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Original Article

Testis-enriched *Spsb1* is not required for spermatogenesis and fertility in mice

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Abstract: Objectives: SPRY (repeats in splA and RyR) domain-containing SOCS (suppressor of cytokine signaling) box protein 1 (SPSB1) is an E3 ligase adaptor protein that has been implicated in various cellular processes and physiological pathways. However, its role in spermatogenesis remains poorly understood. The objective of this study was to investigate the impact of SPSB1 deficiency on spermatogenesis and male fertility in mice. Methods: We generated *Spsb1* knockout (*Spsb1*-KO) mice to explore the effects of SPSB1 deficiency on sperm quality. To assess sperm parameters, we utilized computer-assisted sperm analysis (CASA), which provides precise measurements of sperm motility, concentration, and morphology. Additionally, histological and immunohistochemical analyses were performed to evaluate the influence of SPSB1 deficiency on spermatogenesis. Results: Our results showed no significant differences in semen quality, fertility, or histological findings between *Spsb1*-KO and wild-type (WT) mice. Conclusions: This study demonstrates that SPSB1 is not essential for spermatogenesis or male fertility in mice. These findings provide a valuable resource for future genetic investigations into human fertility and help prevent unnecessary duplication of research efforts in this area.

Keywords: SPSB1, spermatogenesis, knockout, male fertility

Introduction

Spermatogenesis is a highly organized process responsible for producing haploid male germ cells, involving extensive morphological and cellular transformations throughout various stages of development [1-3]. The ubiquitin-proteasome system (UPS) plays a critical role in maintaining protein homeostasis by regulating protein degradation and turnover. This system ensures that specific proteins function effectively during particular stages of spermatogenesis and are subsequently degraded once their function is no longer required [4, 5]. Ubiquitination, the process through which proteins are tagged for degradation, involves three key enzymes: the ubiquitin activating enzyme E1, the ubiquitin ubiquitin-conjugating enzyme E2 and, and the ubiquitin ligase E3. Among these, E3 ligases are particularly important because they confer specificity to the ubiquitination by recognizing target substrates and transferring ubiquitin proteins from E2 to the substrate proteins [6, 7]. Many components of the UPS have been implicated in the regulation of spermatogenesis, influencing various stages of sperm development, maturation, and function. These components play a significant role in maintaining male reproductive health, and their regulatory functions underscore the critical role of the UPS in ensuring that spermatogenesis proceeds efficiently and effectively. Consequently, the UPS is essential for male fertility and overall reproductive success [8].

SPSB proteins are members of the suppressor of cytokine signaling (SOCS) family. The SPSB

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family consists of four members: SPSB 1, SPSB 2, SPSB 3, SPSB 4, all of which contain both SPRY domains and SOCS boxes [9]. SPRY domains are protein interaction modules found in approximately 77 mouse proteins and about 100 human proteins [10]. The SOCS box, a conserved sequence of around 40 amino acids, was first identified in SOCS protein [11]. Subsequently, it was discovered that more than 40 other proteins, spanning nine different protein families-including the SPSB family also contain the SOCS box sequence [12]. In the SPSB family of proteins, the SOCS box plays a key role in binding elongins B/C, which recruit cullin-5 and Rbx-2 to form an active E3 ubiquitin ligase complex [13]. This complex is essential for mediating the ubiquitination and subsequent degradation of target proteins.

Studies have shown that SPSB1 is closely associated with tumorigenesis. For example, SPSB1 protein expression is upregulated during breast cancer recurrence, which enhances c-MET signaling and inhibits apoptosis in breast cancer cells, particularly under chemotherapy treatment or HER2/neu expression suppression [14]. In ovarian cancer cells, SPSB1 interacts with p21, promoting its ubiquitination and degradation, thus driving cancer progression [15]. In glioblastoma cells, SPSB1 suppresses cancer malignancy by maintaining TβRII at low levels [16].

Beyond its role in tumorigenesis, SPSB1 has been implicated in various other biological processes. It has been reported to contribute to myogenic differentiation by targeting the TβRII-Akt-Myogenin pathway [17]. Moreover, through modulating NF-κB activation, SPSB1 plays a role in regulating innate immunity and inflammation [18].

While the involvement of SPSB1 in these biological processes has been well-documented, its specific role in spermatogenesis remains underexplored. To address this gap, our study focuses on understanding the function of SPSB1 in male reproduction. We generated *Spsb1* knockout (*Spsb1*-KO) mice to investigate its potential contribution to spermatogenesis and male fertility. However, our results indicate that the absence of *Spsb1* does not significantly impact spermatogenesis or fertility in male mice.

Materials and methods

Bioinformatic analysis

The scRNA-seq dataset used in this study was sourced from the Gene Expression Omnibus (GEO) database, specifically referenced as GSM5563668. This dataset contains valuable information on normal adult mouse testis samples. Data analysis was performed using the "Seurat" package. To enhance the robustness of the analysis, the scaled data were integrated using the "Harmony" function. To effectively visualize gene expression and its distribution across various cell types, "Dotplot" and "Featureplot" functions were employed.

Animals

The *Spsb1*-KO mice with a C57BL/6J background were purchased from Cyagen Biosciences Inc. (Suzhou, China). CRISPR/Cas9-mediated knockout mice were generated as previously described [19]. The mice used in this study were bred and maintained at the Animal Center of Nanjing Medical University, where they were kept under controlled environmental conditions, with a relative humidity range of 30% to 70% and a stable temperature of 26°C. Prior to commencing the research, the study received approval from the Animal Ethics and Welfare Committee of Nanjing Medical University, as indicated by approval number 2402015. All experimental procedures adhered strictly to the guidelines set forth in the *Guide for the Care and Use of Laboratory Animals*, ensuring the welfare and ethical treatment of the animals involved in the research. The genotypes of the *Spsb1*-associated mice were determined by polymerase chain reaction (PCR) using the following primers: F1 (5'-GTATGCATTTCCTACGCTGAACTG-3'), F2 (5'-AGACTGCACAACCTTACTTGTTCT-3') and R1 (5'-ACTCTTACACTAAGCTTTCTTCCCC-3').

Fertility test

Adult male Spsb1 wild-type (WT, +/+) and knockout (KO, -/-) mice were paired with two WT C57BL/6J females for a duration of three months, during which the sizes of each litter were recorded.

Histology

Following euthanasia via cervical dislocation, testes from both wild-type (WT) and *Spsb1*

knockout (KO) mice (aged 8-12 weeks) were promptly preserved in a modified Davidson's solution for 48 hours. After fixation, the testes were dehydrated using a graded ethanol series, embedded in paraffin, and sectioned at a thickness of 5 μm. The sections were then rehydrated and stained with either periodic acid-Schiff (PAS) or hematoxylin and eosin (H&E), following previously described methods [20-22]. Finally, images were captured using an optical microscope, and histological analysis was performed.

Computer-assisted sperm analysis (CASA)

Sperm samples from adult WT and *Spsb1*-KO mice were collected from the cauda epididymis, suspended in human tubal fluid culture medium (InVitroCare, Inc., Frederick, MD, USA), and incubated at 37°C. Semen quality was then assessed using the Ceros™ II sperm analysis system (Hamilton Thorne, Beverly, MA, USA), as previously described [23-25].

Immunofluorescence

Based on our previous research [22, 26, 27], we made partial adjustments to the immunofluorescence experiment. Tissue sections underwent antigen retrieval using 10 mM citrate buffer (pH 6.0). After blocking with 1% (w/v) bovine serum albumin for 2 hours, the sections were incubated with primary antibodies (Table S1) at 4°C for 12 to 16 hours. The sections were then washed three times with phosphate-buffered saline at room temperature before being incubated with Alexa Fluor secondary antibodies (Thermo Scientific, Waltham, USA) for 1 hour at 37°C. Finally, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured using an LSM-800 confocal microscope (Zeiss, Oberkochen, Germany), followed by fluorescence analysis.

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

Apoptosis was assessed using the TUNEL assay kit (Vazyme, Nanjing, Jiangsu, China) following the manufacturer's instructions. Paraffin-embedded tissue sections were rehydrated and treated with proteinase K before undergoing the TUNEL labeling reaction at 37°C. Fluorescence signals were then visualized using a confocal microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analysis was performed using Prism 8.0 (GraphPad, La Jolla, CA, USA). Differences between groups were assessed using an unpaired Student's t-test or analysis of variance (ANOVA). A p-value of <0.05 was considered statistically significant.

Results

Spsb1 is a testis-enriched gene in mice

We initially analyzed the expression pattern of *Spsb1* using publicly available databases. The BioGPS database (http://biogps.org) revealed that *Spsb1* expression is predominantly localized in the mouse testis (**Figure 1A**). Additionally, analysis of a single-cell RNA sequencing (scRNA-seq) dataset from mouse testis demonstrated that *Spsb1* is highly expressed in spermatids, while its expression in spermatogonia, spermatocytes, and Sertoli cells is barely detectable (**Figure 1B** and **1C**).

Spsb1-KO mice exhibit normal fertility

The first *Spsb1* knockout mouse strain was generated using CRISPR/Cas9 genome editing (**Figures 2A** and S1). To confirm the genotypes of WT, heterozygous, and KO mice, PCR amplification was performed (**Figure 2B**), successfully establishing the *Spsb1*-KO mouse strain. *Spsb1*-KO mice exhibited normal viability and fertility, producing litter sizes comparable to those of WT mice (**Figure 2C**). No significant differences were observed in testis size and weight between WT and *Spsb1*-KO mice (**Figure 2D** and **2E**). Sperm analysis using CASA showed no significant differences in sperm concentration, motility, or progression ratio between WT and *Spsb1*-KO mice (**Figure 2F-H**). Furthermore, H&E staining of sperm morphology revealed no notable differences between WT and *Spsb1*-KO mice (**Figure 2I** and **2J**).

Normal spermatogenesis in Spsb1-KO mice

To assess testicular morphology, we performed hematoxylin and eosin (H&E) staining. In both WT and *Spsb1*-KO mice, the seminiferous tubules remained intact and contained spermatogenic cells at all developmental stages (**Figure 3A**). Additionally, periodic acid-Schiff (PAS) staining was used to further analyze spermatogen-

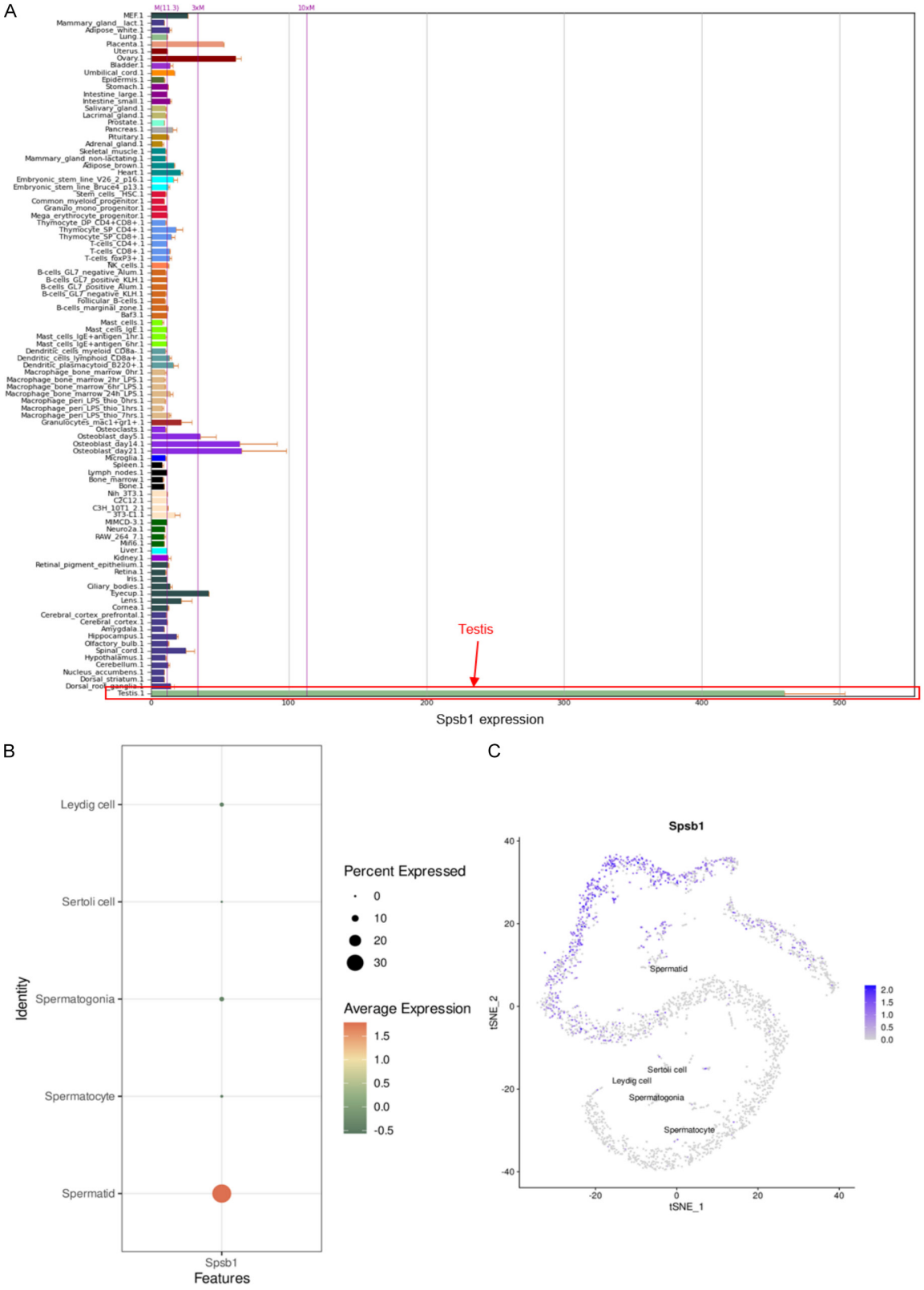


Figure 1. Expression and distribution of *Spsb1* in adult mouse testis samples. (A) Bioinformatic analysis of *Spsb1* expression across various mouse tissues and cell types. Data were obtained from the BioGPS database via microarray analysis (http://biogps.org/). (B) Dot plot illustrating *Spsb1* expression levels across different cell populations within adult mouse testis samples. (C) t-SNE feature plots depicting the distribution of *Spsb1* across various testicular cell types. For (B) and (C), data were obtained from the Gene Expression Omnibus (GEO) database (GSM5563668).

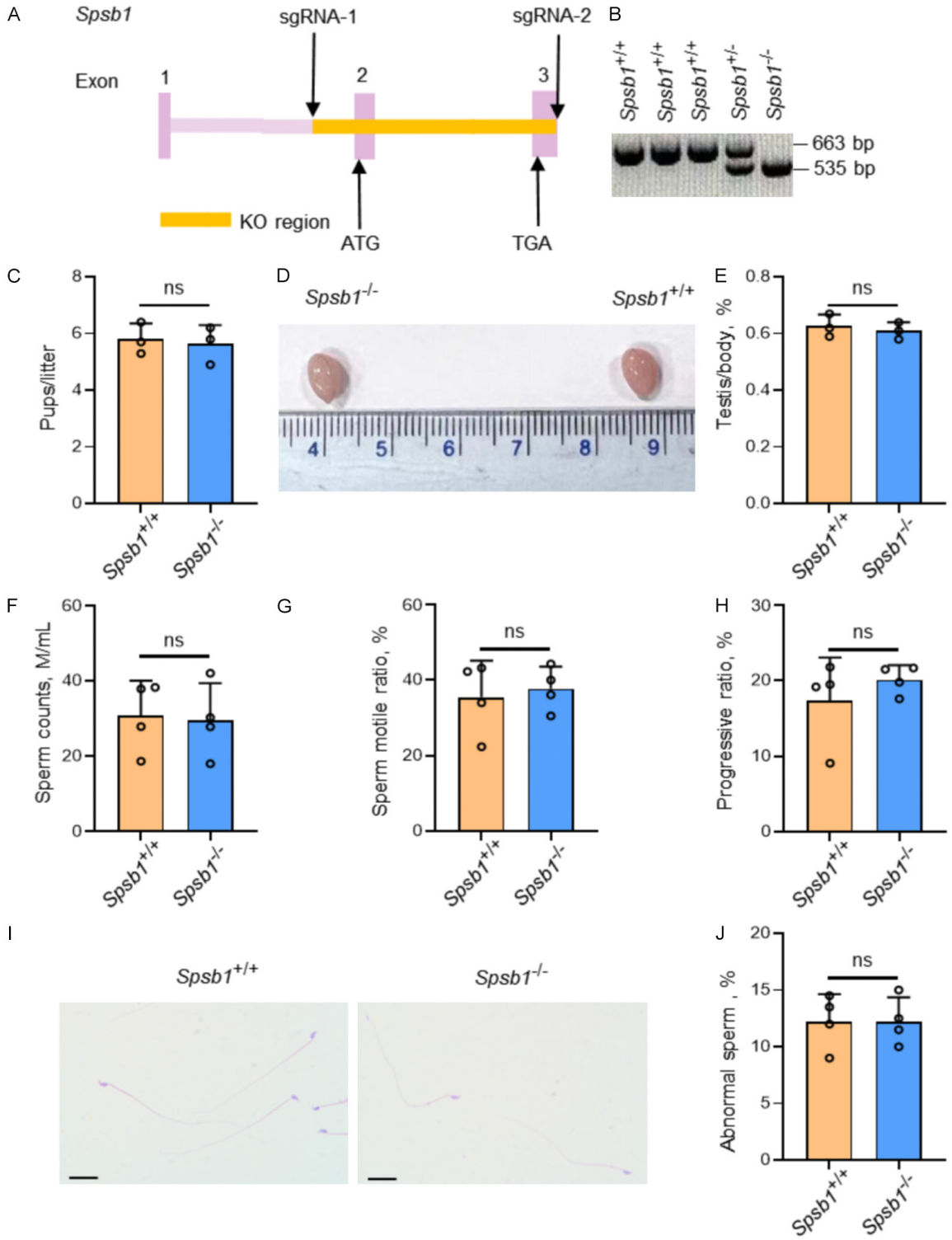


Figure 2. Generation and fertility of *Spsb1*-KO mice. (A) CRISPR/Cas9-mediated gene editing strategy for *Spsb1*. In *Spsb1*-KO mice, exon 2 and 3 of *Spsb1* were deleted. (B) Identification of mouse genotypes by PCR. (C) Fertility testing of (A) CRISPR/Cas9-mediated gene editing strategy for *Spsb1*. In *Spsb1*-KO mice, exons 2 and 3 of *Spsb1* were deleted. (B) PCR-based genotypic identification of WT, heterozygous, and *Spsb1*-KO mice. (C) Fertility assessment of WT and *Spsb1*-KO mice. $n = 3$ per group; $P > 0.05$. (D) Gross morphology of testes from WT and *Spsb1*-KO mice. (E) Testis-to-body weight ratio of WT and *Spsb1*-KO mice. $n = 3$ per group; $P > 0.05$. (F-H) Computer-assisted sperm analysis (CASA) of sperm count (F), motility (G), and progressive ratio (H) in WT and *Spsb1*-KO mice (*M*, million). $n = 4$ per group; $P > 0.05$. (I) H&E staining of sperm in the cauda epididymis of WT and *Spsb1*-KO mice. Scale bar = 20 μm. (J) Quantification of sperm morphology from (I). $n = 4$ per group; $P > 0.05$. ns: not significant.

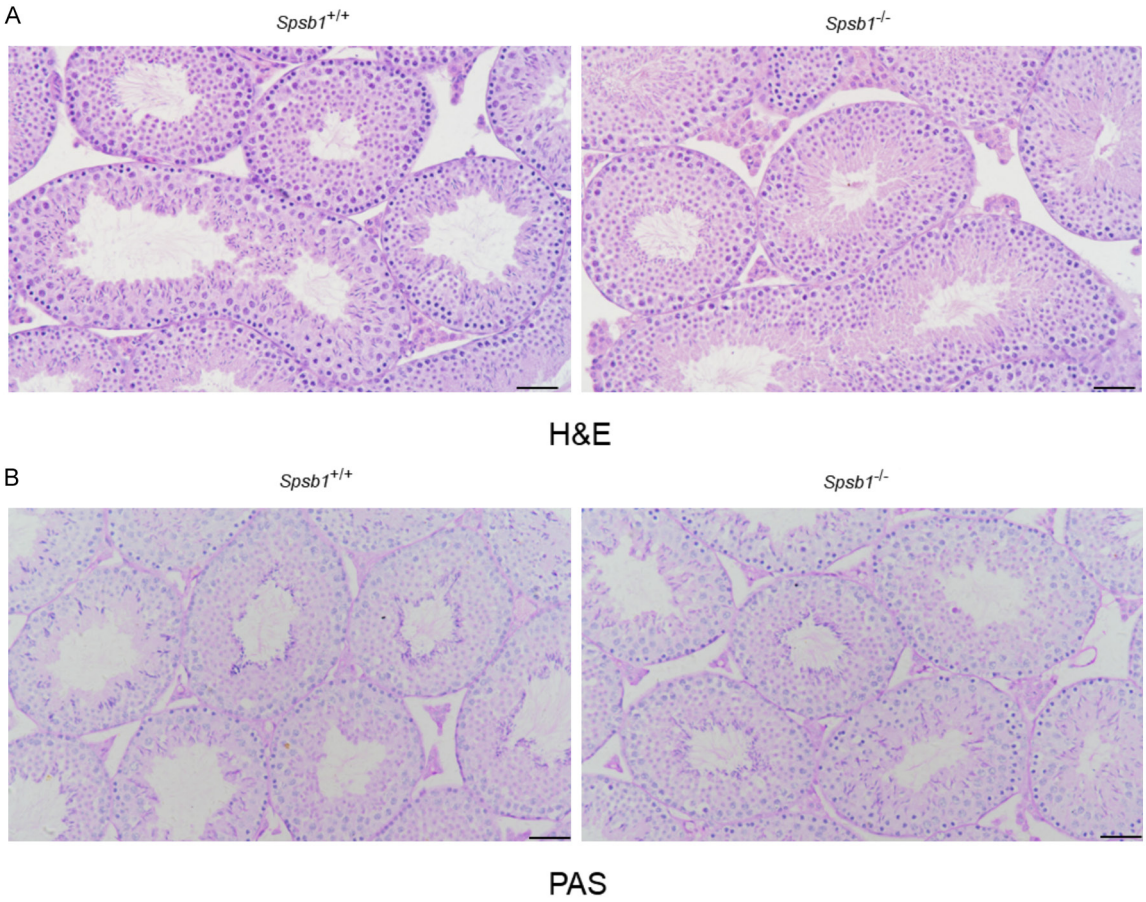
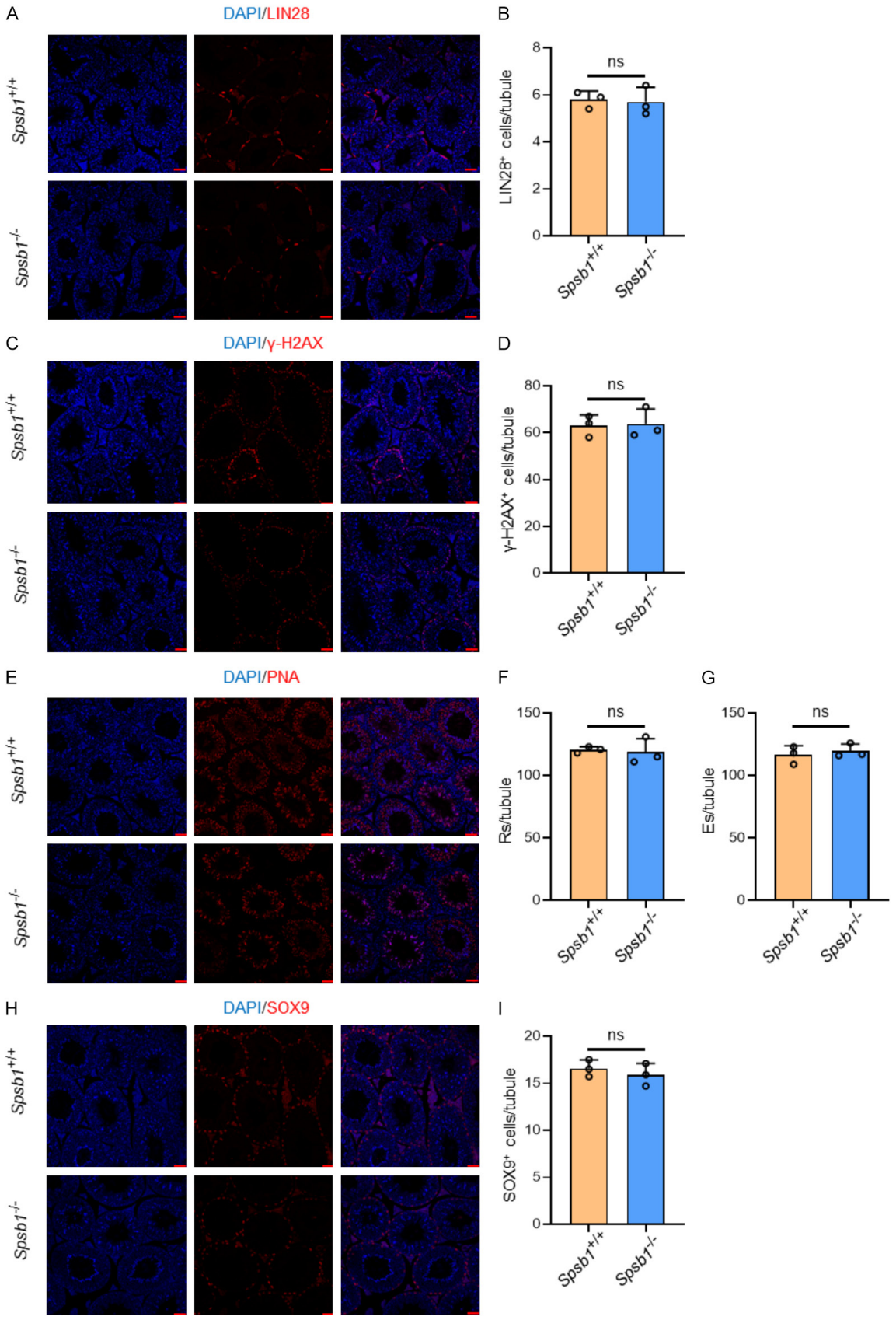


Figure 3. Histological analysis of WT and *Spsb1*-KO testes. A. H&E staining of testis sections from WT and *Spsb1*-KO mice. Scale bar = 50 μm. B. PAS staining of testis sections from WT and *Spsb1*-KO mice. Scale bar = 50 μm.

ic cells within the seminiferous tubules, revealing no apparent morphological differences between the two groups (**Figure 3B**). Spermatogenic cells within the seminiferous tubules are arranged in a highly organized manner based on specific cell connections. To further investigate spermatogenesis, we utilized immunostaining to label distinct cell populations: LIN28 for spermatogonial stem cells (SSCs) (**Figure 4A** and **4B**), γ-H2AX for spermatocytes (**Figure 4C** and **4D**), PNA for spermatids (**Figure 4E-G**), and SOX9 for Sertoli cells (**Figure 4H** and **4I**). Quantitative analysis of positively stained cells showed no significant differences between WT and *Spsb1*-KO mice. Furthermore, apoptosis in testicular tissue was assessed using the TUNEL assay. The results indicated no significant differences in apoptotic cell counts between WT and *Spsb1*-KO mice (**Figure 5A-C**). Collectively, these findings suggest that *Spsb1* is not essential for spermatogenesis in mice.

Discussion

SPSB1 is a critical recognition subunit in the ECS (E3 ligase) complex, playing a pivotal role in maintaining normal cell physiology and morphogenesis. Previous studies have demonstrated that SPSB1 is highly expressed and significantly contributes to brain development in mice [28]. Additionally, during synaptogenesis, the expression of the *Spsb1* gene increases in response to hepatocyte growth factor signaling, suggesting that *Spsb1* is involved in the regulation of synapse formation [29, 30]. Despite its established roles in these processes, the function of SPSB1 in spermatogenesis and male fertility has not been fully explored. In our study, we generated *Spsb1*-KO mice using CRISPR/Cas9 technology to investigate its potential role in spermatogenesis. Our findings indicate that there are no notable differences in spermatogenesis or fertility between the wild type (WT) and *Spsb1*-KO mice, suggesting that


A
DAPI/LIN28
Spsb1+/+
Spsb1-/-
B
ns
LIN28+ cells/tubule
0
2
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6
8
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Spsb1-/-
C
DAPI/γ-H2AX
Spsb1+/+
Spsb1-/-
D
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γ-H2AX+ cells/tubule
0
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80
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Spsb1-/-
E
DAPI/PNA
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Figure 4. Normal spermatogenesis in *Spsb1*-KO mice. (A) Immunostaining and (B) quantification of Lin28 in testes from WT and *Spsb1*-KO mice. (C) Immunostaining and (D) quantification of γ-H2AX in testes from WT and *Spsb1*-KO mice. (E) Immunostaining of PNA, (F) quantification of round spermatids (Rs), and (G) quantification of elongating/elongated spermatids (Es) in testes from WT and *Spsb1*-KO mice. (H) Immunostaining and (I) quantification of SOX9 in testes from WT and *Spsb1*-KO mice. For (A, C, E and H), scale bar = 50 μm. For (B, D, F, G and I), n = 3, P > 0.05, ns: not significant.

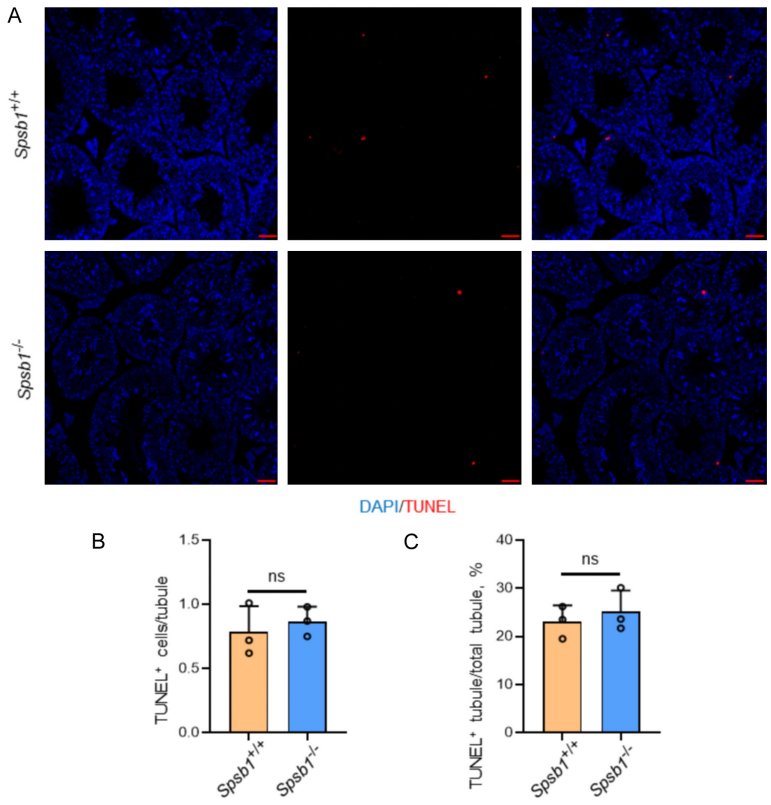

Figure 5. Apoptosis in WT and *Spsb1*-KO testes. A. TUNEL staining of apoptotic cells in WT and *Spsb1*-KO testes. B. Quantification of TUNEL$^+$ cells. C. Quantification of TUNEL$^+$ tubules. Scale bar = 50 μm; n = 3, P > 0.05, ns: not significant.

Spsb1 is not a critical factor for fertility in male mice.

Recent research has shown that many genes involved in encoding ubiquitin enzymes, particularly those expressed in the testis, do not play a crucial role in mouse fertility. Examples of such genes include ankyrin repeat and SOCS box containing 12 (Asb12) [23], Asb15 [24], Asb17 [31], and F-box-only protein 22 (FBXO22) [21]. These findings suggest that there may be functional redundancy or compensatory mechanisms at play, and they highlight the importance of prioritizing research efforts on genes that are essential for fertility.

A potential explanation for the normal spermatogenesis and fertility observed in *Spsb1*-KO mice could be genetic compensation. This phenomenon, where the loss of function of one gene is compensated by the activity of other genes, has been frequently observed in various species [32, 33]. The testis expresses over 1000-2000 genes predominantly, many of which are hypothesized as having vital roles in sperm formation and function. Although some of these genes are essential for male fertility, many others are dispensable for spermatogenesis, likely due to functional redundancy [34]. For example, genes like ASB15 and ASB17, which are expressed in the testis, may compensate for the loss of other related genes [24]. Thus, it is possible that other genes in the testis are compensating for the absence of SPSB1 in the *Spsb1*-KO mice.

There are several limitations to this study that should be acknowledged. First, although we assessed multiple aspects of reproductive function, including fertility outcomes and spermatogenesis, the sample size of mice used may still be insufficient for detecting subtle or age-dependent effects. Second, our study was restricted to male mice, and thus the role of SPSB1 in female fertility remains unexplored. Finally, our research focused on young adult mice (8-12 weeks), and age-related fertility decline in the *Spsb1*-KO model was not assessed. Further studies could address these limitations by expanding the sample size, exploring female fertility, and investigating potential age-related effects in the *Spsb1*-KO model Despite these limitations, our study provides definitive evidence that *Spsb1*-KO mice exhibited normal spermatogenesis and fertility, sug-

gesting that SPSB1 does not play an essential role in these processes. In conclusion, we developed *Spsb1*-KO mice and demonstrated that the absence of SPSB1 does not impair male fertility, thus providing important insights into the genetic regulation of spermatogenesis and male reproduction.

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Disclosure of conflict of interest

None.

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Table S1. Antibody information

Antigen	Species	Source	RRID	Dilution
Lin28	Abcam	Rabbit	AB-776033	1:400
γH2AX	Abcam	Mouse	AB-470861	1:500
PNA	Vector	-	AB-2336642	1:50
SOX9	Millipore	Rabbit	AB-2239761	1:200

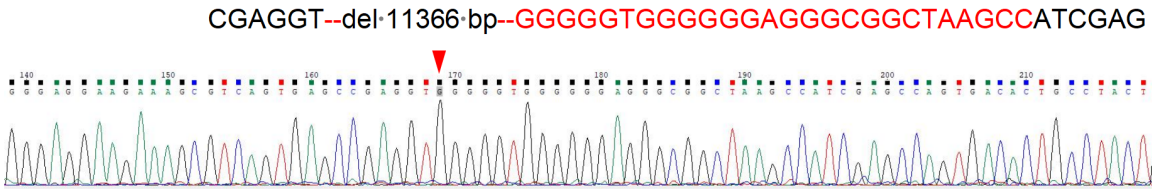

Figure S1. Sanger sequencing of PCR product in *Spsb1*-KO mice.