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Two endoplasmic reticulum proteins (calnexin and calreticulin) are involved in innate immunity in Chinese mitten crab (*Eriocheir sinensis*)

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Calnexin (Cnx) and calreticulin (Crt), which are important chaperones in the endoplasmic reticulum (ER), participate in the folding and quality control of client proteins. *Cnx* and *Crt* identified from Chinese mitten crab (*Eriocheir sinensis*) are designated as *EsCnx* and *EsCrt*, respectively. *EsCnx* and *EsCrt* are expressed in the hemocyte, hepatopancrea, gill, and intestine at the mRNA and protein level. Immunofluorescence analysis indicated that *EsCnx* and *EsCrt* are located in the ER. Moreover, the mRNA and protein expression levels of *EsCnx* and *EsCrt* were altered by challenge with lipopolysaccharides (LPS), peptidoglycans (PGN), *Staphylococcus aureus*, and *Vibrio parahaemolyticus*. Recombinant *EsCnx* and *EsCrt* (r*EsCnx* and r*EsCrt*, respectively) proteins can bind to various Gram-positive and Gram-negative bacteria, as well as to different polysaccharides (LPS and PGN). r*EsCnx* and r*EsCrt* assisted in the clearance of *V. parahaemolyticus in vivo*, and the clearance efficiency was impaired after silencing of *EsCnx* and *EsCrt*. Our results suggest that the two ER proteins are involved in anti-bacterial immunity in *E. sinensis*.

Chaperones perform critical roles in various physiological processes, such as in promotion of cell survival, irreversible aggregation of newly synthesized proteins, prevention of misfolding, maintenance of the unfolded conformation of newly synthesized polypeptides, binding to non-native proteins during cellular stress responses, assembly of large proteins and multiprotein complexes, intracellular signaling, transcription, and differentiation^{1–3}. Calnexin (Cnx) and calreticulin (Crt) are related molecular chaperone proteins localized in the lumen of the endoplasmic reticulum (ER); these proteins are the most extensively studied glycoprotein-specific chaperones⁴. The ER is a dynamic organelle in cells and plays a wide array of functions in the synthesis of membrane and secretory proteins, as well in regulating protein folding, post-translational modification of membrane-associated proteins, secretion, and degradation^{5,6}. This organelle provides an oxidizing environment that, along with chaperone and co-chaperone proteins, induces the formation of disulfide bonds, appropriate folding of nascent glycoproteins, and prevention of improper folding of proteins⁷. As marker proteins, Cnx and Crt preferentially interact with non-native proteins through N-linked oligosaccharide moieties to form relatively stable complexes in the ER^{8,9}; this interaction persists until the proteins are properly folded or until misfolded proteins are degraded^{10,11}.

Cnx, a lectin chaperone that was first isolated from mouse, is transiently associated with partly folded MHC class I molecules¹²; Cnx was subsequently cloned from the liver cDNA library of humans in 1991¹³. Cnx is also associated with incomplete forms of MHC class I molecules, T-cell receptors, and membrane immunoglobulins^{14,15}. In addition, Cnx, with a molecular mass of approximately 90 kDa, is a non-glycosylated type I membrane protein present in many species¹⁶. The protein structure of Cnx contains three functionally distinct domains: a

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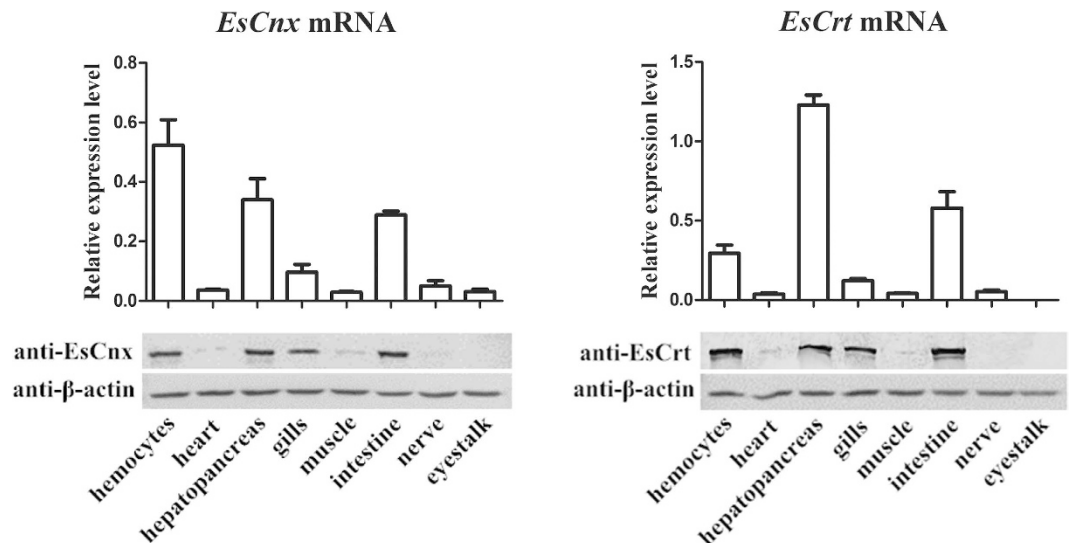


Figure 1. Tissue distributions of *EsCnx* and *EsCrt* at the mRNA level (above) revealed by SYBR Green qRT-PCR and protein expression level (below) revealed by western blot. Vertical bars represent mean \pm S.E. (N = 5) for each tissue. Each bar represents the mean value from five determinations with standard error.

large ER luminal domain, a transmembrane domain, and an ER-retention motif in the cytoplasmic tail¹³. The amino acid sequences of Cnx in plants and animals contain conserved regions in the middle parts and highly diverse regions in the N- and C-termini¹⁷. Cnx and its soluble homolog Crt constitute a dedicated maturation system, known as the Cnx/Crt cycle, which mainly promotes correct folding and oligomerization of newly synthesized glycoproteins before their export from the ER^{18,19}. Furthermore, Cnx plays a role in Ca^{2+} regulation²⁰, phagocytosis (a phylogenetically conserved process)²¹, and cell sensitivity to apoptosis in the ER²².

Crt, a highly conserved ER luminal resident protein, plays important roles in quality control, Ca^{2+} homeostasis, lectin-like chaperoning, gene regulation, cell adhesion, wound healing, cancer, and autoimmunity^{23,24}, as well as in various oxidative stress responses to hydrogen peroxide, hypoxic injury, and iron overload^{25–27}. Crt not only exists inside and outside of the ER lumen but also on the cell surface or extracellular matrix^{28,29}. Crt was first isolated from the sarcoplasmic reticulum of rabbit muscle cells and identified as a high affinity Ca^{2+} -binding protein³⁰; subsequent isolation of this protein in mouse indicated that Crt contains the ER retention/retrieval signal KDEL (Lys-Asp-Glu-Leu) sequence at the C-terminus and is primarily located in the lumen of the ER³¹. Numerous Crt genes have been isolated from diverse organisms³². In nearly all eukaryotes, Crt contains several structural and functional domains, including an N-domain, which is a protein-interacting domain, as well as proline-rich (P) and C domains, which interact with Ca^{2+} (refs 33,34). Crt regulates Ca^{2+} levels by controlling the signaling and homeostasis of this ion and acts as a protein-folding chaperone by forming a complex with the ER protein 57 (ERp57)^{32,35}. In addition, Crt affects biological processes, including growth, reproduction, molting, the immune system, apoptosis, and stress responses^{23,36}.

The roles of molecular chaperones in modulating immune functions have received increased research attention. In this study, *EsCnx* and *EsCrt* were identified from crabs, and their potential functions in innate immunity were investigated. Data obtained in this study will elucidate the roles of Cnx and Crt in crab innate immunity.

Results

cDNA cloning and sequence analysis. The 3175-bp full-length cDNA of *EsCnx* encodes a polypeptide of 598 amino acids; this peptide possesses a predicted molecular weight of 68 kDa and a theoretical isoelectric point of 4.50 (Fig. 1SA). *EsCrt* is 1741-bp long and encodes a protein of 406 amino acids; the calculated molecular weight and theoretical isoelectric point of *EsCrt* are 46.8 kDa and 4.29, respectively.

BLASTP search and multiple sequence alignment showed that *EsCnx* exhibits sequence similarity to other reported crustacean Cnxs, such as 76% identity to that of *Marsupenaeus japonicas* (AIF71174.1) (Fig. 2SA). The deduced amino acid sequence of *EsCrt* shares significant homology with other known Crts, such as 92% similarity to that of *Scylla paramamosain* (AEN94572.1) (Fig. 2SB). The constructed phylogenetic tree showed that *EsCnx*, *M. japonicas* MjCnx, and *Penaecus monodon* PmCnx are clustered into one subgroup (Fig. 3S). Meanwhile, *EsCrt* and nine Crts from other crustaceans and *Culex quinquefasciatus* are clustered into one group (Fig. 4S).

Tissue distribution of *EsCnx* and *EsCrt* and immunofluorescence assay. *EsCnx* and *EsCrt* mRNAs were highly expressed in the hemocyte, hepatopancreas, gill, and intestine (Fig. 1). The highest expression level of *EsCnx* was detected in hemocytes, followed by hepatopancreas and intestines. *EsCrt* was mainly expressed in the hepatopancreas, intestine, and hemocytes of healthy crabs. Western blot analysis showed the presence of *EsCnx* and *EsCrt* in hemocytes, hepatopancreas, gills, and intestine (Fig. 1). Furthermore, immunofluorescence assay combined with confocal microscopy analysis confirmed that *EsCrt* and *EsCnx* were located in the ER (Fig. 2).

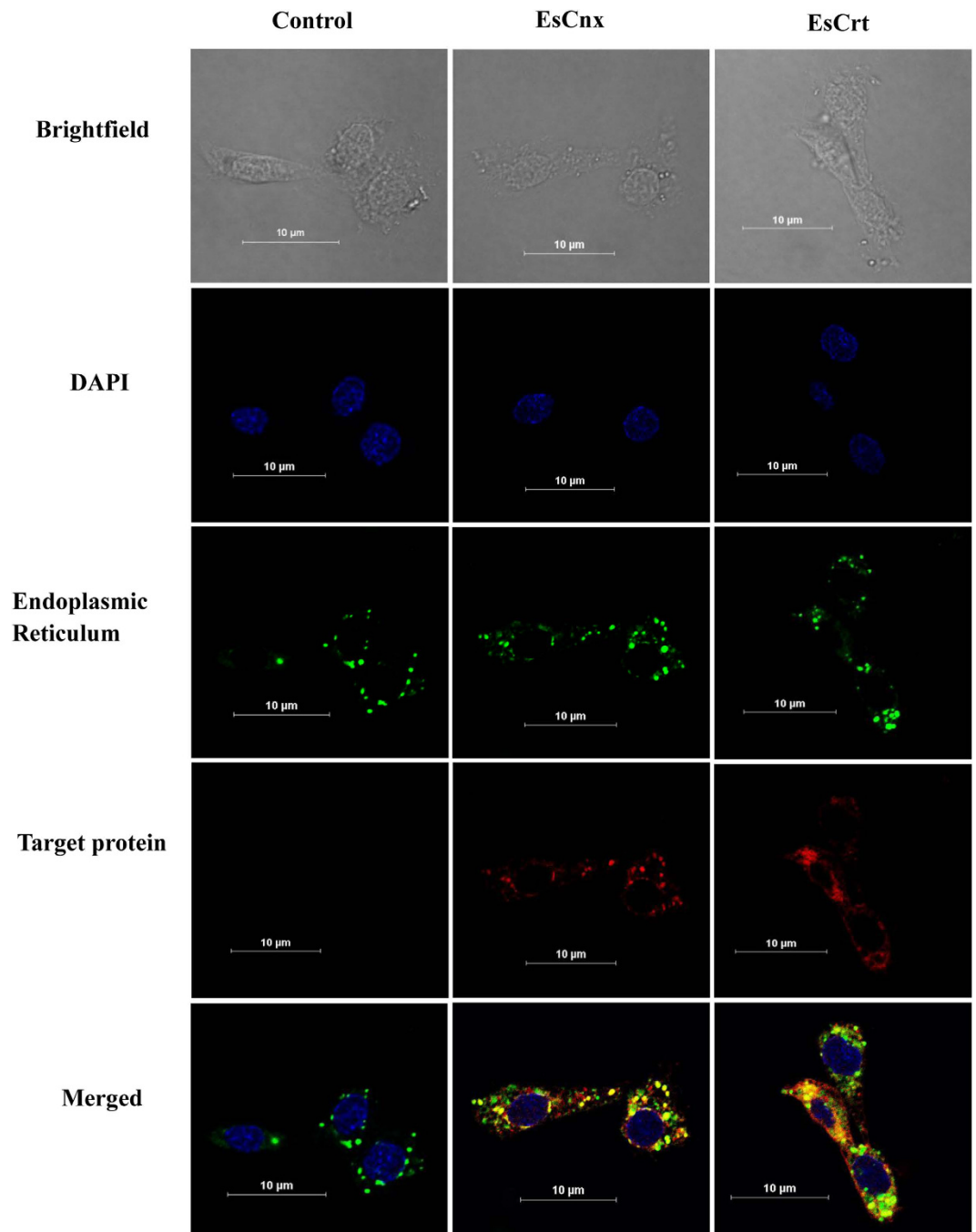


Figure 2. Intracellular localization of EsCrt and EsCnx in crab cells. Hemocytes were stained for EsCrt or EsCnx using rabbit anti-EsCrt antibody (1:1000) or anti-EsCnx (1:1000). DAPI was used to stain the nucleus. DiOC6(3) 3,3'-dihexyloxycarbocyanine iodide is a green fluorescent membrane dye used for staining the endoplasmic reticulum.

Analysis of protein and mRNA expression patterns of *EsCnx* and *EsCrt* after challenge with polysaccharides and microorganisms. When crabs were injected with lipopolysaccharides (LPS), the mRNA expression levels of *EsCnx* from 2 h to 12 h were significantly higher than those in the untreated control and then decreased at 24 h (Fig. 3A). After 2 h of peptidoglycan (PGN) challenge, *EsCnx* was initially upregulated, returned to its original level at 6 h, and then increased at 12 and 24 h (Fig. 3B). The transcript expression of *EsCnx* was initially downregulated 2 h after challenge with *Staphylococcus aureus*, reached the highest level at 6 h, and gradually decreased from 12 h to 24 h (Fig. 3C). After challenge with *Vibrio parahaemolyticus*, the *EsCnx* mRNA expression level gradually increased within 2 h, peaked at 6 h, and then decreased at 12 and 24 h (Fig. 3D). Moreover, the protein expression pattern of EsCnx was similar to that of mRNA expression upon challenge with LPS (Fig. 3A), PGN (Fig. 3B), *S. aureus* (Fig. 3C), and *V. parahaemolyticus* (Fig. 3D).

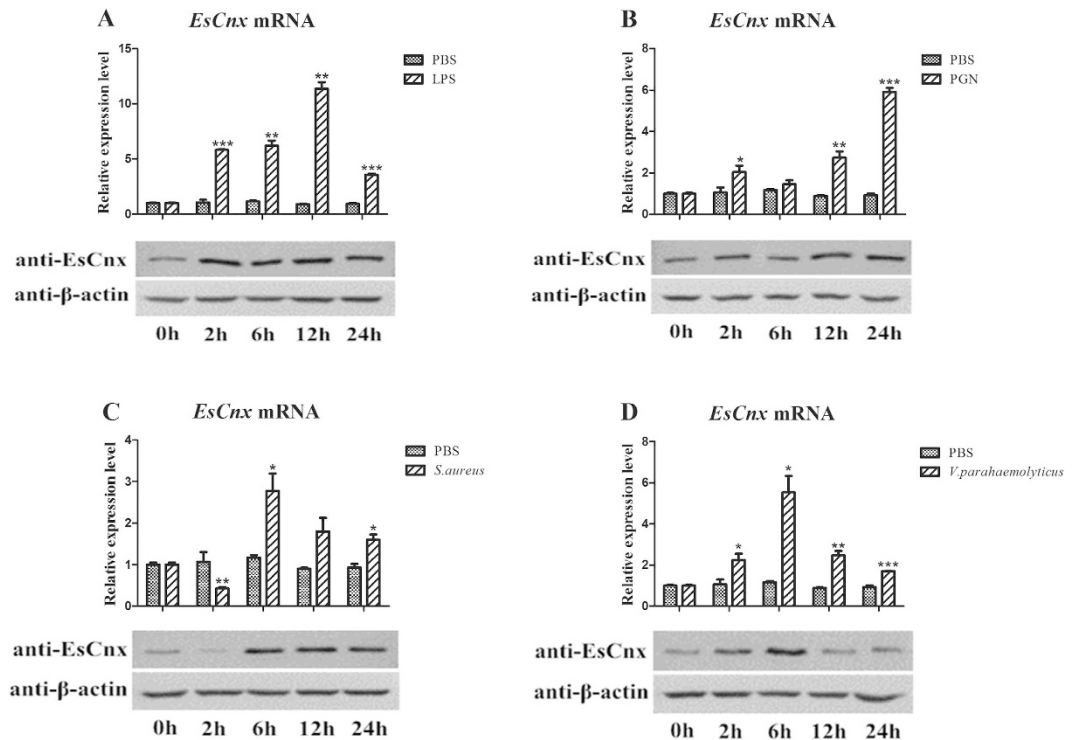


Figure 3. *EsCnx* mRNA and protein expression profile in hepatopancreas after LPS (A), PGN (B), *S. aureus* (C), and *V. parahaemolyticus* (D) challenge as measured by qRT-PCR and western blot. The GAPDH gene was used as internal control to calibrate the cDNA template of all samples. Vertical bars represent mean \pm S.E. (N = 5). Each bar represents the mean value of five determinations with standard deviation. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) compared with the control. The β -actin was used as internal control to calibrate the protein template of all samples.

EsCrt was rapidly upregulated 2 h after LPS challenge, decreased at 6 h, increased at 12 h, and was finally down-regulated again at 24 h (Fig. 4A). The *EsCrt* protein level did not change within 2–12 h after the LPS challenge (Fig. 4A). After 2–6 h of the PGN challenge, *EsCrt* mRNA and *EsCrt* protein expression levels were gradually upregulated, decreased at 12 h, and finally reached the highest levels (Fig. 4B). After 6 h of *S. aureus* challenge, *EsCrt* transcription peaked and then decreased from 12 h to 24 h (Fig. 4C). Upon *S. aureus* challenge, the protein expression pattern of *EsCrt* was similar to that of mRNA expression (Fig. 4C). After *V. parahaemolyticus* challenge, *EsCrt* expression increased from 6 h to 12 h and then recovered to the normal level (Fig. 4D). Furthermore, the protein expression level of *EsCrt* did not evidently change upon *V. parahaemolyticus* challenge (Fig. 4D).

Expression and purification of recombinant proteins. Figure 5A shows a detected band with a molecular weight of approximately 70 kDa, which is consistent with the predicted molecular weight of the Crt region of *EsCnx* (MW: 43.1 kDa) with a 26-kDa GST tag. *EsCrt* bearing the GST tag was approximately 62 kDa (Fig. 5B).

Microbial and carbohydrate binding of rEsCnx and rEsCrt. The bacterial binding assay showed that rEsCrt can bind to all tested microorganisms, including four kinds of Gram-positive bacteria (*S. aureus*, *M. luteus*, *B. subtilis*, and *Bacillus megaterium*) and four kinds of Gram-negative bacteria (*Aeromonas hydrophila*, *V. parahaemolyticus*, *Vibrio anguillarum*, and *Escherichia coli*). rEsCnx weakly bound to *S. aureus* and *A. hydrophila* but strongly bound to the six other microorganisms (Fig. 5C). No visible binding was detected in the negative control.

rEsCnx and rEsCrt demonstrated binding activity to PGN or LPS. The binding activity of rEsCrt to LPS was higher than that of rEsCnx; whereas the binding activity of rEsCrt to PGN was higher than that of rEsCnx. The binding ability of the proteins was found to be dose dependent (Fig. 5D,E). For the control, rArf and GST proteins did not bind to LPS or PGN.

Effect of rEsCnx and rEsCrt on the clearance of *V. parahaemolyticus*. Given that rEsCnx and rEsCrt can bind to the crab pathogen *V. parahaemolyticus*, we determined whether or not rEsCnx and rEsCrt affect the bacterial clearance rate in crabs. The bacterial clearance experiment showed that *V. parahaemolyticus* was cleared more rapidly in the hemolymphs of rEsCnx and rEsCrt injection groups compared with rArf and GST control groups (10 and 15 min, respectively) (Fig. 6A,D).

Clearance assays were also conducted after knockdown of *EsCnx* or *EsCrt*. *EsCnx* or *EsCrt* expression significantly decreased 24 h after injection of *EsCnx*-siRNA or *EsCrt*-siRNA (Fig. 6B,E); moreover, the clearance rate of *V. parahaemolyticus* in the crab significantly decreased after knockdown of *EsCnx* or *EsCrt* (Fig. 6C,F). These findings indicate that *EsCnx* and *EsCrt* play important functions in clearing *V. parahaemolyticus*.

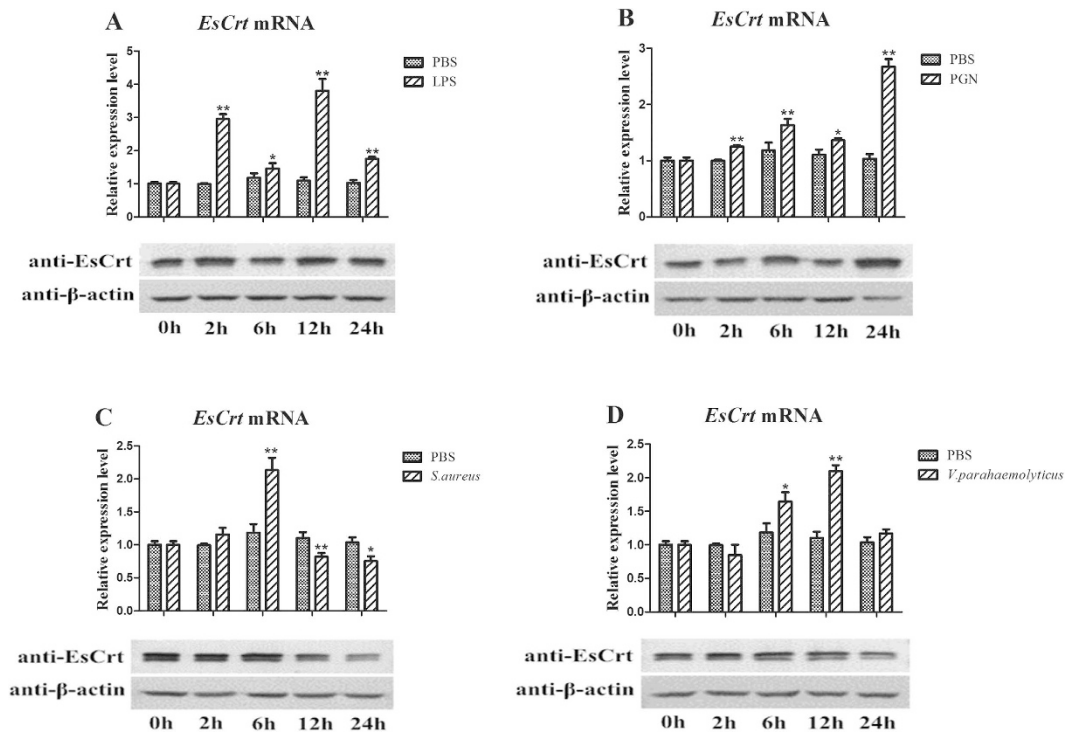


Figure 4. *EsCrt* mRNA and protein expression profile in the hepatopancreas after LPS (A), PGN (B), *S. aureus* (C), and *V. parahaemolyticus* (D) challenge as measured by qRT-PCR and western blot. GAPDH gene was used as an internal control to calibrate the cDNA template for all of the samples. Vertical bars represent mean \pm S.E. (N = 5). Each bar represents the mean value of five determinations with standard deviation. Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) compared with the values of control. The β -actin was used as internal control to calibrate the protein template of all samples.

Discussion

Cnxs and Crts, which are important chaperones in the ER, are involved in translocation, protein folding, and quality control of newly synthesized polypeptides⁴. The roles of Cnxs and Crts in the innate immune system have been investigated in crustaceans. For instance, *Crt* was detected in the ovary and in various molting stages of Chinese white shrimp (*Fenneropenaeus chinensis*). When a host is exposed to heat shock or challenged by white spot syndrome virus (WSSV), *Crt* is rapidly cleaved and the shrimp initiates a defense reaction against the stimuli³². In crayfish, the C1q-binding proteins *Crt* and *gC1qR*, which are highly conserved ubiquitous proteins, can respond to viral infection by forming a complex in the cytoplasm, thereby preventing apoptosis³⁷. Another *Crt* associated with immune defenses against *V. anguillarum* and WSSV was purified from the hemocytes of ridge tail white prawn (*Exopalaemon carinicauda*)³⁸. A novel *MjCnx* protein was also detected in the cytoplasm and on the membrane of hemocytes in kuruma shrimp (*M. japonicas*); *MjCnx* protein not only enhances the clearance of *V. anguillarum* *in vivo* but also promotes phagocytic efficiency of hemocytes³⁹. However, information on the function of *Cnx* and *Crt* in crab immunity remains unavailable⁴⁰. Detecting *Cnxs* and *Crts* from crabs and investigating their immune mechanism can elucidate the functional diversity of crustacean *Cnxs* and *Crts*.

In this study, *EsCnx* and *EsCrt* were identified from *Eriocheir sinensis*. *EsCnx* and *EsCrt* were primarily distributed in hemocytes, hepatopancreas, and intestine; the expression levels of these proteins were altered by challenge with polysaccharides and microorganisms. Moreover, the mRNA transcripts of *MjCnx* from *M. japonicas*³⁹ and *EcCrt* from *E. carinicauda*³⁸ were upregulated in several tissues challenged with *V. anguillarum*. Hemocytes are important tissues involved in crustacean immunity and comprise many immune-related genes⁴¹. As the counterpart of the insect fat body and mammalian liver, the hepatopancreas is important for the humoral immune responses of crustaceans⁴². The arthropod alimentary tract is the first defense barrier against pathogen invasion^{43,44}; furthermore, the intestine, as a crucial part of the alimentary canal, plays an essential role in crab immunity. These results suggest that *EsCnx* and *EsCrt* are involved in crab antibacterial immunity.

Since the discovery of *Cnx* and *Crt* in mammals^{12,30}, the functions of these two proteins have been the subject of most studies. *Cnx* is a lectin chaperone, whereas *Crt* is a lectin-like chaperone^{12,23}. In addition to the chaperone activity, *Cnx* and *Crt* also have nonchaperone functions such as phagocytosis and lectin activity^{21,39}. In this study, crab *rEsCnx* and *rEsCrt* showed the lectin activities of binding diverse bacteria and several polysaccharides. The lectin activities of shrimp *rMjCnx* were also reported³⁹. Lectins, which are pattern-recognition proteins, play crucial functions in recognizing and eliminating pathogens in innate immunity⁴⁵. So, crab *rEsCnx* and *rEsCrt* may play an important role in recognizing and binding bacteria; this phenomenon is the first line of innate immune defense^{46,47}.

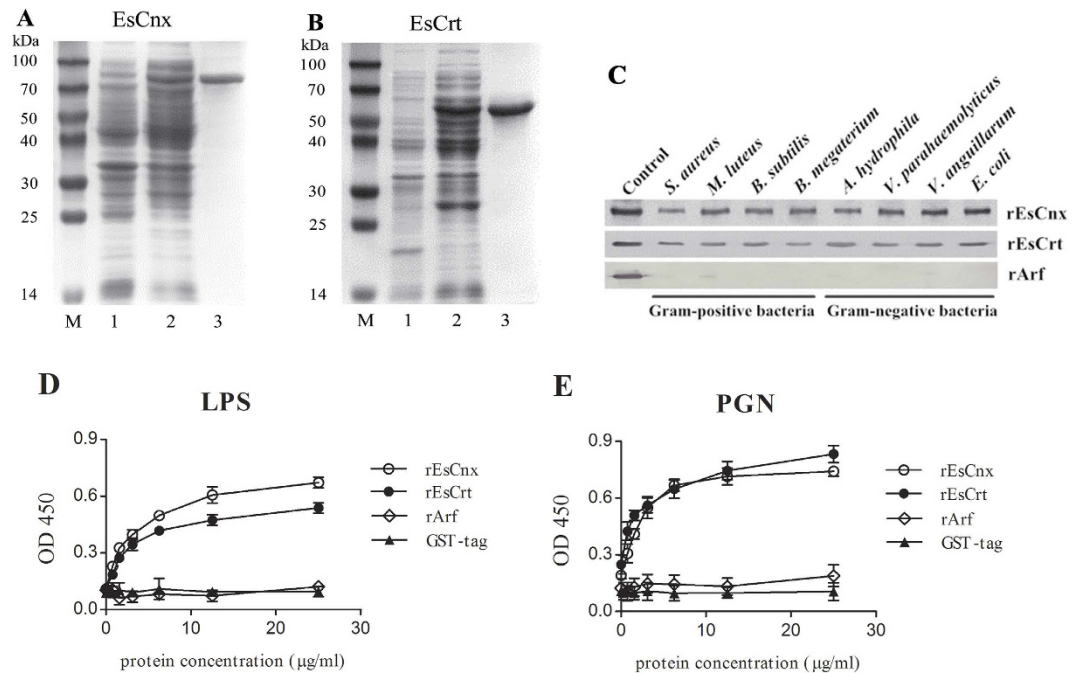


Figure 5. SDS-PAGE analysis of rEsCnx (A) and rEsCrt (B). Lane M: protein molecular weight standards (kDa); lane A1: negative control for rEsCnx (without induction); lane A2: IPTG-induced rEsCnx; lane A3: purified rEsCnx; lane B1: negative control for rEsCrt (without induction); lane B2: IPTG-induced rEsCrt; and lane B3: purified rEsCrt. (C) Direct binding of rEsCnx and rEsCrt proteins to microorganisms (*S. aureus*, *M. luteus*, *B. subtilis*, *B. megaterium*, *A. hydrophila*, *V. parahaemolyticus*, *V. anguillarum*, and *E. coli*). The microorganisms were incubated with rEsCnx or rEsCrt and then washed four times with TBS. ELISA assay was employed to detect the binding of rEsCnx (D) and rEsCrt (E) to LPS and PGN using antiserum against rEsCnx and rEsCrt. All assays were repeated three times.

Furthermore, we assumed that rEsCnx and rEsCrt can accelerate clearance of invading *V. parahaemolyticus*, which is a Gram-negative bacterium and an extremely virulent and prevalent pathogen that affects crab aquaculture⁴⁸. Overexpression of rEsCnx or rEsCrt can enhance clearance of *V. parahaemolyticus in vivo* in crabs and the clearance of bacteria was impaired after knockdown of *EsCnx* or *EsEsCrt*. The rMjCnx also accelerate the removal of bacteria in shrimp³⁹. It was also reported that rMjCnx can promote phagocytosis of bacteria³⁹. Invertebrates exhibit effective innate immune responses, including humoral and cellular responses⁴⁹. Cellular immune responses involve different types of hemocytes, which participate in pathogen clearance through phagocytosis of microorganisms⁵⁰. Recognition of pathogen is the first step of phagocytosis and it needs the participation of receptors. In human, three types of receptors including pattern-recognition receptors (PRRs), opsonic receptors, and apoptotic corpse receptors are involved in phagocytosis⁵¹. The broad binding spectrum of rEsCnx and rEsCrt toward diverse bacteria and pathogen-associated molecular patterns (PAMPs) suggest that *EsCnx* and *EsCrt* may serve as phagocytic receptors.

In conclusion, this work is the first to characterize *EsCnx* and *EsCrt* isolated from *E. sinensis*. *EsCnx* and *EsCrt* expression levels are regulated by polysaccharide or bacterial challenge. Moreover, rEsCnx and rEsCrt proteins not only can bind to various bacteria and different polysaccharides (LPS and PGN) but also facilitate clearance of *V. parahaemolyticus* in crabs. Our results suggest that *EsCnx* and *EsCrt* are involved in anti-bacterial immunity in *E. sinensis*.

Materials and Methods

Cloning of full-length cDNAs of *EsCnx* and *EsCrt*. Two expressed sequence tags were obtained from the hepatopancreas of *E. sinensis* by using Illumina's Solexa Sequencing Technology in the Chinese National Human Genome Center in Shanghai. Two pairs of gene-specific primers (*EsCnx*-F and *EsCnx*-R, as well as *EsCrt*-F and *EsCrt*-R) were designed based on the obtained cDNA sequences of *EsCnx* and *EsCrt* to clone their full-length sequences (the primer sequences are shown in Table 1).

Sequence analysis. The cDNA sequences and deduced protein sequences of *EsCnx* and *EsCrt* were analyzed using the BLAST algorithm of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the Expert Protein Analysis System (<http://web.expasy.org/translate/>). Signal peptide and putative domains were predicted using the Simple Modular Architecture Research Tool (<http://smart.embl-heidelberg.de/>). Molecular weights and isoelectric points of *EsCnx* and *EsCrt* were determined using ExPASy (http://web.expasy.org/compute_pi/). Moreover, MEGA 5.05 was used to produce an unrooted phylogenetic tree based on the deduced full amino acid sequences of *EsCnx* and *EsCrt* and other related proteins by using the neighbor-joining

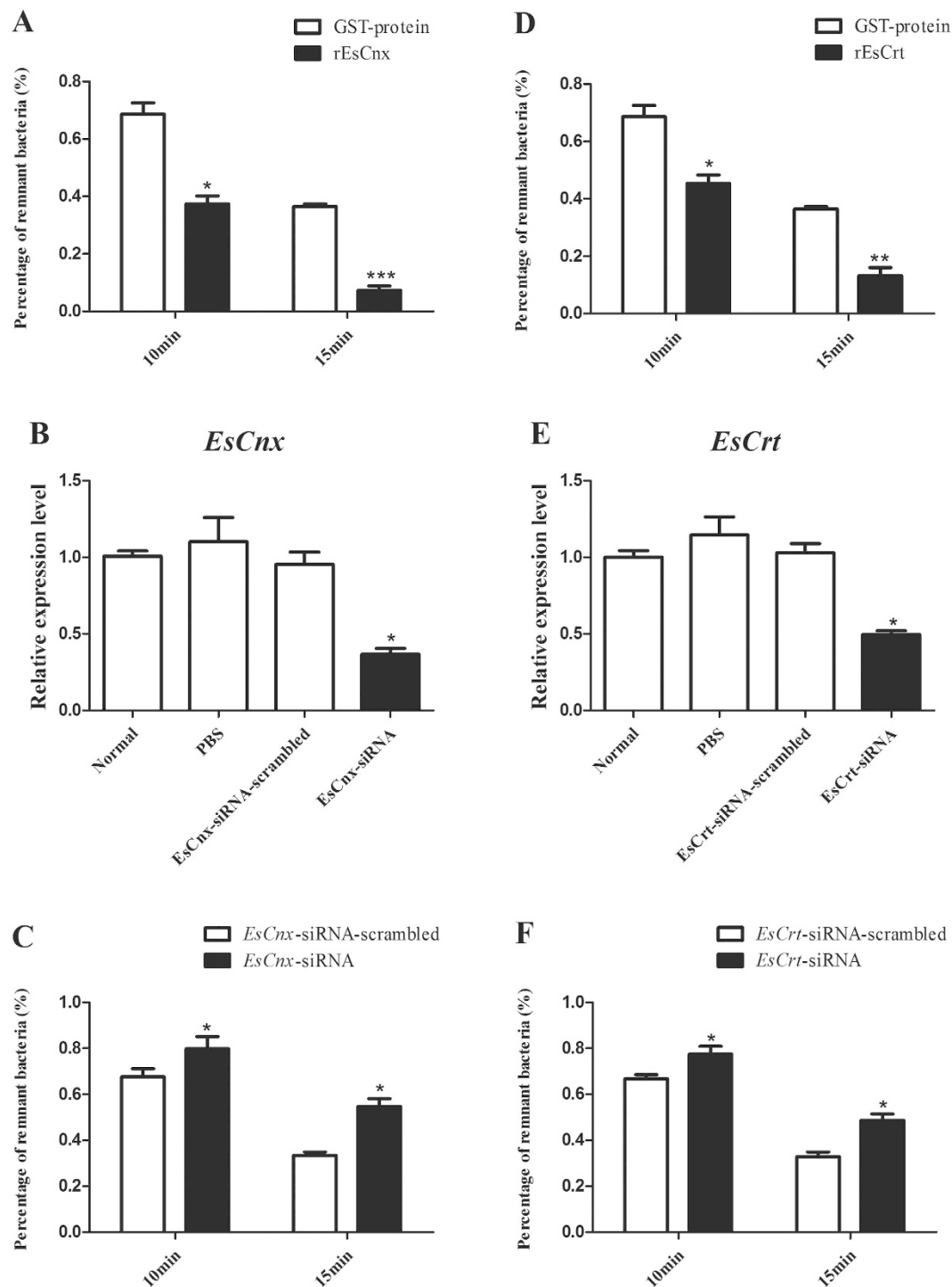


Figure 6. Effect of rEsCnx and rEsCrt in *V. parahaemolyticus* clearance activities in crab. rEsCnx (A) or rEsCrt (D) enhanced the clearance of *V. parahaemolyticus* in vivo. *V. parahaemolyticus* clearance assay was conducted by pre-coating the injected bacteria with rEsCnx, rEsCrt, or GST-tag protein. The number of remnant bacteria was counted at 2, 10, or 15 min post-injection. The percentage of remnant bacteria was calculated as follows: percentage of remnant bacteria = (number of bacteria at 10 min or 15 min/number of bacteria at 2 min) × 100. RNAi of *EsCnx* (B) or *EsCrt* (E) suppressed bacterial clearance in hemocytes. The mRNA transcription level of *EsCnx* 24 h after injection of *EsCnx*-siRNA or *EsCnx*-siRNA-scrambled, as revealed by qRT-PCR analysis. The mRNA transcription level of *EsCrt* 24 h after injection of *EsCrt*-siRNA or *EsCrt*-siRNA-scrambled was revealed by qRT-PCR. The clearance rate of *V. parahaemolyticus* in crabs was impaired by silencing *EsCnx* (C) or *EsCrt* (F) through RNAi. Two groups of crabs were injected with *EsCnx*-siRNA or *EsCrt*-siRNA-scrambled (control), and clearance assay was conducted 24 h post-injection.

Primers name	Sequences (5'–3')
EsCnx-F	GTGACTGACAATACGGCTGAGGCTGACC
EsCnx-R	CCACTCCTTCCTTCTTTGCCTGTGACTTG
EsCrt-F	CCCGAGTACACCCCTGACACAGAAATCT
EsCrt-R	GCTCTTACCTGCCACAAGTCCAAACCGA
EsCnx-RT-F	AAATGGATGGTGAATGGGAGGC
EsCnx-RT-R	TTGGGGATGGTGCGAGGTT
EsCrt-RT-F	GGAGAGCAAGGAGGATGAGGAG
EsCrt-RT-R	AAGGGGATTGACTGAGGAAAGATTA
EsGAPDH-RT-F	CTGCCAAAACATCATCCCATC
EsGAPDH-RT-R	CTCTCATCCCCAGTAAATCGC
EsCrt-ex-F	CCGGAATTCGCTTATGTCATGGAAACATTT
EsCrt-ex-R	TACTCAGCGGCCGCTCCACAATAAGGTTATCAAAAAGG
EsCrt-ex-F	CCGGAATTCGTGTTCTTCCAGGAGACCTTC
EsCrt-ex-R	TACTCAGCGGCCGCTCAATGAGGAAGTTGTGCGAA
EsCnx-siRNA	GCTGGCTGCAGACACTTAT
EsCnx-siRNA-scrambled	ATTGCAGCTGGCAAGCCTT
EsCrt-siRNA	GGTGTGAAAGAACCACAAT
EsCrt-siRNA-scrambled	TGAGGACATGACATGAAAC

Table 1. Primer sequences used in this study. *EcoR* I and *Not* I sites are underlined.

method⁵². One thousand bootstraps were selected for the neighbor-joining tree to assess reliability. MEGA 5.05 and GENDOC were also used to perform multiple sequence alignments.

Animals, polysaccharides, and microbes. Adult Chinese mitten crabs were purchased from a wet market (Nanjing, Jiangsu Province, China) and housed in filtered, aerated freshwater at 20–25 °C for a week before processing. LPS (*E. coli* Serotype 055:B5) and PGN (*M. luteus*) were obtained from Sigma (St. Louis, MO, USA). *S. aureus*, *M. luteus*, *B. subtilis*, *B. megaterium*, *A. hydrophila*, *V. parahaemolyticus*, *V. anguillarum*, and *E. coli* were purchased from the Microbial Culture Collection Center (Beijing, China) and grown in LB broth at 37 °C.

Tissue collection and challenge with polysaccharides and microorganisms. Various tissues (hemocytes, heart, hepatopancreas, gills, muscle, intestine, nerve, and eyestalk from five adult crabs as parallel samples) were collected to determine the tissue distribution of *EsCnx* and *EsCrt* transcripts. The hemolymph was withdrawn using a 1 mL sterile syringe containing an equal volume of anticoagulant solution (ACD-B)⁵³. The samples were immediately centrifuged at 800 × *g* at 4 °C for 15 min to collect hemocytes. A total of 150 crabs in the bacteria-challenged groups were used in the experiment. The crabs were injected with 50 μL of LPS (0.5 μg/μL), PGN (0.5 μg/μL), live *S. aureus* (3 × 10⁷ cells), or *V. parahaemolyticus* (3 × 10⁷ cells) in PBS. Five individual crabs were randomly sampled at 2, 6, 12, and 24 h in the challenge groups during the experiment. In addition, five untreated crabs were used as the untreated control group in the microorganism challenge. Hepatopancreas organs were collected from untreated control and microorganism-challenged treatment groups (LPS, PGN, *S. aureus*, and *V. parahaemolyticus*). All these tissue samples were stored at –80 °C for subsequent RNA extraction.

Total RNA isolation and cDNA synthesis. Total RNA was extracted from the tissues according to the manufacturer's instructions (Spin-column, BioTeke, Beijing, China). First-strand cDNA was obtained using PrimeScript First-strand cDNA synthesis kit with Oligo dT Primer from Takara (Dalian, China). The cDNA mixture was diluted 10 times with PCR-grade water and then stored at –80 °C for qRT-PCR analysis.

Real-time PCR analysis of mRNA expression. The mRNA expression levels of *EsCnx* and *EsCrt* in different tissues and their temporal expression in the hepatopancreas of crabs after the treatments were measured by SYBR Green fluorescent RT-PCR. Two pairs of specific primers (*EsCnx*-RT-F and *EsCnx*-RT-R, as well as *EsCrt*-RT-F and *EsCrt*-RT-R) were used to amplify the corresponding products of *EsCnx* and *EsCrt*. The PCR products were sequenced to verify the specificity of qRT-PCR. Two *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) primers, namely, *EsGAPDH*-RT-F and *EsGAPDH*-RT-R (Table 1), were used for amplifying the 268-bp fragment as an internal control to verify successful transcription and to calibrate the cDNA template for corresponding samples. All samples were analyzed in triplicate in the qRT-PCR analysis. The expression levels of *EsCnx* and *EsCrt* were analyzed using the comparative CT method⁵⁴; the discrepancy between the CT for *EsCnx* or *EsCrt*, and *GAPDH* (Δ CT) was calculated to normalize the variation in the amount of cDNA in each reaction. Moreover, *EsCnx* and *EsCrt* expression levels were calculated by $2^{-\Delta\Delta CT}$. The obtained data were statistically analyzed using an unpaired sample t-test. Significant difference was accepted as $P < 0.05$.

Recombinant expression and purification of *EsCnx* and *EsCrt* in *E. coli*. The cDNA fragments encoding the Crt domains of *EsCnx* and *EsCrt* were amplified using the following primers: *EsCnx*-ex-F and *EsCnx*-ex-R, as well as *EsCrt*-ex-F and *EsCrt*-ex-R (Table 1), with *EcoR* I and *Not* I sites. The amplified products were cloned into pGEX-4T-1 vector (Novagen) and then transformed into competent *E. coli* Rosetta (DE3) cells

(TransGen Biotech). The positive clones were screened through PCR reaction using specific primers and then confirmed by nucleotide sequencing. The cells were grown at 37 °C in LB medium under agitation until an OD₆₀₀ of 0.6–0.8. Recombinant expression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG, 0.5 mmol L⁻¹). The samples were then incubated for another 4 h under the same conditions. rEsCnx and rEsCrt were examined by SDS polyacrylamide gel electrophoresis (SDS-PAGE). In addition, rEsCnx and rEsCrt with a glutathione S-transferase (GST) tag and GST protein was purified using glutathione Sepharose 4B chromatography (Gen-Script, USA) according to the manufacturer's instructions.

Western blot analysis of localization and expression profiles of EsCnx and EsCrt proteins.

Antisera for EsCnx and EsCrt were provided by Dr. Xian-Wei Wang (Shandong University)^{39,55}. The heart, hepatopancreas, gills, muscle, intestine, nerve, and eyestalk of healthy crabs were homogenized in a buffer solution (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 3 mM EDTA, and 1 mM PMSF). The solution was then centrifuged at 10,000 × g for 10 min at 4 °C to collect the supernatant. The hemolymph of normal or challenged crabs was prepared for analysis. The protein concentration was determined using the method described by Bradford. For Western blot analysis, each sample (100 μg protein) separated by 12.5% SDS-PAGE gel was transferred onto a pre-wetted nitrocellulose membrane in electro blotting buffer under a constant current of 80 V for 100 min. The membrane was blocked in 5% non-fat dried milk in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) at room temperature for 2 h. Subsequently, the membrane was incubated with 1/1000 diluted antiserum to EsCnx or EsCrt in TBS at 4 °C overnight and washed three times with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 10 min. The membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG (1/5000 diluted in TBS) at room temperature for 2 h and then washed three times with TBST for 10 min. Protein bands were stained with 3,3'-diaminobenzidine for 10 min, and the reaction was terminated by washing with distilled water.

Immunofluorescence. Healthy crab was swabbed with 75% ethanol, and hemolymph was collected with an equal volume of ACD-B⁵³. The diluted hemolymph was centrifuged at 300 × g at 4 °C for 10 min to collect the cells, which were washed two times in culture medium. The cells were resuspended gently in 2 mL culture medium, and 0.2 mL aliquots were seeded into 2 mL of culture medium on 12-well dishes with glass coverslips and then incubated at 28 °C. After attachment to the coverslips, the cells were washed three times with PBS. Paraformaldehyde solution (4%) in PBS was used to fix the cells for 30 min at room temperature. The cells were then washed in PBS three times and treated with 0.5% Triton X-100 solution for 10 min. After washing three times with PBS, the cells were treated with 5% BSA, blocked for 2 h, and then incubated with 1/1000 diluted rabbit antiserum to EsCnx or EsCrt in PBS at 4 °C overnight. The cells were washed four times with PBST and then incubated with Andy Fluor™ 594 Goat Anti-Rabbit IgG (H+L) Antibodies (Beyotime, China) for 1 h. After washing four times with PBST, the cells were incubated with 500 μL of 25 μM DiOC6(3) (Beyotime, China) diluted in PBS at 37 °C for 10 min. The plates were washed as described above, and the cells were mounted on glass slides containing ProLong gold antifade reagent with DAPI (Invitrogen). After final washing, the cells were incubated in PBS and observed under a confocal laser scanning microscope.

Microbial binding assay. The binding assay was performed as described earlier⁵⁶. Briefly, microorganisms were collected and resuspended in TBS to OD₆₀₀ of 1.0 and then incubated with recombinant EsCnx and EsCrt (600 μg/mL, 50 μL) for 30 min with gentle rotation at room temperature. The microorganisms were pelleted, washed four times with TBS, and then loaded onto 12.5% SDS-PAGE gel. The bound protein was detected by Western blot analysis using anti-GST antiserum. For the control, bacterial cells were incubated with rArf (600 μg/mL) and GST-tagged proteins and then subjected to the same treatments.

Direct binding to PAMPs. ELISA assay was performed to detect the direct binding of EsCnx and EsCrt to LPS and PGN, as described previously⁵⁶. LPS (50 μL) or PGN (80 μg/mL) was used to coat a 96-well microtiter plate. The plate was then incubated at 37 °C overnight, heated at 60 °C for 30 min, and then blocked with 200 μL/well BSA (600 μg/mL) in TBS for 2 h at 37 °C. After washing four times with TBS (200 μL/well), the purified EsCnx, EsCrt, or GST (control) was diluted in TBS containing 0.1 mg/mL BSA to achieve different concentrations (0–50 μg/mL) and added to each well (50 μL/well). Subsequently, the wells were incubated for 3 h at room temperature. The plates were then washed as described above, and anti-GST antiserum (1/2000 dilution) was added to the wells (100 μL/well). After 1 h of incubation at 37 °C, the wells were washed as described above. Approximately 100 μL of peroxidase-conjugated goat anti-rabbit IgG (1/5000 dilution) was added and incubated at 37 °C for 1 h. After final washing, color was enhanced by adding 0.01% 3,3',5,5'-tetramethylbenzidine (Sigma) liquid substrate in citric acid–Na₂HPO₄ buffer. The reaction was terminated by adding 2 M H₂SO₄, and the absorbance was read at 450 nm. The binding assay was performed three times.

V. parahaemolyticus clearance assay. Overnight-cultured *V. parahaemolyticus* was washed and resuspended in PBS. *V. parahaemolyticus* (approximately 3 × 10⁷ cells) was incubated with rEsCnx or rEsCrt (600 μg/mL, 500 μL) at 28 °C for 30 min with rotation. In addition, *V. parahaemolyticus* was incubated with GST (600 μg/mL, 500 μL) as a control. Two groups of mixtures (50 μL each) were injected into the crabs. The hemolymph (500 μL) of three crabs was collected at different post injection times (2, 10, and 15 min). After serial dilution with PBS, the samples (30 μL) were plated onto LB broth plates. The plates were incubated overnight at 37 °C, and the number of bacterial clones in the plate was counted thereafter. Subsequently, the percentage of residual bacteria (i.e., (number of bacteria in plasma at 10 or 15 min/number of bacteria in plasma 2 min after injection) × 100) was determined.

V. parahaemolyticus clearance assay after siRNA interference. Small interfering RNAs (siRNAs), specifically those targeting *EsCnx* or *EsCrt*, were synthesized using an *In vitro* Transcription T7 Kit (Takara, Japan). *EsCnx*-siRNA and *EsCrt*-siRNA were used, and the siRNA sequence was scrambled to generate the control *EsCnx*-siRNA-scrambled or *EsCrt*-siRNA-scrambled (Table 1). Approximately 20 µg of siRNA (or siRNA-scrambled) was injected into the foot of the third appendage at a volume of 50 µL per crab. At 12 h after injection, the siRNA or siRNA-scrambled (20 µg) was injected into the same crab. At 24 h after the last injection, the total RNA of hemocytes from the two groups of crabs was isolated and used to detect the efficiency of RNAi through qRT-PCR. The remaining crabs were used for the clearance assay using the method described above with some modifications. In the modified clearance assay, 25 µL of *V. parahaemolyticus* (approximately 3×10^7 cells), which was not incubated with the recombinant protein, was injected into the crab. The assays described earlier were biologically repeated three times.

References

- Fink, A. L. Chaperone-mediated protein folding. *Physiol Rev* **79**, 425–449 (1999).
- Frydman, J. Folding of newly translated proteins *in vivo*: the role of molecular chaperones. *Annu Rev Biochem* **70**, 603–647 (2001).
- Young, J. C., Agashe, V. R., Siegers, K. & Hartl, F. U. Pathways of chaperonemediated protein folding in the cytosol. *Nat Rev Mol Cell Biol* **5**, 781–791 (2004).
- Williams, D. B. Beyond lectins: the calnexin/calreticulin chaperone system of the endoplasmic reticulum. *J Cell Sci* **119**, 615–623 (2006).
- Chen, X., Karnovsky, A., Sans, M. D., Andrews, P. C. & Williams, J. A. Molecular characterization of the endoplasmic reticulum: insights from proteomic studies. *Proteomics* **10**, 4040–4052 (2010).
- Coe, H. & Michalak, M. ERp57, a multifunctional endoplasmic reticulum resident oxidoreductase. *Int J Biochem Cell Biol* **42**, 796–799 (2010).
- Molinari, M. & Helenius, A. Chaperone selection during glycoprotein translocation into the endoplasmic reticulum. *Science* **288**, 331–333 (2000).
- Rodan, A. R., Simons, J. F., Trombetta, E. S. & Helenius, A. N-linked oligosaccharides are necessary and sufficient for association of glycosylated forms of bovine RNase with calnexin and calreticulin. *Embo J* **15**, 6921–6930 (1996).
- Zapun, A., Jakob, C. A., Thomas, D. Y. & Bergeron, J. J. Protein folding in a specialized compartment: the endoplasmic reticulum. *Structure* **7**, R173–182 (1999).
- Hammond, C., Braakman, I. & Helenius, A. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc Natl Acad Sci USA* **91**, 913–917 (1994).
- Kim, P. S. & Arvan, P. Calnexin and BiP act as sequential molecular chaperones during thyroglobulin folding in the endoplasmic reticulum. *J Cell Biol* **128**(1/2), 29–38 (1995).
- Degen, E. & Williams, D. B. Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. *J Cell Biol* **112**, 1099–1115 (1991).
- Wada, I. *et al.* SSR alpha and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. *J Biol Chem* **266**, 19599–19610 (1991).
- Hochstenbach, F., David, V., Watkins, S. & Brenner, M. B. Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc Natl Acad Sci USA* **89**, 4734–4738 (1992).
- Galvin, K. *et al.* The major histocompatibility complex class I antigen-binding protein p88 is the product of the calnexin gene. *Proc Natl Acad Sci USA* **89**, 8452–8456 (1992).
- Bergeron, J. J., Brenner, M. B., Thomas, D. Y. & Williams, D. B. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. *Trends Biochem Sci* **19**, 124–128 (1994).
- Huang, L., Franklin, A. E. & Hoffman, N. E. Primary structure and characterization of an Arabidopsis thaliana calnexin-like protein. *J Biol Chem* **268**, 6560–6566 (1993).
- Ellgaard, L. & Frickel, E. M. Calnexin, calreticulin, and ERp57: teammates in glycoprotein folding. *Cell Biochem Biophys* **39**, 223–247 (2003).
- Rutkevich, L. A. & Williams, D. B. Participation of lectin chaperones and thiol oxidoreductases in protein folding within the endoplasmic reticulum. *Curr Opin Cell Biol* **23**, 157–166 (2010).
- Roderick, H. L., Lechleiter, J. D. & Camacho, P. Cytosolic phosphorylation of calnexin controls intracellular Ca²⁺ oscillations via an interaction with SERCA2b. *J Cell Biol* **149**, 1235–1248 (2000).
- Müller-Taubenberger, A. *et al.* Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. *Embo J* **20**, 6772–6782 (2001).
- Delom, F., Fessart, D. & Chevet, E. Regulation of calnexin sub-cellular localization modulates endoplasmic reticulum stress-induced apoptosis in MCF-7 cells. *Apoptosis* **12**, 293–305 (2007).
- Gelebart, P., Opas, M. & Michalak, M. Calreticulin, a Ca²⁺-binding chaperone of the endoplasmic reticulum. *Int J Biochem Cell Biol* **37**, 260–266 (2005).
- Michalak, M., Groenendyk, J., Szabo, E., Gold, L. I. & Opas, M. Calreticulin, a multiprocess calcium-buffering chaperone of the endoplasmic reticulum. *Biochem J* **417**, 651–666 (2009).
- Núñez, M. T., Osorio, A., Tapia, V., Vergara, A. & Mura, C. V. Iron-induced oxidative stress up-regulates calreticulin levels in intestinal epithelial (Caco-2) cells. *J Cell Biochem* **82**(4), 660–665 (2001).
- Pinto, J. P. *et al.* Protective role of calreticulin in HFE hemochromatosis. *Free Radic Biol Med* **44**(1), 99–108 (2008).
- Jia, L. *et al.* Novel anti-oxidative role of calreticulin in protecting A549 human type II alveolar epithelial cells against hypoxic injury. *Am J Physiol Cell Physiol* **294**(1), C47–55 (2008).
- Opas, M., Dziak, E., Fliegel, L. & Michalak, M. Regulation of expression and intracellular distribution of calreticulin, a major calcium binding protein of nonmuscle cells. *J Cell Physiol* **149**, 160–171 (1991).
- Sontheimer, R. D., Lieu, T. S. & Capra, J. D. The diverse functional repertoire of a new human autoantigen. *Immunology* **1**, 155–160 (1993).
- Ostwald, T. J. & MacLennan, D. H. Isolation of a high affinity calcium binding protein from sarcoplasmic reticulum. *J Biol Chem* **249**, 974–979 (1974).
- Michalak, M., Corbett, E. F., Mesaali, N., Nakamura, K. & Opas, M. Calreticulin: one protein, one gene, many functions. *Biochem J* **344**, 281–292 (1999).
- Luana, W. *et al.* Molecular characteristics and expression analysis of calreticulin in Chinese shrimp *Fenneropenaeus chinensis*. *Comp Biochem Physiol B Biochem Mol Biol* **147**, 482–491 (2007).
- Liu, H. *et al.* Molecular responses of calreticulin genes to iron overload and bacterial challenge in channel catfish (*Ictalurus punctatus*). *Dev Comp Immunol* **35**, 267–272 (2011).
- Wang, L. *et al.* Molecular cloning and functional study of calreticulin from a lepidopteran pest, *Pieris rapae*. *Dev Comp Immunol* **38**, 55–65 (2012).

35. Visudtiphohle, V., Watthanasurorot, A., Klinbunga, S., Menasveta, M. & Kirtikara, K. Molecular characterization of calreticulin: a biomarker for temperature stress responses of the giant tiger shrimp *Penaeus monodon*. *Aquaculture* **308**, S100–108 (2010).
36. Johnson, S., Michalak, M., Opas, M. & Eggleton, P. The ins and outs of calreticulin: from the ER lumen to the extracellular space. *Trends Cell Biol* **11**, 122–129 (2001).
37. Watthanasurorot, A., Jiravanichpaisal, P., Soderhall, K. & Soderhall, I. A calreticulin/gC1qR complex prevents cells from dying: a conserved mechanism from arthropods to humans. *J Mol Cell Biol* **5**(2), 120–131 (2013).
38. Duan, Y. F. *et al.* Molecular responses of calreticulin gene to *Vibrio anguillarum* and WSSV challenge in the ridgetail white prawn *Exopalaemon carinicauda*. *Fish Shellfish Immunol* **36**, 164–171 (2014).
39. Zhang, Q. *et al.* Calnexin functions in antibacterial immunity of *Marsupenaeus japonicus*. *Dev Comp Immunol* **46**, 356–363 (2014).
40. Wang, X. W. & Wang, J. X. Diversity and multiple functions of lectins in shrimp immunity. *Dev Comp Immunol* **39**(1–2), 27–38 (2013).
41. Wen, R., Li, F. H., Sun, Z., Li, S. H. & Xiang, J. H. Shrimp MyD88 responsive to bacteria and white spot syndrome virus. *Fish Shellfish Immunol* **34**, 574–581 (2013).
42. Gross, P. S., Bartlett, T. C., Browdy, C. L., Chapman, R. W. & Warr, G. W. Immune gene discovery by expressed sequence tag analysis of haemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. *Dev Comp Immunol* **25**, 565–577 (2001).
43. Wang, P. & Granados, R. R. Calcofluor disrupts the midgut defense system in insects. *Insect Biochem Mol Biol* **30**(2), 135–143 (2000).
44. Vallet-Gely, I., Lemaitre, B. & Boccard, F. Bacterial strategies to overcome insect defences. *Nat Rev Microbiol* **6**(4), 302–313 (2008).
45. Huang, Y. *et al.* Function of two novel single-CRD containing C-type lectins in innate immunity from *Eriocheir sinensis*. *Fish Shellfish Immunol* **37**, 313–321 (2014).
46. Janeway, C. A. & Medzhitov, R. Innate immune recognition. *Annu Rev Immunol* **20**, 197–216 (2002).
47. Medzhitov, R. & Janeway, Jr., C. A. Decoding the patterns of self and nonself by the innate immune system. *Science* **296**, 298–300 (2002).
48. Xu, H. S., Shu, M. A., Zhan, X. A. & Wang, S. X. Identification of *Vibrio parahaemolyticus* isolated from cultured *Eriocheir sinensis* and pathogenicity of its extracellular products. *J Fish China* **26**, 357–362 (2002).
49. Gillespie, J. P., Kanost, M. R. & Trenczek, T. Biological mediators of insect immunity. *Annu Rev Entomol* **42**, 611–643 (1997).
50. Jiravanichpaisal, P., Lee, B. L. & Soderhall, K. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology* **211**, 213–236 (2006).
51. Flannagan, R. S., Jaumouille, V. & Grinstein, S. The cell biology of phagocytosis. *Annu Rev Pathol* **7**, 61–98. (2012).
52. Kumar, S., Nei, M., Dudley, J. & Tamura, K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9**, 299–306 (2008).
53. Huang, Y. *et al.* Cloning and characterization of a clip domain serine protease and its homolog (Masquerade) from *Eriocheir sinensis*. *Fish Shellfish Immunol* **35**, 1155–1162 (2013).
54. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(- $\Delta\Delta C(T)$) method. *Methods* **25**, 402–408 (2001).
55. Wang, X. W., Xu, H., Xu, J. D., Zhao, X. F. & Wang, J. X. Collaboration between a soluble C-Type lectin and Calreticulin facilitates White Spot Syndrome Virus infection in Shrimp. *J Immunol* **193**, 2106–2117 (2014).
56. Huang, Y. *et al.* A novel integrin function in innate immunity from Chinese mitten crab (*Eriocheir sinensis*). *Dev Comp Immunol* **52**, 155–165 (2015).

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Author Contributions

Y.H., Q.R., K.M.H., M.J., S.W.Y. and W.W. carried out the experiments. Y.H. and Q.R. designed the experiments and analysed the data. Y.H. and Q.R. wrote the manuscript. All authors have read and approved the manuscript.

Additional Information

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