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A zebrafish (*Danio rerio*) bloodthirsty member 20 with E3 ubiquitin ligase activity involved in immune response against bacterial infection

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ABSTRACT

The tripartite motif (TRIM)-containing proteins exhibit various activities and play important roles in the immune system through regulating signaling pathways. Bloodthirsty gene is a multigene subset of TRIM genes. In this study we identified and characterized a new member of the bloodthirsty subset of TRIM genes, *btr20*, in zebrafish (*Danio rerio*). The gene is located on chromosome 19 and forms a cluster with *btr18*, *btr21*, *btr22* and an E3 ubiquitin ligase TRIM39-like gene. Deduced btr20 represents a RBCC-B30.2 TRIM protein containing 544 amino acids. The mRNA expression level of *btr20* was highest in intestine and gill, followed by in spleen and kidney. Challenge experiment with *Aeromonas hydrophila* strain NJ-1 showed that the levels of *btr20* and *NF-κB* mRNA were remarkably upregulated in the four tissues mentioned above. btr20 was localized in the cytoplasm and formed aggregate in human embryonic kidney cell line 293T. *In vitro* self-ubiquitylation experiment demonstrated that btr20 has E3 ubiquitin ligase activity that can be self-ubiquitylated with most E2 enzymes, especially UbcH6. The results suggested that *btr20* may involve in the anti-microbial activity in the immune system as an E3 ubiquitin ligase.

1. Introduction

Tripartite motif (TRIM)-containing proteins are involved in a variety of biological and physiological processes. Members of the TRIM family protein are induced by type I interferon stimulation and play important roles in the regulation of the signaling pathways involved in innate immune response [1–3]. For example, 26 of the 44 TRIM genes were stimulated by IFNγ and the TLR4 ligand lipopolysaccharide in human monocytes and macrophages [4]. IFN was predominantly mediated through an ISRe (IFN-stimulated response element) sequence on the proximal promoter region and drove the transcription of TRIM5α [5]. TRIM8, a protein induced by IFNγ in epithelial and lymphoid cells, has been shown to interact with suppressor of cytokine signaling 1 (SOCS1) and to inhibit SOCS1-mediated downregulation of IFNγ signaling [6]. NF-κB is a transcription regulator that is activated by stimuli such as cytokines and bacterial or viral products through the pattern recognition receptors (PRRs). Activated NF-κB stimulates the expression of genes, including cytokines, chemokines, adhesion molecules and effectors involved in a wide variety of biological functions [7]. There is increasing evidence that TRIM proteins can activate or inhibit signal pathways downstream of PRRs and TLRs (Toll-like receptors) to modulate NF-κB activation [3]. Mouse TRIM30α negatively regulates TLR-mediated NF-κB activation by targeting TAB2 and TAB3 for degradation, resulting in the abrogation of TAK1 activation [8]. Protein kinases IKKβ and IKKγ are crucial regulators in the NF-κB signal pathway. HuTRIM21/Ro52 interacts with phosphomimetic mutant IKKβ to conjugate monoubiquitin in cooperation with an E2-ubiquitin-conjugating enzyme UbcH5b to downregulate NF-κB signaling [9]. Polyubiquitin conjugation to IKKγ by TRIM23 leads to the activation of downstream signaling of virus-induced IRF3 and NF-κB for antiviral function [10].

The tripartite motif (TRIM) proteins are characterized by a RING domain, one or two B-boxes, and a Coiled-coil region at the N-terminus [11]. The RING and B-box domains containing a zinc-binding motif are located in the N-terminal region of almost all TRIM proteins. Many TRIM proteins have been reported as an E3 ubiquitin ligase based on the RING domain to elicit host antiviral innate immunity in the ubiquitylation process [12–14]. The B-boxes are only found in TRIM proteins and are probably an important determinant of the TRIM family. The Coiled-coil region is approximately 100 residues long and mainly involved in homo-interactions and in promoting the generation of high-molecular-mass complexes [15]. The C-terminal region is highly variable

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and contains specific domains, such as the commonly found B30.2 domain (also known as RFP-like domain) [16]. This 170-residue domain consists of two subdomains PRY and SPRY, and mediates protein–protein interaction.

The TRIM family is ancient and has been greatly diversified in vertebrates, especially in fish. TRIM genes of 208 and 66 were found in zebrafish and pufferfish, respectively [17]. Some of them are orthologues of human, and contain three large multigene subsets: hsl5/TRIM35-like gene (*hltr*), TRIM39/bloodthirsty-like TRIM gene (*btr*) and fish novel large multigene TRIM (*finTRIM/ftr*). These genes encoding RBCC-B30.2 proteins evolve fast, under positive selection, and duplicate to create multigenic families. The *finTRIMs* were unique in fish and upregulated upon viral infection and IFN stimulation, suggesting they were involved in the antiviral immune response through ubiquitylation [18]. *btr* subfamily genes are orthologous to human TRIM39 and relatively dispersed in the zebrafish genome. The first reported bloodthirsty protein, btr25, was involved in erythropoiesis in zebrafish [19]. Recently, an Atlantic cod TRIM protein named Acbloodthirsty was detected in a poly I:C subtractive library [20]. However, functions of other bloodthirsty proteins are still largely unknown. In this study, we reported a zebrafish *btr* subfamily TRIM gene, *btr20*, which was highly expressed in intestine, gill, kidney and spleen and was upregulated after *Aeromonas hydrophila* strain NJ-1 infection. *In vitro* self-ubiquitylation experiment demonstrated that btr20 was an E3 ubiquitin ligase, and may act as a candidate regulator in the innate immune signaling pathways in zebrafish.

2. Materials and methods

2.1. Fish and bacterial challenge

The adult, wild-type zebrafish (*Danio rerio* strain TU) were maintained at 25 °C in a recirculation system with 10% daily exchange of dechlorinated tap water as described in the standard protocols (http://zfin.org/zf_info/zfbook/zfbk.html). For dissection, fish were anesthetized in 0.2% tricaine methanesulphonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA), followed by euthanization in ice water for 15 min [21]. Twelve tissues including muscle, oocyte, sperm, eye, brain, gill, heart, skin, intestine, spleen, kidney and liver were collected from twelve individuals (6 male and 6 female) for expression analysis of the TRIM gene *btr20* mRNA.

A. hydrophila NJ-1 was grown overnight at 30 °C in Luria–Bertani (LB) medium. The culture was then inoculated into a fresh LB medium (1:100 dilution) and grown at 30 °C to 0.8–1.0 at OD_{600nm}. The cells were collected by centrifugation (6000×*g*, 5 min), washed once with PBS buffer, and resuspended in fresh water at a concentration of 7×10^6 cfu mL^{-1}. To study the response to bacterial challenge, fish (30 individuals) were immersed in the *A. hydrophila* NJ-1 suspension, and six of them were randomly withdrawn at 4, 8, 12, 24 and 48 h, respectively. Fish were killed as described above, and tissues of gill, intestine, spleen and kidney from individuals were pooled and frozen in liquid nitrogen for RNA isolation.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from the pooled tissues with the SV Total RNA Isolation System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNA integrity was detected in 1.2% agarose gel electrophoresis, and the concentration of RNA was quantified spectrophotometrically. The first strand cDNA was synthesized from the total RNA using the Rever Tra Ace-α-™ kit (TOYOBO, Osaka, Japan). The cDNA was stored at −20 °C for further experiments.

2.3. Sequence analysis and multiple sequence alignment

The DNA and cDNA sequences of *btr20* were obtained from the Zv9 assembly database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) for intron/exon verification. The features of btr20 domains were predicted by Simple Modular Architecture Reach Tool (SMART) (http://smart.embl-heidelberg.de/). Multiple sequence alignment of btr20 with other TRIM39 proteins including NP_067076.2 (*Homo sapiens*), NP_001006196.3 (*Gallus gallus*) and NP_001085046.1 (*Xenopus laevis*) were performed by ClustalW software.

2.4. Quantitative PCR analysis of the btr20 expression

The real-time quantitative PCR (RT-qPCR) was carried out in an ABI 7500 Real Time Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan). The housekeeping gene *EF-1α* (NM_131263.1) was used as an internal control for cDNA normalization with primers EF-1α-f (5-GCTGGAGGCCAGCTCAAACATG-3) and EF-1α-r (5-ATCA AGAAGAGTAGTACCGCTAGCATTAC-3). The gene-specific primers btr20-f (5-TCCGCTCCATTGAGGGATGTCA-3) and btr20-r (5-GGCCA GTTCCTGGTGTCTGC-3) were used to detect the expression of zebrafish *btr20* (XM_003200513.2) in different tissues. For the challenge experiment, a primer set specific for *NF-κB* (BC122885.1), NF-κB-f (5- GGACGGAGACACGTATCTGC-3) and NF-κB-r (5- ACCAT TTGTGGCTGTTCTGTGAC-3), was designed to detect the immunity responses. Reaction of each sample was performed in triplicate. The comparative C_T method ($2^{-\triangle\triangle CT}$ method) was used to analyze the expression level of *btr20*. All data were given in terms of relative mRNA expression levels as means ± SE. Differences were considered to be significant at $p < 0.05$.

2.5. Subcellular localization of btr20

The coding sequence of *btr20* was amplified by PCR using the zebrafish spleen cDNA as template and primers GFPbtr20-F (5-GAATTCTGATGGCAGAATCTTTACCAACATCGAC-3) and GFPbtr20-R (5-GTCGACTGTTTACTACTTACAGGTGTGATGATT-3). The products were digested by *Eco*RI and *Sal*I and cloned into the corresponding sites of the expression vector pEGFP-N1. HEK293T cells were maintained at 37 °C with 5.0% CO_2 in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Invitrogen), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. HEK293T cells were transfected with the EGFP-btr20 plasmid using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. At 72 h after transfection, cells were fixed in 4% paraformaldehyde for 20 min and then stained with 4,6-diamidino-2-phenylindole (DAPI, Santa Cruz, CA, USA) for 3 min. The cells were washed twice with PBS and taken photo with a fluorescence microscope (IX71, Olympus, Tokyo, Japan).

2.6. Prokaryotic expression and purification of recombinant btr20 for self-ubiquitylation in vitro

The coding sequence of *btr20* was amplified using gene-specific primers GSTbtr20-f (5-GAATTCATGGCAGAATCTTTACCAACATC-GAC-3) and GSTbtr20-r (5-GTCGACTCATTTACTACTTACAGGTGT-GATGATT-3), and subcloned into the pGEX-4T-1 vector as described above. *Escherichia coli* Rosetta cells were transformed with recombinant plasmid GSTbtr20. The positive transformants were cultured in LB medium at 37 °C. When the cultures were grown to OD_{600nm} of 0.8, the expression of GSTbtr20 was induced

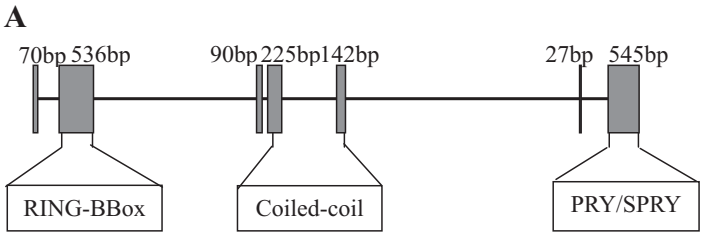
by isopropylthiogalactopyranoside (IPTG) to a final concentration of 0.6 mM for another 8 h at 20 °C with agitation. The cells were harvested by centrifugation at 10,000×*g* for 10 min, and the cells were lysed with the BugBuster Protein Extraction Reagent (Merck KGaA, Darmstadt, Germany). The supernatant was collected by centrifugation and purified by affinity chromatography using Glutathione Sepharose resin. The purity of recombinant btr20 was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

In vitro self-ubiquitylation assay was performed using the Ubiquitylation kit (ENZO) as described previously [22] with some modifications. The ubiquitin-conjugating enzymes (E2) were UbcH1, UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, UbcH8, UbcH10 and UbcH13. The reaction products were analyzed by western blotting using anti-biotin as the antibody. Proteins were electrophoresed on SDS–PAGE (10% gel), and transferred onto a PVDF membrane (Amersham Biosciences, Uppsala, Sweden). The membrane was blocked with BSA/TBS-T blocking buffer (50 mM Tris–HCl, pH 7.4, 200 mM NaCl, 0.1% Tween 20, 1% BSA) for 2 h at room temperature, washed for 3 × 10 min with TBS-T buffer, and incubated with anti-biotin antibody streptavidin-HRP (TIANGEN, Beijing, China) for 1 h at room temperature on a rocking platform. The membrane was washed for another 6 × 10 min with TBS-T buffer on a rocking platform, followed by 1-min incubation in the detection reagent HRP-DAB (TIANGEN). The membrane was examined using the Carestream Gel Logic 212 PRO and Molecular Imaging software system (Carestream Health, Toronto, Canada).

3. Results

3.1. The sequence and structure analysis of the btr20 gene

A *btr20* gene (NC_007130) of 9449 bp, including seven exons and six introns (Fig. 1A), was identified in the genome of zebrafish. The cDNA of *btr20* contains 1635 bp that codes for 544 residues. The deduced btr20 consists of a RING domain, a B-box2 domain, a Coiled-coil domain and a PRY/SPRY domain. Multiple sequence alignment (Fig. 1B) indicated that btr20 shows a high homology with TRIM39 proteins from *H. sapiens*, *G. gallus* and *X. laevis*. The conserved cysteine and histidine residues of TRIM39 proteins were also



B

RING domain

```
H.sapiens     1 -----MAETS----LLEAGASAASTAAALENLQVEASCSVCLEYLKEPVIIECGHNFCKACITRWWEDLERDFPCPVCRKTSR---
G.gallus      1 -----MDEDN----PAES-------------FQDEASCSLCLGFFQDPVSIHCGHNFCRACITRCWEEQEETFSCPRCKATAT---
X.laevis      1 -----MAAANRNPVT---------------ELKEELTCPICLDHFSEPVSIECGHSFCRTCINRSWRGIHSYFECPQCRNVSR---
D.rerio       1 MAESLPTSTKGKAARRRSIDDPPWMSSSSGPLSEELRCSICLEVFTDPVSTPCGHNFCKSCLNKYWNN-SQTCSCPNCKETFTQRP

H.sapiens    78 --------------YRSLRPNR---------------------------------QLGSMVEIAKQLQAVKRKIRDESLCPQHHEA
G.gallus     65 --------------QRNLRPNR---------------------------------ELAKIIEIAKRLS-LRAPSAGERLCERHREV
X.laevis     67 --------------WKFLRPNR---------------------------------LVENMVEITERLAASKISPVSKKQCEKHQEP
D.rerio      86 DLKINTTLREISEHYKEKRPEEKAEVVCDMCEGRKLKALKSCLVCQISYCQTHLEPHSRIAGLKKHKLMDPVRNLEDYICQKHDRP
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B-Box2 domain

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H.sapiens   114 LSLFCYEDQEAVCLICAISHTHRAHTVVPLDDATQEYKEKLQKCLEPLEQKLQEITRCKSSEEKKPGELKRLVESRRQQILREFEE
G.gallus    100 LKLFCEEDQVPICLVCRESHAHRLHAAVPIEEAVEEQKEKIQAHVQILKEKKEKLQGLKEAEEGKSLEFLEKVQQERQKVVLDIKE
X.laevis    103 MKLYCQVDGREICLVCRESVEHRTHTVIPVEESTREFKVQLRDRLQTLRKEAATIIQTKSEDKQKVEKLQKEIEKKRRMLASEFEI
D.rerio     172 LELFCRDDQQIICVSCTDG-DHRNHNTVSLEKTELMIQDTEQMIQDRIKR-IQHIKHSAADRKIYSEKEKAAHVEIFTDLIRSIEG

H.sapiens   200 LHRRLDEEQQVLLSRLEEEEQDILQRLRENAAHLGDKRRDLAHLAAEVEGKCLQSGFEMLKDVKSTLEKNIPRKFGGSLSTICPRD
G.gallus    186 LQQFVEQQERLLLGRLEKLDQEIVRRKEENLAKLLEEISSVSEQIRELEEKCQQPPCEFLQDSRNILSR----------------
X.laevis    189 LREILNDRERELKDRFEVIEKLIIQRTNEKTSKLNEKLSSLQKLIMDIEQNVLPP-SQIYEEPKSAVGR----------------
D.rerio     256 CQIELLEMMEEQQKAAEKQEEELIEELEQEITKLKMRNTKLQQISHTEDLLQIYSSLQCRPADTRNWPEIS---------------
```

PRY domain

```
H.sapiens   286 HKALLGLVKEINRCEKVKTMEVTSVSIELEKNFSNFPRQYFALRKILKQLIADVTLDPETAHPNLVLSEDRKSVKFVETRLRDLPD
G.gallus    272 ------------LENEKSQQAPEILPGAEENPASPPQKNTALKETLMQFRESLTLDPDTAHPRLVLSEDHRCVRWEEAR-HPVPD
X.laevis    274 ---------------------LSQNLSDPQQKPSHG--GTMPATFALKRFTVPVTLDHKTANPNLIISGNRKIVRYEEYP-KNLVN
D.rerio     342 --------------VKTQENLEDLRGALNQLKDTVGEKLTQIELKWMQQYAVDVTLDPDTAHPKLILSDDGKQVKHGDIR-QKLPN
```

SPRY domain

```
H.sapiens   372 TPRRFTFYPCVLATEGFTSGRHYWEVEVGDKTHWAVGVCRDSVSRKGELTPLPETGYWRVRLWN--GDKYAATTTPFTPLHIKVKP
G.gallus    328 NPKRFDSSRCVLGCQGFNAGRHYWEVEVGDGEAWAVGVAKESVERKGRISVKPEVGIWAVGQC---GAQYQALTSPTSPITLHSAP
X.laevis    320 YPERFDMKPCVLGSTGYRSGKRYWEVEVGGGIYWSVGVAAHSVCRKGPFKIEPSGGIWAIGLLGMYTDRYYAFTSPDILLHPRDRP
D.rerio     399 KPQRFDRYAMVLGKEGFSSGRFYFEVQVKGKTKWDLGVVRESVDRKGKITLRPSDGFWTVCLRN--GNQYKAGADSPVSLSLKVKL

H.sapiens   458 KRVGIFLDYEAGTLSFYNVTDRSHIYTFTDTFT--EKLWPLFYPGIRAGRKNAAPLTIRPPTDWE
G.gallus    413 KVIGIYLDYEAGRVAFFDANNEVPIFAYPPTSFGGERVLPLLCLGR------GCRFTLSP-----
X.laevis    405 VVVGVFLNCDEQRVSFFNADSFEHLHTFNDVHS-ADKIFPFFVLEQWEQN---CDLISEFF----
D.rerio     484 QRVGVFVDYEEGLVSFYDVESSSHIYSFTGQTF-TDKLYPYFSPEDNDDGKNSAPLIITPVSSK-
```

Fig. 1. Sequence analysis of *btr20* from zebrafish (*D. rerio*). (A) Genomic structure of *btr20*. Exons are represented by black boxes and lines represent introns; exon lengths are shown on the top of each figure and protein domains are on the bottom. (B) Multiple sequence alignment of deduced Btr20 and TRIM39 proteins from *H. sapiens* (NP_067076.2), *G. gallus* (NP_001006196.3) and *X. laevis* (NP_001085046.1) using the ClustalW software. The predicted domains are marked with a thick line. The conserved residues of the RBB zinc binding motif are marked in gray.

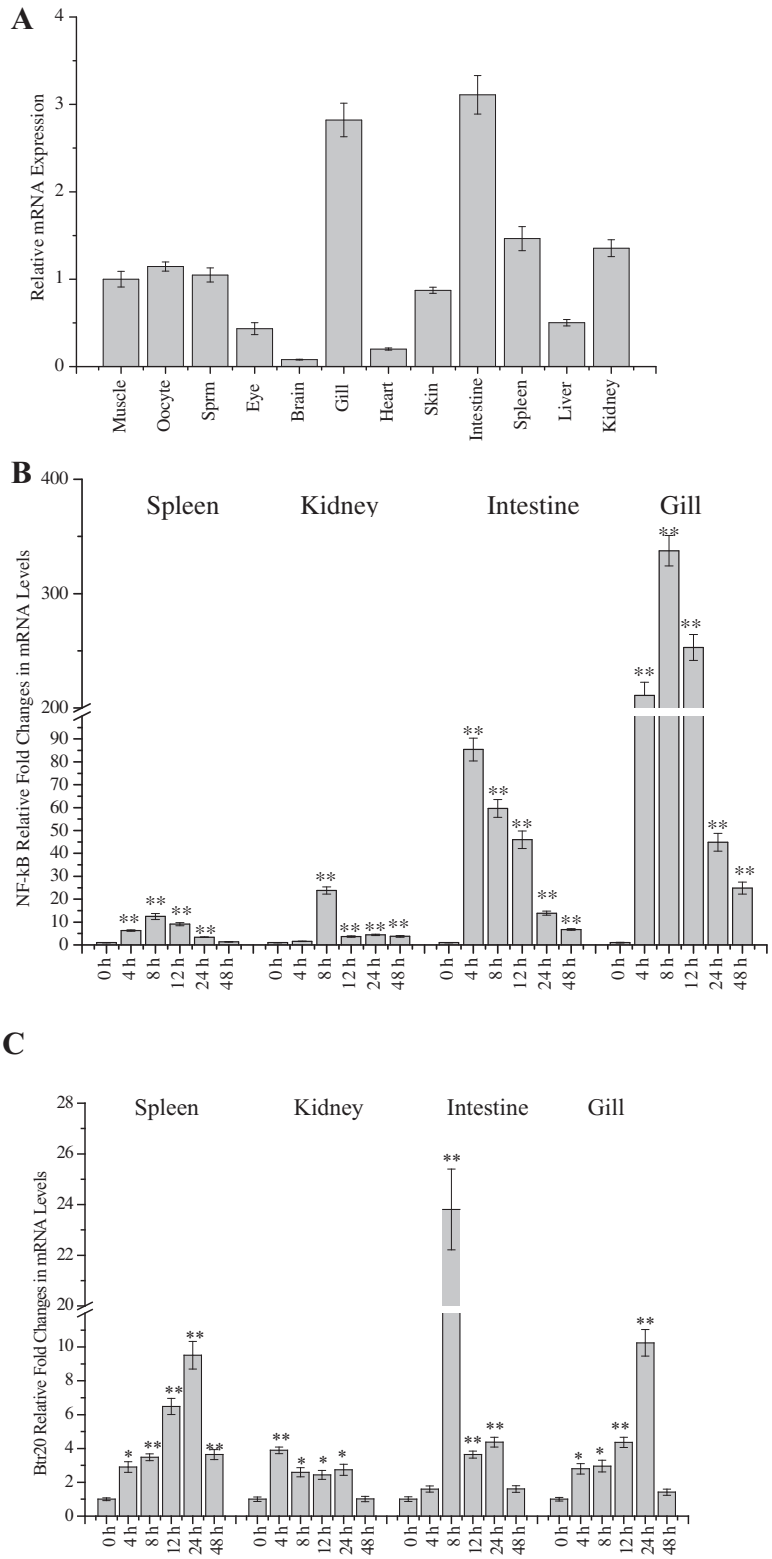

Fig. 2. Expression profiles of *btr20* and *NF-κB* in zebrafish determined by RT-qPCR. (A) *btr20* expression in twelve tissues with the expression level in muscle as 1. (B) *NF-κB* expression in four zebrafish tissues after *A. hydrophila* NJ-1 challenge. (C) *btr20* expression in four zebrafish tissues after *A. hydrophila* NJ-1 challenge. Significant differences were indicated with an asterisk at $p < 0.05$ and two asterisks at $p < 0.01$.

identified in the RING and B-box domains of btr20. The RING domain is a C-x_2-C-$x_{11\text{-}16}$-C-x-H-x_2-C-x_2-C-$x_{7\text{-}74}$-C-x_2-C type and the B-box2 domain is a C-x_2-H-$x_{7\text{-}9}$-C-x_2-[CDHE]-x_4-C-x_2-C-$x_{3\text{-}6}$-H-$x_{2\text{-}4}$-[CH] type (Fig. 1B)[15]. The two domains are encoded as a single exon, which is coincident with TRIM genes from other species (Fig. 1A).

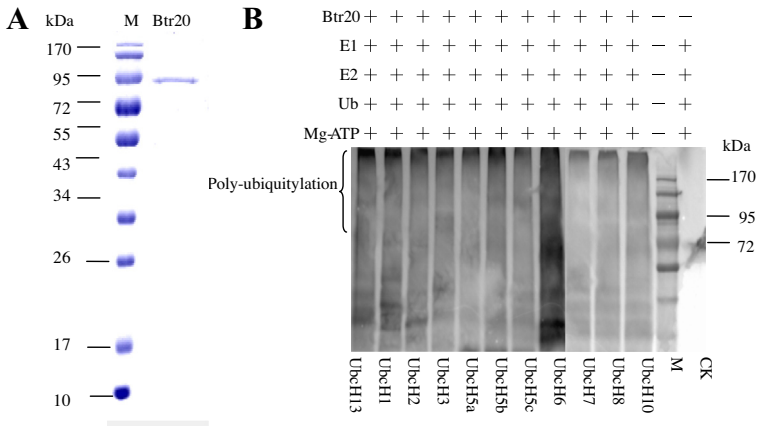

Fig. 3. Heterologous expression of *btr20* in *E. coli* Rosetta cells. (A) SDS–PAGE analysis of recombinant btr20. (B) Western blot analysis of btr20 *in vitro* self-ubiquitylation assay.

3.2. Expression profiles of btr20 in different tissues

RT-qPCR was carried out to analyze the mRNA expression levels of *btr20* in different zebrafish tissues. The expression level of *btr20* was quantified relatively to *EF-1α*, and the level in muscle was set as 1 for calibrator. The *btr20* transcript was detected in all tissues tested with the highest levels in intestine and gill, followed by spleen and kidney. The expression levels in liver, eye, heart and brain were much lower (Fig. 2A).

3.3. Bacterial challenge with A. hydrophila NJ-1

The expression levels of inflammatory cytokine gene *NF-κB* and *btr20* in the gill, intestine, spleen and kidney of zebrafish were analyzed upon the challenge with *A. hydrophila* NJ-1 infection. As show in Fig. 2B, *NF-κB* was up-regulated in the tested tissues in a time-dependent manner, with the maxima at 8 h (12.4-fold) in spleen, 4 h (85.4-fold) in intestine, 8 h (23.8-fold) in kidney, and 8 h (337.5-fold) in gill. The bacterial change also induced the up-regulation of *btr20* expression, reaching the peaks within 24 h (Fig. 2C). In comparison to the control groups, the expression of *btr20* remarkably reached its maximum in spleen and gill at 24 h with a 9.5-fold and 10.2-fold increase, respectively; the most predominant increase occurred in intestine at 8 h with a 23.8-fold increase; the *btr20* expression in kidney was highest at 4 h with a 3.9-fold increase and then maintained in a similar expression level as that of the controls at 48 h (Fig. 2C).

3.4. In vitro self-ubiquitylation of btr20 with different E2s

In order to identify the E3 ubiquitin ligase activity of btr20 *in vitro*, the gene was heterologously expressed in *E. coli* Rosetta cells and purified by affinity chromatography. The purified protein GSTbtr20 yielded a single band of 90 kDa on SDS–PAGE gel (Fig. 3A), in accordance with its predicted molecular mass. The western blot data demonstrated that the recombinant btr20 strongly self-ubiquitylated in the presence of UbcH6, and all the other E2s have a small quantity of accumulation of poly-ubiquitylated products (Fig. 3B). Because the ubiquitylation assay was free of any E3 enzymes other than btr20, btr20 played a role of E3 ubiquitin ligase *in vitro*.

3.5. Intracellular localization of btr20

To study the subcellular localization of btr20, the GFPbtr20 recombinant plasmid was constructed and transfected into HEK293T cells. The subcellular localization of transiently expressed btr20 was investigated after 72 h. The results showed that btr20 were localized in the cytoplasm and formed aggregate as shown in Fig. 4.

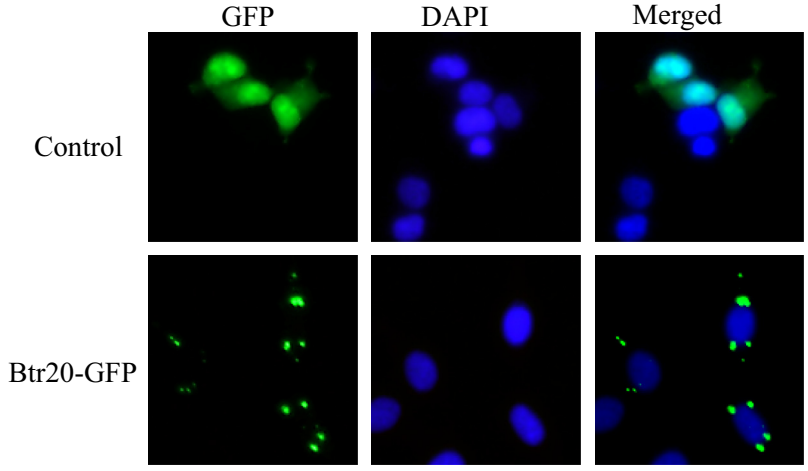

Fig. 4. Subcellular distribution of btr20 in human cell line HEK293T. The nuclei were dyed with DAPI.

4. Discussion

TRIM proteins exert a wide range of activities, including cell proliferation, apoptosis, oncogenesis and the regulation of innate and adaptive immune responses. Most of TRIM proteins have been studied in human and mouse, which are generally induced by IFN and critical for anti-microbial activity. Rapid biotechnology advances in a variety of fields have allowed zebrafish to be a more versatile tool for the study of immunology and host-pathogen interactions. In zebrafish, more than 200 TRIM genes have been identified. The zebrafish *btr* genes are orthologous to human TRIM39, and are relatively dispersed in the zebrafish genome. A preliminary screening of the *btr* genes in kidney after *A. hydrophila* NJ-1 infection (data not shown) showed that some of them were up regulated, especially the *btr20*. Thus we selected *btr20* for further expression and subcellular localization analysis.

A. hydrophila NJ-1 is an opportunistic pathogen of fish and amphibians. When the pathogen invades the fish, the cells firstly reproduce in intestine, and then flow through the artery circulation to the spleen, kidney and other tissues, causing blood disorder and whole body symptoms. In this study, we found that the expression of *btr20* in the immune-related organs (intestine, gill, spleen and kidney) was up-regulated upon *A. hydrophila* NJ-1 infection, suggesting the role of *btr20* in innate immunity against the infection of pathogenic bacteria. NF-κB has a pivotal role in innate immunity, which expression has also been induced by *A. hydrophila*. Similar to phosphorylation, ubiquitylation is involved in the regulation of NF-κB pathway. TRIM proteins as E3 ubiquitin ligases have been revealed as a crucial regulator in regulating the signaling of NF-κB [3,12,23,24]. After bacterial infection we detected the increased expression level of NF-κB mRNA (Fig. 2B). Previous studies have shown that some TRIM proteins, i.e., TRIM19, TRIM21, TRIM23, and TRIM30α, can modulate the various stages of NF-κB activation post PRRs activation [8-10,25]. The similar up-regulation trends of NF-κB and *btr20* upon bacterial infection suggests that btr20 may act as a modulator in the NF-κB signaling pathways to regulate the innate immunity against pathogens infection. It would be interesting to identify the interacting proteins and the mechanisms in the regulation of the signal pathways.

The TRIM family members are also known as RBCC proteins because of the highly conserved order of RING-B-box-Coiled coil domains. This conserved structure makes TRIM proteins of various species form a superfamily. Both the RING and B-boxes are cysteine-rich zinc-binding domains that share a distinct pattern of cysteine and histidine residues. In this study, the btr20 has a C3HC4 type of RING domain that coordinates two zinc atoms. The first atom is mediated by Cys at positions 1, 2, 5, and 6, and the Cys or His at position 3, 4, 7, and 8 interact with the second atom (Fig. 1B). Different pattern in the second potential coordination residue with the RING domain, the B-box domain binds only one atom. The RING finger domain has been found to play a critical role in mediating the transfer of ubiquitin both to substrates as well as to the RING proteins themselves. TRIM proteins have demonstrated E3 ubiquitin ligase activities in the ubiquitylation system. For example, two members of finTRIMs in trout were found to display E3 ubiquitin ligase activity [18], and TRIM3a from zebrafish had demonstrated as a RING domain dependent E3 ubiquitin ligase [22]. btr20 under study also acts as an E3 ubiquitin ligase in zebrafish; its recombinant form has self-ubiquitylation activity *in vitro* in the presence of E2s and has an abundant accumulation of polyubiquitinylated products, especially with UbcH6. These findings showed that the conserved structure of the RING domain accounts for the common function of ubiquitin ligase in TRIM proteins. Protein ubiquitylation is a multi-step process mediated by three enzymes: the ubiquitin-activating enzyme E1 activates ubiquitin, the ubiquitin-conjugating enzyme E2 conjugates the activated ubiquitin and transfers the activated ubiquitin to a substrate with the ubiquitin-ligase E3, and the E3 enzyme mediates the formation of an isopeptide bond between the activated ubiquitin conjugated to E2 and a lysine-residue of the substrate [26]. A comprehensive framework of E2-RING of E3 interactions in human indicated that multiple E2 and E3 enzymes interact with multiple E3s and E2s, respectively [27]. UbcH6 has been identified as an E2 enzyme and regulates proteins in autoimmune disease, immune signal pathways and tumors [28–30]. TRIM21 (Ro52) is a cytoplasmic protein and interacts with UbcH6, while UbcH6 is localized in the nucleus [29]. The different intracellular compartments would depend on changing the intracellular localization of either TRIM21 or UbcH6 induced by an inflammatory mediator. Here we identified btr20 under study was localized in the cytoplasm and formed aggregate. It would be interesting to study further whether btr20 would be relocalized after bacterial infection.

In conclusion, a bloodthirsty member 20 was identified in the genome of zebrafish, and its structural and functional analysis was carried out. Our results showed that *btr20* was highly expressed in intestine, gill, spleen and kidney and showed remarkable up-regulation upon *A. hydrophila* infection. As an E3 ubiquitin ligase, the btr20 could be self-ubiquitylated with most E2 enzymes, especially with UbcH6. Further studies need to be performed to investigate the regulating mechanisms of btr20 in the signal pathways against bacterial infection.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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