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Fish TRIM39 regulates cell cycle progression and exerts its antiviral function against iridovirus and nodavirus

Wei Wang [a, b], Youhua Huang [a], Yepin Yu [a, b], Ying Yang [a, b], Meng Xu [c], Xiuli Chen [a, b], Songwei Ni [a, b], Qiwei Qin [a, **], Xiaohong Huang [a, *]

[a] Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou, 510301, China
[b] University of Chinese Academy of Sciences, Beijing, China
[c] State Key Laboratory Breeding Base for Sustainable Exploitation of Tropical Biotic Resources, College of Marine Science, Hainan University, Haikou, 570228, China

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ABSTRACT

The tripartite motif (TRIM)-containing proteins exert important immune regulatory roles through regulating different signaling pathways in response to different stimuli. TRIM39, a member of the TRIM family, is a RING domain-containing E3 ubiquitin ligase which could regulate cell cycle progression and apoptosis. However, the antiviral activity of TRIM39 is not explored. Here, a TRIM39 homolog from grouper, *Epinephelus coioides* (EcTRIM39) was cloned, and its effects on cell cycle progression and fish virus replication were investigated. The full-length EcTRIM39 cDNA was composed of 2535 bp and encoded a polypeptide of 543 amino acids with 70% identity with TRIM39 homologs from bicolor damselfish. Amino acid alignment analysis indicated that EcTRIM39 contained a RING finger, B-box and SPRY domain. Expression profile analysis revealed that EcTRIM39 was abundant in intestine, spleen and skin. Upon different stimuli *in vivo*, the EcTRIM39 transcript was obviously up-regulated after challenging with Singapore grouper iridovirus (SGIV), and polyinosinic-polycytidylic acid (poly I:C). Using fluorescence microscopy, we found that EcTRIM39 localized in the cytoplasm and formed aggregates in grouper spleen (GS) cells. The ectopic expression of EcTRIM39 *in vitro* affected the cell cycle progression via mediating G1/S transition. Moreover, the RING domain was essential for its accurate localization and effect on cell cycle. In addition, overexpression of EcTRIM39 significantly inhibited viral gene transcription of SGIV and red-spotted grouper nervous necrosis virus (RGNNV) *in vitro*, and the mutant of RING exerted the opposite effect. Together, our results demonstrated that fish TRIM39 not only regulated the cell cycle progression, but also acted as an important regulator of fish innate immune response against viruses.

1. Introduction

The tripartite motif (TRIM) containing proteins, which count over 70 members in humans, have been characterized by the presence of three conserved domains, RING (Really Interesting New Gene), B-Box and coiled-coil (RBCC) [1]. Increased attentions have been attracted on TRIMs due to their important roles in various biological processes, including growth, differentiation, apoptosis and transcription regulation and immune signaling pathway [1–7]. Recently, a large number of studies have uncovered the critical roles of various TRIM proteins in innate antiviral immunity [2,8–12]. These TRIM proteins regulated different antiviral signaling via unique mechanisms. For example, TRIM13 regulated the type I IFN response through inhibition of MDA5 activity [9], while TRIM44 interacted with virus-induced signaling adaptor (VISA) and promoted VISA-mediated antiviral responses [10]. However, the research efforts so far on the function of TRIMs were mainly from the mammalian studies.

TRIM39 was firstly identified as putative cGMP-dependent protein kinase (PKGI)-interactor and observed to regulate important aspects of cellular homeostasis [13]. Further studies indicated that TRIM39 could promote apoptosis signaling through inhibiting the APC/CCdh1-mediated degradation of modulator of apoptosis protein 1 (MOAP-1) [14]. Moreover, TRIM39 not only was able to

* Corresponding author.
** Corresponding author.
E-mail addresses: qinqw@scsio.ac.cn (Q. Qin), huangxh@scsio.ac.cn (X. Huang).

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directly bind and ubiquitylate p53 *in vitro* and *in vivo* which led to p53 degradation [15], but also played a causal role in regulating cell cycle progression and the balance between cytostasis and apoptosis after DNA damage via stabilizing p21 [16]. In addition, TRIM39 was also proved to regulate type I interferon-related pathway and response to viral infection [17]. Although mammalian TRIM39 exerts multiple functions in various biological processes, the roles of its corresponding homolog in fish still remained largely unknown.

Groupers, *Epinephelus* spp. are commercially important marine teleost being widely cultured in China and Southeast Asian countries. However, the emergence of iridoviral and nodaviral pathogens caused heavy economic losses in grouper aquaculture [18–21]. Singapore grouper iridovirus (SGIV) was isolated from diseased groupers and its infection caused more than 90% mortality in grouper and sea bass [19]. Outbreak of viral nervous necrosis usually caused an extremely high mortality of larvae and juveniles, and resulted in great economic losses in grouper aquaculture [18,20]. Although several signaling pathways and immune regulatory molecules have been explored in grouper in response to iridovirus and nodavirus [22–28], no reports focused on TRIM family proteins. Interestingly, bloodthirsty-like TRIM genes (*btr*) were orthologous to human TRIM39 and relatively dispersed in the zebrafish genome [29]. Among them, *btr*25 was involved in erythropoiesis in zebrafish, while *btr*20 was involved in the immune regulation against *Aeromonas hydrophila* strain NJ-1 infection [30,31]. However, little work has been carried out on the role of fish TRIM39-like genes in response to virus infection.

In the present study, we cloned a TRIM39 homolog from grouper and evaluated the roles of EcTRIM39 on cell cycle and fish virus replication. Our data will provide new insights into the function of fish TRIM genes in response to virus infection.

2. Materials and methods

2.1. Fish, cells and viruses

Orange-spotted groupers, *Epinephelus coioides* (50–60 g) were purchased from a marine fish farm, Hainan Province, China. Fish were kept in a laboratory recirculating seawater system at 25–28 °C and for two weeks before use. The GS cells used in this study were grown in Leibovitz's L15 medium containing 10% fetal bovine serum (FBS, Gibco) at 25 °C [27]. The virus stocks of Singapore grouper iridovirus (SGIV) and red-spotted grouper nervous necrosis virus (RGNNV) were prepared and stored at −80 °C until used.

2.2. Cloning of EcTRIM39 and bioinformatic analysis

According to the EST sequence of EcTRIM39 from grouper spleen transcriptome [28], the full length cDNA of EcTRIM39 was amplified with a SMART RACE cDNA amplification kit as described previously [27]. The corresponding primers were listed in Table 1. After sequencing and assembly, the putative open reading frame (ORF) and the deduced amino acid sequences of EcTRIM39 were analyzed using BLAST program (http://www.ncbi.nlm.nih.gov/blast). The conserved protein domains or motifs were predicted using SMART program (http://smart.embl-heidelberg.de/). Multiple amino acid sequences alignment of the EcTRIM39 was carried out ClustalX1.83 software and edited using GeneDoc program. The phylogenetic tree was constructed using Mega 4.0.

2.3. Expression patterns for EcTRIM39 in healthy and challenged grouper

To examine the distribution pattern of EcTRIM39 in different tissues from healthy orange-spotted grouper, including head kidney, heart, liver, spleen, intestine, muscle, brain, skin, gill, stomach and kidney, total RNA was extracted from these tissues using the SV Total RNA Isolation Kit (Promega) as described previously [27]. The transcript of EcTRIM39 in different tissues was detected by quantitative real-time PCR (qRT-PCR) as described following.

To evaluate the expression profiles of EcTRIM39 in response to different stimuli, groupers were injected with PBS, SGIV, poly I:C as described previously [27]. Briefly, poly I:C treated groupers were collected at 0, 3, 6, 12, 24, 48 h post injection, and SGIV treated groupers were collected at 0, 3,12, 24,48, 72 h post injection. At indicated time points, the spleen of different groups ($n > 3$) of challenged grouper were harvested for RNA extraction and qRT-PCR analysis.

Table 1
Primers used in this study.

Name	Sequence (5′–3′)
5′EcTRIM39-R1	ATGGCAACTTCCAGCTGTCTG
5′EcTRIM39-R2	TCACTGACCTGATCCGCTCC
3′EcTRIM39-F1	GTTGTAGGTACAGGGGTTATGATGAG
3′EcTRIM39-F2	CTCTCAATAGACTCTCTGACCACTCC
pcDNA-TRIM39-F	TATAGGTACCGAATGGCAACTTCCAGCTGTCTG
pcDNA-TRIM39-R	AACTCTCGAGGTGTGTTGTAGGTACAGGGGTTATG
pcDNA-ΔRING-F	CGAAAGATCTATGACATTTTCTCCAAGACCTCAAC
pC1-TRIM39-F	AATACTCGAGCTATGGCAACTTCCAGCTGTCTG
pC1-TRIM39-R	AATGGTACCTTAGTGTGTTGTAGGTACAGGGGTTATG
pC1-ΔRING-F	CCACTCGAGCTATGACATTTTCTCCAAGACCTCAAC
RT-Actin-F	TACGAGCTGCCTGACGGACA
RT-Actin-R	GGCTGTGATCTCCTTCTGCA
RT-TRIM39-F	GCAAAGGAGGGGTTCACATCAG
RT-TRIM39-R	CTCATCAAGCCCAAAGGTCCAG
RT-MCP-F	GCACGCTTCTCTCACCTTCA
RT-MCP-R	AACGGCAACGGGAGCACTA
RT-VP19-F	TCCAAGGGAGAAACTGTAAG
RT-VP19-R	GGGGTAAGCGTGAAGACT
RT-RdRp-F	GTGTCCGGAGAGGTTAAGGATG
RT-RdRp-R	CTTGAATTGATCAACGGTGAACA
RT-CP-F	CAACTGACAACGATCACACCTTC
RT-CP-R	CAATCGAACACTCCAGCGACA

2.4. Plasmid construction

To demonstrate the characteristic and function of EcTRIM39 *in vitro*, the full length of open reading frame (ORF) of EcTRIM39 was cloned into pDsRed2-C1 and pcDNA3.1-flag vector as described previously. To further determine the roles of RING domain in the action of EcTRIM39, the truncated fragment, EcTRIM39-ΔRING was also subcloned into these two vectors. All the primers were listed in Table 1, and the constructed plasmids (C1-EcTRIM39, C1-EcTRIM39-ΔRING, Flag-EcTRIM39 and Flag-EcTRIM39-ΔRING) were subsequently confirmed by DNA sequencing.

2.5. Cell transfection

Cell transfection was carried out using Lipofectamine 2000 reagent (Invitrogen) as described previously [27]. Briefly, GS cells were grown in 24-well plates, and then the mixture of Lipofectamine 2000 and plasmids was added for 6 h incubation. After replacing the fresh normal medium, cells were cultured at 25 °C for further study.

To establish stable cells overexpressing EcTRIM39 or EcTRIM39-ΔRING, the recombinant plasmids including (Flag-EcTRIM39 and Flag-EcTRIM39-ΔRING) were transfected into GS cells as described above, and then cells were incubated with medium containing