Integrated analyses of phenotype and quantitative proteome of CMTM4 deficient mice reveal its association with male fertility

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Running Title: CMTM4 association with male fertility
ABBREVIATIONS

KO, knockout; WT, wild type; CKLF, chemokine-like factor; CMTM, CKLF-like MARVEL transmembrane domain-containing family; TM4SF, transmembrane 4 superfamily; BLNK, B-cell linker; ANOVA, one-way analysis of variance; iTRAQ, isobaric tags for relative and absolute quantification; FDR, false discovery rate; GO, gene ontology; EGFR, epidermal growth factor receptor; PD-L1, programmed death-1; OA, obstructive azoospermia; NOA, non-obstructive azoospermia; CRISPR, clustered regularly interspaced short palindromic repeats; CAS9, CRISPR-associated protein 9; sgRNAs, single guide RNAs; PCR, polymerase chain reaction; hCG, human choionic gonadotrophin; PMSG, pregnant mare serum gonadotrophin; PBS, phosphate buffered saline; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; IHC, immunohistochemistry; CASA, computer-aided sperm analysis; DSP, daily sperm production; TEAB, triethylammonium bicarbonate; DTT, dithiothreitol; RP, reversed-phase; LC-MS, liquid chromatograph mass spectrometer; DAVID, database for annotation, visualization and integrated discovery; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris–buffered saline; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; IOD, integrated optical density; DAB, 3,3′-diaminobenzidine; UTR, untranslated region; CDS, coding sequence; PCR, polymerase chain reaction; IVF, in vitro fertilization; VSL, straight line velocity; VCL, curvilinear velocity; GEO, gene expression omnibus; AR, androgen receptor.
SUMMARY

The chemokine-like factor (CKLF)-like MARVEL transmembrane domain-containing family (CMTM) is a gene family that has been implicated in male reproduction. CMTM4 is an evolutionarily conserved member that is highly expressed in the testis. However, its function in male fertility remains unknown. Here, we demonstrate that CMTM4 is associated with spermatogenesis and sperm quality. Using western blotting and immunohistochemical analyses, we found CMTM4 expression to be decreased in poor-quality human spermatozoa, old human testes, and testicular biopsies with non-obstructive azoospermia. Using CRISPR-Cas9 technology, we knocked out the Cmtm4 gene in mice. These Cmtm4 knockout (KO) mice showed reduced testicular daily sperm production, lower epididymal sperm motility and increased proportion of abnormally backward-curved sperm heads and bent sperm midpieces. These mice also had an evident sub-fertile phenotype, characterized by low pregnancy rates upon prolonged breeding with wild type female mice, reduced in vitro fertilization efficiency and a reduced percentage of acrosome reactions. We then performed quantitative proteomic analysis of the testes, where we identified 139 proteins to be down-regulated in Cmtm4-KO mice, 100 (71.9%) of which were related to sperm motility and acrosome reaction. The same proteomic analysis was performed on sperm, where we identified 3588 proteins with 409 being differentially regulated in Cmtm4-KO mice. Our enrichment analysis showed that up-regulated proteins were enriched with
nucleosomal DNA binding functions and the down-regulated proteins were enriched with actin binding functions. These findings elucidate the roles of CMTM4 in male fertility and demonstrates its potential as a promising molecular candidate for sperm quality assessment and the diagnosis or treatment of male infertility.

**Key Words:** CMTM4, Testis, Spermatogenesis, Male fertility, Cas9
INTRODUCTION

The human chemokine-like factor (CKLF)-like MARVEL transmembrane domain-containing (CMTM) family is a gene family encoding proteins that link classical chemokines and the transmembrane 4 superfamily (TM4SF). In humans, the nine members include CKLF and CMTM1-8, which encode proteins that play important roles in the immune system, tumorigenesis, and the male reproductive system (1,2). Our previous studies have reported that CMTM3, CMTM4, CMTM5, and CMTM7 function as tumor suppressors in the development and progression of carcinomas (3-6). CMTM3 and CMTM7 co-localize with RAB5 in early endosomes and facilitate epidermal growth factor receptor (EGFR) internalization and degradation by enhancing RAB5 activity and early endosome fusion (7,8). CMTM3 and CMTM4 mediate cell–cell adhesion by involvement in VE-cadherin turnover, and this process is involved in the regulation of angiogenesis (9,10). CMTM3 and CMTM7 also initiate B-cell linker (BLNK)-mediated signal transduction (11,12). CMTM6 and CMTM4 have been identified as programmed death-1 (PD-L1) regulators that inhibit immune function (13,14). These studies showed that the CMTM family has important regulatory effects on the trafficking, degradation, and signal transduction of membrane molecules. Interestingly, CMTM1, CMTM2, and CMTM4 are highly expressed in the human testis, implying biological roles in male reproduction (2). CMTM1 is predominantly expressed in the human testis, with at least 23 alternative splicing isoforms (15). However, Cmtm1 knockout
(KO) has no significant influence on male fertility (16). CMTM2 is highly expressed in the testis and is closely correlated with spermatogenic defects (17,18). Its two homologues in the mouse, Cmtm2a and Cmtm2b, serve as androgen receptor co-repressor and enhancer, respectively (19-22). Co-expression of Cmtm2a and Cmtm2b is essential for male fertility in mice (16). These findings indicate that CMTM family members may play important roles in spermatogenesis or testicular development.

*CMTM4* is the most conserved member of the CMTM family, and forms a gene cluster with *CKLF* and *CMTM1-3* on chromosome 16q22.1 (2). Our previous studies showed higher expression of CMTM4 in the testis than in other tissues (2, 23), which warrants exploration of its significance in male reproduction. Given its sequence structure and expression characteristics, CMTM4 might also play crucial roles in male fertility as do CMTM2 (16,17). Previous studies have indicated that CMTM4 acts as a tumor suppressor through its involvement in cell growth and cell cycle regulation (4, 23, 24). However, its roles in male reproduction remain unknown.

In the present study, we first assessed the expression of CMTM4 in the spermatozoa and testes of patients with male infertility to characterize its association with spermatogenesis and sperm quality. Because the amino acid sequences are highly homologous between human and mouse CMTM4, the functions of CMTM4 in male fertility were examined in a KO mouse model, and the underlying mechanism was investigated using isobaric tags for relative and
absolute quantification (iTRAQ)-based proteomics. Consistent with the association of CMTM4 expression with sperm quality in patients, Cmtm4 KO mice showed male subfertility with phenotypes of decreased sperm motility and aberrant acrosome reaction. Gene ontology (GO) term analysis revealed that proteins down-regulated in KO mice testis and spermatozoa compared to wild-type (WT) were mainly involved in spermatogenesis and sperm functions including motility, the acrosome reaction, and histone-to-protamine exchange. This study also provided in-depth proteomic mapping of the mouse testis and sperm that will facilitate to understand of spermatogenesis and sperm functions. Combining phenotypic characteristics and proteomic analyses of Cmtm4 KO mice, we have shown that CMTM4 plays key roles in regulating sperm function and male fertility by affecting sperm motility and the acrosome reaction.

**EXPERIMENTAL PROCEDURES**

**Ethical statement**

The present study was approved by the Medical Ethical Committee of the YuHuangDing hospital and all participants provided written informed consent. All experiments were performed in accordance with the guidelines provided by YuHuangDing hospital. All animal experiments were carried out in according with the guidelines of the care and use of laboratory animals. All mice were kept under light/dark cycles of 12/12 hours with free access to food and water.

**Sample preparation**
Samples of human semen were collected from the YuHuangDing hospital, and were classified into four groups without leukocytospermia as follows: the normozoospermia group (24-37 years old, sperm count $>39\times10^6$, progressive motile spermatozoa $>40\%$), asthenozoospermia group (27-36 years old, progressive motile spermatozoa $<32\%$), oligoasthenozoospermia group (27-39 years old, sperm count $<39\times10^6$, progressive motile spermatozoa $<32\%$), and teratozoospermia (27-40 years old, normal morphology $<4\%$). Young testes were obtained from five young fathers (23–27 years old) who died in car accidents, had no history of pathology that could affect reproductive function, and had previously indicated a willingness to donate their bodies to medical research. Donations of their organs for medical research were approved by their immediate family members. Aged testes samples were obtained from five elderly fathers (70–74 years old) who were prostate cancer patients with no anti-androgen treatment before orchiectomy and who had given written informed consent. All procedures were approved by the Ethics Committee of YuHuangDing Hospital. A total of 25 patients with azoospermia (26-37 years old) who underwent surgical testicular sperm extraction were recruited and divided into the obstructive azoospermia (OA) group ($n=5$) and the non-obstructive azoospermia (NOA) group ($n=20$). In OA, spermatozoa were produced normally inside the testicle, while in NOA, spermatogenesis problems were observed with a very low level of sperm production or a total lack of production. Patients with abnormal karyotypes and those who had previously
suffered from an injury to the genitals were excluded. Human testicular quality was evaluated by the modified Johnsen score according to our previous studies (25, 26).

**Protein extraction**

Human sperm samples exhibiting normozoospermia, asthenozoospermia, oligoasthenozoospermia, and teratozoospermia were collected for protein extraction. As in our previous reports (26), after centrifugation at 800 g for 20 min at 4°C in a 50% step Percoll gradient in phosphate buffered saline (PBS) media, the seminal plasma and other contaminating cells in semen were removed. Sperm pellets were dissolved in lysis solution and sonicated for three times at 5 second intervals. Then they were kept at 4°C for 2h before the solution was centrifuged at 12000 g for 45 min, and the supernatant was collected. After determination of protein concentration, protein samples were stored at -80°C.

*In vitro* synthesis of clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) mRNA and sgRNAs

CRISPR-Cas9 constructs were synthesized as described previously (27). Briefly, Cas9 mRNA was synthesized *in vitro* from linear DNA templates by using the mMESSAGE mMACHINE T7 Ultra kit (Thermo Fisher Scientific Inc, CA, USA) according to the manufacturer’s instructions. DNA templates for single guide
RNAs (sgRNAs) were also synthesized in vitro by polymerase chain reaction (PCR); RNA was synthesized from these templates with a MEGA shortscript T7 kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The constructs were diluted with RNase-free injection buffer (0.25 mM EDTA, 10 mM Tris at pH 7.4) before microinjection. The following sequence information was used for sgRNA synthesis: sgRNA#1: AGCGCGCCGCGCAGGTAGTC; sgRNA#2: GAGGAGCTGGACGGCTTCGA. CRISPR-Cas9 constructs and sgRNAs were microinjected into fertilized C57BL/6J oocytes. Knockout of Cmtm4 was confirmed by Sanger sequencing.

Genotyping of Cmtm4 mouse lines

Genotypes were confirmed by the PCR with the following primers:
Cmtm4, forward primer: 5’-GGTCTGGGCTTTTCTTGC-3’, reverse primer: 5’-GCCCAAGGACCTCGGAGTA-3’; Gapdh was internal control, Gapdh, forward primer: 5’-CACTCATTGCCGCCGTTT-3’, reverse primer: 5’-GTCAGGTTTCCCATCCCACATA-3’. PCR was performed as follows: denaturation at 98°C for 2 min, 30 cycles of 98°C for 10 s, 62°C for 10 s and 72°C for 50 s; extension at 72°C for 5 min. Electrophoresis was conducted on 1.2% (w/v) agarose gels and visualized with a Bio-Rad ChemiDOC MP system (Bio-Rad, Hercules, California 94547, USA).

Assessment of KO mouse fertility and fecundity
To assess fertility and fecundity, one littermate male (6 weeks old) was placed in cages with two mature WT females for two months or more. Two littermate females were caged with a WT fertile male for the same period. The number of female mice achieving pregnancy and the number of offspring were recorded.

Mouse sperm suspensions were prepared by mincing the cauda epididymidis of a mature male mouse (WT or KO) in 1 ml IVF medium for about 1 h. Part of this sperm suspension was divided into 30 μl pellets using a micropipette in a plastic culture dish (35 mm × 11 mm) covered with paraffin oil, and the rest was treated by 10 μM calcium ionophore A23187 (Sigma-Aldrich, St. Louis, MO, USA) for assessing the induction of the acrosome reaction. Mature female mice (WT or KO) were induced to super-ovulate by i.p. injection of 10 i.u. pregnant mare serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) 48 h apart. The oocytes were recovered from the oviducts 14 h after injection of hCG and were introduced into each sperm suspension. The sperm concentration was counted by using improved-Neubauer counting chamber, and at least 200 cells were counted. About 10^6 spermatozoa/ml was used for insemination. The development stage and morphology of embryos were recorded at 24 h interval after zygote collection.

**Evaluation of KO mouse sperm quality**
Epididymal spermatozoa was collected from the caudal part of the epididymis. The cauda epididymidis was placed in PBS supplemented with 10% (w/v) bovine serum albumin (BSA) and cut into small pieces followed by incubation on a warming metal plate at 35°C for 5 min. Sperm motility was measured with Computer-aided Semen Analysis system (CASA) System (Medealab™, Erlangen, Germany). For each sample, 12 fields were examined.

To evaluate the acrosome reaction, spermatozoa were suspended in Biggers-Whitten-Whittingham (BWW) and incubated at 37°C, 5% CO2 for 3h to get capacitation. Capacitated sperm was exposed to vehicle alone (DMSO, dimethyl sulfoxide) or calcium ionophore A23187 (10 μM) for 30 min, subjected to Coomassie brilliant blue staining (2% w/v G250) for 3 min, and then washed with PBS three times before mounting on slides for observation. Four hundred spermatozoa were evaluated under a light microscope (×400). Spermatozoa were scored as acrosome-intact when a bright blue staining was observed in the dorsal region of the head or as acrosome-reacted when no labeling was observed.

Daily sperm production (DSP)

DSP was estimated according to a previous report (28). Briefly, after the testes were crushed in 600 μl SMT solution containing 0.05% (v/v) Triton X-100, 0.9% NaCl and 0.01% Azide by ultrasonic pulverizer, the solution was homogenized again. Aliquots of 10 μl were placed on a makler counting chamber and sperm
heads were counted twice under microscopic visualization to determine the average number of spermatids per sample. Sperm heads in 80 small squares were assessed. These values were divided by testicular weight to give the number of spermatids per gram of testis. Because developing spermatids spend 4.84 days in steps 14-16 of spermatogenesis, the number of spermatids per gram of testis was divided by 4.84 to provide the DSP.

**iTRAQ Labeling of mouse testicular samples**

The iTRAQ procedures were described in previous publication (29, 30). Briefly, the testicular or spermatozoa protein samples from the wild and Cmtm4 KO mice were separately pooled. The pH was adjusted to 8.5 by adding 25 μl triethylammonium bicarbonate (TEAB) buffer and samples were treated with 20 mM dithiothreitol (DTT) at 56°C for 60 min and 50 mM iodoacetamide in dark for 30 min. After digestion with 3 μg trypsin (sequencing grade, Promega, Madison, WI, USA) at 37 °C overnight, the peptides were labeled with iTRAQ tags and dried.

**2-D reversed-phase (RP) separation and mass spectrometer data acquisition**

The first dimension RP separation by microLC was performed by using a Durashell RP column (5 μm, 150 Å, 250 mm ×4.6 mm i.d., Agela, Tianjin, China). Mobile phases A (2% v/v acetonitrile, 20 mM ammonium formate, adjusted to pH 10.0 with NH₄OH) and B (98% acetonitrile, 20mM ammonium formate, adjusted to pH 10.0 using NH₄OH) were used to develop a gradient.
The peptides were eluted over 45 min by a gradient of 100% buffer A to 100% buffer B at a flow rate of 800 μl/min. In total, 10 fractions were generated for further LC-MS/MS analysis, for which a nanoflow HPLC instrument (EASY-nLC 1000 system, Thermo Fisher Scientific, Waltham, MA, USA) coupled to an on-line Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a nanoelectrospray ion source (Thermo Fisher Scientific) was used. The chromatography columns were packed in-house with Ultimate XB-C18 3 μm resin (Welch Materials, Shanghai, China). The peptide mixtures were loaded onto the C18 RP column (10-cm length, 75μm inner diameter) with buffer A (99.5% water and 0.5% formic acid, v/v) and separated with a 75-min linear gradient of 5–100% buffer B (99.5% acetonitrile and 0.5% formic acid, v/v) at a flow rate of 300 nl/min. Including the loading and washing steps, the total time for an LC-MS/MS run was 90 min. The electrospray voltage was 2.0 kV. Peptides were analyzed by data-dependent MS/MS acquisition with a dynamic exclusion duration of 18 s. In MS1, the resolution was 70,000, the automatic gain control (AGC) target was 3e6, and the maximum injection time was 20 ms. In MS2, the resolution was 17,500, the AGC target was 1e6, and the maximum injection time was 60 ms. The scan range was 300–1400 m/z, and the top 75 intensive precursor ions were selected for MS/MS analysis.

**Identification and quantification of mouse testicular or spermatozoa proteins**

The raw data were processed by using the proteomic workflow of Proteome Discoverer 1.3. The fragmentation spectra were searched against the UniProt
reviewed mouse database (2017_12, 16950 sequences) by the Mascot search engine (version 2.2.06) with the precursor and fragment mass tolerances set to 15 ppm and 20 milli-mass units (mmu), respectively. The algorithm was set to use trypsin as the enzyme, allowing for two missed cleavage sites. The fixed modification was carbamidomethylation (cysteine), and the variable modifications were oxidation (methionine), acetylation (protein N terminus), and iTRAQ labeling (tyrosine and lysine, N-terminal residues). Peptide ions were filtered from the cut off scores of Percolator based on \( p < 0.01 \). The false discovery rate was set to 1% for peptide identifications. An additional filter was applied with the removal of spectrum matches with scores lower than 10. The iTRAQ quantization values were automatically calculated on the basis of the intensity of the iTRAQ reporter ions in the dissociation scans with higher collision energy using Proteome Discoverer. All protein iTRAQ ratios were exported to an Excel file, the Gaussian distribution of ratios was recalculated manually, and all ratios were transformed to base 10 logarithm values. A confidence interval of 99% (testis proteome, 0.754-1.24; sperm proteome, 0.84-1.19 ) was used to determine the cut off values for statistically significant changes.

**Bioinformatics**

Broad functional classification of molecular function and biological process were performed by using Protein Analysis Through Evolutionary Relationships (PANTHER) tools (http://www.pantherdb.org/). The over-representation analyses of GO (http://www.geneontology.org/) for main biological processes
were performed by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tools (https://david.ncifcrf.gov/). The GO level 2 and 3 categories and a $P$ value cut-off of 0.01 were selected. Knockout and mutant phenotypes related to male reproduction and fertility were obtained by batch query from Mouse Genome Informatics (http://www.informatics.jax.org).

**Western blotting**

For protein separation, 50 μg proteins from each sample were loaded on 12% gels for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS)-PAGE. This was transferred to polyvinylidene difluoride membranes at 100 v for 1 h, blocked with 5% (w/v) skimmed milk for 1h, and incubated with primary antibodies (diluted 1:800 in blocking solution) (Supplementary table 1) at 4°C overnight with gentle agitation. The membranes were washed with 0.5% (v/v) Tween-20 in Tris–buffered saline (TBS) three times and incubated with horseradish peroxidase (HRP)-conjugated anti-IgG for 1 h at room temperature (RT). The immune-reactive complexes were detected by an enhanced chemiluminescence (ECL) kit (Amersham Life Science, Cleveland, OH, USA). The images were analyzed by commercial image analysis software (Gene Tools, version 4.02; Syngene, Cambridge, UK). The integrated optical density (IOD) of positive immune-staining was calculated, and the IOD ratio of target protein to housekeeping proteins was used to express the results of the western blotting analysis.
**Immunohistochemistry (IHC)**

Testicular tissue blocks were fixed in Bouin’s solution for 12 h, and then embedded in paraffin according to conventional methods. Sections of 4 μm thickness were de-waxed. Antigen retrieval was performed in a microwave oven above 94°C for 20 min. Endogenous peroxidases of sections were inhibited by incubation with 3% (v/v) H₂O₂ for 10 min. Then, 3% (v/v) BSA in TBS was used to block non-specific binding with antibodies at RT for 1 h. Sections were then incubated with corresponding primary antibodies (diluted 1:100 in blocking solution) overnight at 4°C. After washing three times with TBS, the sections were incubated with horseradish peroxidase-conjugated IgG (Zhong-Shan Biotechnology, Beijing, China) at a final dilution of 1:400 for 1 h at 37°C. A 3,3′-diaminobenzidine (DAB) kit (Zhong-Shan Biotechnology, Beijing, China) was used to visualize peroxidase activity at the binding sites. Hematoxylin was used to counterstain the sections. After dehydration, the sections were mounted for bright-field microscopy (DM LB2, Leica, Nussloch, Germany). Pre-immune IgG was used as a negative control. Image-Pro analysis software was used to analyze the images of immunostained sections.

**Immunofluorescence analysis**

The mouse spermatozoa were smeared on slides pre-coated with 1% (w/v) gelatin. The air-dried slides were fixed with cold methanol for 10 min, blocked for 1 h at room temperature with 3% (w/v) BSA in PBS and incubated at 4°C overnight with primary antibody against CMTM4, ODF2 (diluted 1:50 in 3%
BSA). After three washes with PBS, the anti-rabbit IgG (Alexa Fluor® 488 Conjugate) (1:100 in 3% BSA) was applied and incubated at room temperature for 1 h; rabbit IgG was used as a negative control. Samples were subsequently washed with PBS. Propidium iodide (0.01 mg/ml, Invitrogen, Carlsbad, CA, USA) counterstaining visualized the nuclei and sections were mounted in 80% (v/v) glycerol. Quantitative assessment of protein expression in spermatozoa was achieved by a Zeiss LSM 510 laser confocal microscope (LSM, Carl Zeiss Microimaging, Thornwood, NY, USA). Slides were systematically examined at 400× magnification according to the World Health Organization manual 2010. Inspection was performed in sequence until a total of 200 spermatozoa had been assessed. By using Zeiss LSM510 Meta software (LSM5 version 3.2, Carl Zeiss Microimaging, Thornwood, NY, USA), the fluorescence intensity value per stained sperm was calculated automatically by subtracting the background fluorescence intensity, which was determined by scanning a sperm-free area.

**Experimental design and statistical rationale**

The expression of CMTM4 in spermatozoa from normozoospermic, asthenozoospermic, oligoasthenozoospermic, and teratozoospermic men was evaluated by western blotting. Its cellular localization and expression intensity in testis from young adult, elderly adult, and NOA patients were detected by IHC. *Cmtm4* KO mice were constructed to investigate CMTM4 function in male infertility. The testis proteomes, epididymal sperm proteome from *Cmtm4* WT and KO mice were compared by iTRAQ, each group was a pool from 10 mice and performed in 2 technical replicates. The MS data were processed by using
Proteome Discoverer 1.3 and searched against the UniProt (2017_12, 16950 sequences) with the Mascot search engine (version 2.2.06). The false discovery rate was set to 1% for peptide identifications. Data are reported as means±SD. Means of two groups were analyzed with Student’s *t*-test, and more than two group means were analyzed by one-way analysis of variance (ANOVA). A commercial software package (SPSS 18.0; SPSS, Chicago, IL, USA) was used to perform the correlation analysis. A value of *P*<0.05 was considered to be statistically significant.

RESULTS

**Association of CMTM4 with human spermatogenesis and sperm quality**

Western blots were performed on protein extracts from the spermatozoa of fertile and infertile men. Spermatozoa from patients diagnosed with asthenozoospermia, oligoasthenozoospermia, or teratozoospermia exhibited obvious poor sperm quality with decreased sperm motility or sperm count (Figure 1A) (Supplementary Table 2). As shown in Figure 1C-D, the expression of CMTM4 in spermatozoa from asthenozoospermic, oligoasthenozoospermic, and teratozoospermic men was significantly lower than that in spermatozoa from normazoospermic men. Especially, significantly decreased expression of CMTM4 was observed in spermatozoa from teratozoospermic patients. An analysis of gene expression omnibus (GEO) data from GSE6968, which shows
gene expression profiles of normozoospermic and teratozoospermic spermatozoa, demonstrated significant down-regulation of CMTM4 in teratozoospermic spermatozoa (Figure 1B). To further explore the association of CMTM4 with spermatogenesis, we compared the expression levels of CMTM4 in the testes of young adults with those of elderly men, who have poor spermatogenesis associated with poor sperm quality as described in our previous study (26,31), and with NOA patients, who have impaired spermatogenesis due to Sertoli cell-only pattern (Figure 2F), maturation arrest (Figure 2G), and hypospermatogenesis (Figure 2H) (Supplementary Table 2). Testes from 10 young adults (including 5 biopsies from OA patients) (score=8), 5 elderly adults (score=6), and 20 NOA patients (score=0) were evaluated as shown in Figure 2A. Morphologically, elderly testes showed hyperplasia of the interstitial layer and vacuolization of seminiferous tubules. Compared with young adults, the testes of elderly adults and NOA patients had significantly decreased CMTM4 expression by immunohistochemical analysis (Figure 2B).

Cmtm4 KO mice were constructed by Cas9 microinjection

To further investigate the role of CMTM4 in spermatogenesis and male fertility, we constructed Cmtm4 KO mice using CRISPR-Cas9. A pair of independent sgRNAs targeting exon 1 of the Cmtm4 locus was designed to establish the homozygous KO mouse model. Co-injection of the two independent sgRNAs with Cas9 mRNA caused a 179-bp deletion spanning the 5’ untranslated region
(UTR) and exon 1 in the open reading frame of the Cmtm4 locus, resulting in a frame-shift mutation and KO of Cmtm4 (Figure 3A). Deletion of the Cmtm4 gene was confirmed by PCR and sequencing analyses (Figure 3B). Western blot analysis confirmed the absence of CMTM4 protein in KO mice (Figure 3C). IHC analysis showed that CMTM4 was mainly localized in the cytoplasm of spermatocytes and round spermatids of WT mice, whereas no signal was detected in the testis of KO mice (Figure 3D, E).

**CMTM4 requirement for male and female fertility**

All germ cells (i.e., spermatogonia, primary spermatocytes, round spermatids, elongated spermatids, and Sertoli cells) were examined in the seminiferous tubules of Cmtm4 KO mouse testes, and no obvious morphological abnormalities were found.

No significant differences were observed in the testis/body weight ratio, cauda epididymal sperm content, or serum testosterone concentration between WT and KO mice (Figure 4A-C). DSP and progressive motility were significantly reduced in adult KO males compared with adult WT males (Figure 4D-G). A higher percentage of abnormal epididymal spermatozoa with backward-curved heads and bent midpieces were observed in Cmtm4 KO mice (Figure 4H,K, Supplementary Table 3).

KO males mating with WT females produced fewer pregnancies after 2 months of continuous cohabitation. The Cmtm4+/− littermates were all fertile,
and produced the same number of offspring as WT littermates. \textit{Cmtm4} KO females gestated normally when mated with WT males (Table 1). We then examined the \textit{in vitro} fertilization (IVF) capacities of spermatozoa obtained from the epididymides of WT and KO male mice. IVF results showed that the spermatozoa of KO mice had low fertilization abilities (Table 2, Figure 4J,L), implying impaired sperm function. There was no difference in the percentage of intact acrosomes between spermatozoa from WT and KO mice, whereas a significantly lower percentage of acrosome reactions was induced by the calcium ionophore A23187 in spermatozoa from KO mice (Figure 4I,M).

\textbf{Differentially expressed proteins in KO mouse testes identified through quantitative proteomics}

To identify testicular proteins and biological processes that were associated with CMTM4 in male fertility, we performed comprehensive differential proteomics analysis of WT and \textit{Cmtm4} KO mouse testes. iTRAQ was applied to compare the proteomes of whole testes from 8-week-old WT and \textit{Cmtm4} KO mice, and each group was pooled from 10 mice and performed in duplicate (Figure 5A). A total of 5394 testicular proteins were identified (Supplementary Table 5). There was an overlap of 1702 (31.6\%) proteins with the reported mouse germ cell proteome (32) (Figure 5B) and 2052 proteins with the reported mouse sperm cell proteome (33) (Figure 5C). A broad functional category classification was performed (Figure 5D,E).
In total, 351 proteins were differentially expressed between WT and Cntm4 KO testes, including 212 up-regulated and 139 down-regulated proteins in the KO testes (Supplementary Table 6). A broad functional classification showed that majority of proteins related to signal transduction (16%), actin/tubulin binding (9%), and protease/protease inhibitor (8%) functions were down-regulated in KO mice, whereas more proteins involved in transport (11%) and DNA binding (9%) functions were up-regulated in KO mice (Figures 6A,B). Enrichment analysis indicated that most of the down-regulated proteins were involved in spermatogenesis and regulation of sperm quality, such as sperm motility and the acrosome reaction (Figure 6C), whereas the up-regulated proteins were mainly involved in metabolic processes (Figure 6D). Several well-known proteins involved in the acrosome reaction (IZUMO1, CRISP1, AKAP1, AKAP3, AKAP4, PLB1, and TRIM36) and sperm motility (ODF1, ODF2, ATP1A4, PGK2, SORD, AKAP3, AKAP4, SMCP, and GAPDHS) were down-regulated in the KO mice (Figure 6E), whereas some up-regulated proteins belonged to the chromatin components involved in the DNA packaging complex (e.g., HIST1H1B, H2AFX, HIST1H3A, H1FX, and H3F3C). Using retrieved GEO dataset GDS3142 that includes mouse transcriptome of different tissues, differentially expressed gene products in the KO testis was grouped into specific, high, wide, and undetectable (low) expression patterns. Notably, 100 (71.9%) of the down-regulated proteins were specifically expressed in the testis (Figures 6F,G). Characterization of germ cell-type specific expression of
the differentially expressed proteins showed that 57% of down-regulated proteins were specifically expressed in round spermatids (Figures 6H,I). The proteomics results indicated that CMTM4 deficiency mainly affected the expression of proteins involved in sperm motility and the acrosome reaction.

The differential expression of selected proteins (PGK2, SORD, HSPA1L, and HSPA9) was confirmed by western blot and IHC analyses. The IHC results indicated that PGK2 and SORD were mainly expressed in round spermatids, whereas HAPA1L and HSPA9 were mainly expressed in spermatocytes (Figure 7). All of these proteins showed down-regulated expression in the KO mouse testis. In agreement with the IHC results, lower levels of these proteins were observed in the KO mice in western blot analysis (Figure 7).

**Differentially expressed proteins in KO mouse sperm identified through quantitative proteomics**

To identify differentially expressed sperm proteins affected by Cmtm4 KO, we next performed proteomic analysis of Cmtm4 KO sperm, and identified 162 up-regulated and 247 down-regulated proteins relative to Cmtm4 WT sperm (Supplementary Table 7). A total of 3588 sperm proteins were identified (Supplementary Table 8), which included 667 (18.6%) and 2103 (58.6%) proteins overlapped with previously reported mouse sperm proteomes (33,34) (Figure 8A,B). 1410 sperm proteins were newly identified in the present study. The majority of these proteins were related to broad functions of metabolism
(30%), catalysis (11%), structural (11%) and energetics (11%), which provided complementary information for mouse sperm proteome (Figure 8C).

Enrichment analysis indicated that the up-regulated proteins were mainly related to functions of DNA binding including histones H2A, H2B, H4 and their variants in KO sperm (Figure 8D). The abnormal up-regulated levels of H2A, H2B, H4 were validated by western blotting. Given that nucleosomal histones were involved histone-to-protamine exchange, the expression of PRM1, PRM2 were also validated. PRM1 and PRM2 showed down-regulated expressions in KO sperm (Figure 9A,B). Down-regulated proteins in KO sperm were mainly related to actin binding functions, which are well-known related to sperm motility and acrosome reaction (Figure 8E). 15 proteins are characterized with male reproductive phenotypes of abnormal sperm function (Supplementary table 9). ODF2, CFL1, MYH11, TPM1, α-tubulin and β-tubulin were validated to be lower expressed in KO sperm. Immunofluorescence analysis demonstrated that Cmtm4 was localized in sperm acrosome and middle piece, and ODF2 showed low intensity in KO sperm (WT sperm:178±18.5; KO sperm:98.6±10.7, P<0.05) (Figure 9C).

DISCUSSION

Sperm quality is the decisive factor in male fertility (35). Most infertile male patients are characterized by poor sperm quality (36, 37,38). Because they are likely to affect spermatogenesis or sperm quality, genes and gene products that
are highly expressed in the testis are promising targets for the diagnosis or
treatment of male infertility (39-42). Our previous studies have identified
CMTM4 is highly expressed in the human testes (2, 23); thus, we hypothesized
that CMTM4 was correlated with spermatogenesis, and its altered testicular
expression would consequently be related to sperm quality. In the present
study, we investigated the association of CMTM4 with spermatogenesis and
sperm quality, and demonstrated a role of CMTM4 in male fertility by
phenotypic and quantitative proteomic analyses of the Cmtm4 KO mouse
model. This study provides novel information in the functional research of the
CMTM family in male reproduction.

In the present study, CMTM4 was observed to be aberrantly expressed in
the spermatozoa of infertile individuals, indicating its association with sperm
quality. As in our previous reports, the elderly testis showed decreased
spermatogenesis, resulting in poor sperm quality (26, 31). Here, IHC analysis
showed down-regulated expression of CMTM4 in elderly and NOA testes, with
especially low expression in NOA testes. The combined results imply that
CMTM4 is a potential key protein in spermatogenesis and sperm quality
regulation.

There is a high homology of the amino acid sequences (95.7%) between
human and mouse CMTM4, and thus the Cmtm4 KO mouse is an ideal model
for investigating the functions of CMTM4 in male reproduction in vivo. The
Cmtm4 KO mouse revealed subfertility in males but no significant
morphological or behavioral abnormalities. No significant difference in serum testosterone level in KO mice compared with WT mice was observed. This indicates that Cmtm4 is not actively involved in androgen regulation, whereas other members of the CMTM family, such as mouse Cmtm2a and Cmtm2b and human CMTM1-v17, CMTM2, and CMTM3, are reported to affect androgen receptor activity (19,22,43). However, Cmtm4 KO mice showed an obvious phenotype of reduced progressive sperm motility, abnormal spermatozoa with backward-curved heads or bent midpieces, leading to male subfertility. A previous study reported that CMTM4 knockdown in HeLa cell resulted in cytokinesis defects (44). Thus, Cmtm4 deficiency may affect male meiotic cytokinesis, thereby resulting in lower DSP and abnormal spermatozoa. These distinct phenotypes between WT and KO mice suggest that they may alter sperm functions in KO mouse. Observation of the acrosome reaction indicated that a significantly reduced percentage of acrosome reactions was induced in KO mice sperm by the calcium ionophore A23187. These findings imply that CMTM4 is actively involved in spermatogenesis and sperm quality regulation. Further in vivo fertility assessment showed that male KO mice were subfertile, whereas female KO mice had normal fertility. In vitro fertility experiments also confirmed that spermatozoa from KO mice was inefficient in producing a two-cell stage. Collectively, these results indicate that Cmtm4 is associated with spermatogenesis and sperm quality, and is essential for male fertility.
Furthermore, quantitative proteomic analyses were performed to detect the molecules and biological processes in testes affected by Cmtm4 deficiency. Our study identified 5840 mouse testis proteins, which provides an inventory of mouse testicular proteins useful for the understanding of spermatogenesis. Of these, 351 proteins showed differential expression between WT and KO mice. Bioinformatic analysis revealed notable effects of Cmtm4 deficiency on male infertility. Down-regulated proteins in KO mice included those that function in spermatogenesis and sperm functions. Of the down-regulated proteins, 100 (71.9%) were testis-specifically expressed, reflecting an association with impaired spermatogenesis, and 57% of down-regulated proteins were exclusively expressed in round spermatids, indicating the association with sperm quality might involve regulation of post-meiotic biological processes. These down-regulated proteins are involved in fertilization, sperm motility, the acrosome reaction, and sperm-oocyte recognition. Several of the down-regulated proteins are known to be involved in spermatogenesis; for example, PKG2 and GAPDHS are more abundant in round spermatids, and are testis-specific glycolytic enzymes positively correlated with sperm motility (45-47). Odf1 and Odf2 are major components of the outer dense fibers that modulate sperm motility (48). Spata20, Spata3, Spata6, Spata7, and Spert are well-known spermatogenesis-related proteins (49). Inactivation of Spata6 in mice leads to acephalic spermatozoa and male sterility (50). IZUMO1, CRISP1, AKAP3, AKAP4, PLB1, and TRIM36 are classified in the literatures by
bioinformatic analyses as having functional involvement in the acrosome reaction (51-54), and their down-regulated expression may contribute to the deficient acrosome reaction of KO mouse spermatozoa. The down-regulation of these spermatogenesis-related proteins may contribute to lower sperm quality and reduced fertility. To further understand the effect of CMTM4 knockout on mouse sperm quality, a quantitative proteomics was performed to detect the affected sperm molecules by Cmtm4 deficiency. A total of 3588 proteins were identified and 1997 proteins were included in current testis proteome, which provides complementary information for sperm protein profiles (Supplementary Figure 1). Chromatin DNA binding function that was overrepresented in up-regulated proteins of KO mouse sperm are known to participate in chromatin remodeling and meiosis during spermatogenesis (55). Particularly, western blotting demonstrated the increased levels of H2A, H2B and H4 in KO sperm, and conversely, reduced levels of PRM1 and PRM2 in KO sperm. The results suggest that impaired histone-to-protamine exchange exists in KO sperm, which may contribute to abnormal sperm morphology and function (56). Most of down-regulated proteins in KO sperm perform actin binding functions, which are well-known to play crucial roles in maintenance of sperm morphology and motility (57). Cofilin in sperm is involved in acrosome reaction and its down-regulation in sperm is correlated with poor sperm quality (58). ODF2 is related to sperm motility (59). Interestingly, α-tubulin and β-tubulin are down-regulated in KO sperm, suggesting the impaired sperm motility and
acrosome function (57). Collectively, proteomic alterations in KO sperm indicate its influence on sperm motility and acrosome reaction, which is consistent with its functions of localization in acrosome and midpiece, and implications of proteomic alterations in KO testes.

This study revealed an important role of CMTM4 in male reproduction. CMTM4 expression level was associated with human spermatogenesis and sperm quality. Phenotypic and quantitative proteomic analyses of Cmtm4 KO mice revealed that CMTM4 mainly affects histone-to-protamine exchange during spermatogenesis process, and functions in sperm motility and acrosome reaction. Proteomic analysis also identified a group of essential proteins as being affected by Cmtm4 deficiency. Their interactions and functions in male fertility are warranted to be further study in the future. This study provides an impetus for investigating the roles of other members of the CMTM family in male reproduction, and also shows that CMTM4 is a promising molecular candidate for the assessment of sperm quality and the diagnosis or treatment of male infertility.

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AUTHOR CONTRIBUTIONS

WenLing Han, FuJun Liu contributed to the research design. XueXia Liu and WenJuan Wang collected the clinical samples; FuJun Liu, XueXia Liu, Xin Liu, Peng Zhu, ZhengYang Liu, Hui Xue, XiULan Yang, Juan Liu performed research. FuJun Liu, XueXia Liu and Xin Liu analyzed data. FuJun Liu and WenLing Han wrote the paper. All authors have read and approved the final manuscript.

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DATA AVAILABILITY: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011936 and 10.6019/PXD011936.

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Table 1. Fertility and fecundity of WT and Cmtm4 KO mice.

| Genotype | Male fertility | Litter numbers | Female fertility | Litter numbers |
|----------|----------------|----------------|------------------|----------------|
| +/+      | 15/15          | 8.46±1.85      | 15/15            | 8.33±2.14      |
| +/-      | 15/15          | 8.60±1.72      | 15/15            | 7.47±1.30      |
| -/-      | 4/15           | 4.00±0.82      | 12/15            | 7.67±2.02      |

Mice of the indicated genotypes were caged with WT mice of the opposite sex. Male or female fertility was indicated as the number of fertile mice/total number of mice, values are shown as mean ± SD.
Table 2. *In vitro* fertilization assay of WT and *Cmtm4* KO mice.

| Group                | Total No. of oocytes inseminated | Total No. of oocytes fertilized | Fertilized oocytes developed to Two cells (%) | Blastocysts (%) |
|----------------------|----------------------------------|---------------------------------|----------------------------------------------|-----------------|
| WT sperm + WT oocyte | 124                              | 73                              | 73 (100)                                     | 37 (50.7)       |
| WT sperm + KO oocyte | 130                              | 82                              | 78 (95.1)                                    | 38 (48.7)       |
| KO sperm + WT oocyte | 148                              | 26                              | 26 (100)                                     | 2 (7.7)         |
| KO sperm + KO oocyte | 132                              | 18                              | 18 (100)                                     | 1 (5.6)         |
Figure legends

Figure 1. Expression of CMTM4 in human spermatozoa.

Human spermatozoa were collected from normozoospermic, asthenozoospermic, oligoasthenozoospermic, teratozoospermic patients and showed significant differences in progressive sperm motility (A); comparison of CMTM4 expression in spermatozoa in normozoospermia and teratozoospermia was analyzed by retrieving GEO data from GSE6968 (B); western blot analysis of CMTM4 in human spermatozoa in normozoospermia, asthenozoospermia, oligoasthenozoospermia and teratozoospermia. The quantification of CMTM4 expression was calculated by normalizing band intensities to the respective TUBB expressions by Image Pro software (C-E). Asth, asthenozoospermia, Olig, oligoasthenozoospermia, Tera, teratozoospermia; Lines (A) indicate mean values, whiskers indicate standard deviation; Data were compared by one-way analysis of variance (ANOVA);*, *P<0.05.

Figure 2. Expression of CMTM4 in human testes.

Johnsen score evaluation of testicular sections from young adults, elderly adults and patients with non-obstructive azoospermia (NOA) (A); quantitative evaluation of CMTM4 expression in testes by Image-Pro (B); representative image of testicular cellular localization of negative control (C) and CMTM4 in young adults (D), elderly adults (E) and NOA patients (F-H); SP, spermatogonia; PS, pachytene spermatocyte; RS, round spermatid; SC, Sertoli cell; Spt, spermatids; Lines (A, B) indicate mean values, whiskers indicate standard deviation; Data
were compared by one-way analysis of variance (ANOVA); * , P<0.05; Each bar represents 20 μm.

**Figure 3. Construction of Cmtm4 KO mice**

Scheme of the genomic target sites in the *Cmtm4* (A). The untranslated region (UTR) of *Cmtm4* is highlighted in green and the coding sequence (CDS) of *Cmtm4* is indicated in blue. 179bp sequences in exon 1 were deleted in *Cmtm4* KO mice. Polymerase Chain Reaction (PCR) (B), western blot (C) and immunohistochemistry analysis (D, E) were performed on mouse testis samples.

WT, wild type; HT, heterozygous; KO, knockout; SP, spermatogonia; PS, pachytene spermatocytes; RS, round spermatids; ES, elongated spermatids. Each bar represents 20 μm.

**Figure 4. Characteristics of fertility of Cmtm4 WT and KO male mice.**

No variance in testicular weight (A), sperm concentration (B) and serum testosterone level (C) between WT and KO mice was observed. Significantly reduced daily sperm production (D) and sperm motility (E,F,G) and increased abnormal sperm morphology (H,K) were observed in Cmtm4 KO mice compared with WT mice. *In vitro* fertility analyses showed a decreased percentage of induced acrosome reactions (I,M), the numbers in brackets represent the number of mice examined, and the decreased percentage of oocytes developed to two-cell stages. The numbers in brackets represent the number of WT oocytes used (J,L). Values are shown as mean ± SD. a, curved back sperm head; b, bent sperm midpiece; WT, wild type; KO, knockout; VSL,
straight line velocity; VCL, curvilinear velocity; Lines (E,F,G) indicate mean values, whiskers indicate standard deviation; Data were compared by one-way analysis of variance (ANOVA);*, P<0.05; **, P<0.01. Each bar represents 20 μm.

**Figure 5. iTRAQ-based quantitative proteomic analysis of Cmtm4 KO mice testis.** Flow chart of iTRAQ analysis of the mouse testicular proteome (A); Venn diagram of the current mouse testis proteome and a previously reported mouse germ cell proteome (B), and the current mouse testicualr proteome and a previously reported mouse sperm proteome (C); broad functional analysis of the current mouse testicular proteome in GO terms of molecular functions (D) and biological processes (E).

**Figure 6. Bioinformatic analysis of differentially expressed proteins in WT and Cmtm4 KO mice testes.** Broad functional classification of down-regulated proteins (A) and up-regulated proteins (B) in KO mice, and biological processes enriched in down-regulated proteins (C) and in up-regulated proteins (D) in KO mice; interactional network between differential proteins and major biological processes of sperm function (E); tissue expression patterns of down-regulated proteins (F) and up-regulated proteins (G); testicular germ cell expression patterns of down-regulated proteins (H) and up-regulated proteins (I) in the normal mouse testis.
Figure 7. Western blot (A,B) and immunohistochemical (C) analysis of PGK2, SORD, HSPA1L and HSPA9 in WT and Cmtm4 KO mice. The integrated optical density (IOD) ratio of target protein to ACTB was used to express the results of the western blot analysis. Lines (C) indicate mean values, whiskers indicate standard deviation; Data were compared by Student’s t-test; *, P<0.05; Each bar represents 50μm.

Figure 8. iTRAQ-based quantitative proteomic analysis of Cmtm4 KO mice sperm. Flow chart of iTRAQ analysis of the mouse sperm proteome (A); Venn diagram of the current mouse sperm proteome and previously reported mouse sperm proteomes (B); broad function of current mouse sperm proteoms (C); enriched molecular functional analysis of down-regulated (D) and up-regulated proteins (E) in KO sperm.

Figure 9. Western blot and immunofluorescence staining of selected proteins. Western blot analysis of H2A, H2B, H4, PRM1, PRM2, MYH11, ODF2, CFL1, TPM1, α-tubulin, β-tubulin, and ACTB serving as a control (A); quantification of blotting intensity is shown as ratio of normalized IOD of each band (KO/WT) (B); quantitative immunofluorescence of CMTM4 and ODF2 in WT and KO sperm (C), intensity was calculated by using Zeiss LSM 510 Meta software.
Figure 1.
Figure 2.

A

Percentage of tubules showing elongated spermatids

Score=8  Score=6  Score=0

Young adults  Elderly adults  NOA

B

Mean value

Young adults  Elderly adults  NOA

C D E F G H

SP  PS  SC  RS  Spl

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Figure 3.

Cmtm4 CKLF-like MARVEL transmembrane domain containing 4 [Mus musculus]

TTTCCGCTGCAGGGCAGCAGCCAGCAGAGAGAGAAGACGAGGGAGCGCG
CGCGAGCGGCCGGCGCGCGCGAGGCAGGCGCGCGCGGGTCGTTGTTGCTGTCGGAC
GGCGATGGGCGGCGGGCGCTCTGAGCCTGCCCAGCTCCAGCCGGCCGGGGAACC
GGCGCGCCGCCAGCGCGCGAGGCAGGACCTGAGCAGCCGGCGGGGGG
AGGGCAGGCGCTGAGCACCTCA

B

784bp
605bp
546bp

WT HT KO

Gapdh Cmtm4

C

29kD
22kD
42kD

WT HT KO

CMTM4

ACTB

D

WT

SP PS RS

E

KO

ES SP RS PS
Figure 4.

A. Testes/body weight (g).
B. Sperm count (×10^6).
C. Testosterone (ng/mL).
D. Daily sperm production per testis (10^6).
E. Progressive motility (%).

F. VSL (μm/sec).
G. VCL (μm/sec).

H. Percentage of abnormal sperm.
I. Induced AR rate (%).
J. Two cell stage (%).

K. WT sperm + WT oocyte vs KO sperm + WT oocyte.

M. Before induction vs After induction.

WT
KO

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Figure 6.

A

B

C

D

E

F

G

H

I

Down-regulated proteins

Up-regulated proteins

Down-regulated proteins

Up-regulated proteins

SP: Spermatogonia; PS: Pachytene spermatocytes; RS: Round spermatids
W: Wide expression; L: Low expression
Figure 7.

A

|   | WT 1 | WT 2 | WT 3 | KO 4 | KO 5 | KO 6 |
|---|------|------|------|------|------|------|
| PGK2 |      |      |      |      |      |      |
| SORD |      |      |      |      |      |      |
| HSPA1L |      |      |      |      |      |      |
| HSPA9 |      |      |      |      |      |      |
| ACTB |      |      |      |      |      |      |

B

- PGK2
- SORD
- HSPA1L
- HSPA9

WT vs. KO

C

- PGK2
- SORD
- HSPA1L
- HSPA9

WT and KO

Mean value

* p < 0.05
Figure 8.
Figure 9.

A

| Protein | WT | KO |
|---------|----|----|
| H2A     |    |    |
| H2B     |    |    |
| H4      |    |    |
| PRM1    |    |    |
| PRM2    |    |    |
| β-actin |    |    |

B

- **β-tubulin**
- **α-tubulin**
- **TPM1**
- **CFL1**
- **ODF2**
- **MYH11**
- **PRM2**
- **PRM1**

Red bars indicate up-regulation, and blue bars indicate down-regulation.

Ratio of normalized IOD of each band (KO/WT)

C

| Protein | WT  | KO  |
|---------|-----|-----|
| CMTM4   | 102.2±20.3 | 0    |
| ODF2    | 176.4±18.5 | 96.6±10.7 |