Identification and characteristics of the testes-specific gene, *Ccdc38*, in mice

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Received July 10, 2015; Accepted May 3, 2016

DOI: 10.3892/mmr.2016.5360

Abstract. Distinguishing the testes-specific genes in different species may disclose key genes associated with testes-specific functions and provide sufficient information for the study and treatment of male infertility. A testes-specific gene, coiled-coil domain containing 38 (*Ccdc38*), was identified by screening UniGene libraries. Systematic bioinformatics analysis demonstrated that the CCDC38 protein was conserved in various mammalian species. It was determined that CCDC38 was exclusively expressed in testes and its expression increased from 2-8 weeks of age. Additional immunohistochemical analysis indicated that CCDC38 was mainly expressed in spermatogonia and spermatocytes. It is of note that, immunofluorescence and co-immunoprecipitation assays demonstrated that CCDC38 interacted with ubiquitinated histone H2A in mouse testes. Therefore, these results suggest that *Ccdc38* is a testes-specific gene, which may be important for mouse spermatogenesis.

Introduction

Infertility affects ~15% of couples worldwide, with half of cases reported to be due to the male partner (1). Treatments for male infertility have not been developed as the associated molecular mechanisms are not well understood (2). A more detailed investigation of the physiological mechanisms of spermatogenesis is required for an improved understanding of infertility prior to the development of therapies for this condition.

Spermatogenesis is a complicated developmental process. Spermatogonia differentiate into spermatocytes and spermatids during two meiotic divisions, which leads to the production of mature sperm (3). Defects at any stage of this process may lead to infertility. The process involves various genes, which encode proteins that in turn are important for specific stages of germ cell development. Theses genes are primarily expressed in spermatogenic cells, and are regulated at the transcriptional or post-transcriptional level. The identification of germ cell-specific or testes-specific genes involved in these unique events provides a means by which to dissect the differentiation program and to study the mechanisms of spermatogenesis. Previous studies have identified a number of testes-specific genes in humans and mice, including fibronectin type 3 and ankyrin repeat domains 1 (4), A kinase (PRKA) anchor protein 3 (5), protein lifeguard 5 (6), protease, serine 41 (7), spermatogenesis associated 33 (2) and testis-specific serine kinase 4 (8). In addition to the aforementioned genes it is possible that additional testis-specific genes exist and provide unique functions.

Coiled-coil domain containing 38 (*Ccdc38*) was selected from the expressed sequence tags (ESTs) obtained through the comparison of testes gene libraries with the libraries of other tissues and cell lines using the differential digital display program (9). The EST profile of *Ccdc38* in Unigene (Mm.477086) indicates that the *Ccdc38* transcript is detected solely in murine testes, which is consistent with the report at BioGPS.org (10). *Ccdc38* orthologs are present in other species, including *Rattus norvegicus* (Gene ID: 500823), *Bos taurus* (Gene ID: 517752), *Pan troglodytes* (Gene ID: 738083) and humans (Gene ID: 120935).

The present study determined that the testes-specific gene, *Ccdc38*, was only expressed in mouse testes. This gene

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Key words: *Ccdc38*, testis, spermatogenesis, ubiquitinated histone H2A

is primarily expressed in spermatogonia and spermatocytes. Additionally, it was identified that CCDC38 may interact with ubiquitinated histone H2A (uH2A) in mouse testes. This suggests that *Ccdc38* is a testes-specific gene, which is important for spermatogenesis in mice.

Materials and methods

Samples. Twenty male and twenty female BALB/c adult mice (25-30 g) were purchased from the Laboratory Animals Center of South Medical University (Guangzhou, China). Mice were treated under a pathogen-free condition at ~22˚C under a 12 h light/dark cycle. All the animals had free access to standard water and chow. Male and female mice were allowed to mate spontaneously, and the day of birth was assigned as day 1. Testes tissues were individually collected from the 1, 2, 3, 4, 6, 8 weeks, and 6 months-old mice following sacrifice by cervical dislocation. The remaining organs, including the heart, brain, lungs, spleen, kidneys, liver, epididymis and bladder from the adult offspring were immediately frozen in RNAlater (Qiagen, Inc., Valencia, CA, USA). The protocol was approved by The Ethics Committee of Peking University Shenzhen Hospital (Shenzhen, China).

Antibodies. The rabbit polyclonal anti-CCDC38 antibody (cat. no. ab170231), the mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (cat. no. ab8245), the mouse monoclonal anti-enhanced green fluorescent protein (EGFP) antibody (cat. no. ab184601) and the rabbit polyclonal anti-hemagglutinin (HA) tag antibody (cat. no. ab137838) were purchased from Abcam (Cambridge, UK). The mouse anti-H2A antibody was purchased from EMD Millipore (Billerica, MA, USA). Anti-rabbit-Alexa Fluor 488 (cat. no. ab150073) and anti-mouse-Cy3 (cat. no. ab97035) were purchased from Abcam.

Construction of plasmids and cell culture. The full length of the *Ccdc38* cDNA was amplified by polymerase chain reaction (PCR) with the forward (F) 5'-ATGGCATCCCAGATGC-3' and the reverse (R) 5'-ACTAAAAAAGTACTCTTCGTC-3' primers, and then inserted into pCDNA3.1/HA plasmids via *Bam*H1 and *Xho*l. The full length of the *H2A* cDNA was amplified by PCR with the F 5'-ATGTCTGGACGTGGCAAA CAG-3' and the R 5'-TTATTTCCCCTTGGCCTTGTGG-3' primers and then inserted into pEGFP-C1 plasmids via *Bam*H1 and *Eco*R1. Reaction condirions were as follows: 98˚C for 2 min; 37 cycles of 98˚C for 10 sec, 55˚C for 30 sec and 72˚C for 2 min; followed by 72˚C for 5 min. The PCR products were cloned and sequenced by Invitrogen; Thermo Fisher Scientific, Inc.. Two cell lines, TM4 and HEK293T were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from mice tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Total RNA (1 μg) was used as a template for the reverse transcription using the PrimeScript RT Enzyme Mix I (Takara Bio, Inc., Shiga, Japan). Reverse and forward oligonucleotide primers were designed using Primer Express version 2.0 software (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The primer sequences were as follows: *Ccdc38*, F 5'-CTTGTC CTGTTAGTCCTGTATAG-3', R 5'-CGTAGAGATGAAGTG TGATGAT-3'; and *Gapdh* (as an internal control) F 5'-AGT GGCAAAGTGGAGATT-3', R 5'-GTGGAGTCATACTGG AACA-3'. The following PCR conditions were used: 98˚C for 2 min; 32 cycles of 98˚C for 10 sec, 55˚C for 30 sec and 72˚C for 30 sec; and 72˚C for 7 min using a LightCycler 480II instrument (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Testes samples obtained at different time points and other tissues were amplified in triplicate. The amplified products were resolved on a 2% agarose gel, stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and visualized with an ultraviolet imaging system (Universal Hood II, Bio-Rad Laboratories, Hercules, CA, USA). Data were normalized to the expression of *Gapdh* and were quantified according to the $2^{-\Delta\Delta Cq}$ method (11).

Western blotting. The protein extracts of various mouse tissues (20 μg) were subjected to 12% sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride membrane (EMD Millipore). Following blocking with 5% non-fat milk, the membranes were incubated with rabbit anti-CCDC38 (1:500, cat. no. ab170231, Abcam) or anti-GAPDH antibody (1:5,000, cat. no. ab181602, Abcam) antibodies overnight at 4˚C. Subsequently, the membranes were washed three times with Tris-buffered saline with Tween-20 buffer for 15 min. Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:2,000, cat. no. ab97051, Abcam, CA, USA) for 1 h at room temperature. Finally, the positive bands were examined using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). The densitometry of each band was analyzed using Image-Pro Plus 6.0 software (Image Pro-Plus 6.0; Media Cybernetics, Silver Spring, MD, USA). The CCDC38 expression level in tested from mice aged 1-8 weeks and 6 months was normalized to GAPDH expression.

Immunohistochemistry and immunofluorescence. Paraffin sections (3-5 μm) of testes samples from 1-8 weeks and 6 months-old mice was used for staining. were prepared as previously described (12). The sections were blocked in 10% goat serum and then incubated with rabbit anti-CCDC38 antibody (dilution, 1:300), rabbit anti-uH2A (dilution, 1:100) antibody overnight at 4˚C. The sections were washed with phosphate-buffered saline (PBS) three times and incubated for 1 h at 37˚C with the anti-rabbit-Alexa Fluor 488 (1:500) and anti-mouse-Cy3 (1:500) secondary antibodies. Subsequently, the slides were incubated with 50 μl of 1X DAPI solution for 5 min at room temperature in the dark, followed by washing with 100 μl PBS three times. The slides were then treated with DAPI for 5 min at room temperature, washed in PBS, mounted, and observed under x100 LSM 710 oil lens (Zeiss GmbH, Jena, Germany). DAB staining was performed according to the manufacturer's recommended protocol (ABC kit; Maixin Biotechnology, Fuzhou, China). The DAB slides

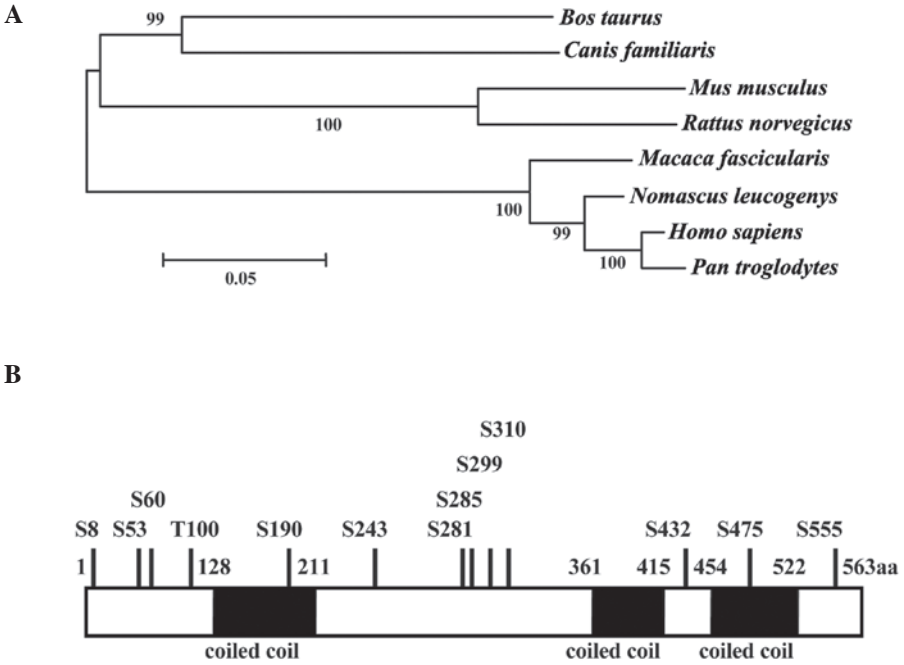

Figure 1. Phylogenetic tree, domains, and modification sites of CCDC38. (A) Phylogenetic tree of CCDC38 in mammals. Phylogenetic analysis was performed with MEGA5. Numbers on the branches represent the bootstrap values from 1,000 replicates obtained using the Neighbor-Joining method. The scale bar corresponds to the estimated evolutionary distance units. GenBank accession numbers are as follows: *Bos taurus*, XP_003586123.2; *Canis familiaris*, XP_854813.2; *Mus musculus*, XP_006513691.1; *Rattus norvegicus*, XP_006241333.1; *Macaca fascicularis*, XP_005571963.1; *Nomascus leucogenys*, XP_003259733.1; *Homo sapiens*, XP_006719292.1; *Pan troglodytes*, XP_001144908.1. (B) Schematic mapping of potential protein domains and post-translational modification sites. The predicted sites for phosphorylation in CCDC38 are indicated. CCDC38, coiled-coil domain containing 38; S/T, Serine/Threonine phosphorylation-sites.

were observed under x40 Leica DM4000B lens (Leica Microsystems GmbH, Wetzlar, Germany). The level of nonspecific staining was determined by omission of the incubation step with the primary antibody.

Hoeschst staining. The Hoechst 33258 staining kit (Invitrogen; Thermo Fisher Scientific, Inc.) was utilized to observe cell nuclear staining in mouse testes. Paraffin sections were prepared as described previously (12). Following culture with primary and secondary antibodies, the slides were incubated with 50 μl of 1X Hoechst 33258 solution for 5 min at room temperature in the dark, followed by washing with 100 μl PBS three times. The results were observed under a LSM 710 oil lens at x100 magnification (Zeiss GmbH, Jena, Germany).

Co-immunoprecipitation (co-IP) assay. Whole testes were prepared with lysis buffer (10 mM Tris pH 7.4, 1.0% Triton X-100, 0.5% NP-40, 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, and 0.2 mM PMSF) supplemented with protease inhibitors. For the *in vivo* co-IP assay, the supernatant was incubated with anti-CCDC38 (10 μg) and anti-uH2A (5 μg) antibodies overnight at 4˚C. Protein A/G beads (60 μl) were then added to each sample, and the mixtures were incubated at 4˚C for 1 h. For the *in vitro* co-IP assay, the pEGFP-H2A overexpression plasmid was synthesized by Invitrogen; Thermo Fisher Scientific, Inc. The CCDC38-pCDNA3.1/HA (Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics; Shenzhen, China) was transfected into HEK293T cells, with pEGFP-H2A (Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics) or the vector control (pEGFP-C1; Invitrogen, Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 transfection reagent (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were harvested 48 h after transfection and extracted with the aforementioned lysis buffer. Subsequently, the supernatant was incubated with anti-EGFP (5 μg) and anti-HA (2 μg) antibodies overnight at 4˚C. Protein A/G beads (60 μl) were also added to every sample, and the mixtures were incubated at 4˚C for 1 h. The beads were then washed three times with lysis buffer, boiled in sample buffer containing 0.2 M dithiothreitol, and analyzed by western blotting as previously described.

Bioinformatic analysis. Bioinformatics related to the genome, chromosome, mRNA and protein products of Ccdc38 were obtained using the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), Mouse Genome Informatics (http://www.informatics.jax.org/) and the UCSC Genome bioinformatics (http://genome.ucsc.edu/). The SIB Bioinformatics Resource Portal (http://www.expasy.org/) was applied to analyze the domains and motifs of CCDC38 protein.

Statistical analysis. Each of the experiments was repeated at least three times. Data are presented as the mean ± standard deviation. Student's t-test was used to compare the difference between two groups. SPSS version 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. $P<0.05$ was considered to indicate a statistically significant difference.

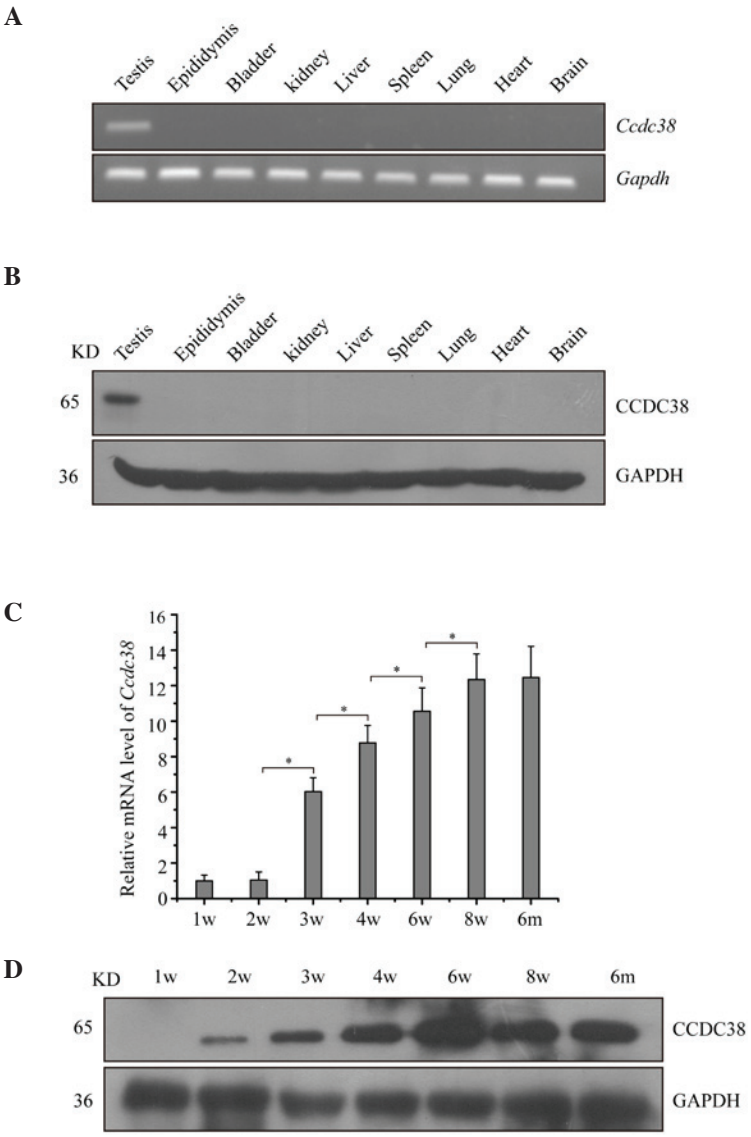

Figure 2. Expression pattern of *Ccdc38* in various tissues of adult mice and in postnatal mice testes. (A) RT-PCR of *Ccdc38* in adult tissues. *Gapdh* was used as the internal control. *Ccdc38* is predominantly expressed in the testis. (B) Expression of the CCDC38 protein in various tissues. Mice tissues were subjected to western blot analysis with antibody against CCDC38. CCDC38 protein was recognized with a band at 65 kDa. The CCDC38 protein is predominantly expressed in the testes. GAPDH was used as the loading control. (C) RT-qPCR of the *Ccdc38* in the testes of mice aged 1 week to 6 months. Error bars indicate the mean ± standard deviation (n=3). *P<0.05. (D) Expression of CCDC38 protein in the testes collected from mice aged 1 week to 6 months. The protein level was markedly increased starting from 2 weeks. GAPDH was used as the loading control. All experiments were performed in triplicate. CCDC38, coiled-coil domain containing 38; RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, RT-quantitative PCR.

Results

Identification of Ccdc38 by in silico screening. The testes-specific gene, *Ccdc38* was identified using the UniGene libraries. To further characterize the expression of *Ccdc38*, its structure and function was analyzed using systematic bioinformatic methods. The *Ccdc38* gene encodes a protein with a predicted molecular weight of ~65 kDa. The homology with other vertebrates in GenBank was also determined, indicating that several predicted homologues only exist in mammals (Fig. 1A), with mouse CCDC38 sharing a high sequence homology with other mammalian homologues. The CCDC38 protein also had three coiled-coil domains in the Pfam database. Sequence analysis indicated that CCDC38 is a serine-rich protein. Two potential sites (12 serine phosphorylation sites and 1 threonine phosphorylation site) were revealed by post-translational modification analysis (Fig. 1B), which suggests that this protein may be important for kinase signaling during spermatogenesis.

Expression of CCDC38 in mice. The mRNA expression of *Ccdc38* in various adult mouse tissues was determined using RT-PCR and RT-qPCR. *Ccdc38* was exclusively expressed in testes (Fig. 2A). To determine its protein level, a polyclonal anti-CCDC38 antibody was used against the coding region of CCDC38 in western blotting. The analysis indicated a unique band at 65 kDa in mouse testes, consistent with the predicted molecular weight by Computing pI/Mw (13). The protein level of CCDC38 was consistent with the mRNA analysis displaying a testis-specific expression pattern (Fig. 2B).

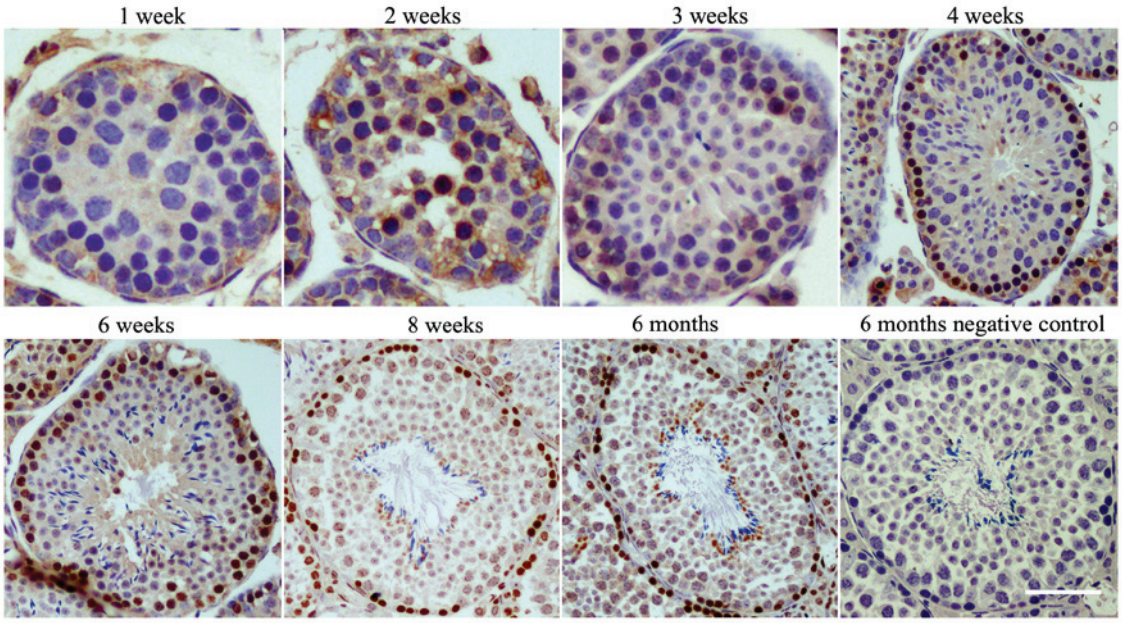

Figure 3. Immunostaining of CCDC38 protein in the aged 1-8 weeks and 6 months testes. CCDC38 is expressed mainly in the spermatogonia and spermatocytes. The signals were observed in nuclei from 2 weeks to 6 months. In controls, no positive signals were observed in 6 months testes sections when anti-CCDC38 antibody was replaced by 10% normal bovine serum. Scale bar, 50 μm. CCDC38, coiled-coil domain containing 38.

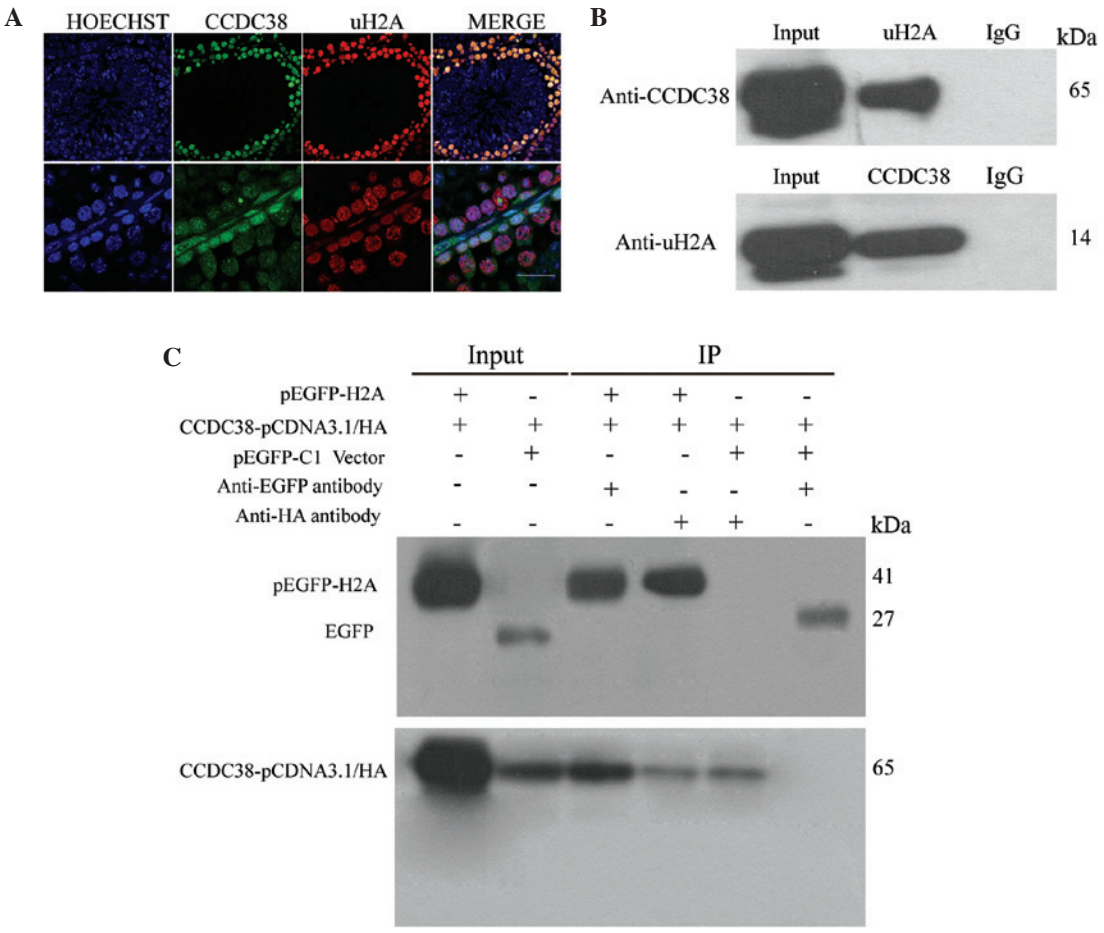

Figure 4. CCDC38 interacts with uH2A in mouse testes. (A) Co-labeling of CCDC38 protein (green) and uH2A (red). Nuclei were stained by Hoechst (blue). Scale bar, 10 μm. Upper panels show lower-magnification views and lower panels show higher-magnification views. (B) Immunopreciptation and immunoblotting of CCDC38 and uH2A in mouse testes protein extracts, IgG was used as a negative control. (C) *In vitro* immunopreciptation of CCDC38 by anti-EGFP antibody. CCDC38-pCDNA3.1/HA was transfected into HEK293T cells together with pEGFP-H2A or the pEGFP-C1 vector control. Anti-EGFP antibody (upper panel) or anti-HA antibody (lower panel) was used for western blot. Expression of CCDC38-pCDNA3.1/HA and pEGFP-H2A in HEK293T cells is indicated in the lanes labeled 'Input'. CCDC38-pCDNA3.1/HA was immunoprecipitated only in the presence of pEGFP-H2A. CCDC38, coiled-coil domain containing 38; uH2A, ubiquitinated histone 2A; HA, hemagglutinin; EGFP, enhanced green fluorescent protein.

Additionally, the timing of mouse CCDC38 expression for mRNA and protein levels during postnatal testis development was investigated. As presented in Fig. 2C, The expression of *Ccdc38* mRNA from 2 to 8 weeks was gradually increased, as compared with that at 1 week. Western blot analysis indicated that the CCDC38 protein was initially expressed at 2 weeks and the expression pattern was consistent with its mRNA expression (Fig. 2D). This indicates that CCDC38 is testes-specific in mice and is developmentally regulated during spermatogenesis.

CCDC38 protein is predominantly expressed in spermatogonia and spermatocytes. Immunocytochemical staining on sections of the immature and adult mouse testes was employed to investigate the expression levels CCDC38 protein in testes. CCDC38 was predominantly expressed in the nucleus of the spermatogonia and spermatocytes of mice aged from 2 weeks to adults (8 weeks) (Fig. 3).

CCDC38 protein interacts with uH2A. CCDC38 and uH2A were examined by immunofluorescence in the testes of adult mice. The results indicated that CCDC38 and uH2A protein were partially co-localized in the nucleus of spermatogonia and spermatocytes (Fig. 4A). The co-IP results indicated that the CCDC38 protein may be co-immunoprecipitated by anti-uH2A and anti-EGFP antibodies *in vivo* and *in vitro*, which demonstrated that CCDC38 interacted with uH2A in mouse testes (Fig. 4B and C).

Discussion

There are ~2,000 genes regulating the process of spermatogenesis (14), and further studies of key genes that regulate this process will aid in the understanding of spermatogenesis. In the present study, the testes-specific gene, *Ccdc38*, was identified, which was associated with spermatogenesis. The association was supported by the multiple tissue analysis of *Ccdc38*, which indicated that it was exclusively expressed in the testes. Further immunohistochemistry analysis indicated that the CCDC38 protein was mainly localized in the nuclei of spermatogonia and spermatocytes of the seminiferous tubules. In addition, the mRNA and protein expression levels of CCDC38 were increased postnatally, from 2-8 weeks in the mice testes. Previous studies have indicated that murine germ cells enter the meiotic prophase ~10 days after birth and proceed with the first wave of meiosis during the remaining 10 days in the mice (15,16). Therefore, it is possible that the developmentally-regulated expression pattern of CCDC38 may contribute to spermatogenic events.

Spermatogenesis involves various histone modifications including ubiquitination, phosphorylation, methylation and acetylation (17). A previous study indicated that a high quantity of uH2A was detected in the pachytene stage of spermatocytes, by immunoblot and immunohistochemical analysis of wild-type mice testes (18). The current study indicated that CCDC38 protein is localized in nuclei of spermatogonia and spermatocytes. CCDC38 may interact with uH2A in murine testes, as indicated by the use of a vector machine, previous experimental results, combined with auto-covariance for prediction of protein-protein interactions from protein sequences (19). In order to test this hypothesis immunocolocalization and co-IP assays were performed. It was determined that CCDC38 and uH2A partially co-localized in the nuclei of spermatogonia and spermatocytes. Additionally, co-IP demonstrated that CCDC38 may interact with uH2A *in vivo* and *in vitro*. H2A ubiquitination contributes to various cellular processes, including DNA damage repair and transcriptional regulation by gene silencing or repression (20-22). Therefore, it is possible that CCDC38 may be important for transcriptional silencing via interactions with uH2A during spermatogenesis.

In conclusion, the present study identified the testes-specific gene *Ccdc38* is conserved in mammalian species. The expression and localization of the CCDC38 protein indicates that it may be important for spermatogenesis. In addition, CCDC38 may also regulate spermatogenesis by interacting with uH2A. The molecular mechanisms of CCDC38 in spermatogenesis require further investigation.

Acknowledgements

The present study was funded by grants from the National Natural Science Foundation of China (grant nos. 31271244 and 31471344) and Shenzhen Project of Science and Technology (grant nos. XB201104220045A and JCYJ20140415162543017).

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