Am J Transl Res 2025;17(3):1814-1823
www.ajtr.org /ISSN:1943-8141/AJTR0163146

Original Article

Spsb3 is not essential for spermatogenesis and male fertility in mice

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Received January 7, 2025; Accepted February 19, 2025; Epub March 15, 2025; Published March 30, 2025

Abstract: Background: Spermatogenesis, the process by which male germ cells develop into mature spermatozoa, is a complex and highly regulated phenomenon crucial for male fertility. Various molecular pathways, including ubiquitination, play critical roles in this process. Ubiquitination regulates multiple stages of spermatogenesis by controlling cell remodeling and protein metabolism. SplA/ryanodine receptor domain and SOCS box containing 3 (SPSB3), a SOCS box protein, interacts with ElonginC/B and recruits Cullin5 to form the ECS E3 ligase complex, which is involved in cell development, proliferation, stress response, and apoptosis. However, the specific role of SPSB3 in spermatogenesis and male reproduction remains poorly understood. Methods: The distribution and expression of *Spsb3* were analyzed using bioinformatics approaches. *Spsb3*-knockout (KO, *Spsb3*$^{-/-}$) mice were generated using CRISPR/Cas9 gene editing. Sperm quality was assessed using a computer assisted sperm analysis (CASA) system. Histological and immunostaining analyses were performed to evaluate the effects of *Spsb3* deletion on mouse testicular structure. Apoptotic cells were detected using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Results: Our findings indicate that *Spsb3* is a testis-enriched gene in mice. However, no significant differences were observed in sperm quality, fertility, or testis histology between *Spsb3*$^{-/-}$ and wild-type (WT) adult mice. Conclusion: This is the first functional study of *Spsb3* in mammalian reproduction. Despite its evolutionary conservation and high testicular expression, *Spsb3* is not essential for mouse spermatogenesis under physiological conditions.

Keywords: *Spsb3*, spermatogenesis, male fertility, testis

Introduction

Spermatogenesis is a multifaceted process that occurs in the seminiferous tubules of the testis, ultimately producing fully mature male gametes. This complex process involves several key stages: spermatogonia proliferate and differentiate into spermatocytes, which then undergo meiosis to produce spermatids. These round spermatids subsequently mature and differentiate into highly specialized spermatozoa, which are released into the lumen of the seminiferous tubules [1, 2]. Each stage is tightly regulated and is essential for the production of functional spermatozoa.

Ubiquitination plays a critical role in various stages of spermatogenesis by regulating cell remodeling and protein metabolism [3-5]. It is a dynamic and multifaceted post-translational modification mediated by a cascade of enzymatic reactions involving E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-ligases [6]. This process maintains intracellular protein homeostasis by covalently attaching ubiquitin (Ub), a highly conserved 76-amino acid protein to target substrate proteins [7, 8]. As the final step in the ubiquitination cascade, E3 ligase facilitates the covalent attachment of ubiquitin and specific substrate lysine residues, significantly

https://doi.org/10.62347/TVPV4242

enhancing the efficiency of ubiquitin transfer [5, 9].

Cullin-RING ligases (CRLs) represent the largest family of E3 ligases in eukaryotes and function as multisubunit protein complexes [10]. The splA/ryanodine receptor domain and SOCS box-containing (SPSB) protein family consists of four members (SPSB1 to SPSB4) that play crucial roles in cell development, proliferation, stress response, and apoptosis [11]. These proteins share a conserved SOCS box at the C-terminus and a central SPRY/B30.2 domain, which is essential for substrate recognition and ubiquitination. SPSBs protein are key components of E3 ubiquitin ligase complexes, where the SPRY/B30.2 domain determines substrate specificity. Previous studies have shown that SPSB3, a SOCS box protein, interacts with ElonginC/B and recruits Cullin5 to form the ECS E3 ligase complex. This complex mediates the polyubiquitination and subsequent proteasome degradation of SNAIL, a key regulator of epithelial-mesenchymal transition [12-14].

Recent studies have also revealed that the CRL5-SPSB3 ubiquitin ligase complex targets nuclear cGAS for degradation, thereby regulating immune response and cell signaling [15]. These findings highlight the significance of SPSB3 in protein homeostasis and cellular signaling; however, its specific role in male reproduction remains unclear. Given its high expression in the testis and established function in ubiquitination, further investigation into the role of SPSB3 in spermatogenesis is warranted.

In this study, we generated *Spsb3*-knockout (KO, *Spsb3*$^{-/-}$) mice using CRISPR/Cas9 gene editing technology to investigate its role in spermatogenesis. Despite the high expression of Spsb3 in mouse testes, our results showed no significant differences in sperm morphology, motility, or concentration between *Spsb3*$^{-/-}$ wild-type (WT) mice. Fertility assessments also revealed no significant changes in mating success and litter size. Furthermore, histopathological analysis and immunostaining demonstrated no abnormalities in testicular structure or in the expression of key spermatogenic markers in *Spsb3*$^{-/-}$ mice. The Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay did not detect a significant increase in apoptotic cells within the testicular tissue of *Spsb3*$^{-/-}$ mice. These findings suggest that *Spsb3* is not essential for mouse spermatogenesis or fertility.

Materials and methods

Bioinformatic analysis

The mouse scRNA-seq dataset (GSM5563668) was obtained from the Gene Expression Omnibus database. Data processing was performed using software packages including “Seurat” and “SingleCellExperiment”. Cell types were identified based on marker genes, and gene expression patterns were visualized using dot plot and feature plot diagrams, as previously described [16].

Animals

Heterozygous *Spsb3*$^{+/-}$ mice were purchased from Cyagen Biosciences Inc. (Suzhou, China). *Spsb3*$^{+/-}$ mice were interbred to generate *Spsb3*$^{-/-}$ (knockout), *Spsb3*$^{+/-}$ (heterozygous), and *Spsb3*$^{+/+}$ (wild-type, WT) offspring. Genotypes were confirmed by PCR amplification followed by agarose gel electrophoresis. The primers involved are as follows: F1: 5’-TGAGAGCATCTTTGGAAGAGTTTG-3’; R1: 5’-CTTGTTATGCAGCACTTGCTTGAG-3’; F2: 5’-GTATGGCACAGACATGGTAAATGG-3’.

All mice were maintained under specific pathogen free (SPF) conditions at Nanjing Medical University. All experimental procedures were approved by the Animal Ethics and Welfare Committee of Nanjing Medical University (No. 2402015).

Fertility test

Adult *Spsb3*$^{-/-}$ and WT male mice were each housed with two adult C57 BL/6J female mice in a 1:2 sex ratio. The number of litters per mating pair and the number of pups per litter were recorded over a three-month period.

Histology

Following euthanasia via cervical dislocation, testicular tissues from 8-week-old *Spsb3*$^{-/-}$ and *Spsb3*$^{+/+}$ mice were collected and fixed in modified Davidson’s fluid for 48 hours. The tissues were then dehydrated using a graded ethanol

series and embedded in paraffin. Sections (5 μm thick) were prepared, rehydrated and stained with hematoxylin and eosin (HE) and periodic acid Schiff (PAS) stains. Stained sections were examined under an optical microscope for histological analysis, as previously described [17, 18].

Computer-assisted sperm analysis (CASA)

Sperm were collected from the epididymis of 8-week-old mice and suspended in human tubal fluid culture medium at 37°C. Sperm samples were diluted, loaded onto 80 μm slides, and analyzed for sperm concentration and motility using the Hamilton Thorne's Ceros II analyzer (Beverly, MA, USA), as previously described [19, 20].

Immunofluorescence

Immunostaining was performed following a standard protocol [21]. Briefly, tissue sections were incubated overnight (12-16 hours) at 4°C with the following primary antibodies: Anti-LIN28 (1:200, Abcam), anti-γH2AX (1:1000, Abcam), PNA (1:500, Vector), anti-SOX9 (1:200, Millipore) and anti-HSD-3β (1:500, Santa Cruz Biotechnology). Following three washes with PBS at room temperature, sections were incubated with AlexaFluor-conjugated secondary antibodies (Thermo Scientific, Waltham, USA) at 37°C for 1 hour. Nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI) and images were captured using a Zeiss LSM800 confocal microscope for fluorescence analysis.

Terminal deoxynucleotidyl transferase-dUTP nick-end labeling (TUNEL) assay

Tissue sections were treated with 20 μg/mL protease K at room temperature for 10 minutes, followed by incubation in equilibration buffer for 30 minutes. Samples were then incubated with BrightRed labeled reaction buffer at 37°C in the dark for 60 minutes to minimize non-specific signals. After three PBS washes, nuclei were counterstained with DAPI. The reaction buffer (BrightRed), was optimized with a Tris-HCl buffering system (pH 7.4-7.6) to maintain enzyme stability and efficiency. The assay was conducted in a humidified chamber (> 70% humidity) to prevent reagent evaporation and ensure consistent results. Images were obtained using a Zeiss confocal microscope (Oberkochen, Germany).

Statistical analysis

Data are presented as mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad, La Jolla, CA, USA). Tests results from an unpaired Student's t-test with $P < 0.05$ were considered statistically significant.

Results

Distribution and expression of Spsb3 in adult mice

According to the BioGPS database (http://biogps.org), *Spsb3* is predominantly expressed in the adult mouse testis (**Figure 1A**). To further clarify the distribution of *Spsb3* across different testicular cell types, we performed bioinformatic analyses using the publicly available single cell RNA sequencing (scRNA-seq) dataset (GSM 5563668). The results showed that *Spsb3* expression was minimal in Leydig cells, Sertoli cells, and spermatogonia, but predominantly localized in spermatocytes and spermatids (**Figure 1B**, **1C**).

Generation of Spsb3$^{-/-}$ mice

To investigate the physiological function of *Spsb3*, we employed CRISPR/Cas9-based genome editing to delete exons 3-6 of *Spsb3* using two different guide RNAs (gRNAs) (**Figures 2A** and S1). The genotypes of *Spsb3$^{-/-}$*, *Spsb3$^{-/+}$* and *Spsb3$^{+/+}$* mice were confirmed by PCR amplification followed by agarose gel electrophoresis (**Figure 2B**).

Fertility assessment of Spsb3$^{-/-}$ mice

Fertility tests revealed no significant differences in the number of litters between *Spsb3$^{-/-}$* and *Spsb3$^{+/+}$* adult mice (**Figure 2C**). Additionally, testis size and weight were comparable between *Spsb3$^{-/-}$* and *Spsb3$^{+/+}$* adult male mice (**Figure 2D** and **2E**). Computer-assisted sperm analysis (CASA) demonstrated no significant differences in sperm concentration, motility, or progression ratio between *Spsb3$^{-/-}$* and *Spsb3$^{+/+}$* mice (**Figure 2F-H**). Furthermore, hematoxylin and eosin (HE) staining of sperm

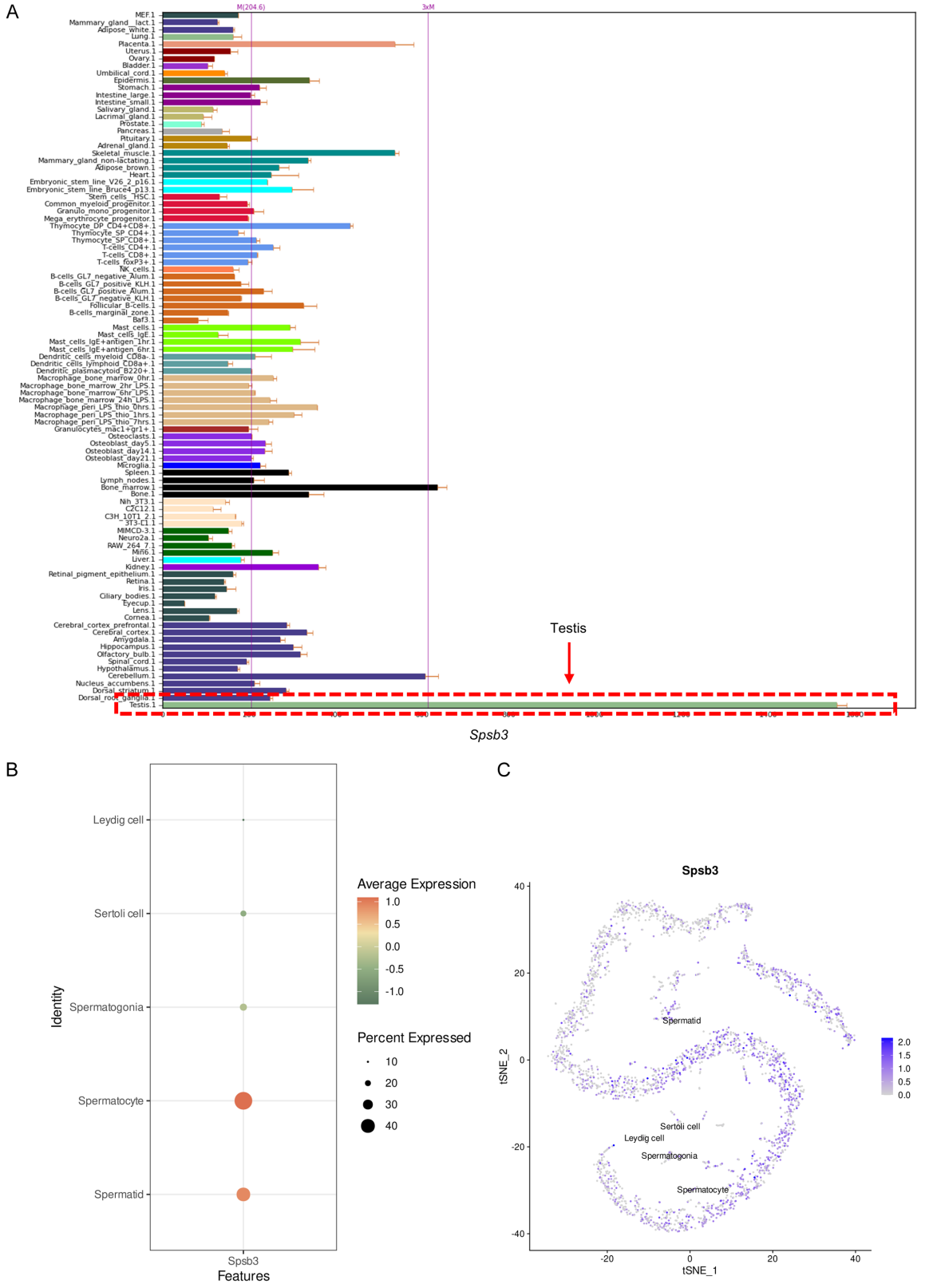

Figure 1. Distribution and expression of *Spsb3* in normal adult mice. A. Bioinformatics analysis of *Spsb3* expression across various tissues and cell types in mice. Data were obtained from the BioGPS database based on microarray analysis (http://biogps). B. Dot plot showing *Spsb3* expression in different cell types within the testicular sample. C. t-SNE diagram illustrating the distribution of *Spsb3* among different cell types in the testicular sample.

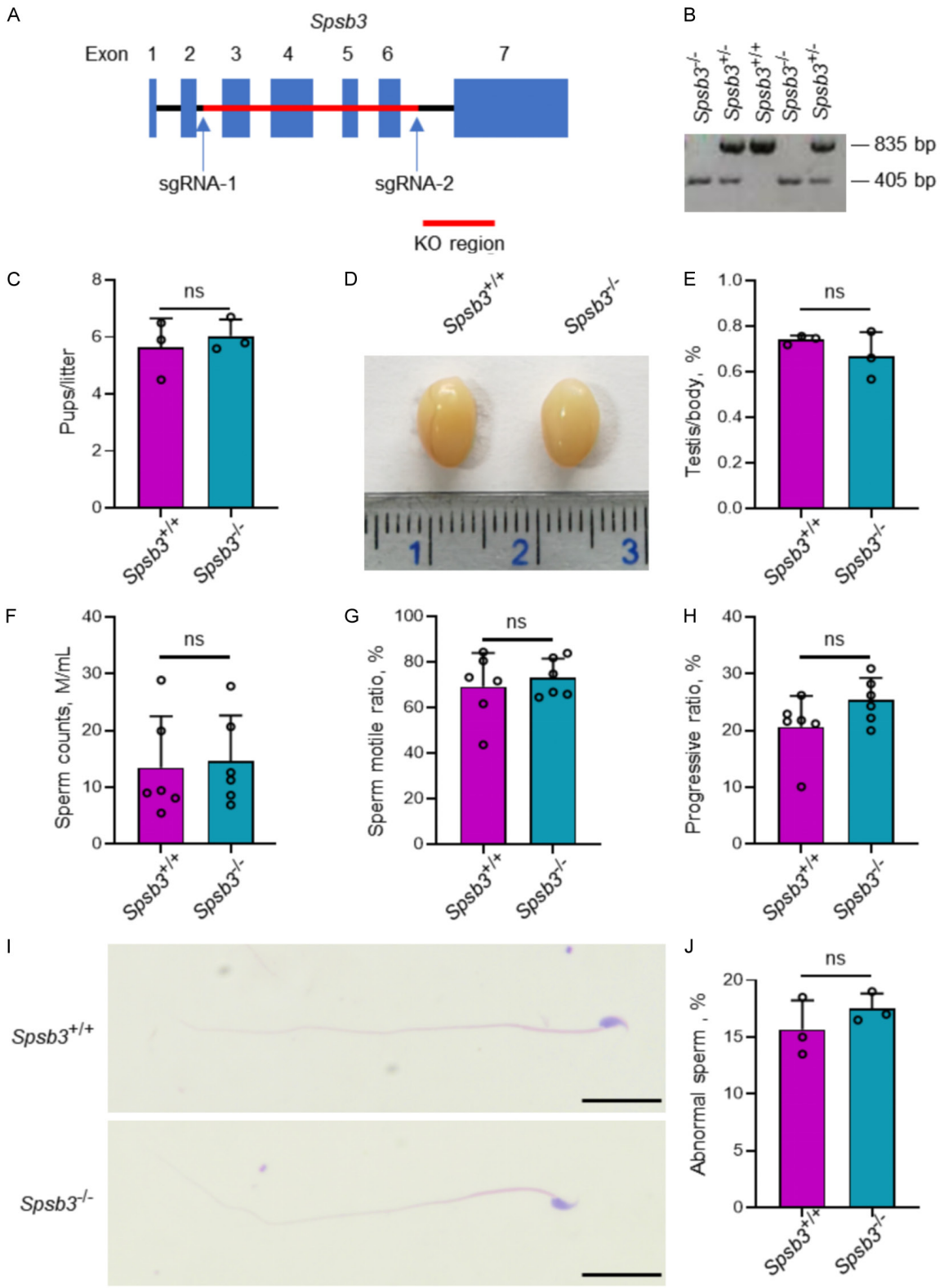

Figure 2. Generation and fertility tests in *Spsb3*$^{-/-}$ mice. (A) CRISPR/cas9-mediated genome editing of *Spsb3*. (B) Verification of mouse genotypes by PCR. (C) Fertility test of *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ male mice. n = 3 per group. P > 0.05. (D) Gross morphology of testes from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. (E) Testis/body weight ratios of *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. n = 3 per group. P > 0.05. (F-H) CASA results for sperm count (F), motility (G), and progressive motility ratio (H) in *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. M, million. n = 6 per group. P > 0.05. (I) H&E staining of sperm in the cauda epididymis of *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. Scale bar = 20 μm. (J) Quantification of (I). n = 3 per group. P > 0.05.

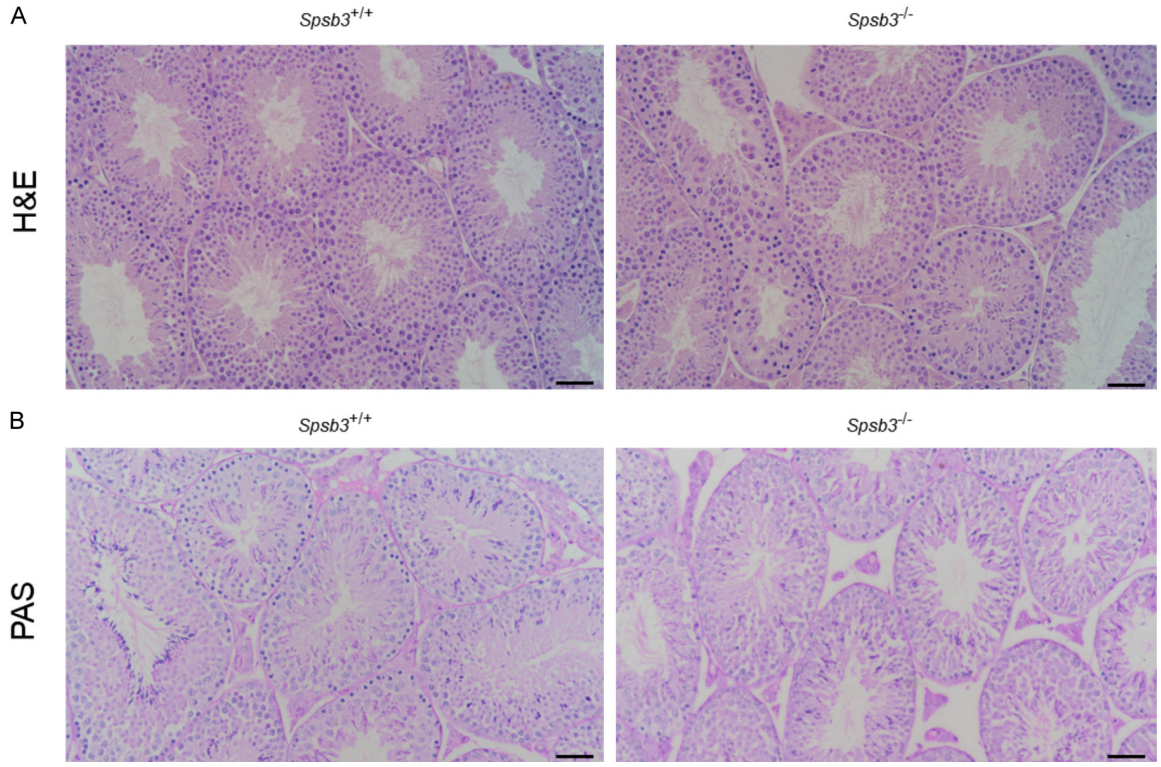

Figure 3. Histological analysis of the testes. A. HE staining of testis sections from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. B. PAS staining of testis sections from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. Scale bar = 50 μm.

revealed no morphological difference between *Spsb3*$^{-/-}$ and *Spsb3*$^{+/+}$ mice (**Figure 2I**, **2J**).

Normal spermatogenesis in Spsb3$^{-/-}$ *mice*

Testicular morphology was assessed using HE and periodic acid-Schiff (PAS) staining. Both *Spsb3*$^{-/-}$ and *Spsb3*$^{+/+}$ mice exhibited intact seminiferous tubules containing all stages of spermatogenic cells (**Figure 3A**, **3B**). To further evaluate the impact of *Spsb3* deletion on spermatogenesis, we quantified spermatogonial stem cells (SSCs), spermatocytes, spermatids, Sertoli cells, and Leydig cells in testes using immunostaining for Lin28, gamma-H2AX, PNA, SOX9, and 3β-HSD signals, respectively. The results showed no significant differences in the number of these cell types between *Spsb3*$^{-/-}$ and *Spsb3*$^{+/+}$ mice (**Figure 4**). Finally, TUNEL analysis revealed no significant differences in the number of apoptotic cells between the two groups (**Figure 5**).

Discussion

Previous studies have identified a total of 2,300 testis-enriched genes in mice, which may play crucial roles in spermatogenesis [22, 23]. However, some genes that are abundantly expressed in the testes may not directly influence male fertility [24, 25]. The *Spsb3* gene, which is essential for germ cell development and stress response in planarian Dugesia japonica [26], was identified as a testis-enriched gene in this study. Given the relatively conserved evolutionary nature of spermatogenesis [27], we generated a *Spsb3* gene knockout mouse model using CRISPR/Cas9-based gene editing. Through a series of experiments, we confirmed that *Spsb3* deletion (*Spsb3*$^{-/-}$) has no significant impact on spermatogenesis or fertility in mice. While the absence of notable reproductive phenotypic changes in knockout mice is often overlooked, sharing such data can help prevent redundant experiments and conserve research resources and time.

The maintenance of normal spermatogenesis and fertility in *Spsb3*$^{-/-}$ mice can reasonably be attributed to functional redundancy among genes. Specifically, genetic compensation following gene loss is a widely observed phenomenon across species, wherein homologous

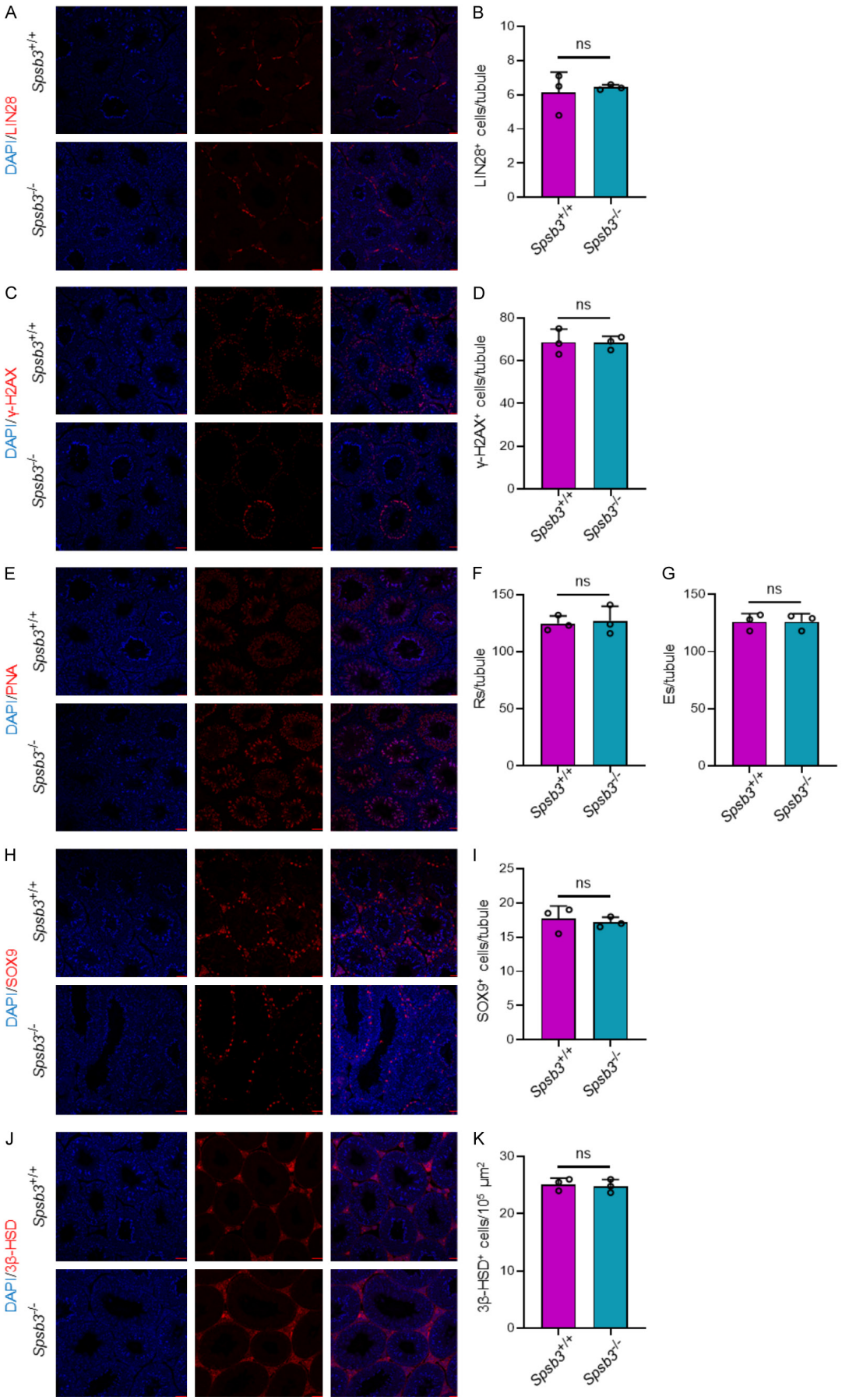
A
DAPI/LIN28
Spsb3+/+
Spsb3-/-
B
LIN28+ cells/tubule
ns
C
DAPI/γ-H2AX
D
γ-H2AX+ cells/tubule
E
DAPI/PNA
F
Rs/tubule
G
Es/tubule
H
DAPI/SOX9
I
SOX9+ cells/tubule
J
DAPI/3β-HSD
K
3β-HSD+ cells/10⁵ μm²

Figure 4. Immunostaining of stage-specific markers during spermatogenesis in *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. (A) Immunostaining of LIN28 in paraffin-embedded testicular sections from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. Scale bar = 50 μm. (B) Quantification of LIN28^{+} cells in (A). n = 3 per group. P > 0.05. (C) Immunostaining of γ-H2AX in paraffin-embedded testicular sections from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. Scale bar = 50 μm. (D) Quantification of γ-H2AX^{+} cells in (C). n = 3 per group. P > 0.05. (E) Immunostaining of PNA in paraffin-embedded testicular sections from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. Scale bar = 50 μm. (F) Quantification of round spermatids (Rs) in (E). n = 3 per group. P > 0.05. (G) Quantification of elongating/elongated spermatids (Es) in (E). P > 0.05. (H) Immunostaining of SOX9 in paraffin-embedded testicular sections from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. Scale bar = 50 μm. (I) Quantification of SOX9^{+} cells in (H). n = 3 per group. P > 0.05. (J) Immunostaining of 3β-HSD in paraffin-embedded testicular sections from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. Scale bar = 50 μm. (K) Quantification of 3β-HSD^{+} cells in (J). n = 3 per group. P > 0.05.

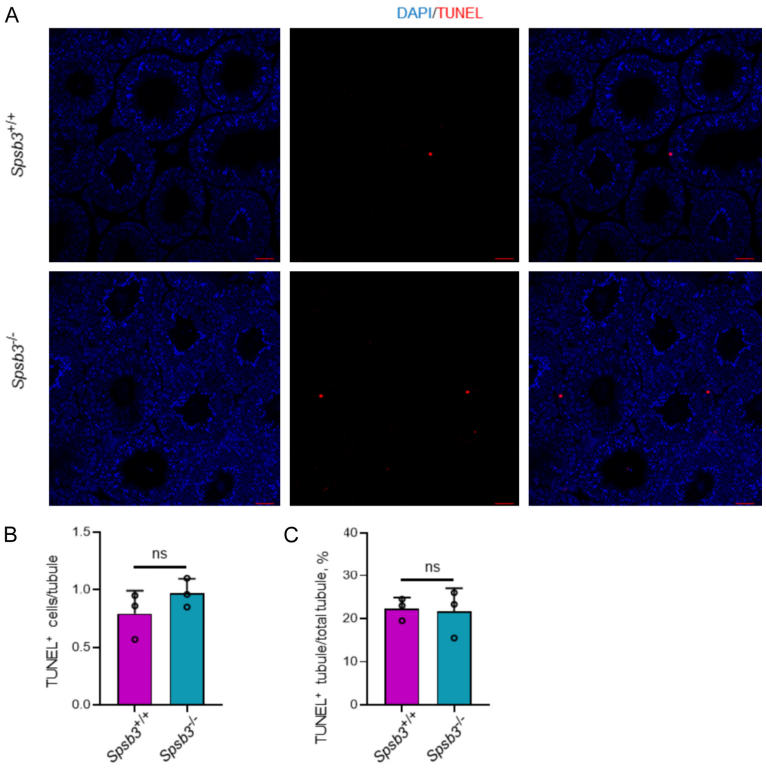

Figure 5. TUNEL analysis for detecting apoptotic cells in two groups of mice (A) TUNEL assay performed on paraffin-embedded testicular sections from *Spsb3*$^{+/+}$ and *Spsb3*$^{-/-}$ mice. (B) Quantification of TUNEL^{+} cells in (A). n = 3 per group. P > 0.05. Scale bar: 50 μm. (C) Quantification of TUNEL^{+} tubules in (A), P > 0.05. Scale bar: 50 μm.

genes with overlapping functions compensate for the missing gene. This mechanism helps maintain normal physiological functions despite gene loss [28-30]. For example, in *Slc26a1*-knockout mice, the mRNA levels of its family members, *Slc26a5* and *Slc26a11*, were upregulated, ensuring that male fertility and spermatogenesis remained unaffected [31]. Similarly, in *Asb12*-KO mice, compensatory increases were observed in the expression levels of multiple Asb family members, including *Asb1, 2, 3, 4, 5, 7, 8, 9, 11, 14, 15, 17*, and *18* [32]. Additionally, deletion of *Ubqln3* led to the upregulation of *Ubqln1*, *Ubqln2*, and *Ubqln4* in mouse testes, while deletion of *UbqlnL* significantly increased the expression of *Ubqln1* and *Ubqln4* [33, 34].

Although we confirmed that *Spsb3* is a testis-enriched gene, our findings indicate that it does not significantly affect fertility or spermatogenesis in mice. Consequently, *Spsb3* can be ruled out as a potential target for male infertility treatment. Moving forward, this data will help researchers better prioritize candidate genes, optimize research strategies, and focus on genes with a more pronounced impact on male fertility, ultimately driving more effective solutions for infertility treatment.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32370903 to X.H.), and the Gusu Health Talent Program of Suzhou (GSWS2023056 to C.S.).

Disclosure of conflict of interest

None.

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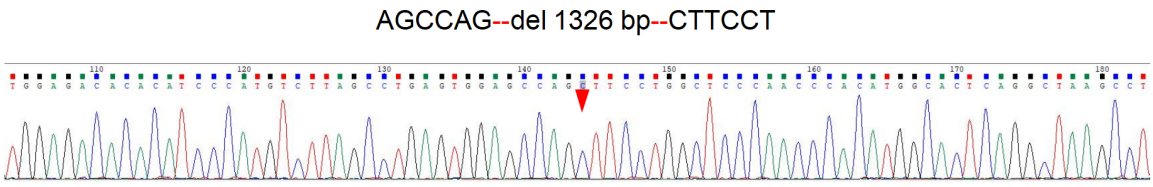

Figure S1. Sanger sequencing of PCR product in *Spsb3*-KO mice.