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A novel RING finger protein *Cq*RNF152-like with self-ubiquitination activity inhibits white spot syndrome virus infection in a crustacean *Cherax quadricarinatus*

Shu-cheng Zheng[a,1], Xue-jiao Chang[a,1], Wei-dong Li[a], Hao Wang[a], Li-mei Guo[a], Ke-jian Wang[a], Hai-peng Liu[a,b,*]

[a] State Key Laboratory of Marine Environmental Science, State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen University, Xiamen, 361102, Fujian, China
[b] Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology (Qingdao), China

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ABSTRACT

Really Interesting New Gene (RING) finger proteins are highly conserved molecules that participate in a variety of biological processes such as regulation of development, apoptosis and antiviral immunity in vertebrates. However, the functions of RING finger proteins are still poorly understood in crustaceans. Previously, we found that the transcript of a homolog of RING finger protein 152 (*Cq*RNF152-like) was up-regulated in a differentially expressed transcriptome library of the haematopietic tissue (Hpt) cells from red claw crayfish *Cherax quadricarinatus* upon white spot syndrome virus (WSSV) infection, which is one of the most devastating viral diseases for crustaceans like shrimp and crayfish. The full-length cDNA sequence of *CqRNF152-like* was then identified with 975 bp, including an ORF of 685 bp that encoded a 195 amino acids protein, a 5′- UTR of 180 bp, and a 3′-UTR with a poly (A) tail of 207 bp. The conserved domain prediction showed that *Cq*RNF152-like contained a conserved RING-finger domain. Gene expression analysis showed that *CqRNF152-like* was distributed in all tissues examined and the transcript is significantly up-regulated after WSSV challenge both *in vivo* in Hpt tissue and *in vitro* in cultured Hpt cells. Furthermore, the transcripts of both an immediate early gene *ie1* and a late envelope protein gene *vp28* of WSSV were clearly increased in the Hpt tissues, hemocytes and cultured Hpt cells after gene silencing of *Cq*RNF152-like, which were further proved to be significantly decreased after overloading of recombinant *Cq*RNF152-like protein in Hpt cell cultures. Meanwhile, *Cq*RNF152-like was found to bind with WSSV envelope protein VP28 by proteins pull-down assay. Similar to most of RNF proteins, *Cq*RNF152-like protein sequence contained a conserved RING-finger domain and showed self-ubiquitination activity in a RING finger domain dependent manner. Taken together, *Cq*RNF152-like is likely to function as an antiviral molecular against WSSV infection through interaction with the envelope protein VP28 in a crustacean *C. quadricarinatus*. This is the first report that a RING finger protein with directly antiviral functions via interaction with viral protein and self-ubiquitination activity in crustacean, which sheds new light on the molecular mechanism of WSSV infection and the control of white spot disease.

1. Introduction

RING finger proteins (RNF) are a family proteins that characterized by a RING finger domain, which generally serves as E3 ubiquitin ligase strongly associated with ubiqutination [1]. It's well-known that ubiqutination is one of the most important post translation modification, in which E3 ubiquitin ligase directly recognizes specific protein substrates in collaboration with E1 ubiquitin-activating enzyme and E2 ubiquitin-conjugating enzyme to transfer Ubiquitin (Ub) to target proteins, leading to proteasome-mediated degradation or changing of function and location [2]. Ubiqutination has been found to be involved in a broad range of physiological processes, including development, oncogenesis, apoptosis and innate immunity [3]. As one of the crucial E3 ubiquitin ligase family, RING finger proteins are also involved in innate immune response [4]. Accumulating evidences have shown that RING finger proteins play important roles in host immune responses to viral

* Corresponding author. State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen, 361102, Fujian, China.
E-mail address: Haipengliu@xmu.edu.cn (H.-p. Liu).
[1] Contribute equally to this work.

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infection through either inhibiting virus replication by directly targeting viral components for proteasome-mediated degradation, or regulating the innate immune signaling pathway and the subsequent cytokine induction. For instance, porcine RNF114 inhibits classical swine fever virus (CSFV) replication via targeting and catalyzing the K27-linked polyubiquitination of the nonstructural protein NS4B of CSFV for proteasome-dependent degradation [5]. In another case, various innate immune signaling pathways or antiviral effectors have also been found to be regulated by RING finger proteins via ubiquitination of specific target proteins. RNF5 targets both virus-induced signaling adaptor (VISA) and stimulator of interferon genes (STING) for K48-linked polyubiquitination and degradation mediated by proteasome to negatively regulate the virus-triggered induction of type I IFNs and cellular antiviral response [6,7]. However, most of studies associated with immune response in viral infection of RING finger proteins were focused on in humans, which are involved in RNA virus infection [8,9]. How the RING finger proteins function in DNA virus infection in invertebrate are rarely reported.

RNF152 was initially identified as a lysosome localized E3 ubiquitin ligase with pro-apoptotic activities [10]. Previously, RNF152-mediated K63-linked polyubiquitination of RagA GTPase was found to strongly inhibit mTORC1 activation [11]. A recent study further proved that RNF152 catalyzes GTP-bound Rheb GTPase ubiquitination followed by inactivating Rheb and mTORC1 to regulate tumor growth [12]. These studies revealed a crucial role of RNF152 in metabolism and tumorigenesis. In addition, RNF152 was also involved in development of the eyes, midbrain and hindbrain in zebrafish [13]. Nevertheless, the function implicated in immune response in viral infection of RFN152 has not been reported.

White spot syndrome virus (WSSV) is one of the most devastating crustacean viral pathogen with a wide host range, including all species of shrimps and other crustaceans such as crayfish, crab, and spiny lobster [14]. A great deal of work has been made on the immune regulatory molecules from shrimp and crayfish, however, due to the lacking of a shrimp cell line for WSSV propagation, the molecular regulatory mechanisms involved in the WSSV-host interaction have not been well interpreted [15]. It has been described that both signal crayfish *Pacifastacus leniusculus* and the red claw crayfish *Cherax quadricarinatus* can be infected by WSSV and its haematopietic tissue (Hpt) cell culture is suitable for WSSV replication [16,17]. Besides, several functional studies of anti-WSSV genes have been carried out in this useful cell culture model [18–21]. Previously, we found that *Cq*RNF152-like gene was up-regulated in a differentially expressed transcriptome library of Hpt cells from *C. quadricarinatus* post WSSV infection, indicating that *Cq*RNF152-like gene may participate in immune response in viral infection [22]. In the present study, molecular characterization, self-ubiquitination activity and anti-WSSV effect of *Cq*RNF152-like were studied. Our data provide the first evidence that *Cq*RNF152-like exhibited self-ubiquitination activity and exerted the antiviral function via interaction with WSSV envelope protein VP28 in a crustacean, which will definitely contribute to understand the molecular mechanism of antiviral immunity in crustacean and further enrich our knowledge of the function of RING finger proteins in invertebrate.

2. Materials and methods

2.1. Animals, Hpt cell cultures and virus

Freshwater red claw crayfish, *C. quadricarinatus*, were obtained from Source Sentai Agricultural Science and Technology Co., Ltd of Zhangzhou, Fujian Province, China and cultivated in aerated tanks at 26 °C. Only inter-molting healthy crayfish were used in this study. Hpt cells were isolated from *C. quadricarinatus* and cultured as described in previous study. WSSV was kindly provided by Prof. Xun Xu (Third Institute of Oceanography, SOA, Xiamen, Fujian, China). The virus was prepared as described by Xie et al. and quantified via absolute quantification by PCR [23].

2.2. Tissues collection, RNA extraction and cDNA synthesis

After acclimation in aerated freshwater at 26 °C for one week, crayfish tissues were collected. Haemocytes were obtained with a sterile needle and centrifuged with 1000 × g for 10 min at 4 °C. Other tissues such as haematopietic (Hpt), hepatopancreas, gill, heart, stomach, epithelium, gonad, muscle, nerve, intestine, and eyestalk were sampled from three random individuals for total RNA isolation accordingly.

Total RNA from tissues was isolated by using Trizol reagent (Roche, Germany) according to the manufacturer's instruction. RNase-Free DNase I (Ambion, USA) was used to eliminate genomic DNA contamination in the extracted RNA following the manufacturer's protocol. The extracted RNA was evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Total RNA extracted from Hpt was reversely transcribed using the SMARTer™ RACE cDNA Amplification kit (Clontech, USA) and PrimeScriptTM RT Reagent Kit (TaKaRa) for full-length cDNA cloning according to the manufacturer's instruction.

2.3. Gene cloning of the full-length cDNA of CqRNF152-like and bioinformatics analysis

A partial *CqRNF152-like* cDNA sequence of *C. quadricarinatus* was obtained from a transcriptome library upon WSSV infection in our lab (unpublished data). The gene-specific primers for RACE were shown in Table 1. The PCR conditions were as follows: 5 min at 94 °C; 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s; and 72 °C for 10 min. All amplified PCR products were gel-purified using a Gel Extraction Kit (Dongsheng Biotech) and the expected DNA fragments were ligated into a pMD18-T vector (TaKaRa) for sequencing at Sangon Biotech (Shanghai) Co., Ltd. Then, the full-length cDNA sequence of *CqRNF152-like* was assembled using DNAMAN software.

The *CqRNF152-like* of Open Reading Frame (ORF) and amino acid sequence was analyzed by using the ExPASy translate tool (http://web.expasy.org/translate/). The homologous conserved domains were identified by SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de). The 3D domain structure of *Cq*RNF152-like was constructed by using SWISS-MODEL server (http://swissmodel.expasy.org/). The alignment of the RING domain in *CqRNF152-like* was carried out by DNAMAN6.0. The phylogenetic tree was constructed with Mega 6.06 using the Neighbour-Joining method based on sequence alignments of *CqRNF152-like* with other homologous amino acid sequences.

Table 1
Primer sequences used in this study.

Primers	Sequence
RNF152-like-F	ATGTCTTTGTATCCCACG
RNF152-like-R	AGGATTAGTATTTTCCATGA
RNF152-like-GSP1	ACAGTGTATTGGTAGTAGCAGGGAT
RNF152-like-GSP2	GTTTCTTACACTCGTTTTTCTTGAC
UPM	CTAATACGACTCACTATAGGGC
qRNF152-like-F	AGTGTATCCACCAGTTGC
qRNF152-like-R	CGTAGTTTGCCATAGTCA
16S–F	AATGGTTGGACGAGAAGGAA
16S-R	CCAACTAAACACCCTGCTGATA
dsRNF152-like-F	TCTTTGTATCCCACGTTACCAGACG
dsRNF152-like-R	TAGTTGAGTAGGTTGCATCGACAGC
dsGFP-F	CGACGTAAACGGCCACAAGT
dsGFP-R	TTCTTGTACAGCTCGTCCATGC
IE1-F	CTGGCACAACAACAGACCCTACC
IE1-R	GGCTAGCGAAGTAAAATATCCCCC
VP28–F	AAACCTCCGCATTCCTGT
VP28-R	GTGCCAACTTCATCCTCATC

2.4. Distribution profile of CqRNF152-like in different tissues from the red claw crayfish

Twelve tissues were obtained from three random healthy crayfish and prepared for total RNA isolation and cDNA synthesis as described above. To determine the *CqRNF152-like* mRNA transcript levels in the different tissues, qRT-PCR was performed using an ABI PCR machine (Applied Biosystems 7500, UK). A pair of specific primers (Q-F/Q-R in Table 1) was designed by using Primer Premier 5.0 to amplify a target product from the cDNA, and the crayfish 16S rRNA gene (Genbank no: AF135975.1) was employed as an internal standard. *CqRNF152-like* expression levels relative to 16S ribosomal gene expression were quantified by using the $2^{-\triangle\triangle Ct}$ method [24]. The primers designed for 16S rRNA gene amplification were shown in Table 1. The qRT-PCR reactions were performed in a 96-well PCR plates. The reaction volume consisted of 20 μL of mixture containing 10 μL of SYBR Green Master (2 ×) (Roche, USA), 0.5 μL of each primer (10 mM), 5.0 μL of cDNA, and 4 μL of sterile water. The qRT-PCR program was as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The amplification specificity of *CqRNF152-like* and 16S rRNA was confirmed by analysing the melt curves following PCR reactions. Each reaction was performed in triplicates.

2.5. The gene expression of CqRNF152-like in vivo and in vitro upon WSSV challenge

A total of 36 healthy red claw crayfish (10 g ± 2 g with body weight and 5 cm ± 1 cm in body length) were divided into 2 groups (18 specimens per group). The experimental group was infected by injection of 10^5 copies of WSSV/10g body weight with sterile syringe in the fourth walking leg. The control group was injected with the equivalent volume of crayfish saline (CFS). Haemocytes from three crayfishes were collected from each group at indicated time points (1 h, 6 h, 12 h, 24 h and 48 h after injection) accordingly. Hpt cells from *C. quadricarinatus* were isolated from three random individuals and cultured in a 24-well plate followed by infected with WSSV (MOI = 1) or incubated with the equivalent CFS, respectivley. Hpt cell cultures were collected with cell lysis from each group at indicated time points for RNA extraction and qRT-PCR.

2.6. RNA interference assay

RNA interference assay was carried out by double-strand RNA (dsRNA) injection into the animals or transfection into Hpt cell cultures. The primers for PCR amplification of target sequence were shown in Table 1. The dsRNA was synthesized by using the MegaScript kit (Ambion, USA) according to the manufacturer's instructions and purified with the Trizol Isolation Reagent (Roche, USA).

For RNAi assay *in vivo*, a total of 12 healthy red claw crayfish were divided into 2 groups (6 specimens per group). The individual was injected with 1 μg dsRNA/g body weight with sterile syringe followed by WSSV infection (500 copies/g) in the fourth walking leg. Injection of the equivalent green fluorescent protein (GFP) dsRNA was served as the negative control. Haemocyte and hematopoietic tissue from three individual crayfish were collected from each group at indicated time points (6 h and 12 h post WSSV injection) for further analysis.

For RNAi assay *in vitro*, Hpt cells were seeded into 24-well culture plates and cultured in 500 μL of L15 medium (HyClone, USA). One hundred nanogram for per 1×10^5 cells of dsRNA was transfected with Cellfectin II Reagent (Life Technologies, USA) in Hpt cells according to the manufacturer's protocol. After 36 h, Hpt cells were infected with WSSV (MOI = 1) and collected at indicated time points for further analysis. The GFP dsRNA treated cells were used as negative control treatment. The experiments were repeated in triplicates.

2.7. Expression and purification of CqRNF152-like recombinant protein

*Cq*RNF152-like recombinant protein fused with MBP tag and His tag was expressed from pMAL-c2X with minor modification and VP28 fused with GST tag was expressed from pGEX-4T-1 in *E. coli* BL21 (DE3), which were both induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 20 h at 16 °C. Then, the MBP-His-*Cq*RNF152-like recombinant protein was purified with Dextrin Beads 6FF followed by purification with a Ni-NTA affinity column and washed by washing buffer (50 mM Tris-HCl, 500 mM NaCl, pH 7.4) with 40 mM, 80 mM and 120 mM of imidazole in series. Finally, the purified MBP-His-*Cq*RNF152-like recombinant protein was collected by elution buffer (50 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, pH 7.4) and dialyzed by 1 × PBS.

2.8. Proteins pull-down assay

WSSV envelope components were prepared as described previously [23]. Briefly, the purified virus suspension was centrifuged at 20,000 × g for 30 min at 4 °C and the pellets were resuspended in 0.4 ml salt-containing buffer TMN (20 mM Tris-HCl, 150 mM NaCl, 2 mM MgCl2, pH 7.5). Then, the supernatant was collected after centrifugation at 20,000 × g for 20 min at 4 °C and analyzed by SDS-PAGE and staining with Coomassie brilliant blue. Equal amounts of the WSSV envelope proteins were divided into each tube. Five microgram of MBP-His-*Cq*RNF152-like or MBP control protein and 20 μL of dextrin beads was added into each tube and rotated for 2 h at 4 °C with end-over-end mixing. After incubation, the beads were washed with PBS for 5 times. The binding proteins were eluted for 2 h at 4 °C by incubation with 15 μL of maltose elution buffer (10 mM maltose in PBS buffer). The protein sample was denatured by boiling for 10 min and analyzed by electrophoresis in 12% SDS-PAGE gels.

2.9. Antiviral assay with CqRNF152-like recombinant protein

Hpt cells were seeded into 24-well culture plates and cultured in 500 μL of L15 medium (HyClone, USA). Briefly, 4 μL of PULSin (Polyplus, France), 100 μL of 20 mM Hepes and 1 μg of MBP-His-*Cq*RNF152-like were used to prepare the mixture of solution. The mixed solution was incubated at room temperature for 15 min, and then added into each well. After 4 h incubation, the medium was removed and cells were washed with PBS. Then, the Hpt cells were infected with WSSV (MOI = 1) followed by collection with SDS sample loading buffer (Solarbio, China) for Western blot analysis or collection with cell lysis (Sigma, USA) for RNA extraction and qRT-PCR.

2.10. Expression of CqRNF152-like wild type and mutants in Baculovirus expression system

CqRNF152-like ORF with restricted digestion sites *Bam*H I and *Xba* I was subcloned into modified pFastBac1 Baculovirus transfer vector. Meanwhile, point mutations of *CqRNF152-like* ORF were introduced by overlapped PCR to generate the *CqRNF152-like* mutants C50A (cysteine-to-alanine) and H50A (histidine-to-alanine), respectively. Recombinant pFastBac1 vectors were transformed to *E. coli* DH10Bac and the positive recombinant Baculovirus were transfected in a monolayer culture of *Spodoptera frugiperda* cells (Sf9) that grown at 26 °C in SIM SF insect SF9/SF21 cell serum-free medium (SinoBiological, China) supplemented with a final concentration of 1% penicillin and streptomycin (Solarbio, China). The recombinant Baculovirus was harvested at 96 h post transfection. Plaque assays were then performed on the supernatants to determine the titer of recovered recombinant virion particles. The Sf9 cells were infected with recombinant Baculovirus at 10 multiplicities of infection (MOI) and harvested at 72 h post infection for co-immunoprecipitation, immunobloting and ubiquitination assay.

2.11. Co-immunoprecipitation, immunoblotting and ubiquitination assays

Sf9 cells cultured in 10-cm^2 dishes were infected with the indicated recombinant Baculovirus. After 72 h, the infected cells were collected and lysed with cell lysis buffer (20 mM Tris (pH7.5), 150 mM NaCl, 1% Triton X-100) supplemented with protease inhibitor (APExBIO, USA). The cell supernatant was collected and incubated with Ni^{2+}-NTA His Bind Resin (Smart-lifesciences, China) at 4 °C overnight. After washing with lysis buffer for five times, the samples were boiled with SDS sample loading buffer (Solarbio, China) for 10 min. The protein samples were prepared for SDS-PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, USA). The membranes were blocked in 5% non-fat milk in 1 × Tris-buffered saline with 0.1% Tween 20 (TBST) and then incubated with diluted primary antibodies, including anti-His monoclonal antibody (Proteintech, USA), anti-FLAG monoclonal antibody (Sigma, USA), anti-β-actin monoclonal antibody (Proteintech, USA) and anti-VP28 monoclonal antibody accordingly at room temperature for 1 h. After washing with TBST for three times, anti-mouse IgG monoclonal antibody conjugated to horseradish peroxidase (TransGen Biotech, China) were used as secondary antibodies. After washing with TBST for three times, an enhanced chemiluminescence system was used for detection. Ubiquitination was determined by Western blotting using the anti-Ub monoclonal antibody (Santacruz, USA).

2.12. Statistical analysis

The data were analysed by Student's *t*-test and presented as the mean ± SD from three independent assays for comparison between two groups by using SPSS ver. 18.0 software, in which the $p < 0.05$ was considered as statistically significant.

3. Results

3.1. CqRNF152-like is a novel RING finger protein

Previously, we found that a strong up-regulation of *Cq*RNF152-like gene in a differentially expressed transcription library from red claw crayfish Hpt cells upon WSSV infection, indicating that *Cq*RNF152-like gene was likely to be involved in the cellular response against WSSV infection [22]. However, the role of *CqRNF152-like* gene in host-WSSV interactions was yet unknown which needs for further studies. In order to elucidate the role of *CqRNF152-like* gene in host-WSSV interaction, we then cloned the full length cDNA of *CqRNF152-like* gene (Genbank no: AEL23143.1) by using RACE technology based on the partial cDNA sequence of *CqRNF152-like* from the red claw crayfish transcriptome (unpublished data). As shown in Fig. 1A, the full-length cDNA of *CqRNF152-like* was 975 bp, including an ORF of 685 bp that encoded a 195 amino acids protein, a 5′-Untranslated Regions (UTR) of 180 bp, and a 3′-UTR with a poly (A) tail of 207 bp. The calculated protein molecular weight was about 21.5 kDa with pI of 4.67. The conserved domain prediction and 3D structure analysis showed that *Cq*RNF152-like contained a conserved RING-finger domain, suggesting that *Cq*RNF152-like may serve as an E3 ubiquitin ligase (Fig. 1B). Meanwhile, *Cq*RNF152-like exhibited 60.6% identity of amino acid sequence with RNF152-like from *Penaeus vannamei*, but was with lower identity to RNF152 homologs from other species, including *Pangasianodon hypophthalmus*, *Ictalurus punctatus*, *Danio rerio* and *Mus musculus* (Fig. 2A). Surprisingly, *Cq*RNF152-like also exhibited 34%–36% identity of amino acid sequence with a member of another E3 ubiquitin ligase family Tripartite motif (TRIM) 32 from various species. Phylogenetic analysis further showed that *Cq*RNF152-like and *Penaeus vannamei* RNF152-like are presented as an independent branch, but more close to TRIM32 from other species (Fig. 2B). Collectively, these data may reveal a complex evolution status of *Cq*RNF152-like and exhibit the species-specific RING finger proteins in crustacean.

(A)

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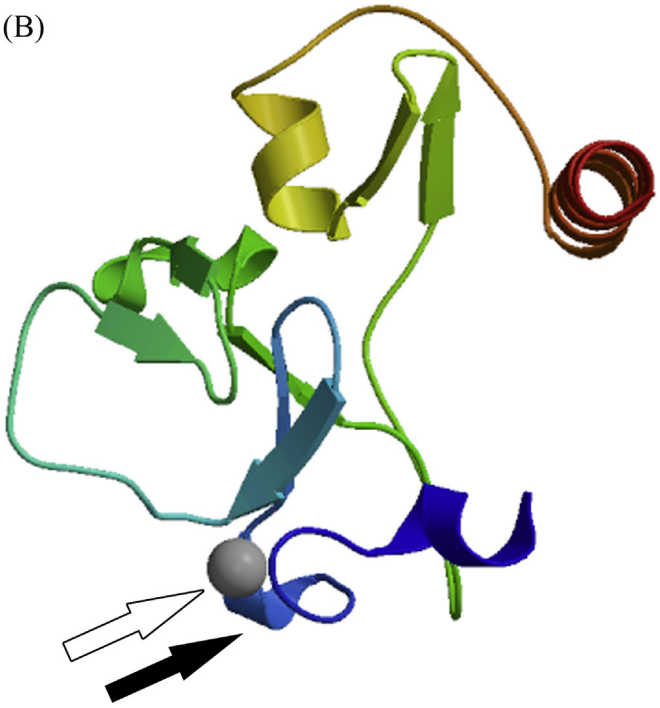
1     GAGTCTGAGTGTTGACGCCAGTGGTGTAAGGTGTGGTGGTGCTGCAGGCAAAAATTCCAA
61    TATGAAGTACAGCCATGGAAAATACCGAGTGACTGATAAATTGTGAAATACAGTATATTA
121   AAAAATAAGTCAAGAAAAACGAGTGTAAGAAACTTGGAAAGAGACAAAACGAAAAAAAG
181   ATGTCTTTGTATCCCACGTTACCAGACGATGCTTCGGTTCTGAGAGAGAGCAAGTCTTGC
61     M  S  L  Y  P  T  L  P  D  D  A  S  V  L  R  E  S  K  S  C
241   GAGGCCTCACACTCCCTCCTGCCAACTTGCTCAGTGTGCCTGGATGAATTTTGCGAGAAG
81     E  A  S  H  S  L  L  P  T  C  S  V  C  L  D  E  F  C  E  K
301   AGGCTCCCAAAATACCTTACATGTCATCACACTTTCTGTGATGAGTGTATCCACCAGTTG
101    R  L  P  K  Y  L  T  C  H  H  T  F  C  D  E  C  I  H  Q  L
361   CTCAAGTTTGGCAAGATTGAGTGTCCTCTGTGTCGCAAGATCACTCAAGTTACCTCAGCT
121    L  K  F  G  K  I  E  C  P  L  C  R  K  I  T  Q  V  T  S  A
421   GACGTCCTCCAGACCAACAATGATGTTCTGACTATGGCAAACTACGGAAATCTTTTTGTT
141    D  V  L  Q  T  N  N  D  V  L  T  M  A  N  Y  G  N  L  F  V
481   AATCTGTGGTGTCATGACTGCCAAGACATACCCAAGGCCACCTGCATGACCCACAACGTT
161    N  L  W  C  H  D  C  Q  D  I  P  K  A  T  C  M  T  H  N  V
541   AGCAACCGGTCAGCCACCTCCTGCGCTACCATTGGTCAACAGGTGGACAGAACTGTGGAA
181    S  N  R  S  A  T  S  C  A  T  I  G  Q  Q  V  D  R  T  V  E
601   GCTTGGACCCCCAGTGTAATGCGCCTAGTAGAGACGACACAAGGCTGGGCTCTCATCGCT
201    A  W  T  P  S  V  M  R  L  V  E  T  T  Q  G  W  A  L  I  A
661   GTCGATGCAACCTACTCAACTATAACTAGCATTTCAGAATATGTAACTCCATTAACATCT
221    V  D  A  T  Y  S  T  I  T  S  I  S  E  Y  V  T  P  L  T  S
721   TATATCACTGACTGGTTTAATTCTTTCATGGAAAATACTAATCCTTGATGAAAAATAAAT
241    Y  I  T  D  W  F  N  S  F  M  E  N  T  N  P  *
781   TCAAGGACATAGATTAAATGTATTGTTACTAGTATATACAGTGTATTGGTAGTAGCAGGG
841   ATTAGCAGATTTATTTGTAAAATAATATACACGTTAACTGTATAATATTACAGTTTATTG
901   TATTCTTGAATGTCACAACTAATAAATAAAATTATGTGATTTTGCAAAAAAAAAAAAAAA
961   AAAAAAAAAAAAAA
```

(B)



(caption on next page)

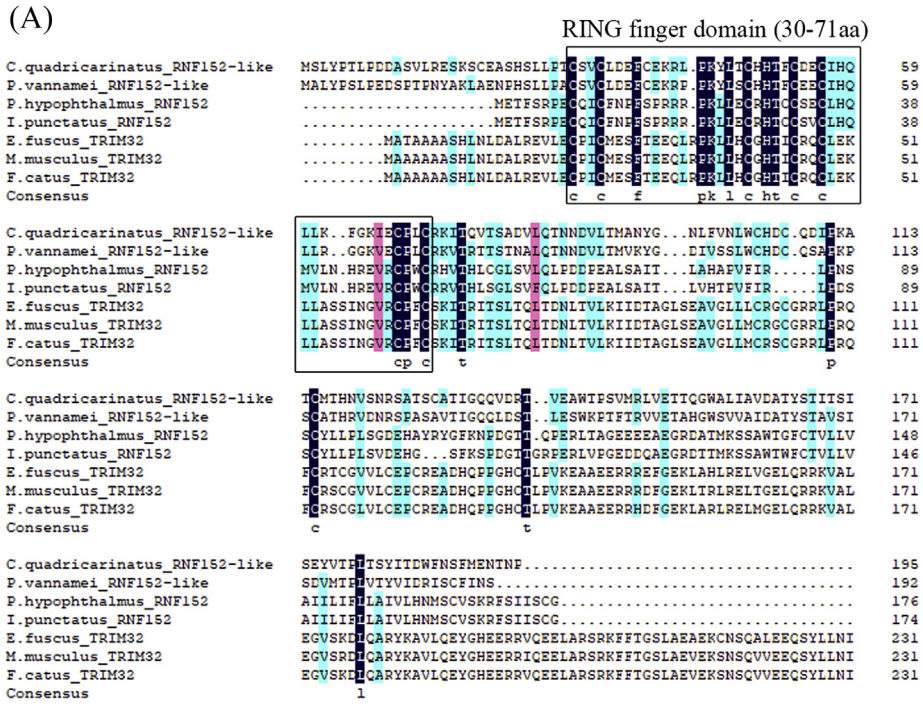
Fig. 1. The full-length cDNA sequence, deduced amino acid sequence and 3D structure model of *CqRNF152-like* from *C. quadricarinatus*. (A) The full-length cDNA sequence and deduced amino acid sequence. The conserved RING finger domain is underlined. The asterisk stands for the stop codon. (B) The 3D structure model of *CqRNF152-like*. The SWISS-MODEL template library was searched with Blast and HHBlits for evolutionary related structures matching the target sequence. The hollow arrow indicates the Zinc ion and the solid arrow indicates RING finger that binds to Zinc ion.

3.2. Tissue distribution and expression profile of CqRNF152-like post WSSV infection

To analyze the distribution of *CqRNF152-like* transcript in different tissues of red claw crayfish, the relative mRNA expression of *CqRNF152-like* was determined by qRT-PCR in selected samples, including haemocyte, stomach, gonad, muscle, nerve, intestine, heart, Hpt, hepatopancreas, gill, epithelium and eyestalk from the red claw crayfish. As shown in Fig. 3A, the transcript of *CqRNF152-like* was predominantly detected in muscle, nerve, gonad and hematopoietic tissue, especially with the highest expression in muscle. To further examine whether *CqRNF152-like* was in responsive to WSSV infection, the transcript of *CqRNF152-like* in both Hpt tissue *in vivo* and in Hpt cell culture *in vitro* were detected from red claw crayfish after WSSV challenge at different time points accordingly. As shown in Fig. 3B, the gene expression of *CqRNF152-like* was significantly increased at both 24 and 48 h post WSSV infection in Hpt tissue *in vivo*. Meanwhile, the expression of *CqRNF152-like* was also significantly up-regulated at 12 h and 24 h in Hpt cell cultures *in vitro* post WSSV infection (Fig. 3C), which was consistent with that from our previous study in that *CqRNF152-like* was significantly up-regulated at 12 h in Hpt cell cultures *in vitro* after WSSV infection [22], suggesting that *CqRNF152-like* was responsive to the WSSV infection both *in vivo* and *in vitro* in red claw crayfish. This finding clearly demonstrates that *CqRNF152-like* gene is likely to be involved in the WSSV infection in red claw crayfish.

3.3. CqRNF152-like inhibits WSSV replication both in vivo and in vitro

To further identify how *Cq*RNF152-like functioned in WSSV



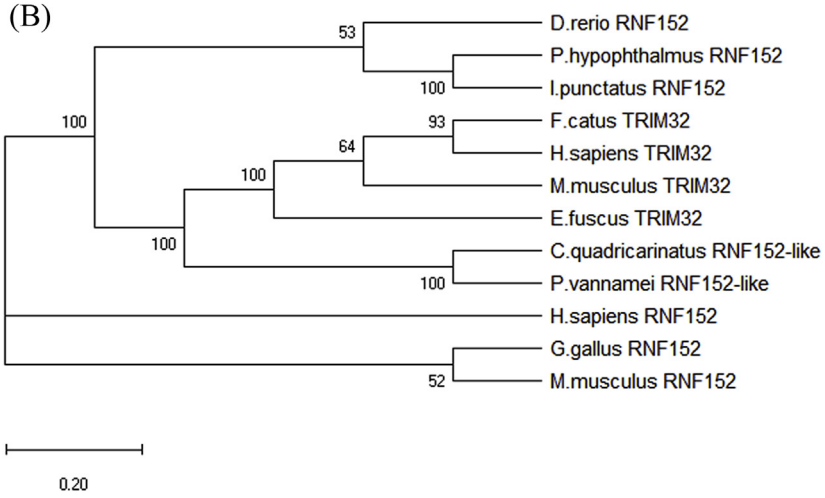


Fig. 2. Multiple sequences alignment and the phylogenetic analysis of *CqRNF152-like*. A: Multiple sequences alignment of putative amino acid sequences with highly similar homolog from various species based on Basic Local Alignment Search Tool (BLAST). The conserved RING finger domain is shown in box. The amino acid sequences of homolog of RNF152 or TRIM32 were selected from *C. quadricarinatus* (AEL23143.1) and other species, including *Penaeus vannamei* RNF152-like (XP_027208566.1), *Pangasianodon hypophthalmus* RNF152 (XP_026796755.1), *Ictalurus punctatus* RNF152 (XP_017351929.1), *Eptesicus fuscus* TRIM32 (XP_008150332.1), *Mus musculus* TRIM32 (AAO13297.1) and *Felis catus* TRIM32 (NP_001295976.1). (B) The phylogenetic tree of *CqRNF152-like* with homolog of RNF152 or TRIM32 within various species. In addition to the highly similar species above, other homologs are shown as follows: *Homo sapiens* RNF152 (NP_775828.1), *Danio rerio* RNF152 (NP_001014380.2), *Gallus gallus* RNF152 (NP_001291963.1) and *Mus musculus* RNF152 (NP_001153840.1).

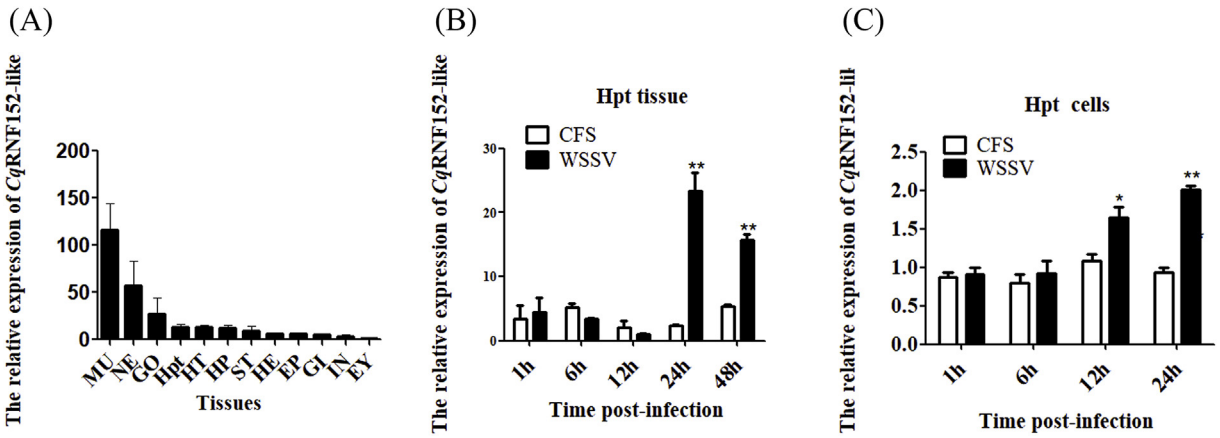


Fig. 3. Tissue distribution and expression profile of *CqRNF152-like* post WSSV infection. (A) The relative expression of *CqRNF152-like* gene in different tissues from *C. quadricarinatus*. Muscle (MU), nerve (NE), gonad (GO), hematopoietic tissue (Hpt), heart (HT), hepatopancreas (HP), stomach (ST), haemocyte (HE), epithelial tissue (EP), gill (GI), intestine (IN), eyestalk (EYE). The eyestalk was employed as an internal control group. (B) The expression profile of *CqRNF152-like in vivo* upon WSSV challenge. CFS injected crayfish was used as the control treatment. (C) The expression profile of *CqRNF152-like in vitro* in Hpt cell cultures post WSSV infection. CFS treatment was used as control group. This experiment was performed in biological triplicates. The significant difference is indicated by asterisk in comparision with those of controls (*$p < 0.05$, **$p < 0.01$).

infection, the viral transcripts were determined in crayfish after gene silencing of *CqRNF152-like* followed by WSSV infection. As shown in Fig. 4A, the gene knockdown efficiency of *CqRNF152-like* was about 60% in Hpt tissue after injection of dsRNA targeted *CqRNF152-like* compared with those of negative controls. Moreover, the transcript of the viral immediate early gene *ie1* was significantly increased at 6 h and 12 h post WSSV infection in Hpt tissue after gene silencing of *CqRNF152-like* when compared to those of negative controls (Fig. 4B). Meanwhile, a late viral gene *vp28* also showed similar expression profile after WSSV infection in *CqRNF152-like* silenced Hpt tissue, in which the viral transcript was markedly increased at both 6 h and 12 h compared to those of controls (Fig. 4C). Similar result was also observed in hemocyte that is critical for immune clearance of invading pathogens in invertebrates (Fig. 4D, E and 4F). Taken together, these results clear suggest that *CqRNF152-like* plays an antiviral function against WSSV replication *in vivo*.

To further prove above findings, RNA interference assay was also performed *in vitro* in Hpt cell cultures. As shown in Fig. 4G, the gene expression of *CqRNF152-like* was significantly reduced about 95% compared with that of the control cells during WSSV infection till 12 h tested, indicating that *CqRNF152-like* was efficiently silenced in Hpt cell cultures. Similarly, the transcript of *ie1* gene was significantly increased in *CqRNF152-like* silenced Hpt cells than those of control groups at both 3 h and 6 h after WSSV infection (Fig. 4H). Meanwhile, *vp28* gene also showed similar expression profile after WSSV infection in *CqRNF152-like* silenced Hpt cells, which transcript was markedly increased at both 6 h and 12 h compared to those of control cells (Fig. 4I). These data confirm the speculation above that *CqRNF152-like* has anti-WSSV property.

3.4. Antiviral activity of recombinant CqRNF152-like protein

To further prove the antiviral function of *Cq*RNF152-like during WSSV infection, *Cq*RNF152-like recombinant protein fused with MBP and His tag were expressed and purified from *E. coli*. As shown in Fig. 5A, the purified *Cq*RNF152-like recombinant protein was successfully obtained, which was in agreement with the calculated molecular mass of *Cq*RNF152-like protein and was confirmed by MALDI-TOF/TOF mass spectrometry analysis as several peptide fragments were corresponding to the deduced protein sequences of *Cq*RNF152-like (data not shown). Then, the MBP-His-*Cq*RNF152-like recombinant protein was overloaded into Hpt cell cultures by protein delivery kit followed by infection with WSSV. The extra MBP-His-*Cq*RNF152-like recombinant protein was detected in Hpt cells by Western blotting, indicating a successful overloading delivery of recombinant protein into Hpt cells (Fig. 5B). Importantly, both of the transcripts of WSSV *ie1* and *vp28* were markedly decreased in Hpt cell cultures after overloading of MBP-His-*Cq*RNF152-like recombinant protein when compared to those of control groups (Fig. 5C–D). Taken together, both the gene silencing assay and the recombinant protein overloading assay results strongly suggest that *Cq*RNF152-like inhibits WSSV replication.

3.5. CqRNF152-like binds with WSSV envelope protein VP28

In the case of WSSV, VP28 is the most abundant envelope protein which has been found to interact with various cellular factors to facilitate the viral infection or as the target of antiviral effectors [23,25]. To identify whether *Cq*RNF152-like protein could also bind to VP28, an *in vitro* MBP pull-down assay between WSSV envelope components and MBP-His-*Cq*RNF152-like recombinant proteins was performed. As shown in Fig. 6A, MBP-His-*Cq*RNF152-like recombinant protein was found to bind with VP28 from WSSV envelope components by Western blotting with anti-VP28 monoclonal antibody. To further prove the above result, MBP-His-*Cq*RNF152-like recombinant protein and VP28 with GST-tag were used in MBP pull-down assay. As shown in Fig. 6B. GST-VP28 was shown to be bound to MBP-His-*Cq*RNF152-like protein. Consistently, MBP-His-*Cq*RNF152-like protein was also found to bind with GST-VP28 in a GST pull-down assay (Fig. 6C). Thus, these results strongly demonstrate that *Cq*RNF152-like could specifically bind with the viral envelope protein VP28, in which the subsequently biological effect of this binding during WSSV infection is worthy of furthermore investigations.

3.6. CqRNF152-like protein exhibits self-ubiquitination activity in a RING finger domain-dependent manner

Most of RNF proteins have been defined as E3 ubiquitin ligase due to the presence of RING finger domain in the N-terminal, which mediates self-ubiquitination or substrate ubiquitination [1]. Meanwhile, E3 ubiquitin ligase-mediated ubiquitination has been found to participate in innate immune against viral infection [4]. To determine whether *Cq*RNF152-like had self-ubiquitination activity and whether it was dependent on the RING finger domain, the ubiquitination assay was perform in Sf9 cells, which were co-infected with His-*Cq*RNF152-like wild type, C30A mutant (cysteine-to-alanine) or H50A mutant (histidine-to-alanine) recombinant Baculovirus with HA-Ub, respectively.

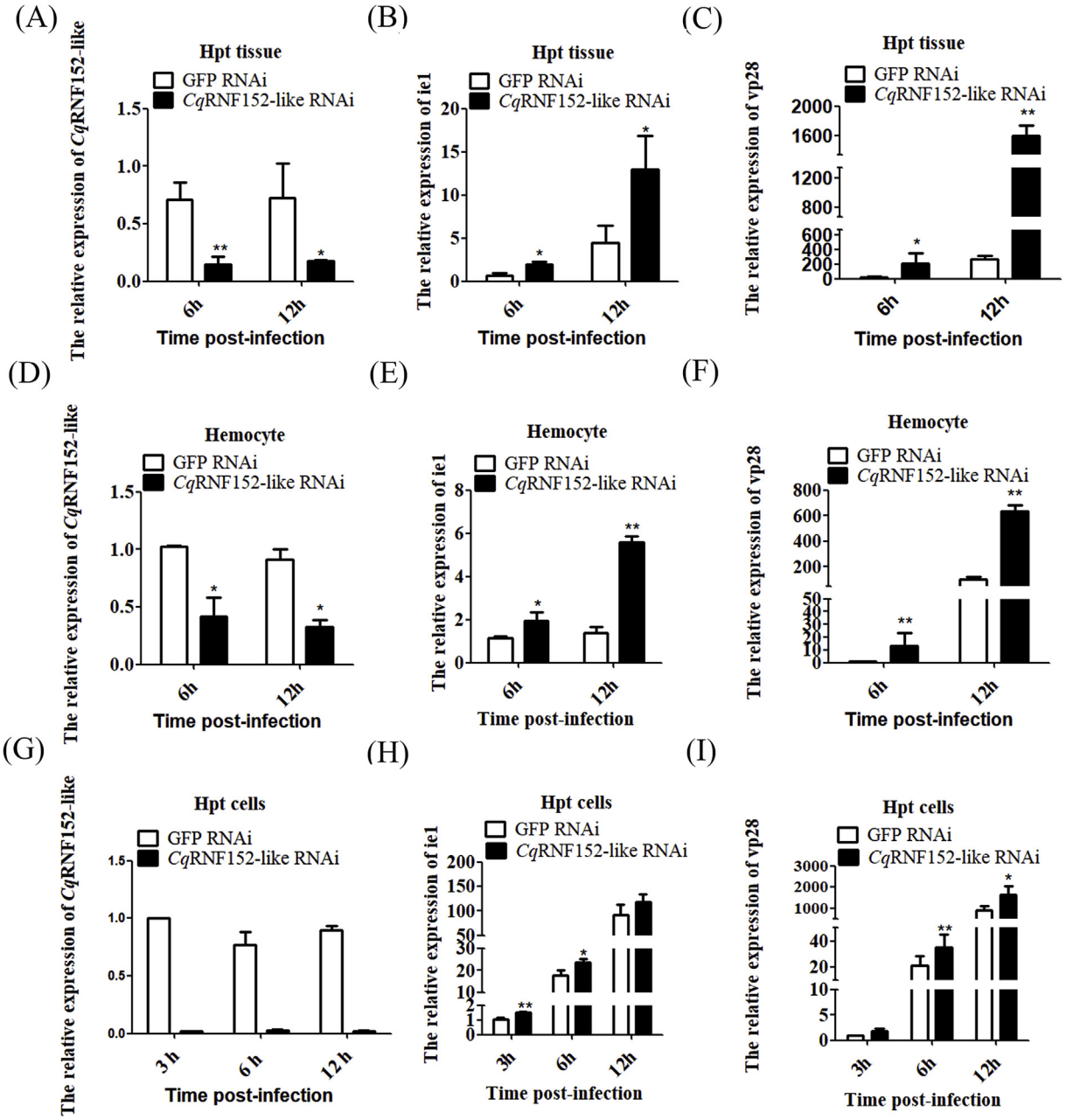


Fig. 4. Increased WSSV replication by gene silencing of *CqRNF152-like* both *in vivo* and *in vitro*. The relative gene expression of *CqRNF152-like*, viral gene *ie1* and *vp28* were determined in Hpt tissue (A-C) and Hemocyte (D-F), respectively, from crayfish injected with *CqRNF152-like* dsRNA followed by WSSV challenge. GFP dsRNA was used in the control groups. (G-I) The relative gene expression of *CqRNF152-like*, viral gene *ie1* and *vp28* was determined in Hpt cell cultures transfected with *CqRNF152-like* dsRNA followed by WSSV infection. The GFP dsRNA was served as control treatment. This experiment was repeated for three times. The asterisk indicates the significant difference compared with those of controls (*$p < 0.05$, **$p < 0.01$).

Both of these two point mutants were located in the RING finger domain, which resulted in an incomplete RING finger domain. As shown in Fig. 7, the ubiquitination level was strongly detected in the presence of His-*Cq*RNF152-like if compared to that of negative controls. Meanwhile, the ubiquitination level was significantly decreased in the Sf9 cells co-infected with C30A mutant or H50A mutant and HA-Ub (Fig. 7), respectively, which demonstrated that *Cq*RNF152-like self-ubiqutination was dependent on the presence of RING finger domain. Although self-ubiquitination of *Cq*RNF152-like still could be observed in the absence of intact RING finger domain, we speculate that the low ubiquitination level might be catalyzed by other ubiquitin ligases originated from Sf9 cells as backgrounds. Taken together, these data suggest that *Cq*RNF152-like showed self-ubiquitination activity, of which RING finger domain is essential for this activity.

4. Discussion

E3 ubiquitin ligases were categorized into two families, including the RING-finger-containing protein family and the homologous to E6-AP COOH terminus (HECT) family, of which RING-type E3 ubiquitin ligase constitutes the large majority of the more than 600 E3 ubiquitin ligases in mammalian cells [26]. As a matter of fact, a growing number of RING-type E3s are implicated in a variety of biological processes, such as development, apoptosis, tumorigenesis and innate immune [4]. Due to its importance in health and disease, RING-type E3s have been becoming an increasingly studied proteins family. Previously, some

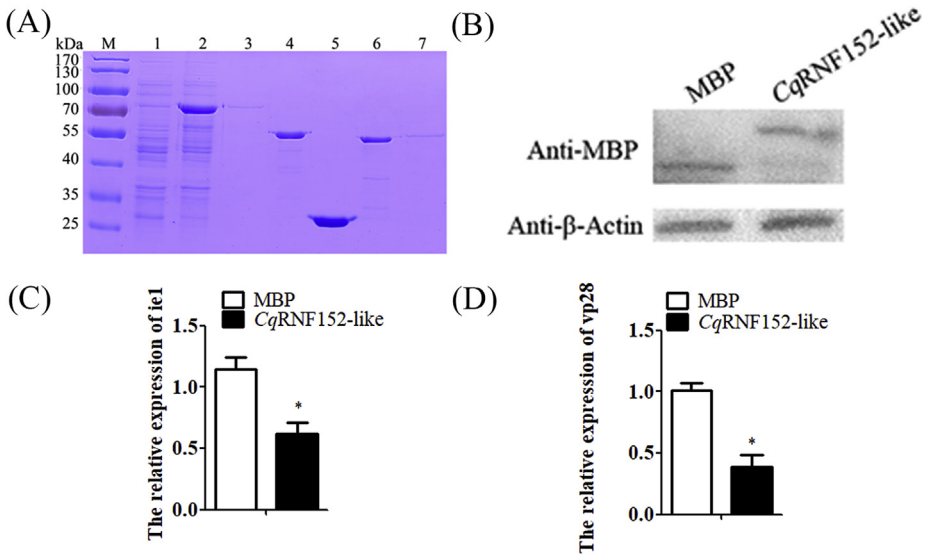


Fig. 5. Reduced WSSV replication by overloading of recombinant *Cq*RNF152-like protein in Hpt cell culture. (A) Expression and purification of recombinant proteins. M: molecular weight marker (kDa); 1–2: recombinant MBP-His-*Cq*RNF152-like protein induced by 0 and 0.1 mM IPTG in *E. coli* BL21 (DE3), respectively. 3: The purified recombinant MBP-His-*Cq*RNF152-like protein. 4–6: The purified recombinant MBP, GST and GST-VP28 proteins, respectively. (B) Western blotting analysis of overloading delivery of recombinant MBP and MBP-His-*Cq*RNF152-like protein into Hpt cells by using anti-MBP antibody. The β-actin was served as the internal control. (C-D) The relative gene expression of both *ie1* and *vp28* in Hpt cells after the overloading delivery of recombinant MBP and MBP-His-*Cq*RNF152-like protein, respectively, into Hpt cells followed by WSSV infection for 6 h. This experiment was repeated for three times. The asterisk indicates the significant difference compared with those of controls ($^{*}p < 0.05$, $^{**}p < 0.01$).

RING finger proteins that involved in WSSV infection were also reported in various species in crustaceans, such as Pellino and Tumor necrosis factor receptor-associated factor 6 (TRAF6) [27–29]. However, we still known less about their precise roles that mediated by ubiquitination in response to viral infection. In this study, we identified a RING finger domain-containing protein *Cq*RNF152-like in a crustacean *C. quadricarinatus*, which functioned in restraining WSSV replication and showed self-ubiquitination activity.

No doubt that *Cq*RNF152-like is a typical RING finger protein due to the presence of the conserved RING finger domain. Canonical RING motifs have been categorized into two major subclasses, RING-HC (C3HC4-type) and RING-H2 (C3H2C3-type), while the noncanonical RING motifs have been defined as various virants, including C4C4-, C3HC3D-, C2H2C4-, and C3HC5-type [26]. However, unlike to above characteristic, the RING finger domain of *Cq*RNF152-like was defined as C4H2C2H2-type, which exhibited specific RING finger domain in *C. quadricarinatus*. In addition, *Cq*RNF152-like shared relatively high identity to *P. vannamei* RNF152-like and RNF152 homolog from other species, but a relatively lower identity was also shown with TRIM32 from various species in vertebrates. TRIM proteins are defined as

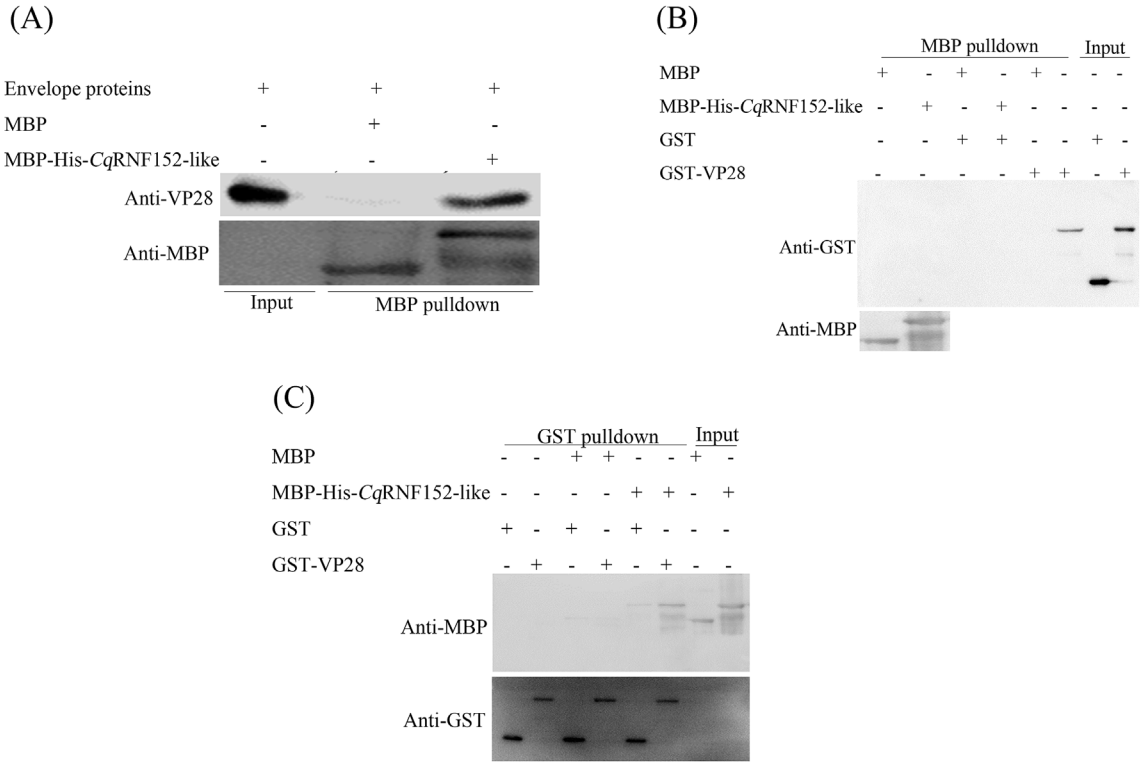


Fig. 6. *Cq*RNF152-like bound to the WSSV envelope protein VP28. **(A)** Pull-down assay between WSSV envelope components and recombinant MBP-*Cq*RNF152-like protein was performed followed by Western blotting analysis with anti-VP28 monoclonal antibody. **(B)** MBP pulldown assay between recombinant MBP-*Cq*RNF152-like protein and recombinant GST-VP28 protein was further conformed by Western blotting analysis with anti-GST antibody. **(C)** GST pulldown assay between recombinant MBP-*Cq*RNF152-like protein and recombinant GST-VP28 protein was further confirmed by Western blotting analysis with anti -MBP antibody. MBP and GST were served as negative controls. This experiment was performed in biological triplicates.

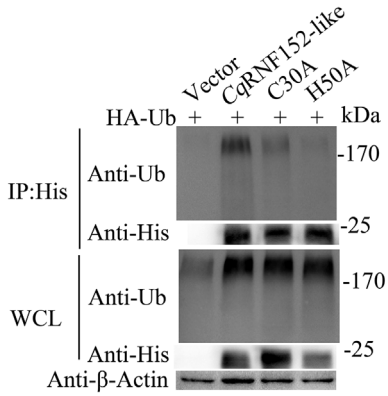


Fig. 7. Self-ubiquitination assay of *Cq*RNF152-like protein. Ubiquitination assay was performed in Sf9 cells that co-infected with His-*Cq*RNF152-like wild type, C30A mutant or H50A mutant recombinant Baculovirus and HA-Ub recombinant Baculovirus, respectively. The infected cells were collected and assessed by IP with Ni^{2+}-NTA His Bind Resin followed by immunoblotting analysis with anti-His monoclonal antibody and anti-Ub monoclonal antibody at 72 h post infection. The β-actin was served as internal control. WCL: whole cells lysate.

another subfamily of the RING type E3 ubiquitin ligase, which contain more than 80 members in human [30]. Generally, typical TRIM proteins are composed of a RING finger domain, one or two B-BOX domain and a coiled-coil region in the N-terminal and various specific domains in the C-terminal, though eight TRIM proteins in human are lacking of RING finger domain [31]. Since only one conserved RING finger domain present in the *Cq*RNF152-like and without B-BOX domain and a coiled-coil region, it appears to not strictly to classify this *Cq*RNF152-like into TRIM proteins family. However, due to less known about the domain organization and function of TRIM proteins in crustaceans, the classification of RING finger domain-containing proteins and the relationship between RNF family and TRIM proteins family in crustacean are needed to be further investigated.

In our present study, the transcript of *Cq*RNF152-like was up-regulated upon WSSV infection both *in vivo* and *in vitro*, suggesting that *Cq*RNF152-like was responsive to WSSV infection. Then, both gene silencing assay and recombinant protein overloading assay proved that *Cq*RNF152-like functioned in antiviral effect during WSSV infection. Some previous studies have found that the viral envelope protein VP28 plays a critical role in the entry of WSSV into host cells and could be recognized by several host cell proteins, such as *Cq*GABARAP [17], *Cq*Laminin receptor [25] and *Cq*Ns1abp-like protein [32] in a crustacean red claw crayfish *C. quadricarinatus*, and anti-LPS-factor (ALF) and lysozyme (LYZ) of *P. vannamei* [33]. Thus, based on its abundance and importance in viral infection, we used anti-VP28 specific antibody to determine whether *Cq*RNF152-like interacted with VP28. In agreement with our speculation, *Cq*RNF152-like protein could specifically bind with envelope protein VP28, suggesting the key role of *Cq*RNF152-like involved in host-WSSV interaction is probably associated with this binding to viral envelope that is worthy of furthermore study.

Most of RING finger domain-containing proteins have E3 ubiquitin ligase activity and could be polyubiquitinated by themselves, which were first utilized as a means of evaluating their potential to function with E2 conjugating enzymes [34]. In the present study, we found that *Cq*RNF152-like also showed self-ubiqtination activity. Both of point mutants in C30A and H50A, leading to an incomplete of RING finger domain, resulting in a significantly decreased self-ubiquitination, which further proved that RING finger domain is essential for self-ubiquitination of *Cq*RNF152-like. Previously, self-ubiqutination of a RING domain E3 ubiqutin ligase Mdm2 was found to enhance the recruitment of the E2 ubiquitin-conjugating enzyme and stimulate Mdm2 substrate ubiquitination activity [35]. In contrast, another E3 ubiquitin ligase Rsp5/Nedd4 underwent self-ubiquitination to trigger oligomerization and concomitant decrease of ligase activity [36]. Importantly, our finding here provides the first report that RING finger protein of crustacean exhibited self-ubiquitination activity. Therefore, whether self-ubiquitination of *Cq*RNF152-like affects its substrate ubiquitin ligase activity or autologous conformational change is needed to be further proved. Meanwhile, most of RING finger proteins interact with target protein and then catalyze polyubiquitination mediated by E3 ubiquitin ligase activity. In the case of RNF with antiviral function, pRNF114 catalyzes CSFV NS4B protein K27-linked polyubiquitination for proteasome-dependent degradation [5]. However, whether the *Cq*RNF152-like protein-mediated VP28 protein ubiquitination is occurred during WSSV infection needs to be further investigated, if so, which linkage of ubiquitination and degradation pathway is employed by the *Cq*RNF152-like protein is waiting for further elucidation.

In summary, we identified a RNF152 homolog with self-ubiquitination activity in red claw crayfish. Gene silencing of *CqRNF152-like* gene clearly promoted the viral replication, whereas overloading of recombinant *Cq*RNF152-like protein inhibited the viral replication in the crayfish Hpt cells. Meanwhile, *Cq*RNF152-like could restrain WSSV replication by binding to viral envelope protein VP28. Taken together, these data strongly suggest that *Cq*RNF152-like acts as an antiviral molecule to inhibit WSSV infection, which will benefit the further study of molecular mechanism underlying antiviral immunity and provide a new thought of the disease control of WSSV infection.

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