1 *PmTBC1D20*, a Rab GTPase-activating protein from the black tiger
2 shrimp, *Penaeus monodon*, is involved in white spot syndrome virus
3 infection

4 Wanchart Yingvilasprasert^a, Premruethai Supungul^{a,b}, Anchalee Tassanakajon^{a,*}

5

6 ^a *Center of Excellence for Molecular Biology and Genomics of Shrimp, Department of*
7 *Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*

8 ^b *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science*
9 *and Technology Development Agency (NSTDA), Pathumthani 12120, Thailand*

10

11

12

13

14

15

16

17

18

19 ^{*}Corresponding author. Tel.: +66 2 218 5439; Fax: +66 2 218 5414.

20 E-mail address: anchalee.k@chula.ac.th (A. Tassanakajon)

ABSTRACT

TBC (TRE2/BUB2/CDC16) domain proteins contain an ~200-amino-acid motif and function as Rab GTPase-activating proteins that are required for regulating the activity of Rab proteins, and so, in turn, endocytic membrane trafficking in cells. TBC domain family member 20 (TBC1D20) has recently been reported to mediate hepatitis C virus replication. Herein, *PmTBC1D20* identified from the black tiger shrimp, *Penaeus monodon*, was characterized and evaluated for its role in white spot syndrome virus (WSSV) infection. The full-length cDNA sequence of *PmTBC1D20* contains 2,003 bp with a predicted 1443 bp open reading frame encoding a deduced 480 amino acid protein. Its transcript levels were significantly up-regulated at 24 and 48 h by ~2.3- and 2.1-fold, respectively, after systemic infection with WSSV. In addition, depletion of *PmTBC1D20* transcript in shrimps by double stranded RNA interference led to a decrease in the level of transcripts of three WSSV genes (*VP28*, *ie1* and *wsv477*). This suggests the importance of *PmTBC1D20* in WSSV infection. This is the first report of TBC1D20 in a crustacean and reveals the possible mechanism used by WSSV to modulate the activity of the host protein, *PmTBC1D20*, for its benefit in viral trafficking and replication.

Keywords: TBC domain, *Penaeus monodon*, white spot syndrome virus, shrimp, Rab-GTPase-activating protein

1. Introduction

The Black Tiger shrimp, *Penaeus monodon*, is one of the most important commercial crustacean aquaculture species worldwide and especially in Thailand, the leading producer and exporter of shrimp products (~550,000 and ~350,000 metric tons, respectively, in 2012), with the exports providing ~3,330 million US dollars (~100,000 million bahts) each year. However, large scale intensive monocultures have led to disease problems worldwide, including in Thailand, that cause serious yield and economic losses in the shrimp farming industry due to poor shrimp growth, quality and even complete farm or region wide deaths of the farm stocks caused by various microbes (Flegel, 2006). Of the pathogenic diseases of farmed shrimps, viruses are especially prevalent, infectious and account for the most significant losses of worldwide shrimp value and production, and include the White Spot Syndrome Virus (WSSV). WSSV symptomatically affects a diverse array of crustaceans and most cultivated marine shrimp species worldwide and causes significant and rapid mortality, ranging from 70-100% mortality in 3 to 10 days. For *P. monodon*, WSSV infection typically leads to 100% mortality within 7 days of infection with an entire cultivation pond being wiped out. This rapid and high mortality level of infected shrimps induces a high economic loss in the shrimp farming industry (Chou *et al.*, 1995), which combined with its worldwide distribution and diverse host range, makes control strategies of great importance but are currently limited to essentially unavailable. WSSV is a 293-307 kbp dsDNA virus belonging to the *Nimaviridae* family and monotypic genus *Whispovirus*. This rod-shaped or ovoid/ellipsoid to bacilliform virus with a flagella-like tail is about 120-150 nm in diameter at the center by 270-290 nm in length (van Hulten *et al.*, 2001). Shrimps will typically die within 2-7 days after infection and no effective control method or treatment has been used in the field (Chou *et al.*, 1995). Although siRNA and anti-viral coat antibody based neutralization immunization have been proposed as potential therapeutic agents for WSSV

66 treatment, these approaches are costly and laborious to the point of currently being
67 logistically impractical, whilst the principal also suffers from that such approaches are often
68 host-species or viral strain specific. Therefore, a greater knowledge of the shrimp immune
69 system and virus infection mechanism would be likely to help to find an effective treatment
70 for this infectious and often devastating disease of cultured shrimps, if not groups of viral
71 infections that share common traits for infection or replication methods.

72 Rabs are a large group of GTP-binding proteins that are an apparent key regulator in
73 the endocytotic and exocytotic transport processes, controlling membrane trafficking, and in
74 the identity of cellular compartments (Barnekov et al., 2009; Zerial and McBride, 2001).
75 Many viruses usurp host Rab proteins for directing the endocytosis, trafficking and
76 assembling of their proteins (Fukuda, 2008; Krishnan et al., 2007; Sklan et al., 2007a, 2007b;
77 Stenmark, 2009). In the Rab cycle, the activity of the Rab proteins is regulated by members
78 of the two specific key families, namely the GDP-GTP exchange factors (GEFs), which
79 activate the inactive state (GDP-bound) of Rabs to the active state (GTP-bound), and the
80 GTPase activating proteins (GAPs), which terminate Rab activity by hydrolyzing the bound
81 GTP and so switching the active GTP-bound form to the inactive GDP-bound form (Barr and
82 Lambright, 2010; Fukuda, 2011). Several human Rabs have been identified and
83 characterized, whilst the Rab effectors remain less well characterized.

84 GAPs have an important role in promoting the hydrolysis of GTP bound to specific
85 GTPases that results in inactivation of the GDP-bound GTPase. In the active (GTP-bound)
86 state, GTPases that are membrane bound promote the assembly of effector complexes, which
87 can be cytosolic or membrane proteins, at the membrane surface. These effectors display
88 various biological functions that are important for signalling, such as vesicle formation and
89 fusion (Barr, 2009). The Rab GAP, GYP6, was the first GAP identified in *Saccharomyces*
90 *cerevisiae* (Strom et al., 1993), and subsequently some yeast Ypt GAPs have been identified.

From their sequences it is apparent that these Rab GAP proteins share a common protein domain, the TBC (TRE2/BUB2/CDC16) domain (Neuwald, 1997; Richardson and Zon, 1995). TBC domain proteins contain an ~200-amino-acid motif that was initially identified in the TRE2/BUB2/CDC16 genes (Richardson and Zon, 1995). The TBC domain has three signature motifs: Rxxxw (motif A), IxxDxxR (motif B), and YxQ (motif C), which are located at the N-terminus of the domain. Like all TBC family members, TBC1 domain family member 20 (TBC1D20) contains a Rab GAP homology domain (Barr, 2009). In *Caenorhabditis elegans*, the TBC domain has been shown to confer a role in cytokine systems and the innate immunity (Alper et al., 2008). Human TBC1D20 has been implicated in mediating Hepatitis C virus (HCV) replication by binding to the viral NS5A protein that is involved in replicating the viral genome. Depletion of TBC1D20 severely impaired HCV replication and prevented new infection, with a minimal effect on the host cell viability (Sklan et al., 2007a). Subsequently, TBC1D20 was found to be a Rab GAP for the Rab1 GTPase that is located in the endoplasmic reticulum (ER) (Sklan et al., 2007b). Rab1 GTPase regulates the anterograde transport of cargo between the ER and the Golgi apparatus (Barnekov et al., 2009). Thus, TBC1D20 seems to be the GAP that blocks the delivery of secretory cargo from the ER to the cell surface (Haas et al., 2007).

Recently, a homologue of TBC1D20 was identified in the expressed sequence tag (EST) libraries of *P. monodon*, and cDNA microarray analysis revealed that *PmTCB1D20* was up-regulated in response to WSSV infection (unpublished data). Herein, a full-length cDNA sequence of *PmTBC1D20* was obtained and the protein domains characterized from the deduced encoding amino acid sequence. The induction of *PmTBC1D20* transcript levels by WSSV infection in the hemocytes was verified by quantitative real-time RT-PCR, whereas double stranded (ds)RNA interference (RNAi)-mediated gene silencing was performed to study the effect of *PmTBC1D20* depletion on WSSV-infected shrimps. In

116 addition, fluorescence-labeling along with confocal laser scanning microscopy (CLSM) was
117 used to localize the *PmTBC1D20* protein in the shrimp hemocytes. This is the first report of
118 TCB1D20 in a crustacean and demonstrates the possible role of *PmTBC1D20* in WSSV
119 infection.

2. Materials and methods

2.1. Obtaining the full length *PmTBC1D20* cDNA sequence

The 5' and 3' RACE libraries were prepared from *P. monodon* using the SMART RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's protocol and then used as templates for the 5' and 3' RACE-PCR using the gene specific primers 5R6817 and 3R6817, respectively (Table 1). These primers were designed from the partial cDNA sequence of *PmTBC1D20* (Hc-N-N01-2807-LF) obtained from the *P. monodon* EST libraries. The RACE-PCR product was ligated into the pGEM®-T Easy vector (Promega, USA) and transformed into JM109 cells, as per the manufacturer's protocol. After selection and cloning of transformants, the recombinant plasmid DNA was extracted and then commercially sequenced (Macrogen Inc., Korea). The whole fragment of *PmTBC1D20* gene was amplified by PCR using fullTBC_F and fullTBC_R primers (Table 1).

2.2. Sequence analysis

The cDNA sequence of *PmTBC1D20* was analyzed for its likely identity and similarity to other known sequences in the NCBI GenBank database using the online BLASTX algorithm (Altschul et al., 1990). Domain identification of the deduced encoded amino acid sequence was analyzed by the PROSITE (<http://prosite.expasy.org>) and SmartTM databases (<http://smart.embl-heideber.de/>), whilst the signal peptide was predicted using the online SignalP 3.0 program (<http://www.cbs.dtu.dk/services/SignalP/>) (Bendtsen et al., 2004). The predicted amino acid sequences of TBC1D20 homologs from vertebrates and invertebrates (including insects and *P. monodon*) in the GenBank database were used to identify and compare the TBC domain by PROSITE and SMART databases. Then, the TBC domains were multiply aligned using the CLUSTAL-X program (Thompson et al., 1997).

2.3. Shrimp samples, total RNA extraction, and cDNA synthesis

Sub-adult *P. monodon* shrimps of about 16 g wet (live) weight were obtained from a local shrimp farm in Thailand, and were then acclimatized at a salinity of 15 ppt for at least 7 days in laboratory culture before use in the experiments. Shrimps were separated into two groups; a WSSV-infected group and an uninfected control group. In the WSSV-infected group, shrimps were injected with 100 μ L of a 1:8000 dilution of the WSSV stock in lobster hemolymph medium (LHM; 15 mM CaCl₂, 10 mM KCl, 5 mM MgCl₂, 8.1 mM MgSO₄, 486 mM NaCl, 0.5 mM Na₂HPO₄, 36 mM NaHCO₃ and 0.05% (w/v) dextrose in Minimum Essential Medium (Invitrogen)), a dose which was found to cause 100% shrimp mortality within 4 days (data not shown). The control shrimps were treated in the same manner except they were injected with 100 μ L of WSSV-free LHM. The hemolymph was collected from shrimps using syringes containing 10% (w/v) sodium citrate as the anti-coagulant at 0.25, 12, 24, and 48 h post infection (hpi) from three individual shrimps per group at each time point. The hemolymph was immediately centrifuged (800 x g; 10 min, 4 °C), the hemocyte pellet harvested and total RNA extracted using the TRI Reagent[®] (Molecular Research Center). DNA contamination was removed by RNase-free DNase I (Promega) treatment. One μ g of DNase-treated total RNA was used as the template to synthesize the first strand cDNA using the First Strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. The synthesized cDNA was stored at -80 °C until used.

2.4. Tissue distribution of *PmTBC1D20* transcript

In order to investigate the tissue distribution of *PmTBC1D20* transcript in *P. monodon*, total RNAs were extracted from lymphoid organ, eye stalk, gill, heart, hemocyte, hepatopancreas, intestine, epipodite, antennal gland and stomach of healthy shrimp using TRI reagent[®] (Molecular Research Center). Total RNAs were removed the DNA contamination and subjected to synthesize the first strand cDNA as described in section 2.3. The expression

of *PmTBC1D20* mRNA was analyzed by semi-quantitative RT-PCR using the sg6817F/sg6817R primers (Table 1). EF-1 α was used as the internal reference control was detected with the gene specific primers, EF-1 α F and EF-1 α R (Table 1). One microliter of the ten-fold diluted cDNA was subjected to PCR in a 25 μ L reaction volume containing 10 mM KCl, 2 mM MgSO₄.7H₂O, 20 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 10 mM (NH₄)₂SO₄, 0.1mg/ml BSA, 0.2 mM of each dNTP, 0.2 μ M of each specific primers and 1.5 unit of Taq DNA polymerase (RBC Bioscience). The reaction was predenatured at 94 °C for 2 min following by 30 cycles of denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. Amplification product was analyzed by resolution through a 1.5% (w/v) agarose gel and visualized by UV transillumination after ethidium bromide visualization.

2.5. Gene expression profile of *PmTBC1D20* in *P. monodon* hemocytes upon WSSV infection by quantitative real-time RT-PCR

To investigate the expression levels of *PmTBC1D20* mRNA in *P. monodon* hemocytes after WSSV infection, quantitative real-time RT-PCR (qrt-RT-PCR) was performed. The specific primers, sg6817real_F and sg6817real_R primers (Table 1) were used to amplify an expected 171 bp product from the cDNA. A partial fragment (150 bp) of the EF-1 α gene transcript was amplified using the EF-1 α F/EF-1 α R primers (Table 1) and used as the internal reference control. A standard curve for amplification of the *PmTBC1D20* fragment was produced and compared to that of the EF-1 α fragment. Qrt-RT-PCR was carried out in a real-time thermal cycler (Bio-Rad, USA) with iQTM SYBR[®] Green Supermix (Bio-Rad, USA). The reaction mixture (20 μ L) consisted of 5 μ L of the 1:10 diluted cDNA template, 0.2 μ M of each primer and 1 $\times$ iQTM buffer containing DNA polymerase. Thermal cycling was performed at 95 °C for 9 min, followed by 40 cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 45 sec. For each sample, the ΔC_t was calculated from the threshold

PCR cycle (C_t) of the TBC1D20 gene normalized relative to the C_t of the reference EF-1 α gene in the same sample. The $\Delta\Delta C_t$ value was then calculated from the ΔC_t of the WSSV-infected group - ΔC_t of the control group (LHM injected). Each qrt-RT-PCR reaction was performed in triplicate and the results are presented as the relative expression ratios derived from $2^{-\Delta\Delta C_t}$. The data were subjected to one-way analysis of variance (one-way ANOVA) (Steel and Torrie, 1980) followed by Duncan's new multiple range test (Duncan, 1955) using the SPSS statistics 17.0 program. Significance was accepted at the $P < 0.05$ level.

2.6. Immunolocalization of the PmTBC1D20 protein in shrimp hemocytes

To investigate the localization of PmTBC1D20 protein in shrimp hemocytes, the anti-PmTBC1D20 antibody was produced. Briefly, the TBC domain sequence of PmTBC1D20 gene was clone into the pET19b with a 6-His tag at the amino-terminus. The recombinant plasmid was then transformed into an *E. coli* Rosetta (DE3) for overexpression of recombinant TBC domain (rTBC). The rTBC protein was purified using a Ni-Sepharose column (GE). After confirmation with western blot analysis, TBC domain protein was further used to generate rabbit polyclonal antibodies by a commercial service at Biomedical Technology Research Unit, Chiang Mai University, Thailand.

The hemolymph was collected from six individual healthy shrimps, and then fixed in 4% (w/v) paraformaldehyde for 10 min. The fixed hemolymph was centrifuged at $800 \times g$ at 4 °C for 10 min to separate the hemocytes. The hemocyte pellet was resuspended in phosphate buffered saline (PBS) and attached onto the poly-L-lysine coated slide by centrifugation at $1000 \times g$ for 10 min. Slides were blocked with blocking buffer (10% (v/v) fetal bovine serum in PBS) at room temperature for 1 h, and then probed with purified rabbit polyclonal antibody against PmTBC1D20 and purified mouse monoclonal antibody against VP28 for 1 h at 37 °C. The slides were washed three times with washing buffer (0.05% (v/v)

Tween-20 in PBS) and subsequently incubated with Alexa 488 conjugated goat-anti-rabbit antibody (Invitrogen) at room temperature for 1 h. The slides were then washed three times with washing buffer to remove non-specific binding, incubated with TO-PRO-3 iodide (Invitrogen) to stain the nuclear DNA and washed once with PBS. ProLong Gold antifade (Invitrogen) mounting medium was applied, and the slides were examined by CLSM under a Nikon confocal laser scanning microscope and collecting bright field and fluorescence images.

2.7. RNAi-mediated knock-down of PmTBC1D20 and WSSV infection

2.7.1. Double-stranded RNA synthesis (dsRNA)

DsRNA of the *PmTBC1D20* gene, and that for the GFP gene as a control, were synthesized using the T7 Ribomax™ Express Large Scale RNA production System (Promega). The specific primers containing the 5' T7 promoter sequence of sg6817_FT7 and sg6817_RT7 for the *TBC1D20* gene, and GFP_FT7 and GFP_RT7 for the GFP gene (Table 1) were used to amplify single stranded (ss)RNA from each respective plasmid DNA template (1 µg). Both PCR reactions were performed with 0.02 units/µL of Hotstart Phusion Taq DNA Polymerase (New England Biolabs) with PCR cycle parameters of an initial denaturation at 98 °C for 30 s, followed by 39 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 20 s, and with a final extension at 72 °C for 10 min. After purification, one microgram of each template was used in an in vitro transcription of T7 RiboMAX™ Express Large Scale RNA Production Systems (Promega, USA), according to the manufacturer's protocol. An equal amount of each respective pair of ssRNA was then annealed to form the dsRNA and the DNA template was eliminated by DNaseI treatment, as detailed in the manufacturer's protocol. An aliquot of the dsRNA was analyzed by 1.5% (w/v) agarose-TBE gel electrophoresis to confirm its yield and purity.

2.7.2. PmTBC1D20 gene silencing by RNAi

To investigate the function of *PmTBC1D20* in response to WSSV infection, RNAi mediated gene knock-down of *PmTBC1D20* was performed by double injections (24 h apart) of 10 µg of dsRNA per 1 g shrimp wet weight. Juvenile *P. monodon* shrimps of 4 g wet weight were used and split into three groups, each of nine individual shrimps. A total of 40 µg of *PmTBC1D20* dsRNA or GFP dsRNA were dissolved in 0.85% (w/v) NaCl to a final volume of 30 µL and were injected into each of the nine shrimps in the respective group (*PmTBC1D20* or GFP dsRNA) in the lateral area of the fourth abdominal segment using a 0.5-mL insulin syringe with a 29-gauge needle. The third group was injected as above but with only 0.85% (w/v) NaCl. After 24 h the second injection of the same dsRNA (10 µg per 1 g shrimp) or 0.85% (w/v) NaCl was injected into each shrimp. After a further 24 h the hemolymph of three individual shrimps from each group were collected and used for total RNA extraction and first strand cDNA synthesis, as described above. The effectiveness of the RNAi-mediated gene knock-down of the *PmTBC1D20* gene was analyzed by semi-quantitative RT-PCR using the sg6817F/sg6817R primers (Table 1) as mentioned in section 2.4. EF-1α was used as the internal reference control was detected with the gene specific primers, EF-1αF and EF-1αR as mentioned in section 2.4.

2.7.3. Effect of *PmTBC1D20* depletion on WSSV infection in *P. monodon* hemocytes

In order to investigate the effect of *PmTBC1D20* transcript suppression on WSSV infection, the transcription levels of three WSSV genes were determined in the RNAi *PmTBC1D20*-knock-down shrimps after WSSV infection. Juvenile shrimps of 4 g wet weight were double injected with *PmTBC1D20* dsRNA, GFP dsRNA or 0.85% (w/v) NaCl as outlined in section 2.7.2, except that the second injection also contained WSSV (10² copies). The hemolymph was collected 24 h after this second injection (24 hpi with WSSV). Transcript levels of the *VP28*, *ie1*, and *wsv477* genes of WSSV were analyzed using qrt-RT-PCR. The qrt-RT-PCR was performed using an equal amount of cDNA template in an

267 iCycler iQTM Real-Time Detection system using iQTM SYBR Green Supermix (Bio-Rad). The
268 condition for *VP28* (VP28QF/VP28QR) (Table 1), *ie1* (ie1-qrtF/ie1-qrtR) (Table 1) and
269 *wsv477* (477-qrtF/477-qrtR) (Table 1) genes amplification was the same conditions as that
270 detailed for the sg6817real primers in section 2.5. The calculation of the relative expression
271 level was also performed as described in section 2.5. All samples were normalized relative to
272 the C_t of the reference EF-1 α gene in the same sample.

273

3. Results

3.1. Full-length cDNA of *PmTBC1D20*

A partial cDNA sequence of TBC1D20 was previously obtained from the EST library of WSSV-challenged *P. monodon* hemocytes (<http://pmonodon.biotec.or.th>) (Tassanakajon et al., 2006). The complete sequence of *PmTBC1D20* was obtained in this study using 5'- and 3' RACE and was found to contain a 5'-untranslated region (UTR) of 226 bp and a 3' UTR of 337 bp with a putative polyadenylation signal sequence and a poly(A) tail. The deduced open reading frame of *PmTBC1D20* contained 1443 bp and was predicted to encode for 480 amino acids (Fig. 1). No signal peptide was found using the on-line SignalP 3.0 program. BLASTX searching of GenBank search indicated that this protein shared a 54% significant amino acid sequence similarity to the TBC1 domain family, member 20-like of the acorn worm, *Saccoglossus kowalevskii*. Protein structural analysis using the SMART program revealed the TBC domain in the middle of this protein and also found a transmembrane region at the C-terminus, as illustrated in Fig. 1.

To evaluate the relationships among the TBC1D20 proteins, the predicted amino acid sequence of the TBC domain from *PmTBC1D20* was compared with those of known TBC1D20 members in the GenBank database from vertebrates (human (*Homo sapiens*), house mouse (*Mus musculus*), zebrafish (*Danio rerio*), and lancelet (*Branchiostoma floridae*)), and invertebrates of echinoderm (sea urchin (*Strongylocentrotus purpuratus*)), hemichordate (acorn worm (*Saccoglossus kowalevskii*)) and insects (yellow fever mosquito (*Aedes aegypti*), fruit fly (*Drosophila melanogaster*), and red flour beetle (*Tribolium castaneum*)) by using the CLUSTAL-X program to align the amino acid sequences, as illustrated in Fig. 2. The multiple sequence alignment revealed a high similarity of these domains between vertebrates and invertebrates, with 26% identity and 57% semi-conserved

sequence similarity across all ten phylogenetically diverse sequences. The % amino acid sequence identity between more closely related members was much higher. The TBC domain of *PmTBC1D20*, in common with other TBC domain members, showed two conserved catalytic residues (arginine¹⁸⁷ and glutamine²²⁶) and three highly conserved fingerprint sequence motifs, ¹⁴⁸RxxxW, ¹⁸¹VxxDxxR, and ²²⁴YxQ.

3.2. Tissue distribution of *PmTBC1D20* transcript in *P. monodon*

Ten tissues of healthy shrimp including lymphoid organ, eye stalk, gill, heart, hemocyte, hepatopancreas, intestine, epipodite, antennal gland and stomach were analyzed the expression of the *PmTBC1D20* mRNA levels by RT-PCR. This gene transcript was expressed in all tissues, especially in intestine followed by hemocyte, gill and antennal gland (Fig. 3). Expression levels were lower in lymphoid organ, heart, hepatopancreas and epipodite and lowest in eye stalk (Fig. 3).

3.3. Up-regulation of *PmTBC1D20* transcript levels in *P. monodon* hemocytes after WSSV infection, as determined by Qrt-RT-PCR

A previous microarray analysis of genes with altered expression levels in hemocytes of *P. monodon* identified *PmTBC1D20* as one of the genes that was up-regulated in response to WSSV infection (unpublished data). Here, the expression level of *PmTBC1D20* transcripts in *P. monodon* hemocytes after WSSV infection was examined by qrt-RT-PCR. *PmTBC1D20* transcript levels in hemocytes were revealed to be significantly increased at 24 and 48 hpi with WSSV, by about 2.3- and 2.1- fold, respectively, compared to the control uninfected shrimps (Fig. 4).

3.4 Localization of *PmTBC1D20* protein in shrimp hemocytes by immunofluorescence microscopy

To examine the expression of *PmTBC1D20* protein in shrimp hemocytes, immunocytochemistry with CLSM was performed. Hemocytes, collected from the healthy shrimps were visualized for the presence of the host *PmTBC1D20* using the respective specific antibodies the respective Alexa 448 fluorophore-conjugated secondary antibodies. Consequentially, the location of *PmTBC1D20* protein was observed as green color. The hemocyte nucleus was stained with To-Pro-3 iodide and the emission spectral filter adjusted to blue color. A positive signal for *PmTBC1D20* protein was observed in the cytoplasm of all types of shrimp hemocytes as showed in Fig.5.

3.5. *Suppression of PmTBC1D20 transcript levels reduces WSSV replication in hemocytes*

RNAi-mediated-gene knock-down was used to study the effect of suppression of the *PmTBC1D20* transcript level on the WSSV infection in hemocytes. First, the efficiency of the *PmTBC1D20* knock-down was ascertained. Shrimps were double-injected with either *PmTBC1D20* dsRNA or, as controls, with GFP dsRNA or 0.85% (w/v) NaCl alone, and then 24 h later the hemolymph from each shrimp was collected for RNA extraction and the expression level of *PmTBC1D20* transcripts determined by qrt-RT-PCR. Suppression of the *PmTBC1D20* transcript was observed in the ds*PmTBC1D20*-injected shrimps (Fig. 6A) but not in either control groups (dsGFP- or NaCl-injected shrimps).

To determine whether suppression of the *PmTBC1D20* transcript level affected WSSV infection, shrimps were injected twice (24 h apart) with *PmTBC1D20* dsRNA, or GFP dsRNA or NaCl for the two control groups, and also with WSSV on the second injection. Then 24 hpi with WSSV the hemolymph was collected for determination of the expression level of three viral gene transcripts by qrt RT-PCR. The expression levels of the WSSV immediate early gene (*ie1*), early gene (*wsv477*), and late gene (*VP28*) were all significantly decreased in the *PmTBC1D20* knock-down hemocytes, by more than 90%, when compared to the control group (Fig. 6B). Thus, RNAi-mediated knock-down of

346 *PmTBC1D20* transcript levels in shrimps appeared to decrease the WSSV infection level, at
347 least in hemocytes.

348

4. Discussion

There are several potential pathways for viruses to enter animal cells, including endocytosis (Gruenberg, 2009). In humans, many viruses, such as HCV, influenza virus, human immunodeficiency virus and dengue virus, enter host cells by endocytosis (Krishnan et al., 2007; Sieczkarski and Whittaker, 2003; Sklan et al., 2007a, 2007b; Vidricaire and Tremblay, 2005). Most intracellular membrane trafficking for endocytosis, intracellular movement and secretion is mediated via members of the Rab GTPase superfamilies, which are regulated by the specific GEF and GAP regulators (Mizuno-Yamasaki et al., 2012). However, the current understanding of these interactions is far from complete, especially in crustaceans. Therefore, further study on these regulators will likely lead to a better understanding of the mechanisms of viral infection.

TBC domain proteins, members of the GAP family, are one of the three effectors that regulate the activity of the Rab GTPases. They interconvert Rab between an active GTP-bound form and inactive GDP-bound form (Barr, 2009). The cycling between GTP and GDP can lead to many processes in cells, including in the control of membrane trafficking to transport any membrane-bound protein or cargo within the cells from one organelle to another for processing or for secretion (Barr, 2009). TBC1D20, one of TBC domain family proteins, is specific for Rab1 GTPase. However, although TBC1D20 functions to inactivate Rab1 GTPase in normal cells, it is also usurped by some viruses to mediate aspects of their replication, such as HCV (Sklan et al., 2007a, 2007b). The interaction between the N terminus of the HCV nonstructural protein NS5A and the host *Hs*TBC1D20 may subvert the host cell machinery for mediating the endocytosis, trafficking and also sorting of the viral proteins (Sklan et al., 2007b). In the case of herpes simplex virus, two Rab GTPases (Rab

1a/b and Rab43) and their respective partners (TBC1D20 and RN-tre) are important for the trafficking of the virus envelope proteins from the ER to the Golgi (Zenner et al., 2011).

In shrimps, most reports to date have focused only on the Rab GTPase, which are also involved in WSSV infection, but not their effectors. The *PjRab* (Rab6A) protein of *Peneaus japonicus* was found to be up-regulated upon WSSV infection (Wu and Zhang, 2007), whereas the Rap GTPase from the Chinese white shrimp, *Fenneropenaeus chinensis*, was reported to be up-regulated upon both *Vibrio harveyi* (bacteria) and WSSV infections (Ren et al., 2012). Indeed, *in vitro* pull-down assays indicated that Rap GTPases interact with VP28, the envelope protein of WSSV (Ren et al., 2012). RNAi-mediated gene knock-down of Rab7 in *P. monodon* was found to affect WSSV, YHV and Leam-Singh virus infections (Ongvarrasopone et al., 2008, 2010; Sritunyalucksana et al., 2006). Nevertheless, the mechanism of virus entry into shrimp cells is still not well-understood.

In the present study, the cDNA encoding *PmTBC1D20* was initially identified from *P. monodon* EST libraries and cDNA microarray analysis revealed that the *PmTBC1D20* transcript was up-regulated at 6 hpi with WSSV infection (Pongsomboon et al., 2011). This is the first report of a TBC1D20 homolog in a crustacean. Like all TBC domain proteins, *PmTBC1D20* contains the three conserved signature motives (motifs A-C) that are important for promoting the hydrolysis of GTP bound to the Rab protein. The TBC domain has been proposed to be involved in catalyzing GTP hydrolysis by a dual-finger mechanism (Pan et al., 2006), where the conserved glutamine (Q) in motif C positions the nucleophilic water molecule required for GTP hydrolysis and the conserved arginine (R) in motif B then promotes GTP hydrolysis by polarizing the developing partial negative charge of the gamma phosphate of GTP (Bos et al., 2007; Pan et al., 2006). Up-regulation of *PmTBC1D20* mRNA after WSSV-infection indicated that *PmTBC1D20* is likely to be involved in WSSV infection. In addition, depletion of *PmTBC1D20* levels by RNAi-mediated gene knock-down

followed by WSSV infection resulted in a significant decrease in the WSSV infection, as demonstrated by the decrease in the viral gene transcript levels of the intermediate early (*ie1*), early (*wsv477*), and late (*VP28*) genes, indicting the likely crucial role of *PmTBC1D20* in WSSV infection in the hemocytes. This result is similar to that previously reported in humans where suppression of *HsTBC1D20* resulted in a dramatically decreased HCV infection level (Sklan et al., 2007a, 2007b). Furthermore, it has been reported that numerous intact virions and incompletely assembled viral particles are found in the nucleus of heavily infected semigranular cells (SGCs) and also found intact virions in the cytoplasmic vacuoles of this cell type (Wang et al., 2002). On the contrary, intact virions are found in the cytoplasmic vacuoles, but not in the nuclei of granular cells (GCs) (Wang et al., 2002). It seems to be that virus replication take place in the SCGs and perchance due to virus infection/replication, phagocytosis or endocytosis in the cytoplasmic of the GCs (Wang et al., 2002). Thus, it is likely that *PmTBC1D20* functions in the trafficking of viral particles and so the depletion of *PmTBC1D20* could prevent formation of new virions and so prevent infection of other cells in the shrimp and quench the infection. The discovery of this Rab GAP protein that is likely to be involved in WSSV infection, and potentially other pathogenic shrimp viruses that share the same infection pathway, should provide insights into host-pathogen interactions by which WSSV and mechanistically related viruses hijack the cellular proteins of shrimps for their own benefit in virus infection and replication.

Conclusions

A Rab GTPase-activating protein, *PmTBC1D20*, was identified from *P. monodon* hemocytes. Expression of *PmTBC1D20* transcript was up-regulated upon WSSV infection. Suppression of *PmTBC1D20* mRNA in shrimp led to a reduction of the expression of WSSV, *ie1*, *wsv477* and *VP28* transcripts. These suggest the important role of *PmTBC1D20* in WSSV infection.

Acknowledgements

We would like to thank Prof. Dr. Paisan Sithigorngul at Srinakharinwirot University for kindly provide the mouse monoclonal antibody specific to VP28. We also thank Dr. Robert Douglas John Butcher at the Publication Counseling Unit (PCU), Faculty of Science, Chulalongkorn University, for English language corrections of this manuscript. The present work was supported by grants from the National Research University Project of CHE, the Ratchadaphiseksomphot Endowment Fund (FW643A), and The Thailand Research Fund (TRF Senior Research Scholar number RTA5580008). A student fellowship granted to W. Yingvilasprasert by the Thailand Graduate Institute of Science and Technology (TGIST) program of National Science and Technology Development Agency (NSTDA) is gratefully acknowledged. Equipment was supported by JST/JICA, SATREPS.

References

- Alper, S., Laws, R., Lackford, B., Boyd, W.A., Dunlap, P., Freedman, J.H., Schwartz, D.A., 2008. Identification of innate immunity genes and pathways using a comparative genomics approach. *PNAS*. 105, 7016-7021.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215(3), 403-410.
- Barnekov, A., Thyrock, A., Kessler, D., 2009. Rab protein and their interaction partners. *Int. Rev. Cell Mol. Biol.* 274, 235-265.
- Barr, F.A., 2009. Rab GTPase function in Golgi trafficking. *Sem. Cell Dev. Biol.* 20, 780-783.
- Barr, F., Lambright, D.G., 2010. Rab GEFs and GAPs. *Curr. Opin. Cell Biol.* 22(4), 461-470.
- Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: signalP 3.0. *J. Mol. Biol.* 340, 783-795.

446 Bos, J.L., Rehmann, H., Wittinghofer, A., 2007. GEFs and GAPs: Critical Elements in the
447 Control of Small G Proteins, *Cells* 129, 865-877.

448 Chou, H., Huang, C., Wang, C., Chiang, H., Lo, C., 1995. Pathogenicity of a baculovirus
449 infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. *Dis.*
450 *Aquat. Org.* 23, 165-173.

451 Duncan, D.B., 1955. Multiple range and multiple F test. *Biometrics* 11, 1-42.

452 Flegel, T.W., 2006. Detection of major penaeid shrimp viruses in Asia, a historical
453 perspective with emphasis on Thailand. *Aquaculture* 258, 1-33.

454 Fukuda, M., 2008. Regulation of secretory vesicle traffic by Rab small GTPases. *Cell Mol.*
455 *Life Sci.* 65, 2801-2813.

456 Fukuda, M., 2011. TBC proteins: GAPs for mammalian small GTPase Rab? *Biosci. Rep.* 31,
457 159-168.

458 Gruenberg, J., 2009. Viruses and endosome membrane dynamics. *Curr. Opin. Cell Biol.* 21,
459 582-588.

460 Haas, A.K., Yoshimura, S., Stephens, D.J., Preisinger, C., Fuchs, E., Barr, F.A., 2007.
461 Analysis of GTPase-activating proteins: Rab1 and Rab43 are key Rabs required to
462 maintain a functional Golgi complex in human cells. *J. Cell Sci.* 120, 2997-3010.

463 Krishnan, M.N., Sukumaran, B., Pal, U., Agaisse, H., Murray, J.L., Hodge, T.W., Fikrig, E.,
464 2007. Rab 5 is required for the cellular entry of dengue and West Nile viruses. *J. Virol.*
465 81, 4881-4885.

466 Mizuno-Yamasaki, E., Rivera-Molona, F., Novick, P., 2012. GTPase networks in membrane
467 traffic. *Annu. Rev. Biochem.* 81, 637-659.

468 Neuwald, A.F., 1997. A shared domain between a spindle assembly checkpoint protein and
469 Ypt/Rab-specific GTPase-activators. *Trends Biochem. Sci.* 22, 243-244.

470 Ongvarrasopone, C., Chanasakulniyom, M., Sritunyalucksana, K., Panyim, S., 2008.
 471 Suppression of PmRab7 by dsRNA Inhibits WSSV or YHV Infection in Shrimp. *Mar.*
 472 *Biotechnol.* 10, 374-381.

473 Ongvarrasopone, C., Chomchay, E., Panyim, S., 2010. Antiviral effect of PmRab7 knock-
 474 down on inhibition of Laem-Singh virus replication in black tiger shrimp. *Antiviral*
 475 *Res.* 8, 116-118.

476 Pan, X., Eathiraj, S., Munson, M., Lambright, D.G., 2006. TBC-domain GAPs for Rab
 477 GTPase accelerate GTP hydrolysis by a dual-finger mechanism. *Nature* 442, 303-306.

478 Ren, Q., Zhou, J., Jia, Y.P., Wang, X.W., Zhao, X.F., Wang, J.X., 2012. Cloning and
 479 characterization of Rap GTPase from the Chinese white shrimp *Fenneropenaeus*
 480 *chinensis*. *Dev. Comp. Immunol.* 36(1), 247-252.

481 Richardson, P.M., Zon, L.I., 1995. Molecular cloning of a cDNA with a novel domain
 482 present in the tre-2 oncogene and the yeast cell cycle regulators BUB2 and cdc16.
 483 *Oncogene* 11, 1139.

484 Sieczkarski, S.B., Whittaker, G.R., 2003. Differential requirements of Rab5 and Rab7 for
 485 endocytosis of influenza and other enveloped viruses. *Traffic* 4, 333-343.

486 Sklan, E.H., Serrano, R.L., Eina, S., Pfeffer, S.R., Lambright, D.G., Glenn, J.S., 2007b.
 487 TBC1D20 is a Rab1-GTPase-activating protein that mediates Hepatitis C virus
 488 replication. *J. Biol. Chem.* 282, 36354-36361.

489 Sklan, E.H., Staschke, K., Oakes, T.M., Elazar, M., Winters, M., Aroeti, B., Danieli, T.,
 490 Glenn, J.S., 2007a. A Rab-GAP TBC domain protein binds Hepatitis C virus NS5A and
 491 mediates viral replication. *J. Virol.* 81, 11096-11105.

492 Sritunyalucksana, K., Wannapapho, W., Lo, C.F. Flegel, T.W., 2006. PmRab7 Is a VP28-
 493 Binding Protein Involved in White Spot Syndrome Virus Infection in Shrimp *J. Virol.*
 494 80(21), 10734-10742.

495 Steel, R.G.D., Torrie, J.H., 1980. Principles and Procedures of Statistics. 1st Edn., McGaw
496 Hill Book Co. Inc., New York, USA.

497 Stenmark, H., 2009. Rab GTPases as coordinators of vesicle traffic. Nat. Mol. Cell Biol. 10,
498 513-525.

499 Strom, M.P., Vollmer, P., Tan, T.J., Gallwitz D., 1993. A yeast GTPase-activating protein
500 that interacts specifically with a member of the Ypt/Rab family. Nature. 361, 736-739.

501 Tassanakajon, A., Klinbunga, S., Paunglarp, N., Rimphanitchayakit, V., Udomkit, A.,
502 Jitrapakdee, S., Sritunyalucksana, K., Phongdara, A., Pongsomboon, S., Supungul, P.,
503 Tang, S., Kuphanumart, K., Pichyangkura, R., Lursinsap, C., 2006. *Penaeus monodon*
504 gene discovery project: the generation of an EST collection and establishment of a
505 database. Gene 384, 104-112.

506 Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The
507 CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment
508 aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.

509 van Hulten, M.C., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers
510 M., Sandbrink, H., Lankhorst, R.K., Vlak, J.M., 2001. The white spot syndrome virus
511 DNA genome sequence. Virol. 286(1),7-22.

512 Vidricaire, G., Tremblay, M.J., 2005. Rab5 and Rab7, but not ARF6, govern the early events
513 of HIV-1 infection in polarized human placental cells. J. Immunol. 175, 6517-6530.

514 Wang, Y.T., Liu, W., Seah, J.N., Lam, C.S., Xiang, J.H., Korzh, V., Kwang, J., 2002.
515 White spot syndrome virus (WSSV) infects specific hemocytes of the shrimp *Penaeus*
516 *merguiensis*. Dis. Aquat. Organ. 52(3),249-59.

517 Wu, W., Zhang, X., 2007. Characterization of a Rab GTPase up-regulated in the shrimp
518 *Penaeus japonicus* by virus infection. Fish Shellfish Immunol. 23(2), 438-445.

519 Zenner, H.L., Yoshimura, S., Barr, F.A., Crump, C.M., 2011. Analysis of Rab GTPase-
520 activating proteins indicates that Rab1a/b and Rab43 are important for HSV-1
521 secondary envelopment. *J. Virol.* 85(16), 8012-8021.

522 Zerial, M., McBride, H., 2001. Rab proteins as membrane organizations. *Nat. Rev. Mol. Cell.*
523 *Biol.* 2, 107-117.

524

Table 1

List of primers used for the PCR in this study

Primer name	Sequence (5'→3')
5R6817	AAGACCGCCATCGCCAATTGCAAGTT
3R6817	TGCGGCCAACAATGGAGAAGACCACA
Sg6817real_F	AGTCAGCGTTTAGAGAGGTT
Sg6817real_R	GCTCGAACTCTCCACTCTC
EF-1αF	GGTGCTGGACAAGCTGAAGGC
EF-1αR	CGTTCGGGTGATCATGTTCTTGATG
fullTBC_F	ACGCAGAGTGATGTTGTG
fullTBC_R	GGCTAGTGGCAATGTATG
Sg6817F	CAATTGGCGATGGCGGTCTT
Sg6817R	TGTTGGCCGCATGAACTCAC
Sg6817-FT7	GGATCCTAATACGACTCACTATAGGCAATTGGCGATGGCGGTCTT
Sg6817-RT7	GGATCCTAATACGACTCACTATAGGTGTTGGCCGCATGAACTCAC
GFP_F-FT7	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGGGAGGA
GFP_R-FT7	GGATCCTAATACGACTCACTATAGGTACTTGTACAGCTCGTCCA
VP28QF	GGGAACATTCAAGGTGTGGA
VP28QR	GGTGAAGGAGGAGGTGTTGG
ie1-qRTF	AGCAAGTGGAGGTGCTATGT
ie1-qRTR	CCATGTCGATCAGTCTCTTC
477-qRTF	GGCCAAGTCATGGAGATCTA
477-qRTR	CCATCCACTTGGTTGCAGTA

Fig. 1. The full-length cDNA sequence of *PmTBC1D20*, deduced ORF and predicted amino acid sequences (shown in single letter code (capitals)). The predicted TBC domain is indicated in grey highlight and the transmembrane region is underlined at the C-terminus. The ATG start codon is shown in bold, whilst an asterisk indicates the TGA stop codon. The potential AATAAA polyadenylation signal is shown in bold italic upper case letters.

Fig. 2. Multiple sequence alignment of the predicted amino acid sequences of the TBC domain of *PmTBC1D20* with that of various organisms. The three conserved signature motifs are shown. Motif A (RxxxW) is boxed and in bold characters. Motif B with the catalytic arginine residue (VxxDxxR) is shown in grey highlight. Motif C (YxQ) is shown in black highlight. Amino acid conservation across alignments is shown as (*), (:), and (.) for identical, conserved and semi-conserved, respectively. The abbreviations represent the species' scientific names; *Hs-Homo sapiens* (acc. No. NM_144628.2), *Mm-Mus musculus* (acc. No. BC040089.1), *Dr-Danio rerio* (acc. No. NM_001025172.1), *Sp-Strongylocentrotus purpuratus* (acc. No. XM_001178307.1), *Sk-Saccoglossus kowalevskii* (acc. No. XM_002736113.1), *Bf-Branchiostoma floridae* (acc. No. XM_002612327.1), *Aa-Aedes aegypti* (acc. No. XM_001653340.1), *Dm-Drosophila melanogaster* (acc. No. NM_001042984.1), *Tc-Tribolium castaneum* (acc. No. XM_961552.2) *Pm-Penaeus monodon* (acc. No, KF039720).

Fig. 3. The expression of *PmTBC1D20* transcript levels in 10 healthy shrimp tissues by RT-PCR. The expression of EF-1 α transcript levels was used as the internal control.

Fig. 4. Analysis of *PmTBC1D20* transcript levels in the hemocytes of *P. monodon* shrimps at different times (0.25, 12, 24 and 48 hpi) after WSSV infection, as determined by qrt-RT-PCR. Data are shown as the mean ($\pm$ 1 SD) of three replicates and represent the fold change of *PmTBC1D20* after normalization relative to the EF-1 α transcript levels and then compared

relative to the control group (set as = 1). Significant differences ($p < 0.05$) are marked with an asterisk.

Fig. 5. Localization of the *PmTBC1D20* protein in *P. monodon* hemocytes by immunofluorescence CLSM. *PmTBC1D20* protein was localized in the cytoplasm of hemocytes of the healthy shrimps. The fluorescence was developed using secondary antibodies conjugated with Alexa 488 (green) for TBC1D20. Nuclei (adjusted to blue color) were stained with TO-PRO-3 iodide. BFs are bright field images. Bars = 10 μ m. Higher magnifications of the shrimp hemocyte cells were inserted. GC indicates the granular cells, SGC indicates the semigranular cells and HC indicates the hyaline cells.

Fig. 6. RNAi-mediated *PmTBC1D20* transcript suppression reduced WSSV gene transcripts in the hemocytes of WSSV-infected *P. monodon*. (A) ds*PmTBC1D20* or dsGFP or NaCl were double-injected into shrimps and 24 h later the *PmTBC1D20* transcript level was determined by RT-PCR using EF-1 α as the control. (B) ds*PmTBC1D20* or dsGFP or NaCl were double-injected, along with purified WSSV on the second injection, and 24 h after the second injection (24 hpi with WSSV) the transcript expression levels of the three indicated WSSV genes were analyzed using qrt-RT-PCR. Data are shown as the mean ($\pm$ 1 SD) of three replicates, each of three shrimps, and are shown as the fold change of viral gene expression levels after normalization relative to the EF-1 α transcript levels and then compared relative to the GFP dsRNA injected shrimps (set as = 1). Significant differences ($p < 0.05$) are marked with an asterisk.

Figure 1

ACGCGGGGAACGCAGAGTGATGTTGTGATGAGACCCCTCCAAGGCAGGATTTGAGTGACTAGTCTTCCCAGACCATCACATCAGCACCTAG	90
GGACGTCATCCCCCATAGGATGTGATAGCATGATCTCGTGCACATGGAAGGACAGTGCATAGGAGAGGCATACCAAATCATCCTGTGATG	180
CTTTTTTATCTCTTTTAATTTTAACAACAGTTTTTAATTGATTCAAG ATG GAAGAAAATAAAAAGTGAAACAGCCAGAGATTCAAGTGGAGT	270
M E E N K S E T A R D S S G V	
GAAGCCCAACCCAAAAGATGAGACCAATGGTGCATGCTCATCCCCAGAGGACCAAAAAGATTACAAACAAGGGAACAAGTGATGGGGACCA	360
K P N P K D E T N G A C S S P E D Q K I T N K G T S D G D H	
TCTTGCTGAGACAGGCAAGGAAGTGGAGGACCGCTGTAATGGGGAAATTTACAAGAAGAAAGATTTTTTCAGAAGGGCAGAAAGTGGAGGA	450
L A E T G K E V E D R C N G E I Y K K K D F S E G Q K V E D	
TGTAACAGGATCATCATCGGAAAATGATGCTTCAGAAACGGCTGAGAAAACCATGAAGAGAAGAAAGAAAAGGCCCAAGAATGTATCGCA	540
V T G S S S E N D A S E T A E K T M K R R K K R P K N V S Q	
AGAGGCTCAGGACCGTCAAAAGAAAATGCAGCAAATTCAGATGTACTGTCTTCCACTCCACTAGATTTAGAAGTCCTTCGAAAACCTTGC	630
E A Q D R Q K K M Q Q I Q D V L S S T P L D L E V L R K L A	
AATTGGCGATGGCGGTCTTATAAAAGACGAGTTACGCCGCAAGGCTTGGCCGCGCCTCATGATGATTGAGCATATGGAAGTGACCCCCAA	720
I G D G G L I K D E L R R K A W P R L M M I E H M E V T P K	
ACCCACCCTGGACACGATCAAAAGCCACAAGGATTACCAGCAAGTGGTGTGGATGTCAACCGGTCCCTCAAACGCTTCCCTCCTGGAAT	810
P T L D T I K S H K D Y Q Q V V L D V N R S L K R F P P G I	
AGATGATGATTACAGGCTGGTTCTGATGGACCAGCTCACCCTCTCATTATTAGGATTCTCATGAAGCATCCCGAACTGAATTATTACCA	900
D D D Y R L V L M D Q L T T L I I R I L M K H P E L N Y Y Q	
GGGGTTTCATGATGTTGCTATAACATTCCCTTCTGGTCATGGGTGAAGATGTTGGTTATGAGATGGTGGAGAAGCTCTCCGTCACACACCT	990
G F H D V A I T F L L V M G E D V G Y E M V E K L S V T H L	
GAGTGAGTTCATGCGGCAACAATGGAGAAGACCACATATTACCTCACCTACATATATCCCATTCTTCGAAGGGCAGACACCAAGCTGTA	1080
S E F M R P T M E K T T Y Y L T Y I Y P I L R R A D T K L Y	
CCAGTTTTTTGATAGATTCAGGTGTAGGAACAGTGTTTTGTGGCCCTGGCTTATTACATGGTTTCGCTCACACACTTTCTGACTACCGCAA	1170
Q F L I D S G V G T V F C L P W L I T W F A H T L S D Y R N	
TGTGGTGAGGCTATACGACTGCTTCTTGGCTTCCCCTTATCTCATGCCCATGTACATAGCTGCAGCAATTGTTTTACACAAGAAAGAAGA	1260
V V R L Y D C F L A S P Y L M P M Y I A A A I V L H K K E D	
TGTTTTAGCTGCAGAATGTGATATGGCAATGCAACATTATATCTTTTCACAGGTCCTGATACATTACCTCTGGAGCGCATCCTAGTAGA	1350
V L A A E C D M A M Q H Y I L S Q V P D T L P L E R I L V D	
TGCAGGGGAATTATATAACCTTTATCCACCTGACACTTTTACAAGAGGAAGCCAACGAGTTTCTGCAGAAAAAGGCTGAAGAAGAGAAGCT	1440
A G E L Y N L Y P P D T L Q E E A N E F L Q K K A E E E K L	
GGAGAAGCAGCAAGTCCTCGAGAGACGTAAGAAGTTTGAAGCAGCAAGGAGAGGTCAGAATTACCGGGGGATCCTTGCAACGGTGTTTCAG	1530
E K Q Q V L E R R K K F E A A R R G Q N Y R G I L A T V F R	
ATACCTTCCACTGGTGCCAGCTCAGAGGAACATGCTTGTGAAGGTGGCTGCCTTTGCAGTAACAGCATAACATTGCCACTAGCCTTTATAG	1620
Y L P L V P A Q R N <u>M L V K V A A F A V T A Y I A T S L Y S</u>	
CTACTATTCCAATTCAGCTCTTGTGTTTCCTTTTGTAGCAAAACGATGATAATATATGAATTACAGTGCATAGCTCTTAGTTCCCTAGTCA	1710
<u>Y Y</u> S N S A L V F P F V A K R *	
GTTTCATCACCTAGTCTTTCACTCTGCTCGATTATTTTAAAAAATTGAATGATGGGTAGTAACTTTATTATCTTTTTGCTCTGTAAAC	1800
TAATAAAATAATGGAAGAAGTATGTTAAATGTATATAACATAACTATATTGTACAGAGTTTTAGAGTTTTTTGTTTTTTGTATTTTCAG	1890
AATATATGATATTAGCATGTCAGTTTGTATATTCAGGGCTCATAACTTAGAGTTTTTGTAAAGTTTACTATGG AATAAA GTGGTTGTTT	1980
GAAAAAAAAAAAAAAAAAAAAA	2002

Figure 2

HsTBC1D20	EGGLLTDEI	RRKVV	PKLLNVNANDPPPISGKNLRQMSKDYQQVLLDVRRSLRRFPPGMPE	117		
MmTBC1D20	EGGLLTDEI	RCQVW	PKLLNVNTSEPPPVSRKDLRDMSKDYQQVLLDVRRSLRRFPPGMPD	116		
DrTBC1D20	EGGLMTDEI	RRLVW	PKLLNVSEENIPEELDPVDRENNKDFNQVLLDVQSLRRFPPGMPD	114		
SpTBC1D20	RGGLLTDEI	RRKAW	PKLLNVNVFDPPPK-----LKHRDYNQVVLDVNRSLKRFPPGMQD	114		
SkTBC1D20	HGGLLNDEI	RRKAW	PKLLNVNVFEIPPKPADV-TSHRDYHQVVLDVNRSLRRFPPGMRN	99		
BfTBC1D20	DGGLLRDDI	RRKAW	PKLMNVNVFEIPPKPAN--IRAHRDYRQVVLDVERSMMRRFPANMKE	127		
AaTBC1D20	EYGLINDDI	RRKVV	PLLVGVDPKLVDPAAPSLEELNSHPEYNQVVLDVNRSLKRFPPGIPY	118		
DmTBC1D20	PYGLVNDDI	RRILW	PQLAAVDINHLGQAPTLDKFQCHPEYNQVVLDVNRSLKRFPPGIPY	111		
TcTBC1D20	--GLVGDDI	RCKVW	PLLLELDPNNLEKTPLEEELSSHSEYEQVVLDVNRSLKRFPPGIPY	115		
PmTBC1D20	DGGLIKDEI	RRKAW	PRLMMIEHMEVTPKPTLDTIKSHKDYQQVVLDVNRSLKRFPPGIDD	198		
	:	*::*	* * :	...*:*.**::***...		
HsTBC1D20	EQREGLQEELIDIILLILERNPQLH	YYQ	GYHDIVVTFLLVVGGERLATSLVEKLSTHHLRD	177		
MmTBC1D20	EQREGLQEELIDIILLVLD RNPQLH	YYQ	GYHDIVVTFLLVVGGERLATSLVEKLSTHHLRD	176		
DrTBC1D20	EQREGLQEELTDIILRVLVKNPQLH	YYQ	GYHDIVVTFLLVLGERLATALVEKLSTHHLRD	174		
SpTBC1D20	EQREVLQDQLVDVIVRVLINNDQLH	YYQ	GYHDIVVTFLLVVGKEKSFSLVDKLSTHHLRD	174		
SkTBC1D20	DQREVLQEQLINVIVRVLLRNKQLH	YYQ	GYHDICVTFLLVVGQDLAFALIEKLSTHHLRD	159		
BfTBC1D20	EQRSRLQDQLVDIILRVLVRHPELH	YYQ	GYHDICVTFLLVVGEDLAFAIMDKLSEYHLRD	188		
AaTBC1D20	EQRVALQDQLTVLILRVIIKYPHLK	YYQ	GYHDVAITFLLVVGEEVAFHVMEILSTNHLVE	178		
DmTBC1D20	EQRIALQDQLTVLILRVIIQKYPNLR	YYQ	GYHDVAITFLLVVGEEIAFAIMEQLSTTHFSE	171		
TcTBC1D20	KQRLALQDQLTVLILRVIIKYPHLR	YYQ	GYHDVAITFLLVVGGEALGFSIMERLSTDHLRE	175		
PmTBC1D20	DYRLVLMDQLTTLIIRILMKHPELN	YYQ	GFHDVAITFLLVMGEDVGYEMVEKLSVTHLSE	258		
	. *	* ::*	*: : : . . *	****:***:*****:*. : : : *		
HsTBC1D20	FMDPTMDNTKHILNYLMPIIDQVN	PELHDFMQSAE	VGTFALS	WLITWFGHVLSDFRHVV	237	
MmTBC1D20	FMDPTMDNTKHILNYLMPIIDQV	SPELHDFMQSAE	VGTFALS	WLITWFGHVLMDFRHVV	236	
DrTBC1D20	FMDPTMDNTKHILNYLMPIIERV	NPEVDFMQQA	EVGTIFALS	WLITWFGHVLSDFRHVV	234	
SpTBC1D20	FMDRTMDRTKHMLNYLLPIIRKAN	PKLHDFMERSE	VGTVFAL	AWLITWYGHVLNDFHSIL	234	
SkTBC1D20	FMDRTMDSTKHILNYLLPIIGKAN	PELREFMDR	STVGT	VFALAWLITWYGHVLSDFRSVV	219	
BfTBC1D20	FMDESMDRTRHILGYLMPIVGKA	HPELRDFLERS	SDVGT	IFALS	WLITWYGHVLTDIRHIV	248
AaTBC1D20	CMQETMEPTQRRLMFIYPLVR	RENAALCDYLER	STVGT	LFALPWYLTWFGHSLNSYRSVV	238	
DmTBC1D20	CMQETMEATQRRLMFIWPIIKFEN	PELYQFLQNST	VGT	LFSLPWYLTWFGHSLTSYRTVV	231	
TcTBC1D20	CMEPTMEKTSYRLTYIYPLLSRV	DPQLYEFMDRAT	VGT	MFALPWFLTWFGHSLNQYKDVV	235	
PmTBC1D20	FMRPTMEKTTYLTYIYPI	LRRADTKLYQFLID	SGVGT	VFCLPWLTWFAHTLSDYRNVV	318	
	*	:*:	* *	: : * :	*.*.* :*:.* * . : :	
HsTBC1D20	RLYDFFLACHPLMPIYFAA	IVLYREQEVLDCD	270			
MmTBC1D20	RLYDFFLACHPLMPIYFAA	IVLYREQEVLDCD	269			
DrTBC1D20	RLYDFFLACHPLMPIYFAA	IVLHREEEVLDCD	267			
SpTBC1D20	RLYDFFLACHPLMPVYLA	AAVIIYRNAD-----	272			
SkTBC1D20	RLYDFFLACHPLMPVYLA	AAIVLHRETEVLSGE	252			
BfTBC1D20	RLYDFFLACHPLMPIYLA	AQIVLYRSEDILRVE	280			
AaTBC1D20	RLYDYFLASDFLLPIYVT	SAIVLYRQNEIFRED	271			
DmTBC1D20	RLYDYFLASPIYTSIFV	TAAILLYRSDEILKED	264			
TcTBC1D20	RLYDFFLATPPLMPLYV	AAALVVERREEVFEQG	298			
PmTBC1D20	RLYDCFLASPYLMPMYIA	AAIVLHKKEDVLAEE	351			
	****	***	: : : : : :	:		

Figure 3

Figure 4

Figure 5

Figure 6A

Figure 6B