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The ubiquitin ligase subunit β-TrCP in Sertoli cells is essential for spermatogenesis in mice

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ABSTRACT

β-TrCP is the substrate recognition subunit of an SCF-type ubiquitin ligase. We recently showed that deletion of the genes for both β-TrCP1 and β-TrCP2 paralogs in germ cells of male mice resulted in accumulation of the transcription factor DMRT1 and spermatogenic failure, whereas systemic β-TrCP1 knockout combined with β-TrCP2 knockdown had previously been shown to lead to disruption of testicular organization and accumulation of the transcription factor SNAIL. Here we investigated β-TrCP function in Sertoli cells by generating mice with targeted deletion of the β-TrCP2 gene in Sertoli cells on a background of whole-body β-TrCP1 knockout. Loss of β-TrCP in Sertoli cells caused infertility due to a reduction in the number of mature sperm. Whereas spermatogonia were not affected, male germ cells entered meiosis prematurely and the number of round spermatids was reduced in the mutant mice. Extracts of Sertoli cells and of the testis from the mutant mice manifested accumulation of SNAIL, and expression of the SNAIL target gene for E-cadherin was down-regulated in Sertoli cells from these animals. Our results indicate that β-TrCP in Sertoli cells regulates Sertoli cell–germ cell interaction through degradation of SNAIL, with such regulation being critical for sperm development.

1. Introduction

Protein ubiquitylation is essential for a wide variety of cellular regulatory processes (Glickman and Ciechanover, 2002; Nakagawa and Nakayama, 2015). Proteins subject to ubiquitylation are selected by E3 ubiquitin ligases that are either single polypeptides or multisubunit complexes. One of the best studied types of multisubunit E3 ligase is the SCF (SKP1–CUL1–F-box protein) complex, in which the scaffold proteins SKP1 and CUL1 bridge ROC1 and an interchangeable F-box protein. ROC1 interacts with a loaded ubiquitin-conjugating E2 enzyme whereas the F-box protein binds to its respective substrates, resulting in juxtaposition of ubiquitin and the substrate protein and consequent ubiquitylation of the latter. The targeting of its substrate proteins for proteasomal degradation by the SCF complex is required for many cellular processes including proliferation and differentiation (Petroski and Deshaies, 2005).

β-Transducin repeat–containing protein (β-TrCP) is one of the ~25 F-box proteins that are conserved from insects to humans (Jin et al., 2004). Mammalian cells express two distinct paralogs of β-TrCP—β-TrCP1 (also known as BTRC) and β-TrCP2 (also known as FBXW11)—that had been thought to function redundantly as a result of their targeting of the same substrates (Frescas and Pagano, 2008; Fuchs et al., 2004; Nakayama and Nakayama, 2006). To investigate the physiological functions of β-TrCP, we and others previously generated mice with targeted deletion of either the β-TrCP1 or β-TrCP2 gene. The mice deficient in β-TrCP1 either appeared normal (Nakayama et al., 2003) or manifested moderate disruption of spermatogenesis and subfertility in males (Guardavaccaro et al., 2003), whereas those deficient in β-TrCP2 died during embryogenesis as a result of abnormal development of extraembryonic tissue (Nakagawa et al., 2015). These observations were thus indicative of a functional distinction between β-TrCP paralogs, at least in some tissues.

Mammalian spermatogenesis can be divided into three phases: a mitotic expansion phase of diploid spermatogonia, a meiotic phase of spermatocytes, and a postmeiotic reconstruction (spermiogenesis) phase of spermatids, all of which lead to the production of mature sperm (Cooke and Saunders, 2002; Jan et al., 2012). In mice, the first wave of spermatogenesis begins with the appearance of spermatogonia in the testis shortly after birth (Meikar et al., 2011). Spermatogonia, the self-renewing spermatogenic stem cells that are maintained throughout life, differentiate into spermatocytes that first enter meiosis around day 10 postpartum (10 dpp). Meiosis, which takes about 10 days, generates round spermatids at around the time of weaning (20 dpp). Round spermatids then turn into elongated spermatids

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during the next 2–3 weeks, with mature sperm being released into the lumen of the seminiferous tubules from ~35 dpp.

All of these steps of spermatogenesis depend on the structural and nutritive support provided by Sertoli cells that surround the male germ cells (Franca et al., 2016). The association of germ cells with Sertoli cells is mediated by several adhesion complexes (Cheng and Mruk, 2012; Goossens and van Roy, 2005). The combination of β-TrCP1 knockout and β-TrCP2 conditional knockdown in the whole body of adult male mice was previously shown to result in pronounced disruption of testicular organization. This testicular phenotype was attributed to testicular accumulation of the β-TrCP substrate SNAIL, given that it was reversed by knockdown of this protein alone (Kanarek et al., 2010). However, we recently found that loss of both β-TrCP paralogs in germ cells of male mice did not result in the accumulation of SNAIL in these cells, even though these mice were completely sterile. Indeed, we identified DMRT1 (Doublesex- and Mab3-related transcription factor 1) as a novel substrate of β-TrCP and showed that the accumulation of DMRT1 in germ cells of the mutant mice was responsible at least in part for the spermatogenic failure (Nakagawa et al., 2017). Given the widespread expression of β-TrCP1 and β-TrCP2 (β-TrCP1/2) in the various testicular cell types as well as the apparent discrepancy between our previous findings and those of others, we hypothesized that SNAIL might be regulated by β-TrCP1/2 in testicular cells other than germ cells and that β-TrCP1/2 in such cells might also play a key role in male germ cell development via regulation of cell-cell contact or of the production of paracrine factors.

To examine the possibility that β-TrCP1/2 in Sertoli cells participate in germ cell development, we generated mice in which the genes for both paralogs are deleted specifically in Sertoli cells. Although we did not detect any abnormality in Sertoli cells of the mutant mice by optical microscopy, the loss of β-TrCP1/2 in these cells gave rise to sterility associated with a marked reduction in the number of male germ cells. We also observed premature entry of male germ cells into meiosis as well as a pronounced reduction in the number of spermatids in the mutant mice. Loss of β-TrCP1/2 resulted in the accumulation of SNAIL in isolated Sertoli cells as well as the down-regulation of E-cadherin expression at the periphery of seminiferous tubules. Our results indicate that β-TrCP1/2 regulate the interaction between Sertoli cells and germ cells through degradation of SNAIL in Sertoli cells, with such regulation being critical for male germ cell development.

2. Materials and methods

2.1. Generation of mutant mice

β-TrCP1 knockout mice and mice with a floxed exon 5 of the β-TrCP2 gene on the C57BL/6J background were described previously (Nakagawa et al., 2015; Nakayama et al., 2003). *Amh-Cre* [129 S.FVB-Tg(Amh-cre)8815Reb/J] mice (Holdcraft and Braun, 2004) and ROSA26-EYFP [B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J] mice (Srinivas et al., 2001) were obtained from The Jackson Laboratory. All mice investigated in the present study were on the mixed background. Mice were maintained under the specific pathogen–free condition at the Institute of Animal Experimentation, Tohoku University Graduate School of Medicine, with water and rodent chow provided ad libitum. They were treated according to the regulations of the Standards for Humane Care and Use of Laboratory Animals of Tohoku University and the Guidelines for Proper Conduct of Animal Experiments of the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

2.2. Histology and immunofluorescence analysis

Testes were dissected, fixed for 16 h at 4 °C with 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated, embedded in paraffin, and sectioned at a thickness of 5 μm. For E-cadherin staining, 0.5 mM $CaCl_2$ was added in the fixation solution. The sections were subjected to periodic acid–Schiff (PAS) staining, to TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling) staining, or to immunofluorescence analysis as previously described (Nakagawa et al., 2017). For immunostaining, sections were boiled for 5 min in 20 mM sodium citrate buffer (pH 6.0) and then incubated consecutively for 16 h at 4 °C in PBS containing 3% bovine serum albumin and 0.3% Triton X-100, for 16 h at 4 °C with primary antibodies, and for 1 h at room temperature with Alexa Fluor 488– or Alexa Fluor 546–conjugated secondary antibodies (A-11008 and A-11030, respectively, Thermo Fisher Scientific). The sections were washed with PBS containing 0.1% Triton X-100, incubated for 10 min at room temperature with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/ml), and then examined with a BZ9000 fluorescence

Table 1
Fertility of male β-TrCP knockout mice.

Genotype	Number of fertile mice/total	Litter size (mean ± SEM)[a]
Control	7/7	6.33 ± 2.09
β-TrCP2 CKO	7/7	7.00 ± 2.28
β-TrCP1/2 DKO	0/7	0

[a] Resulting from crosses of 8-week-old male mice of the indicated genotypes with WT females.

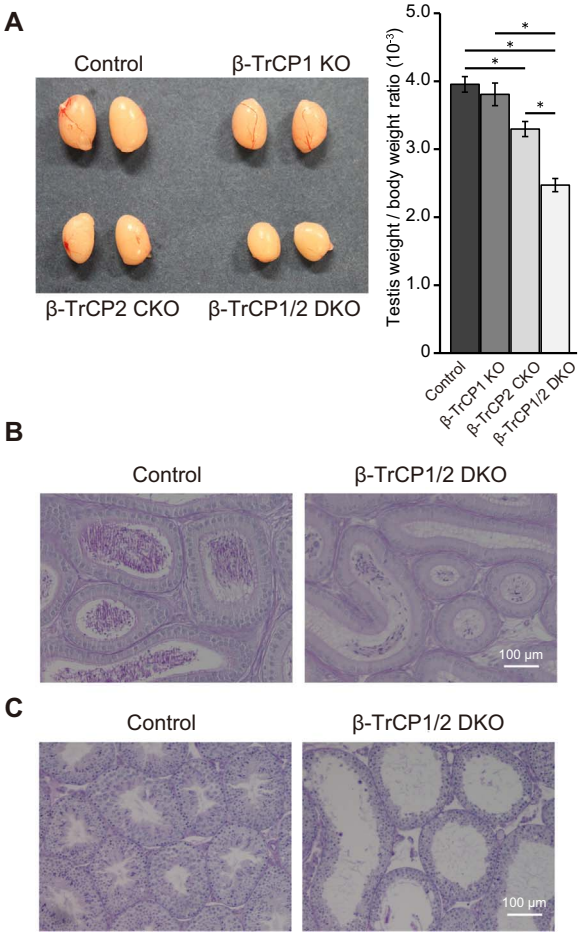

Fig. 1. Spermatogenic defect in male β-TrCP1/2 DKO mice. (A) Testis size and weight for mice of the indicated genotypes (control, *Amh-Cre* transgene only) at 8 weeks of age. Quantitative data are means ± SEM for five mice of each genotype. *p < 0.05 (one-way ANOVA followed by Tukey's post hoc test). (B, C) PAS staining of epididymis tubules (B) and seminiferous tubules (C) of 8-week-old control (*Amh-Cre*) and β-TrCP1/2 DKO mice. Scale bars, 100 μm.

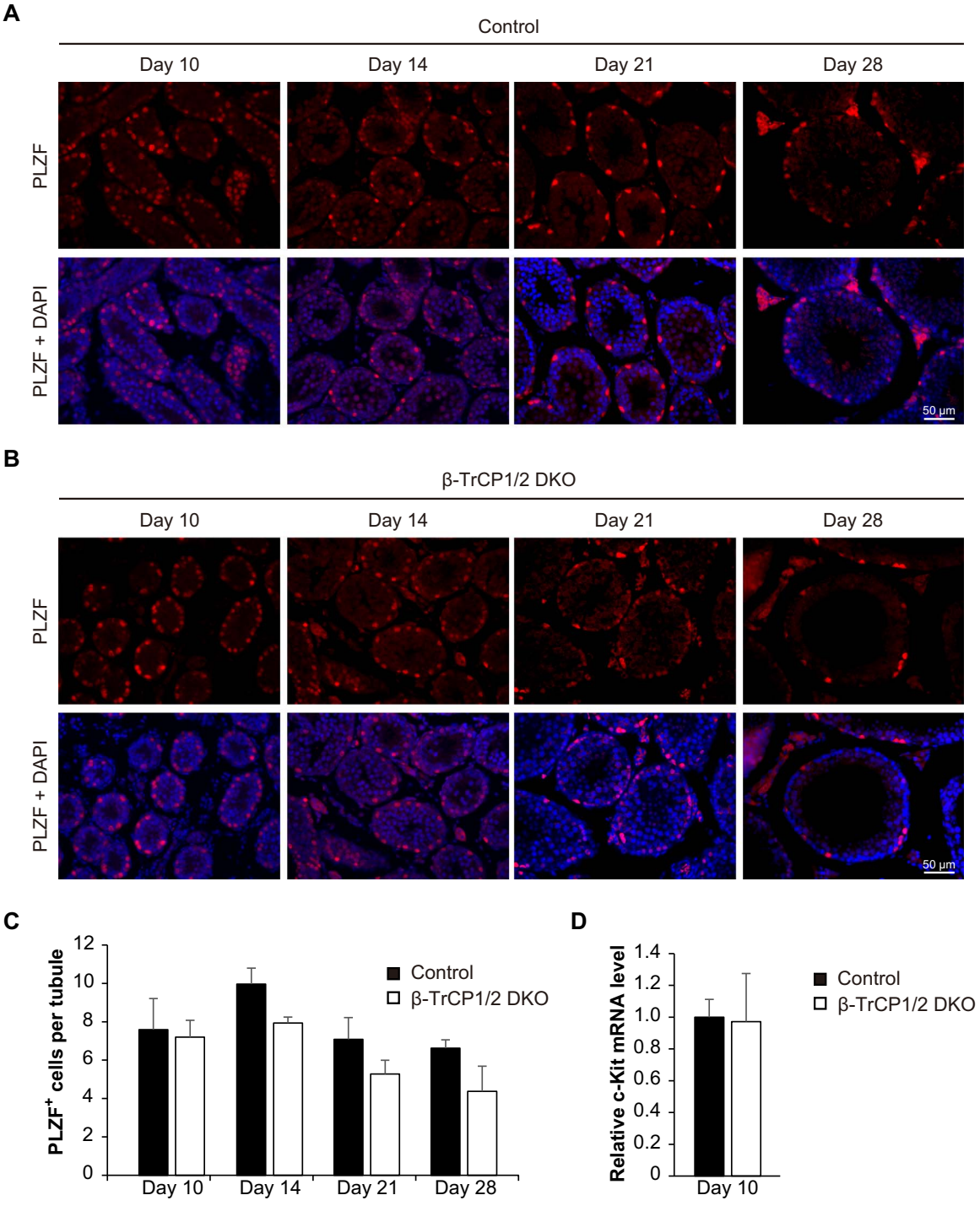

Fig. 2. Lack of effect of β-TrCP1/2 loss on spermatogonia in seminiferous tubules of male mice. (A, B) Immunofluorescence staining of PLZF alone (upper panels) or together with DAPI staining of nuclei (lower panels) in seminiferous tubules of control (*Amh-Cre*) (A) or β-TrCP1/2 DKO (B) male mice at 10, 14, 21, and 28 dpp. Scale bars, 50 μm. (C) Number of $PLZF^+$ cells per seminiferous tubule for control and β-TrCP1/2 DKO mice at the indicated ages as determined from images similar to those in (A) and (B). Data are means ± SEM for three mice of each group. Statistically significant differences were not detected by two-way ANOVA. (D) RT-qPCR analysis of c-Kit mRNA abundance (normalized by that of β-actin mRNA) in the testis of β-TrCP1/2 DKO mice at 10 dpp. Data are expressed relative to the value for control (*Amh-Cre*) mice and are means ± SEM for three mice of each genotype.

microscope (Keyence) or a DeltaVision fluorescence microscopy system (GE Healthcare). Antibodies to PLZF (sc-22839) were obtained from Santa Cruz Biotechnology; those to SYCP3 (ab97672), to STRA8 (ab49602), and to WT1 (ab89901) were from Abcam; those to TNP1 (17178-1-AP) were from Proteintech; those to E-cadherin (610181) were from BD Biosciences and Takara Bio (ECCD-2); those to GFP (598) were from MBL; and those to ZO-1 (61–7300) were from Thermo Fisher Scientific.

2.3. Whole mount immunofluorescence

Whole mount immunofluorescence of seminiferous tubules was performed as described previously (Nakagawa et al., 2010; Endo et al., 2015) with some modification. Seminiferous tubules were dissected from testes and fixed for 16 h at 4 °C with 4% paraformaldehyde and 0.5 mM $CaCl_2$ in phosphate-buffered saline (PBS). The samples were attached to MAS-coated slide glass by half-drying. After incubation in

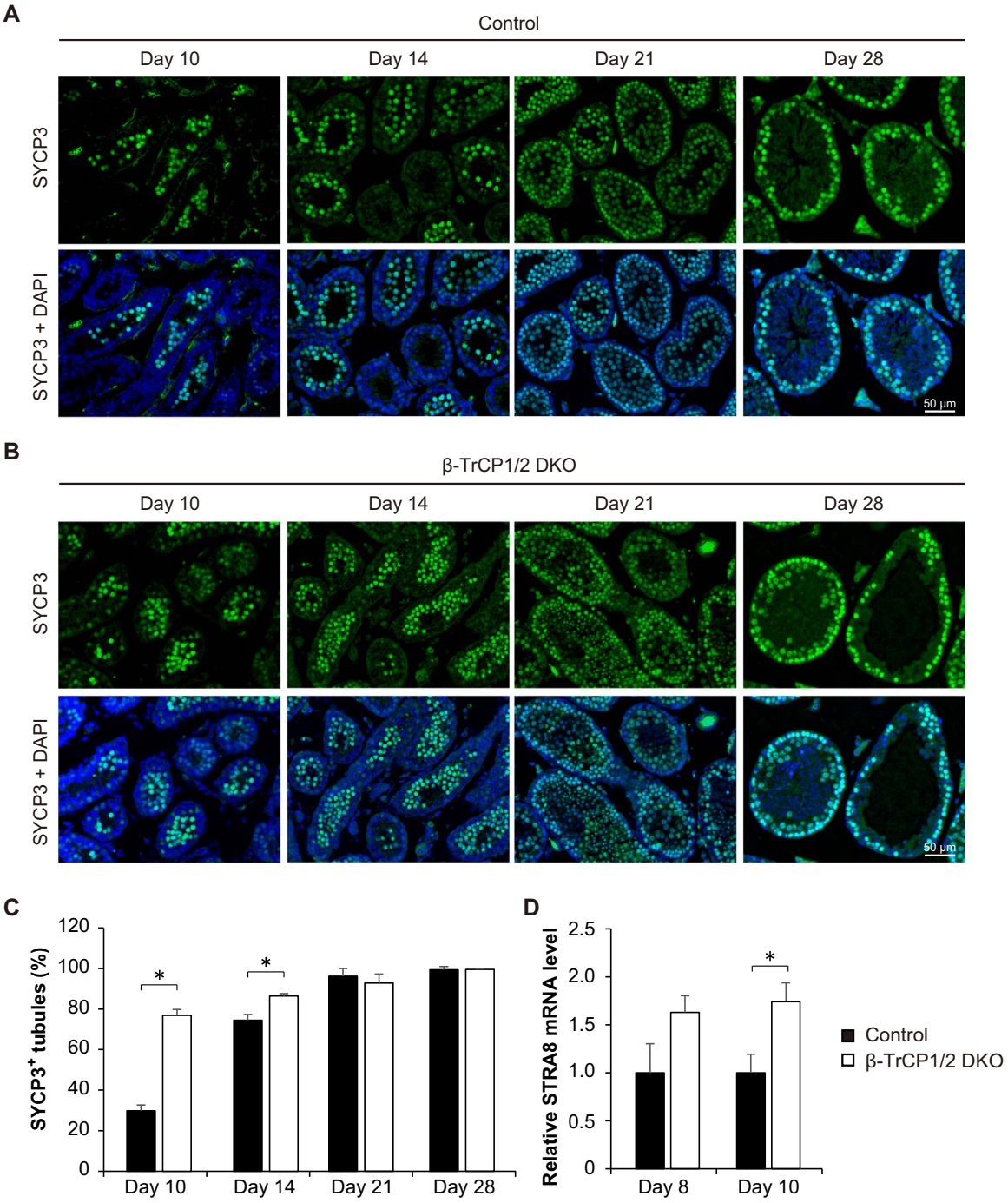

Fig. 3. Early entry of germ cells into meiosis in seminiferous tubules of β-TrCP1/2 DKO mice. (A, B) Immunofluorescence staining of SYCP3 alone (upper panels) or together with DAPI staining of nuclei (lower panels) in seminiferous tubules of control (*Amh-Cre*) (A) or β-TrCP1/2 DKO (B) male mice at 10, 14, 21, and 28 dpp. Scale bars, 50 μm. (C) Number of seminiferous tubules containing $SYCP3^+$ cells for control and β-TrCP1/2 DKO mice at the indicated ages as determined from images similar to those in (A) and (B). Data are means ± SEM for three mice of each group. *$p < 0.05$ (two-way ANOVA followed by Tukey's post hoc test). (D) RT-qPCR analysis of STRA8 mRNA abundance (normalized by that of β-actin mRNA) in the testis of β-TrCP1/2 DKO mice at 8 and 10 dpp. Data are expressed relative to the value for control (*Amh-Cre*) mice and are means ± SEM for three (8 dpp) or four (10 dpp) mice of each genotype.

0.2% NP40 in PBS for 20 min, the samples were dehydrated through a methanol series (25%, 50%, 75%, and 100% in PBS containing 0.1% Tween 20 [PBS-T]) on ice, followed by rehydration in PBS-T. After blocking with 1% bovine serum saline (BSA) and 4% donkey serum in PBS-T for 1 h, specimens were incubated in 1% BSA, 4% donkey serum and 0.5 mM $CaCl_2$ in PBS-T containing anti-E-cadherin antibody (1:50, ECCD-2, Takara) and anti-PLZF antibody (1:50, sc-22839, Santa Cruz Biotechnology) at 4 °C overnight. After washing with PBS-T, Alexa Fluor 488– or Alexa Fluor 546–conjugated secondary antibodies (A-11008 and A-11030, respectively, Thermo Fisher Scientific) were applied to the samples at room temperature for 2 h. After washing with PBS-T, specimens were mounted in ProLong Gold Antifade mountant (P36935, Thermo Fisher Scientific) and then examined with a LSM780 confocal microscope (Zeiss).

2.4. Isolation of RNA and RT-qPCR analysis

RNA was isolated and purified with the use of an SV Total RNA

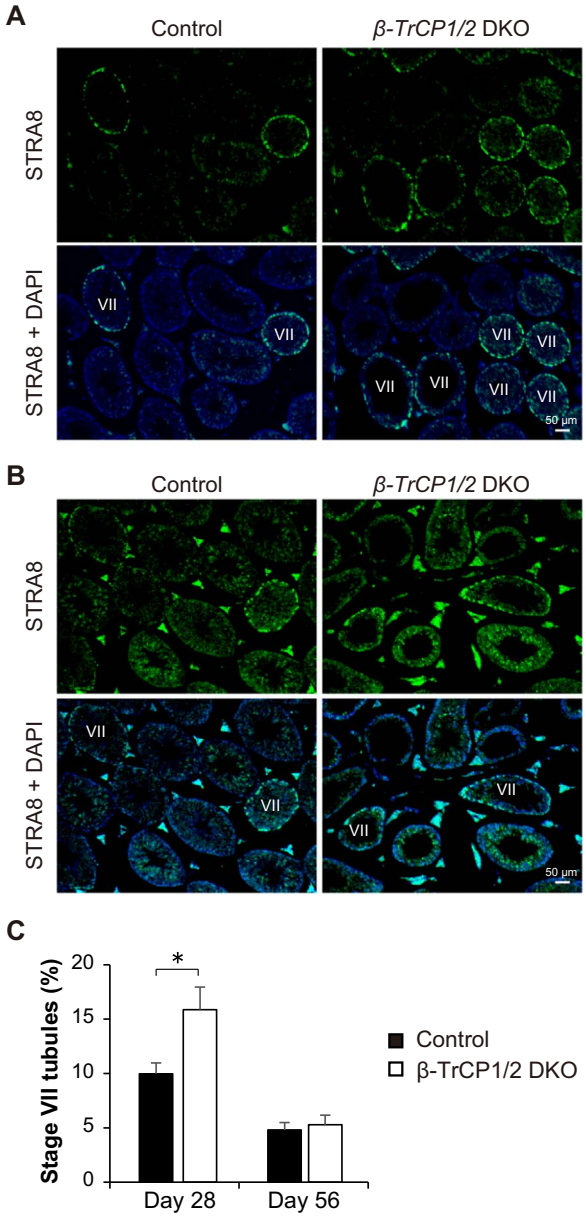

Fig. 4. Increased number of stage VII seminiferous tubules in β-TrCP1/2 DKO mice at 28 dpp. (A, B) Immunofluorescence staining of STRA8 alone (upper panels) or together with DAPI staining of nuclei (lower panels) in seminiferous tubules of control (*Amh-Cre*) or β-TrCP1/2 DKO male mice at 28 dpp (A) and 56 dpp (B). Seminiferous tubules at stage VII of development are indicated. Scale bars, 50 μm. (C) Number of stage VII seminiferous tubules for control and β-TrCP1/2 DKO mice at the indicated ages as determined from images similar to those in (A) and (B). Data are means ± SEM for four mice of each group. *p < 0.05 (unpaired Student's *t*-test).

Isolation System (Promega) and was then subjected to reverse transcription (RT) with the use of a PrimeScript RT reagent kit (Takara Bio) followed by real-time polymerase chain reaction (PCR) analysis with a StepOnePlus Real Time PCR System (Life Technologies) and Fast SYBR Green Master Mix (Life Technologies). Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method and were normalized by the amount of β-actin or WT1 mRNA. The sequences (5′-3′) of PCR primers (forward and reverse, respectively) were GCCACGTCTCAGCCATCTG and GTCGCCAG CTTCAACTATTAACT for c-Kit, TTCCTGCGTGTTCCACAAGT and ACTGGGTTGGTTGCCTTCTC for STRA8, AGTGTGACGTTGACA TCCGTA and GCCAGAGCAGTAATCTCCTTCT for β-actin, TGCT GACCGCTCCAACCT and CTTCACATCCGAGTGGGTTTG for SNAIL, AGCTGCCCCGAAAATGAAA and GATCTGAACCAGGTTCTTTGGAAA for E-cadherin, GCAAGACCCTAGGCAAGCTGT and TCTGGGTGG TCTTTCTTGTGC for SOX8, AGGAAGCTGGCAGACCAGTA and CG TTCTTCACCGACTTCCTC for SOX9, and CCAGTGTAAAACTTG TCAGCGAAA and ATGAGTCCTGGTGTGGGTCTTC for WT1.

2.5. Isolation and primary culture of Sertoli cells

Sertoli cells were isolated from the testis of mice at 14 dpp as previously described (Hasegawa et al., 2012; Karl and Griswold, 1990) and were then cultured at 34 °C in Dulbecco's modified Eagle's medium (DMEM)–F12 supplemented with insulin (10 μg/ml) (Thermo Fisher Scientific), transferrin (5 μg/ml) (Sigma), and epidermal growth factor (5 ng/ml) (Becton Dickinson). The medium was refreshed after culture for 2 days, and the cells were harvested for analysis at day 4.

2.6. Sperm count and motility

The cauda epididymis was dissected from 10-week-old male mice, and spermatozoa were isolated in TYH medium [119.37 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.07 mM $NaHCO_3$, 1.0 mM sodium pyruvate, 5.56 mM glucose, 0.4% bovine serum albumin, penicillin (50 U/ml), streptomycin (50 μg/ml), 0.1% phenol red]. After incubation for 30 min at 37 °C under 5% CO_2, the sperm suspension was collected and diluted for quantitative assessment. Movies of motile spermatozoa were acquired with a BZ9000 microscope (Keyence).

2.7. Immunoblot analysis

Testes were homogenized in a solution containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 1 mM EDTA, protease inhibitors [aprotinin (10 μg/ml) (Sigma), leupeptin (10 μg/ml) (Peptide Institute), 1 mM phenylmethylsulfonyl fluoride], and phosphatase inhibitors [0.4 mM sodium orthovanadate, 0.4 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate], and the homogenate was then diluted 1:1 with 2x radioimmunoprecipitation assay (RIPA) buffer [1x: 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease and phosphatase inhibitor cocktails]. Total lysates of Sertoli cells were prepared with 1x RIPA buffer. Crude tissue homogenates and cell lysates were centrifuged at 20,000×*g* for 15 min at 4 °C, and the resulting supernatants were subjected to SDS-polyacrylamide gel electrophoresis for immunoblot analysis. Primary antibodies included those to SNAIL (3895) from Cell Signaling Technology, those to EMI1 (sc-50927) and to DMRT1 (sc-377167) from Santa Cruz Biotechnology, and those to WT1 (ab89901) and to β-actin (ab8227) from Abcam. Immune complexes were detected with horseradish peroxidase–conjugated antibodies to mouse (W4021) or rabbit (W4011) immunoglobulin (Promega).

2.8. Statistical analysis

Data are presented as means ± SEM and were compared with the unpaired Student's *t*-test or by one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. β-TrCP1/2 ablation in Sertoli cells gives rise to sterility and impaired spermatogenesis

To study β-TrCP1/2 function in Sertoli cells, we set out to generate Sertoli cell–specific β-TrCP1/2 double-knockout (DKO) mice. To this end, we first generated Sertoli cell–specific β-TrCP2 conditional knockout (β-TrCP2 CKO) animals by crossing mice in which exon 5 of the β-TrCP2 gene is flanked by loxP sequences (β-TrCP2$^{F/F}$ mice) (Nakagawa et al., 2015) with *Amh-Cre* mice, in which Cre recombinase is expressed under the control of the mouse anti-Müllerian hormone gene promoter in pre-Sertoli

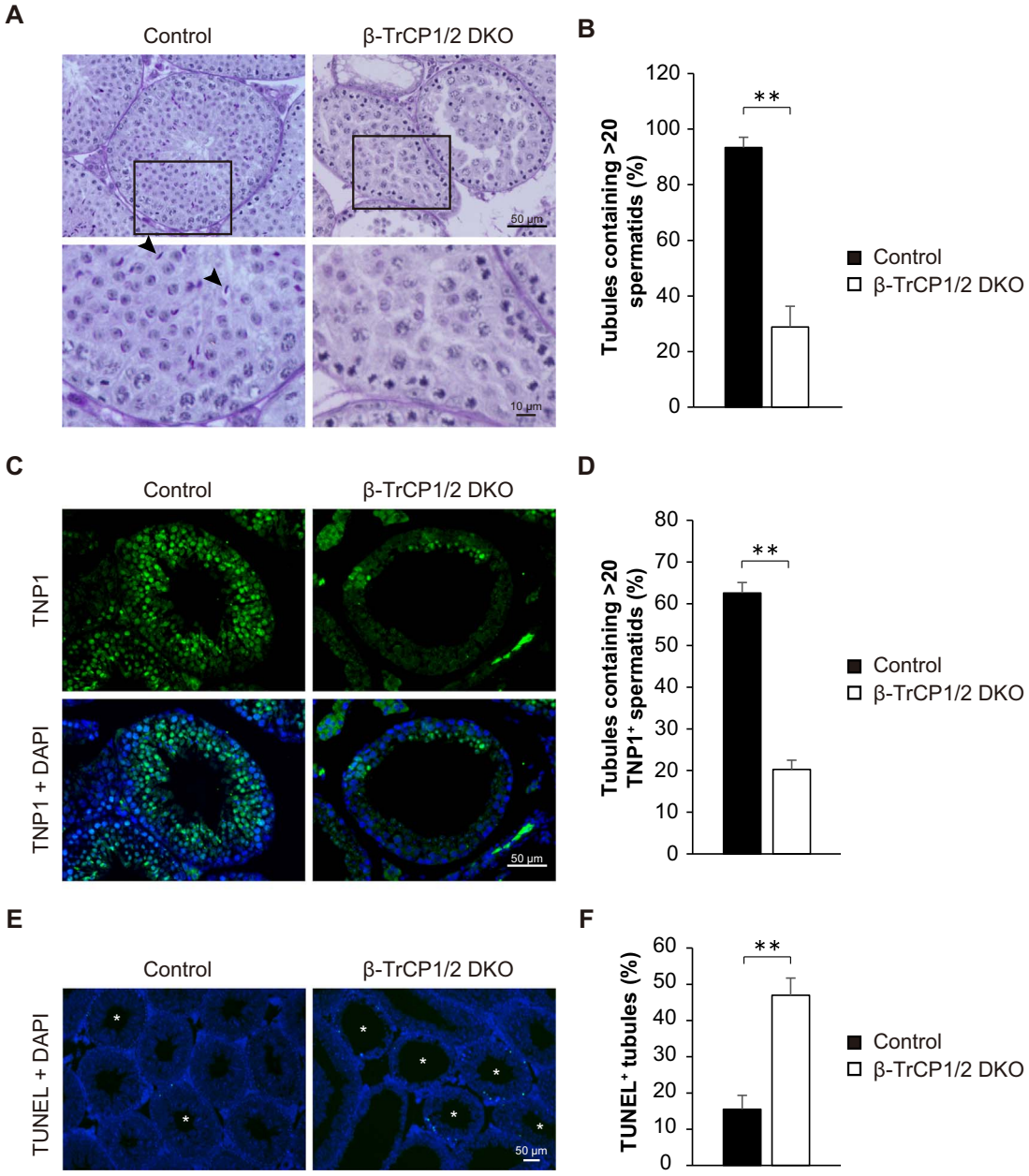

Fig. 5. Reduced number of spermatids in seminiferous tubules of β-TrCP1/2 DKO mice. (A) PAS staining of seminiferous tubules of control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 28 dpp. The boxed regions of the upper panels are shown at higher magnification in the lower panels. Arrowheads indicate elongated spermatids. Scale bars, 50 μm (upper panels) or 10 μm (lower panels). (B) Number of seminiferous tubules containing > 20 spermatids determined from images similar to those in (A). Data are means ± SEM for four mice of each group. **p < 0.01 (unpaired Student's *t*-test). (C) Immunofluorescence staining of TNP1 alone (upper panels) or together with DAPI staining of nuclei (lower panels) in seminiferous tubules of control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 28 dpp. Scale bar, 50 μm. (D) Number of seminiferous tubules containing > 20 $TNP1^+$ spermatids determined from images similar to those in (C). Data are means ± SEM for four mice of each group. **p < 0.01 (unpaired Student's *t*-test). (E) TUNEL and DAPI staining of seminiferous tubules in control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 28 dpp. Asterisks indicate TUNEL-positive tubules. Scale bar, 50 μm. (F) Percentage of TUNEL-positive tubules determined from images similar to those in (E). Data are means ± SEM for four mice of each group. **p < 0.01 (unpaired Student's *t*-test).

and mature Sertoli cells from embryonic day 14.5 (Holdcraft and Braun, 2004). Exon 5 of the β-TrCP2 gene encodes the F-box domain required for binding to the SKP1-CUL1 scaffold, and its deletion results in a frameshift and generation of a premature stop codon. Mating of β-TrCP2 CKO male mice with wild-type (WT) females gave rise to similar numbers of live pups as did that of control (*Amh-Cre* transgene only) male mice (Table 1), indicating that loss of β-TrCP2 function in Sertoli cells did not affect spermatogenesis, likely as a result of functional compensation by β-TrCP1. We then crossed $\beta\text{-TrCP2}^{F/F}$ mice, $\beta\text{-TrCP1}^{-/-}$ mice (Nakayama et al., 2003), and *Amh-Cre* mice to yield $\beta\text{-TrCP1}^{-/-}$;$\beta\text{-TrCP2}^{F/F}$;*Amh-Cre* (β-TrCP1/2 DKO) male mice. Mating of these males with WT female mice revealed that they were completely sterile (Table 1). Furthermore, their testes were significantly smaller than those of control and β-TrCP2 CKO mice (Fig. 1A). Histological analysis revealed a marked reduction in the number of sperm in the epididymis (Fig. 1B) and seminiferous tubules (Fig. 1C) of male β-TrCP1/2 DKO mice. On the basis of these data, we concluded that β-TrCP in Sertoli cells is essential for spermatogenesis and that the β-TrCP paralogs are functionally redundant with regard to this function.

3.2. Loss of β-TrCP1/2 in Sertoli cells does not affect spermatogonia

To determine at which developmental phase loss of β-TrCP1/2 impairs spermatogenesis, we examined male germ cells at distinct

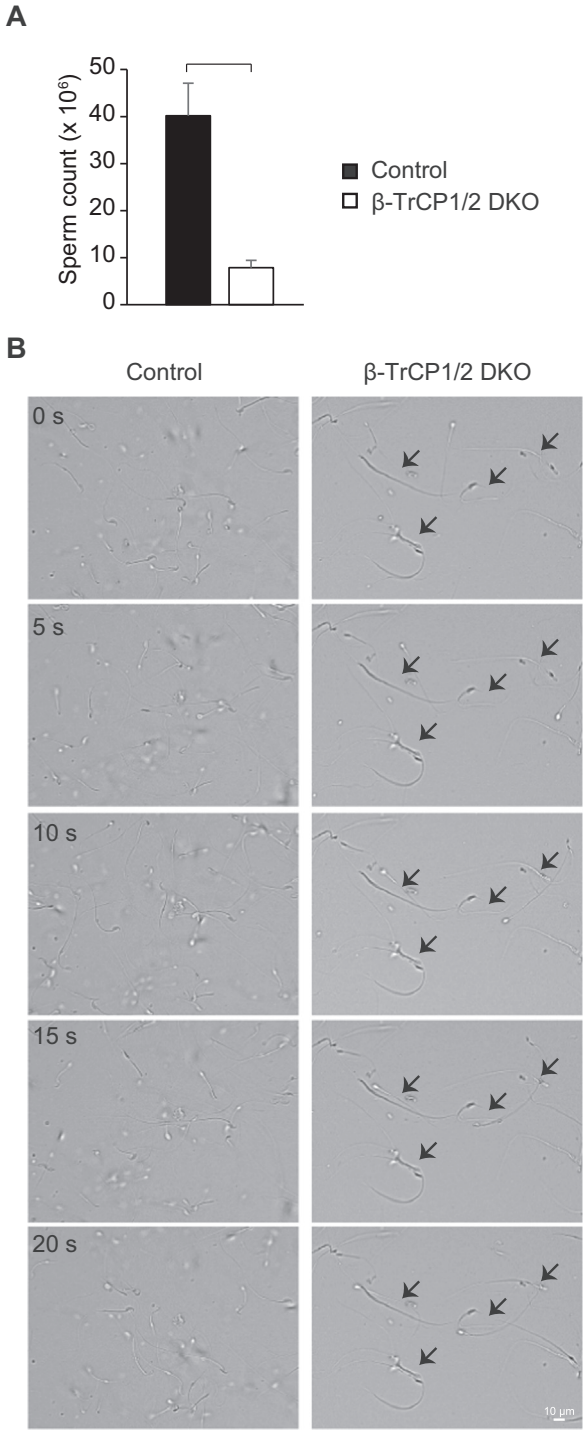

Fig. 6. Reduced number and immotility of spermatozoa in β-TrCP1/2 DKO mice. (A) Sperm count for the cauda epididymis of control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 70 dpp. Data are means ± SEM for three mice of each group. *p < 0.05 (unpaired Student's *t*-test). (B) Time-lapse phase-contrast microscopic images at 5-s intervals of spermatozoa isolated as in (A). Arrows indicate immotile spermatozoa. Scale bar, 10 μm.

differentiation stages characterized by marker expression. Immunofluorescence staining showed that PLZF (promyelocytic leukemia zinc finger), a marker of undifferentiated spermatogonia, was expressed similarly in germ cells of control and β-TrCP1/2 DKO male mice from 10 to 28 dpp (Fig. 2A–C). RT and quantitative PCR (qPCR) analysis revealed that expression of the gene for c-Kit, a marker of differentiating spermatogonia, was also not altered in the testis of β-TrCP1/2 DKO mice (Fig. 2D). These data thus suggested that β-TrCP1/2 in Sertoli cells are not required for the production, maintenance, or differentiation of spermatogonia.

3.3. Loss of β-TrCP1/2 in Sertoli cells advances meiotic entry of male germ cells

We next examined spermatocyte meiosis in β-TrCP1/2 DKO mice at 10–28 dpp. Male germ cells positive for SYCP3 (synaptonemal complex protein 3), a marker of meiotic prophase, were detected in seminiferous tubules of both control and β-TrCP1/2 DKO mice (Fig. 3A, B). However, the number of SYCP3-positive cells was increased markedly in β-TrCP1/2 DKO mice at 10 dpp and moderately but significantly at 14 dpp (Fig. 3C). The amount of STRA8 (stimulated by retinoic acid 8) mRNA also tended to be increased at 10 dpp and was significantly increased at 14 dpp in the mutant mice (Fig. 3D), with STRA8 being required for meiotic entry (Anderson et al., 2008; Mark et al., 2008). Increased meiotic entry as evidenced by the number of stage VII seminiferous tubules (Matson et al., 2010) was also apparent in the mutant mice at 28 dpp, but not at 56 dpp (Fig. 4). These data suggested that β-TrCP1/2 in Sertoli cells are necessary to restrain male germ cells from premature entry into meiosis at the early stage of spermatogenesis during development. The number of meiotic cells was not reduced in adult β-TrCP1/2 DKO mice, indicating that spermatogenesis is impaired after the meiotic stage.

3.4. Loss of β-TrCP1/2 in Sertoli cells reduces the number of spermatids

The first postmeiotic male germ cells are round spermatids that differentiate further into elongated spermatids. Compared with control mice, round spermatids at 28 dpp were found to be scarce in the testis of β-TrCP1/2 DKO mice (Fig. 5A, B). Whereas some seminiferous tubules contained elongated spermatids at this stage in control mice (Fig. 5A), such cells were rarely detected in the testis of β-TrCP1/2 DKO mice, indicative of impairment of the differentiation of round spermatids into elongated spermatids in the mutant animals. Immunostaining of TNP1 (transition protein 1), a marker of elongating spermatids, confirmed these observations made with histological staining (Fig. 5C, D). The TUNEL assay revealed that the number of apoptotic cells was significantly increased in the β-TrCP1/2 DKO mouse testis (Fig. 5E, F). Together, these results thus suggested that β-TrCP1/2 in Sertoli cells are important for the survival of spermatids. These phenotypes were similar to those of mice with simultaneous whole-body β-TrCP1 knockout and β-TrCP2 knockdown (Kanarek et al., 2010).

3.5. Loss of β-TrCP1/2 in Sertoli cells reduces the number and motility of mature sperm

We then isolated mature sperm from the cauda epididymis of male mice at 70 dpp. Consistent with the observed decrease in the number of spermatids in seminiferous tubules (Fig. 5A), the number of mature sperm was also significantly reduced in β-TrCP1/2 DKO mice (Fig. 6A). Although we could detect mature sperm in the mutant mice, most of these cells were immotile (Fig. 6B), suggesting that β-TrCP1/2 in Sertoli cells are essential for the generation of motile sperm.

3.6. Loss of β-TrCP1/2 in Sertoli cells does not affect Sertoli cell survival

We next examined whether the reduction in the number of spermatids in β-TrCP1/2 DKO mice might result from a loss or dysfunction of Sertoli cells. Immunostaining of seminiferous tubules for the Sertoli cell marker WT1 (Wilms tumor 1) did not reveal any difference between control and β-TrCP1/2 DKO mice (Fig. 7), indicating that β-TrCP1/2 are not essential for the survival of Sertoli cells but

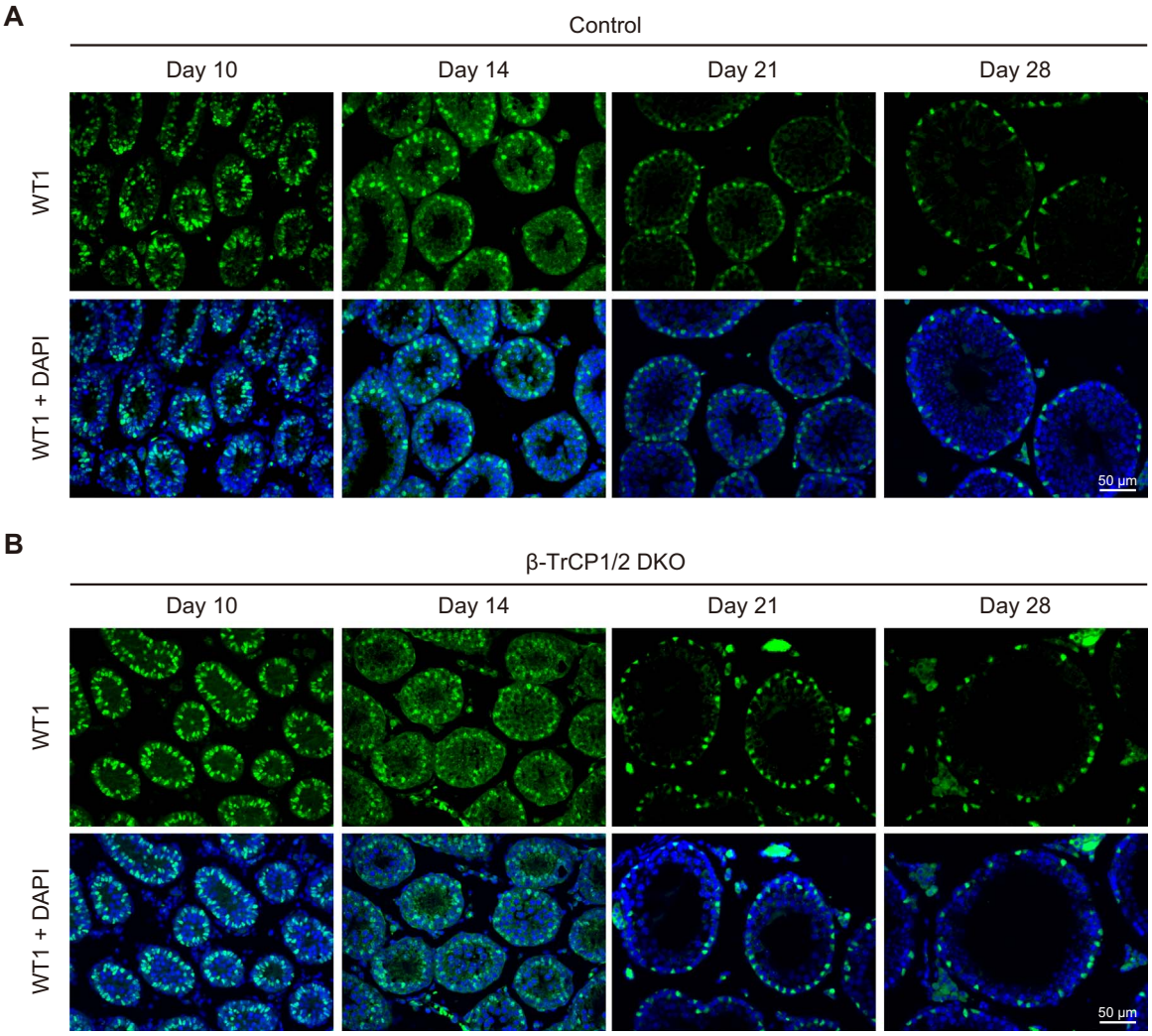

Fig. 7. No difference in Sertoli cell number between β-TrCP1/2 DKO and control mice. Immunofluorescence staining of WT1 alone (upper panels) or together with DAPI staining of nuclei (lower panels) was performed for seminiferous tubules of control (*Amh-Cre*) (A) and β-TrCP1/2 DKO (B) mice at 10, 14, 21, and 28 dpp. Scale bars, 50 μm.

rather for their function with regard to the support of round spermatids.

3.7. Loss of β-TrCP1/2 in Sertoli cells results in the accumulation of SNAIL

SNAIL is a major substrate of β-TrCP1/2 in adult mouse testis, as indicated by the observations that SNAIL accumulated in the testis of β-TrCP1/2–deficient mice and that knockdown of SNAIL was sufficient to rescue the generation of mature sperm in these mice (Kanarek et al., 2010). However, we were not able to detect the accumulation of SNAIL in β-TrCP1/2–deficient male germ cells (Nakagawa et al., 2017), raising the possibility that SNAIL is targeted for degradation by β-TrCP1/2 in Sertoli cells. To test this possibility, we isolated and cultured Sertoli cells from β-TrCP1/2 DKO mice and then examined the abundance of SNAIL by immunoblot analysis. The amount of SNAIL in β-TrCP1/2–deficient Sertoli cells was significantly increased compared with that in control Sertoli cells (Fig. 8A, B). We also detected the accumulation of DMRT1 in β-TrCP1/2–deficient Sertoli cells, similar to our previous observation with male germ cells (Nakagawa et al., 2017), whereas EMI1 (early mitotic inhibitor 1), a substrate of β-TrCP1/2 expressed in the testis (Verschuren et al., 2007), as well as β-actin, which is not a substrate of β-TrCP1/2, showed no such accumulation. The activity of accumulated SNAIL in the β-TrCP1/2–deficient Sertoli cells was indicated by the associated down-regulation of the expression of the E-cadherin gene, which is subject to negative transcriptional regulation by SNAIL (Fig. 8C). In contrast, expression of the DMRT1 target genes for SOX8 and SOX9 (Matson et al., 2011), which are positively regulated by DMRT1, was not increased in the mutant cells (Fig. 8C), indicating that the activity of DMRT1 was not enhanced in these cells. The amount of SNAIL mRNA was also not altered in Sertoli cells from β-TrCP1/2 DKO mice (Fig. 8C), consistent with the notion that β-TrCP1/2 mediate the degradation of SNAIL. The accumulation of SNAIL was also apparent in extracts prepared from the testis of β-TrCP1/2 DKO mice to an extent similar to that observed in isolated Sertoli cells (Fig. 8D, E).

E-cadherin is reported to be a marker of spermatogonial stem cells (Tokuda et al., 2007; Tolkunova et al., 2009). Consistently, immunofluorescence analysis showed the expression of E-cadherin at the periphery of PLZF-positive spermatogonia, including lateral region where spermatogonia were aligned and luminal region where spermatogonia were not linked (Fig. 8F). With the use of genetic marking of Sertoli cell cytoplasm by ROSA26-EYFP mice (Garcia et al., 2014), we found E-cadherin at luminal side of spermatogonia was located at the boundary of spermatogonia and Sertoli cells (Fig. 8G). A reduction of E-cadherin was apparent at the boundary of both spermatogonium-spermatogonium and spermatogonium-Sertoli cells in β-TrCP1/2 DKO mice, indicating that loss of β-TrCP1/2 in Sertoli cells causes reduction of E-cadherin in not only Sertoli cells but also male germ cells. Since it is hypothesized that E-cadherin is involved in interconnection of spermatogonia, we examined the spermatogonial connectivity with

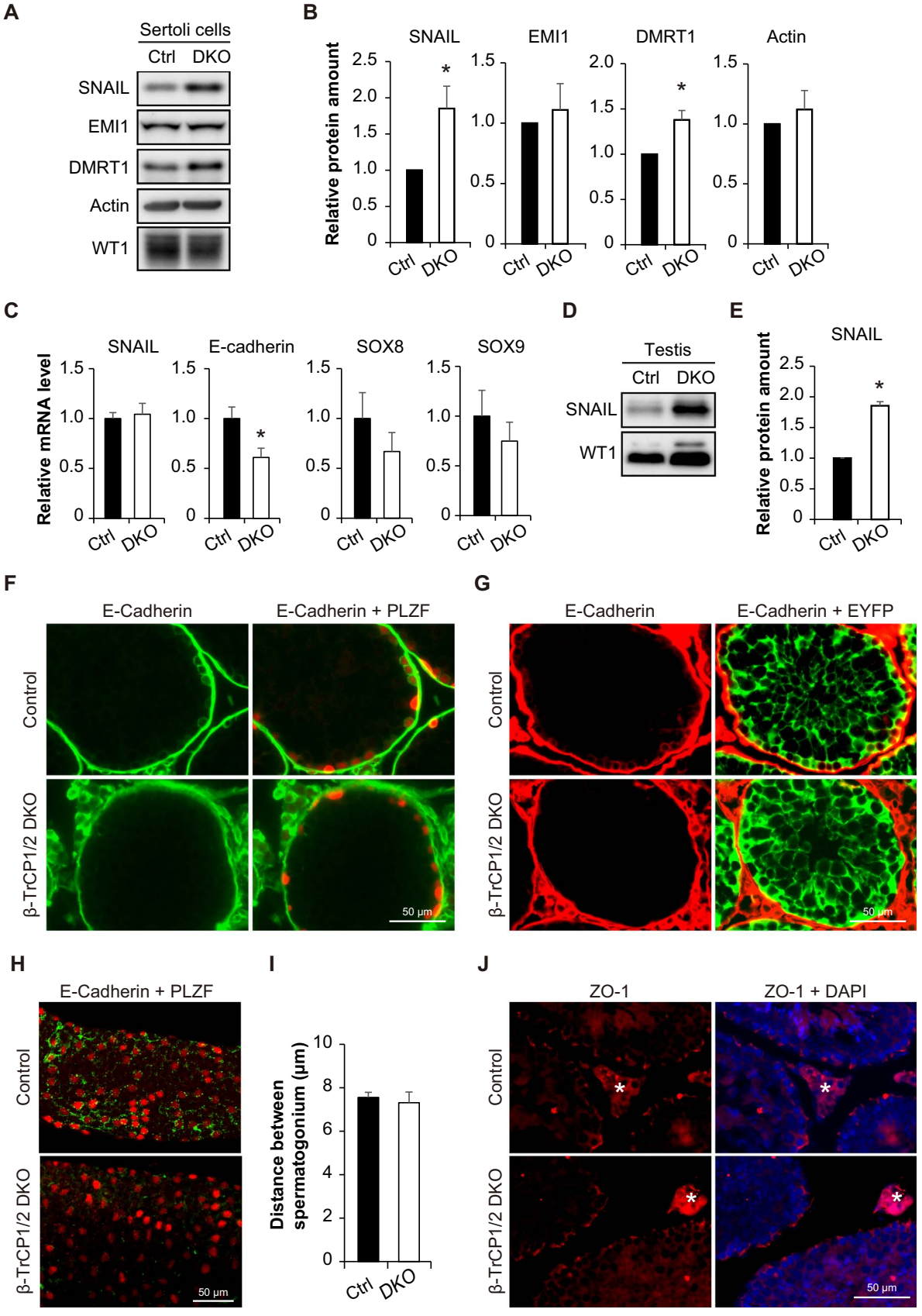
A
Sertoli cells
Ctrl DKO
SNAIL
EMI1
DMRT1
Actin
WT1
B
SNAIL
EMI1
DMRT1
Actin
Relative protein amount
Ctrl DKO
C
SNAIL
E-cadherin
SOX8
SOX9
Relative mRNA level
D
Testis
Ctrl DKO
SNAIL
WT1
E
SNAIL
Relative protein amount
F
E-Cadherin
E-Cadherin + PLZF
Control
β-TrCP1/2 DKO
50 μm
G
E-Cadherin
E-Cadherin + EYFP
H
E-Cadherin + PLZF
I
Distance between spermatogonium (μm)
J
ZO-1
ZO-1 + DAPI

Fig. 8. Molecular analysis of the spermatogenic defect of β-TrCP1/2 DKO mice. (A) Immunoblot analysis of the indicated proteins in primary Sertoli cells isolated from control (*Amh-Cre*, Ctrl) and β-TrCP1/2 DKO mice at14 dpp. Testes isolated from three mice were combined for preparation of one batch of Sertoli cells. (B) Quantification of band intensities normalized by that for WT1 in immunoblots similar to that in (A). Data are expressed relative to the corresponding value for control cells and are means ± SEM for three batches of primary Sertoli cells of each genotype. *p < 0.05 versus control (unpaired Student's *t*-test). (C) RT-qPCR analysis of the abundance of SNAIL, E-cadherin, SOX8, and SOX9 mRNAs (normalized by that of WT1 mRNA) in primary Sertoli cells isolated from control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 14 dpp. Data are expressed relative to the corresponding value for control cells and are means ± SEM for four batches of primary Sertoli cells of each group. *p < 0.05 versus control (unpaired Student's *t*-test). (D) Immunoblot analysis of SNAIL and WT1 in the testis of control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 14 dpp. (E) Quantification of the band intensity for SNAIL normalized by that for WT1 in immunoblots similar to that in (D). Data are expressed relative to the value for control mice and are means ± SEM for three mice of each group. *p < 0.05 versus control (unpaired Student's *t*-test). (F) Immunofluorescence staining of E-cadherin alone (left panels) or together with PLZF (right panels) in seminiferous tubules of control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 28 dpp. Scale bars, 50 μm. (G) Immunofluorescence staining of E-cadherin alone (left panels) or together with EYFP (right panels, a marker of cytoplasm of Sertoli cells) in seminiferous tubules of control (*Amh-Cre;ROSA26-EYFP*) and β-TrCP1/2 DKO (*Amh-Cre;ROSA26-EYFP;β-TrCP1*$^{-/-}$*;β-TrCP2*$^{F/F}$) mice at 28 dpp. Scale bars, 50 μm. (H) Whole mount immunostaining of E-cadherin (green) and PLZF (red) in seminiferous tubules of control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 28 dpp. Scale bars, 50 μm. (I) Quantification of distance between spermatogonium. Data are means ± SEM for three mice of each group. (J) Immunofluorescence staining of ZO-1 alone (left panels) or together with DAPI staining of nuclei (right panels) in seminiferous tubules of control (*Amh-Cre*) and β-TrCP1/2 DKO mice at 28 dpp. Scale bars, 50 μm. Asterisks indicate the interstitium.

whole mount staining of seminiferous tubules. Although E-cadherin was reduced, we could not detect the loosening of spermatogonia in alignment in β-TrCP1/2 DKO testis (Fig. 8H, I), implicating that E-cadherin is not essential for interconnection of spermatogonia. We speculate that the intercellular bridges can compensate for the loss of E-cadherin to tie spermatogonia (Greenbaum et al., 2011). In contrast to E-cadherin, the tight junction protein ZO-1 (zonula occludens-1) was preserved in the testis of our β-TrCP1/2 DKO mice (Fig. 8J), similar to observations with the β-TrCP1 knockout, β-TrCP2 knockdown mice described previously (Kanarek et al., 2010). Overall, our data thus indicated that the spermatogenic defect previously described for mice with systemic deficiency of β-TrCP1/2 (Kanarek et al., 2010) was due to the accumulation of SNAIL in Sertoli cells.

4. Discussion

We have here shown that β-TrCP1/2 in Sertoli cells play an essential role in male germ cell development. We found that, whereas the number of spermatogonia was not affected in our β-TrCP1/2 DKO mice, these animals manifested an earlier entry of male germ cells into meiosis and a significant reduction in the number of round spermatids compared with control mice. Our results are consistent with previous findings of impaired spermatogenesis accompanied by cell adhesion failure and SNAIL accumulation in the testis of β-TrCP1/2–deficient mice (Kanarek et al., 2010), but they narrow down β-TrCP1/2 regulation of SNAIL to Sertoli cells.

The SNAIL protein is present in both Sertoli cells and germ cells (data not shown), but it is regulated by β-TrCP primarily in Sertoli cells rather than other cell types present in the testis. The reason why SNAIL in Sertoli cells is especially sensitive to β-TrCP ablation remains unclear. Given that β-TrCP recognizes SNAIL that has been phosphorylated by glycogen synthase kinase (GSK)–3β (Yook et al., 2005; Zhou et al., 2004), it is possible that GSK-3β activity determines the location and timing of SNAIL degradation. Consistent with this notion, GSK-3β was previously shown to be expressed in mouse Sertoli cells (Guo et al., 2003).

With regard to the molecular mechanism by which β-TrCP and SNAIL regulate Sertoli cell function, it will be important to determine which SNAIL target genes contribute to such regulation. We and others (Kanarek et al., 2010) have shown that expression of the SNAIL target gene for E-cadherin was significantly down-regulated as a result of β-TrCP1/2 loss, indicating that the SNAIL protein that accumulates in response to such loss is active with regard to suppression of E-cadherin gene transcription. Therefore, the loss of E-cadherin is likely responsible at least in part for the aberrant spermatogenesis in β-TrCP1/2–deficient mice. Analysis of the effects of conditional knockout of E-cadherin in Sertoli cells will be required to confirm this conclusion and whether reduction of E-cadherin is responsible for premature meiotic entry observed in our β-TrCP1/2 DKO mice.

SNAIL plays an important role in embryogenesis as well as in the epithelial-mesenchymal transition (Wang et al., 2013), suggesting that SNAIL target genes other than that for E-cadherin also might contribute to the spermatogenic defect associated with β-TrCP ablation. Indeed, E-cadherin is expressed only at the basal side of seminiferous tubules (Fig. 8F, G) (Tokuda et al., 2007; Tolkunova et al., 2009), whereas spermatids are present at the apical side. Given that we detected increased apoptosis in seminiferous tubules of β-TrCP1/2 DKO mice at 28 dpp, we conclude that loss of β-TrCP in Sertoli cells results in impaired survival of spermatids. Given that E-cadherin is not expressed in the vicinity of spermatids, the down-regulation of this adhesion protein is likely not the only mechanism underlying the spermatogenesis defect of β-TrCP–deficient mice. It is also possible that proteins other than SNAIL accumulate in Sertoli cells in response to β-TrCP loss and contribute to this abnormality.

We detected early entry of male germ cells into meiosis in β-TrCP1/2 DKO mice during the first postnatal month. A similar phenotype has been described for germ cell–specific DMRT1 knockout mice (Matson et al., 2010). However, in contrast to these mice, we did not detect a loss of differentiating spermatogonia positive for c-Kit mRNA, suggesting that "skipping" of spermatogonial differentiation is unlikely to account for the early meiotic entry of germ cells in our β-TrCP1/2 DKO mice. Further analysis of the molecular functions of β-TrCP1/2 in Sertoli cells may provide insight into the mechanism underlying the timing or rate of meiosis in male germ cells.

Finally, our results suggest that the functions of β-TrCP1 and β-TrCP2 in Sertoli cells are redundant. Whether the same is true for other tissues remains to be determined by analysis of tissue-specific β-TrCP2 CKO and β-TrCP1/2 DKO mice. We conclude that the ubiquitin ligase subunit β-TrCP in Sertoli cells is required for male germ cell differentiation as a result of its targeting of the transcription factor SNAIL for degradation.

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Competing interests

No competing interests declared.

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