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TRAF3 enhances STING-mediated antiviral signaling during the innate immune activation of black carp

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ABSTRACT

Tumor necrosis factor receptor-associated factor 3 (TRAF3) is a main regulator of antiviral and anti-inflammatory pathways in mammals, which is considered to induce type I interferon (IFN) activation and negatively regulate the activation of the canonical and non-canonical NF-κB pathways. To elucidate its function in teleost fish, TRAF3 homologue of black carp (Mylopharyngodon piceus) has been cloned and characterized in this study. The open reading frame (ORF) of black carp TRAF3 (bcTRAF3) consists of 1722 nucleotides and bcTRAF3 contains 574 amino acids. bcTRAF3 protein migrated around 65 KDa in immunoblot analysis of both EPC and HEK293T cells. bcTRAF3 was identified as a cytosolic protein and suggested to form aggregates or be associated with vesicles scattering in the cytoplasm. It was interesting that both NF-κB and IFN transcription was activated by bcTRAF3 in reporter assay. When co-expressed with black carp STING (bcSTING), bcTRAF3 was redistributed in the cytoplasm and its subcellular location overlapped with that of bcSTING no matter what the cells was infected with GCRV or not, which suggested the association between these two molecules. bcSTING-mediated IFN production was up-regulated by bcTRAF3 in a dose dependent manner in reporter assay. Accordingly, EPC cells transfected with both bcSTING and bcTRAF3 showed enhanced antiviral activity comparing EPC cells expressing bcSTING alone. Taken together, the data generated in this paper supported the conclusion that bcTRAF3 was recruited into host innate immune activation and positively regulated bcSTING-mediated antiviral signaling.

1. Introduction

The innate immune system, an evolutionarily conserved mechanism, is the first line of host defense against pathogens, which collaborates with the adaptive immune system to help vertebrates to survive from the disadvantage environments (Jiang, 2010). The recognition of the invading microbes is crucial for the activation of host innate immune system, which relies on a limited number of germline-encoded receptors (Akira et al., 2006). These receptors have evolved to recognize the conserved products of microbial metabolism produced by microbial pathogens, but not their hosts (Janeway et al., 2002). The detection of pathogen-associated molecular patterns (PAMPs) is carried out by the pattern recognition receptors (PRRs), which include membrane-bound Toll-like receptors (TLRs) and cytosolic receptors such as RIG-I-like receptors (RLRs). PRRs activate NF-κB and interferon regulated-factor 3/7 (IRF3/7) through downstream signaling; subsequently trigger the production of pro-inflammatory cytokines, IFNs and IFN stimulated genes (ISGs), which finally initiate host innate immune response (Akira et al., 2006; Zhong et al., 2006).

Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) constitute a family of adapter proteins (TRAF1–7), which were originally discovered and characterized as signaling adaptor molecules coupled to the cytosolic regions of multiple TNF receptors (TNFRs) upon their activation. TRAF members are involved in a wide spectrum of cellular responses including cell proliferation, apoptosis, and differentiation; however, emerging evidence demonstrated that members of TRAF family played vital roles in the signaling during host immune response (Zapata et al., 2009).

Like other TRAF family members, TRAF3 exhibits a modular structure with well-defined functional domains, including a conserved TRAF domain, several Zinc finger (ZF) motifs and a RING domain (Häcker et al., 2011). Normally, TRAF3 functions as K63-specific ubiquitin ligases, which alter the function of target proteins through their non-degradative, site-specific ubiquitination activity and activate
several downstream proteins (Guven-Maiorov et al., 2016). Notably, the C-terminal TRAF domain (also known as MATH domain) and the N-terminal RING domain play the critical role in TRAF3 functioning (Häcker et al., 2011; Ni et al., 2000). Previous studies demonstrated that MAVS (Mitochondrial antiviral signaling protein) (Belgnaoui et al., 2012) interacted with the MATH-TRAF3 domain, and the RING domain with TRIF/IRAK1, as well as TBK1/IKKε, suggesting that TRAF3 served as a critical linker between TLR adaptors and downstream regulatory kinases important for IRF activation (Oganesyan et al., 2006). In RLR signaling, the activation of type-I IFNs is a critical linker between TLR adaptors and downstream regulatory kinases important for IRF activation (Oganesyan et al., 2006). In RLR signaling, the activation of type-I IFNs is a critical linker between TLR adaptors and downstream regulatory kinases important for IRF activation (Oganesyan et al., 2006).

| Table 1 | Primers used in the study. |
|---|---|
| Primer name | Sequence(5'-3') | Amplicon length (nt) and primer information |
| **CDS** | | |
| bcTRAF3-F | ATTCGCCGAGGGGTAATTG | Ex vivo q-PCR |
| bcTRAF3-R | TGGGGTCTGGGAGGTC | 1722bp |
| **Expression construct** | | |
| HA-bcTRAF3-F | ACTGACGCTACATGTGCGGAGGGT |
| HA-bcTRAF3-R | ACTGACGCGTACAGGTCGAGG |
| bcTRAF3-HA-F | ACTGACGCTACAGGTCGAGG |
| bcTRAF3-HA-R | ACTGACGCTACATGTGCGGAGGGT |
| Flag-bcTRAF3-F | ACTGCGGCTACATGTGCGGAGGGT |
| Flag-bcTRAF3-R | ACTGACGCTACATGTGCGGAGGGT |
| **q-PCR** | | |
| bc Q actin-F | TGGCCGCGCTGCCCTCTC |
| bc Q actin-R | TTGGGTAACAGGGATG |
| bcTRAF3-Q-F | GTCACCAGCATGACTGTCCA |
| bcTRAF3-Q-R | GGCCTCTAGTGTGGTGTTCC |
| bcMAVS-Q-F | ATGACAGGATCAGGGGAAT |
| bcMAVS-Q-R | ATGTTGGAAGGGGGATG |
| bcSTING-Q-F | TGTTGTTGCGCCTTTC |
| bcSTING-Q-R | TGGTGTTGCGCCTTTC |
| bcIFNa-Q-F | AAGCGGAGGACCCAGTGAGAT |
| bcIFNa-Q-R | GACTCCTTATGTGATGGCTTGT |
| bcViperin-Q-F | CCAAAGAGCAGAAAGGGACC |
| bcViperin-Q-R | TCAATAGGCAAGACGAACGAGG |
| bcIL-1β-Q-F | ACCCCTTCCTCAACATAC |
| bcIL-1β-Q-R | TACCACAGTCATCCACCA |
| bcgig2-Q-F | AACCAGGAACCGAAGTCAG |
| bcgig2-Q-R | GCAATCCATTTTTAGAGGG |
| bcgig2-Q-F | AACCAGGAACCGAAGTCAG |
| bcgig2-Q-R | GCAATCCATTTTTAGAGGG |

**2. Materials and methods**

**2.1. Cells and plasmids**

HEK293T, *Epithelioma papulosum cyprini* (EPC), *Ctenopharyngodon idella* kidney (CIK) and *Mylopharyngodon piceus* fin (MFP) cells were kept in the lab (Zhou et al., 2015). HEK293T cells were cultured at 37 °C with 5% CO₂; EPC, CIK and MFP cells were cultured at 25 °C with 5%
CO2. All the 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 HEK293T transfection and Lipomax (SUDGEN) was used for EPC and MPF transfection (Chen et al., 2017a).

cDNA5/FRT/TO (Invitrogen), pcDNA5/FRT/TO-HA-bcSTING, pRL-TK, Luci-NF-κB (for human NF-κB promoter activity analysis), Luci-bcIFNa (for black carp IFNa promoter activity analysis), DrIFNφ1pro-luc, DrIFNφ2pro-luc and DrIFNφ3pro-luc (for zebrafish IFNφ1/2/3 promoter activity analysis accordingly) were kept in the lab (Jiang et al., 2017). The recombinant expression vector pcDNA5/FRT/TO-HA-bcTRAF3, pcDNA5/FRT/TO-bcTRAF3-HA and pcDNA5/FRT/TO-Flag-bcTRAF3 were constructed by cloning the open reading frame (ORF) of bcTRAF3 fused with an HA (Flag) tag at its N-terminus or C-terminus into pcDNA5/FRT/TO (Invitrogen) respectively.

2.2. Cloning the cDNA of bcTRAF3

Degenerate Primers (Table 1) were designed to amplify the cDNA of bcTRAF3 based on the sequences of TRAF3 of common carp (C. carpio) and zebrafish (D. rerio). 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) of bcTRAF3 was cloned by using the degenerate primers. The amplified fragments were cloned into pMD18-T vector and sequenced by X. Wang et al. (2018).
Table 3

| Gene           | Species name and abbreviation | Accession number |
|----------------|-------------------------------|------------------|
| TRAF1          | Homo sapiens (Hs)             | EAW87479.1       |
|                | Mus musculus (Mm)             | NP_001313530.1   |
|                | Patagonas fasciata monilis (Pfm) | OPJ76461.1    |
|                | Danio rerio (Dr)              | NP_001121853.1   |
|                | Camphorophyngodon idella (Ci) | ABF96697.1       |
| TRAF2          | Homo sapiens                  | ADQ89802.1       |
|                | Mus musculus                  | EDL08257.1       |
|                | Gallus gallus (Gg)            | XP_015135109      |
|                | *black carp (bc)              | ASO96650.1       |
|                | Camphorophyngodon idella      | ABF96696.1       |
| TRAF3          | Homo sapiens                  | AAH75087.1       |
|                | Mus musculus                  | EDL18642.1       |
|                | Amazona australis (Aa)        | KJR77667.1       |
|                | *black carp                   | AUP40798.1       |
|                | Cyprinus carpio (Cc)          | ADZ55454.1       |
| TRAF4          | Homo sapiens                  | AAH101769.1      |
|                | Mus musculus                  | NP_033449.2      |
|                | Patagonas fasciata monilis    | OPJ76818.1       |
|                | Danio rerio                   | CAH89006.1       |
|                | Salmo salar (Ss)              | NP_001167069.1   |
| TRAF5          | Homo sapiens                  | CAG38794.1       |
|                | Mus musculus                  | BAA11218.1       |
|                | Gallus gallus                 | NP_898950.1      |
|                | Danio rerio                   | XP_021252328.1   |
|                | Epinephelus coioides (Ec)     | AME21334.1       |
| TRAF6          | Homo sapiens                  | NP_004611.1      |
|                | Mus musculus                  | BAA12705.1       |
|                | Patagonas fasciata monilis    | OPJ82827.1       |
|                | *black carp                   | AUW56621.1       |
|                | Camphorophyngodon idella      | AGS16781.1       |
| TRAF7          | Homo sapiens                  | AA568363.1       |
|                | Mus musculus                  | AAH108598.1      |
|                | Patagonas fasciata monilis    | OPJ87475.1       |
|                | Danio rerio                   | NP_001075654.1   |
|                | Epinephelus coioides          | AME21335.1       |

Invitrogen.

2.3. Sequence analysis

The searches for amino acid sequence of TRAF3 and other TRAFs family members from different species were conducted at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Sequence analysis and prediction of domains of bcTRAF3 cdna were accomplished by online tools of ExPasy (https://www.expasy.org/). The amino acid alignment of TRAF3 from five different species, including human (H.sapiens), mouse (M.musculus), pigeon (C.livia), zebrafish (D.rerio) and black carp (M.piecus), was built with MEGA 5.05 and Genedoc program. Phylogenetic tree was constructed based on the deduced amino acid sequences by the Maximum-likelihood algorithm with 500 bootstrap replicates embedded in MEGA 5.05 program.

2.4. Virus production and titration

SVCV (strain: SVCV741) and GCRV (strain: GCRV106) were kept in the lab and propagated in EPC or CIK cells separately at 25 °C in the presence of 2% fetal bovine serum, which was kindly provided by Dr. Yong’an Zhang (Institute of Hydrobiology, CAS). Virus titers were determined by plaque assay on EPC cells as previously described (Chen et al., 2017). Brieﬂy, the 10-fold serially diluted virus supernatants were added onto EPC cells and incubated for 2 h at 25 °C. The supernatant media was replaced with fresh DMEM containing 2% FBS and 0.75% methylcellulose (Sigma) after incubation. Plaques were counted at day 3 post-infection.

2.5. Quantitative real-time PCR

The relative bcTRAF3 mRNA level in the selected tissues of black carp or MF cells was examined by quantitative real-time PCR (q-PCR). The primers for bcTRAF3 and β-actin (as internal control) were listed in Table 1. The primer sequences were: 1 cycle of 50 °C/2 min, 1 cycle of 95 °C/10 min, 40 cycles of 95 °C/15 s, 60 °C/1 min, 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-△△CT method.

2.6. Luciferase reporter assay

HEK293T cells in 24-well plate were co-transfected with pRL-TK (25 ng), Luciferin-β (200 ng), bcTRAF3 or the empty vector; EPC cells in 24-well plate were co-transfected with pRL-TK (25 ng), luciferin-β; bcIFNa (luciferin-β), DrfIFNq1/2/3pro-luc (200 ng), bcTRAF3 and/or bcSTING. For each transfection, the total amount of plasmid DNA (425 ng) was balanced with the empty vector. The cells were harvested and lysed by renilla luciferase lysis buffer (Promega) on ice at 24 h post transfection. The centrifuged supernatant was used to measure the firefly luciferase activity and the renilla luciferase activity according to the instruction of the manufacturer (Promega) as described previously (Yan et al., 2017).

2.7. Immunoblotting

HEK293T or EPC cells were transfected with HA-bcTRAF3, bcTRAF3-HA or the empty vector separately. The transfected cells were harvested at 48 h post-transfection and lysed for immunoblot assay as previously described (Xiao et al., 2017). 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 (1:1000; Sigma). Target proteins were visualized with BCIP/ NBT Alkaline Phosphatase Color Development Kit (Sigma).

2.8. Immunofluorescence microscopy

HEK293T or EPC cells were transfected with HA-bcTRAF3, bcTRAF3-HA, or the empty vector separately; or co-transfected with Flag-bcTRAF3 and HA-bcSTING; Flag-bcTRAF3, respectively. 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 immunofluorescent staining as previously described (Chen et al., 2017a). Mouse monoclonal anti-HA antibody (Sigma) was probed at the ratio of 1:400; rabbit polyclonal anti-Flag antibody (Sigma) was probed at the ratio of 1:250; Alexa 594-conjugated secondary antibody (Invitrogen) and Alexa 488-conjugated secondary antibody (Invitrogen) were probed at the ratio of 1:250; DAPI was used for nucleus staining.

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 ANOVA and post-hoc analysis.
3. Results

3.1. Molecular cloning and sequence analysis of bcTRAF3

To study the role of bcTRAF3 in black carp, the cDNA of TRAF3 was cloned from the spleen of black carp and the coding sequence of bcTRAF3 consists of 1722 nucleotides (NCBI accession number: AUP40798.1). Initial sequence analysis of bcTRAF3 cDNA (predicted by online tools of CDS in NCBI: https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) predicts that bcTRAF3 contains 574 amino acid residues (aa), including a RING-Finger domain (43–83), two zinc finger (ZF) domains (139–194 and 198–253), and a MATH-TRAF3 domain (383–568). These functional domains are conserved among TRAF proteins from human, mouse, pigeon, zebrafish and black carp, especially the N-terminal RING domain and C-terminal MATH-TRAF3 domain showed prominently high conservation (Fig. 1A and B). bcTRAF3 has a calculated molecular weight of 65 kDa and an isoelectric point of 7.91.

To gain insight into TRAF3 evolution, amino acid sequence of bcTRAF3 was subjected to multiple alignments with those of TRAF3 proteins from different species (Table 2). The amino acid sequence of bcTRAF3 showed more than 75.2% identity with other fish TRAF3, and even as high as 74.3% identity with human TRAF3, suggesting the highly structural conservation across vertebrate TRAF protein (Table 2). A phylogenetic tree was constructed using a maximum-likelihood method with 500 bootstrap replicates based on the multiple alignments of seven members of TRAF family from various species (Fig. 1A and B). In the phylogenetic tree, bcTRAF3 was clustered with other TRAF3 homologues and bcTRAF6 was clustered with other TRAF6 homologues. The data showed that bcTRAF3 was clustered tightly with common carp TRAF3 (C. carpio), which correlated with the identity (94.6% identity) shown in Table 2.

3.2. bcTRAF3 expression ex vivo in response to different stimuli

To examine the expression of bcTRAF3 during host innate immune activation, MPF cells were treated with LPS or poly (I:C) at different concentrations and used for q-PCR. In the poly (I:C) treated group, the transcription of bcTRAF3 was activated after poly (I:C) treatment (2 h), recovered at 12 h, and raised again at 24 h for all doses (Fig. 2A). Similar to that of poly (I:C) treatment, bcTRAF3 mRNA level increased right after LPS treatment (10 μg/ml and 25 μg/ml), decreased, recovered then decreased within 48 h post stimulation. However, in the group of 1 μg/ml, bcTRAF3 mRNA level decreased after stimulation and increased from 24 h point (Fig. 2B). The varied transcription level of bcTRAF3 in MPF cells suggested that this fish TRAF3 homologue might play a significant role in the host innate immune response initiated by both virus and bacteria.

To further explore the bcTRAF3 mRNA profile during host antiviral innate immune response, MPF cells were infected with SVCV/GCRV and bcTRAF3 transcription post infection was examined by q-PCR. In SVCV infected MPF cells, bcTRAF3 mRNA level was stable (even decreased) within the first 12 h post-infection and increased obviously from 24 h point post-infection. It was interesting that relative bcTRAF3 level of 1 MOI group at 24 h point was much higher than those of 0.1
and 0.01 MOI groups, however, relative bcTRAF3 level of 1 MOI group at 48 h point was much lower than other two groups (Fig. 2C). Similar phenomena were seen in GCRV infected MPF cells, bcTRAF3 mRNA level was stable (even decreased) within the first 12 h post-infection and increased obviously from 24 h point post-infection (for 0.01 MOI group and 0.1 MOI group). However, relative bcTRAF3 level of 1 MOI group increased obviously from 8 h point post-infection (Fig. 2D). bcTRAF3 transcription in MPF cells varied post GCRV or SVCV infection, demonstrated this protein was involved into host antiviral innate immune response. Different mRNA profile of bcTRAF3 in host cells post infection suggested that this protein was recruited into different mechanisms during host innate immune activation initiated by GCRV or SVCV. However, bcTRAF3 level was very low (even decreased) in the early stage post both GCRV and SVCV infection (within 12 h), implied the invading virus utilized unknown mechanisms to suppressed the expression of host immune related molecules, such as TARF3.

3.3. Protein expression and subcellular distribution of bcTRAF3

EPC cells or HEK293T cells were transfected with HA-bcTRAF3, bcTRAF3-HA or the empty vector separately and used for immunoblot assay to investigate the protein expression of bcTRAF3. A specific band of ~65 KDa was detected in the whole cell lysate of both EPC and HEK293T cells transfected with HA-bcTRAF3 or bcTRAF3-HA but not in the control cells, which matched its predicted molecular weight (Fig. 3A and B). Our data demonstrated that the position of the HA tag did not impact the expression of bcTRAF3, which was similar with that of the other two black carp TRAF family members, bcTRAF2 and bcTRAF6 (Chen et al., 2017a; Jiang et al., 2017). To determine the subcellular distribution of bcTRAF3, both EPC cells and HEK293T cells were transfected with HA-bcTRAF3, bcTRAF3-HA or the empty vector separately and used for immunofluorescence staining. The immunofluorescence microscopy data of both EPC cells and HEK293T cells showed clearly that bcTRAF3 was mainly distributed in cytoplasm, especially near the nucleus (Fig. 3C and D).

3.4. bcTRAF3 mediated signaling

In order to elucidate bcTRAF3-mediated signaling, HEK293T cells and EPC cells were transfected with bcTRAF3 and used for luciferase reporter assay. Our data showed that over-expressed bcTRAF3 in EPC cells induced the transcription of black carp IFNa, DrIFNφ2 and DrIFNφ3 in a dose-dependent manner (Fig. 4A–C). It was interesting that bcTRAF3 showed the ability to activate the transcription of NF-κB (Fig. 4D). Studies on mammalian TRAF3 showed that TRAF3 played a critical role of the activation of type I IFNs in both TLR and RLR signaling besides its function as an inhibitor of NF-κB signaling pathway (Häcker et al., 2011). To investigate if the expression of IFNs, ISGs and NF-xβ related genes in host cells were influenced by bcTRAF3, MPF cells were transfected with bcTRAF3, then infected with GCRV before q-
PCR analysis. For NF-κB related genes, the relative expression level of bcp50, p52, p65, IL-1β and IkBα were all up-regulated in MPF cells expressing bcTRAF3 by comparison of that in the cells transfected with the empty vector, no matter GCRV treatment or not (Fig. 5B∼F), which matched the result of reporter assay (Fig. 4D). It is clearly that bcTRAF3, different from mammalian TRAF3, plays a positive role in NF-κB activation in the context of viral infection. In addition, the expression level of IFNs-related genes: bcMAVS, bcSTING and bcIFNa (Huang et al., 2015), ISGs: bcMx1(Xiao et al., 2016) and bcViperin, and the GCRV-induced gene 2 (bcgig2) in MPF cells were holistically higher when the cells were transfected with bcTRAF3, despite the infection with GCRV or not (Fig. 5I∼L). Both bcMAVS and bcSTING were proved to be the critical antiviral signaling proteins in previous studies, which led to the investigation of the expression of these two genes in MPF cells in the condition of bcTRAF3 overexpression. The mRNA level of bcMAVS did not changed much in the cells transfected with bcTRAF3, despite the infection with GCRV or not (Fig. 5I 1∼I). Both bcMAVS and bcSTING were proved to be the critical antiviral signaling proteins in previous studies, which led to the investigation of the expression of these two genes in MPF cells in the condition of bcTRAF3 overexpression. The mRNA level of bcMAVS did not changed much in the cells transfected with bcTRAF3, despite the infection with GCRV or not; however, exogenous bcTRAF3 prominently up-regulated the transcription of bcSTING (Fig. 5G and H). Thus, the q-PCR data implied the collaboration between bcTRAF3 and bcSTING when the host cell was subjected to pathogen invasion.

3.5. Induced subcellular redistribution of bcTRAF3 by bcSTING

In mammals, human and fish, STING is responsible for the recruitment of TBK1 and IRF3 and the activation of both type I IFNs and proinflammatory cytokines (Ran et al., 2014; Sun et al., 2011). There were few reports about the interaction of STING and TRAF3; however, evidence showed that the formation of STING-TRAF3-TBK1 complex is crucial for the innate antiviral response (Chen et al., 2014). To investigate the relationship between bcTRAF3 and bcSTING, EPC (Fig. 6A–C) and MPF (Fig. 6D–F) cells were transfected with bcTRAF3 and/or bcSTING separately and used for immunofluorescence staining. When co-expressed with bcSTING, the previous brilliant dots (green in Fig.3D, 6A and D, representing bcTRAF3) in EPC or MPF cells disappeared, bcTRAF3-expressing region (green) matched that of bcSTING pattern (red) very well, no matter what the cells was infected with GCRV or not (Fig. 6B, C, E, F). Thus, the data implied that bcTRAF3 associated with bcSTING and was recruited into the signaling mediated by bcSTING.

3.6. Up-regulated bcSTING-mediated antiviral signaling by bcTRAF3

Our previous data has identified the bcSTING-mediated antiviral signaling during host innate immune response initiated by GCRV invasion. To characterize the relationship between bcTRAF3 and bcSTING, EPC cells were transfected with bcSTING and/or bcTRAF3 and used for reporter assay. The results showed that both DrIFNφ1 and DrIFNφ3 fold induction by bcSTING were up regulated by bcTRAF3 in a
dose dependent manner (Fig. 7A and B), which demonstrated that bcTRAF3 functioned positively in bcSTING-mediated IFN production. To further examine if bcTRAF3 affected the antiviral activity of bcSTING, EPC cell were transfected with bcTRAF3 and/or bcSTING and subjected to GCRV and SVCV infection. The viral titer in the media of EPC cells expressing bcSTING or bcTRAF3 was higher than that of EPC cells expressing both bcSTING and bcTRAF3 (Fig. 7C and D), which clearly demonstrated that bcTRAF3 upregulated bcSTING-mediated antiviral activity. Taken together, our data demonstrated clearly that bcTRAF3 functioned positively in bcSTING-mediated signaling and up-regulated its antiviral activity.

4. Discussion

In 1994, similar to the method of discovering TRAF1 and TRAF2, a new protein with zinc fingers and a TRAF domain was discovered through yeast two-hybrid screening and this protein could interact with CD40 and help transduce downstream signaling, which was named as TRAF3 later (Hu et al., 1994). Notably, TRAF3 is a master regulator at the crossroads of antiviral and anti-inflammatory pathways; its deletion in myeloid cells leads to inflammatory diseases and cancer in mice (Guven-Maiorov et al., 2016). Black carp contributes importantly to the freshwater industry of China; however, its innate immune system remains much unknown. In our previous study, MDA5 (melanoma differentiation associated gene 5), LGP2 (Laboratory of Genetics and Physiology 2), MAVS, STING, TBK1 and IKKε were cloned and characterized from this cyprinid fish (named as bcMDA5, bcLGP2, bcMAVS, bcSTING, bcTBK1 and bcIKKε accordingly). These black carp RLR signaling components, like their mammalian counterparts, activate the production of interferon (IFN) and boast their antiviral activity in EPC cells. At the same time, the roles of bcTRAF2 and bcTRAF6, two other black carp TRAF members, during host innate immune activation have been identified, which functions positively in bcMAVS-mediated antiviral signaling (Chen et al., 2017a; Jiang et al., 2017). In the IF data of this study, brilliant green dots (bcTRAF3 expression area) were scattering in the cytoplasm, and the similar phenomenon was seen in the immunofluorescence microscopy data of both bcTRAF2 and bcTRAF6 (Chen et al., 2017a; Jiang et al., 2017), which implied that these three TRAF family members of black carp may have the similar mechanism to associated with vesicle or form aggregates.

Although all TRAF members share structural similarities, TRAF proteins play non-overlapping roles in signaling, in which both their adaptor and E3 ubiquitin ligase function are important for signal transduction downstream of different receptors (Häcker et al., 2011). The investigation of mammalian TRAF3 pointed out that TRAF3 was found associated with markers of the ER-Exit-Sites (ERES), ER-to-Golgi...
apparatus and cis-Golgi apparatus in non-infected cells (van Zuylen et al., 2012). Importantly, deletion of TRAF3 has identified it as a critical mediator involved in the induction of the type I IFNs by the RLR pathway and other report has suggested that TRAF3 functions as a negative regulator of NF-κB (He et al., 2007; Liao et al., 2004).

bcTRAF3, like bcTRAF2 and bcTRAF3, scattered in the cytoplasm presenting as the brilliant dots in IF data, which co-related with the data of mammalian TRAF3 (van Zuylen et al., 2012). However, bcTRAF3 showed the ability in NF-κB activation in reporter assay like bcTRAF2/6 and q-PCR data, which is different to mammalian TRAF3, an inhibitor in NF-κB pathway (He et al., 2006, 2007; Liao et al., 2004) and showed that bcTRAF3 is mainly involved in the activation of non-canonical NF-κB (RelB-p52) pathway in its host. Similar to TRAF3 of mammals, bcTRAF3 showed much stronger ability than that of bcTRAF2/6 in IFN activation in reporter assay of EPC cells, which suggested the different roles of TRAF members of black carp (Chen et al., 2017a; Jiang et al., 2017).

Besides its role in the DNA sensing signaling, STING is now considered an important adaptor of innate immune signaling in response to RNA viruses (Ran et al., 2014; Sun et al., 2011). STING is an originally...
found ER-resident protein, which in response to dsDNA treatment, has been verified to traffic from the ER to the Golgi giving rise to punctate structure formation (van Zuylen et al., 2012). In the previous study, the subcellular distribution of bcSTING was overlapped with that of bcMAVS, when these two proteins were co-expressed in EPC cells or the cells were subjected to poly (I:C) stimulation or virus infection (Lu et al., 2017). There were few in vitro and in vivo reports investigating the association between STING and TRAF3 in human and mammals. It had been discovered that SARS coronavirus could inhibit type-I interferon signaling pathway through disturbing the interaction of STING and TRAF3 (Chen et al., 2014). Also, it was reported that the Gn proteins cytoplasmic tail (Gn-T) of hantavirus interferes with the formation of STING-TBK1-TRAF3 complexes required for IRF3 activation and IFN induction (Matthys and Mackow, 2012). However, up to now, there is no evidence showed the relation between STING and TRAF3 in fish. In our study, the IF data of this paper showed that bcSTING-expressing region matched that of bcTRAF3-expressing region very well when the EPC cells were co-transfected with both bcSTING and bcTRAF3 (Fig. 6), which is discovered in teleost fish for the first time. However, the direct associate on between bcTRAF3 and bcSTING could not be detected through co-immunoprecipitation method in this lab. What is more, bcTRAF2/6 functioned positively in bcMAVS-mediated antiviral signaling and bcTRAF2/6 overlapped with bcMAVS in IF data of EPC cells (Chen et al., 2017a; Jiang et al., 2017). The study on bcTBK1 showed that bcTRAF6 associated with bcTBK1 in the IF data and bcTRAF6 up-regulated bcTBK1-mediated IFN production (Yan et al., 2017). Thus, it is implied that a signaling complex including bcMAVS, bcSTING, bcTRAF2/3/6 and bcTBK1 was formed during host innate immune response initiated by GCRV/SVCV, which is similar to that of human and mice (Jiang, 2010; Zhao et al., 2016; Zhong et al., 2008). The recruiting mechanism of these factors during the innate immune activation of black carp will be further investigated and it is speculated that the membranous network consisted of the ER, Golgi and mitochondria constitutes a convenient platform on which antiviral cell-signaling complexes are arranged and optimally activated like its mammalian counterparts (van Zuylen et al., 2012).
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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dci.2018.07.009.

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