

Characterization and Expression of Mouse *Cdc50c* during Spermatogenesis

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Abstract Cdc50p is a transmembrane protein required for polarized growth in *Saccharomyces cerevisiae*. The knowledge on physiological functions of its mammalian homologs, however, is limited. Using database analysis, we identified one mouse testis expressed sequence tag, named *Cdc50c*, encoding a previously uncharacterized homolog of Cdc50p. Similar to yeast Cdc50p, the putative Cdc50c protein contains three transmembrane spanning regions. Its orthologs are present in many species such as fish, avian and human, suggesting its evolutionary conservation. In multitissue reverse transcription-polymerase chain reaction analyses the mRNA for Cdc50c was predominately detected in testis. The onset of the gene expression coincides with the first appearance of spermatocytes during testicular development. *In situ* hybridization analyses revealed that *Cdc50c* mRNA localized in pachytene spermatocytes and round and elongated spermatids. Our data suggest that *Cdc50c* might play important roles during spermatogenesis.

Keywords spermatogenesis; infertility; testis; cell polarity; *Cdc50c*

Cell polarity is an essential feature of many animal cells. It is the ultimate reflection of complex mechanisms that establish and maintain functionally specialized domains in the plasma membrane and cytoplasm. The specific proteins assembling a polarized and specialized cortical actin cytoskeleton are vital for the asymmetric organization along an appropriate axis [1,2].

How cells regulate actin cytoskeleton assembly is a topic studied widely in budding yeast, a commonly used model system. Recently, a novel transmembrane protein Cdc50p, localized to the late endosome, was found to be required for polarized cell growth in yeast [3]. Further studies suggest that Cdc50p functions in cell polarity by regulating phospholipid asymmetry and sterol structural integrity [4–7]. It is proposed that the lipid environment might be an important determinant of the sites onto which the actin patches are assembled [5].

However, it remains unclear whether mammalian homologs of Cdc50p have roles in reproduction. By searching for mouse homologs using database screening,

we found an expressed sequence tag named *Cdc50c* with high similarity to Cdc50p in the testis library. Here we report that *Cdc50c* was predominantly expressed in adult testis of mouse. Its mRNA expression level was up-regulated gradually from the second week after birth when the spermatocytes appear, suggesting that *Cdc50c* plays a role during and after meiosis. Furthermore, *in situ* hybridization revealed the localization of Cdc50c mRNA in pachytene spermatocytes and spermatids. Our data provide evidence that mouse Cdc50c is involved in spermatogenesis.

Materials and Methods

Animals

ICR mice were maintained at a constant temperature (22 °C) in a standard animal facility with free access to pelleted food and water (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). All experimental procedures were carried out according to the guidelines of the Institutional Animal Care and Use Committee of the Institute of Biochemistry

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Database analysis

The National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) was screened to identify orthologs of yeast Cdc50p in other species including rat, dog, chimpanzee, and human. Both cDNA and protein sequences were used as baits to search the NCBI nucleotide and protein databases, respectively.

Putative Cdc50c orthologs were then aligned using the ClustalW program (<http://www.ebi.ac.uk/Tools/clustalw>). The phylogenetic tree was constructed using the neighbor-joining algorithm in MEGA software [8]. Statistical support values for internal branches of the tree were obtained from the analysis of 1000 bootstrap samples.

Semiquantitative reverse transcription-polymerase chain reaction

The reverse transcription-polymerase chain reaction (RT-PCR) was carried out in a 20 μ l final volume containing 2 μ g total RNA, 1 \times RT buffer, 100 pmol random primer, 1 mM deoxynucleoside triphosphate, 10 U RNase inhibitor RNasOUT, and 200 U Moloney murine leukemia virus reverse transcriptase at 42 °C for 1 h. All RT reagents were purchased from Fermentas (Hanover, USA). Aliquots (2 μ l) of cDNA synthesized above were used as templates for a semiquantitative PCR analysis. Primers (upstream, 5'-TCTCTTCCTTTT-ACCTTCC-3'; downstream, 5'-CATACTTATCTG-TCCACCAT-3') were used, and a cDNA fragment of 316 bp was amplified. As a loading control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified using a primer set (upstream, 5'-CGGAGTCAACGGATTTGGTTCGTAT-3'; downstream, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3').

In situ hybridization

In situ hybridization was carried out as described previously [9]. Briefly, 4% paraformaldehyde fixed and paraffin embedded testes were cut into sections of 10 μ m. A fragment of nucleotides corresponding to *Cdc50c* nucleotides 625–1125 was subcloned into a pCS2+ vector. Digoxigenin-UTP-labeled sense and antisense riboprobes were generated using a Riboprobe *in vitro* Transcription System (Roche Applied Science, Indianapolis, USA). Hybridization signals were detected by color development of NBT (5-bromo-4-chloro-indolylphosphate) and BCIP (nitroblue tetrazolium chloride). The slides were then fixed and counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride.

Results

Characteristics of mouse *Cdc50c*

To obtain the clones of murine homologues of Cdc50p, the NCBI mouse database was analyzed by the BLAST program using yeast Cdc50p as a query sequence (GenBank accession No. NP_010018.1), and three homologs named *Cdc50a*, *Cdc50b* and *Cdc50c* were found (data not shown) [10]. We focused on *Cdc50c* (GenBank accession No. AK016597) because its cDNA was found only in the testis library. We isolated it by RT-PCR using oligonucleotide primers corresponding to this sequence (data not shown). It contained a 1026 bp open reading frame encoding a polypeptide of 342 amino acids [Fig. 1(A)]. The result of sequence alignment showed that mouse Cdc50c has high similarity with yeast Cdc50p (32%).

The structural prediction of putative Cdc50c shows that it contains three transmembrane regions with a large loop region [Fig. 1(B)]. Its topology is similar to that of yeast Cdc50p, except that the predicted third transmembrane domain is localized in the C-terminus [11].

As there are three Cdc50 homologs in mammals, they seem to originate from a common ancestor through gene duplication [12]. To gain insight into the evolution of the Cdc50 protein, we downloaded all protein sequences similar to Cdc50p. All of them were aligned and an unrooted phylogenetic tree was constructed as described in "Materials and Methods". As shown in Fig. 1(C), Cdc50c and Cdc50a are clustered as a group, indicating that they might have evolved through gene duplication, whereas Cdc50b is clustered as a separate branch. Moreover, Cdc50c orthologs are present in the genomes of many vertebrates, including fish, avian and mammalian, suggesting that it is evolutionary conserved [Fig. 1(C)].

Expression of mouse *Cdc50c* mRNA

To reveal the expression pattern of *Cdc50c* in adult mouse tissues, we used RT-PCR analysis. As shown in Fig. 2(A), *Cdc50c* was predominately present in testis with a faint band observed in brain.

Based on the tissue-specific expression pattern of *Cdc50c*, we then examined the expression levels of *Cdc50c* mRNA in testis during testis development using a semiquantitative RT-PCR analysis. Low expression of *Cdc50c* was observed in 2-week-old immature testis [Fig. 2(B)]. After 3 weeks, a major increase in expression was observed, keeping its expression till adult [Fig. 2(B)]. This onset of its expression correlates with the accumulation

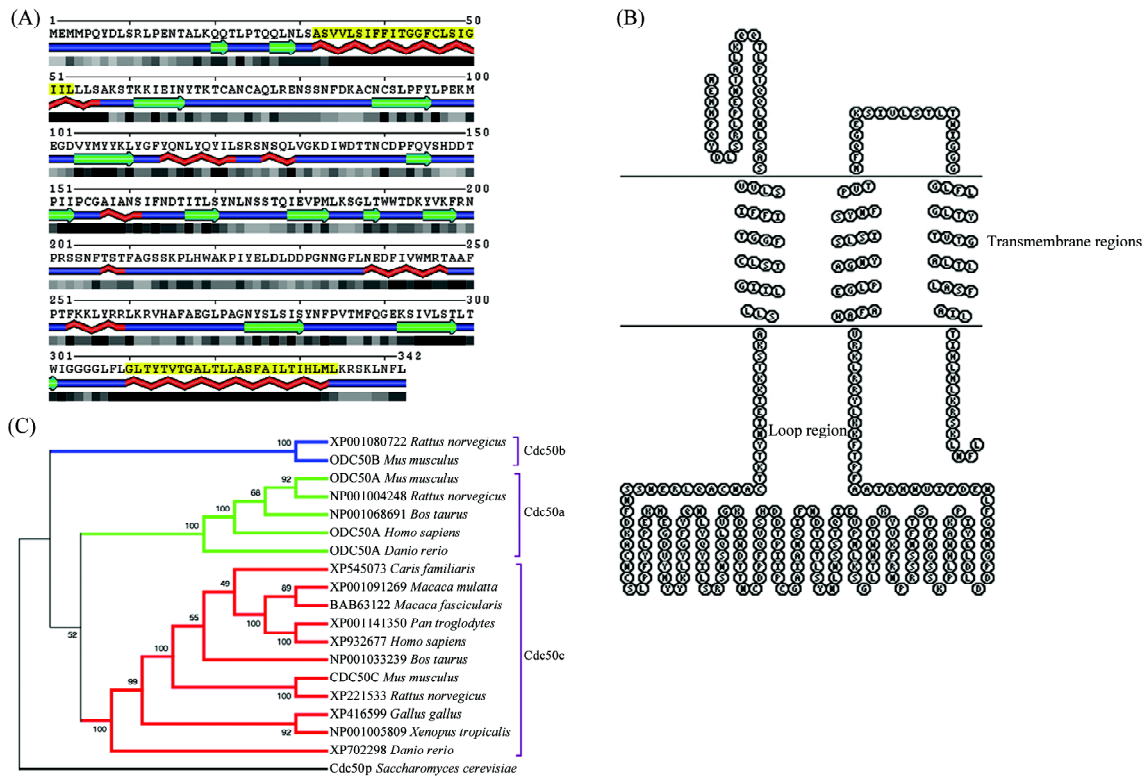


Fig. 1 Secondary structure analyses and alignments of orthologous *Cdc50c* proteins in different species

(A) *Cdc50c* protein secondary structure was predicted by the MINNOU server (<http://minnou.cchmc.org/>). α -Helices are marked by red lines, β -strands are marked by green arrows, and the C-coil structure is marked by the blue line. The transmembrane regions are marked in yellow shade. (B) Predicted membrane topology of *Cdc50c*, showing three transmembrane regions and one long loop. (C) Polygenetic tree of the *Cdc50* family. The tree was constructed by the neighbor-joining method and the values at branch points are bootstrap percentages with 1000 replications. The scale indicates the number of amino acid substitutions. The *Cdc50c* orthologs are marked with red.

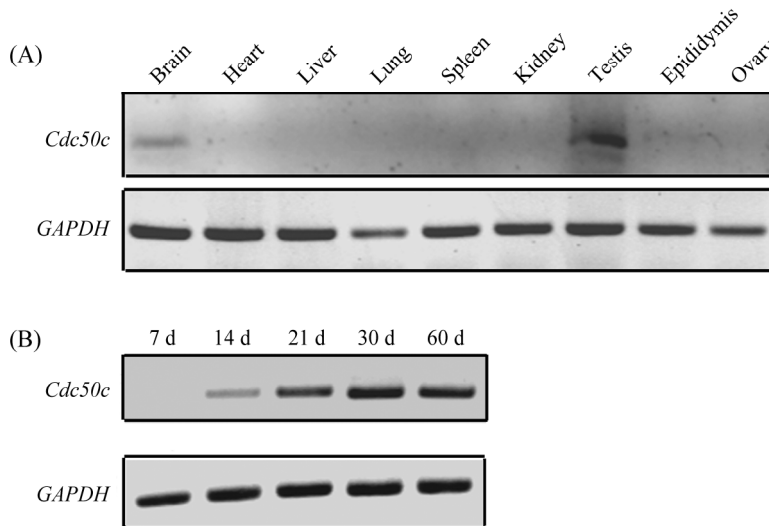


Fig. 2 Expressions of *Cdc50c* mRNA in mouse tissues

(A) Reverse transcription-polymerase chain reaction (RT-PCR) analyses of *Cdc50c* mRNA in multiple mouse tissues. PCR for *Cdc50c* was run for 35 cycles, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as a loading control) was amplified for 25 cycles. (B) Semiquantitative PCR analyses of mouse *Cdc50c* mRNA during testicular development. Mouse cDNAs from 7 day-old, 14 day-old, 21 day-old, 30 day-old, and 60 day-old testes were used as the templates. GAPDH was amplified as a loading control.

of late pachytene spermatocytes and round spermatids [Fig. 2(B)], indicating that *Cdc50c* might play a role during meiosis and post-meiosis stages of spermatogenesis [13].

Finally, we carried out *in situ* hybridization analyses using a probe against *Cdc50c* to precisely clarify the spatial expression pattern of *Cdc50c* mRNA in the mouse testis. In the fully mature testis (8 weeks post-partum), expression was observed in the seminiferous epithelium, where the level of mRNA was high in some individual tubule cross-sections but hardly detectable in others [Fig. 3(A,B)]. In positive tubule sections, the whole epithelium was not labeled, but the most intense signal was localized close to the center region [Fig. 3(A,B)]. The hybridization was specific because there was no labeling in the control group [Fig. 3(C,D), sense probe]. The extensive labeling over the basal part of the epithelium suggested that *Cdc50c* mRNA was expressed only in germ cells.

One cycle of spermatogenesis, which occurs in synchronized waves within the seminiferous epithelia, is divided into 12 stages in mouse [14–16]. As shown in Fig. 3(A,B), the hybridization signal was maximal at stages VII–XII of the cycle, weaker at stages IV–VI, and very low or undetectable at stages I–III. This suggested that the transcription of *Cdc50c* initiated between stages IV and VI reached its maximum between stages VIII and X in each epithelial cycle.

The dynamic distribution of *Cdc50c* mRNA during the epithelial cycle suggests that it might localize in late spermatocytes and post-meiotic cells [17], which is coincident with the RT-PCR results shown above [Fig. 2(B)]. More detailed analysis showed that the most intense hybridization signals were confined to pachytene spermatocytes in stage X [Fig. 3(E,F)], whereas no labeling was detected in zygotenes. Similarly, strong labeling was also detected in pachytene spermatocytes, but no signal was detected in leptotene spermatocytes in stage VIII [Fig. 3(G,H)]. In stages VIII and X, moderate signals were detected in the round spermatids (St8) and weak signals were observed in elongated spermatids (St10 and St15). No signals were found in the Sertoli cells or Leydig cells, indicating that *Cdc50c* mRNA specifically expresses in germ cell. Therefore, *Cdc50c* is first expressed in pachytene spermatocytes and remains expressed in round and elongated spermatids during spermatogenesis.

Discussion

In this report, we analyzed the mouse *Cdc50c* mRNA

expression pattern during spermatogenesis. Based on the highly similar sequence to yeast *Cdc50p*, as well as phylogenetic analysis, we found that *Cdc50c* is the yeast *Cdc50p* homolog in mice. *Cdc50c* mRNA is predominantly present in the mouse testis. Its expression level gradually increases during spermatogenesis. Furthermore, *Cdc50c* mRNA is present in pachytene spermatocytes and spermatids as revealed by *in situ* hybridization. In summary, our results suggest that *Cdc50c* is involved in rodent spermatogenesis.

Cdc50c potential function in male fertility

Mammalian spermatogenesis includes three phases: germ stem cell (spermatogonia) proliferation; and two rounds of meiosis and spermiogenesis in which the spermatids differentiate to form spermatozoa [18,19]. In the last stage, the nuclei are compacted, new organelles such as the acrosome, manchette, post-acrosomal lamina and perinuclear ring are formed, coupled with the shedding of a significant amount of cytoplasm [14,15]. Male germ cell polarity is established in this critical stage. Some reports show that the junctional adhesion molecule is essential for the polarization of round spermatids by playing a role in the recruitment and organization of a cell polarity complex [20]. However, the molecular mechanism of establishing the polarity of round spermatids is largely unknown.

In yeast, *Cdc50p* is a key player during the establishment of cell polarity. It is an integral membrane protein and a member of the yeast protein family that includes LEM3 [3,8]. Knock-out of yeast *Cdc50* causes retention of flippase *Drs2p* in the endoplasmic reticulum. The $\Delta cdc50$ and $\Delta drs2$ mutants show a defect in polarized cell growth after shifting to the non-permissive growth temperature of 18 °C for 12 h [7,11]. In our study, *Cdc50c*, one mammalian homolog of *Cdc5p*, is expressed in the pachytene spermatocytes and spermatids. The specific expression suggests that *Cdc50c* might play a role in cell polarity formation during spermatogenesis.

Structure and conservation of Cdc50c sequence

Sequence alignment shows that *Cdc50c* is evolutionary conserved. However, *Cdc50c* is highly diverse in the hominoids due to the mutations in the splicing and/or poly(A) signal sites [21]. The mouse *Cdc50c* mRNA encodes a full-length protein. Therefore, it seems that mouse *Cdc50c* is a better model for studying its functions during spermatogenesis.

The predicted mouse *Cdc50c* protein has three trans-membrane regions, however, it is generally believed that

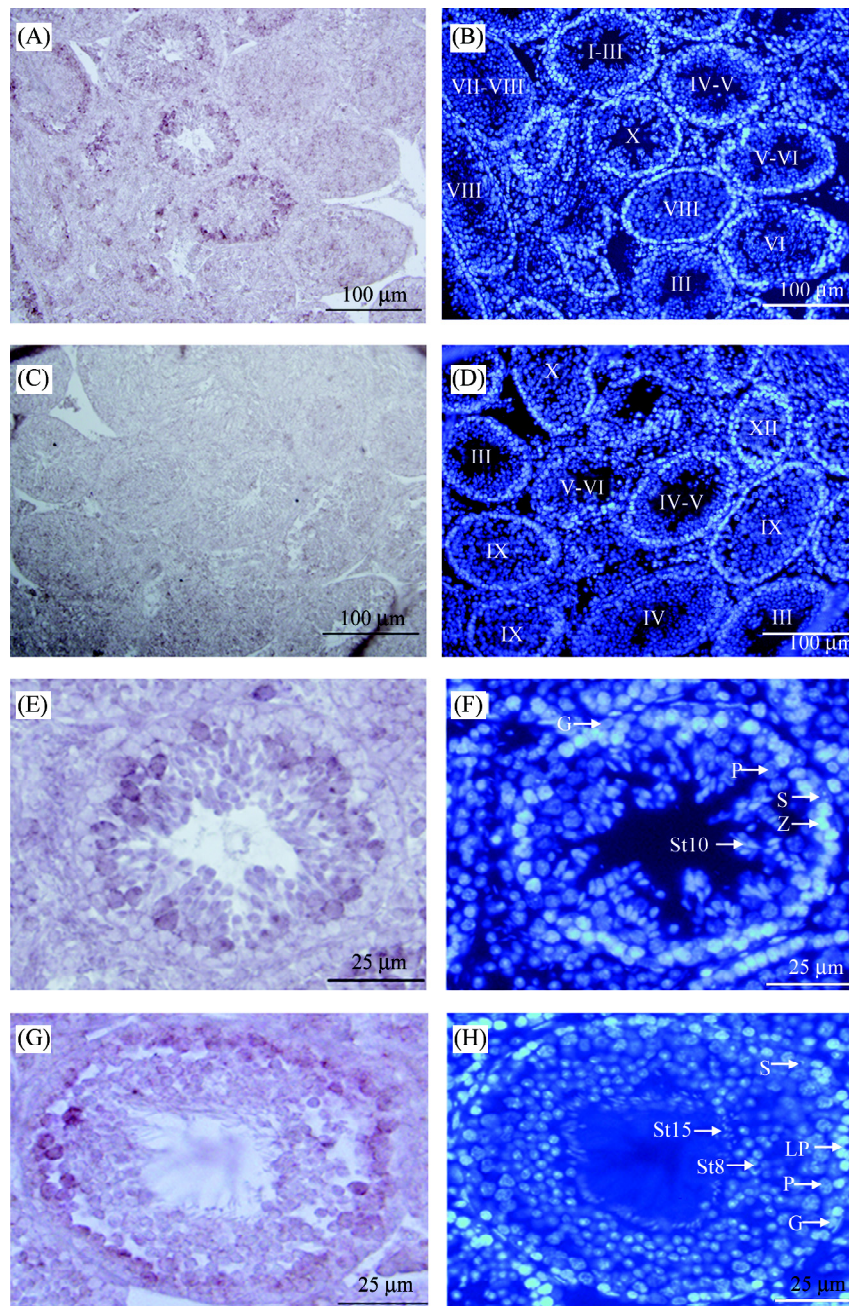


Fig. 3 Localizations of *Cdc50c* mRNA in mouse testis detected by *in situ* hybridization

(A) Localization of *Cdc50c* mRNA in mouse testis. Bright field (A, C, E, G) and 4',6'-diamidino-2-phenylindole dihydrochloride (B, D, F, H) images are shown. The hybridization signals are confined to stages VIII–X. The sense control is shown in (C). Roman numerals represent stages of the tubules. High magnification images (E, F) show one stage X tubules with intensive hybridization signals in pachytene spermatocytes and step 10 (St10) spermatids. The other high magnification images (G, H) show one stage VIII tubule with intensive hybridization signals in pachytene spermatocytes, moderate signals in step 8 (St8) spermatids and very weak in step 15 (St15) spermatids. G, spermatogonia; LP, leptotene spermatocytes; P, pachytene spermatocytes; S, Sertoli cells; Z, zygotene spermatocytes.

the *Cdc50* family proteins belong to two-transmembrane-span proteins. As shown in **Fig. 1(B)**, additional transmembrane regions exist in the N-terminus of *Cdc50c*. Whether modification of membrane topology affects the

function of *Cdc50c* requires further study.

In conclusion, our results show that *Cdc50c* is expressed in the meiosis and post-meiosis stages, implying a unique function in spermatogenesis.

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