



## Full length article

## Black carp TAB1 up-regulates TAK1/IRF7/IFN signaling during the antiviral innate immune activation

Ziqi Zou<sup>1</sup>, Xinchu Xie<sup>1</sup>, Wanzhen Li, Xuejiao Song, Yaqi Tan, Hui Wu, Jun Xiao, Hao Feng\*

State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha, 410081, China

## ARTICLE INFO

## Keywords:

TAB1  
TAK1  
IRF7  
Black carp  
Interferon

## ABSTRACT

TAK1-binding protein 1 (TAB1) forms the protein complex with TAK1 and enhances its kinase activity in human and mammals. To elucidate the role of TAB1 in the innate immunity of teleost fish, the TAB1 homologue of black carp (*Mylopharyngodon piceus*) (bcTAB1) has been cloned and characterized in this paper. bcTAB1 is composed of 498 amino acids and contains a typical PP2Cc domain like its mammalian counterpart. The transcription of bcTAB1 gene *in vivo* and *ex vivo* varied in response to different stimuli; and the immunofluorescence staining showed that bcTAB1 was distributed in both cytoplasm and nucleus of host cell. The reporter assay showed that neither bcTAB1-expression alone nor co-expression of bcTAB1 and bcTAK1 could activate the transcription of IFN in EPC cells. Accordingly, EPC cells expressing bcTAB1 or co-expressing bcTAB1 and bcTAK1 showed no improved antiviral activity against grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV). However, EPC cells co-expressing bcTAB1, bcTAK1 and bcIRF7 showed fiercely increased IFN-inducing ability in reporter assay and obviously improved antiviral activity in plaque assay compared with EPC cells co-expressing bcTAK1 and bcIRF7. The subsequent co-immunoprecipitation assay identified that bcTAB1 associated with bcTAK1 but not interacted with bcIRF7. Based on our previous finding that bcTAK1 up-regulates bcIRF7-mediated IFN signaling during host innate immune activation, the data generated in this study support the conclusion that bcTAB1 interacts with bcTAK1 and boosts bcTAK1-activated bcIRF7/IFN signaling during host antiviral innate immune response against GCRV and SVCV.

## 1. Introduction

Innate immunity provides vertebrates the first line of immune defense against pathogenic microorganisms through detecting diverse microbial pathogen-associated molecular patterns (PAMPs) by utilizing a variety of germline-encoded pattern recognition receptors (PRRs), which 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 [1,2]. After recognizing invading pathogens, PRRs recruit downstream signaling and trigger the activation of transcription activators IRF3/7 and NF- $\kappa$ B. Activated NF- $\kappa$ B and IRF3/7 translocate into nucleus and initiate the transcription of pro-inflammatory cytokines and IFNs, which lead to the anti-pathogenic state of the host cells finally [3–5].

Transforming growth factor- $\beta$  activated kinase1 (TAK1) plays crucial roles in the pro-inflammatory and innate immune signaling in human and mammals, such as TNF receptor, IL-1R, and TLR signaling, in which TAK1 functions as a vital regulator of NF- $\kappa$ B [6]. TAB1 (also

known as MAP3K7IP1) has been identified as a binding protein and activator of TAK1, which is a key intermediate in several cytokine signaling pathways including TGF- $\beta$ , TNF and IL-1 signaling [7]. TAB1 associates with and activates the kinase activity of TAK1, in which TAB1 is facilitated for TAK1 to regulate JNK/p38 MAPKs and IKK signaling, inducing the activation of transcription factors AP-1 and NF- $\kappa$ B [8,9]. It has also been proven that the TAK1-TAB1 complex induces the nuclear translocation of NF- $\kappa$ B (p50/p65) heterodimer accompanied by the degradation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  [10,11]. The global inactivation of mice TAB1 causes embryonic lethality with several developmental dysregulations like heart and lungs defect [12]. What is more, xTAB1 and xTAK1 play a synergistic role in the BMP signal transduction pathway in *Xenopus* embryos [13]. However, the roles of TAB1 and TAK1 in the antiviral innate immunity are still obscure. Especially, there is no report about the role of TAB1-TAK1 complex in IFN signaling during host innate immune activation initiated by virus.

Compared with its mammalian counterpart, TAB1 homologues have been identified only in several teleost species till now and the role of

\* Corresponding author. ,

E-mail address: [fenghao@hunnu.edu.cn](mailto:fenghao@hunnu.edu.cn) (H. Feng).<sup>1</sup> These authors contribute equally to this paper.

TAB1 in teleost still remains largely unknown. For instance, grass carp (*Ctenopharyngodon idella*) TAB1 was first reported to play an important role in the innate immune response to parasitic infection [14]. TAB1 of the large yellow croaker (*Larimichthys crocea*) plays an important role in inducing NF- $\kappa$ B activation via forming a complex with TAK1 [15]. TAB1 of orange-spotted grouper (*Epinephelus coioides*) has recently been cloned and preliminary characterized [16].

Black carp (*Mylopharyngodon piceus*) is an economically important fresh water species, which is one of the “Four Domesticated Fish” in Chinese freshwater aquaculture [17]. Black carp is subjected to bulk of pathogenic microorganisms such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV), which are two major RNA viruses threatening fresh water industry in China [18,19]. Among all known PRR families, the RLR family is a group of core receptors capable of discriminating self-RNA from non-self-RNA and plays a vital role in detecting pathogens of RNA virus infection to initiate early innate antiviral response [20–22]. Thus, the RLR/MAVS/IRF signaling of black carp has been studied in this lab, which is aimed to elucidate RLR signaling in host innate immunity against GCRV and SVCV [23–25]. In particular, our previous study demonstrated that black carp TAK1 (bcTAK1) positively regulated black carp IRF7 (bcIRF7)-mediated antiviral signaling against both SVCV and GCRV, which was the first reporter about the role of TAK1 in IRF7/IFN signaling in vertebrates [26]. To illuminate the role of TAB1 in bcTAK1/bcIRF7/IFN signaling, bcTAB1 has been cloned and characterized in this paper, which was found to significantly enhanced bcTAK1's ability of up-regulating both bcIRF7-induced IFN production in reporter assay and bcIRF7-induced antiviral activity in plaque assay. And the direct association between bcTAB1 and bcTAK1 was identified sub-sequentially. Thus, our data has further explored the mechanism of TAB1-TAK1 complex in IRF7/IFN signaling against RNA virus, which is reported for the first time in vertebrates.

## 2. Materials and methods

### 2.1. Cells and plasmids

HEK293T cells, *Epithelioma Papulosum Cyprinid* (EPC) cells, *Ctenopharyngodon idella* kidney (CIK) cells and *Mylopharyngodon piceus* kidney (MPK) cells were kept in the lab [27]. HEK293T cells were cultured at 37 °C with 5% CO<sub>2</sub>; while EPC, CIK, and MPK cells were cultured at 26 °C with 5% CO<sub>2</sub>. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Transfection was done as previously described, calcium phosphate was used for HEK293T transfection, Lipomax (Sudgen, China) was used for EPC transfection and MPK transfection [24].

pcDNA5/FRT/TO (Invitrogen, USA), pRL-TK, Luci-eIFN (for fathead minnow IFN promoter activity analysis), Luci-DrIFN $\phi$ 1/2/3 (for zebrafish IFN $\phi$ 1/2/3 promoter activity analysis accordingly), and Luci-bcIFN $\alpha$  (for black carp IFN $\alpha$  promoter activity analysis) were kept in the lab [26]. The recombinant expression vector pcDNA5/FRT/TO-Flag-bcTAB1 and pcDNA5/FRT/TO-bcTAB1-Flag were constructed by cloning the open reading frame (ORF) of bcTAB1 fused with a Flag tag at its N-terminus/C-terminus into pcDNA5/FRT/TO, respectively.

### 2.2. Cloning the cDNA of bcTAB1

Degenerate primers (Table 1) were designed to amplify the cDNA of bcTAB1 based on the TAB1 sequences of grass carp (*C. idella*) (KJ184547.1) and zebrafish (*D. rerio*) (XP\_002662286.4). Total RNA was isolated from the spleen of black carp and the first-strand cDNA were synthesized by using the Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). The coding sequence (CDS) of bcTAB1 was cloned at the first attempt by using the degenerate primers. The amplified fragments were cloned into pMD18-T vector (TaKaRa, Japan) and sequenced by Invitrogen.

### 2.3. Virus produce and titration

SVCV (strain: SVCV741) and GCRV (strain: GCRV106) were kept in the lab and propagated in EPC or CIK separately at 26 °C in the presence of 2% fetal bovine serum. EPC or CIK cells were infected with SVCV or GCRV accordingly; the cells and the supernatant media were collected together when the cytopathic effect (CPE) was about 50% and stored at –80 °C. After freezing and thawing for three times, the mixture was used for virus titer mensuration. Virus titers were determined by plaque assay on EPC cells as previously described [27]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 26 °C. The supernatant was replaced with fresh DMEM containing 2% FBS and 0.75% methylcellulose (Sigma, USA) after incubation. Plaques were counted at day 3 post infection.

### 2.4. LPS and poly (I:C) treatment

MPK cells were seeded in 6-well plate ( $2 \times 10^6$  cells/well) at 16 h before treatment. Poly (I:C) (Sigma, USA) was used for synthetic dsRNA stimulation, which was heated to 55 °C (in PBS) for 5 min and cooled at room temperature before use. MPK cells were replaced with fresh media containing poly (I:C) at the final concentration of 5 µg/ml, 25 µg/ml, and 50 µg/ml and harvested at different time points post treatment. bcTAB1 mRNA level in the MPK cells was examined by quantitative real-time PCR (q-PCR). For LPS (Sigma, USA) treatment, MPK cells in 6-well plate ( $2 \times 10^6$  cells/well) were replaced with fresh LPS-containing media (1 µg/ml, 10 µg/ml, and 50 µg/ml) separately and harvested at different time points (2 h, 8 h, 12 h, 24 h, and 48 h) post stimulation as above.

### 2.5. Quantitative real-time PCR

The relative bcTAB1 mRNA level in the selected tissues of black carp was determined by quantitative real-time PCR (q-PCR). Black carps of 11 months (weight of ~100 g) were injected intraperitoneally with GCRV ( $5 \times 10^5$  pfu/fish) or sterile PBS separately and cultured at 25 °C. The injected black carps were collected at 24 h, 48 h, 72 h, 96 h or 120 h post injection and total RNA was isolated from spleen, liver and kidney independently. Three fish were collected for each injected group and three RNA samples for each tissue were combined in each group and used for cDNA synthesis. The primers for bcTAB1 and  $\beta$ -actin (as internal control) (Table 1) were used for the detection of bcTAB1 mRNA in the above tissues and MPK cells treated with different stimuli. The q-PCR program was: 1 cycle of 95 °C/10min, 40 cycles of 95 °C/15s, 60 °C/1min, followed by dissociation curve analysis (60 °C–95 °C) to verify the amplification of a single product. The threshold cycle (CT) value was determined by using the manual setting on the Applied Biosystems Fast 7500 Real-Time PCR System (ABI, USA) and exported into a Microsoft Excel spreadsheet for subsequent data analysis where the relative expression ratios of target gene in treated groups versus those in control group were calculated by  $2^{-\Delta\Delta CT}$  method [25].

### 2.6. Immunoblotting

HEK293T cells or EPC cells in 6-well plate ( $2 \times 10^6$ ) were transfected with plasmid expressing bcTAB1 or the empty vector separately. The transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described [27]. In brief, the whole cell lysates were isolated by 10% SDS-PAGE and transferred to PVDF membrane. The transferred membranes were probed with mouse monoclonal anti-Flag antibody (1:3000; Sigma, USA), which were followed by the incubation with goat-anti-mouse IgG (1:30000; Sigma, USA). The target proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma, USA).

**Table 1**  
Primers used in the study.

| Primer name                 | Sequence(5'–3')                     | Amplicon length (nt) and primer information |
|-----------------------------|-------------------------------------|---------------------------------------------|
| <b>CDS</b>                  |                                     |                                             |
| bcTAB1-F                    | ATGGCGGCGCAGCGCAG                   | 1497bp                                      |
| bcTAB1-R                    | TCACTGCGGTCCCCTCTCACC               | bcTAB1 CDS cloning                          |
| <b>Expression construct</b> |                                     |                                             |
| bcTAB1-N-F3                 | ACTGACGATATCATGGCGGCGCAGCGCAG       |                                             |
| bc-TAB1-N-R                 | ACTGACCTCGAGTCACTGCGGTCCCCTCTCACC   | FRT-To-Flag-bcTAB1                          |
| bcTAB1-C-F                  | ACTGACGATATCGCCACCATGGCGGCGCAGCGCAG | FRT-To-bcTAB1-Flag                          |
| bcTAB1-C-R                  | ACTGACCTCGAGCTGCGGTCCCCTCTCACC      |                                             |
| <b>q-PCR</b>                |                                     |                                             |
| bc Q actin-F                | TGGGCACCGCTGCTTCCT                  |                                             |
| bc Q actin-R                | TGTCCGTCAGGCAGCTCAT                 | <i>in vivo</i> & <i>ex vivo</i> q-PCR       |
| QTAB1-1-F                   | TTAGAGCAGGAGGTTTCAGGT               |                                             |
| QTAB1-1/2-R                 | CATCTCTGTTGTCGGTTGT                 | <i>in vivo</i> & <i>ex vivo</i> q-PCR       |

## 2.7. Immunofluorescence microscopy

MPK cells in 24-well plate ( $3 \times 10^5$  cells/well) were transfected with plasmid expressing bcTAB1 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 used for immunofluorescence (IF) staining as previously described [23]. Mouse monoclonal anti-Flag antibody (Sigma, USA) was probed at the ratio of 1:300; Alexa 488-conjugated secondary antibody (Invitrogen, USA) was probed at the ratio of 1:800 and DAPI was used for nucleus staining.

## 2.8. Luciferase reporter assay

EPC cells in 24-well plate ( $3 \times 10^5$  cells/well) were co-transfected with pRL-TK (25 ng), Luci-eIFN (Luci-bcIFN $\alpha$  or Luci-DrIFN $\eta$ 1/2/3) (250 ng), pcDNA5/FRT/TO-Flag-bcTAB1, pcDNA5/FRT/TO-HA-bcTAK1 and/or pcDNA5/FRT/TO-HA-bcIRF7. For each transfection, the total amount of plasmid DNA was balanced with the empty vector. The cells were harvested at 24 h post transfection and lysed by renilla luciferase lysis buffer (Promega, USA) on ice. The centrifuged supernatant was used to measure firefly luciferase activity and renilla luciferase activity according to the instruction of the manufacturer (Promega, USA) [26].

## 2.9. Co-immunoprecipitation (co-IP)

HEK 293T cells in 10 cm plate were co-transfected with pcDNA5/FRT/TO-Flag-bcTAB1 and pcDNA5/FRT/TO-HA-bcTAK1, or pcDNA5/FRT/TO-Flag-bcTAB1 and bcIRF7-pEGFP-N1. The transfected cells were harvested at 48 h post-transfection and lysed for immunoprecipitation (IP) assay as previously described [24]. The whole cell lysates of the transfected cells was incubated with protein A/G agarose beads at 4 °C for 2 h. Flag-conjugated (HA-conjugated) protein A/G agarose beads were added in the supernatant after pre-clearing and incubated with the supernatant media at 4 °C for 4 h. Flag-conjugated (HA-conjugated) protein A/G agarose beads were boiled in 6 x sample buffer after 3–5 times of wash and the eluted proteins were used for IB as above.

## 2.10. 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 ( $\pm$  SEM) of three independent experiments. Asterisk (\*) stands for  $p < 0.05$ . Two-tailed Student's *t*-test was used for all statistical analyses with the GraphPad Prism 4.0 software (GraphPad Prism, USA).

## 3. Results

### 3.1. Molecular cloning and sequence analysis of bcTAB1

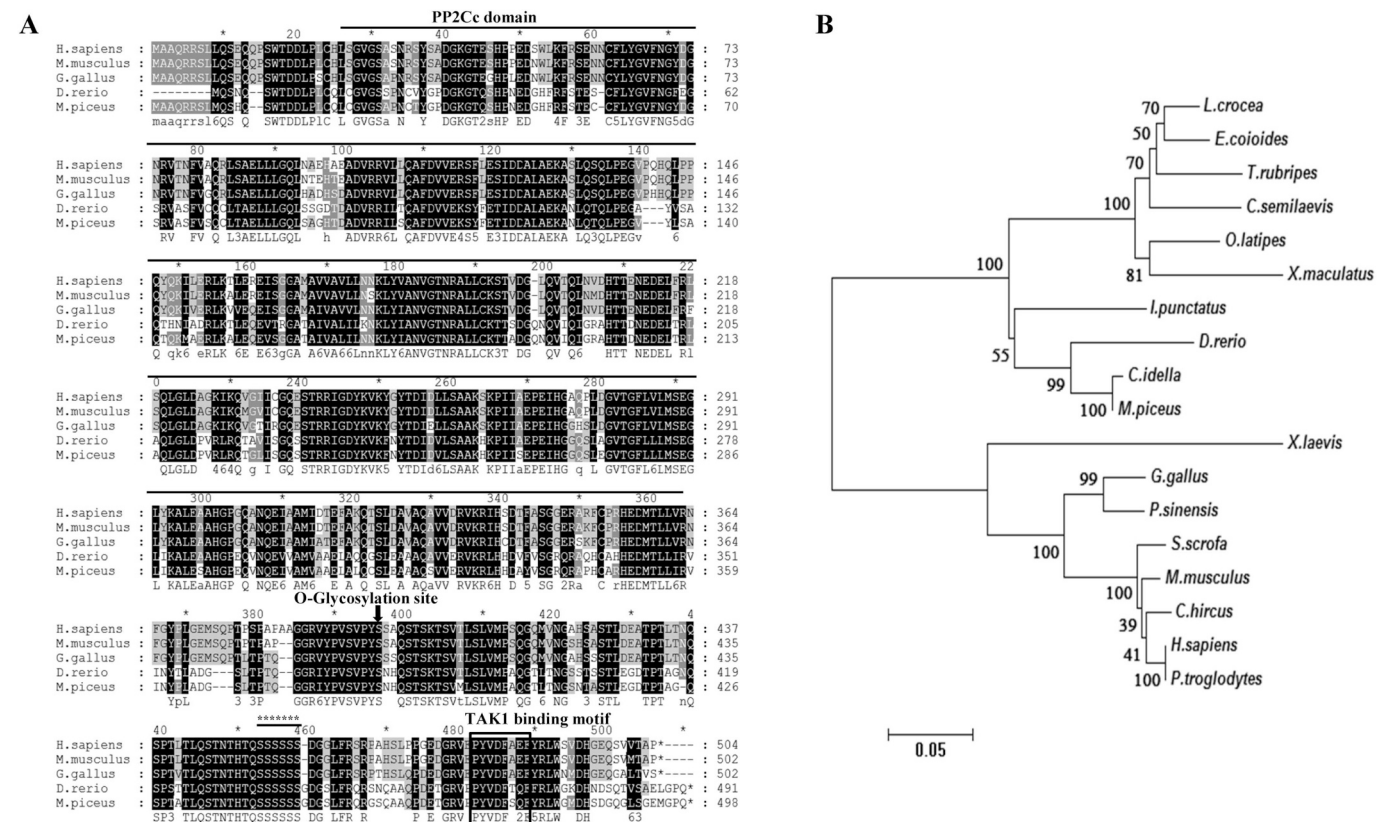
To study the role of bcTAB1 in black carp, cDNA of bcTAB1 gene was cloned from the spleen of black carp. The coding sequence of bcTAB1 cDNA consists of 1497 nucleotides (NCBI accession number: MK424333). The predicted bcTAB1 protein contains 498 amino acids, which contains a typical PP2C domain (24–358aa), a suspected conservative O-Glycosylation site (S<sub>385</sub>), three suspected N-Glycosylation sites (N<sub>32</sub>, N<sub>246</sub> and N<sub>409</sub>), several conserved serine residues (441–446aa) and a C-terminal conserved TAK1 binding motif (Fig. 1A and Supplementary Fig. 1). bcTAB1 has a calculated molecular weight of 53.7 kDa and an isoelectric point of 5.60 (calculated by EXPASY Compute PI/Mw).

To gain insight into TAB1 evolution, amino acid sequence of bcTAB1 has been subjected to multiple alignments with those of TAB1 proteins from human (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*) and zebrafish (*D. rerio*), which indicates that TAB1 is a conserved protein in vertebrates (Fig. 1A). The comparison of bcTAB1 with TAB1 proteins from other known species shows that bcTAB1 shares high identity with grass carp TAB1 (99.4%) (Table 2). Phylogenetic analysis of TAB1 from the selected species demonstrates that these TAB1 homologues could be divided into four groups that consist of mammal, bird, reptile and piscine branches, in which bcTAB1 is clustered together with grass carp TAB1 (Fig. 1B).

### 3.2. bcTAB1 expression *in vivo* and *ex vivo*

To investigate bcTAB1 transcription *in vivo*, mRNA level in spleen, liver or kidney of the black carp injected with GCRV was examined separately by q-PCR analysis. In general, the increase rate of bcTAB1 transcription in liver and kidney was obviously higher than that in spleen. Specifically, bcTAB1 mRNA level in spleen increased significantly right after GCRV infection and reached the highest point (6.2-fold of the control) at day 1 post infection, and then fell below the control level until a minor recovery on day 5 post infection. bcTAB1 mRNA level in liver presented a fluctuant rise in the first day to the fourth day after infection and reached the highest value at day 5 post infection (81.5-fold of the control). bcTAB1 transcription in kidney was increased right after infection and reached the peak at day 3 (72.5-fold of the control), then was decreased significantly (Fig. 2A).

To learn bcTAB1 mRNA profile during host innate immune response, MPK cells were subject to different stimuli and bcTAB1 transcription was examined by q-PCR. In LPS treated MPK cells, bcTAB1 mRNA level was increased right after stimulation and the highest relative bcTAB1 mRNA level was 6.3-fold of the control (24 h, 10  $\mu$ g/ml). However, bcTAB1 transcription was not obviously increased post poly (I:C) treatment and even decreased bcTAB1 mRNA level was detected



**Fig. 1.** Evolution study of bcTAB1.(A): Comparisons of bcTAB1 with other vertebrate TAB1 by using MEGA 6.0 program and GeneDoc program, which including: *H. sapiens* (NP\_006107.1), *M. musculus* (NP\_079885.2), *G. gallus* (NP\_001006240.2), *D. rerio* (XP\_002662286.4) and *M. piceus*. The protein domains were predicted by CDS (Conserved Domain Search) of NCBI (<http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi>), and Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de>). (B): Phylogenetic tree of vertebrate TAB1. The amino acid sequence of bcTAB1 was aligned with TAB1 from different species by using MEGA 6.0 software, which included (GenBank accession number): *H. sapiens* (NP\_006107.1), *P. troglodytes* (XP\_016794684.1), *M. musculus* (NP\_079885.2), *C. hircus* (XP\_017904274.1), *S. scrofa* (NP\_001230996.1), *G. gallus* (NP\_001006240.2), *P. sinensis* (XP\_014429632.1), *X. laevis* (XP\_018116700.1), *D. rerio* (XP\_002662286.4), *T. rubripes* (XP\_011618322.1), *L. crocea* (XP\_010737364.2), *O. latipes* (XP\_011476721.2), *E. coioides* (KF768018.1), *C. idella* (KJ184547.1), *I. punctatus* (XM\_017482336.1), *X. maculatus* (XP\_023189039.1), *C. semilaevis* (XP\_024914324.1) and *M. piceus* (MK424333). The bar stands for scale length and the numbers on different nodes stand for bootstrap value.

**Table 2**  
Comparison of bcTAB1 with other vertebrate TAB1 (%).

| Species                         | Full-length sequence |          |
|---------------------------------|----------------------|----------|
|                                 | Similarity           | Identity |
| <i>Mylopharyngodon. piceus</i>  | 100                  | 100      |
| <i>Ctenopharyngodon. idella</i> | 99.8                 | 99.4     |
| <i>Danio. rerio</i>             | 93.2                 | 88.0     |
| <i>Ictalurus. punctatus</i>     | 90.2                 | 84.1     |
| <i>Epinephelus. coioides</i>    | 88.2                 | 81.5     |
| <i>Oryzias. latipes</i>         | 88.2                 | 80.5     |
| <i>Takifugu. rubripes</i>       | 88.0                 | 81.4     |
| <i>Cynoglossus. semilaevis</i>  | 87.0                 | 80.3     |
| <i>Larimichthys. crocea</i>     | 85.7                 | 79.7     |
| <i>Xiphophorus. maculatus</i>   | 83.6                 | 75.8     |
| <i>Gallus. gallus</i>           | 78.7                 | 67.4     |
| <i>Mus. musculus</i>            | 78.8                 | 66.4     |
| <i>Capra. hircus</i>            | 78.3                 | 65.6     |
| <i>Pelodiscus. sinensis</i>     | 78.1                 | 66.9     |
| <i>Sus. scrofa</i>              | 78.1                 | 66.3     |
| <i>Homo. sapiens</i>            | 78.1                 | 65.8     |
| <i>Pan. troglodytes</i>         | 78.1                 | 65.8     |
| <i>Xenopus. laevis</i>          | 76.3                 | 62.1     |

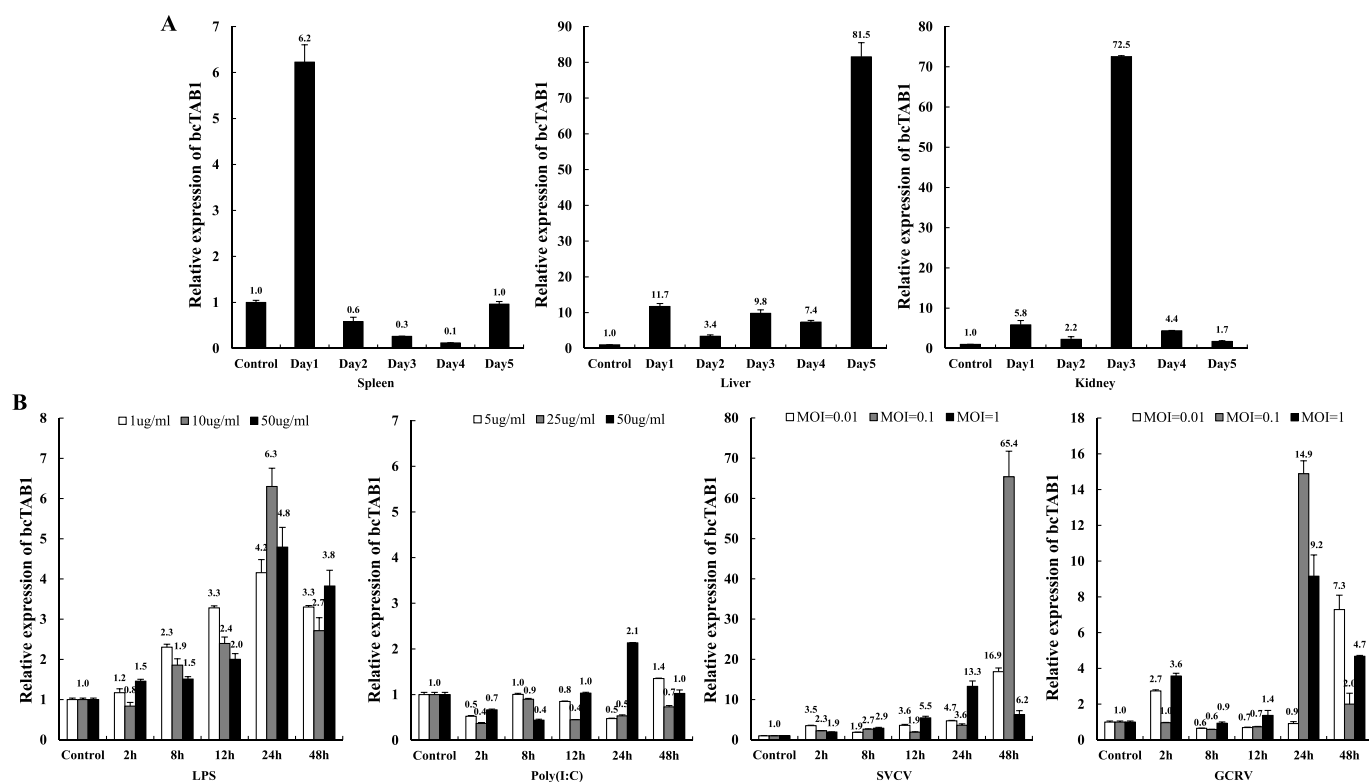
The IDs of TAB1 proteins in the table were the same as those in Fig. 1.

right after stimulation (Fig. 2B). In SVCV infected MPK cells, bcTAB1 mRNA level was increased right after infection and the highest relative bcTAB1 mRNA level within 48 h post infection (hpi) was 65.4-fold of

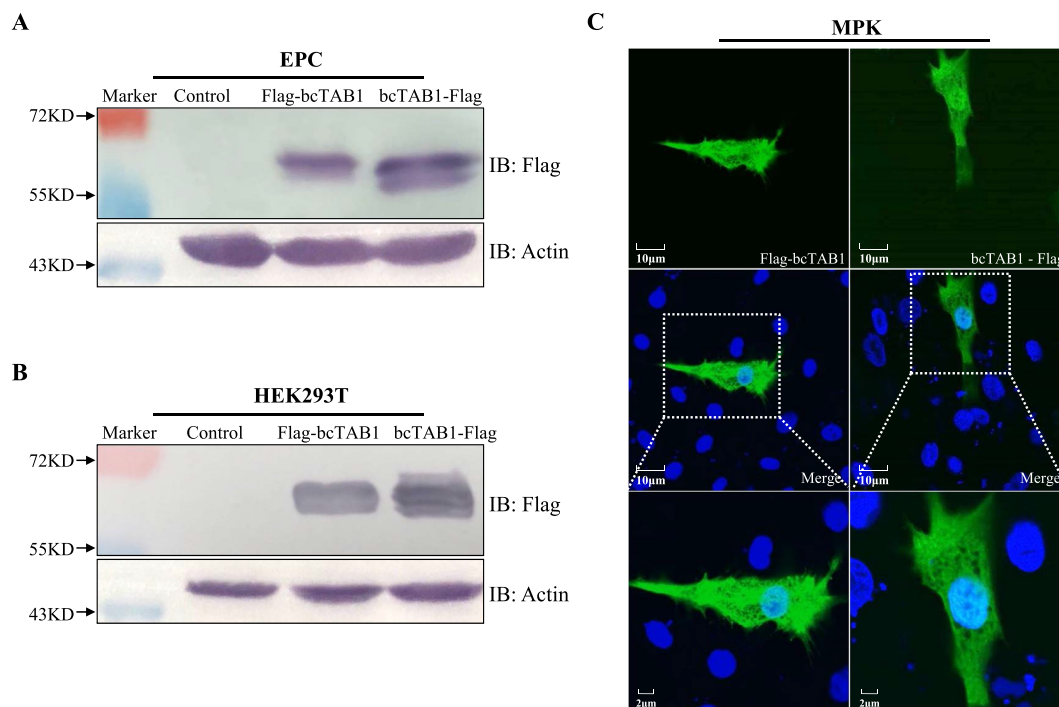
the control (48 h, 0.1MOI). However, in GCRV infected MPK cells, bcTAB1 mRNA level of 0.01MOI and 1MOI groups was increased right after infection (2hpi) and was decreased from 8hpi. bcTAB1 transcription was increased from 24hpi again and the highest relative bcTAB1 mRNA level within 48hpi was 14.9-fold of the control (24 h, 0.1MOI) (Fig. 2B). bcTAB1 transcription varied *in vivo* and *ex vivo* in response to different stimuli suggested that this fish protein was involved in host innate immune response initiated by these stimuli.

3.3. Protein expression and subcellular distribution of bcTAB1

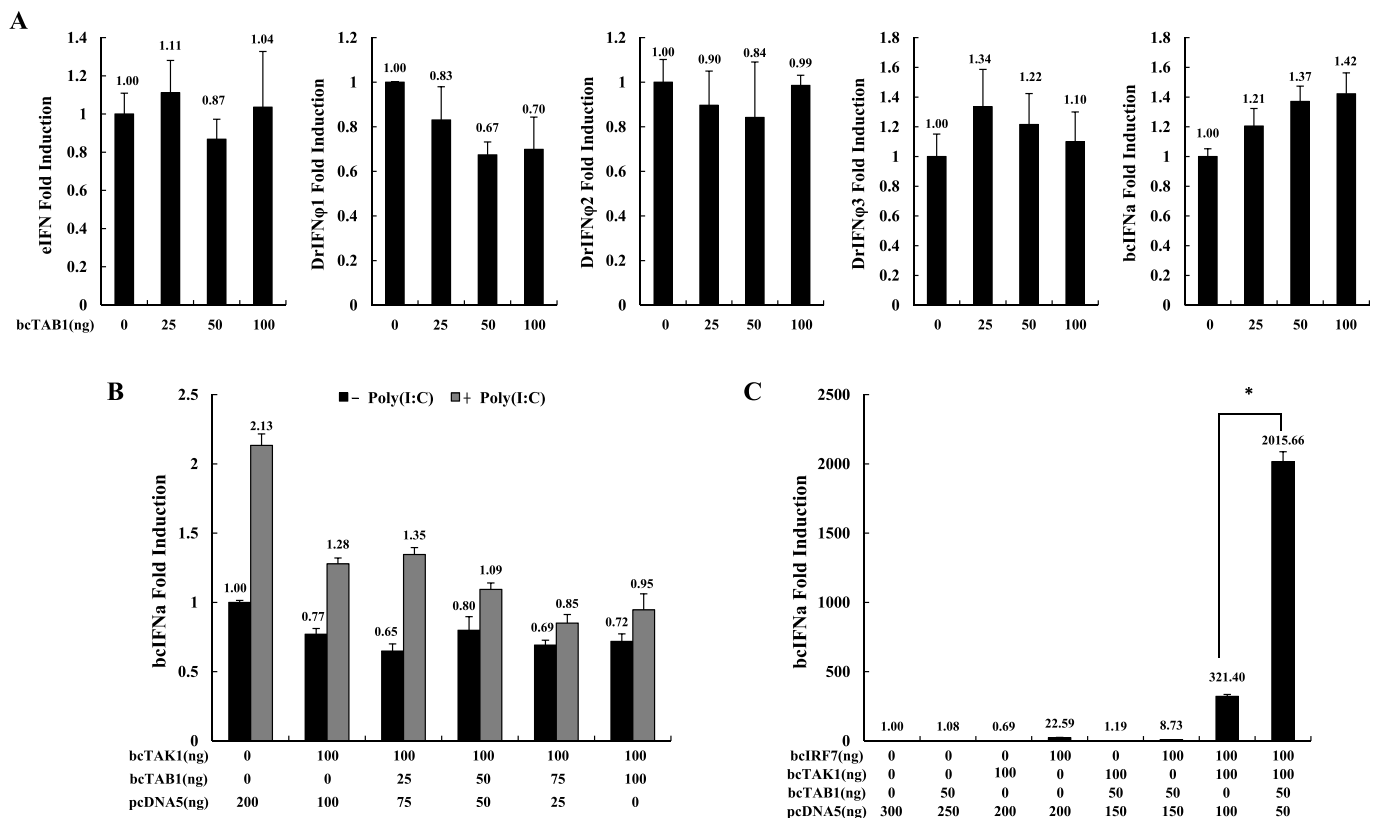
HEK293T cells or EPC cells were transfected with plasmids expressing bcTAB1 and used for immunoblotting (IB) assay to investigate bcTAB1 protein expression, in which mouse anti-Flag antibody was used to detect the overexpressed bcTAB1. The specific bands of ~62 kDa were detected in the whole cell lysate of both HEK293T cells and EPC cells transfected with bcTAB1 but not in the control (Fig. 3A and B). In the IB data, the migration of bcTAB1 (~62 kDa) was larger than its predicted molecular size (53.7 kDa) and more than one specific band were detected. It is speculated that the increased molecular weight of bcTAB1 is attributed to the post-translationally modification, for instance, three asparagine (N<sub>32</sub>, N<sub>246</sub>, N<sub>409</sub>) of bcTAB1 have been found in the conserved motif (N-X-S/T) of N-linked glycosylation. To determine the subcellular distribution of bcTAB1, MPK cells were transfected with plasmids expressing bcTAB1 and used for immunofluorescence staining (IF). The IF data showed clearly that the green color representing bcTAB1 was detected in both cytoplasm



**Fig. 2.** bcTAB1 expression *in vivo* and *ex vivo*. (A): Black carps of three months (weight of  $\sim 100$  g) were injected intraperitoneally with GCRV ( $5 \times 10^5$  pfu/fish) or sterile PBS separately and cultured at 25 °C. The injected black carps were sacrificed at 24 h, 48 h, 72 h, 96 h or 120 h post injection and total RNA was isolated from spleen, liver and kidney independently. Three fish were collected for each injected group at different time points. In each group (three fish), three RNA samples were combined and used for cDNA synthesis. Relative mRNA level of bcTAB1 was detected by q-PCR. The numbers above the error bars stand for the average bcTAB1 mRNA level, error bars represent the standard error of the mean ( $\pm$  SEM) of three independent experiments. (B): MPK cells in 6-well plate ( $2 \times 10^6$  cells/well) were treated with poly (I:C) or LPS at indicated concentration separately; or infected with SVCV or GCRV at indicated MOI separately. The cells were harvested at indicated time points post stimulation separately and used for RNA isolation. The relative bcTAB1 mRNA level was examined by q-PCR. The numbers above the error bars stand for average bcTAB1 mRNA level.



**Fig. 3.** Protein expression and subcellular distribution of bcTAB1. EPC (A) or HEK293T (B) cells were transfected with plasmids expressing bcTAB1 and used for immunoblot (IB) assay. (C): MPK cells were transfected with plasmids expressing bcTAB1 and the transfected cells were used for immunofluorescence staining according to the methods. Flag-bcTAB1: pcDNA5/FRT/TO-Flag-bcTAB1; bcTAB1-Flag: pcDNA5/FRT/TO-bcTAB1-Flag; The bars stand for the scale of 2  $\mu$ m or 10  $\mu$ m.



**Fig. 4.** The role of bcTAB1 in TAK1/IRF7-mediated IFN signaling. (A): EPC cells in 24-well plate were co-transfected with pRL-TK, Luci-bcIFNa (Luci-eIFN, Luci-DrIFNp1, Luci-DrIFNp2, and Luci-DrIFNp3) (for IFN promoter activity analysis), bcTAB1 or the empty vector separately and used for luciferase reporter assay according to methods. (B): EPC cells in 24-well plate were co-transfected with pRL-TK, Luci-bcIFNa, bcTAB1 and/or bcTAK1 and treated with poly (I:C) at the concentration of 25  $\mu$ g/ml, then used for luciferase reporter assay at 12 h after treatment according to methods. (C): EPC cells in 24-well plate were co-transfected with pRL-TK, Luci-bcIFNa, bcTAB1, bcTAK1 and/or bcIRF7, and used for reporter assay.

and nucleus, which demonstrated that bcTAB1 was a protein that expressed throughout the cells (Fig. 3C).

### 3.4. IFN signaling regulated by bcTAB1

To investigate the effect of bcTAB1 on IFN signaling, EPC cells were transfected with plasmids expressing bcTAB1 and/or bcTAK1 and used for dual luciferase reporter assay. In general, neither bcTAB1 expression alone nor co-expression of bcTAB1 and bcTAK1 could induce the transcription of IFN promoters (Fig. 4A and B). The bcIFNa promoter induction in the EPC cells expressing bcTAK1 was increased slightly after poly (I:C) treatment. However, compared with the control (EPC cells transfected with the empty vector), the lower bcIFNa promoter-inducing activity was observed in the EPC cells expressing bcTAK1 alone or co-expressing bcTAK1 and bcTAB1 after poly (I:C) treatment (Fig. 4B).

Our previous study demonstrated that bcIRF7 played a vital role in antiviral innate immunity and bcTAK1 up-regulated bcIRF7-mediated IFN signaling [25,26]. To explore whether bcTAB1 function on bcTAK1/bcIRF7/IFN signaling, EPC cells co-expressing bcTAB1, bcTAK1 and/or bcIRF7 were recruited for reporter assay. It was clear that the fold induction of bcIFNa promoter in EPC cells co-expressing three proteins (bcTAB1, bcTAK1 and bcIRF7) (2015-fold of the control) was obviously higher than that in EPC cells co-expressing bcTAK1 and bcIRF7 (321-fold of the control), which demonstrated that bcTAB1 up-regulated bcTAK1/bcIRF7/IFN signaling (Fig. 4C).

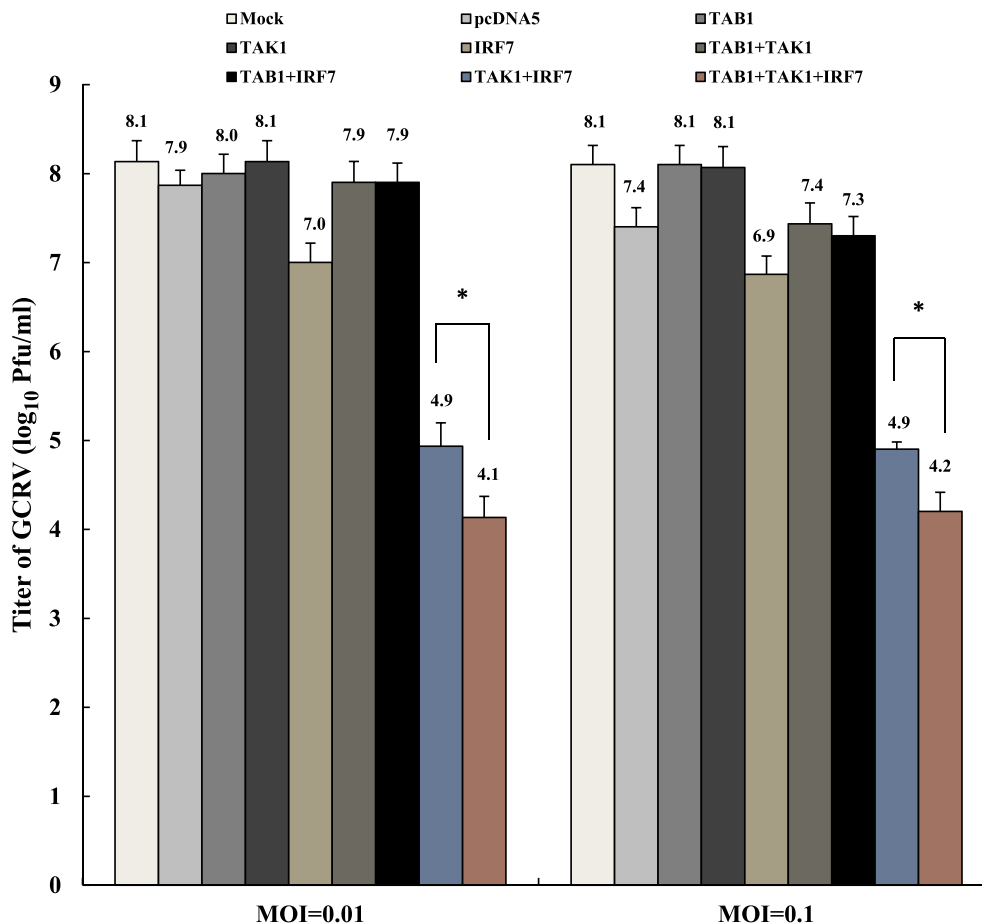
### 3.5. The role of bcTAB1 in TAK1/IRF7/IFN antiviral signaling

Our previous study demonstrated that bcTAK1 up-regulated bcIRF7-mediated antiviral activity [26]. To further explore the role of bcTAB1

on the up-regulation of bcIRF7-mediated antiviral activity by bcTAK1, EPC cells were transfected with plasmid expressing bcTAB1, bcTAK1 and/or bcIRF7, then subject to GCRV infection or SVCV infection separately. In GCRV infection group, the viral titer in the media of EPC cells co-expressing bcTAK1 and bcIRF7 was obviously lower than that of the EPC cells expressing bcIRF7 alone, which was in line with our previous research [26]. The plaque assay showed clearly that neither EPC cells expressing bcTAB1 nor EPC cells co-expressing bcTAB1 and bcTAK1 showed improved antiviral ability in contrast to the control group. However, EPC cells co-expressing these three proteins (bcTAB1/bcTAK1/bcIRF7) showed obviously improved antiviral activity compared with EPC cells co-expressing bcTAK1 and bcIRF7 (Fig. 5). Similar to GCRV infected group, EPC cells co-expressing these three molecules (bcTAB1/bcTAK1/bcIRF7) showed much stronger antiviral activity against SVCV than EPC cells co-expressing bcTAK1 and bcIRF7 (Fig. 6). Combined with the reporter assay results, our data demonstrated that bcTAB1 up-regulated TAK1/IRF7/IFN signaling during antiviral innate immune activation.

### 3.6. bcTAB1 interacts with bcTAK1 but not bcIRF7

In order to explore the mechanism of bcTAB1 in bcTAK1/bcIRF7/IFN antiviral signaling, two groups of co-immunoprecipitation (co-IP) experiments were recruited separately, which were aimed at the possible association between bcTAB1 and bcTAK1 (or bcIRF7). In the co-IP of bcTAB1 and bcTAK1, specific bands (red arrow indicated) representing bcTAB1 were detected in the precipitated proteins by bcTAK1, which demonstrated the direct binding between bcTAB1 and bcTAK1 *in vitro* (Fig. 7A). It was interesting that the amount and smear level of bcTAB1 in the HEK293T cells co-expressing bcTAB1 and



**Fig. 5.** bcTAB1 up-regulated TAK1/IRF7/IFN antiviral signaling against GCRV. EPC cells in 24-well plate ( $2 \times 10^5$  cells/well) were co-transfected with bcTAB1, bcTAK1 and/or bcIRF7. The transfected cells were infected with GCRV at 24 h post-transfection and the virus titers in the supernatant media were determined by plaque assay at 48 h post-infection. The numbers above the error bars stand for average virus titer. Mock: cells without transfection; pcDNA5: cells transfected with pcDNA5/FRT/TO; TAB1: pcDNA5/FRT/TO-Flag-bcTAB1; TAK1: pcDNA5/FRT/TO-HA-bcTAK1; IRF7: pcDNA5/FRT/TO-HA-bcIRF7.

bcTAK1 were obviously higher than that in HEK293T cells co-expressing bcTAB1 and the empty vector (Fig. 7A, middle panel), which suggested that bcTAK1 promoted both the expression level and post-translational modification level of bcTAB1. However, no specific band representing bcTAB1 was detected in the precipitated proteins by bcIRF7, which demonstrated that there was no direct association between bcTAB1 and bcTAK1 *in vitro* (Fig. 7B).

#### 4. Discussion

In humans and mammals, TAB1 is found to be constitutively associated with TAK1 and serves as a crucial activator in TAK1 signaling since TAK1 is not activated in TAB1<sup>-/-</sup> MEFs stimulated by IL-1 or TNF $\alpha$  [28]. Except for TAK1, TAB1 also directly binds to and activates p38 $\alpha$  and the combination is involved in the feedback regulation of TAK1 kinase activity [29,30]. An evolutionarily conserved motif at the C-terminus of TAB1 is essential for its constitutively binding to and activating the kinase domain of TAK1. The mutation analysis has demonstrated that Phe-484 of TAB1 is the most important single residue for binding TAB1 to TAK1 [31,32]. Co-expression of TAB1 and TAK1 induces the oligomerization and autophosphorylation of TAK1 at several residues (Thr-184, Thr-187 and Ser-192) in the kinase activation loop and thereby activates TAK1 *in vitro* [33,34]. However, other report has showed that the fully TAB1-dependent manner of TAK1 activation is limited to certain stress responses such as osmotic stress but not in TAK1-mediated cytokine signaling [35].

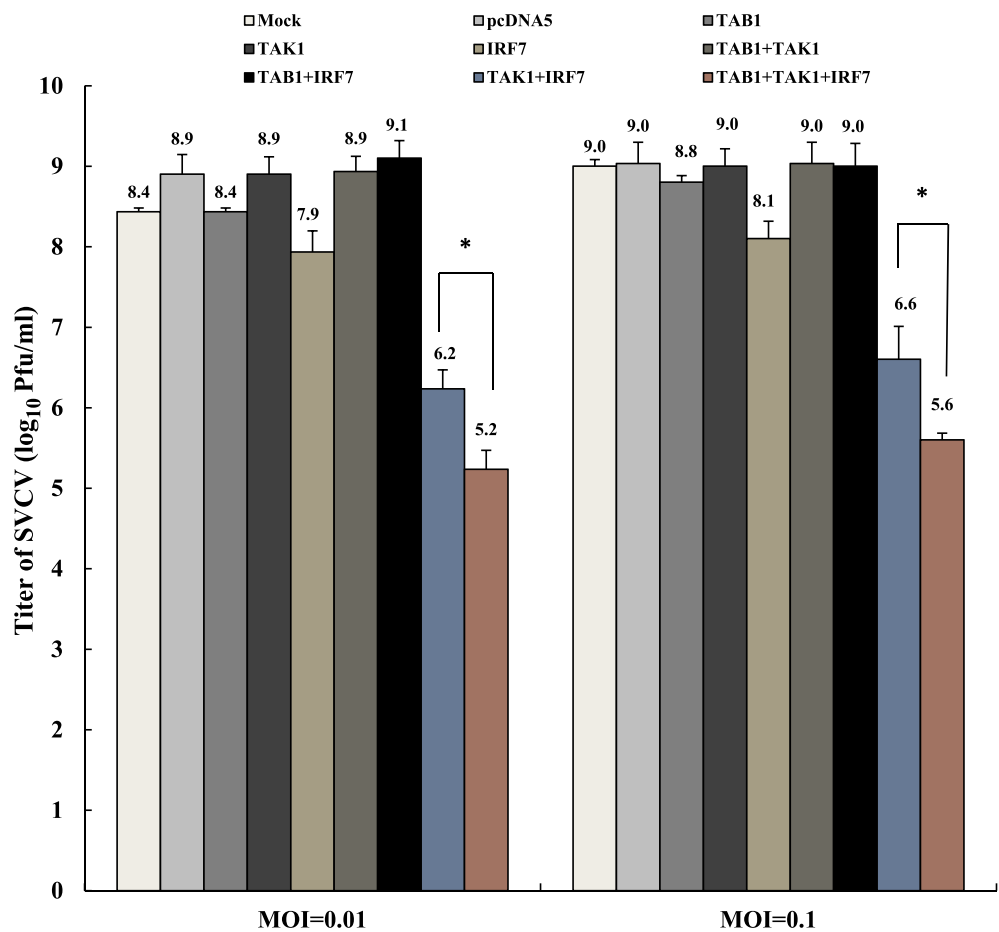
Phosphorylation sites (aa 452–457) of human TAB1 have been identified to promote a primarily cytosolic localization of TAB1, which are phosphorylated by TAK1 as well as by p38 MAPK. The data suggests that most TAB1 in cells is kept un-phosphorylated by serine/threonine phosphatases and is found in the nucleus [36]. Similar serine clusters

have been found in bcTAB1 (aa 441–446) by sequences alignment (Fig. 1A), which implies that these sites may be related to the nuclear importation of bcTAB1 (Fig. 3C).

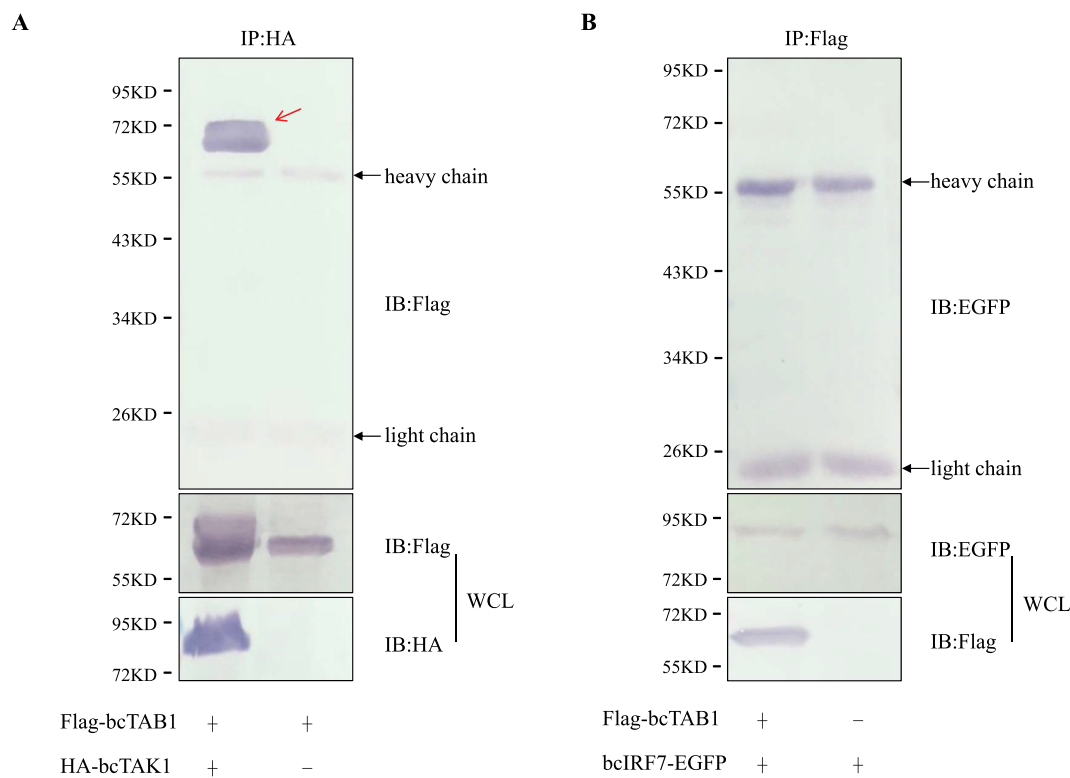
TAB1 is a key innate immunity-signaling O-glycan protein and a well-characterized OGT glycosylation substrate, which can be dynamically O-glycosylated at Ser395 in the C-terminal domain. What is more, the single O-glycosylation site on TAB1 has been proved to be related to the full activation of TAK1 [37,38]. The IB data of this study showed that more than one specific band (larger than the predicted molecular size of bcTAB1) were detected in the whole cell lysate of cells transfected with plasmid expressing bcTAB1 (Fig. 3A and B). Additionally, a suspected O-glycosylation site (S<sub>385</sub>) and three suspected N-glycosylation sites (N<sub>32</sub>, N<sub>246</sub>, N<sub>409</sub>) have been found in bcTAB1 (Fig. 1A and Supplementary Fig. 1), which suggested that this fish protein was modified with glycosylation. However, whether bcTAB1 is modified with glycosylation or not needs further research.

A positive feedback loop between TAB1 and pTAK1 has been hypothesized on the data that the expression of TAB1 has been reduced in the epidermis of TAK1 knockout mice, in which TAB1 induces the oligomerization and autophosphorylation of TAK1 and the phosphorylated TAK1 induces production of TAB1 at a post-transcriptional level [34,39]. Similar to this, in the co-IP data of our study, different expression levels of bcTAB1 have been detected in the HEK293T cells transfected with bcTAB1 alone or co-transfected with bcTAB1 and bcTAK1 (Fig. 7), which indicates that there is a high probability of positive regulation between bcTAB1 and bcTAK1.

In teleost fish, TAB1 was involved in the anti-parasitic innate immune response and inducing the activity of NF- $\kappa$ B by forming a complex with TAK1 [14,15]. The up-regulation of TAB1 expression by LPS stimulation indicates that TAB1 is involved in the host's natural immune response in amphioxus (*Branchiostoma belcheri*) [40], and TAB1 plays a



**Fig. 6.** bcTAB1 up-regulated TAK1/IRF7/IFN antiviral signaling against SVCV. EPC cells in 24-well plate ( $2 \times 10^5$  cells/well) were co-transfected with bcTAB1, bcTAK1 and/or bcIRF7. The transfected cells were infected with SVCV at 24 h post-transfection and the virus titers in the supernatant media were determined by plaque assay at 48 h post-infection. The numbers above the error bars stand for average virus titer. Mock: cells without transfection; pcDNA5: cells transfected with pcDNA5/FRT/TO; TAB1: pcDNA5/FRT/TO-Flag-bcTAB1; TAK1: pcDNA5/FRT/TO-HA-bcTAK1; IRF7: pcDNA5/FRT/TO-HA-bcIRF7.



**Fig. 7.** The association between bcTAB1 and bcTAK1. HEK293T cells were co-transfected with bcTAB1 and bcTAK1 (A), or bcTAB1 and bcIRF7 (B), and used for co-immunoprecipitation (co-IP) assay. IB: immunoblot; WCL: whole cell lysate; Flag-bcTAB1: pcDNA5/FRT/TO-Flag-bcTAB1; HA-bcTAK1: pcDNA5/FRT/TO-HA-bcTAK1; bcIRF7-EGFP: bcIRF7-pEGFP.

protective role against bacterial infection in shrimp (*Litopenaeus vannamei*) [41]. However, there is no report on TAB1 in the antiviral innate immunity so far in teleost. In general, TAB1 binds to TAK1 and activates the kinase activity of TAK1, which is primarily involved in regulating JNK/p38 MAPKs and IKK signaling and inducing the activation of AP-1 and NF- $\kappa$ B [8,9], while IRF7 triggers the transcription of IFNs in response to viral invasion by phosphorylated by the complex of IKK $\epsilon$  and TBK1 [42]. In this study, the synergistic relationship between bcTAB1 and bcTAK1 in the antiviral state in black carp has been identified, which boosts the IFN signaling mediated by bcIRF7 during host innate immune activation against GCRV and SVCV. Based on these studies, it is hypothesized that bcTBK1/bcIKK $\epsilon$  complex attracts and phosphorylates most bcIRF7 molecules after the recognition of invading RNA viruses by PRRs of black carp, which transfer into nuclear and trigger the transcription of IFNs. And few bcIRF7 molecules are recruited by bcTAK1 at the same time, in which bcTAK1 phosphorylates bcIRF7 through similar or different mechanism to that of bcTBK1/bcIKK $\epsilon$ . bcTAB1 binds and activates bcTAK1 during this process, which leads to the enhanced TAK1/IRF7 signaling (Figs. 5 and 6). It is speculated that the affinity between bcTAK1 and bcIRF7 is weaker than that between bcTBK1/bcIKK $\epsilon$  complex and bcIRF7, which leads to that black carp TBK1/IKK $\epsilon$ /IRF signaling functions majorly in the innate immune activation. And it is reasonable that the limited endogenous IRF7 molecules are not “satisfied by” exogenous bcTAK1/bcTAB1 complexes in EPC cells, which lead to the non-enhanced antiviral activity of EPC cells co-transfected with bcTAB1 and bcTAK1 and the obviously improved antiviral activity of EPC cells co-transfected with bcTAB1, bcTAK1 and bcIRF7.

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (81471963), Training Program for Excellent Young Innovators of Changsha (kp1802013), Hunan province college students research learning and innovative experiment project (201810542033), Hunan Normal University undergraduates innovative experiment project (2018126, 2018127) and the Cooperative Innovation Center of Engineering and New Products for Developmental Biology of Hunan Province (20134486).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.04.040>.

## References

- [1] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, *Cell* 124 (4) (2006) 783–801.
- [2] C.A. Thaiss, M. Levy, S. Itav, et al., Integration of innate immune signaling, *Trends Immunol.* 37 (2) (2016) 84–101.
- [3] X. Jiang, L.N. Kinch, C.A. Brautigam, et al., Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response, *Immunity* 36 (6) (2012) 959–973.
- [4] A. Pichlmair, C.R. e Sousa, Innate recognition of viruses, *Immunity* 27 (3) (2007) 370–383.
- [5] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, *Cell* 140 (6) (2010) 805–820.
- [6] S. Aashaq, A. Batool, K.I. Andrabi, TAK1 mediates convergence of cellular signals for death and survival, *Apoptosis* (2018) 1–18.
- [7] Y. Hirata, M. Takahashi, T. Morishita, et al., Post-translational modifications of the TAK1-TAB complex, *Int. J. Mol. Sci.* 18 (1) (2017) 205.
- [8] H. Sakurai, A. Nishi, N. Sato, et al., TAK1–TAB1 fusion protein: a novel constitutively active mitogen-activated protein kinase kinase kinase that stimulates AP-1 and NF- $\kappa$ B signaling pathways, *Biochem. Biophys. Res. Commun.* 297 (5) (2002) 1277–1281.
- [9] S.R. Mihaly, S. Morioka, J. Ninomiya-Tsuji, et al., Activated macrophage survival is coordinated by TAK1 binding proteins, *PLoS One* 9 (4) (2014) e94982.
- [10] H. Sakurai, N. Shigemori, K. Hasegawa, et al., TGF- $\beta$ -activated kinase 1 stimulates NF- $\kappa$ B activation by an NF- $\kappa$ B-inducing kinase-independent mechanism, *Biochem. Biophys. Res. Commun.* 243 (2) (1998) 545–549.
- [11] Y. Lu, Y. Zhang, L. Li, et al., TAB1: a target of triptolide in macrophages, *Chem. Biol.* 21 (2) (2014) 246–256.
- [12] M. Inagaki, Y. Komatsu, G. Scott, et al., Generation of a conditional mutant allele for Tab1 in mouse, *Genesis* 46 (8) (2008) 431–439.
- [13] H. Shibuya, H. Iwata, N. Masuyama, et al., Role of TAK1 and TAB1 in BMP signaling in early Xenopus development, *EMBO J.* 17 (4) (1998) 1019–1028.
- [14] F. Zhao, Y.W. Li, H.J. Pan, et al., TAK1-binding proteins (TAB1 and TAB2) in grass carp (*Ctenopharyngodon idella*): identification, characterization, and expression analysis after infection with *Ichthyophthirius multifiliis*, *Fish Shellfish Immunol.* 38 (2) (2014) 389–399.
- [15] S.Y. Bao, Q.X. Sun, C.L. Yao, The interaction of TAK1 and TAB1 enhances LPS-induced cytokine release via modulating NF- $\kappa$ B activation (*Larimichthys crocea*), *Fish Shellfish Immunol.* 74 (2018) 450–458.
- [16] Y.Z. Hu, X. Li, R. Han, et al., Molecular identification and expression analysis of TAB1 from orange-spotted grouper (*Epinephelus coioides*), *Dev. Comp. Immunol.* 90 (2019) 152–156.
- [17] X. Zhang, Y. Shen, X. Xu, et al., Transcriptome analysis and histopathology of black carp (*Mylopharyngodon piceus*) spleen infected by *Aeromonas hydrophila*, *Fish Shellfish Immunol.* 83 (2018) 330–340.
- [18] H. Chen, J. Xiao, J. Li, et al., TRAF2 of black carp upregulates MAVS-mediated antiviral signaling during innate immune response, *Fish Shellfish Immunol.* 71 (2017) 1–9.
- [19] Y. Teng, H. Liu, J.Q. Lv, et al., Characterization of complete genome sequence of the spring viremia of carp virus isolated from common carp (*Cyprinus carpio*) in China, *Arch. Virol.* 152 (8) (2007) 1457–1465.
- [20] Y.M. Loo, M. Gale Jr., Immune signaling by RIG-I-like receptors, *Immunity* 34 (5) (2011) 680–692.
- [21] M. Chang, B. Collet, P. Nie, et al., Expression and functional characterization of the RIG-I like receptors MDA5 and LGP2 in rainbow trout *Oncorhynchus mykiss*, *J. Virol.* 85 (16) (2011) 8403–8412.
- [22] S.N. Chen, P.F. Zou, P. Nie, Retinoic acid-inducible gene I (RIG-I)-like receptors (RLR s) in fish: current knowledge and future perspectives, *Immunology* 151 (1) (2017) 16–25.
- [23] J. Liu, J. Li, J. Xiao, et al., The antiviral signaling mediated by black carp MDA5 is positively regulated by LGP2, *Fish Shellfish Immunol.* 66 (2017) 360–371.
- [24] W. Zhou, J. Zhou, Y. Lv, et al., Identification and characterization of MAVS from black carp *Mylopharyngodon piceus*, *Fish Shellfish Immunol.* 43 (2) (2015) 460–468.
- [25] J. Li, Y. Tian, J. Liu, et al., Lysine 39 of IKK $\epsilon$  of black carp is crucial for its regulation on IRF7-mediated antiviral signaling, *Fish Shellfish Immunol.* 77 (2018) 410–418.
- [26] C. Wang, J. Peng, M. Zhou, et al., TAK1 of black carp positively regulates IRF7-mediated antiviral signaling in innate immune activation, *Fish Shellfish Immunol.* 84 (2019) 83–90.
- [27] X. Wang, X. Song, X. Xie, et al., TRAF3 enhances STING-mediated antiviral signaling during the innate immune activation of black carp, *Dev. Comp. Immunol.* 88 (2018) 83–93.
- [28] H. Mendoza, D.G. Campbell, K. Burness, et al., Roles for TAB1 in regulating the IL-1-dependent phosphorylation of the TAB3 regulatory subunit and activity of the TAK1 complex, *Biochem. J.* 409 (3) (2008) 711–722.
- [29] P.C.F. Cheung, D.G. Campbell, A.R. Nebreda, et al., Feedback control of the protein kinase TAK1 by SAPK2a/p38 $\alpha$ , *EMBO J.* 22 (21) (2003) 5793–5805.
- [30] L. Richardson, C.L. Dixon, L. Aguilera-Aguirre, et al., Oxidative Stress-Induced TGF- $\beta$ /TAB1-mediated p38MAPK activation in human amnion epithelial cells, *Biol. Reprod.* (2018).
- [31] K. Ono, T. Ohtomo, S. Sato, et al., An evolutionarily conserved motif in the TAB1 C-terminal region is necessary for interaction with and activation of TAK1 MAPKKK, *J. Biol. Chem.* 276 (26) (2001) 24396–24400.
- [32] K. Brown, S.C.M. Vial, N. Dedi, et al., Structural basis for the interaction of TAK1 kinase with its activating protein TAB1, *J. Mol. Biol.* 354 (5) (2005) 1013–1020.
- [33] H. Sakurai, H. Miyoshi, J. Mizukami, et al., Phosphorylation-dependent activation of TAK1 mitogen-activated protein kinase kinase by TAB1, *FEBS Lett.* 474 (2–3) (2000) 141–145.
- [34] E. Omori, M. Inagaki, Y. Mishina, et al., Epithelial transforming growth factor  $\beta$ -activated kinase 1 (TAK1) is activated through two independent mechanisms and regulates reactive oxygen species, *Proc. Natl. Acad. Sci. Unit. States Am.* 109 (9) (2012) 3365–3370.
- [35] M. Inagaki, E. Omori, J.Y. Kim, et al., TAK1-binding protein 1, TAB1, mediates osmotic stress-induced TAK1 activation but is dispensable for TAK1-mediated cytokine signaling, *J. Biol. Chem.* 283 (48) (2008) 33080–33086.
- [36] A. Wolf, K. Beuerlein, C. Eckart, et al., Identification and functional characterization of novel phosphorylation sites in TAK1-binding protein (TAB) 1, *PLoS One* 6 (12) (2011) e29256.
- [37] S. Pathak, V.S. Borodkin, O. Albarbarawi, et al., O-GlcNAcylation of TAB1 modulates TAK1-mediated cytokine release, *EMBO J.* 31 (6) (2012) 1394–1404.
- [38] K. Rafie, O. Raimi, A.T. Ferenbach, et al., Recognition of a glycosylation substrate by the O-GlcNAc transferase TPR repeats, *Open biology* 7 (6) (2017) 170078.
- [39] R. Singh, Model predicts that MKP1 and TAB1 regulate p38 $\alpha$  nuclear pulse and its basal activity through positive and negative feedback loops in response to IL-1, *PLoS One* 11 (6) (2016) e0157572.
- [40] D. Yin, W. Li, M. Fu, et al., Identification and characterization of a TAB1 gene involved in innate immunity of amphioxus (*Branchiostoma belcheri*), *Gene* 575 (2) (2016) 294–302.
- [41] S. Wang, M. Li, B. Yin, et al., Shrimp TAB1 interacts with TAK1 and p38 and activates the host innate immune response to bacterial infection, *Mol. Immunol.* 88 (2017) 10–19.
- [42] J. Zhang, M. Tian, Z. Xia, et al., Roles of I $\kappa$ B kinase  $\epsilon$  in the innate immune defense and beyond, *Virol. Sin.* 31 (6) (2016) 457–465.