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# Mice deficient in Dmrt7 show infertility with spermatogenic arrest at pachytene stage

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**Abstract** **Genes including DM domain regulate sexual development in diverse metazoan phyla. One of these genes, *Dmrt7*, was expressed only in testes of adult mice. To determine the role of *Dmrt7* in mice, we generated *Dmrt7*-knockout mice (*Dmrt7*$^{-/-}$). Although the *Dmrt7*$^{-/-}$ showed normal growth, null males were infertile. No sperm was detected in the epididymis of *Dmrt7*$^{-/-}$ adult males. Absence of spermatids in a histological analysis, decreased expression of *Ccna1* mRNA and the accumulation of SCP3-positive spermatocytes showed the arrest of spermatogenesis at the pachytene stage in the *Dmrt7*-knockout mice.**


*Keywords:* Dmrt7; Knockout mouse; Male infertility; Spermatogenesis; Testis

## 1. Introduction

Most genes controlling sexual development show no evidence of conserved function among phyla. However, the *doublesex* (*dsx*) gene of *Drosophila* and the *mab-3* gene of *Caenorhabditis elegans* encode proteins with conserved DM DNA binding domains and those genes have been shown to direct similar aspects of sexual differentiation [1]. *dsx* is alternatively spliced to generate distinct proteins ($Dsx^M$ and $Dsx^F$) each of which functions in one of the two sexes [2], but *mab-3* is required only in males [3]. $dsx^F$ can compensate for the loss of function of *mab-3* in *C. elegans* [4]. The DM domain-containing genes are also present in human and mouse, and are called *Dmrts* (*doublesex* and *mab-3* related transcription factors). As in *C. elegans* and *Drosophila*, *Dmrt* genes have been implicated in sex determination. For example, in medaka fish, mutations in the *Dmy* gene, in which the DM domain is conserved, cause male sex reversal, and in humans, deletions at or near the cluster of DM domain containing genes on chromosome 9 (containing *Dmrt1*, *Dmrt2* and *Dmrt3*) cause XY sex reversal [5]. Studies in mouse have not yet revealed the essential function for individual DM-containing genes in sex determination. However, *Dmrt1*, which is expressed only in testes in adult mice [6], is required for proper male sexual differentiation [7]. A recent study revealed that targeted disruption of *Dmrt2* resulted in perinatal lethality due to abnormal rib and sternal development, leading to an inability to breathe [8]. Thus, the *Dmrt* genes may have important roles in the development of various tissues.

In addition to *Dmrt1*, *Dmrt7* is expressed only in testis and ovary of mouse embryo [9]. In this study, we detected *Dmrt7* mRNA only in testis but not in ovary in adult mice. The expression pattern was similar to that of *Dmrt1* in mice. In order to define the function of *Dmrt7* in males, we generated Dmrt7-deficient mice by gene targeting.

## 2. Materials and methods

### 2.1. Southern and Northern blot analysis

For Southern blot analysis, 10 μg of genomic DNA extracted from the tail were digested with XbaI and loaded on 1% agarose gels. For Northern blot analysis, total RNA was extracted from different organs using RNA Stat 60 (Leedo Medical Laboratories, USA), and poly(A)$^+$ RNAs were purified by using oligotex-dT 30/super (Takara Bio, Otsu, Japan). Two micrograms of poly(A)$^+$ RNA were loaded on formaldehyde-agarose gels. These DNA and RNA samples were subjected to electrophoresis and transferred onto Byodyne B nylon membrane (Pall BioSupport, USA), and blocked before hybridization. The probes were labeled with Mega-prime DNA labeling system (Amersham Biosciences, Japan) with [$^{32}$P] dCTP.

### 2.2. RT-PCR analysis of Dmrt7 transcript

RNAs were obtained from testes and cDNAs were produced from two micrograms of total RNA according to a previous study [10]. For RT-PCR, 1 μl of the cDNA product was used to amplify the cDNAs for the *dmrt7* or *glyceraldehyde-3-phosphate dehydrogenase* (*Gapd*). The primer sequences used for the PCR reactions were: *Dmrt7*-F: 5′-ccctaacctgcctcacacat-3′; *Dmrt7*-R: 5′-tttgtcccagtgaggtagcc-3′; *Gapd*-F: 5′-atggtgaaggtcggtgtgaacg-3′; *Gapd*-R: 5′-aaacatgggggcatcggcagaa-3′. PCR reaction was performed by using a rTaq polymerase (Takara Bio, Otsu, Japan) in a 20 μl reaction volume containing 1 μl of the cDNA mixture. After 2 min of denaturation at 95 °C, 25 cycles of amplifications consisting of 95 °C, 10 s; 65 °C, 10 s; 72 °C, 20 s were performed.

### 2.3. Generation of Dmrt7$^{-/-}$ mutant mice and genotyping

To construct the targeting vector, an oligo-nucleotide harboring multiple cloning sequences was inserted into the NotI site of the 3′ flanking sequence of an IRES-Cre-pA-FRT-PGK-Neo-pA-FRT expression cassette (gift of R.R. Behringer; S. Jamin et al. [11]). The inserted linker sequence, 5′-ggccgacgtacgtcgacggcccatatggccgcggccgcatttaaatacgcgtgt-3′; 5′-ggccacacgcgtatttaatgcggccgcggccatatgggccgtcgacgtacgtc-3′, contained BsiWI-SalI-NdeI-SacII-NotI-BstZI-SwaI-DraI-MluI restriction enzyme sites. A part of the 5′ homologous arm (1.7 kb) of the KO vector, with a newly generated PacI site at its 5′ end, was obtained by PCR using primer 17: 5′-ttaattaacgttcctcttcgccactgtgtc-3′ and primer 18: 5′-attaattacttttggagacaagatctcac-3′. A 3′ right arm (4.9 kb) was also obtained by PCR using primer 23: 5′-cgtacgcgaaattagtaccaacttgggcccagtg-3′, and primer 24: 5′-ggccatatgggccgcaacactgagcctatgtttcttcctggag-3′, with the addition of the BsiWI site at the 5′ end and the SfiI

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site at the 3′ end by PCR-mediated mutagenesis, respectively. PCR was performed by using a *Pfu Ultra*™ High-Fidelity DNA Polymerase (STRATAGENE, La Lolla, CA) and a 129/Sv strain-derived mouse genome was used as the template DNA. The Dmrt7 coding sequence of exon 2 was fused in the frame with the Cre recombinase gene followed by the neomycin resistance gene under the control of the PGK promotor (PGK-Neo). This KO vector can remove most of exon 2 and all the exon 3 sequences after homologous recombination. An MC1 promotor-herpes simplex virus-thymidine kinase cassette (MC1-TK) used for negative selection was inserted into the NotI site of the KO vector. After completion of the vector construct, we linearized the KO vector with SwaI and electroporated into E14TG2a embryonic stem cells. G418 and FIAU (Moravek Biochemicals, Brea, CA) doubly resistant clones were screened by Southern blot analysis. Chimeric mice generated by microinjection of heterozygous embryonic stem cell clone (clone No. 10) into C57BL/6J blastocysts were mated with C57BL/6J females and heterozygous mice ($Dmrt7^{+/-}$) were obtained. Offspring from intercrossing of heterozygous littermates were genotyped by Southern blot analysis (Fig. 2) to obtain *Dmrt7* null mutants. Animals of mixed genetic background (C57BL/6J and 129/Sv) were analyzed in this study. The care and use of mice in this study were approved by the Institutional Animal Care and Use Committee of Tohoku University. The animals were killed by cervical dislocation and the testes were isolated.

### 2.4. Histology and immunostaining

Fresh mouse testis and epididymis were fixed in Bouin's solution, and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin.

In immunohistochemical analysis, antigen retrieval was performed to incubate these dewaxed slides in 0.1 M Tris–HCl (pH 9.5) for 20 min at 100 °C in an autoclave, and blocked for 1 h with 5 % goat serum in TBS. The anti-Scp3 immune serum (kindly provided by Dr. S. Chuma) was diluted to 1/500 with 1% BSA in TBS and used for 2 h at room temperature. HRP-conjugated goat anti rabbit IgG (Vector Laboratory, Burlingame, CA) was used for the second antibody. Nuclei were counterstained with hematoxylin.

### 2.5. Statistics

The results of the experiments are expressed as the means ± S.E.M. ANOVA was used for statistical analysis of the results and a *P*-value less than 0.05 was accepted as being significantly different.

## 3. Results and discussion

*Dmrt7* may play an important role in the development or differentiation in tissues since *Dmrt1* or *Dmrt2* is essential for testis differentiation or somite patterning, respectively [7,8]. In embryogenesis, the transcription of *Dmrt7* was detected only in gonads of both sexes [9]. On the other hand, little information about *Dmrt7* expression has been reported in adult mouse tissues. We examined the expression of *Dmrt7* by Northern blot analysis in various tissues of adult mice at 3 months. *Dmrt7* was expressed only in testis but not in ovary (Fig. 1A). There were two forms of *Dmrt7* transcripts (Fig. 1A). The long transcript consists of the short one and an intron1 (unpublished sequence data). The tissue-specific expression of *Dmrt7* in adult mice was similar to that of *Dmrt1* [6]. *Dmrt1* deficient males are infertile for the defects of postnatal testis differentiation [7]. To study the function of *Dmrt7* in male reproduction in vivo, we generated mice deficient in the *Dmrt7* gene.

In the *Dmrt7* mutant mice, exons 2 and 3 were replaced with Cre recombinase and PGK-Neo cassettes. The vector construction was shown in Fig. 2A. The region of exons 2 and 3 includes the DM domain, a zinc finger-like DNA-binding motif [4,12]. Using a 5′ external probe (Fig. 2A, left), 14

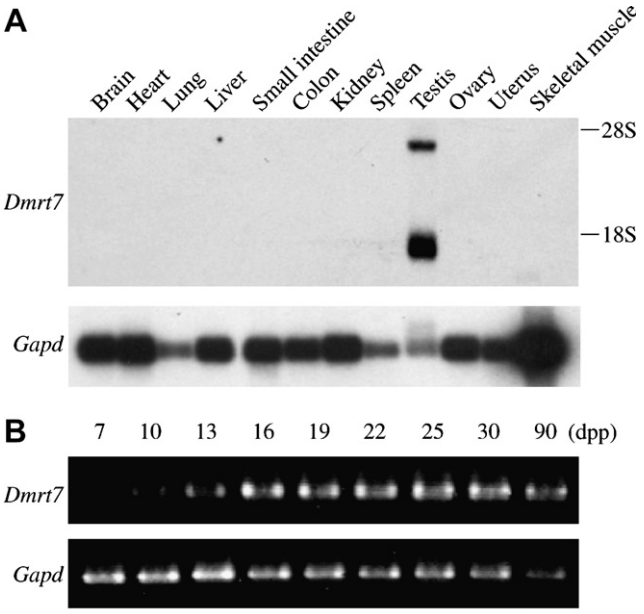


Fig. 1. Expression analysis of *Dmrt7*. (A) Northern blot analysis of *Dmrt7* expression in adult tissues at 3 months. Two micrograms of poly(A)$^+$ RNAs were loaded. The *Dmrt7* transcripts were detected only in the testes. The probe contained the exons 2 and 3 region of *Dmrt7* mRNA. *Gapd* is shown as a control. (B) RT-PCR analysis of *Dmrt7* expression in testes. cDNAs obtained from testes at indicated days after birth were amplified with primers for *Dmrt7* and *Gapd*.

(8.75%) of the 160 ES cell clones analyzed were correctly targeted at the *Dmrt7* locus. Five ES cell clones (clone No. 4, 5, 10, 11, 12) were further analyzed by a Southern blot with 5′ (Fig. 2B, top) and 3′ (Fig. 2B, bottom) external probes (Fig. 2A, right), and all showed two bands with correct molecular size in both blots. $Dmrt7^{+/-}$ offspring were born from intercrossing C57BL/6J females with chimeric males obtained from heterozygous ES cell clone No. 10 (Fig. 2B). $Dmrt7^{+/-}$ mice were viable and fertile. A 1:2:1 Mendelian distribution of progeny from $Dmrt7^{+/-}$ intercrosses was observed ($Dmrt7^{+/+}$:$Dmrt7^{+/-}$:$Dmrt7^{-/-}$, 93:168:84), indicating that *Dmrt7* was not required for embryonic development. Southern blot analysis using 5′ external (Fig. 2C, top) and internal probe containing exon 2 (Fig. 2E, bottom) clarified that targeted disruption of *Dmrt7* gene was correctly occurred. Northern blot analysis with testicular RNA revealed the absence of *Dmrt7* mRNA (Fig. 2D).

$Dmrt7^{-/-}$ mice were grossly indistinguishable from their littermates and lived to the adult stage. Whereas adult $Dmrt7^{-/-}$ females were fertile, adult $Dmrt7^{-/-}$ males were infertile, despite the normal formation of copulation plugs in mated females ($n = 15$). To define the causes of infertility in $Dmrt7^{-/-}$ males, adult testes were analyzed grossly and histologically. Testes from adult $Dmrt7^{-/-}$ males were significantly smaller ($P < 0.001$) than $Dmrt7^{+/+}$ and $Dmrt7^{+/-}$ males (Fig. 3A). $Dmrt7^{-/-}$ testes (32.7 ± 1.7 mg, $n = 4$) were around 41.3% the weight of $Dmrt7^{+/+}$ testes (79.2 ± 7.2 mg, $n = 3$) in seven week-old animals. The weight of $Dmrt7^{+/-}$ testes (73.9 ± 6.0 mg, $n = 3$) was similar to that of $Dmrt7^{+/+}$ ones. In contrast to the presence of sperm in fluid prepared from epididymis of adult $Dmrt7^{+/+}$ males, those prepared from $Dmrt7^{-/-}$ males ($n = 4$) showed absence of sperm under a microscope.

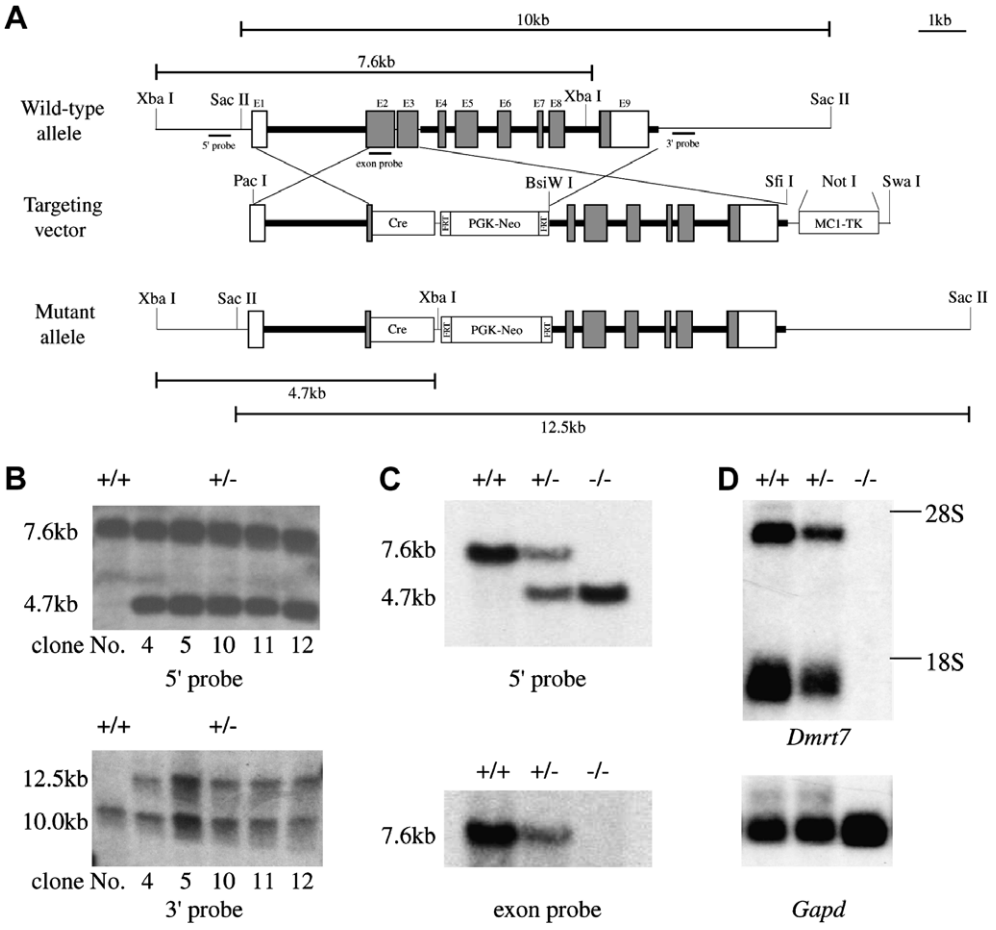


Fig. 2. Generation of *Dmrt7*$^{-/-}$ mice. (A) The wild-type and mutant *Dmrt7* loci and gene targeting construct. Exons (E) are indicated by boxes (white boxes, 5′ and 3′ uncoding regions; gray boxes, coding regions). The positions of the restriction enzyme sites and the probes used for Southern blot analysis are shown. (B, C) Southern blot analysis of genomic DNAs from ES cell clones (B) or from littermate progenies from *Dmrt7*$^{+/-}$ crosses (C). XbaI-digested (B-top, C) or SacII-digested (B-bottom) DNA is hybridized with the radiolabeled probes indicated in A. (D) Northern blot analysis of poly(A)$^{+}$ RNA (2 μg per lane) from the testes of *Dmrt7*$^{+/+}$, *Dmrt7*$^{+/-}$ and *Dmrt7*$^{-/-}$ adult mice. The blot was sequentially hybridized with *Dmrt7* and *Gapd* cDNA probes.

In addition, in the histological analysis, sperm was completely absent in the sections of epididymis of adult males ($n = 4$; Fig. 3F), while the epididymal tubules were filled with sperm in *Dmrt7*$^{+/+}$ males (Fig. 3E). Furthermore, elongating spermatids were hardly detected in the seminiferous tubules of *Dmrt7*$^{-/-}$ males ($n = 5$), whereas round spermatids, spermatocytes and spermatogonium were visible (Fig. 3D). The number of round spermatids was far fewer in *Dmrt7*$^{-/-}$ (Fig. 3D) than that in *Dmrt7*$^{+/+}$ ($n = 3$, Fig. 3C). Sertoli cell vacuolization, which might be caused by sever germ cell depletion through endocytosis [13], was observed in *Dmrt7*$^{-/-}$ testes (Fig. 3D).

Cyclin A1 (*Ccna1*) mRNA is known as a marker for the late pachytene spermatocytes [13–15]. Spermatogenesis was completely disrupted before the first meiotic division in *Ccna1* deficient mice [16]. In contrast, there were trace numbers of survived spermatids in *Dmrt7*$^{-/-}$ testes. *Ccna1* was abundant in *Dmrt7*$^{+/+}$ testes but slightly detectable in *Dmrt7*$^{-/-}$ testes (Fig. 3B). This result corresponds to the existence of few germ cells passed through the late pachytene stage in *Dmrt7*$^{-/-}$ testes. A synaptonemal complex protein (SCP3) is a component of the synaptonemal complex and a marker for all primary spermatocytes [17,18]. In the immunohistochemical analysis, accumulation of SCP3-positive spermatocytes were observed in the seminiferous tubules of *Dmrt7*$^{-/-}$ males compared to those of *Dmrt7*$^{+/+}$ males (Fig. 3G,H), which might be due to the block of differentiation into the spermatids. At 17 days post partum (dpp), the most advanced germ cells in the seminiferous tubules are at the mid-pachytene stage of the first wave of spermatogenesis [16]. There were not significant differences between in the seminiferous tubules of *Dmrt7*$^{+/+}$ and *Dmrt7*$^{-/-}$ testes at 16 dpp (data not shown). Thus, the present data suggested spermatogenic arrest at the early-mid pachytene stage in *Dmrt7*$^{-/-}$ testes. Importance of *Dmrt7* at first meiotic prophase agrees with our observation that the *Dmrt7* mRNA in testes increased from 10 dpp (Fig. 1B), when the first wave of spermatogenesis begins [19].

In the present study, we demonstrated that *Dmrt7* is essential for male fertility. This testicular lesion in *Dmrt7*$^{-/-}$ mice is similar to that in *Dmrt1* deficient mice, and suggests the essential roles of the DM domain for sexual development and differentiation among phyla. We plan further study to elucidate the mechanisms by which DMRT7 facilitates spermatogenesis.

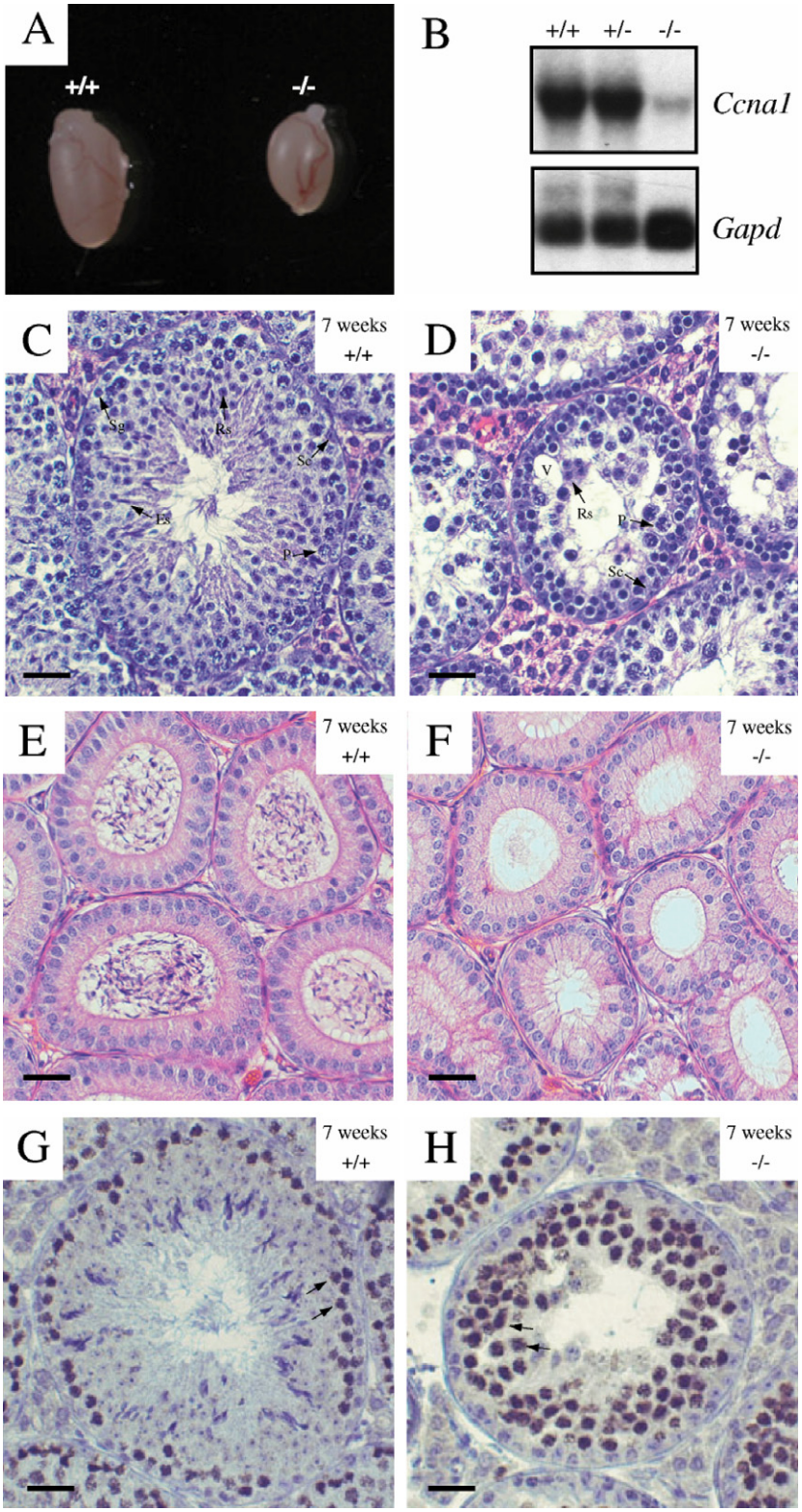


Fig. 3. Analysis of postnatal testes and epididymis. (A) Gross analysis of adult testes from 7-week-old *Dmrt7*$^{+/+}$ and *Dmrt7*$^{-/-}$ mice. (B) Northern blot analysis of 7-week-old *Dmrt7*$^{+/+}$, *Dmrt7*$^{+/-}$ and *Dmrt7*$^{-/-}$ testes. *Ccna1* probe was re-hybridized after the striping of the membrane used in the Fig. 2D. *Gapd* was shown as a control. (C–F) Histological analysis of testes (C, D) or epididymis (E, F) of 7-week-old *Dmrt7*$^{+/+}$ (C, E) and *Dmrt7*$^{-/-}$ (D, F) mice. The sections were stained with hematoxylin and eosin. Scale bars show 30 μm. Es, elongating spermatids; P, pachytene spermatocyte; Rs, round spermatids; Sc, Sertoli cells; Sg, spermatogonium; V, vacuoles. (G, H) Immunohistochemical analysis of testes of 7-week-old *Dmrt7*$^{+/+}$ (G) and *Dmrt7*$^{-/-}$ (H) mice. SCP3-antibody was used and nuclei were counterstained with hematoxylin. Arrows: SCP-positive germ cells. Scale bars show 30 μm.

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