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Characterization of the black carp TRAF6 signaling molecule in innate immune defense

Shu Jiang [a, b], Jun Xiao [b], Jun Li [b], Hui Chen [b], Chanyuan Wang [b], Chaoliang Feng [b], Hao Feng [b, *]

[a] The Second Xiangya Hospital, Central South University, Changsha 410011, China
[b] State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha 410081, China

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ABSTRACT

Tumor necrosis factor receptor-associated factor 6 (TRAF6) plays a vital role in the innate immune response of higher vertebrates. To elucidate its function in teleost fish, TRAF6 homologue of black carp (*Mylopharyngodon piceus*) has been cloned and characterized in this study. Black carp TRAF6 (bcTRAF6) transcription in *Mylopharyngodon piceus* fin (MPF) cells was up-regulated in response to both poly (I:C) treatment and viral infection, but was suppressed by LPS stimulation. bcTRAF6 migrated around 72 KDa in immunoblot analysis and was identified as a cytosolic protein suggested to be associated with vesicles scattering in the cytoplasm. Reporter assay demonstrated that NF-κB instead of IFN was activated by bcTRAF6; and EPC cells expressing bcTRAF6 presented the same cytopathic effect (CPE) ratio to that of control cells. When co-expressed with bcMAVS, bcTRAF6 was redistributed and overlapped with the subcellular location of bcMAVS. It was interesting that bcMAVS mediated the IFN induction was up-regulated by low input of bcTRAF6 but down-regulated by high input of bcTRAF6. Taken together, the data generated in this paper supported the conclusion that bcTRAF6 associated with bcMAVS and was recruited into bcMAVS mediated signaling during host innate immune response.

1. Introduction

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 [1,2]. More and more studies demonstrated that members of TRAF family played vital roles in the signaling during host immune response [3,4]. TRAF family is composed of seven members (TRAF1~7), which were originally discovered and characterized as signaling adaptor molecules coupled to the cytoplasmic regions of receptors of the TNF-R superfamily [5,6]. All family members except TRAF1 contain a RING finger domain localized at their amino-terminus, followed by one or more zinc finger domains. The RING finger domain is common to many E3 ubiquitin ligases, constituting the core of the ubiquitin ligase catalytic domain [1].

TRAF6 is broadly expressed in tissues of higher vertebrates and well conserved across species [7]. TRAF6 was identified through two independent efforts, which showed that TRAF6 was capable of bridging signaling by TNFR and TLR/IL-1R superfamilies [8,9]. TRAF6$^{-/-}$ mice survive less than two weeks after birth, which demonstrates that TRAF6 possesses critical biological functions [10]. Bone marrow chimera mice derived from TRAF6$^{-/-}$ donor bone marrow showed disorganized lymphoid tissues and inflammatory autoimmune disease, which implied the important roles of TRAF6 in the mammal immune system [11].

Mammalian TRAF6 protein is highly conserved and binding to TRAF6 interaction motifs is believed to activate TRAF6 by promoting oligomerization. These interaction motifs can link TRAF6 directly to the intracellular domains of transmembrane receptors, such as TGFβRI [12,13], or to signaling receptors indirectly via cytoplasmic intermediaries, such as MAVS [14]. MAVS harbors binding motifs for TRAF2, TRAF3, TRAF5, and TRAF6, in which two motifs, PGENSE (153–158) and PEENEY (455–460) bind TRAF6. Mutations of these motifs that disrupted MAVS binding to TRAF6 will abrogate its ability to activate IRF3 in mammals; IRF3 activation was also abolished in cells lacking TRAF6 [14].

Identification of zebrafish TRAF6 was the first report in teleost

* Corresponding author.
E-mail address: fenghao@hunnu.edu.cn (H. Feng).

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fish [15], in which zebrafish TRAF6 was found to stimulate NF-KB activation and its transcription were up-regulated when exposed to snakehead rhabdovirus (SHRV). TRAF6 orthologs was cloned and identified in other fishes in recent years, such as Cyprinidae and Epinephelus [16–19]. Similar to TRAF6 of higher vertebrates, TRAF6 of grass carp (*Ctenopharyngodon idella*) also contains one RING domain, two zinc fingers, one coiled-coil region, and one MATH domain. High similarity of sequence and structural domains between the fish and mammalian TRAF6 proteins implies the conserved function of fish TRAF6 [17,20].

As an important freshwater industrial species in China, black carp (*Mylopharyngodon Piceus*) is among the "Four Domesticated Fish" for at least a thousand years. Black carp is subject to some pathogenic microorganisms in natural or aquacultural conditions such as grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV), however, its innate immune system remains much unknown. In our previous study, MAVS, IKKε and LGP2 were cloned and characterized from this cyprinid fish (named bcMAVS, bcIKKε and bcLGP2 accordingly). Just like their mammalian counterparts, those RLR signaling components of black carp activated the promoter activity of zebrafish interferon (IFN) and fathead minnow IFN [21–23]. bcIFNa, one type I IFN and the downstream effector of RLR signaling of black carp, were also cloned and showed strong antiviral ability against both SVCV and GCRV [24]. To investigate the role of TRAF6 in the antiviral innate immune response in this cyprinid fish, bcTRAF6 was cloned and characterized in this paper. This fish TRAF6 homologue activated NF-κB but not IFN transcription. On account of the fact that MAVS polymers recruit TRAF6 through distinct TRAF-binding motifs in mammals [14], we extended our research to explore the relationship between bcTRAF6 and bcMAVS during the host immune response. Our data demonstrated clearly that bcTRAF6 was recruited onto mitochondria by bcMAVS and regulated bcMAVS mediated signaling in a dose dependent manner.

2. Materials and methods

2.1. Cells and plasmids

HEK293T, HeLa, NIH3T3, *Epithelioma papulosum cyprini* (EPC), *Ctenopharyngodon idella* kidney (CIK), *Mylopharyngodon piceus* kidney (MPK) and *Mylopharyngodon piceus* fin (MPF) cells were kept in the lab [24]. HEK293T and HeLa cells were cultured at 37 °C; EPC, CIK and MPF cells were cultured at 25 °C. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. 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 [25].

pcDNA5/FRT/TO-HA, pcDNA5/FRT/TO-Flag, pcDNA5/FRT/TO-HA-bcMAVS, EGFP-bcMAVS, pRL-TK, Luci-NF-κB, Luci-eIFN (for fathead minnow IFN promoter activity analysis) and Luci-zIFN3 (for zebrafish IFN3 promoter activity analysis) were kept in the lab [21]. The recombinant expression vector pcDNA5/FRT/TO-HA-bcTRAF6 and pcDNA5/FRT/TO-bcTRAF6-HA were constructed by cloning the open reading frame (ORF) of bcTRAF6 fused with an HA tag at its N-terminus or C-terminus into pcDNA5/FRT/TO (Invitrogen), respectively.

2.2. Cloning the cDNA of bcTRAF6

Degenerate Primers (Table 1) were designed to amplify the cDNA of bcTRAF6 based on the sequences of TRAF6 of *C. auratus* (KF76099.1), *C. idella* (KC465198.1), *C. Carpio* (HM535646.1) and *D. rerio* (NM_001044752.1). 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). The coding sequence (CDS) was cloned at the first attempt by using the degenerate primers. Rapid amplification of cDNA ends (RACE) was

Table 1
Primers used in the study.

Primer name	Sequence(5′-3′)	Ampcicon length (nt) and primer information
TRAF6-F2	ATGGCTTGCARYGACATGGAGAAG	1629
TRAF6-R1	TCAAAGTGAAGGTTCTGGGCCCCGAG	Gene cloning
3'race		
TRAF6-3R1	TGAGGTCACACCACGAATC	3′ UTR 1st PCR
TRAF6-3R2	GGGTTTTGGCTATGTTACTTTCC	3′ UTR 2nd PCR
UPM-longer	CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT	
UPM-Short primer	CTAATACGACTCACTATAGGGC	
5'race		
TRAF6-5R1	CCATCACAGCAGGCATCATCAAG	5′ UTR 1st PCR
TRAF6-5R2	CATCATAGCCTTGCTGGTCTG	5′ UTR 2nd PCR
5'race out primer	CATGGCTACATGCTGACAGCCTA	
5'race inner primer	CGCGGATCCACAGCCTACTGATG ATCAGTCGATG	
Expression construct		
TRAF6-N-F	ACTGACGGTACCATGGCTTGCAGTGACAT	
TRAF6-N-R	ATTTGCGGCCGCTCAAAGTGAAGGTTCT	HA-TRAF6
TRAF6-C-F	ACTGACGGTACCGCCACCATGGCTTGCAGTG	
TRAF6-C-R	ATTTGCGGCCGCTTAAGTGAAGGTTCTGGGC	TRAF6-HA
q-PCR		
bc Q actin-F	TGGGCACCGCTGCTTCCT	
bc Q actin-R	TGTCCGTCAGGCAGCTCAT	
TRAF6-qF1	GGTTGTCGTGAAAAGATGC	
TRAF6-qR1	TATTGAGAGATTGGCTGCG	

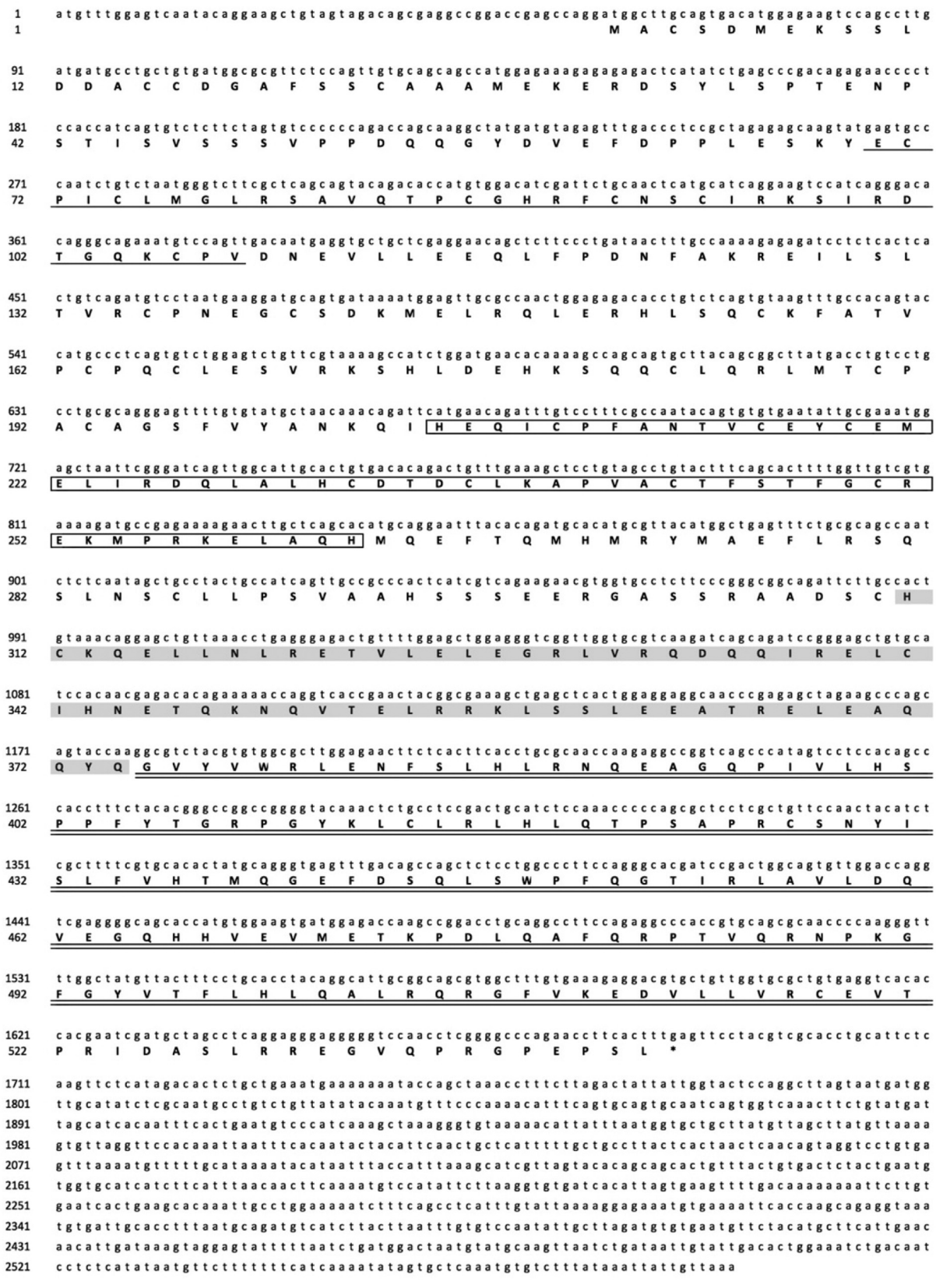

Fig. 1. The full-length cDNA of bcTRAF6. N-terminal RING domain is underlined (70–108), zinc finger-TRAF domain (205–262) is boxed, domain of coil-coiled (311–374) is marked in grey, MATH_TRAF6 c-terminal domain is indicated by double line (375–521). The protein domains were predicted by CDS (Conserved Domain Search) of NCBI (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi), and Simple Modular Architecture Research Tool (SMART) (http://smart.emble-heidelberg.de).

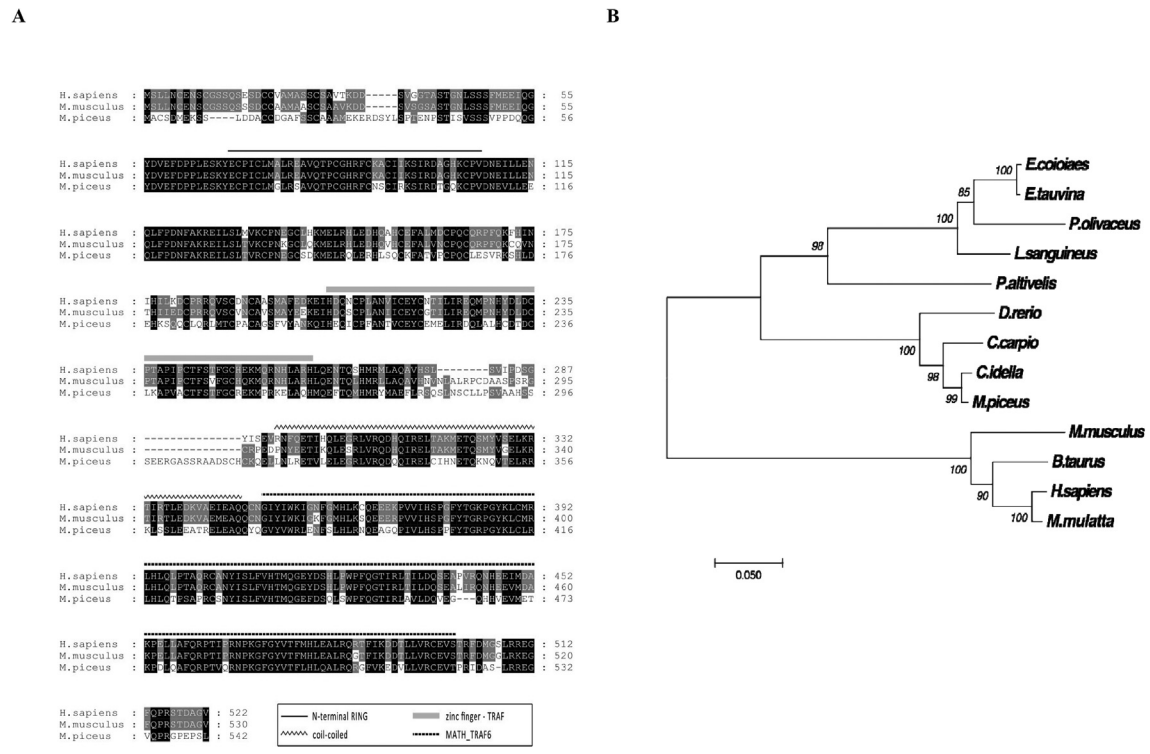

Fig. 2. Evolution of vertebrate TRAF6. A. Comparisons of bcTRAF6 with other vertebrate TRAF6 protein sequences by using MEGA 6.0 program and GeneDoc program, which including: *H. sapiens* (AY228337.1), *M. musculus* (D84655.1) and *M. piceus*. The protein domains were predicted by CDS (Conserved Domain Search) of NCBI (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi), and Simple Modular Architecture Research Tool (SMART) (http://smart.emble-heidelberg.de). **B.** By using MEGA 5.0 program, maximum likelihood phylogenetic tree was generated from vertebrate TRAF6 of different species which include (GenBank accession number): *E. coioiaes* (KF137656.1), *E. tauvina* (KJ000381.1), *P. olivaceus* (KM655804.1), *L. sanguineus* (KF279358.1), *P. altivelis* (AB516401.1), *D. rerio* (NM_001044752.1), *C. Carpio* (HM535646.1), *C. idella* (KC465198.1), *M. musculus* (D84655.1), *B. taurus* (DQ471669.1), *H. sapiens* (AY228337.1), *M. mulatta* (NM_001135796.1). The bar stands for scale length and the numbers on different nodes stand for bootstrap value.

Table 2
Comparison of bcTRAF6 with other vertebrate TRAF6 (%).

Species	Full-length sequence of protein	
	Identity	Similarity
M. piceus	100.0	100.0
C.idella	98.5	99.3
C.carpio	93.2	96.3
D.rerio	89.9	94.6
P.altivelis	69.0	78.0
L.sanguineus	65.5	77.4
E.tauvina	64.6	76.0
E.coioides	64.6	76.0
P.olivaceus	63.3	75.2
M.mulatta	57.3	70.4
H.sapiens	56.5	70.4
B.taurus	57.0	69.5
M.musculus	56.1	69.4

performed to obtain 5′UTR and 3′UTR of bcTRAF6 cDNA by using 5′Full RACE kite and 3′Full RACE kite separately (TaKaRa). The amplified fragments were cloned into pMD18-T vector and sequenced by Invitrogen.

2.3. Virus produce and titration

SVCV and GCRV were kept in the lab and propagated in EPC or CIK separately at 25 °C in the presence of 2% fetal bovine serum. Virus titers were determined by plaque assay on EPC cells as previously described [21]. Briefly, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 25 °C. The supernatant was replaced with DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) after incubation. Plaques were counted at day 3 post infection.

2.4. LPS and poly (I:C) treatment

MPF cells were seeded in 6-well plate (2 × 106 cells/well) 16 h before treatment. Poly(I:C) (Sigma) was used for synthetic dsRNA stimulation, which was heated to 55 °C (in PBS) for 5min and cooled at room temperature before use. MPF cells were replaced with 1 ml fresh media containing poly (I:C) and harvested at different time points (2 h, 8 h, 24 h) post treatment. bcTRAF6 mRNA level in the MPF cells was determined by quantitative real-time PCR (q-PCR). For LPS treatment, MPF cells in 6-well plate (2 × 106 cells/well) were treated with LPS separately and harvested at different time points post stimulation as above.

2.5. Quantitative real-time PCR

The relative bcTRAF6 mRNA level in the selected tissues of black carp or MPF cells was determined by quantitative real-time PCR. The primers for *bcTRAF6* gene and *β-actin* gene (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 spreadsheet for subsequent data analysis where the relative expression ratios of target gene in treated groups versus those in control group were calculated by $2^{-\triangle\triangle CT}$ method.

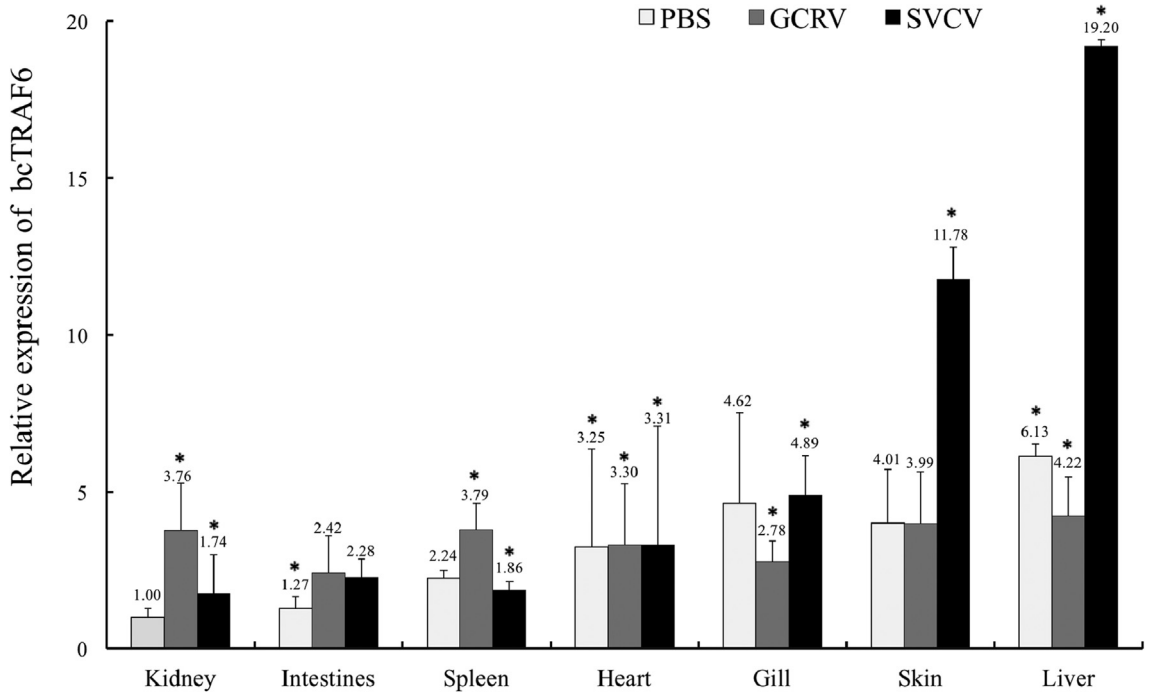

Fig. 3. Tissue-specific mRNA expression of bcTRAF6. Black carp of six months (weight of 120 g) were injected intraperitoneally with GCRV (2.52×10^6 pfu/fish), SVCV (2.43×10^6 pfu/fish) or sterile PBS (as healthy control) separately and cultured at 25 °C. Three fish were collected for each injected group (PBS, SVCV or GCRV). The injected black carp were sacrificed at 33 h post injection and total RNA was isolated from tissues of heart, liver, spleen, kidney, intestine, skin and gill independently. Relative mRNA level of bcTRAF6 after virus challenge or in healthy condition was detected by q-PCR separately. The numbers above the error bars stand for average bcTRAF6 mRNA level, error bars denote standard deviation.

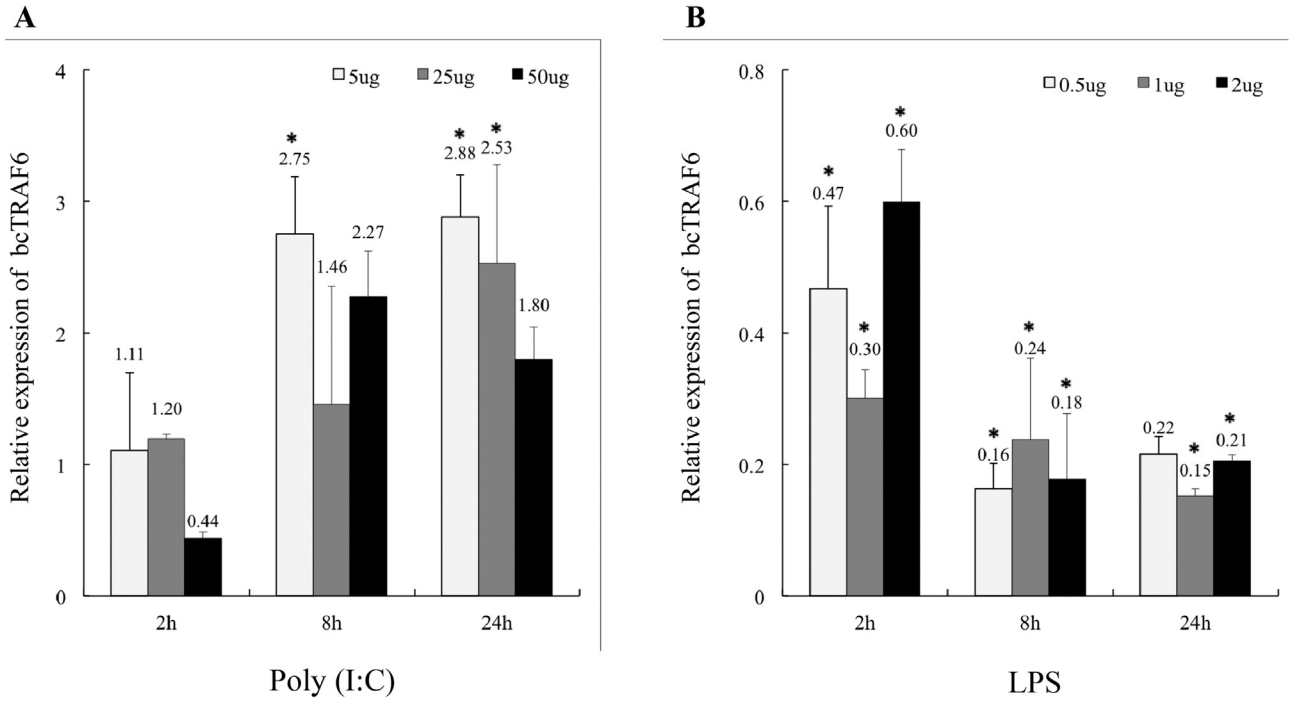

Fig. 4. The mRNA expression of bcTRAF6 post poly (I:C) or LPS stimulation. MPF cells in 6-well plate (2×10^6 cells/well) were treated with poly (I:C) (**A**) or LPS (**B**) at the indicated concentration separately. The cells were harvested at 2 h, 8 h or 24 h post stimulation separately and used for RNA isolation. The relative bcTRAF6 mRNA level was examined by q-PCR. The numbers above the error bars stand for average bcTRAF6 mRNA level, error bars denote standard deviation and asterisk (*) stands for $p < 0.05$.

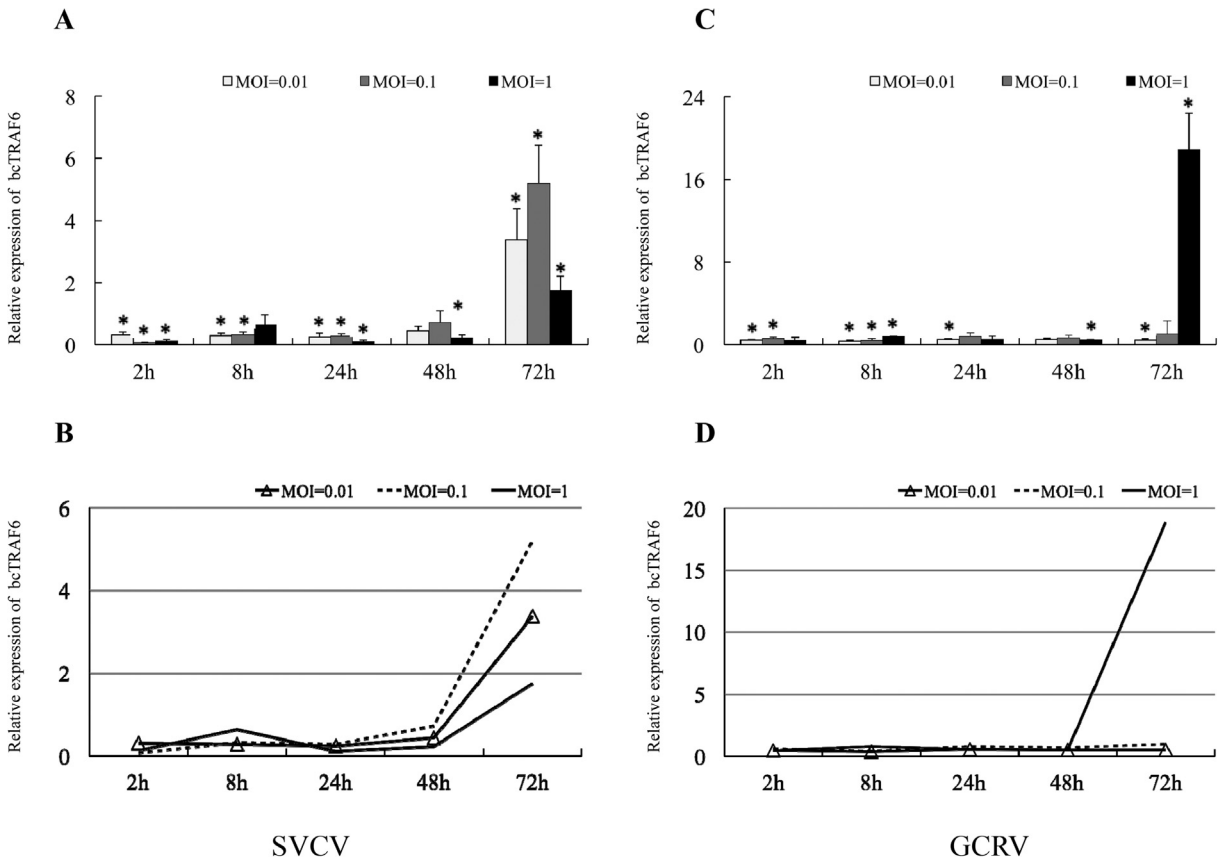

Fig. 5. SVCV or GCRV activated bcTRAF6 transcription in MPF cells. MPF cells in 6-well plate (2×10^6 cells/well) were infected with SVCV or GCRV at indicated MOIs separately and the cells were harvested at indicated time point post infection independently. bcTRAF6 transcription was examined by q-PCR. **A&C**. The bar chart of relative bcTRAF6 mRNA level in MPF cells after SVCV or GCRV infected. **B&D**. The trend chart of relative bcTRAF6 mRNA level in MPF cells after SVCV or GCRC infected. Error bars denote standard deviation and asterisk (*) stands for $p < 0.05$.

2.6. Luciferase reporter assay

HEK293T cells in 24-well plate were co-transfected with pRL-TK (25 ng), Luci-NF-κB (250 ng), bcTRAF6 or the empty vector; EPC cells in 24-well plate were co-transfected with pRL-TK (25 ng), Luci-eIFN or Luci-zIFN3 (250 ng), bcTRAF6 and/or bcMAVS, or the empty vector. For each transfection, the total amount of plasmid DNA was balanced with the empty vector. The cells were harvested and lysed on ice at 24 h post transfection. The centrifuged supernatant was used to measure firefly luciferase and renilla luciferase activities according to the instruction of the manufacturer (Promega) as described previously [25].

2.7. Immunofluorescence microscopy

HeLa cells or EPC cells were transfected with HA-bcTRAF6, bcTRAF6-HA, the empty vector separately; or co-transfected with HA-bcTRAF6 and Flag-bcMAVS; HA-bcTRAF6 and EGFP-bcMAVS separately. Transfected HeLa and EPC 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 immune-fluorescent staining as previously described [23]. Mouse monoclonal anti-HA antibody (Sigma) was probed at the ratio of 1:300; Alexa 594-conjugated secondary antibody (Invitrogen) was probed at the ratio of 1:200 and Alexa 488-conjugated secondary antibody (Invitrogen) was probed at the ratio of 1:1000; DAPI was used for nucleus staining.

2.8. Immunoblotting

HEK293T or EPC cells were transfected with HA-bcTRAF6, bcTRAF6-HA or the empty vector separately. Transfected cells were harvested at 48 h post-transfection and lysed for immunoblot (IB) assay as previously described [22]. Briefly, whole cell lysates were isolated by 10% SDS-PAGE and transferred to PVDF membrane. The transferred membrane was probed with mouse monoclonal anti-HA antibody (1:3000; Sigma). Target proteins were visualized with BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma).

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 bcTRAF6

To study the role of bcTRAF6 in black carp, the cDNA of TRAF6 was cloned from the spleen of black carp. The full-length cDNA of bcTRAF6 consists of 2590 nucleotides including 5′UTR, coding sequence, 3′UTR and poly (A) tail. The open reading frame (ORF) of

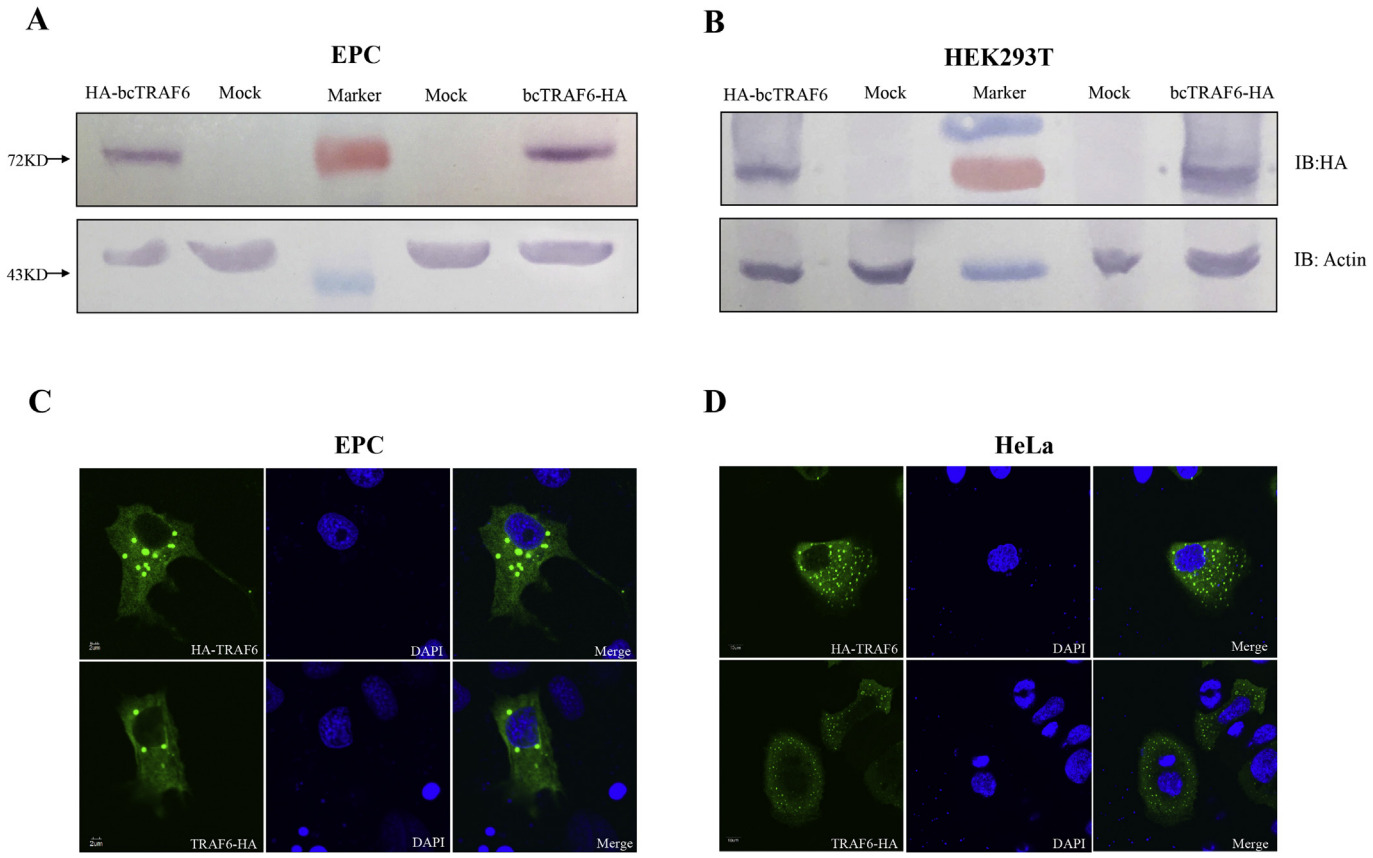

Fig. 6. Protein expression and subcellular distribution of bcTRAF6. EPC (**A**) or 293T (**B**) cells were transfected with pcDNA5/FRT/TO-HA-bcTRAF6, pcDNA5/FRT/TO-bcTRAF6-HA or the empty vector separately. The transfected cells were harvested and lysed at 48 h post transfection. Whole cell lysate was used for immunoblot (IB) assay in which bcTRAF6 was detected by anti-HA antibody. Mock: 293T or EPC cells transfected with empty vector, HA-bcTRAF6: pcDNA5/FRT/TO-HA-bcTRAF6, bcTRAF6-HA: pcDNA5/FRT/TO-bcTRAF6-HA. Both EPC cells (**C**) and HeLa cells (**D**) were transfected with pcDNA5/FRT/TO-HA-bcTRAF6, pcDNA5/FRT/TO-bcTRAF6-HA or the empty vector separately; the transfected cells were fixed at 36 h post transfection and used for immunofluorescence staining according to the methods. TRAF6 (green) indicates intracellular expression of bcTRAF6, DAPI (blue) indicates nucleus of EPC (**C**) or HeLa (**D**); the bar stands for the scale of 2 μm (EPC) or 10 μm (HeLa). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bcTRAF6 cDNA is 1629 bp, which commences at nucleotide of 57 and terminates at nucleotide of 1685. Initial sequence analysis of bcTRAF6 cDNA (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; http://smart.emble-heidelberg.de) predicts that bcTRAF6 contains 543 amino acid residues (aa), including a N-terminal RING domain (70–108), an zinc finger-TRAF (205–262), a coil-coiled domain (311–374), a MATH_TRAF6 c-terminal domain (375–521), which is similar to mammal TRAF6 (Fig. 1).

To gain insight into TRAF6 evolution, amino acid sequence of bcTRAF6 was subjected to multiple alignments with those of TRAF6 from human and mouse, which demonstrated that TRAF6 is a conserved protein in vertebrates (Fig. 2A). Phylogenetic analysis was applied to bcTRAF6 and TRAF6 of other known species (Fig. 2B). bcTRAF6 shares high protein sequence similarity with grass carp (*C. idella*) TRAF6 (99.3%) and common carp (*C. carpio*) (96.3%) and is clustered tightly with grass carp and common carp TRAF6, which correlates with the closest genetic relationship of these cyprinid fishes (Fig. 2B and Table 2).

3.2. Tissue specific mRNA expression of bcTRAF6

To investigate the transcription of bcTRAF6 *in vivo*, total RNA was isolated separately from heart, liver, spleen, kidney, intestine, skin and gill of the black carp injected with GCRV, SVCV or PBS. bcTRAF6 transcription in different tissues was examined by q-PCR, in which the q-PCR of β-actin was recruited as the inside parameter. bcTRAF6 transcription was detected in all the selected tissues and bcTRAF6 mRNA level in liver and gill was comparatively higher than those of other tissues. In GCRV injected group, bcTRAF6 mRNA level in kidney, spleen, intestine increased post viral infection, significantly in kidney and spleen. In SVCV injected group, bcTRAF6 mRNA level in liver, kidney, intestine, skin and gill increased post SVCV injection, significantly in liver and skin (Fig. 3). Our data suggested that bcTRAF6 was recruited into the innate immune response of black carp initiated by GCRV or SVCV.

3.3. bcTRAF6 mRNA expression ex vivo in response to different stimulations

MPF cells were treated with LPS or poly (I:C) at different concentrations and used for q-PCR to examine bcTRAF6 mRNA variation. bcTRAF6 mRNA level in MPF cells slightly increased after poly (I:C) treatment (Fig. 4A), which was relevant to that of *in vivo* data of GCRV or SVCV group, and suggested that bcTRAF6 had been involved in the mechanism against RNA virus infection (Fig. 3). It was interesting that bcTRAF6 mRNA level in MPF cells after LPS treatment decreased and the lowest level was only 15% of the control (1 μg dose/24 h point), which was contrary to that of poly (I:C) stimulation (Fig. 4B).

To obtain bcTRAF6 mRNA profile during host innate immune response, MPF cells were infected with SVCV or GCRV and bcTRAF6 transcription at different time points post infection was examined

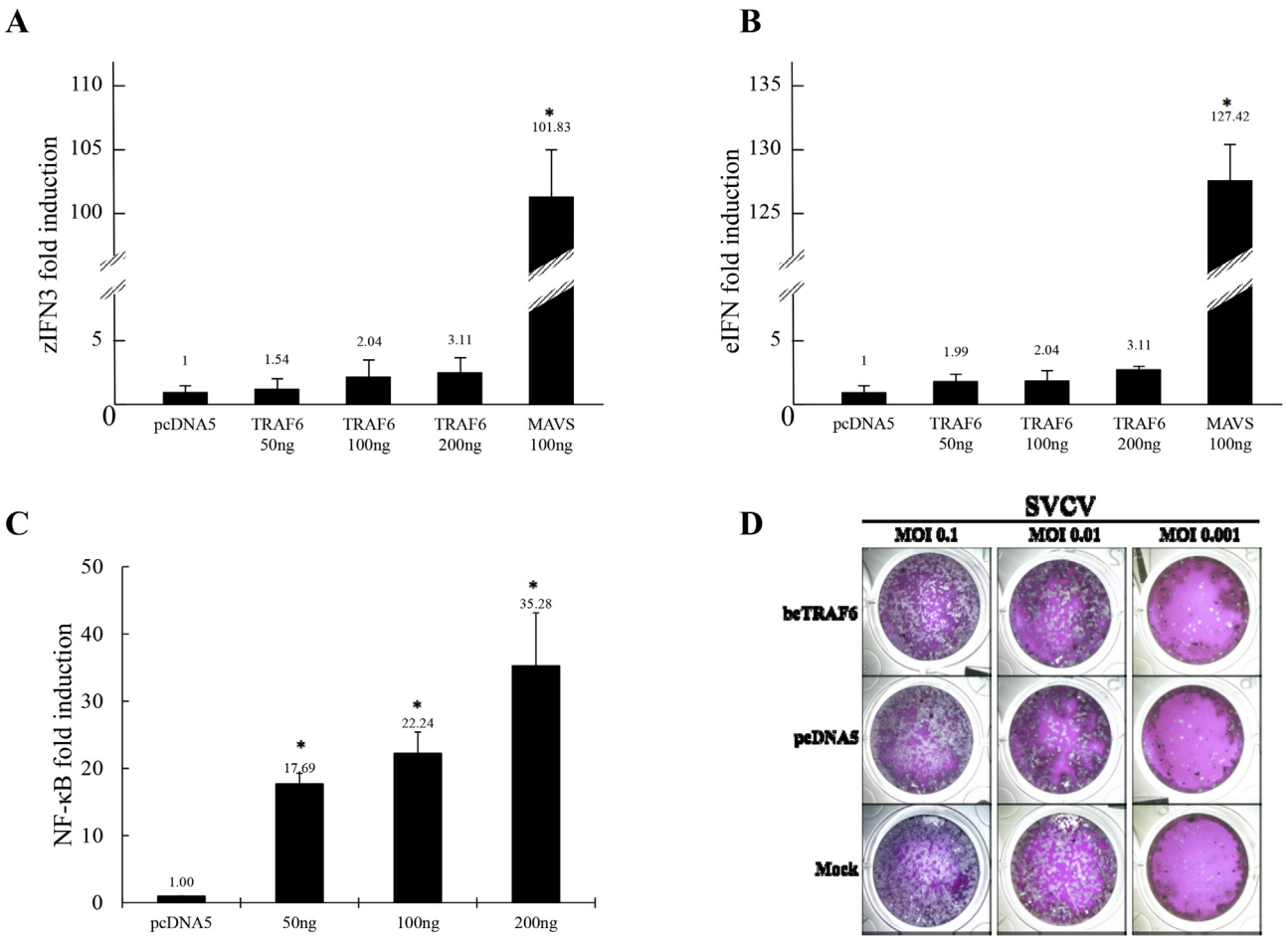

Fig. 7. Signaling and antiviral ability of bcTRAF6. EPC cells or HEK293T cells in 24-well plate were co-transfected with pRL-TK, Luci-zIFN3 or Luci-eIFN or Luci-NF-κB, bcTRAF6 or the empty vector separately and applied to luciferase reporter assay according to methods. **A**. Promoter activity of zebrafish IFN3 induced by bcTRAF6. **B**. Promoter activity of fathead minnow IFN induced by bcTRAF6. **C**. Promoter activity of NF-κB induced by bcTRAF6. **D**. EPC cells in 24-well plate were transfected with bcTRAF6 or the empty vector and infected with SVCV at the indicated MOIs at 24 h post transfection. The cell monolayers were stained with crystal violet at 48 h post-infection. The error bars represent the standard deviation and data represent three independent experiments. Asterisk (*) stands for $p < 0.05$. bcTRAF6: pcDNA5/FRT/TO-HA-bcTRAF6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by q-PCR. In SVCV group, bcTRAF6 mRNA level in MPF cells varied little before 48 h post infection (hpi) and obviously increased from 48 h hpi for infection at all doses (0.01, 0.1 and 1 MOI). The highest relative bcTRAF6 mRNA level was 5.2 folds (MOI = 0.1, 72 h point) of control within the 72 hpi (Fig. 5A and B). In GCRV group, bcTRAF6 mRNA level in MPF cells remained unchanged within 72 hpi for infection at both 0.01 MOI and 0.1 MOI. However, the mRNA level increased fiercely from 48 hpi when MPF cells suffered the infection of 1 MOI (Fig. 5C and D). In our previous data, bcMAVS transcription in MPF cells was enhanced at 24 h post SVCV infection (1, 0.1 and 0.01 MOI) and reached the peak at 48 hpi; and the mRNA level of bcMAVS was enhanced at 24 hpi (for 1 MOI) or 48 hpi (for 0.1 and 0.01MOI) separately in the GCRV infected group, which all reached the highest peak at 72 hpi [22]. The data generated in this study implied that bcTRAF6, similar to its mammalian counterpart, was a downstream signaling molecule of bcMAVS.

3.4. Protein expression and subcellular distribution of bcTRAF6

HEK293T cells or EPC cells were transfected with HA-bcTRAF6, bcTRAF6-HA or the empty vector separately and used for immunoblot (IB) assay to investigate the protein expression of bcTRAF6, in which mouse anti-HA antibody was used to detect the exogenous bcTRAF6. A specific band of ~72 KDa was detected in the whole cell lysate of 293T cells transfected with HA-bcTRAF6 or bcTRAF6-HA but not in the control cells, which matched the predicted molecular weight of this fish protein. Similar to the data of 293T cells, the specific band of ~72 KDa was detected in the EPC cells transfected with HA-bcTRAF6 or bcTRAF6-HA (Fig. 6A and B). Our data suggested that the position of the fused HA tag did not impact the expression level of bcTRAF6, which was different from that of other members of RLR signaling of black carp, e.g. MAVS and IKKε [21,22].

To determine the subcellular location of bcTRAF6, both EPC cells and HeLa cells were transfected with HA-bcTRAF6, bcTRAF6-HA or the empty vector separately and used for immunofluorescence staining (IF). The IF data in both EPC cells and HeLa cells showed clearly that bcTRAF6 expression region (green) surrounded tightly the nucleus (blue), which demonstrated that bcTRAF6 was mainly distributed in cytoplasm. Especially, brilliant green dots were widely scattered the cytoplasmic region, which implied that bcTRAF6 associated with the vesicle or formed aggregates itself or with other molecules in both EPC and HeLa cells (Fig. 6C and D).

3.5. Signaling and antiviral ability of bcTRAF6

To elucidate the signaling mediated by bcTRAF6, EPC cells were

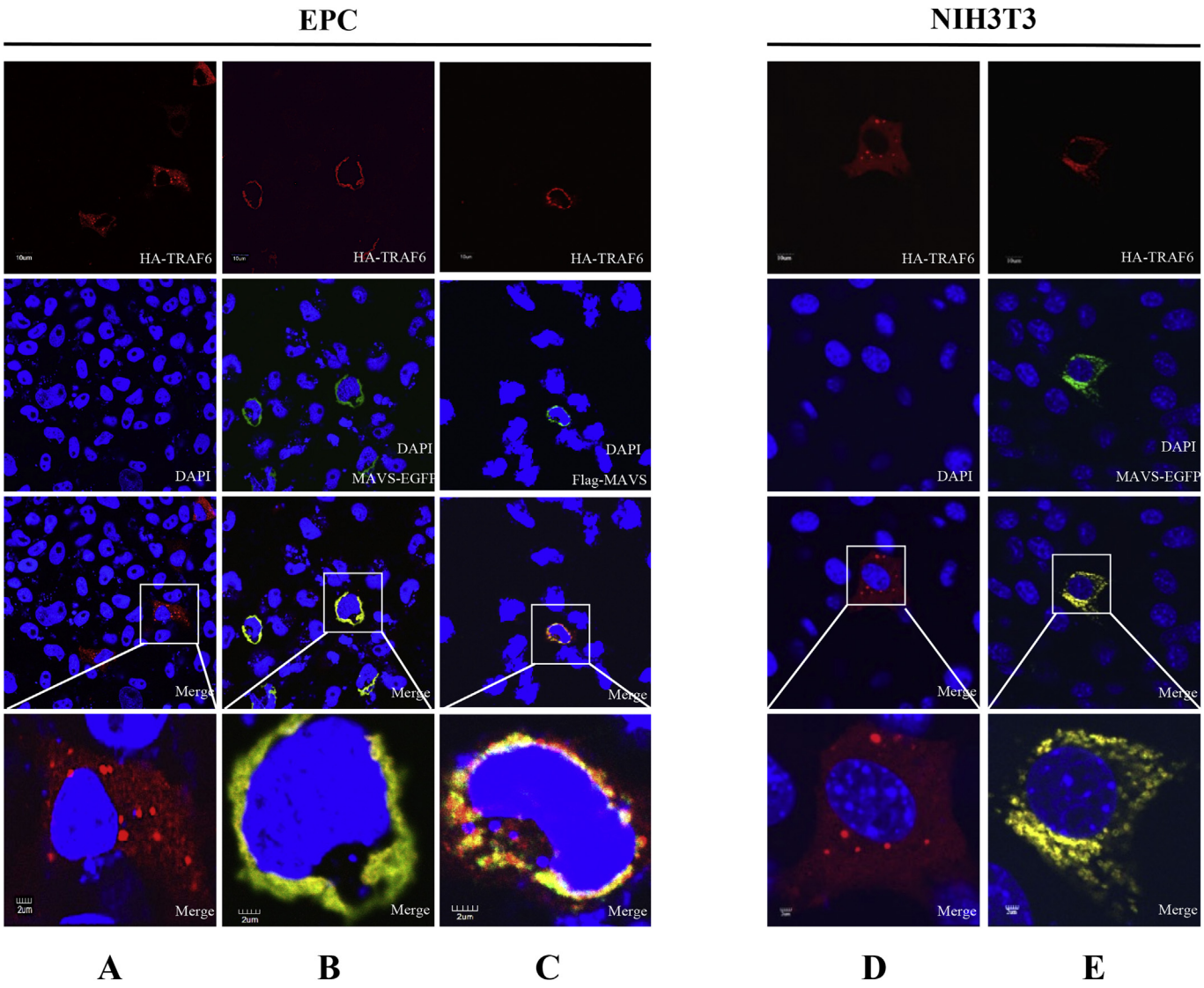

Fig. 8. Induced subcellular redistribution of bcTRAF6 by bcMAVS. Both EPC and NIH3T3 cells transfected with bcTRAF6 and/or bcMAVS and used for immunofluorescence staining. **A&D.** HA-bcTRAF6 transfection alone. **B&E**. HA-bcTRAF6 and bcMAVS-EGFP cotransfection. **C**. HA-bcTRAF6 and Flag-bcMAVS cotransfection. The bars stand for the scale of 2 μm (button panel) or 10 μm (upper panel) separately. HA-bcTRAF6: pcDNA5/FRT/TO-HA-bcTRAF6. Flag-bcMAVS: pcDNA5/FRT/TO-Flag-bcMAVS.

co-transfected with bcTRAF6, Luci-NF-κB or Luci-eIFN or Luci-zIFN3 and applied to luciferase reporter assay. Our data showed clearly that expression of bcTRAF6 in EPC cells had no effect on the induction of either zebrafish IFN3 or fathead minnow IFN (Fig. 7A and B). And the q-PCR data of MPK cells over-expressing bcTRAF6 with/without GCRV infection demonstrated that the expression of IFN and IFN stimulated genes (ISGs) was not changed obviously by the overexpressed bcTRAF6 (Supplementary Fig. 1). However, exogenous bcTRAF6 in human cells triggered the transcription of human NF-κB in a dose dependent manner (Fig. 7C). To test the antiviral activity of bcTRAF6, EPC cells were transfected with bcTRAF6 at 24 h before SVCV infection and applied to crystal violet staining. However, cytopathic effect (CPE) ratio of the EPC cells expressing bcTRAF6 was similar to that of control cells, which demonstrated that exogenous bcTRAF6 alone could not help the antiviral ability of EPC cells and correlated with the reporter assay (Fig. 7D).

3.6. bcMAVS signaling influenced by bcTRAF6

In mammals and human, MAVS activates the downstream TBK1/IKKε through the recruiting TRAF members, which then phosphorylate IRF3/7 to initiate the induction of type I IFNs [14]. To explore the relationship between TRAF6 and MAVS in blackcarp innate immune response, EPC and NIH3T3 cells were transfected with bcMAVS and/or bcTRAF6 separately and applied to immunofluorescence staining. When co-expressed with bcMAVS, the brilliant dots of bcTRAF6 (green in Fig. 6 and red in Fig. 8A and D) in the cytoplasm disappeared, bcTRAF6 expression pattern (red) matched bcMAVS display pattern (green) very well, which demonstrated clearly that subcellular distribution of bcTRAF6 was identical to that of mitochondria-associated bcMAVS (Fig. 8B, C and E). Our data implied that bcMAVS "dragged" bcTRAF6 to the outer membrane of mitochondria of EPC cells, which suggested that bcTRAF6 was recruited into the signaling mediated by bcMAVS.

To further explore if bcTRAF6 is related to the signaling mediated by bcMAVS, EPC cells transfected with bcMAVS and/or bcTRAF6 and applied to reporter assay. It was very interesting that bcTRAF6 regulated bcMAVS signaling either positively or negatively in a dose dependent manner. When co-transfected with bcTRAF6 at the amount less than 25 ng, both zebrafish IFN3 and fathead

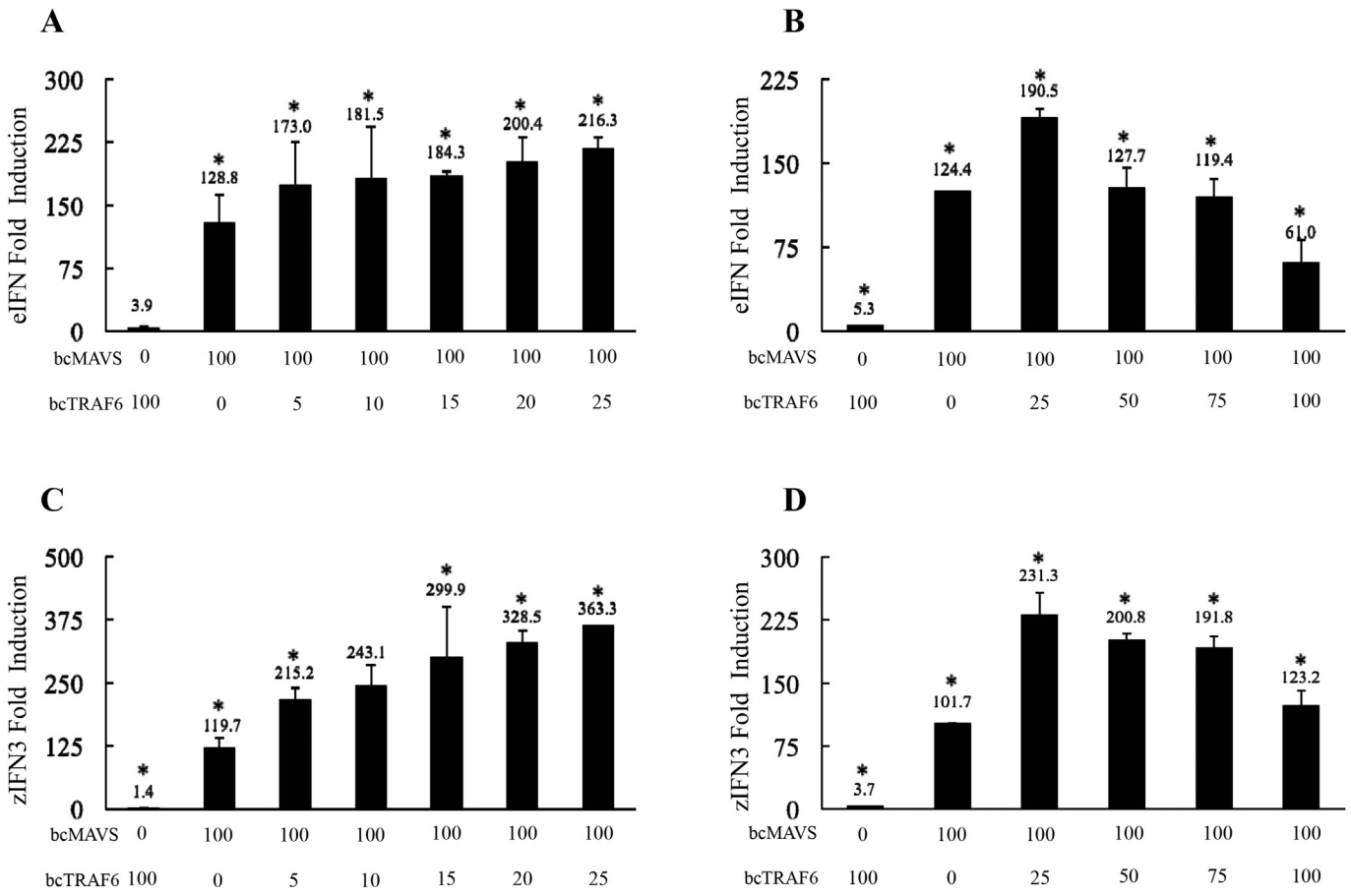

Fig. 9. Regulated bcMAVS signaling by bcTRAF6. EPC cells in 24-well plate were co-transfected with pRL-TK, Luci-eIFN/Luci-zIFN3, bcMAVS and/or bcTRAF6 and applied to reporter assay to examine the IFN induction. **A.** Fathead minnow IFN promoter activity in EPC cells transfected with bcMAVS (100 ng) and low amount of bcTRAF6 (≤25 ng). **B.** Fathead minnow IFN promoter activity in EPC cells transfected with bcMAVS (100 ng) and high amount of bcTRAF6 (≥25 ng). **C.** Zebrafish IFN3 promoter activity in EPC cells transfected with bcMAVS (100 ng) and low amount of bcTRAF6 (≤25 ng). **D.** Zebrafish IFN3 promoter activity in EPC cells transfected with bcMAVS (100 ng) and high amount of bcTRAF6 (≥25 ng). The numbers above the error bars stand for average bcTRAF6 mRNA level; error bars denote standard deviation and asterisk (*) stands for $p < 0.05$.bcTRAF6: pcDNA5/FRT/TO-HA-bcTRAF6; bcMAVS: pcDNA5/FRT/TO-HA-bcMAVS; pcDNA5: pcDNA5/FRT/TO-HA.

minnow IFN induction by bcMAVS (100 ng) were up regulated by bcTRAF6 in a dose dependent manner (Fig. 9A and C). However, when the bcTRAF6 input over 25 ng, both zebrafish IFN3 and fathead minnow IFN induction by bcMAVS (100 ng) were down-regulated in a dose dependent manner (Fig. 9B and D). Our data suggested that bcMAVS recruited bcTRAF6 into its signaling, however, excess bcTRAF6 dampened the signaling mediated by bcMAVS through a unknown mechanism.

4. Discussion

TRAF family members are primarily involved in the regulation of inflammation, antiviral responses and apoptosis, but TRAF proteins play non-overlapping roles in signaling [26–28]. Mammalian TRAF6 mediates the signaling not only from the members of the TNF receptor superfamily, but also from the members of the Toll/IL-1 family [8]. TRAF6 functions as a signal transducer in the NF-κB pathway downstream of RIG-I, in which it activates IκB kinase (IKK) and induces phosphorylation of IκBα inhibitor [29–33]. Similar to mammals, teleost TRAF6 also plays distinct roles in different species [15,34].

To further elucidate the innate immune system of black carp, bcTRAF6 was cloned and characterized in this paper. The full-length cDNA of bcTRAF6 consists of 2590 nucleotides and the predicted bcTRAF6 protein contains 542 amino acids (Fig. 1). The immunoblot analysis of both fish and human cells showed this fish protein migrated around 72 KDa, which was a little bigger than its predicted molecular weight and might be related to the post-translational modification, such as phosphorylation and ubiquitination (Fig. 6). bcTRAF6 shares high similarity with grass carp TRAF6 (99.3%) and is clustered tightly with grass carp TRAF6 in phylogenetic tree (Fig. 2), which correlates with our previously identified proteins, such as bcMAVS, bcIKKε, bcLGP2 [21–23]. It is interesting and worthy further exploration that these two genetically related cyprinid fishes possess total different life styles and feeding habits.

The transcription of bcTRAF6 gene was constitutively detected in all the examined tissues of black carp and bcTRAF6 mRNA level in these tissues varied right after virus infection (Fig. 3), which was similar to that of grass carp TRAF6 and suggested that bcTRAF6 is a novel cellular protein essential for early stage of innate immune response against virus infection [17]. TRAF6 was found to express predominantly in the gill of zebrafish, in the liver of common carp, in the kidney of India major carp and in the head kidney of grass carp [17]. In our study, bcTRAF5 expression was high in the liver, gill and skin (Fig. 3).

It is very interesting that LPS stimulation led to the obviously decreased bcTRAF6 transcription in MPF cells (Fig. 4B). TRAF6 activates NF-κB signaling in mammals and our data showed bcTRAF6 induced NF-κB expression through reporter assay (Fig. 7C). Thus, it

is speculated that LPS suppressed the expression of bcTRAF6 in host cells to avoid the activation of NF-κB signaling, which was crucial for the induction of inflammatory factors against pathogen invasion. However, the mechanism behind this suppression of bcTRAF6 needs further exploration. The reporter assay and plaque-forming assay demonstrated that bcTRAF6 showed little IFN induction ability and antiviral activity alone in EPC cells (Fig. 7), which was different with that of grouper TRAF6. Our data suggested that TRAF6 from different species of teleost utilizes different mechanism to function in host innate immune response against pathogenic microbes invasion [34].

The IF data in both EPC cells and HeLa cells demonstrated that bcTRAF6 at bcTRAF6 presented as brilliant green dots widely scattering the cytoplasmic region, which implied that bcTRAF6 associated with the vesicle or formed aggregates itself or with other molecules (Fig. 6). *Ex vivo* human TRAF6 was able to associates with huntingtin protein and increase its both the number and size of intracellular aggregates via an atypical mode of ubiquitination [35], which suggested that bcTRAF6 formed aggregates with other molecules in both human and fish cells. Thus, our data implied that bcTRAF6 might function as an ubiquitin E3 ligase in NF-κB activation down stream of RLR signaling of black carp like its mammalian counterparts [36]. However, overexpressed bcMAVS "eliminated" these brilliant dots of bcTRAF6 and "dragged" bcTRAF6 to the outer membrane of mitochondria (Fig. 8). Taken the phenotype together that bcMAVS mediated IFN induction was regulated by the input of bcTRAF6 in a dose dependent manner (Fig. 9), our data demonstrated that bcMAVS recruited ubiquitin E3 ligase bcTRAF6 to activate antiviral signaling cascades like its human counterpart [14,37]. However, the mechanism behind the association between bcMAVS and bcTRAF6 needs further investigation.

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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.2017.06.011.

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