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this work.Specific deficiency of *Plzf* paralog,
Zbtb20, in Sertoli cells does not affect
spermatogenesis and fertility in miceXiaohua Jiang^{1*}, Huan Zhang^{1*}, Shi Yin¹, Yuanwei Zhang¹, Weimei Yang¹, Wei Zheng¹, Liu Wang¹,
Zheng Wang¹, Ihtisham Bukhari¹, Howard J. Cooke^{1,2}, Furhan Iqbal¹ & Qinghua Shi¹¹Laboratory of Molecular and Cell Genetics, CAS Key Laboratory of Innate Immunity and Chronic Disease, CAS Hefei Institutes of Physical Science, Hefei National Laboratory for Physical Sciences at Microscale, School of Life Sciences, University of Science & Technology of China, Hefei, China, ²MRC Human Genetics Unit and Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, United Kingdom.

Zbtb20 is a POK family transcription factor and primarily functions through its conserved C2H2 Krüppel type zinc finger and BTB/POZ domains. The present study was designed to define the function of the *Zbtb20*, in vivo, during mouse spermatogenesis. Immunohistochemical studies revealed that ZBTB20 protein was localized specifically in the nuclei of Sertoli cells in seminiferous tubules. To investigate its role during spermatogenesis, we crossed *Amh-Cre* transgenic mice with *Zbtb20* floxp mice to generate conditionally knockout mice (cKO) in which *Zbtb20* was specifically deleted in Sertoli cells. The cKO mice were fertile and did not show any detectable abnormalities in spermatogenesis. Taken together, though specific deletion of transcription factor *Zbtb20* in Sertoli cells has no apparent influence on spermatogenesis, its specific localization in Sertoli cells makes *Zbtb20* a useful marker for the identification of Sertoli cells in seminiferous tubules.

Transcription factors are regulatory proteins that are mainly involved in transcription activation (or rarely in inhibition) by binding to DNA sequences^{1,2}. They are considered to be the most important regulators in gene expression^{2,3}. Transcription factors work in germ and somatic cells (especially in Sertoli cells) to control spermatogenesis in mammals^{4,5}. So far, only relatively few transcription factors have been identified in spermatogenesis. Studies of all the transcription factors functioning in spermatogenesis indicate that transcription factors expressing in germ cells are more likely to function in specifically developmental stages of germ cells, while transcription factors expressing in Sertoli cells are often required during all the stages of gametogenesis in seminiferous tubules^{4,5}. ZBTB [zinc finger and BTB (Broad complex, Tramtrack, and Bric-à-brac)] proteins, also known as Poxvirus and zinc finger (POZ) and Krüppel-type (POK) proteins, are a family of transcription factors that play critical roles in development, differentiation and oncogenesis^{6–12}. POK/ZBTB proteins exhibit C-terminal zinc fingers and an N-terminal BTB/POZ domain. The former recognizes and binds to specific DNA sequences, while the BTB/POZ domain mediates homodimerization and/or heterodimerization and interacts with other proteins^{13–15}. In the mouse genome, 44 genes encode different POK/ZBTB proteins and a few of them have been reported to be essential for spermatogenesis. For example, *Zbtb16*, also known as *Plzf* (promyelocytic leukemia zinc finger), expresses in gonocytes and undifferentiated spermatogonia and it is the first gene reported to be essential for spermatogonial stem cell self-renewal in mouse testis^{9,16}.

Besides *Zbtb16*, functional roles of other POK/ZBTB genes, such as *Zbtb20*, remain unclear during spermatogenesis. *Zbtb20* overexpression in transgenic mice under the control of a forebrain-specific promoter (D6 promoter) leads to the formation of hippocampus-like neuronal structures and behavioral abnormalities¹⁷, which indicates that *Zbtb20* can control the organ development and differentiation. Another study shows that *Zbtb20* null mice were infertile, however, the underlying mechanisms remained unknown due to pleiotropic defects such as growth retardation, prolonged hypoglycemia and postnatal lethality before 12 weeks of age of these mice¹⁸.

The aim of this study was to define the function of the *Zbtb20* in vivo during spermatogenesis of mice. We found that ZBTB20 specifically expressed in the nuclei of Sertoli cells, making it a useful marker for the identification of Sertoli cells in seminiferous tubes. However, mice homozygous for a null mutation of *Zbtb20*, specifically in Sertoli cells, do not exhibit any detectable abnormalities in spermatogenesis and fertility, indicating that *Zbtb20* is dispensable for spermatogenesis and male fertility in mice.



Methods

Experimental animals. *Zbtb20*^{fl/fl} mice homozygous for a floxop allele of *Zbtb20*, *Amh-Cre* mice and *mT/mG* reporter mice were used in the present study and were described previously^{19–21}. For fertility testing, 8 to 12-week old *Zbtb20*^{fl/fl} (control) and *Amh-Cre*; *Zbtb20*^{fl/fl} (cKO) males were separately housed with wild-type C57BL/6 females for 6 months. The size of litters generated by these males was recorded. All mice were housed under controlled photoperiod conditions (lights on 08:00–20:00) and supplied with food and ddH₂O *ad libitum*. All experimental protocols and animal handling procedures were conducted in accordance with the guidelines and procedures approved by Institutional Animal Care Committee of University of Science and Technology of China.

Sertoli cell isolation. A modified previously described method was used to isolate Sertoli cells from the testes of 21-dpp mice^{22,23}. Briefly, testes were decapsulated under a dissection microscope. The seminiferous tubules were pooled and washed with phosphate-buffered saline (PBS) thrice and incubated with 2 mg/ml collagenase I (Sigma, C7661, MO, USA) and 0.5 mg/ml DNase I (Sigma, D4527) in DMEM: F12 (HyClone, SH40007-13, UT, USA) for 15 minutes at 37°C on a shaker, then washed twice with DMEM: F12 and further digested with 2 mg/ml collagenase I, 0.5 mg/ml DNase I and 1 mg/ml hyaluronidase (Sigma, H3506) for 15 minutes at 37°C. The tubules were allowed to settle and were then washed twice with DMEM: F12 before being digested with 2 mg/ml collagenase I, 0.5 mg/ml DNase I, 2 mg/ml hyaluronidase and 1 mg/ml trypsin (Sigma, T8003) for 30 minutes at 37°C. These dispersed cells were then washed twice with DMEM: F12 medium and placed into culture dishes in DMEM: F12 containing 10% fetal bovine serum (HyClone, SV30087-02) and were incubated at 37°C with 5% CO₂. After 1 day of culturing, the solution was removed, and the cells were treated with a hypotonic solution (20 mM Tris, pH 7.4) for 1 minutes for the removal of remaining germ cells, if any, and harvested for further analyses.

Sperm counting. The unilateral epididymides and vasa deferentia were removed from 70-dpp control and cKO mice, incised several times and incubated in 1 ml buffer containing 75 mM NaCl, 24 mM EDTA and 0.4% bovine serum albumin (Sigma, A2058) at 37°C with 5% CO₂ for 30 minutes to allow sperm release from the epididymides. Sperm were collected after a nylon-mesh filtration and counted with a haemocytometer.

Histological examination and immunohistochemistry. The control and cKO mice were euthanized by cervical dislocation and testes were immediately fixed in Bouin's solution for hematoxylin and eosin (H & E) staining or in 4% formaldehyde in PBS for immunohistochemistry. For immunohistochemistry, anti-SOX9 (1 : 100; Millipore, AB5535, MA, USA), anti-3β-HSD (1 : 100; Santa Cruz, SC-30820, CA, USA), anti-α-ACTIN2 (1 : 100; Abcam, AB68167) or anti-DDX4 (1 : 100; Abcam, AB13840, Cambridge, UK) were co-incubated with anti-ZBTB20 (1 : 500, donated by Prof. Weiping Zhang) respectively and the ZBTB20 signals were detected using a tyramine amplification kit (Invitrogen, T20948, MA, USA) according to the manufacturer's instructions. All images were captured using a microscope (Nikon Eclipse 80i microscope) equipped with a digital camera (Nikon DS-Ri1 for H & E staining or Hamamatsu C4742-80 for immunohistochemistry).

Real-time PCR. RNA isolation and real-time PCR were performed as previously described²⁴. All PCR primers used are listed in Supplementary Table 1. For real-time PCR analysis, C_T values of samples were normalized to the corresponding C_T values of *Gapdh*. Quantification of the fold change in gene expression was determined by the comparative C_T method.

Western blot. Protein samples from Sertoli cells were prepared in lysis buffer (50 mM Tris/HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (Roche). Western blot was carried out as described previously²⁴ and primary antibodies against ZBTB20 (1 : 500) and GAPDH (1 : 1000; Millipore, MAB374, MA, USA) were used to detect the protein bands.

Statistical analysis. Protein and mRNA levels, testis weights, testis/body weight ratios, sperm number and litter size between control and cKO mice were analyzed by using the Student's *t*-test. Results are presented as mean ± SEM. Statistical significance was set at *P* < 0.05.

Results

Expression of ZBTB20 in mouse testes. In seminiferous tubules of adult testes (70-dpp) (Fig. 1A), ZBTB20 protein was detected only in the nuclei of Sertoli cells, as it was co-localized with SOX9, a Sertoli cell nucleus specific marker (Fig. 1A) and it was not detected in germ cells as ZBTB20 staining was absent in the cells stained positive by DDX4, a germ cell specific marker (Fig. 1B). In order to more precisely evaluate ZBTB20 expression in Sertoli cells during testicular development, we performed immunohistological analysis of ZBTB20 in testes during early developmental stages (at 7- and 14-dpp) and observed that ZBTB20 was expressed at all the studied

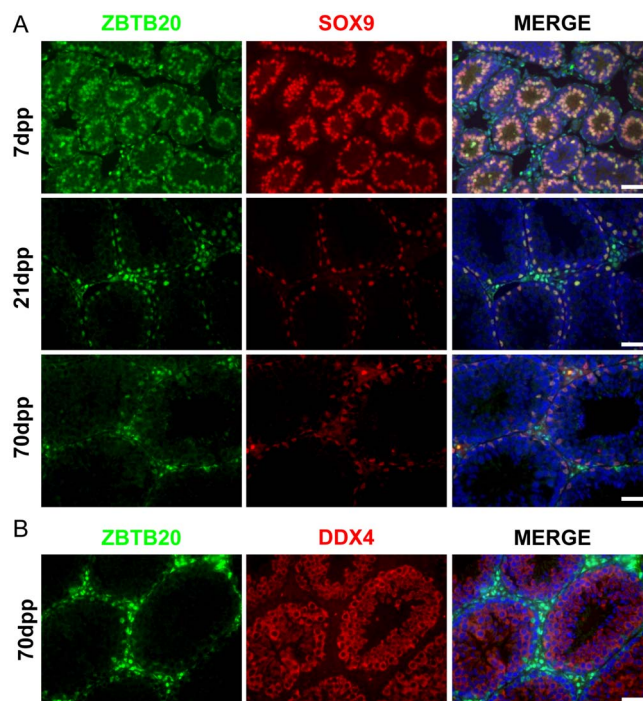


Figure 1 | ZBTB20 protein localization in mouse testes. (A). Cellular localization of ZBTB20 and SOX9 in testes at indicated ages. Localization of ZBTB20 (green) and SOX9 (red, a Sertoli cell marker) in sections of testes from 7-, 14- and 70-dpp mice was displayed by co-immunofluorescent staining for these proteins, showing that in seminiferous tubules, ZBTB20 just localized in the nuclei of Sertoli cells of all developmental stages examined. The images shown are representative results of experiments that were repeated thrice using samples from different sets of testes and yielded similar results. *Scale bars*, 50 μm. (B). Cellular localization of ZBTB20 and DDX4 in adult testes. Localization of ZBTB20 (green) and DDX4 (red, a germ cell marker) was observed in sections of testes from 70-dpp mice by immunofluorescence. In seminiferous tubules, opposite staining of ZBTB20 and DDX4 revealed that ZBTB20 did not localize in germ cells. The data shown are representative images of experiments that were repeated thrice using samples from different sets of testes and yielded similar results. *Scale bars*, 50 μm.

stages in Sertoli cells (Fig. 1A). Besides in Sertoli cells, ZBTB20 was also found in interstitial regions between seminiferous tubules in testis (Fig. 1A). Co-immunostaining of ZBTB20 with 3β-HSD, a Leydig cell cytoplasm specific marker, revealed that ZBTB20 was localized in Leydig cells (Supplementary Fig. S1A). Additionally, co-immunostaining of ZBTB20 with α-ACTIN2, a peritubular myoid cell marker, indicated that ZBTB20 also localized in some peritubular myoid cells (Supplementary Fig. S1B).

Zbtb20 deletion in Sertoli cells. In order to assess the *Zbtb20* functions in Sertoli cells during spermatogenesis, we generated mice in which the *Zbtb20* gene was specifically disrupted in testicular Sertoli cells. We combined a conditional floxop *Zbtb20* allele with the *Cre*-expressing line *Amh-Cre* (Fig. 2A). To study the *Cre* recombinase activity, *Amh-cre* mice were crossed with fluorescence reporter mice (*mT/mG*), in which *Amh-Cre* mediated recombination resulted in excision of the RFP cassette and expression of the GFP reporter²¹. Based on the analysis of *Amh-Cre* recombined *mT/mG* reporter mice, we confirmed that the *Amh-Cre* mice had strong recombinase activities in Sertoli cells (Supplementary Fig. S2). *Zbtb20* deletion efficiency in Sertoli cells was analyzed by detecting the *Zbtb20* mRNA and protein levels in the isolated Sertoli cells (Supplementary Fig. S3). We found that the

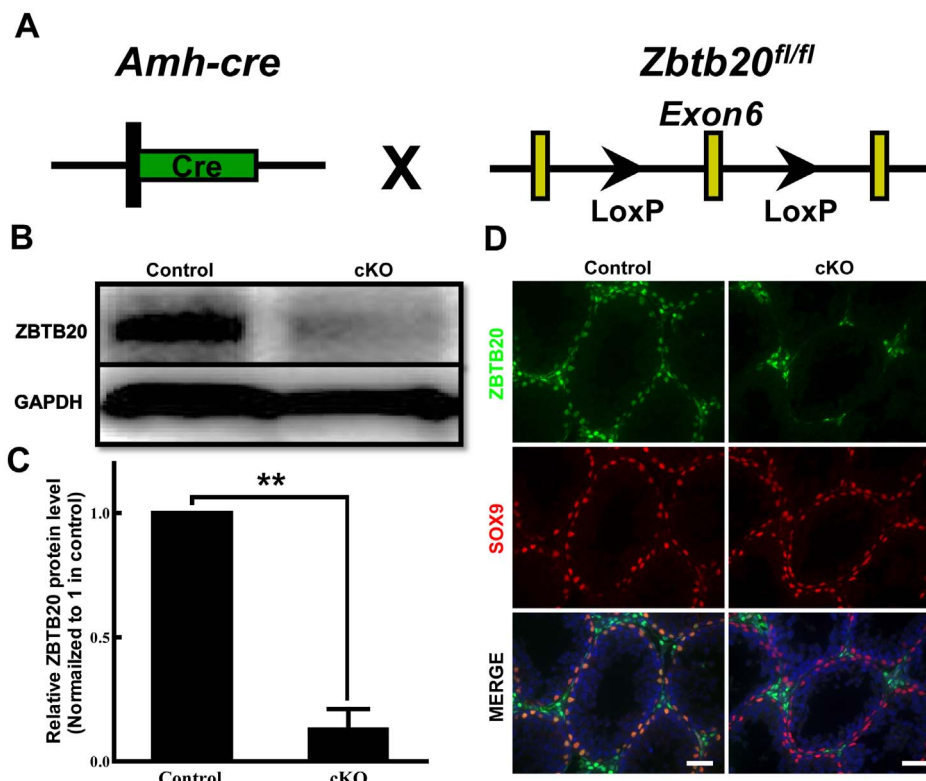


Figure 2 | Conditional deletion of *Zbtb20* in Sertoli cells. (A). Hybrid scheme used to develop *Zbtb20* cKO mice. (B). Western blot analysis of ZBTB20 in isolated Sertoli cells from 21-dpp control and cKO mice. GAPDH is served as a protein loading control. The image shown is a representative result from three independent experiments. (C). Quantitative results of Western blot experiments shown in (B). The ZBTB20 protein level was normalized and plotted against GAPDH, and was arbitrarily set as 1 in controls. Data are presented as mean \pm SEM from three independent experiments. ** $P < 0.01$, Student's *t*-test. (D). Cellular localization of ZBTB20 and SOX9 in sections of control and cKO testes. Localization of ZBTB20 (green) and SOX9 (red) in sections of testes of 21-dpp mice were shown by immunofluorescence. ZBTB20 localization was lost in cKO Sertoli cells. The shown are representative images from experiments that were repeated thrice using samples from different sets of testes and yielded similar results. Scale bars, 50 μ m.

Zbtb20 disruption resulted in a drastic reduction in *Zbtb20* mRNA and protein levels in the cKO Sertoli cells (Supplementary Fig. S4 and Fig. 2B–C). As shown in Fig. 2D, immunofluorescent analysis of the cKO testes revealed that the localization of ZBTB20 in Sertoli cell was abolished (Fig. 2D) without affecting its localization in Leydig cells (Supplementary Fig. S5), which further confirms the efficient and specific disruption of *Zbtb20* in Sertoli cells.

Normal spermatogenesis in *Zbtb20* cKO mice. After successfully generating a Sertoli cell *Zbtb20* knockout mouse model, we investigated spermatogenesis in the cKO mice. The testes from control and cKO littermates of different ages were analysed. We did not find any significant differences in testis morphology, weight and testis/body weight ratio between the two groups (Fig. 3A–B; Supplementary Fig. S6). As shown in Fig. 3C, both cKO and control testes exhibited typical seminiferous tubule morphology with all stages of spermatogenic cells (from spermatogonia to spermatozoa), suggesting that spermatogenesis was normal in cKO mice. We also analyzed the sperm in epididymides and found that cKO and control mice produced similar number of sperm (Fig. 3C–D). Finally, the breeding performance of the cKO males was found similar to that of control males as litters produced by mating cKO males with wild type females showed a size similar to those produced by control males (Fig. 3E). Taken together, these results indicated that *Zbtb20* conditional knockout in Sertoli cells does not affect spermatogenesis or fertility of mice.

Discussion

Our present study describes the expression of transcription factor *Zbtb20* in mouse testis. We have observed that ZBTB20 protein was

specifically localized in the nuclei of Sertoli cells locating within seminiferous tubules along with Leydig and some peritubular myoid cells present between seminiferous tubules in mouse testes. As compared to its paralogue gene, *Zbtb16*, which is restricted to gonocytes and undifferentiated spermatogonia^{9,16}, *Zbtb20* was specifically expressed in testicular somatic cells (Fig. 1A–B; Supplementary Fig. S1). In the interstitial compartment Leydig and some peritubular myoid cells showed a positive ZBTB20 staining (Supplementary Fig. S1). In contrast, all Sertoli cells showed a constant and intense expression of *Zbtb20* in all seminiferous tubules independent of the age of animals, which makes it a useful molecular marker for the identification of Sertoli cells (Fig. 1A–B). This expression pattern was in agreement with the expression of previously described Sertoli cell transcriptional factors, such as SOX9 and GATA4, which have been shown to play essential roles in spermatogenesis^{25–32}.

To define the function of *Zbtb20* in Sertoli cells, we generated mice with the *Zbtb20* being specifically disrupted in these cells by crossing *Amh-Cre* transgenic mice with *Zbtb20* floxed mice (Fig. 2A). Note that, as the *Amh-Cre* transgene is expressed from embryonic day 14.5 onwards²⁰, excision of *Zbtb20* occurs long before the onset of spermatogenesis at 5-dpp³³. Consistently, based on the *Amh-Cre* recombined *mT/mG* reporter mice, we confirmed that *Amh-Cre* mice had strong recombinase activity in Sertoli cells (Supplementary Fig. S2). In the isolated Sertoli cells, we observed that the *Zbtb20* deletion using *Amh-Cre* resulted in a drastic reduction of *Zbtb20* mRNA and protein level in the Sertoli cells of cKO mice (Fig. 2B–C; Supplementary Fig. S3–4). Furthermore, the Sertoli cell expression pattern of ZBTB20 was abolished in the cKO testes based on immunofluorescence analysis, further confirming efficient deletion of *Zbtb20* in these cells (Fig. 2D and Supplementary Fig. S5).

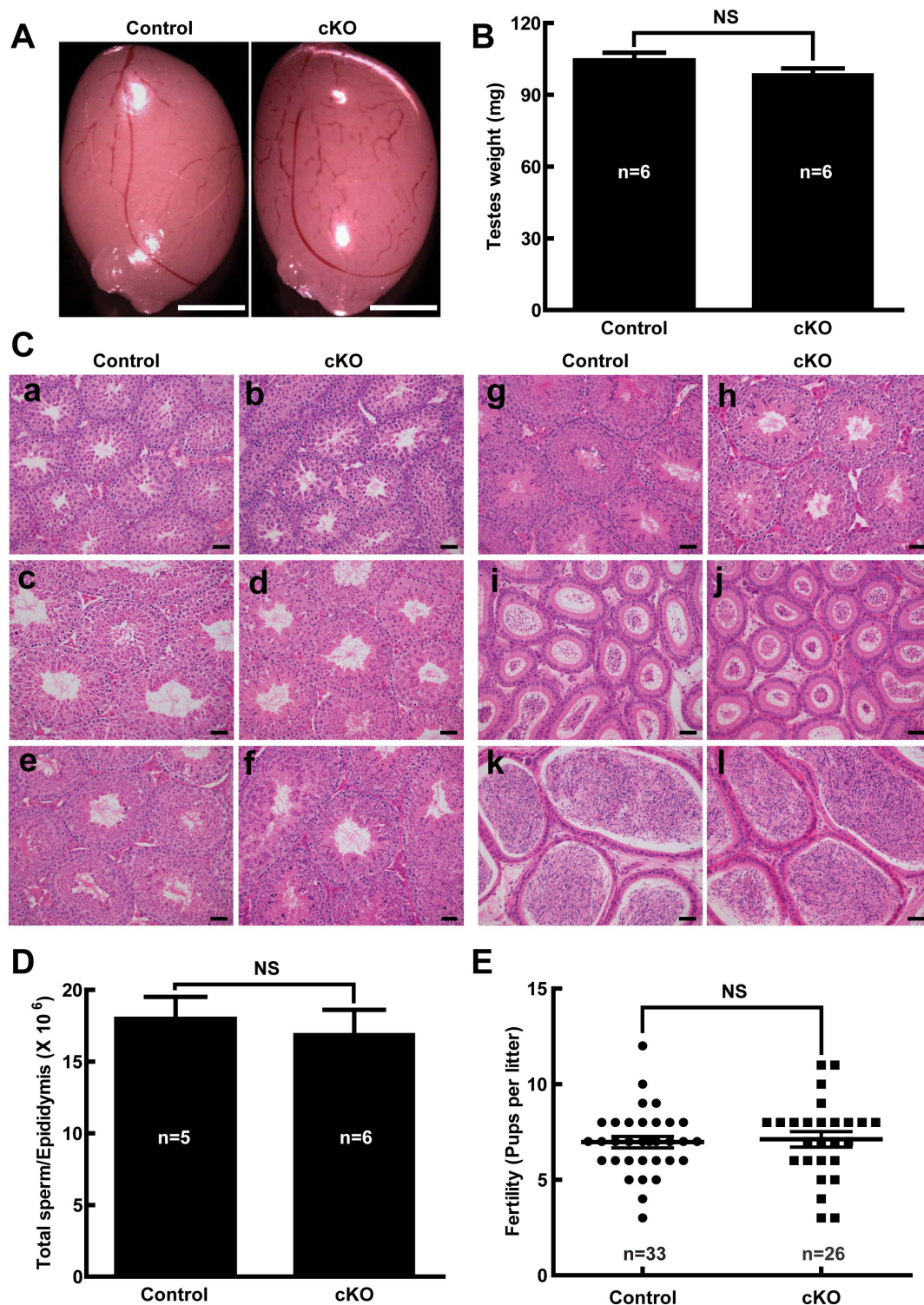


Figure 3 | Normal spermatogenesis in cKO mouse testes. (A). Representative images of testes from 70-dpp control and cKO mice. Scale bars, 2 mm. (B). Mean testis weight of 70-dpp control and cKO mice. n, the number of animals. Error bars represent SEM. (C). H&E staining of testes and epididymides from control and cKO mice at indicated ages. a–h: Testes from control and cKO mice of age at 21-(a, b), 42-(c, d), 70-(e, f) and 140-(g, h) dpp. i–l: Epididymides caput (i, j) and cauda (k, l) from 70-dpp control (i, k) and cKO mice (j, l). Scale bars, 50 μ m. (D). Total sperm per epididymis from 70-dpp control and cKO mice. n, the number of animals. Error bars represent SEM. (E). The number of pups per litter from control and cKO mice. Data shown are from 6 control or 5 cKO males. Dots and squares represent the number of pups of each litter for control and cKO mice, respectively. n, the number of litters. Error bars represent SEM.

However, the phenotype of the *Zbtb20* deficient mice indicates that *Zbtb20* in Sertoli cells was not required for spermatogenesis (Fig. 3A–E; Supplementary Fig. S6).

The lack of phenotype in mice that *Zbtb20* was specifically disrupted in Sertoli cells may result from functional redundancy in the *Zbtb* family. In this scenario, expression of *Zbtb20* target genes in



Zbtb20 null Sertoli cells would be regulated by other *Zbtb* factors, such as *Zbtb32*, *Zbtb37* and *Zbtb44*, which are highly expressed in testes³⁴. We thus hypothesize that some of the unidentified ZBTB proteins may have compensated for the loss of *Zbtb20* function in Sertoli cells. ZBTB20 was also expressed in Leydig and peritubular myoid cells, however, the significance of this protein in these cells remains unclear. Thus, future studies on Leydig- or peritubular myoid cell-specific knockout animals for *Zbtb20* may further shed light on the role of this transcription factor in the testes.

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Author contributions

Conceived and designed the experiments: Q.S., X.J., H.Z. and W.Y. Performed the experiments: X.J., H.Z., S.Y., Y.Z., W.Y., W.Z., L.W. and Z.W. Analyzed the data: X.J., S.Y. and W.Y. Paper wrote up: Q.S. and X.J. Modification of the manuscript: I.B. F.I. and H.J.C.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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