



Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Fish species-specific TRIM gene FTRCA1 negatively regulates interferon response through attenuating IRF7 transcription

Min Wu^{a,b}, Cheng Dan^a, Jian-Fang Gui^{a,b,c}, Yi-Bing Zhang^{a,b,c,d,*}^a State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, China^b University of Chinese Academy of Sciences, Beijing, 10049, China^c The Innovation Academy of Seed Design, Chinese Academy of Sciences, Wuhan, 430072, China^d Key Laboratory of Aquaculture Disease Control of Ministry of Agriculture, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, China

A B S T R A C T

Keywords:

finTRIM

FTRCA1

RLR signaling

IFN antiviral response

Negative regulation

In mammals and fish, emerging evidence highlights that TRIM family members play important roles in the interferon (IFN) antiviral immune response. Fish TRIM family has undergone an unprecedented expansion leading to generation of finTRIM subfamily, which is exclusively specific to fish. Our recent results have shown that FTRCA1 (finTRIM *C. auratus* 1) is likely a fish species-specific finTRIM member in crucian carp *C. auratus* and acts as a negative modulator to downregulate fish IFN response by autophagy-lysosomal degradation of protein kinase TBK1. In the present study, we found that FTRCA1 also impedes the activation of crucian carp IFN promoter by IRF7 but not by IRF3. Mechanistically, FTRCA1 attenuates IRF7 transcription levels likely due to enhanced decay of IRF7 mRNA, leading to reduced IRF7 protein levels and subsequently reduced fish IFN expression. E3 ligase activity is required for FTRCA1 to negatively regulate IRF7-mediated IFN response, because ligase-inactive mutants and the RING-deleted mutant of FTRCA1 lose the ability to block the activation of crucian carp IFN promoter by IRF7. These results together indicate that FTRCA1 is a multifaceted modulator to target different signaling factors for shaping fish IFN response in crucian carp.

1. Introduction

Innate interferon (IFN) immune response is the first line of vertebrate defense against virus infection. As a kind of very simple microbes, viruses harbor few unique features suitable for detection by host cells. Therefore, virus genomic nucleic acids or viral-derived nucleic acids during viral replication, as conserved portions of microbes called pathogen associated molecular patterns (PAMPs), are generally recognized by pattern recognition receptors (PRRs) of host cells, such as cytosolic receptors, namely retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) that are composed of RIG-I, MDA5 and LGP2 [1]. Once recognition, signaling cascades are initiated through recruitment and activation of downstream adaptor protein mitochondrial antiviral signaling (MAVS) and further activating cytoplasmic protein kinases TANK-binding kinase 1 (TBK1). The activated TBK1 in turn phosphorylates and activates IFN regulatory factors 3/7 (IRF3/7) to turn on the

transcription of type I IFNs, which subsequently induce the expression of IFN-stimulated genes (ISGs) through JAK-STAT signaling pathway, thereby eliminating the viral invaders [1,2].

In mammals, IRF3 is constitutively expressed in diverse cell types and in response to viral infection, it is primarily responsible for turning on the expression of the early phase IFNs, such as IFN β [3]. Unlike IRF3, IRF7 is constitutively expressed at extreme low levels in most cells but at high levels in macrophages and plasmacytoid dendritic cell (pDCs) [4]. As a typical ISG, IRF7 is significantly induced by the secreted IFN β in early stage and activated by virus infection; the activated IRF7 then dramatically induces the production of the later phase IFNs, including most IFN α s [5]. This notion is evidenced by gene knockout analyses in mice, showing that IRF3 deficiency significantly obstructs the production of both IFN α and IFN β but in IRF7-deficient cells, there is no IFN α expression and only decreased amounts of IFN β detected [6,7]. Similar to mammals, Fish have the pivotal molecules of RLR

Abbreviations: FTRCA1, (finTRIM *Carassius auratus* 1); IRF3/7, IFN regulatory factor 3/7; ISG, IFN-stimulated gene; ISRE, IFN-stimulated regulatory element; LGP2, Laboratory of genetics and physiology 2; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma-differentiation-associated gene 5; MITA, mediator of IRF3 activation; RIG-I, retinoic acid-inducible gene I; RLRs, RIG-I like receptors; TBK1, TANK-binding kinase 1

* Corresponding author.. State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, 430072, China.

E-mail address: ybzhang@ihb.ac.cn (Y.-B. Zhang).

<https://doi.org/10.1016/j.fsi.2019.04.297>

Received 18 March 2019; Received in revised form 25 April 2019; Accepted 27 April 2019

Available online 29 April 2019

1050-4648/© 2019 Published by Elsevier Ltd.

Table 1
Primers used in the study.

Primer names	Sequences (5'to3')
IRF7-VT-F	CCACTGTGCTGGATATCTG
IRF7-VT-R	CATTGGCTTTGTCGTTAG
FTRCA1-VT-F	TCTGGCACAATAACATACGC
FTRCA1-VT-R	AGATGAGTTTTTGTTCGGGC
CAB-IRF7-F	CAACGAGCACCTAACGA
CAB-IRF7-R	CCACCTGGCTGAGCAATT
EPC-IRF3-F	GGACGAGGAAAGCGTGTCTC
EPC-IRF3-R	GTGAAATCTGCCCAAACACC
EPC-IFN-F	ATGAAAACCTCAAATGTGGACGTA
EPC-IFN-R	GATAGTTCCACCCATTTCTTAA
EPC-Actin-F	CACGTGCCCACATCTACGAG
EPC-Actin-R	CCATCTCTGCTCGAAGTC

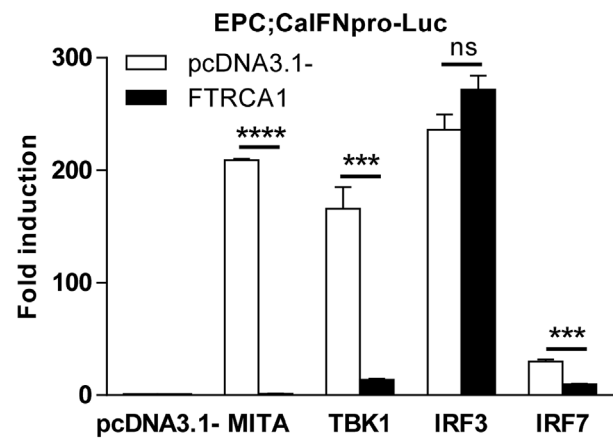
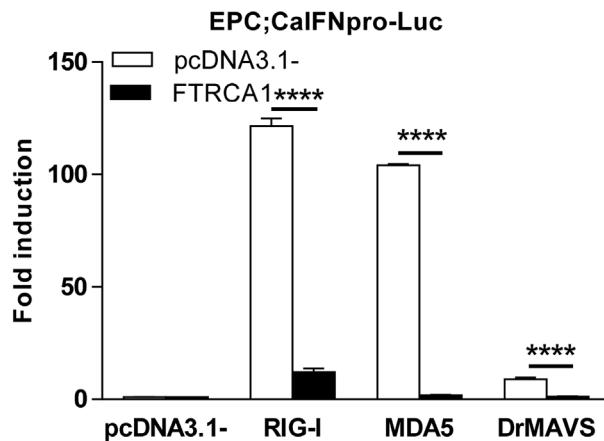
signaling pathway including RIG-I, MDA5, MAVS, MITA, TBK1 and IRF3/7, all of which are involved in fish type I IFN response [8,9]. However, unlike mammalian IRF3/7 [5], fish IRF3 and IRF7 are two IFN-stimulated genes (ISGs), and they both synergistically regulate the expression of different fish IFN genes [10–13].

Whilst IFNs are essential for clearing virus infection, overproduction

of IFNs leads to the development of immunopathological conditions; therefore, multiple mechanisms have been developed to precisely modulate the IFN signaling [14]. In mammals, emerging evidence has revealed the pivotal roles of some tripartite motif (TRIM) proteins in IFN antiviral response [15]. For example, TRIM4 positively regulates RIG-I-mediated IFN induction by targeting RIG-I for K63-linked ubiquitination [16]; TRIM40 promotes K27- and K48-linked polyubiquitination of MDA5 and RIG-I for proteasomal degradation [17]. In line with the regulatory function of TRIMs, many TRIM genes are virus- or IFN-induced genes [15,18]. TRIM family proteins are sequentially comprised of really interesting new gene (RING) domain, one or two B-box domains, coiled-coil domain and diverse C-terminal domain. RING domain endows most TRIM proteins with E3 ubiquitin ligase activity [14]. Varied number of TRIM family members are observed in the organisms of different evolutionary degrees in that there are > 80 members in human, ~64 members in mice, ~20 in worms and < 10 in flies [19]. An unprecedented expansion of TRIM family has occurred in fish, which results in the occurrence of a novel TRIM subfamily specific to fish (finTRIM, FTR) [20].

Recently, we identified a TRIM homolog from UV-inactivated GCRV-infected crucian carp (*C. auratus*) blastulae embryonic (CAB) cells and found that it is likely a fish species-specific TRIM. Since it

A



B

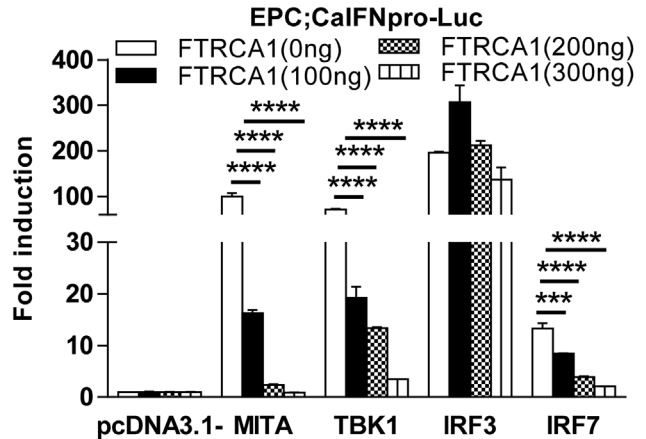
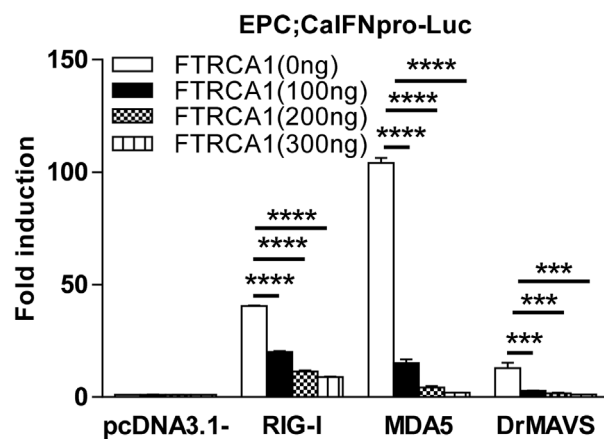


Fig. 1. FTRCA1 inhibited the activation of crucian carp IFN promoter by IRF7 but not by IRF3. EPC cells seeded in 24-well plates overnight were cotransfected with crucian carp IFN promoter plasmid (IFNpro-Luc) (200 ng), together with each of pivotal molecules of RLR signaling pathway including RIG-I, MDA5, MAVS, MITA, TBK1, IRF3 and IRF7 (200 ng), and FTRCA1 at a constant dose (200 ng) (A) or at increasing amounts (0, 100, 200, 300 ng; B). At 48 h post-transfection, the cells were collected for luciferase assays. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

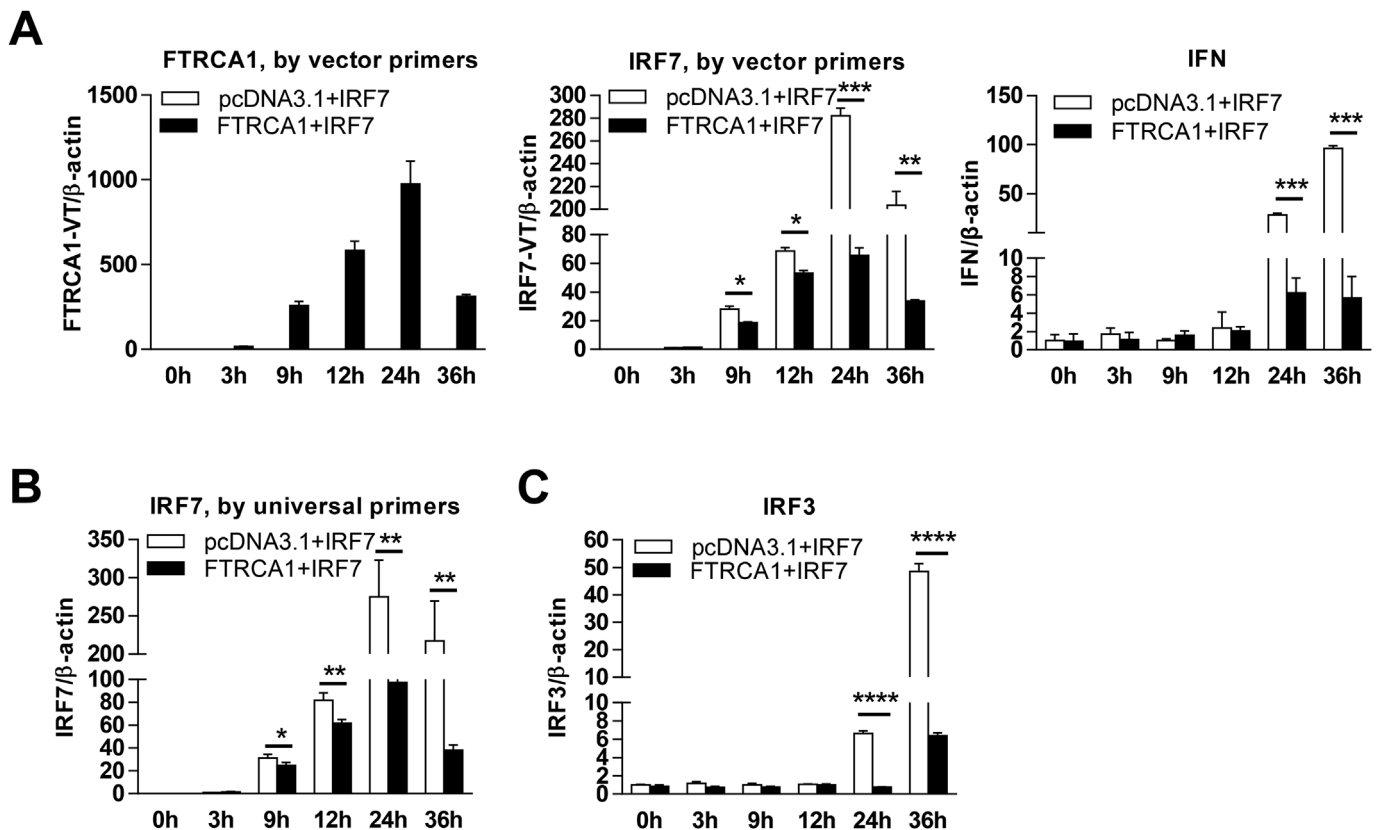


Fig. 2. FTRCA1 downregulated IRF7-directed IFN expression by attenuating IRF7 transcription. EPC cells seeded in 3.5 cm² dishes were transfected with IRF7-myc alone or with IRF7-myc and Flag-FTRCA1 together (1 μg each) for the indicated time points, followed by RT-qPCR analysis of the mRNAs derived from the transfected plasmid FTRCA1 (left panel in A), the transfected plasmid IRF7 (middle panel in A), and the endogenous cellular IFN (right in A), or by RT-qPCR analysis of the mRNAs from both the transfected IRF7 and endogenous IRF7 (B), the endogenous cellular IRF3 (C). The relative expression was normalized to the expression of β-actin and represented as fold induction relative to the expression level in control cells that was set to 1. Error bars represent SD obtained by measuring each sample in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

belongs to finTRIM family, therefore named FTRCA1 (finTRIM *C. auratus* 1) [21]. Function analysis revealed that FTRCA1 is an E3 ligase, whereby it negatively regulates fish IFN response through autophagy-lysosomal degradation of TBK1 [21]. In the present study, we found that FTRCA1 downregulated fish IFN response by attenuating IRF7 transcription. Reduced IRF7 transcription levels is likely as result of enhanced decay of IRF7 mRNA, leading to reduced IRF7 protein levels and reduced IFN expression. Our results indicate that FTRCA1 is a multifaceted modulator to target different signaling factors for shaping fish IFN response in crucian carp.

2. Materials and methods

2.1. Cells, reagents and plasmids

Crucian carp (*C. auratus* L.) blastula embryonic cells (CAB) and epithelioma papulosum cyprini cells (EPC) were cultured as described previously [10,22]. Chloroquine was purchased from Cell Signaling Technology (USA), and MG132 was from Calbiochem (Germany). All expression plasmids, including crucian carp IFN promoter-driven luciferase plasmids (CaIFNpro-Luc), RIG-I-myc, MDA5-myc, MITA, TBK1-myc, IRF3-myc, IRF7-myc, and zebrafish MAVS (DrMAVS), were described previously [10,11,23]. Flag-FTRCA1, FTRCA1-ΔRING, and eight ligase-inactive mutants of FTRCA1 including C13A, C16A, C28A, H30A, C33A, C36A, C52A and mut7A were described in Ref. [21].

2.2. Transfection and luciferase activity assays

Transfection assays were performed with FuGENE HD Transfection

Reagent (Promega) according to the manufacturer's protocol or by our previous reports [10,11,21,22]. Briefly, CAB or EPC cells seeded in 24-well plates or 3.5 cm² dishes overnight were transfected with the indicated plasmids, and the ratio of plasmids and FuGENE HD Transfection Reagent (Promega) is 1:3 per well. Luciferase activity assays were performed by a Junior LB9509 luminometer (Berthold, Pforzheim, Germany) and normalized to the amounts of Renilla luciferase activities as described previously [10,11,22,24].

2.3. RNA extraction, cDNA synthesis, and real time-PCR

Total RNA was extracted by SV Total RNA Isolation System (Promega). First-strand cDNA was synthesized using oligo dT and M-MLV reverse transcriptase (Promega). Real-time PCR (RT-qPCR) was performed in a 20 μl volume containing SYBR Green I Dye. All samples were analyzed in triplicate and the expression values, unless indicated, were normalized to β-actin. Primers used for RT-PCR analysis are listed in Table 1.

2.4. Western blotting

Western blots were performed as previously described [10,11,22]. The tag-specific Abs, anti-myc for IRF7 and anti-Flag for FTRCA1, were purchased from Cell Signaling Technology.

2.5. Statistical analysis

The student's *t*-test is used for statistical analysis of the results from luciferase assays and RT-PCR assays.

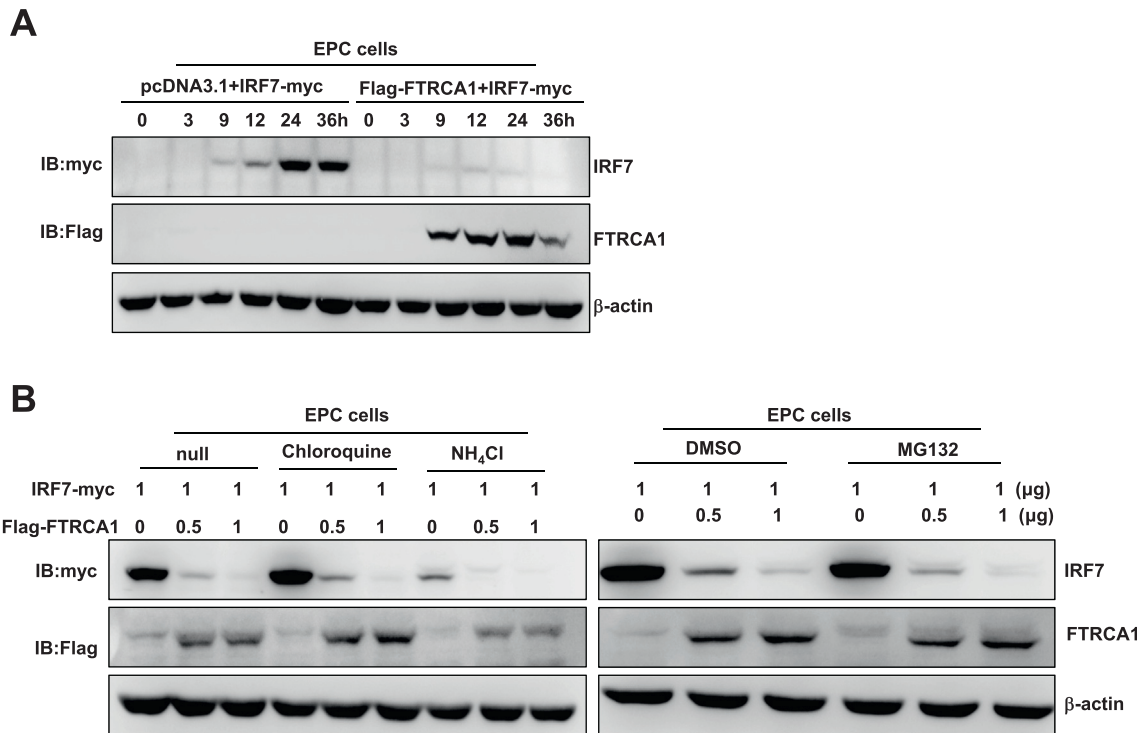


Fig. 3. FTRCA1-mediated decrease of IRF7 protein is not due to protein degradation. EPC cells seeded in 3.5 cm² dishes were transfected with IRF7-myc alone or with IRF7-myc and Flag-FTRCA1 together (1 μ g each) for the indicated time points (A), or EPC cells were cotransfected with IRF7-myc and Flag-FTRCA1 at the indicated amounts for 24 h, followed by treatment with or without 50 μ M Chloroquine, 50 mM NH₄Cl, or 20 μ M MG132 for additional 9 h (B). Western blotting was used to analyze the expression of IRF7 and FTRCA1 proteins using corresponding Abs.

3. Results

3.1. FTRCA1 inhibits the activation of crucian carp IFN promoter by IRF7 but not by IRF3

Similar to our previous results [21], luciferase assays showed that overexpression of pivotal components of RLR signaling pathway, including RIG-I, MDA5, MAVS, MITA, TBK1, IRF3 and IRF7, significantly induced the activity of crucian carp IFN promoter-driven luciferase (IFNpro-luc); however, this activation was inhibited by simultaneous overexpression of FTRCA1, with an exception that IRF3-directed activation was not influenced (Fig. 1A). Consistently, increasing inhibition was observed when increasing doses of FTRCA1 were transfected together with a constant dose of each of RLR signaling factors, including IRF7 but except IRF3 (Fig. 1B). These results indicated that FTRCA1 down-regulates RLR pathway-directed IFN expression and interestingly, although both IRF3 and IRF7 are two crucial transcription factors involved in RLR signaling [11,24], only IRF7-mediated IFN promoter activation is inhibited by FTRCA1.

3.2. FTRCA1 down-regulates IRF7-directed IFN expression by attenuating IRF7 transcription

To further determine the functional role of FTRCA1 on IRF7-mediated IFN response, RT-PCR was used to detect the transcription of IRF7 and IFN in EPC cells transfected with fish IRF7 and FTRCA1 together. Using primers to amplify the mRNAs derived only from the transfected plasmids expressing FTRCA1 or IRF7, we found that FTRCA1 transcription was detected initially at 3 h post transfection, peaked at 24 h post transfection and decreased thereafter, showing an expression pattern similar to IRF7 gene transcription in IRF7 alone-transfected cells (Fig. 2A). However, transfection of FTRCA1 and IRF7 together resulted in significantly decreased expression of IRF7 in a time-dependent fashion, and this decrease was initially detected at 9 h post

transfection (Fig. 2A). Similarly, endogenous EPC IFN gene expression was induced by transfection of IRF7 alone in a time-dependent fashion, and this induction was significantly inhibited by co-transfection of FTRCA1, being detected initially at 24 h post transfection. These results indicated that FTRCA1 negatively regulates IRF7-directed IFN gene expression likely through attenuating IRF7 transcription.

Using a pair of primers to amplify IRF7 mRNAs from both endogenous IRF7 and transfected IRF7 plasmids, similar downregulation of IRF7 transcription was observed, with initial detection also at 9 h post transfection (Fig. 2B). Interestingly, endogenous IRF3 gene transcription was downregulated by FTRCA1, being detected initially at 24 h post transfection, a time similar to endogenous IFN transcription but later than IRF7 transcription (Fig. 2C). Considering that fish IRF3 is a typical ISG [10], these results indicated that the observed down-regulation of IRF3 gene transcription is likely due to the attenuated IFN expression by FTRCA1.

3.3. FTRCA1-mediated decrease of IRF7 protein is not due to protein degradation

Western blotting was used to compare IRF7 protein levels in the presence and absence of FTRCA1 transfection in EPC cells. As shown in Fig. 3A, compared to transfection of IRF7 alone, co-transfection of IRF7 and FTRCA1 resulted in consistent reduction of IRF7 protein in a time-dependent fashion. Ubiquitin-proteasome pathway and autophagy-lysosome pathway are two mainly pathways for protein degradation [25]. To exclude the possibility that protein degradation is involved in FTRCA1-mediated reduction of IRF7 protein, we investigated the effect of MG132 (inhibitor of ubiquitin-proteasome-dependent degradation pathway) and NH₄Cl, Chloroquine (inhibitors of autophagy-lysosome-dependent degradation pathway) on FTRCA1-mediated downregulation of IRF7 protein in EPC cells. Western blotting showed that addition of these inhibitors could not block the decrease of IRF7 protein by FTRCA1 (Fig. 3B). These results indicated that the reduction of IRF7

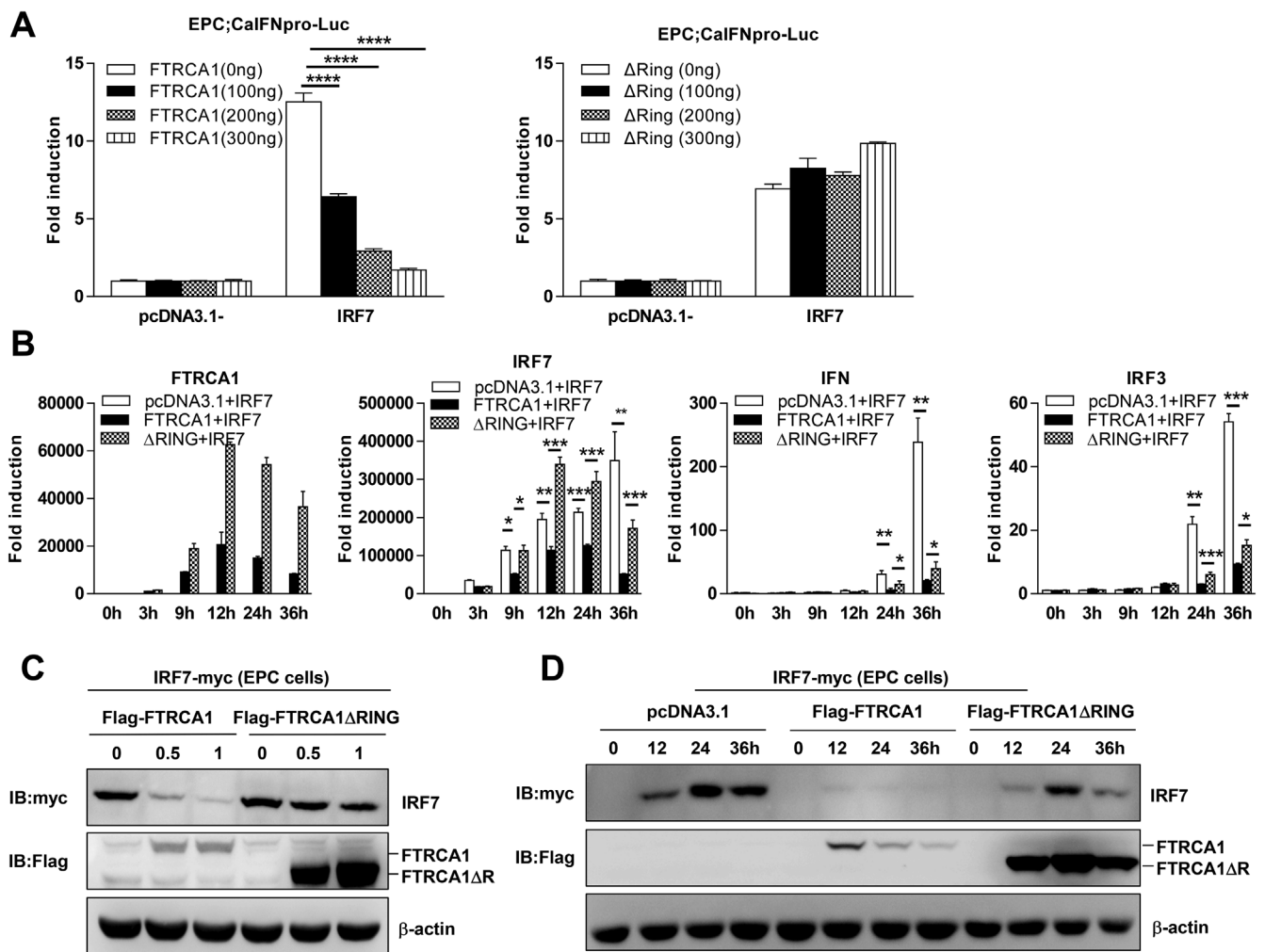


Fig. 4. FTRCA1 inhibited IRF7-directed IFN expression dependent on the N-terminal RING domain. **A.** FTRCA1-ΔRING failed to negatively regulate IFN promoter activation by IRF7. EPC cells seeded in 24-well plates overnight were cotransfected with CaIFNpro-Luc, IRF7 (200 ng each), and FTRCA1 (left panel) or FTRCA1-ΔRING (right panel) at increasing amounts (0, 100, 200, 300 ng). 48 h later, the cells were collected for luciferase assays. **B.** FTRCA1-ΔRING lost the potential to inhibit IRF7 transcription. EPC cells seeded in 3.5 cm² dishes were transfected with IRF7-myc, together with Flag-FTRCA1 or FTRCA1-ΔRING (1 μg each) for different time points followed by RT-qPCR detection of transcription of the transfected FTRCA1 and IRF7, the endogenous IFN and IRF3 genes. **C and D.** IRF7 protein expression was almost not influenced by FTRCA1-ΔRING. EPC cells seeded in 3.5 cm² dishes were transfected with IRF7-myc (1 μg), together with Flag-FTRCA1 or FTRCA1ΔRING at increasing amounts (0, 0.5, 1 μg) for 48 h (C), or at a constant amount (1 μg each) for different time points (D). Western blotting was used to analyze the expression of TBK1 and FTRCA1 proteins using corresponding Abs. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

protein by FTRCA1 is ascribed from the attenuated IRF7 gene transcription, but not from protein degradation through ubiquitin-proteasome pathway or autophagy-lysosome pathway.

3.4. FTRCA1 inhibits IRF7-directed IFN expression dependent on the N-terminal RING domain

FTRCA1 belongs to TRIM family featuring a typical RING domain in N-terminus [21]. To determine the role of RING domain of FTRCA1 in IRF7-directed IFN expression, we firstly compared the inhibitory effects between wild type FTRCA1 and RING-deleted mutant of FTRCA1 (FTRCA1-ΔRING). As shown in Fig. 4A, unlike wild type FTRCA1, FTRCA1-ΔRING failed to block the activation of IFN promoter by IRF7. Compared to transfection of empty vector, transfection of wild type FTRCA1 resulted in reduced IRF7 transcription levels, but transfection of FTRCA1-ΔRING did not (Fig. 4B). Similar to that in Fig. 2, the transcription levels of endogenous IFN and IRF3 were significantly reduced by transfection of wild type FTRCA1, being initially detected at 24 h post transfection; however, this reduction was significantly alleviated in FTRCA1-ΔRING-transfected cells (Fig. 4B). As expected, the

levels of IRF7 protein were reduced in wild type FTRCA1-transfected EPC cells but nearly not in FTRCA1-ΔRING-transfected cells, by dose-response assays (Fig. 4C) and time-course assays (Fig. 4D). These results indicated that the N-terminal RING domain is essential for FTRCA1 downregulating IRF7-directed IFN response.

3.5. E3 ligase activity of FTRCA1 is required for negative regulation of IRF7-directed IFN response

FTRCA1 has E3 ligase activity dependent of RING domain [21]. Previously we have constructed eight ligase-deficient mutants of FTRCA1, including seven single-site mutants C13A, C16A, C28A, H30A, C33A, C36A, C52A, and a multiple-site mutant mut7 [21]. To explore whether the E3 ligase activity is critical for FTRCA1 function, EPC cells were transfected with IRF7 together with each of these mutants followed by luciferase assays. Unlike wild-type FTRCA1, these mutants all failed to block IRF7-mediated activation of fish IFN promoter (Fig. 5). These results indicated that E3 ligase activity of FTRCA1 is required for blocking IRF7-directed IFN response.

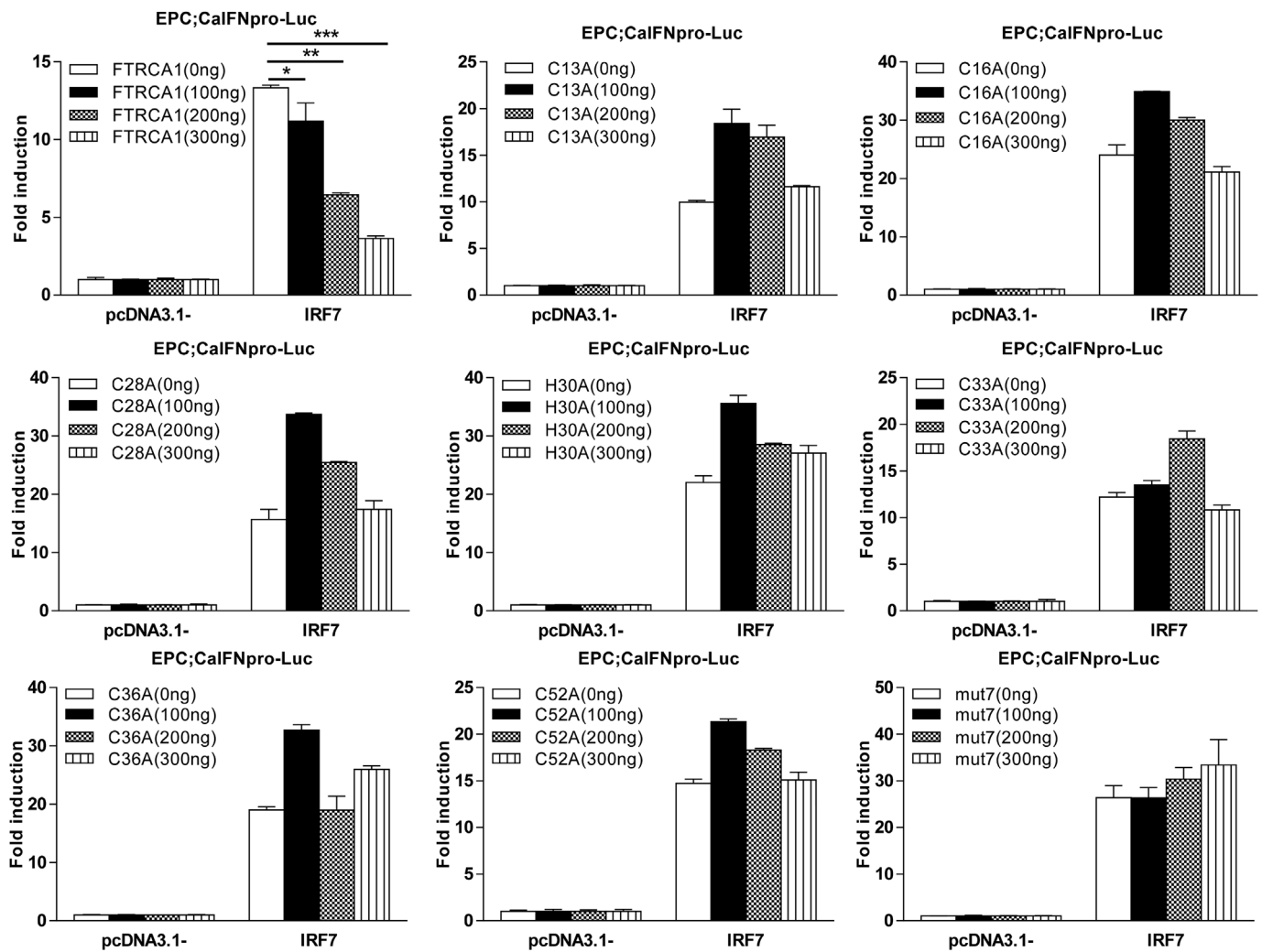


Fig. 5. Ligase-inactive mutants of FTRCA1 lost the ability to block the activation of crucian carp IFN promoter by IRF7.

EPC cells seeded in 24-well plates overnight were cotransfected with CaIFNpro-Luc, IRF7 or pcDNA3.1 (200ng each), together with increasing amounts (0, 100, 200, 300ng) of FTRCA1 or each of ligase-inactive mutants of FTRCA1 (including C13A, C16A, C28A, H30A, C33A, C36A, C52A). 48h later, the cells were collected for luciferase assays. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

In mammals, many TRIM family genes are significantly upregulated by IFN or by virus infection in a type I IFN-dependent manner [26] and actually, these genes are typical ISGs [27]. This expression characteristic correlates with the pivotal roles of these TRIMs in innate IFN antiviral response, acting directly as antiviral effectors [28,29], indirectly as modulator of IFN response [30–32], or as both [33]. In the past years, emerging studies have shown that fish TRIM genes are involved in fish IFN antiviral response [34–36], although the molecular mechanisms involved remain largely known. In fish, besides the existence of orthologs of human TRIM genes, there is a fish-specific TRIM subgroup, named finTRIM or FTR [20]. Recent studies have shown that FTR members function as modulators during fish IFN antiviral response [21,37,38]. Interestingly, zebrafish FTR83 is not an ISG and still potentiates antiviral response by upregulation of IFN and ISGs [37], indicating that the IFN induction feature is not a prerequisite for TRIM function during IFN response.

Notably, there is greatly varied gene number of finTRIM family among fish species, indicating that genus-specific or even species-specific gene expansion of TRIM family has happened during teleost fish radiation [20,21,37,39]. Similar phenomenon occurs in mammals. For example, mouse and human genomes have species-specific TRIM genes

[40], some of which are even specific to different human populations [41], indicating a constant and on-going expansion of TRIM genes. Such fast expansion benefits to serve as a TRIM gene reservoir, allowing a given species to easily acquire new antiviral genes towards selective pressures from viral infection [42]. FTRCA1 might be crucian carp-specific member of finTRIM, because so far no “one to one” orthologue has been found in other fish species, even in gibel carp (*C. auratus gibelio*), a species belonging to a same genus *Carassius* with crucian carp (*C. auratus*) [21]. FTRCA1 is upregulated by IFN and virus infection and importantly, it downregulates RLR-triggered IFN response through lysosomal degradation of TBK1 [21]. In the present study, we extended this notion and found that FTRCA1 exhibits an ability to reduce IRF7 transcription levels, thereby downregulating IRF7-mediated IFN response. Since IRF7 is a master transcription factor involved in RLR-IFN signaling, these results indicate that FTRCA1 targets at least two substrates TBK1 and IRF7 to negatively modulate IFN response.

Although overexpression of IRF7 and FTRCA1 together results in a reduced IRF7 protein level compared to overexpression of IRF7 alone, the reduction of IRF7 protein should be due to the attenuated IRF7 transcription level but not to IRF7 protein degradation (Figs. 2A and 3). This is very different from FTRCA1 targeting TBK1 protein for degradation, because TBK1 transcription is not changed by similar co-transfection assays [21]. In addition, although both IRF7 and TBK1

proteins are reduced in FTRCA1-overexpressing cells, TBK1 protein reduction is blocked by Chloroquine and NH₄Cl (inhibitors of autophagy-lysosome-dependent degradation pathway) [21] and on the contrary, neither MG132 (inhibitor of ubiquitin-proteasome-dependent degradation pathway) nor Chloroquine and NH₄Cl can rescue IRF7 protein levels (Fig. 3). These results together strongly indicate that FTRCA1 promotes TBK1 protein degradation through lysosomal-dependent pathway and instead, it destabilizes IRF7 transcription resulting in a decreased protein level. Finally, FTRCA1 interacts with TBK1 but not with IRF7 [21], strengthening the notion that FTRCA1 cannot target IRF7 protein for degradation. It is notable that despite of different mechanisms used for TBK1 and IRF7, E3 ligase activity is required for FTRCA1 function (Figs. 4 and 5).

An interesting question is how FTRCA1 attenuates IRF7 transcription level. In the present study, both plasmids expression IRF7 and FTRCA1 are transfected into fish cells, and the transcription levels from either the transfected plasmid IRF7 or both the plasmid IRF7 and cellular IRF7 gene are decreased (Figs. 2 and 4B). Since the transcription of the plasmid IRF7 is driven by a constitutive promoter CMV that is located in the commercial vector pcDNA3.1; therefore, the observed reduction of IRF7 transcription should be resulted from an enhanced decay of IRF7 mRNA by FTRCA1 at posttranscriptional step, and the targeted site for IRF7 mRNA decay is probably in open reading frame (ORF) of IRF7 gene. It is well known that regulation of mRNA decay in the cytoplasm is tightly controlled by sequence-specific RNA binding proteins (RBPs), which often bind to the sequences of targeted mRNA in either the 5'UTR, ORF, or more commonly the 3' UTR dependent on conditions [43]. Based on these findings, we speculate that FTRCA1 attenuates IRF7 transcription levels likely due to enhanced decay of IRF7 mRNA, which directly leads to reduced IRF7 protein levels and subsequently reduced fish IFN expression, thus downregulating IFN antiviral response.

Another interesting question is how FTRCA1 selects IRF7 rather than IRF3 to downregulate IFN response. IRF3 and IRF7 are two crucial transcription factors responsible for induction of different IFN genes and they are very homologous in amino acid sequences. Our previous results have excluded the possibility that FTRCA1 has regulatory selectivity on distinct fish IFN gene expression through IRF7 and IRF3, respectively [21]. In the present study, although a reduced IRF3 transcription level is detected in FTRCA1-overexpressing cells, the time point for initial detection of transcription reduction is later than IRF7 but similar to IFN (Figs. 2 and 4B). Since both fish IRF3 and IRF7 are ISGs, the observed downregulation of IRF3 gene transcription is likely due to the attenuated IFN expression by FTRCA1. However, it is still hard to understand why FTRCA1 interacts with IRF3 but not with IRF7 [21]. Regardless of these unresolved issues, the data in the current study support that FTRCA1 is a multifaceted modulator to target different signaling factors for shaping fish IFN response, because at least two substrates, fish TBK1 and IRF7, have been confirmed as targets of FTRCA1.

Acknowledgments

This work was supported by grants from the National Key R&D Program of China (2018YFD0900302), the National Natural Science Foundation of China (31572646 and 31772875), and the Freshwater Ecology and Biotechnology Laboratory (2019FBZ04).

References

- [1] M. Schlee, G. Hartmann, Discriminating self from non-self in nucleic acid sensing, *Nat. Rev. Immunol.* 16 (2016) 566–580.
- [2] A. Zevini, D. O'Laughlin, J. Hiscott, Crosstalk between cytoplasmic RIG-I and STING sensing pathways, *Trends Immunol.* 38 (2017) 194–205.
- [3] J. Hiscott, Triggering the innate antiviral response through IRF-3 activation, *J. Biol. Chem.* 282 (2007) 15325–15329.
- [4] A. Iwasaki, E.F. Foxman, R.D. Molony, Early local immune defences in the respiratory tract, *Nat. Rev. Immunol.* 17 (2017) 7–20.
- [5] K. Honda, H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, et al., IRF-7 is the master regulator of type-I interferon-dependent immune responses, *Nature* 434 (2005) 772–777.
- [6] S. Sharma, B.R. tenOever, N. Grandvaux, G.P. Zhou, R. Lin, J. Hiscott, Triggering the interferon antiviral response through an IKK-related pathway, *Science* 300 (2003) 1148–1151.
- [7] B. Hatesuer, H.T. Hoang, P. Riese, S. Trittel, I. Gerhauser, H. Elbahesh, et al., Deletion of *Irf3* and *Irf7* genes in mice results in altered interferon pathway activation and granulocyte-dominated inflammatory responses to influenza infection, *J. Innate Immun.* 9 (2017) 145–161.
- [8] Y.B. Zhang, J.F. Gui, Molecular regulation of interferon antiviral response in fish, *Dev. Comp. Immunol.* 38 (2012) 193–202.
- [9] P. Boudinot, C. Langevin, C.J. Secombes, J.P. Levrault, The peculiar characteristics of fish type I interferons, *Viruses* (2016) 8.
- [10] F. Sun, Y.B. Zhang, T.K. Liu, L. Gan, F.F. Yu, Y. Liu, et al., Characterization of fish IRF3 as an IFN-inducible protein reveals evolving regulation of IFN response in vertebrates, *J. Immunol.* 185 (2010) 7573–7582.
- [11] F. Sun, Y.B. Zhang, T.K. Liu, J. Shi, B. Wang, J.F. Gui, Fish MITA serves as a mediator for distinct fish IFN gene activation dependent on IRF3 or IRF7, *J. Immunol.* 187 (2011) 2531–2539.
- [12] H. Feng, Q.M. Zhang, Y.B. Zhang, Z. Li, J. Zhang, Y.W. Xiong, et al., Zebrafish IRF1, IRF3, and IRF7 differentially regulate IFN β 1 and IFN β 3 expression through assembly of homo- or heteroprotein complexes, *J. Immunol.* 197 (2016) 1893–1904.
- [13] Y. Ding, J. Ao, X. Huang, X. Chen, Identification of two subgroups of type I IFNs in perciforme fish large yellow croaker *larimichthys crocea* provides novel insights into function and regulation of fish type I IFNs, *Front. Immunol.* 7 (2016) 343.
- [14] M. van Gent, K.M.J. Sparrer, M.U. Gack, TRIM proteins and their roles in antiviral host defenses, *Annu. Rev. Virol.* 5 (2018) 385–405.
- [15] G.A. Versteeg, R. Rajsbaum, M.T. Sanchez-Aparicio, A.M. Maestre, J. Valdiviezo, M. Shi, et al., The E3-ligase TRIM family of proteins regulates signaling pathways triggered by innate immune pattern-recognition receptors, *Immunity* 38 (2013) 384–398.
- [16] J. Yan, Q. Li, A.P. Mao, M.M. Hu, H.B. Shu, TRIM4 modulates type I interferon induction and cellular antiviral response by targeting RIG-I for K63-linked ubiquitination, *J. Mol. Cell Biol.* 6 (2014) 154–163.
- [17] C. Zhao, M. Jia, H. Song, Z. Yu, W. Wang, Q. Li, et al., The E3 ubiquitin ligase TRIM40 attenuates antiviral immune responses by targeting MDA5 and RIG-I, *Cell Rep.* 21 (2017) 1613–1623.
- [18] R. Rajsbaum, A. Garcia-Sastre, G.A. Versteeg, TRIM immunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity, *J. Mol. Biol.* 426 (2014) 1265–1284.
- [19] K. Ozato, D.M. Shin, T.H. Chang, H.C. Morse, TRIM family proteins and their emerging roles in innate immunity, *Nat. Rev. Immunol.* 8 (2008) 849–860.
- [20] L.M. van der Aa, J.P. Levrault, M. Yahmi, E. Laurent, V. Briolat, P. Herbolme, et al., A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish, *BMC Biol.* 7 (2009) 7.
- [21] M. Wu, X. Zhao, X.Y. Gong, Y. Wang, J.F. Gui, Y.B. Zhang, FTRCA1, a species-specific member of finTRIM family, negatively regulates fish IFN response through autophagy-lysosomal degradation of TBK1, *J. Immunol.* 202 (8) (2019) 2407–2420.
- [22] T.K. Liu, Y.B. Zhang, Y. Liu, F. Sun, J.F. Gui, Cooperative roles of fish protein kinase containing Z-DNA binding domains and double-stranded RNA-dependent protein kinase in interferon-mediated antiviral response, *J. Virol.* 85 (2011) 12769–12780.
- [23] H. Feng, Y.B. Zhang, Q.M. Zhang, Z. Li, Q.Y. Zhang, J.F. Gui, Zebrafish IRF1 regulates IFN antiviral response through binding to IFN β 1 and IFN β 3 promoters downstream of MyD88 signaling, *J. Immunol.* 194 (2015) 1225–1238.
- [24] Q.M. Zhang, X. Zhao, Z. Li, M. Wu, J.F. Gui, Y.B. Zhang, Alternative splicing transcripts of zebrafish LGP2 gene differentially contribute to IFN antiviral response, *J. Immunol.* 200 (2018) 688–703.
- [25] I. Dikic, Proteasomal and autophagic degradation systems, *Annu. Rev. Biochem.* 86 (2017) 193–224.
- [26] R. Rajsbaum, J.P. Stoye, A. O'Garra, Type I interferon-dependent and -independent expression of tripartite motif proteins in immune cells, *Eur. J. Immunol.* 38 (2008) 619–630.
- [27] K. Asaoka, K. Ikeda, T. Hishinuma, K. Horie-Inoue, S. Takeda, S. Inoue, A retrovirus restriction factor TRIM5 α is transcriptionally regulated by interferons, *Biochem. Biophys. Res. Commun.* 338 (2005) 1950–1956.
- [28] S.D. Barr, J.R. Smiley, F.D. Bushman, The interferon response inhibits HIV particle production by induction of TRIM22, *PLoS Pathog.* 4 (2008) e1000007.
- [29] K. Wang, C. Zou, X. Wang, C. Huang, T. Feng, W. Pan, et al., Interferon-stimulated TRIM69 interrupts dengue virus replication by ubiquitinating viral nonstructural protein 3, *PLoS Pathog.* 14 (2018) e1007287.
- [30] W. Zeng, L. Sun, X. Jiang, X. Chen, F. Hou, A. Adhikari, et al., Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity, *Cell* 141 (2010) 315–330.
- [31] P. Wang, W. Zhao, K. Zhao, L. Zhang, C.J. Gao, TRIM26 negatively regulates interferon-beta production and antiviral response through polyubiquitination and degradation of nuclear IRF3, *PLoS Pathog.* 11 (2015).
- [32] Y. Ran, J. Zhang, L.L. Liu, Z.Y. Pan, Y. Nie, H.Y. Zhang, et al., Autoubiquitination of TRIM26 links TBK1 to NEMO in RLR-mediated innate antiviral immune response, *J. Mol. Cell Biol.* 8 (2016) 31–43.
- [33] T. Pertel, S. Hausmann, D. Morger, S. Zuger, J. Guerra, J. Lascano, et al., TRIM5 is an innate immune sensor for the retrovirus capsid lattice, *Nature* 472 (2011) 361–365.
- [34] Y.H. Huang, M. Yang, Y.P. Yu, Y. Yang, L.L. Zhou, X.H. Huang, et al., Grouper

- TRIM13 exerts negative regulation of antiviral immune response against nodavirus, *Fish Shellfish Immunol.* 55 (2016) 106–115.
- [35] W. Wang, Y. Huang, Y. Yu, Y. Yang, M. Xu, X. Chen, et al., Fish TRIM39 regulates cell cycle progression and exerts its antiviral function against iridovirus and nodavirus, *Fish Shellfish Immunol.* 50 (2016) 1–10.
- [36] J. Kim, J.W. Kim, D.G. Kim, B.H. Nam, Y.O. Kim, J.Y. Park, et al., Molecular characterization of *Rhodeus uyekii* tripartite motif protein 1 (TRIM1) involved in IFN-gamma/LPS-induced NF-kappaB signaling, *Fish Shellfish Immunol.* 79 (2018) 42–51.
- [37] C. Langevin, E. Aleksejeva, A. Houel, V. Briolat, C. Torhy, A. Lunazzi, et al., FTR83, a member of the large fish-specific finTRIM family, triggers IFN pathway and counters viral infection, *Front. Immunol.* 8 (2017) 617.
- [38] B. Chen, S. Huo, W. Liu, F. Wang, Y. Lu, Z. Xu, et al., Fish-specific finTRIM FTR36 triggers IFN pathway and mediates inhibition of viral replication, *Fish Shellfish Immunol.* 84 (2019) 876–884.
- [39] P. Boudinot, L.M. van der Aa, L. Jouneau, L. Du Pasquier, P. Pontarotti, V. Briolat, et al., Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish, *PLoS One* 6 (2011) e22022.
- [40] M. Sardiello, S. Cairo, B. Fontanella, A. Ballabio, G. Meroni, Genomic analysis of the TRIM family reveals two groups of genes with distinct evolutionary properties, *BMC Evol. Biol.* 8 (2008) 225.
- [41] K. Han, D.I. Lou, S.L. Sawyer, Identification of a genomic reservoir for new TRIM genes in primate genomes, *PLoS Genet.* 7 (2011) e1002388.
- [42] Q.Y. Zhang, J.F. Gui, Diversity, evolutionary contribution and ecological roles of aquatic viruses, *Sci. China Life Sci.* 61 (2018) 1486–1502.
- [43] K.A. Braun, E.T. Young, Coupling mRNA synthesis and decay, *Mol. Cell Biol.* 34 (2014) 4078–4087.