Fish and Shellfish Immunology 84 (2019) 1041–1049

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

NEDD8-conjugated Cullin4 positive regulates antimicrobial peptides expression in *Eriocheir sinensis*

Lei Yang, Zechao Ruan, Xuejie Li, Lu Li, Qun Wang*, Weiwei Li**

Laboratory of Invertebrate Immunological Defence & Reproductive Biology, School of Life Sciences, East China Normal University, Shanghai, China

ARTICLE INFO

Keywords:
Eriocheir sinensis
NEDD8
Cullin4
Antimicrobial peptides
Neddylation
RNA interference

ABSTRACT

The ubiquitin-proteasome system is involved in numerous cellular processes, such as signal transduction, autophagy, cell cycle control, embryogenesis, and regulation of immune response. Neural precursor cell expressed developmentally downregulated 8 (NEDD8) is a ubiquitin-like protein that activates Cullin-RING ligases and modifies substrates via neddylation. However, there is limited information on how neddylation regulates innate immunity in crustaceans. In the present study, we identified the evolutionarily conserved NEDD8 with the ubiquitin homologue domain in the Chinese mitten crab (*Eriocheir sinensis*), named it *Es*NEDD8. Then, we analyzed the expression patterns and cellular location of its substrate, *Es*Cullin4. qRT-PCR showed that both *Es*NEDD8 and *Es*Cullin4 were widely expressed in all the selected tissues, and *Es*Cullin4 was significantly upregulated in hemocytes after bacterial stimulation. Moreover, silencing of *Es*Cullin4 significantly suppressed the expression of antimicrobial peptides (AMPs) in the hemocytes after bacterial stimulation, and inhibition of *Es*Cullin4 neddylation by treatment with the NEDD8-activating enzyme inhibitor MLN4924 significantly inhibited the expression of the AMPs. Thus, the results show that *Es*NEDD8-modified *Es*Cullin4 could control antimicrobial activities via regulation of AMPs expression in the Chinese mitten crab.

1. Introduction

Ubiquitination is a common type of post-translational modification, and it plays crucial roles in various life processes such as DNA repair, cell cycle regulation, inflammation immunity, and signal transduction [1,2]. Ubiquitination has a fundamental role in the immune system [3–5], and it is accompanied by a cascade of enzymes such as ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) [6]. Over the past few decades, ubiquitin-like proteins such as SUMO, neural precursor cell expressed developmentally downregulated 8 (NEDD8), ATG8, Ufm1, ISG15, FAT10, and URM have been identified [7].

The ubiquitin-like proteins play crucial roles in cell cycle control and embryogenesis. Covalent attachment of the proteins to substrates requires prior activation by the E1 complex and linkage to the E2 enzyme. The attachment of NEDD8 to Cullins, which are a family of structurally related proteins containing an evolutionarily conserved Cullin domain, via the neddylation pathway activates the associated E3 activity and, thus, promotes polyubiquitination and proteasomal degradation of cyclins and other regulatory proteins [8–10]. To date, the most well-studied substrates of NEDD8 are Cullins [11], each member of the Cullin family can be modified by NEDD8 [8]. MLN4924, the NEDD8-activating enzyme (NAE) inhibitor, inactivates Cullin-RING-based E3 ubiquitin ligases (CRLs) through inhibition of Cullin neddylation, leading to the accumulation of multiple CRLs substrates [12].

Several studies have examined the importance of ubiquitin-like post-translational modifiers. NEDD8 with a high homology to ubiquitin is covalently linked to all members of the Cullin family through an enzymatic cascade analogous to ubiquitination [13]. Unlike ubiquitin, which mostly acts as a degradation signal for the target proteins, NEDD8 acts as an activation signal for Cullin-based ubiquitin-protein ligases [14]. CRLs, which are responsible for almost 20% of ubiquitin-dependent proteins subjected to proteasome degradation, are the largest class of E3 ubiquitin ligases [15].

CRLs have three major components: a Cullin protein; Roc1 or Roc2, which recruits the E2 enzyme; and a substrate adaptor that transfers ubiquitin from E2 to the substrates. Cullins have been conserved during evolution from yeast to humans, and at least five Cullins are present in metazoans [16,17]. Cullin4, a member of the Cullin family, typically serves as a scaffold protein in CRLs. In mammalian immune cells, Cullin4a was found to affect the differentiation of granulocytes and cell cycle [18]. Cullin4b maintains the mRNA expression of TNF-a [19] and

* Corresponding author. School of Life Science, East China Normal University, 500 Dongchuan Road, Shanghai, China. Tel.: +86 021 24206934.
** Corresponding author. School of Life Science, East China Normal University, 500 Dongchuan Road, Shanghai, China. Tel.: +86 021 54345415.
E-mail addresses: qwang@bio.ecnu.edu.cn (Q. Wang), wwli@bio.ecnu.edu.cn (W. Li).

https://doi.org/10.1016/j.fsi.2018.10.077
Received 8 July 2018; Received in revised form 1 October 2018; Accepted 27 October 2018
Available online 28 October 2018
1050-4648/

plays a negative role in regulating lipopolysaccharide signaling in macrophages [20]. To date, most studies on Cullin4 have been conducted in mice and humans. However, to the best of our knowledge, the immune functions of Cullin4 in invertebrates, especially the Chinese mitten crab (*Eriocheir sinensis*), are still unknown.

The Chinese mitten crab, a major economic aquaculture species in southeast Asia, is threatened by bacterial diseases [21]. Previous studies have shown that antimicrobial peptides (AMPs) play a fundamental role in the immune reactions of crustaceans [22], However, the mechanism underlying the regulation of AMPs by CRL4 E3 ligase is still unclear. In this study, the cDNA of NEDD8 was obtained from the Chinese mitten crab, *E sinensis*, named it *Es*NEDD8, which was widely expressed in various tissues. Then, we analyzed the expression patterns and cellular location of its substrate, *Es*Cullin4. Subsequently, we investigated the mRNA expression levels of *Es*Cullin4 in hemocytes after stimulation with bacterial pathogens, and the relationship between *Es*Cullin4 and AMPs was explored. In addition, we observed that a neddylation inhibitor, MLN4924, significantly suppressed the expression of AMPs in hemocytes, indicating the vital function of the neddylation pathway in innate immune defense.

2. Materials and methods

2.1. Experimental animals and sample preparation

Healthy adult Chinese mitten crabs (n = 135; wet weight = 100 ± 15 g) were purchased from the Xin'An Agricultural Market in Shanghai, China. The crabs were acclimated in filtered, aerated freshwater for 1 week at 20–25 °C. For RNA extraction, hemocytes and tissues of stomach, hepatopancreas, brain, gill, muscle, intestine, and heart were collected and frozen rapidly in liquid nitrogen and stored at −80 °C.

2.2. Immune challenge and sample collection

For the bacterial challenge, the crabs were randomly divided into three groups (Gram-negative bacterium group, Gram-positive bacterium group, and phosphate-buffered saline [PBS] group). The Gram-positive bacterium (*Staphyloccocus aureus* BYK0113) and Gram-negative bacterium (*Vibrio parahaemolyticus* BYK00036) were obtained from the National Pathogen Collection Center for Aquatic Animals (Shanghai Ocean University, Shanghai, China). Bacterial stains were cultured in Luria-Bertani medium. After centrifugation at 4500 × *g* for 5 min, the supernatant was discarded and the precipitate was washed three times with PBS and adjusted to 1×10^6 CFU/ml. Then, the bacterial suspension (100 μl) was injected into the last pair of walking legs before sterilized with 75% alcohol in the Gram-positive bacterium (*S. aureus*) group and Gram-negative bacterium (*V. parahaemolyticus*) group. The PBS group (100 μl) was the control. More than five mitten crabs from each group were collected randomly at specific time intervals (0, 2, 6, 12, 24, 36, and 48 h) after the challenge. Healthy crabs were lightly anesthetized in an ice bath for about 5–10 min before the experiment. Hemocytes were collected from the hemocoel in the last pair of walking legs from each crab by using a 10 ml sterile syringe preloaded with 5 ml of anticoagulant solution (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl, and 10 mM EDTA) [23]. Then, the supernatant was discarded after centrifugation (800 × *g* at 4 °C for 10 min), and the hemocytes were stored at −80 °C.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from the tissues and hemocytes by using the TRIzol® reagent (RNA Extraction Kit, Invitrogen, and Carlsbad, CA, USA), according to protocol. The RNA concentration was evaluated using NanoDrop 2000 (Thermo Fisher Scientific, USA), and the RNA quality was analyzed using 1% agarose gel electrophoresis. The cDNA for real-time PCR was obtained using the PrimeScript™ cDNA synthesis Kit (TaKaRa, Japan). The *Es*NEDD8 partial cDNA sequence was obtained from the *E. sinensis* hemocyte cDNA library (unpublished), and gene-specific primers (Table 1) were designed to clone the EST sequence. The following PCR procedures were: 94 °C for 5 min; 33 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; and 72 °C for 10 min. Finally, the PCR products were purified with the PCR & DNA Fragment Purification Kit (GeneStart, Beijing), then inserted into the pEASY-transT1 vector (TransGen, Beijing) for sequencing.

Table 1
List of primers used for the analyses.

Primer name	Sequence (5′-3′)
cDNA cloning	
*Es*NEDD8 F	ATGCTCATCAAAGTGAAG
*Es*NEDD8 R	CTAAGAGGAGGCCATTCC
Real-time quantitative PCR	
β-actin qF	GCATCCACGAGACCACTTACA
β-actin qR	CTCCTGCTTGCTGATCCACATC
*Es*NEDD8qF	AGCGGGTGGAGGAGAAGGA
*Es*NEDD8 qR	CCAGCACCAGATGAAGCAC
*Es*Cullin4 F	AGGGACTGGATTCTCTCT
*Es*Cullin4 R	CCTTATCTTTCTCTGGGTT
*Es*DWD1 qF	ACGGGTCGTCAACGAAACTG
*Es*DWD1 qR	GGTCACTGGGTTACCATAGCG
Lys qF	CTGGGATGATGTGGAGAAGTGC
Lys qR	TTATTCGGTGTGTTATGAGGGGT
*Es*ALF1 qF	GACGCAGGAGGATGCTAAC
*Es*ALF1 qR	TGATGGCAGATGAAGGACAC
*Es*ALF2 qF	GACCCTTTGCTGAATGCTTGA
*Es*ALF2 qR	CTGCTCTACAATGTCGCCTGA
*Es*ALF3 qF	ACGAGGAGCAAGGAAAGAAAG
*Es*ALF3 qR	TTGTGCCATAGACCAGAGACTT
*Es*Crus1 qF	GCTCTATGGCGGAGGATGTCA
*Es*Crus1 qR	CGGGCTTCAGACCCACTTTAC
*Es*Crus2 qF	GCCCACCTCCCAAACCTAT
*Es*Crus2 qR	GCAAGCGTCACAGCAGCACT
RNAinterference	
si*Es*Cullin4 F	GCCAUGACCUUAGUUUGUUTT
si*Es*Cullin4 R	AACAAACUAAGGUCAUGGCTT

2.4. Tissue expression analysis

The distribution of *Es*Cullin4 in different tissues was evaluated using semi-quantitative RT-PCR. Two gene-specific primers of *Es*Cullin4 were designed using the Primer 5.0 software (Table 1), and they were used to amplify a 165-bp fragment. The relative abundance of *Es*NEDD8 in the selected tissues was also determined using RT-PCR with target-specific primers (Table 1). β-actin (Table 1) was used as the internal control. The experiment was performed using a final volume of 25 μl containing 12.5 μl of 2 × Easy Taq PCR SuperMix (Transgen, Beijing), 2 μl of 10 mM primer pairs, 9.5 μl of sterile deionized water, and 1 μl of first-strand cDNA from the selected tissues. The PCR amplification protocol was as follows: 95 °C for 5 min; 33 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and a final 72 °C for 10 min. Each experiment was performed in triplicate.

2.5. Real-time quantitative PCR

The mRNA expression was analyzed using real-time quantitative PCR with the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). SYBR Premix Ex Taq™ (TaKaRa, Japan) was used to perform real-time PCR according to the following protocol: 95 °C for 1 min, followed by 39 cycles at 95 °C for 5 s and 58 °C for 30 s. The relative data were analyzed using CFX Manager™ and quantified with the comparative CT ($2^{-\Delta\Delta Ct}$) method [24]. All data for the analysis were obtained from three independent experiments.

2.6. Immunofluorescence analysis

The hemocytes were isolated from the healthy male crabs, as mentioned in section 2.2, and counted using an automated cell counter (Invitrogen Countess). The hemocytes were seeded onto a 24 × 24 mM cell climbing slice (WHB, Shanghai, China) and cultured for 24 h in 60-mM dishes in vitro, according to established primary culture techniques [25]. Then, the hemocytes were collected and washed twice with 1 × PBS (Solarbio, Beijing), fixed in 4% paraformaldehyde for 10 min, and immediately incubated with 0.2% Triton X-100 in PBS for 15 min. Subsequently, the cell slides were blocked with 3% BSA for 2 h and incubated with the rabbit anti-*Es*Cullin4 polyclonal antibody or anti-Dorsal antibody (antibodies produced by the laboratory using recombinant protein) at a dilution of 1:100 in 3% BSA overnight at 4 °C. The next day, the cell slides were washed with PBST to remove the antibodies. Then, the cell slides were stained with FITC-conjugated secondary antibodies (at a dilution of 1:500 in PBST; the antibody was purchased from Tiangen, Beijing) for 2 h at 37 °C in the dark. Finally, the cell slides were stained with the nuclear counterstain DAPI (Beyotime, China) and observed under a fluorescence microscope (Leica, Germany).

2.7. Hemocyte culture and RNA interference in vitro

Small interfering RNA (siRNA) duplexes for the *Es*Cullin4 RNA interference experiment were synthesized by GenePharma (Shanghai, China), and the RNA oligonucleotides (si*Es*Cullin4 and siGFP) used are listed in Table 1. SiGFP was used as the negative control. Hemocytes were isolated from the healthy male crabs, as described in section 2.2, before the RNAi experiment and counted using an automated cell counter (Invitrogen Countess, USA). The hemocytes were cultured for 24 h in 60 mM dishes, according to established primary culture techniques [25]. Then, the siRNA, which was dissolved in RNase-free water, was transfected into cultured primary hemocytes by using the siRNA mate reagent at a final concentration of 5 nM. Total RNA was extracted to evaluate the RNAi efficiency of the target genes by using qRT-PCR.

2.8. Expression of AMP genes

After silencing *Es*Cullin4, as described in section 2.7, the cultured hemocytes were challenged with different strains for 24 h, then, total RNA was extracted, as described in section 2.3. Real-time quantitative PCR was used to evaluate the expression level of AMP genes. The following AMP genes were selected on the basis of previous studies: *Es*Crus1 [26] and *Es*Crus2 [27], lysoz-yme gene (LYS) [28], *Es*DWD1 [29], anti-lipopolysaccharide factor (*Es*ALF1) [30], second anti-lipopolysaccharide factor (*Es*ALF2) [31], and third anti-lipopolysaccharide factor (*Es*ALF3) [21]. All primers used to detect the expression levels of the AMPs were listed in Table 1, and all experiments were performed in triplicate.

2.9. Inhibition assay

MLN4924, an NEDD8-activating enzyme inhibitor that can inactivate CRLs by inhibiting NEDD8-modified Cullins, was obtained from a previous study [32]. Before the experiment, hemocytes were isolated from the healthy male crabs, as described in section 2.2, and cultured in vitro for 24 h in dishes, as described in section 2.7. The cultured cells were pretreated with 1 μM MLN4924 for 6 h, prior to the bacterial challenge; the same volume of DMSO was added to the control group. Next, the cultured hemocytes were stimulated with different heat-killed bacteria for 24 h. Subsequently, total RNA was extracted to detect AMP expression levels by using qRT-PCR.

2.10. Bioinformatic analysis

The online search tool BLASTX (http://www.ncbi.nlm.nih.gov/) was used to analyze the nucleotide sequences, and ORFfinder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi) was used to identify the open reading frame (ORF). SMART (http://smart.embl-heidelberg.de/) was used to predict the protein functional domains. Multiple sequence alignment was performed using ClustalX2.0 program and DNAMAN software. A phylogenetic tree based on *Es*NEDD8 was constructed using the neighbor-joining method with MEGA6 software.

2.11. Statistical analysis

SPSS software and GraphPad Prism 5 were used for the statistical analyses. The error bars represented standard deviation (SD). One-way analysis of variance (ANOVA) and post-hoc Duncan's multiple range tests were performed, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Gene cloning and sequence analysis of EsNEDD8

The cloned partial *Es*NEDD8 cDNA has a 243 bp ORF (GenBank accession: MH171299) encoding a protein with 80 amino acids (Fig. 1A). *Es*NEDD8 was significantly conserved during evolution (Fig. 1B), and it has been determined to contain only a ubiquitin homologue (UBQ) domain by using the SMART program (Fig. 1A). We found some NEDD8 sequences from GenBank for the phylogenetic analysis, and BLASTX analysis showed that *Es*NEDD8 has 95% identity with the NEDD8 of *Scylla paramamosain* (Fig. 2). The relationships shown in the phylogenetic tree are in accordance with their taxonomic classifications, which have closer relations with invertebrate compared with vertebrate.

3.2. Tissue expression pattern analysis and subcellular location

qRT-PCR were used to analyze the transcript levels of *Es*Cullin4 and *Es*NEDD8 in the different tissues (Fig. 3). The data showed that *Es*Cullin4 expression was the highest in the brain and lower in the heart and hemocytes (Fig. 3A). Similarly, *Es*NEDD8 expression was widely detected in all the selected tissues. The expression level of *Es*NEDD8 was the lowest in the stomach and the highest in the gill (Fig. 3B). *Es*Cullin4 was found localized in the cytoplasm and mainly around the nucleus in the hemocytes (Fig. 4).

3.3. Expression profiles of EsCullin4 in hemocytes after bacterial challenge

The temporal expression pattern of *Es*Cullin4 after different types of bacterial challenge was determined using real-time qPCR. The expression levels of *Es*Cullin4 were upregulated at 12 h and 24 h ($P < 0.05$) after response to *S. aureus* infection (Fig. 5A). In response to *V. parahaemolyticus* infection, the expression level of *Es*Cullin4 was upregulated from 24 to 48 h, especially at 48 h (Fig. 5B). The control group (injected with PBS) showed non-significant variations in *Es*Cullin4 expression level.

3.4. Detection efficiency of EsCullin4 RNA

To evaluate the expression of related immune genes after silencing of *Es*Cullin4 by RNA interference, the interference efficiency of the primary cultured hemocytes was detected. The expression of *Es*Cullin4 was significantly downregulated ($P < 0.05$) by siRNA with or without bacterial challenge (Fig. 6). Transfection of si*Es*Cullin4 for 24 h led to a more than 65% decrease in the mRNA level of *Es*Cullin4 (Fig. 6A). After bacterial challenge, the silencing efficiency reached 50% (Fig. 6B and C).

Fig. 1. (A) Nucleotide sequence of *Es*NEDD8 and the amino acid sequence are shown to the left and right, respectively. The open reading frame that encodes a protein of 80 amino acids has 243 bp. The ubiquitin homologue domain is highlighted in red. **(B)** Multiple alignment of *Es*NEDD8 and 14 other species from GenBank. The sequences and their accession numbers are as follows: *Sp*NEDD8, *Scylla paramamosain*, AFP23394.1; *Hs*NEDD8, *Homo sapiens*, NM_006156.2; *Mm*NEDD8, *Mus musculus*, NM_008683.3; *Bt*NEDD8, *Bos taurus*, NM_008683.3; *Dr*NEDD8, *Danio rerio*, EF178472.1; *Am*NEDD8, *Alligator mississippiensis*, XM_006259722.3; *Sc*NEDD8, *Serinus canaria,* XM_009100480.2; *Ma*NEDD8, *Macaca mulatta*, NM_001266888.2; *Gj*NEDD8, *Gekko japonicus*, XM_015406368.1; *Ph*NEDD8, *Pseudopodoces humilis*, XM_014262707.1; *Dm*NEDD8, *Drosophila melanogaster*, NM_001299141.1; *Xt*NEDD8, *Xenopus tropicalis*, NM_001016973.2:30–263; *Ss*NEDD8, *Sus scrofa*, XM_003128544.3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Effects of EsCullin4 silencing on the expression of AMPs

To check whether *Es*Cullin4 regulates the expression of AMPs, the mRNA levels of immune-related genes, namely, *Es*ALF1, *Es*DWD1, *Es*ALF2, *Es*ALF3, *Es*Crus2, *Es*Crus1, and Lys, in *Es*Cullin4-silenced hemocytes were analyzed after *S. aureus* challenge (Fig. 7A) or *V. parahaemolyticus* challenge (Fig. 7B). The results showed that the expression levels of *Es*ALF3, Lys, *Es*Crus1, *Es*Crus2, and *Es*DWD1 were significantly downregulated after *S. aureus* stimulation (Fig. 7A). The expression levels of *Es*DWD1, *Es*Crus2, *Es*Crus1, Lys, and *Es*ALF2 were significantly downregulated after *V. parahaemolyticus* stimulation (Fig. 7B).

3.6. MLN4924 inhibits the expression of AMPs in vitro

MLN4924, a potent inhibitor of the activation of CRL E3 ligase, was used to investigate the regulation of AMPs by *Es*Cullin4 [15,33]. The mRNA expression levels of Lys, *Es*DWD1, *Es*Crus1, and *Es*Crus2 were detected in the MLN4924-treated hemocytes after *S. aureus* stimulation or *V. parahaemolyticus* stimulation (Fig. 8). Treatment with MLN4924 dramatically suppressed the expression of Lys (Fig. 8C), *Es*Crus1 (Fig. 8A), and *Es*DWD1 (Fig. 8D) after *S. aureus* stimulation, but it had no effect on the expression of *Es*Crus2 (Fig. 8B). The expression of *Es*DWD-1 was also suppressed after *V. parahaemolyticus* stimulation (Fig. 8H) (See Fig. 9).

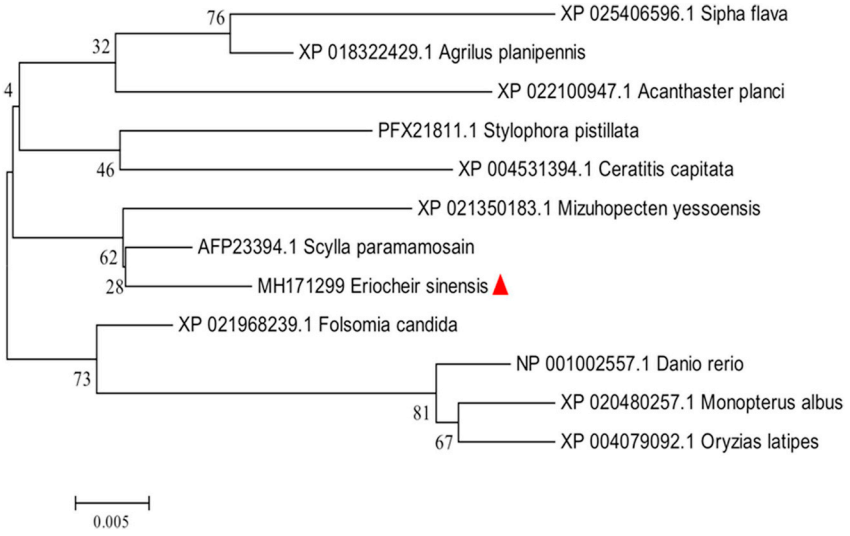

Fig. 2. A phylogenetic tree of *Es*NEDD8 and proteins from 11 other species from GenBank, constructed using MEGA6. The red triangle (▲) indicates *Es*NEDD8 from *Eriocheir sinensis.* (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

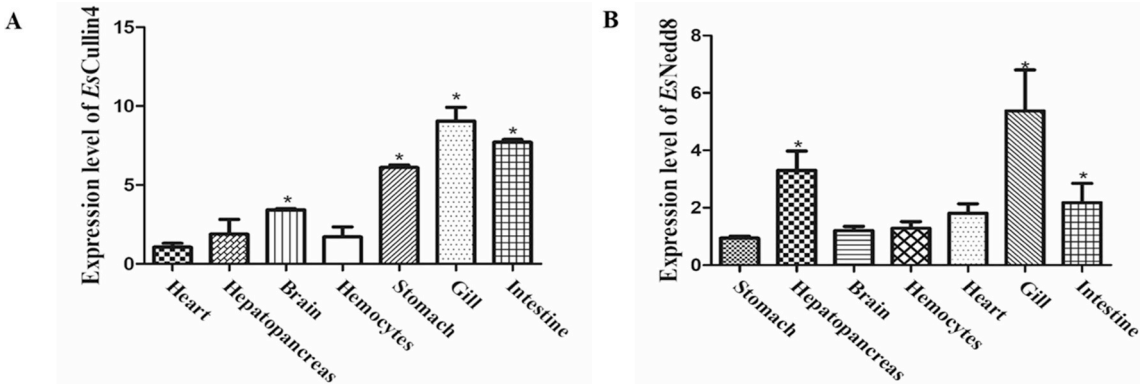

Fig. 3. qRT-PCR analysis of *Es*Cullin4 **(A)** and *Es*NEDD8 **(B)** expression levels in seven different tissues of unchallenged crabs. The X-axis and Y-axis represent different tissues and relative expression levels, respectively. The error bars represent standard deviation (SD). Three independent repeats were performed with at least three crab for each sample. The data were analyzed using one-way analysis of variance and post-hoc Duncan's multiple range tests, and statistical significance was set at $^{*}P < 0.05$.

3.7. Neddylation inhibition with MLN4924 dampens EsDorsal nuclear translocation

To investigate the regulation of Toll pathway in *Eriocheir sinensis* during bacterial challenge, we first to explored the translocation of *Es*Dorsal. as is shown, the results showed that the distribution of *Es*Dorsal in untreated group was mainly observed in the cytoplasm, at 1 h after *S. aureus* challenge, almost dorsal protein was translocated form the cytoplasm into the nucleus. In 1 μM of MLN4924 treated hemocytes *Es*Dorsal did not translocate into the nucleus upon *S. aureus* challenge, still mainly in the cytoplasm.

4. Discussion

In this study, we identified a ubiquitin-like protein in the Chinese mitten crab and named it *Es*NEDD8; we obtained a 243bp ORF that encodes a protein of 80 amino acids, and the predicted weight of the protein was 10 kDa. These results are highly similar to those obtained using *S. paramamosain.* Only one functional domain was predicted for *Es*NEDD8, the UBQ domain, using SMART analysis, which is highly consistent with ubiquitin [34]. Previous studies have suggested that the conjugation of NEDD8 with Cullins is a necessary perquisite for the activation of CRLs. Modification of Cullins by NEDD8 is termed neddylation [8,35,36]. Unlike in ubiquitination, UBC12 is the only known E2 involved in neddylation, and transfer of NEDD8 from E2 to the substrates is an essential process to activate the substrates [14].

The multiple sequence alignment showed that the molecular structure of NEDD8 was highly conserved, which indicates similar functions in different species. The phylogenetic tree analysis instructed that *Es*NEDD8 protein was closer to the NEDD8 protein in *S. paramamosain* and other invertebrate species, compared with fish. Thus, the relationships in the phylogenetic tree corresponded to normal taxonomic classifications. Currently, the most well studied substrates in the neddylation pathway are Cullins. Cullins function as scaffold proteins and have been evolutionarily conserved [37]. Knockdown of Cullin4B reduced TNF-a production in mouse macrophage cells [37], and knockdown of Cullin5 resulted in lower secretion levels of IL-1b, IL-6, and TNF-a [38]. Interestingly, in *Culex* mosquitoes, *cx*Cul4 function as proviral by degradation of STAT via the ubiquitination pathway in the Jak-STAT pathway [39]. However, there is limited information on the role of Cullins in the innate immunity of invertebrates, especially crustaceans.

The classification and structure of *Es*Cullin4 in the Chinese mitten crab have been reported [40]; however, little is known about *Es*Cullin4

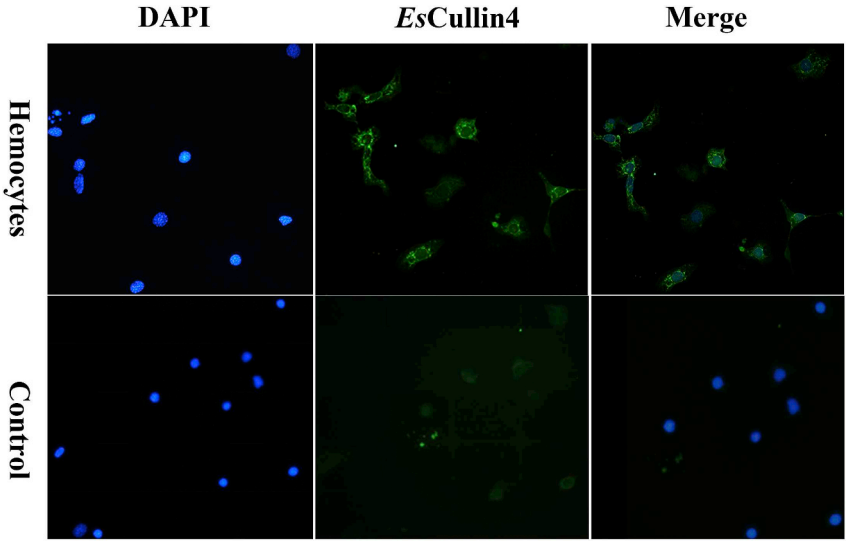

Fig. 4. Immunofluorescence of *Es*Cullin4 in hemocytes. The blue fluorescence signal represents the nuclear DNA labelled with DAPI, and the green signal indicates the target protein. Scale bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

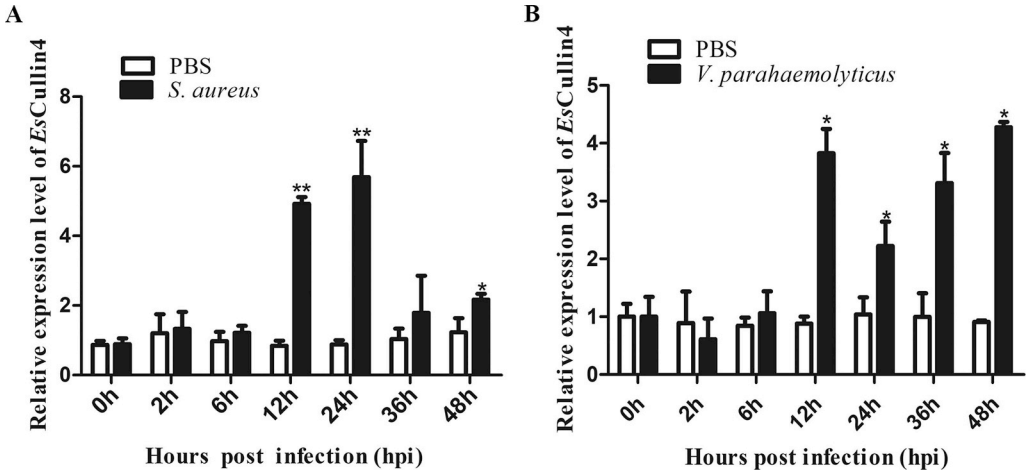

Fig. 5. Relative expression levels of *Es*Cullin4 were determined using qRT-PCR at 0, 2, 6, 12, 24, 36, and 48 h post-challenge. The left column represents the control group, and the right column, the experimental groups: *S. aureus* stimulation group **(A)** and *V. parahaemolyticus* stimulation group **(B)**. Three independent repeats were performed with at least three crab for each sample. The data are mean ± SD values of three independent experiments and analyzed using post-hoc Duncan's multiple range tests. $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with the control.

in hemocytes. The results show that both *Es*Cullin4 and *Es*NEDD8 ubiquitously expressed in all the tested tissues and indicate that *Es*Cullin4 and *Es*NEDD8 participate in the regulation of a multitude of cellular functions. In this study, *Es*Cullin4 was found to be abundantly expressed whether it is simulated by *S. aureus* or *V. parahaemolyticus*, suggesting that both Gram-positive and Gram-negative bacteria could induce the gene transcription of *Es*Cullin4. Previous studies have demonstrated that AMPs function as effector molecules that quickly identify and eradicate invading pathogens in the innate immune system [41,42]. The role of AMPs is particularly important in invertebrates. Thus, to prove whether *Es*Cullin4 can regulate the expression of AMPs, we detected AMPs expression after silencing of *Es*Cullin4 and subsequent stimulation by bacteria. The results showed that the mRNA expression levels of some AMPs significantly decreased, which indicates that *Es*Cullin4 can regulate AMP expression via a still unknown mechanism. *Es*Cullin4 was found localized in the cytoplasm and mainly around the nucleus in the hemocytes, which indicates that *Es*Cullin4 may play an important role in the transport of surface signals from the cytoplasm to the nucleus. On the basis of these results, we speculate the regulation of AMPs by *Es*Cullin4 via an intracellular signaling pathway. However, there is limited information on intracellular signaling cascades triggered by Cullin4 or the expression of AMPs regulated by Cullin4 in invertebrates.

Five Cullins have been identified in the Chinese mitten crab, and neddylation is necessary to activate Cullins [32]. The neddylation pathway was first found to be involved in the degradation of an interferon regulatory factor (IRF3) induced by the Sendai virus [43]. Previous studies have shown that some viruses could hijack Cullin-based ubiquitin ligases to inhibit interferon signaling [44–47]. However, a recent study has shown that the neddylation pathway regulates the production of type I interferons in vertebrates [48]. Therefore, we tried to determine the functions of the neddylation pathway in the regulation of AMPs in invertebrates. Our results showed that MLN4924 blocked the expression of most AMPs in the cultured hemocytes. Thus, *Es*NEDD8-modified *Es*Cullin4 may be necessary for its regulatory effect on AMP expression.

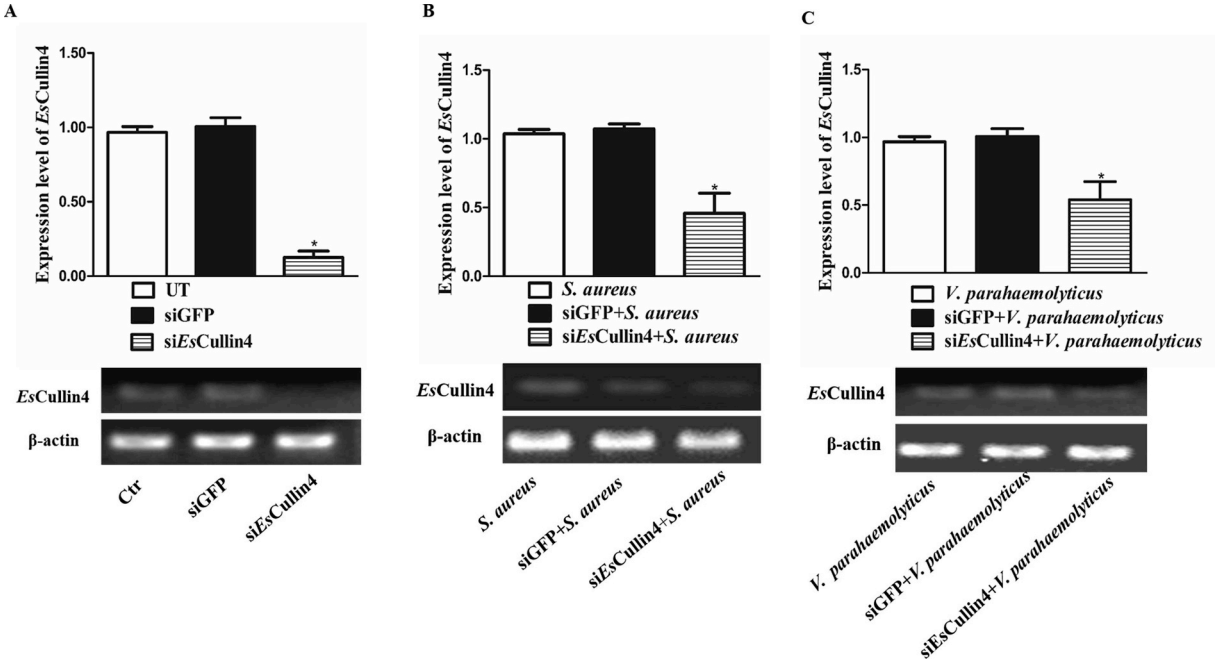

Fig. 6. RNAi efficiency of *Es*Cullin4. **(A)** The mRNA expression level of *Es*Cullin4 detected in hemocytes after transfection with *Es*Cullin4 siRNA or siGFP. **(B)** The mRNA expression level of *Es*Cullin4 detected using real-time PCR and RT-PCR 24 h after transfection and *S. aureus* stimulation **(B)** or *V. parahaemolyticus* stimulation **(C)**. The data were analyzed using post-hoc Duncan's multiple range tests. $^{*}P < 0.05$.

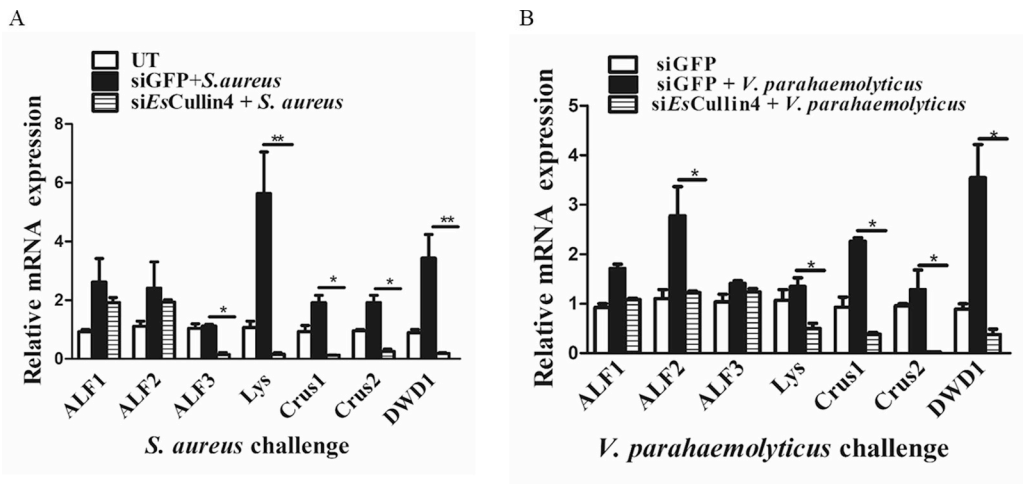

Fig. 7. Expression levels of seven selected AMPs were detected using real-time PCR after effective knockdown of *Es*Cullin4 and 24 h of stimulation with *S. aureus* **(A)** or *V. parahaemolyticus* **(B)**. the data are mean ± SD values of three independent experiments and were analyzed using post-hoc Duncan's multiple range tests. $^{*}P < 0.05$.

The Toll pathway has significant roles in the innate immunity of invertebrates and vertebrates. Toll-like receptors (TLRs) directly identify PAMPs from pathogens and activate NF-κB, resulting in acute responses to resistant pathogens [49]. A previous study has demonstrated that, in shrimp, PAMPs can directly bind to TLRs, resulting in the degradation of Cactus and translocation of Dorsal into the nucleus, thereby initiating the expression of AMPs [50]. Thus, to evaluate the relationship between neddylation and Toll pathway, we detected the nuclear translocation of Dorsal after treatment with MLN4924. The results showed that post *S. aureus* stimulation, the amount of Dorsal in the cytoplasm of MLN4924 treated hemocytes were greater than in DMSO treated hemocytes. This data indicates that the neddylation pathway could positive regulate the translocation of Dorsal. In previous study, Cullins inactivated by MLN4924 could block the degradation of IκBα [51]. Cactus, the IκBα homologue, is required for degradation after activation of the Toll signal pathway [52], although the molecular mechanism underlying the degradation of Cactus by certain CRLs in crabs is still unclear. ERK is the upstream signal of Dorsal, and

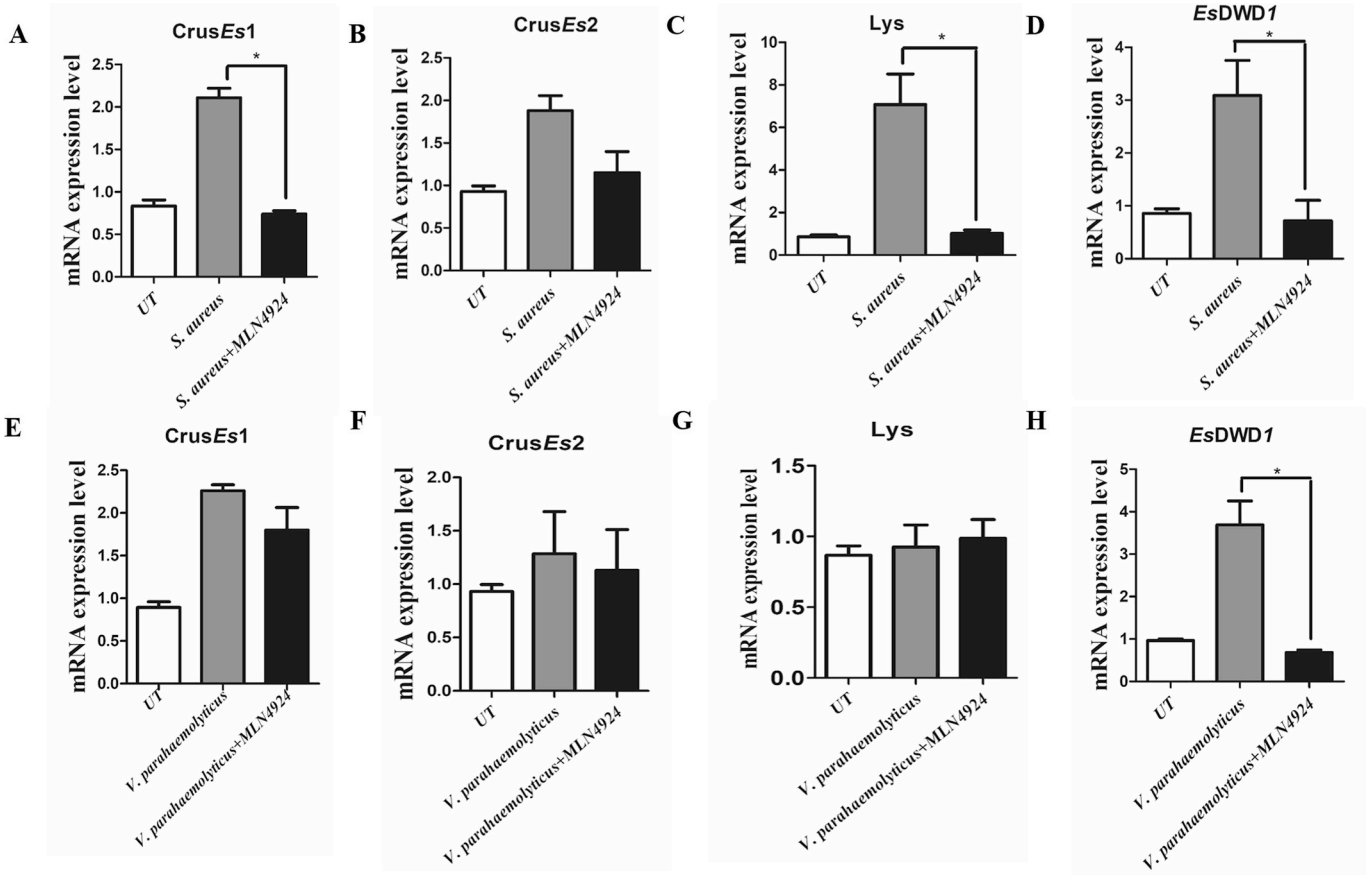

Fig. 8. (A) MLN4924 suppressed the expression of AMPs in vitro. Hemocytes cultured in vitro were treated with MLN4924 at a dose of 1 μM for 6 h before *S. aureus* stimulation (1×10^7 CFU/ml). **(B)** The expression levels of *Es*Crus1, *Es*Crus2, Lys, and *Es*DWD-1 in the hemocytes of the crabs treated with the neddylation inhibitor MLN4924 at a dose of 1 μM for 6 h before *V. parahaemolyticus* stimulation (1×10^7 CFU/ml). The untreated cells served as controls. The mRNA levels of *Es*Crus1, *Es*Crus2, Lys, and *EsDWD*-1 were measured using real-time PCR. All the data were obtained from three independent experiments. One-way analysis of variance and post-hoc Duncan's multiple range tests were performed, and $P < 0.05$ was considered statistically significant ($^{**}P < 0.01$).

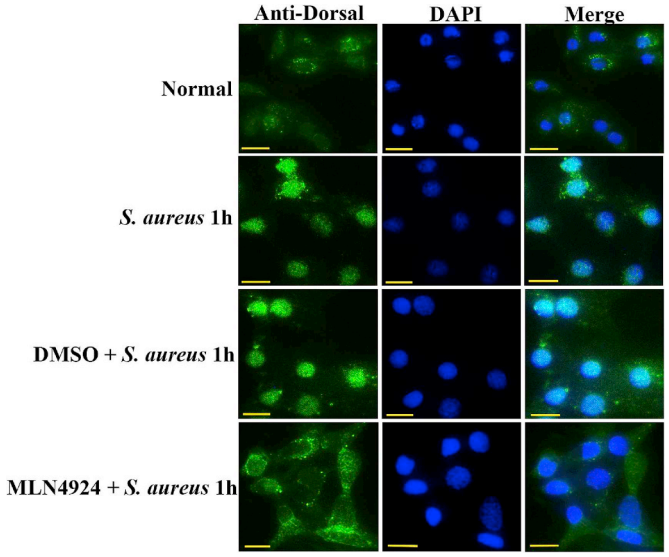

Fig. 9. MLN4924 dampens *Es*Dorsal nuclear translocation. The distribution of *Es*Dorsal was detected using an immunocytochemical assay with *Es*Dorsal antibody. 4′6-diamidino-2-phenylindole (DAPI) was used to reveal the nucleus, and the green signal indicates the distribution of *Es*Dorsal. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phosphorylated ERK regulates the translocation of Dorsal into the nucleus to promote the production of AMPs in shrimp [53]. In mammals, the neddylation pathway is essential for ERK activation in T cells [54], and the expression of cytokines and chemokines is significantly blocked when neddylation of Cullins is inhibited; the potential molecular mechanism is through inhibition of NF-κB and MAPK signal pathways [55]. However, few studies have focused on the relationship between the neddylation and MAPK pathways in invertebrates, and there is limited information on the relationship between the MAPK and Toll pathways. Thus, the role of the Cullin family in innate immunity of *E. sinensis*, especially in regulating key molecules in the intracellular signal pathway, needs to be investigated.

In summary, the cDNA of *Es*NEDD8 was obtained from the Chinese mitten crab. Both *Es*Cullin4 and *Es*NEDD8 were widely observed in all detected tissues. In hemocytes, *Es*Cullin4 was found localized in the cytoplasm and mainly around the nucleus. Furthermore, the expression of *Es*Cullin4 was upregulated significantly in the hemocytes after bacterial stimulation. Moreover, our study showed that *Es*Cullin4 regulates AMPs expressions and neddylation of *Es*Cullin4 by *Es*NEDD8 is necessary. In addition, the translocation of Dorsal was suppressed after treated with MLN4924 in hemocytes. However, the immune functions of the Cullin family, especially the relationship between neddylation and intracellular signaling pathways in invertebrates, need to be studied further.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grant No. 31602189 and 31672639). We thank the Instruments Sharing Platform of School of Life Sciences, East China Normal University for the equipment support.

References

[1] A. Hershko, A. Ciechanover, A. Varshavsky, The ubiquitin system, Annu. Rev. Biochem. 67 (1) (2000) 425–479.
[2] M. Gao, M. Karin, Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli, Mol. Cell 19 (5) (2005) 581–593.
[3] M. Karin, Y. Benneriah, Phosphorylation meets ubiquitination: the control of NF-κB activity, Annu. Rev. Immunol. 18 (1) (2003) 621–663.
[4] Y. Ben-Neriah, Regulatory functions of ubiquitination in the immune system, Nat. Immunol. 3 (1) (2002) 20–26.
[5] A. Ciechanover, A. Orian, AL.. Schwartz, Ubiquitin-mediated proteolysis: biological regulation via destruction, Bioessays 22 (5) (2015) 442–451.
[6] S. Dupré, D. Urbangrimal, R. Haguenauertsapis, Ubiquitin and endocytic internalization in yeast and animal cells, Biochim. Biophys. Acta Mol. Cell Res. 1695 (3) (2004) 89–111.
[7] U.F. Cajee, R. Hull, M. Ntwasa, Modification by ubiquitin-like proteins: significance in apoptosis and autophagy pathways, Int. J. Mol. Sci. 13 (9) (2012) 11804–11831.
[8] D.M. Duda, L.A. Borg, D.C. Scott, H.W. Hunt, M. Hammel, B.A. Schulman, Structural insights into NEDD8 activation of cullin-RING ligases: conformational control of conjugation, Cell 134 (6) (2008) 995–1006.
[9] J. Jones, K. Wu, Y. Yang, C. Guerrero, N. Nillegoda, Z.Q. Pan, L. Huang, A targeted proteomic analysis of the ubiquitin-like modifier Nedd8 and associated proteins, J. Proteome Res. 7 (3) (2008) 1274–1287.
[10] D.P. Xirodimas, A. Sundqvist, A. Nakamura, L. Shen, C. Botting, R.T. Hay, Ribosomal proteins are targets for the NEDD8 pathway, EMBO Rep. 9 (3) (2008) 280.
[11] K.E. Coleman, M. Békés, J.R. Chapman, S.B. Crist, M.J. Jones, B.M. Ueberheide, T.T. Huang, SENP8 limits aberrant neddylation of NEDD8 pathway components to promote cullin-RING ubiquitin ligase function, Elife 6 (2017).
[12] T.A. Soucy, P.G. Smith, M.A. Milhollen, A.J. Berger, J.M. Gavin, S. Adhikari, J.E. Brownell, K.E. Burke, D.P. Cardin, S. Critchley, An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer, Nature 458 (7239) (2009) 732–736.
[13] J. Merlet, J. Burger, J.E. Gomes, L. Pintard, Regulation of cullin-RING E3 ubiquitin-ligases by neddylation and dimerization, Cellular & Molecular Life Sciences Cmls 66 (11–12) (2009) 1924.
[14] D.T. Huang, M. Zhuang, O. Ayrault, B.A. Schulman, Identification of conjugation specificity determinants unmasks vestigial preference for ubiquitin within the NEDD8 E2, Nat. Struct. Mol. Biol. 15 (3) (2008) 280.
[15] T.A. Soucy, P.G. Smith, M.A. Milhollen, A.J. Berger, J.M. Gavin, S. Adhikari, J.E. Brownell, K.E. Burke, D.P. Cardin, S. Critchley, An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer, Nature 458 (7239) (2009) 732–736.
[16] S. Nayak, F.E. Santiago, H. Jin, D. Lin, T. Schedl, E.T. Kipreos, The Caenorhabditis elegans skp1-related gene family, Curr. Biol. 12 (4) (2002) 277–287.
[17] E.J. Bennett, J. Rush, S.P. Gygi, J.W. Harper, Dynamics of cullin-RING ubiquitin ligase network revealed by systematic quantitative proteomics, Cell 143 (6) (2010) 951.
[18] B. Li, F.C. Yang, D.W. Clapp, K.T. Chun, Enforced expression of CUL-4A interferes with granulocytic differentiation and exit from the cell cycle, Blood 101 (5) (2003) 1769.
[19] J.R. Pfeiffer, S.A. Brooks, Cullin 4B is recruited to tristetraprolin-containing messenger ribonucleoproteins and regulates TNF-α mRNA polysome loading, J. Immunol. 188 (4) (2012) 1828.
[20] M.H. Hung, Y.R. Jian, C.C. Tsao, S.W. Lin, Y.H. Chuang, Enhanced LPS-induced peritonitis in mice deficiency of cullin 4B in macrophages, Gene Immun. 15 (6) (2014) 404.
[21] Y. Zhang, L. Wang, L. Wang, J. Yang, Y. Gai, L. Qiu, L. Song, A new anti-lipopolysaccharide factor (EsALF-3) from Eriocheir sinensis with antimicrobial activity, Afr. J. Biotechnol. 10 (77) (2011) 945–952.
[22] F. Li, J. Xiang, Recent advances in researches on the innate immunity of shrimp in China, Dev. Comp. Immunol. 39 (1–2) (2013) 11–26.
[23] V.J. Smith, K. Söderhäll, Induction of degranulation and lysis of haemocytes of the freshwater crayfish Astacus astacus by components of the prophenoloxidase activating system in vitro, Cell Tissue Res. 233 (291–303) (1983) 295–303 Cell & Tissue Research 233(2).
[24] T.D. Schmittgen, Real-time quantitative PCR, Methods 25 (4) (2001) 383.
[25] Y.T. Zhu, D. Li, X. Zhang, X.J. Li, W.W. Li, Q. Wang, Role of transglutaminase in immune defense against bacterial pathogens via regulation of antimicrobial peptides, Dev. Comp. Immunol. 55 (2016) 39–50.
[26] C. Mu, P. Zheng, J. Zhao, L. Wang, H. Zhang, L. Qiu, Y. Gai, L. Song, Molecular characterization and expression of a crustin-like gene from Chinese mitten crab, Eriocheir sinensis, Dev. Comp. Immunol. 34 (7) (2010) 734–740.
[27] C. Mu, P. Zheng, J. Zhao, L. Wang, L. Qiu, H. Zhang, Y. Gai, L. Song, A novel type III crustin (CrusEs2) identified from Chinese mitten crab Eriocheir sinensis, Fish Shellfish Immunol. 31 (1) (2011) 142–147.
[28] L.Q. Pan, Y. Feng, J.J. Miao, Z. Lin, L. Jian, Molecular cloning and characterization of a novel c-type lysozyme gene in swimming crab Portunus trituberculatus, Fish Shellfish Immunol. 29 (2) (2010) 286.
[29] S. Li, X.K. Jin, X.N. Guo, A.Q. Yu, M.H. Wu, S.J. Tan, Y.T. Zhu, W.W. Li, Q. Wang, A Double WAP Domain-Containing Protein Es-DWD1 from Eriocheir sinensis Exhibits Antimicrobial and Proteinase Inhibitory Activities, PloS One 8 (8) (2013) e73563.
[30] C. Li, J. Zhao, L. Song, C. Mu, H. Zhang, Y. Gai, L. Qiu, Y. Yu, D. Ni, K. Xing, Molecular cloning, genomic organization and functional analysis of an anti-lipopolysaccharide factor from Chinese mitten crab Eriocheir sinensis, Dev. Comp. Immunol. 32 (7) (2008) 784–794.
[31] Z. Ying, L.L. Wang, L.L. Wang, J.L. Yang, Y.C. Gai, L.M. Qiu, L.S. Song, The second anti-lipopolysaccharide factor (EsALF-2) with antimicrobial activity from Eriocheir sinensis, Dev. Comp. Immunol. 34 (9) (2010) 945–952.
[32] Y.L. Wang, D. Li, H.D. Yang, L. He, W.J. Sun, Z.L. Duan, Q. Wang, The E3 ubiquitin ligase CRL4 regulates proliferation and progression through meiosis in Chinese mitten crab Eriocheir sinensis1, Biol. Reprod. 94 (3) (2016) 65.
[33] J.I. Toth, L. Yang, R. Dahl, M.D. Petroski, A gatekeeper residue for NEDD8-

activating enzyme inhibition by MLN4924, Cell Rep. 1 (4) (2012) 309.
[34] C. Raonaik, W. Delacruz, J.M. Laplaza, S. Tan, J. Callis, A.J. Fisher, The rub family of ubiquitin-like proteins. Crystal structure of Arabidopsis rub1 and expression of multiple rubs in Arabidopsis, J. Biol. Chem. 273 (52) (1998) 34976–34982.
[35] A. Saha, R. Deshaies, Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation, Mol. Cell 32 (1) (2008) 21–31.
[36] R.J. Deshaies, E.D. Emberley, A. Saha, Control of Cullin-ring Ubiquitin Ligase Activity by Nedd8, Springer New York, 2010.
[37] J.R. Pfeiffer, S.A. Brooks, Cullin 4B is recruited to tristetraprolin-containing messenger ribonucleoproteins and regulates TNF-Î ± mRNA polysome loading, J. Immunol. 188 (4) (2012) 1828–1839.
[38] Z. Zhu, L. Wang, R. Hao, B. Zhao, L. Sun, R.D. Ye, Cutting edge: a cullin-5-TRAF6 interaction promotes TRAF6 polyubiquitination and lipopolysaccharide signaling, J. Immunol. 197 (1) (2016) 21–26.
[39] P.N. Paradkar, J.B. Duchemin, J. Rodriguezandres, L. Trinidad, P.J. Walker, Cullin4 is pro-viral during west nile virus infection of Culex mosquitoes, PLoS Pathog. 11 (9) (2015) e1005143.
[40] W. Yuan-Li, L. Qing, X. Jing, Z. Ming, S. Wen-Juan, H. Lin, W. Qun, Involvement of the single Cul4 gene of Chinese mitten crab Eriocheir sinensis in spermatogenesis, Gene 536 (1) (2014) 9.
[41] K. Radek, R. Gallo, Antimicrobial peptides: natural effectors of the innate immune system, Semin. Immunopathol. (2007) 27–43.
[42] O.E. Sørensen, N. Borregaard, A.M. Cole, Antimicrobial peptides in innate immune responses, Contrib. Microbiol. 15 (2008) 61.
[43] A. Bibeau-Poirier, S.P. Gravel, J.F. Clément, S. Rolland, G. Rodier, P. Coulombe, J. Hiscott, N. Grandvaux, S. Meloche, M.J. Servant, Involvement of the IkappaB kinase (IKK)-related kinases tank-binding kinase 1/IKKi and cullin-based ubiquitin ligases in IFN regulatory factor-3 degradation, J. Immunol. 177 (8) (2006) 5059.
[44] C.M. Ulane, C.M. Horvath, Paramyxoviruses SV5 and HPIV2 assemble STAT protein ubiquitin ligase complexes from cellular components, Virology 304 (2) (2002) 160–166.
[45] M. Barry, K. Früh, Viral modulators of cullin RING ubiquitin ligases: culling the host defense, Sci. STKE 2006 (335) (2006) pe21.
[46] L.C. Platanias, E.N. Fish, Signaling pathways activated by interferons, Exp. Hematol. 27 (11) (1999) 1583–1592.
[47] C.M. Horvath, Weapons of STAT destruction. Interferon evasion by paramyxovirus V protein, FEBS J. 271 (23–24) (2004) 4621–4628.
[48] Zhang Xueying, Yujun Zhenjie, Qingyang Guihua, Yunlu Wang, Shen Beifen, Neddylation is required for herpes simplex virus type I (HSV- 1)-induced early phase interferon-beta production, Chinese Journal of Immunology 13 (5) (2016) 577–583.
[49] C. Pasare, R. Medzhitov, Toll-like receptors: linking innate and adaptive immunity, Microb. Infect. 6 (15) (2004) 1382–1387.
[50] J.J. Sun, S. Xu, Z.H. He, X.Z. Shi, X.F. Zhao, J.X. Wang, Activation of Toll pathway is different between kuruma shrimp and Drosophila, Front. Immunol. 8 (2017) 1151.
[51] R.E. Amir, H. Haecker, M. Karin, A. Ciechanover, Mechanism of processing of the NF-kappa B2 p100 precursor: identification of the specific polyubiquitin chain-anchoring lysine residue and analysis of the role of NEDD8-modification on the SCF (beta-TrCP) ubiquitin ligase, Oncogene 23 (14) (2004) 2540.
[52] N.Q. Fernandez, J. Grosshans, J.S. Goltz, D. Stein, Separable and redundant regulatory determinants in Cactus mediate its dorsal group dependent degradation, Development 128 (15) (2001) 2963–2974.
[53] J.J. Sun, J.F. Lan, X.Z. Shi, M.C. Yang, G.J. Niu, D. Ding, X.F. Zhao, X.Q. Yu, J.X. Wang, β-Arrestins negatively regulate the Toll pathway in shrimp by preventing dorsal translocation and inhibiting dorsal transcriptional activity, J. Biol. Chem. 291 (14) (2016) 7488–7504.
[54] H.S. Jin, L. Liao, Y. Park, Y.C. Liu, Neddylation pathway regulates T-cell function by targeting an adaptor protein Shc and a protein kinase Erk signaling, Pnas 110 (2) (2013) 624–629.
[55] D. Qi, J. Zhang, Y. Gao, X. She, Y. Wang, Y. Wang, G. Xin, MLN4924 protects against bleomycin-induced pulmonary fibrosis by inhibiting the early inflammatory process, Am. J. Tourism Res. 9 (4) (2017) 1810.