The novel white spot syndrome virus-induced gene, \textit{PmERP15}, encodes an ER stress-responsive protein in black tiger shrimp, \textit{Penaeus monodon}

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\section*{ABSTRACT}
By microarray screening, we identified a white spot syndrome virus (WSSV)-strongly induced novel gene in gills of \textit{Penaeus monodon}. The gene, \textit{PmERP15}, encodes a putative transmembrane protein of 15 kDa, which only showed some degree of similarity (54–59\%) to several unknown insect proteins, but had no hits to shrimp proteins. RT-PCR showed that \textit{PmERP15} was highly expressed in the hemocytes, heart and lymphoid organs, and that WSSV-induced strong expression of \textit{PmERP15} was evident in all tissues examined. Western blot analysis likewise showed that WSSV strongly up-regulated \textit{PmERP15} protein levels. In WSSV-infected hemocytes, immunofluorescence staining showed that \textit{PmERP15} protein was colocalized with an ER enzyme, protein disulfide isomerase, and in Sf9 insect cells, \textit{PmERP15}-EGFP fusion protein colocalized with ER -Tracker\textsuperscript{TM} Red dye as well. GRP78, an ER stress marker, was found to be up-regulated in WSSV-infected \textit{P. monodon}, and both \textit{PmERP15} and \textit{GRP78} were up-regulated in shrimp injected with ER stress inducers tunicamycin and dithiothreitol. Silencing experiments showed that although \textit{PmERP15} dsRNA-injected shrimp succumbed to WSSV infection more rapidly, the WSSV copy number had no significant changes. These results suggest that \textit{PmERP15} is an ER stress-induced, ER resident protein, and its induction in WSSV-infected shrimp is caused by the ER stress triggered by WSSV infection. Furthermore, although \textit{PmERP15} has no role in WSSV multiplication, its presence is essential for the survival of WSSV-infected shrimp.

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1. Introduction

The endoplasmic reticulum (ER) is a vital eukaryotic organelle responsible for multiple functions, such as the synthesis, folding and posttranslational modification of membrane and secretory proteins; lipid and sterol biosynthesis; and storage of intracellular calcium (Bravo et al., 2013; Schröder, 2008). Various stressful conditions, including hypoxia, hypoglycemia, hyperthermia, oxidative stress and viral infection, can strongly influence and disturb ER functions, and thereby seriously compromise the protein folding process (Schönthal, 2012). Accumulation of misfolded or unfolded proteins induces ER stress and leads to activation of a specific cellular process called the unfolded protein response (UPR). Through transcriptional and translational controls, the UPR increases the ER’s protein-folding capacity, attenuates global protein translation, and enhances the ER-associated degradation (ERAD) of misfolded proteins, all of which aim at adapting ER to stressful conditions and restoring ER function. However, when adaptation fails, the UPR leads to activation of apoptotic pathways and elimination of dysfunctional cells (Logue et al., 2013; Schönthal, 2012).

White spot disease (WSD), a devastating viral shrimp disease, has seriously impacted the shrimp aquaculture industry worldwide over the last two decades (Leu et al., 2009). The causative pathogen is the white spot syndrome virus (WSSV), which is the type species of the genus \textit{Whispovirus} in the family \textit{Nimaviridae} (Vlak et al., 2004). WSSV has a large (80–120 × 250–380 nm), enveloped, rod-shaped virion with a unique, tail-like extension at one end (Sánchez-Paz, 2010). Its genome is a circular double-stranded DNA of about 300 kbp in size, encoding more than 181 open reading
frames, of which most encode proteins showing no significant homology to known proteins (Yang et al., 2001). WSSV has a broad host range among crustaceans, but is highly lethal only to penaeid shrimp (Flegel, 2006; Lo et al., 1996), and acute WSSV infection usually causes shrimp to die within 3–7 days (Chou et al., 1995). WSSV targets a wide range of tissues/organisms, and after infection, it multiplies in the nucleus, producing a lytic infection (Leu et al., 2009).

The huge economic impact caused by WSD has intensified the efforts of researchers to investigate WSSV–host interaction, with the goal of developing effective strategies for preventing, controlling, and even curing the disease. Although progress has been hampered by the fact that most WSSV-encoded proteins show no significant homology to other viral proteins, recent studies have unveiled that the WSSV–host interactions are, in many aspects, similar to other virus–host interactions. For example, WSSV can modulate apoptosis to facilitate its multiplication (Leu et al., 2013), uses IRES (internal ribosomal binding sites) for regulating the translation of some proteins (Kang et al., 2009, 2013), takes advantage of the Warburg effect to meet the need for energy and basic building blocks during viral genome replication (Chen et al., 2011a), and hijacks the immune-related JAK-STAT and NF-κB pathways in order to promote the expression of the immediately early gene 1 (Chen et al., 2011b; Liu et al., 2007).

Although numerous WSSV–host interactions have been identified, a lot of unanswered questions remain. To gain more insight into how P. monodon responds to WSSV infection, we applied microarray analysis to compare the gene expression changes in normal and WSSV-infected gills. These experiments revealed distinctive patterns of gene expression in WSSV-infected gills and identified a diverse array of differentially expressed genes (our unpublished data). Among the WSSV-inducible transcripts, a transcript with no significant homology to any sequence in the NCBI database was further investigated, because it was one of the highly up-regulated transcripts identified by the microarray analysis. In this paper, we characterize this previously unknown gene PmERP15; the results show this gene encodes a novel ER stress-responsive protein (ERP) that we are documenting for the first time. Through the identification of this novel gene, we further confirmed that WSSV infection induces ER stress.

2. Materials and methods

2.1. Virus, viral inoculum and experimental shrimp

The virus used in this study was WSSV Taiwan isolate T-1 (GenBank Accession Number AF440570) (Wang et al., 1995). The WSSV stock and working inoculum were prepared as described previously (Wang et al., 2007). The P. monodon shrimp used in this study were obtained from culture ponds at the Biotechnology Division of the Fisheries Research Institute in southern Taiwan or from the Aquatic Animal Center at National Taiwan Ocean University.

2.2. Microarray analyses

The oligonucleotide-based microarray platform developed by Agilent Technologies was used in this study. Firstly, for oligonucleotide probe design, P. monodon genes and ESTs were collected from the NCBI nucleotide non-redundant (nr) and EST databases, respectively, and their corresponding sequences were analyzed with the Bio301 program (Chen et al., 2012a) for automatic sequence assembly and annotation. The assembled tentative unique sequences were then analyzed with Unique Probe Selector (UPS; http://array.iis.sinica.edu.tw/ups/) to design the oligonucleotide probes. The oligonucleotide probe sequences were then sent to Agilent Technologies for P. monodon microarray manufacture.

Adult P. monodon shrimps (mean weight, ~40 g) were challenged with WSSV by intramuscular injection. At the indicated times post infection (0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 18, 24, 30, 36, 48 hour), three shrimps were randomly collected, and the gills were harvested and stored in liquid nitrogen. Total RNAs were extracted from the gills using TRIzol reagent (Invitrogen, USA) following the procedure provided by the supplier. After quantification at A260, the three RNA samples at the same time point were pooled at equal amounts. Total RNA of 0.5 μg was amplified by a Fluorescent Linear Amplification Kit (Agilent Technologies, USA) and labeled with Cy3–CTP or Cy5–CTP (PerkinElmer, USA) during the in vitro transcription process. Labeled cRNA (2 μg) was fragmented and then hybridized to the P. monodon microarray. After washing and drying, microarrays were scanned with an Agilent microarray scanner (Agilent Technologies) and the scanned images were analyzed by Feature Extraction Software 8.1 (Agilent Technologies). Normalization and data analysis were conducted using R and Bioconductor.

2.3. Quantitative real time RT-PCR

P. monodon shrimps were challenged with WSSV by intramuscular injection and at the indicated times post infection (0, 5, 12, 24, 36, 48 h), three shrimp were randomly collected. Total RNAs were extracted from the gills using TRIzol reagent (Invitrogen, USA). After quantification at A260, 1 μg of total RNAs was treated with DNase I, primed with oligo-dt-anchor primer (Roche) and reverse-transcribed with SuperScript II (Invitrogen, USA) at 42°C for 50 min. Aliquots of this cDNA were then used for quantitative real time PCR analysis. The qRT-PCR amplifications were carried as previously described (Leu et al., 2008b). In brief, the reaction was performed in duplicate for each sample using SYBR Green 2× Supermix (Applied Biosystems, USA). The qRT-PCR program was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. After PCR, the specificity of each PCR product was checked by a dissociation curve analysis (95°C for 15 s, 60°C for 30 s, 95°C for 15 s). The Ct values for PmERP15 and EF-1α genes were determined for each sample. The expression levels of PmERP15 gene in WSSV-infected shrimp relative to normal shrimp were then determined using the 2−ΔΔCT method (Livak and Schmittgen, 2001). The PmERP15 primer pair used in this analysis was 1306-59F and 1306-122R (Table 1). Pmmonodon EF-1α was used as the internal control gene, and the corresponding primers were P2547-F and P2548-R (Table 1).

2.4. 5′/3′ RACE to isolate full-length PmERP15 cDNA

Total RNA extracted from the gills of P. monodon infected with WSSV for 48 h was subjected to poly A+ RNA purification using the QuickPrep Micro mRNA Purification Kit (GE Healthcare). The purified mRNA was subjected to 5′/3′ RACE using the SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) according to protocol supplied by the manufacturer. For 5′ RACE, the synthesized 5′-RACE-Ready cDNA was subjected to nested PCR using two gene-specific primers (P32-R1 and P33-R2; Table 1) and universal primers supplied in the kit. For 3′ RACE, the synthesized 3′-RACE-Ready cDNA was subjected to PCR using the gene-specific primer (P30-F; Table 1) and universal primer provided in the kit. The PCR was performed using the Advantage 2 Polymerase Mix (Clontech Laboratories, Inc.) and the PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced.

2.5. RT-PCR analysis of PmERP15 and GRP78 genes in tissues from normal and WSSV-infected P. monodon shrimp

Tissues were collected from normal shrimp and WSSV-infected shrimp at 48 hpi. TRIzol reagent (Invitrogen, USA) was used to extract total RNAs. One microgram total RNA was treated with DNase I and
reverse-transcribed as described earlier. An aliquot of this cDNA was used for PCR analysis. The PmERP15 primers (P53-Bam-F3, P54-Hin-R3), GRP78 primers (P133-F, P134-R) and the EF-1α gene primers (P116-F, P117-R) used in this analysis are listed in Table 1.

2.6. Preparation of PmERP15 recombinant protein and antibodies

The entire PmERP15 coding region was amplified by PCR (primer P53-Bam-F3, P54-Hin-R3; Table 1), and cloned into pET-28b(+) plasmid. The resulting clones were transformed into Escherichia coli strain BL21(DE3). A single colony was inoculated into the Luria–Bertani medium supplemented with 50 μg/ml kanamycin and the bacteria were grown overnight at 37 °C. The bacteria were inoculated into new medium at a ratio of 1:300 and grown at 37 °C for 1.5–2 h. After the addition of 1 mM IPTG, the bacteria were cultured for another 3 h. The induced bacteria were collected by centrifugation, suspended in ice-cold phosphate-buffered saline (PBS) containing 10% glycerol and a protease inhibitor cocktail tablet (Roche Molecular Biochemicals) and sonicated on ice. The insoluble debris then was sent to Seeing Bioscience Co., Ltd. for injection into rabbits. Antibodies, the protein bands were sliced from the gel, minced, and followed by Coomassie blue staining. For the production of PmERP15 recombinant protein, the resin was then washed five times with ice-cold wash buffer (1M NaCl, 10mM Tris–HCl, pH7.5). The resin was then sent to Seeing Bioscience Co., Ltd. for injection into rabbits.

2.7. Western blot analysis to detect PmERP15 protein in shrimp gills

The gills collected from uninfected shrimp and from WSSV-infected shrimp at 48 hpi were homogenized in lysis buffer (Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP–40) containing protease inhibitor (Roche) on ice. After clearing the lysates by centrifugation at 13,680 × g for 5 min at 4 °C, the protein content of the cleared lysates were quantified with Bradford reagent (BioRad). Twenty-five micrograms of the lysates was mixed with SDS sample buffer, separated by SDS–PAGE, and transferred to Immobilon-P membranes (Millipore). The membrane was blocked with blocking buffer (3% skim milk in TBS [50 mM Tris–HCl, pH 7.5, 200 mM NaCl]) for 1 h at room temperature and then incubated with anti-PmERP15 antibody (1:2500 in blocking buffer) at 4 °C overnight. After washing three times in TBST (TBS containing 0.5% Tween–20), the membrane was incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (1:5000 in blocking buffer). Lastly, the membrane was washed three times with TBST and detected with Western Lightning Plus ECL (Perkin Elmer, Inc.).

2.8. Indirect immunofluorescence assay of PmERP15 in shrimp hemocytes

Hemolymph was collected from normal shrimp and from WSSV-infected shrimp at 48 hpi using a syringe containing cold anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid and 10 mM EDTA, pH 7.5). The hemolymph was added onto glass coverslips placed in the wells of a 24-well plate and then left for 1 h to settle the hemocytes. The attached hemocytes were washed with PBS, fixed in 4% paraformaldehyde in PBS for 10 min at 4 °C, treated with 0.1% Triton X-100 in 4% paraformaldehyde/PBS for 3 min at 4 °C, and then washed twice with PBS. After blocking with PBS containing 5% bovine serum albumin and 2% normal goat serum for 16 h at 4 °C, the cells were incubated for 3 h at room temperature with the mouse anti-PmERP15 antibody (1:2500 in PBS) or with the mouse anti-β-actin antibody (1:200 in PBS; Abcam) or with both. The cells were then washed three times with PBST (PBS containing 0.2% Tween–20) and reacted either with carboxymethylindocyanine (Cy3) dye-conjugated goat anti-rabbit IgG antibody (1:1000 in PBS; Sigma) or with Alexa Fluor® 488 dye-conjugated goat anti-mouse IgG antibody (1:1000 in PBS; Invitrogen), or else reacted with both for 2 h at room temperature. The nucleus was counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories). After washing three times (10 min each time) with PBST, the coverslips were air dried and mounted with anti-fade mounting medium (Sigma). Fluorescence signals were examined using a fluorescence microscope (Olympus BX51) and a confocal microscope (Leica TCS SP5).

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Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Primer sequence (5′–3′)</th>
<th>Usage</th>
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<td>P64-126SP</td>
<td>5′-CTACTTTTCTACCAATGCTTG-3′</td>
<td>RT-PCR</td>
</tr>
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</table>

*The sequence of the T7 promoter is underlined; italicized letters indicate restriction enzyme cutting sites.*
2.9. Expression and localization of PmERP15-EGFP fusion protein in Sf9 cells

S. frugiperda Sf9 insect cells were maintained at 27 °C in SF-900 II SFM medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories). To construct the expression plasmid, the PmERP15 coding region was PCR amplified (primer P80-Hin-F4 and P81-Bam-R4), restricted with HindIII and BamHI enzymes, and cloned into pDhsP/V5-His, an expression vector driven by the heat inducible Drosohila heat shock protein 70 gene (hsp70) promoter (Leu et al., 2008a). Then, the PCR product of EGFP DNA fragment (primer P266-F-Not and P267-R-Not) was digested with NotI enzyme, and cloned downstream of PmERP15 coding region to generate the expression plasmid, pHsP-PmERP15-EGFP/V5-His. Sf9 cells seeded in 24-well plates (2 × 10^5/well) were transfected with pHsP-PmERP15-EGFP/V5-His (or with the pHsP-EGFP/V5-His control plasmid) using Cellfectin II reagent (Invitrogen) following the protocol provided by the supplier. After transfection for 16–18 h, the cells were heat shocked in a 42 °C water bath for 30 min and then returned to 27 °C. To determine the expression of PmERP15-EGFP fusion protein, at 16–18 h post heat shock, the cells were washed with PBS, lysed with SDS sample buffer and then subjected to SDS–PAGE followed by Western blot analysis probing with anti-EGFP antibodies (Bioman Scientific Co., Ltd.). To determine the localization of PmERP15-EGFP fusion proteins in live Sf9 cells, at 16–18 h post heat shock, the cells were washed with PBS and stained with 0.5 μM ER-Tracker™ Red dye (Invitrogen) in PBS for 30 min. After washing with PBS, the cells were replenished with culture medium and examined using a fluorescence microscope (Olympus BX51). For confocal microscopic analysis, the cells seeded onto glass coverslips were transfected, heat shocked and stained with ER-Tracker™ Red dye as described earlier. Then after washing with PBS, the cells were fixed in 4% paraformaldehyde in PBS for 2 min at 37 °C, washed several times with PBS and then examined using the confocal microscope Leica TCS SP5.

2.10. The effect of tunicamycin and dithiothreitol on the expression of PmERP15 and GRP78 in P. monodon

Tunicamycin (Sigma) was prepared first as a stock solution in dimethyl sulfoxide (DMSO) at 10 mg/ml. For injection, tunicamycin was further diluted in PBS and then injected intramuscularly into shrimp (~20 g) at a dosage of 2 μg/g shrimp body weight. Dithiothreitol (DTT; Sigma) was prepared as a 1 M stock solution in dimethyl sulfoxide (DMSO or H2O) in PBS served as negative controls for tunicamycin or DTT treatments, respectively. At 24 h post injection, the gills and hemocytes were collected and subjected to RT-PCR analysis. The PmERP15 primers (P53-Bam-F3, P54-Hin-R3), GRP78 primers (P133-F, P134-R) and the EF-1α gene primers (P116-F, P117-R) used in this analysis are listed in Table 1.

2.11. Knock-down of PmERP15 in P. monodon by dsRNA-mediated RNA interference

DNA templates for dsRNA synthesis were prepared by PCR using gene specific primers incorporated with T7 promoter at 5’ ends; the primers for preparing PmERP15 and Luciferase dsRNAs are listed in Table 1. The T7 RiboMAX™ Express RNAi System (Promega) was used to synthesize the dsRNAs according to the manufacturer’s instructions. After synthesis, the dsRNAs were treated with RNase A and DNase I, and then extracted with Trizol reagent. After alcohol precipitation, the dsRNAs were verified by agarose gel electrophoresis. Then precipitated dsRNAs were quantified by UV spectrophotometry and stored at −80 °C for later use. For dsRNA injection, the shrimp (~5 g) were intramuscularly injected with the corresponding dsRNA (0.5 μg/g shrimp) contained in 50 μl of PBS. Two days later, the shrimp were injected again with 50 μl of WSSV inoculum. Two days after WSSV injection, the gills were collected from surviving shrimp and subjected to RT-PCR to check the silencing efficiency. The PmERP15 primers (P53-Bam-F3, P54-Hin-R3), WSSV ice1 primers (P63-126F, P64-126SP) and the EF-1α gene primers (P116-F, P117-R) are listed in Table 1.

2.12. The effect of PmERP15 silencing on WSSV replication

The shrimp (~5 g) were intramuscularly injected with PmERP15 dsRNA, Luciferase dsRNA, or PBS. At 2 days after injection, all the shrimp were injected with WSSV. Two more days later, the gills (n = 4) were collected from surviving shrimp and subjected to DNA extraction using EasyPure Genomic DNA Spin Kit (Bioman Scientific Co., Ltd.) according to the protocol supplied by the company. The purified genomic DNAs were subjected to agarose gel electrophoresis to check quality and then quantified using NanoDrop® ND-1000 (Thermo Scientific). WSSV copy numbers in the purified DNA were determined using quantitative real-time PCR according to methods in previous reports (Lin et al., 2011). Cumulative mortality after WSSV injection in PmERP15-silenced shrimp

There were six shrimp groups in this experiment and each group contained 15–18 animals. Shrimp (~5 g) were intramuscularly injected with PmERP15 dsRNA, Luciferase dsRNA, or PBS. Two days later, the shrimp were again injected with either PBS or WSSV. After injection, cumulative mortality was recorded daily. Three independent experiments were performed and data represent the average results of the three experiments.

3. Results

3.1. Identification of the WSSV-inducible gene PmERP15 from the gills of WSSV-infected P. monodon shrimp

After microarray screening, we identified several assembled P. monodon tentative unique sequences (TUS) that showed increased expression levels in WSSV-infected gills. After RT-PCR (data not shown) and qRT-PCR analyses (Fig. 1), three of them, TUS2785, TUS1306, and TUS2365, were definitely confirmed to have a WSSV-inducible expression pattern. As shown in Fig. 1, after WSSV infection, the expression levels of the three unique sequences increased, and at 48 hpi, the expression of TUS2785 and TUS1306 was strongly up-regulated by about 1000-fold, and TUS2365 by about 30-fold, compared to uninfected normal shrimp. Sequence annotation results showed that TUS2785 encodes the triosephosphate isomerase (TPI, a glycolytic enzyme), whereas TUS2365 and TUS1306 were unknown. TUS1306 was chosen for further study due to its high induction level and unknown function. This novel gene was named PmERP15 (Penaeus monodon ER stress-responsive protein).

3.2. Cloning and sequence analysis of the full-length PmERP15 gene

5’/3’ RACE was performed to isolate the full-length sequence of PmERP15 cDNA. As shown in Fig. 2, PmERP15 cDNA is 732 bp, comprising a 5’ untranslated region (UTR) of 120 bp, an open reading frame (ORF) of 414 bp and a 3’ UTR of 198 bp. The PmERP15 ORF encodes a polypeptide consisting of 137 amino acids with a calculated molecular mass and pI value of 15 kDa and 8.45, respectively. Amino acid sequence analysis revealed that the predicted protein is a transmembrane protein with a single transmembrane region from residues 42–64 and a cytosolic C-terminus (http://www.cbs.dtu.dk/services/TMHMM/), without signal peptide (http://www
A homology search using BLASTP against the NCBI nr database showed that PmERP15 protein has 54–59% similarity to several unknown insect proteins, such as *Drosophila willistoni* GK10098 (XP_002071654), and 50% similarity to a hypothetical crustacean protein predicted from *Daphnia pulex* genome (EFX85211). Surprisingly, however, PmERP15 protein had no matches to any shrimp proteins. The nucleotide and deduced amino acid sequences have been submitted to the NCBI database under the accession number KF041007 (Fig. 2).

### 3.3. Expression analysis of PmERP15 gene in normal and WSSV-infected *P. monodon* shrimp

To investigate the tissue distribution of the PmERP15 transcript, total RNAs were extracted from various *P. monodon* tissues and subjected to RT-PCR analysis. Fig. 3 shows that PmERP15 transcript was expressed in all analyzed tissues with various expression levels. The PmERP15 transcript was highly expressed in the hemocytes, heart, and lymphoid organ. Expression levels were lower in the intestines, nerves and stomach, and lowest in the pleopod, hepatopancreas, and gills. Next we investigated whether or not WSSV infection induces the expression of PmERP15 in various shrimp tissues. As shown in Fig. 4, PmERP15 expression was strongly up-regulated in all assayed tissues after infection, suggesting that the up-regulation of PmERP15 expression in *P. monodon* is a systemic response following WSSV infection. Please note that the PCR cycling number for PmERP15 differs between Figs. 3 and 4.

To investigate whether PmERP15 protein also increases after WSSV infection, we produced a rabbit anti-PmERP15 antibody raised against the purified, full-length recombinant PmERP15 protein and performed Western blot analysis. Tissue lysates extracted from the gills of normal or WSSV-infected *P. monodon* were first separated through a SDS–PAGE gel, and then stained with Coomassie blue to ensure equal loading of the two samples (Fig. 5A). Next, the protein levels of PmERP15 in the lysates were analyzed with Western blot probing with anti-PmERP15 antibody. As shown in Fig. 5B, a protein band of 15 kDa, which is consistent with the predicted molecular weight, was observed in the WSSV-infected tissue lysate.
weight of PmERP15 protein, was only detected in lysates from gills of WSSV-infected shrimp but not in gill lysates of normal shrimp. We further used the antibody against the major WSSV structural protein VP28 to confirm the infection of shrimp with WSSV and anti-GAPDH antibody to confirm the equal loading of the two lysates. In summary, these experiments showed that WSSV infection systemically induces up-regulation of PmERP15 at both RNA and protein levels in P. monodon shrimp.

3.4. Localization of PmERP15 protein in WSSV-infected P. monodon hemocytes

To determine the subcellular localization of PmERP15 protein, we subjected infected and uninfected P. monodon hemocytes to indirect immunofluorescence staining using the anti-PmERP15 antibody. The results showed that strong immunofluorescence signals could only be detected in WSSV-infected hemocytes but not in uninfected hemocytes (Appendix S1), and the signals in WSSV-infected hemocytes were detected in the cytoplasm, mainly in the perinuclear region (Fig. 6A). To determine whether the perinuclear region corresponds to perinuclear ER, the antibody against the ER marker, protein disulfide isomerase (PDI), was used to co-stain the hemocytes with anti-PmERP15 antibody, and the hemocytes were then observed using a confocal microscope. As shown in Fig. 6B, PmERP15 signals were co-localized with PDI signals, suggesting that PmERP15 is located in the ER.

3.5. Expression and localization of PmERP15-EGFP fusion protein in insect cells

The experiment discussed earlier located the PmERP15 protein to the hemocyte ER. To further confirm its ER residence property, we expressed PmERP15 as an EGFP fusion protein and observed its localization in insect Sf9 cells; EGFP alone served as a control. The expression of PmERP15-EGFP fusion proteins in Sf9 cells was first confirmed by Western blot analysis detected with anti-EGFP antibody (data not shown). Then to locate the fusion protein in Sf9 cells, the transfected cells were stained with ER-Tracker™ Red dye and observed under a fluorescence microscope. As shown, the green fluorescence of PmERP15-EGFP fusion protein was mainly located in the perinuclear region and colocalized with the ER-Tracker™ Red dye (Fig. 7A), whereas the fluorescence of EGFP alone was evenly distributed throughout the cell (Fig. 7B). The colocalization of PmERP15-EGFP with the ER-Tracker™ Red dye was further confirmed by confocal microscopy (Fig. 7C). Therefore, in both shrimp

Fig. 4. Up-regulation of PmERP15 gene expression in various P. monodon tissues after WSSV infection. Pleo: pleopod; Intes: intestine; Hp: hepatopancreas; Hemo: hemocyte; Lo: lymphoid organ; Sto: stomach. H2O was used as negative control for the PCR reaction. EF-1α gene was used as an internal control for the experiment. N: normal shrimp; I: WSSV-infected shrimp. H2O was used as a negative control for the PCR reaction. The PCR cycling number for PmERP15 gene was 28 in this experiment.

Fig. 5. Western blot analyses of PmERP15 in normal and WSSV-infected P. monodon shrimp gills. (A) Equal amounts (25 μg) of tissue lysates extracted from normal (lane 1) and WSSV-infected (lane 2) shrimp gills were subjected to SDS–PAGE and Coomassie blue staining. (B) The same tissue lysates were subjected to Western blot analyses probing with anti-PmERP15, anti-WSSV VP28, or anti-GAPDH antibodies. The anti-VP28 antibody was used to confirm that the shrimp was infected with WSSV. The anti-GAPDH antibody was used as an internal control to ensure equal loading of the two shrimp tissue lysates.

Fig. 6. Immunofluorescence staining of PmERP15 in P. monodon hemocytes. (A) The hemocytes infected with WSSV were reacted with rabbit anti-PmERP15 antibody and then detected with Cy3-conjugated goat anti-rabbit IgG antibody. DAPI was used to counterstain the nuclear DNA. Scale bar equals 25 μm. (B) Colocalization of PmERP15 with PDI in WSSV-infected P. monodon hemocytes by confocal microscopy. PmERP15 (red) was visualized as described earlier and PDI (green) was visualized with mouse anti-PDI antibody and Alexa Fluor® 488 dye-conjugated goat anti-mouse IgG antibody. DAPI was used to counterstain the nuclear DNA. Scale bar equals 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
hemocytes and insect cells, PmERP15 protein was found to be associated with ER.

### 3.6. PmERP15 is an ER stress-responsive protein

The results discussed earlier showed that PmERP15 is an ER-resident protein and its expression is strongly induced by WSSV infection; based on that, we hypothesized that PmERP15 is an ER stress responsive protein and its expression is induced by ER stress caused by WSSV infection. To prove that, we at first investigated whether WSSV infection induces ER stress in *P. monodon* by analyzing the expression levels of *GRP78* in WSSV-infected *P. monodon*. *GRP78* is the master regulator of ER stress response and its expression is induced by ER stress (Samali et al., 2010). We found a *P. monodon* EST (GE615917) is homologous to *Fenneropenaeus chinensis* GPR78 (Luan et al., 2009), and based on its sequence, a primer pair was designed for RT-PCR. As shown in Fig. 8, after WSSV infection, *GRP78* transcripts were strongly up-regulated in every assayed tissue, suggesting that WSSV infection induces ER stress and activates UPR in *P. monodon*. We then investigated whether PmERP15 is a general ER stress response protein. To that end, two well-characterized ER stress inducers, tunicamycin and DTT, were injected into the *P. monodon*, and then the expressions of *GRP78* and *PmERP15* were analyzed with RT-PCR. As shown in Fig. 9, injection of either tunicamycin or DTT strongly induced the expression of both *PmERP15* and *GRP78* transcripts in gills and hemocytes. Therefore, the *PmERP15* expression was induced upon exposure to ER stress.

### 3.7. Silencing PmERP15 increases the mortality of *P. monodon* after WSSV infection but has no effect on WSSV replication

To characterize the role of PmERP15 during WSSV infection, a silencing experiment was performed. We first checked the silencing efficiency of PmERP15 expression in WSSV-infected shrimp. The shrimp were injected with PBS or dsRNAs (*PmERP15* or *Luciferase*) 2 days before being challenged by injection with WSSV. The gills were collected at 48 hpi and subjected to RT-PCR analysis. As shown in Fig. 10, when shrimp were pretreated with *PmERP15* dsRNA for 2 days, the strong up-regulation of *PmERP15* transcription by WSSV was not observed, which indicated that the injected *PmERP15* dsRNA efficiently silenced the expression of *PmERP15* in WSSV-infected shrimp. We also checked the expression of WSSV ie1 gene to confirm the successful infection with WSSV. Then, the effect of inhibiting PmERP15 expression on WSSV replication was investigated. At 48 hpi, the WSSV copy numbers in PmERP15-silenced shrimp showed no significant difference to those in *Luciferase* dsRNA- or PBS-pretreated shrimp (Fig. 11). Thus, the data showed that knocking down PmERP15 expression had no effect on WSSV replication. We next determined the effect of silencing PmERP15 on the cumulative mortality of WSSV-challenged shrimp. As shown in Fig. 12, compared to *Luciferase* dsRNA- and PBS-pretreated shrimp, PmERP15

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**Fig. 7.** Localization of PmERP15-EGFP protein in Sf9 insect cells. Sf9 cells were transfected with PmERP15-EGFP (A and C) or EGFP (B) expression plasmids. After heat shock to induce protein expression, the cells were stained with ER-Tracker™ Red dye and observed in a fluorescence microscope (A and B) and in a confocal microscope (C). Scale bar equals 20 μm.

**Fig. 8.** Up-regulation of *GRP78* gene expression in various *P. monodon* tissues after WSSV infection. Pleo: pleopod; Intes: intestine; He: hepatopancreas; Hemo: hemocyte; Lo: lymphoid organ; Sto: stomach. H2O was used as a negative control for the PCR reaction. *EF-1α* gene was used as an internal control for the experiment. N: normal shrimp; I: WSSV-infected shrimp. H2O was used as a negative control for the PCR reaction.

**Fig. 9.** Induced expression of PmERP15 and GRP78 in *P. monodon* injected with the ER stress inducers tunicamycin (TM) and DTT. At 24 h post injection, the gills and hemocytes (Hemo) were collected and subjected to total RNA extraction and RT-PCR analysis. *EF-1α* gene was used as an internal control. The shrimp injected with the appropriate vehicle (DMSO or H2O) in PBS were used as negative controls for tunicamycin or DTT treatments, respectively.
dsRNA-pretreated shrimp succumbed to WSSV infection very quickly: the cumulative mortality of the \( PmERP15 \) dsRNA group at 1, 2, 3 days post infection was about 30%, 43% and 78%, respectively, whereas that of the Luciferase dsRNA group was about 4%, 13% and 50%, respectively. Therefore, although \( PmERP15 \) showed no role in WSSV multiplication, it enhanced the survival of shrimp after WSSV infection.

This is not the first study to show that UPR is involved in WSSV–host interaction. For Chinese shrimp \( F.\) chinensis, WSSV infection has been found to modulate the expression of \( CRP78 \) \((Luan et al., 2009)\), \( catrcticulin \) \((Luana et al., 2007)\) and protein disulfide isomerase genes \((Ren et al., 2011)\). In the Pacific white shrimp \( Litopenaeus vannamei\), the \( IRE1-XBP1 \) UPR pathway has been shown to be activated by WSSV infection \((Chen et al., 2012b)\). Further, the two UPR signaling pathway transcription factors, \( XBP1 \) and \( LaATFa\), have been shown to activate the promoters of WSSV \( wsv083 \) and \( wsv023 \) genes, respectively, and silencing the expression of the transcription factors by dsRNAs led to decreased cumulative mortality of \( L.\) vannamei after WSSV infection \((Li et al., 2013)\). Therefore, although our study showed that the ER stress-related \( PmERP15 \) is not involved in WSSV multiplication, results obtained by \( Li et al. (2013)\) suggested that WSSV might exploit UPR signaling pathways to support their multiplication in shrimp.

Although the role of \( PmERP15 \) in ER stress is currently unknown, its importance is revealed by the fact that silencing its expression increased the cumulative mortality of shrimp following WSSV infection (Fig. 12). This suggests that \( PmERP15 \) is essential for shrimp to survive ER stress conditions. UPR signaling pathways exert transcriptional and translational controls to eliminate ER stress and restore or enhance ER function; however, if ER stress is too severe, UPR induces apoptotic pathways to eliminate damaged cells \((Logue et al., 2013; Schönthal, 2012)\). In mammals, a large number of genes are transcriptionally up-regulated by UPR, and these genes include ER chaperones, ERAD components, enzymes that regulate oxidative stress and stabilize redox homeostasis of the ER, proteins that are involved in ER biogenesis and so on \((Cao and Kaufman, 2012; Schönthal, 2012)\). Because \( PmERP15 \) is strongly up-regulated by UPR and the protein is located in ER, we predict that \( PmERP15 \) protein should play some role in relieving ER stress, and that when the protein is depleted through RNAi, the ER stress caused by WSSV infection cannot be alleviated, which finally leads to the death of the shrimp.

Similarity search against NCBI databases revealed that \( PmERP15 \) shows some similarity to a group of functionally unknown genes in various insects and to a predicted \( Daphnia \) protein, but has no similarity to any penaeid shrimp genes. The presence of \( PmERP15 \)-similar proteins in various insects, including \( Drosophila \), mosquitoes and bees, suggests that this group of proteins plays an important role in insects. The failure to identify \( PmERP15 \)-homologous genes in other penaeid shrimp in the NCBI databases was surprising, but
not impossible, as complete genome sequences are currently not available for any penaeid shrimp, and penaeid shrimp ESTs are fewer in comparison with those of insects. Based on our own experience, homologous genes between *P. monodon* and *L. vannamei* are highly similar to each other, and primers designed specifically for *P. monodon* genes can usually successfully amplify the PCR product from *L. vannamei* genes. However, when we used *PmERP15* gene primers to perform PCR with WSSV-infected *L. vannamei* gill cDNA as a template, we were unable to amplify the expected PCR product. *PmERP15* antibody also failed to detect the presence of the protein in WSSV-infected *L. vannamei*. Therefore, whether the *PmERP15* gene exists only in *P. monodon* or the corresponding homologous gene in *L. vannamei* is too divergent to be detected by either PCR or antibody must await further investigation.

To be localized in the ER, the transmembrane proteins usually depend on a short stretch of amino acids, the ER targeting or retention/retrieval signal, which is located in the cytosolic domain of a protein and interacts with the COP1 vesicle coat (Michelsen et al., 2005; Teasdale and Jackson, 1996). The most thoroughly studied targeting signal for ER transmembrane proteins is the di-lysine motif, KXXX or XOKK, which is located near the C-terminus of type I transmembrane proteins, where the lysine residues must be located at positions -3 and -4 or -3 and -5 from the C-terminus (Teasdale and Jackson, 1996). Another known targeting signal is the di-arginine motif, which has the consensus sequence ΦΨY/R-Ψ-R, where ΦΨ represents an aromatic or bulky hydrophobic residue and X denotes any amino acid. In contrast to the C-terminal di-lysine motif, the di-arginine motif is not necessarily located at the distal termini of any transmembrane proteins (Michelsen et al., 2005). Other less well understood mechanisms of ER targeting have been identified. For example, some transmembrane proteins use their transmembrane domain (TMD) for ER retention, such as cytochrome P450 (Honsho et al., 1998) and the novel ER molecular chaperone Cosmc (Sun et al., 2011). In insect SF9 cells, *PmERP15* protein could still exhibit ER-resident properties, suggesting that the ER targeting mechanism of *PmERP15* protein is conserved between crustaceans and insects. Therefore, it is plausible to identify the ER-targeting mechanism of *PmERP15* in SF9 cells. We can rule out the possibility of using di-lysine motif as targeting signal, because *PmERP15* has no di-lysine motif at the C-terminus and the C-terminal EGFP fusion protein remains targeted to ER. A putative di-arginine motif is located between amino acids 89 and 92, and we will determine whether this putative motif could target *PmERP15* to the ER or whether the ER localization of *PmERP15* is directed by TMD or else.

During the preparation of this paper, Vatanavicharn et al. (2014) reported the identification of a novel viral responsive protein, *PmVRP15*, from *P. monodon* hemocytes after WSSV infection. In fact, *PmERP15* and *PmVRP15* are the same protein: their amino acid sequences are almost the same (137 aa), with only two differences at amino acids 16 (Q vs. E) and 26 (V vs. I). Their major findings include: (1) *PmVRP15* was highly induced in hemocytes after WSSV infection; (2) in the hemocytes, *PmVRP15* was “localized in the cytoplasm near to the nuclear membrane” (i.e., the perinuclear region; please see fig. 5 in their paper); and (3) silencing of *PmVRP15* in *P. monodon* significantly decreased WSSV propagation and the cumulative mortality rate of WSSV-infected shrimp compared to the control shrimp injected with *GFP* dsRNA. Obviously, the first two points are consistent with our results but the last point is completely opposite. We speculate this discrepancy between our findings is caused by different dsRNA injection schemes. Compared to our method, they injected a much greater amount of dsRNA into the shrimp (10 μg vs. 0.5 μg/g shrimp) and injected it twice. It is known that in shrimp, injection of dsRNA induces not only the RNAi mechanism that can specifically silence the expression of the target gene, but also innate antiviral immunity in a sequence-independent manner (Robalino et al., 2004, 2005). Considering that a high amount of dsRNA was injected twice, a strong antiviral response might be evoked, and although a control shrimp group injected with GFP dsRNA was used for comparison, a shrimp group injected with PBS (and then WSSV) should be included to evaluate the extent of nonspecific antiviral response triggered by the dsRNA. As presented in our results (Fig. 12), the cumulative mortality rate of the Luciferase dsRNA WSSV shrimp group showed no significant difference from the PBS + WSSV shrimp group, showing that our dsRNA injection method had no discernible effect on WSSV infection.

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**Appendix: Supplementary material**

Supplementary data to this article can be found online at doi:10.1016/j.dci.2014.12.001.

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