the activity of human NF-κB promoter) were kept in the lab. Luci-ISRE (ISRE promoter of *Epithelioma Papulosum Cyprinid*) was a gift from Dr. Yongan Zhang (Institute of Hydrobiology, CAS) (Lu et al., 2016). The knockdown shRNA plasmids targeting bcRNF5 was constructed according to the reference (Yan et al., 2023). Detailed primers are displayed on Table 1.

2.2. Virus and reagents

Titer of spring viremia of carp virus (SVCV) was detected by viral plaque assay as previously described (Liu et al., 2022). EPC cells were infected with the virus supernatant diluted 10 times in sequence at 26 °C. After incubation for 1 h, the semisolid medium (DMEM with 2% FBS and 0.75% methylcellulose) were added, the plaques were observed and counted after 72 h infection.

Mouse anti-HA (Sigma), mouse anti-Myc (Affinity), Mouse anti-Flag (Sigma), Alexa Fluor 594 goat anti-rabbit (Invitrogen), Alexa fluor 488 goat anti-mouse (Invitrogen) and Mouse anti-Flag conjugated protein A/G agarose beads (Sigma) were used as requested of the manufacturers' instructions. MG132 was purchased from Selleck (S2619).

2.3. Luciferase reporter assay

To investigate the effect of bcRNF5 on bcSTING-mediated IFN or NF-κB signaling pathway, EPC or HEK293T cells were co-transfected with plamids expressing the vector or different qualities of bcRNF5, pRL-TK, bcSTING, Luci-DrIFNφ1 or Luci-bcIFNa or Luci-ISRE or Luci–NF–κB in 24-well plates for 24 h, and the PLB-lysed cells were measured for luciferase reporter assay according to the reference (Yan et al., 2023).

2.4. Quantitative real-time PCR (q-PCR)

q-PCR was performed to detect the mRNA transcription level of SVCV proteins (G, M, N, P) and type I IFN/ISGs (IFN, Viperin, ISG15, MX1, PKR, IL1-β) in EPC or MPF cells after SVCV infection. The primers for amplification of these proteins were displayed in Table 1. The program is as follows: 95 °C/10 min (1 cycles), 95 °C/15 s (40 cycles), 60 °C/1 min. Threshold cycle (CT) values were manually set and determined on the 7500 Real-Time PCR System (ABI, USA), and the above date were calculated for $2^{-\triangle\triangle CT}$ method (Liu et al., 2022).

2.5. Immunoblotting and co-immunoprecipitation

Immunoblotting (IB) was used to test the effect of bcRNF5 on expression of bcSTING with or without proteasomal inhibitor MG132 (20 μmol/L), and the knockdown efficiency of bcRNF5 was also verified by this method. Specifically, HEK293T cells were co-transfected with bcRNF5 and/or bcSTING or bcRNF5-shRNA-1/2/3 for 48 h, and subsequently lysed cells were used for IB experiments, which IB assays were performed according to the reference (Liu et al., 2022).

Co-immunoprecipitation (Co-IP) was used to explore whether bcRNF5 interacts with bcSTING and whether bcRNF5 has the effect on ubiquitination of bcSTING. bcSTING and/or bcRNF5 and HA-Ub/HA-Ub-K48O/HA-Ub-K63O were co-transfected into HEK293T cells by PEI, and cells were used for Co-IP experiments after 48 h, which were performed as the reference (Song et al., 2019).

2.6. Immunofluorescence microscopy

The HeLa cells were co-transfected with bcRNF5 and bcSTING or empty vector by using LipoMax. 24 h after transfection, the cells were treated with 4% histiocyte fixative, 0.2% Triton X-100 and 10% FBS as the reference (Song et al., 2019), and then the cells were incubated with a percentage of 1:500 mouse monoclonal antibody anti-FLAG and 1:1000 alexa 488-conjugated secondary antibody for 1 h, respectively. Finally, 6 μl DAPI was added for nuclear staining and air-dried slides were photographed using laser scanning confocal microscope (Olympus

Table 1
Primers used in the study.

Name	Sequence (5′-3′)	Information
q-PCR		
epc-actin-F	AAGGAGAAGCTCTGCTATGTGGCT	*ex vivo* q-PCR
epc-actin-R	AAGGTGGTCTCATGGATACCGCAA	*ex vivo* q-PCR
epc-MX1-F	TGGAGGAACCTGCCTTAAATAC	*ex vivo* q-PCR
epc-MX1-R	GTCTTTGCTGTTGTCAGAAGATTAG	*ex vivo* q-PCR
epc-ISG15-F	TGATGCAAATGAGACCGTAGAT	*ex vivo* q-PCR
epc-ISG15-R	CAGTTGTCTGCCGTTGTAAATC	*ex vivo* q-PCR
epc-Viperin-F	GCAAAGCGAGGGTTACGAC	*ex vivo* q-PCR
epc-Viperin-R	CTGCCATTACTAACGATGCTGAC	*ex vivo* q-PCR
epc–IFN–F	ATGAAAACTCAAATGTGGACGTA	*ex vivo* q-PCR
epc–IFN–R	GATAGTTTCCACCCATTTCCTTAA	*ex vivo* q-PCR
SVCV-G-F	GATGACTGGGAGTTAGATGGC	*ex vivo* q-PCR
SVCV-G-R	ATGAGGGATAATATCGGCTTG	*ex vivo* q-PCR
SVCV-P-F	AACAGGTATCGACTATGGAAGAGC	*ex vivo* q-PCR
SVCV-P-R	GATTCCTCTTCCCAATTGACTGTC	*ex vivo* q-PCR
SVCV-N-F	GGTGCGAGTAGAAGACATCCCCG	*ex vivo* q-PCR
SVCV-N-R	GTAATTCCCATCATTGCCCCAGAC	*ex vivo* q-PCR
SVCV-M-F	CGACCGCGCCAGTATTGATGGATAC	*ex vivo* q-PCR
SVCV-M-R	ACAAGGCCGACCCGTCAACAGAG	*ex vivo* q-PCR
bc-RNF5-F	GTGTAAAGCAGGAATCAGTCG	*ex vivo* q-PCR
bc-RNF5-R	CACGCTCAGCATCCAGAA	*ex vivo* q-PCR
bc-actin-F	TGGGCACCGCTGCTTCCT	*ex vivo* q-PCR
bc-actin-R	TGTCCGTCAGGCAGCTCAT	*ex vivo* q-PCR
bc-IFNa-F	AAGGTGGAGGACCAGGTGAAGTTT	*ex vivo* q-PCR
bc-IFNa-R	GACTCCTTATGTGATGGCTTGTGG	*ex vivo* q-PCR
bc-IFNb-F	GACCACGTTTCCATATCTTT	*ex vivo* q-PCR
bc-IFNb-R	CATTTTTTCTTCATCCCACT	*ex vivo* q-PCR
bc-Viperin-F	CCAAAGAGCAGAAAGAGGGACC	*ex vivo* q-PCR
bc-Viperin-R	TCAATAGGCAAGACGAACGAGG	*ex vivo* q-PCR
bc-IL1β-F	GTATGGAAGCGGTTGAGGTA	*ex vivo* q-PCR
bc-IL1β-R	CAGACACACAGGCTGGGATG	*ex vivo* q-PCR
bc-MX1-F	GACTCCTTATGTGATGGCTTGTGG	*ex vivo* q-PCR
bc-MX1-R	TGAGCGTAGGCATTAGCAC	*ex vivo* q-PCR
bc-PKR-F	GAGCGGACTAAAAGGACAGG	*ex vivo* q-PCR
bc-PKR-R	AAAATATATGAGACCCAGGG	*ex vivo* q-PCR
shRNA		
shbcRNF5-F1	CCGGGAGCCACTTTCGAGTGTAACACTCGAGTGTTACACTCGAAAGTGGCTCTTTTTG	bcRNF5-shRNA-1
shbcRNF5-R1	AATTCAAAAAGAGCCACTTTCGAGTGTAACACTCGAGTGTTACACTCGAAAGTGGCTC	
shbcRNF5-F2	CCGGGCAGGAATCAGTCGAGATAAACTCGAGTTTATCTCGACTGATTCCTGCTTTTTG	bcRNF5-shRNA-2
shbcRNF5-R2	AATTCAAAAAGCAGGAATCAGTCGAGATAAACTCGAGTTTATCTCGACTGATTCCTGC	
shbcRNF5-F3	CCGGGCAATGCCAACAACGGCAACACTCGAGTGTTGCCGTTGTTGGCATTGCTTTTTG	bcRNF5-shRNA-3
shbcRNF5-R3	AATTCAAAAAGCAATGCCAACAACGGCAACACTCGAGTGTTGCCGTTGTTGGCATTGC	

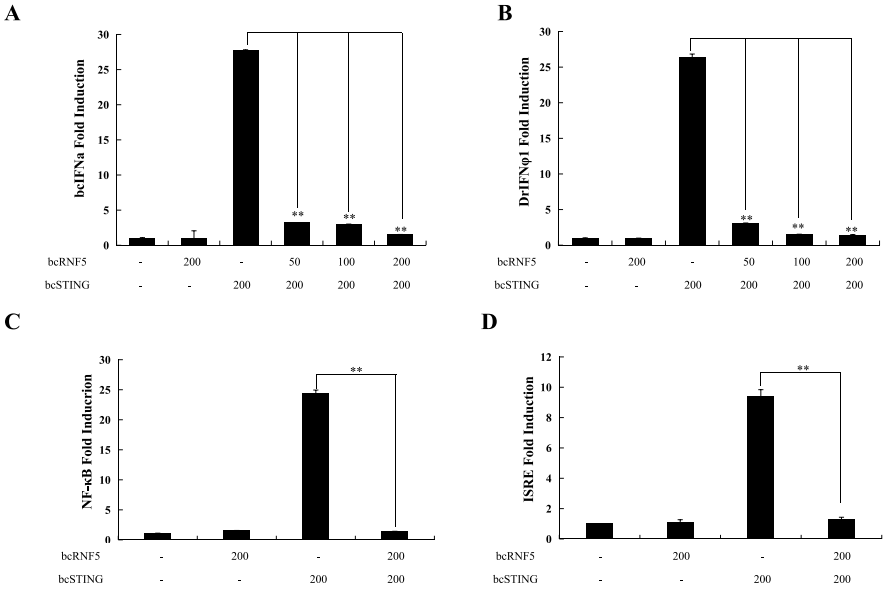

Fig. 1. bcRNF5 suppresses bcSTING-mediated IFN signaling.
EPC cells or HEK293T cells in 24-well plates were co-transfected with the reporter plasmids pRL-TK, Luci-bcIFNa (A) or DrIFNφ1 (B) or NF-κB (C) or ISRE (D) and the indicator plasmids bcRNF5 and bcSTING, respectively, and then used for reporter assay at 24 h post-transfection. bcRNF5: pcDNA5/FRT/TO/HA-bcRNF5; bcSTING: pcDNA5/FRT/TO/Flag-bcSTING. The data represent three independent experiments. *P < 0.05, **P < 0.01.

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3. Results

3.1. bcRNF5 inhibits bcSTING-mediated IFN signaling

Earlier studies have found that mammalian RNF5 negatively regulated IFN signaling through targeting STING to inhibit IFNβ production (Zhong et al., 2009). To investigate the effect of bcRNF5 in STING/IFN signaling pathway, EPC cells over-expressing bcRNF5 and/or bcSTING were performed by the reporter assays. As showed in Fig. 1, over-expressed bcSTING significantly induced the transcription activity of bcIFNa, DrIFNφ1, NF-κB and ISRE promoters. Moreover, over-expression of bcRNF5 alone had few effects on transcription level of these promoters. However, the transcription activity of bcIFNa (Fig. 1A), DrIFNφ1 (Fig. 1B), NF-κB (Fig. 1C) and ISRE (Fig. 1D) were strongly inhibited in cells co-expressing bcRNF5 and bcSTING. The results showed that bcRNF5 had an inhibitory effect on bcSTING-mediated IFN/NF-κB cascade in the innate immunity.

3.2. bcRNF5 down-regulated bcSTING-mediated antiviral activity

In mammals, STING acts as a core adapter in the innate immunity triggered by pathogenic DNA/RNA, and its antiviral activity was strongly inhibited by RNF5 (Zhong et al., 2009). Our recent study has also shown that STING could exert antiviral effects in host cells against SVCV/GCRV (Lu et al., 2017). To investigate whether the antiviral capacity of bcSTING is also limited by bcRNF5, EPC cells co-expressing bcRNF5 and bcSITNG were infected with different doses of SVCV (MOI = 0.01/0.1/1), and then the collected supernatant was used for viral titer assay, the pellets were performed for crystal violet staining experiments or q-PCR. The above results found that the cells co-expressing bcSTING and bcRNF5 had a higher viral titer and mortality than that of cells transfected with bcSTING alone (Fig. 2A&B). Moreover, EPC cells co-expressing bcSTING and bcRNF5 had higher mRNA transcription levels of SVCV-encoded proteins (P, N, M, G) than those expressing bcSITNG alone (Fig. 3A–D). On the contrary, the transcription levels of IFN/ISGs (epcIFN, epcMx1, epcViperin and epcISG15) in EPC cells co-expressing bcSTING and bcRNF5 were significantly lower than those in EPC cells transfected with bcSTING alone (Fig. 3E–H). The above results prominently indicated that bcRNF5 extremely inhibited bcSTING-mediated antiviral capacity.

3.3. Knockdown of bcRNF5 potentiated host IFN signaling

Recently studies have reported that RNF5 silencing in the mouse could effectively promote type I IFN expression, inhibiting HSV-1 virus replication (Liu et al., 2022). To explore the antiviral capacity in host cells with bcRNF5 knockdown, shRNA recombinant plasmids targeting bcRNF5 were constructed and their knockdown efficacy were detected for IB assay, which showed that bcRNF5-shRNA-3 significantly decreased the expression of exogenous bcRNF5 (Fig. 4A). Meanwhile, q-PCR results revealed that the mRNA level of endogenous RNF5 in MPF cells expressing bcRNF5-shRNA-3 was lower (Fig. 4B). Subsequently, MPF cells expressing bcRNF5-shRNA-3 were infected with SVCV, and titer in the culture medium was detected by the plaque assays, the transcription levels of SVCV encoding proteins G, bcIFNa, bcIFNb, bcViperin, bcMX1, bcIL1β and bcPKR in MPF cells were detected by qPCR. After SVCV infection, the viral titer and the mRNA level of SVCV encoding protein G in the bcRNF5 knockdown group was significantly reduced than the control group (Fig. 4C and D). On the contrary, the mRNA levels of IFNs/ISGs (*bcIFNb, bcIFNa, bcPKR, bcMX1, bcIL1β* and *bcViperin*) in the bcRNF5 knockdown group were obviously enhanced compare with the control group (Fig. 4E). To sum up, these results revealed that RNF5 inhibited the host cellular antiviral responses.

3.4. The interaction between bcRNF5 and bcSTING

To further determine the relationship between RNF5 and STING in black carp, HEK293T cells and HeLa cells co-expressing bcSTING and bcRNF5 were performed for Co-IP and IF assay. The Co-IP assay found that a specfic band representing bcRNF5 (about 22 kDa) was tested in the Flag-bcSTING-precipitated proteins (Fig. 5A). Furthermore, the IF data clearly showed the green fluorescence (bcSTING) coincides with the red fluorescence (bcRNF5), indicating that the subcellular distribution of these two proteins is consistent (Fig. 5B). Thus, these results demonstrate that bcRNF5 interacts with bcSTING.

3.5. bcRNF5 mediated proteasome-dependent degradation of bcSTING

The above data indicated that bcRNF5 interacted with bcSTING and had a negative regulatory effect on bcSTING-mediated IFN signaling. To explore the regulation mechanism of bcRNF5, we detected whether bcRNF5 affects the protein level of bcSTING. HEK293T cells co-expressing bcSTING and different doses of bcRNF5 (2 μg, 2.5 μg) were collected and used by IB assay. The IB data revealed that the expression level of bcSTING protein in HEK293T cells co-transfected with plasmids expressing bcRNF5 and bcSTING was observably decreased compared with bcSTING single transfected group (Fig. 6A), indicating that the degradation of bcSTING was caused by bcRNF5. To further investigate the mechanism of bcRNF5-induced degradation of bcSTING, bcRNF5 and bcSTING were co-transferred in HEK293T, and cells were treated with proteasomal inhibitor MG132 (20 μmol/L) for IB assays. We observed that the reduction of bcSTING induced by bcRNF5 was profoundly blocked by MG132 treatment (Fig. 6B), suggesting that bcRNF5 triggered bcSTING degradation through the proteasome pathway.

3.6. bcRNF5 enhanced K48-linked ubiquitination of bcSTING

Ubiquitination has emerged as one of the most prevalent post-translational modifications in the regulation of antiviral immunity (Bhoj and Chen, 2009). Previously study reported that mammalian RNF5 restrained STING/IFN signaling by ubiquitin-proteasome pathway (Zhong et al., 2009). To further investigate whether bcRNF5 mediates

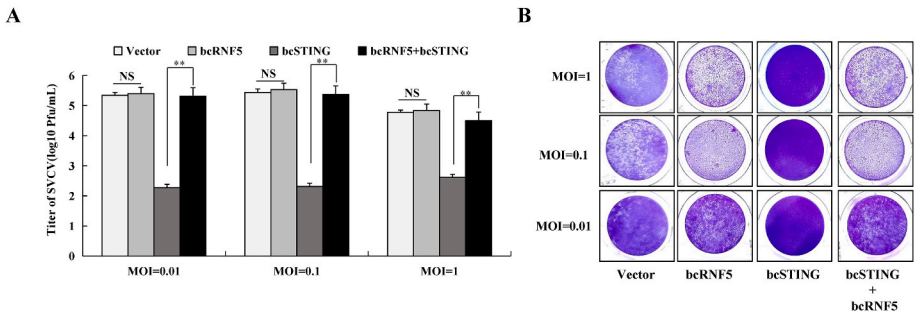

Fig. 2. Down-regulated antiviral capacity of bcSTING by bcRNF5.
EPC cells were transfected with plasmids expressing vector or bcRNF5 or bcSTING or bcRNF5 and bcSTING in 24-well plates, and then infected with SVCV (MOI = 0.01, 0.1, 1) respectively. 24 h after infection, the harvested supernatant was used for viral titer assays (A) and the monolayer cells were examined by crystal violet staining (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

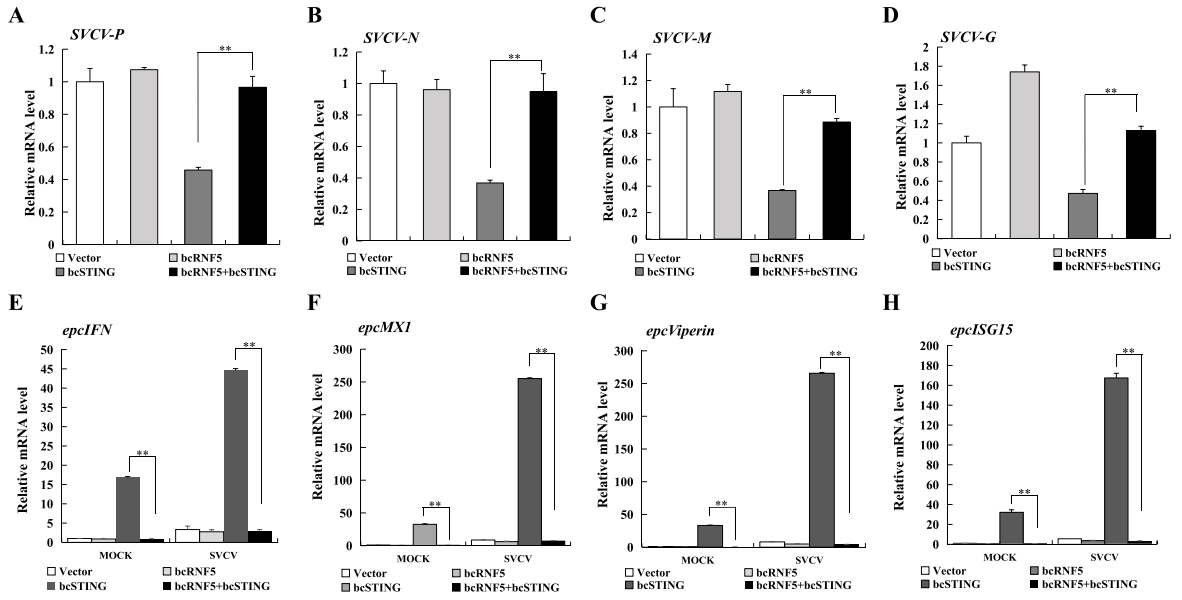

Fig. 3. The mRNA expression of cytokines and viral protein in EPC cells during SVCV infection.
EPC cells co-expressing vector or bcRNF5 or bcSTING or bcRNF5 and bcSTING in 24-well plates were infected with SVCV (MOI = 0.1) respectively. 8 h after infection, the harvested cells were used for RNA isolation. The mRNA expression of SVCV-P (A), N (B), M (C), G (D) and epcIFN (E), epcMX1 (F), epcViperin (G), epcISG15 (H) were tested for q-PCR assay.

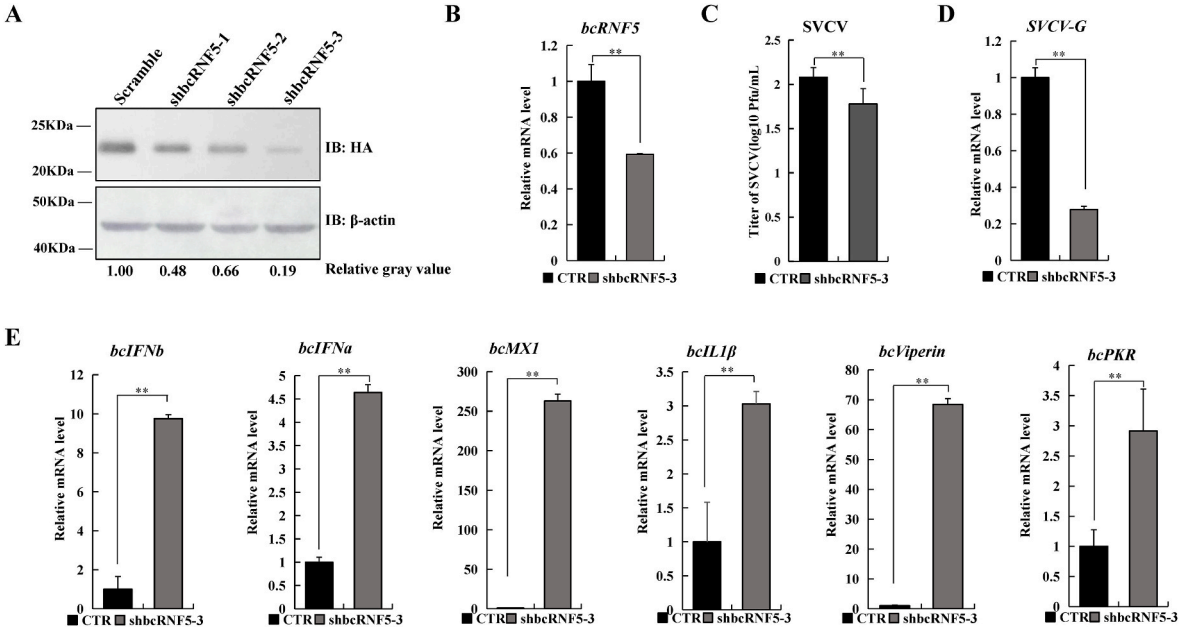

Fig. 4. bcRNF5 knockdown improved the antiviral ability of host cells.
HEK293T cells co-expressing bcRNF5 and shRNA targeting bcRNF5 (shbcRNF5-1, shbcRNF5-2, shbcRNF5-3) or Scramble in 6-well plates were used for IB assay at 48 h post-transfection (A). MPF cells expressing shRNA-bcRNF5 or control (Scramble) in 6-well plates were infected with SVCV (MOI = 0.1) after 24 h. The collected supernatants were tested by viral titer assay (C), and the harvested cells were used by q-PCR (B, D, E) at 24 h post-infection. shbcRNF5-1: PLKO-shRNA-bcRNF5-1; shbcRNF5-2: PLKO-shRNA-bcRNF5-2; shbcRNF5-3: PLKO-shRNA-bcRNF5-3; Scramble: PLKO-shRNA-scramble.

bcSTING ubiquitination, we co-transfected HA-Ub, bcSTING with/-without bcRNF5 in HEK293T cells, and precipitated bcSTING protein respectively to detect its ubiquitination changes. The Co-IP data showed that the ubiquitination level of bcSTING was markedly enhanced in bcSTING and bcRNF5 co-transfected group compared with bcSTING single transfected group (Fig. 7A). To further confirm the ubiquitination type of STING, HA-Ub-K48O/K63O plasmids, bcSTING and with/-without bcRNF5 respectively were co-transfected into HEK293T, which revealed that the K63-linked ubiquitination of bcSTING was similar between bcSTING singly expressed group and bcSTING/bcRNF5 co-expressed group. However, in the bcRNF5 and bcSTING co-expressed group, K48-linked ubiquitination of bcSTING was observably increased compared with the bcSTING singly expressed group (Fig. 7B). All the above results suggested that bcRNF5 could negatively regulate antiviral responses for degradation of STING by K48-linked ubiquitin-proteasome pathway.

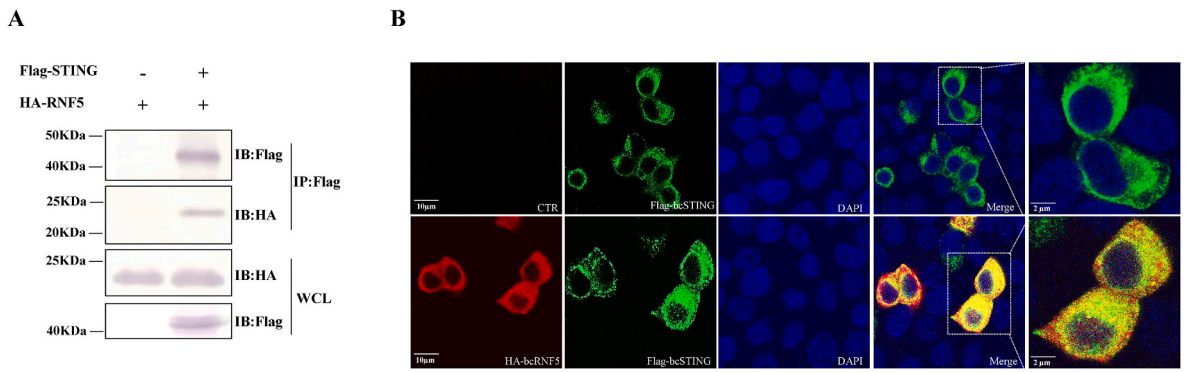

Fig. 5. bcRNF5 interacted with bcSTING.
HEK293T cells in 10 cm dish were co-transfected with bcSTING and bcRNF5 or vector and bcRNF5. The harvested cells were used by Co-IP assay at 48 h post transfection (A). IF staining of bcSTING and bcRNF5 in HeLa cells. The bars stand for the scale of 10 μm and 2 μm (B). IP: immunoprecipitation; IB: immunoblot; WCL: whole cell lysate.

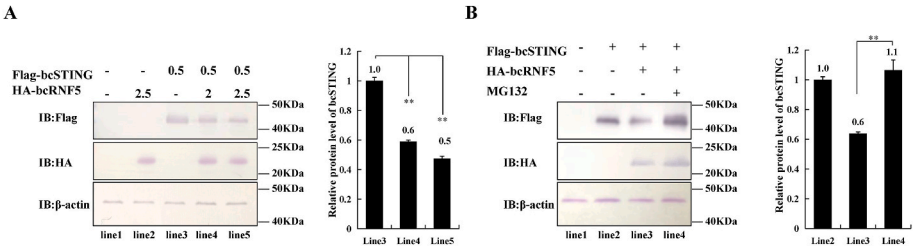

Fig. 6. bcRNF5 triggered bcSTING degradation.
HEK293T cells were co-transfected with plasmids co-expressing bcSTING and different doses of bcRNF5 (2 μg, 2.5 μg) in 6-well plates. Then the cells were collected for IB assay at 48 h post transfection (A). HEK293 cells in 6-well plates were co-transfected with bcSTING and bcRNF5, the transfected cells were treated with or without MG132 (20 μmol/L) at 8 h before harvest, and then the harvested cells were used for IB assay at 48 h post transfection (B). The histogram is the quantitative analysis of related bcSTING levels.

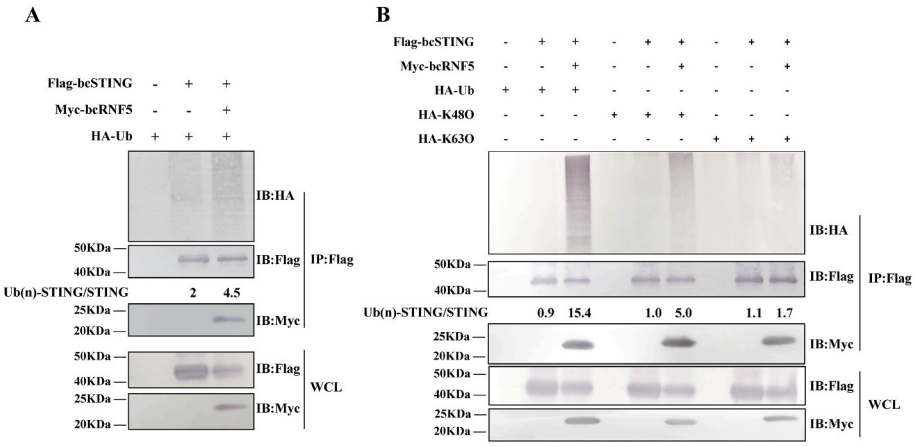

Fig. 7. bcRNF5 enhanced K48-linked ubiquitination of bcSTING.
HEK293T cells were co-transfected with HA-Ub, bcSTING and/or bcRNF5. The cells were harvested for Co-IP assay at 48 h post transfection (A). HEK293T cells co-expressing HA-Ub (HA-Ub-K48O or HA-Ub-K63O), bcSTING and/or bcRNF5 were collected for Co-IP assay at 48 h post transfection (B). The ubiquitin level of bcSTING was tested by IB assays.

4. Discussion

Abnormal production of IFN in innate and adaptive immunity is deleterious and may be disastrous to the host. Hence, avoiding excessive production of IFN is essential to maintain the homeostasis of the host (Jefferies, 2019). The cGAS–STING signaling axis detects pathogenic or endogenous damage DNA to induce innate immune responses involving strong IFN production (Hopfner and Hornung, 2020). The PTM of STING, such as ubiquitination and phosphorylation, exert a crucial role in STING mediated IFN production. In previous studies, TBK1 could phosphorylate STING at Ser366, providing the docking site for IRF3 activation and IFN production (Tanaka and Chen, 2012). TRIM56 could induce K63-linked ubiquitination of STING at K150 for activating STING/IFN signaling pathways (Tsuchida et al., 2010). The autocrine motility factor receptor (AMFR) could promote the activation of STING through inducing the K27-linked ubiquitination of STING at multiple sites (Wang et al., 2014). On the contrary, USP13 inhibited SITNG/IFN signaling by eliminating the K27-linked ubiquitination of STING to reduce its interaction with TBK1 (Sun et al., 2017). In this context, our data show that bcRNF5 could enhance K48-linked ubiquitination modification of bcSTING and affect its stability, thus negatively regulating the STING/IFN signaling in black carp.

As the key to executive function, the stability of STING is strictly regulated by multiple mechanism. Degradation of STING resulted in inhibition of its activity, and inhibition of proteasomal activity can increase the stability of STING and enhance IFN/ISG expression (Gui et al., 2019; Gonugunta et al., 2017). In mammalians, RNF5 restrained the excessive IFN expression by inducing the K48-linked ubiquitination of STING at K150, resulting in its proteasomal degradation (Zhong et al., 2009; Liu and Xia, 2022). In contrast to that, deubiquitinase CYLD

removed the K48-linked ubiquitination of STING at K150 to promote the stability of STING protein (Zhang et al., 2018). Besides, the desumoylation enzyme SENP2 was reported to decrease the stability of STING protein by desumoylation of STING at K338 and negatively regulated STING/IFN signaling (Hu et al., 2016). Here, our data have expounded that bcRNF5-mediated ubiquitination can induce degradation of bcSTING through the proteasome pathway (Figs. 5 and 6), which was consistent with its mammalian counterpart.

The STING activity was strictly regulated by diversiform ubiquitination modification in innate immunity, such as K11, K48 and K63 linked ubiquitination, and the above polyubiquitin chains are also regulated by different kinds of E3 ubiquitin ligase (Hopfner and Hornung et al., 2020). RNF26 up-regulated the stability of STING through facilitating K11 linked polyubiquitination of STING at K150, furthermore, RNF26 could inhibit the degradation of STING resulting from RNF5-mediated ubiquitination (Qin et al., 2014). TRIM32 catalyzed the K63-linked ubiquitination of STING at multiple Lysine sites to induce IFN production (Zhang et al., 2012). In this manuscript, the K48 linked ubiquitination of bcSTING was dramatically increased by bcRNF5, which may be essential for the activity of bcSTING as it is in mammalian counterpart (Fig. 7B). In view of the fact that previous studies in fish mainly focused on the regulatory function of fish STING on downstream signaling (Biacchesi et al., 2012; Feng et al., 2014), here our study revealed an important regulatory way for STING in teleost.

E3 ubiquitin ligase is one of the necessary factors to determine the type of ubiquitination of substrate protein. Therefore, the function of E3 ubiquitin ligase family has been widely studied in recent decades. Recently, black carp RNF5 could interact with MAVS and negatively regulate MAVS/IFN signaling (Yan et al., 2023). Nevertheless, the immunomodulatory mechanisms of RNF5 in fish is still unclear. Informed studies have reported that knockdown of RNF5 enhances Sev-induced IFNβ in mammals (Zhong et al., 2009). Similarly, our data revealed that knockdown of RNF5 in MPF cells significantly enhanced the transcription level of IFN/ISGs, and decreased the transcription level of SVCV encoded protein, and the titer of SVCV also markedly decreased (Fig. 4). Moreover, over-expression of RNF5 obviously reduced STING-mediated IFN expression and antiviral activity (Figs. 1 and 2). These results collectively demonstrate that black carp RNF5 is an inhibitor in STING-mediated innate immunity responses, which expanded understanding of the regulation of RNF5 in vertebrate.

In summary, this study found that RNF5 of black carp restrains the STING-mediated IFN signaling pathway, and knockdown of bcRNF5 has stronger antiviral capacity in host cell. Moreover, bcRNF5 promotes the degradation of bcSTING through K48-linked ubiquitin-proteasome pathway. Thus, our research provides a new direction for host to maintain appropriate immune responses in black carp.

Data availability

The data that has been used is confidential.

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