Fish and Shellfish Immunology 86 (2019) 474–479

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

RNF135 is a positive regulator of IFN expression and involved in RIG-I signaling pathway by targeting RIG-I

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ARTICLE INFO

Keywords:
RNF135
Zebrafish
RIG-I
IFN
Ubiquitination

ABSTRACT

RIG-I-like receptors (RLRs) play a key role in antiviral and inflammatory responses. Increasing evidence indicates that ubiquitination is crucial for regulation of RIG-I signaling pathway. Several ubiquitin ligases were reported to be involved in RIG-I-mediated signal transduction. In the present study, we demonstrated zebrafish RING finger protein 135 (zbRNF135) was a critical player in the regulation of RIG-I signaling pathway. *zbRNF135* mRNA was widely expressed in different tissues of zebrafish. The expression of *zbRNF135* was up-regulated post poly(I:C) treatment *in vivo* and in *vitro*. Furthermore, the expression profiles of RIG-I signaling pathway related genes (*LGP2*, *MDA5*, *RIG-I*, *MAVS*, *TRAF3*, *IRF3* and *IRF7*), together with its downstream molecules (*IFN1*, *ISG15*, *Mx* and *PKR*), were up-regulated by overexpression of zbRNF135 in ZF4 cells. Luciferase and ubiquitination assays revealed that overexpression of zbRNF135 facilitated zebrafish RIG-I (zbRIG-I)-mediated IFN1 promoter activation by mediating K63-linked ubiquitination of zbRIG-I. The co-immunoprecipitation assay showed that zbRNF135 specifically interacted with zbRIG-I. Our study indicated that zbRNF135 participated in innate immune response through modulating RIG-I signaling pathway.

1. Introduction

Innate immunity is the first line of host defense against infection, its activation requires the recognition of pathogen-associated molecular patterns through pattern-recognition receptors (PRRs). Several types of PRRs could recognize viral nucleic acid to activate innate immunity [1]. RIG-I like receptors (RLRs), as an intracellular PRR, mainly sense cytosolic viral nucleic acids, and then initiate a series of signaling events that lead to the production of type I interferon (IFN−I) to elicit a cellular antiviral response [2]. Insufficient IFN production causes chronic infection, whereas excessive innate immune response gives rise to pathogenesis of autoimmune and/or inflammatory diseases [3]. Thus, it is of great value to better understand the molecular mechanism of negative or positive regulation of innate immunity which is critical for efficient viral clearance.

It is well known that RLRs activities are regulated by post-translational modifications, such as ubiquitination and phosphorylation [4]. The ubiquitination of proteins plays an important role in regulating RLRs signaling pathway [5]. E3 ubiquitin ligases play central regulatory roles in the ubiquitination cascade reactions and function as negative or positive regulators of RLRs signaling pathway [6]. For instance, in human, TRIM25 positively regulated RIG-I signaling pathway via mediating K63-linked polyubiquitination of RIG-I [7]. Human Triad3A negatively regulates RIG-I-mediated signaling pathway through K48-linked, ubiquitin-mediated degradation of the tumor necrosis factor receptor-associated factor 3 (TRAF3) adapter [8].

In mammals, as the major families of E3 ubiquitin ligase, RING finger proteins (RNF) have been involved in the regulation of RIG-I signaling pathway. For example, RNF122 could mediate K48-linked ubiquitination of mouse RIG-I, consequently suppressing RIG-I-dependent antiviral response [9]. Mouse RNF114 targeted MAVS for proteasomal degradation to inhibit RLRs-mediated IFN production [10]. RNF123 functions as an inhibitor of RIG-I signaling pathway by interacting with RIG-I and MDA5 [11]. RNF135 facilitates RIG-I ubiquitination to up-regulate RIG-I-mediated IFN signaling [12].

In teleost fish, several homologous of RNFs have been cloned and characterized from zebrafish, Atlantic salmon and Chinese sturgeon [13–16]. Among these genes, RNF114 homologous from Chinese sturgeon was significantly up-regulated in poly(I:C)-treated Chinese sturgeon and proved to possess a potential antiviral role through

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https://doi.org/10.1016/j.fsi.2018.11.070
Received 3 October 2018; Received in revised form 21 November 2018; Accepted 29 November 2018
Available online 30 November 2018

modulating RIG-I signaling pathway [13]. Previous studies have shown that fish RIG-I signaling pathway related genes were associated with virus infection and played a major role in antiviral immunity [17,18]. Like mammal RLRs signaling, RLRs signaling in fish is also strictly controlled by several molecules to maintain immune homeostasis [19]. In this study, we demonstrate that zebrafish RNF135 (zbRNF135) acts as a positive regulator of RIG-I signaling pathway by targeting zebrafish RIG-I (zbRIG-I) and mediating its K63-linked ubiquitination.

2. Materials and methods

2.1. Cells and reagents

Anti-HA polyclonal, anti-Flag monoclonal and anti-Myc monoclonal antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Anti-*β*-actin antibodies were purchased from Invitrogen Corporation (Carlsbad, CA). PCDNA3.1-HA-Ub-K63 plasmid was purchased from Huzhen Corporation (Shanghai, China).

ZF4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 nutrient mix supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin-glutamine solution, at 28 °C, 5% CO_2. HEK293T cells were maintained in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 incubator.

2.2. Tissue distribution patterns of zbRNF135

One-year-old male and female AB line wild-type zebrafish were raised under an artificial photoperiod of 10 h darkness to 14 h light at 28 °C. All procedures carried out with zebrafish were approved by the Ethics Committee of Institutional Animal Care and Use Committee of Guangzhou Medical University.

For the tissue distribution analysis of zbRNF135, poly(I:C) was injected into the muscle of healthy zebrafish. Tissues samples of heart, intestine, muscle, gill, liver, brain, kidney and spleen from three individuals were collected at 24 h post-injection. The control fish were injected with phosphate-buffered saline (PBS).

2.3. Cell treatments with poly(I:C)

ZF4 cells cultivated on flasks (10^6 cells) were treated with 500 μl of poly(I:C) at the concentration of 6 mg/ml at 28 °C. The control was treated with the same volume of PBS. Cells from triplicates were harvested at 3, 6, 12, 24 and 48 h post poly(I:C) stimulation.

To assess the effect of zbRNF135 overexpression on induction of RIG-I signaling pathway related gene and its downstream factors, ZF4 cells were transfected with pCMV-myc vector or pCMV-myc-zbRNF135. At 24 h post transfection, cells were treated with poly(I:C) for 24 h, and harvested for total RNA isolation.

2.4. Plasmid constructions

The encoding region of zbRNF135 and zbRIG-I was amplified by PCR using primers listed in Supplementary Table S1 and cloned into pCMV-myc and pCMV-flag vector, respectively.

2.5. RNA extraction and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was isolated from various tissues of zebrafish (heart, intestine, muscle, gill, liver, brain, kidney, and spleen) or ZF4 cells using TRIzol Reagent (Takara) and cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara) according to the manufacturer's instructions. QRT-PCR was performed using SYBR Green I (Takara) and the LightCycler 480 System (Roche, Switzerland) as described previously [20]. Primers were listed in Supplementary Table S1. Zebrafish *β-actin* was used as a reference gene. Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Each sample was tested in triplicate.

2.6. Luciferase activity assay

Luciferase activity assay was performed as described previously [20]. HEK293T cells seeded overnight at 10^5 cells perwell in 24-well plates were cotransfected with various plasmids (pCMV-myc-zbRIG-I or pCMV-myc, DrIFN1 pro-Luc, pRL-TK) at a ratio of 10:10:1 (pCMV-myc-zbRIG-I or pCMV-myc:DrIFN1 pro-Luc:pRL-TK) using Lipofectamine™ 3000 at the same time. At 48 h post transfection, cells were harvested and lysed according to the Dual-Luciferase Reporter Assay System (Promega). Luciferase activities were measured by a Junior LB9509 luminometer (Germany) and normalised to the amounts of Renilla Luciferase activities. Results were expressed as mean ± SD from three independent experiments performed in triplicates. Luciferase activities were expressed as the fold stimulation.

2.7. Co-immunoprecipitation and western blot

HEK293T cells were seeded in 75 cm^2 dishes overnight and transfected with a total of 10 μg pCMV-myc-zbRNF135 and pCMV-flag-zbRIG-I plasmids. At 48 h posttransfection, cells were lysed on ice with 1 ml RIPA lysis buffer containing protease inhibitors (Calbiochem, Germany) for 30 min. The cell lysates were centrifuged at 12,000 g for 10 min at 4 °C, and then the supernatant was transferred to a fresh tube and incubated with protein A/G plus agarose (Calbiochem, Germany) together with 1 μg anti-Flag antibodies overnight at 4 °C with constant agitation. Immunoprecipitated proteins were collected by centrifugation at 5,000 g for 3 min at 4 °C, washed five times with lysis buffer. Samples were boiled with 1% (wt/vol) SDS sample buffer. The immunoprecipitates and whole cell lysates were analyzed by immunoblotting with anti-Myc and anti-Flag antibodies.

2.8. Ubiquitination assay

HEK293T cells were transfected with pCMV-Myc-zbRNF135 and pCDNA3.1-HA-Ub-K63 or pCMV-Flag-zbRIG-I and pCMV-Myc vector, respectively. Twenty-four hours after transfection, cells were treated with poly(I:C) and subjected to immunoprecipitation with anti-Flag antibodies and immunoprecipitates were analyzed by immunoblots with anti-HA and anti-Flag antibodies. The whole cell lysates were analyzed by immunoblots with anti-Flag, anti-Myc and *β-actin* antibodies.

2.9. Statistical analysis

Statistics were carried out using SPSS version 20. Differences between control and treatment groups were assessed by one-way ANOVA. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Inducible expression of zbRNF135 in vivo and in vitro

As shown in Fig. 1A, *zbRNF135* was ubiquitously expressed in all tissues tested, including kidney, liver, brain, gill, muscle, heart, eye, intestine and spleen, with the highest expression in the spleen. To investigate the induction pattern of *zbRNF135* in response to poly(I:C), zebrafish and ZF4 cells were stimulated with poly(I:C). In zebrafish, the expression of *zbRNF135* was significantly up-regulated in all examined tissues post poly(I:C) stimulation (Fig. 1B). In ZF4 cells, *zbRNF135* mRNA expression increased at 3 h and peaked at 24 h with the highest value of 7.2-fold higher than that of the control, followed by a slow decrease at 48 h (Fig. 1C).

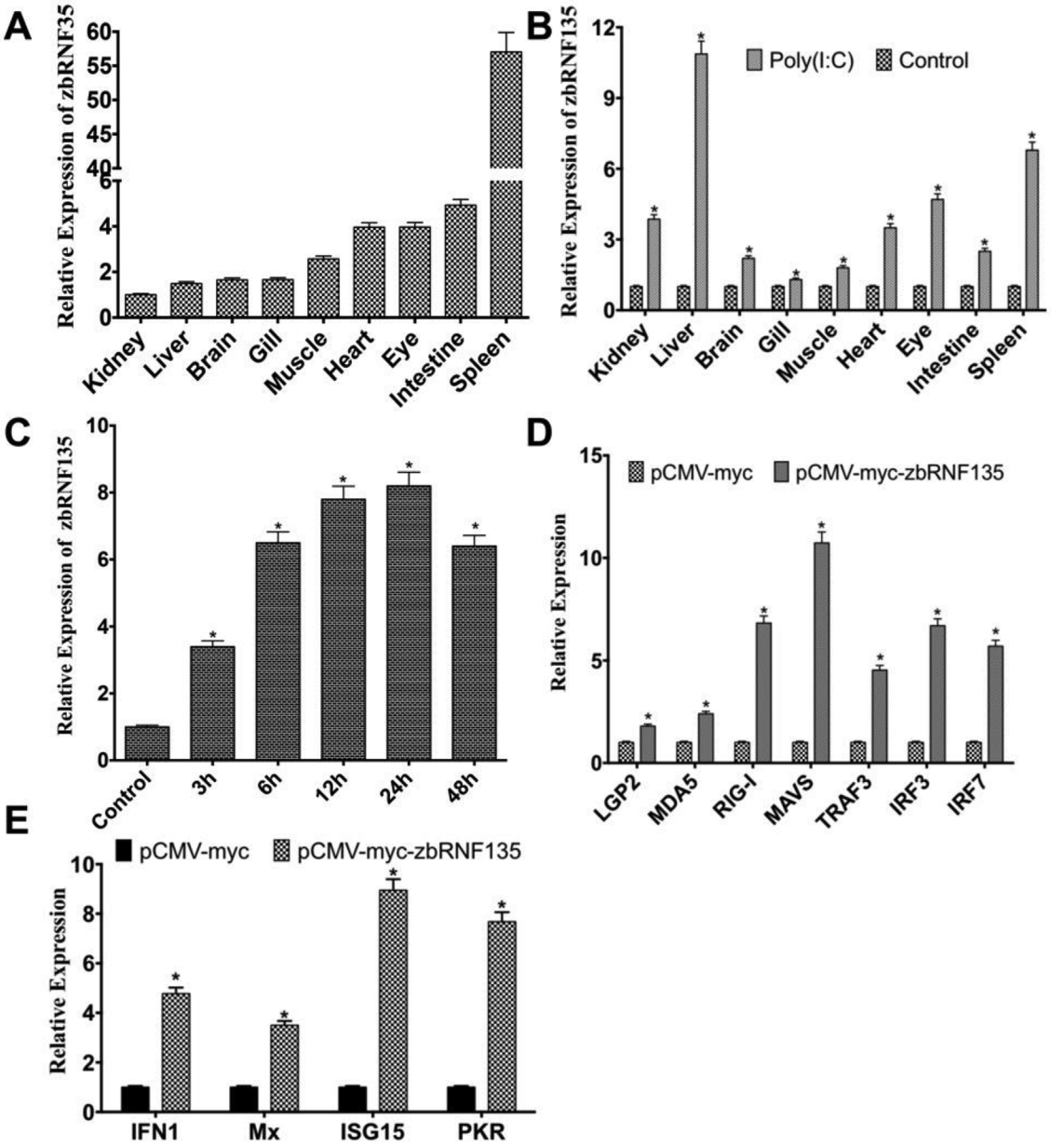

Fig. 1. Expression levels of *zbRNF135* and its related genes. (A) Expression analysis of *zbRNF135* mRNA in different tissues of zebrafish. The ordinate shows the fold-change in expression relative to kidney tissue. (B) Expression analysis of *zbRNF135* mRNA in poly(I:C) and PBS injected zebrafish. The ordinate shows the fold-change in expression relative to tissues of zebrafish injected with PBS. (C) Expression analysis of *zbRNF135* mRNA in ZF4 cells post poly(I:C) treatment from 3 to 48 h. (D) Expression levels of RIG-I signaling pathway related genes and its downstream factors in zbRNF135 overexpressing ZF4 cells post poly(I:C) treatment. The ordinate shows the fold-change in expression relative to ZF4 cells treated with PBS. (E) Expression levels of *IFN1* and *ISGs* in zbRNF135 overexpressing ZF4 cells post poly(I:C) treatment. The ordinate shows the fold-change in expression relative to ZF4 cells treated with PBS. Results were expressed as mean ± SD from three independent experiments performed in triplicates. Asterisks indicate significant differences between groups (*, $p < 0.05$).

3.2. *zbRNF135 promotes RIG-I signaling in ZF4 cells*

As shown in Fig. 1D, the mRNA expressions of zebrafish *LGP2*, *MDA5*, *RIG-I*, *MAVS*, *TRAF3*, *IRF3* and *IRF7* were up-regulated in zbRNF135 overexpressing ZF4 cells by 0.8-fold, 1.4-fold, 5.8-fold, 9.7-fold, 3.5-fold, 5.7-fold and 4.7-fold compared with the control group post poly(I:C) treatment, respectively. Furthermore, expressions of *IFN1*, *Mx*, *IFN-stimulated gene 15* (*ISG15*) and *PKR* were up-regulated in zbRNF135 overexpressing ZF4 cells post poly(I:C) stimulation (Fig. 1E).

3.3. *zbRNF135 promotes RIG-I-mediated IFN1 promoter activation*

Considering the relationship between RIG-I signaling pathway and zbRNF135, we examined the effect of zbRNF135 on IFN-I activity after stimulation with poly(I:C) by using a luciferase reporter gene driven by the zebrafish IFN1 promoter. Reporter activation was enhanced in zbRNF135 overexpressing HEK293T cells compared with the control during poly(I:C) stimulation (Fig. 2A). Co-expression of zbRNF135 with zbRIG-I potentiated activation of the IFN1 promoter in a dose dependent manner (Fig. 2A). Overexpression of K63-linked ubiquitin alone has no effect on zbRIG-I-mediated activation of IFN1 promoter activity. Co-expression of K63-linked ubiquitin and zbRNF135 enhanced the promotion effect of zbRNF135 on zbRIG-I-mediated activation of IFN1 promoter activity (Fig. 2B).

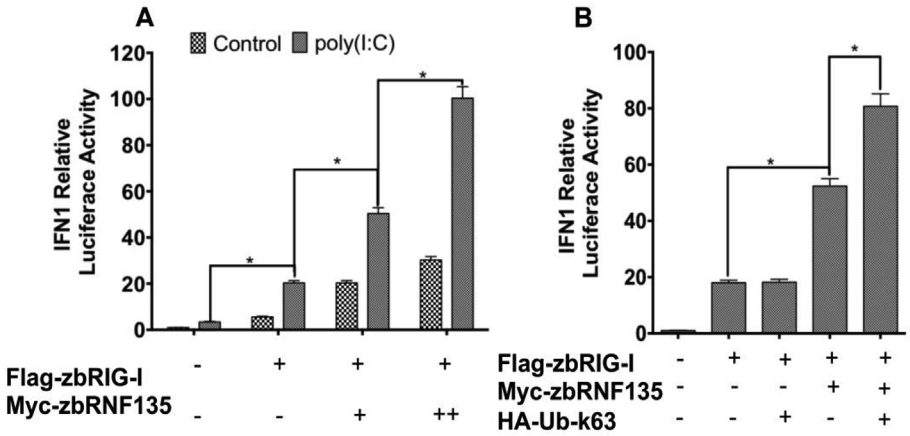

Fig. 2. zbRNF135 enhanced zbRIG-I-mediated zebrafish IFN1 promotor activity *in vitro*. (A) HEK293T cells were transfected with *Flag-zbRIG-I* plasmid, along with increasing amounts of *Myc-zbRNF135* plasmid. At 24 h after transfection, luciferase activities were measured. (B) HEK293T cells were transfected with the indicated plasmids for 24 h, then luciferase activities were measured. All data were from three independent experiments and expressed as mean ± SD. Asterisks indicate significant differences between groups (*, $p < 0.05$).

3.4. zbRNF135 interacts with zbRIG-I

PCMV-flag-zbRIG-I (Flag-zbRIG-I) plasmid was co-transfected with either *pCMV-myc-zbRNF135* (Myc-zbRNF135) or pCMV-myc plasmid in HEK293T cells to explore the conservation of RNF135/RIG-I interaction in zebrafish. Immunoprecipitation against the Flag tag revealed that Flag-zbRIG-I was co-immunoprecipitated with Myc-zbRNF135 (Fig. 3A), indicating the interaction of zbRNF135 with zbRIG-I.

3.5. zbRNF135 promotes K63-linked ubiquitination of zbRIG-I

To investigate whether zbRNF135 facilitated RIG-I-mediated IFN-I induction via ubiquitinating zbRIG-I, we further examined the effect of zbRNF135 on zbRIG-I ubiquitination. First, the expression of Flag-zbRIG-I, Myc-zbRNF135 and β-actin proteins was confirmed by western blot. Then, co-immunoprecipitation experiments showed that the K63-ubiquitination of zbRIG-I was markedly increased in the presence of zbRNF135 expression plasmid (Fig. 3B).

4. Discussion

In mammals, ubiquitin ligases have been reported to play central roles in the host defense against viral infection [21,22]. Studies in recent years have demonstrated that many fish ubiquitin ligases also have the similar functions. For example, Liao et al. reported that Chinese sturgeon RNF114 might participate in the innate immune response via mediating the RIG-I signaling pathway [13]. A HECT-type ubiquitin ligase, cloned from mandarin fish *Siniperca chuatsi,* might be involved in infectious spleen and kidney necrosis virus infection [23]. Fish TRIM proteins have important roles in innate immune response against viral infection [24,25]. However, the mechanism by which ubiquitin ligases work in teleosts still needs to be clearly demonstrated. Here, we demonstrated that zebrafish RNF135 was a key player in inducing IFN-I expression via regulating RIG-I signaling pathway.

Firstly, we examined *zbRNF135* mRNA expression *in vivo* and found it to be ubiquitously expressed in all the tissues tested. Similarly, previous study has shown that *RNF135* mRNA is also widely expressed in various tissues of human and mouse [26]. *zbRNF135* was highly expressed in spleen, indicating its association with the immune response. However, *zbRNF135* mRNA expression was low in other immune organ, such as kidney and liver. Studies have revealed that RNF135 play important roles in many different biological processes [27–30], such as proliferation, tumorigenesis and immunity, we speculated that the difference of zbRNF135 expression level between different immune organ might be closely associated with its role in different biological processes. It has been previously reported that *RNF135* mRNA is

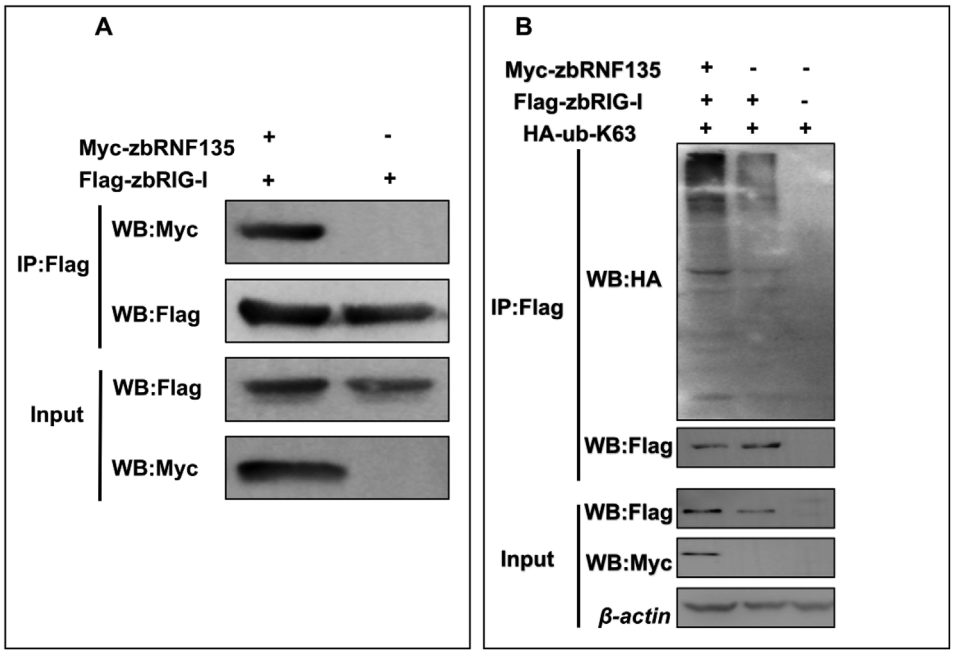

Fig. 3. zbRNF135 targeted zbRIG-I and promoted K63-linked ubiquitination of zbRIG-I. (A) Interaction of zbRIG-I and zbRNF135. *PCMV-myc-zbRNF135* or *pCMV-myc* and *pCMV-flag-zbRIG-I* plasmids were transfected into HEK293T cells for 48 h. Cells were lysed, Flag-tagged zbRIG-I was immunoprecipitated with anti-Flag antibodies. And the precipitates and cell lysates (input) were analyzed by western blot using anti-Flag or anti-Myc antibodies. (B) zbRNF135 mediated K63-linked poly-ubiquitination of zbRIG-I. *PCMV-myc-zbRNF135* and *pCMV-flag-zbRIG-I* plasmids were transfected into HEK293T cells together with HA-tagged K63 ubiquitin expression vector. At 24 h after transfection, transfected cells were treated with poly(I:C) and then lysated and immunoprecipitated with anti-Flag antibodies. The precipitates and cell lysates (input) were evaluated by immunoblot with anti-Myc, anti-Flag, anti-HA and *β-actin* antibodies.

induced by poly(I:C) stimulation in mammalian cells [12]. In fish, previous studies have reported that poly(I:C) can induce the expression of immune related gene, such as MDA5, IRF3 and IFN [31–33]. Thus, poly(I:C) was used to investigate the induction of *zbRNF135* mRNA in this study *in vivo* and *in vitro*. Gene expression analyses suggested that *zbRNF135* mRNA was up-regulated post poly(I:C) stimulation. Poly (I:C), a mimic of viral double stranded RNA, has been well known as a potent inducer of IFN-I. Therefore, our results indicated that zbRNF135 expression could be modulated by virus pathogens infection and zbRNF135 might be associated with the immune response.

RNF135 was reported to positively regulate RIG-I signaling pathway to promote IFN-β induction during the early phase of viral infection [27]. Thus, we further explored the effect of overexpression of zbRNF135 on the RIG-I signaling pathway in ZF4 cells. Overexpression of zbRNF135 significantly enhanced poly(I:C) induced expression of RIG-I signaling pathway related genes and its downstream factors, suggesting that zbRNF135 might induce IFN-I production through modulating the RIG-I signaling pathway. We next sought to determine the molecular mechanisms by which zbRNF135 facilitated IFN-I signaling. RIG-I is a well-known cytosolic viral RNA sensor that triggers a cascade of signaling events leading to the transcription of IFN and pro-inflammatory cytokines [34]. Considering that RIG-I is crucial in the production of IFN-I, its activation must be tightly regulated to maintain immune homeostasis, such as ubiquitination and phosphorylation [4]. Recently, multiple ubiquitin-like proteins have been reported to regulate RIG-I activation, such as RNF123, RNF125, RNF135 and RNF122 [9,11,26]. For instance, RNF122 promoted RIG-I proteasomal degradation by K48-linked ubiquitination. RNF135 has also been shown to promote K63-linked ubiquitination of RIG-I. Firstly, we characterized the role of zbRNF135 in RIG-I-mediated IFN-I inducing signaling by reporter gene analyses. Luciferase activity was enhanced by the ectopic expression of zbRIG-I together with zbRNF135 in a dose-dependent manner and the enhancement of zbRIG-I-mediated IFN1 promoter activity by zbRNF135 was further strengthen by overexpression of K63-linked ubiquitin, indicating zbRNF135 might function as a positive regulator of RIG-I signaling by mediated K63-linked polyubiquitination of zbRIG-I as in mammals. It was also revealed through ubiquitin assay that K63-ubiquitination of RIG-I induced by poly(I:C) was accelerated in cells co-transfected with zbRNF135. Mutational analysis demonstrated that Lys-788 within the RIG-I repressor domain (RD) was critical for mouse RNF135-mediated K63-linked polyubiquitination [27]. The exact locus for zbRNF135-mediated zbRIG-I ubiquitination needs to be further studied.

Previously, Oshiumi and colleagues firstly reported that RNF135 binded the RIG-I C-terminal RD and mediated the K63-linked polyubiquitination of RIG-I RD [27]. Differently, Gao and colleagues demonstrated that RIG-I N-terminal Caspase Activation and Recruitment Domains was required for interaction with RNF135 [35]. In our study, zbRIG-I was also identified as a target for zbRNF135-mediated polyubiquitination, indicating that the interaction of RIG-I and RNF135 was conserved in mammals and bony fish. The interaction sites of zbRNF135 and zbRIG-I need to be further elucidated.

In this study, we provided evidence that RNF135 functioned as a positive regulator of RIG-I signaling pathway in teleost. zbRNF135 promoted zbRIG-I ubiquitination and targeted zbRIG-I, exerting a pivotal role in the regulation of innate immune response. This study will contribute to our understanding of the regulatory mechanism of RIG-I signaling pathway in teleost.

Acknowledgments

This research program was financially sponsored by the National Natural Science Foundation of China (81400013, 81873419), Science and Technology Planning Project of Guangdong Province, China (2014A20212329), Department of education of GuangDong Province, China (2016KTSCX110), Medical and Health Plan Project in Huangpu District of Guangzhou City, China (201616) and Science and Technology Program of Guangzhou, China (201804010018). This work was completed in the Central Laboratory of the Fifth Affiliated Hospital of Guangzhou Medical University. We thank all members from the department of central laboratory, the Fifth Affiliated Hospital of Guangzhou Medical University for their invaluable help.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.070.

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