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Negative regulation of the interferon response by finTRIM82 in the orange spotted grouper

Shunyou Lv[a], Ya Zhang[a], Jiaying Zheng[a], Xiaohong Huang[a], Youhua Huang[a,**], Qiwei Qin[a,b,*]

[a] College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

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ABSTRACT

Tripartite motif (TRIM) proteins have been demonstrated to exhibit critical functions in multiple cellular processes, including development, carcinogenesis, and programmed cell death, and are also widely recognized to be important antiviral restriction factors or modulators of immune and inflammatory signaling pathways. However, in teleosts, additional TRIM members have been identified and their functions remain largely unknown. Here, a novel finTRIM gene from orange spotted grouper (EcfinTRIM82) was cloned and characterized. Sequence analysis indicated that EcfinTRIM82 encoded a 575 amino acid peptide which shared 94% and 82% identity with Asian sea bass (*Lates calcarifer*), and zebrafish (*Danio rerio*) finTRIM82, respectively. EcfinTRIM82 contained three conserved domains, including a RING, B-Box, and SPRY domain. Using fluorescence microscopy, we found that green fluorescence aggregates were observed in the cytoplasm of EcfinTRIM82-EGFP transfected grouper spleen (GS) cells. As the infection proceeded, EcfinTRIM82 transcription was significantly upregulated in Singapore grouper iridovirus (SGIV) or red-spotted grouper nervous necrosis virus (RGNNV) infected GS cells. This suggests that EcfinTRIM82 might be involved in fish virus infection. The *in vitro* overexpression of EcfinTRIM82 in GS cells significantly enhanced the replication of SGIV and RGNNV, evidenced by increased expression of viral genes, including the SGIV major capsid protein (MCP), VP19, ICP-18, RGNNV coat protein (CP), and RNA-dependent RNA polymerase (RdRp). Furthermore, the ectopic expression of EcfinTRIM82 significantly decreased the expression of interferon (IFN)-related signaling molecules, including interferon regulatory factor 3 (IRF3), IRF7, interferon stimulated gene 15 (ISG15), ISG56, IFP35, and myxovirus resistance gene (MXI), suggesting that EcfinTRIM82 regulated viral replication via the negative regulation of the host IFN response. In addition, EcfinTRIM82 overexpression substantially decreased the level of proinflammatory cytokine transcription. Furthermore, the ectopic expression of EcfinTRIM82 significantly weakened the melanoma differentiation-associated protein 5 (MDA5), mediator of IRF3 activation (MITA) and mitochondrial antiviral-signaling (MAVS) protein-induced IFN response by detecting the transcription of interferon related cytokines and the promoter activity of IFN. Together, our results demonstrate that finTRIM82 negatively regulates the innate antiviral immune response against grouper virus infection.

1. Introduction

Tripartite motif (TRIM) proteins have attracted increased attention due to the multiple roles they play in various cellular processes, including cell differentiation, development, carcinogenesis, cell death, and the innate immune response [1,2]. TRIM44 functions as a positive regulator of virus-triggered immune responses by enhancing the stability of virus-induced signaling adaptor (VISA) [3]. In addition, was it was demonstrated that TRIM8 functions as a novel therapeutic target to enhance p53 tumor suppressor activity [4]. To date, substantial progress has been made in elucidating the role of the TRIM proteins in mammals and some TRIM family members in lower vertebrates have also been cloned and characterized in different fish species [5–9]. For example, grouper TRIM25 and TRIM35 display antiviral functionality through enhancing the interferon response against fish iridovirus and nodavirus infections [8,9]. Moreover, zebrafish TRIM69 was found to participate in p53-mediated apoptosis during zebrafish development [10]. Interestingly, a new subset of TRIM genes termed finTRIM (*fi*sh *n*ovel *TRIM*) have recently been predicted to share low identity with mammal TRIM homologs; however, they are widely present in various

* Corresponding author. College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, China.
** Corresponding author. College of Marine Sciences, South China Agricultural University, Guangzhou, 510642, China.
E-mail addresses: huangyh@scau.edu.cn (Y. Huang), qinqw@scau.edu.cn (Q. Qin).

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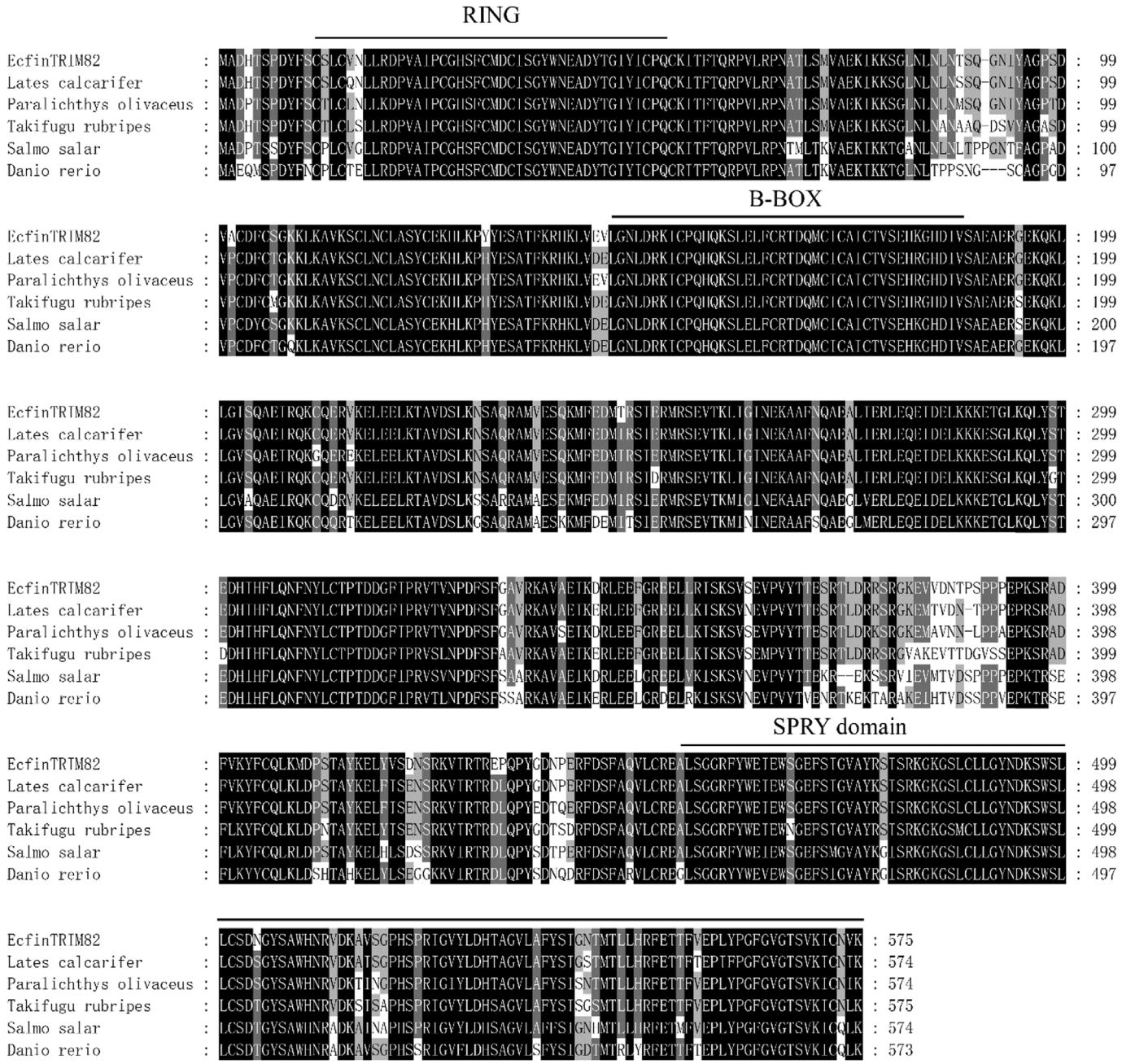

Fig. 1. Amino acid alignment of EcfinTRIM82 and other finTRIM82 homologs from different fish species. The conserved domains, including the RING, B-Box, and SPRY domains are underlined.

teleost species [11]. Among these genes, zebrafish finTRIM83 was able to induce interferon and interferon-stimulated gene expression, as well as confer protection against different enveloped and non-enveloped RNA viruses [12]. In addition, finTRIM12, finTRIM51, finTRIM67, finTRIM83, and finTRIM84 have been proposed to exert a crucial role in the innate immune response, as evidenced by their upregulated level of expression during grass carp reovirus (GCRV) and spring viremia of carp virus (SVCV) infection [13]. However, the detailed function of the majority of the finTRIM genes and the potential mechanisms remain poorly understood.

Grouper (*Epinephelus* spp.) comprises a group of important economic marine fish species that are widely cultured in tropical and subtropical regions, particularly in south-east Asia; however, cultured grouper have encountered an increasing number of infectious diseases, including different bacterial, parasitic, and viral pathogens in recent years [14–18]. Among the viral pathogens, Singapore grouper iridovirus (SGIV) or red spotted grouper nervous necrosis virus (RGNNV) infection typically cause mass mortality in fry to the juvenile stage of grouper [14,19,20]. To clarify the mechanism of the action of grouper viruses, a large number of immune genes were cloned and their roles in viral infection were elucidated. For example, some immune-related genes, such as interferon, interferon-stimulated gene (ISG)15, mitochondrial antiviral-signaling protein (MAVS), myxovirus resistance gene MX, and interferon regulatory factor (IRF) 3 exhibited different antiviral activity against SGIV and RGNNV infection [21–23]. In particular, based on transcriptome or genome annotation, a minority of genes from grouper or other fish were predicted to share a low identity with mammalian homologs or were only present in fish species [11]. Whether some of these genes also exerted crucial roles against fish virus infection remain largely unknown.

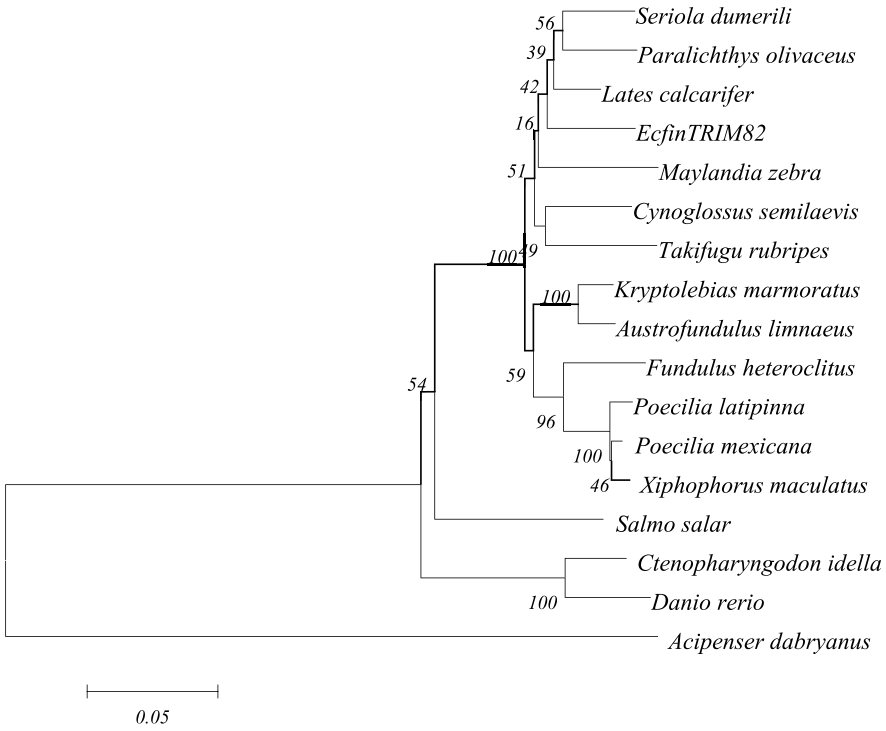

Fig. 2. Phylogenetic analysis of EcfinTRIM82. A phylogenetic tree was constructed using MEGA 5.0 using the neighbor-joining (NJ) method.

In the present study, a novel finTRIM gene from orange spotted grouper (EcfinTRIM82) was cloned and characterized. The role of EcfinTRIM82 in response to fish virus infection and the potential mechanism underlying the function of EcfinTRIM82 were investigated in grouper spleen (GS) cells. Together, our results demonstrated for the first time, that finTRIM82 negatively regulates the innate antiviral immune response against fish viruses.

2. Materials and methods

2.1. Fish, viruses, and cells

Orange-spotted groupers, *E. coioides* (50 g-60 g) were purchased from Hainan Province, China and housed in a recirculating seawater system. Grouper spleen (GS) cells were cultured at 28 °C in Leibovitz's L15 medium containing 10% fetal bovine serum (FBS, Gibco) [24]. SGIV and RGNNV were used as the viruses in the present study, which were prepared as previously described [24,25].

2.2. Cloning and sequence analysis of EcfinTRIM82

Based on the assembly of EST sequences from the grouper spleen transcriptome [26], the full-length cDNA of EcfinTRIM82 was amplified by PCR amplification and verified with DNA sequencing. The deduced amino acid sequences of EcfinTRIM82 were subjected to BLAST program in the NCBI database. Multiple sequence alignment of finTRIM82 homologs was carried out using ClustalX1.83 software and edited using the GeneDoc program. A phylogenetic tree was constructed using Mega 5.0 software [27].

2.3. EcfinTRIM82 expression patterns

To clarify the distribution pattern of EcfinTRIM82, the total RNA was extracted from different grouper tissues, including the head, kidney, heart, liver, spleen, intestine, muscle, brain, skin, gill, stomach, fin, and kidney as described previously [21]. The level of EcfinTRIM82 expression was assessed using quantitative real-time PCR (qRT-PCR) as described below.

To analyze the expression profiles of EcfinTRIM82 against fish virus infection, GS cells were infected with SGIV or RGNNV at the indicated time points. The cells were then collected for RNA extraction and further qRT-PCR analysis.

2.4. Plasmid construction

To elucidate the potential function of EcfinTRIM82 during fish virus infection, EcfinTRIM82 was cloned into pEGFP-C1 and pcDNA3.1-Flag vectors. The corresponding recombinant plasmids, including pEGFP-EcfinTRIM82 and Flag-EcfinTRIM82, respectively, were confirmed by DNA sequencing.

2.5. Cell transfection and luciferase assay

GS cells were seeded into 24-well plates, and transfection was carried out using the transfection reagent, Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Briefly, the expression plasmids were mixed with Lipofectamine 2000 and then added to the cells for a 6 h incubation. After replacing the reagent with fresh medium, the cells were cultured at 25 °C for further study.

To detect the interferon promoter activity induced by EcfinTRIM82, GS cells were co-transfected with reporter constructs containing the interferon (IFN)-β promoter (IFN-Luc or ISRE-Luc), NF-κB promoter (NF-κB-Luc), or *Renilla* luciferase (internal control) together with the empty vector (control), expression vector EcfinTRIM82, or melanoma differentiation-associated protein 5 (MDA5), mediator of IRF3 activation (MITA) or mitochondrial antiviral-signaling (MAVS) plasmids. At 48 h post-transfection, cells were collected and lysed for the luciferase assay using the Dual-Luciferase Reporter Assay system (Promega) as described previously [9].

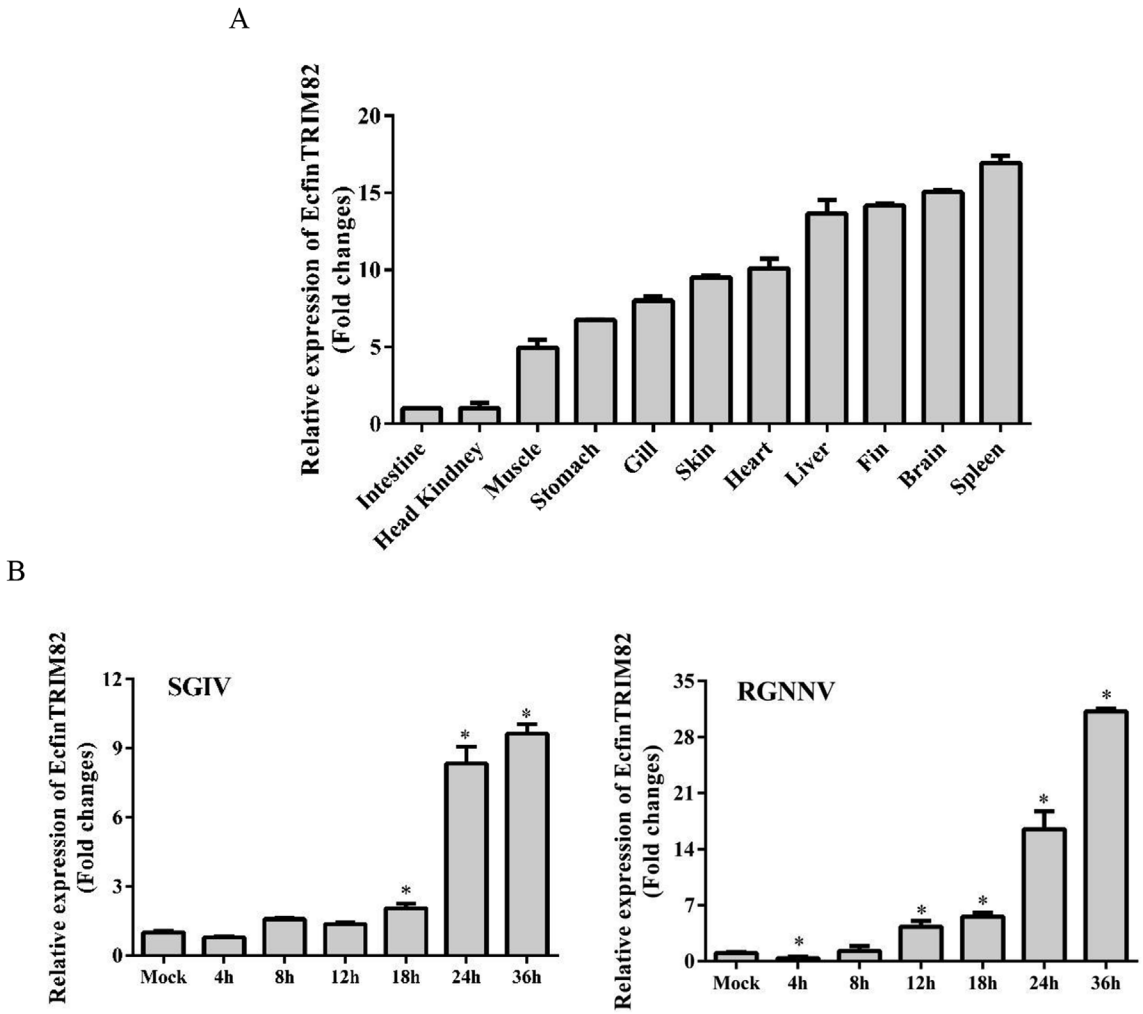

Fig. 3. Expression patterns of EcfinTRIM82. (A) The expression profiles of EcfinTRIM82 in different tissues isolated from groupers. (B) The level of EcfinTRIM82 expression in grouper cells in response to SGIV or RGNNV infection.

2.6. Fluorescence microscopy

To examine the subcellular localization of EcfinTRIM82 in grouper cells, pEGFP-C1 and pEGFP-EcfinTRIM82 plasmids were transfected into GS cells as described above. At 48 h post transfection, the cells were fixed and stained with DAPI. Samples were observed under a fluorescence microscope.

2.7. RNA extraction and qRT-PCR

Total RNA from grouper tissues or cells was extracted using a SV Total RNA Isolation System (Promega, USA) and reverse-transcribed using a ReverTraAce qPCR RT kit (Toyobo) as described previously [5]. qRT-PCR was carried out in a Roche 480 Real Time Detection System (Roche, German) as described previously [9]. The level of viral gene expression, including SGIV, major capsid protein (MCP), ICP-18, VP19 and RGNNV coat protein (CP), and RNA-dependent RNA polymerase (RdRp) were detected to evaluate the replication of SGIV or RGNNV. The levels of interferon relative gene expression, including IRF3, IRF7, ISG15, ISG56, IFP35, myxovirus resistance gene (MXI), and pro-inflammatory factors, including TNFα, IL-8, and IL-1β were also examined to assess the effect of EcfinTRIM82 on the host interferon and inflammatory response. For qRT-PCR analysis, each assay was performed in triplicate using the following cycling conditions: 94 °C for 5 min, followed by 45 cycles at 94 °C for 5 s, 60 °C for 10 s, and 72 °C for 15 s. The primers used in the present study are listed in supplemental file 1. The level of target gene expression was normalized to β-actin and the data was calculated as the fold change compared to that of empty vector. The data ware represented as the mean ± SD. Statistical significance was determined with Student's *t*-test and the significance level was defined as $p < 0.05$ (*).

3. Results

3.1. Molecular characterization of EcfinTRIM82

According to the EST sequences from the transcriptome data, the full-length cDNA of EcfinTRIM82 was obtained using PCR amplification and validated by sequencing. The sequence analysis revealed that EcfinTRIM82 encoded a 575 amino acid peptide which shared 94% and 82% identity with Asian sea bass (*Lates calcarifer*) and zebrafish (*Danio rerio*) finTRIM82, respectively. However, EcfinTRIM82 only showed 25% identity with the human (homo sapiens) TRIM proteins. Amino acid alignment indicated that EcfinTRIM82 and finTRIM82 homologs from other fish contained three conserved domains, including a RING, B-BOX, and SPRY domain (Fig. 1). The phylogenetic analysis indicated that EcfinTRIM82 shared the closest relationship with Asian sea bass (Fig. 2).

3.2. EcfinTRIM82 expression profiles

As shown in Fig. 3A, EcfinTRIM82 transcripts were ubiquitously

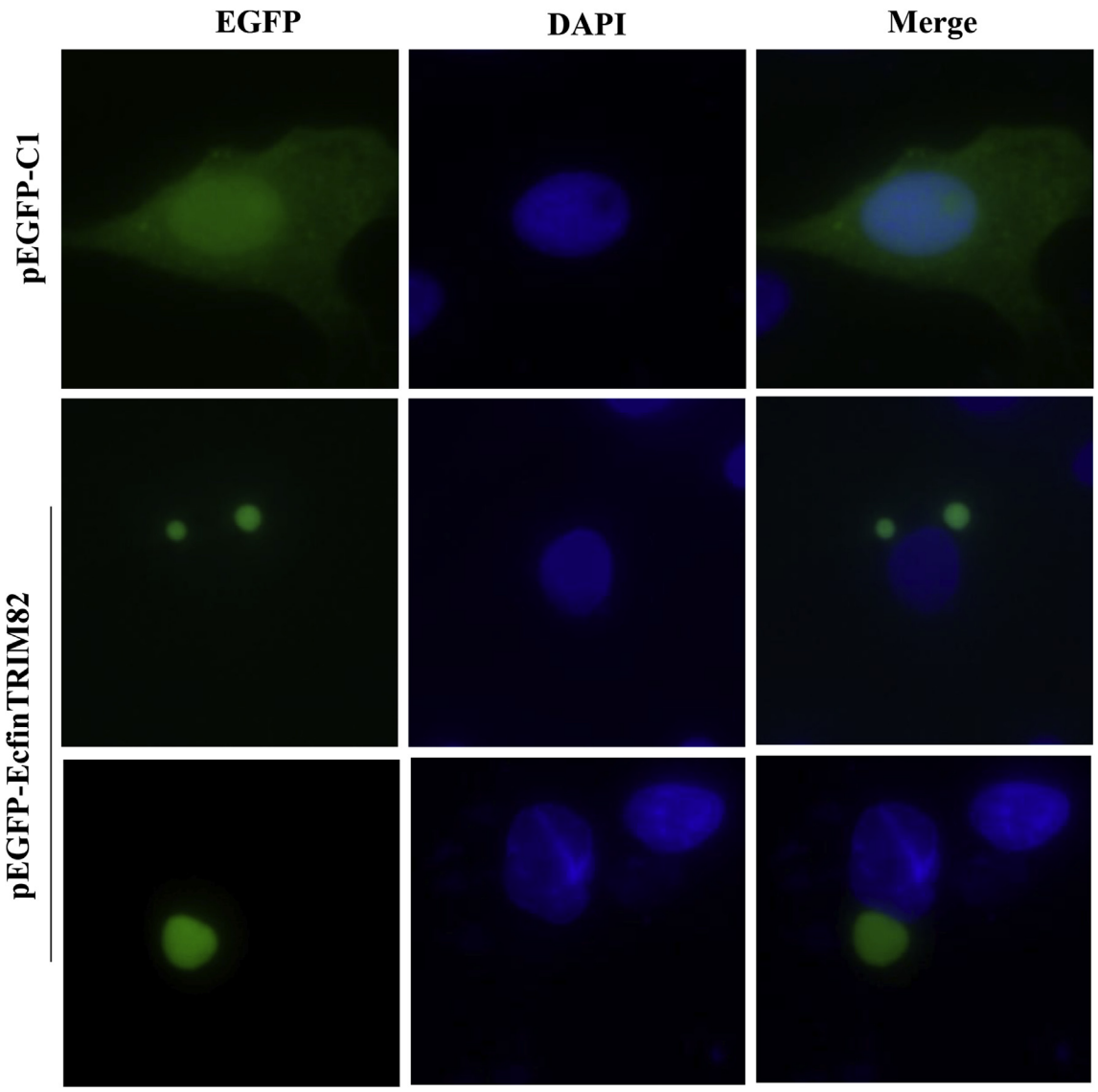

Fig. 4. Subcellular localization of EcfinTRIM82 in grouper cells. pEGFP-C1 and pEGFP-EcfinTRIM82 were transfected into GS cells and subsequently stained with DAPI. The fluorescence was observed using fluorescence microscopy.

detected in all of the investigated tissues, with predominant expression in the kidney, spleen, brain, and liver. To examine the expression patterns of EcfinTRIM82 in response to viral infection *in vitro*, the level of EcfinTRIM82 transcription was detected following RGNNV and SGIV infection of GS cells at different time points. As shown in Fig. 3B, the level of EcfinTRIM82 expression was downregulated at 4 h post-infection (p.i.) and gradually increased after 18 h p.i. with SGIV. In RGNNV infected cells, the expression level of EcfinTRIM82 was increased from 8 h p.i. and reached a peak up to 31.2 folds compared to that of mock cells at 36 h p.i.

3.3. *EcfinTRIM82 encodes a cytoplasmic protein*

To clarify the subcellular localization of EcfinTRIM82 *in vitro*, pEGFP-EcfinTRIM82 and pEGFP-C1 were transfected into grouper spleen cells and the fluorescence was observed under fluorescence microscopy. As shown in Fig. 4, green fluorescence aggregates were observed in the cytoplasm of the EcfinTRIM82-transfected cells. No fluorescence was observed in the nucleus. In contrast, in the pEGFP-C1-transfected cells, green fluorescence was distributed throughout both the cytoplasm and nucleus. Thus, these findings indicated that EcfinTRIM82 encoded a cytoplasmic protein.

3.4. *EcfinTRIM82 overexpression enhanced fish virus replication*

Due to the low identity between EcfinTRIM82 and human TRIM family members, the function of EcfinTRIM82 remained uncertain. We firstly evaluated the potential effects of EcfinTRIM82 on fish virus infection. As shown in Fig. 5A, the severity of CPE induced by SGIV and RGNNV were both significantly enhanced in EcfinTRIM82 overexpressing cells when compared with control vector transfected cells (Fig. 5A). Consistently, the expressions of viral genes, including SGIV MCP, VP19 and ICP-18, were all significantly increased in SGIV-infected EcfinTRIM82 overexpression cells, compared to those in control vector transfected cells (Fig. 5B). Meanwhile, our results also showed that the overexpression of EcfinTRIM82 significantly increased the transcription level of RGNNV CP and RdRp during RGNNV infection (Fig. 5C). Thus, EcfinTRIM82 was speculated to act as a negative regulator against fish virus infection.

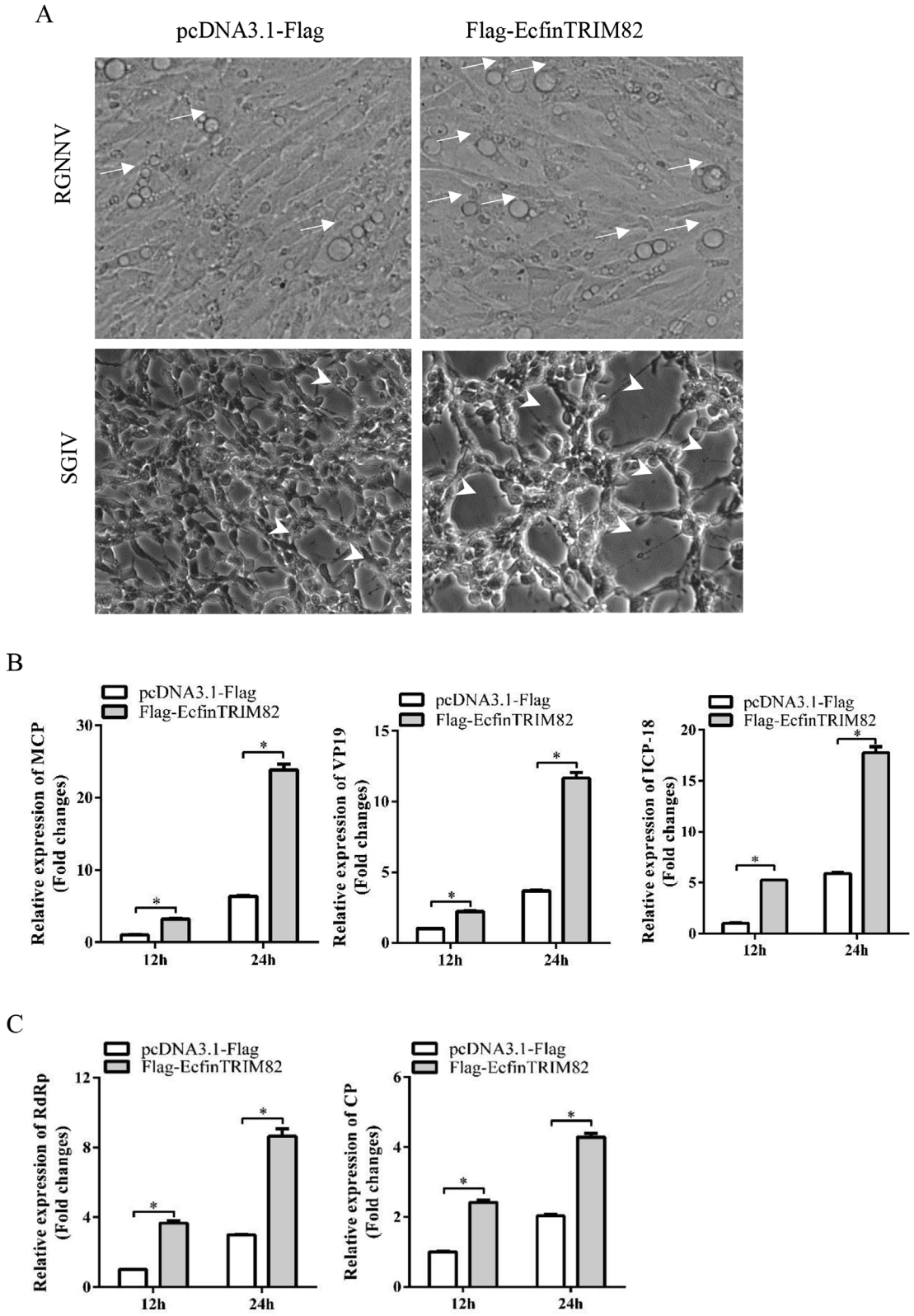

Fig. 5. The effect of EcfinTRIM82 overexpression on fish virus replication. (A) EcfinTRIM82 overexpression enhanced the severity of CPE induced by SGIV and RGNNV. (B) EcfinTRIM82 overexpression significantly increased the viral gene transcription of SGIV, including MCP, VP19, and ICP-18. (C) EcfinTRIM82 overexpression significantly increased the viral gene transcription of RGNNV, including CP and RdRp.

3.5. EcfinTRIM82 ectopic expression downregulates interferon and inflammatory signaling molecule expression

To demonstrate the potential mechanism underlying the enhanced activity of EcfinTRIM82 against fish virus infection, we examined the level of host interferon and inflammatory factor transcription in EcfinTRIM82 overexpressing cells. As shown in Fig. 6A, the transcripts of IRF3, IRF7, ISG15, ISG56, IFP35, and MXI were also significantly

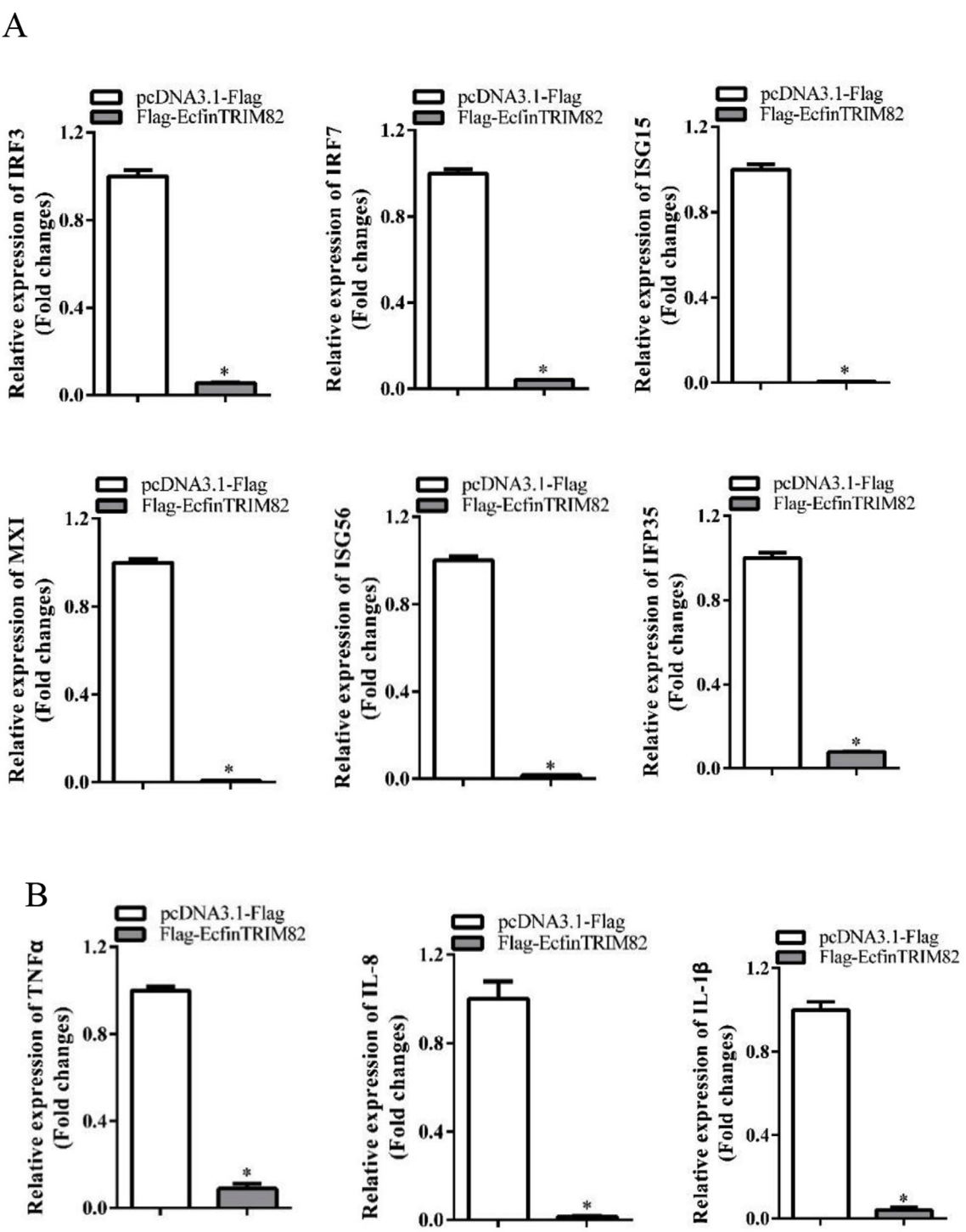

Fig. 6. EcfinTRIM82 overexpression inhibits the interferon and inflammatory response. (A) EcfinTRIM82 overexpression decreased the level of interferon-related cytokine and effector expression. (B) The ectopic expression of EcfinTRIM82 down-regulated the level of proinflammatory factor expression.

downregulated in EcfinTRIM82-overexpressing cells compared to the control vector-transfected cells. For example, the ectopic expression of EcfinTRIM82 decreased the level of IRF3 and IRF7 expression up to 5.60% and 4.16%, respectively. Furthermore, EcfinTRIM82 overexpression also significantly decreased the transcription of the pro-inflammatory factors TNFα, IL-1β, and IL-8 (Fig. 6B). Together, these findings suggested that EcfinTRIM82 overexpression negatively regulated the host interferon and inflammatory response.

3.6. EcfinTRIM82 negatively regulates the MDA5, MITA, and MAVS-induced interferon response

To determine whether the MDA5, MITA, or MAVS signaling molecules were associated with EcfinTRIM82, we determined the regulatory role of EcfinTRIM82 in the immune response induced by MDA5, MITA, or MAVS, respectively. In EcfinTRIM82 and MDA5, MITA, or MAVS co-transfected cells, the level of the above mentioned immune-related gene expression was evaluated. As shown in Fig. 7 – 9, the overexpression of MITA, MDA5, or MAVS alone induced the expression of interferon-

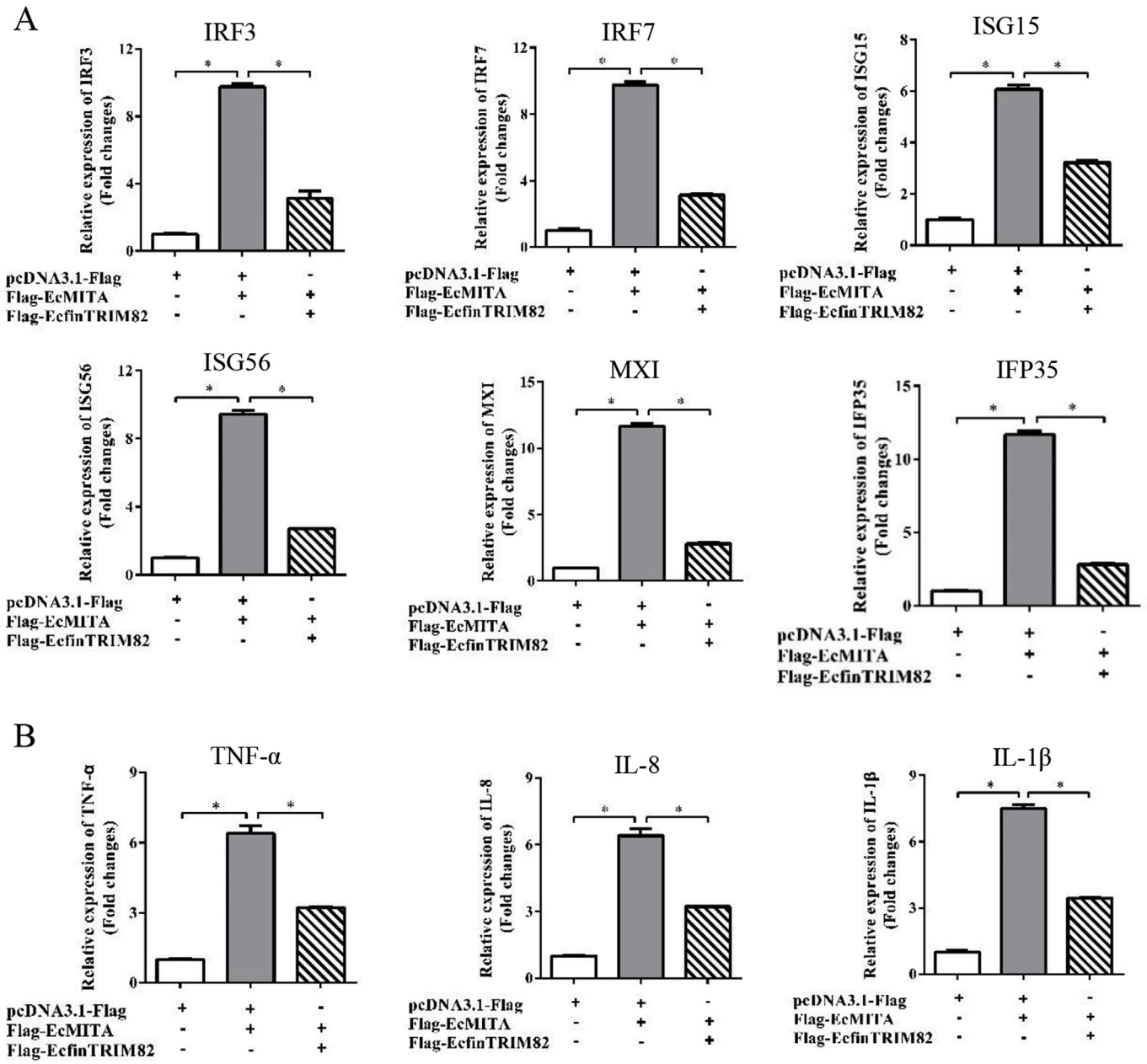

Fig. 7. The ectopic expression of EcfinTRIM82 weakened the interferon or pro-inflammatory response-induced by MITA. EcfinTRIM82 was co-transfected with MITA, and the level of interferon-related cytokines or effectors (A) and pro-inflammatory cytokine transcription (B) was detected with qRT-PCR.

related signaling molecules and proinflammatory cytokines (IRF3, IRF7, ISG15, ISG56, MXI, IFP35, TNFα, IL-8 and IL-1β) compared to the empty vector. However, co-transfection with EcfinTRIM82 significantly decreased the expression of all the detected host genes. Our results demonstrated that the ectopic expression of EcfinTRIM82 significantly weakened the MDA5, MITA, and MAVS-induced interferon response.

To further confirm the negative regulation of EcfinTRIM82 on the interferon response induced by different effectors, the impact of EcfinTRIM82 overexpression on interferon (i.e., IFN-1 and ISRE) or NF-κB promoter activity induced by different effectors was determined using a dual-luciferase reporter assay. As shown in Fig. 10A, the luciferase activity of the ISRE, IFN-1, and NF-κB promoters was significantly lower in the EcfinTRIM82-overexpressing cells compared to that of the cells transfected with the empty vector. In contrast, in MITA, MDA5, or MAVS-transfected cells, the luciferase activity of ISRE, IFN-1, and NF-κB was significantly increased compared to the cells transfected with the control vector. Notably, the luciferase activity of ISRE, IFN, and NF-κB induced by MITA, MDA5, or MAVS was significantly decreased in the EcfinTRIM82 co-transfected cells (Fig. 10B–D). Thus, we speculated that EcfinTRIM82 negatively regulated MITA, MDA5, or the MAVS-induced interferon response.

4. Discussion

An increasing number of studies have demonstrated that numerous TRIM family members exhibit regulatory functions in innate and inflammatory responses [28–32]. Interestingly, several fish-specific finTRIM genes were found to be differentially regulated in response to fish virus infection, which suggests that finTRIM genes might play a critical role in the antiviral innate immune response [13]. However, few reports have focused on the detailed function of finTRIM proteins in response to viral infection [12]. In the present study, a finTRIM82 homolog from orange spotted grouper was investigated. The sequence analysis indicated that finTRIM82 homologs from different fish species

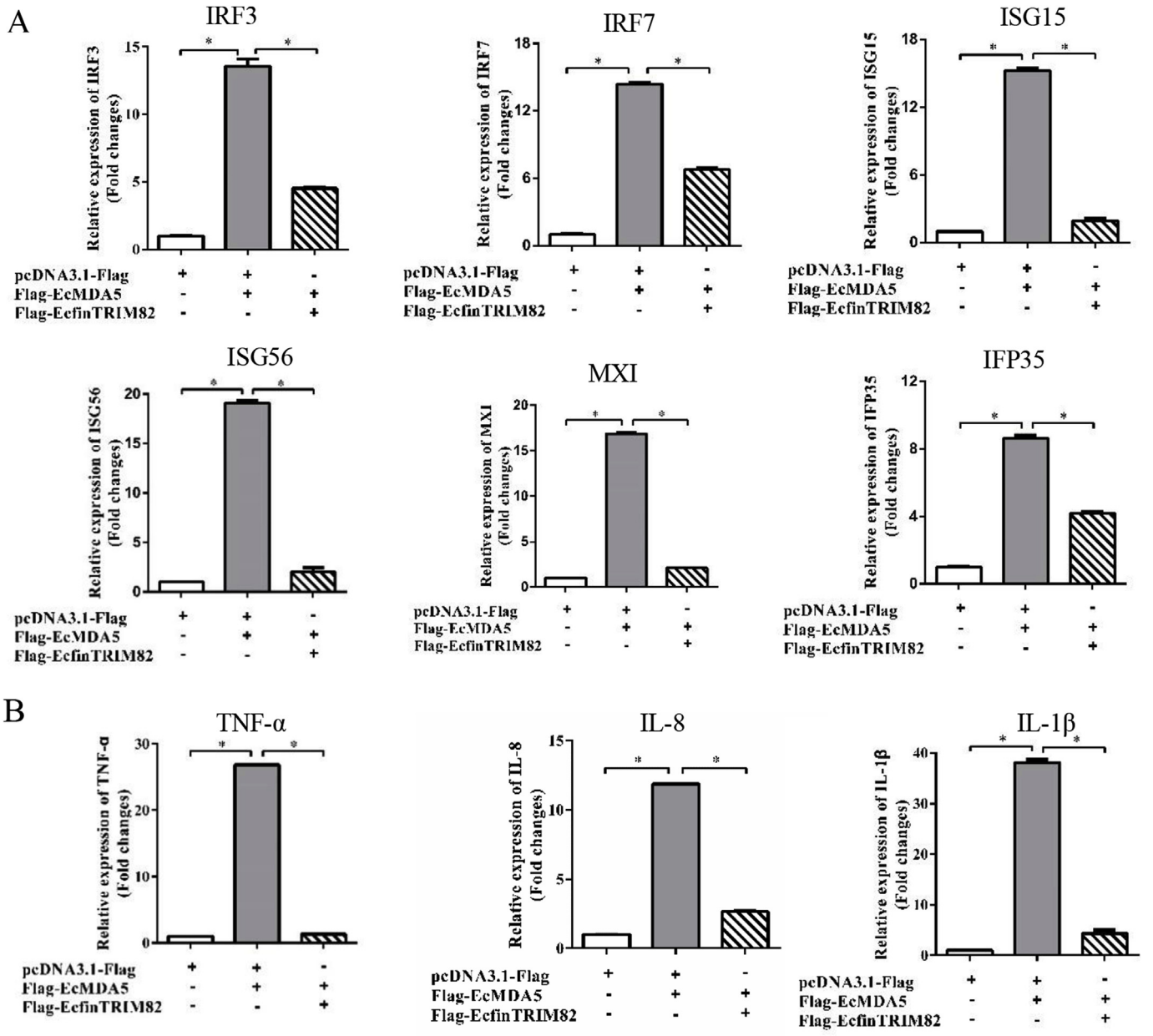

Fig. 8. EcfinTRIM82 overexpression negatively regulates the MDA5-induced interferon or proinflammatory response. EcfinTRIM82 was co-transfected with MDA5 and the level of interferon-related cytokine or effectors (A) and pro-inflammatory cytokine transcription (B) was detected with qRT-PCR.

all contained three conserved domains, including a RING, B-Box, and SPRY domain. Notably, EcfinTRIM82 shared an 82% sequence identity with zebrafish (*Danio rerio*) finTRIM82, and certain homologs were found in other fish species. However, zebrafish finTRIM82 and EcfinTRIM82 only displayed a 27% and 25% identity, respectively with the human TRIM protein. Thus, we proposed that EcfinTRIM82 and other finTRIM82 represented a novel conserved finTRIM protein that is absent in humans and other mammals.

Subcellular localization has contributed to both the exploration of the key functional attributes of investigated proteins and is helpful for understanding disease mechanisms and developing novel drugs [33]. Notably, despite sequence and structural similarity, the TRIM proteins are localized to different subcellular compartments and are not functionally interchangeable [34]. In this study, we found that EcfinTRIM82 formed cytoplasmic bodies in grouper cells, which was similar to grouper TRIM32 and TRIM35, but differed from grouper TRIM16L [9,35,36]. Moreover, during SGIV or RGNNV infection, the level of EcfinTRIM82 expression was significantly upregulated, which suggested that EcfinTRIM82 might be involved in fish virus infection.

Our previous studies found that several grouper TRIM proteins are involved in the antiviral immune response, including TRIM26, TRIM62, and TRIM32 [7]. Recent studies have also demonstrated that the overexpression of zebrafish finTRIM83 drastically inhibited viral growth for both infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) *in vitro* [12]. To clarify the potential function of EcfinTRIM82, we further evaluated the role of EcfinTRIM82 in fish virus replication. Our results showed that EcfinTRIM82 overexpression significantly increased the level of viral gene transcription during SGIV or RGNNV infection. Therefore, we speculated that EcfinTRIM82 functioned enhanced virus replication against infection with both fish RNA and DNA viruses. Similarly, studies have reported that grouper TRIM62 and TRIM35 significantly enhanced the level of RGNNV gene transcription *in vitro* [7,9]. In contrast, grouper TRIM25 and TRIM8 were found to exert an antiviral function against

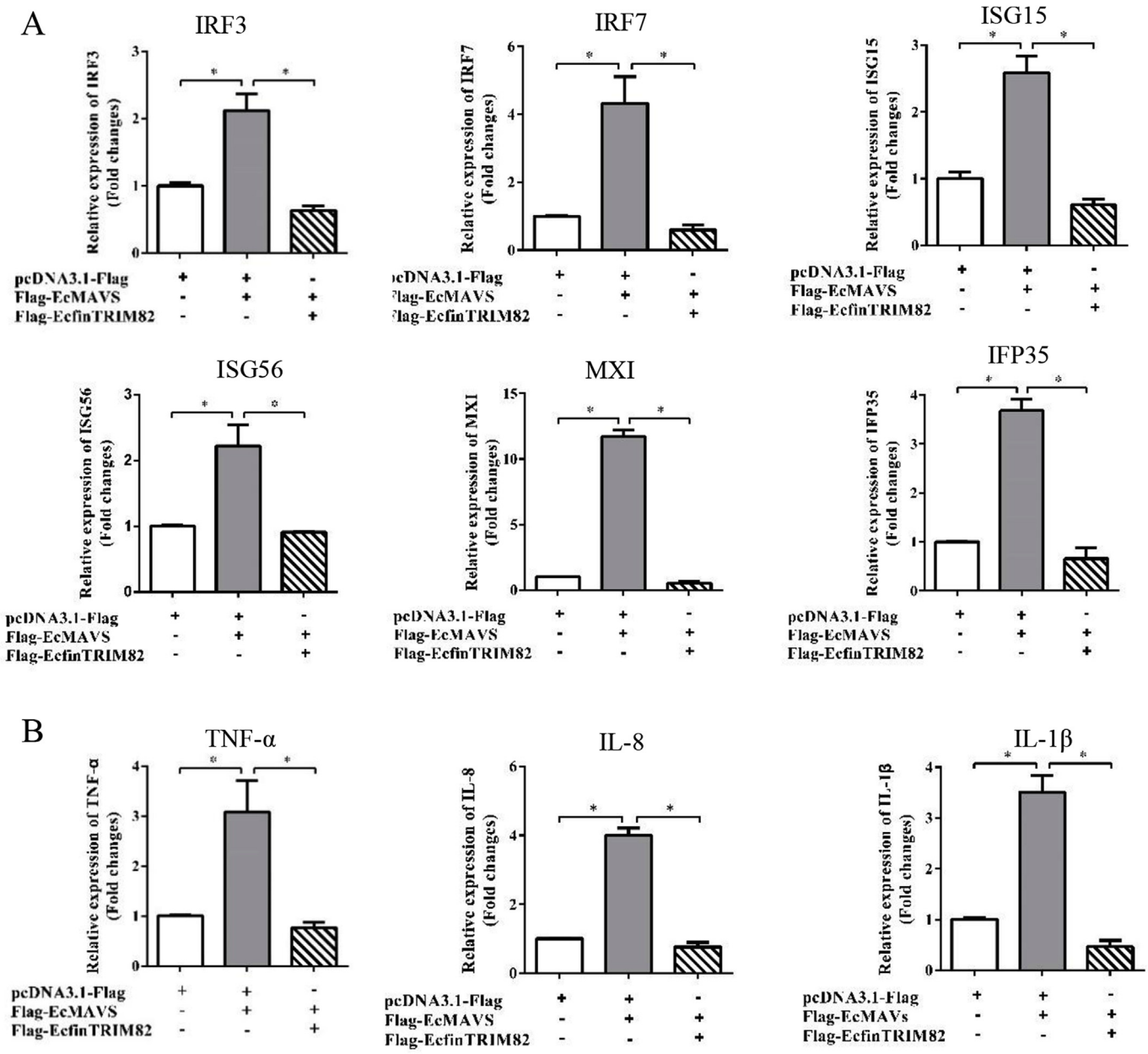

Fig. 9. The ectopic expression of EcfinTRIM82 down-regulated the MAVS-induced interferon and pro-inflammatory response. GS cells were co-transfected with EcfinTRIM82 and MAVS, and the level of interferon-related gene (A) and pro-inflammatory cytokine transcription (B) was detected with qRT-PCR.

SGIV or RGNNV [6,8].

To explore the potential mechanism of EcfinTRIM82 on virus replication, we investigated the effect of EcfinTRIM82 on the host interferon response, including interferon-related cytokines or effectors, as well as interferon promoter activity. The ectopic expression of EcfinTRIM82 significantly decreased the transcription of interferon-related cytokines or effectors, including IRF3, IRF7, ISG15, MXI, IFP35 and ISG56. Consistently, EcfinTRIM82 overexpression significantly decreased interferon promoter activity. In our previous study, we also found that these cytokines or effectors were upregulated (e.g., IRF3, IRF7, and ISG15) and exhibited antiviral activity against RGNNV or SGIV infection [21,37,38]. Furthermore, the ectopic expression of EcfinTRIM82 significantly weakened the MDA5, MITA, or MAVS-induced interferon response, as evidenced by the decreased expression of interferon-related cytokines or effectors and interferon promoter activity. Moreover, it has been demonstrated that MITA exhibits crucial antiviral activity against both fish DNA and RNA virus infection [39]. Grouper TRIM62 was found to negatively regulate an MDA5 but not an MITA-induced interferon response [7]. Although grouper TRIM35 weakens the MAVS-, MITA-, or TBK1-induced interferon response, it did not affect the MDA5-induced immune response [9]. Taken together, these findings indicated that EcfinTRIM82 negatively regulated the antiviral immune response against grouper fish viruses. However, whether EcfinTRIM82 interacts with MDA5, MITA, or MAVS requires further investigation.

In summary, we cloned a novel finTRIM82 gene and demonstrated its role during fish virus infection. Our results revealed that EcfinTRIM82 encoded a cytoplasmic protein and functioned as a critical negative antiviral factor against SGIV and RGNNV infection. Moreover, the overexpression of EcfinTRIM82 significantly decreased the expression of interferon-related signaling molecules or effectors, as well as proinflammatory cytokines. Moreover, EcfinTRIM82 was shown to negatively regulate the MDA5-, MITA-, MAVS-induced interferon response. Thus, our data demonstrated for the first time that grouper

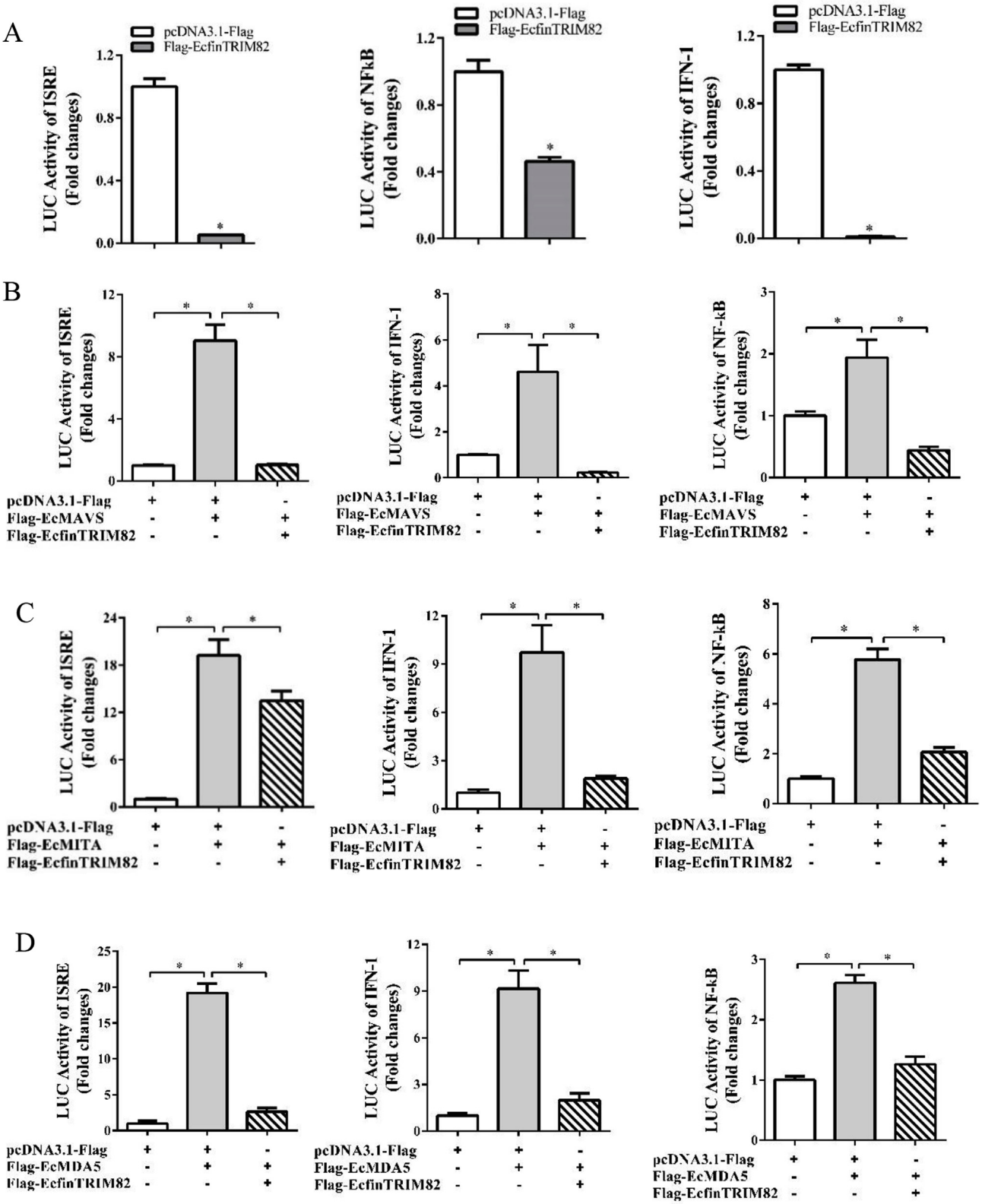

Fig. 10. EcfinTRIM82 overexpression down-regulated the interferon or NF-κB promoter activities induced by MDA5, MITA, or MAVS. (A) The ectopic expression of EcfinTRIM82 inhibited the activities of the IFN-1, ISRE, and NF-κB promoters. (B–D) EcfinTRIM82 overexpression reduced the MAVS-, MITA-, or MDA5-induced activities of the IFN-1, ISRE, and NF-κB promoters.

finTRIM82 exerted negative antiviral activity via reducing the host cell interferon response. Thus, these findings provide novel insight into the role of finTRIM proteins in the modulation of immune pathways during vertebrate evolution.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.004.

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