PPP2R1B is modulated by ubiquitination and is essential for spermatogenesis

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Abstract
The serine-threonine protein phosphatase 2A (PP2A) is a heterotrimeric enzyme complex that regulates many fundamental cellular processes. PP2A is involved in tumorigenesis because mutations in the scaffold subunit, PPP2R1B, were found in several types of cancers. However, the biological function of PPP2R1B remains largely unknown. We report here that homozygous deletion of Ppp2r1b in Mus musculus impairs meiotic recombination and causes meiotic arrest in spermatoocytes. Consistently, male mice lacking Ppp2r1b are characterized with infertility. Furthermore, heterozygous missense mutations in the Homo sapiens PPP2R1B gene, which encodes PPP2R1B, are identified in azoospermia patients with meiotic arrest. We found that PPP2R1B mutants are susceptible to degradation by an E3 ligase CRL4A DCAF6, and resistant to de-polyubiquitylation by ubiquitin-specific protease 5 (USP5). In addition, heterozygous mutations in PPP2R1B reduce stability of the wild-type PPP2R1B. Our results demonstrate an essential role of PPP2R1B in spermatogenesis and identify upstream regulators of PPP2R1B.

KEYWORDS
DCAF6, meiotic arrest, meiotic recombination, spermatogenesis, USP5

Abbreviations: CHX, protein synthesis inhibitor cycloheximide; DAPI, 4′,6-diamidino-2-phenylindole; DCAF6, DDB1-CUL4 associated factor 6; DDB1, damaged DNA-binding protein 1; DUB, Deubiquitinase; H&E, Hematoxylin and eosin; IF, immunofluorescence; MG132, Carbobenzoxy-Leu-Leu-leucinal; MLH1, MutL homolog 1; MS, mass spectrometry; Neo, neomycin resistance gene; PP2A, the serine-threonine protein phosphatase 2A; SC, synaptonemal complex; siRNA, short interfering RNA; SYCP1, synaptonemal complex protein 1; SYCP3, synaptonemal complex protein 3; Ub, ubiquitin; USP5, ubiquitin-specific protease 5; WT, wild-type.

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PP2A is a ubiquitously expressed serine threonine phosphatase family protein that is involved in many fundamental cellular processes, such as cell proliferation, signal transduction, DNA repair, and apoptosis. It is a heterotrimer composed of a dimeric core enzyme that consists of a scaffold (A) and catalytic (C) subunit and a third variable regulatory subunit (B). The scaffold subunit PP2A-A provides a base for physical assembly that facilitates interaction of the catalytic subunit and the regulatory subunit, as well as interaction with other substrates. PP2A-A has two paralogous isoforms, PPP2R1A and PPP2R1B, which have 87% sequence identity and are encoded by two distinct genes, PPP2R1A and PPP2R1B. PPP2R1A is predominant, being found in ~90% of PP2A-A complexes, and it is highly abundant in all tissues and regulates various cellular processes. Previous studies have identified mutations of both of these isoforms in a variety of human malignancies, including breast, lung, skin and ovary cancer, suggesting that they play a suppressive role in cancer. However, the physiologic function of PPP2R1B in vivo is unknown.

More than 20 million men worldwide are afflicted with infertility, which is a perplexing disorder. Azoospermia, which is defined as the absence of sperm in the ejaculate, accounts for up to 20% of cases of male infertility and is often untreatable. Non-obstructive azoospermia is characterized by spermatogenic failure, and mainly consists of Sertoli-cell-only syndrome and meiotic arrest. Male infertility is a complex phenotype, and there are many genetic and environmental causes of spermatogenic impairment. Genetic factors such as chromosome aberration and microdeletions in the azoospermia factor region are causes of male infertility. However, the genetic alterations and molecular basis of the majority of cases of azoospermia are unknown. More than 3,000 genes associated with spermatogenesis have been identified, and more than 400 mutant mouse models with a reproductive phenotype have been established. These models have provided information regarding genes and mechanisms involved in infertility. However, only a few of these genes, including SOHLH1, SYCP3, SYCE1, TEX15, and TDRD9, have shown alterations in individuals with azoospermia. Although many of these genes are involved in the process of spermatogenesis, only a few are critical for the direct promotion of spermatogenesis. Therefore, understanding of the genetic events underlying spermatogenesis will offer potential clues for the development of diagnostic tools and approaches to the treatment of infertile men.

Ubiquitinated proteins are involved in many important cellular processes such as embryonic development, cell cycle control, immune response, intracellular signaling pathways, Amerik and Hochstrasser et al. Ubiquitination can be divided into two types, monoubiquitination and polyubiquitination. They are classified based on the number of ubiquitin molecules. Monoubiquitination is responsible for signal transduction, receptor endocytosis and DNA repair, and polyubiquitination is essential for signal transduction and protein degradation, which is the well-known function of ubiquitination. Ubiquitination is mainly conducted by ubiquitin-activating enzymes, ubiquitin-conjugating enzymes, and ubiquitin ligases. Deubiquitination is accomplished by deubiquitilase. Therefore, these two processes play important roles in protein stability. Spermatogenesis is composed of a number of complex processes and includes several dramatic morphological changes. Meiotic proteins and histones are dynamic during spermatogenesis, and thus ubiquitin-proteasome system is suggested to participate in the regulation of these proteins. Although there is report that ubiquitination of histones by RNF20 promotes DNA double-strand break repair and histone replacement, the function of ubiquitination in spermatogenesis needs further investigation.

The goal of this study was to broaden our understanding of male infertility. We found that germ-line Ppp2r1b-deficient male mice are infertile, and spermatocytes in these mice accumulate in the pachytene stage of meiosis. We investigated the function of PPP2R1B in spermatogenesis and found that the meiotic recombination is abnormal. Whole exome sequence analysis was carried out in blood samples obtained from four generations of a family, where two members were azoospermic. This analysis yielded the identification of a PPP2R1B gene mutation. Next, we performed direct Sanger sequencing of the PPP2R1B gene open reading frame from 257 patients with azoospermia, and identified other heterozygous missense mutations in the PPP2R1B gene. We found that mutations in the PPP2R1B gene identified in meiotic arrest-mediated azoospermia result in unstable PPP2R1B mutants due to high binding affinity with an E3 ligase CRL4A DCAF6. In addition, the instability of PPP2R1B mutants almost could not be reversed by USP5. Moreover, PPP2R1B protein was almost undetectable in patients with PPP2R1B heterozygous mutants due to the influence of mutants on the wild-type PPP2R1B protein. These results demonstrate that PPP2R1B plays a critical role in mediating meiotic progression during spermatogenesis.

2 | MATERIALS AND METHODS

2.1 | Experimental model and subject details

2.1.1 | Human study

The human studies were conducted in accordance with the Declaration of Helsinki and the International Conference
on Harmonization Good Clinical Practice guidelines and were approved by the ethics committees at Peking University Third Hospital. The approval number is 2017S2-034. Informed consent was obtained from all subjects or their relatives. A four-generation family in which two male individuals were infertile due to meiotic arrest was brought to our attention by the Department of Urology at Peking University Third Hospital. Five members of this family, including the two members with azoospermia, were recruited. Blood samples obtained from this family were subjected to whole-exome sequence analysis. A mutation was identified in the gene PPP2R1B, which showed maternal transmission. Next, a cohort of 257 patients selected for azoospermia and 258 normal control men were recruited from the Center for Reproductive Medicine at Peking University Third Hospital. This cohort included 120 patients with meiotic arrest and 137 with Sertoli-cell-only syndrome. Sanger sequencing was used to screen for PPP2R1B gene mutations in these 257 patients and 258 controls.

A diagnosis of non-obstructive azoospermia in patients was confirmed by semen analysis performed according to the guidelines of the World Health Organization. Based on the histologic examination of testicular biopsies, azoospermia was defined as Sertoli-cell-only (germ cells absent) or meiotic arrest (absence of maturation beyond spermatocytes). Men with known causes of infertility, including other chromosomal abnormalities and Y-chromosome microdeletions, were excluded from this study.

2.1.2 | Generation of the Ppp2r1b knockout mice

Mice were used in accordance with mandated standards of care, and studies were approved by the Institutional Animal Care and Use Committee at Peking University. C57BL6J and 129S6 were purchased from Jackson Laboratories. EIIa-Cre mice were obtained from Dr Chyuan-Sheng Lin in Columbia University as a gift. To generate Ppp2r1b<sup>lox<sup>−</sup> lox<sup>−</sup></sup> mice, C57BL6J/129S6 hybrid mouse ES cells were electroporated with a targeting vector containing the floxed promoter and exon 1. ES cells were selected by G418 treatment and were screened for homologous recombination by PCR. Positive ES clones were injected into C57BL/6J blastocysts, and the blastocysts were implanted into pseudopregnant females to generate chimeras. The male chimeras were bred with female Actin-Flpe transgenic mice to delete the FRT-flanked neomycin resistance gene (Neo) from the targeted allele. Neo-deleted Ppp2r1b<sup>lox<sup>−</sup> lox<sup>−</sup></sup> mice were genotyped by PCR. Two heterozygous mice were mated to generate Ppp2r1b<sup>lox<sup>−</sup> lox<sup>−</sup></sup> mice. Ppp2r1b<sup>lox<sup>−</sup> lox<sup>−</sup></sup> mice were backcrossed with C57BL6J for six generations and were then mated with EIIa-Cre transgenic mice to delete the floxed promoter and exon 1. Genotyping of mice by PCR was performed using the following two pairs of primers: 5′-ACCGAACTGGGTGAGTAAAACGT-3′; 5′-CAAGGACCCTTTAACACGTATTG-3′ and 5′-CGTCGTCTGGGACCGG-3′; 5′-GCACGTCTCATTGCGGACG-3′. Mice were housed in a pathogen-free facility. Both female and male mice, 2-6 months of age, were used for the phenotypic analysis. Male mice at 8-50 days postpartum were used to investigate the temporal expression patterns of PPP2R1B and PPP2R1A.

2.1.3 | Cell lines

HeLa (RRID:CVCL_0030) and HEK293T (RRID:CVCL_0063) cells were purchased from ATCC, authenticated by STR profiling and tested for mycoplasma contamination by GENEWIZ. These cells were cultured in DMEM (CORNING) supplemented with 10% FBS.

2.1.4 | Cell transfection, immunoprecipitation and immunoblotting

Cells were transfected with various plasmids using Lipofectamine 3000 (Invitrogen) reagent according to the manufacturer’s protocol. For immunoprecipitation assays, cells were lysed with RIPA lysis buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, and 1% SDS) supplemented with protease-inhibitor cocktail (Roche). Immunoprecipitation was performed at 4°C using S-protein beads (catalogue no. 69704; Novagen), protein A/G agarose beads (catalogue no. IP10, Calbiochem), ANTI-FLAG® M2 Affinity Gel (catalogue no. A2220, Sigma), and Ni-NTA agarose (catalogue no. 3010; QIAGEN). Immunocomplexes were washed with RIPA lysis buffer four times. Both lysates and immunoprecipitates were probed with the indicated primary antibodies, which was followed by detection with the appropriate secondary antibody.

2.1.5 | Protein half-life assay

For PPP2R1B half-life assays, Lipofectamine 3000 transfection was performed with HEK293T cells in 2 cm plates at ~60% confluence. Plasmids expressing WT PPP2R1B and PPP2R1B mutants (R471Q, T513P, R194Q), USP5, DCAF6 or siRNAs were used for transfection as indicated in individual experiments. Twenty-four hours later, the cells were treated with the protein synthesis inhibitor cycloheximide (Sigma, 10 µg mL<sup>−1</sup>) for the indicated periods of time before collection.
2.1.6 | In vivo PPP2R1B ubiquitylation assay

For in vivo PPP2R1B ubiquitylation assays, HA-DCAF6, HA-CUL4A, Myc-USP5, His-ubiquitin, S-HA-PPP2R1B WT and PPP2R1B mutants (R471Q, T513P, R194Q) were transfected into HEK293T cells with Lipofectamine 3000. Twenty-four hours later, the cells were treated with a 20 µmol/L dose of the proteasome inhibitor MG132 (catalogue no. 474790, Calbiochem) for 8 hours. Cells were washed with PBS, pelleted, and lysed in RIPA buffer plus 20 µmol/L MG132 and protease-inhibitor cocktail. Lysates were centrifuged to obtain cytosolic proteins, and then they were incubated with S-protein beads for an additional 8 hours at 4°C. The beads were then washed three times with RIPA buffer. Proteins were released from the beads by boiling in SDS-PAGE sample buffer and were analyzed by immunoblotting with an anti-His monoclonal antibody (ZSGB-BIO).

2.1.7 | Pull-down MS/MS analysis

Proteins were precipitated with a TCA-acetone method that was followed by in-solution digestion. Peptides were dissolved in 10 µL of 0.5% acetic acid and were autosampled directly onto a 100-µm × 10-cm fused silica emitter, which was made in-house and was packed with reversed-phase ReproSil-Pur C18-AQ resin (3 µm and 120 Å; Ammerbuch). Samples were eluted for 50 minutes with linear gradients of 5%-32% acetonitrile in 0.5% acetic acid at a flow rate of 300 nL/min. Mass spectrum data were acquired with an LTQ Orbitrap Elite mass spectrometer (ThermoFisher) equipped with a nanoelectrospray ion source (Proxeon Biosystems). Fragmentation in the LTQ was performed by high-energy collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; activation time, 10 milliseconds) with a target value of 3000 ions. The raw files were searched with the SEQUEST engine against a database from the UniProt protein sequence database. Parameters were set as follows: protein modifications were carbamidomethylation (C) (fixed) and oxidation (M) (variable); the enzyme specificity was set to trypsin; the maximum missed cleavages was set to 2; the precursor ion mass tolerance was set to 10 ppm; and the MS/MS tolerance was 0.02 Da.

2.1.9 | Sperm counting

The cauda epididymides and the vas deferens were carefully removed and placed in a dish containing 1 × PBS. After adipose tissue and blood vessels were removed, tissues were transferred to a new dish containing 1 mL of 1 × PBS. The ductus deferens were squeezed gently with forceps to extrude sperm. The cauda was lightly minced and squeezed gently and then incubated at 37°C for 30 minutes to allow remaining sperm to swim out into the medium. Tissue and medium were collected in a microcentrifuge tube. After allowing this mixture to stand for 3 minutes, the supernatant was collected. For fixation, an equal volume of 4% paraformaldehyde was added to the supernatant, and the sample was incubated for 10 minutes. Fixed spermatozoa were mounted on blood cell counting plates.

2.1.10 | RNA preparation and quantitative real-time PCR analysis

Different mouse tissues were lysed in Trizol (Invitrogen). Total RNA was recovered from tissues following the manufacturer’s protocol. Two micrograms of purified RNA from each sample was reverse transcribed to generate single-stranded cDNA with All-In-One RT MasterMix (catalogue no. G486, abm). The newly synthesized cDNA was mixed with TransStart Top Green qPCR SuperMix (catalogue no. AQ131, Transgen Biotech) in a volume of 20 µL. For quantitative PCR, a real-time PCR detection system (ABI 7500) was used to detect each gene in triplicate. Fold changes were analyzed (quantified) relative to an internal control gene, GAPDH, using the 2−ΔΔCT method.

2.1.11 | Spermatocyte spreading

Spermatocyte spreading was conducted using the drying-down technique. Testes were decapsulated, and the tubules were washed with phosphate-buffered saline at room temperature. Tubules were then placed in a hypotonic extraction buffer (30 mmol/L Tris, 50 mmol/L sucrose, 17 mmol/L trisodium citrate dihydrate, 5 mmol/L EDTA, 0.5 mmol/L DTT, and 0.5 mmol/L phenylmethylsulfonyl fluoride pH 8.2) for 30 minutes. The tubules were minced in 100 mmol/L sucrose, pH 8.2, and mixed. Suspensions were placed on slides, which were treated with fixation buffer (1% paraformaldehyde, pH 9.2, 0.15% Triton X-100) in advance. The slides were allowed to air dry for 2 hours in a humid box. Finally, the slides were rinsed twice with 0.4% Phtotof (Kodak, 1464510) for 2 minutes and dried at room temperature. Slides were stored at −80°C.
2.1.12 | Immunofluorescence and confocal microscopy

For immunofluorescence-based evaluation of subcellular localization in testes, samples were fixed with 10% formalin for two days. The testes were then deparaffinized and rehydrated. Antigen retrieval was carried out using 1 mmol/L EDTA buffer (pH 9.0) in a microwave oven. For exact subcellular localization by immunofluorescence in spermatocyte spreads, meiotic spreading slides were rinsed with PBS twice. The following steps were then identical for cells, tissue, and meiotic spreads. Following permeabilization in 0.2% Triton X-100 (PBS), testes or cells were blocked with 5% BSA and incubated with specific primary antibodies against SYCP3 (Santa Cruz, sc-74569, 1:200), SYCP1 (Abcam, ab15090, 1:200), MLH1 (BD pharmingen, 550838, 1:25), RAD51 (Santa Cruz, sc-8349, 1:200), human PPP2R1B (Sigma, HPA018908, 1:200), or mouse PPP2R1B (Ruiying Biological, RLT3826, 1:200) at 4°C for 8 hours. This was followed by incubation with a secondary antibody (1:200 dilution; AlexaFluor 488 and AlexaFluor 555; Invitrogen) at room temperature for 1 hour and staining with 0.5 µg/mL DAPI for 10 minutes. Glass cover slips were applied, and the results were evaluated with fluorescence microscopy. A Nikon TCS A1 microscope was used for confocal microscopy.

2.1.13 | Spermatogenic single-cell suspension preparation and testicular germ cell chromosome spread

Testes were obtained from mice 15 days after birth. After the tunica albuginea was removed, the tissues were placed in PBS containing 0.1% collagenase (Gibco) and were incubated at 37°C, 5% CO₂ for 15 minutes with occasional inversion. Tissue and PBS were then transferred to a 5 mL microcentrifuge tube and allowed to settle for 10 minutes. The pellet was collected, and this process was repeated, followed by incubation with 10 mL of enzymatic solution (PBS, 1.5 mg mL⁻¹ hyaluronidase [catalogue no. H-3506, Sigma], and 0.125 mg mL⁻¹ trypsin [catalogue no. T1300, Solarbio]). Tubules were incubated for 10 minutes at 35°C. The solution was then transferred to a 100 mm dish with DMEM containing 10% fetal bovine serum and 1% myllicin. After incubation for 30 minutes, the solution was centrifuged at 100 g for 3 minutes in a centrifuge tube. The supernatant was collected. 1.5 mL of DMEM was added to the supernatant, and the single-cell suspension was prepared for spreading.

The suspension was transferred to an Eppendorf tube and centrifuged for 1 minutes at 18 000 g. The pelleted cells were resuspended and incubated at 37°C for 20 minutes in a hypotonic solution (0.075 mol/L KCl). Cells were spun down and resuspended in the fixative (methanol/acetic-acid, 3:1). Approximately 30 µL of this cell suspension was dropped onto a wet glass slide. The specimen was then dried at room temperature and immediately stained with Giemsa.

2.1.14 | Flow cytometry

The spermatogenic single-cell suspension preparation protocol was performed as described above, but the mice were six months old. Cells fixed overnight with cold 70% ethanol were digested with RNase at 37°C for 30 minutes and stained with PI, which was followed by flow cytometry analysis with a BD FACSVersetm instrument.

2.1.15 | TUNEL

TUNEL staining was used to quantify apoptotic cells. Sections were deparaffinized and rehydrated, and antigen retrieval was carried out as described above. Then, sections were incubated with 20 mg/mL proteinase K for 15 minutes at room temperature and were permeabilized in 0.2% Triton X-100 (PBS). Fluorescein was used to detect apoptotic cells using an In Situ Cell Death Detection Kit (catalogue no. 11684795910, Roche). The labeling procedure and control preparation were carried out according to the manufacturer's protocol.

2.1.16 | RNA interference

Smart pool siRNAs against DCAF6 (siRNA 1, 5'-GUUGGA GGUGACAGAUAUU-3'; and siRNA 2, 5'-UUAGAAG GCCGCUAGUAUUU-3') and USP5 (siRNA 1, 5'-CCC GGAUGGCUAGUGUACUCU-3'; and siRNA 2, 5'-CCGAUGGAGCUAGUGUACUCUUA-3') were purchased from Shanghai Genepharma. Transfection was carried out according to the manufacturer's protocol. After 48 hours, the cells were washed with phosphate-buffered saline, lysed directly into RIPA lysis buffer and resolved by 10% SDS-polyacrylamide gel electrophoresis.

2.1.17 | Plasmids and antibodies

HA-tagged PPP2R1B WT, R194Q, R410H, R471Q, and T513P mutants were cloned into pSA-vectors. CUL4A, CUL4B and DCAF6 were inserted into pSA-HA and pCMV-Flag vectors as indicated. USP5 and Ub related plasmids were gifts from Dr Lingqiang Zhang. Antibodies used are anti-PPP2R1B (Ruiying Biological, RLT3826, 1:1000); anti-human PPP2R1B (Sigma,
HPA018908, 1:200, RRID:AB_1855655) and anti-PP2A-Cα (Ruiying Biological, RLT3830, 1:2000), anti-Flag (Sigma, F3165, 1:2000, RRID:AB_259529), and anti-DCAF6 (Sigma, SAB2700894, 1:2000); anti-SYCP3 (Santa Cruz, sc-74569, 1:200 for IF, RRID:AB_2197353); anti-SYCP1 (Abcam, ab15090, 1:200 for IF, RRID:AB_301636); anti-MLH1 (BD pharmingen, 550838, 1:25 for IF, RRID:AB_2297859); anti-USP5 (Proteintech, 15158-1-AP, 1:1000, RRID:AB_2213856) and anti-actin (ZSGB-BIO, TA-09, 1:2000, RRID:AB_2636897), anti-His (ZSGB-BIO, TA-02, 1:2000, RRID:AB_2801388) and anti-GAPDH (ZSGB-BIO, TA-08, 1:2000, RRID:AB_2747414); anti-Myc (SUNGENE BIOTECH, KM8003, 1:5000); anti-RAD51 (Santa Cruz, sc-8349, 1:200 for IF, RRID:AB_2253533); and anti-S tag (EASYBIO, BE2041, 1:5000).

2.1.18 | Isolation of genomic DNA

Genomic DNA was isolated from whole blood and testicular biopsies from 257 patients using a Gentra Puregene kit (Qiagen). A total of 258 blood samples and testicular biopsies obtained from men with normal sperm concentrations (>40 × 10⁶ sperm per milliliter) were used as normal controls.

2.1.19 | Genome and Sanger sequencing

Genomic DNA was extracted from peripheral blood leukocytes by standard procedures. Exome isolation was performed using a 50 mb All Exon kit v5 (Agilent Technology). Whole-exome sequencing library construction was performed according to Illumina’s standard protocol. The prepared libraries were sequenced on Illumina’s HiSeq X Ten system with 76 bp paired end reads for whole-exome sequencing.

Burrows-Wheeler Aligner software (v0.5.9) was used to align the sequence reads to the human reference genome NCBI build 37 (hg19). Picard tools (v1.115) were used to remove PCR duplicates. Our pipeline first recalibrated the base qualities and realigned the sequence reads around indels to obtain more accurate quality scores and to ensure a better alignment in regions with indels. GATK Unified Genotyper was then used to perform consensus calling and identify SNVs. We compared our variants against common germline polymorphisms present in the dbSNP 131 and 1000 Genomes databases (frequency below 0.1%) to discard common germline SNPs.

A variant of the PPP2R1B gene was considered to be a candidate mutation because it was present in the proband, his mother and grandmother, but it was absent in his brother and father.

All fifteen exons of the candidate gene, PPP2R1B, from patients were subjected to PCR amplification using Ex Taq DNA polymerase (TAKARA). All PCR products were sequenced on a 3730XL sequencer (Applied Biosystems) according to the manufacturer’s instructions.

2.1.20 | Quantification and statistical analysis

The nonparametric Mann-Whitney U test or Student’s t-test were used to determine significant differences between mouse groups. Two-way ANOVA was performed to analyze differences in half-life of proteins. Statistical differences in patients were assessed by Fisher’s exact test. P < .05 was considered statistically significant. Data represent the mean ± SD. Statistical analysis was performed using GraphPad Prism 6 (GraphPad software).

3 | RESULTS

3.1 | PPP2R1B is specifically expressed in testis

PP2A is a well-known phosphatase, and the distribution of PPP2R1B, the scaffold subunit of PP2A, is also investigated. According to the tissue expression data in THE HUMAN PROTEIN ATLAS (https://www.proteinatlas.org/ENSG0000137713-PPP2R1B/tissue), PPP2R1B is only highly expressed in testis. As maturation of spermatogenesis is age-dependent, we examined expression of PPP2R1B in different tissues at the transcriptional level in various stages of development and observed that PPP2R1B was significantly increased at 15 days postpartum in mouse testis, and its peak was at 50 days postpartum (Figure S1A). Of note, PPP2R1A expression exhibited no specific pattern of distribution in mouse tissues and gradually decreased following birth before ultimately stabilizing (Figure S1B). Moreover, PPP2R1B was found to be existed in the pachytene/diplotene stage, as shown by immunofluorescence (IF). SYCP3, which is the axial element of the synaptonemal complex, was used as positive controls (Figure S1C).

3.2 | Ppp2r1b-deficient mice exhibit azoospermia

It is reported that PPP2R1B acts as a tumor suppresser. To evaluate the physiologic role of PPP2R1B in vivo, gene targeting by homologous recombination was performed to disrupt PPP2R1B function in mice. The exon 1 of the Ppp2r1b was replaced with a neomycin resistance gene (Figure 1A). This targeted disruption of the Ppp2r1b gene and the generation of germ-line knockout mice were confirmed by evaluation of Ppp2r1b in genomic DNA and mRNA in MEF cells by PCR (Figure 1B). The PPP2R1B protein status in these
mice was determined by western blotting of testicular tissues (Figure 1C). Although Ppp2rla (17A3.2) and Ppp2r1b (9A5.3) have extensive sequence similarity, this targeted disruption had no off-target effect (Figure 1D).

We next closely analyzed the phenotype of the Ppp2r1b-deficient mice. Ppp2r1b deficiency did not lead to embryonic lethality, and Ppp2r1b-deficient mice grew to adulthood with no gross abnormalities and no increase in mortality. The body weight of adult mice without PPP2R1B was not significantly different from that of wild-type (WT) mice (Figure S1D), and no tumors or behavioral abnormalities were observed in adult PPP2R1B null mice. However, as shown in Figure 1E,F,
mice. In contrast, the epididymis of Ppp2r1b-deficient mice were affected. To this end, we measured fertility by continuously mating 10-week-old Ppp2r1b−/− male mice with WT female mice of identical age at a 1:2 male-to-female sex ratio for ten weeks. The number of litters and average number of pups per litter were calculated. Ppp2r1b−/− male mice exhibit male infertility. H, Calculation of the total number of sperm in the epididymis of ten-week-old Ppp2r1b−/− and WT males (n = 10 for each genotype). I, Hematoxylin & Eosin staining of the epididymis from ten-week-old WT and Ppp2r1b−/− mice to show mature sperm. The outlined areas in the images on the left are magnified on the right. Scale bar, 25 μm.

We next conducted computer-assisted sperm analysis to count the mature sperm in samples harvested from the caudal epididymis of Ppp2r1b−/− and Ppp2r1b+/+ mice.28 No sperm were found in Ppp2r1b−/− male mice, compared with WT male mice, which showed counts of 60 million/mL (Figure 1H, n = 6). Hematoxylin and eosin (H&E)-stained sections of the epididymis of Ppp2r1b−/− male mice showed only a few round-shaped cells with large nuclei that were likely immature spermatids, and no mature spermatozoa were found (Figure 1I). This result was consistent with the infertility observed in these mice. In contrast, the epididymis of Ppp2r1b+/+ male mice contained large numbers of mature spermatozoa (Figure 1I).

Taken together, the experiments and analysis show that the absence of Ppp2r1b in mice leads to azoospermia.

3.3 | Ppp2r1b deficiency leads to meiotic arrest

Since spermatogenesis is a complex process, a variety of cells are involved, such as spermatocytes, spermatogonia, Sertoli cells and interstitial cells.29 We thus examined all of these cells. As reported in Figure 2A, there were no histologic differences in spermatogonia, Sertoli cells or interstitial tissues in H&E stained sections of testis in Ppp2r1b−/− and Ppp2r1b+/+ male mice. Seminiferous tubules from control testes showed cells in various stages of spermatogenesis, whereas Ppp2r1b−/− were diagnosed with non-obstructive azoospermia characterized by primary spermatocytes and few abnormal haploid cells (Figure 2A). Testicular cells were labeled with propidium iodide and were assayed with flow cytometry. Haploid cells were predominant (68.6%) in WT mouse testes; however, Ppp2r1b−/− testes predominantly contained diploid and tetraploid cells, and there were few haploid cells (<3%, Figure 2B), suggesting that Ppp2r1b deficiency causes meiotic arrest.

To corroborate the results, we performed immunofluorescence to evaluate spermatocytes in meiotic prophase I in seminiferous tubules with anti-SYCP3 antibody,30 which was followed by DAPI staining for DNA. As shown in Figure S2A,B, it seems no differences in Ppp2r1b−/− and Ppp2r1b+/+ mouse testes before 20 postnatal days. Spermatocytes completed the first meiotic division in control testes at 20 days after birth and then formed round spermatids. Ppp2r1b−/− tubules showed an accumulation of primary spermatocytes and contained no round spermatids at 20 postnatal days. (Figure 2C). At 30 postnatal days, elongating spermatids were readily identifiable in Ppp2r1b−/− testes but were not found in Ppp2r1b+/− testes; Ppp2r1b−/− testes instead revealed an accumulation of primary spermatocytes, in particular those in pachynema (Figure 2C). To further validate the meiotic arrest, exact meiotic stages were determined in meiotic spreads with antibodies against SYCP3 and SYCP1.31,32 As described in Figure 2D, the main meiotic stages are shown in WT and Ppp2r1b−/− mice. Compared to the WT group, the diplotene and diakinesis stages of prophase I were almost lost in Ppp2r1b−/− mice. Furthermore, SYCP1 and SYCP3 signals were continuous both in WT and Ppp2r1b−/− mice. These results demonstrate that Ppp2r1b deletion results in meiotic arrest at the pachytene stag, and the synopsis is essentially normal in Ppp2r1b-deficient mice.

3.4 | Ppp2r1b deficiency induces impaired meiotic recombination

After homologous chromosomes pairing, synaptonemal complex starts to assemble and interhomolog crossover is promoted by SC.34 To investigate whether crossover is compromised...
FIGURE 2  Ppp2r1b deficiency leads to meiotic arrest in mouse testes. A, Histologic appearance of seminiferous tubules in Ppp2r1b−/− vs WT males at age of three months (Hematoxylin & Eosin). The boxed areas are magnified on the right. Arrow, spermatogonia cell; arrowhead, Sertoli cell; asterisk, interstitial cell; red circle, abnormal haploid cell. Scale bar: 50 μm (main); 25 μm (magnification). B, Testicular cells from two-month-old Ppp2r1b−/− and WT male mice were marked with PI and subject to flow cytometry analysis. C, Spermatocytes in prophase I of meiosis I, stained with DAPI for DNA and with a SYCP3 antibody in sections of seminiferous tubes in Ppp2r1b−/− and WT testes obtained from males at 20 or 30 d postpartum. The boxed areas are displayed at 4× magnification. Scale bar: 50 μm (main); 12.5 μm (magnification). D, Spermatocytes spreads from a two-month-old Ppp2r1b−/− mouse and its WT littermate stained for SYCP3 (green) and SYCP1 (red). Scale bars, 5 μm.
in Ppp2r1b<sup>−/−</sup> mice, metaphase spreads were conducted. Spermatocytes for spreads were isolated from Ppp2r1b<sup>−/−</sup> and Ppp2r1b<sup>+/+</sup> testes, which were stained with DAPI to visualize chromosomes. As shown in Figure 3A, most control spermatocytes had 20 pairs of homologous chromosomes at metaphase I, whereas in Ppp2r1b<sup>−/−</sup> cells, univalents were frequently found (N = 60 from three animals), indicating that crossover formation in Ppp2r1b<sup>−/−</sup> spermatocytes is compromised. In order to further verify the absence of crossover in Ppp2r1b<sup>−/−</sup> mice, we stained MLH1, a typical marker of meiotic crossover.35
As shown in Figure 3B,C, MLH1 is decreased dramatically in Ppp2r1b<sup>−/−</sup> mice compared with wild-type mice. Meiotic recombination is important for crossover formation, while defects in meiotic recombination are vital causes of pachytene arrest. RAD51 is an important marker molecule for meiotic recombination. Invasion of homologous chromosome strands is accomplished with the help of RAD51, and after single strand invasion, double-strand break repair is compromised.

To investigate whether recombination is abnormal in Ppp2r1b<sup>−/−</sup> mice, we detected RAD51 distribution and found that RAD51 foci decreased dramatically owing to the depletion of Ppp2r1b in meiotic spreads (Figure 3D,E), suggesting that RAD51 recruited to the recombination foci is insufficient in Ppp2r1b<sup>−/−</sup> mice, and that programmed double-strand break repair is compromised.

It is known that impaired meiotic recombination or synapsis in mouse spermatocytes likely induces apoptosis, then we operate the TUNEL analysis in Ppp2r1b<sup>−/−</sup> and Ppp2r1b<sup>+/+</sup> testes. Indeed, as described in Figure 3F, an average of 30% of spermatocytes were clearly apoptotic in Ppp2r1b<sup>−/−</sup> testes in comparison with 6% in WT testes. Only ~1.5 apoptotic cells per seminiferous tubule section were found on average in Ppp2r1b<sup>+/+</sup> testes, whereas an average of 9.3 apoptotic cells per tubule occurred in Ppp2r1b<sup>−/−</sup> testes (Figure 3F,G). This accounts for the infertility of Ppp2r1b<sup>−/−</sup> male mice and the absence of mature sperm cells in their testes. Together, these data reveal that Ppp2r1b deficiency induces compromised crossover formation and meiotic recombination.

3.5 | Ppp2r1b mutations are identified in the cases of human non-obstructive azoospermia

Although the above results demonstrate a role for Ppp2r1b in the spermatogenesis, the physiological function of genes between human and mouse does not always keep the same, especially with regard to infertility. To this end, we explored whether there was a correlation between Ppp2r1b mutation and non-obstructive azoospermia. A family with four generations exhibiting dominant transmission of infertility was evaluated through whole-exome sequencing. In this family, a 34-year-old patient, patient 5, and his uncle (maternal brother) were diagnosed with non-obstructive azoospermia (Figure S3A), and the clinical testing for known causes of infertility, including chromosomal abnormalities and Y-chromosome microdeletions in this case produced negative results. In order to verify the symptom in patient 5, we performed H&E staining of the human testicular biopsy. As shown in Figure S3B, the testicular tissue from patient 5 exhibited accumulation of primary spermatocytes and contained no spermatids. However, control seminiferous tubules showed cells in various stages of spermatogenesis (Figure S3B). There were no histologic differences in the spermatogonia and Sertoli cells of the proband and control (Figure S3B). Whole-exome sequencing of blood samples obtained from five members of this four-generation family identified 12 candidate mutations through bioinformatic analysis (Table S1). For confirming whether any of these gene mutations may be related to azoospermia, THE HUMAN PROTEIN ATLAS (https://www.proteinatlas.org/) and Mouse Genome Informatics (http://www.informatics.jax.org/) were used for examining the tissue expression patterns and related mouse models of 12 gene mutations, respectively. Results show that 7 genes (PPP2R1B, BCKDHA, EPB41L4A, GOLGA6L10, SHPK, ST3GAL6, and UT52R) are expressed in testis, but none of these mouse models is correlated with fertility except Ppp2r1b-deficient mice designed by ourselves. To further explore the role of these 12 genes, we have done literature review, and the result shows that none of related literatures is reported on fertility. Thus, PPP2R1B mutation is likely correlated to non-obstructive azoospermia.

The PPP2R1B mutation was a heterozygous missense mutation located at chromosome 11q23.1 (designated c.581G → A (p.R194Q)) (Figure S3A), and was characterized by maternal transmission and co-segregated with male infertility (Figure S3A). To further validate the relationship between human male infertility and PPP2R1B mutations, we next evaluated this gene in testicular biopsy samples collected from 120 unrelated infertile Chinese patients with meiotic arrest, 137 patients with Sertoli-cell-only syndrome and 258 normal controls. Sanger sequencing was performed...
to sequence exons of PPP2R1B, and three types of heterozygous missense mutations were identified in the coding region of PPP2R1B on chromosome 11q23.1 in 120 patients with meiotic arrest (Figure S3C,D and Table S2). One of these missense mutations, c.581G → A (p.R194Q), was in exon 5 and was predicted to result in the substitution of a positively charged arginine residue for an uncharged polar glutamine residue in the predicted PP2A subunit B binding region (Figures S3D and S4A). An additional two missense mutations c.1412G → A (p.R471Q) and c.1537A → C
FIGURE 4  CRL4A\textsuperscript{DCAF6} polyubiquitylates and degrades PPP2R1B. A, PPP2R1B and indicated mutants were each similarly transfected into HEK293T cells for 48 h, and cell lysates were subject to western blotting with anti-HA antibody. B, HEK293T cells transfected with the indicated plasmids were treated with cycloheximide (10 μg/mL), and lysates were collected at the indicated times for western blot analysis. Quantification of PPP2R1B and mutant protein levels relative to β-actin are shown. The results are shown as the mean ± SD, n = 3 independent experiments. P < .0001, Two-way ANOVA test. C, Half-life analysis of mutant R410H in HEK293T cells transfected with PPP2R1B or mutant R410H. D, HEK293T cells transfected with the indicated plasmids were treated with or without the proteasome inhibitor MG132 (20 μmol/L, 8 h), and proteins were analyzed with western blot. F, Increasing amounts of DCAF6 were transfected into HeLa cells, and endogenous expression of PPP2R1B was evaluated. G, PPP2R1B protein levels were analyzed in HeLa cells transfected with individual DCAF6 siRNAs. H, Half-life analysis of PPP2R1B was performed in HEK293T cells transfected with PPP2R1B or the indicated siRNA. NC, negative control. J, K, The proteasome inhibitor MG132 (20 μmol/L, 8 h) was adopted in HEK293T cells transfected with the indicated plasmids. Flag-DCAF6 was extracted with anti-Flag beads, eluted with Flag-peptides and incubated with S-tagged PPP2R1B/PPP2R1A WT, S-tagged PPP2R1B mutants or S-tag-vector immunoprecipitated with S-beads. Proteins retained on S-beads were blotted with the indicated antibodies. L, PPP2R1B ubiquitylation was analyzed in MG132 (20 μmol/L, 8 h) treated HEK293T cells, and the cells were transfected with the CRL4 E3 ligase CUL4A or DCAF6 together with PPP2R1B or the R194Q mutant.

(p.T513P), were found in exon 12 and were located in the predicted PP2A subunit C binding region (Figures S3D and S4A). These mutations affect a highly conserved amino acid and were predicted to be deleterious by SIFT software and PolyPhen-2 (PPH2) software (Figure S4B). No mutations of the PPP2R1B gene were identified in 258 controls with normal sperm concentration based on the sequencing analysis of coding exons. However, a heterozygous missense mutation c.1229G → A (p.R410H) was found in one of 137 patients with Sertoli-cell-only syndrome (Figure S3C,D and Table S2).

A query of these four mutations in the Genome Aggregation Database (gnomAD; https://gnomad.broad institute.org/) showed that the allele frequency of mutations c.581G → A (p.R194Q) and c.1412G → A (p.R471Q) are 0.000004 (1/251402) and 0.000004 (1/247134) in general population, and the mutations c.1229G → A (p.R410H) and c.1537A → C (p.T513P) have been identified at frequencies of 0.000004 (12/251266) and 0.000002 (5/250222) in general population, respectively (Table S2). The locations of the affected residues are shown in Figure S4B.

In total, PPP2R1B mutations were present in 3 of 120 men with meiotic arrest (P = .03145) and in 1 of 137 men with Sertoli-cell-only syndrome (P = .3468) (Table S3). Thus, these data support the hypothesis that PPP2R1B mutations may be related to the arrest of spermatogenesis in infertile men.

3.6 | PPP2R1B mutants are unstable

In order to determine the cause of azoospermia in patient 5 carrying the R194Q mutation, we investigated whether the PPP2R1B mutants were unstable. First, each of the PPP2R1B mutations was cloned into a pSAA vector and were transfected into HEK293T cells. Three PPP2R1B mutants R194Q, R471Q and T513P from patients with meiotic arrest were unstable, and the T513P mutant was particularly unstable and was almost undetectable (Figure 4A, lanes 2, 4, 5 vs lanes 1, 3 and S5a-S5c). In contrast, the protein level of mutation R410H, which was identified in Sertoli-cell-only syndrome, remained unchanged in comparison with WT PPP2R1B (Figure 4A, lane 1 vs lane 3 and Figure S5B, lane 3 vs lane 2). Next, cells were treated with the protein synthesis inhibitor cycloheximide (CHX) to determine how these mutations affected protein stability. The results obtained by CHX treatment are summarized in Figure 4B,C and Figure S5D-G. Notably, the half-lives of R194Q, R471Q and T513P mutants were less than 2 hours. In contrast, the half-life of the Sertoli-cell-only syndrome mutation R410H was equivalent to that of WT PPP2R1B at ~10 hours (Figure 4C and Figure S5G). The R194Q, R471Q and T513P mutations did not significantly influence PPP2R1B structural or conformational integrity (Figure S5H). However, the reduction of protein levels of these mutants was reversed by the proteasome inhibitor MG132 (Figure 4D, lanes 2, 4 vs lanes 3, 5 and 4e, lanes 1, 3, 5 vs lanes 2, 4, 6), indicating that these mutants undergo degradation through the ubiquitin-proteasome pathway. It was of particular interest that the PP2A-C-binding affinity of the R194Q, R471Q, and T513P mutants was impaired (Figure S5I, lane 2 vs lanes 3, 5, 6), suggesting that the formation of PP2A holoenzyme was likely compromised in testes with mutants.

3.7 | PPP2R1B mutants promote their own degradation by CRL4A\textsuperscript{DCAF6}

Ubiquitin (Ub) E3 ligases are substrate specific; thus, an in vitro pull-down assay was performed to screen for the E3 ligase that specifically targets PPP2R1B and its mutants for degradation. As shown in Figure S5J, mass spectrometry (MS) revealed that the R194Q mutant associates specifically with damaged DNA-binding protein 1 (DDB1) and DDB1-CUL4 associated factor 6 (DCAF6), which mediates CRL4-E3 ligase binding with its substrates.\textsuperscript{50}
We next validated the role of CRL4\textsuperscript{DCAF6} as an E3 ligase acting on PPP2R1B. As described in Figure S5K,L, overexpression of CRL4 components CUL4A, DDB1, DCAF6 or ROC1 reduced protein levels of PPP2R1B (lane 1 vs lanes 2, 3, 4), and downregulation of PPP2R1B by CRL4 was blocked by MG132 treatment (Figure S5M, lanes 2, 4 vs lanes 3, 5). However, overexpression of CUL4B, which shares extensive sequence similarity and functional redundancy with its homologous protein CUL4A,\textsuperscript{51} did not significantly reduce PPP2R1B (Figure S5N, lane 2 vs lane 3). Importantly,
Deubiquilinase (DUB) maintains the protein stability by cleaving ubiquitin-protein bonds. To identify the DUB responsible for PPP2R1B de-ubiquitylation, purified PPP2R1A or PPP2R1B from HEK293T cells were incubated separately with testicular tissue lysate followed by MS analysis. Ubiquitin-specific protease 5 was found in the pull-down list for PPP2R1B (Figure S5J).

To validate whether USP5 is the DUB of PPP2R1B, we conducted the following experiments. As shown in Figure 5A, ectopic expression of USP5 increased levels of PPP2R1B protein in a dose-dependent manner. Depletion of USP5 by two independent siRNA led to a significant reduction of PPP2R1B protein levels (Figure 5B), which was reversible by MG132 treatment (Figure 5B). These results demonstrate that USP5 specifically stabilizes PPP2R1B protein in cells. Interestingly, USP5 just slightly stabilized PPP2R1B R194Q mutant compared to wild-type PPP2R1B. However, USP5 significantly stabilized PPP2R1B R194Q mutant when compared to wild-type PPP2R1B and the other DUB containing USP, ZnF-UBP, and UBA domains like USP13, was used as a negative control. In accordance, elimination of USP5 rather than USP13 increased PPP2R1B ubiquitylation in cells (Figure 5G, lanes 2, 3 vs lane 4). The results indicate that USP5 specifically de-polyubiquitylates and stabilizes PPP2R1B.

To confirm the effect of E3 and DUB on PPP2R1B mutants, we performed ubiquitylation assay on PPP2R1B mutants with USP5 or CRL4A<sub>DCAF6</sub>. As shown in Figure 5H, the three mutants identified in patients with meiotic arrest were more likely to be targeted by CRL4A<sub>DCAF6</sub> compared with WT PPP2R1B and the mutant identified in patients with Sertoli-cell-only syndrome (Figure 5H, lanes 2, 6 vs lanes 4, 8, 10). However, USP5 acting as a DUB of PPP2R1B suppressed ubiquitylation of wild type PPP2R1B induced by the E3 ligase CRL4A<sub>DCAF6</sub> (Figure 5H, lane 2 vs lane 3), but not that of R194Q, R471Q, and T513P mutants (Figure 5H, lanes 4, 8, 10 vs lanes 5, 9, 11). Collectively, the R194Q, R471Q, and T513P mutants are more susceptible to ubiquitin-proteasome degradation.
due to their enhanced binding with the specific E3 ligase CRL4ADCAF6, and degradation is almost not reversible by USP5.

### 3.9 PPP2R1B mutants reduce the stability of the wild-type PPP2R1B protein

PPP2R1B mutants identified in patients with meiotic arrest were susceptible to degradation likely due to high binding affinity with E3 ligase CRL4ADCAF6. However, these PPP2R1B mutants are heterozygous in azoospermia patients with meiotic arrest, and there is a wild-type allele ofPPP2R1B in these patients. Given the fact that phenotypes of patients withPPP2R1B heterozygous mutants andPpp2r1b+/− mice are different, it is possible that the wild-type PPP2R1B protein is affected byPPP2R1B heterozygous mutants. Therefore, we next detected the levels of total PPP2R1B protein in patients through immunofluorescence analysis. As described in Figure 6A, the total level of PPP2R1B expression was nearly absent in seminiferous tubules of patient 5 with the R194Q mutation compared with control seminiferous tubules, which was similar toPpp2r1b knockout mouse model.

To further verify the effect ofPPP2R1B heterozygous mutants on wild-type PPP2R1B protein, wild-type PPP2R1B protein and mutants were co-transfected into the same cells. As shown in Figure 6B, wild-type PPP2R1B protein became unstable when mutants were present (lanes 2, 4, 5 vs lane 1), suggesting that PPP2R1B mutants can promote the degradation of wild-type PPP2R1B protein. Then, ubiquitylation assay was performed to confirm the degradation ofPPP2R1B protein. As expected, wild-type PPP2R1B protein co-transfected with mutants was highly ubiquitinated (Figure 6C, lanes 3, 5, 6 vs lanes 1, 2, 4). The fact that wild-type PPP2R1B protein is degraded through ubiquitin-proteasome system indicates an interaction between wild-type PPP2R1B protein and PPP2R1B mutants. Indeed, we found that PPP2R1B mutants could form complex with wild-type PPP2R1B protein (Figure 6D, lanes 2-6). These data indicate that the level of PPP2R1B mutant and wild-type protein is reduced in PPP2R1B heterozygous mutants owing to the ubiquitin-proteasome system directly or indirectly.

### 4 DISCUSSION

PPP2R1B is located at chromosome 11q23.1, where loss of heterozygosity has been identified in 25%-50% of lung, cervical, breast, ovary and stomach cancers, as well as in melanoma and B-cell chronic lymphocytic leukemia.42-46 PPP2R1B is also mutated in many cancers, including breast, colon, neck and lung carcinomas and melanoma.27,57,58 Therefore, PPP2R1B has been proposed previously to act as a tumor suppressor that plays a role in tumorigenesis.27,59,60 However, neither heterozygous nor homozygous Ppp2r1b-deficient mice develop spontaneous tumors, suggesting thatPPP2R1B is not critical in tumor suppression.

The starting point of this study is thatPpp2r1b-deficient males produced no mature spermatozoa, and detection on autoimmune disease of testis, androgen level, and lactate level all produce negative results. Therefore, we focus our attention on the process of meiosis. The testes of adultPpp2r1b knockout mice contain increased numbers of pachytene primary spermatocytes. Meiotic recombination is also impaired byPpp2r1b deletion. However, the mechanism needs further investigation. According to the latest reports about protein phosphatase 6 regulating spermatogenesis,31 the phenotype ofPpp6cKO mice is similar toPpp2r1b−/− mice. Therefore, it is possible that similar protein phosphatase, such asPPP2R1B, promotes the chromatin relaxation to regulate meiotic recombination. Interestingly, some spermatocytes have successfully passed the first division, and this may be due to the little compensation ofPPP2R1A whenPpp2r1b is deleted.

PPP2R1B is present only in vertebrates with XY-type sexual reproduction, indicating that this gene has a unique role in the evolution of these species. In this study,PPP2R1B R410H mutation was identified in 0.7% of male patients with Sertoli-cell-only syndrome at the P value of .3468, which is not significant. In the following test of binding affinity or protein stability, the mutation was consistent with wild-type PPP2R1B protein. Thus, the relationship betweenPPP2R1B R410H mutation and infertile men with Sertoli-cell-only syndrome is unclear according to our data. However, we have identifiedPPP2R1B mutations in 2.5% of infertile men with meiotic arrest, and an autosomal pattern of inheritance found in one family. Besides,PPP2R1B mutations identified in meiotic arrest-mediated azoospermia are more readily degraded by the novel E3 ligase CRL4ADCAF6 than WT protein. Our data indicate that these mutants may induce instability of wild-type PPP2R1B protein due to the physical interaction with wild-type PPP2R1B protein. PP2A-C subunit binding affinity is also compromised in these mutants, which likely leads to higher binding affinity of the mutants with E3 ligase CRL4ADCAF6. Thus, the protein level ofPPP2R1B in testis is almost undetectable in azoosperma patients withPPP2R1B heterozygous mutations, and the formation ofPP2A holoenzyme is likely impaired in testes of these patients. These findings in azoospermia patients withPPP2R1B mutations are in accordance with the phenotype found inPpp2r1b-deficient mice.

Under normal physiological conditions, spermatogenesis consists of a series complex events with high order, then meiosis related proteins are regulated precisely, especially kinase and phosphatase, such asPP2A in this case. Our data indicates thatPPP2R1B has slight binding affinity with DCAF6 at a basal level, but it may be modified somehow to bind DCAF6 with high affinity. Coincidentally, the three-dimensional
FIGURE 6 The stability of the wild-type PPP2R1B protein is affected by PPP2R1B mutants. A, Germ cells stained with DAPI for DNA and with an anti-PPP2R1B antibody in sections of seminiferous tubes in normal individuals and Patient 5. The boxed areas are displayed at 4× magnification. Scale bars: 10 μm (main); 2.5 μm (magnification). B, Flag-PPP2R1B protein levels were evaluated in HEK293T cells co-transfected with HA-DCAF6 and wild-type HA-PPP2R1B protein or mutants. C, Flag-PPP2R1B protein co-transfected with HA-DCAF6 and wild-type HA-PPP2R1B protein or mutants was analyzed in HEK293T cells. Cells were treated with MG132 (20 μmol/L) for 8 h prior to collection. D, HEK293T cells co-transfected with Flag-PPP2R1B protein and wild-type HA-PPP2R1B protein or mutants were treated with MG132 (20 μmol/L) for 8 h prior to collection. The cell lysates were subject to immunoprecipitation with anti-S beads and then blotted with anti-Flag antibodies.
structure of PPP2R1B mutants may be similar to the modified state of PPP2R1B. Hence, these PPP2R1B mutants are dramatically degraded. Because wild-type PPP2R1B protein and PPP2R1B mutants form a complex, it is possible that PPP2R1B mutants are physically associated with wild-type protein, leading to degradation of wild-type PPP2R1B protein.

Taken together, our work demonstrates the biological function and necessary role of PPP2R1B in mice spermatogenesis. In addition, our work has identified CRL4A$	ext{DCAF6}$ and USP5 as the upstream regulators of PPP2R1B, and contributes to uncover the progress of spermatogenesis.

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CONFLICT OF INTEREST
The authors have declared that no conflicts of interest exist.

AUTHORS’ CONTRIBUTIONS
Y. Yin, Z. Zhang, and L. Yuan conceived of the study. L. Yuan performed some experiments and interpreted some of the data. Most of the experiments were performed by M. Du and Z. Zhang. Z. Zhang and M. Zhu generated the knockout mouse model and identified phenotypes. C. Zhang developed and performed bioinformatics and statistical analysis. Zhe. Zhang collected information on patients and X. Zhao performed mass spectrometry analysis. Y. Li, R. Li, and H. Liang helped with some experiments. H. Jiang, and J. Qiao collected and analyzed samples from patients with azoospermia. Y. Yin supervised the project, interpreted the data, and wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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