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TRIM25 negatively regulates IKKε-mediated interferon signaling in black carp

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ABSTRACT

IKKε plays an important role in the activation of IRF3/IRF7 and the production of interferon (IFN), however, its regulation remains obscure in human. E3 ligase TRIM25 has been reported to manipulate the K63-linked ubiquitination of RIG-I, leading to the activation of RIG-I/IFN signaling. To elucidate the role of TRIM25 in teleost, a TRIM25 homolog (bcTRIM25) was cloned and characterized from black carp (*Mylopharyngodon piceus*). bcTRIM25 contains 653 amino acids, possessing conservative RING, B-box and SPRY domain, which is highly expressed in muscle, spleen and skin. bcTRIM25 knock-down enhanced the antiviral ability of host cells. bcTRIM25 over-expression alone in EPC cells attenuated bcIFNa promoter transcription in the reporter assays and impeded *PKR* and *MX1* expression in qRT-PCR. Interestingly, co-IP assays indicated that bcTRIM25 interacted with bcIKKε and the induced bcIFNa promoter transcription by bcIKKε was notably hindered by bcTRIM25. Furthermore, bcIKKε-induced expression of interferon stimulated genes (ISGs) and antiviral activity were dampened by bcTRIM25. Further exploration showed that bcTRIM25 visibly enhanced the ubiquitination of bcIKKε but significantly attenuated the phosphorylation of bcIKKε. Thus, our data demonstrate for the first time in vertebrate that TRIM25 negatively regulates IKKε through enhancing its ubiquitination, which sheds a light on the regulation of IKKε/IFN signaling.

1. Introduction

Innate immunity is an intrinsic baseline defense in cells, which plays key roles in host defense against pathogens such as virus and bacteria invasion [1]. The innate immune system recognizes pathogens by a series of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type Lectins receptors (CLRs), NOD-like receptors (NLRs), Retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and cytosolic DNA sensors etc [2]. RLRs are essential for the innate recognition of cytosol viral components and are involved in the restriction of viral replication and dissemination. After activated by viral RNA, RLRs members such as RIG-I and melanoma differentiation-associated gene 5 (MDA5) recruit the adaptor protein mitochondrial antiviral signaling (MAVS) to activate downstream kinases TANK-binding kinase 1 (TBK1)/IkB kinase ε (IKKε) [3], which lead to the phosphorylation and nuclear translocation of interferon regulatory factor 3/7 (IRF3/7) to induce type I interferon (IFN) production [4,5]. Subsequent IFNs then trigger a variety of IFN-stimulated genes (ISGs) to limit the virus replication.

IKKε is a member of IKKs family involved in IRF3/7 activation that plays critical roles in the regulation of type I IFN signaling pathways. IKKε is initially defined as activators of NF-κB and acted on multiple NF-κB members and effectors, and is later found to be involved in antiviral innate immunity by activating IRF3/IRF7 through its kinase activity [6]. IKKε belongs to the non-canonical IKK family member which has a kinase domain at the N-terminus, a ubiquitin-like domain, and a C-terminal SDD domain [7]. Similar to another non-canonical IKK family member TBK1, the activities of IKKε are tightly regulated to maintain immune homeostasis. Several studies have shown that ubiquitination modification plays important roles in regulating IKKε mediated

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Table 1
Primers used in the study.

Primer name	Sequence (5′-3′)	Primer information
CDS		
bcTRIM25-F	ATGGCGGAAAATATGTCTTTA	For bcTRIM25
bcTRIM25-R	TCAGTTGTTGAGCTTGCACAG	CDS cloning
Expression vector		
bcTRIM25-F	ACTGACGGGCCCATGGCGGAAAATATGTC	For expression vector construction
bcTRIM25-R	ACTGACGGTACCTCAGTTGTTGAGCTTGC	
qRT-PCR		
q- bcTRIM25-F	AAACCCGAACTCAAGAAGAA	*ex vivo* qRT-PCR
q- bcTRIM25-R	CTCGCAATAAGACGCCATAC	
q-SVCV-*M*-F	CGACCGCGCCAGTATTGATGGATAC	
q-SVCV-*M*-R	ACAAGGCCGACCCGTCAACAGAG	
q-SVCV-N-F	GGGTCTTTACAGAGTGGG	
q-SVCV-N-R	TTTGTGAGTTGCCGTTAC	
q-SVCV-P-F	AACAGGTATCGACTATGGAAGAGC	
q-SVCV-P-R	GATTCCTCTTCCCAATTGACTGTC	
q-SVCV-G-F	GATGACTGGGAGTTAGATGGC	
q-SVCV-G-R	ATGAGGGATAATATCGGCTTG	
q-eViperin-F	ATGAAAACTCAAATGTGGACGTA	
q-eViperin-R	GATAGTTTCCACCCATTTCCTTAA	
q-ePKR-F	ACCTGAAGCCTCCAAACATA	
q-ePKR-R	GCATTCGCTCATCATTGTC	
q-eMx1-F	TGGAGGAACCTGCCTTAAATAC	
q-eMx1-R	GTCTTTGCTGTTGTCAGAAGATTAG	
q-eISG15-F	TGATGCAAATGAGACCGTAGAT	
q-eISG15-R	CAGTTGTCTGCCGTTGTAAATC	
q-eActin-F	AAGGAGAAGCTCTGCTATGTGGCT	
q-eActin-R	AAGGTGGTCTCATGGATACCGCAA	
shRNA		
bcTRIM25-shRNA-1-F	CCGGGCAAACTGATGGAGTTCTACTCTCGAGAGTAGAACTCCATCAGTTTGCTTTTTG	PLKO.1-shbcTRIM25 construction
bcTRIM25-shRNA-1-R	AATTCAAAAGCAAACTGATGGAGTTCTACTCTCGAGAGTAGAACTCCATCAGTTTGC	
bcTRIM25-shRNA-2-F	CCGGGCAGATGAGCGGCGCTTTAAACTCGAGTTTAAAGCGCCGCTCATCTGCTTTTTG	
bcTRIM25-shRNA-2-R	AATTCAAAAGCAGATGAGCGGCGCTTTAAACTCGAGTTTAAAGCGCCGCTCATCTGC	
bcTRIM25-shRNA-3-F	CCGGGGACCAAATCTGAGATCAACACTCGAGTGTTGATCTCAGATTTGGTCCTTTTTG	
bcTRIM25-shRNA-3-R	AATTCAAAAGGACCAAATCTGAGATCAACACTCGAGTGTTGATCTCAGATTTGGTCC	

signaling. For instance, the IKKε-mediated tumorigenesis requires the K63-linked polyubiquitination by cIAP1/cIAP2/TRAF2 E3 ligase complex [8]. Unanchored K48-linked synthesized by E3-ubiquitin ligase TRIM6 can bind and activate IKKε for STAT1 phosphorylation, promoting IKKε mediated antiviral response [9]. In addition, the formation of the complex between IKKε and TBK1 is important for their kinase activity. Several molecules have been identified as having a negative regulatory effect on type I IFN production by influencing the TBK1/IKKε complex through various mechanisms. For example, suppressor of IKBKE (SIKE), which has been identified as a suppressor of IKKε, blocks type I IFN signaling by disrupting the interaction of IKKε or TBK1 with TRIF and IRF3 [10]. DDX19 has been reported to inhibit IFN production by disrupting TBK1-IKKε-IRF3 interactions [11]. However, compared with TBK1, whose control mechanism has been widely reported, the regulatory mechanism of IKKε still remains largely unknown.

Protein ubiquitination is an important post-translational modification that attaches ubiquitin molecules to the substrate lysine residue in the form of monoubiquitin or polyubiquitin chains [12–14]. A large number of proteins are regulated by ubiquitin-dependent processes, which means that almost all cellular functions are affected by these pathways, including the regulation of life activities such as cell cycle, proliferation, apoptosis, signaling, inflammation, etc [15–18]. E3 ligases play a pivotal role in the process of ubiquitination on account of their substrate-specificity [19]. TRIM25, as an E3 ubiquitin ligase, is involved in a variety of biological processes, including cell growth, necrosis, and tissue homeostasis. In mammals, the role of TRIM25 in antiviral immune response has been widely reported [20]. For example, TRIM25 can inhibit viral replication of infectious bursal disease by targeting VP3 for ubiquitination and degradation [21]. TRIM25 interacts with EBOV vRNP, resulting in self-ubiquitination and ubiquitination of viral nucleoproteins (NPs), allowing NPs to separate from EBOV vRNPs to inhibit Ebola virus replication [22]. In addition, TRIM25 is found to have a dual role in RIG-I regulation. As the first reported protein that is capable of delivering the polyubiquitin moiety to RIG-I, TRIM25 is crucial for RIG-I mediated type I IFN production by K63-linked polyubiquitination in many cases [23–25]. In contrast, TRIM25 also plays a negative role in RIG-I activation by stabilizing FAT10 through the inhibition of its proteasome-dependent degradation [26].

In various teleost species, the regulation roles of TRIM25 in antiviral immune response have been reported. TRIM25 inhibits virus infection and replication by positively regulating antiviral signaling in *Cyprinus carp* and *Danio rerio* [27,28], which relies on promoting K63-Linked ubiquitination of RIG-I. The two TRIM25 isoforms and four alternative splice variants have been cloned in *Larimichthys crocea* and *Parallichthys olivaceus,* respectively [29,30], and both were demonstrated to regulate innate immunity. In orange spotted grouper, the ring domain of TRIM25 is reported to be necessary for the regulation of antiviral effects [31]. However, whether TRIM25 can play a negative role in RIG-I mediated antiviral activity is not illustrated. In this paper, black carp TRIM25 was cloned and the relationship with IFN signaling axis was explored. Our data clearly demonstrate that bcTRIM25 inhibits RLRs mediated IFN signaling by target bcIKKε, which attributes a previously unrecognized regulation mechanism of RIG-I/IKKε/IFN signaling.

2. Materials and methods

2.1. Cells and virus

HEK293T cells, *Epithelioma papulosum* cyprini (EPC) cells, and *Mylopharyngodon piceus* kidney (MPK) cells were maintained in the laboratory. HEK293T cells were cultured at 37 °C and EPC, MPK cells were cultured at 26 °C, all with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% FBS. HEK293T and EPC cells were transfected with PEI, while Lipomax were used to

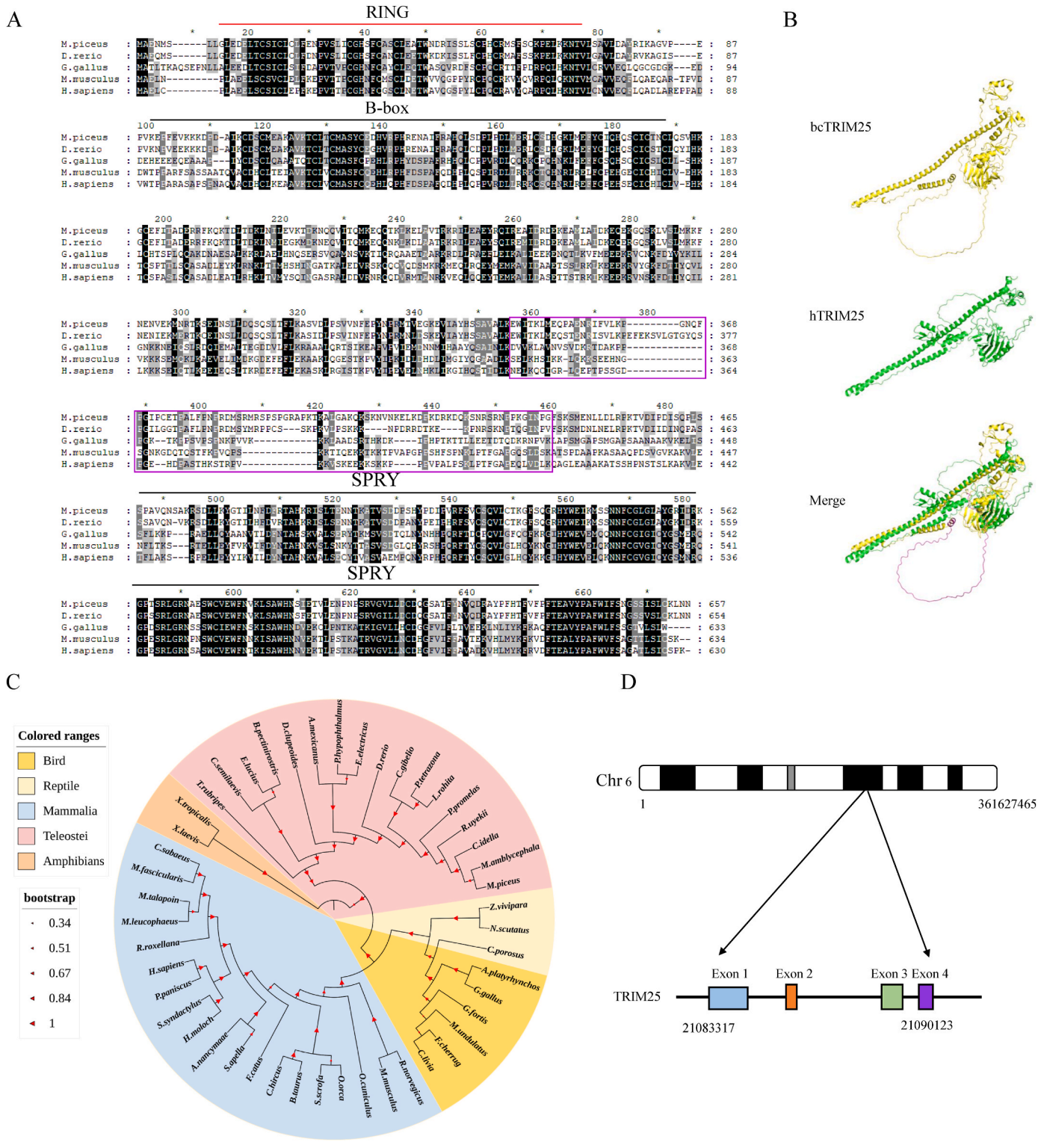

Fig. 1. Homology comparison and phylogenetic analysis.
A: MEGA6 and GeneDox software were used to compare cyprinid TRIM25 with human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*) and zebrafish (*Danio rerio*) TRIM25 homologs. The tagged structural domains were obtained by prediction from the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/guide/domains-structures/). B: Structural prediction of bcTRIM25 and comparison of structural differences with human TRIM25 (hTRIM25). Alpha-Fold site was used for the structure prediction. The Pymol website was used to compare the structure of the human TRIM25 and bcTRIM25. The purple region is the structure of the bcTRIM25 unconcerned region compared to human TRIM25, which corresponds to the amino acid sequence in the purple box in Fig. 1A. C: Phylogenetic tree constructed by MEGA6 software using several representative species of mammals, birds, reptiles, amphibians and fish. Genebank accession numbers are given in Table 2. D: Chromosomal distribution and exon information of the *bcTRIM25* gene.

transfect MPK cells to improve the transfection efficiency respectively according to the manufacturers' instructions. EPC cells were used to produce spring viremia of carp virus (SVCV/strain: SVCV741), while virus titers were determined by plaque assay on EPC cells as previously described [32]. Briefly, the collected virus supernatant was 10-fold serially diluted and added into EPC cells in 24-well plates. The media was removed after incubated for 1 h and fresh DMEM containing 2% FBS and 0.75% methylcellulose (Sigma, USA) was added and then the plaques were measured 72 h post infection.

2.2. Plasmids

pcDNA5/FRT/TO, pcDNA5/FRT/TO-bcIRF3-HA, pcDNA5/FRT/TO-bcIRF7-HA, pcDNA5/FRT/TO-HA-Ub, pcDNA5/FRT/TO-HA-bcRIG-I, pcDNA5/FRT/TO-Flag-bcIKKε, pcDNA5/FRT/TO-bcIKKε-Flag, pRL-TK, Luci-bcIFNa (for black carp IFNa promoter activity analysis) were kept in the lab. The knockdown vector targeting bcTRIM25 was constructed by insert the shRNAs into PLKO.1, which was designed according to the protocol on the website (http://rnaidesigner.thermofisher.com/rnaiexpress).

2.3. Sequence and phylogenetic analysis

ClustalX was utilized for conducting the multiple sequence alignment. The TRIM25 protein sequences from various species were collected and combined in a single fasta file, which was then utilized for conducting the phylogenetic analysis. The MEGA6 program was employed for aligned all of those protein sequences, and the neighbor-joining technique with 1000 replications of bootstrap was used to construct the phylogenetic tree. The genome structure of bcTRIM25 was obtained from the black carp genome database (data unpublished). Additionally, protein three-dimensional structure of bcTRIM25 and human TRIM25 (hTRIM25) was predicted by the Alpha-Fold online tool.

2.4. Immunoblot (IB) and Co-immunoprecipitation (co-IP) analysis

The transfected EPC or HEK293T cells were harvested and lysed for immunoblot (IB) assay as previously described [33]. Briefly, whole cell lysate was transferred to polyvinylidene difluoride (PVDF) membrane after being separated by 10% SDS-PAGE. The membranes were probed with monoclonal primary antibody and secondary antibody. Target proteins were visualized in membrane with BCIP/NBT Alkaline Phosphatase Color Development Kit. For co-immunoprecipitation (co-IP) assay, HEK293T cells seeded in 100 mm cell culture dishes the day before transfection and the cells were harvested at 48 h post transfection (hpt) as previously described [34]. The cellular debris was removed by centrifugation at 10,000×*g* for 5 min at 4 °C. The supernatant was pre-cleared with Protein Agarose A/G beads for 1 h and incubated overnight with anti-Flag/HA agarose beads on a rocker platform at 4 °C. These samples were boiled in SDS sample buffer after 5 times of wash and the eluted proteins were used for IB as above.

2.5. qRT-PCR

Total RNA was isolated for qRT-PCR analysis to measure mRNA abundance of the indicated genes. The relative mRNA expression levels of indicated genes derived from EPC and MPK cells were normalized to the corresponding β-actin. The indicated gene-specific primers were listed in Table 1. The relative expression ratio of the target gene was calculated by $2^{-\Delta\Delta CT}$method.

2.6. Luciferase reporter assays

EPC cells in 24-well plate were transfected with indicated expressing plasmids, pRL-TK, Luci-bcIFNa or Luci-DrIFNφ3. The reporter gene assays were performed using the dual luciferase reporter assay kit (Promega) according to the instruction of the manufacturer. Firefly luciferase activities were normalized on the basis of Renilla luciferase activities.

Table 2
Comparison of bcTRIM25 with other TANK congeners (%).

Specie	Accession ID	Full length sequence	
		Identity	Similarity
Mylopharyngodon piceus	OR145914	100.00	100.00
Megalobrama amblycephala	XP_048054074.1	96.10	97.45
Ctenopharyngodon idella	XP_051754066.1	93.15	94.22
Rhodeus uyekii	AFP48364.1	89.64	93.09
Pimephales promelas	XP_039529483.1	86.90	90.63
Labeo rohita	XP_050967838.1	85.76	90.50
Carassius gibelio	XP_052414268.1	82.50	86.32
Danio rerio	NM_200175.1	82.28	87.69
Puntigrus tetrazona	XP_043098361.1	81.67	86.80
Pangasianodon hypophthalmus	XP_034169530.2	56.93	68.29
Electrophorus electricus	XP_026868177.2	56.24	68.28
Denticeps clupeoides	XP_028827611.1	54.56	65.92
Esox lucius	XP_010887621.2	52.81	65.98
Astyanax mexicanus	XP_007234460.3	52.72	66.25
Boleophthalmus pectinirostris	XP_020795470.2	48.17	60.53
Cynoglossus semilaevis	XP_024914408.1	46.06	59.58
Takifugu rubripes	XP_029706118.1	41.14	55.86
Crocodylus porosus	XP_019397983.1	32.84	47.49
Callorhinchus milii	XP_007909819.2	32.69	49.56
Notechis scutatus	XP_026536611.1	32.06	45.74
Zootoca vivipara	XP_034959729.1	31.73	47.08
Felis catus	NP_001277180.1	31.11	43.85
Gallus gallus	NM_001318458.2	31.03	47.94
Melopsittacus undulatus	XP_030902235.2	30.15	46.57
Capra hircus	XP_005709605.3	29.88	44.82
Homo sapiens	NM_005082.5	29.73	44.53
Hylobates moloch	XP_032018708.1	29.59	44.38
Saimiri boliviensis	XP_039332391.1	29.51	43.60
Anas platyrhynchos	AVP72049.1	29.45	45.72
Symphalangus syndactylus	XP_055114793.1	29.44	44.38
Mandrillus leucophaeus	XP_011834625.1	29.44	44.53
Chlorocebus sabaeus	XP_008009564.2	29.29	44.23
Pan paniscus	XP_003817454.1	29.29	44.38
Macaca fascicularis	XP_005583855.1	29.29	44.23
Miopithecus talapoin	APC26187.1	29.29	44.08
Aotus nancymaae	XP_012315895.1	29.23	43.77
Orcinus orca	XP_012389219.1	29.20	43.81
Sapajus apella	XP_032135252.1	29.19	43.56
Rattus norvegicus	AAH61749.1	29.11	42.65
Bos taurus	NP_001093806.1	28.99	43.79
Sus scrofa	XP_005657028.3	28.99	43.34
Rhinopithecus roxellana	XP_010378950.1	28.99	44.08
Mus musculus	NM_009546.2	28.84	43.92
Oryctolagus cuniculus	XP_051681443.1	28.80	42.98
Falco cherrug	XP_027664054.1	24.22	38.57
Columba livia	PKK25645.1	23.94	37.44
Geospiza fortis	XP_030916770.1	23.81	38.54
Xenopus laevis	XP_018097229.1	21.97	36.32
Xenopus tropicalis	XP_012810659.2	21.52	35.29
Mesocricetus auratus	XP_040605982.1	20.58	33.47
Etheostoma cragini	XP_034734772.1	13.14	25.00

2.7. Immunofluorescence microscopy

The transfected cells were fixed with 4% paraformaldehyde at 24 hpt then permeabilized with Triton X-100 (0.2% in PBS) and stained indicated antibody as previously described. The 100x oil lens of the Olympus confocal microscope was used for fluorescence observation of stained cells.

2.8. Statistics analysis

All statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or Dunnett's post-hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ was

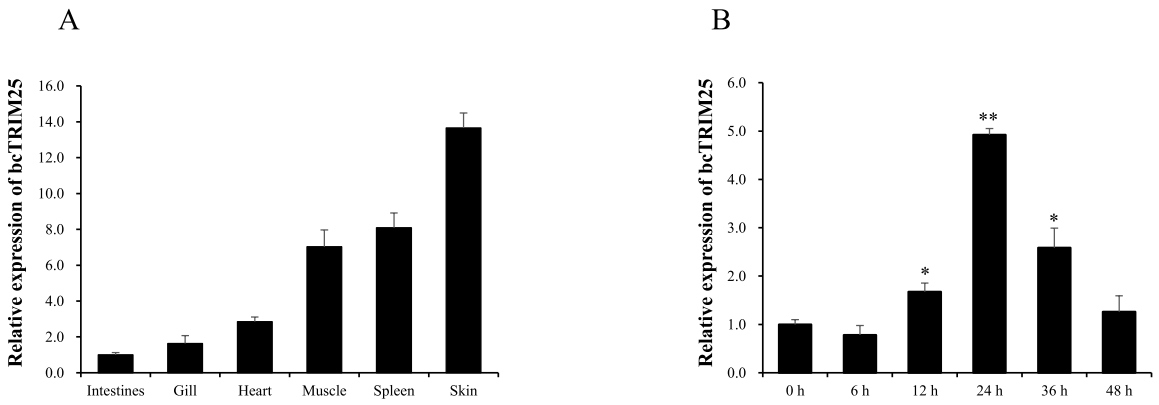

Fig. 2. bcTRIM25 expression patterns.
A: Tissue distribution exhibited by bcTRIM25 in healthy black carp. B: Expression profile of bcTRIM25 in MPK cells treated with indicated MOI of SVCV infection for indicated times.

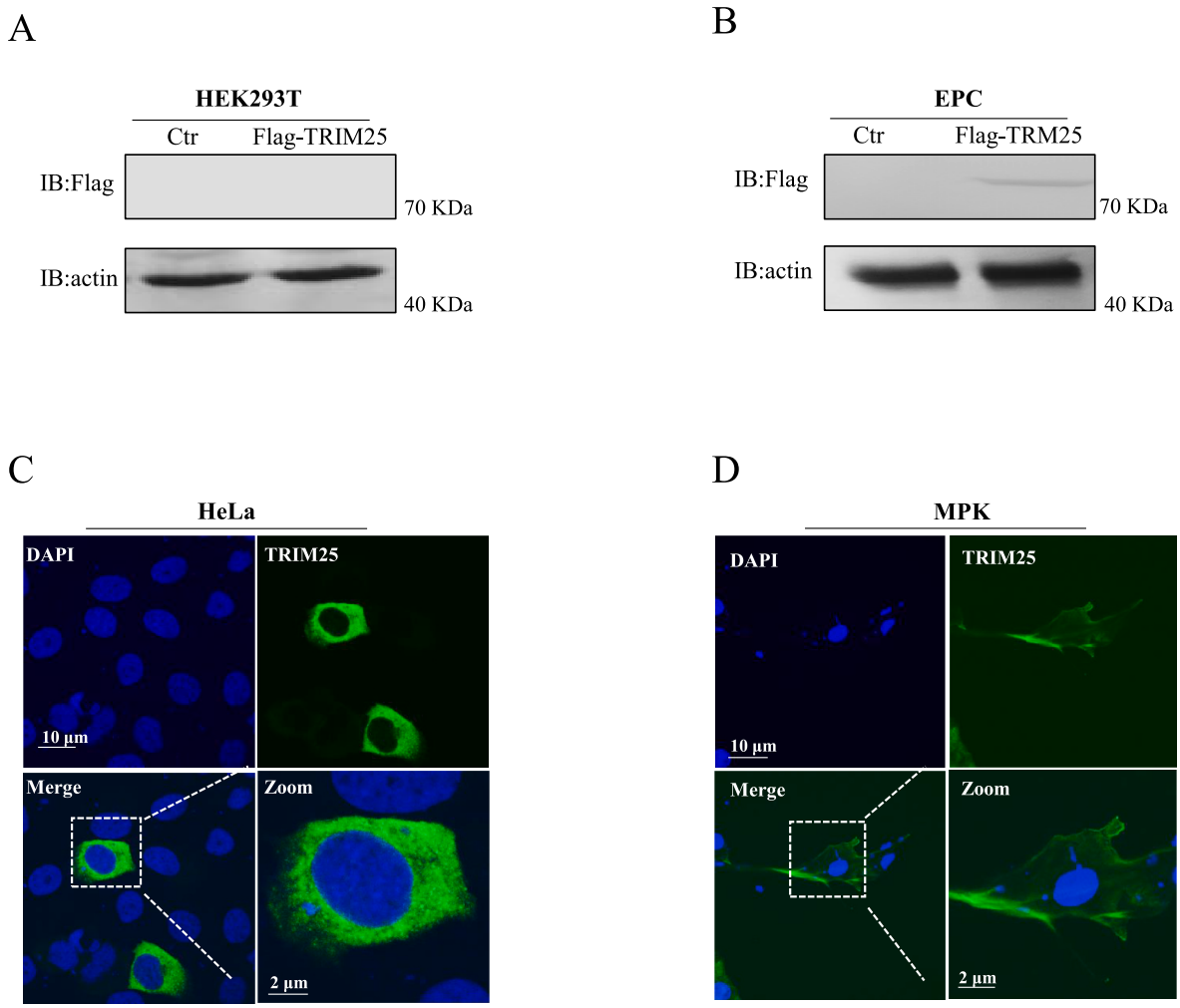

Fig. 3. Protein expression and intracellular localization of bcTRIM25.
A: bcTRIM25 recombinant vector was over-expressed in HEK293T (A) and EPC (B), and then the protein expression of bcTRIM25 was detected by WB assay, and β-actin was used as an internal reference. Intracellular localization of bcTRIM25 was investigated using immunofluorescence assays after over-expression of bcTRIM25 in HeLa (C) and MPK (D) cells, respectively.

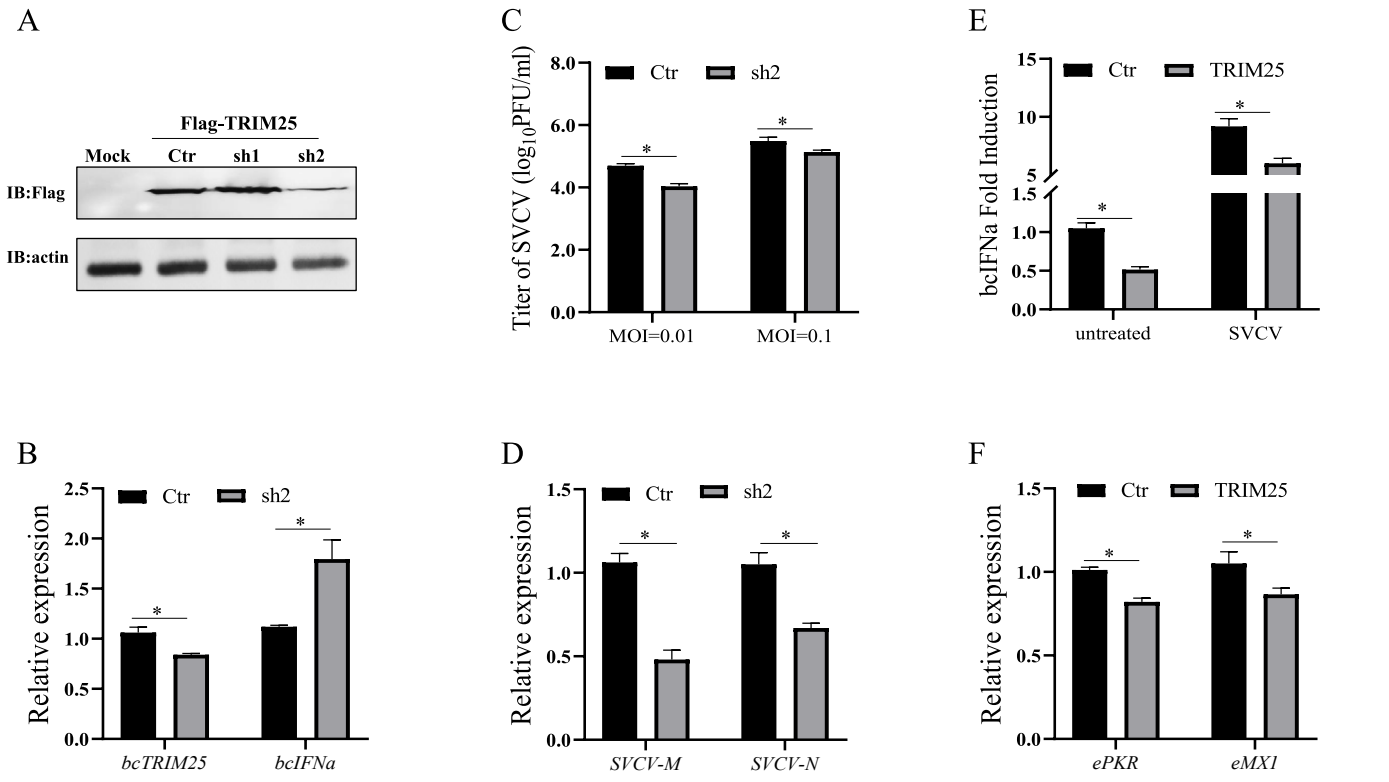

Fig. 4. bcTRIM25 knock-down improved the antiviral activity.
A: PLKO.1-bcTRIM25-shRNA 1 (sh1) and PLKO.1-bcTRIM25-shRNA 2 (sh2) were co-expressed with pcDNA/FRT/TO-Flag-TRIM25 in HEK293T cells to measure the knockdown efficiency by WB assay. PLKO.1-scramble was used as control. B–D: MPK cells were over-expressed with control plasmids or PLKO.1-bcTRIM25-shRNA 2, respectively. One set of the transfected cells was used for qRT-PCR assay to detect bcTRIM25 and bcIFNa 24 hpt (B). Another set of the transfected cells was infected with SVCV (MOI = 0.01&0.1) 24 hpt. The supernatant was used for virus titer determination (C) and the cell pellet (infected with SVCV at the MOI of 0.1) was used for qRT-PCR assays to detect *SVCV-M&N* mRNA levels 24 h post infection (D). E: EPC cells were transfected with bcTRIM25 and the reporter genes vectors, and the cells were infected with SVCV (MOI = 0.1) 24 hpt, then harvested for reporter assays. F: EPC cells were expressed bcTRIM25 control vector and transfected cells were harvested for qRT-PCR assay to detect endogenous expression of *PKR* and *MX1*.

marked as significant.

3. Result

3.1. Molecular cloning and sequence analysis of bcTRIM25

The cDNA of bcTRIM25 was cloned from spleen of black carp, which contains 1959 nucleotides. The predicted bcTRIM25 protein size is 74 KDa, which is conserved with other vertebrate congeners, particularly in RING (8–77 aa), B-box (103–189 aa) and SPRY (485–653 aa) domain (Fig. 1A&B). The structure prediction shows that the structure of black carp TRIM25 and human TRIM25 are similar, but there are certain differences in spatial folding, especially the non-conserved 355–460 amino acids (Fig. 1B). Phylogenetic analysis classifies the listed TRIM25 homolog into five categories, mammalian, avian, reptilian, amphibian and piscine branch. In the piscine branch, bcTRIM25 is closely related to TRIM25 homolog of *Megalobrama amblycephala* and *Ctenopharyngodon idella* (Fig. 1C and Table 2), which are also cyprinids fishes. Genomic information indicates that the bcTRIM25 gene is located on chromosome 6 (21083317–21090123 bp), with four exons and three introns (Fig. 1D).

3.2. mRNA expression of bcTRIM25 in vivo and ex vivo

To explore bcTRIM25 transcript in various tissues, the mRNA levels of bcTRIM25 were tested by qRT-PCR in the healthy black carp intestine, gill, skin, muscle, spleen and heart. Among the six tissues listed, the transcriptional levels of bcTRIM25 were comparatively higher in muscle, spleen and skin and were relatively lower in intestine, gill and heart (Fig. 2A). Subsequently, the temporal expression profile of bcTRIM25 in MPK cells were detected at different times post SVCV infection (Fig. 2B). After viral infection, the mRNA level of bcTRIM25 gradually increased until it reached its highest value at 24 h. Then, mRNA levels of bcTRIM25 gradually decrease as the infection time increasing. These data suggest that bcTRIM25 responds to SVCV infection and imply that it may play different roles at different periods in the virus infection.

3.3. Protein expression and cellular localization of bcTRIM25

Immunoblot assays data showed that a specific band about 75 KDa was detected in cells transfected with bcTRIM25, which indicated that the expression vector of TRIM25 could be successfully expressed both in HEK293T and EPC cells (Fig. 3A&B). To explore the subcellular structure localization of bcTRIM25, HeLa and MPK cells were transfected bcTRIM25 and used for immunofluorescence. The results showed that bcTRIM25 (green signal) was mainly located in cytoplasm (Fig. 3C&D), which suggested that bcTRIM25 is a cytoplasmic protein.

3.4. bcTRIM25 diminished IFN signaling

The shRNA targeting bcTRIM25 was constructed to investigate role of bcTRIM25 in antiviral signaling. The IB results showed that bcTRIM25 shRNA 2 (sh2) could significantly inhibit the protein expression level of over-expressed bcTRIM25 (Fig. 4A). Corresponding to this, the qRT-PCR results showed that over-expressing bcTRIM25

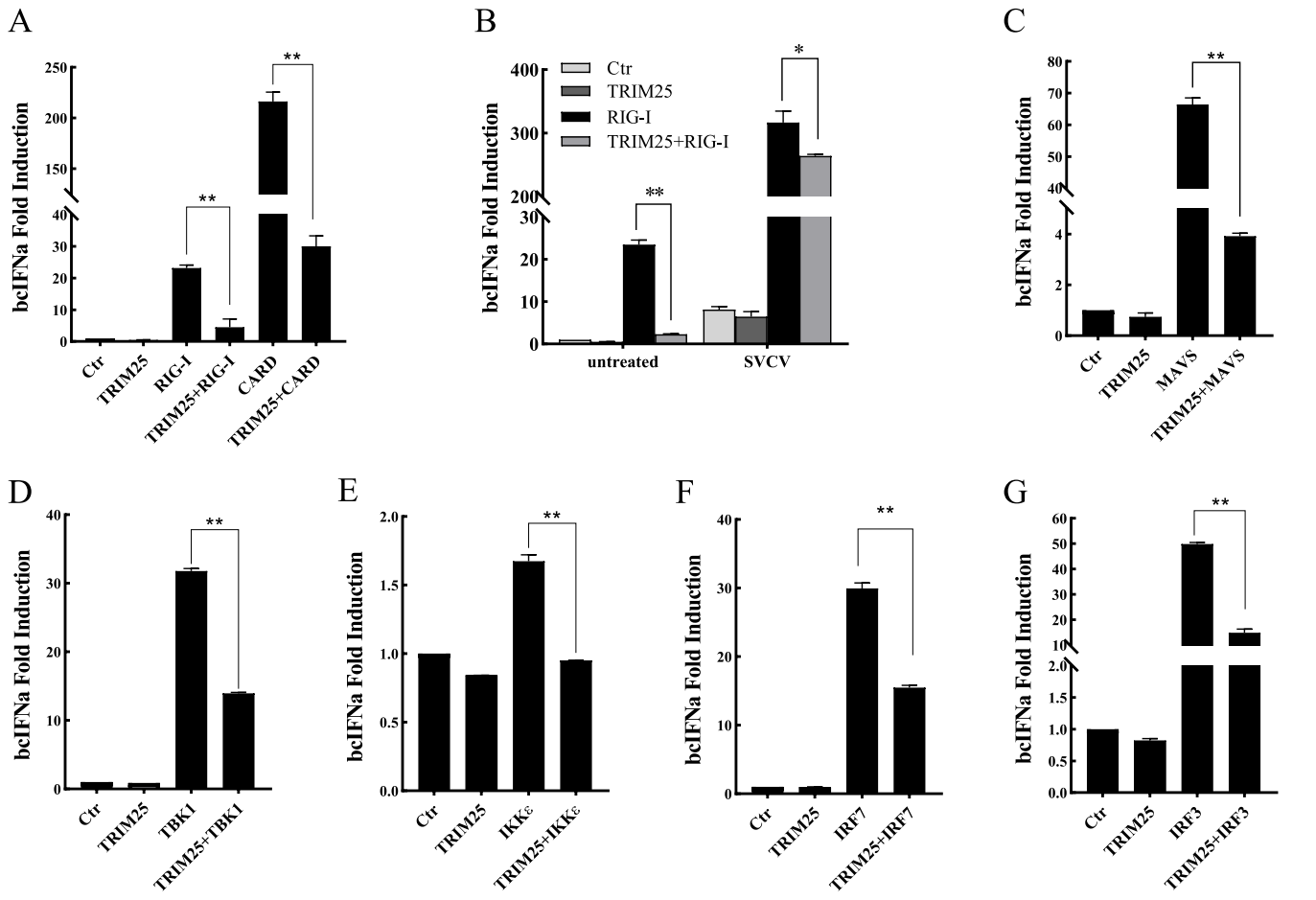

Fig. 5. bcTRIM25 overexpression attenuated IFN signaling.
Plasmids expressing bcTRIM25 and bcRIG-I, bcRIG-I-CARD (A), bcMAVS (C), bcTBK1 (D), bcIKKε (E), bcIRF3 (F) and bcIRF7 (G) were singly or co-expressed in EPC cells with the indicated reporter genes vectors. Cells were harvested for reporter assays 24 hpt. bcTRIM25 and bcRIG-I singly or co-expressing cells were infected with SVCV (MOI = 0.1) and used for reporter assays 24 hpt (B).

shRNA 2 in MPK cells could inhibit the transcription level of endogenous bcTRIM25, however, promoting the mRNA expression level of bcIFNa (Fig. 4B). It was interesting that knocking down bcTRIM25 in MPK cells significantly reduced the viral titers in cell supernatant after virus infection and also decreased the mRNA expression levels of SVCV M and N proteins in the cell pellet (Fig. 4C&D). On the contrary, overexpression of bcTRIM25 in cells significantly inhibited the bcIFNa promoter transcriptional activity and impaired the mRNA expression levels of PKR/MX1 (Fig. 4E&F). All these data suggest the negative role of bcTRIM25 in antiviral response.

Previously studies in mammals have showed that TRIM25 enhanced RIG-I antiviral signaling [23]. In order to explore the mechanism behind the negative regulation role of TRIM25 in the antiviral immunity in black carp, the regulatory relationship between bcTRIM25 and bcRIG-I was studied through reporter assay. Unlike its mammalian counterpart, results showed that bcTRIM25 suppresses bcRIG-I or CARD-mediated IFN promoter activity (Fig. 5A). And SVCV infection does not relieve this detraction effect (Fig. 5B). Furthermore, the regulatory relationship

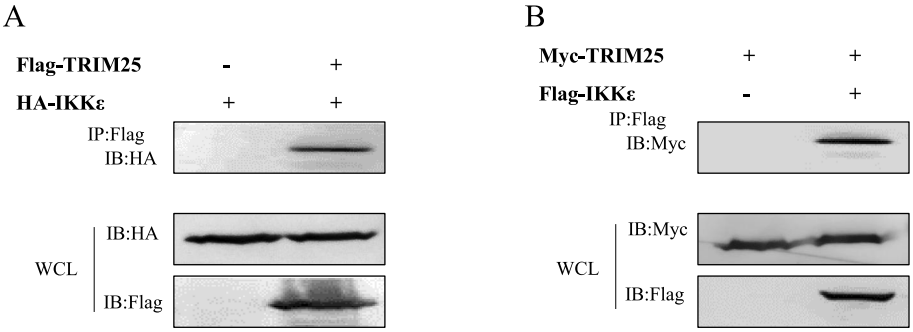

Fig. 6. bcTRIM25 interacted with bcIKKε.
A&B: HEK293T cells were over-expressed with bcTRIM25 and/or bcIKKε and harvested for co-IP assay at 48 hpt. The histogram was the quantitative analysis of related ubiquitination of bcIKKε.

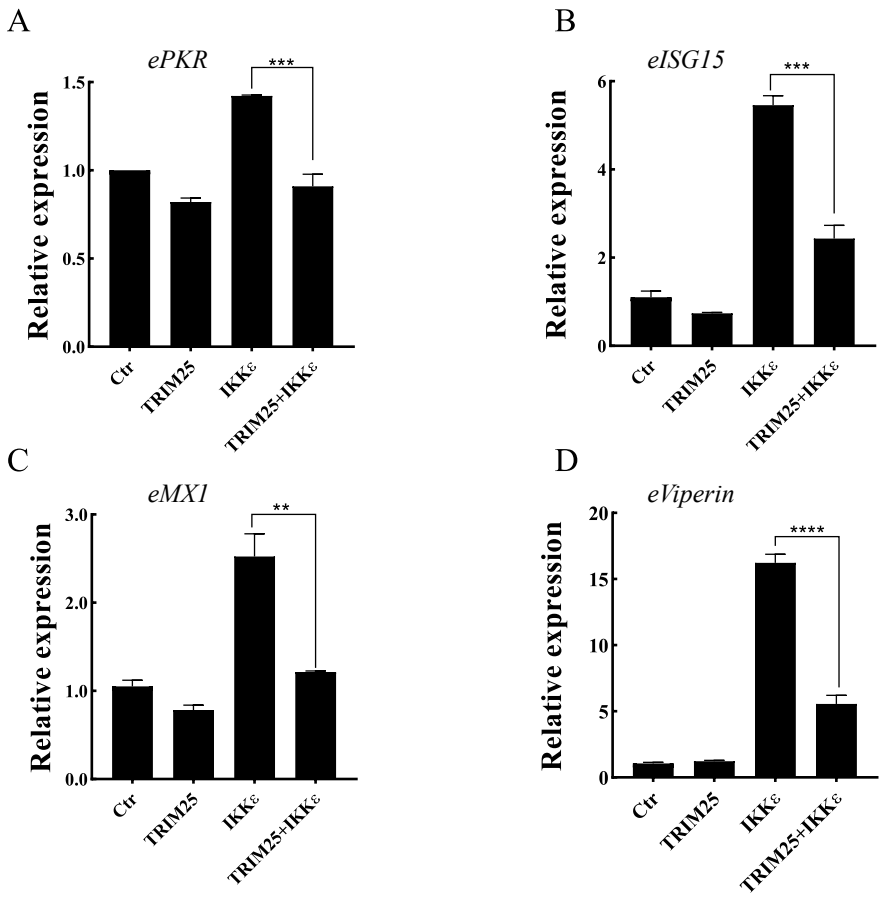

Fig. 7. bcTRIM25 attenuated bcIKKε-induced ISGs expression.
bcTRIM25 and/or bcIKKε was over-expressed in EPC cells and the transfected cells were harvested at 24 hpt. The relative mRNA levels of *PKR*, *ISG15*, *MX1* and *Viperin* were detected by qRT-PCR assays.

between bcTRIM25 and other key factors of RLR signal pathway was studied with reporter assay. The data showed that bcTRIM25 significantly inhibits bcMAVS/TBK1/IKKε/IRF3/IRF7-mediated IFN promoter transcriptional activity (Fig. 5C–G). These data suggest that bcTRIM25 negatively regulates RLR mediated antiviral signaling.

3.5. *bcTRIM25 attenuated bcIKKε-mediated antiviral response*

To explore the targets of bcTRIM25 in regulating RLR signaling, the interaction between TBK1/IKKε/IRF3/IRF7 were examined by co-IP assays. The results showed that bcTRIM25 interacted with bcIKKε, but not bcTBK1, bcIRF3 and bcIRF7 (Fig. 6&SFig1). These indicate that bcTRIM25 may target IKKε to participate in the inhibition of IFN signaling.

To confirm the regulatory role of bcTRIM25 on bcIKKε mediated IFN signaling, bcIKKε were over-expressed in EPC cells with or without bcTRIM25, and the mRNA levels of ISGs genes, including *PKR*, *ISG15*, *MX1* and *Viperin* were examined by qRT-PCR. The results showed that bcIKKε expression significantly induced transcription of the above ISGs (Fig. 7). However, bcIKKε-induced transcription of these ISGs were all impaired when co-expressed with bcTRIM25 (Fig. 7). Furthermore, bcIKKε over-expression signally reduced the virus titer in the supernatant of cells after SVCV infection, while bcTRIM25 co-expression prominently debilitated this effect (Fig. 8A&B). Similarly, the reduced mRNA expression levels of SVCV proteins by bcIKKε in EPC cells were also rescued to some degree by bcTRIM25 co-expression (Fig. 8C–F). These data illustrate that bcTRIM25 observably inhibits bcIKKε-mediated IFN antiviral signaling.

3.6. *The regulated ubiquitination and phosphorylation of bcIKKε by bcTRIM25*

To investigate the regulatory mechanism of bcTRIM25 on bcIKKε mediated antiviral response, the influence of bcTRIM25 on the post-translational modification of bcIKKε, including ubiquitination and phosphorylation, were examined. The ubiquitination level of bcIKKε in the group of bcIKKε expression alone was obviously lower than that of bcIKKε in the group of bcIKKε/bcTRIM25 co-expression, which demonstrated that bcTRIM25 enhanced the ubiquitination modification of bcIKKε, considering that co-expressed bcTRIM25 did not change the protein expression level of bcIKKε (Fig. 9A). It was interesting that the phosphorylation level of bcIKKε in the group of bcIKKε expression alone was obviously higher than that of bcIKKε in the group of bcIKKε/bcTRIM25 co-expression, which indicated that bcTRIM25 dampened the ubiquitination modification of bcIKKε. Thus, our data suggest that bcTRIM25 negatively regulates IFN signaling by regulating phosphorylation and ubiquitination of bcIKKε.

4. Discussion

Recent studies have revealed a significance of a variety of TRIM proteins in the regulation of fine-tuning of innate immune responses [35,36]. Subsequently, multiple TRIM family members are explored to play vital regulatory role in antiviral signaling, such as TRIM8 and TRIM22

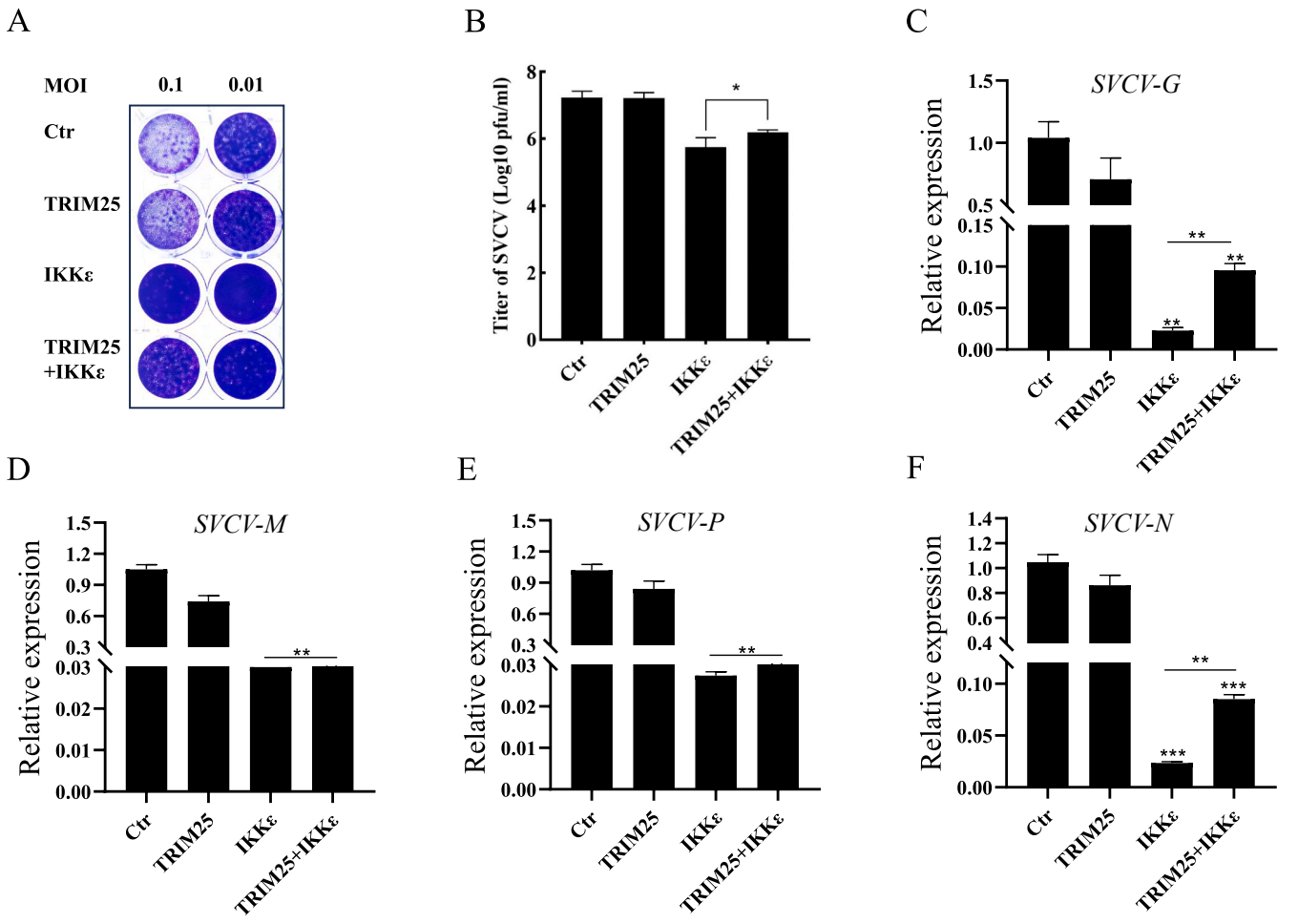

Fig. 8. bcTRIM25 inhibited bcIKKε-mediated antiviral ability.
EPC cells were transfected with bcTRIM25 and/or bcIKKε, then infected with SVCV (MOI = 0.1 or 0.01). The infected cells were cultured with semi-solid medium until used for crystalline violet staining, respectively (A). The other groups were infected with SVCV at MOI = 0.01 and cultured with liquid medium for 24 h. The supernatant was used for titer determination (B) and the cell pellet was used for qRT-PCR assay to detect the mRNA expression levels of viral proteins (C–F).

[37–39]. In contrast, some members of TRIM family also negatively regulate signaling pathways in innate immunity, which have been selected by some viruses as targets for immune escape sites. For example, TRIM19 and TRIM23 [40–42]. In this study, TRIM25 was identified as a negative regulatory factor RLR/IFN signaling [26]. The results showed that knock-down of bcTRIM25 enhanced the antiviral ability of host cells and overexpression of bcTRIM25 in EPC cells not only attenuated bcIFNa promoter transcription activity but also impeded mRNA expression of antiviral genes. In addition, our results indicated TRIM25 negatively regulates interferon signaling through binding and promoting ubiquitination of IKKε in black carp, which expanded the understanding of the regulation mechanism of TRIM25 in vertebrate.

As an important member of the TRIM family, TRIM25 is reported to play positive or negative role in regulating IFN signaling. Numerous studies have identified that TRIM25 plays positive role in RIG-I-mediated antiviral activity [24]. In mammalian, TRIM25 is able to catalyze the K63 linked ubiquitination at the Lys 172 residue of RIG-I, leading to a robust increase in RIG-I downstream signaling [43]. In teleost, both the zebrafish and common carp TRIM25 could promote the K63-linked polyubiquitination of RIG-I, positively regulating RIG-I mediated antiviral signaling [27]. For its negative role in IFN signaling, there was only one report showing that TRIM25 attenuated the activation of RIG-I by modulating the stability of FAT10, a negative regulator of RIG-I mediated inflammatory response [26]. However, in this study, bcTRIM25 was proved to diminish bcRIG-I mediated type I IFN production in cells both at the resting state or after virus challenge. Our study provides direct evidence for the negative role of bcTRIM25 in RIG-I mediated antiviral signaling.

Previous studies show that TRIM25 inhibits viral replication mainly by two ways. On the one hand, TRIM25 promotes the degradation of the structural protein or destroys virus ribonucleoprotein complex and thus attenuates viral replication [21,22]. On the other hand, it can also bind RIG-I and TRAF2 to promote its induced activation of IFN signaling and inflammatory signaling [44]. In this study, bcTIRM25 was found to interact with bcIKKε and restricted bcIKKε-mediated antiviral signaling by promoting its ubiquitination, which is different from its mammalian counterpart. As a key kinase that phosphorylates IRF3/7 and subsequently promotes IFN production, the kinase activity of IKKε is tightly regulated to achieve immune homeostasis [45].

Numerous molecules play a role as negative regulators in the regulation of type I interferon (IFN) production by influencing the TBK1/IKKε complex through various mechanisms [47] [[46,47]]. However, the regulation mechanisms of IKKε by ubiquitination are poorly understood. Especially in teleost fish, the regulatory mechanism of IKKε is largely unknown. In our previous article, bcIKKε was identified to play a positive role in bcIRF3/7-mediated antiviral activity. The results showed that the K39 of bcIKKε was independent for its kinase activity, which is highly conserved in vertebrates [33]. In the present study, we demonstrated that bcTRIM25 inhibited bcIKKε-mediated antiviral activity by

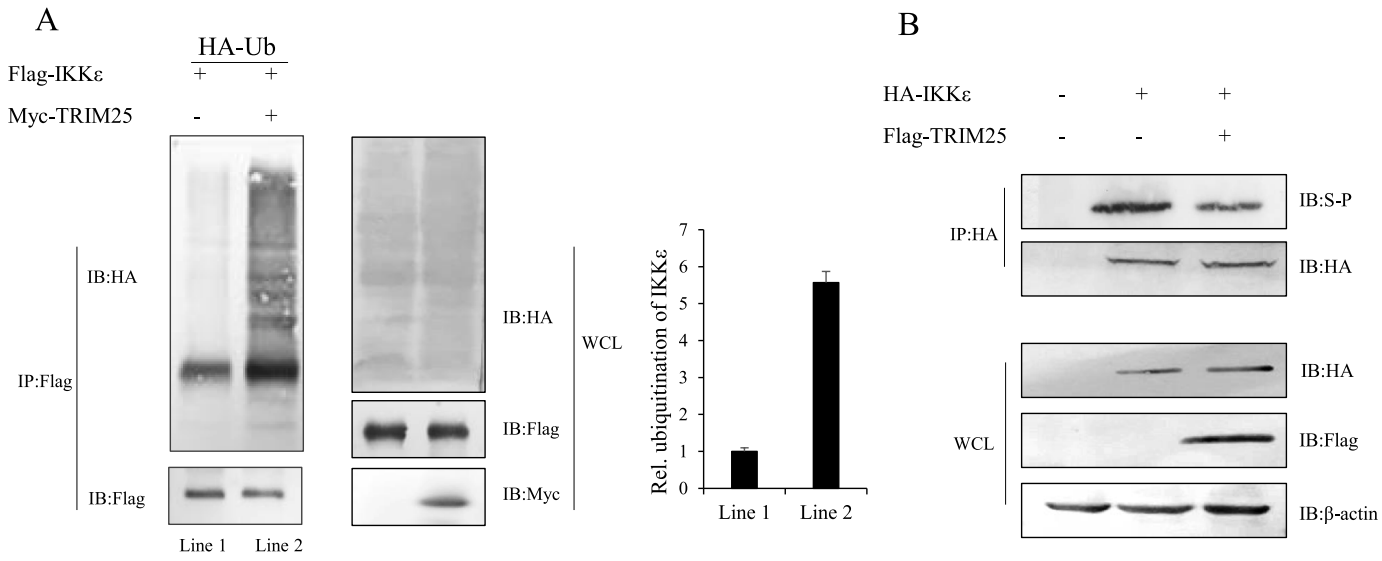

Fig. 9. bcTRIM25 regulated the post-translation modifications of bcIKKε.
A: The ubiquitin recombinant expression vector and the plasmid were over-expressed in HEK293T cells and used for ubiquitination modification assays at 48 hpt. B: bcIKKε and/or bcTRIM25 were over-expressed in HEK293T cells and the transfected cells were harvested to detect the phosphorylation level of bcIKKε at 48 hpt. S–P: Phosphoserine Monoclonal Antibody (ABM40198) purchased from Abbkine.

regulating the ubiquitination and phosphorylation of bcIKKε (Fig. 9). To our knowledge, this is the first report in vertebrate that TRIM25 negatively regulates IKKε through enhancing its ubiquitination. Based on the previous studies on IKKε in mammalian, it is reasonable to believe that the activity of bcIKKε is greatly related to its phosphorylation, and ubiquitination also plays an important role in its activation. However, the relationship between phosphorylation and ubiquitination of bcIKKε needs further study.

Overall, our data presented a new perspective on the regulatory role of TRIM25 in teleost, which showed that bcTRIM25 was involved in the negative regulation of the RIG-I antiviral signaling axis. In addition, it was also found for the first time that TRIM25 could target IKKε to promote its ubiquitination modification and inhibit its phosphorylation modification, thus achieving attenuated IFN signaling and maintaining immune homeostasis.

CRediT authorship contribution statement

Can Yang: Investigation, and, Writing – original draft. **Juanjuan Shu:** Methodology, and, Visualization. **Yujia Miao:** Visualization. **Xiaoyu Liu:** Software. **Tianle Zheng:** Investigation. **Ruixin Hou:** Methodology. **Jun Xiao:** Conceptualization, and, Writing – review & editing. **Hao Feng:** Writing – review & editing.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2023.109095.

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