Research paper

A core component of the CUL4 ubiquitin ligase complexes, DDB1, regulates spermatogenesis in the Chinese mitten crab, _Eriocheir sinensis_

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**A B S T R A C T**

Studies in mammals have shown that damaged DNA-binding protein 1 (DDB1) is a multifunctional protein that recognizes UV-induced DNA lesions and activates nucleotide excision repair process, and could also be a linker protein for Cullin4 in ubiquitination to regulate cell cycle progression. However, there are few studies of DDB1 in crustaceans. In this study, a cDNA representing the DDB1 gene from _Eriocheir sinensis_ (Es-DDB1) was cloned successfully. The full length Es-DDB1 cDNA comprises 4871 nucleotides, and encodes an open-reading frame (ORF) of 1137 amino acid residues. Bioinformatics' analysis showed that the domains and structure of Es-DDB1 have been highly conserved during evolution. Antibodies against Es-DDB1 and Es-Cul4 were raised using a prokaryotic expression system. Moreover, a co-immunoprecipitation assay showed that Es-DDB1 could bind Es-Cul4 in the testis of _Eriocheir sinensis_. Furthermore, quantitative real-time PCR and Western blotting showed high expression in the testis, particularly during the spermatocyte stage. Immunofluorescence assays showed that Es-DDB1 was mainly distributed in the cytoplasm in the early and middle developmental stages. These results indicated that Es-DDB1 might play a key role in spermatogenesis of _E. sinensis_.

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1. Introduction

The Chinese mitten crab (_Eriocheir sinensis_) is economically important in Chinese aquaculture. In recent years, developmental precociousness has become a serious problem, causing significant economic losses in the crab aquaculture industry (Hou et al., 2010). Studies on spermatogenesis of _Eriocheir sinensis_ might identify the molecular mechanisms underlying this developmental precociousness, which should lead to solutions to this problem.

The male reproductive system of _E. sinensis_ includes a pair of testes, vasa deferens, seminal vesicles, accessory glands and ejaculatory ducts (Du, 1988a). Based on morphological and histological analyses, testis development in _E. sinensis_ can be divided into five stages, which take one year to produce mature testes: spermatogonium stage (May–June), spermatocyte stage (July–August), spermatid stage (September–October), sperm stage (October–April in the following year) and the dormant stage (April–May) (Du, 1988b). In other words, rapid development of the testes occurs mainly for the spermatogonia (early stage), primary spermatocytes and secondary spermatocytes (middle stage), and spermatids and spermatozoa (late stage) (Sun et al., 2012).

Damaged DNA-binding protein 1 (DDB1), which was first identified as a subunit of a heterodimeric DNA damage-binding complex, is a conserved protein that is involved in the global genome-nuclear excision repair pathway (Tang et al., 2000). DDB1 is also a component of the Cul4 ubiquitin ligase complex (Jackson and Xiong, 2009), which regulates the ubiquitination that is essential for nucleotide excision repair of the DDB1-CUL4 ligase complex (Jackson and Xiong, 2009), the ubiquitylation of p21, which is a substrate of Cul4-A–DDB1 complex, is involved in the expression of cell cycle regulatory proteins (Iovine et al., 2011). The DDB1-CUL4 ligase complex also directs the DNA replication licensing factor (Cdt1) to regulate DNA damage (Higa et al., 2003; Hu et al., 2004), is involved in transcription-regulated cell growth via degradation of c-Jun and STAT proteins (Wertz et al., 2004; Precious et al., 2005) and regulates cell cycle progression (Cang et al., 2006; Kim et al., 2008). The ubiquitylation of p21, which is a substrate of Cul4A–DDB1-E3-ligase, dependent on its interaction with proliferating cell nuclear antigen (PCNA) and represents another efficient DNA repair mechanism (Abbas et al., 2008). DDB1 has been highly conserved during evolution, from fission yeast to humans (Tang and Chu, 2002). Deletion of the DDB1 gene in fission yeast led to an increased rate of spontaneous mutations and was essential for somatic cells differentiation into meiotic cells (Holmberg et al., 2005). Loss of DDB1 in fruit flies caused early lethality in development (Takata et al., 2004). Moreover, DDB1 deletion...
in the mouse brain led to genomic instability and caused early embryonic lethality (Cang et al., 2006). To date, most studies of DDB1 have been conducted in higher vertebrates, especially in humans and mice; however, the functions of DDB1 in invertebrates, especially in crustaceans, remain unknown.

Based on recent research in our laboratory, the results showed that Es-CUL4 plays a key role in gametogenesis and reproductive success in E. sinensis (Wang et al., 2014). However, DDB1’s role in spermatogenesis in Eriocheir sinensis is unknown. Thus, in the present study, we cloned the DDB1 cDNA from Eriocheir sinensis and investigated its expression patterns, cellular location and potential roles during different testis development stages and in different tissues of E. sinensis. In addition, we identified a protein–protein interaction between DDB1 and Cul4 in E. sinensis.

2. Materials and methods

2.1. Animal and tissue preparation

Healthy, adult Chinese mitten crabs were purchased from Xin’an aquaculture market (Shanghai, P.R. China) once a month from July 2015 to January 2016. The crabs used in experiments were lightly anesthetized using an ice-bath. Nine tissues (heart, hepatopancreas, stomach, muscle, gill, intestine, hemocytes, accessory gland and testis) were excised and frozen in liquid nitrogen immediately, and then stored at −80 °C before nucleic acid and protein extraction. For use of Chinese mitten crab, no approval is needed in China.

2.2. RNA extraction and cloning of the full length cDNA

Total RNA was extracted from E. sinensis tissues using the Trizol reagent (Invitrogen, USA), according to the manufacturer’s protocol. The concentration and quality, respectively, of the total RNA were estimated using a NanoDrop2000 (Thermo scientific) instrument at a wavelength of 260 nm and by agarose gel electrophoresis (Bio-Rad PowerPac Basic, USA).

Total RNA (1 μg) isolated from the testes was reverse transcribed using the 3′-Full RACE Core Set with PrimeScript RTase (Takara) and SMARTer™ RACE cDNA Amplification Kit (Clontech) for rapid amplification of cDNA ends (RACE) cloning. Gene-specific primers were designed for 3′ and 5′ RACE (Table 1), and the RACE-PCR products were purified and inserted into the pMD19T vector (Takara). The 3′ and 5′ terminal fragments were spliced together using DNAman (Lynnon Biosoft) to obtain the full-length cDNA of Es-DDB1.

2.3. Structural characterization of the DDB1 gene

The data for the genomic DNA of the DDB1 were obtained from genomic data of E. sinensis, which were sequenced on the Illumina HiSeq 2000 platform (unpublished data) and evaluated via transcriptome data (He et al., 2012). To check the accuracy of the predicted gene model, we designed primers to re-clone the full-length cDNA. The gene organization (intron/exon boundaries) was predicted using Genscan software (http://genes.mit.edu/GENSCAN.html) (Burge and Karlin, 1997) and the schematic diagram of the Es-DDB1 gene organization was constructed according to the predicted results (Wang et al., 2016).

2.4. Bioinformatic analysis of Es-DDB1

The nucleotide sequences were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI: http://blast.ncbi.nlm.nih.gov/Blast.cgi). ORF finder (http://www.ncbi.nlm.nih.gov/orffinder) was used to identify the open reading frame (ORF). To predict the Es-DDB1 protein structure and functional domains, we used SMART (http://smart.embl-heidelberg.de/). The ProtParam tool of ExPASy (http://web.expasy.org/protparam/) was used to predict the protein molecular weight and theoretical isoelectric point. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict whether the protein contains a signal peptide. The PSORT WWW Server (http://psort.hgc.jp/) predicted the protein subcellular localization. The phylogenetic tree of DDB1 proteins from different species was constructed using MEGA6 software and the alignment generated by clustalx2.1. A 3D homology model of Es-DDB1 was generated using SWISS-MODEL (http://swissmodel.expasy.org/) (Biasini et al., 2014). Using the crystal structure of human DDB1 (PDB ID: 4Es54-1A) as the template (Yeh et al., 2012; Li et al., 2006), the 3D structure of Es-DDB1 was visualized using the PyMOL molecular graphics system (Delano, 2002).

2.5. Real-time PCR expression analysis of Es-DDB1

Expression of Es-DDB1 in different testis development stages (July 2015 to January 2016) was analyzed by real-time PCR, by using a CFX96TM Real-Time System (Bio-Rad). First-strand cDNA was prepared. Two PCR primers (RT-DDB1; Table 1) specific for Es-DDB1 were designed based on the Es-DDB1 cDNA sequence and samples were normalized to the expression of the β-actin gene (for primers, see Table 1). Real-time PCR was performed in triplicate for each sample and amplifications were carried out in a final volume of 25 μL, including 1 μL diluted cDNA template (500 ng/μL), 10.5 μL PCR grade water, 0.5 μL of each primer, and 12.5 μL 2 × SYBR Premix Ex Taq (Takara). PCR conditions were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 56 °C for 30 s, and 95 °C for 15 s.

Gene expression data were analyzed using CFX Manager™ software (version 1.0), and quantified using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) based on Ct values for both Es-DDB1 and β-actin (Schmittgen and Livak, 2008). The data obtained from real-time PCR analysis were shown as the mean ± SD. Es-DDB1 expressions in different tissues were also analyzed by real-time PCR, using the same reaction conditions as detailed above.

2.6. Construction of the pET28a-DDB1 and pET28a-Cul4 expression vectors

DNAs encoding the complete MMS1-N domain of Es-DDB1 and the complete CULLIN domain of Es-Cul4 (GenBank accession number: JX110658) were amplified using forward primers (DDB1-F, Cul4-F Table 1) and reverse primers (DDB1-R, Cul4-R Table 1) designed by Primer Premier 5.0. The PCR products were purified and sequenced by Majorbio (Shanghai), and then cloned separately into the pMD19-T vector to form pMD19-T-DDB1 and pMD19-T-Cul4. Recombinant vector pMD19-T-DDB1 and expression vector pET28a were digested with BamHI and Xhol, and then ligated together using T4 DNA ligase at 16 °C overnight to form pET28a-DDB1. pMD19-T-Cul4 was treated in the same way to form pET28a-Cul4. The resulting expression vectors, pET28a-DDB1 and pET28a-Cul4, were sequenced by Majorbio (Shanghai).

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’–3’)</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>3′ Es-DDB1 OUTER</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Forward</td>
</tr>
<tr>
<td>3′ Es-DDB1 INNER</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Forward</td>
</tr>
<tr>
<td>5′ Es-DDB1</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Reverse</td>
</tr>
<tr>
<td>RT-DDB1</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Forward</td>
</tr>
<tr>
<td>RT-DDB1</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Reverse</td>
</tr>
<tr>
<td>DDB1-F</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Forward</td>
</tr>
<tr>
<td>DDB1-R</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Reverse</td>
</tr>
<tr>
<td>Cul4-F</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Forward</td>
</tr>
<tr>
<td>Cul4-R</td>
<td>CCGAGGAAGGACAAAGCAGAAG</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

Note: BamHI and Xhol restriction sites are underlined; the stop codon is shown in bold.
2.7. Expression and purification of the recombinant proteins from *Escherichia coli*

The recombinant expression plasmids pET28a-DDB1 and pET28a-Cul4 were transformed separately into *E. coli* transetta (DE3) and cultured overnight at 37 °C. The expression of the fusion protein was induced as follows: 1 mM IPTG for 3 h at 37 °C, 1 mM IPTG for 3 h at 30 °C, 0.25 mM IPTG for 3 h at 37 °C, and 0.25 mM IPTG for 3 h at 30 °C. Cells were collected by centrifugation at 7000 rpm for 5 min at 4 °C. The precipitate were washed twice with 1 M PBS, and resuspended in PBS. The samples (80 μl) with 20 μl 5× SDS-PAGE loading buffer were boiled together at 100 °C for 5 min. The products were detected by SDS-PAGE to determine the best induction conditions (Wu et al., 2007).

The cell lysates were pelleted by centrifugation and the pellet was resuspended in PBS (20 ml/g thallus) with 1% TritonX-100 before being lysed by ultrasonication. Proteins expressed in inclusion bodies were washed with 100 mM PBS containing 50 mM NaCl and 2 M Urea twice, followed by denaturation with 8 M Urea, before being resuspended in binding buffer. The samples were then loaded onto a Ni-NTA HisTrap™ FF crude (GE Healthcare) and washed with imidazole elution buffer (20 mM Tris-HCl, 50 mM NaCl, 8 M Urea, × mM imidazole (x = 100, 200, 300, 500)) at 1 ml/min (Li et al., 2014). Fractions were collected and examined by 10% SDS-PAGE.

2.8. Production of polyclonal antibodies

The purified pET28a-DDB1 and pET28a-Cul4 recombinant proteins were used to produce polyclonal antibodies in rabbits. Rabbits were immunized every two weeks as described by Li et al. (2014). After eight weeks, the antiserum was collected and an enzyme linked immunosorbent assay (ELISA) was used to check the titer. Western blotting was performed to verify the presence of the recombinant proteins (Fig. 1).

**Fig. 1.** Sequence analysis of the cDNA and predicted peptide sequences of *Es*-DDB1. The numbering of the nucleotide and amino acid sequences is shown to the left and right, respectively. The start codon and stop codon are indicated in red; the polyadenylation site (AATAAA) is indicated in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
tein abundance levels. The expression intensities of were determined using Western blotting with

\[ 14 \]

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then used to determine specificity of the antibodies for the purified protein.

2.9. Western blotting

Total proteins were collected from different testis development stages (Jul, Aug, Sep, Oct, Nov, Dec and Jan.) with RIPA Lysis Buffer and quantified using the BCA method (BCA Protein Assay Kit, Beyotime). Each sample was subjected to separation by SDS-PAGE and transferred to PVDF membranes. The membrane was blocked with 5% non-fat powdered milk (Sangon, Shanghai) for 1 h at room temperature. Rabbit anti-Es-DDB1 polyclonal antibody and a \( \beta \)-actin antibody were diluted 1:5000 and 1:1000 with PBST, respectively, and incubated overnight at 4 °C. The following day, sections were incubated with HRP-conjugated goat anti-rabbit IgG (Transgen, China) anti-

\[ 20 \]

to PVDF membranes. The membrane was blocked with 5% non-fat powdered milk for 1 h at room temperature. Rabbit anti-DDB1 polyclonal antibody and a \( \beta \)-actin antibody were diluted 1:200 with 3% BSA, and incubated overnight at 4 °C. The following day, sections were incubated with HRP-conjugated goat anti-rabbit IgG (Transgen, China) anti-

\[ 500 \]

The SPSS software (Ver11.0) was used for statistical analysis. Statistical significance was calculated by post hoc Duncan multiple range tests and one-way analysis of variance. Significance for the mean values was set at \( P < 0.05 \).

3. Results

3.1. Gene cloning and bioinformatic analysis of Es-DDB1

The full-length Es-DDB1 cDNA sequence (GenBank accession: KX785224) (Fig. 1) cloned from E. sinensis testis was 4871 bp, which contained a 3414 bp ORF, and 47 bp 5′- and 1398 bp 3′-untranslated regions. The genetic structure of Es-DDB1 comprised 24 exons (Fig. 2A) (GenBank accession: KX785223), which is similar to the number in mammalian genes (about 27). As predicted in ProtParam, the putative protein comprised 1137 amino acids and had a calculated molecular mass of 125 kDa, with a pl of 5.06 (ExPASy). SMART predicted an MMS1_N domain, a CPSF_A domain and a low complexity region (Fig. 2B). MMS1 belongs to the DDB1 family and the two proteins are homologous. The CPSF family is involved in mRNA polyadenylation and might be involved in RNA/DNA binding. These two domains were conserved among diverse species. SignalIP did not predict the presence of a signal peptide, indicating that Es-DDB1 is not a secreted protein. Furthermore, TMHMM 2.0 did not predict any transmembrane regions. The predicted protein
subcellular localization revealed that the Es-DDB1 protein was 60.9% likely to be located in the cytoplasm, 30.4% likely to be in the nucleus and 4.3% likely to be in the mitochondria and vesicles of secretory system, respectively. (PSORT).

Multiple protein sequence alignment analysis of the Es-DDB1 amino-acid sequence with those from 16 other species (Fig. 3) revealed that Es-DDB1 has conserved functional domains compared with mammalian proteins. A phylogenetic tree was constructed using Mega6 of representative invertebrate and vertebrate sequences from protein Blast searches (Fig. 4). The relationships displayed in the phylogenetic tree corresponded to their taxonomic classifications.

The result of 3-D protein structure model prediction of Es-DDB1 by SWISS-MODEL demonstrated that the protein structure included the MMS1-N domain (red) and CPSF-A (yellow) domains (Fig. 5A). The multidomain structure consisted of seven-bladed β propellers (referred to as BPA to BPC) and a C-terminal helical domain (CTD) (Li et al., 2006; Yeh et al., 2012). No Es-DDB1 crystal structure is currently available in PDB, and Human DDB1 (PDB ID: 4e54.1.A) is the closest homolog for which a structure has been determined; therefore, the human protein was used as a template to generate a homology model of Es-DDB1. The Es-DDB1 model was then compared with a resolution of 4e54.1 Å (Fig. 5B).

3.2. Expression analysis of Es-DDB1 by real-time PCR

Real-time PCR analysis was used to determine the transcription levels of Es-DDB1 in the different tissues in Eriocheir sinensis (Fig. 6). The data showed that the expression level of Es-DDB1 was obviously highest in the testis compared with the other tissues (heart, gill, hepatopancreas, intestine, stomach, muscle).
Real-time PCR was then used to determine the expression level of Es-DDB1 in *E. sinensis* in different testis development stages (Fig. 7). The highest levels of Es-DDB1 in testis were observed in July and August, corresponding to the spermatocyte stage; the expression level then gradually decreased in the spermatids and spermatozoa stages (September to January).

### 3.3. Expression and purification of Es-DDB1 and Es-Cul4 proteins

The expression vector pET28a-DDB1 contained a 1644 bp nucleotide sequence (from nucleotide 69–1712 of the cDNA) from *Es*-DDB1 (Fig. 8), which encoded 548 amino acids. The predicted molecular mass of the recombinant protein was about 66 kDa, and the isoelectric point was 5.06.

The recombinant protein was induced in four different conditions and showed the highest expression using 1 mM IPTG at 30 °C (Fig. 9A), which was adopted for further experiments. The molecular mass of the recombinant protein was about 68 kDa, which agreed with the predicted size. The recombinant protein was expressed in inclusion bodies. After denaturation in 8 M urea and purification by Ni-NTA column affinity chromatography, a relatively pure protein was obtained, the concentration of pure protein was 0.63 mg/mL. Detection with Western blotting showed that the antibody was specific (Fig. 9C), and the titer of antibody was 1:128,000.

Similarly, pET28a-Cul4 contained a 1017 bp nucleotide sequence (nucleotides 1553–2569) and expression from this construct produced a recombinant protein of about 47 kDa (Fig. 9B), with an isoelectric point of 8.90, the concentration of pure protein was 0.61 mg/mL. The recombinant proteins were used successfully to raise specific antibodies in rabbits (Fig. 9C), the antibody titer was 1:128,000.

### 3.4. Expression of Es-DDB1 proteins in different tissues and different testis development stages of *E. sinensis*

Es-DDB1 was detected at its highest levels in the testis, and at lower levels in the hepatopancreas, hemocyte, intestines, heart, stomach, and lowest in gill and accessory sex gland (Fig. 10). Western blotting of different testicular developmental stages in *E. sinensis* showed the highest abundance in samples from August and September. After that, the abundance decreased until January, corresponding the spermatids and spermatozoa stages (Fig. 11).

### 3.5. Subcellular location of Es-DDB1 protein during development stages in the testis

To examine the role of Es-DDB1 in spermatogenesis, immunofluorescence microscopy was used to observe its specific location in the
3.6. Es-DDB1 could bind Es-Cul4

Testis proteins were incubated with the anti-Es-Cul4 antibody to capture the antigen–antibody complexes. The products were detected using Western blotting, and a 125 kDa band was visualized, which was detected by the anti-Es-DDB1 antibody (Fig. 13A lane2). Conversely, for proteins incubated with the anti-Es-DDB1 antibody, Western blotting showed a protein band of 92 kDa, which was detected by the anti-Es-Cul4 antibody (Fig. 13B lane2). IgG purified from rabbit was used as a control (Fig. 13 lane 3). Therefore, we tentatively concluded that DDB1 and Cul4 bind to each other in *E. sinensis*, possibly forming a complex that functions in spermatogenesis.

4. Discussion

In this study, we obtained the 4871 bp full-length *Es*-DDB1 cDNA, containing a 3414 bp ORF, and 47 bp 5′- and 1398 bp 3′-untranslated regions. The genetic structure of *Es*-DDB1 is similar to mammalian genes. The putative *Es*-DDB1 protein contains 1137 amino acids, with a predicted molecular weight of 125 KDa, which is similar to that of Drosophila (126 KDa).

NCBI and SMART predicted two conserved domains, MMS1-N and CPSF-A. These two domains were conserved among diverse species. Comparison of the homology model of *Es*-DDB1 with the Human DDB1 (PDB ID: 4e54.1.Å) showed that the multidomain structure consisted of seven-bladed (5 propellers (referred to as BPA to BPC) and a C-terminal helical domain (CTD), which revealed that the two species’ proteins were basically similar to each other.

Using DNAMAN software, we found that the molecular structure was conserved between 16 species. This result indicated that its function may be similar across species. The phylogenetic tree revealed two distinct clades, representing vertebrates and invertebrates. Thus, the relationships in the phylogenetic tree corresponded to their taxonomic classifications.

By establishing prokaryotic expression vectors, the *Es*-DDB1 and *Es*-Cul4 proteins were successfully expressed in *E. coli*. The two recombinant protein were purified from inclusion bodies and used to prepare rabbit antibodies.

Real-time PCR showed that *Es*-DDB1 is mainly present in the testis, with lower expression in the hepatopancreas, stomach, and intestines of *E. sinensis*. Western blotting analysis of *Es*-DDB1 in different tissues of *E. sinensis* showed a peak in the testis. Thus, the protein level was consistent with the transcription level.

DDB1 plays an important role in controlling levels of cell cycle regulators and maintaining genomic stability (Cang et al., 2007), and deletion of DDB1 abolished the self-renewing capacity of hepatocytes, thereby inducing liver tumorigenesis (Yamaji et al., 2010). Therefore, we speculated that DDB1 might play a role in spermatogenesis of *E. sinensis*. Accordingly, we performed real-time PCR analysis in different developmental stages of the testis. The expression level of *Es*-DDB1 mRNA was high in the spermatocyte stage (July and August) and decreased in the spermatozoa stage (November–January of the following year). Meanwhile, we examined the expression of *Es*-DDB1 in different developmental stages by Western blotting and observed high expression at the spermatocyte stage (July and August). In recent years, many studies have shown that DDB1 participates in development. In Drosophila, DDB1 had roles in cell proliferation and development, with its highest levels in the early embryogenesis stages, and decreasing as development proceeds (Takata et al., 2002). Deletion of DDB1 in mice resulted in female infertility, increased rates of apoptosis and poor responses to ovulation signals (Yu et al., 2015). Moreover, DDB1 is also required for the maintenance of spermatogonial stem cells (SSCs) (Yu et al., 2016). Previously, we reported that *Es*-Cul4 might play an important role in regulating spermatogenesis (Wang et al., 2014). Initially, we identified *Es*-DDB1 and *Es*-Cul4 as interacting proteins, and the expression pattern of *Es*-Cul4 in spermatogenesis was similar to that of *Es*-DDB1. Therefore, we hypothesized that *Es*-DDB1 is highly active in testis and might play important roles in essential cellular processes, including spermatogenesis, possibly as a complex with *Es*-Cul4.

Testes undergo five developmental stages during spermatogenesis (spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid and spermatozoon), whose morphological and histological structures can be differentiated (Du, 1988b; Wang et al., 2014). We used immunofluorescence analysis to confirm the subcellular

![Fig. 7](image.png)

**Fig. 7.** Real-time PCR analysis of *Es*-DDB1 expression in different development stages of the testis in *E. sinensis*. The expressions of *Es*-DDB1 and β-actin were measured and data are means ± SD of triplicate experiments. Significant differences among development stages (*P* < 0.05) are indicated by lowercase letters (a, b, and c).

![Fig. 8](image.png)

**Fig. 8.** (A) Electrophoretic analysis of *Es*-DDB1 and *Es*-Cul4 on an agarose gel (1% w/v). Lane M: 100–2000 bp DNA size marker. Lane 1 and 2: 1644 bp cDNA amplification product. Lane 3 and 4: 1017 bp cDNA amplification product. (B) Electrophoretic analysis of recombinant plasmid pMD19-T-DDB1 and recombinant plasmid pMD19-T-Cul4 on an agarose gel (1% w/v). Lane M: 100–2000 bp DNA size markers. Lane 1: Double digestion of recombinant plasmid pMD19-T-cav-1 with BamHI and Xhol restriction enzymes. Lane 2: Double digestion of recombinant plasmid pMD19-T-Cul4 with BamHI and Xhol restriction enzymes. (C) Electrophoretic analysis of expression plasmid pET-28a-DDB1 and pET-28a-Cul4.
localization of DDB1 in each of the five stages of spermatogenesis. We detected Es-DDB1 in the cytoplasm of sperm cells during most of the developmental stages in the testis, and it was highly expressed in the spermatogonia and spermatocytes. However, its expression decreased slightly in the subsequent stages and showed weakened or no expression in spermatids and mature sperm. Meanwhile, we detected that the Es-DDB1 protein was translocated from the cytoplasm to nucleus, and was located especially in the acrosomal tubule and apical vesicle.
Fig. 11. (A) Western blotting analysis of the expression of the Es-DDB1 protein in different development stages of the testis in E. sinensis. The band intensities of the Es-DDB1 protein were normalized against that of β-actin. (B) Values expressed as relative values. Statistical significance is indicated with lowercase letters (a, b, and c).

Fig. 12. Immunofluorescence of Es-DDB1 in the testis of Eriocheir sinensis. Nuclei were stained with DAPI (blue), and target proteins were reacted with specific Es-DDB1 antibodies (green). a, Spermatogonium b, Spermatocytes Spermatid d, Spermatozoa. Scale bar in A, B and C = 8 μm (under 40× magnification), scale bar in D = 3 μm (under 100× magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
In Drosophila, a study also observed that DDB1 was transferred from cytoplasm to nucleus during spermatogenesis (Takata et al., 2002). Our results, together with those of previous studies, suggested strongly that Es-DDB1 plays a crucial role in early crustacean spermatogenesis.

In summary, the full-length cDNA of the Es-DDB1 gene from E. sinensis was obtained and registered in GenBank (GenBank accession: KX785224). We developed specific anti-Es-DDB1 and anti-Es-Cul4 antibodies. Both real-time PCR and Western blotting analysis indicated the Es-DDB1 was highly expressed in the testis of E. sinensis, particularly in the spermatocyte stage, but decreased in subsequent stages, which agreed with immunofluorescence analysis. In addition, we found that Es-DDB1 and Es-Cul4 interact in E. sinensis, probably forming a complex. Thus, we believe that Es-DDB1 plays a key role in early spermatogenesis development in E. sinensis.

Conflict of interest

We declare that we have no conflict of interest statement.

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