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Molecular characterization of a CpTRIM35-like protein and its splice variants from whitespotted bamboo shark (*Chiloscyllium plagiosum*)

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ABSTRACT

The tripartite motif (TRIM) proteins play important roles in a broad range of biological processes, including apoptosis, cell proliferation and innate immunity response. In this study, a TRIM gene and its three splice variants were cloned from an elasmobranch fish—whitespotted bamboo shark (*Chiloscyllium plagiosum* Bennett). Phylogenetic analysis indicated that the gene was closely related to TRIM35 homologs, thus termed CpTRIM35-like. Deduced CpTRIM35 has a RBCC-PRY/SPRY structure typical of TRIM proteins, and its splice variants (CpTRIM35-1–3) have different truncations at the C-terminus. The gene products were constitutively expressed in adult sharks with the highest levels in spleen and kidney. The different subcellular locations, upregulation upon LPS and poly I:C stimulation, and significant E3 ubiquitin ligase activities suggested their different roles in immune responses as an E3 ubiquitin ligase. This is the first TRIM protein ever characterized in elasmobranch fish.

1. Introduction

Tripartite motif-containing (TRIM) proteins are widely distributed in insects, teleosts and higher vertebrates. The proteins, also known as RBCC proteins, share a common structure that consists of a RING domain, one or two B-box (B-box 2 or both B-box 1 and B-box 2) and a Coiled-coil region [1–3]. The RING domain confers E3 ubiquitin ligase activity in ubiquitylation or sumoylation [4,5]. The B-box 2 domain has shown effects on the recognition of virus capsid [6]. The coiled-coil region mediates protein–protein interactions and defines specific subcellular location. TRIM proteins are classified according to the C-terminal structures, which include PRY/SPRY domain, C-terminal subgroup one signature (COS), fibronectin type 3 (FN3), plant homeodomain (PHD), HT2A and LIN-41 (NHL) repeats domain, etc. The PRY/SPRY domain is most commonly found in TRIM proteins, which mediates protein–protein interactions or RNA binding [7]. For example, of 75 human (*Homo sapiens*) TRIM proteins known so far, 24 have PRY/SPRY domain at the C-terminus [8].

The TRIM proteins play multifarious roles in biological and physiological processes, including innate immune response, cell differentiation, apoptosis, oncogenisis and autoimmunity [2,5]. For example, Versteeg et al. had demonstrated that half of the human TRIM-family members showed enhanced innate immune responses at both pre- and post-transcriptional levels [8]. One remarkable character was that many TRIM proteins can be induced by interferons (IFNs) that are critical for anti-microbial activity [9]. Furthermore, TRIM proteins can activate or inhibit the downstream signal pathways of pattern recognition receptors (PRRs) and further modulate various stages of NF-κB activation [8,10].

In comparison to the exhausting discovery of TRIM genes in human, studies on fish TRIM genes are limited and sporadic. TRIM33 (TIF1γ) from zebrafish is the first reported TRIM protein in fish, which is an essential regulator for embryonic and adult hematopoiesis [11]. From then on, three more TRIM genes, including the bloodthirsty (*bty*) TRIM gene, the ortholog of human TRIM71 gene, and the TRIM3a gene, have been reported to be involved in erythropoiesis [12], embryonic development [13] and brain development [14]. By using the deep sequencing technology, 58 and 240 of TRIM/TRIM-like sequences had been identified in pufferfish and zebrafish, respectively, most of which are poorly related to human TRIM genes [15]. Moreover, the multiple TRIM genes (finTRIM/ftr) found in rainbow trout and zebrafish had no obvious orthologs of higher vertebrates and might be evolved in strong positive selection [16]. These results indicate that TRIM

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genes in fish might separate from those of higher vertebrates at an early stage.

Cartilaginous fishes are a class of aquatic, gill-breathing, finned vertebrates, equivalent to the bony fishes. The chondrichthyes are classified into two subclasses: Elasmobranchii (sharks, rays and skates) and Holocephali (chimaeras). Recently, the whole genome of a holocephalan fish elephant shark (*Callorinchus milii*) has been sequenced [17], and several TRIM genes were identified. Owing to their phylogenetic position, chondrichthyans provide a critical reference for our understanding of vertebrate evolution. In this study, we cloned a TRIM35-like gene and its three splice variants from an elasmobranch fish. Their expression profiles, subcellular localizations and biological roles were also determined. To our knowledge, this is the first TRIM protein ever characterized in elasmobranch fish.

2. Materials and methods

2.1. Fish and sample collection

The whitespotted bamboo sharks (~1.5 kg) were purchased from the Jingshen seafood market (Beijing, China). Fish were maintained in 200 L tanks with aerated seawater at 28 °C and fed twice daily for two weeks. For the challenge experiment, fish were anaesthetized in tricaine methanesulfonate (MS-222, 50 mg L^{-1}, Argent Chemical Laboratories) for infection. Before the dissection, fish were killed by an overdose of MS-222. The samples were collected and immediately frozen in liquid nitrogen and stored at −70 °C before use.

2.2. Total RNA extraction and cDNA cloning

Total RNA was extracted using the SV Total RNA Isolation System (Promega). The single strand cDNA was then synthesized with the First Strand cDNA Synthesis kit Rever Tra Ace-α-™ (TOYOBO) and used as the template. Based on the conserved regions of zebrafish TRIM proteins, two degenerate primers TRIM-F and TRIM-R (Supplementary Table S1) were designed and a touch down PCR was conducted to amplify the partial TRIM gene. The flanking fragments were obtained by 5'- and 3'-RACE-PCR with several gene-specific primers (Supplementary Table S1) using the SMARTer RACE cDNA Amplification Kit (Clontech). The gene segments were ligated into the pGEM-T Easy Vector (Promega), sequenced, and assembled with the known sequence. The primer set CpTRIM35-F and CpTRIM35-R (Supplementary Table S1) was used to amplify the cDNA of full-length gene by using a high yield and high fidelity kit Advantage 2 PCR Kit (Clontech).

2.3. Structural and phylogenetic analysis

The open reading frames (ORFs) were predicted using the ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Blastn and blastp programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to analyze the nucleotide and deduced amino acid sequences, respectively. Multiple sequence alignment of CpTRIM35 and its splice variants or other TRIM proteins was performed using the ClustalW software. The phylogenetic tree was constructed by the Neighbor-joining (NJ) method with 1000 replicates using MEGA 4.1 software.

2.4. Expression profiles of CpTRIM35 and its splice variants

The expression profiles of *CpTRIM35* and its splice variants in twelve tissues, including muscle, spleen, kidney, gill, pancreas, liver, eye, gut, skin, brain, heart and blood, were determined by semi-quantitative PCR. The *β-actin* gene as an internal control was amplified with the primers actin-F and actin-R (Supplementary Table S1). The PCR products were visualized in a 1.5% agarose gel under UV light.

For challenge experiment, eighteen sharks were randomly divided into two challenge groups and one control group, and each group (six fish) was placed in one individual tank. Before intraperitoneal injection, the sharks were anesthetized as described above, and injected with 1 mL kg^{-1} PBS buffer, 5 mg kg^{-1} LPS (*Escherichia coli* O55:B5; Sigma) or poly I:C (Sigma) in PBS buffer, respectively. Spleen was collected at 12 h after the stimulation, pooled within the group, frozen in liquid nitrogen, and then stored at −70 °C for RNA isolation and cDNA synthesis. Semi-quantitative PCR was performed as described above.

2.5. Subcellular localization

Human embryonic kidney cell line 293T (HEK293T) cells was grown in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. The ORFs of *CpTRIM35* and its splice variants were cloned by expression primers (Supplementary Table S1) and inserted into plasmid pEGFP-N1 (Clontech), respectively. HEK293T cells were transfected with the four recombinant plasmids using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. After 72 h, the cells were fixed in 4% buffered paraformaldehyde (Beyotime) for 20 min. Nuclei were stained with 1 mg mL^{-1} of 4,6-diamidino-2-phenylindole (DAPI, Sigma) for 2 min, followed by fluorescence microscopy observation (OLYMPUS IX71).

2.6. Self-ubiquitylation of CpTRIM35 in vitro

The ORFs of *CpTRIM35*, truncated *CpTRIM35* with removal of RING domain (*CpTRIM35*△R) and its splice variants were inserted into pGEX-4T-1 vector. Correct constructs were transformed into *E. coli* Rosetta competent cells. The recombinant proteins were purified using affinity chromatography with Glutathione Sepharose resin (National Engineering Research Center for Biotechnology, Beijing, China). The protein concentrations were determined with a Protein Assay Kit (Bio-Rad).

In vitro self-ubiquitylation assay was performed using the Ubiquitylation kit (ENZO, Farmingdale, NY, USA) as described previously with some modifications [14]. The ubiquitin-conjugating enzyme (E2) was UbcH5a. PBS instead of CpTRIM35 proteins was the control group. Proteins were separated on 12% SDS–PAGE and transferred onto a PVDF membrane (Amersham Biosciences). The membrane was blocked overnight at 4 °C with BSA/TBS-T blocking buffer (50 mM Tris–HCl, 200 mM NaCl, 1% BSA, 0.1% Tween 20, pH 7.4), followed by three 10-min washes in TBS-T buffer. The membrane was incubated in streptavidin-HRP solution (Tiangen) for 1 h at room temperature and washed with TBS-T buffer (6 × 10 min). The membrane was then incubated in detection reagent HRP-DAB (Tiangen) for 1 min. The emitted signals were detected and imaged by the CCD imaging instrument.

3. Results

3.1. Cloning of the cDNAs of CpTRIM35-like gene and its splice variants

A gene fragment of 828 bp was obtained by using the degenerate primers TRIM-F and TRIM-R with spleen derived cDNA as the template. The 5′ and 3′ flanking regions were obtained by RACE-PCR. A total of four full-length transcripts were isolated, including 1935-bp *CpTRIM35* (KF356200), 1564-bp *CpTRIM35-1* (KF356201),

1289-bp *CpTRIM35-2* (KF356202) and 1032-bp *CpTRIM35-3* (KF356203). Excluding the 3′ and 5′ UTR sequences, the ORFs of *CpTRIM35*, *CpTRIM35-1*, *CpTRIM35-2*, and *CpTRIM35-3* are 1614 bp, 1134 bp, 1095 bp and 711 bp in length, respectively, which encode 236–537 amino acid residues.

3.2. Structural and phylogenetic analysis

Multiple alignments of the deduced amino acid sequences of CpTRIM35 and its three splice variants revealed that these proteins were highly conserved at the N-termini (1–170 residues) but varied a lot in the C-terminal regions (Fig. 1). The C-terminal sequence of CpTRIM35-3 was identical to that of CpTRIM35, but different from those of CpTRIM35-1 and CpTRIM35-2. Blastp analysis indicated that CpTRIM35 has a classic TRIM motif composed of a RBCC domain at the N-terminus and a C-terminal PRY/SPRY domain. CpTRIM35-1 consisted of a RBCC region and an incomplete PRY/SPRY domain, while CpTRIM35-2 and -3 only contained the RB domain. The results revealed that *CpTRIM35-13* are three splice variants of *CpTRIM35*. Phylogenetic analysis of CpTRIM35 against other TRIM proteins (Supplementary Fig. S1) showed that CpTRIM35 was far related to TRIM35 proteins of teleost and higher vertebrates like mammals and birds. The result indicates that CpTRIM35 may evolve divergently at an early stage.

3.3. Expression profiles of CpTRIM35 and its splice variants

The constitutive expressions of *CpTRIM35* and its splice variants were determined by semi-quantitative RT-PCR with *β-actin* as the internal control. As shown in Fig. 2A, *CpTRIM35* was detected in all examined tissues except for pancreas. The three splice variants showed differential expression patterns. *CpTRIM35-1* was detected in all tested tissues except for muscle, pancreas and liver. *CpTRIM35-2* was absent from muscle and gut. *CpTRIM35-3* was absent from muscle and pancreas. The expression levels of *CpTRIM35* and *CpTRIM35-1* were higher than that of *CpTRIM35-2* and *CpTRIM35-3*. Moreover, the genes showed higher expression levels in immunity-related tissues like spleen and kidney. The results indicate that the TRIM proteins might be involved in innate immunity response.

The immune responses of *CpTRIM35* and its splice variants to LPS and poly I:C challenges were determined in spleen. All testes genes showed the increase of expression with LPS treatment, while poly I:C only induced the expression of *CpTRIM35* (Fig. 2B). The expression levels of *CpTRIM-1/2* were decreased after stimulation with poly I:C. Previous work showed that different stimuli like LPS and poly I:C can recognize various toll-like receptors (TLR4, TLR3), and have distinct signal pathways to influence the expression levels of TRIM genes [8]. *CpTRIM35* and its three splice variants had different expression levels after LPS and poly I:C challenge, indicating a promising evidence of distinct involvement of *CpTRIM35* and its three splice variants in defense response of sharks against pathogens.

3.4. Intracellular localization of CpTRIM35 and its splice variants

Fused proteins of CpTRIM35 and its splice variants with GFP at the C-terminus were successfully transfected into HEK293T cells. As shown in Fig. 3, CpTRIM35 and CpTRIM35-2 were predominantly localized in cytoplasm, and CpTRIM35-2 was also observed in some granules. CpTRIM35-1 and CpTRIM35-3 were diffuse, and were detected in both cytoplasm and nucleus. The difference of subcellular localization further confirms their diverse biological roles in cells.

3.5. In vitro self-ubiquitylation assay

TRIM protein confers E3 ubiquitin ligase activity in ubiquitylation due to its RING domain at the N-terminus. To test the E3 ubiquitin ligase activities in CpTRIM35 and its variant proteins, pure CpTRIM35, RING domain deleted mutant CpTRIM35△R and its variant proteins were produced in *E. coli* Rosetta cells and purified by affinity chromatography (Fig. 4A). In the presence of ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) UbcH5a, high-molecular-weight ubiquitin products were detected after treatment with CpTRIM35 and its variant proteins but not in the blank control (PBS buffer) and CpTRIM35△R (Fig. 4B). The results indicate that the homologous TRIM35-like proteins from whitespotted bamboo shark have a RING domain dependent E3 ubiquitin ligase activity in cooperation with UbcH5a.

4. Discussion

TRIM proteins have emerged as important mediators in multiple biological processes. A large variety of TRIM proteins have been identified in insects, fish and mammals. In this study, we obtained a full-length TRIM gene *CpTRIM35* and its three splice variants by using degenerate PCR and RACE-PCR from elasmobranch fish. Phylogenetic analysis indicates that CpTRIM35 and zinc-binding protein A33-like protein from *Latimeria chalumnae* are clustered in one small branch. *L. chalumnae* is one of two extant species of coelacanth, a rare order of vertebrates, also known as a "living fossil". The close relationship of CpTRIM35 to that of *L. chalumnae* but far from bony fish TRIM35/like proteins indicates that CpTRIM35 is an ancient protein. The evolution of the CpTRIM35 and bony fish TRIM35/like has been going on independently like the cartilaginous fishes and bony fishes. The results are consistent to previous studies that the TRIM family is ancient and has been greatly diversified in vertebrates and especially in fish [15,16]. Furthermore, compared with higher vertebrates that have only one *TRIM35* gene, a multigene subset of *TRIM35* has been identified in teleost [15], i.e. 37 in zebrafish and 6 in pufferfish. However, by using the degenerate primers designed from the conserved TRIM motifs of zebrafish, we only found one *TRIM35* gene fragment from whitespotted bamboo shark while no *TRIM35* gene was found in the elephant shark genome. Further studies are required to discover whether there is a subfamily of TRIM35 genes in elasmobranch fish.

The deduced CpTRIM35 has the typical TRIM protein structure (RBCC-PRY/SPRY), while the structures of its splice variants are incomplete (RB or RBCC with incomplete PRY/SPRY). Similar splicing pattern has been reported in human HsTRIM35 (Supplementary Fig. S2), in which variant A consists of the RB domain, variant B has an integrate RBCC domain and a PRY/SPRY domain, and variant C has an incomplete PRY/SPRY domain. This splicing pattern is different from that of chicken TRIM35 (Supplementary Fig. S3), in which the missing parts are mainly located in the BCC region. In human, 90% of TRIM genes have more than one splice variant and 52% of the splice forms lack potential key domains such as RING or SPRY domains [8]. In this study, three splice variants of CpTRIM35 are different in PRY/SPRY domain, which may relate to their different functions.

The expression profiles of *CpTRIM35* and its splice variants indicate that *CpTRIM35* is a constitutive gene in both immune- and non-immune-related organs of adult sharks. Of all tested tissues in mouse, brain, heart, spleen, thymus and muscle are the major sites for *TRIM35* expression as reported previously [18,19]. Previous studies had shown that TRIM proteins are involved in the antibacterial and antiviral immune responses. For example, a TRIM protein named Acbloodthirsty was detected in the poly I:C subtractive cDNA library of Atlantic cod [12], and the finTRIM genes were

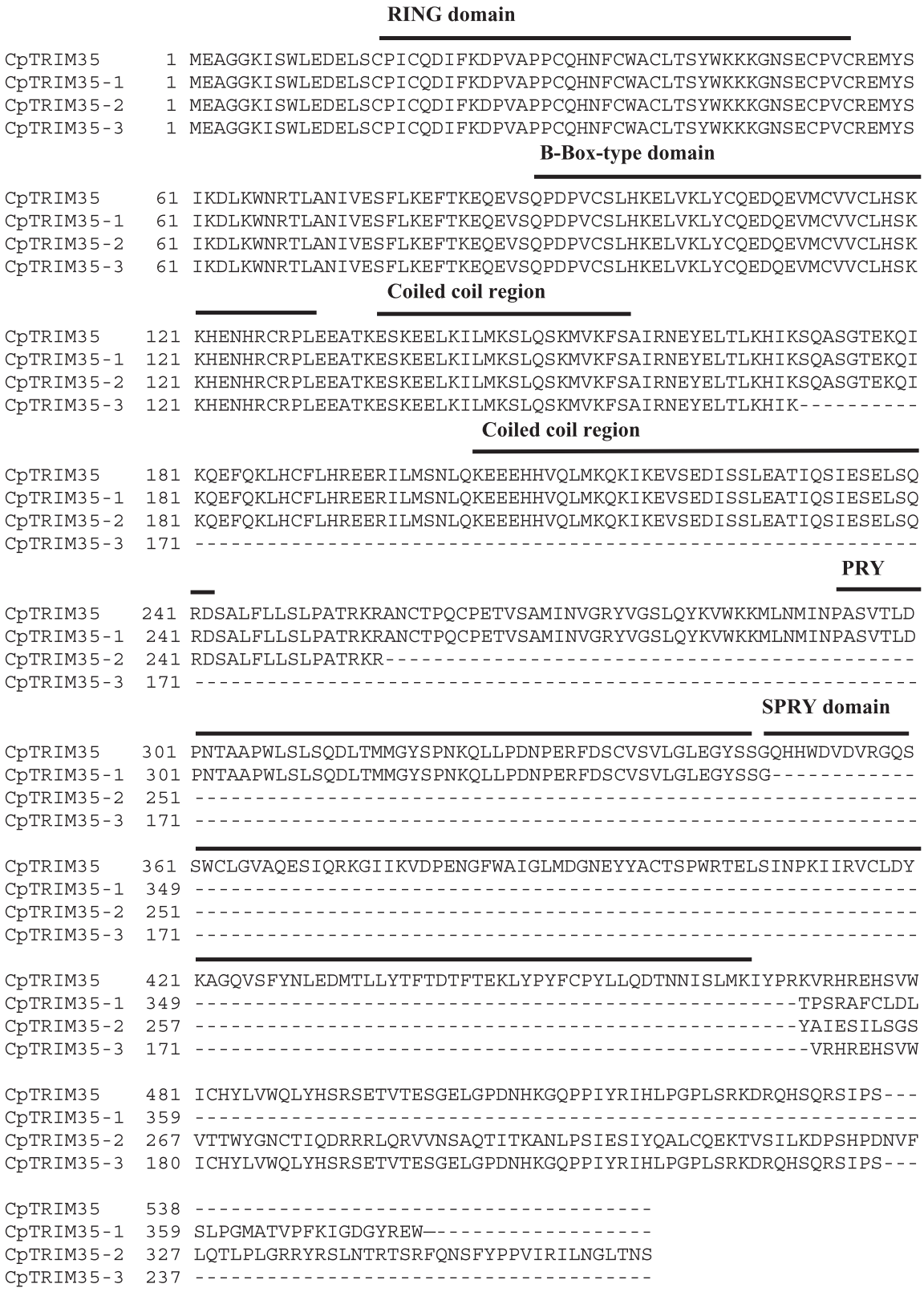

Fig. 1. Multiple sequence alignment of deduced CpTRIM35 and its three splice variants, CpTRIM35-1-3, by using the ClustalW.

observed to function in the antiviral immune response through ubiquitylation in rainbow trout [20]. In this study, we found that LPS and poly I:C could induce the expression of *CpTRIM35* and its splice variants with different levels. The results suggest that CpTRIM35 and its variant proteins varied in antibacterial and antiviral immune responses.

Different subcellular location of a single protein might imply its specific biological function. The great majority of TRIM proteins were reported to be present in both cytoplasm and nucleus [1,8].

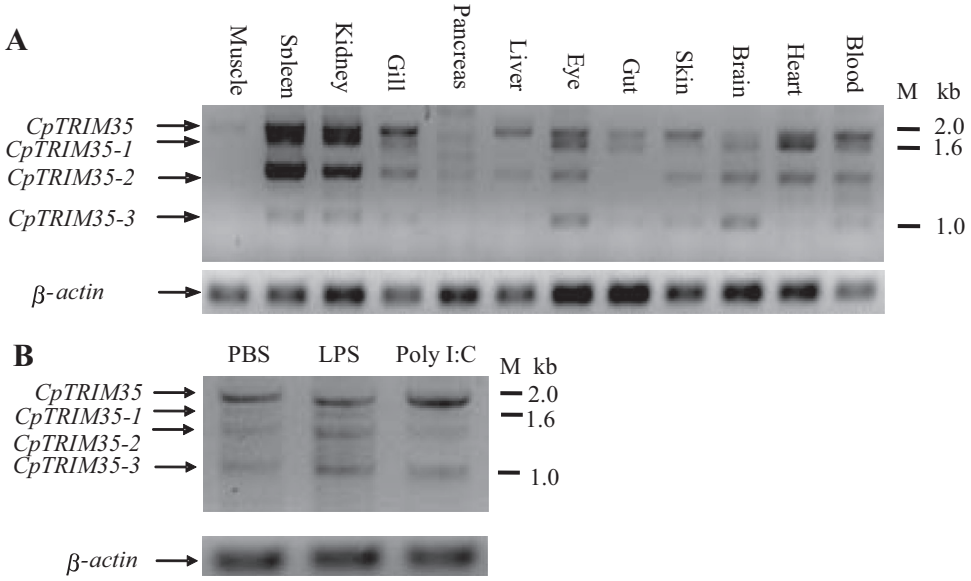

Fig. 2. Expression profiles of *CpTRIM35* and its splice variants in twelve tissues of *C. plagiosum* (A) and in spleen cells of *C. plagiosum* after challenge with LPS and poly I:C (B). *β-Actin* (KF555655) was included as the internal control. PBS was injected as a control.

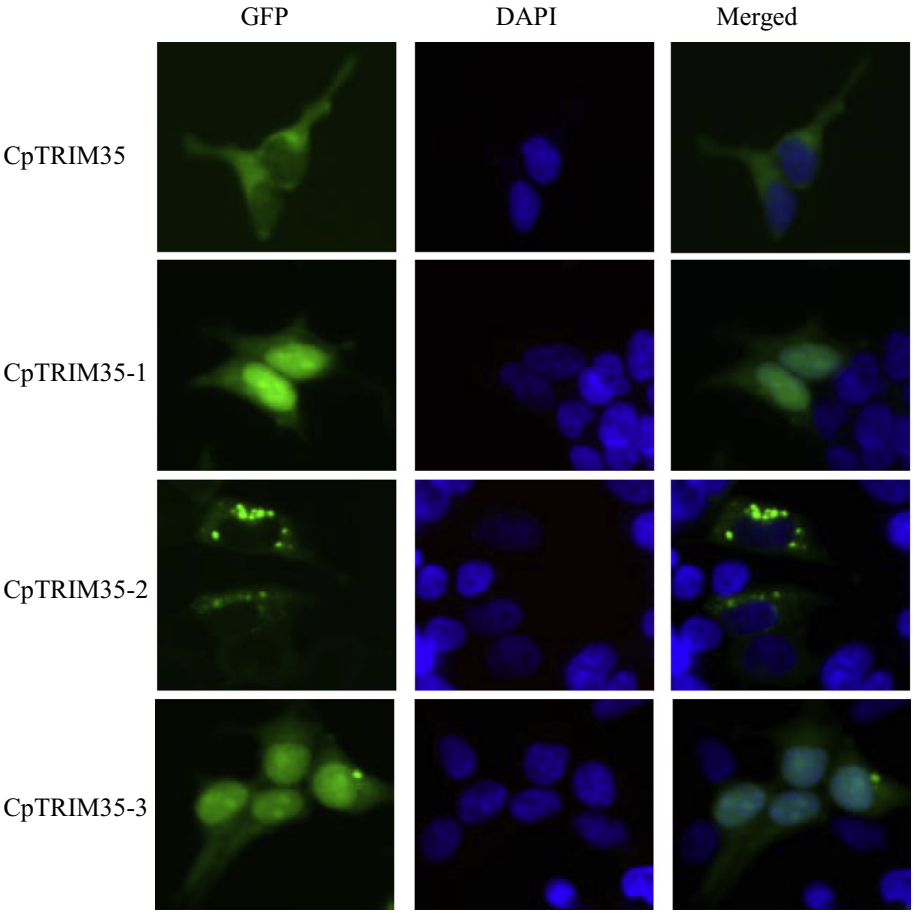

Fig. 3. Subcellular distribution of CpTRIM35 and its variants in human cell line HEK293T. The nuclei of cells are dyed with DAPI.

Their cellular locations were defined by different protein structures, i.e. the RB domains or the CC region. Considering the different structures of CpTRIM35 and its splice variants, they might have the propensity to form different high-order molecular structures in cells and vary in biological functions. In addition, CpTRIM35 and its variant proteins acted as E3 ligases in the ubiquitylation system. It is well-known that the poly-ubiquitylation of a protein might serve as a signal for its degradation in the proteome or allow the coordi-

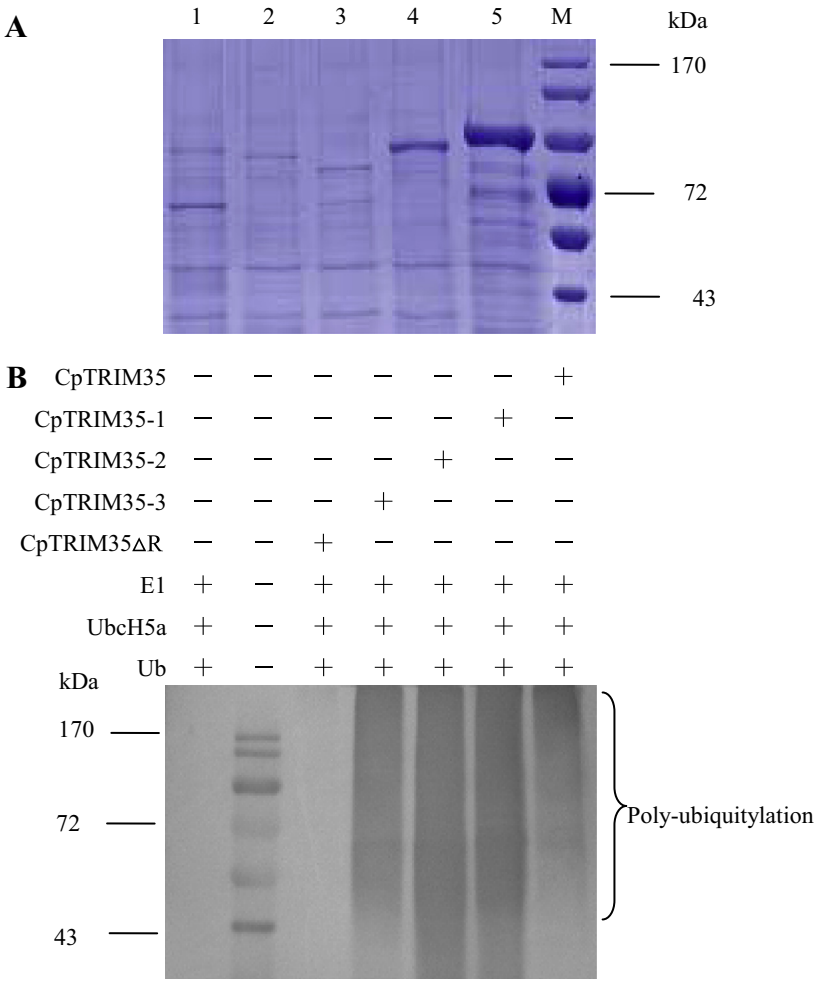

Fig. 4. Heterologous expression in *E. coli* Rosetta cells. (A) SDS–PAGE analysis. Lane M: protein marker; lane 1: CpTRIM35-3; lane 2: CpTRIM35-1; lane 3: CpTRIM35-2; lane 4: CpTRIM35△R; lane 5: CpTRIM35. (B) Western blot analysis of CpTRIM35 and its variants *in vitro* self-ubiquitylation activities.

nation of other processes such as endocytic trafficking, inflammation, translation and DNA repair [21,22]. Thus CpTRIM35 and its variant proteins may function as the substrate recognition modules and interact with both E2 enzyme and substrate to protect shark against pathogen infection.

In summary, a *TRIM35-like* gene *CpTRIM35* and its three splice variants were identified in whitespotted bamboo shark. The genes were constitutively expressed in almost all of the tested tissues with the highest expression levels in spleen and kidney. Subcellular localization analysis, challenging test with LPS and poly I:C and *in vitro* self-ubiquitylation assay revealed that CpTRIM35 and its variants might have specific functions as an E3 ligase in the immune system and protect host against pathogen.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.139.

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