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TBK1 of black carp plays an important role in host innate immune response against SVCV and GCRV

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ABSTRACT

Tank-binding kinase 1 (TBK1) plays a pivotal role in the induction of type I IFNs in higher vertebrates. To explore the function of TBK1 in teleost, TBK1 of black carp (*Mylopharyngodon Piceus*) was cloned and characterized in this paper. The full-length cDNA of black carp TBK1 (bcTBK1) consists of 2857 nucleotides and the predicted bcTBK1 protein contains 727 amino acids, which includes an N-terminal kinase domain (KD), an ubiquitin-like domain (ULD) and two C-terminal coiled-coils. The transcription of bcTBK1 was constitutively detected in all the selected tissues and bcTBK1 mRNA level was increased in all selected tissues in response to SVCV or GCRV infection except that in muscle post GCRV invasion. The transcription of bcTBK1 in *Mylopharyngodon Piceus* fin (MPF) cells was up-regulated by the stimulation of SVCV, GCRV or poly (I:C) but not by LPS treatment. bcTBK1 migrated around 80 kDa in immunoblot assay and was identified as a cytosolic protein by immunofluorescence staining. bcTBK1 showed strong IFN-inducing ability in reporter assay and presented strong antiviral activity against both GCRV and SVCV in EPC cells. The reporter assay demonstrated that TRAF6 of black carp (bcTRAF6) up-regulated bcTBK1-induced IFN expression and the subcellular distribution of bcTBK1 overlapped with that of bcTRAF6 when these two proteins were co-expressed in EPC cells. Taken together, our study support the conclusion that bcTBK1 plays an important role in the antiviral innate immune response of black carp against SVCV and GCRV, in which its activity was positively regulated by bcTRAF6.

1. Introduction

The innate immune system is an evolutionally conserved mechanism, which has been found protecting the host by representing the first line of defense against pathogenic microbes. However, innate immunity differs among vertebrates in different aspects [1]. As lower vertebrate, fishes do not developed full adaptive immunity, innate immunity becomes indispensable for them surviving from the disadvantage environment [2–4]. The innate immune system recognizes conserved pathogen-associated molecular patterns (PAMPs), such as bacterial peptidoglycans, lipopolysaccharides (LPS) and viral components, via limited number of pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and cytosolic DNA sensors [5]. Upon the effective detection of invading virus, host cells are activated to express cytokines, chemokines and type I interferons (IFNs) to initiate the antiviral immune responses [6–8].

In RLR pathways, RIG-I and MDA5 recognize viral dsRNA in the cytosol and signal through the adaptor protein MAVS, which localize mainly on the outer mitochondrial membrane [9]. Triggered by RIG-I/MDA5, MAVS recruits TBK1 and inhibitor of NF-κB kinase ε (IKKε), two non-canonical members of the IκB kinases (IKKs) family, to phosphorylate and activate IRF3/7, which translocate into the nucleus to regulate the transcription of type I IFNs and IFN-stimulated genes (ISGs) [5,10,11].

Increasing evidence implies that ubiquitination plays a pivotal role in the activation of TBK1 by tumor necrosis factor receptor-associated factors (TRAFs), which are recruited by mitochondria

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antiviral signaling protein (MAVS) to form a complex with TBK1, such as TRAF3 and TRAF6 [12,13]. The mutations that targeted the DLAIS, a highly conserved motif of MAVS, abolished mindbomb E3 ubiquitin protein ligase2 (MIB2) binding, attenuated the K63-linked ubiquitination of TBK1, and decreased the phosphorylation-mediated activation of IRF3/7 [14].

To date, TBK1 has been cloned from only few teleost, such as zebrafish (*Danio rerio*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), crucian carp (*Carassius auratus*), Atlantic cod (*Gadus morhua*), Atlantic salmon (*Salmo salar*) and tilapia (*Oreochromis niloticus*) [15,16]. In contrast to the deep understanding of innate immune system in mammals, the mechanism in fish how TBK1 functions as a regulator in MAVS-mediated activation of IRF3/7 still remains poorly understood [16]. To further explore the role of teleost TBK1 in host antiviral innate immune response, TBK1 ortholog from black carp (*Mylopharyngodon Piceus*) has been identified and characterized in this study.

Black carp is among the "four famous domestic fishes" for over a thousand years in China and is the most highly esteemed and expensive food fish among the four domestic fishes. This industrial important species is subjected to bulk of pathogenic microbes in both natural and aquaculture condition such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV). In our previous study, MAVS and IKKε orthologs (bcMAVS and bcIKKε) had been cloned and characterized from black carp, and similar to their mammalian counterparts, bcMAVS and bcIKKε were recruited into in the host antiviral immune responses against SVCV and GCRV [17,18]. Both caspase recruitment domain (CARD) domain and transmembrane (TM) domain of bcMAVS played the key role in its self-association and antiviral ability [19], what is more, TRAF6 of black carp (bcTRAF6) was recruited by bcMAVS into its mediated antiviral signaling [20]. In this paper, the role of bcTBK1 in host antiviral signaling was explored and our data demonstrated that bcTBK1 played an important role in the innate immune response against both SVCV and GCRV, in which bcTRAF6 functioned as a positive regulator of bcTBK1.

2. Materials and methods

2.1. Cells and plasmids

HEK293T (293T) cells, HeLa cells, Epithelioma Papulosum Cyprinid (EPC) cells, *C. idella* kidney (CIK) cells and *Mylopharyngodon piceus* fin (MPF) cells were kept in the lab [21]. All the cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 u/ml penicillin and 100 μg/ml streptomycin. HEK293T cells and HeLa cells were cultured 37 °C with 5% CO_2; while fish cells were cultured at 26 °C with 5% CO_2. Transfection was done as previously described, calcium phosphate was used for 293T transfection, Fugene®6 (Promega) was used for EPC transfection and Lipofectamine®2000 was used for HeLa transfection [22].

pcDNA5/FRT/TO-HA, pcDNA5/FRT/TO-Flag, pcDNA5/FRT-TO-HA-bcTRAF6, pRL-TK, Luci-zIFN3 (for zebrafish IFN3 promoter activity analysis) and Luci-eIFN (for fathead minnow IFN promoter activity analysis) were kept in the lab [23]. The recombinant vectors expressing bcTBK1 were constructed by inserting the open reading frame (ORF) of black carp TBK1 (bcTBK1) into pcDNA5/FRT/TO-HA or pcDNA5/FRT/TO-Flag between *Kpn*I and *Xho*I restriction sites separately, with a HA tag at its N-terminus for pcDNA5/FRT/TO-HA-bcTBK1 and C-terminus for pcDNA5/FRT/TO-TBK1-HA, for pcDNA5/FRT/TO-Flag-bcTBK1 and pcDNA5/FRT/TO-bcTBK1-Flag, the Flag tag was fused at the N-terminus and C-terminus accordingly.

2.2. Cloning of bcTBK1 cDNA

Total RNA was isolated from the spleen of black carp and first-strand cDNA were synthesized by using the Revert Aid First Strand cDNA Synthesis Kit (Thermo). Degenerate Primers were designed for amplifying bcTBK1 cDNA basing on the TBK1 sequences of *C. carpio* (HQ850442.1) and *C. auratus* (JF970228.1) and *D. rerio* (DQ860098.1). The coding sequence (CDS) was cloned at the first attempt by using the degenerate primers. Rapid amplification of cDNA ends (RACE) was performed to obtain 5′UTR and 3′UTR of

Table 1
Primers used in the study.

Primer name	Sequence(5′-3′)	Primer information
5′RACE		
bcTBK1-5′UTR-R1	GGAAGGTCTGAGCCTGACGGTTG	5′ UTR 1st PCR
bcTBK1-5′UTR-R3	GGACGTCCAGCGGACGCAGGAAGC	5′ UTR 2nd PCR
outer primer	CATGGCTACATGCTGACAGCCTA	
inner primer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG	
3′RACE		
bcTBK1-3′UTR-F1	GAAGAAGGTGATGCGGCTGTCC	3′ UTR 1st PCR
bcTBK1-3′UTR-F3	AACCGCAGGCGTATCTCAGTCC	3′ UTR 2nd PCR
UPM-longer	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UPM-Short primer	CTAATACGACTCACTATAGGGC	
ORF		
bcTBK1-F	ATGCAGAGTACGGCGAACTACC	ORF cloning
bcTBK1-R	TCACATCCGATCCACGGCCCTG	
Expression construct		
bcTBK1-N-F	ACTGACGGTACCGCCACCATGCAGAGTACGGCG	FRT-TO-bcTBK1-HA, FRT-To-Flag-bcTBK1
bcTBK1-N-R	ACTGACCTCGAGTCACATCCGATCCACGGC	
bcTBK1-C-F	ACTGACGGTACCGCCACCATGCAGAGTACGG	
bcTBK1-C-R	ACTGACCTCGAGCATCCGATCCACGGCCCT	
q-PCR		
Fish actin F	CATGTTCGAGACCTT	*In vivo* Semi-q-PCR
Fish actin R	AGGCAGCTCATAGCT	
bcTBK1-S-F2	AAGTACAGCCACGCTACGAC	*In vivo* Semi-q-PCR
bcTBK1-S-R2	TGAGATACGCCTGCGGTTTG	
bc Q actin-F	TGGGCACCGCTGCTTCCT	*Ex vivo* q-PCR
bc Q actin-R	TGTCCGTCAGGCAGCTCAT	
bcTBK1-R-F1	TCAAACCGCAGGCGTATCTC	*Ex vivo* q-PCR
bcTBK1-R-R1	TCCTTCACCACTCCCTCCAT	

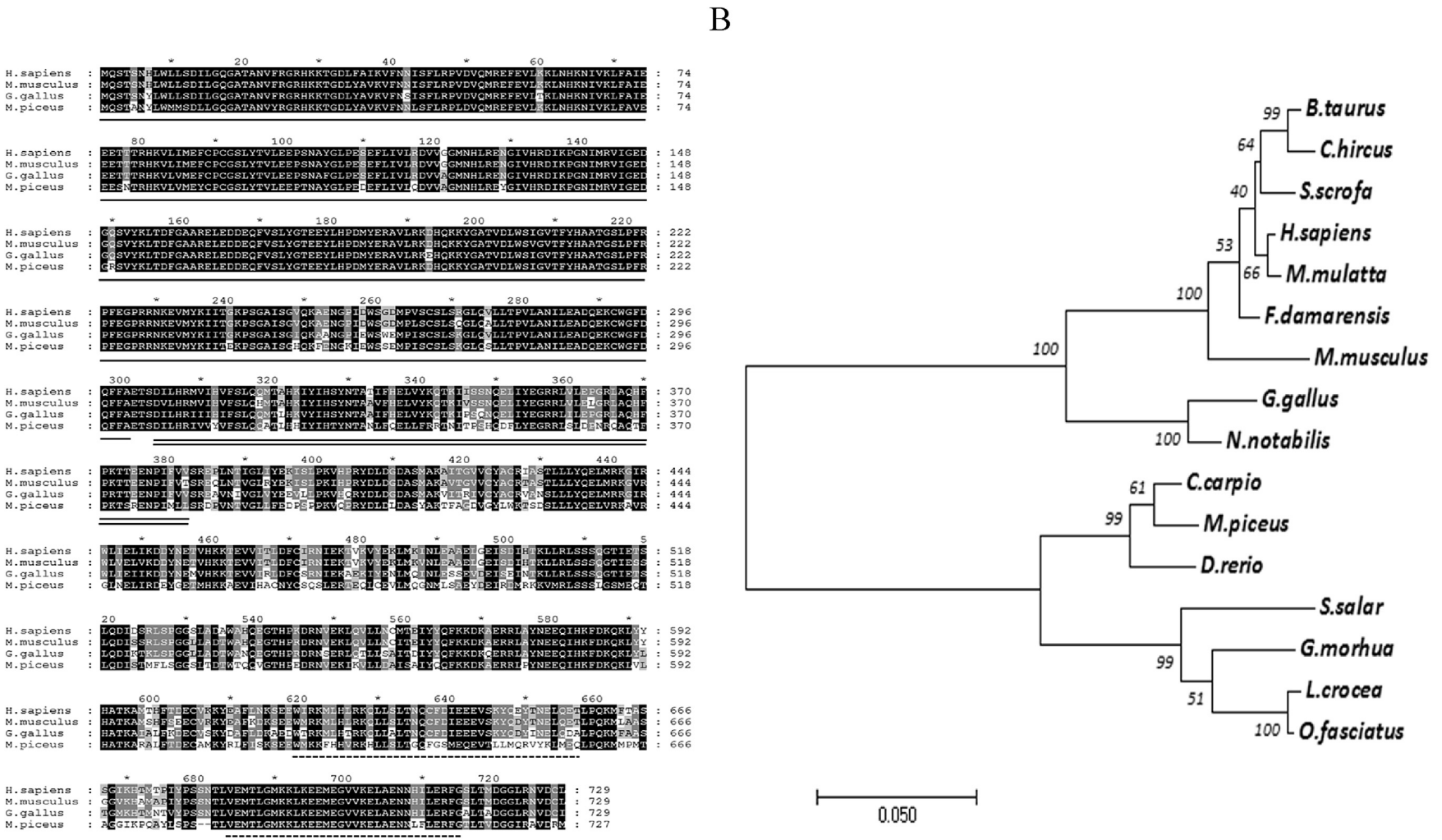

Fig. 1. Evolution of bcTBK1.

(A) Comparisons of bcTBK1 with other vertebrate TBK1 protein sequences by using MEGA 6.0 program and GeneDoc program. N-terminal kinase domain (KD) is underlined; an ubiquitin-like domain (ULD) is marked by double line and two C-terminal coiled-coils are indicated by dash line. (**B**) Phylogenetic tree of vertebrate TBK1. The amino acid sequence of bcTBK1 was aligned with TBK1 from different species by using MEGA 7.0 software, which included (GenBank accession number): *B. Taurus* (NP_001179684.1), *C. hircus* (XP_017903478.1), *S. scrofa* (NP_001098762.1), *F. damarensis* (KFO24499.1), *H. sapiens* (NP_037386.1), *M. mulattan* (NP_001248122.1), *M. musculus* (NP_062760.3), *G. gallus* (NP_001186487.1), *N. notabilis* (XM_010014393.1), *C. carpio* (HQ850442), *D. rerio* (NP_001038213.2), *S. salar* (NP_001243651.1), *G. morhua* (GU937850.1), *L. crocea* (XM_019271185), *O. fasciatus* (KF267454). The bar stands for scale length and the numbers on different nodes stand for bootstrap value.

Table 2
Comparison of bcTBK1 with other vertebrate TBK1 (%).

Species	Full-length sequence of protein	
	Similarity	Identity
Mylopharyngodon. piceus	100	100
Bos. Taurus	83.8	71.6
Capra. hircus	83.8	71.5
Sus. scrofa	83.4	70.8
Homo. sapiens	84.1	71.6
Macaca. mulatta	84.0	71.6
Fukomys. damarensis	83.6	71.4
Mus. musculus	83.7	71.5
Gallus. gallus	82.6	70.8
Nestor. notabilis	59.0	51.0
Cyprinus. carpio	98.1	96.4
Danio. rerio	97.1	93.9
Salmo. salar	92.9	84.4
Gadus. morhua	92.0	83.5
Larimichthys. crocea	92.7	85.4
Oplegnathus.fasciatus	92.7	85.3

bcTBK1 cDNA by using 5′Full RACE kit and 3′Full RACE kit separately (TaKaRa). The amplified fragments were cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titer

SVCV and GCRV were kept in the lab [23]. SVCV and GCRV were propagated in EPC and CIK separately at 25 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque forming assay on EPC cells separately as previously described [23]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 25 °C. The supernatant was removed after incubation and DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) was added. Plaques were counted at day 3 post-infection.

2.4. Semi-quantitative RT-PCR

Black carp of six months (weight of 120 g) were injected intraperitoneally with GCRV (2.52×10^6 pfu/fish) SVCV (2.43×10^6 pfu/fish) or sterile PBS separately and cultured at 25 °C. Three fish were collected for each injected group (PBS, SVCV or GCRV). The injected black carps were sacrificed at 33 h post injection and total RNA was isolated from heart, liver, spleen, kidney, gill, intestine, skin and muscle independently. Total RNA was isolated from the indicated tissues independently. In each group (three fish), three RNA samples for each tissue were combined and used for cDNA synthesis. bcTBK1-S-F and bcTBK1-S-R were used for the detection of bcTBK1 mRNA in the above tissues; PCR of β-actin was performed as internal control. The semi-quantitative RT-PCR program was: 95 °C for 5min, then 30 cycles of 95 °C/30s, 55 °C/30s and 72 °C/1min, finally extension at 72 °C for 7min. The infection and semi-quantitative RT-PCR were repeated three times.

2.5. Quantitative real-time PCR

The relative bcTBK1 mRNA level in MPF cells was determined by quantitative real-time PCR (q-PCR). The primers for bcTBK1 and β-actin (as internal control) were listed in Table 1. The program was: 1 cycle of 50°C/2min, 1 cycle of 95°C/10min, 40 cycles of 95°C/15s, 60°C/1min, followed by dissociation curve analysis (60°C-95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the 7500 Real-Time PCR System and exported into a Microsoft Excel Sheet for subsequent data analyses where the relative expression ratios of target gene in treated group versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method.

2.6. Immunoblotting

HEK293T cells in 6-well plate in 6-well plate (1×10^6 cells/well) were transfected with bcTBK1 or the empty vector separately and the transfected cells were harvested at 48 h post transfection. The whole cell lysates were used for immunoblot assay as previously [23]. In brief, the proteins were isolated by 10% SDS-PAGE and the transferred membrane was probed with Mouse-*anti*-Flag or Mouse-*anti*-HA monoclonal antibody (1:4000; Sigma) separately, which was followed by incubation with goat-*anti*-mouse IgG (1:30000; Sigma). Target protein was visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.7. Immunofluorescence microscopy

HeLa cells or EPC cells were transfected with bcTBK1 and/or bcTRAF6, or the empty vector separately. The transfected cells were fixed with 4% (v/v) paraformaldehyde at 24 h post transfection. The fixed cells were permeabilized with Triton X-100 (0.2% in PBS) and applied to immune-fluorescent staining as previously described [21]. Mouse-*anti*-HA antibody (Sigma) was probed at the ratio of 1:300 and Alexa 488-conjugated secondary antibody (Invitrogen) was probed at the ratio of 1:1000; and DAPI (4, 6-diamidino-2-phenylindole) were used for the nucleus staining.

2.8. Luciferase reporter assay

EPC cells in 24-well plate were co-transfected with bcTBK1and/or bcTRAF6, pRL-TK, Luci-zIFN3 or Luci-eIFN. For each transfection, the total amount of DNA was balanced with the empty vector. The cells were harvested and lysed at 24 h post transfection. The centrifuged supernatant was used to measure the activities of firefly luciferase and renilla luciferase according to the instruction of the manufacturer (Promega) as previously [24].

2.9. Statistics analysis

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

3. Results

3.1. Molecular cloning and sequence analysis of bcTBK1

To study the role of bcTBK1 in the innate immune response of black carp, the full-length cDNA of bcTBK1 was cloned from the spleen of black carp. The full-length cDNA of bcTBK1 consists of 2857 nucleotides and the open reading frame (ORF) of bcTBK1 is composed of 2184 nucleotides (NCBI accession number: KX881379.1). The predicted protein of bcTBK1 contains 727 amino acids, including an N-terminal kinase domain (KD), an ubiquitin-like domain (ULD) and two C-terminal coiled-coils, which are conserved among TBK1 from human, mouse and jungle (Supplementary Fig. 1 and Fig. 1A) [25]. The calculated molecular weight of bcTBK1 is 83.7 kDa and the theoretical isoelectric point of this fish protein is 6.45 (Calculated by EXPASy Compute PI/Mw).

To gain insight into bcTBK1 evolution, amino acid (aa) sequence

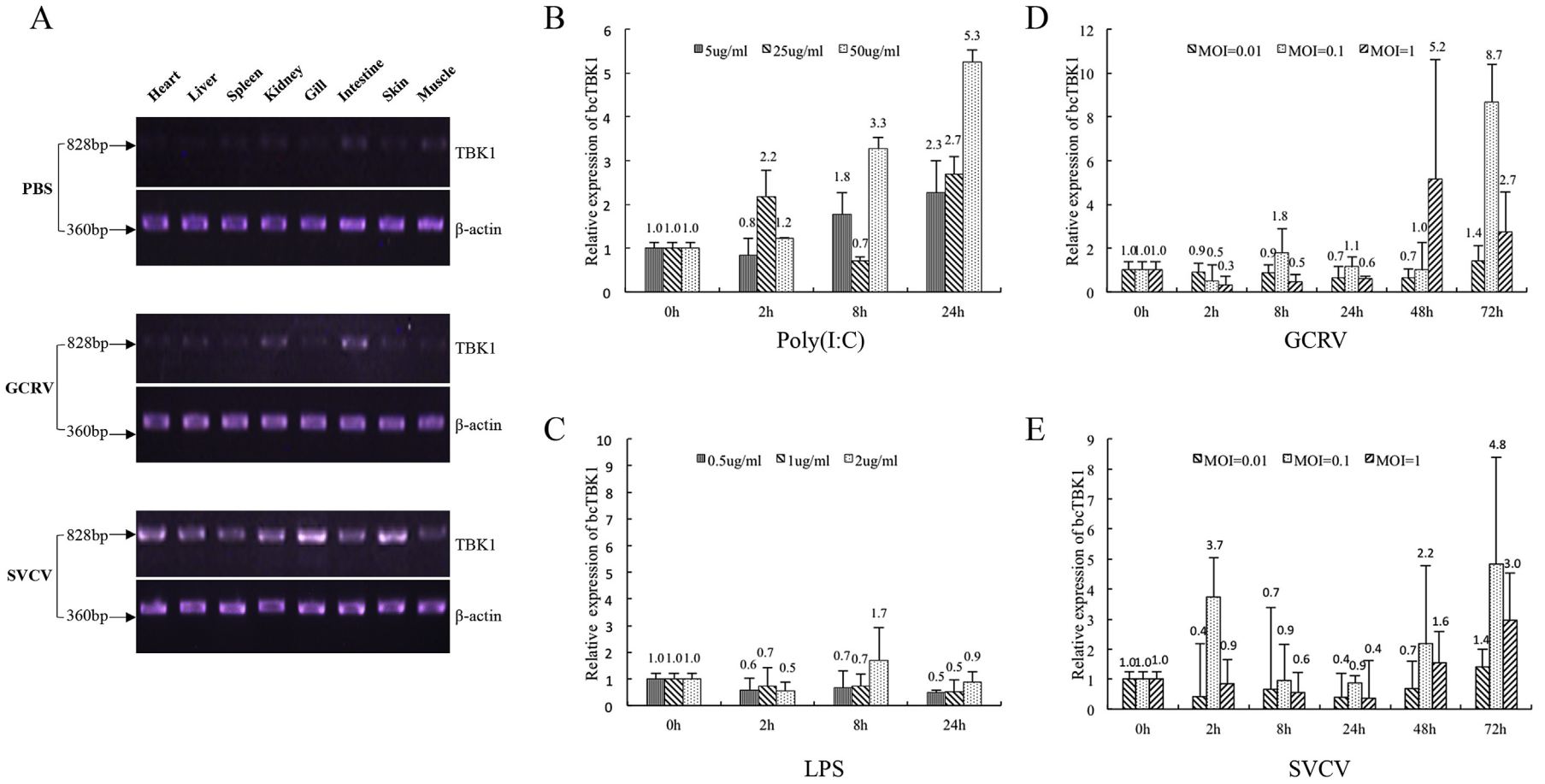

Fig. 2. bcTBK1expression *in vivo* and *ex vivo*.
(**A**) Tissue specific expression of bcTBK1. (**B**)&(**C**) MPF cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before stimulation. The cells were treated with poly (I:C) or LPS at the indicated concentration separately and harvested for q-PCR independently at the indicated time points post stimulation. **(D)&(E)** MPF cells were seeded in 6-well plate (2×10^6 cells/well) at 16 h before viral infection. The cells were infected with GCRV or SVCV at indicated MOI separately and harvested for q-PCR independently at the indicated time points post infection. The number above the error bar represents average bcTBK1 mRNA level.

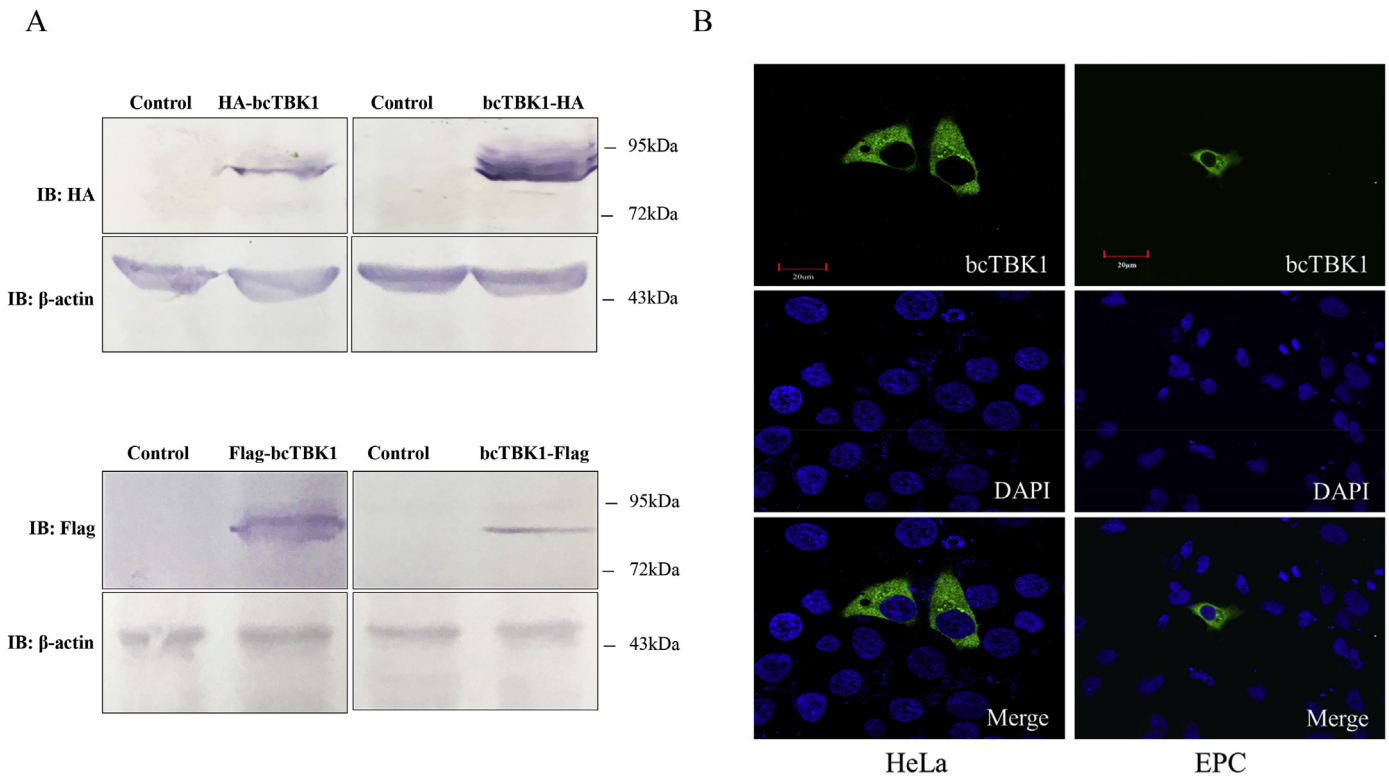

Fig. 3. Protein expression and subcellular distribution of bcTBK1.
(**A**) Immunoblot assay of bcTBK1 in HEK293T cells. Control: cells transfected with empty vector, HA-bcTBK1: pcDNA5/FRT/TO-HA-bcTBK1, bcTBK1-HA: pcDNA5/FRT/TO-bcTBK1-HA, Flag-bcTBK1: pcDNA5/FRT/TO-Flag-bcTBK1, bcTBK1-Flag: pcDNA5/FRT/TO-bcTBK1-Flag, IB: immunoblot. (**B**) Immunofluorescence staining of bcTBK1 in EPC cells and HeLa cells. bcTBK1: pcDNA5/FRT/TO-bcTBK1-Flag, and the bar stands for the scale of 20 μm.

of bcTBK1 was subjected to multiple alignments with that of different species, in which most sequences were quoted from NCBI. Phylogenetic analysis of TBK1 from the selected species demonstrates that these homologue proteins could be divided into three groups that consist of mammal, bird and fish branches (Fig. 1B). In phylogenetic analysis, bcTBK1 shares the high protein sequence identity with common carp (*C. carpio*; 96.4%) and zebrafish (*D. rerio*; 93.9%), which indicates a closer genetic relationship among these cyprinid fishes (Table 2).

3.2. Tissue specific expression of bcTBK1

To investigate the distribution of bcTBK1 mRNA *in vivo*, total RNA was isolated from heart, liver, spleen, kidney, gill, intestine, skin and muscle of the black carp injected with sterile PBS or SVCV or GCRV separately and applied to RT-PCR analysis. A specific band of 828bp was detected in all the selected tissues of black carp injected with PBS, which indicated that bcTBK1 was constitutively transcribed in these tissues of black carp under healthy condition. bcTBK1 mRNA level in kidney, intestine and muscle was slightly higher than those of other tissues (Fig. 2A, **upper panel**). bcTBK1 mRNA level in all tissues but muscle was slightly increased post GCRV injection especially in kidney and intestine (Fig. 2A, **middle panel**). bcTBK1 mRNA level in all the selected tissues was obviously increased post SVCV injection especially that in gill and skin (Fig. 2A, **lower panel**). The phenotype that bcTBK1 mRNA level in different tissues from black carps under different conditions shifted immediately after virus infection implied that bcTBK1 was involved in the black carp innate immune response initiated by GCRV or SVCV.

3.3. bcTBK1 expression ex vivo in response to different stimulation

To learn the profile of bcTBK1 mRNA expression in host cells during the innate immune response, MPF cells were subjected to different stimuli and applied to q-PCR analysis. For poly(I:C) stimulation, bcTBK1 mRNA in MPF cells increased after treatment at all doses, and the highest relative bcTBK1 mRNA level was up to 5.3 folds (50μg/ml; 24 h point). It was interesting that bcTBK1 mRNA level decreased (30% attenuation) at 8 h point for the 25 ug/ml dose treatment (Fig. 2B). However, LPS treatment did not induce obvious bcTBK1 expression for all doses (0.5 ug/ml, 1 ug/ml, 2 ug/ml) at most time points (Fig. 2C). These data suggest that bcTBK1 is an important component of host defense mechanism responsible for virus infection; however, whether this teleost RLR member is involved in the recognition of bacteria invasion or not needs further investigation. Higher dose LPS input and longer time of treatment will be recruited in our future study to explore the function of bcTBK1 in the host innate immune response initiated by bacterial invasion.

In GCRV infected MPF cells, bcTBK1 mRNA level varied post infection (pi) and was elevated at different time points (48 hpi for 1MOI, 72 hpi for 0.1MOI and 0.01MOI) (Fig. 2D). In SVCV infected MPF cells, bcTBK1 mRNA level varied post viral invasion and was elevated at 72 hpi for all three doses (except a robust at 2hpi in 0.1 MOI group) (Fig. 2E). These data indicated that bcTBK1 had been recruited into host antiviral innate immune response, such as GCRV and SVCV.

3.4. Protein expression and intracellular distribution of bcTBK1

HEK293T cells were transfected with HA-bcTBK1, Flag-bcTBK1,

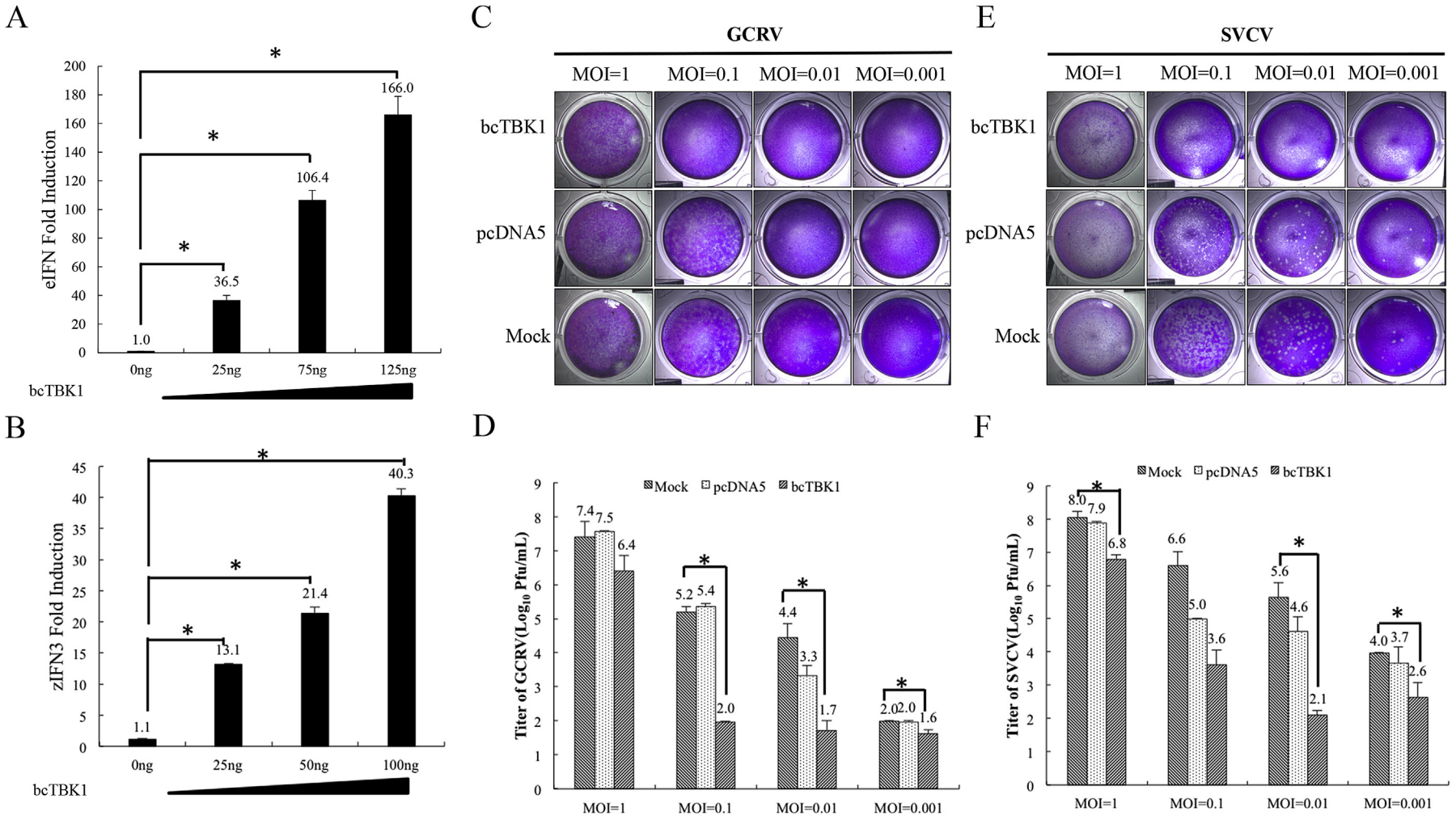

Fig. 4. bcTBK1-mediated antiviral signaling.

(**A**)&(**B**) Fathead minnow IFN promoter activity and zebrafish IFN3 promoter activity induced by bcTBK1. bcTBK1: pcDNA5/FRT/TO-HA-bcTBK1. The numbers above the error bars stand for average IFN folds induction. (**C ~ F**) Antiviral activity of bcTBK1. EPC cells in 24-well plate were transfected with bcTBK1 or the empty vector separately and infected with GCRV/SVCV at indicated MOI at 24 h post transfection. The cell monolayers were stained with crystal violet (**C&E**) and virus titers in the supernatant media were determined by plaque assay at 24 h post-infection. The numbers above the error bars stand for average virus titers. (**D&F**). Mock: EPC cells without transfection; TBK1: EPC cells transfected with pcDNA5/FRT/TO-HA-bcTBK1; pcDNA5: EPC cells transfected with pcDNA5/FRT/TO-HA.

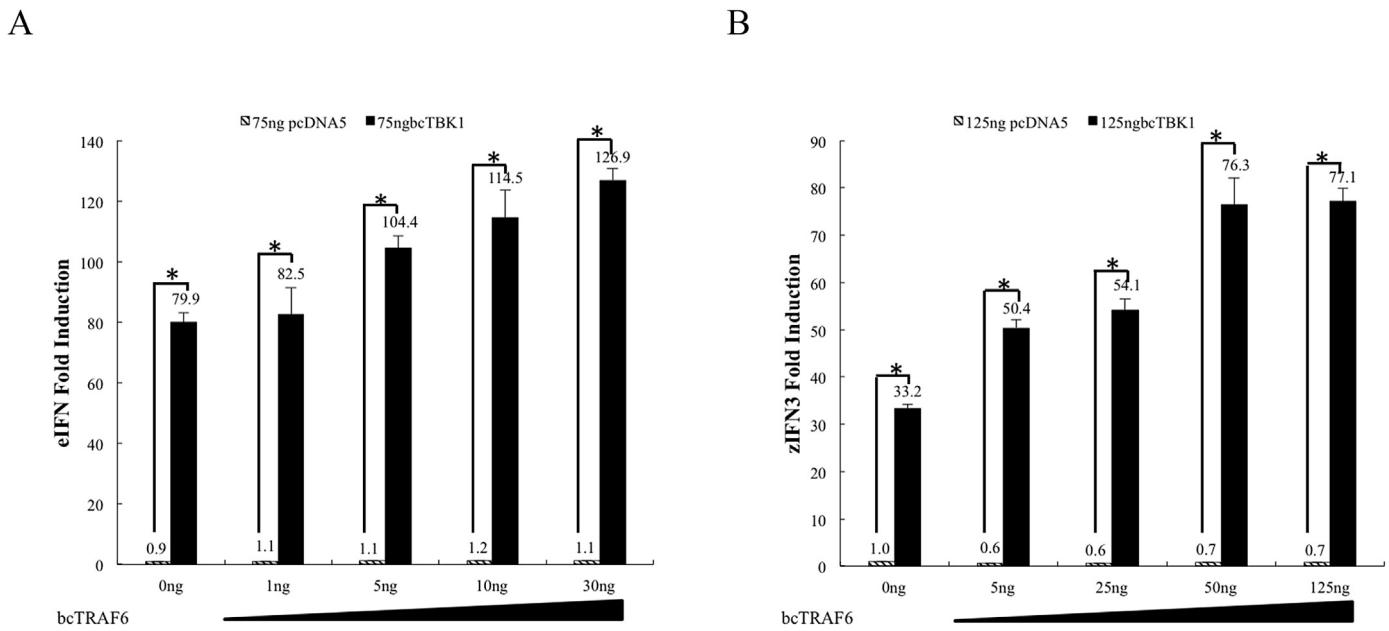

Fig. 5. IFN-inducing activity of bcTBK1 was up-regulated by bcTRAF6.
EPC cells in 24-well plate were co-transfected with bcTBK1 or empty vector, bcTRAF6, pRL-TK, Luci-zIFN3 or Luci-eIFN, and the cells were harvested at 24 h post transfection and applied to reporter assay. For each transfection, the total amount of DNA was balanced with the empty vector. (**A**) Fathead minnow IFN promoter activity induced by bcTBK1 and bcTRAF6. (**B**) Zebrafish IFN3 promoter activity induced by bcTBK1 and bcTRAF6. TBK1: pcDNA5/FRT/TO-HA-bcTBK1, TRAF6: pcDNA5/FRT/TO-HA-bcTRAF6, pcDNA5: pcDNA5/FRT/TO empty vector. The numbers above the error bars stand for average IFN folds induction.

bcTBK1-HA or bcTBK1-Flag separately and applied to immunoblot assay to investigate the protein expression of bcTBK1. A specific band of ~80 KDa was detected in the whole cell lysate of HEK293T cells transfected with plasmids expressing bcTBK1; and the immunoblott assay data demonstrated bcTBK1 was expressed in mammalian cell system whatever tag was fused to its N-terminus or C-terminus (Fig. 3A). To further examine the subcellular distribution of bcTBK1, both EPC cells and Hela cells were transfected with HA-bcTBK1 or the empty vector separately and used for immunofluorescence staining assay. The immunofluorescence staining data of both fish cell and mammalian cells showed clearly that bcTBK1-expressing space (green) surrounded tightly the nucleus (blue), which indicates that bcTBK1 is a cytosolic protein (Fig. 3B).

3.5. IFN-inducing activity of bcTBK1

In the antiviral innate immune response of human, activated TBK1 phosphorylates IRF3/7 after PRRs recognizing viral components, which transfer into nuclear and initiate the transcription of IFNs [8]. To determine the IFN-inducing activity of bcTBK1, EPC cells were co-transfected with bcTBK1, pRL-TK, Luci-zIFN3 or Luci-eIFN, and applied to dual luciferase reporter assay. For the induced promoter activity of fathead minnow IFN, as the bcTBK1 input increased (25 ng, 50 ng, 100 ng), the eIFN fold induction increased as well and the highest value was 166 folds of that of control (Fig. 4A). Similar to that of fathead minnow IFN, the zebrafish IFN3 fold induction increased as the bcTBK1 input increased (25 ng, 50 ng, 100 ng) and the highest value was 40 folds of that of control (Fig. 4B). Our data demonstrated clearly that expression of bcTBK1 in EPC cells triggered downstream signaling and induced the transcription of both zebrafish IFN3 and fathead minnow IFN.

3.6. Antiviral activity of bcTBK1 against SVCV and GCRV

To interpret the role of bcTBK1 in the innate immune response under viral invasion, EPC cells were transfected with bcTBK1 or the empty vector and applied to virus infection at 24 h post transfection. In GCRV infected group, both CPE ratio and viral titer in the supernatant media of the EPC cells expressing bcTBK1 were obviously lower than those of empty vector transfected cells or untransfected cells (Fig. 4C and D). The viral titer in the media of bcTBK1-expressing cells showed the biggest reduction (>1000 times) when the cells were infected with GCRV at the dose of 0.1 MOI; and the smallest reduction happened at the dose of 0.001 MOI (Fig. 4D). The data of SVCV group was similar to that of GCRV group; both CPE ratio and viral titer in the media of the EPC cells expressing bcTBK1 obviously lower than those of control cells (Fig. 4E and F). The viral titer in the media of bcTBK1-expressing cells showed the biggest reduction (>100 times) when the cells were infected with SVCV at the dose of 0.01 MOI (Fig. 4F). Thus, our data presented clearly that exogenous bcTBK1 enhanced the antiviral ability of EPC cells against both SVCV and GCRV, which demonstrated that that this fish TBK1 ortholog functioned as an important factor in host antiviral innate immune response.

3.7. Up-regulated bcTBK1 signaling by bcTRAF6

In our previous study, the subcellular location of bcMAVS matched well that of bcTRAF6 when these two proteins were co-expressed in EPC cells, and bcTRAF6 was recruited into bcMAVS-mediated IFN production [20]. Thus, it is speculated that bcTRAF6 might be a potential E3 ligase of ubiquitination of bcTBK1, which led to the exploration of the relationship between bcTRAF6 and bcTBK1. To investigate if the influence of bcTRAF6 on bcTBK1 mediated IFN induction, EPC cells were co-transfected with bcTBK1 and bcTRAF6, and applied to dual luciferase reporter assay. For the induced promoter activity of fathead minnow IFN, as the bcTRAF6 input increased (5 ng, 25 ng, 50 ng, 125 ng), the eIFN fold induction by bcTBK1 increased as well (Fig. 5A). Similar to that of fathead minnow IFN, the zIFN3 fold induction by bcTBK1 increased as the bcTRAF6 input increased (Fig. 5B).

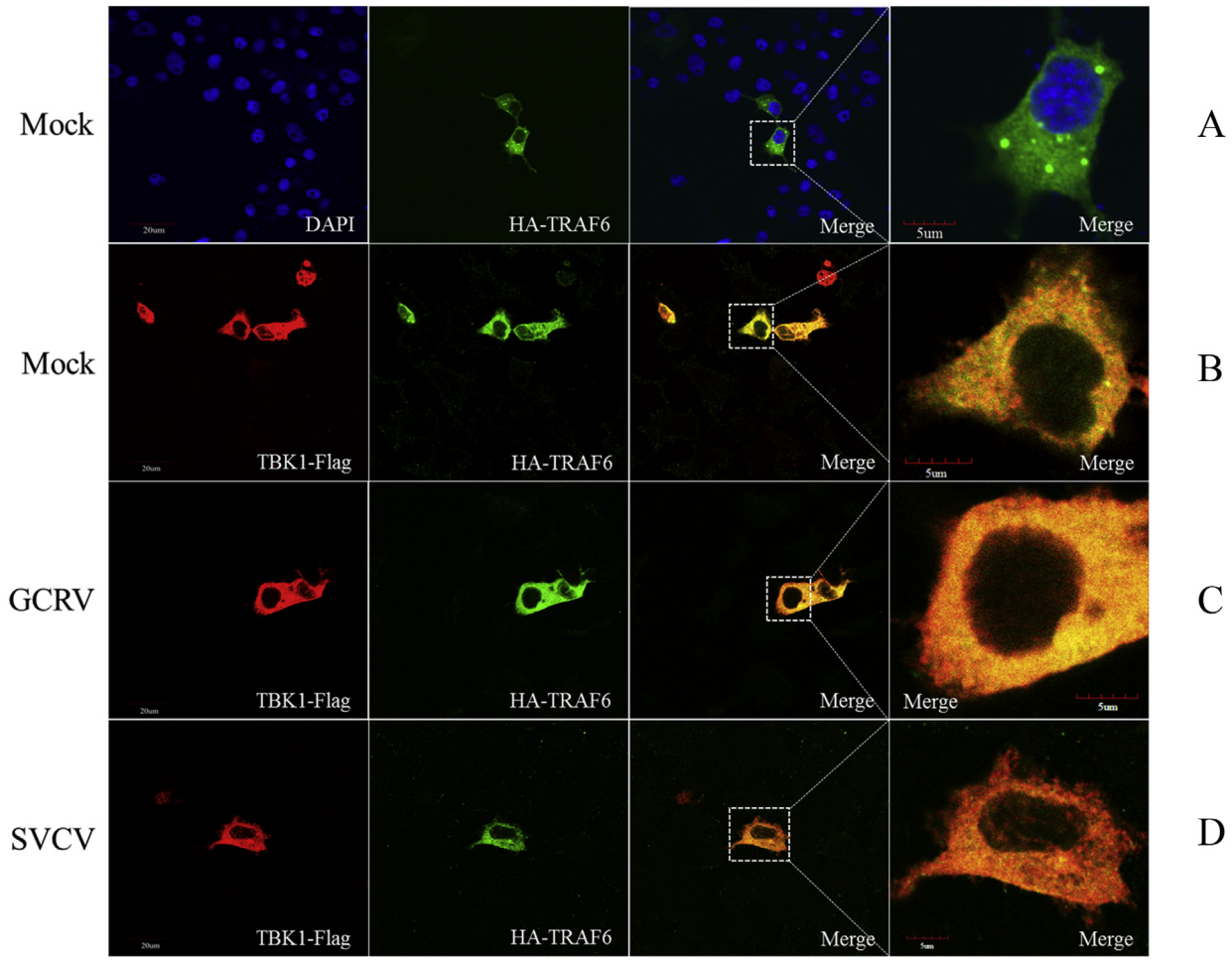

Fig. 6. Co-expressed bcTRAF6 and bcTBK1 presented identical subcellular distribution.
EPC cells were transfected with bcTRAF6 or/and bcTBK1 separately and infected with SVCV or GCRV (MOI = 1) at 24 h post transfection independently. The cells were fixed for immunofluorescence staining at 12 h post infection. Mock: EPC cells without viral infection. TBK1-Flag: pcDNA5/FRT/TO-bcTBK1-Flag; HA-TRAF6: pcDNA5/FRT/TO-HA-bcTRAF6; the bars stand for the scale of 20 μm and 5 μm accordingly.

In the immunofluorescence staining data, bcTRAF6-expressing region (green) surrounded tightly the nucleus (blue) and brilliant green dots were widely scattered the cytoplasmic region (Fig. 6A and reference [20]). However, when co-expressed with bcTBK1, the brilliant green dots (bcTRAF6-expressing area) were disappeared and the subcellular distribution of bcTRAF6 (green) matched that of bcTBK1 (red) very well whatever the cells was infected with virus or not (Fig. 6B, C and D), which demonstrated the same subcellular location of bcTRAF6 and bcTBK1 and suggested the functional association between these two proteins [26,27].

4. Discussion

IKKε and TBK1 are newly found IKK-related kinases and have a pivotal role in coordinating the activation of IRF3/7 and NF-κB as well as inducing IFN-β in innate immune responses [28]. As one of the major downstream substrates of TBK1-mediated IFN expression, IRF3 is phosphorylated by TBK1, followed by IRF3 dimerization, nuclear translocation and transcriptional activation [29]. TBK1 activity can be regulated by multiple molecules in a variety of ways, while ubiquitination and phosphorylation tend to be crucial for TBK1 activation [12,13,30]. USP38-mediated K33-deubiquitination and DTX4/TRIP-mediated K48-ubiquitination tightly control the activity of TBK1 during antiviral immune response [31]. In interferon-mediated innate immune response induced by viruses, TBK1 becomes associated with glycogen synthase kinase 3 beta (GSK3B) to enhance its self-oligomerization and autophosphorylation [32]. TBK1 constitutively forms complexes with TRAF family member-associated NF-κB activator (TANK), nucleosome assembly protein 1(NAP1) and similar to NAP1 TBK1 adaptor (SINTBAD), which also links TBK1 to virus-activated signaling cascades [33,34]. Suppressor of IKKε 1 (SIKE1) associates with TBK1 under physiological condition and dissociates from TBK1 upon viral infection or TLR3 stimulation, which functions as an inhibitory regulator of the TBK1/AKT axis [35,36].

Recent identifications of TBK1 of some teleost fish, however, indicated superficially similar characteristics between mammals and fish, when few features of TBK1 of fish were seriously exploited [16,37–40]. It's necessarily important to study TBK1 of fish in details, thus the distinctions of TBK1 of mammals and that of fish could be dissected afterward. In this study, we identified TBK1 from black carp (bcTBK1), which is one of the most important freshwater fish in China, and characterized its function in antiviral innate immune defense against RNA viruses. bcTBK1 shows highly protein sequence similarity with those of mammals and birds, and possesses the same structure including N-terminal kinase domain (KD), ubiquitin-like domain (ULD) and the C-terminal coiled-coil (Fig. 1), which demonstrates that these functional domains are

conserved in vertebrates and suggests that TBK1 of teleost fish might be highly consistent with its mammalian counterparts in structure and functions.

In this study, bcTBK1 showed strong IFN-inducing ability and EPC cells expressing bcTBK1 obtained much improved antiviral activity although the mRNA level of bcTBK1 *in vivo* and *ex vivo* did not increase (Figs. 2 and 4) as high as those of bcMDA5 and bcLGP2 in our pervious study in response to both GCRV and SVCV (21, 22). It is reasonable that bcTBK1 functions as a regulator in the RLR signaling during the host innate immune response initiated by viral infection such as GCRV and SVCV liking its mammalian counterpart. Overexpression of bcTBK1 in EPC cells enhanced its anti-viral ability. Dual luciferase reporter assay showed that bcTBK1 upregulated the IFN expression in EPC cells, in which zebrafish IFN3 promoter and EPC IFN promoter analysis system were recruited. The mechanisms of activation, abrogation and suppression of bcTBK1 in host during host innate immune response is still to be finished, knock-out or know-down cell line should be established to explain the relationships between bcTBK1 and surrounding factors.

TRAFs are a group of adaptor proteins that couple tumor necrosis factor receptor (TNFR) family to signaling pathways, which are involved in a wide spectrum of cellular responses including cell proliferation, apoptosis, and differentiation [41,42]. TRAF6 is broadly expressed in tissues of higher vertebrates and well conserved across species [41]. bcTRAF6 was cloned and characterized in our previous study. Our data demonstrated clearly that bcTRAF6 was recruited onto mitochondria by bcMAVS and regulated bcMAVS mediated signaling in a dose dependent manner. In this paper, bcTRAF6 subcellular distribution overlapped with that of bcTBK1 when these two proteins were expressed in EPC cells whatever with or without viral infection (Fig. 6). Although the direct association between bcTBK1 and bcTRAF6 needs to be further identify, it is reasonable that bcTRAF6 functions importantly in bcTBK1 activation through ubiquitination since this assumed fish E3 ligase enhanced the IFN-inducing ability of bcTBK1 in reporter assay (Fig. 5). Taken these data together, it is speculated that bcMAVS, bcTRAF6 and bcTBK1 are recruited into a complex on the mitochondria post viral infection, which is crucial for the IFN production just like their mammalian counterparts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.08.016.

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