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Research Article

Sertoli cells require TDP-43 to support spermatogenesis†

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Abstract

TAR DNA binding protein of 43 kD (TDP-43) is an evolutionarily conserved, ubiquitously expressed transcription factor and RNA-binding protein with major human health relevance. TDP-43 is present in Sertoli and germ cells of the testis and is aberrantly expressed in the sperm of infertile men. Sertoli cells play a key role in spermatogenesis by offering physical and nutritional support to male germ cells. The current study investigated the requirement of TDP-43 in Sertoli cells. Conditional knockout (cKO) of TDP-43 in mouse Sertoli cells caused failure of spermatogenesis and male subfertility. The cKO mice showed decreased testis weight, and low sperm count. Testis showed loss of germ cell layers, presence of vacuoles, and sloughing of round spermatids, suggesting loss of contact with Sertoli cells. Using a biotin tracer, we found that the blood-testis barrier (BTB) was disrupted as early as postnatal day 24 and worsened in adult cKO mice. We noted aberrant expression of the junction proteins connexin-43 (gap junction) and N-cadherin (ectoplasmic specialization). Oil Red O staining showed a decrease in lipid droplets (phagocytic function) in tubule cross-sections, Sertoli cells cytoplasm, and in the lumen of seminiferous tubules of cKO mice. Finally, qRT-PCR showed upregulation of genes involved in the formation and/or maintenance of Sertoli cell junctions as well as in the phagocytic pathway. Sertoli cells require TDP-43 for germ cell attachment, formation and maintenance of BTB, and phagocytic function, thus indicating an essential role for TDP-43 in the maintenance of spermatogenesis.

Summary Sentence
Sertoli cells require the DNA/RNA binding protein TDP-43 to support spermatogenesis. In the absence of TDP-43, Sertoli cells failed to maintain germ cell attachment and the blood-testis barrier was compromised.

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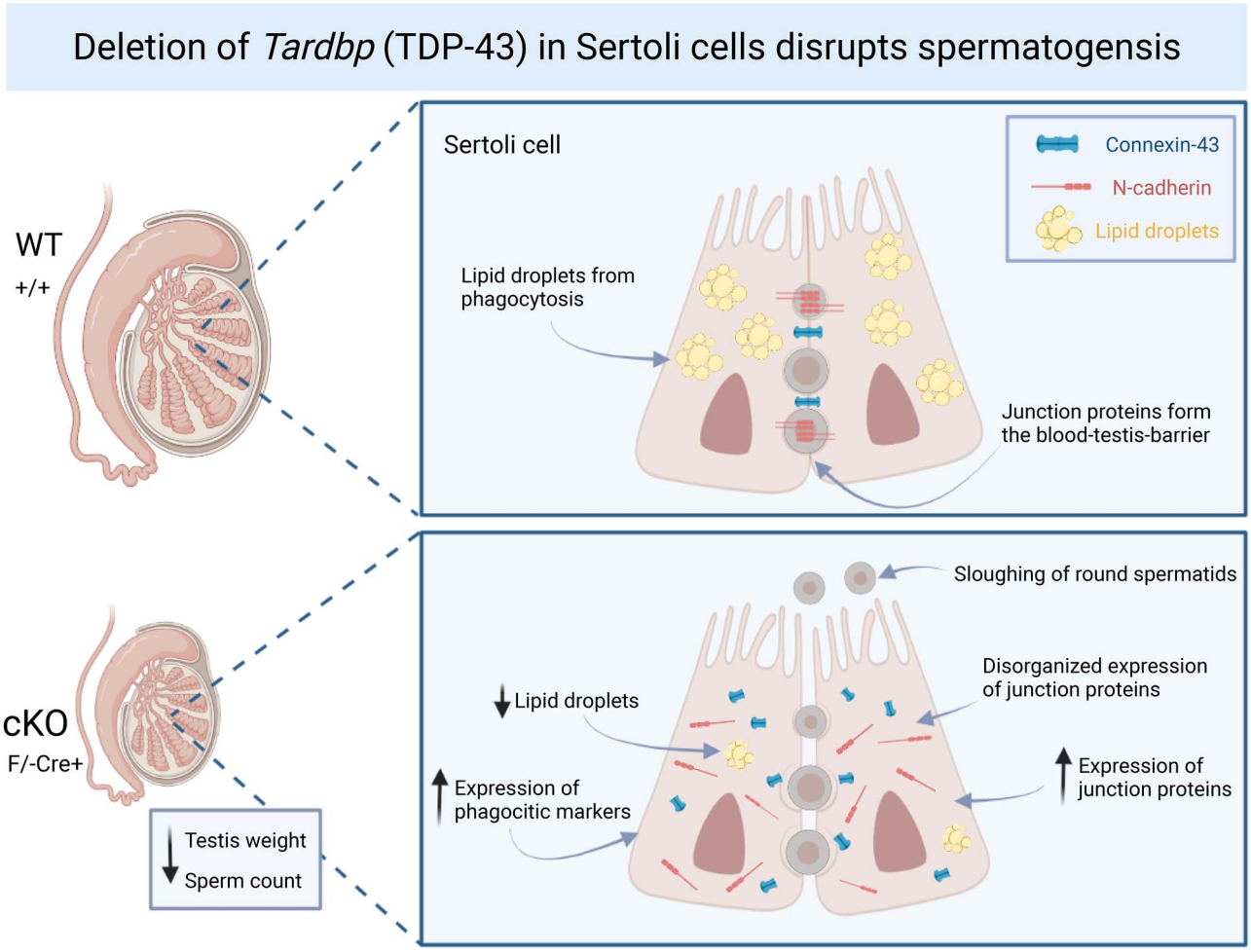
Graphical Abstract



Keywords: conditional knockout, TAR DNA binding protein of 43 kD, testis, junctions, infertility, male

Introduction

Infertility affects one in six couples of the reproductive age group. Of these, male factor infertility accounts for approximately half the number of cases. Currently, there are no treatment options for male infertility due to the idiopathic nature of the condition [1]. Therefore, identification of proteins and cellular pathways critical for male fertility is important. In this work, we focus on the role of the transcription factor/splicing protein, TAR DNA binding protein of 43 kilodaltons (TDP-43; gene symbol: *Tardbp*), in male fertility. TDP-43 is an evolutionarily conserved and ubiquitously expressed multifunctional protein with roles in transcription, alternative splicing of mRNA, translation, microRNA biogenesis, mRNA transport, and stability [2, 3]. Knowledge about the function of TDP-43 primarily comes from the field of neuroscience because it has been studied extensively in the context of brain pathology. We cloned TDP-43 from a mouse testis cDNA library as a promoter-binding transcription factor and showed that it regulates gene transcription in the seminiferous epithelium [4]. Our work also showed that TDP-43 is aberrantly expressed in germ cells and spermatozoa of infertile men [5].

TDP-43 is a protein of major relevance for human health because of its association with a variety of neurodegenerative disorders [6–8]. TDP-43 is depleted from the nuclei and found as part of cytoplasmic aggregates in the neurons and glial cells of patients with amyotrophic lateral sclerosis and frontotemporal dementia [3, 6]. Loss-of-function as well as gain-of-function mechanisms of TDP-43 pathogenicity have been extensively studied in the field of neuroscience [8–11]. However, the role of TDP-43 outside the nervous system remains largely unknown.

Our previous work has established that TDP-43 acts as a repressor involved in maintaining spatiotemporal gene expression during spermatogenesis [12]. Subsequently, we characterized the domains of TDP-43 required for transcriptional repression [13]. Recently, we showed that loss of TDP-43 in germ cells of the postnatal testis causes meiotic arrest and male infertility [14]. Nevertheless, our work thus far primarily focused on the role of TDP-43 in germ cells. TDP-43, however, is abundantly expressed in the Sertoli cells as well [4].

Sertoli cells are in close contact with the germ cells in the seminiferous epithelium and play critical roles in spermatogenesis. They provide physical support for the germ cells, phagocytoze residual bodies and apoptotic germ cells, release spermatids during spermiation, secrete tubular fluid, deliver nutrients and growth factors, and establish the blood-testis barrier (BTB) [15, 16]. Given the multiple roles of TDP-43 in cellular functions, we hypothesized that it plays a critical

Table 1. Sequence of primers used for PCR.

Primer	Forward	Reverse
Tardbp Flox	AACTTCAAGATCTGACACCCTCCCTCCCC	GGCCCTGGCTCATCAAGAACTG
Tardbp Null	TCTTACAATGCCTGGCGTGGTG	CGTGGTTGCGCACCCTAACTATAA
Amh Cre	AGTACGTGAGATATCTTTAACCCT	GCGGTCTGGCAGTAAAAACTATC

role in regulating Sertoli function(s) and spermatogenesis. To investigate the role of TDP-43 in Sertoli cells, we have generated conditional knockout (cKO) mice lacking TDP-43 only in Sertoli cells. We found that loss of TDP-43 in Sertoli cells leads to testicular degeneration, sloughing of round spermatids, disruption of the BTB, and decreased lipid droplets in the seminiferous tubules. Together, these findings indicate that TDP-43 is essential for Sertoli cell functions and for the successful completion of spermatogenesis.

Materials and methods

TDP-43 conditional knockout

All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC #18250). To generate the Sertoli cell-specific cKO mouse, we first crossed a C57/BL6 background *Tardbp* (Exon 3) F/F mouse [17] with a Stra8-Icre line [18] to delete the *Tardbp* in germ cells and create a null allele. Then, a *Tardbp* F/− mouse was crossed with the Amh-Cre deleter line [19] to generate mice of genotypes *Tardbp* F/+ Amh Cre + and *Tardbp* +/− Amh Cre+, which were crossed to generate the mouse *Tardbp* F/− Amh Cre + herein designated cKO, as summarized in Figure 1a. As controls, littermates with genotype F/+ or age-matched F/+, F/F or +/+ mice were used. Mice were maintained on a C57/Bl6 background.

Mouse genotyping

Routine genotyping was carried out by PCR using specific primers (Table 1). To detect wild-type versus floxed *Tardbp* alleles, *Tardbp* primers were used and yielded amplicons of 159 bp (wild type) and 300 bp (floxed). The expected band size for Amh-Cre was 160 bp, and *Tardbp* null was 575 bp. Reactions were run for 30 cycles of 94, 60, and 72 °C for 1 min each and visualized after separation by electrophoresis in a 1.2% agarose gel.

Fertility test

To test the fertility of TDP-43 cKO mice, 2 MO cKO male mice were mated with 2 MO C57/Bl6 females (one male and one female per cage). Wild-type breeding pairs (C57/Bl6, 2 MO) were used as controls. Cages were monitored for the presence and number of pups for 4 months.

Testicular weight and sperm analysis

Animals were euthanized by CO_2 at different ages, from postnatal day (PND) 12 to 10 months old (MO). Their testes were harvested, photographed, and weighed before fixation with Bouin's solution or flash freezing in liquid nitrogen for subsequent analysis. The epididymis cauda were collected, minced, and incubated in phosphate-buffered saline (PBS) at 37 °C for 20 min for the release of spermatozoa. Sperm in the supernatants were morphologically analyzed and counted using a hemocytometer under an optical microscope (Laxco, WA).

Histology and immunohistochemistry

For hematoxylin–eosin (HE) staining and immunohistochemistry, testes and epididymis from mice of different ages were fixed in Bouin's solution overnight at room temperature (RT). The fixed tissues were washed in ice-cold 70% ethanol and paraffin-embedded. Then, 4-μm thickness sections were deparaffinized and rehydrated and stained with HE or treated with EDTA pH 9.0 (TDP-43 antibody) or citrate buffer pH 6.0 (other antibodies) in a steamer (Oster, FL) for 1 h for antigen retrieval. After cooling, slides were blocked with protein block (Abcam, UK) and incubated in the primary antibody for 2 h at RT. Primary antibodies used were rabbit polyclonal anti-TDP-43 (1:500, Proteintech (Rosemont, IL), 12892), mouse monoclonal anti-SOX-9 (1:200, Millipore, Burlington, MA AB5535), guinea-pig polyclonal anti-SP-10 (in house, 1:400, described in [20]), rabbit polyclonal anti-N-cadherin (1:200, Proteintech (Rosemont, IL), 22018–1-AP), and mouse monoclonal anti-connexin-43 (1:400, Santa Cruz, Carlsbad, CA sc-271837). The binding of the primary antibody was detected using specific HRP polymers (BioCare, CA). Nuclei were visualized using Harris hematoxylin counterstaining. The primary antibody was omitted in the negative control to determine nonspecific binding of the HRP polymer. Immunohistochemistry was performed on at least two sections from no less than four animals per genotype. For the evaluation of Sertoli cell number, at least 50 seminiferous tubules cross-sections from each biological replicate were quantified by SOX-9. For the evaluation of Sertoli junctions, 50–70 seminiferous tubules cross-sections per animal were evaluated by the pattern of N-cadherin and connexin-43 expression.

Morphometric analysis

Testes were histopathologically evaluated by HE. The diameter of the seminiferous tubules cross-sections in cKO and littermate controls were compared by measuring the shortest diameter distance in at least 15 random tubules per biological replicate of each genotype using ImageJ software (National Institutes of Health). The percentage of affected tubules was counted on HE stained slides at 10× magnification (at least 50 tubules per replicate, $N = 3$ per timepoint). The penetrance of target gene deletion was evaluated by counting the percentage of Sertoli cells nonimmunostained for TDP-43 in adult (3–10 MO) testis cross-sections (four fields at 20× per replicate, $N = 3$).

Flow cytometry

To analyze the cellular composition within the seminiferous tubules, testicular cells were stained with a nuclear stain and subject to flow cytometry for classification based on DNA content, as described previously [21]. Briefly, 3 MO

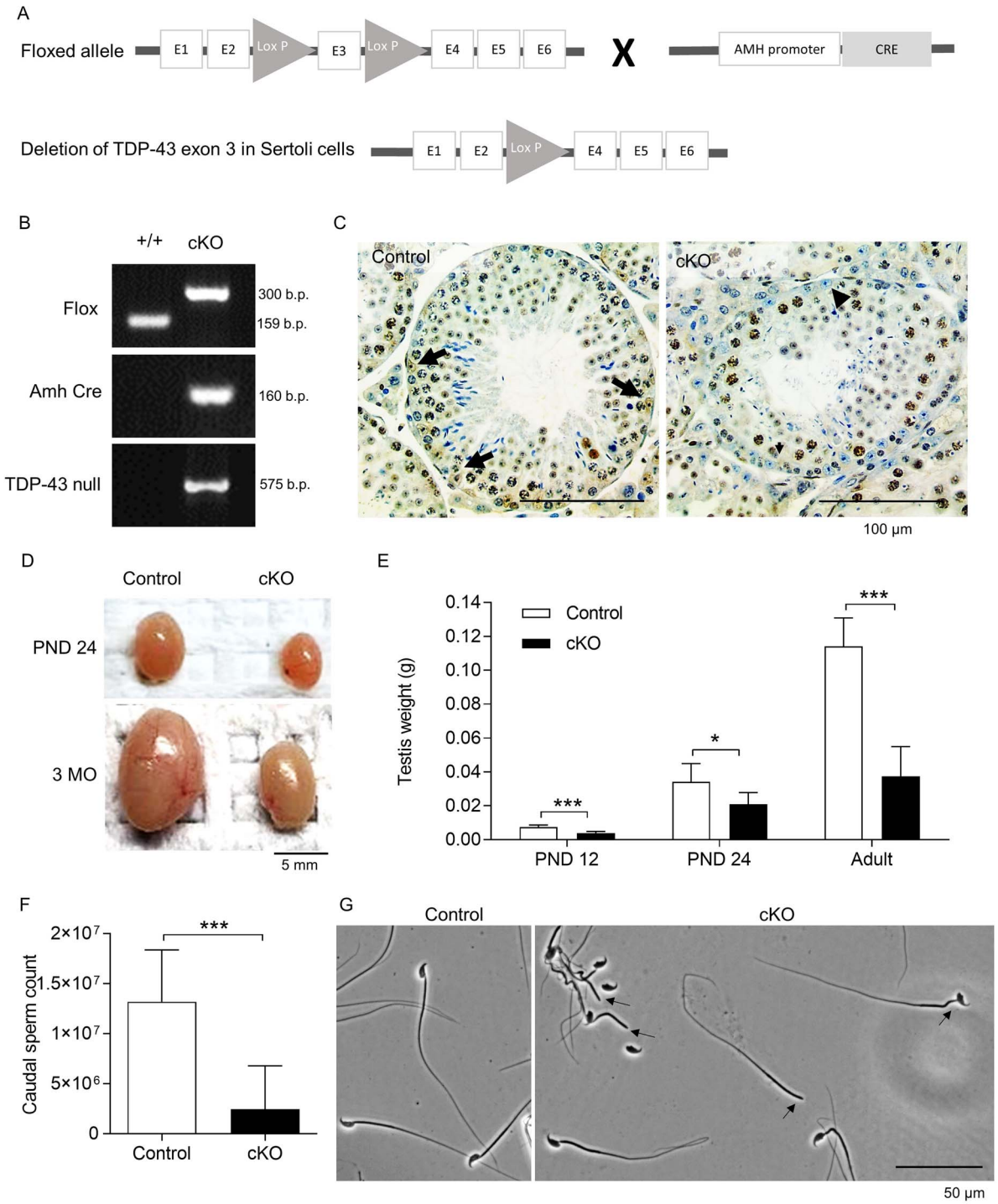


Figure 1. Loss of TDP-43 in Sertoli cells leads to decreased testis weight and sperm counts and testicular atrophy. (A) Schematic representation of the deletion of TDP-43 exon 3 with Amh-Cre- mediated recombination for the generation of mutant mice lacking TDP-43 in Sertoli cells. (B) Genotype confirmation of cKO mice and controls by PCR. (C) Immunohistochemistry for TDP-43 showing lack of TDP-43 specifically in Sertoli cells in the cKO mouse. Black arrows: expression of TDP-43 in the nuclei of Sertoli cells in littermate controls; black arrowhead: Sertoli cells lacking TDP-43 expression in the cKO mouse. Note that TDP-43 expression remains normal in germ cells of the cKO mouse. (D) Testis size at PND 24 and 3 MO adults; (E) testis weight in the prepubertal stage (PND 12), young (PND 24), and adults (3–10 MO); (F) sperm count; (G) sperm from cKO mice showing abnormalities. Arrows highlight sperms with detached heads.* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by Student's *t*-test.

testes were decapsulated, tubules were minced, and washed two times with Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, MA) to eliminate interstitial cells. Tubule pieces were incubated for 20 min at 34.5 °C with collagenase type IV (2 mg/ml; Sigma, MO) and DNAse I (40 μg/ml, Sigma, St. Louis, MO). Then, the tubules were washed two times in DMEM and resuspended in trypsin (10 mg/ml), collagenase IV (1 mg/ml), hyaluronidase (1.5 mg/ml), and DNase I (40 μg/ml) for 15 min at 34.5 °C. The cells were passed through a 45-mm nylon mesh, centrifuged at 300 g for 5 min, and suspended

in PBS. Finally, cells were fixed in – (PFA) 4%, washed with PBS, stained with Hoechst (1:500, Thermo Fisher, Waltham, MA), and analyzed using a flow cytometer (Cytek Aurora, Cytek, CA).

Apoptosis assay

Adult tubule cross-sections (3–10 MO) were subject to TUNEL analysis to identify apoptotic cells using ApopTag Peroxidase *in Situ* Apoptosis Detection Kit S7100 (EMD Millipore, MA) following the manufacturer's instructions. Tubules' cross-sections containing apoptotic cells were quantified from at least two sections of four animals of each genotype (cKO and age-matching +/+ controls).

Staging of the cycle of seminiferous epithelium

To determine the progression of the seminiferous epithelium cycle, immunohistochemistry was performed on testis cross-sections from 2-month-old cKO and control mice to identify stages. The SP-10 antibody, which stains the acrosome and distinguishes its morphology in steps 2–16 spermatids, was used for the identification of stages as reported [20]. Tubule cross-sections showing stages II and VII/VIII were counted for comparing the progression of the seminiferous cycle between the cKO and control mice. Stage II was identified by the presence of round spermatids with small acrosomal granules rather than acrosomal caps over the nuclei, and stage VII–VIII was characterized by the presence of round spermatids containing fully formed acrosome and elongated spermatids lining lumen of the tubule. At least, 100 tubule cross-sections from three animals of each genotype (cKO and age-matching +/+ controls) were counted.

Biotin-tracer assay

The permeability of the BTB was assessed using EZ-Link Sulfo-NHS-LC-Biotin (10 mg/ml in PBS/0.01 M CaCl2, Thermo Fisher, Waltham, MA) as previously described [22]. The testes of PND 24 or 3 MO cKO and littermate controls were collected, and the tunica albuginea was punctured once with a 26-gauge needle. Then, the testes were incubated in NHS-linked biotin for 30 min at RT. After incubation in the tracer, the testes were fixed in Bouin's solution overnight at RT and processed for paraffin embedding. For visualization, 4-μm thick sections were deparaffinized, rehydrated, and incubated in streptavidin AlexaFluor-488 conjugate (1:500, S32354, Life Technologies, CA) and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Thermo Fisher, Waltham, MA) for 1 h. Slides were mounted in Fluoromount (Southern Biotech, AL) and photographed using a camera (Retiga Teledyne, CA) coupled to an inverted fluorescent microscope (Leica DMI 4000B, Germany). At least, 100 seminiferous tubules cross-sections from seven biological replicates of each genotype were evaluated for the presence of biotin tracer in the lumen.

Oil Red

To evaluate lipid droplets in the seminiferous tubules, testes of 3 MO cKO and littermate controls were stained with Oil Red O (Sigma, St. Louis, MO). Briefly, fresh collected testes were flash-frozen in OCT Compound (Tissue Plus, Fisher Health Care, MA) in dry ice and 100% ethanol and sectioned at 15-μm-thick sections in a cryostat. Slides were kept at RT for 10 min, fixed with PFA 4% for 15 min, and rinsed with PBS. Then, sections were rinsed with 60% isopropanol, incubated in Oil Red O for 15 min at RT, and rinsed again with 60% isopropanol. Slides were counterstained with Harris hematoxylin for 30 s, rinsed in water, and mounted. At least, 50 tubules seminiferous cross-sections of four biological replicates of each genotype were evaluated by the presence of lipid droplets in the lumen or in the basal region of the tubules (Sertoli cells cytoplasm).

qRT-PCR analyses of target genes

Total RNA from the adult testes (cKO and age-matching +/+ controls) was isolated using Trizol reagent (Ambion, Life Technologies, Carlsbad, CA). To examine mRNA levels, 1 μg of total RNA was reverse transcribed using a first-strand cDNA synthesis kit (Takara, Japan). qRT-PCR was conducted using the SYBR Green qRT-PCR kit (Takara) on an Applied Biosystems 2500 Real-Time PCR machine (Applied Biosystems, CA). qRT-PCR was carried out in a 20 μl reaction mixture, which consisted of 10 μl of 2 × SYBR Green Mix, 0.2 μl of forward and reverse primers (20 μM of each primer), and 2 μl diluted cDNA. Cycling conditions were denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 34 s at 60 °C, and extension for 30 s at 72 °C, followed by a melting step. All qPCR plates included a negative control, actin as the reference gene for each sample, and all reactions were performed in triplicate. Relative gene transcription changes were calculated using the $2-\Delta\Delta$Ct method [23]. All results are the averages of four independent *Tardbp* cKO mice samples compared with controls. Data are expressed as the mean ± SEM for a 95% confidence interval. Statistical comparisons of $\Delta\Delta$Ct were made by Student's *t* test. Statistically significant difference was indicated with $P < 0.05$. Primers used are described in Table 2.

Statistical analysis

Statistical significance was evaluated by Student's *t*-test or two-way ANOVA followed by Bonferroni post-test using Prism (GraphPad, CA) software when appropriate. All experiments were performed with at least four biological replicates of each genotype. Data were described as mean ± standard deviation (SD), with differences considered significant when $P < 0.05$.

Results

Lack of TDP-43 in Sertoli cells leads to subfertility in mice

To assess the impact of loss of TDP-43 in Sertoli cells on fertility, male cKO mice were housed individually with a single wild-type female for 4 months, beginning at 2 months of age. Mice lacking TDP-43 in Sertoli cells produced fewer litters (a 2.75-fold decrease) and a smaller number of pups per litter (2.77-fold less) compared to controls, indicating that in the absence of TDP-43, Sertoli cells are unable to provide optimal support for the differentiating spermatogenic cells ultimately affecting their fertilization capability (Table 3).

Ablation of TDP-43 in Sertoli cells led to testicular atrophy, decreased testis weight and sperm counts

Mice bearing one *Tardbp* floxed allele, one *Tardbp* null allele, and the Amh Cre transgene (genotype: F/-Cre + mice) would be deficient for TDP-43 in Sertoli cells (hereafter referred to as cKO mice). Genotype of the cKO mice used in the study

Table 2. Sequences of primers for RT-qPCR.

	Forward	Reverse	Amplicon length (nt)
Cdh2 (N-cadherin)	TTGCTTCAGGCGTCTGTGGAG	ACATCCTTCGGTAAGACTGCG	95
Gja1 (Connexin-43)	CAGGTCTGAGAGCCCGAACT	GTCTGGGCACCTCTCTTTCAC	110
Tjp1 (Zonula occludens-1)	GAGACAAGATGTCCGCCAGG	AAACCCAGGAGCCCTGTGAA	104
Cldn11 (Claudin-11)	TCCTCATCCTTCCAGGCTAC	GATGCAGGGGAGAACTGTCA	107
Ocln (Occludin)	AGAGTACATGGCTGCTGCTG	TTTGCTCTTCAACTGCTTGC	103
Elmo1 (Elmo-1)	CTGGCGCCTACCCTAAACTC	GCAAGAGACCACCCATCACA	81
RhoG	AGGGCACCAGGTCACTACTT	ACCACCACACACTTGATGCT	104
Rac1 (Rac-1)	CGCAGACAGTTGGAGACACAT	GATGCAGGACTCACAAGGGAA	106
Tyro3 (Tyro-3)	GGCAAAGGAGAGTTTGGATCAG	TCGCTTGAGGCAATGATGTC	110
Adgrb1 (BAI-1)	CCAGCTCATGACCGACTTTGA	GCTGCAATGTCTTTGTTCAGCA	87
Actin (Actb)	GTGGATCAGCAAGCAGGAGTA	AAGAAAGGGTGTAAAACGCAGC	101

Table 3. Fertility of male cKO mice.

	Control +/+	cKO	*P*-value
Number of tested males	6	5	
Average number of litters	3.3 ± 1.2 SD	1.2 ± 1 SD	0.014
Average pups per litter	8.6 ± 2.7 SD	3.3 ± 3.1 SD	0.001

was verified by PCR (Figure 1B). We performed immunohistochemistry using TDP-43 antibody to assess Sertoli cell-specific loss of TDP-43. Figure 1C shows that while the control testis cross-section present TDP-43 staining in both Sertoli and germ cells, cKO testis display immunoreactivity in germ cells but not Sertoli cells, thus confirming cell-specific loss of TDP-43. Quantification of Sertoli cells negative to TDP-43 immunostaining showed a 76% ± 18.2 SD penetrance of the targeted deletion. To evaluate the impact on testis growth, we collected testes from mice of different ages (prepubertal [PND 12], young [PND 24], and adult [3–10 MO]). Testes of cKO mice showed reduced size and weight at all ages. There was approximately a 2-fold reduction at the prepubertal stage, 1.6-fold reduction at young age, and 3-fold reduction in the adults (Figure 1D and E). Loss of TDP-43 in Sertoli cells led to a 5-fold decrease in cauda epididymal sperm count in adult mice ($2.45 \times 10^6 \pm 4.3 \times 10^5$ SD in cKO versus $13 \times 10^6 \pm 5.2 \times 10^6$ SD in littermate controls, Figure 1F), and sperm from the cKO mice showed multiple morphological aberrations including detached heads (Figure 1G).

Histopathological evaluation showed signs of testicular atrophy beginning at PND 24, which persisted into adult testes with a significant reduction in the diameter of tubule cross-section (1.27-fold at PND 24 and 1.5-fold in the adults, Figure 2A and B). Furthermore, several other abnormalities, including the presence of vacuoles (Figure 2A, arrowheads) and loss of germ cells layers (Figure 2A, asterisks) within the seminiferous epithelium, were observed as early as PND 24 and consistently found until 10 months of age, which was the oldest cKO mouse analyzed. At early ages, not all tubule cross-sections showed the phenotype (about 65.7% ± 11.0 SD at PND 24) but the severity became worse with advanced age (92.4% ± 6.1 SD at 10 MO, Figure 2C).

Targeting TDP-43 in Sertoli cells altered testicular cell composition

To obtain a quantitative assessment of the cell types affected by the loss of TDP-43, we evaluated the whole testis cell composition of 3 MO cKO mice by flow cytometry using Hoechst nuclear staining (Figure 3A and B). In the wild-type control, round spermatids (1C) represented 79.5% ± 8.2 SD of the total testicular cells. Spermatogonia, secondary spermatocytes, and somatic cells together (2C) constituted 10.3% ± 4.4 SD. In contrast, the cKO mice showed a decreased percentage of round spermatids (56% ± 4 SD, $P < 0.01$) and an elevated presence of 2C cells (35.4% ± 7.4 SD, $P < 0.001$). Both control and cKO mice showed similar percentages of primary spermatocytes (4C). Our procedure for germ cell preparation for flow cytometry analysis eliminates interstitial cells. Thus, elevation of the 2C population and decrease in round spermatids suggest increased proportion of Sertoli cells to germ cells, meiotic arrest, loss of round spermatids, or a combination of these.

Sloughing of round spermatids in TDP-43 cKO mouse

In order to characterize the germ cell loss, we performed immunohistochemistry using the acrosome-specific SP-10 antibody, which marks post-meiotic round spermatids. Control testis showed several layers of SP-10 positive round spermatids in the seminiferous epithelium, whereas the cKO testis showed thinning of the inner layers of germ cells and loss of round spermatids (Figure 3C). This suggests that loss of TDP-43 in Sertoli cells might cause round spermatids to detach. Cross-sections of the epididymis showed heads of spermatozoa in control mice, whereas the cKO mice showed round cells in the lumen of the tubules of the epididymis which were positively stained by the SP-10 antibody (Figure 3D) indicating that sloughed cells were composed mostly of round spermatids. This suggests that loss of TDP-43 compromised the Sertoli cell's ability to provide attachment to round spermatids.

Loss of TDP-43 induced apoptosis, but Sertoli cell number remained normal

Next, we evaluated cellular apoptosis in the seminiferous tubules cross-sections by TUNEL assay. Loss of TDP-43 in Sertoli cells caused an increase in tubules containing apoptotic

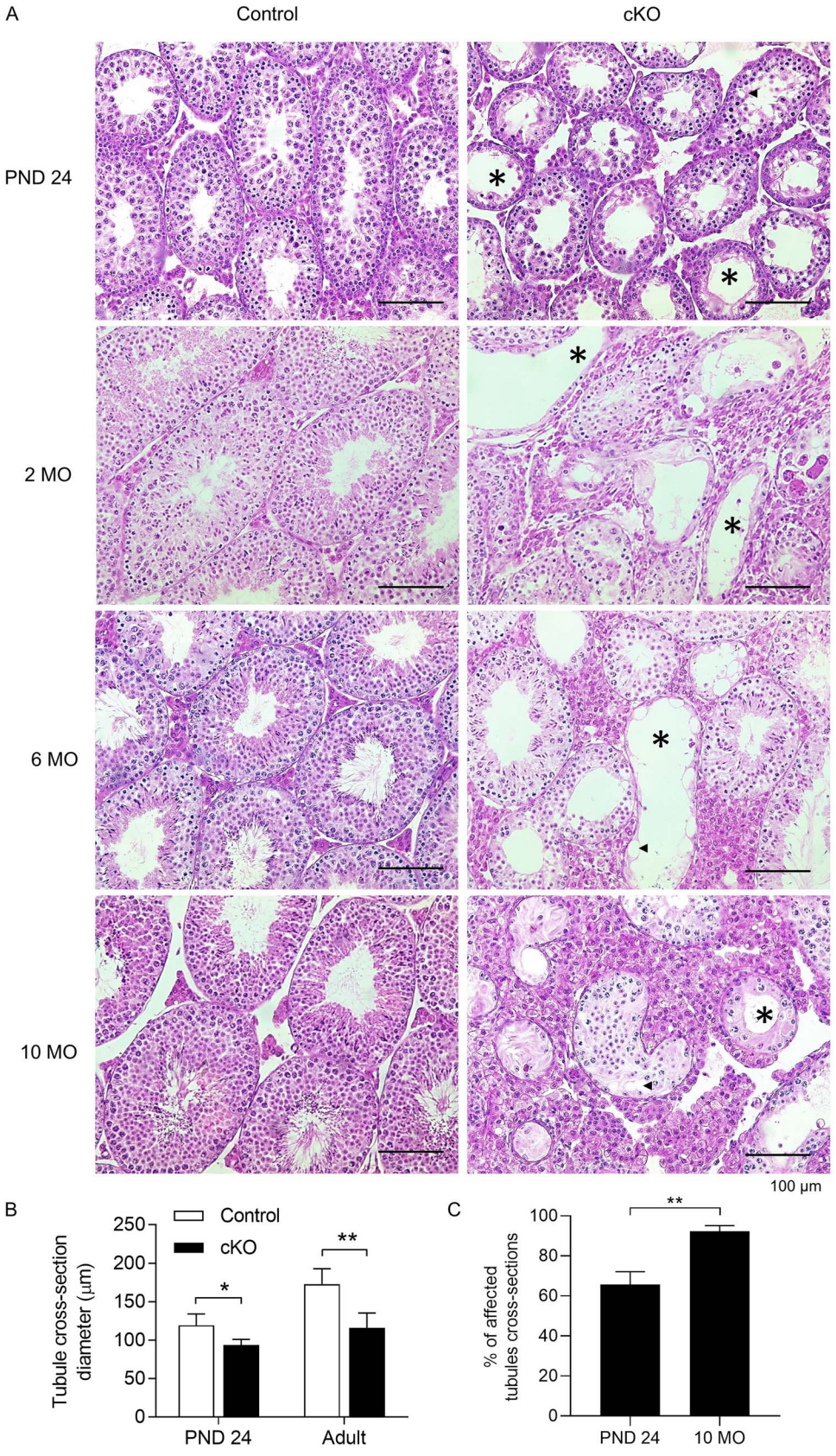


Figure 2. Deletion of TDP-43 in Sertoli cells leads to multiple degenerating alterations. (A) Hematoxylin–eosin staining in testis from PND 24, 2 MO, 6 MO, and 10 MO cKO mice and controls. Several alterations are observed, such as loss of germ cell layers (asterisks) and vacuoles (arrowheads), beginning at PND 24 and sustained in the adults. (B) Tubule cross-section diameter at PND 24 and 3 MO adults. (C) Percentage of tubules cross-sections presenting degeneration at PND 24 and 10 MO cKO mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t*-test.

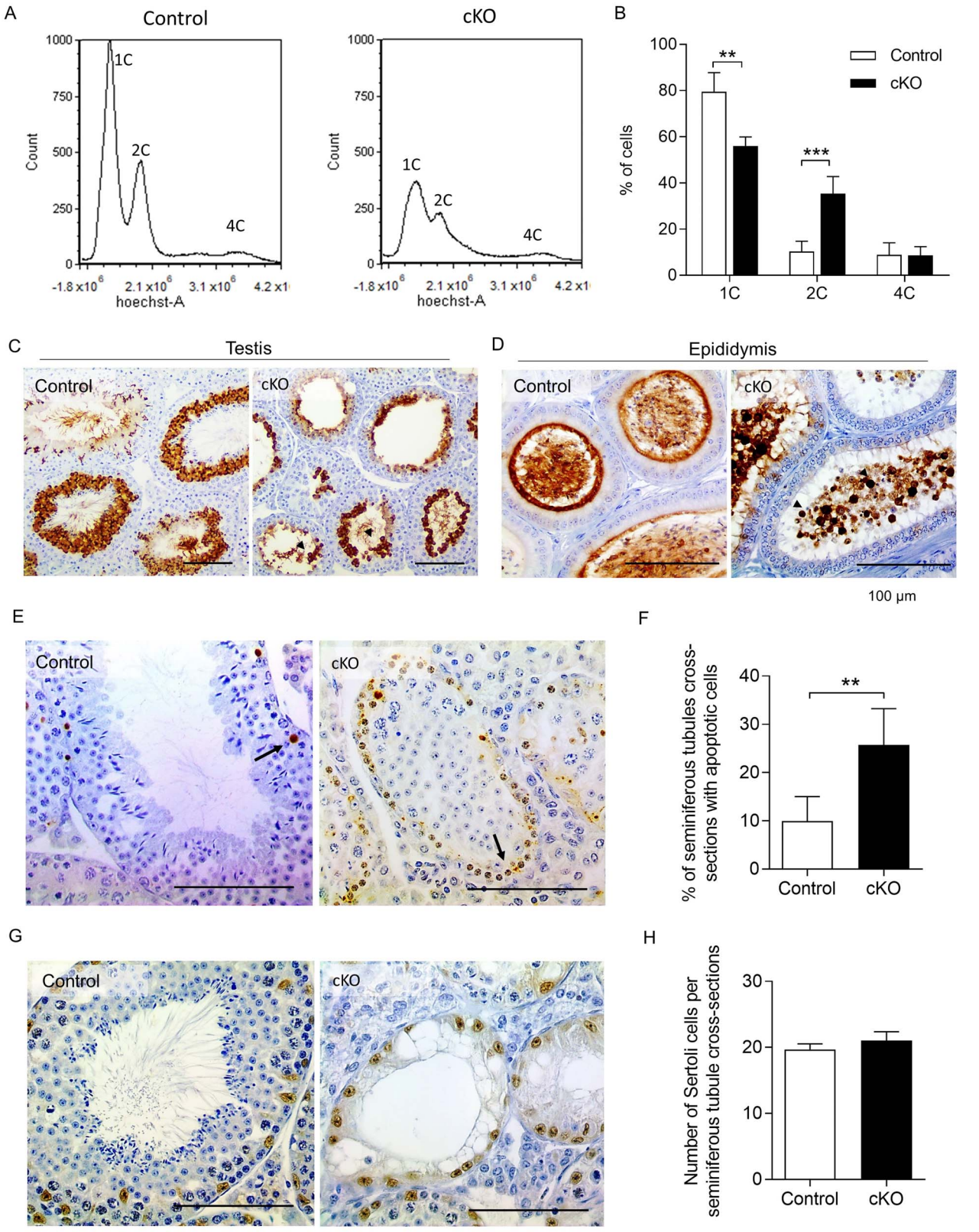


Figure 3. Ablation of TDP-43 in Sertoli cells leads to increased apoptosis of germ cells and sloughing of round spermatids but do not affect Sertoli cell number. (A) Flow cytometry histogram and (B) analysis of DNA content of adult testicular cells revealed a lower percentage of 1C cells (round spermatids) and a higher percentage of 2C cells (indicative of spermatogonia, secondary spermatocytes, and somatic cells) in the cKO mice in comparison to wild-type controls. $^{**}P < 0.01$ and $^{***}P < 0.001$ by two-way ANOVA followed by Bonferroni post-test. (C) Immunohistochemistry for the acrosomal marker SP-10 in testis and (D) epididymis, showing sloughing of round spermatids in the lumen of the epididymis of cKO mice. (E) Testis cross-sections subjected to TUNEL analysis showing brown-stained apoptotic cells (black arrows) and (F) quantification of tubules cross-sections containing apoptotic cells. $^{**}P < 0.01$ by Student's *t*-test. (G) Immunohistochemistry for SOX-9, a nuclear Sertoli cell marker, and (H) quantification of SOX-9+ Sertoli cells per tubule cross-section.

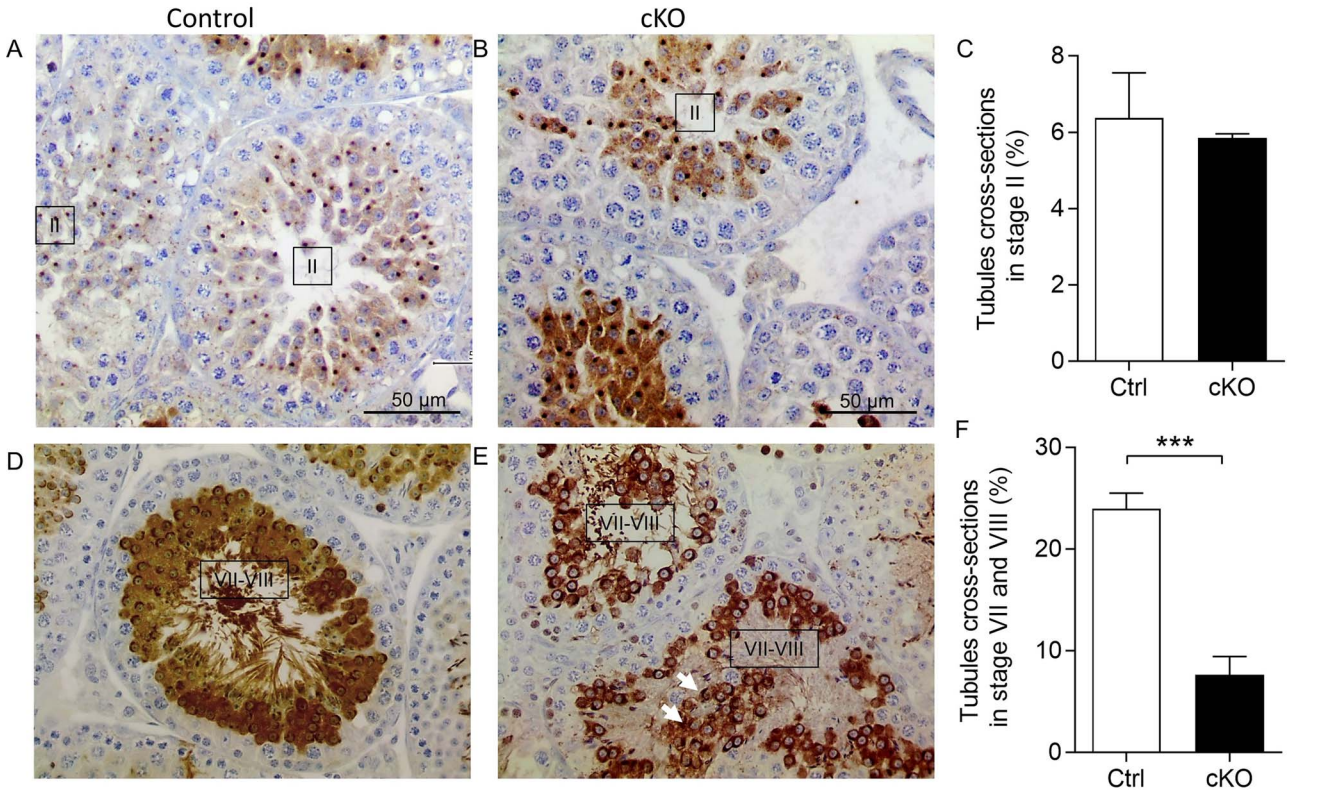


Figure 4. Requirement of TDP-43 in Sertoli cells is dependent on the stage of the seminiferous cycle. Immunohistochemistry of testis cross-sections of 2 MO cKO and control mice was performed using the SP-10 antibody. There was no difference at stage II of the cycle (A, B) but fewer stage VII/VIII cross-sections (D, E) were observed in the cKO mice. Quantification shown in C and F, respectively. Sloughed round spermatids (E, arrows) likely contributed to the absence of elongated spermatozoa in cKO mice at stage VII/VIII. ***$P < 0.001$ by Student's *t*-test.

cells in the adult testes (Figure 3E and F). Most of the TUNEL-positive cells were in the basal layer of the seminiferous epithelium (arrows, Figure 3E). Based on nuclei morphology, apoptotic cells were germ cells rather than Sertoli cells. This was confirmed by quantifying the number of SOX-9 positive cells (marker of Sertoli cells), which revealed that Sertoli cell numbers remained similar between cKO and littermate controls (Figure 3G and H). These findings suggest that TDP-43 is not required for the persistence of Sertoli cells in the testis, but the loss of TDP-43 in the Sertoli cells affects germ cell survival.

Lack of TDP-43 in Sertoli cells disrupted the progression of the seminiferous cycle

To evaluate if loss of TDP-43 in Sertoli cells affected spermatogenesis early or later in the cycle of the seminiferous epithelium, we quantified tubules cross-section at stage II and stages VII-VIII (Figure 4). The tubules of cKO mice often showed disorganized morphology, hampering a more detailed staging. Control and cKO mice presented similar number of tubules cross-sections representing stage II (control: 6.4% ± 1.2 SD, cKO: 5.8% ± 0.1 SD, $P > 0.05$). In contrast, cKO mice showed a significant decrease in tubules cross-sections at stages VII–VIII (control: 24% ± 1.5 SD, cKO: 7.6% ± 1.8 SD, $P < 0.001$), indicating loss and/or abnormal cycle progression.

To further characterize the impact of TDP-43 loss on Sertoli cell function, we evaluated the effect of knockout on the barrier function and phagocytosis.

Inactivation of TDP-43 in Sertoli cells disrupted the BTB

One of the main functions of the Sertoli cells is to create a barrier separating the luminal and basal compartments of the seminiferous tubules, which is accomplished by the formation of tight junctions between adjacent Sertoli cells. We used a biotin tracer to evaluate if loss of TDP-43 compromised Sertoli cell tight junctions and the intactness of the BTB. In this assay, seepage of biotin from the interstitium into the lumen of the seminiferous tubule indicates a breach in BTB. In cKO mice, we found an increased number of seminiferous tubule cross-sections with biotin within the lumen, both at PND 24 (28.5% ± 13.8 SD in cKO, versus 4.4% ± 3.2 SD in the littermate controls, $P = 0.01$) and in adults (42.7% ± 7.1 SD in cKO, versus 7.5% ± 5.4 SD in the littermate controls, $P < 0.0001$, Figure 5). The observation of biotin leakage into the lumen of cKO seminiferous tubules cross-sections as early as PND 24 and persistence till the adult age indicates that loss of TDP-43 in Sertoli cells leads to disruption of the BTB and suggests a direct or indirect role of TDP-43 in its formation and/or maintenance.

Altered expression of N-cadherin and connexin-43 in TDP-43 cKO mice

N-cadherin and connexin-43 are key proteins of the ectoplasmic specializations and gap junctions, respectively, and play fundamental roles in Sertoli-Sertoli and Sertoli-germ cells attachment [24]. Therefore, we evaluated the expression of these proteins in adult cKO mice testis by

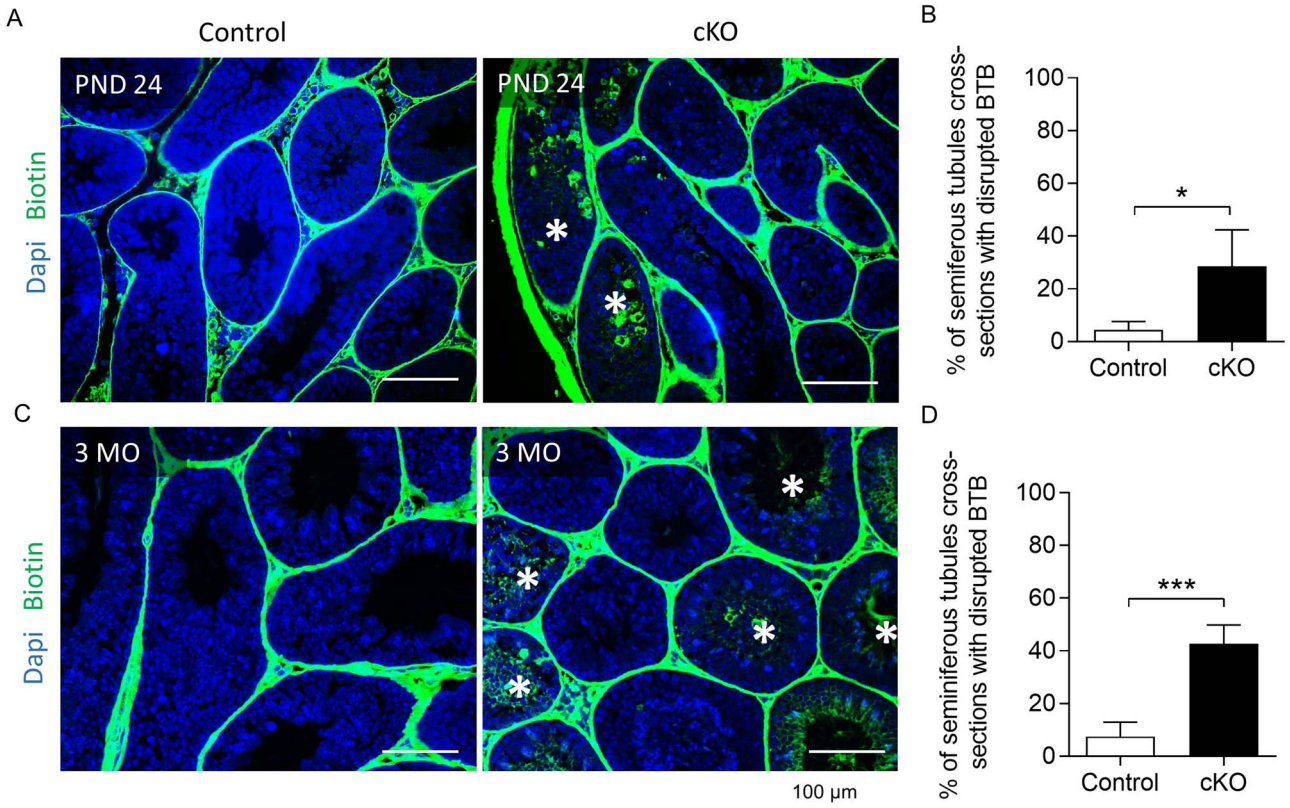


Figure 5. Lack of TDP-43 in Sertoli cells leads to disruption of the BTB. We injected a biotin tracer in the interstitium of freshly harvested testes to investigate possible leakage in the BTB. The biotin remained trapped outside of the tubules in the littermate controls, both at PND 24 (A, B) and 3 MO (C, D). In contrast, several tubules cross-sections of cKO mouse had staining of biotin in their lumen, indicating disruption of BTB. $^{*}P < 0.05$ and $^{***}P < 0.001$ by Student's *t*-test.

immunohistochemistry. Both N-cadherin and connexin-43 showed altered patterns of expression in affected tubules. In control mice, N-cadherin was expressed in the basal ectoplasmic specializations across the BTB and in the apical ectoplasmic specializations between Sertoli cells and elongated spermatids, with some variation between different stages of the cycle of the seminiferous epithelium (Figure 6A). In contrast, the cKO mice showed disorganized deposition of N-cadherin, with evident overexpression of this protein in a portion of the affected tubules. N-cadherin's abnormal expression was observed in 56.25% ± 25.6 SD of the seminiferous tubules in cKO mice and 5% ± 4.9 SD in the littermate controls ($P = 0.0006$, Figure 6C).

Connexin-43 was located in gap junctions between Sertoli cells and between Sertoli and germ cells in control mice (Figure 6B). Similar to N-cadherin, connexin-43 expression was disorganized in mice lacking TDP-43 in Sertoli cells. Most of the affected tubules showed increased expression of connexin-43, with an altered appearance in 73.7% ± 15.1 SD of the tubules (versus 2.6 ± 2.5 SD in control littermates, $P < 0.0001$, Figure 6D). Taken together, these findings suggest that the loss of TDP-43 in Sertoli cells leads to altered expression of key junction proteins.

Loss of TDP-43 leads to decreased lipid droplets in the seminiferous tubules

Next, we aimed to investigate if the lack of TDP-43 in Sertoli cells disrupted their phagocytic function. To this end, we evaluated the lipid droplets by Oil Red O staining of testis cross-sections of adult cKO mice (Figure 7). The presence of lipid droplets has been reported within (1) the interstitial space (indicative of cholesterol esters storage for steroidogenesis in Leydig cells [25], activated macrophages [26]), (2) cytoplasm of Sertoli cells (indicative of phagocytosis of residual bodies and apoptotic germ cells [27]), and (3) lumen of the seminiferous tubules (from residual bodies and cytoplasmic droplets [27]). Consistent with the above, Oil Red stained lipid droplets could be observed in control mice within the interstitium, lumen of the seminiferous tubule, and in Sertoli cell cytoplasm (Figure 7A and C). In contrast, mice lacking TDP-43 in Sertoli cells showed fewer tubule cross-sections containing lipid droplets in Sertoli cells ($P < 0.001$), or lumen ($P < 0.05$), or in both ($P < 0.01$). Lipid droplets were absent in 5.4% ± 3.4 SD of tubule cross-sections of control mice, versus 33.3% ± 6.25 SD of the cKO mice ($P < 0.001$) (Figure 7B, D, and E). Taken together, these findings suggest a role for TDP-43 in Sertoli cell phagocytosis.

Evaluation of Sertoli cell function in cKO mice by measurement of mRNA for junction proteins and proteins involved in phagocytosis

To further investigate how the loss of TDP-43 affected Sertoli cell junctions and phagocytic function, we quantified the mRNA expression of different factors involved in Sertoli cell junctions and the phagocytic pathway by RT-qPCR (Figure 8). We found that mRNA for *Cdh2* and *Gja1*, which code for N-cadherin and connexin-43, respectively, was upregulated

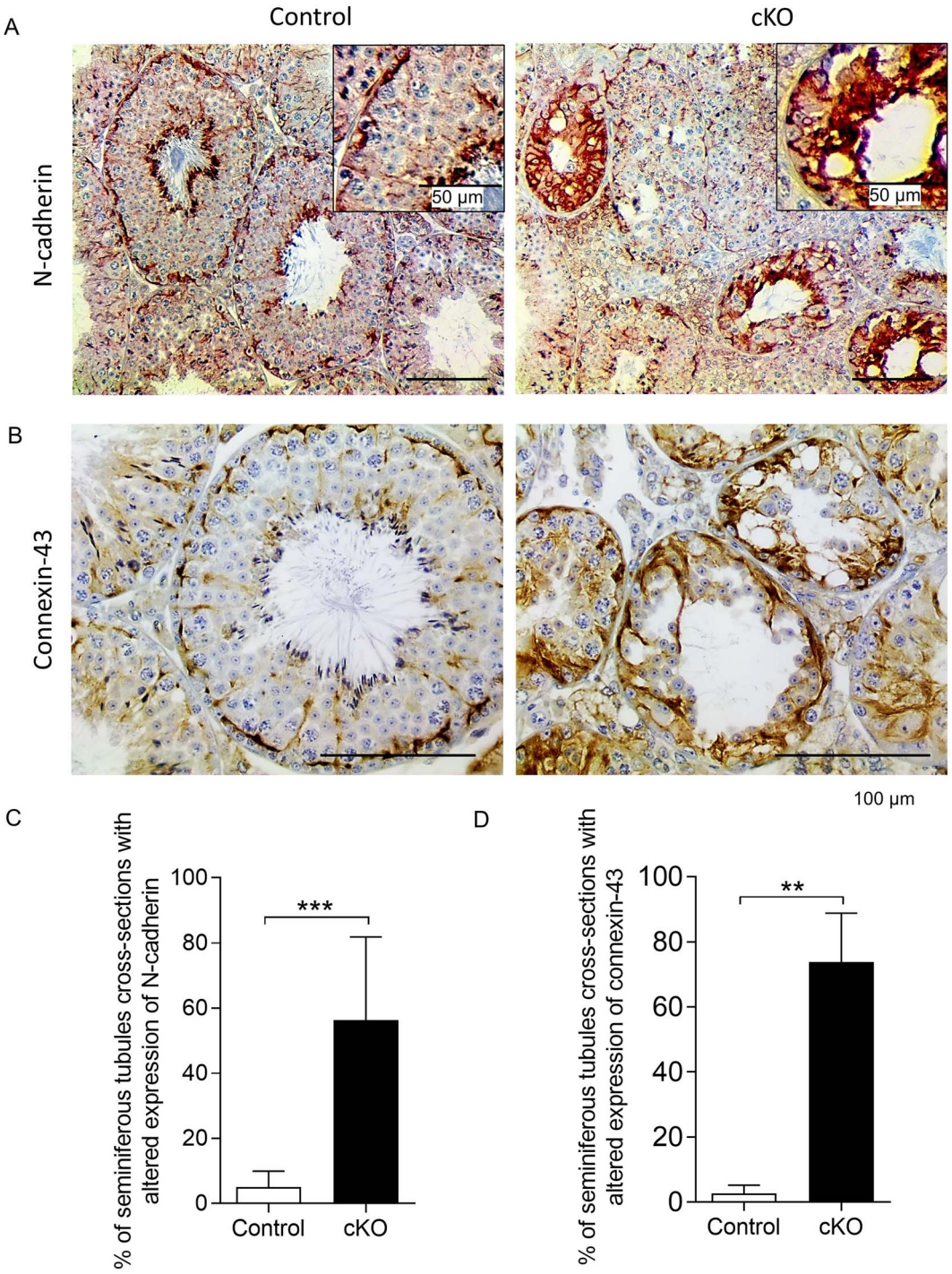


Figure 6. Ablation of TDP-43 in Sertoli cells causes altered expression of N-cadherin and connexin-43. (A) Immunohistochemistry revealed that the lack of TDP-43 in Sertoli cells caused the disorganized deposition of N-cadherin (top panel and detail) and connexin-43 (lower panel) in the adult testes. (B) Quantification of tubules cross-sections with altered expression of N-cadherin and (C) connexin-43. $^{**}P < 0.01$ and $^{***}P < 0.001$ by Student's *t*-test.

in cKO mice (Figure 8A). This is consistent with the aberrantly high level of protein detected by immunohistochemistry (Figure 6). Transcripts for *Tjp1* (Zonula Occludens-1), an essential tight junction protein, were also increased in comparison with control. Additionally, several genes involved in Sertoli cell phagocytosis, including *Elmo1*, *Rac1*, *Tyro3*, and *Adgrb1*, were significantly upregulated in cKO mice (Figure 8B). These results suggest a direct or indirect role for TDP-43 in transcriptional regulation of several genes essential for the normal physiology of Sertoli cells.

Discussion

The evolutionarily conserved and ubiquitously expressed DNA/RNA binding protein TDP-43 performs multiple functions in mammalian cells, including transcriptional repression,

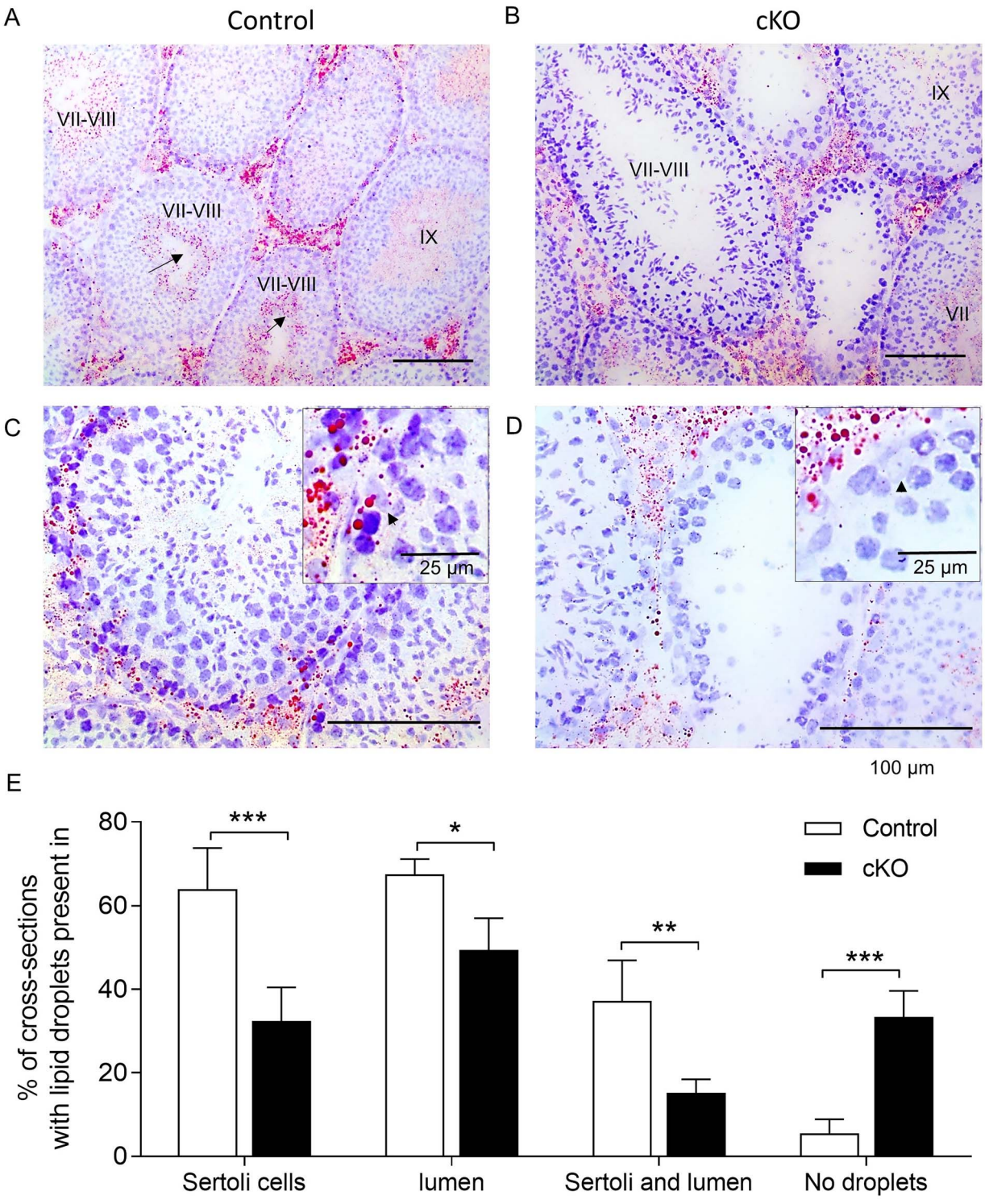


Figure 7. Lack of TDP-43 in Sertoli cells reduces lipid droplets in the seminiferous tubules. (A) Oil Red lipid staining on adult testes showed decreased lipid droplets in the cKO mouse. Arrows indicate lipid droplets in the lumen of the tubules, and arrowheads indicate Sertoli cells (details). (B) Quantification of tubules containing lipid droplets within the Sertoli cell cytoplasm, lumen, in both or neither. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by two-way ANOVA followed by Bonferroni post-test.

mRNA splicing, micro-RNA biogenesis, and mRNA stability, and is also found in the stress granules. TDP-43 has been extensively studied in the context of neurodegenerative disorders, and all of the above functions have been shown in mouse and human neuronal cells. We are the first to explore the function of TDP-43 in the testis. TDP-43 is expressed by the germ cells as well as somatic cells of the testis [4]. Our previous work has established that TDP-43 functions as a transcriptional repressor and regulates spatiotemporal gene expression within the seminiferous epithelium. Conditional

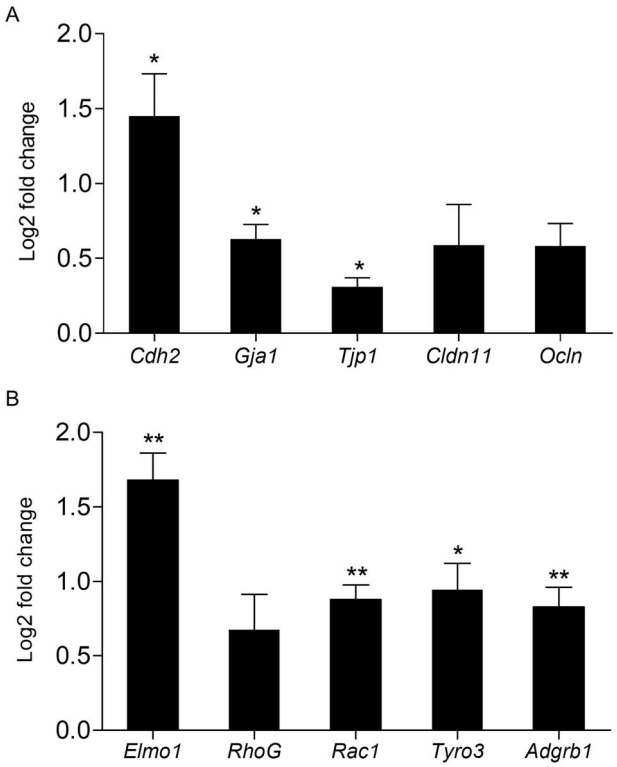


Figure 8. Loss of TDP-43 in Sertoli cells induces the upregulation of several transcripts involved in cell junctions and phagocytosis. (A) mRNA expression fold change in relation to wild-type controls in adult cKO mice for cell junction and (B) phagocytosis transcripts. $^{*}P < 0.05$ and $^{**}P < 0.01$ by *t*-test.

knockout of *Tardbp* in spermatogonia showed that TDP-43 is essential for the completion of prophase I of meiosis in mice [14]. Here we demonstrate an essential role for TDP-43 in Sertoli cell function and spermatogenesis. Using a cKO mouse model, we show that loss of TDP-43 in Sertoli cells causes testicular atrophy and severely reduced sperm counts, leading to subfertility. Loss of TDP-43 affected critical Sertoli cell functions, including the maintenance of the BTB, attachment of round spermatids, and phagocytic pathway. We observed aberrant expression of multiple junction proteins and genes involved in phagocytosis. Together, these findings indicate a non-cell autonomous requirement of TDP-43 for spermatogenesis.

The Amh-Cre transgene starts to express Cre recombinase in Sertoli cells between ~E11.5 and E14.5, around the time of Sertoli cell specification [19]. In agreement with that, deletion of TDP-43 in Sertoli cells caused early effects in the testis, with a significant reduction of testicular weight starting at the prepubertal stage (PND 12), and vacuoles and loss of germ cells layers observed within the first wave of spermatogenesis and sustained in the adults. Multiple testicular alterations resulted in subfertility.

To note, the cKO mice of this study showed different levels of abnormalities, and a small number of normal-looking tubules were often observed. Interestingly, we noticed that the overall phenotype turned milder when the initial mixed background was bred into a pure C57/bl6 line. Phenotypic variability on different mouse genetic backgrounds has been well documented and could be explained, most likely, by a variation in the transgene's penetrance [28, 29]. Therefore, it is possible that the subfertility (rather than infertility) phenotype resulted from viable sperm generated by the tubules that escaped the CRE activity. Nevertheless, our findings indicate that TDP-43 plays a crucial role in the Sertoli cells, and that its deletion triggers an atrophic degenerative process in the seminiferous epithelium.

Immunohistochemistry showed that the number of Sertoli cells remained the same in cKO versus controls, despite a marked increase in apoptotic germ cells in cKO mice. This suggests that TDP-43 is not required for the development and persistence of Sertoli cells in the testis, but that the loss of TDP-43 in Sertoli cells hampers the survival of germ cells. Comparison of the progression of the seminiferous cycle in 2 MO cKO and control mice showed that the Sertoli cells requirement of TDP-43 is more pronounced at stages VII–VIII compared to earlier in the cycle. This is consistent with the notion of stage-specific physiology of Sertoli cells [30]. Flow cytometry showed that loss of TDP-43 led to a change in the testicular composition, with a relative increase in the percentage of 2C cells, a population that includes spermatogonia, secondary spermatocytes, and somatic cells, and decrease of 1C cells, a population composed of round spermatids. The presence of sloughed off round spermatids within the lumen of the seminiferous tubules as well as the epididymis explains in part the observation that 1C population decreased in cKO mice.

Use of the acrosomal protein marker SP-10 confirmed that the sloughed off cells within the lumen of the epididymis of the cKO mice are in fact round spermatids. These cells are normally attached to Sertoli cells by specialized cell junctions such as ectoplasmic specializations, composed mainly of N-cadherin [24]. It is interesting to note that N-cadherin as well as Connexin-43, another junction protein involved in Sertoli-germ cells and Sertoli-Sertoli interactions, are more abundant and aberrantly distributed within the seminiferous epithelium of the cKO mice. Consistent with this, qRT-PCR showed that mRNAs coding for N-cadherin, connexin-43, and zonula occludens-1 was overexpressed in cKO mice testes. These findings showed that the deletion of TDP-43 affects the expression of multiple junction proteins. In Sertoli cells, these junction proteins are responsible for crucial functions, such as the attachment of germ cells and the formation and maintenance of the BTB.

The BTB isolates the meiotic and post-meiotic germ cells in the adluminal compartment from the interstitium, blood, and immune system while undergoing extensive reorganization to allow the migration of germ cells from the basal region of the tubules to the lumen during the cycle of the seminiferous epithelium [24]. Thus, the BTB junction proteins must be tightly regulated to ensure proper function of this dynamic structure [24]. We found disruption of BTB as early as at PND 24, around the time of BTB formation, which persisted in adults. Thus, TDP-43 appears to regulate Sertoli-germ cell attachments as well as the formation and/or maintenance of the BTB.

Interestingly, a previous study reported that the blood-spinal cord barrier, which shares several junction proteins with the BTB, such as occludin and ZO-1, was disrupted in cKO mice lacking TDP-43 in spinal cord motor neurons [31]. Although TDP-43 was reported to play different roles in distinct cell types [32, 33], the disruption of the blood-tissue barrier in the testis and blood-spinal cord barrier in the nervous systems indicates that TDP-43 plays a direct or indirect role in controlling junction proteins across different

systems of the body. Future studies investigating the function of TDP-43 in other blood-tissue barriers, such as the blood-placenta, the blood-retina, and the blood-thymus barrier, will be interesting to confirm this hypothesis. In addition, the specific targets of TDP-43 leading to the disruption of BTB or the blood-spinal cord barrier remain to be elucidated, and their identification will be valuable to understand the pathways and mechanisms of action of TDP-43.

In the final stages of spermatogenesis, the elongation of spermatids is followed by a significant cytoplasmic volume reduction and the formation of residual bodies. These densely packed structures are readily phagocytized by Sertoli cells and can be identified by neutral lipid stains [25, 34, 35]. Lipid droplets are also observed following the phagocytosis of apoptotic germ cells [36]. Herein, mice lacking TDP-43 in Sertoli cells showed decreased lipid droplets in the seminiferous tubules, both in the lumen and in Sertoli cell cytoplasm. The luminal lipid droplets indicate residual bodies, and their absence may be a consequence of its diminished formation due to the high apoptosis rate and sloughing of germ cells. Accordingly, reduced lipid droplets in the Sertoli cell cytoplasm may be due to less substrate available after the establishment of testis degeneration in the adults or indicate impaired phagocytosis by the direct or indirect influence of TDP-43 in the phagocytic pathway and/or lipid droplet formation. Consistent with this, we found dysregulation of mRNAs for key proteins involved in phagocytosis; they were upregulated in TDP-43 cKO mice. This increase in mRNA levels could reflect loss of their transcriptional repression or inappropriate splicing leading to increased stability. Aberrant splicing, in particular, could result in loss of protein production and therefore loss of phagocytic activity. It has been previously described that TDP-43 is required for proper lysosomal activity, and lysosomes are essential for the scavenger function of Sertoli cells [24, 36, 37]. Also, it has been reported that TDP-43 controls energy metabolism and lipid storage in adipocytes [17, 38]. These reports suggest a role for TDP-43 in lysosome and lipid droplet formation and offer a plausible explanation for the lower rates of lipid droplets in cKO Sertoli cells in our study. The mechanism by which TDP-43 participates in Sertoli cell phagocytic function remains to be investigated.

TDP-43 has been shown to be a transcription factor as well as a splicing regulator. We observed that 7 out of 10 Sertoli cell transcripts investigated are upregulated in the cKO testes. Alleviation of the previously reported transcriptional repressor function of TDP-43 might be one reason for the upregulated mRNA levels. Alternatively, aberrant splicing leading to irregular exon inclusion/exclusion, as shown for other transcripts, could potentially have caused the upregulation of Sertoli cell transcripts. There could also be indirect effects in which loss of TDP-43 could alter the expression of proteins regulating mRNA processing and stability. Future studies using primary Sertoli cells isolated from cKO mice or RNAi in Sertoli cell lines will be essential to address the mechanism by which TDP-43 regulates gene expression in Sertoli cells.

Overall, our findings suggest that TDP-43 is required in Sertoli cells for the completion of spermatogenesis, formation, and maintenance of the BTB, and the phagocytic function, thus indicating an essential role for TDP-43 in male fertility. Given that 30–40% of male infertility is idiopathic, it will be important to determine the extent to which TDP-43 proteinopathies contribute to male infertility.

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Conflict of interest

The authors have declared that no conflict of interest exists.

Authors' contributions

H.D.Z.: designed experiments, collected and analyzed the data, and wrote the paper; H.O., A.C., J.M.R., M.T.: collected and analyzed the data; P.P.R.: designed and supervised the study analyzed the data, and wrote the paper.

Data availability

The data underlying this article are available in the article.

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