Fatty acid binding proteins (FABPs) are members of the lipid binding protein superfamily and play crucial role in fatty acid transport and lipid metabolism. In macrophages, Adipocyte-type FABP is an important mediator of inflammation. However, the immune functions of FABPs in invertebrates are not well understood; here, we obtained the gene structure of Eriocheir sinensis FABP 3 and FABP 9 (EsFABP 3 and EsFABP 9), and compared with EsFABP 10. The mRNA expression profiles show that all three FABPs were significantly up-regulated in hemocytes after being challenged with bacteria. Of the three, EsFABP 3 was the most stable and also the most highly up-regulated. Further studies showed that knockdown of EsFABP 3 led to higher bacterial counts in the hemocyte culture medium and a significant decrease in the mRNA expression of some antimicrobial peptides following bacterial stimulation. Moreover, a subcellular study demonstrated that EsFABP 3 can affect nuclear translocation of the dorsal after Gram-positive bacterial stimulation in hemocytes. These findings support the notion that EsFABP 3 could inhibit bacterial proliferation by regulating antimicrobial peptides expression via the Toll signaling pathway. © 2017 Published by Elsevier Ltd.
**FABPs also play important roles in modulating signal transduction and gene transcription** [18]. In lipid transport and metabolism, FABPs influence lipid sensing and response mechanisms by delivering lipids to nuclear receptors, such as PPARs, which mediate transcriptional programs. FABPs also play a role in regulating FA absorption [19,20]. In the tumor stroma, E-FABP—expressing tumor-associated macrophages (TAM) produce high levels of IFN-β through up-regulation of lipid droplet formation in response to tumors. E-FABP—mediated IFN-β signaling can further enhance recruitment of tumoricidal effector cells, in particular natural killer cells, to the tumor stroma for antitumor activities [14]. Furthermore, in mice A-FABP forms a finely tuned positive loop between JNK and activator protein-1 (AP-1) that exacerbates lipopolysaccharide (LPS)-induced inflammatory responses in macrophages [21]. However, it is still unknown if FABPs regulate immune function or antimicrobial activity (through the MAPK pathway like in mammals) in invertebrates.

In this study, we determined the gene structure of two FABP isoforms in the Chinese mitten crab and compared them with another published FABP (EsFABP 10) [31]. Then, the mRNA expression profiles of EsFABP 3, EsFABP 9, and EsFABP 10 were analyzed following stimulation with *Vibrio parahaemolyticus* and *Staphylococcus aureus*. The quantity of bacterial cells in the culture medium was significantly increased with the knockdown hemocyte of EsFABP 3; this may be caused by the expression of positively regulated EsFABP 3 antimicrobial peptides. Furthermore, the translocation of Esdorsal was inhibited in the knocked down hemocyte of EsFABP 3, which suggests that FABP regulates Toll signaling in the Chinese mitten crab.

### 2. Materials and methods

#### 2.1. Experimental animals and bacterial infection

Healthy adult Chinese mitten crabs (*E. sinensis*; n = 200; wet weight = 80 ± 20 g) were purchased from the Xin’An Market in Shanghai, China and acclimated to filtered freshwater in the aquaculture center for 1 week under constant temperature (20–25 °C).

*V. parahaemolyticus* (BYK00036) and *S. aureus* (BYK0113) were isolated from the National Pathogen Collection Center for Aquatic Animals (Shanghai Ocean University, Shanghai, China). The strains were obtained by centrifugation (5000 × g for 3 min), washed three times in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4), resuspended in PBS, and plated for colony counting. For immune challenging, 330 crabs were then randomly divided into three groups (sex ratio in PBS, and plated for colony counting. For immune challenging, collected hemocytes (10 crabs for each group) for RNA extraction at 4 h [32]. After that, hemocytes were obtained by centrifuging (800 rpm, 5 min). Bacteria were then collected by centrifugation (3000 rpm, 17 °C, 20 min) and resuspended in Luria-Bertani (LB) medium, and bacteria were cultured overnight in Luria-Bertani (LB) medium, and bacteria were then collected by centrifugation (3000 rpm, 17 °C, 20 min). The strains were then cultured in LB medium, and bacteria were then collected by centrifugation (3000 rpm, 17 °C, 20 min).

#### 2.2. Primary culture of *E. sinensis* hemocyte

Primary culture of *E. sinensis* hemocytes was performed according to a previously reported method [33]. Crab hemocytes were isolated from hemolymph by centrifugation (300 × g for 10 min at 4 °C), resuspended in Leibovitz L-15 medium (Gibco, Carlsbad, CA, USA) supplemented with 1% antibiotics (10,000 U/ml penicillin and 10,000 µg/ml streptomycin [Gibco]) and 0.2 mM NaCl (676 ± 5.22 mOsm/kg) at pH 7.2–7.4, seeded (4 mL) in 60-mm dishes (Corning, Corning, NY, USA) at a density of 7 × 10^5 cells/mL, and incubated at 28 °C without CO2 for 12 h.

#### 2.3. Genomic DNA extraction and cloning genes of FABP3, FABP9 DNA was extracted from *E. sinensis* hemocytes using the Axysprep™ Multisource Genomic DNA Mini-prep Kit (Axogen) according to the manufacturer’s protocol. Gene-specific primers (Table 1) corresponding to the cDNA of FABP3 and FABP9 were used to obtain the FABP3 and FABP9 genomic DNA sequences [16,34]. The PCR reaction was performed in a 25 µL volume containing 2.5 µL 10× KOD-Plus-Ver.2 buffer, 2.5 µL 2 mM dNTPs, 1.5 µL 25 mM MgSO4, 0.75 µL 10 µm primer, 16 µL RNA free water, 0.5 µL (1 U/µL) KOD-Plus-PCR Cloning Enzyme (Toyobo), and 0.5 µL template (i.e., genomic DNA extracted from hemocytes). The PCR conditions were 94 °C for 2 min followed by 30 cycles of 98 °C for 10 s and 58 °C for 3 min. Amplicons of the expected size were gel-purified and ligated into a pGEM-T easy vector (Promega) with T4 DNA ligase. Positive clones containing the predicted size inserts were sequenced using T7 and SP6 primers (Table 1).

#### 2.4. Total RNA extraction and real-time quantitative PCR analysis

Total RNA was extracted using the TRIzol reagent (Invitrogen, Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
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<tr>
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<tr>
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</tr>
<tr>
<td>SP6</td>
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</tr>
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<td>EsFABP9</td>
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</tr>
<tr>
<td>Real-Time-PCR</td>
<td>Real-Time-PCR</td>
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<td>EsFABP10-RT-R</td>
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<td>EsALF-1-RT-R</td>
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Carlsbad, CA, USA) according to the manufacturer's protocol and previous publications [35]. The total RNA concentration and quality were evaluated by measuring absorbance on a NanoDrop™ 2000 (Thermo Scientific, Waltham, MA, USA) and agarose-gel electrophoresis, respectively. Total RNA was reverse-transcribed using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan).

Real-time quantitative PCR (qPCR) was performed using SYBR® Premix Ex Taq™ (TaKaRa) with conditions of 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 58°C for 30 s. The alternate gene-specific primers were designed based on the cDNA sequences; β-actin was used as an internal control (Table 1). RT-qPCR was performed in triplicate using independently extracted RNA, and the relative expression level was calculated using the 2^ΔΔCt comparative Ct method [36].

2.5. RNA interference in vitro and bacterial challenging

The dsRNA of EsFABP3 and GFP (i.e., control) were prepared as previously described [37–39]. Briefly, EsFABP3 fragments were amplified by PCR with specific primers (Table 1). After purification, the T7 RNA polymerase-binding site was incorporated into each DNA template using PCR and single-stranded RNAs were synthesized by using the T7 RibomAX Express Large Scale RNA Production System (Promega). To generate dsRNA, the corresponding complementary single-stranded RNAs were mixed, annealed, and purified by phenol/chloroform/isoamyl alcohol extraction. Then, the dsRNA (4 μg) was transfected into primary E. sinensis cultured hemocytes for 2 h using the Lipofectamine transfection kit [35]. Total RNA was extracted to evaluate the knockdown effect of the target genes using RT-qPCR and semi-quantitative RT-PCR.

To test the gene expression of EsFABP3 and related AMPs, the cultured hemocytes were transfected with dsEsFABP3 (4 μg) for 24 h and stimulated with V. parahaemolyticus and S. aureus (1 × 10^5 CFU, heat-killed at 72°C for 20 min) for 24 h. The mRNA expression levels of Es-DWD1 [40,41], CrusEs1 [41,42], CrusEs2 [41,43], EsALF1 [41,44], EsALF2 [41,45], and EsALF3 [41,46] were analyzed using RT-qPCR; dsGFP was used as the control.

2.6. Bacterial counts after RNAi of EsFABP3

Live V. parahaemolyticus and S. aureus were prepared as described (Section 2.1) and diluted to 1 × 10^5 CFU/mL. After post-transfection with EsFABP3 dsRNA or GFP dsRNA for 24 h (control was left untreated), the 100 μL bacterial suspension was added into the hemocyte culture medium. At 0 and 24 h, 100 μL of medium was diluted and plated on LB agar. Following incubation, the CFUs were counted. Five dishes from five crabs were analyzed at each time point.

2.7. Immunofluorescence detection of dorsal after EsFABP3 knockdown and S. aureus stimulation

Primary cultures of hemocytes (prepared as previously described in Section 2.1 with slight modifications) were seeded onto a cover glass (24 × 24 mm cell climbing slice culture plate, purchased from WHB, Shanghai, China) in 60-mm dishes. The cultured hemocytes were transfected with dsEsFABP3 (4 μg) for 24 h and stimulated with 100 μL 1 × 10^5 CFU of S. aureus (heat-killed at 72°C for 20 min) for 24 h. Following incubation, the medium was removed and the cells were washed twice with PBS. The hemocytes were fixed using 4% paraformaldehyde in PBS for at least 1 h, exposed to 0.5% Triton X-100 for at least 30 min, and washed with PBS at least three times. The hemocytes were blocked overnight using 5% BSA in PBS and incubated in the first antibody (goat anti-dorsal, purchased from Santa Cruz, sc-26907, 1:200 in blocking buffer). The hemocytes were then washed three times with PBS (0.2% Tween 20 in PBS), allowed to react with dye-Alexa Fluor® 488 conjugated anti-lgG antibody (1:500 in blocking buffer; the antibody was obtained from Abcam, item number 150129) for 2 h at 37°C. After counterstaining with DAPI, the glass was taken out of the 60 mm dishes for observation. Fluorescent signals were observed with a fluorescence microscope (Leica, Germany).

2.8. Statistical analysis

SPSS software (Ver11.0) was used for statistical analysis, and the data are presented as mean ± standard error values. Statistical significance was set at P < 0.05, which determined by using one-way analysis of variance and post hoc Duncan multiple range tests.

3. Results

3.1. Comparison of the genetic structure of FABP3, EsFABP9, and EsFABP10

We compared the genomic DNA sequences of EsFABP3 and EsFABP9 obtained in this study with the genetic structure of EsFABP10 [Fig. 1] [31]. All three genes are comprised of three introns and four exons, which similar to mammalian most FABPs [47]. The length of four exons in EsFABP3 are 73 bp, 173 bp, 105 bp, and 51bp; the exons are separated by three introns (8641 bp, 1320 bp and 2153 bp). The four exons of EsFABP10 are 70 bp, 173 bp, 114 bp, and 54 bp in length, and are separated by three introns (23624 bp, 826 bp, and 2246 bp). All three FABPs appear to have four exons separated by introns of varying lengths.

3.2. Expression profiles of EsFABPs in hemocytes after the immune challenge

S. aureus and V. parahaemolyticus were used to represent Gram-negative and Gram-positive bacteria, respectively [48]. After S. aureus injection, the expression of EsFABP10 was down-regulated at 12 and 24 h; the levels returned to normal after 36 h (Fig. 2A). EsFABP9 activity was ~9 times greater in the treatment group than in the control (P < 0.05) at both 24 h and 36 h (Fig. 2B). EsFABP9 activity was ~11 times greater in the treatment group than in the control (P < 0.05) at 24 h; the levels returned to normal at 36 h (Fig. 2C). Following V. parahaemolyticus injection, EsFABP10 was up-regulated at both 12 h and 36 h to nearly 2 and 3 times above the control, respectively (P < 0.05) (Fig. 2D). EsFABP3 activity was three times greater in the treatment group than in the control at 24 h and 36 h after injection, respectively (P < 0.05) (Fig. 2E). EsFABP9 activity was ~11 times greater in the treatment group than in the control at 36 h (Fig. 2F). All hemocyte EsFABPs could be induced with bacteria, but at different levels. mRNA expression of EsFABP3 was the most stable and sensitive.

3.3. Expression profiles of EsFABP3 in hemocytes after bacteria stimulation

EsFABP3 was chosen for further investigation, the results of which indicated that EsFABP3 transcription was significantly up-regulated (P < 0.05) in primary cultured hemocytes with S. aureus and V. parahaemolyticus stimulation after 8, 12, 24, 48, and 72 h. The increase in transcription levels was much as 9 times (Fig. 3A) and 3.6 times (Fig. 3B) for S. aureus and V. parahaemolyticus, respectively. These results demonstrate the potential immune function of EsFABP3, which varies rapidly and significantly after bacterial stimulation. No significant variation in expression was observed in the control samples.
3.4. The detection efficiency of EsFABP3 RNA in vitro

To investigate the possible regulatory effects of EsFABP3 on AMPs and cellular signaling, dsEsFABP3 was transfected into primary cultured hemocytes to knockdown EsFABP3 expression. Transfection of dsEsFABP3 for 24 h led to a greater than 50% decrease in the EsFABP3 mRNA levels compared to the control (dsGFP; Fig. 4A). The knockdown efficiency also reach 50% compared with dsGFP after infection with S. aureus and V. parahaemolyticus (Fig. 4B).

3.5. The impact of EsFABP3 on bacterial proliferation

To investigate the impact of EsFABP3 on bacterial proliferation, EsFABP3 in cultured hemocytes was knocked down by dsEsFABP3 and live bacteria were added into the medium. Compared with the dsGFP and normal (Ctr) cultures, the number of bacteria in the hemocyte culture medium increased significantly 24 h after dsEsFABP3 transfection (Fig. 5A and B). This result indicates that EsFABP3 could significantly inhibit bacterial proliferation.

3.6. Regulation of AMP expression by EsFABP3

After dsEsFABP3 transfection and bacterial challenging, we investigated the possible regulatory function of EsFABP3 cellular signaling. The decrease in EsFABP3 expression following exposure to S. aureus was accompanied by significant reductions in CrusEs1, CrusEs2, EsALF1, EsALF2, and Es-DWD1 expression; however, EsALF3...
Fig. 3. Precise temporal expression profiles of EsFABP3 in response to the bacterial challenges. (A) Crabs were injected with 100 μL of *Staphylococcus aureus* (1 × 10^5 CFU/mL) or PBS (control). (B) Crabs were injected with 100 μL of *V. parahaemolyticus* (1 × 10^5 CFU/mL) or PBS (control). The mRNA expression of EsFABP3 was determined by RT-qPCR at 0, 4, 8, 12, 24, 48, and 72 h post-challenge relative to the mRNA expression of β-actin. The p-value was calculated using post hoc Duncan multiple range tests, and significant differences were accepted when p < 0.05 (*).

Fig. 4. RNAi efficiency of EsFABP3. Primary cultured hemocytes were transfected with 4 μg of EsFABP3 dsRNA (dsFABP3), and the expression of EsFABP3 was analyzed at 24 h post-transfection using RT-qPCR and semi-quantitative RT-PCR: (A) control, and; (B) following *S. aureus* and *V. parahaemolyticus* challenges. The p-value was calculated using post hoc Duncan multiple range tests, and significant differences (p < 0.05) are represented by * and letters.

Fig. 5. Effect of EsFABP3 RNAi on bacteria counts. The bacterial CFU/mL were determined in LB medium at 0 and 24 h following addition of EsFABP3 dsRNA. GFP dsRNA (dsGFP) and normal hemocytes (Ctr) were used as controls. The effect of EsFABP3 RNAi on the CFU of *Staphylococcus aureus* (A) and *V. parahaemolyticus* (B). Each bar represents the mean and S.D. from five independent replicates. Statistical significance was determined by post hoc Duncan multiple range tests (*P < 0.05).
expression was not affected (Fig. 6A). The decrease in EsFABP3 expression following exposure to V. parahaemolyticus was accompanied by significant reductions in EsALF1 and EsALF2 expression, however, other AMPs expression was not affected (Fig. 6B). These results suggest that EsFABP3 could regulate AMP expression, especially following treatment with S. aureus.

3.7. EsFABP3 regulates the translocation of the dorsal protein in the Toll pathway

In normal (i.e., untreated) E. sinensis hemocytes, dorsal protein could be observed in the cytoplasm and nucleus; much of the dorsal protein was observed in the cytoplasm (Fig. 7A). There was no change in the distribution of dorsal protein in dsGFP transfection hemocytes (Fig. 7B). In EsFABP3-knockdown hemocytes, most of the...
dorsal protein was observed in the cytoplasm (Fig. 7C). In the S. aureus stimulation hemocytes, most of the dorsal protein was translocated into the nucleus, and only few dorsal proteins could be detected in the cytoplasm (Fig. 7D). In the dsEsFABP3 transfected group challenged by S. aureus, more dorsal protein was detected in the cytoplasm (Fig. 7E). These results revealed that EsFABP3 can regulate the transport of dorsal protein from the cytoplasm to then nucleus, which suggests that EsFABP3 could be regulated by AMP expression via the Toll pathway [49].

4. Discussion

FABPs are multifunctional proteins that participate in different processes, such as the cell growth and differentiation, lipid metabolism, and inflammation [19,50–52]. However, to this point most studies have been focused on vertebrates, and our knowledge of FABPs is still limited in invertebrates, especially crustaceans. In Eriocheir sinensis, the full-length cDNA of FABP3, FABP9, FABP10 have been reported previously [3,16,34]; based on those reports, we analyzed the gene sequences of these FABPs and found that all of those FABPs have three introns and four exons. Furthermore, the lengths of exons are relatively stable, however, unlike exons the length of introns is not conserved. The intron lengths vary greatly in size, this may be driven natural selection since long introns could enhance gene recombination and introduce mutations into adjacent exons [53,54].

Studies have found that expression of several invertebrates FABPs varies during immune response. In Drosophila, the expression of FABPs was up-regulated following exposure to LPS [55]. In Fenneropenaeus chinensis, the expression of FcFABP increased after being challenged with WSSV, but decreased following infection with V. anguillarum [56]. In Eriocheir sinensis, Es-FABP3, Es-FABP9, and Es-FABP10 mRNA expression were all altered in some primary immune tissues after being challenged with LPS and PGN [16,17]. However, these results in crustaceans were all following the injection of pathogen associated molecular patterns (PAMPs), and the expression of FABPs after PAMP injection is not stable [16,17]. Furthermore, bacteria often contain more than one type of antigen, and a single PAMP cannot entirely replace bacteria in an immune challenge [57–59]. Thus, our work exploring the changes in FABP

Fig. 7. Effects of EsFABP3 RNAi on the translocation of dorsal proteins. Translocation of dorsal proteins from the cytoplasm to the nucleus after S. aureus stimulation. DAPI was used to counterstain the nuclear DNA, and Alexa Fluor® 488 was used to reveal the subcellular location of dorsal protein in hemocytes with 200× magnification; nuclei are indicated by the arrows: (24 hpi) (A) normal cells, (B) dsGFP-addition cells, (C) dsEsFABP3-addition cells, (D) S. aureus-stimulation cells, and (E) S. aureus-stimulation after addition of dsEsFABP3 cells.
expression after the injection of different bacterial species into hemocytes to reveal the real response of FABPs in crabs after immune challenge is important. The results shown that the up-regulation of FABP expression after being challenged with bacteria is more significant and stable than that seen following LPS and PGN. Additionally, EsFABP3 was more sensitive following bacterial stimulation.

Es-FABP3 expression was more significantly altered following PGN stimulation than with LPS. However, in our study, Es-FABP3 expression was more significantly up-regulated following the S. aureus (a Gram-positive bacteria) stimulation than following the V. parahaemolyticus (a Gram-negative bacteria) stimulation. Some bacteria binding assays in shrimp and crab have demonstrated that FABPs can bind directly to bacteria and inhibit growth [16,17,56], which suggests that FABPs are an important antibacterial protein.

Invertebrate hemocytes can inhibit the growth of or directly kill bacteria [60–63]. To investigate the immune function of EsFABP3, the EsFABP3 levels in hemocytes were measured following treatment with either V. parahaemolyticus or S. aureus. dsEsFABP3 was then transfected to and incubated with living bacteria; this led to a significant increase in the bacterial cell density, which indicates that the knockdown of EsFABP3 decreased the bacteriostasis or bactericidal capability of E. sinensis. Hence, we speculate that EsFABP3 (an intracellular protein) may impede the proliferation of bacteria by modulating the expression of some antimicrobial substances or phagocytosis-related molecules [62,63].

As an essential component of the innate immune defense system, antimicrobial peptides (AMPs) rapidly and efficiently effect invading microorganisms [63,64]. Therefore, we investigated the expression of the reported AMPs in E. sinensis following EsFABP3 knockdown and bacterial stimulation. Our results show that the expression of some AMPs decreased significantly in EsFABP3-silenced hemocytes following bacterial stimulation, which indicates that some AMPs are positive regulated by EsFABP3. After being challenged with S. aureus, the expression of AMPs in EsFABP3 knockdown hemocytes was decreased further, which suggests that EsFABP3 may regulate AMP expression via the Toll pathway.

Recent studies have found that FABPs play an important role in inflammation by modulating some signal pathways. For example, in mice macrophages A-FABP enhances a positive feedback loop which can modulate A-FABP expression and some A-FABP-related pro-inflammatory cytokines [21]. The Toll and Imd are regarded as the major pathways controlling innate invertebrate immune response [22–24]. In Drosophila, the Toll and the Imd pathways integrate the signals from the PRRs and activate the transcription of a nuclear factor-kB (NF-kB) family, this leads to the production of antimicrobial peptides (AMPs) [25–27]. Gram-positive and fungal infections trigger Toll activation, which then activate downstream factors, including Tube and MyD88 adaptors, the Pelle kinase, Cactus (the Drosophila homolog of I-kB), and the Dorsal and Dif transactivators [28–30]. In particular, dorsal proteins, a transcription factor that only exists in Toll signaling pathways, could regulate AMP expression by translocation from the cytoplasm to the nucleus [27,49,65]. As previously discussed, to confirm whether EsFABP3 regulates Toll signaling, we investigated the nuclear translocation of dorsal proteins after EsFABP3 knockdown. The results showed that following S. aureus stimulation, the amount of dorsal proteins in the cytoplasm of dsEsFABP3 transfected hemocytes were greater than in dsGFP transfection cells. This indicates that EsFABP3 could positive regulate the nuclear translocation of dorsal proteins. However, the mechanism by which EsFABP3 regulates the translocation of dorsal proteins in crabs is still unclear. It is possible that EsFABP3 regulates the nuclear translocation of dorsal proteins by activating some other signaling molecules in Toll signaling pathway, such as MyD88, Pelle, Tube, or Cactus [26,28,66,67], via direct binding with signaling molecules or using another protein as a bridge.

In summary, we obtained and compared the gene sequences and structures of EsFABP10, EsFABP3, and EsFABP9. The mRNA expression of EsFABP3 in hemocytes suggests and important role in immunological defense. Furthermore, the enhancement of bacterial CFU in EsFABP3 knockdown cell culture medium was found caused by the down-regulation of AMPs expression by EsFABP3. Furthermore, Esdorsal protein translocation was suppressed in EsFABP3 knockdown hemocytes; this could explain why this gene can regulate AMP expression. Our collective results indicate that EsFABP3 could inhibit the bacterial proliferation by regulating antimicrobial peptides expression via the Toll signaling pathway, however, the mechanism by which EsFABP3 regulates the nuclear translocation of dorsal proteins is still unclear.

Acknowledgements

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