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Original Article

WSB1 is dispensable for mouse spermatogenesis and male fertility

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Abstract: Purposes: WSB1 (WD repeat and SOCS box-containing protein 1) is part of the E3 ligase complex Elongin B/C-Cullin 2/5-SOCS box E3 ubiquitin ligase (ESC) complex. Our previous studies and those of others have confirmed that some SOCS box proteins, including ASB1 and ASB9, are important for the development of male germ cells. Given that WSB1 is a member of the same protein family, we hypothesized that it might also be involved in spermatogenesis. Although WSB1 has been implicated in tumorigenesis and cancer progression in other tissues due to its E3 ligase activity, its roles and mechanisms in spermatogenesis remain unknown. Methods: Conditional knockout (cKO) mice with deletion of the *Wsb1* gene were generated using CRISPR/Cas9 genome editing. Sperm quality was analyzed using a computer-assisted sperm analysis (CASA) system. To assess the impact of WSB1 deletion, cauda epididymal sperm morphology and testicular tissue structure and organization were studied. Apoptotic cells in the testes were assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Findings: Compared with wild-type (WT) mice, *Wsb1*-cKO mice showed no significant differences in fertility, semen quality, tissue morphology, or rates of apoptosis of germ cells. Nevertheless, *Wsb2* was distinctly upregulated. These findings suggest that normal male reproduction does not depend on WSB1 and that increased WSB2 may represent a compensatory mechanism due to the loss of WSB1. This compensatory effect may explain the absence of an overt phenotype and may guide future research by focusing on other candidate genes and providing context for studies in human reproductive genetics.

Keywords: WSB1, spermatogenesis, male fertility, E3-ubiquitin ligase

Introduction

Spermatogenesis is an elaborate and complex network of processes all of which take place in the seminiferous tubules (ST) of the testis [1]. It begins with the differentiation of spermatogonial stem cells into spermatocytes via mitosis and continues with the division of tetraploid spermatocytes into haploid spermatids via meiosis. Finally, haploid round spermatids develop into mature sperm [2-4]. Ubiquitin (Ub) is a ubiquitously expressed 76-amino-acid protein found in all eukaryotic tissues. Ubiquitination is important in cellular homeostasis and protein turnover [5, 6]. Ubiquitination is a highly regulated, ATP-dependent cascade involving covalent modification of target proteins with ubiquitin, leading to their degradation. This pathway is also known as the ubiquitin-proteasome system (UPS) [7, 8]. Three types of enzymes orchestrate the ubiquitination cascade: ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3). Through their sequential action, the carboxyl terminus of ubiquitin is conjugated to lysine residues on the target substrate protein [9-11].

E3 ubiquitin ligase WD repeat and SOCS box-containing protein 1 (WSB1) is part of the elongin B/C-Cullin 2/5-SOCS box complex (ECS

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complex). It contains a C-terminal SOCS box and seven WD40 repeats and is a putative target of multiple oncogenes [12-14]. WSB1 has been implicated in the development of many tumors. For example, WSB1 and EZH2 can drive c-Myc through WNT/β-catenin signaling, which may promote prostate cancer [15]. WSB1 deficiency greatly reduces bleomycin (BLM)-induced fibrosis, suggesting that WSB1 enhances BLM-induced lung injury [16], and WSB1 promotes degradation of DIO2 via ubiquitination, which is important in colorectal cancer (CRC) [17]. Recent studies show that several substrate-recognition domains of ECS-type ubiquitin ligases - ASB1, ASB9, and ASB17 - are important for sperm production. *Asb1* knockout in mice causes abnormal reactive oxygen species and hydrogen sulfide accumulation in testicular tissue, leading to widespread sperm DNA damage and male infertility [18]. ASB9 mutations disrupt sperm neck formation and lead to structural defects in the sperm head and infertility [6]. Conversely, *Asb17* knockout mice do not show fertility defects; however, they have significantly lower sperm counts consistent with oligospermia [19]. WSB1 is another ECS ligase adaptor that has been postulated to regulate spermatogenesis due to its structural and functional similarity to other ASB-family proteins [20-22]. Despite advances in understanding of WSB1 in different tumor diseases, its involvement in mouse spermatogenesis is not well understood.

This study aimed to clarify the role of WSB1 in murine spermatogenesis. Using CRISPR/Cas9 technology, we generated conditional knockout mice targeting *Wsb1* (*Wsb1*-cKO). Subsequent analyses showed that WSB1 loss does not significantly affect spermatogenesis or male reproductive capacity.

Materials and methods

Bioinformatic analysis

Data were downloaded from published studies and analyzed using an online single-cell analysis tool (The Male Health Atlas database) [23].

Animals

Wsb1 mice were obtained from Cyagen Biosciences Inc. (Suzhou, China), and *Amh/Ddx4*-Cre transgenic mice were provided by the State Key Laboratory of Reproductive Medicine and Offspring Health at Nanjing Medical University. The institutional animal facility was maintained under specific pathogen free conditions for all mice. The mice in this study were bred and maintained at the Nanjing Medical University Animal Center, in which the environmental conditions were closely controlled--relative humidity was kept between 30 and 70 percent, and ambient temperature was kept at 26°C. The institutional Animal Ethics and Welfare Committee gave approval to the experimental protocol (Approval No. 2402015).

Polymerase chain reaction (PCR) genotyping

PCR followed by agarose gel electrophoresis was used to genotype wild-type (WT), *Wsb1* germ cell-specific (*Wsb1*-gKO), and *Wsb1* Sertoli cell-specific (*Wsb1*-sKO) mice. The primer sequences used were as follows: F1: 5'-GTCTGAGGAGGTCAGAGTGTTAGA-3', R1: 5'-AGAAAGGCCTTCAATATCAGCCAC-3'.

Fertility testing

A three-month breeding trial was conducted to assess reproductive capacity. Control and *Wsb1*-cKO males aged eight weeks old were each paired with two WT females, and litter sizes were recorded.

Computer-assisted sperm analysis (CASA)

WT, *Wsb1*-gKO and *Wsb1*-sKO mice aged eight weeks old were used to obtain sperm samples, which were suspended in human tubal fluid medium (In Vitro Care, Inc., Frederick, MD, USA). After five minutes of incubation at 37°C, semen quality parameters were evaluated using a Ceros II sperm analysis system (Hamilton Thorne, Beverly, MA, USA) [18, 24].

RNA extraction and real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Testicular total RNA was isolated using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After reverse transcription into complementary DNA (cDNA), samples were analyzed on a real-time PCR platform (Applied Biosystems, Foster City, CA, USA) for quantitative analysis. The housekeeping gene 18S rRNA was used as an

internal reference. The primers for each gene are as follows: *Wsb2*: forward primer, 5'-AGCACGGTAAGCAGATCCAG-3'; reverse primer, 5'-TGCTCCACAGAAAGACCGAC-3'; product length, 123. 18s rRNA: forward primer, 5'-AAACGGCTACCACATCCAAG-3'; reverse primer, 5'-CCTCCAATGGATCCTCGTTA-3'; product length, 155.

Histology

Mice were euthanized by carbon dioxide exposure and then subjected to cervical dislocation before subsequent experiments. Testes were harvested from adult mice of the indicated genotypes (WT, *Wsb1*-gKO and *Wsb1*-sKO) and immersion-fixed in modified Davidson's solution for 48 hours. After stepwise dehydration in graded ethanol, tissues were embedded in paraffin blocks. Five-micrometer sections were prepared, paraffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Histological evaluation performed by light microscopy [25, 26].

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

Apoptosis was detected using a TUNEL assay kit (Vazyme, Nanjing, China) as described previously [27, 28]. Tissue sections were deparaffinized and rehydrated, followed by proteinase K treatment at room temperature for 20 minutes. After 1 hour incubation in equilibration buffer, it was labeled with BrightRed solution at 37°C for another hour. Two phosphate-buffered saline (PBS) washes were followed by counterstaining the nuclei with Hoechst 5 min at room temperature. Slides were then imaged.

Immunofluorescence

Paraffin-embedded tissue sections were deparaffinized and rehydrated, and antigens were retrieved using citrate buffer. Sections were preincubated in 1% (w/v) bovine serum albumin (BSA) to reduce the binding of non-specific antibody [29, 30]. The following antibodies were incubated overnight at 4°C: anti-LIN28 (1:200, Abcam), anti-gamma H2A.X variant histone (γ-H2AX) (1:200, Abcam), peptide nucleic acid (PNA, 1:400, Vector), anti-SRY-box transcription factor 9 (SOX9) (1:200, Millipore), anti-GATA4 (1:200, R&D Systems) and anti-3-hydroxysteroid (3β-HSD) (1:500, Santa Cruz). Following three rinses in PBS, sections were incubated for 1 hour at 37°C with Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, USA). Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI), and imaging was performed using an LSM 800 confocal laser scanning microscope (Zeiss).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, USA). Differences between groups were assessed using one-way analysis of variance (ANOVA). Statistically significant values were considered to be below 0.05.

Results

Expression pattern of WSB1 and WSB2 in human and mouse testes

We performed single-cell data analysis using a public tool (The Male Health Atlas database). The analysis showed that WSB1 expression was highest in differentiated spermatogonia in both humans and mice and was comparatively lower in partial spermatogonial stem cells (SSC), spermatocytes (SPC) and spermatids (SPT). Additionally, WSB2 expression was highest in spermatogonial stem cells in both humans and mice (**Figure 1**).

Generation of Wsb1 reproductive system conditional knockout (Wsb1-cKO) mice

The *Wsb1*-cKO model was generated using CRISPR/Cas9 technology to insert loxp sites flanking exons 5-7. Subsequent Cre-mediated recombination deleted these exons, which was essential for constructing the *Wsb1*-cKO mouse model (**Figure 2A**). Given that WSB1 was expressed as early as the spermatogonia stage but its expression and function in somatic cells remain unclear, we crossed *Wsb1*-floxed mice with *Ddx4*-Cre and *Amh*-Cre transgenic mice, respectively. This strategy yielded *Wsb1*-gKO and *Wsb1*-sKO mice (**Figure 2B**). Successful targeting was confirmed by PCR-based genotyping, validating generation of the *Wsb1*-cKO strain (**Figure 2C**).

Wsb1-cKO mice are fertile

Over a three-month breeding trial, average litter sizes were similar between WT and *Wsb1*-cKO mice (**Figure 3A**). CASA detected no statistically

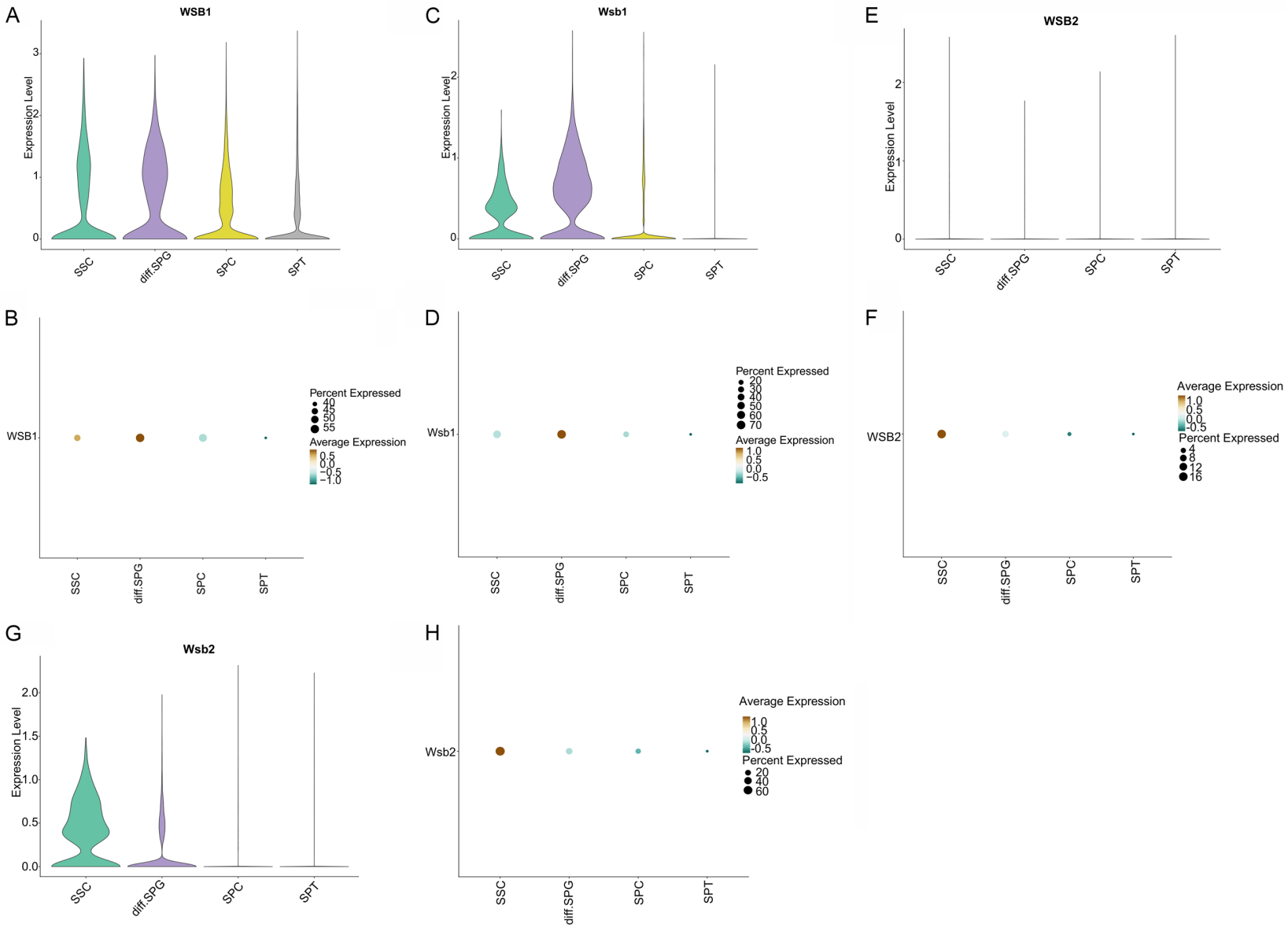

Figure 1. Expression of WSB1 and WSB2 in human and mouse germ cells. A. Bioinformatic analysis of WSB1 expression across human germ cell subtypes. B. Dot plot illustrating WSB1 expression levels in germ cell populations from human testes samples. C. Bioinformatic analysis of *Wsb1* expression across mouse germ cell

subtypes. D. Dot plot illustrating *Wsb1* expression levels in germ cell populations from mouse testis samples. E. Bioinformatic analysis of WSB2 expression across human germ cell subtypes. F. Dot plot illustrating WSB2 expression levels in germ cell populations from human testis samples. G. Bioinformatic analysis of *Wsb2* expression across mouse germ cell subtypes. H. Dot plot illustrating *Wsb2* expression levels in germ cell populations from mouse testis samples. All data were obtained from The Male Health Atlas database.

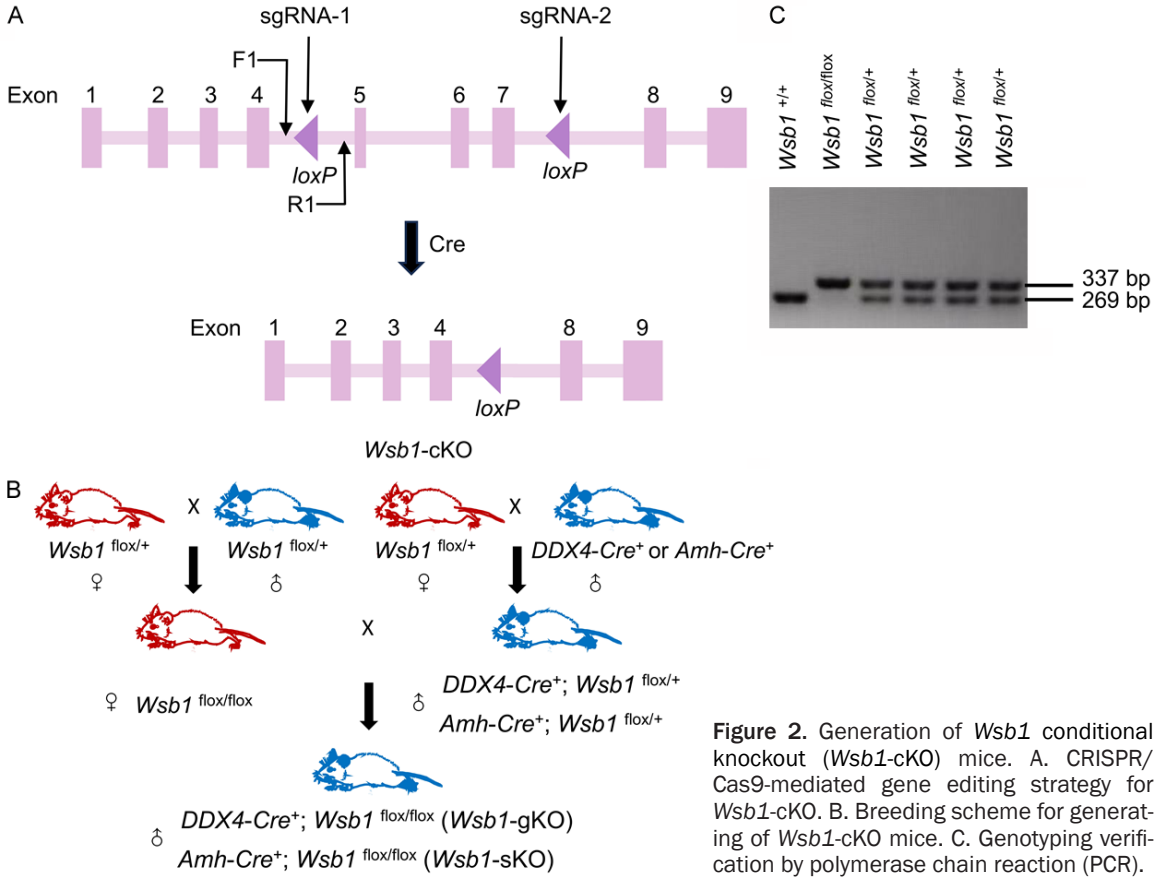

Figure 2. Generation of *Wsb1* conditional knockout (*Wsb1*-cKO) mice. A. CRISPR/Cas9-mediated gene editing strategy for *Wsb1*-cKO. B. Breeding scheme for generating of *Wsb1*-cKO mice. C. Genotyping verification by polymerase chain reaction (PCR).

significant changes in sperm concentration, overall motility, or progressive motility (**Figure 3B-D**). Morphological examination using H&E staining and immunofluorescence, together with quantitative assessment, further showed that sperm structure was indistinguishable among the three genotypes (**Figure 3E-G**).

Wsb1-cKO mice exhibit normal spermatogenesis

H&E staining of testicular sections showed intact seminiferous tubules containing germ cells at all developmental stages in both WT and *Wsb1*-cKO mice (**Figure 4A**). The TUNEL assay showed that the number of apoptotic cells and the percentage of TUNEL-positive tubules were comparable among the three genotypes (**Figure 4B-D**). To further evaluate spermatogenesis, we assessed spermatogonial stem cells (identified by LIN28), spermatocytes (identified by 7-H2AX) and acrosomes (identified by PNA) in WT and *Wsb1*-cKO testes (**Figure 5**). Markers (SOX9, GATA4 and 3β-HSD) were used to evaluate somatic cell populations (**Figure 6**). No differences were observed across these measures (**Figures 5**, **6**). Then, the RNA levels of *Wsb2* in the WT, *Wsb1*-gKO and *Wsb1*-sKO were examined by RT-qPCR. The findings demonstrated that the levels were both upregulated in the *Wsb1*-gKO and *Wsb1*-sKO groups. This indicates that knocking out *Wsb1* triggers some compensatory increase in *Wsb2* (**Figure 7**). Collectively, these results indicate that WSB1 deficiency does not interfere with spermatogenic development in mice.

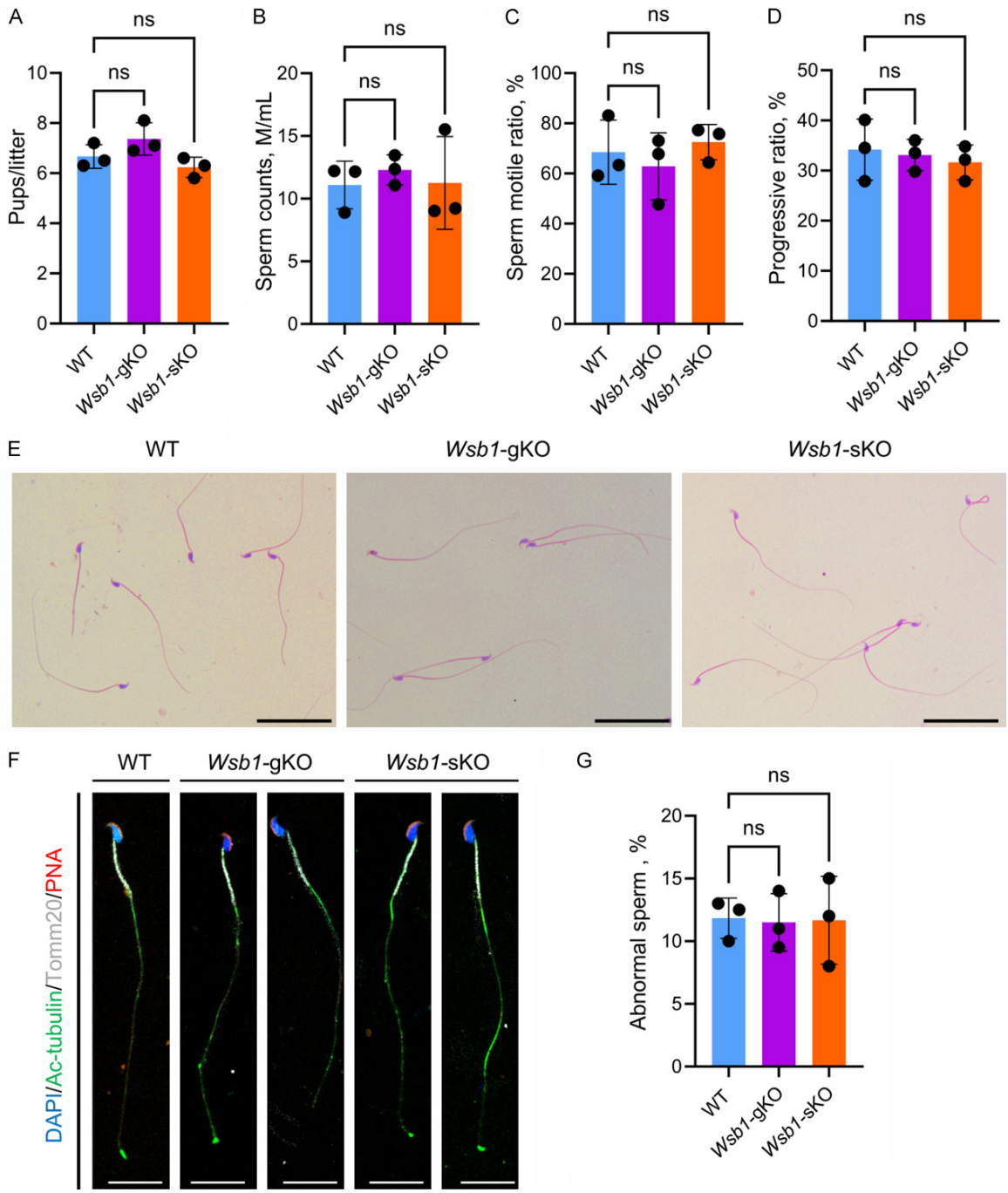

Figure 3. *Wsb1*-cKO mice exhibit normal fertility. (A) Fertility assessment of WT, *Wsb1* germ cell-specific (*Wsb1*-gKO) and *Wsb1* Sertoli cell-specific (*Wsb1*-sKO) mice. n = 3 per group. (B-D) Computer-assisted sperm analysis (CASA) of sperm count (B), total motility (C), and progressive motility (D) in WT, *Wsb1*-gKO and *Wsb1*-sKO mice. M, million. n = 3 per group. ns, not significant. (E) Hematoxylin and eosin (H&E) staining of spermatozoa from the cauda epididymis of WT, *Wsb1*-gKO and *Wsb1*-sKO mice. Scale bar = 50 μm. (F) Immunofluorescence staining of spermatozoa from the cauda epididymis of WT, *Wsb1*-gKO and *Wsb1*-sKO mice. Tomm20 serves as a mitochondrial sheath marker, PNA as an acrosome marker, and ac-tubulin as a flagellum marker. Scale bar = 20 μm. (G) Quantitative analysis of the immunofluorescence staining results presented in (F). n = 3 per group. ns, not significant.

Discussion

In mammals, spermatogenesis is a complex process that requires tight regulation. This gradual program of cellular growth and differentiation leading to sperm formation involves multiple steps: spermatogonia (mitotic precursors), spermatocytes (meiotic divisions), and haploid

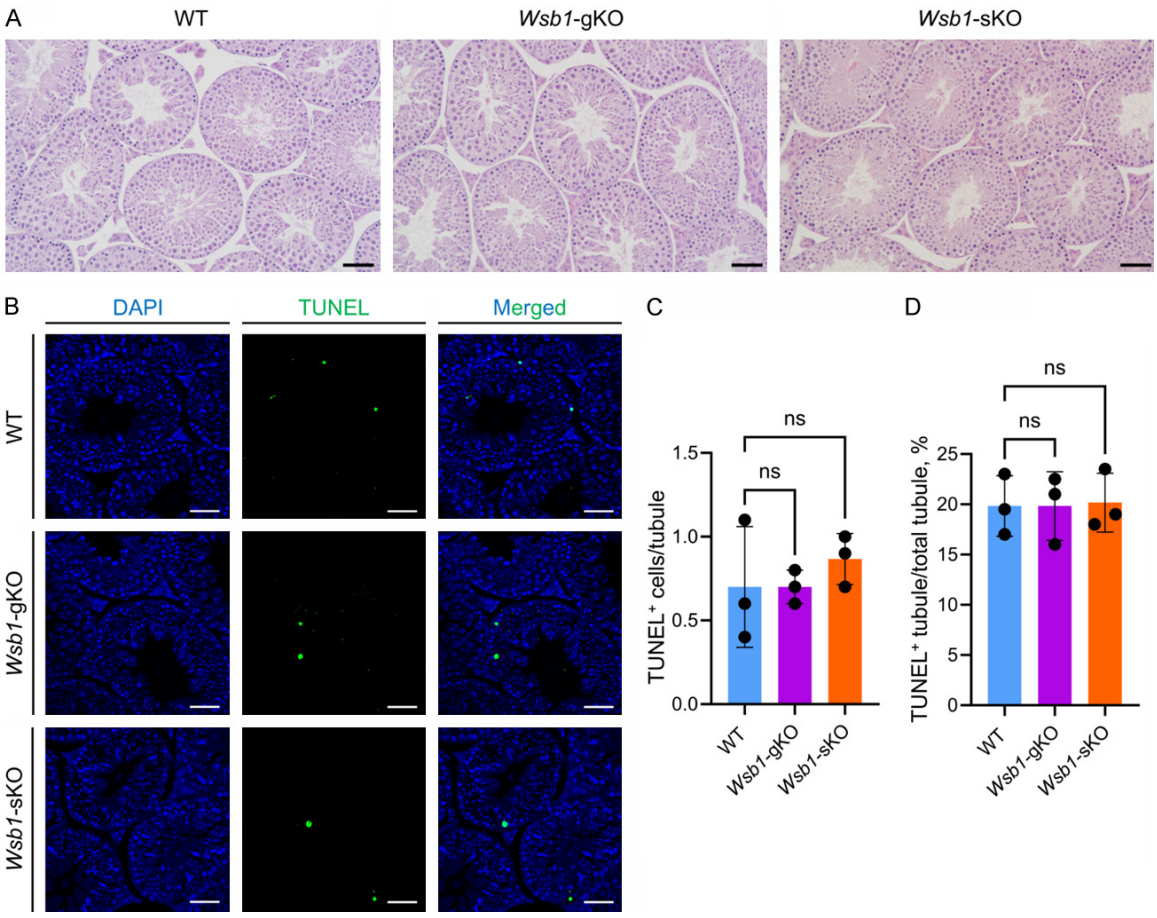

Figure 4. Testicular histomorphology and terminal deoxynucleotidyl transferase - dUTP nick-end labeling (TUNEL) analysis of WT, *Wsb1*-gKO and *Wsb1*-sKO mice. A. H&E staining of testicular tissue sections from WT, *Wsb1*-gKO and *Wsb1*-sKO mice. Scale bar = 50 μm. B. Detection of apoptotic cells via TUNEL assay. Scale bar = 50 μm. C. Quantification of TUNEL-positive (TUNEL$^+$) cells (a marker of apoptosis) in testes from WT, *Wsb1*-gKO and *Wsb1*-sKO mice using the TUNEL assay. n = 3 per group. ns, not significant. D. Quantification of the ratio of TUNEL$^+$ seminiferous tubules to total seminiferous tubules (expressed as a percentage) in testes of WT, *Wsb1*-gKO and *Wsb1*-sKO mice via the TUNEL assay. n = 3 per group. ns, not significant.

round spermatids (rSD), which elongate to become elongated spermatids (eSD) [31]. Ubiquitination is an important regulatory process in this cascade and is coordinated by E1, E2 and E3 enzymes [32]. Among these, E3 ligases are distinguished by their substrate specificity and their temporal and spatial regulation in testicular tissue, making them key determinants of the regulatory network [33-35]. These include WSB1, an E3 ubiquitin ligase capable of recognizing numerous substrates and facilitating their ubiquitination, thereby promoting proteasomal degradation [36]. WSB1 has been reported to promote tumor metastasis [17, 37, 38], inhibit glycolytic metabolism in liver cancer cells [39], and contribute to the development of cancer and other functions [14, 15, 40, 41]. Nevertheless, its function in the male reproductive system has yet to be elucidated.

Bioinformatic analysis revealed elevated WSB1 expression in differentiating spermatogonia in both human and mouse testes. To explore its role in spermatogenesis, we established a *Wsb1*-cKO model using CRISPR/Cas9 technology. The findings did not support to our original hypothesis: *Wsb1*-cKO male mice showed normal fertility, with litter sizes statistically indistinguishable from those of WT controls. Testis and sperm morphology, the structure of the seminiferous tubule structure, and sperm quality (including quantity, vitality and forward motility) were unaffected. No significant abnormalities were observed in apoptosis, germ cell

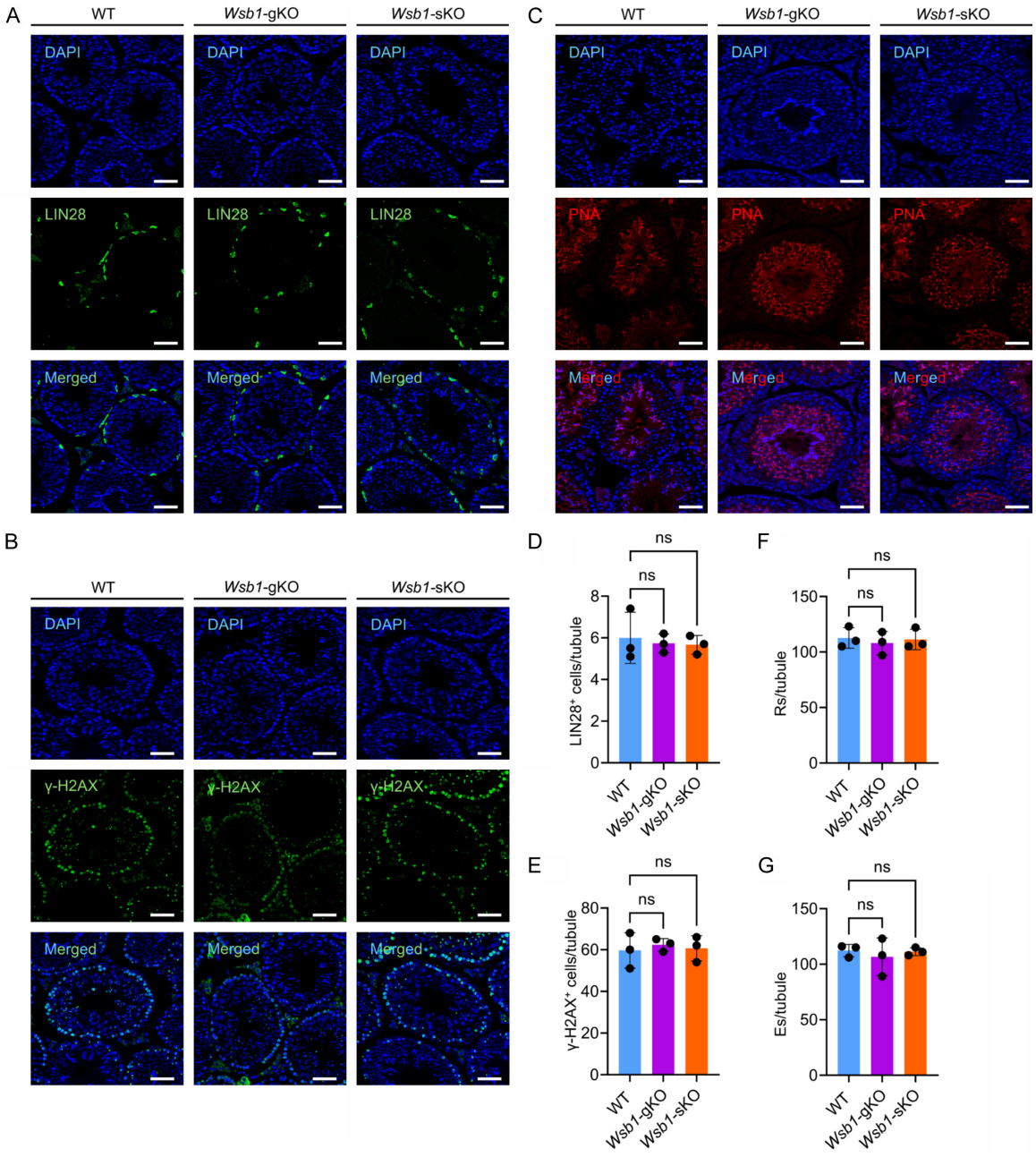

Figure 5. Immunostaining of stage-specific markers of spermatogenesis in WT, *Wsb1*-gKO and *Wsb1*-sKO mice. A. Immunostaining of LIN28 in spermatogonial stem cells from WT, *Wsb1*-gKO and *Wsb1*-sKO mice. B. Immunostaining of γ-H2AX in spermatocytes from WT, *Wsb1*-gKO and *Wsb1*-sKO mice. C. Immunostaining of PNA in acrosomes from WT, *Wsb1*-gKO and *Wsb1*-sKO mice. D-G. Quantification showed no significant differences among genotypes. n = 3 per group; ns, not significant.

markers, or somatic cell markers. The absence of WSB1 does not affect spermatogenesis, possibly due to compensation by WSB family members or paralogous genes with overlapping functions [42]. A comparable compensatory response was noted in our earlier investigations. Specifically, knockout of *Ubqln3* triggered a marked increase in *Ubqln1*, *Ubqln2*, and *Ubqln4* expressions. Similarly, deletion of *Asb12* in mice induced upregulation of other members of the Asb protein family [42]. Among WD-40 repeat-containing proteins harboring a SOCS box, two members - WSB1 and WSB2 - have been identified [43]. WSB1 and

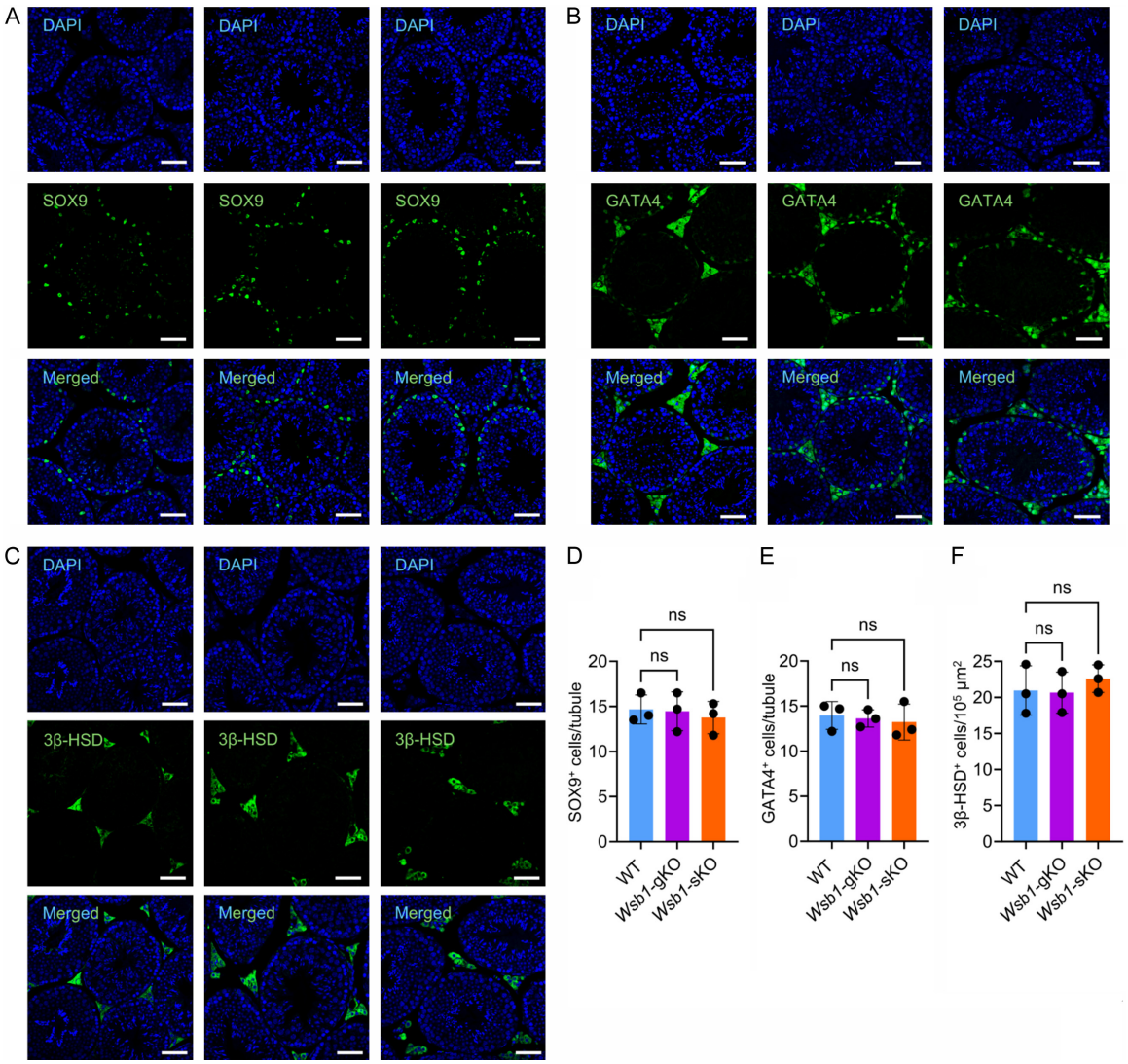

Figure 6. Immunostaining of testicular somatic cell-specific markers in WT, *Wsb1*-gKO and *Wsb1*-sKO mice. A. Immunostaining of SOX9 in testicular Sertoli cells from WT, *Wsb1*-gKO and *Wsb1*-sKO mice. B. Immunostaining of GATA4 in Sertoli cells from WT, *Wsb1*-gKO and *Wsb1*-sKO mice. C. Immunostaining of 3β-HSD in Leydig cells of WT, *Wsb1*-gKO and *Wsb1*-sKO mice. D-F. Quantification showed no significant differences among genotypes. n = 3 per group; ns, not significant.

WSB2 share 65% similarity at the protein level and thus may have some functional homology [44]. It is worth noting that WSB2 has been reported to be expressed in Sertoli cells, spermatogonia and spermatocytes in mice. This may suggest that it plays a specific role in male reproductive development [36, 44]. Moving forward, our investigations will extend to other members of the SOCS box family - particularly those within the WSB subfamily - to assess whether any of these proteins are indispensable for spermatogenesis.

In summary, this study demonstrates that despite prominent WSB1 expression in differentiated spermatogonia in both humans and mice, WSB1 does not influence male reproductive capacity in mice. These observations provide a basis for subsequent investigations.

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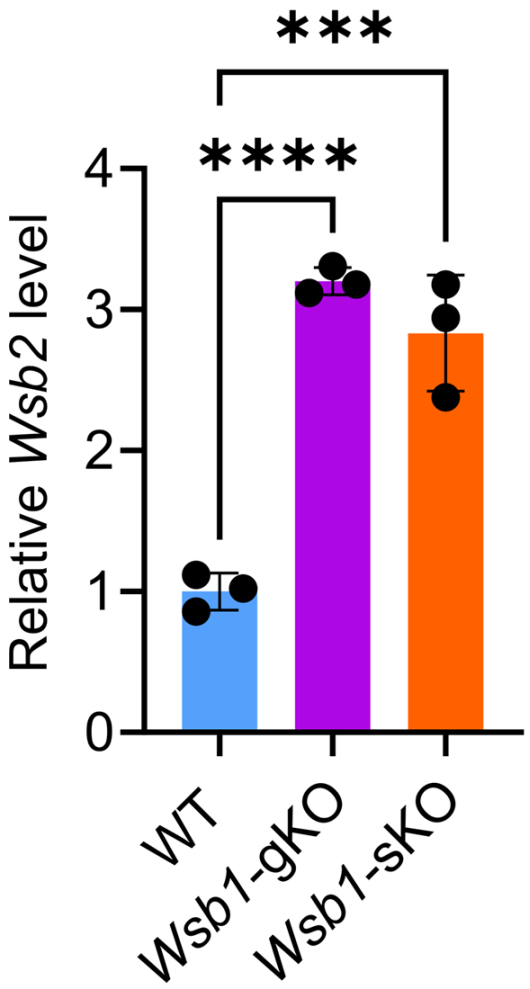

Figure 7. *Wsb1*-cKO mice exhibit normal spermatogenesis. Expression levels of *Wsb2* in testes from wild-type (WT), *Wsb1* germ cell-specific (*Wsb1*-gKO) and *Wsb1* Sertoli cell-specific (*Wsb1*-sKO) mice were analyzed by RT-qPCR. ***, P<0.001; ****, P<0.0001. These results show that *Wsb2* expression is upregulated in *Wsb1*-gKO and *Wsb1*-cKO.

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Disclosure of conflict of interest

None.

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