Fish and Shellfish Immunology 84 (2019) 876–884

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Fish-specific finTRIM FTR36 triggers IFN pathway and mediates inhibition of viral replication

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ARTICLE INFO

Keywords:
FTR36
finTRIM
Antiviral
Interferon

ABSTRACT

The tripartite motif (TRIM) family involves many cellular processes, including fundamental functions in antiviral immunity. Antiviral activities of TRIMs are reported in a variety of patterns, and one of the most significant channels is related to the activation of the type-I interferon (IFN) pathway. In this study, we described a *fintrim (ftr)* gene named *ftr36*, which is mainly expressed in the gills, skin, and intestines. This study shows that *ftr36* encodes a protein affording a potent antiviral effect. *In vitro*, overexpression of FTR36 mediated an upregulated pattern of recognition receptor retinoic acid–inducible gene I (RIG-I), interferon regulatory factor 3/7(IRF3/7), IFN, and IFN-stimulated genes (ISGs) expression. Thereby, FTR36 expression could afford host defense against the spring viremia of carp virus (SVCV) and the giant salamander iridovirus (GSIV). With the deletion of the RING domain or B30.2 domain separately, the antiviral ability of FTR36 was abolished partially and almost lost its ability to activate the IFN-pathway. These findings indicate that both RING and B30.2 domains are indispensable for the antiviral activity of FTR36. Altogether, this study described a finTRIM FTR36, which can activate IFN-pathways and stimulate ISGs to provide host defense against viral infections.

1. Introduction

Natural immunity is an essential host defense against pathogenic infections. Activation of natural immune signal transduction pathways in cells are regulated by various transcriptional or translational modifications, as well as post-translational regulations including phosphorylation, acetylation, and ubiquitination. TRIM proteins were reported to be involved in many cellular processes, such as developmental processes, tumor suppression, and cell cycle regulation [1]. Some TRIM proteins are associated with antiviral defense via different mechanisms, often induced by type-I IFN or in response to viral infection as modulators and enhancers [2]. In mammals, the earliest studies showed that TRIM5α was related to natural immunity through targeting retroviruses and lentiviruses. The PRY/SPRY domain of TRIM5α is directly related to the core protein nucleocapsid of the virus, causing the degradation of the virus protein. TRIM5α is also known to activate the transcription factor nuclear factor-kappa B (NF-κB) signaling pathway by activating TAK1, thus inhibiting the virus infection [3]. Some TRIM proteins can be targeted by the viruses themselves, enabling them to evade the surveillance of the host immune system. TRIM25 can interact with the nonstructural protein NS1 of the influenza virus and inhibit the ubiquitination of RIG-I K63 chain, thereby inhibiting the generation of IFN mediated by RIG-I [4]. TRIM25 is also able to inhibit p53's transcriptional activity and dampen its response to DNA damage [5]. TRIM52 was also reported to interact with JEV nonstructural protein 2A (NS2A). This interaction degrades NS2A in a proteasome-dependent manner via E3 ligase activity of TRIM52 [6]. Polyubiquitination of the K63 ubiquitin chain of the NS5 protein, mediated by TRIM23, also inhibits signal transduction and activation of transcription 2 (STAT2), indicating that the yellow fever virus also uses the natural immune system for its replication and survival [3].

The TRIM protein family contains a RING finger domain, one or two B-box domains and a coiled-coil (CC) region, as well as a highly variable C-terminus such as the PRY/SPRY domain, also known as the B30.2 domain or the NHL domain [7,8]. In mammals, the E3 ubiquitin ligase activity of the RING domain of TRIM5α contributes to the potency of virus restriction [9]. The coiled-coil domain of human TRIM40 combines with RIG-I and MDA5 through their CARD domain [10].

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https://doi.org/10.1016/j.fsi.2018.10.051
Received 16 July 2018; Received in revised form 15 October 2018; Accepted 22 October 2018
Available online 23 October 2018

Moreover, the B30.2 domain of human TRIM21 is critical for the modulation of IRF3 function [11].

FinTRIM is a large new subfamily of TRIMs in teleosts, formed of nearly identical RING/B-box regions and C-termini of variable length. The long variants include a B30.2 domain, which was identified in rainbow trout as virus-induced transcripts [12]. From several latest studies about finTRIMs, many finTRIM genes were predominantly expressed in the spleen, gill, and head kidney. These results indicate that the grass carp finTRIM genes are involved in diverse cellular processes, including innate immune responses [13]. A recent study has demonstrated the probable important roles of *ftr12*, *ftr51*, *ftr67*, *ftr82*, *ftr83*, and *ftr84* in innate immune responses and nonimmunity-related tissues in zebrafish [14]. FTR83 induced IFN and IFN-stimulated gene expression afforded protection against different enveloped and non-enveloped RNA viruses [15].

Spring viremia of carp (SVC) is a serious disease responsible for high mortality caused by spring viremia of carp virus (SVCV), which is a member of the genus *Sprivivirus* belonging to *Rhabdoviridae* family. SVCV contains a linear, negative-sense, ssRNA genome, which encodes five viral proteins and causes severe losses in aquaculture [16–18]. Giant salamander iridovirus (GSIV), a dsDNA virus, belongs to genus *Ranavirus* in *Iridovirus* family and is so far the only viral pathogens found in the giant salamander. The disease is widely prevalent throughout the country and causing major economic losses [19]. In this study, we described a finTRIM gene *ftr36*, which could inhibit viral replication and positively regulate the IFN pathway. This work proved that antiviral function is one of the characteristics of finTRIMs and might provide new insight for exploring the fundamental functions of the TRIM family in terms of antiviral effect in future.

2. Meterials and methods

2.1. Ethics statement

All experimental animals were handled in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal infection tests did not involve any endangered or protected species. Experiments using zebrafish were performed under the approval of the Animal Ethics Committee of Huazhong Agriculture University (HZAU). The infection and dissection experiments were performed under 3-Aminobenzoic acid ethyl ester methane sulfonate (MS-222) (Sigma, USA) anesthesia to minimize fish suffering.

2.2. Fish cell lines, virus and zebrafish

The fat head minnow (FHM) cell (ATCC® CCL-42™) line and the epithelioma papulosum cyprini (EPC) cell (ATCC® CRL-2872™) line were maintained at 28 °C in 90% Medium 199 (M199, Hyclone, USA) or MEM (Hyclone, USA) with 10% fetal bovine serum (FBS, Gibco, Australia). The zebrafish embryonic fibrablast cell (ATCC® CRL-2050™) line were maintained in 90% DMEM/F-12 (DMEM, Hyclone, USA) with 10% fetal bovine serum (FBS, Gibco, Australia) at 28 °C. The SVCV (ATCC: VR-1390) was propagated in FHM cells until virus-induced cytopathic effects (CPE) appeared at 28 °C. The GSIV, isolated from diseased andrias davidianus, was propagated in EPC cells until the CPE appeared at 25 °C. The cell samples were then frozen and thawed. The supernatant was collected and stored at −80 °C after centrifugation at 10000*g*. Adult wild-type (WT) AB strain male zebrafish were purchased from the China Zebrafish Resource Centre (CZRC) to ensure a clear genetic background and no pathogenic infection.

2.3. In vivo assay

After acclimating to low temperature (17 °C) for 7 days (d), the experimental zebrafish were randomly divided into two groups: SVCV-infected and non-SVCV-infected groups. For the virus-infected group, each fish was intraperitoneally inoculated with 10 μL of SVCV at the dose of 10^5 PFU/ml. The injection experiments were performed using anesthesia by MS-222. The experimental zebrafish were continuously observed 2–3 times a day. No treatment was performed on the healthy group. All the zebrafish still left from the infected and healthy groups were killed using an over-dosage of MS-222 after 0, 1, 2, 3 and 5 days. Three zebrafish tissues were collected and pooled together as one sample, were snap-frozen in liquid nitrogen, and stored at −80 °C for RNA extraction.

2.4. Sequence analysis

The CDs of zebrafish *ftr36* was 1674bp in length and encoded 557 amino acids. The alignment was generated using ClustalX2 and presented using ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The phylogenetic tree was constructed based on the amino acid sequences of FTR36 using the Neighbor-joining (NJ) algorithm (MEGA version 5.1).

2.5. RNA extraction, reverse transcription, and quantitative real-time PCR

The brain, spleen, liver, kidney, heart, skin, gill, eye, muscle, and intestine tissues of 3 healthy or infected zebrafish were collected and stored at −80 °C with TRIzol reagent (TAKARA) for RNA isolation. Total RNA was extracted according to the manufacturer's instruction. The reverse transcription was carried out using the ReverTra Ace qPCR RT kit (TAKARA). The relative expression of each cDNA was determined by absolute quantitative real-time PCR using SYBR Green Realtime PCR Master Mix (TAKARA). Fluorescent signals were analyzed by a Light Cycler/Light Cycler 480 System (Roche). PCR conditions were as follows: 95 °C for 5 min, then 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. All primers for qPCR were shown in Table 1. The relative fold changes were calculated by comparing them to the corresponding controls using the $2^{-\Delta\Delta CT}$ (where CT is threshold cycle) method. Relative mRNA level was normalized to vector in Fig. 5. Three independent experiments were conducted for statistical analysis.

2.6. Plasmid conduction

The finTRIM family member 36 (*ftr36*) was cloned in fusion with a HIS tag in pcDNA4. The full length CDs of *ftr36* were amplified using primers of *ftr36*-F and *ftr36*-R, *Kpn* I and *Xho* I were added at the primers, and the RING or B30.2 domain deleting fragment of *ftr36* was amplified using primers *ftr36*-ΔRING/ΔB30.2-F and *ftr36*-ΔRING/ΔB30.2-R.

2.7. Plasmid transfection, virus infection and immune fluorescence

The recombinant plasmid was transfected into FHM cells using the FuGENE HD (Roch, Germany) transfection reagent when the cells were approximately 70%–80% confluent, according to the manufacturer's protocol. For the antiviral assay, FHM cells seeded in 6-well plates were transfected with 2 μg pcDNA4-*ftr36*-HIS or the empty plasmid (vector). At 24 h post transfection (p.t.), cells were infected with SVCV at MOI 0.05, cells were infected at 28 °C for 1 h, then washed with PBS for three times. After 6 h, 12 h, 24 h, and 36 h post infection (p.i.), aliquots of the supernatant were harvested for determination of virus titers, and cell monolayers were harvested for qPCR or western blot. For immune fluorescence, after 24 h p.t., cells were washed with PBS two times, and then fixed with methanol for 10 min. They were then incubated with mouse anti-His antibodies (ABclonal, China, 1:3000 dilution) for 2 h, followed by FITC-conjugated goat anti-mouse antibodies (ABclonal, China, 1:2000 dilution) for 45 min. Afterwards, the cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) and observed under laser scanning confocal fluorescence microscopy (Leica, Germany) or

Table 1
Primers of this study.

Application	Prime Name	Sequence (5′–3′)
qPCR	TBP-F	TTACCCACCAGCAGTTTAG
qPCR	TBP-R	ACCTTGGCACCTGTGAGTA
qPCR	qftr36-F	GATTGTTGGTGGCAGGTG
qPCR	qftr36-R	GACGGAGGGTTTAGGGAG
qPCR	SVCV-G-F	CGACCTGGATTAGACTTG
qPCR	SVCV-G-R	AATGTTCCGTTTCTCACT
qPCR	SVCV-M-F	TACTCCTCCCACTTACGA
qPCR	SVCV-M-R	CAAGAGTCCGAGAAGGTC
qPCR	SVCV-N-F	GCGGTTTTCTGTATGTGTCTC
qPCR	SVCV-N-R	CTCTGCCAAATCACCATACTC
qPCR	qIRF3-F	GTTTAGAGGGACAATTAACTGGACTA
qPCR	qIRF3-R	GAGGGTCCACTCTTTGAAAATG
qPCR	qIRF7-F	CCATTCATTGCTGACATCTACAGT
qPCR	qIRF7-R	GTTCGTCTCAAAGTTGCTCCTC
qPCR	qIFN1-F	AACGCAGCACAATGGAAC
qPCR	qIFN1-R	TGATGGATGGTGGTATCG
qPCR	qISG15-F	TAATGCCACAGTCGGTGAA
qPCR	qISG15-R	AGGTCCAGTGTTAGTGATGAGC
qPCR	qMX1-F	ATCTGGTGGATAAGGGAAC
qPCR	qMX1-R	CATCCTCTGTTAATGTGGC
qPCR	qPKR-F	ACCTGAAGCCTCCAAACATA
qPCR	qPKR-R	GCATTCGCTCATCATTGTC
qPCR	qViperin-F	GCAAAGCGAGGGTTACGAC
qPCR	qViperin -R	CTGCCATTACTAACGATGCTGAC
qPCR	qRIG-I-F	TGCTGGACCGGATGTGTTATCT
qPCR	qRIG-I-R	TGGTGATCGATGGTTCGATTCT
qPCR	qGSIV-MCP-F	GACTTGGCCACTTATGAC
qPCR	qGSIV-MCP-R	GTCTCTGGAGAAGAAGAA
qPCR	qNF-κB P65-F	GGCAGGTGGCGATAGTGTT
qPCR	qNF-κB P65-R	CATTCCTTCAGTTCTCTTGCG
qPCR	qAP-1-F	GGATTAAAGCCGAGAGGAAGC
qPCR	qAP-1-R	GTTCTGCGACTTTAGGTTCTTG
Plasmid construction	ftr36-F	CGGGGTACCCCGATGGCAGAATCCAGTCTTTC
Plasmid construction	ftr36-R	CCGCTCGAGCGGCACTGTCAGATCACAGAGCT
Plasmid construction	ftr36-ΔRING	CCGCTCGAGCGGAATGATGCTAATGCTGCTCACTCT
Plasmid construction	ftr36-ΔB30.2	CGGGGTACCCCGATGCCCGCTCCTGAACCACA

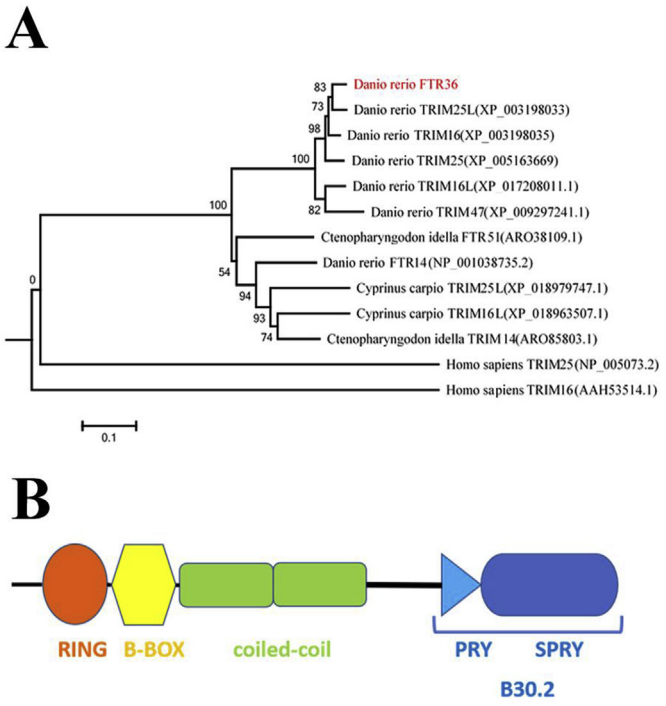

Fig. 1. FTR36 is a member of the finTRIM family in teleost fish. (A) Neighbor-joining tree of FTR36 and the most similar homologs in several fish species, and some similar homologs in fish and human. (B) Schematic structure of FTR36 domains.

fluorescence microscopy (Leica, Germany).

2.8. Western blot assay

For western blotting assays, cells (10^6) were treated with RIPA lysis buffer (Beyotime, China), and subjected to SDS-PAGE. The samples were transferred onto polyvinylidene fluoride membrane (PVDF) (Bio-Rad, USA). After blocking in 2% albumin from bovine serum (BSA) at room temperature for 1 h, the membranes were incubated with mouse anti-HIS antibodies (ABclonal, China, 1:1000 dilution) or mouse anti-SVCV-M (Laboratory preparation and preservation) for 2 h [20]. Rabbit anti-β-actin antibodies (ABclonal, China, 1:10000 dilution) were used as internal control. After washing thrice with TBST, the membranes were incubated with Horseradish peroxidase (HRP) conjugated secondary antibodies, including goat anti-mouse and anti-rabbit IgG (ABclonal, China, 1:2000 dilution) for 45 min, and then the results were detected using chemical luminescence substrate (General Electric, USA) with Amersham Imager 600 (GE, USA).

2.9. Plaque formation assay

The SVCV titer was determined by plaque assay. FHM cells were seeded in 12-well plates. When the cells grew to 100% confluence, the recovered virus was 10-fold diluted and then used to infect the monolayers. After 1 h of virus adsorption, the infection medium was removed, and infected FHM cells were then overlaid with M199 culture medium containing 5% FBS and 1.5% carboxymethyl cellulose (CMC, Sigma-Aldrich) at 28 °C. At 60 h p.i., cells were fixed with 10% formaldehyde overnight (10–12 h) and stained with 0.5% crystal violet for 3 h. After washing with tap water, visible plaques were counted and

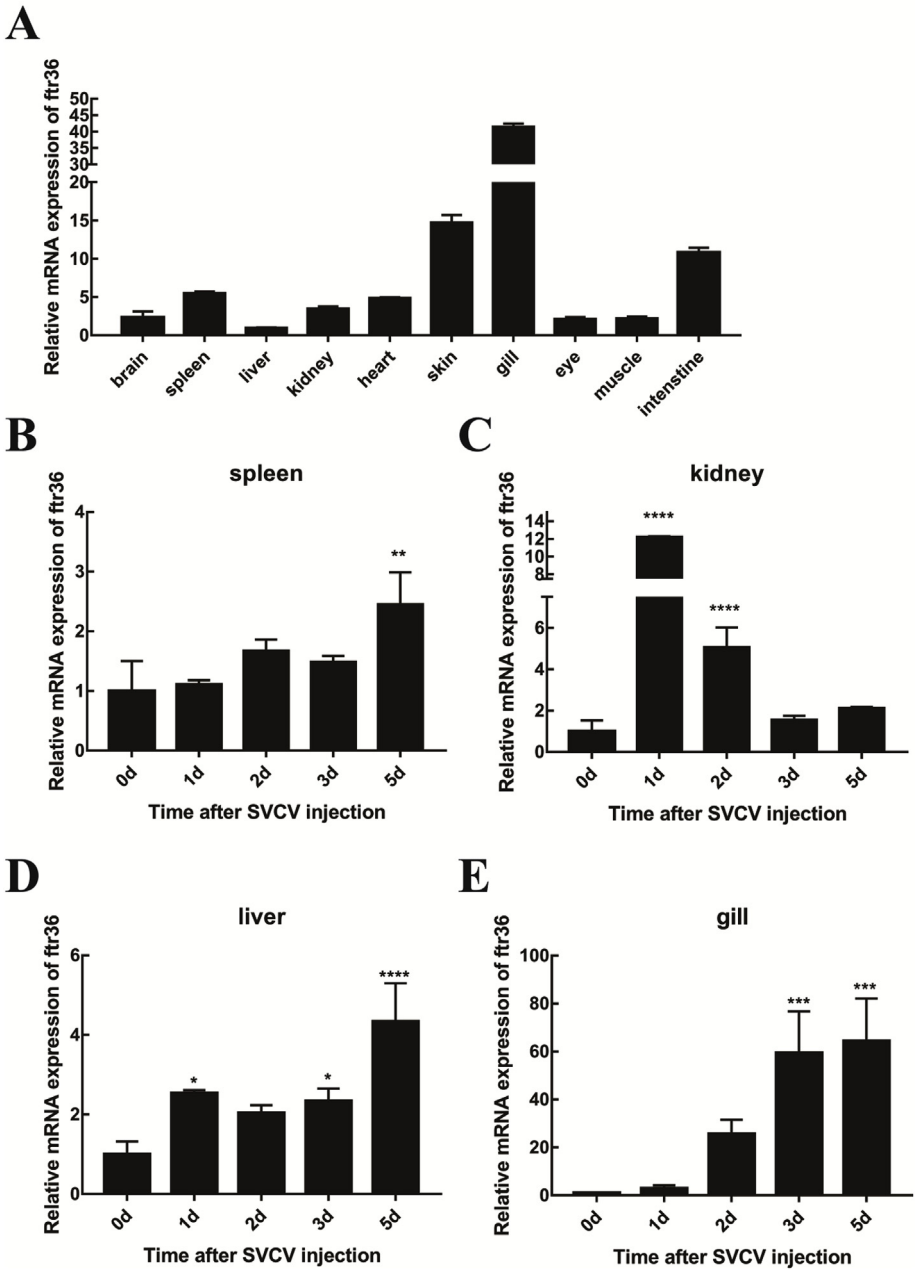

Fig. 2. The expression pattern of *ftr36* mRNA in test and control (healthy) groups. (A)The relative expression level of *ftr36* in different tissues of healthy fish. (B, C, D, and E) The expression level of *ftr36* in tissues spleen, kidney, liver, and gill detected by qRT-PCR. Statistical analyses were performed using a two-way ANOVA test. Error bars are the SDs in triplicate. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

viral titers were calculated. All data were expressed as the mean of triplicated samples.

2.10. Statistics analysis

Data was expressed as means ± standard deviations (SDs) of results from three independent experiments. The significance of the variability between different treatment groups was determined by two-way ANOVA tests of variance. A p value of < 0.05 was considered statistically significant and marked with *, $p < 0.01$ was marked with **, $p < 0.001$ was marked with *** and $p < 0.0001$ was marked with ****. All statistical analyses and calculations were done using GraphPad Prism 7.0 (GraphPad Software Inc, USA).

3. Results

3.1. ftr36 is a member of the large fish finTRIM family

Phylogenetic analysis of TRIM sequences from different fishes shows that zebrafish (*Danio rerio*) *ftr36* sequences cluster with zebrafish *trim25* and *trim16*, distinguished from human (*Homo sapiens*) *trim25* and *trim16*. Comparative analysis has revealed that *ftr36* does not share highly homologous sequences in humans, but shares paralogues among zebrafish, grass carp (*Ctenopharyngodon idella*), and common carp (*Cyprinus carpio*) (Fig. 1A). These results indicated that *ftr36* is a novel molecule in the finTRIMs family. The TRIM protein family contains a RING domain followed by one or two B-box domains, and a predicted coiled-coil (CC) region, as well as a highly variable C-terminus such as the B30.2 domain. Also, *ftr36* has the typical domain structure of the

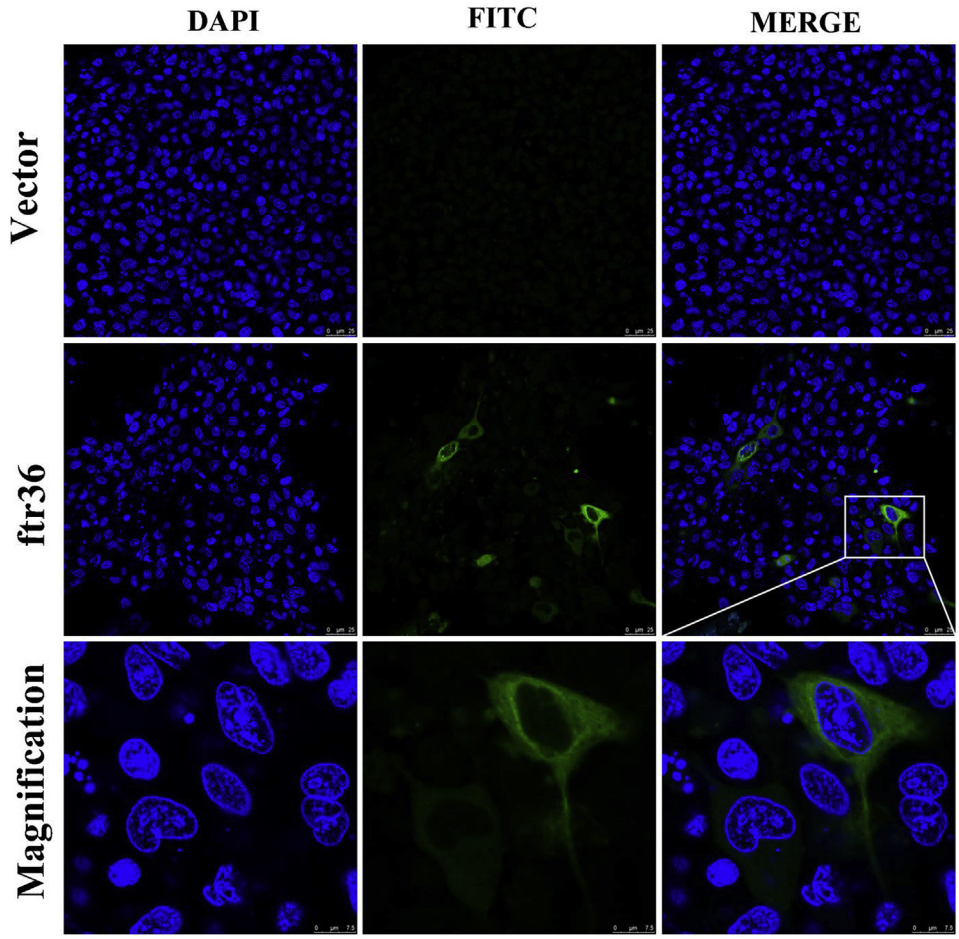

Fig. 3. Subcellular localization of FTR36 in FHM cells. FHM cells were transfected with *ftr36* or vector plasmids. After 36 h p.t., subcellular localization of FTR36 was examined using immune fluorescence assay. Cell nuclei were stained with DAPI, samples were observed under the laser scanning confocal microscope.

finTRIM family containing a RING/B-Box/Coiled coil and a typical B30.2 domain. This study shows that *ftr36* has two coiled-coil domains (Fig. 1B), which makes it different from other *trim* members. Zebrafish FTR36 protein sequences are 93% similar to zebrafish TRIM25, 93% similar to zebrafish TRIM16, 70% similar to zebrafish FTR14, and 65%–70% similar to common carp and grass carp TRIM proteins, but only 30%–31% similar to human TRIM25 and TRIM16 (supplementary materials Fig. 1).

3.2. Inducible expression pattern of ftr36 in vivo

To determine the relative expression pattern of *ftr36 in vivo,* different tissues of zebrafish were evaluated using qRT-PCR. The relative expression level of *ftr36* was assessed in brain, spleen, liver, kidney, heart, skin, gill, eye, muscle, and intestine. The data showed that *ftr36* is distributed mainly in the gills, skin, intestine, and spleen (Fig. 2A), indicating that *ftr36* may primarily play a significant role in innate immunity. To further investigate the inducible expression level of *ftr36* in tissues, the kidney, spleen, liver, and gill from the zebrafish infected with SVCV were sampled for qRT-PCR. All tissues could detect the virus infection, and the amount of SVCV increased gradually and significantly increased ($p < 0.0001$) after 5 days p.t. (supplementary materials Fig. 2). In the spleen, *ftr36* was significantly induced ($p < 0.01$) by SVCV after 5 days p.i. (Fig. 2B). In the kidney, *ftr36* was significantly up-regulated at day 1 ($p < 0.0001$) and 2 p.i. ($p < 0.0001$) (Fig. 2C). In the liver, *ftr36* was also induced at day 1 p.i. ($p < 0.05$), day 3 p.i. ($p < 0.05$) and day 5 p.i. ($p < 0.0001$) with the highest level of *ftr36* expression on day 5 p.i. (Fig. 2D). In gill tissue, the mRNA expression of *ftr36* was up-regulated gradually with SVCV infection, significantly induced at day 3 p.i. ($p < 0.001$) and reached a peak at day 5 p.i. ($p < 0.001$) (Fig. 2E). *ftr36* differential expression occurred in different tissues, indicating that *ftr36* might exert different levels of functionality in the progression of anti-SVCV infection.

3.3. The subcellular localization of FTR36

To clarify the subcellular localization of FTR36, the recombinant plasmid pcDNA4-*ftr36*-HIS and empty vector pcDNA4 were transfected into FHM cells in plates. Following 36 h p.t., cells were fixed with methanol, which was followed by the immune fluorescence process. Then, the treated samples were observed with a confocal microscope. The subcellular localization showed that the green fluorescence of FTR36 was evenly distributed in the cytoplasm around the nucleus. In the empty vector group of control, no obvious fluorescence could be detected (Fig. 3). Similarly, the subcellular location of FTR36 also distributed in cytoplasm in ZF4 cells (supplement materials Fig. 3).

3.4. FTR36 affords protection against viral infection

To investigate whether FTR36 has the ability to defend against viral infection, we detected the impact of FTR36 overexpression in EPC cells to clarify the susceptibility of two kinds of viral infection by SVCV and GSIV.

In this test, *ftr36* was transfected in EPC cells. After 24 h p.t., cells were infected with SVCV at MOI of 0.05 or GSIV at MOI of 1.0. Samples were collected after 24 h p.i. and empty plasmid were used as a

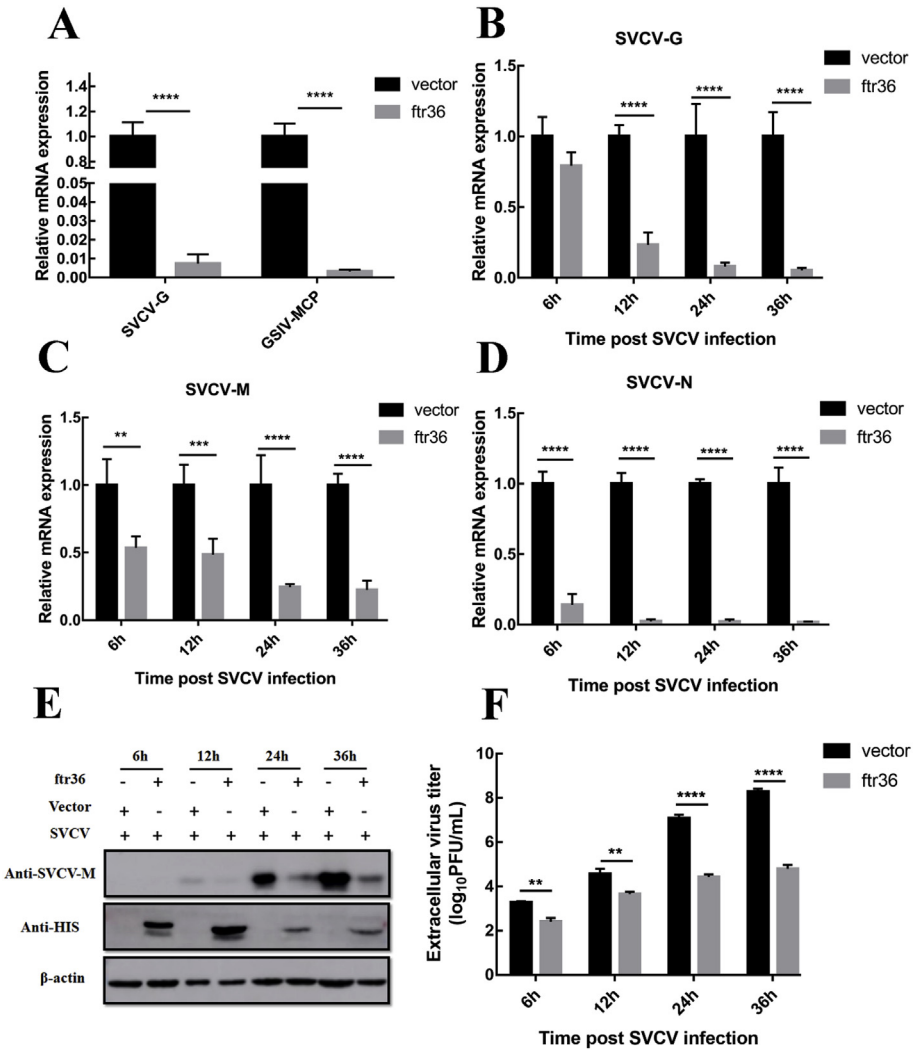

Fig. 4. The significant antiviral activity of FTR36. (A)The relative expression level of SVCV-G and GSIV-MCP after transfected with *ftr36* or vector plasmids in EPC cells infected with SVCV or GSIV respectively. (B, C, and D) The relative expression mRNA level of SVCV-G, SVCV-M, SVCV-N after transfection with *ftr36* or vector plasmids. (E) The protein level of SVCV-M after transfected with *ftr36* or vector plasmids. FHM cells were seeded in 6-well plates. Each well was transfected with 2 μg of *ftr36* or control vector. The cells were harvested for western blot analysis, (F) and the supernatant was collected and viral titer was determined by plaque formation assay. Statistical analyses were performed using a two-way ANOVA test. Error bars are the SDs in triplicate. $^{**}p < 0.01$, $^{****}p < 0.0001$.

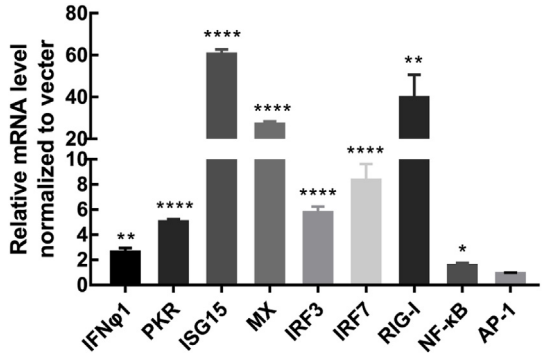

Fig. 5. FTR36 triggers the IFN signaling pathway. FHM cells were transfected with *ftr36* or control vector plasmid and several important genes of the IFN pathway were analyzed after 36 h post transfection. IFNϕ1, Molecular sensor RIG-I, key transcription factors as interferon regulatory factors irf3 and irf7 were up-regulated by the overexpression of FTR36. ISGs (MX, PKR, ISG15) also stimulated by FTR36 overexpression. NF-κB and AP-1 were detected after overexpressing FTR36, using the empty vector as control. Relative mRNA levels were normalized to the control vector. Statistical analyses were performed using an unpaired Student's t-test. Error bars are the SDs in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

negative control. qRT-PCR was used to detect the mRNA level of SVCV-G (the glycoprotein of SVCV) and GSIV-MCP (major capsid protein, MCP). The results showed that the relative mRNA level of SVCV and GSIV were significantly inhibited ($p < 0.0001$) with the FTR36 overexpression (Fig. 4A).

We further verified these findings on the FHM, another susceptible host cell line to SVCV. The recombinant plasmid was transfected into FHM cells to overexpress FTR36. At 24 h p.t., cells were treated with SVCV at MOI of 0.05. Then samples were harvested at p.i. times 6 h, 12 h, 24 h, and 36 h and analyzed by qRT-PCR. The results demonstrated that the mRNA level of viral genes including SVCV-G, SVCV-N (Nucleoprotein of SVCV), and SVCV-M (Matrix protein of SVCV) notably decreased at 24 h ($p < 0.0001$) and 36 h ($p < 0.0001$) p.i. as compared to the control group transfected vector (Fig. 4B, C, 4D). This data confirmed the results obtained in EPC cells. To detect the protein level of SVCV after overexpressing FTR36 as described above, whole cells were collected and assessed for the protein level of SVCV-M using western blotting assay. The supernatant was collected to measure the viral titer. Consistent with the data above, the protein level of SVCV-M was down-regulated with the overexpression of FTR36 at 12 h, the inhibition effect is most evident at 36 h as compared with the negative control (Fig. 4E). The viral titer of SVCV was significantly inhibited ($p < 0.01$) as well (Fig. 4F and supplementary materials Fig. 4). These results indicate that FTR36 could significantly inhibit virus replication *in vitro*.

3.5. FTR36 stimulates the relative expression of key genes of the IFN pathway

Since FTR36 can play a significant role in antiviral effects, it is reasonable to ponder upon the antiviral mechanism of FTR36. Based on previous studies of TRIMs [15,21], FTR36 might reach the antiviral immune purpose through the IFN pathway. Therefore, we assessed the relative expression of IFN and IFN-stimulated genes (ISGs).

FTR36 was overexpressed in FHM cells 36 h after transfection in 6-well plates. Cells were then collected for qRT-PCR analysis. Type-I IFNϕ1, ISGs (PKR, ISG15 and MX) were detected. The results indicate that the relative expression of IFNϕ1 was upregulated by 2-fold or more, and the expression of ISGs were up-regulated by 5–50-flod.

Furthermore, to investigate FTR36 through which pathway to induce IFNϕ1 and ISGs, we assessed molecular sensor namely RIG-I (also known as ddx58), two key transcription factors IRF3 and IRF7, NF-κB and activator protein 1 (AP-1). Our data showed that RIG-I was significantly upregulated by FTR36 overexpression (by more than 30-fold). IRF3/7 were also induced by more than 5-fold. NF-κB was up-regulated by about 1.5-fold, whereas, AP-1 was not up-regulated by the overexpression of FTR36 (Fig. 5). To summarize, these observations provide the evidence that FTR36 can trigger IFNϕ1 production and the induction of ISGs.

3.6. RING and B30.2 domains are prerequisites for antiviral activity of FTR36

After clarifying that FTR36 activates the IFN pathway, we wanted to figure out which domain is required for the FTR36 antiviral effects. To address this, *ftr36*-ΔRING and *ftr36*-ΔB30.2 were constructed by fusing with HIS tag as represented in Fig. 6A. After the same treatment as described above with *ftr36*, *ftr36*-ΔRING, *ftr36*-ΔB30.2 or empty vector, infected cells were collected for qRT-PCR, and the supernatant was collected to determine viral production. FHM cells overexpressing FTR36-ΔRING and FTR36-ΔB30.2 did not show a notable change of the mRNA level of SVCV-G as compared with the control cells (Fig. 6B). Accordingly, no obvious change of the SVCV-induced CPE could be observed in cells expressing FTR36-ΔRING or FTR36-ΔB30.2 as compared to control cells (Fig. 6C). The mRNA expression of RIG-I, IFNϕ1, and Viperin were detected to clarify FTR36-mediated affection of the IFN pathway. The data showed that FTR36-ΔRING and FTR36-ΔB30.2 did not significantly trigger IFN pathway compared with full-length *ftr36* (Fig. 6 D, E, F). These results support that the synergistic role of RING and B30.2 domain of FTR36 revealing that both RING and B30.2 domain are required for its antiviral activity.

4. Discussion

TRIM proteins have received intense research interest because of their roles in a wide range of cellular biological processes, especially, as a key subset in immune response [2,8,22]. Human TRIM56 is known to dictate antiviral restriction of influenza A and B viruses by impeding viral RNA synthesis [23]. Human TRIM52 exerted antiviral activity against Japanese encephalitis virus (JEV) infection by targeting and degrading viral NS2A [6]. *Epinephelus coioides* TRIM8 (EcTRIM8) could exert antiviral function through the regulation of the expression of proinflammatory cytokines and interferon related transcription factors in response to fish viruses, thus significantly inhibiting the replication of SGIV and red spotted grouper nervous necrosis virus (RGNNV) [22]. EcTRIM32 regulates interferon immune and inflammation response and contributes critical roles in SGIV infection. The expression level of EcTRIM32 was upregulated after the GSIV challenge and activated interferon signaling pathway [24]. The FTR83 is able to upregulate type-I IFN responses and afford protection against RNA virus infections [15]. To the best of our knowledge, no study has been reported to focus on the molecular functions of fish FTR36 until now. In this study, zebrafish FTR36 also could inhibit SVCV and GSIV replication. Here we showed that SVCV could induce zebrafish *ftr36* mRNA expression in zebrafish tissues. This suggests that *ftr36* is induced by SVCV through IFN-independent signaling pathways. Thereby, this study was aimed to figure out the function of zebrafish FTR36 in viral infection.

From the N-terminus to C-terminus, TRIM proteins are comprised of a RING zinc finger domain, one or two B-boxes, a coiled-coil domain, and a juxtaposition of a PRY and a SPRY domain, known as B30.2 domain [7,25]. Therefore, TRIM proteins are also known as RBCC proteins. FTR83 and FTR82 contain a RING zinc finger domain, two B-boxes, a coiled-coil domain, a B30.2 domain [15]. Unlike these TRIM proteins, FTR36 has only one B-box domain, but two coiled-coil domains. Previous studies have shown that the RING domain and B30.2 domain of TRIM proteins play important roles in the response to virus infection.

The RING domain is an indispensable part of TRIM proteins, which was proved to have some relationship with E3 ubiquitin ligases activities. The E3 ubiquitin ligase activity of the RING domain of human TRIM5α is linked with the potency of HIV-1 restriction [26]. Anti-influenza virus activity of TRIM56 was also independent of the E3 ligase activity. Moreover, expression of a short segment within the C-terminal tail of TRIM56 inhibited the replication of influenza viruses as effectively as that of full-length TRIM56 by specifically targeting viral RNA synthesis [23]. Similarly, the RING domain was also indispensable for the antiviral action of fish EcTRIM25 against iridovirus and nodavirus infection [27]. Recent research has demonstrated that the RING domain is essential for EcTIRM32 in response to both DNA and RNA virus infection [24]. B30.2 domain-mediated RNA-binding activity of human TRIM25 is required for E3 ubiquitin ligase enzymatic activity [28]. Lacking RING or B30.2 domain of zebrafish, FTR83 was unable to trigger IFN-independent signaling pathways and thus unable to reduce IHNV or VHSV infection and trigger IFN-independent signaling pathways [15]. In this study, we constructed plasmids lacking RING and B30.2 domains respectively and showed that the RING domain and B30.2 domain are both required for the antiviral immunity of FTR36. Accordingly, we proved that the antiviral activity of FTR36 is related to its molecular structure.

The antiviral TRIMs have been involved in a variety of mechanisms that affect its function, and TRIMs frequently function as modulators rather than direct effectors in antiviral immunity [29]. The research on zebrafish finTRIMs indicated that FTR83 significantly increases basal IFN expression and modulates expression of ISGs, mediating a potent antiviral activity against RNA viruses *in vitro* and *in vivo* [15]. Our study has shown that FTR36 mediates notable antiviral activity against different kinds of viruses, including SVCV and GSIV. These results revealed that the antiviral activity is a fundamental function of finTRIMs. Some TRIM family proteins were reported to trigger IFN-independent pathways [11,15,21,22,30], and thus, we examined several important genes involved in IFN pathway, including significant pattern recognition receptor RIG-I, IRF3/7, IFNϕ1 and ISGs (MX, PKR, ISG15 and Viperin). The relative mRNA level of these genes was upregulated obviously when FTR36 is overexpressed. These results indicate that FTR36 exerts its antiviral activity through the IFN pathway. EcTRIM8 could exert antiviral function through the regulation of the expression of both proinflammatory cytokines and interferon-related transcription factors in response to fish viruses [22]. Thus, interferon-related transcription factors NF-κB and AP-1 was detected and FTR36 slightly increase the expression of NF-κB but had no effect on the expressions of AP-1. These new pieces of evidences suggest that FTR36 are able to promote IFN response to achieve antiviral immunity. It should be noticed that although this study has demonstrated that FTR36 is related to IFN-pathway, the interaction between FTR36 and the molecules involved in the signaling pathway is still unknown. Further research is required in this area.

In summary, this study reports the antiviral functions of FTR36 for the first time. Our *in vitro* and *in vivo* test results have shown that FTR36

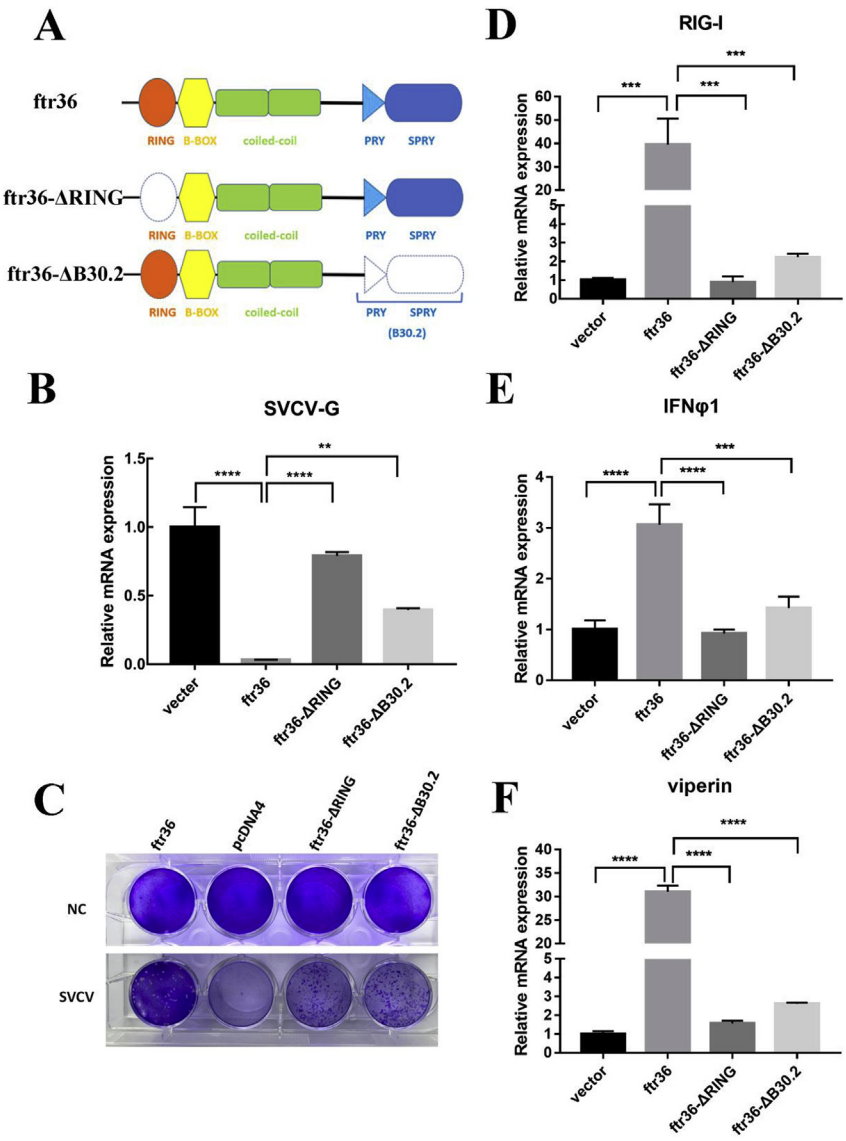

Fig. 6. RING and B30.2 domains are required in viral restriction. (A) Schematic representations of FTR36 domain. (B) The relative mRNA expression of SVCV-G was not notably inhibited with the deletion of RING or B30.2 domain of *ftr36*. FHM cells were transfected with full-length *ftr36*, *ftr36*-ΔRING, *ftr36*-ΔB30.2 or empty plasmid. After 24-h transfection, cells were treated with MOI 0.05 SVCV for 24 h qRT-PCR was used for detecting. Results of qRT-PCR were normalized on the TBP expression. Statistical analyses were performed using a one-way ANOVA test. Error bars are the SDs in triplicate. $^{**}p < 0.01$ $^{****}p < 0.0001$. (C) FHM cells were transfected with full-length *ftr36*, *ftr36*-ΔRING, *ftr36*-ΔB30.2 or empty plasmid. At 24 h post transfection, cells were treated with MOI 0.05 SVCV or non-infected control. The supernatant was collected after 24 h viral infection and viral-induced CPE was assessed by crystal violet staining at 60 h p.i. Mock-transfected cells (Ctrl) and non-infected cells (NI) were also used as controls. (D, E and F) RIG-I, IFNϕ1 and Viperin mRNA expression were not significantly induced while deleted RING or B30.2 domain of *ftr36*. FHM cells were transfected with full-length of *ftr36*, *ftr36*-ΔRING, *ftr36*-ΔB30.2, or empty vector plasmid. Following 36 h transfection, cells were collected for qRT-PCR analysis. Results were normalized on the TBP expression. Statistical analyses were performed using a one-way ANOVA test. Error bars are the SDs in triplicate. $^{***}p < 0.001$ $^{****}p < 0.0001$.

is able to inhibit SVCV and GSIV replication significantly. In the early and later stages of SVCV replication, FTR36 could notably inhibit viral infection. Furthermore, FTR36 could significantly induce the production of IFNϕ1, and ISGs thought IFN pathway. Finally, compared with the full-length *ftr36*, lacing the RING and B30.2 domain could impair the inhibitory effect of FTR36 on SVCV replication and weaken the induction of IFNϕ1 and ISGs expression. Therefore, findings from this study will provide new information for the development of new approaches for enhanced prevention and control of viral diseases in world aquaculture.

Acknowledgements

This work was supported by Natural Science Foundation of China (31172433), Fundamental Research Funds for the Central Universities (2662018YJ022), and a Science & Technology Supporting Program from Hubei Province (2015BBA234).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.051.

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