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# Involvement of the single *Cul4* gene of Chinese mitten crab *Eriocheir sinensis* in spermatogenesis

Yuan-Li Wang [1], Qing Li [1], Jing Xie, Ming Zhu, Wen-Juan Sun, Lin He *, Qun Wang *

*School of Life Science, East China Normal University, Shanghai, China*

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The Cullin-RING finger ligases (CRLs) are involved in the ubiquitin-mediated degradation of cell cycle regulators and play an important role in gametogenesis. Cullin 4 (CUL4) is a conserved core component of a new class of ubiquitin E3 ligase, and participates in the proteolysis of several regulatory proteins through the ubiquitin–proteasome pathway. The mammals encode two paralogs of CUL4, CUL4A and CUL4B, and the two *Cul4* genes are functionally redundant. However, Drosophila or other metazoans only contain one *Cul4* gene. Here we cloned the *Cul4* gene and confirmed that there is only one protein of CUL4 in *Eriocheir sinensis*, a full length *Cul4* comprised of 2777 nucleotides, an open-reading frame of 2373 bp encoding 790 amino acid residues. The expression level of *Cul4* mRNAs, as demonstrated by quantitative real-time PCR, varied significantly during testis development, with the greatest transcript levels found at an early stage. Localization analysis using antibodies against CUL4A/4B in the reproductive system showed that *Es*CUL4 mainly distribute in spermatogonia and primary spermatocytes, and gradually reduced during the development and maturation of sperm. The results indicated that a single CUL4 protein may play a role in spermatogenesis in *E. sinensis*.

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## 1. Introduction

The ubiquitin proteasome pathway (UPP) plays a critical role in a large number of fundamental biochemical processes, including regulation of the cell cycle, apoptosis, organelle biogenesis, tissue remodeling and signal transduction (Pickart, 2001; Pickart and Eddins, 2004; Varshavsky, 1997). Ubiquitin-dependent proteolysis is essential for virtually all steps of mammalian spermatogenesis (Sutovsky, 2003). The different steps of gametogenesis require a high turnover of the proteins that are involved in the control of meiosis and gamete maturation. The E3 ubiquitin ligases are the most diverse enzymes in the ubiquitination pathway due to their specificity in recognizing thousands of substrates. Database mining indicates that E3 ligase complexes can be grouped into three main families: Homologous to E6AP Carboxy Terminus (HECT), Really Interesting New Gene (RING) and UFD2 homology (U-box) proteins (Pickart and Eddins, 2004). The function of RING finger complexes is to catalyze the covalent attachment of ubiquitin to protein substrates to mark them for proteolysis (Tyers and Jorgensen, 2000). The Cullin-RING E3 ligases (CRLs) constitute the largest family of ubiquitin ligases in eukaryotic cells, due to the great diversity of their substrate-receptor subunits, and serve as vital regulators of a wide array of cellular processes (Petroski and Deshaies, 2005). There are three Cullins in yeast, five in *Drosophila* and *Arabidopsis* (CUL1 to CUL5), six in *Caenorhabditis elegans* (CUL1-6), and up to nine in humans. The human genome encodes six canonical human Cullin proteins (CUL1, CUL2, CUL3, CUL4A, CUL4B, and CUL5) (Sarikas et al., 2011; Skaar et al., 2007) and three atypical Cullin proteins [CUL7, CUL9 (also known as PARC) and APC2 (anaphase promoting complex subunit 2)], which are partially conserved with the canonical human Cullins (Nikolaev et al., 2003; Yu, 1998; Zachariae, 1998).

CRL activity can be regulated by numerous mechanisms, such as the turnover of substrate receptors and the reversible attachment of the ubiquitin-like protein Nedd8 to Cullins (neddylation). Of note, Nedd8 modification has now emerged as a regulatory pathway of fundamental importance for cell cycle control and for embryogenesis in metazoans (Higa et al., 2003; Hori et al., 1999). The CRLs are involved in the ubiquitin-mediated degradation of cell cycle regulators, and Cullin family members have been implicated in the proteolytic degradation of $G_1$-S regulators in *C. elegans*, supporting the possibility that the ubiquitin-associated genes induced at this time might participate in checkpoint regulation (Feng et al., 1999; Kipreos et al., 1996; Zhong et al., 2003). Recent findings suggested a similar function for CUL4, which regulates $G_1$ cell cycle progression by primarily targeting the CDK inhibitor, and these events appear to be conserved from *Drosophila* to humans (Higa et al., 2006).

*Abbreviations:* CRLs, Cullin-RING finger ligases; Nedd8, neural precursor cell-expressed developmentally downregulated 8; CUL4, Cullin 4; qRT-PCR, quantitative real-time PCR; UPP, the ubiquitin proteasome pathway; E3, ubiquitin-ligases; EST, expressed sequence tag; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; PFA, paraformaldehyde; IHC, immunohistochemistry; IF, immunofluorescence; H&E, hematoxylin and eosin; DAB, 3, 3′N-diaminobenzidine tertrahydrochloride; DAPI, 6-diamidine-2-phenylidole-dihydrochlo-ride; ORF, open reading frame; RACE, rapid amplification of cDNA end; SE, standard error; GSP, gene-specific primer.

* Corresponding authors. Tel.: +86 2124206934, +86 21 24206614.
*E-mail addresses:* lhe@bio.ecnu.edu.cn (L. He), qwang@bio.ecnu.edu.cn (Q. Wang).

[1] Both authors contributed equally to this work.

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Most related studies that emerged in the last decade have emphasized the role of ubiquitin-dependent proteolysis in fertilization and sperm maturation during the epididymal passage, while more current work has focused on the essential role of ubiquitin-dependent proteolysis for virtually all steps of mammalian spermatogenesis (Sutovsky, 2003; Sutovsky et al., 2001). *Cul4* silencing by RNAi in *C. elegans* results in DNA re-replication in blast cells, and male germ cells undergo premature spermatogenesis, since CUL4 prevents aberrant re-initiation of DNA replication by facilitating the degradation of CDT-1 (Zhong et al., 2003). Recent findings have also suggested that CUL4A functions in murine spermatogenesis and showed that germline deletion of *Cul4A* leads to male infertility (Kopanja et al., 2011; Yin et al., 2011). In mammals, two *Cul4* genes, *Cul4A* and *Cul4B* sharing 80% sequence homology, are broadly co-expressed and assemble structurally similar ubiquitin ligases with some functional redundancy (Higa et al., 2003; Hu et al., 2004; Kipreos et al., 1996). However, such as *D. melanogaster* and *C. elegans* only contain one *Cul4* gene. Several Cul4 genes exciting in crustaceans have not been reported in any literature. Here, we cloned the *Cul4* gene from the testes of *Eriocheir sinensis* and characterized specific aspects of the role of the CUL4 in gametogenesis, particularly in spermatogenesis.

The Chinese mitten crab (*E. sinensis*) is a catadromous crustacean and one of the most important aquaculture species in China, with a high commercial value as a food source (Wang et al., 2006). Relative to mammals, mitten crabs require more complicated environments to induce mating and spawning, and unique regulatory mechanisms are involved in crustacean reproduction (Rudnick et al., 2003). However, the problem of precociousness is prevalent and leads to large economic losses for the crab aquaculture industry (Hou et al., 2010). Hence, a better understanding of the molecular mechanisms of spermatogenesis to improve *E. sinensis* product quality has become an immediate research priority. Based on previous work in our lab, we have determined that ubiquitin/ribosomal fusion and SUMOylation genes play key roles in gametogenesis and reproductive success in *E. sinensis* (Wang et al., 2012a, 2012b). We had successfully constructed an expressed sequence tag (EST) cDNA library (Jiang et al., 2009; Zhang et al., 2011) and transcriptome library (He et al., 2012) from the testis in *E. sinensis*. Despite the essential role of the ubiquitin/proteasome pathway (UPP) system in reproduction, however, the description of Cullin proteins in development of the testis, vas deferens and seminal vesicle of crustaceans is not yet well established.

The aim of the current study was to determine the number and potential roles of *Cul4* genes in the male reproductive system of *E. sinensis*, which consists of a pair of testes, vas deferens, seminal vesicles, accessory glands and ejaculatory ducts. The spermiogenesis of *E. sinensis* has three major stages, including early stage, middle stage and late stage (Wang and Yang, 2010). The testis of the immature crab during rapid development mainly contains spermatogonia (early stage), primary spermatocytes and secondary spermatocytes (middle stage), while the majority of the spermatogenic cells in the mature crab are spermatids and spermatozoa (late stage) (Sun et al., 2012). After obtaining the full-length cDNA of *Cul4* from *E. sinensis* (*EsCul4*), we tested its expression pattern in the developing testis and localization during different developmental stages of spermatozoon in the testis by histological analysis using a monoclonal antibody against CUL4A/4B. *Es*CUL4 was mainly detected in spermatogonia and primary spermatocytes. The results indicated that a single CUL4 protein may be involved in regulating spermatogenesis in *E. sinensis*. Such insight into the *EsCul4* gene and its expression during spermatogenesis is important for understanding the molecular mechanisms of *E. sinensis* development and reproductive biology.

## 2. Materials and methods

### 2.1. Animal and tissue preparation

Healthy sexually mature female and male *E. sinensis* (100–150 g) were obtained from a commercial crab farm (Caojing Town special aquaculture farm in the Jinshan District near Shanghai, China). We obtained three crabs every month during July, August, September, October, November, December in 2012, and January in 2013, respectively. Crabs were placed in an ice bath for 3–5 min until lightly anesthetized prior to sacrifice. The following ten tissues from three different individuals were removed surgically and frozen in liquid nitrogen immediately for storage at −80 °C prior to nucleic acid and protein extraction: stomach, thoracic ganglia, muscle, hepatopancreas, gill, heart, intestine, hemocytes, testis, and ovary (testes and ovaries were all obtained in September 2012 during the rapid growth period for testis and ovarian development).

Based on the testicular classification described by Du et al. (1988), spermatogenesis in seminiferous tubules can be divided into five stages: I, spermatogonium stage (May to June); II, spermatocyte stage (July to August); III, spermatid stage (August to October); IV, sperm stage (October to April); and V, rest stage (April to May). For the present study, testicular stages were further sub-divided based on the month of dissection: Stage II-1 (July); Stage II-2 (August), Stage III-1 (September); Stage III-2 (October); Stage IV-1 (November); Stage IV-2 (December); and Stage IV-3 (January).

### 2.2. RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from *E. sinensis* tissues according to the manufacturer's protocol of the RNA Extraction Kit (Axygen, USA). The concentration and quality of total RNA were estimated by spectrophotometry at an absorbance of 260 nm (Eppendorf Biophotometer Plus, Germany) and agarose gel electrophoresis (Bio-Rad PowerPac Basic, USA). Total RNA (5 μg) isolated from the testes was reverse transcribed using the SMART™ cDNA kit (Clontech, USA) for rapid amplification of cDNA end (RACE) cloning. For quantitative real-time PCR (qRT-PCR) expression analysis, total RNA (3 μg) was reverse transcribed using the PrimeScript™ Real-time PCR Kit (TaKaRa, Japan).

### 2.3. Putative Cul4B cDNA full-length RACE cloning

To obtain the total coding sequence of the potential *E. sinensis* Cul4B (tentatively named according to analysis by the BLAST X program) cDNA sequence, 5′ and 3′ RACE (SMART™ cDNA kit, Clontech) were performed using gene-specific primers (Cul4B-5′, Cul4B-3′; Table 1) designed according to the testis transcriptome library from *E. sinensis* (He et al., 2012). Using a Mastercycler gradient thermal cycler (Eppendorf), PCR reactions were performed in a final volume of 25 μL, containing 2.5 μL of 10× PCR buffer, 2 μL of 10 mM dNTP mix, 2 μL of Universal Primer A Mix (UPM, Clonetech), 1 μL of 10 mM gene-specific primer,

**Table 1**
Primer sequences.

| Primer description | Sequences (5′–3′) | Primer name |
|---|---|---|
| Sequencing | ATTTAGGTGACACTATAGAA | SP6 |
| | TAATACGACTCACTATAGG | T7 |
| Clontech™ Kit primers | CTAATACGACTCACTATAGGGCAAGC | Long |
| Universal primer A mix | −AGTGGTATCAACGCAGAGT | |
| | CTAATACGACTCACTATAGGGC | Short |
| Nested universal primer | AAGCAGTGGTATCAACGCAGAGT | |
| Primers for RACE PCR | | |
| 5′GSP primer | CTGATGCGGAAGAGTTGATTGGTG | Cul4B-5′ |
| 3′GSP primer | AAGAACGGTCAGGAGAAACCATCG | Cul4B-3′ |
| 5′GSP primer | CGGTCGATGAGGGACTCTAT | Cul4A-5′ |
| 3′GSP primer | TGGAACGGGACAAGGACA | Cul4A-3′ |
| Degenerate primers targeting Cul4A | | |
| Forward primer | TGTTCCAGGACCGGcartaycarat | Cul4A-DF |
| Reverse primer | TGGGGTTGTCCTTGTCCCkytccatrtart | Cul4A-DR |
| Primers for qRT-PCR analysis | | |
| CUL4 5′ primer | TTGATGCTGCCATTGTTCGG | Cul4-RT-F |
| CUL4 3′ primer | CTCGCTCCATGTAGTCTCGGTC | Cul4-RT-R |
| β-actin 5′ primer | CTCCTGCTTGCTGATCCACATC | Actin-F |
| β-actin 3′ primer | GCATCCACGAGACCACTTACA | Actin-R |

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