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Molecular characteristics and function study of TNF receptor-associated factor 5 from grouper (*Epinephelus coioides*)

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ABSTRACT

Tumor necrosis factor receptor-associated factor 5 (TRAF5) is a key adapter molecule that participates in numerous signaling pathways. The function of TRAF5 in fish is largely unknown. In the present study, a TRAF5 cDNA sequence (EcTRAF5) was identified in grouper (*Epinephelus coioides*). Similar to its mammalian counterpart, EcTRAF5 contained an N-terminal RING finger domain, a zinc finger domain, a C-terminal TRAF domain, including a coiled-coil domain and a MATH domain. The EcTRAF5 protein shared relatively low sequence identity with that of other species, but clustered with TRAF5 sequences from other fish. Real-time PCR analysis revealed that EcTRAF5 mRNA was broadly expressed in numerous tissues, with relatively high expression in skin, hindgut, and head kidney. Additionally, the expression of EcTRAF5 was up-regulated in gills and head kidney after infection with *Cryptocaryon irritans*. Intracellular localization analysis demonstrated that the full-length EcTRAF5 protein was uniformly distributed in the cytoplasm; while a deletion mutant of the coiled-coil domain of EcTRAF5 was observed uniformly distributed in the cytoplasm and the nucleus. After exogenous expression in HEK293T cells, TRAF5 significantly activated NF-κB. The deletion of the EcTRAF5 RING domain or of the zinc finger domain dramatically impaired its ability to activate NF-κB, implying that the RING domain and the zinc finger domain are required for EcTRAF5 signaling.

1. Introduction

Tumor necrosis factor receptor-associated factor family members (TRAFs) are intracellular adaptors that mediate cellular effects by binding to their cognate cellular receptors, which include tumor necrosis factors receptors, Toll-like receptor, RIG-I-like receptor, and NOD-like receptor. TRAFs activate nuclear factor κB (NF-κB) and Jun N-terminal kinase [1–4]. They are important regulators of a wide range of biological functions, including cell proliferation, apoptosis, differentiation, and survival [5–8].

TRAFs (except TRAF1) share a common structure, which includes a conserved N-terminal RING finger domain and a variable number of zinc finger domains that are important for downstream signaling [9]. At the C-terminus, TRAFs (except TRAF7) have a coiled-coil domain and a MATH domain, which mediate interactions with other signaling molecules and the formation of homo- and hetero-dimers with other TRAFs [10].

TRAF5 plays a crucial role in regulating cell proliferation, apoptosis, differentiation, and survival. In TRAF5-deficient B cells, the production of IgM and IgG2 is significantly impaired, and JNK activation is decreased. The proliferation of TRAF5-deficient T cells is also significantly impaired [11]. Additionally, TRAF5 participates in many signal pathways in mammals. In RLR, the signal pathway TRAF5 is required for IFNβ expression in human bronchial epithelial cells and in pulmonary alveolar epithelial cells [12]. In mouse B cells, TRAF5 participates in the regulation of TLR-mediated production of IL-6, TNF-α, and IgM [13]. TRAF5, together with TRAF1, TRAF2, TRAF3, and TRAF6, also interacts directly or indirectly with members of TNFR [14]. TRAF5

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Table 1
Primers used in this study.

Primer	Sequence (5′to 3′)
TRAF5 F	ATGGCGACAGCAGACACAAAGCC
TRAF5 R	CTACAGCTGCTCTAAACCTGCCATGTCC
TRAF5 F1	CGCTGAAGGAGGAGTTTGTTGGAGCCGTGATCACACCAGC
TRAF5 R1	GCTGGTGTGATCACGGCTCCAACAAACTCCTCCTTCAGCG
TRAF5 F2	CATTACGCCACCTACAGGAGCACATGCTGCTGGTCCTGCG
TRAF5 R2	CGCAGGACCAGCAGCATGTGCTCCTGTAGGTGGCGTAATG
TRAF5 F3	TGGAGGAGCTGGAGCAGCTGCTAAAGCATCACTCGGGTCT
TRAF5 R3	AGACCCGAGTGATGCTTTAGCAGCTGCTCCAGCTCCTCCA
TRAF5F4	AACTGATCTGGAAGATCGAGGTCAAAGAGGACACGTTGTT
TRAF5R4	AACAACGTGTCCTCTTTGACCTCGATCTTCCAGATCAGTT
TRAF5 F5	GGGAATTCGGCGCCACCATGGGAGCCGTGATCACACCAG
TRAF5 R5	GGGGATCCTCCAGCTGCTCTAAACCTGCC
TRAF5R5′	GGGGATCCTCCTACAGCTGCTCTAAACCTGCC
TRAF5F6	CCGCAGGTCGTGTCAGTA
TRAF5R6	CAGGTGTTGGTCAGGTGTTC
β-actin F	TGCTGTCCCTGTATGCCTCT
β-actin R	CCTTGATGTCACGCACGAT

Table 2
Amino acid identity (%) of TRAF5 between grouper and other vertebrates.

Species	Identity (%)				
	Full-length	RING finger domain	Zinc finger domain	Coiled-coil domain	MATH domain
Homo sapiens	39	43	39	83	50
Mus musculus	40	45	39	57	50
Bos Taurus	39	43	39	57	48
Gallus gallus	38	40	33	83	51
Danio rerio	43	45	40	54	54
Lepisosteus oculatus	48	52	45	44	61
Scleropages formosus	45	52	47	58	60

activates NF-κB (induced by CD40 [15]) and the lymphotoxin-β receptor [16], but it has a dominant negative effect on CD30 [17] and CD27 signaling [18]. Moreover, CD40-mediated activation of B cells is impaired in TRAF5-deficient mice [19].

In recent years, TRAFs have been identified in teleost fish, and the function of some TRAF proteins has been described. For example, TRAF2 isolated from rock bream (*Oplegnathus fasciatus*) acts as a transducer of TNF-α-induced NF-κB activity [20]. TRAF3 isolated from common carp (*Cyprinus carpio* L.), and from humphead snapper (*Lutjanus sanguineus*), plays important roles in immune responses to virus and bacteria [21,22]. In black carp (*Mylopharyngodon piceus*), TRAF2 and TRAF6 participate in antiviral signaling [23,24]. TRAF6 from rock bream [25], from grass carp (*Ctenopharyngodon idella*) [26], and from

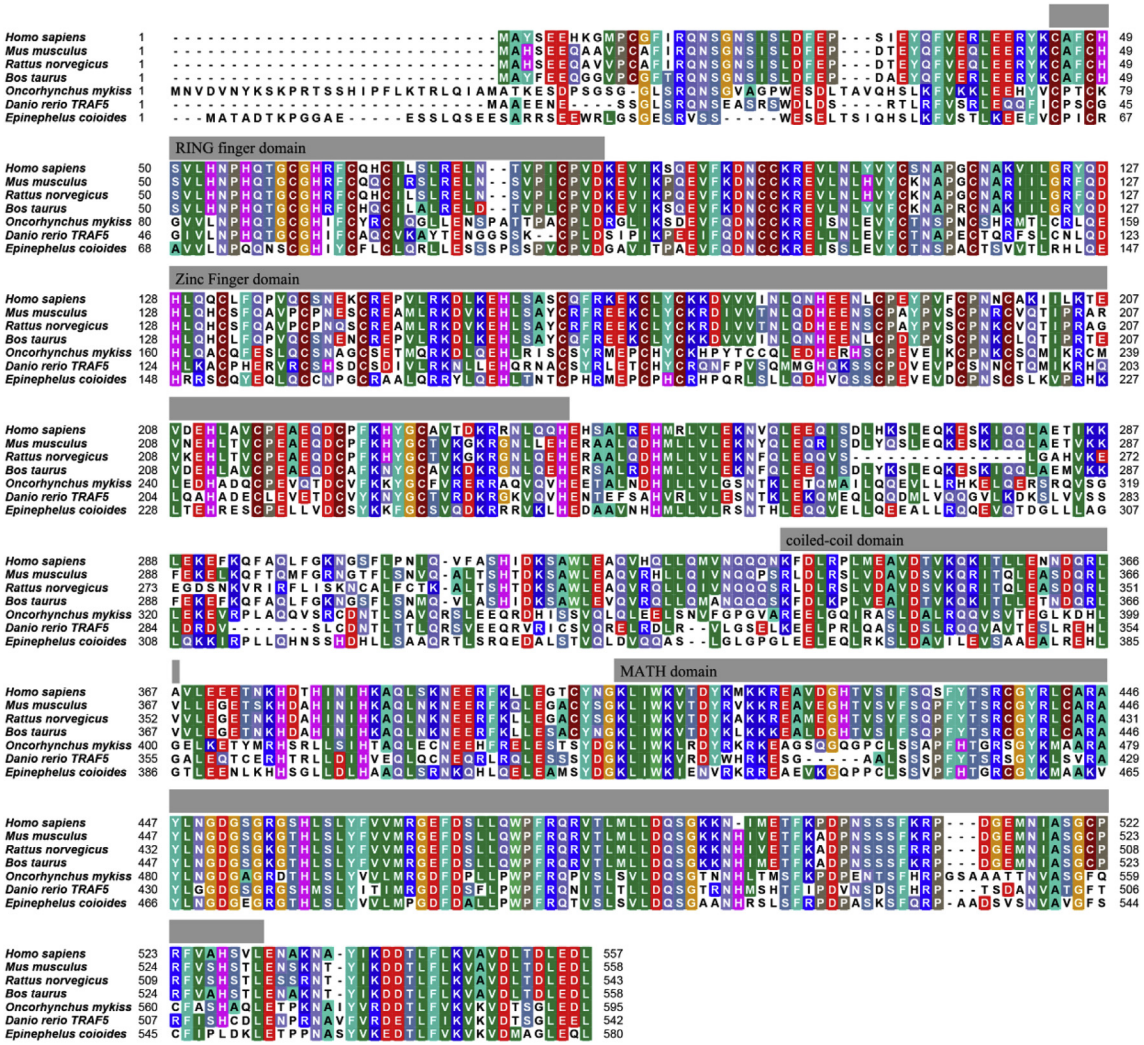

Fig. 1. Multiple alignment of predicted amino acid sequences of EcTRAF5 and TRAF5 of other species. The conserved domains including the ring finger, the zinc finger, the coiled-coil domain, and the MATH domain are underlined.

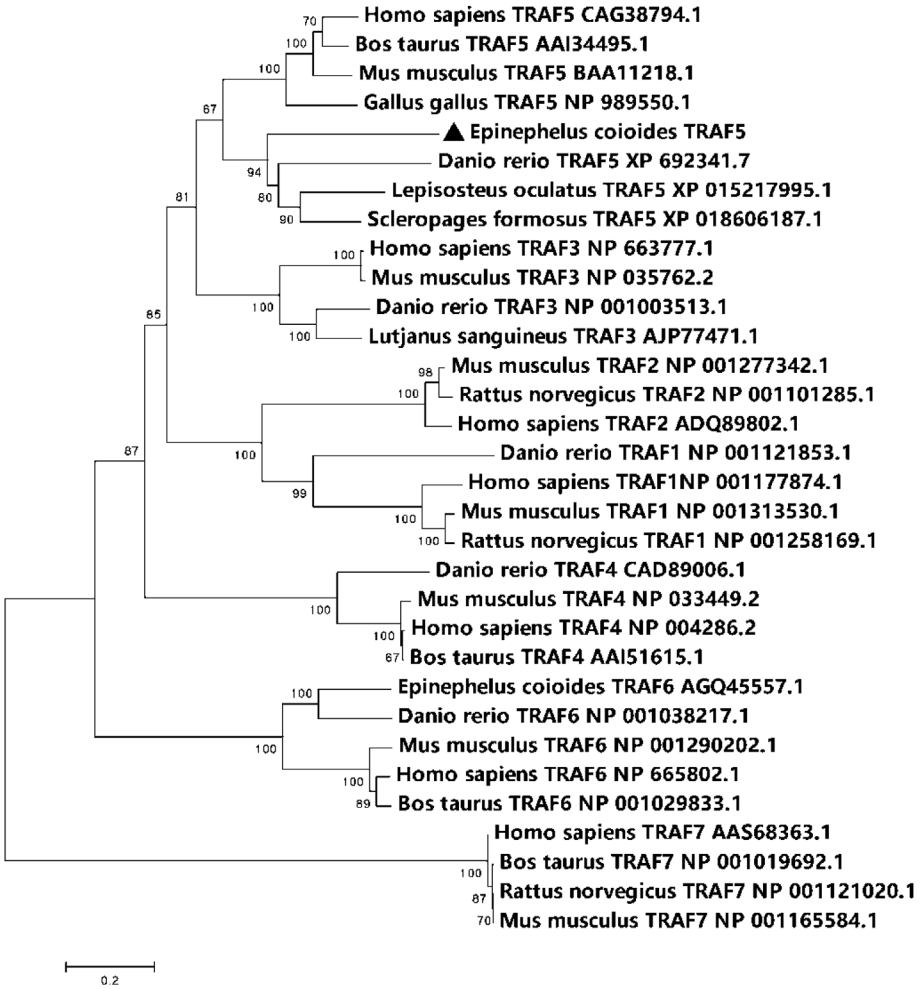

Fig. 2. Phylogenetic analysis of TRAF proteins. A phylogenetic tree was constructed with the neighbor-joining method using the MEGA 5.0 program. Numbers on the lines indicate the percentage of bootstrap values for 1,000 replicates.

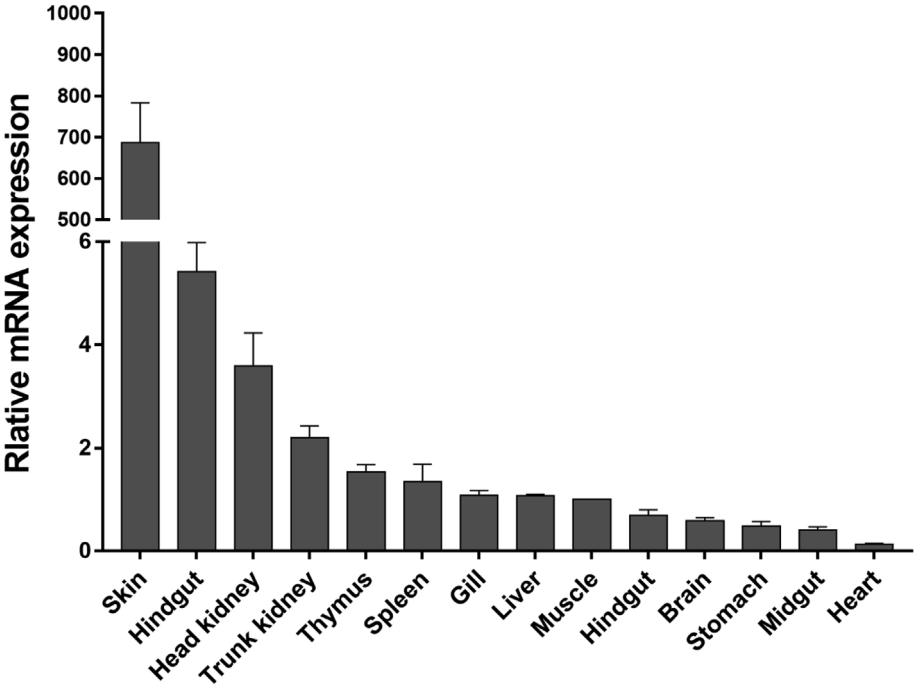

Fig. 3. Expression of EcTRAF5 in different tissues of healthy grouper. The levels of mRNA expression were normalized to β-actin. Data are mean ± SE (N = 3).

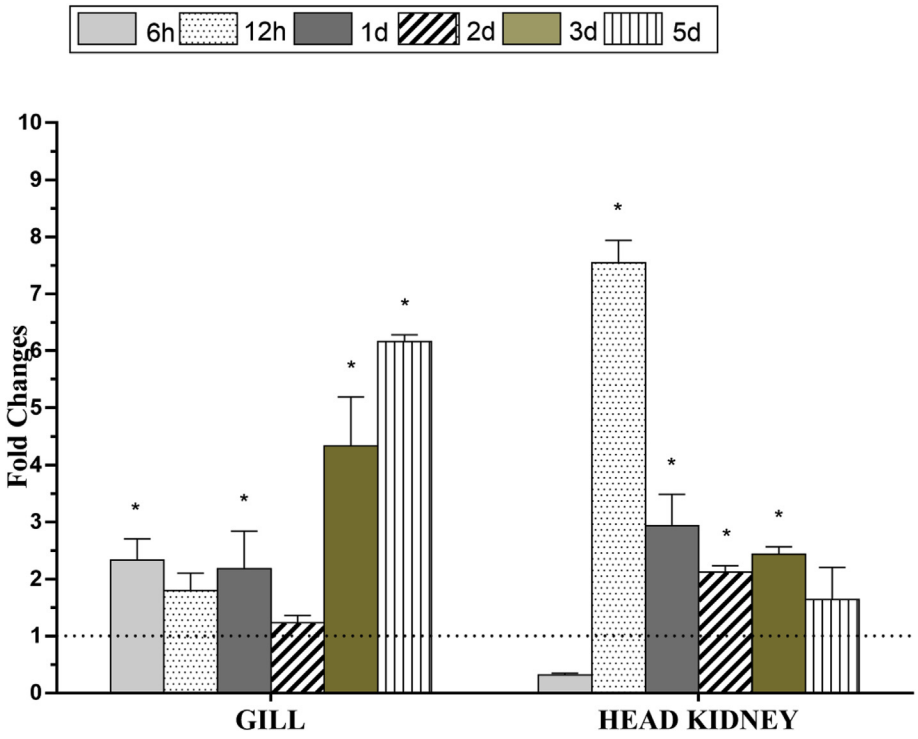

Fig. 4. Expression of EcTRAF5 in the gills and head kidney after *C. irritans* infection. The levels of mRNA expression were normalized to β-actin, and then to the corresponding control for each time point. Data are mean ± SE (N = 3). Significant differences of gene expression between control fish and fish infected with *C. irritans* are indicated with asterisks (*) ($P < 0.05$).

grouper (*Epinephelus coioides* and *E. tauvina*) [27,28], is involved in antiviral and anti-parasite immune responses.

The piscine TRAF5 DNA sequence is available in NCBI GenBank, but no study has addressed its function. In the present study, we cloned a TRAF5 cDNA sequence from orange-spotted grouper (*E. coioides*), and analyzed its expression profile in healthy fish, and in fish infected with *Cryptocaryon irritans*. The subcellular location and signal transduction function of EcTRAF5 were also analyzed.

2. Materials and methods

2.1. Fish rearing, fish infection, and tissue sampling

Healthy grouper (60.3 ± 7.3 g) were purchased from the Marine Fisheries Development Center of Guangdong Province, Guangdong, China. Fish were reared at 25 °C in an aerated flow-through water system for two weeks; they were fed daily with commercial grouper feed. For TRAF5 expression analysis, samples of skin, gill, muscle, thymus, head kidney, liver, spleen, heart, stomach, foregut, midgut, hindgut, trunk kidney, and brain were collected from three healthy groupers. Groupers were exposed to *C. irritans* at a dose of 25,000 theronts per fish. Control fish were treated in the same way as infected fish but were not infected with *C. irritans*. Samples of gill and spleen were collected from both groups at 6 and 12 h, and at 1, 2, 3, and 5 d after infection. All samples were immediately frozen in liquid nitrogen and preserved at −80 °C until RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from tissues using a HiPure Universal RNA Mini Kit (Magen, China). The quality of total RNA was assessed by electrophoresis on 1% agarose gels and OD260/280 analysis. After treatment with RNase-free DNase I (Thermo Fisher Scientific, USA), 1 μg of total RNA was used to synthesize the first-strand cDNA using a RevertAid Frist Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer instructions.

2.3. Cloning of grouper TRAF5

An unigene sequence was retrieved from transcriptome data of grouper skin, gill, spleen, and head kidney after *C. irritans* infection (unpublished data). This unigene had high sequence identity with TRAF5 of other species. Analysis with the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) indicated that the unigene contained the complete ORF of TRAF5. Based on the unigene sequence, primers of TRAF5F/R were designed to amplify the ORF of grouper TRAF5 (named EcTRAF5 hereafter) (Table 1). The PCR reaction was performed as follows: 35 cycles of 98 °C for 10 s, 58 °C for 15 s, and 72 °C for 40 s, plus one step of 72 °C for 10 min. The PCR product was ligated to the pEASY-blunt simple cloning vector (TransGen Biotech, China), transformed into DH5α, and then sequenced.

2.4. Bioinformatics analysis

Nucleotide and amino acid sequences were analyzed using the BLAST program (http://blast.ncbi.nlm.nih.gov/). The isoelectric point (pI) and molecular weight (Mw) were calculated using an online program (http://web.expasy.org/compute_pi/). Protein structure analysis was performed using the simple modular architecture research tool (http://smart.embl-heidelberg.de/). Multiple amino acid sequence alignments were constructed using ClustalW2 (http://www.ebi.ac.uk/Tools/clusta-lw2/index.html). A phylogenetic tree was constructed using the MEGA 5.0 program by the neighbor-joining method and 1,000 bootstrap replications.

2.5. Gene expression analysis

Real-time PCR was used to analyze the expression of TRAF5 using β-actin as a reference gene. Primers TRAF5F6/R6 (TRAF5) and β-actinF/R (β-actin) are listed in Table 1. Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, USA) and a Roche Light Cycler480 (Roche) apparatus, following the manufacturer instructions. The PCR reaction volume contained 0.5 μl of cDNA, 0.3 μl of each primer, 5 μl of iTaq Universal SYBR Green Supermix (Bio-Rad, USA), and 3.9 μl of water. The PCR amplification was as follows: one step of

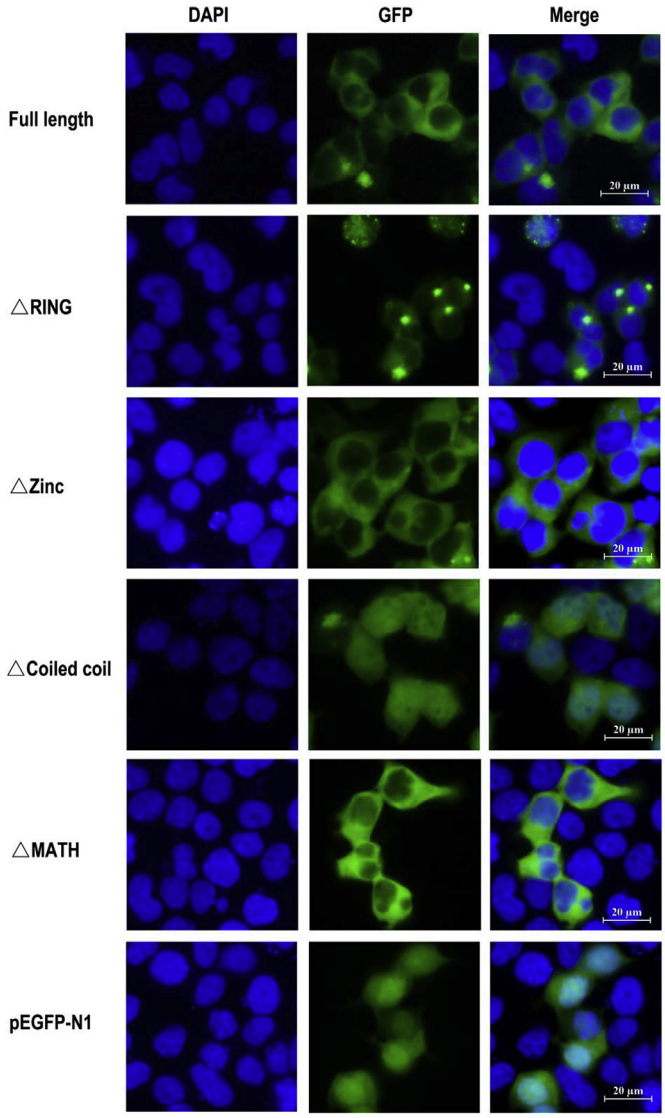

Fig. 5. Subcellular localization of full-length EcTRAF5 and of EcTRAF5 domain deletion mutants in HEK293T cells. HEK293 cells were transfected with each plasmid, the nucleus was stained with DAPI, and the intracellular localization of EcTRAF5 was detected by fluorescence microscopy.

95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. The specificity of the PCR products was confirmed by melting curve analysis and by sequencing. Each sample was analyzed in triplicate. The mRNA expression of the target gene was analyzed using the $2^{-\triangle\triangle CT}$ method [29].

2.6. Construction of expression plasmids

PCR was used to generate expression vectors encoding full-length TRAF5, and deletion mutants of the RING finger domain (△RING; deletion of residues 63–104), of the zinc finger domain (△Zinc; deletion of residues 148–261), of the coiled-coil domain (△Coiled-coil; deletion of residues 358–386), and of the MATH domain (△MATH; deletion of residues 424–581). The primers used were: TRAF5F1/R1/R1′, TRAF5F1/R1/F5/R5/R5′, TRAF5F2/R2/F5/R5/R5′, TRAF5F3/R3/F5/R5/R5′, TRAF5F4/R4/F5/R5/R5' (Table 1). The PCR products contained restriction enzymes sites for *Eco*R I at the 5′-terminus, and *Bam*H I at the 3′-terminus. The PCR products were subjected to double enzyme digestion, sub-cloned into pEGFP-N1 vector, and transfected into DH5α. After sequencing, the positive clone plasmids were purified using the E.Z.N.A. Endo-free Plasmid Mini Kit (Omega, USA) according to the manufacturer protocol.

2.7. Subcellular localization of EcTRAF5

HEK293T cells were cultured at 37 °C with 5% (v/v) CO_2 in DMEM supplemented with 10% fetal bovine serum (Gibco, USA). Cells were seeded into 24-well plates with L-lysine-treated coverslips, and cultured for 24 h. Cells were washed with Opti-MEMI Reduced Serum Medium (Gibco), and then transfected with 1 μg of the target plasmids using Lipofectamine 2000 reagent (Invitrogen, USA). Six hours later, the medium was replaced with complete medium. After 24 h, cells were fixed with 500 μl of immunostaining fixative (Beyotime, China) for 10 min, treated with PBST for 10 min, and stained with 1 mg/ml of DAPI for 5 min. Cells were washed with PBS three times after each step. Finally, a drop of anti-fade mounting medium was added to microscope slides (Beyotime), and protein localization was analyzed and recorded using a Zeiss LSM DUO confocal laser scanning microscope (Carl Zeiss, Germany).

2.8. Luciferase reporter assays

HEK293T cells were seeded in a 96-well plate, and transfected with 200 ng of plasmids, including 150 ng of target plasmids, 40 ng of NF-κB reporter plasmids, and 10 ng of pRL-TK *Renilla* reference plasmid. After 24 h, cells were washed with PBS and lysed with passive lysis buffer (Promega, USA). Firefly and *Renilla* luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer instructions. Each experiment was performed in triplicate. Relative luciferase activity was calculated as the activity of firefly luciferase relative to *Renilla* luciferase.

2.9. Statistical analysis

Data are expressed as mean ± standard error and the significance of differences between samples was determined using Duncan's test. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Sequence of EcTRAF5 and phylogenetic analysis

The ORF of EcTRAF5 (GenBank no. KR005610) was 1,743 bp encoding 581 amino acids. Its theoretical Mw and pI were 64.9 kD and 6.37, respectively. As shown in Fig. 1, the predicted EcTRAF5 protein contained an N-terminal RING finger domain (63–104 aa), a zinc finger domain (148–261 aa), a coiled-coil domain (358–386 aa), and a C-terminal MATH domain (424–552 aa).

The predicted amino acid sequence of EcTRAF5 had 39%, 40%, 39%, 38%, 43%, 48%, and 45% sequence identity with TRAF5 of *Homo sapiens*, *Mus musculus*, *Bos taurus*, *Gallus gallus*, *Danio rerio*, *Lepisosteus oculatus*, and *Scleropages formosus*, respectively (Fig. 1 and Table 2). The RING finger domain, zinc finger domain, coiled-coil domain, and MATH domain of EcTRAF5 had 40%–52%, 33%–47%, 44%–83%, and 51%–61% sequence identity with that of other species, respectively (Table 2).

A phylogenetic tree was constructed to analyze the evolutionary relationship between TRAF5 of grouper and of other species. Fig. 2 shows that all TRAF5s, including EcTRAF5, were clustered into one group. On the TRAF5 branch, EcTRAF5 fell into the same cluster with other teleost TRAF5 (with 94% bootstrap support) that was separate from mammals.

3.2. Expression analysis of EcTRAF5

EcTRAF5 transcripts were detected in all tested tissues by real-time

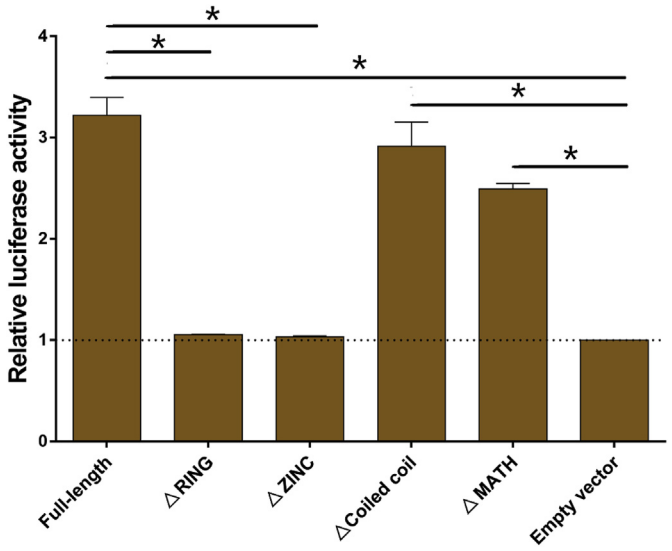

Fig. 6. NF-κB activity induced by full-length EcTRAF5 and EcTRAF5 domain deletion mutants. Results are expressed as mean ± SE (N = 3). Significant differences are indicated with asterisks (*) ($P < 0.05$).

PCR (Fig. 3). The expression level of EcTRAF5 was significantly higher in the skin, hindgut, and head kidney, and lower in heart, midgut, stomach, and brain (Fig. 3).

Fig. 4 shows the expression profile of EcTRAF5 in gill and head kidney after *C. irritans* infection. The expression level of EcTRAF5 increased in both tissues during the course of *C. irritans* infection. In gills, EcTRAF5 expression was up-regulated after *C. irritans* infection, reaching a peak at day 5 (about 6-fold higher than uninfected controls). In head kidney, the expression level of EcTRAF5 was down-regulated at 6 h (about 0.3-fold) after infection, and then up-regulated from 12 h to 5 d; it reached a peak at 12 h (about 7.5-fold relative to the uninfected control).

3.3. Intracellular localization of EcTRAF5

To determine the subcellular distribution of EcTRAF5, the full-length EcTRAF5 was inserted into the pEGFP-N1 vector and transfected into HEK293T cells. As shown in Fig. 5, EcTRAF5 was uniformly distributed in the cytoplasm. Four expression vectors with deletion of the RING finger domain, the zinc finger domain, the coiled-coil domain, or the MATH domain of EcTRAF5 were also transfected into HEK293T cells. As shown in Fig. 5, EcTRAF5 deletion of the zinc finger domain or of the MATH domain had the same cellular location as EcTRAF5. The protein with a deletion of the RING finger was located in the cytoplasm, but with some aggregates. In addition, the TRAF5 deletion of the coiled-coil domain was observed uniformly in the cytoplasm and the nucleus.

3.4. Signal transduction of EcTRAF5

To determine the signaling role of EcTRAF5, full-length or deletion mutants of EcTRAF5 with stop codons at their 3′-terminus were transfected into HEK293T cells. Full-length EcTRAF5 significantly induced NF-κB activation, but the deletion of the RING finger domain or of the zinc finger domain significantly impaired the ability of EcTRAF5 to activate NF-κB (Fig. 6). Deletion of the coiled-coil domain or of the MATH domain reduced EcTRAF5 ability to activate NF-κB, but changes were not significant relative to the full-length EcTRAF5.

4. Discussion

Although TRAFs have been extensively studied in mammals, functional studies of fish TRAFs are scarce. In this paper, we identified a TRAF5 from orange-spotted grouper, and analyzed its expression and signaling functions.

EcTRAF5 had 581 amino acids, which contained four conserved domains, including a RING finger domain, a zinc finger domain, a coiled-coil domain, and a MATH domain, similar to mammalian TRAF5 [15,16,30]. Full-length EcTRAF5 shared relatively lower sequence identity than the coiled-coil domain and MATH domain, suggesting both domains may play a crucial role in TRAF5 function. Phylogenetic analysis indicated that TRAF5 from grouper and from other species clustered into one group; However, EcTRAF5 showed a closer relationship with other fish species.

In mice, TRAF5 is expressed in all major visceral organs, with the relatively high expression in lungs and spleen [16]. TRAF5 transcript is abundantly expressed in spleen and bursa of Fabricius in chichens, but not detected in lung, liver, bone marrow, and heart [31]. We found that EcTRAF5 was ubiquitously expressed in all tested tissues with relatively high expression in skin, head kidney, and hindgut. The skin and the gut are mucosa-associated lymphoid tissues in teleost fish, which not only act as a physical barrier, but also generate innate and adaptive immune responses [32,33]. In addition, the head kidney is the largest hematopoiesis site in teleost fish, which also performs immune functions [34,35]. Therefore, high expression of EcTRAF5 in these cutaneous and immune organs may help fish against invading pathogens.

Previous studies indicated that TRAF5 is involved in host defense against virus and bacteria [36–38]. However, whether TRAF5 is need for host defense against parasitic infections is not clear. Cai et al., found that FOSL1 knockout chimeric mice had lower levels of malaria parasitemia by reducing K63 ubiquitination of TRAF3/TRIF and by blocking interaction of TRAF3/TRIF with TBK1 [39]. When infected with *C. irritans* or *Ichthyophthirius multifilii*, the expression of TRAF6 was up-regulated in grouper and grass carp, implying that TRAF6 may be involved in fish defense against parasites [26,40]. In order to study the involvement of EcTRAF5 in anti-parasitic infection, we studied its expression profile after *C. irritans* infection. After infection with *C. irritans*, EcTRAF5 expression was up-regulated in local infection sites (gills) and in immune organs (spleen), which implies that EcTRAF5 may play an important role in resistance to *C. irritans* infection.

Horie et al. have shown that TRAF5 is aggregated in the cytoplasm in cell lines derived from H-RS cells, and that it is uniformly distributed in the cytoplasm of other cell lines [41,42]. Consistent with this study, we found that EcTRAF5 was uniformly localized in the cytoplasm of HEK293T cells. Further studies indicated that the subcellular distribution of EcTRAF5 was affected by its coiled-coil domain, as the deletion of this domain caused EcTRAF5 to be diffusely distributed in both the cytoplasm and the nucleus. From the latter research, we conclude that changing the subcellular location of EcTRAF5 has no effect on its downstream signaling. However, whether the deletion of coiled-coil impaired TRAF5's ability to interact with upstream molecules in the pathway is not clear.

Previous studies have indicated that TRAF5 cooperates with TRAF2 in TNF-induced and OX40-induced NF-κB activation [43,44]. Furthermore, over-expression of mammalian TRAF5 activated NF-κB signaling in HEK293T cells [15,16,45]. In the present study, we found that full-length EcTRAF5 also had the ability to activate NF-κB in HEK293T cells, suggesting that there is a functional conservation of TRAF5 in vertebrates. TRAF proteins have four conserved domains with specific roles in signal transduction. To identify the EcTRAF5 domain required for NF-κB activation, four domain-deletion vectors were constructed; the deletion of the RING finger domain or of the zinc finger domain significantly reduced the ability of EcTRAF5 to activate NF-κB, but deletion of the coiled-coil domain or the MATH domain had no effect on activation of NF-κB. Our results were consistent with previous reports. For example, Ishida et al. have shown that TRAF5 lacking the RING finger domain and part of the zinc finger domain suppresses CD40-induced CD23 expression [15]. Nakano et al., also demonstrated that the

RING finger domain and the zinc finger domain are required for TRAF5 to activate NF-κB [16], which implies that the function of this domain of TRAF5 is conserved in vertebrates.

In conclusion, TRAF5 was ubiquitously expressed in numerous tissues; grouper infection with *C. irritans* induced the expression of EcTRAF5 in gills and head kidney. Additionally, EcTRAF5 was localized in the cytoplasm, and its distribution was dependent on the coiled-coil domain. Finally, over-expression of EcTRAF5 significantly activated NF-κB signaling, and this function was dependent on the RING finger domain and on the zinc finger domain.

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