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Involvement of the fusome-related protein Add1 in spermatogenesis of the Chinese mitten crab (*Eriocheir sinensis*) by organization of cytoskeleton

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Abstract
The sperm nucleus of the decapod crustacean Chinese mitten crab, *Eriocheir sinensis*, is loose and fibrous, which is a non-condensed nucleus. Our purpose was to analyze the structural distribution of the fusome-related protein Add1 during the spermatogenesis in *E. sinensis* to better understand the non-condensed nucleus of *E. sinensis*. RT-qPCR and western blot results showed that Add1 was expressed at the transcriptional and translational level during spermatogenesis of *E. sinensis*. Immunofluorescence results showed that Add1 was widely distributed in the process of spermatogenesis, including spermatogonia, spermatocytes, spermatids, and sperm. In the initial stage of sperm nucleus decondensation of *E. sinensis*, Add1 was widely expressed in the cytoplasm of spermatogonia. In the process from stage III spermatids to sperm, Add1 is mainly expressed in the nucleus. With the continuous differentiation of cells, Add1 gradually transferred from the cytoplasm to the nucleus. Our results indicated that the fusome-related protein Add1 might be involved in the spermiogenesis through the construction of a new nuclear skeleton to participate in the decondensation of sperm nuclei and the maintenance and protection of non-condensed chromatin.

Keywords Add1 · Cytoskeleton · *Eriocheir sinensis* · Spermatogenesis · Decondensed sperm nuclei

Spermatogenesis is the process of differentiation of reproductive stem cells into sperm, which requires the participation of a variety of cells, genes, hormones, and epigenetic regulation (Neto et al. 2016). Sertoli cells provide nutrition and structural support during spermatogenesis. They also participate in the metamorphosis process of germ cells through tyrosine-phosphorylation of their microtubule cytoskeleton (Usik and Ogneva 2018;

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Dunleavy et al. 2019). Fusome and spectrosome are derived from the endoplasmic reticulum, which are the same organelle with different shapes at different stages of cell differentiation (Isabel Mandelbaum 1980). Spectrosome appears spherical in germline stem cells, while fusome is a network of branch-shaped membranous tubules and generates in differentiated germ cells (Thomas Vaccari and Ephrussi 2002). Fusome/spectrosome is taken part in the differentiation of germ cells in *drosophila* testis as well as ovary (Erik L Snapp et al. 2004; Lisa N Petrella et al. 2007). Add1 is an important component of fusome and participates in the cytoskeleton organization. Add1 is able to interact with Myo10 (myosin X) to be involved in mitosis and meiosis in germ cells (Brieno-Enriquez et al. 2017). The fusome-related protein Add1 also is a protein binding to both actin and spectrin complex, which may impact on the stability of the skeletal protein through Ca^{2+} and calmodulin, thereby determining the morphology of the cell (Kim and Spiegelman 1996; Kiang and Leung 2018; Gupta et al. 2019). The *Add1* gene was first studied in the differentiation of adipocytes. It was also found to be involved in the biological process of osteoblasts, blood vessel cells, and blood cells (Robledo et al. 2008; Zhao et al. 2011; Gemini-Piperni et al. 2014). Abnormal phosphorylation and genetic variation including alternative splicing of Add1 cause the pathogenic process, such as cardiovascular diseases (Kiang and Leung 2018; Gupta et al. 2019). Add1 binds to mRNAs and some non-coding RNAs which are modified by N6-methyladenosine (m6A) (Dominissini et al. 2012; Wang et al. 2014). The sperm nucleus of decapod crustacean Chinese mitten crab, *Eriocheir sinensis*, is non-condensed and its genetic material is loose (Wu et al. 2015). Unlike mammalian condensed nuclear sperm that protect the stability of genetic material through a condensed state, the non-condensed-nuclear sperm are very susceptible to damage due to the relatively loose genetic material. Therefore, this type of sperm may have their own unique protective mechanism. As a special organelle composed of membrane and skeletal proteins, the fusome has the protective function of membrane and skeleton. Therefore, as an important component of fusome, Add1 may participate in the protection of the genetic material during the spermatogenesis by organization of cytoskeleton. However, the study on Add1 in the *E. sinensis* testes has not yet been reported. Accordingly, this study intends to analyze the distribution of Add1 in *E. sinensis* testes and its possible functions, using RT-qPCR, western blot, and immunofluorescence (IF) techniques and *E. sinensis* testes as the research material. The research results can provide the theoretical basis for the molecular mechanism of cell differentiation and specialization in spermatogenesis, as well as provide theoretical data for healthy reproduction and breeding protection.

Materials and methods

Material

Mature male *E. sinensis* were purchased from Baise City, China. Thirty crabs of 100–150 g were selected, which were healthy, vigorous. The animals were anesthetized by cooling on ice under the condition of − 20 ℃ for about 10 min, and then immediately dissected to obtain testes. The study of this *E. sinensis* in China does not require any official approval because the crabs are common aquatic animals used as food in China.

Main reagents and instruments Total RNA extraction kit was purchased from Beijing Soleibao Technology Co., Ltd. (Beijing, China), Thermo Scientific RerertAid First-strand cDNA Synthesis kit was purchased from Thermo Fisher Technology Co., Ltd. (shanghai,

China), Blaze Taq SYBR® Green qPCR mix was purchased from Yijin Biotechnology Co., Ltd. (Guangzhou, China). Anti-alpha Adducin antibody [EP734Y] (ab40760), anti-beta-actin antibody [mAbcam 8226]-Loading Control (ab8226), anti-Ddx4/MVH antibody [mAbcam27591] (ab27591), goat anti-mouse IgG H&L (HRP)(ab6789), goat anti-mouse (Alexa Fluor 594) (ab150116), goat anti-rabbit IgG H&L (HRP) (ab6721), and goat anti-rabbit IgG H&L (Alexa Fluor488) (ab150077) were purchased from Abcam company (Shanghai, China). The primer of *Add1* and *β-actin* for RT-PCR were designed by Primer-blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and then were synthesized by the Sangon company (Shanghai, China). RT-PCR was carried out using Roche LightCycler96 RT-qPCR instrument (Roche Enterprise Co., Ltd., USA). Immunofluorescence localization was performed using ultra-sensitive multi-function imager (Situofan, USA) and laser confocal microscope (Olympus, Japan).

Method

RNA extraction and sequencing

Take 100 mg of each testis tissue from the sample, extract total RNA using RNA extraction kit, and send it to Shenzhen Huada Gene Co., Ltd. for sequencing. RNA quality detection, reverse transcription, library construction, and processing of sequencing data were performed according to standard procedures.

Add1 protein tertiary structure prediction and phylogenetic tree construction

The tertiary structure model of the protein was constructed using the SWISS-MODEL homology modeling method (https://swissmodel.expasy.org/interactive). BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) alignment of the protein sequence was performed, sequence analysis was performed using MAGA 7.0 software, and the evolutionary history was inferred using the neighborhood connection method to construct a system Evolutionary tree.

RT-qPCR to detect the expression of Add1 gene

The total RNA in the testis tissues of each sample was reverse-transcribed to synthesize cDNA and then detected by real-time fluorescent quantitative PCR. Setting procedure: two-step method, pre-denaturation at 95 °C for 30 s; denaturation at 95 °C for 5 s; annealing/extension at 60 °C for 30 s; 40 cycles. The melting curve reaction program is set according to the program recommended by the Roche LightCycler96 RT-qPCR instrument, and the

Table 1 The primers of genes used in the study for RT-qPCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Add1	CTCATTCTGCGGAATCACGGACTC	TGGATCTCACATGCAACCACA AGG
β-actin	ACCTCGGTTCTATTTTGTCGG	ATGCTTTCGCAGTAGTTCGTC

Fig. 1 The mRNA sequence of *Add1* and its translated amino acid sequence of *Eriocheir sinensis* testes ▶

relative quantitative value is calculated by $2^{-\Delta\Delta Ct}$. The primers used here has been listed in the Table 1.

Western blotting to detect the expression of Add1 protein

Lysate of 1 ml was added into each 50–100 mg of testis tissue to lyse the tissue cells. And then the cell lysates were centrifuged at 12000 *g* for 10 min at 4 °C to extract total protein solution. The protein solution was quantified by BCA method. The SDS-PAGE and western blotting referred to the method Ling (Ling et al. 2020). The ultra-sensitive multifunction imager was used for imaging and subsequent quantitative analysis.

Immunofluorescence localization to detect the expression of add1 protein

Take fresh adult *E. sinensis* testis tissues and fix them with formaldehyde immediately, and make paraffin sections. After deparaffinization, rehydration, and antigen retrieval, the slides were incubated overnight at 4 °C with the primary antibody and then incubated with a fluorescent secondary antibody for 1 h in the dark under room temperature. The sections were observed under a confocal microscope, following staining with fluorescent cell nuclear dye.

Statistical analyses

SPSS 13.0 statistical software was used for analysis, and the measurement data were expressed as mean ± sd. After normal distribution and chi-square test, two independent samples *t*-test were used. The mean fluorescence intensity was compared between different cells using ANOVA for a two-by-two comparison. The statistically significant difference was considered at $P < 0.05$ in the present study.

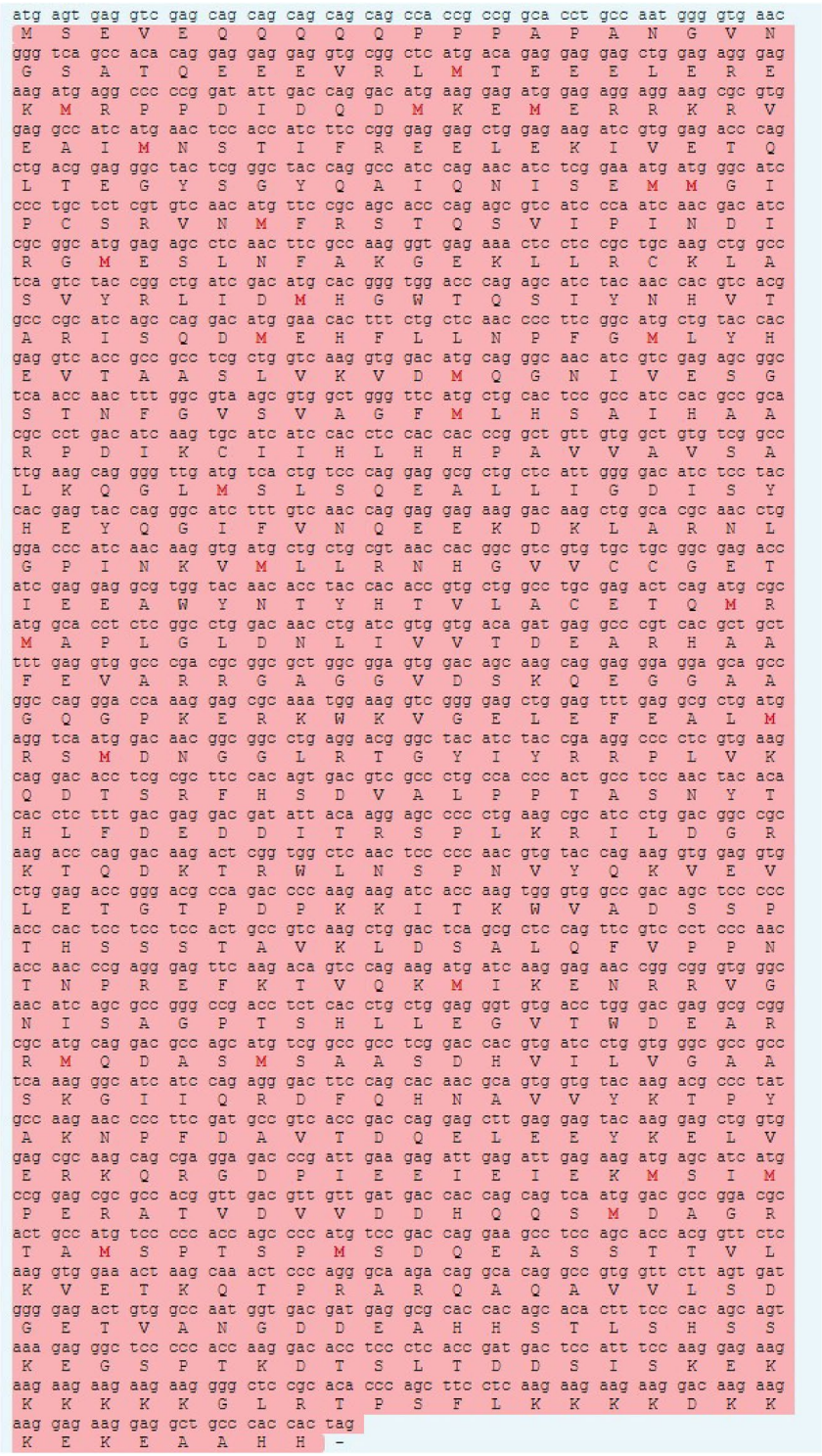
Results

The *Add1* mRNA sequence with 2247 bases was obtained by sequencing (Fig. 1). The ORF of *Add1* is translated into a protein and contains 748 amino acids, using the Swiss model. A three-dimensional model of *Add1* protein was construed (Fig. 2).

Phylogenetic tree analysis showed that Decapoda *E. sinensis* and Decapod *Penaeus vannamei* clustered together, *Hymenoptera* bees clustered with *Coleoptera Cerambycidae*, and *Diptera fruit flies* clustered with *Diptera mosquitoes*. It is one group. All of the above species are grouped with *lepidopteran hanging silkworms* and then grouped with *lepidopteran moths* and *butterflies*. Among them, the Add1 protein of Chinese mitten crab has the highest homology with *Penaeus vannamei*, followed by *Cerambycidae*, bees, mosquitoes, etc.

The expression of Add1 in the testis tissues of *E. sinensis* RT-qPCR results showed that Add1 mRNA was expressed in the adult crab testes (Fig. 3). Similar to the RT-qPCR results, the WB results showed that Add1 protein was also expressed in the adult *E. sinensis* testes (Fig. 3).

Immunofluorescence localization results showed that the fluorescence of Add1 protein antibody was widely distributed in various types of cells in the adult *E. sinensis* testis, but the fluorescence intensity of different cells was diverse (Fig. 4). The fluorescence intensity of various types of cells was from strong to weak: spermatogonia, stage I spermatids,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G S A T Q E E E V R L M T E E E L E R E
aag atg agg ccc ccg gat att gac cag gac atg aag gag atg gag agg agg aag cgc gtg
K M R P P D I D Q D M K E M E R R K R V
gag gcc atc atg aac tcc acc atc ttc cgg gag gag ctg gag aag atc gtg gag acc cag
E A I M N S T I F R E E L E K I V E T Q
ctg acg gag ggc tac tcg ggc tac cag gcc atc cag aac atc tcg gaa atg atg ggc atc
L T E G Y S G Y Q A I Q N I S E M M G I
ccc tgc tct cgt gtc aac atg ttc cgc agc acc cag agc gtc atc cca atc aac gac atc
P C S R V N M F R S T Q S V I P I N D I
cgc ggc atg gag agc ctc aac ttc gcc aag ggt gag aaa ctc ctc cgc tgc aag ctg gcc
R G M E S L N F A K G E K L L R C K L A
tca gtc tac cgg ctg atc gac atg cac ggg tgg acc cag agc atc tac aac cac gtc acg
S V Y R L I D M H G W T Q S I Y N H V T
gcc cgc atc agc cag gac atg gaa cac ttt ctg ctc aac ccc ttc ggc atg ctg tac cac
A R I S Q D M E H F L L N P F G M L Y H
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S T N F G V S V A G F M L H S A I H A A
cgc cct gac atc aag tgc atc atc cac ctc cac cac ccg gct gtt gtg gct gtg tcg gcc
R P D I K C I I H L H H P A V V A V S A
ttg aag cag ggg ttg atg tca ctg tcc cag gag gcg ctg ctc att ggg gac atc tcc tac
L K Q G L M S L S Q E A L L I G D I S Y
cac gag tac cag ggc atc ttt gtc aac cag gag gag aag gac aag ctg gca cgc aac ctg
H E Y Q G I F V N Q E E K D K L A R N L
gga ccc atc aac aag gtg atg ctg ctg cgt aac cac ggc gtc gtg tgc tgc ggc gag acc
G P I N K V M L L R N H G V V C C G E T
atc gag gag gcg tgg tac aac acc tac cac acc gtg ctg gcc tgc gag act cag atg cgc
I E E A W Y N T Y H T V L A C E T Q M R
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M A P L G L D N L I V V T D E A R H A A
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F E V A R R G A G G V D S K Q E G G A A
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G Q G P K E R K W K V G E L E F E A L M
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R S M D N G G L R T G Y I Y R R P L V K
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cgc atg cag gac gcc agc atg tcg gcc gcc tcg gac cac gtg atc ctg gtg ggc gcc gcc
R M Q D A S M S A A S D H V I L V G A A
tca aag ggc atc atc cag agg gac ttc cag cac aac gca gtg gtg tac aag acg ccc tat
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E R K Q R G D P I E E I E I E K M S I M
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P E R A T V D V V D D H Q Q S M D A G R
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aag gtg gaa act aag caa act ccc agg gca aga cag gca cag gcc gtg gtt ctt agt gat
K V E T K Q T P R A R Q A Q A V V L S D
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K E G S P T K D T S L T D D S I S K E K
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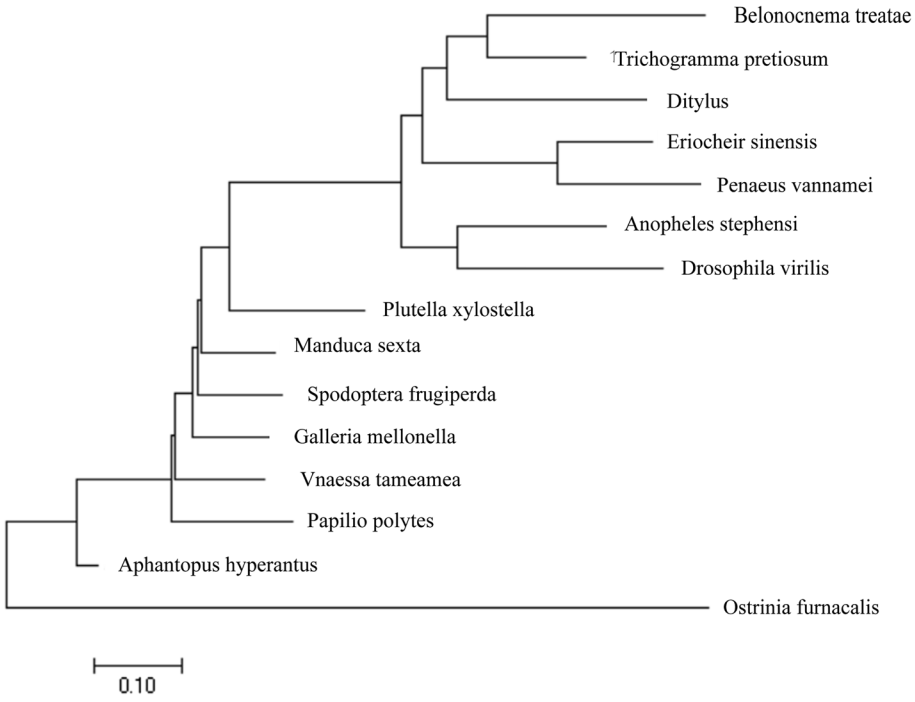

Fig. 2 Phylogenetic tree analysis of Add1 protein in *Eriocheir sinensis* testes

spermatocytes, stage II spermatids, stage III spermatids, and sperm. Between the 6 type of cells, except for between spermatocytes and stage I spermatids or stage II spermatids, between stage I spermatids and stage II spermatids, between stage II spermatids and stage III spermatids, between sperm, stage III spermatids and sperm, the differences between any two cell fluorescence intensity were statistically significant ($n=3$, $P<0.05$). This result indicated that the Add1 protein in the adult *E. sinensis* testis had the most expression in spermatogonia, and the others in descending order were stage I spermatids, spermatocytes, stage II spermatids, stage III spermatids, and sperm. As for the subcellular location, Add1 in spermatogonia, spermatocytes, stage I spermatids, and Add1 in stage II spermatids were expressed in the cytoplasm, cell membrane, intercellular substance, extracellular matrix, and a few in the nucleus. Add1 in stage III spermatids was mainly located in the nucleus and hardly in the cytoplasm. In sperm, the Add1 protein was mainly expressed in the middle of the nucleus and few in the cytoplasm. Figure 5 showed the pattern of spermatogenesis at each stage, and the orange rectangle shows the distribution of Add1 protein.

Discussion

The spermatogonial stem cells divide and then differentiate from spermatogonia to spermatocytes to spermatids and finally metamorphose and specialize to sperm (Sperry 2012). In this process, the morphology and structure of cells undergo obvious changes, and the cytoskeleton synchronously undergoes dramatic changes. The fusome/

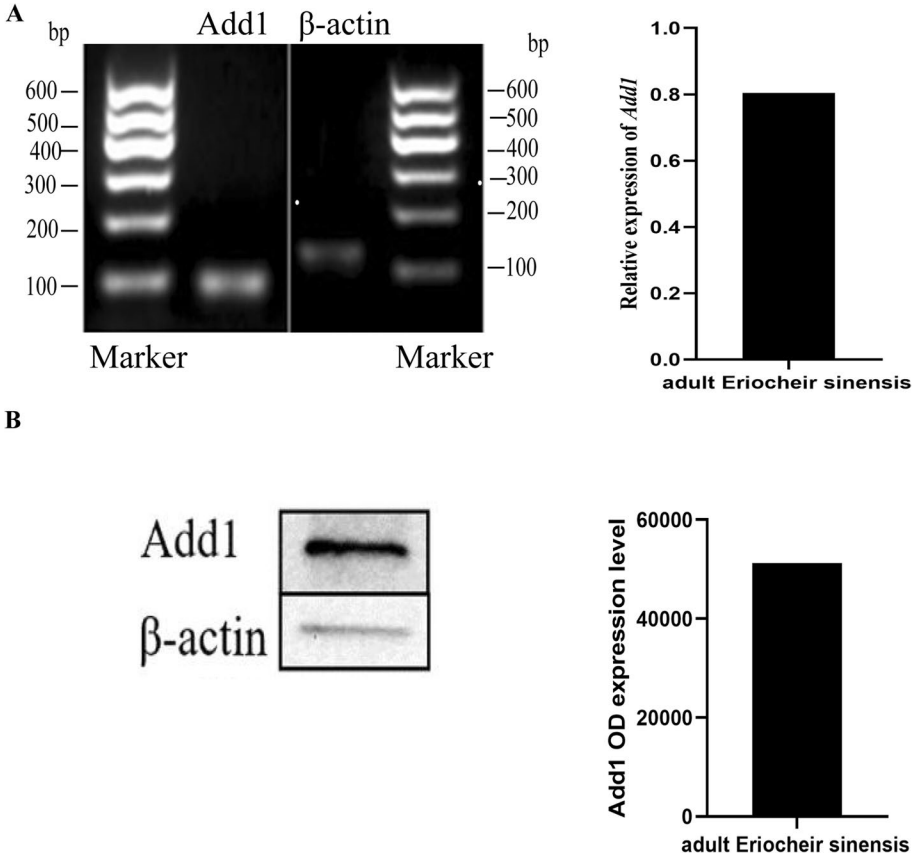

Fig. 3 Agarose gel electrophoresis results of RT-qPCR product of *Add1* gene (**A**) and WB of Add1 protein (**B**) in *Eriocheir Sinensis* testes ($n=3$)

spectrosome is composed of cytoskeleton proteins and membrane tubules. The cytoskeleton proteins include the adductor-like protein Hu-li tai shao (encoded by Hts). Ankyrin and α and β spectrin together construct the core of fusome/spectrosome (Lighthouse et al. 2008; Lin et al. 1994; Lin and Spradling 1995). Fusome and spectrosome are germ cell-specific organelles, which alter from sphere of spectrosome to branch shape of fusome during germ cell differentiation (McKearin, 1997). Mutations in both Hts and α-Spc genes can cause fusome/spectrosome to disappear, indicating that fusome/spectrosome is a key component that regulates syncytial mitosis and cell differentiation (McKearin, 1997). Add1 is a member of the basic helix-loop-helix leucine zipper (TFE bHLH-LZ) transcription factor family. Add1 is directly involved in the biogenesis of cell morphology as a cytoskeletal protein. The simultaneous expression of Add1 with peroxisome proliferator-activated receptor γ (PPARγ) in adipocytes enhances transcriptional activity of the adipogenic nuclear hormone receptor and participates in the differentiation of adipocytes (Kim and Spiegelman 1996). Phosphorylation of *Add1* and activation of Cdk5 can cause epidermal growth factor (EGF) to induce cell migration and invasion (Su et al. 2019). Add1 is distributed in the nucleus, nucleoplasm, cytoplasm, cytoskeleton, plasma membrane, cell adhesion junction, focal adhesion, and

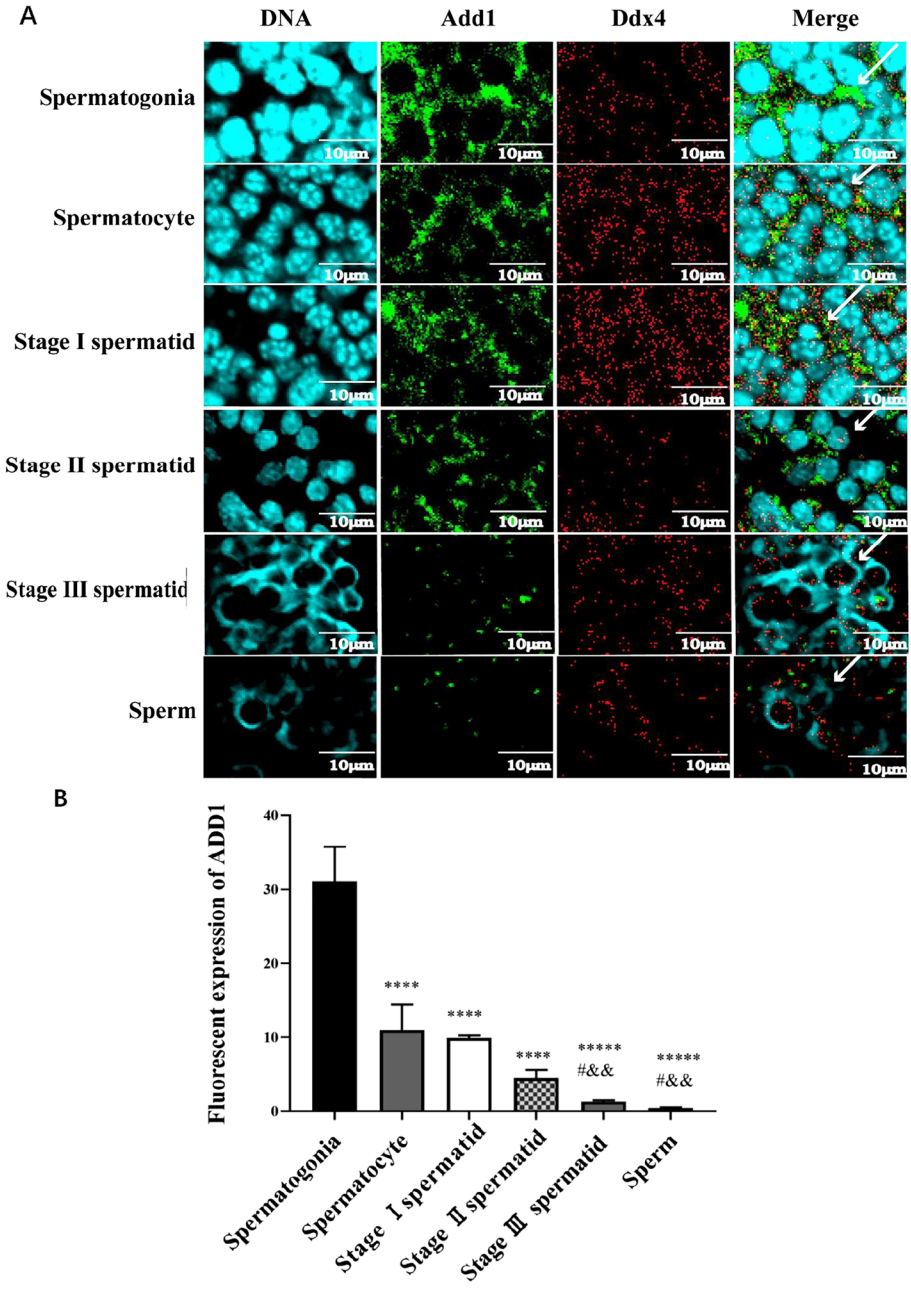

Fig. 4 Expression level and location of Add1 in diversity cells in *Eriocheir sinensis* testes. Note: **A** Immunofluorescence localization; **B** immunofluorescence quantitative analysis results. *Compared with spermatogonial: $*P<0.05$, $**P<0.01$, $****P<0.001$; #Compared with spermatocyte: ## $P<0.01$, ### $P<0.001$; & Compared with stage I spermatid: & $P<0.01$, && $P<0.01$; Ddx4 is a marker protein of male germ cells; the scale of immunofluorescence was 10 μm. IF of Add1 in *Eriocheir sinensis* testes ($n=3$)

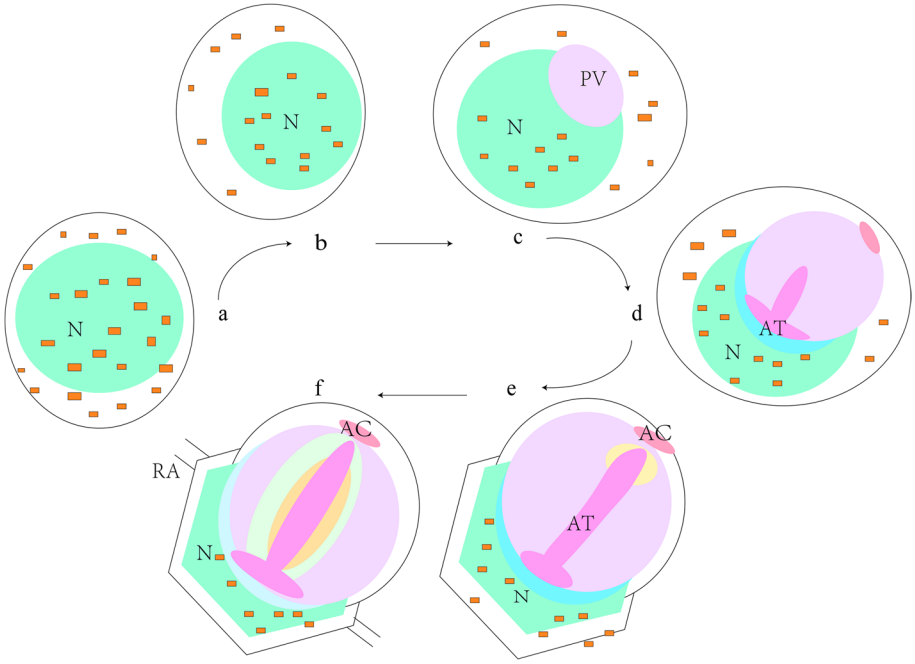

Fig. 5 Schematic diagram of Add1 positioning in *Eriocheir sinensis* testes. Note: N, nucleus; PV, proacrosomal vesicle; AC, acrosome cap; AT, acrosome tube; MC, membrane complex; RA, radical arm

other cellular components. It provides physical support for the plasma membrane and mediates signal transduction in various cellular physiological processes after combining with spectrin and then being regulated by protein kinase C and calcium/calmodulin-dependent pathways (Kiang and Leung 2018). Add1 is involved in biological processes of cell morphogenesis, embryonic development in the uterus, cell volume homeostasis, hemoglobin metabolism, red blood cell differentiation, negative regulation of actin filament polymerization, positive regulation of protein binding, multicellular biological growth, cell–cell adhesion, and so on.

Our results showed that the distribution of Add1 were different between the developmental stages of male germ cells in the testis tissues in the Chinese mitten crab. Add1 is mainly expressed in the cytoplasm of spermatogonia and spermatocytes. The first- and second-stage spermatids are mainly expressed in the cytoplasm. In the third stage of the process from spermatids to sperm, Add1 is mainly expressed in the nucleus and at a point in the sperm nucleus in the sperm. Therefore, in the early stage of sperm maturation, Add1 is likely to promote chromatin on the cytoskeleton in the sperm nucleus. Protein synthesis, after the sperm matures, gathers in the sperm nucleus to play a role. The non-condensed state of the sperm nucleus of *E. sinensis* is also closely related to the synthesis of chromatin. Therefore, we speculate that Add1 may play a role in the process of the loose state of the sperm nucleus of *E. sinensis*.

In the process from stage III spermatids to sperm, Add1 is mainly expressed in the nucleus. Therefore, Add1 is likely to participate in the spermiogenesis, and through the construction of a new nuclear skeleton to participate in the decondensation of sperm nuclei and the maintenance and protection of non-condensed chromatin.

Add1 is an actin binding protein. The interaction of Add1 phosphorylated at S726 with the Xklp2 target protein (targeting protein for Xklp2, TPX2) can maintain the polarity and integrity of the spindle during mitosis (Hsu et al. 2018). After being phosphorylated by cyclin-dependent kinase 1, ser12 and ser355 on Add1 play an important role in the assembly of mitotic spindle by binding to myosin X. The correct assembly of the spindle is closely related to the correct development of mitosis (Chan et al. 2014). Add1 often functions as a heterodimer and can also bind to Add2 or Add3 subunits (Kiang and Leung 2018). These complexes can bind to fascin-1 (FSCN1) (Tang et al. 2016). FSCN1 is a membrane cytoskeleton protein. Both Add1 and FSCN1 are involved in inducing the formation and stabilization of F-actin (filamentous actin). F-actin is involved in cell division, cytoplasmic flow, and regulation of activity of actin filaments. In addition, cell division depends on the cytoskeleton during spermatogenesis, involving regulatory proteins that regulate cytoskeletal organization (Li et al. 2017). During the spermatogenesis process, the transport of spermatogenic cells across the seminiferous epithelium requires cooperation of cell connections, signal proteins, and cytoskeleton based on F-actin and microtubules (MT). Add1 interacts with MTPN (myotrophin) to promote NF-κB (nuclear factor-κB) subunit dimerization and regulates the activity of NF-κB transcription factors, and regulates the growth of actin filaments. Actin is an important part of a peripheral protein, and peripheral protein and integrin together constitute membrane protein (Chan et al. 2014; Wilson 2005). Membrane proteins are embedded in the cell membrane; Add1, integrin, and peripheral protein together participate in the membrane cortex skeleton and cell–cell adhesion (Schrier, 1985). Add1 is expressed in spermatogonia, spermatocytes, spermatids, and sperm in the *E. sinensis.* It also shows that Add1 participates in a wide range of biological functions, and is generally involved in the construction of the cytoskeleton during spermatogenesis. Add1 protein may also be co-expressed with Casp3, Add2, Add3, Capza2, Srebf1, Capg, and other co-expressed proteins to participate in diversity biological processes. The wide and differentiated expression of Add1 protein in the *E. sinensis* testes demonstrated that Add1 played important roles during spermatogenesis and provided a material basis for diversity male germ cells to construct specific cell membrane skeletons, cytoplasmic skeletons, and even nuclear skeletons and adapt to meet the various biological processes during spermatogenesis.

The analysis of phylogenetic tree indicated that *E. sinensis* had the highest homology with *Penaeus vannamei*, followed by *Oedemeridae*, bees, and mosquitoes in the phylogeny, and the Add1 protein can be used as a basis for phylogenetic evolutionary analysis.

Add1 is a cytoskeletal protein, which can construct the cytoskeleton for attachment, transportation, and stabilization of other molecules, so it may play an important role in the protection of loose genetic material in the sperm nucleus of the Chinese mitten crab. However, it is unclear what kind of structure it constitutes in protecting non-enriched nuclear genetic material, whether it is FUSOME or other new skeleton structures. Due to the particularity of the protection mechanism of the nuclear genetic material of non-condensed nuclear sperm, a clear study of this mechanism will help to provide a theoretical basis for the germplasm protection of non-condensed nuclear sperm species and the development of aquaculture. Therefore, this will be a very interesting and meaningful research content, and it will be a direction for our further research.

In summary, Add1 was widely distributed in the process of spermatogenesis, including spermatogonia, spermatocytes, spermatids, and sperm. In the initial stage of sperm nucleus decondensation of *E. sinensis*, Add1 was widely expressed in the cytoplasm of spermatogonia. In the process from stage III spermatids to sperm, Add1 is mainly expressed in the

nucleus. With the continuous differentiation of cells, Add1 gradually transferred from the cytoplasm to the nucleus. Our results indicated that the fusome-related protein Add1 might be involved in the spermiogenesis through the construction of a new nuclear skeleton to participate in the decondensation of sperm nuclei and the maintenance and protection of non-condensed chromatin.

Author contribution SL performed sequencing PCR experiment and analyzed data and writing. NA performed most experiments and data analysis. TY and LS were responsible for analyzing the experimental data. LG conceived and designed the study, provided guidance of experiment and paper writing, and prepared the experimental reagents and materials.

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Data availability All data during this study are available from the author.

Code availability Not applicable.

Declarations

Ethical approval. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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