Original Article

The testis-specifically expressed gene *Trim69* is not essential for fertility in mice

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Abstract

Protein ubiquitination is essential for diverse cellular functions including spermatogenesis. The tripartite motif (TRIM) family proteins, most of which have E3 ubiquitin ligase activity, are highly conserved in mammals. They are involved in important cellular processes such as embryonic development, immunity, and fertility. Our previous studies indicated that *Trim69*, a testis-specific expressed TRIM family gene, potentially participates in the spermatogenesis by mediating testicular cells apoptosis. In this study, we investigated the biological functions of Trim69 in male mice by established *Trim69* knockout mice with CRISPR/Cas9 genomic editing technology. Here, we reported that the male *Trim69* knockout mice had normal fertility. The adult knockout mice have shown that the appearance of testes, testis/body weight ratios, testicular histomorphology, and the number and quality of sperm were consistent with wild-type mice. These results indicated that the E3 ubiquitin ligase protein Trim69 was not essential for male mouse fertility, and it might be compensated by other TRIM family members such as Trim58 in *Trim69*-deficiency testis. This study would help to elucidate the functions of tripartite motif protein family and the regulation of spermatogenesis.

Keywords: Trim69, gene knockout, spermatogenesis, E3 ubiquitin ligase, male fertility

Introduction

Spermatogenesis is an extremely ordered multistep process and requires delicate regulation of cell division and differentiation[1]. From spermatogonial stem cell to mature sperm, any dysfunction of the process would cause arrest of spermatogenesis and sperm abnormality, even male infertility[2–3]. The entire process of spermatogenesis is regulated by many factors, including cytokines[4], hormones growth factors[5], and a set of genes functioning in various processes, such as transcription, translation, and post-translational regulation[6–7].

Increasing evidence show that ubiquitination participated in spermatogenesis and spermatogonial differentiation[8–9]. The tripartite motif (TRIM) family was defined as one of the largest subfamilies of E3 ubiquitin ligases. Until now, about 80 proteins have been identified as TRIM family proteins, and many of them participate in diverse important biological processes, such as cell differentiation, proliferation, apoptosis, and autophagy[10]. TRIM family proteins were also considered critical for spermatogenesis and embryogenesis. For example, Trim27 was reported to

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regulate meiosis by regulating XY body formation and germ cell proliferating during spermatogenesis[11]. Trim28 played an important role in demethylation of DNA in embryos, preventing embryo from developmental abnormalities[12].

Trim69 (also known as the Ret finger protein, RFP) is a member of the TRIM family and contains a TRIM motif that consists of a RING domain[13]. Previous studies have shown that Trim69 had E3 ubiquitin ligase activity, and could inhibit cataractogenesis by negatively regulating p53[13–14]. And it has been reported that *Pnldc1* knockout caused defects in P-element-induced wimpy testis (PIWI)-interacting RNA 3′ end trimming, transposon gene silencing and spermatogenesis in mice, thus eventually leading to male infertility[15]. Our earlier studies showed that Trim69 was an important downstream target of Pnldc1, and it was significantly downregulated in *Pnldc1* knock-out mice. We also found Trim69 was specifically expressed in testis in normal mice. Nevertheless, the exact role of Trim69 in spermatogenesis is still unclear. This study aimed to examine the expression and biological functions of Trim69 in testes. Here, we used the CRISPR/Cas9 system to establish *Trim69* knockout mice. The phenotype analysis showed that, compared with wild-type control mice, the proportion of apoptotic cells, the number and quality of sperm in *Trim69* knockout mice showed no difference. Detection of other members of the TRIM family genes showed slightly increased mRNA expression of Trim58 in *Trim69* knockout mice, which might partially explain the normal spermatogenic process after *Trim69* knockout. Thus, the knockout of *Trim69* does not affect the normal spermatogenic process in testis. The research provides a basis for future mechanistic studies of spermatogenesis.

Materials and methods

Animals

C57/BL6 mice were housed in a specific pathogen-free (SPF) environment, given sufficient water and food, and followed the circadian rhythm of the mice. We carried out animal breeding and experiments following the requirements of the Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC-1601117).

Generation of *Trim69* mutant mice by CRISPR/Cas9

To generate *Trim69* mutant mice, the mMESSAGE mMACHINE T7 Ultra Kit (Ambion, USA, AM1345) and a RNeasy Mini Kit (Qiagen, Germany, 74104) were used to produce Cas9 mRNA by *in vitro* transcription and purifying it. The sgRNAs sequences are as follow: 5′-TCAGGTTCTCTCCATGCTCTG-GG-3′ and 5′-GCTTTCAATGCAAGGATGCACGG-3′ which the PAM sequences are GGG and CGG. SgRNA oligonucleotides were ligated with pUC57-T7-sgRNA plasmid which has been *Bsa* I (NEB, USA, R0535S)-digested. The plasmid was digested with *Dra* I (NEB, R0129S) and purified using MinElute PCR Purification Kit (Qiagen, 28004). SgRNAs were produced using the MEGAshortscript Kit (Ambion, AM1354) and purified using the MEGAclear Kit (Ambion, AM1908). We collected 128 fertilized eggs from C57/BL6 mice, which were subjected to Cas9 mRNA and sgRNA injection, and transferred into the ampullary-isthmic junction of the oviducts of 5 adult female recipient mice. The recipient mice were placed on a heated table to maintain body temperature at 37 °C. Ten pups were finally born, and all of them survived.

Breeding strategy

C57/BL6 mice were used for all the experiments. The mice with frameshift deletion were backcrossed to wild-type C57/BL6 mice for at least 5 generations. And heterozygous mice were mated to produce homozygous mice, which were used for phenotype analysis.

Fertility test and sperm analysis

The fertility test was performed by mating each male with two wild-type C57/BL6 females. All the mice used for the fertility test were 8 weeks old. The number of pups and the sex ratio were recorded.

We performed a sperm analysis to determine sperm quality. Cauda epididymis was cut into pieces with scissors in a cell culture dish containing 100 μL of modified human tubal fluid medium (mHTF) (Irvine Scientific, USA, 90126) supplemented with 10% fetal bovine serum at 37 °C, filtered through 200 mesh nylon membrane, and washed by additional 200 μL of mHTF into a 1.5 mL EP tube to collect the sperm. After incubated at 37 °C for 5 minutes, sperm motility and number were analyzed with Hamilton Thorne IVOS II system (Hamilton Thorne, USA).

RNA extraction, RT-PCR and qRT-PCR

Total wild type and homozygous testicular RNA were extracted with Trizol Reagent (Invitrogen, USA, 15596-018). We obtained cDNA by reversing transcription of total RNA (2 μg) using Prime Script

RT Master Mix (TaKaRa, Japan, RR047A). We used AceQ qPCR SYBR Green Master Mix (Low ROX Premixed) (Vazyme, USA, Q131-02) to perform quantitative reverse transcription PCR (qRT-PCR) in a StepOne Real-Time PCR System (Applied Biosystems). The qRT-PCR reaction was carried out following the steps on the reagent instructions and 18S was used as an internal control. The primers used in the experiment were arranged in *Table 1*.

Isolation of spermatogenic cells

We used the STA-PUT method to sort different spermatogenic cells in mouse testes, including spermatogonia, pachytene spermatocytes, round spermatids, and elongated spermatids[16]. Mice between postnatal day 6 to 8 were selected for spermatogonia sorting because the percentage of spermatogonia in this period was high. Other cell types were obtained from adult mice. First, the testicular tissue was digested into small tubes by collagenase (1 mg/mL) (Invitrogen, 17104-019), and then the small tubes were further digested into a single cell suspension with 0.25% Trypsin containing DNase I (1 mg/mL) (Ruibio, China, TD3212).

After the single cell suspension flowed into the sedimentation tank, the single cell suspension was lifted up with a 2% to 4% bovine serum albumin (BSA) (Beyotime, China, ST023) gradient. After about 2 hours of sedimentation, cells of different types would distribute in different positions of the BSA gradient.

Western blotting

Freshly taken testicles were placed in a dish containing PBS to rinse the blood, and then RIPA lysate with protease inhibitor cocktail tablets (Roche, Switzerland, 11873580001) was added. In order to thoroughly lysis, tissues were cut into small pieces with scissors and sonicated to homogenate and then centrifuged at 4 degrees for 40 minutes to obtain supernatant. Using the bicinchoninic acid (BCA) (Beyotime, P0012) kit to measure protein concentration and determine sample loading amount. Equal protein amounts (30 µg) per lane were separated by 12% SDS-PAGE in electrophoresis buffer and were transferred onto polyvinylidene difluoride membranes (Bio-Rad, USA, 1620177) in transfer buffer at 90 mA at 4 °C for 2 hours. The membrane was blocked in 5% nonfat dry milk in TBS for 2 hours and then incubated overnight at 4 °C. The next day, we conducted secondary antibody incubation (1:1000 dilution) and exposure. Primary antibodies used were listed in *Table 2*.

Table 1 **List of primer sequences**

Primer name	Sequence (5′ to 3′)
Trim69-WT-F	GCCACAACTTCTGCCAAGACTG
Trim69-WT-R	GAGGACTCTGAGCGTTCTCGTTAT
Trim69-RT-F	TTCTCTCTCCAGGCCCATCTC
Trim69-RT-R	GAGGTGAAGCCTTTTGAGCC
Trim5-RT-F	CCATCGCCAGGGAACAAAGAAAGT
Trim5-RT-R	CACCTGAACCCAGTAGCGTTGAG
Trim7-RT-F	CAGGAGGGTCGGCTTCTAAG
Trim7-RT-R	GAGACTGTGGTTGGCTTGGA
Trim10-RT-F	GGAACACGGGGAGAAAATCTAC
Trim10-RT-R	AGACACACGAGACACTTCTGT
Trim11-RT-F	CCTGGATTACTTCACCGACCC
Trim11-RT-R	AGGCGTCGTGCCATTTCTG
Trim15-RT-F	CAGGTGGAGGAGCTAGAGGAGA
Trim15-RT-R	GTGGAGGTTTCGGATCTTCTTGAC
Trim17-RT-F	CTTGCCAGACGGTTACAAGAG
Trim17-RT-R	CTCAGCCACTTTTGTCAGGAG
Trim21-RT-F	GGAGAGGAGGATTCGTGGTTCAGA
Trim21-RT-R	GCGGTGTTGCGATCCAGAGTAAT
Trim25-RT-F	ATGGCTCAGGTAACAAGGGAG
Trim25-RT-R	GGGAGCAACAGGGGTTTTCTT
Trim26-RT-F	GAAAGCCATCCCTCACATGGTTAA
Trim26-RT-R	GCCAGTATAGGTCACACACTTCC
Trim27-RT-F	GGAGCAAATCCAGAACCGACT
Trim27-RT-R	GCCCCGTTGATGCTGTTATAG
Trim34a-RT-F	CTCAGGAGCATCGTGGTCAC
Trim34a-RT-R	CCTTGTCCAGGATTCTTCTAAGC
Trim34b-RT-F	GCGGCGTGAGTGGTCAGATA
Trim34b-RT-R	AAATGTGAAGTTGTCCCAGTAGGC
Trim35-RT-F	TTCCGGGCCAAGTGTAAGAAC
Trim35-RT-R	CCAAGTCGTTTGCACCTCA
Trim38-RT-F	ATGGGCTCAGACTTTAGCACG
Trim38-RT-R	CTGTTTTTGGGCTGACATTGC
Trim39-RT-F	GCAAAGCCTCCGAAGAGAAGAA
Trim39-RT-R	GAAGTCGCTGTAGAATGTCCTGTT
Trim41-RT-F	ATGAGCCGCATGTTTTGTCAG
Trim41-RT-R	GCCCCTAGTACACAGCAGT
Trim43a-RT-F	TGGTGCAGGACTTGGATGACTTGT
Trim43a-RT-R	GGTCTGACTGAGGTGTAGCCGAAC
Trim43c-RT-F	TGAAGCTACGATAGTCCACGAGAG
Trim43c-RT-R	GGCACGGAGTCTTGGTATCATAC

Table 1 List of primer sequences (continued)

Primer name	Sequence (5′ to 3′)
Trim47-RT-F	GAGGACCGCATGGATGAACT
Trim47-RT-R	AACTCTGAAGGGTGGCTGTG
Trim50-RT-F	CCCATTTGCCTGGAGGTCTTC
Trim50-RT-R	CAGGACAGCATAGCTCGGAG
Trim58-RT-F	CTGGCCCAACAGAACAAGG
Trim58-RT-R	CTCACATCGTGTCAAGACTCC
Trim60-RT-F	CTGCCTCCGTTTGCTCTGG
Trim60-RT-R	GAGCCTAATGGTTTCAGTCATGT
Trim62-RT-F	TGCGAGCACTACTTCTGCC
Trim62-RT-R	CTTGACCTTGTCGTGAGCC
Trim65-RT-F	GAGGACGTGGTGACTTGCTC
Trim65-RT-R	ACGCCATGAATCCTGGATGC
Trim68-RT-F	TCCCAGAACTTGAGCTACACC
Trim68-RT-R	AGACGGACCTTGTCTACAACA
Trim72-RT-F	CCGCAGGCTCTAAGCACTAAC
Trim72-RT-R	CTGCTCGCAGTAGATGCTCA
Trim75-RT-F	ATAGCAGCATTAGAACACCACG
Trim75-RT-R	ATGAGCTGACGCCTACAATTC
Trim6-RT-F	CGGATTCCAGATTGGATTCTTTT
Trim6-RT-R	TGATGCAGACCTGGCAGAAG
Triml1-RT-F	TGGAGAACCTCAGGGAAGAGC
Triml1-RT-R	GAGGAGGCACATCAGACAAAA
Trim69-exon1-3-RT-F	CTCCTCCTCTCATGGGCTCT
Trim69-exon1-3-RT-R	GCTCCTTCAGGGTTGTCTGA
18s-RT-F	TAACGAACGAGACTCTGGCAT
18s-RT-R	CGGACATCTAAGGGCATCACAG

Immunofluorescence

The testicular tissue was fixed with modified Davidson's fluid (MDF) for 24 hours, cut in half and fixed with MDF for an additional 24 hours. After dehydration and entrapment, sections were cut at 5 μm thickness. After dewaxed with xylene and hydrated with gradient alcohol, testicular paraffin section was washed with PBS. Subsequent acidic antigen retrieval of the sections was conducted in the microwave oven in 10 mm citrate buffer (pH 6.0) with high fire for 3 minutes and low fire for 7 minutes. We blocked the slides with 5% BSA at room temperature for 2 hours, then diluted the antibodies according to the ratio of antibody instructions and incubated slides at 4 °C overnight. The next day, secondary antibody and Hoechst 33342 were incubated at room temperature for 2 hours and 5 minutes, respectively. Finally, confocal photographs were taken after slides sealing with glycerol (Carl Zeiss, LSM800, Germany).

Spermatozoa obtained from cauda were air-dried and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The other operation steps were consistent with the paraffin section. Antibodies were listed in **Table 2**.

Paraffin section preparation, and Hematoxylin and Eosin staining

The testicular and epididymal tissues were fixed with MDF for 24 hours, cut in half and fixed with MDF for an additional 24 hours. After dehydration entrapment of mouse testes or epididymis, sections were cut at a 5 μm thickness. After deparaffinization, slides were stained with Hematoxylin and Eosin (H&E) for histological analysis.

TUNEL assay and chromosome spread

The paraffin section of the testis can be used for detecting the apoptosis signal by using the TUNEL BrightRed Apoptosis Detection Kit (Vazyme, A113-03) according to the manufacturer's instructions.

The process of prophase I spermatocytes was observed by chromosome spreads. Primary antibodies were as follows: anti-SYCP3 (1:150; Proteintech, USA, 23024-1-AP) and anti-γH2AX (1:200; Abcam, UK, ab26350). Mounted the slides with glycerol after staining, then took pictures with a confocal microscope.

Transmission electron microscopy

Cauda epididymides were dissected and fixed in Gluta solution (Sigma, USA, P1126). Post-fixation with 2% (wt/vol) OsO_4, tissue blocks were embedded in epoxy resin. Transmission electron microscope (TEM) sections were shot after ultra-thin sectioning (FEI Tecnai G2 Spirit Bio TWIN, FEI Company, USA).

Statistical analysis

SPSS 19.0 statistical software was used for data analysis. Chi-square test was used for statistics of counting data, and one-way ANOVA was used for group comparison. The measurement data were expressed in the form of mean±SD. The comparison between the two groups was analyzed by the Student's t-test. P values less than 0.05 were considered statistically significant.

Table 2   **List of antibodies**

Antibodies	Source	Catalog number	Dilution
Rabbit Polyclonal Anti-TRIM69	Proteintech Group	12951-1-AP	1:500
Rabbit Polyclonal Anti-Lin28	Abcam	ab46020	1:200
Mouse Monoclonal Anti-gamma H2A.X	Abcam	ab26350	1:200
Rabbit Polyclonal Anti-SCP3	Abcam	ab15093	1:150
Rabbit polyclonal Anti-Sox9	EMD Millipore Corp	AB5535	1:200
Mouse Monoclonal Anti-Tubulin, Acetylated	Sigma	T6793	1:200
Rabbit Polyclonal Anti-bax	Cell signaling technology	2772	1:1000
Rabbit Polyclonal Anti-Bcl2	Proteintech Group	26593-1-AP	1:500
Mouse Monoclonal Anti-Caspase 3/p17/p19	Proteintech Group	66470-2-Ig	1:1000
Rabbit Polyclonal Anti-beta Tubulin	Abways	AB0039	1:5000
Lectin PNA Conjugate (Alexa Fluor 568)	Thermo Fisher	L-32458	1:1000
Goat Anti-Rabbit IgG H&L (HRP)	Thermo Fisher	31460	1:1000
Goat Anti-Mouse IgG H&L (HRP)	Thermo Fisher	31430	1:1000
Donkey Anti-Rabbit IgG H&L (Alexa Fluor 555)	Thermo Fisher	A-31572	1:1000
Donkey Anti-Mouse IgG H&L (Alexa Fluor 488)	Thermo Fisher	A-21202	1:1000
Donkey Anti-Mouse IgG H&L (Cy3-AffiniPure)	Jackson	715-165-150	1:1000

Results

Expression pattern analysis of Trim69 among tissues and developmental stages

We examined the expression pattern of Trim69 among 15 mouse tissues including the testis and ovary, and the results showed *Trim69* as a testis-specific gene (***Fig. 1A***), which suggested that Trim69 might play important roles in spermatogenesis. To determine temporal expression of Trim69 during testis development, we performed qRT-PCR at different postnatal time points. Trim69 was detected initially on the 17th day, when late pachytene spermatocytes could be observed, and the expression level gradually increased to a high level on the 35th day, when full spermatogenesis achieved (***Fig. 1B***). This indicated that Trim69 began to express in pachytene spermatocytes. To further confirm the exact germ cell stages in which Trim69 was expressed, we purified spermatogonia, pachytene spermatocyte, round spermatids, and elongated spermatids by STA-PUT, with cell purity >80% as assessed by morphology and fluorescent staining, with spermatogonia stained by LIN28, pachytene spermatocytes stained by γH2AX, round and elongated spermatids stained by PNA (***Fig. 1C***). qRT-PCR analysis of Trim69 indicated that Trim69 was predominantly expressed in pachytene

spermatocytes and spermatids (***Fig. 1D***). This special expression pattern of Trim69 suggests that it is likely to play important roles in the process from meiosis to sperm metamorphosis and maturation.

Generating *Trim69* knockout mice using CRISPR/Cas9

To study the function of Trim69 in testis, we used CRISPR/Cas9 gene editing technology to generate *Trim69*$^{-/-}$ mice. We first obtained *Trim69* mutants chimera founders and crossed them with C57/BL6 mice to purify background and produce mutant mice. Wild type, heterozygous and homozygous genotypes could be distinguished *via* Sanger sequencing, and we finally obtained the homozygous mouse with frameshift by 8 bp deletion of exon 2 (***Fig. 2A*** and ***B***).

Trim69$^{-/-}$ mice are fertile with no apparent spermatogenesis defects

We mated heterozygous male and female mice to obtain homozygous knockout (*Trim69*$^{-/-}$) mice. We designed forward primer in exon 1 and reverse primer in exon 3, with the product sequence spanned exon 2. The results of RT-PCR and Sanger sequencing showed 8 bp deletion, which could result in frameshift mutation, but not the exon 2 skipping (***Fig. 2C*** and ***D***). Western blotting analysis showed the Trim69 protein with the expected molecular weight of 57 kDa in
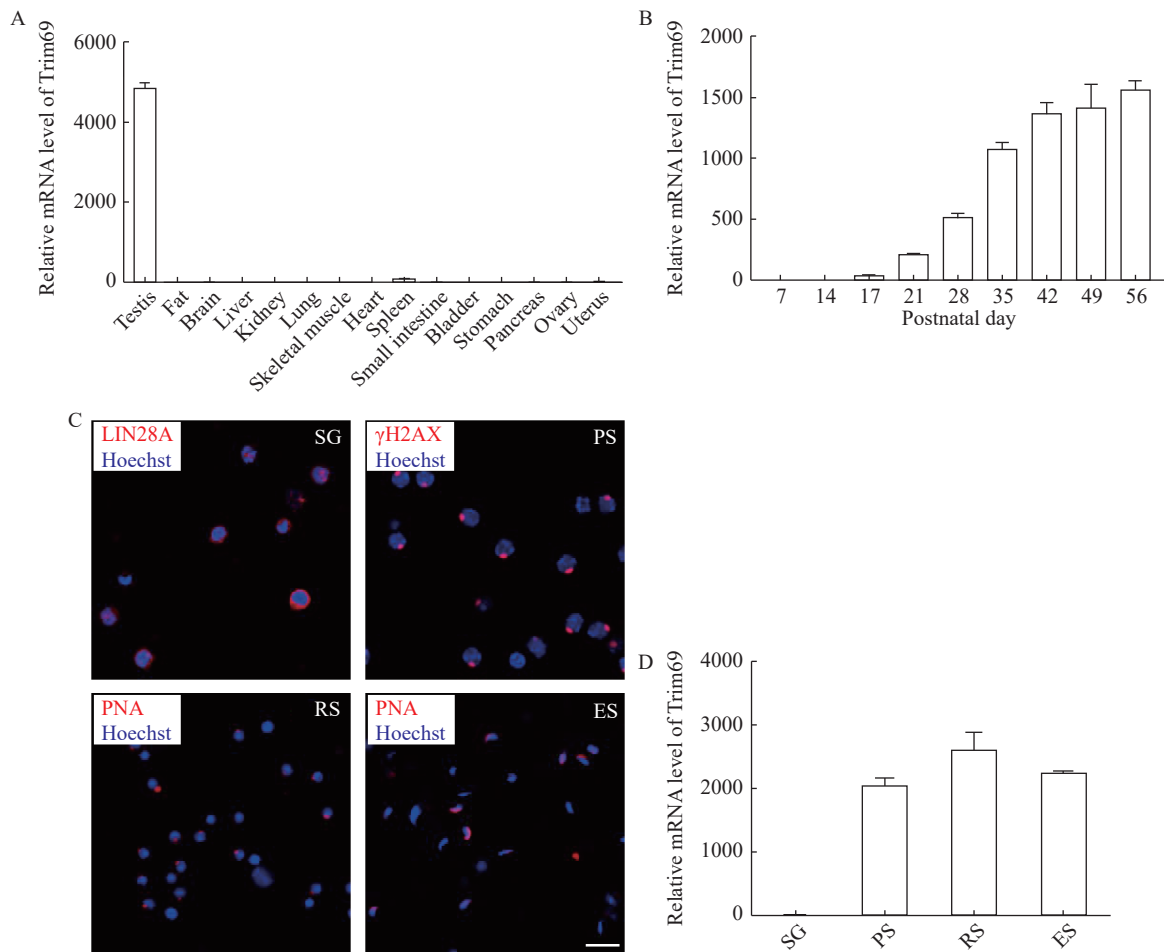
Fig. 1 Expression of Trim69 among tissues and different developmental stages of germ cells in mouse. A and B: qRT-PCR analysis of Trim69 expression in 15 mouse tissues (A) and testis at different timepoints of mice after birth (B). Organs used were from male mice except for the ovary and uterus. n=3. C: The morphologies of mouse spermatogonia, pachytene spermatocytes, round spermatids, and elongated spermatids purified by STA-PUT, and stained by LIN28 (red), γH2AX (red), and PNA (red). DNA was counterstained with Hoechst (blue). Scale bar, 20 μm. D: Trim69 mRNA level in spermatogonia, pachytene spermatocytes, round spermatids and, elongated spermatids. SG: spermatogonia; PS: pachytene spermatocytes; RS: round spermatids; ES: elongated spermatids. n=3.

controls, which could not be detected in *Trim69⁻/⁻* testis (***Fig. 2E***), confirming successful deletion of *Trim69*.

Trim69⁻/⁻ males and females both showed no obvious abnormalities in body development or behaviors. In addition, we examined the fertility of *Trim69⁻/⁻* males and females by mating 8-week-old *Trim69⁻/⁻* males or females with wild type mice. Our results showed that homozygous mice showed normal fertility for both females (***Fig. 2F***) and males (***Fig. 2G***). In adult *Trim69⁻/⁻* male mice, the size and weight of the testes are basically the same as those of the wild types (***Fig. 2H*** and ***I***).

Histological examination of *Trim69⁻/⁻* testes showed normal morphology. H&E staining of testicular sections exhibited that seminiferous tubules with spermatogenic cells in different phases were normal (***Fig. 3A***).

Immunostaining for the spermatogonia marker LIN28, spermatocyte marker γH2AX and Sertoli cell marker SOX9 showed that testicular lumen of *Trim69⁺/⁺* and *Trim69⁻/⁻* mice contained equivalent number of spermatogonia, spermatocytes and Sertoli cells with normal morphologically (***Fig. 4A–F***). Chromosomal spread data confirmed the normal meiotic prophase I of *Trim69⁻/⁻* testes by double staining with SCP3 and γH2AX, by which leptotene, zygotene, pachytene, diplotene spermatocytes could be identified, with similar ratio distributions to the controls (***Fig. 5A*** and ***B***).

Furthermore, we examined the apoptosis, and found no significant change of apoptotic signals in *Trim69⁻/⁻* testes. The number of apoptotic cells per tubule and the number of tubules containing apoptotic cells were similar in knockout and wild-type mice (***Fig. 6A−C***).
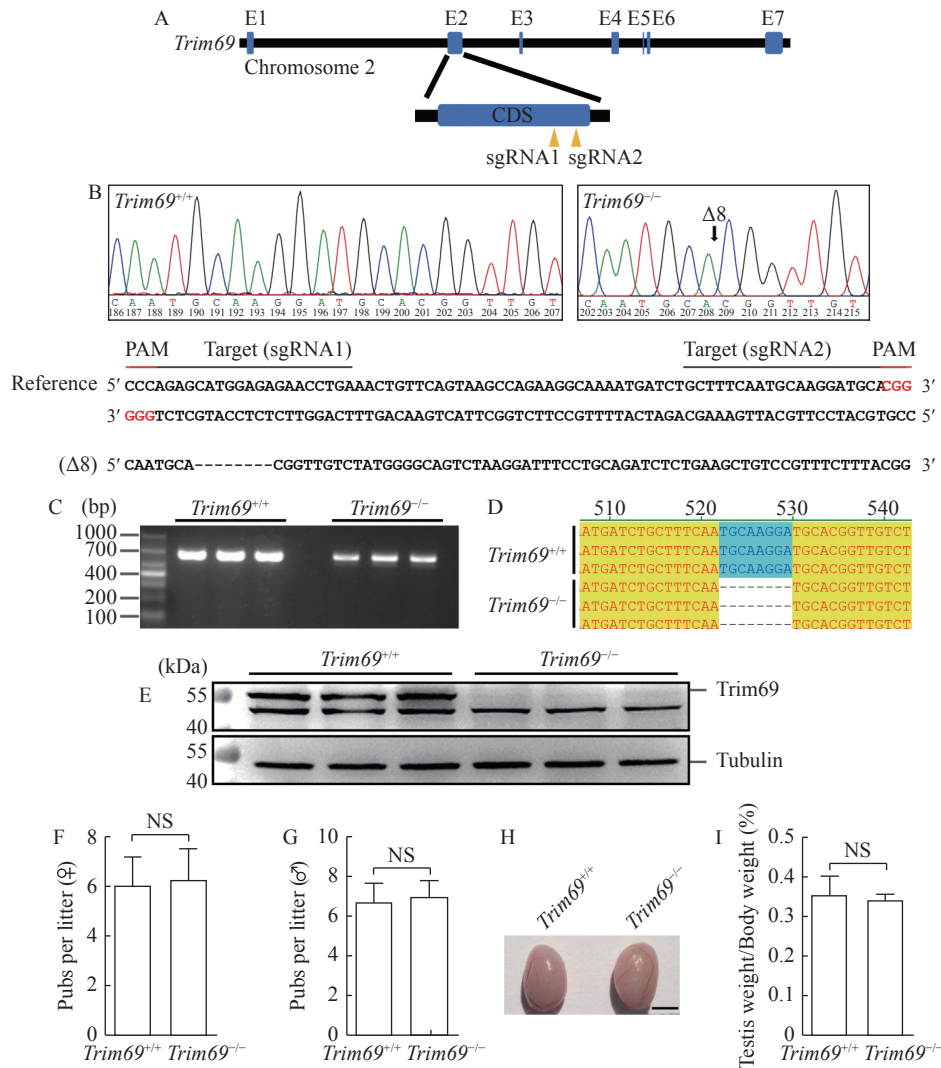
Fig. 2 **Generation of *Trim69⁻/⁻* mice and fertility analysis.** A: Diagram illustrating the CRISPR/Cas9 targeting strategy, including position and sequences of guide RNAs (sgRNAs). E: exon. B: Sequencing results showing that mice with deletion of 8 bases in *Trim69* were successfully obtained. C and D: RT-PCR and sequencing detecting frameshift mutations of *Trim69*. n=3. E: Western blotting analysis of TRIM69 protein in testis after *Trim69* knockout using TUBULIN as a loading control with molecular weights of markers shown on the left. n=3. F and G: Average pups per litter of wild-type and *Trim69⁻/⁻* female (F, n=4) and male (G, n=4) mice. H: Representative images of 8-week-old *Trim69⁺/⁺* and *Trim69⁻/⁻* testes. Scale bar, 3 mm. I: Testis/Body weight ratios of *Trim69⁺/⁺* and *Trim69⁻/⁻* mice. Data were presented as mean±SD. NS: no significance (P>0.05), n=4.

Besides, apoptosis-related genes, BAX, CASPASE3 and BCL-2 showed similar expression levels between *Trim69⁺/⁺* and *Trim69⁻/⁻* testes (***Fig. 6D***).

H&E staining of *Trim69⁻/⁻* epididymis displayed the cauda filled with spermatozoa (***Fig. 7A***). There was no significant difference in morphology between *Trim69⁺/⁺* and *Trim69⁻/⁻* sperm (***Fig. 7B*** and ***E***), and sperm tail also showed similar AC-tubulin distribution (***Fig. 7C***). Moreover, we examined axonemes using transmission electron microscopy. The sperm head had normal ultrastructure with condensed chromatin and normal acrosome, and the across sections of the midpieces showed normal mitochondrial sheath, outer dense fibers and the axoneme both in *Trim69⁺/⁺* and

Trim69⁻/⁻ flagella (***Fig. 7D***). Consistent with the morphological analysis, epididymal sperm count and the ratio of motile and progressive motile sperm of homozygotes were equivalent to those of wild types (***Fig. 7F–H***).

Expression changes of TRIM family genes in *Trim69⁻/⁻* testis

As a testis-specific developmentally regulated gene, *Trim69* was shown to be not essential for spermatogenesis, so we suspected that other genes in TRIM family might compensate the function of *Trim69* after *Trim69* knockout, making up for the defects in spermatogenesis. To test this hypothesis, we
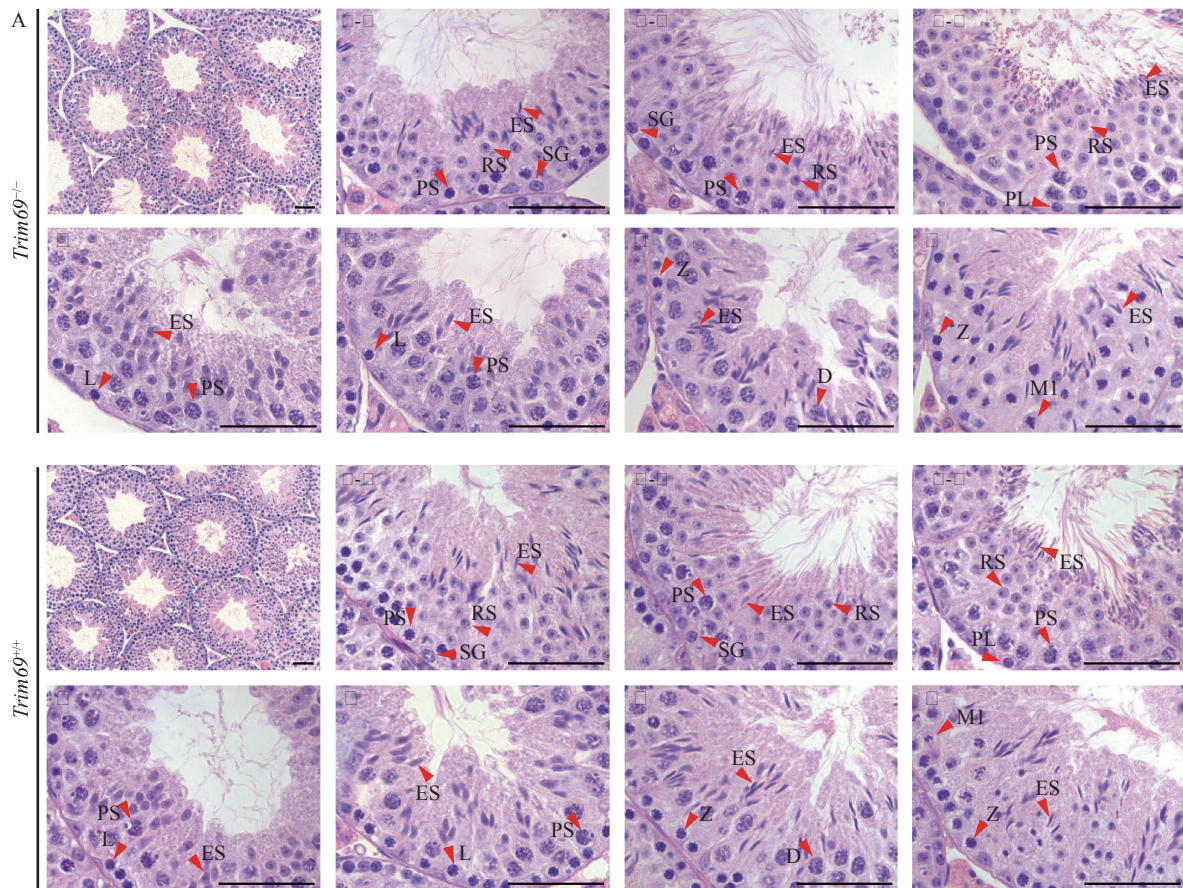
Fig. 3 Different stages of seminiferous tubules of *Trim69⁻/⁻* and control testes. Hematoxylin and Eosin staining of various stages of testicular seminiferous tubules from wild-type and homozygous mice. Red arrows indicating major spermatogenic cells in different stages. SG: spermatogonia; PL: preleptotene spermatocytes; L: leptotene spermatocytes; Z: zygotene spermatocytes; PS: pachytene spermatocytes; D: diplotene spermatocytes; M1: post-prophase I meiotic phase; RS: round spermatids; ES: elongated spermatids. Scale bar, 50 μm.

performed qRT-PCR analysis of other genes from the TRIM family in *Trim69⁺/⁺* and *Trim69⁻/⁻* testes. Analysis results showed that the overall expression levels of TRIM family did not change significantly in *Trim69⁻/⁻* testes (***Fig. 8A***), except that Trim58 was up-regulated (***Fig. 8B***). The domain of Trim58 was similar to Trim69 because they collectively contained the RING-finger domain, coiled-coil domain and SPRY domain, which suggested a similar function of them. Trim58 was thought to stimulate the degradation of the dynein holoprotein complex and its upregulation in *Trim69* knockout mice might play a compensatory role.

Discussion

Previous studies have estimated that more than 1000 genes might participate in the production of functional sperm[5]. But our knowledge of the testis-predominantly expressed genes' function in

spermatogenesis is still very limited[17]. Among post-translational modifications (PTMs)[18], it was reported that the highest rate of ubiquitination among all the rats' examined organs was in the testis, which implies that functional spermatogenesis is particularly relying on ubiquitin-regulated protein function and stability[19].

Ubiquitination is dependent on ubiquitin enzymes, according to their amino acid sequence and the mechanism by which they transfer the ubiquitin molecule, there are E1, E2 and E3 ubiquitin enzymes. E3 ubiquitin ligase recognizes the target protein and provides the specificity for ubiquitination[20]. E3 ubiquitin ligase family can be divided into three categories: protein family with HECT domain, protein family with RING domain, and family with U-box domain[21]. RING E3 ligases act as scaffold proteins, bringing the E2 conjugating enzyme and target protein in close proximity and transferring the ubiquitin from one to the other[22]. Up to now, many researches have shown that E3 ligases play a role in some important
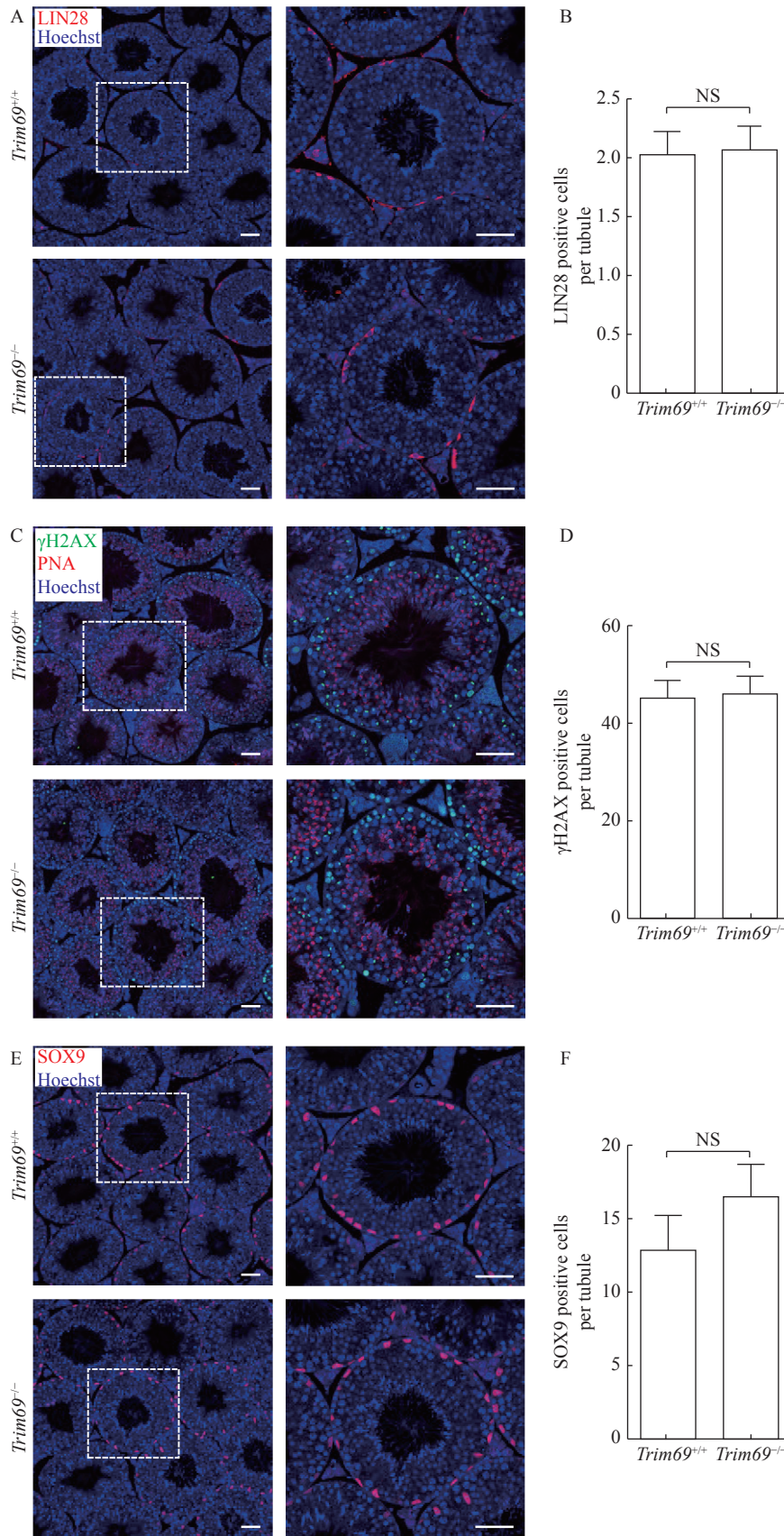
***Fig. 4* Immunofluorescence analysis of *Trim69⁻/⁻* and control testes.** Immunofluorescence detection of LIN28 (A and B), γH2AX (C and D) and SOX9 (E and F) in *Trim69⁺/⁺* and *Trim69⁻/⁻* testes. Scale bar, 50 μm. The statistics (*n*=3) were shown on the right of the corresponding figures. Data were presented as mean±SD. NS: no significance (*P*>0.05).
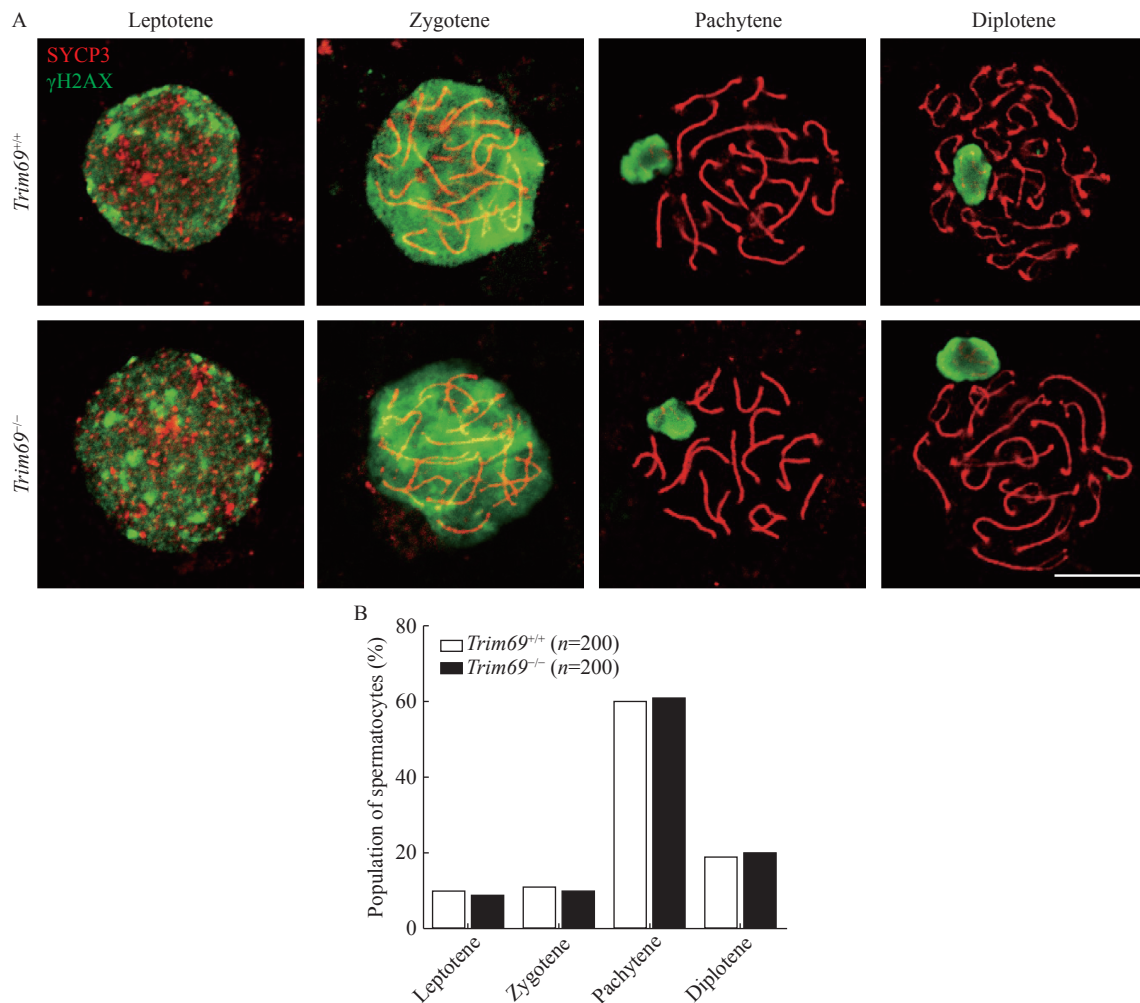
Fig. 5 **Chromosome spread analysis of *Trim69^−/−* and control testicular spermatocytes.** A: Immunostaining of surface-spread spermatocytes from 8-week-old *Trim69^+/+* and *Trim69^−/−* mice for SYCP3 (green) and γH2AX (red). Scale bar, 10 μm. B: Percentages of spermatocytes at different stages of meiotic prophase I, including leptotene, zygotene, pachytene and diplotene, as shown in A.

steps of spermatogenesis. For example, the *Ube2j1* knockout mice show the defects of late spermatogenesis such as incomplete cytoplasm, which leads to sterility[23]. Cul4A E3 ubiquitin ligase plays a significant role in DNA replication, chromatin concentration and cell cycle. The *Cul4A* knockout mice show decreased sperm number, reduced sperm motility and defective acrosome formation, resulting in male sterility[24].

Trim69 was first identified from human testis and could mediate ubiquitination *in vivo*[13]. Zebrafish Trim69 regulates embryo development *via* down-regulation of the c-Jun and p53 pathways[14]. Some researchers suggest that Trim69 participates in the regulation of virus infection[25]. However, the functions of Trim69 in human or mice reproduction are poorly understood. We speculated that Trim69

may ubiquitinate its particular substrates during spermatogenesis.

To investigate the function of the Trim69 in spermatogenesis, we generated *Trim69* knockout mice and found that they showed normal fertility compared with the control. The deletion of *Trim69* did not cause a notable change in sperm count and morphology. Besides, no impairment was noted in the seminiferous tubules, and we could observe all stages of germ cells from spermatogonia to mature sperm in the testis. Furthermore, the analysis of sperm meiosis demonstrated similar sub-stages in *Trim69^+/+* and *Trim69^−/−* testes.

As Trim69 regulated p53 pathway and apoptosis[14], we further detected the level of apoptosis in knockout testis by TUNEL staining, but found no change in apoptosis level. Considering that TUNEL staining
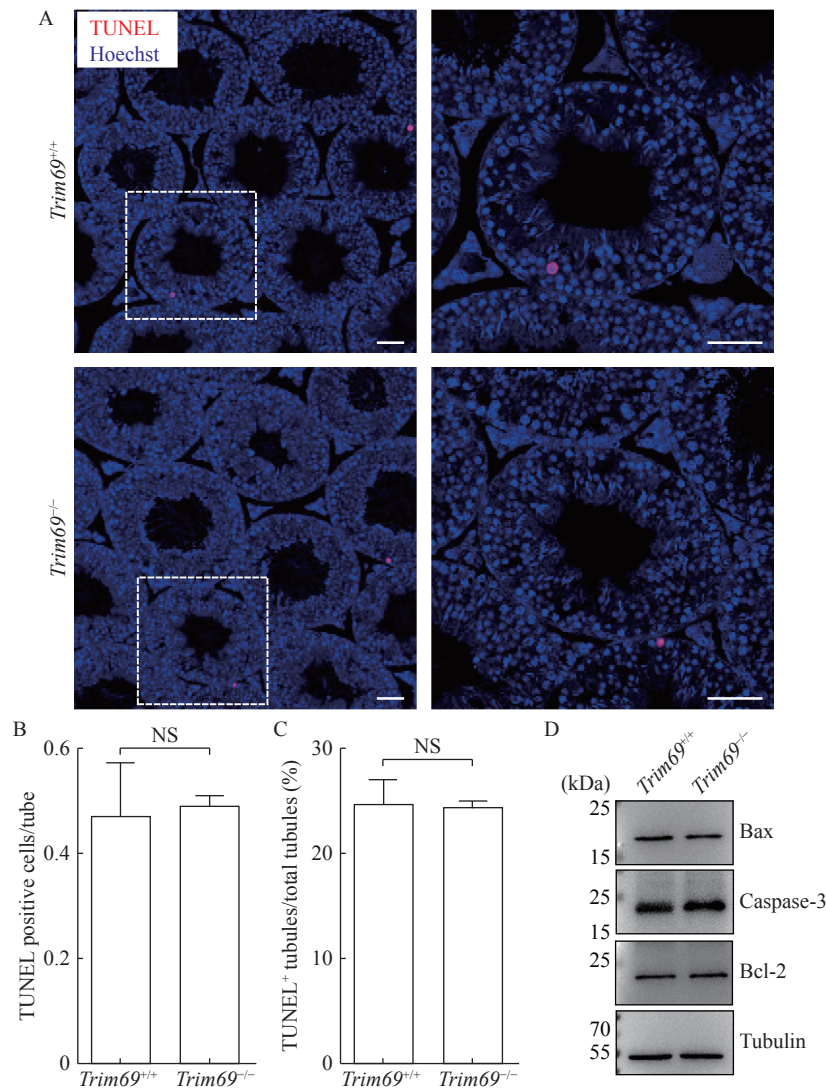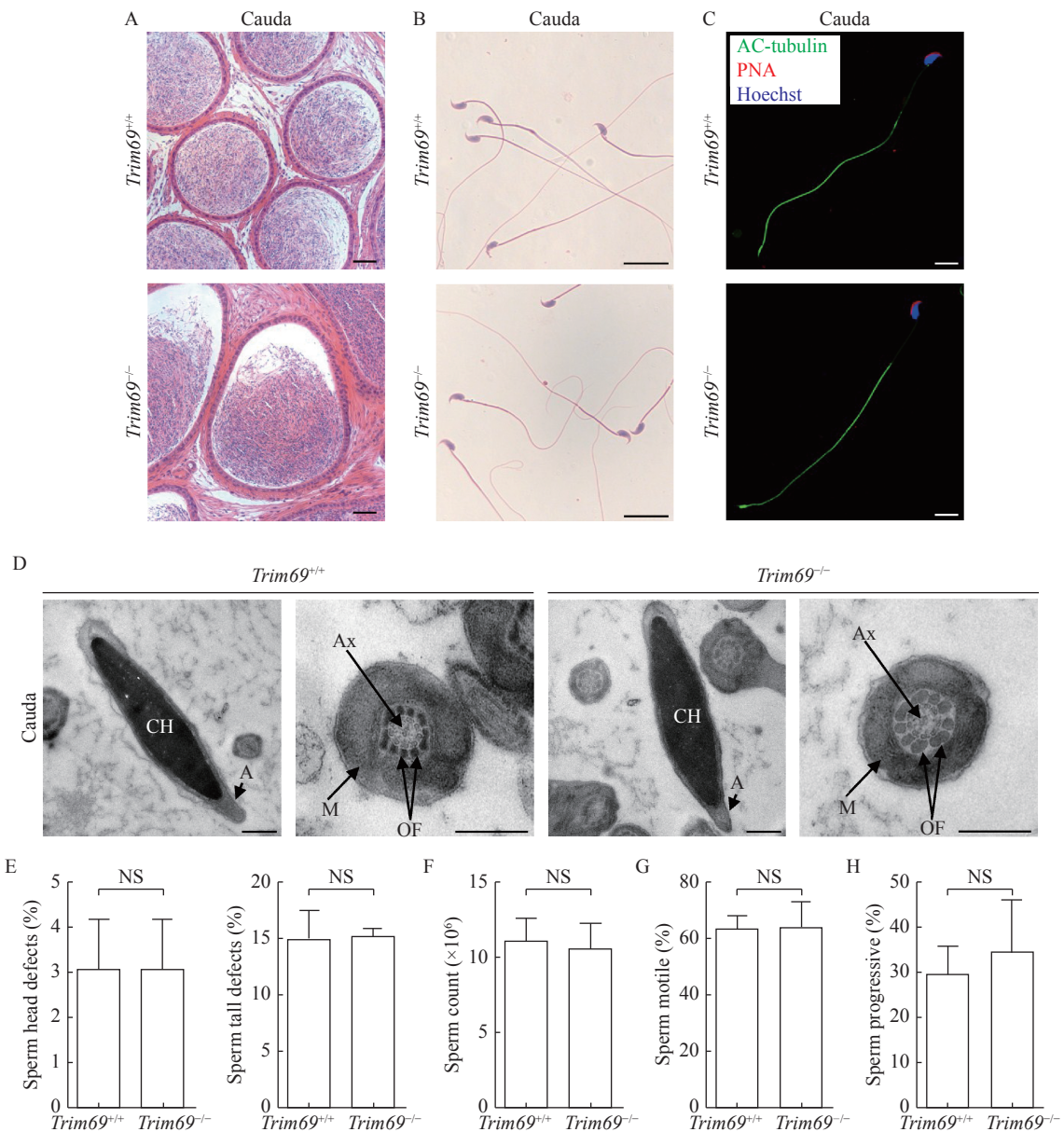
Fig. 6 **Analysis of apoptosis in *Trim69⁺/⁺* and *Trim69⁻/⁻* testis.** A: TUNEL assay (red) and Hoechst (blue) staining of wild-type and *Trim69⁻/⁻* testis. Scale bar, 50 μm. B and C: Number of apoptotic cells per tubule (B) and ratios of TUNEL-positive tubules to total tubules (C) in adult *Trim69⁺/⁺* and *Trim69⁻/⁻* testis. Data were presented as mean±SD. NS: no significance (*P*>0.05). D: Western blotting analysis of Bax, Caspase 3 and Bcl-2 in wild-type and *Trim69⁻/⁻* testes with Tubulin as a loading control.

could only show late apoptosis, we detected the level of BAX, BCL-2 and CASPASE3 in testis, which were important markers of apoptosis signaling pathways. BAX, considered to be an apoptosis regulator, could accelerate programmed cell death by binding to, and antagonizing the apoptosis repressor BCL-2, and promotes activation of CASP3, thus leading to apoptosis.

A possible explanation for our results is that Trim69 is dispensable for male fertility despite of its specific expression in the testis, which might be functional redundancy. Genetic compensatory mechanism has been widely found in certain gene knockout mice. Compared with wildtype mice, these

mice have no obvious phenotypic defects. For example, *Calpain11* is a testes high expression gene, and is thought to have closely relationship with the acrosome reaction. Although *Capn11⁻/⁻* mice are fertile, other calpain paralog, such as calpain-1 and calpain-2, may work to substitute for calpain-11 deficiency and compensate its roles[26]. Considering proteins from the TRIM family might have redundant functions, we detected the expression of TRIM family in knockout mice. The results showed that though most members of TRIM family had no significant changes, Trim58 was up-regulated. Trim58 and Trim69 belong to the Ring finger protein family, and their structures are very similar[27]. We speculated that

Fig. 7 Analysis of sperm from cauda epididymis in _Trim69_+/+ and _Trim69_−/− mice. A: Hematoxylin and Eosin (H&E) staining of cauda epididymis sections from _Trim69_+/+ and _Trim69_−/− mice. Scale bar, 50 μm. B and C: H&E staining (B) and immunofluorescent staining (C) of sperm stained with AC-tubulin (green), PNA (red) and Hoechst (blue). Scale bar, 20 μm. D: Electron micrographs of sperm head and flagella in wild-type and _Trim69_−/− mice. A: acrosome; CH: condensed chromatin; M: mitochondria; OF: outer dense fibers; Ax: axoneme. Scale bar, 500 nm. E–H: Statistics of sperm head and tail abnormalities (E), sperm count (F), motility (G) and progressive motility (H) of _Trim69_+/+ and _Trim69_−/− mice. Data were presented as mean±SD. NS: no significance ($P>0.05$).

the two genes might have very close functions. Recently, it has been reported that Trim58 may play a role in many tumorigeneses through ubiquitination[28]. But there are few reports about the roles of TRIM family proteins in gametogenesis. In the future, the role of TRIM family in reproduction, especially in spermatogenesis, deserves further study.

Anyway, Trim69 is specifically expressed in testis and is conserved in the eutherians. The _Trim69_ knockout mice exhibited no apparent impairment in fertility. This is the first study demonstrating that Trim69 is not essential for male mice fertility. In the next step, we plan to further study the role of Trim69 and its family members in spermatogenesis by constructing multi-targets knockout mice. Our studies will help to improve the understanding of TRIM protein family in testes and our data will help researchers in the field of reproductive genetics in prioritizing the target genes.
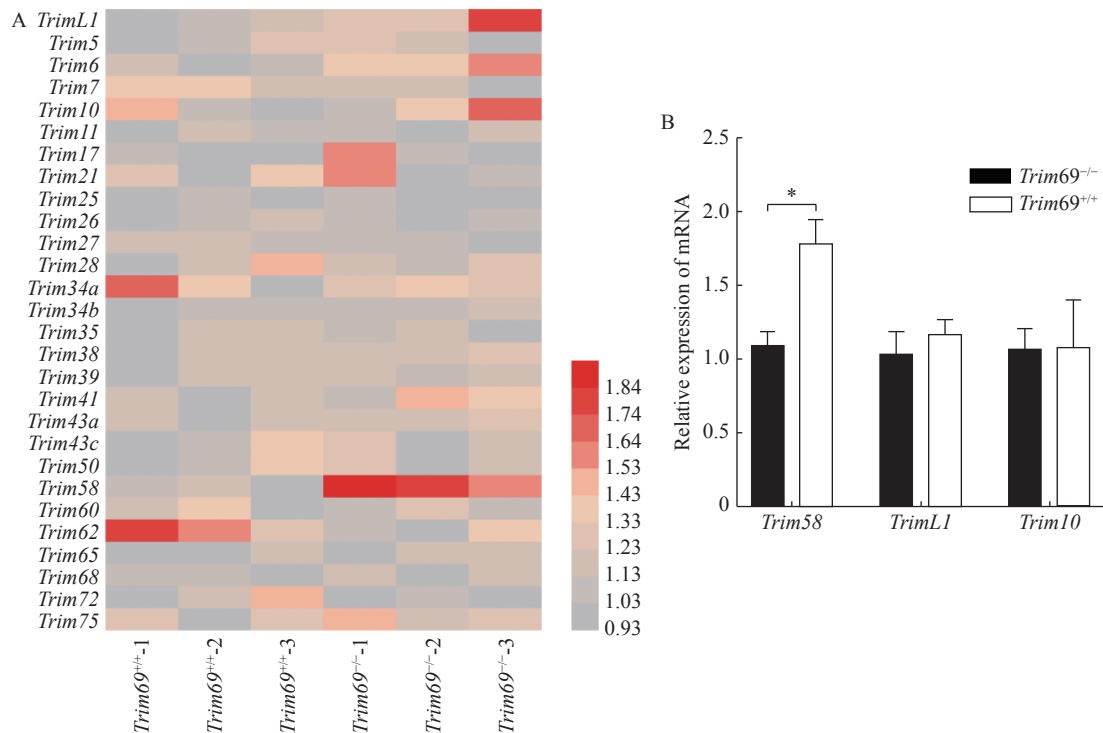
Fig. 8 **Expression analysis of TRIM family gene members in *Trim69*⁺/⁺ and *Trim69*⁻/⁻ testis.** A: Heatmap of mRNA expression levels of TRIM family gene members in *Trim69*⁺/⁺ and *Trim69*⁻/⁻ testis. B: Bar plot of expression of Trim58, TrimL1, and Trim10 in testis by qRT-PCR analysis (*n*=3). Data were presented as mean±SD. *$P<0.05$.

Acknowledgments

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