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Cell type-autonomous and non-autonomous requirements for *Dmrt1* in postnatal testis differentiation

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Abstract

Genes containing the DM domain, a conserved DNA binding motif first found in *Doublesex* of *Drosophila* and *mab-3* of *C. elegans*, regulate sexual differentiation in multiple phyla. The DM domain gene *Dmrt1* is essential for testicular differentiation in vertebrates. In the mouse, *Dmrt1* is expressed in pre-meiotic germ cells and in Sertoli cells, which provide essential support for spermatogenesis. *Dmrt1* null mutant mice have severely dysgenic testes in which Sertoli cells and germ cells both fail to differentiate properly after birth. Here we use conditional gene targeting to identify the functions of *Dmrt1* in each cell type. We find that *Dmrt1* is required in Sertoli cells for their postnatal differentiation, and for germ line maintenance and for meiotic progression. *Dmrt1* is required in germ cells for their radial migration to the periphery of the seminiferous tubule where the spermatogenic niche will form, for mitotic reactivation and for survival beyond the first postnatal week. Thus *Dmrt1* activity is required autonomously in the Sertoli and germ cell lineages, and *Dmrt1* activity in Sertoli cells also is required non-autonomously to maintain the germ line. These results demonstrate that *Dmrt1* plays multiple roles in controlling the remodeling and differentiation of the juvenile testis.

Keywords

DMRT1; conditional targeting; testis; Sertoli; gonocyte; spermatogenesis; autonomous

INTRODUCTION

The seminiferous epithelium of the mammalian testis is a complex and highly dynamic tissue comprised of germ cells and Sertoli cells surrounded by a basal lamina and a layer of peritubular myoid cells. During early postnatal development and puberty the Sertoli and germ cells undergo tremendous changes and interact closely to establish a germ line stem cell population, supported by the Sertoli cells, that can produce mature spermatozoa for the duration of reproductive life.

Primordial germ cells (PGCs) appear around 7 days post-coitum (dpc) in the extraembryonic mesoderm (Ginsburg et al., 1990) and migrate through the allantois to the hindgut, reaching

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the genital ridge by 9.5-11 dpc (Anderson et al., 2000). After PGCs arrive in the gonadal primordium they are enclosed by pre-Sertoli cells, which coalesce during this period to form testis cords that later elongate into seminiferous tubules (McLaren, 1998). Sertoli cells play an important role in controlling germ cells in the embryo, arresting male germ cell mitosis in G1 and blocking meiotic initiation (Adams and McLaren, 2002; Bowles et al., 2006; McLaren and Southee, 1997). The mitotically-arrested PGCs differentiate into gonocytes, which remain mitotically quiescent until around birth in the mouse (de Rooij and Russell, 2000; Nagano et al., 2000; Vergouwen et al., 1991).

During the first postnatal week, the seminiferous tubules are remodeled in preparation for spermatogenesis. At birth, the tubules have the embryonic arrangement, with Sertoli cells at the periphery and gonocytes in the center. Starting perinatally, the gonocytes migrate radially from their initial central location to the basal lamina at the periphery (Nagano et al., 2000). Explant culture experiments suggest that the c-KIT receptor and its ligand may play a role in this process (Orth et al., 1997). Expression patterns and transgenic studies also have implicated ALCAM and short-type PB-cadherin as potential mediators of gonocye migration (Ohbo et al., 2003; Wu et al., 2005), but so far no gene has been shown to be essential in gonocytes for their postnatal migration. By postnatal day 6 (P6), ~ 90% of gonocytes are located at the basal lamina in what will become the spermatogonial stem cell niche (McLean et al., 2003; Nagano et al., 2000). Germ cells that complete the radial migration rapidly differentiate into germ line stem cells (A spermatogonia) that are first apparent by P6 in the mouse, whereas germ cells that fail to migrate die (de Rooij, 2001). The spermatogonia undergo proliferative cell divisions and begin to differentiate into spermatocytes that commence meiosis.

The early postnatal period also is a critical time for Sertoli cell development. Sertoli cells proliferate only in the fetal and pre-pubertal periods, and by the end of the second postnatal week they begin to undergo maturation, losing their proliferative capacity and acquiring functions that will enable them to support spermatogenesis (Sharpe et al., 2003). Sertoli cell support is crucial for germ cell differentiation, meiosis and spermiogenesis, and signals from the Sertoli cells are essential for the maintenance of the spermatogonial stem cell niche in adults (Griswold, 1995; Hess et al., 2006). Although the cellular behaviors of germ cells and Sertoli cells in the early postnatal testis that lead to the establishment of the spermatogenic stem cell niche are well described, very little is known of how these processes are regulated. However, as described below, the *Dmrt1* gene is required for these events and provides an entry point for the elucidation of their control.

Dmrt1 (*doublesex* and *mab-3* related transcription factor 1) is a critical and conserved regulator of postnatal testis differentiation (Raymond et al., 2000). *Dmrt1* is expressed in the testis of all vertebrates so far examined, starting at the genital ridge stage and continuing throughout adult life (Kettlewell et al., 2000; Marchand et al., 2000; Raymond et al., 1999a; Raymond et al., 2000; Smith et al., 1999). In humans, deletion of the region of chromosome 9p containing *DMRT1* results in testicular dysgenesis (Crocker et al., 1988; Ion et al., 1998; Ogata et al., 1997; Raymond et al., 1999b; Raymond et al., 1998), while amplification of *DMRT1* is associated with a form of testicular germ cell cancer, spermatocytic seminoma (Looijenga et al., 2006). *Dmrt1* mutant mice have severely abnormal testis development starting at about P2 and resulting in severely dysgenic testes resembling those of 9p deletion patients (Fahrioglu et al., 2007; Raymond et al., 2000). *Dmrt1* encodes a protein with a DM domain, a DNA binding motif first identified in the sexual regulators Doublesex of *Drosophila* and the MAB-3 of *C. elegans* (Erdman and Burtis, 1993; Raymond et al., 1998). DM domain-encoding genes have been shown to regulate various aspects of sexual differentiation in insects, nematodes, and mammals, suggesting an ancient involvement of these genes in sexual regulation (Zarkower, 2001).

Targeted deletion of *Dmrt1* in the mouse demonstrated that the gene is essential for postnatal testis differentiation (Raymond et al., 2000). *Dmrt1* is unusual in that it is expressed specifically in both Sertoli cells and in germ cells, starting as soon as the genital ridge forms, and its loss affects the development of both cell types. Germ line defects in *Dmrt1* null mutant mice include the failure of germ cells to undergo radial migration, to reactivate mitosis, to enter meiosis, and to survive beyond P10. Sertoli cell defects include developmental arrest and over-proliferation (Fahrioglu et al., 2007; Raymond et al., 2000). In addition, *Dmrt1* mutant adults are incompletely virilized (J. Balciuniene and D.Z., unpublished). Based on mRNA expression analysis, loss of *Dmrt1* affects both Sertoli cells and germ cells at least as early as P1 (Fahrioglu et al., 2007).

Although *Dmrt1* is expressed in Sertoli cells and germ cells and is required for their postnatal development, the essential role of Sertoli cells in supporting germ cell development has made it unclear in which cells *Dmrt1* performs which of its functions. To find out, we have conditionally targeted *Dmrt1* in the Sertoli cell and germ cell lineages using cell type-specific *Cre* transgenes. This approach reveals specific requirements for *Dmrt1* in both cell lineages as well as a cell non-autonomous role for *Dmrt1* in germ cell development. We find that *Dmrt1* is required in germ cells but not Sertoli cells for radial migration of gonocytes to the basal lamina, for their mitotic reactivation, and for germ cell survival beyond the first postnatal week. *Dmrt1* activity in Sertoli cells is required for their proper organization and differentiation, and for survival and differentiation of wild type germ cells beyond the second postnatal week.

MATERIALS AND METHODS

Generation of SCDmrt1KO and GCDmrt1KO mutant mice

Cell type-specific *Dmrt1* knockout mice were generated using Cre-mediated recombination of *loxP* sites in a functional *Dmrt1* allele, $Dmrt1^{floxed}$, whose construction was previously described (Raymond 2000). $Dmrt1^{+/-}$ animals (129/Sv and C57BL/6 mixed background) were crossed either with *Desert hedgehog–Cre* (*Dhh-Cre*) mice expressing *Cre* selectively in Sertoli cells (Lindeboom et al., 2003), or with *Tissue Non-specific Alkaline Phosphatase Cre* (*TNAP-Cre*) animals expressing Cre in primordial germ cells (PGCs) (Lomeli et al., 2000). $Dmrt1^{floxed/floxed}$ animals were crossed with *Dhh-Cre*; $Dmrt1^{+/-}$ to generate *SCDmrt1KO* mutants (*Dhh-Cre;* $Dmrt1^{floxed/-}$) or with *TNAP-Cre*; $Dmrt1^{+/-}$ animals to produce *GCDmrt1KO* mutants (*TNAP-Cre;* $Dmrt1^{floxed/-}$). As controls, we used $Dmrt1^{floxed/-}$ or $Dmrt1^{floxed/+}$ littermates.

Genotyping

Genotyping was performed as described previously (Fahrioglu et al., 2007; Raymond et al., 2000). Tail-clip DNA was amplified for 35 cycles. The wild-type *Dmrt1* allele ($Dmrt1^{+}$) was detected by PCR with CR92/CR99, with an annealing temperature of 55°C. The $Dmrt1^{floxed}$ allele was detected by PCR with KOS2/KOS3N, with an annealing temperature of 62°C. The deleted allele $Dmrt1^{-}$ was detected with KOS1N/KOS3N with an annealing temperature 65° C. *Cre* transgenes were detected by PCR with CreF/CreR, with annealing temperature of 62° C. PCR with CR92/CR99 contained 10% DMSO.

Primers

CreF: 5'-CCTGATGGACATGTTCAGGGATCG -3'

CreR: 5'-TCCATGAGTGAACGAACCTGGTCG -3'

CR92: 5'-CAGCTCCATGGCGAACGACGACACATTCGG-3'

CR99: 5'- CTGCAGCGAGCGCATTTGGGCAGC-3'

KOS2: 5'- TGCACACGTGCACCCTCGCCATCG -3'

KOS1N: 5' GATCTATCTGGAGCCAGGTGGTAG -3'

KOS3N: 5'- TCATGGCAGCTCTCCCAGTGGAGC -3'

Histological analysis

Dissected testes were fixed in Bouin's fixative or phosphate-buffered formalin overnight at 4° C, progressively dehydrated in a graded ethanol series and embedded in paraffin wax. Sections (6 μm) were deparaffinized, rehydrated, and stained with hematoxylin and eosin.

Tissue immunofluorescent staining

Slides with paraffin sections were washed in PBT (0.1% Tween 20 in phosphate buffered saline [PBS]) and autoclaved in 10 mM citric acid (pH 6.0) to retrieve antigenicity. Slides were blocked in 5% serum (matched to the species of the secondary antibody) in PBS for 1 h at room temperature and incubated with primary antibodies overnight at 4°C prior to detection with secondary antibodies.

Antibodies

Primary antibodies used for immunofluorescence were rat anti-GATA1 (1:200, Santa Cruz Biotechnology, sc-265), goat anti-GATA4 (1:200, Santa Cruz Biotechnology, sc-1237), rabbit anti-AR (1:50, Santa Cruz Biotechnology, sc-816), rabbit anti phosphohistone H3 (1:200, Upstate 06-570), rat anti-TRA98 (1:200, gift of H. Tanaka and Y. Nishimune), rat anti-BC7 (1:100, gift of H. Tanaka and Y. Nishimune), rat anti-TRA369 (1:1000, gift of H. Tanaka and Y. Nishimune) and rabbit anti-DMRT1 (1:400; (Raymond et al., 2000)). Secondary antibodies used were goat anti-rabbit Alexa 488, goat anti-rabbit Alexa 594, and goat anti-rat Alexa 594 (Molecular Probes), all used at 1:250. Donkey anti-goat FITC (Jackson) and donkey anti-rabbit Texas Red (Jackson) were used at 1:50 according to the manufacturer's instructions.

RESULTS

Generation of Sertoli cell-specific Dmrt1 knockout mice

To test whether *Dmrt1* is required autonomously in each of the two cell lineages that express the gene, we generated cell-type specific *Dmrt1* knockout mice. We started by targeting *Dmrt1* in the Sertoli cell lineage. These experiments employed mice with the null deletion allele $Dmrt1^{-}$ (Raymond et al., 2000), or a conditional allele, $Dmrt1^{floxed}$, in which the same segment that is deleted in $Dmrt1^{-}$ is instead flanked by *loxP* sites. Cre-mediated recombination of these *loxP* sites removes the transcriptional start site and proximal promotor and the first exon, which contains the DM domain, and results in a protein- and mRNA-null mutation (Raymond et al., 2000). For conditional targeting we used the *Dhh-Cre* transgene (gift of Dr. Dies Meijer), which has been shown to recombine reporter transgenes in pre-Sertoli cells very efficiently by 18 dpc (Lindeboom F, Gillemans N et al., 2003). The breeding scheme is described in Materials and Methods. In each experiment we compared *Dhh-Cre;*$Dmrt1^{floxed/-}$ (*SCDmrt1KO*) animals, with *Dmrt1* deleted in Sertoli cells, to $Dmrt1^{floxed/-}$ or $Dmrt1^{floxed/+}$ control animals containing one or two functional copies of the gene in Sertoli cells. Comparison of these controls with each other and with $Dmrt1^{+/+}$ revealed no phenotypic differences (data not shown).

To confirm that *Dmrt1* is efficiently and specifically deleted in Sertoli cells of *SCDmrt1KO* mice, we stained P5 testis sections with antibodies to DMRT1 and either the Sertoli cell marker GATA4 or the germ cell marker TRA98 (Figure 1). GATA4 normally is expressed in juvenile Sertoli cells but not in germ cells, and is expressed in *Dmrt1* mutant Sertoli cells (Sharpe, R

et al., 2003;Raymond et al, 2000). DMRT1 expression was absent from virtually all Sertoli cells in *SCDmrt1KO* mice, while remaining in the GATA4-negative germ cells (Figure 1A). Staining with the germ cell-specific TRA98 antibody and anti-DMRT1 confirmed that DMRT1 expression was unaffected in virtually all germ cells of *SCDmrt1KO* mice (Figure 1B). Together these results confirm that *Dmrt1* is deleted efficiently and specifically in the Sertoli cells of *SCDmrt1KO* testes.

Abnormal organization of seminiferous tubules in SCDmrt1KO testes

To evaluate testicular development in *SCDmrt1KO* mice, we first histologically examined the testes of *SCDmrt1KO* mice and control littermates during the initial period of spermatogenesis, from P5 to P28. By P5, prior to meiosis, most germ cells normally have migrated radially from their initial position in the center of the seminiferous tubule into close apposition with the basal lamina (Figure 2A; closed arrowheads). In *SCDmrt1KO* testes, a few germ cells could be found in the center of tubules (Figure 2A, open arrowheads), but most had successfully migrated to the periphery (closed arrowheads). By P7, the approximate time of meiotic initiation, germ cells were exclusively peripheral both in control and *SCDmrt1KO* testes (Figure 2B), suggesting that Sertoli cell *Dmrt1* expression is not required for germ cell radial migration, although the process may be slightly delayed in *SCDmrt1KO* testes. At P9, in control testes the Sertoli cells were organized in a ring either just inside the ring of germ cells at the periphery or mixed among the germ cells at the periphery, but in *SCDmrt1KO* mutants the Sertoli cells were more randomly distributed (Figure 2C). By P14, control tubules had begun to form lumens and had accumulated primary spermatocytes in the adluminal compartment (Figure 2D). In *SCDmrt1KO* testes at P14, however, the pattern was very different, with no luminal space apparent and tubules filled with a poorly organized mix of Sertoli cells and differentiating germ cells (Figure 2D). By P28, the control seminiferous tubules contained polarized Sertoli cells properly adjoining the basal lamina, and normally developing germ cells, including spermatogonia, primary spermatocytes, round spermatids, and elongated spermatids. In contrast, the *SCDmrt1KO* tubules at P28 contained no apparent germ cells and were filled with randomly localized unpolarized Sertoli cells of immature morphology (Figure 2E).

Dmrt1 activity in Sertoli cells is not required for germ cell mitotic reactivation

Germ cells in *Dmrt1* null testes fail to migrate to the tubule periphery, do not reactivate mitosis or initiate meiosis, and die before P14 (Fahrioglu et al., 2007; Raymond et al., 2000). As just described, although germ cells in *SCDmrt1KO* testes migrate to the basal lamina in the first postnatal week and initiate meiosis, they are lost by P28. We therefore examined other aspects of early germ cell development in *SCDmrt1KO* mutants. Specifically, we asked whether wild type germ cells surrounded by mutant Sertoli cells can reactivate mitosis, and we more closely examined their meiotic progression (see below).

To examine mitosis, we used an antibody to the mitotic marker phospho-histone H3 (P-H3; Figure 3). P-H3 also is expressed in meiotic cells, so we also stained for the meiotic marker BC7 (not shown). We found that control and *SCDmrt1KO* testes had similar numbers of mitotic germ cells both at P5 (not shown) and P7 (Figure 3). This contrasts with *Dmrt1* null mutants, in which germ cell mitosis was absent at these stages (Fahrioglu et al., 2007). Because gonocyte mitotic reactivation requires *Dmrt1* but is unaffected by elimination of *Dmrt1* in Sertoli cells, we conclude that the requirement is likely to be germ line-autonomous.

Meiotic progression, but not initiation, requires Dmrt1 activity in Sertoli cells

To examine the onset of the defects in *SCDmrt1KO* testes, we used antibodies to TRA98 and GATA4 to follow the fates of germ cells and Sertoli cells, respectively, between P5 and P14 (Figure 4). As shown in Figure 2, germ cell migration appears slightly delayed at P5 in *SCDmrt1KO* mutants (Figure 4A), but it does occur by P7 (Figure 4B), and otherwise little

difference was apparent between control and *SCDmrt1KO* testes up to P7. However, by P9 the distribution of TRA98-positive germ cells was highly abnormal in *SCDmrt1KO* mutants, with many more cells in the center and many fewer at the periphery of the tubules relative to controls (Figure 4C). This confirms the histology in Figure 2 showing that the mutant germ cells, although able to migrate to the periphery, fail to maintain proper organization with Sertoli cells. At P14 in control tubules Sertoli cell nuclei were intercalated among spermatogonia at the periphery, and there was an adluminal compartment contained differentiating meiotic germ cells. This indicates that Sertoli cell polarization was occurring and separate populations of spermatogonia (peripheral) and spermatocytes (adluminal) were being established. In contrast, in the *SCDmrtr1KO* seminiferous tubules there was no organized peripheral ring of Sertoli cells and spermatogonia; instead the tubules were filled with TRA98-positive germ cells and lacked a lumen, and many Sertoli cells were located away from the basal lamina (Figure 4D). In addition, some tubules had very few spermatogonia at the periphery at P14, suggesting that *Dmrt1* in Sertoli cells may play a role in helping maintain the spermatogonial population.

Although meiosis can initiate in *SCDmrt1KO* testes, germ cells are absent by P28, as shown earlier. To examine meiotic progression, we used two stage-specific germ cell antibody markers: BC7, which recognizes leptotene to zygotene spermatocytes (Koshimizu et al., 1993); and TRA369, which recognizes pachytene spermatocytes through elongated spermatids (Watanabe et al., 1992). At P14, some *SCDmrt1KO* tubules contained many BC7-positive cells, indicating that initiation of meiotic prophase does not require *Dmrt1* in Sertoli cells (Figure 5A). However, very few cells in *SCDmrt1KO* testes were TRA369-positive, suggesting a possible meiotic prophase arrest (Figure 5B). We also examined P21 testes, in case meiotic progression was delayed rather than arrested, but also found very few TRA369-positive cells (data not shown). From these results we conclude that germ cells in *SCDmrt1KO* testes efficiently enter meiosis, but most of them arrest at or before pachynema.

The results described so far indicate that Sertoli cells are present in *SCDmrt1KO* mutants and that germ cells move and develop appropriately until approximately P7 and can reactivate mitosis and enter meiotic prophase in approximately normal numbers. After this stage, however, *SCDmrt1KO* testes begin to exhibit a number of abnormalities. These defects include aberrant organization of germ cells and Sertoli cells, failure to establish a spermatogonial population at the periphery, failure of Sertoli cell polarization and lumen formation, and meiotic arrest leading to germ cell death.

Dmrt1 is required in Sertoli cells for their differentiation

In *Dmrt1* null mutants, Sertoli cells over-proliferate and fail to adopt a differentiated morphology (Raymond et al., 2000). Genome-wide mRNA expression analysis in *Dmrt1* mutant testes has identified a number of Sertoli cell markers that are underexpressed at P1 and P2, further indicating that Sertoli cell differentiation is abnormal in *Dmrt1* mutants (Fahrioglu et al., 2007). As described above, Sertoli cells in *SCDmrt1KO* mutants also appear incompletely differentiated and fail to support germ cell development.

We further investigated Sertoli cell differentiation in *SCDmrt1KO* mutants using two maturation markers, the androgen receptor (AR) (Figure 6) and GATA1 (Figure 7). In rodents AR protein is expressed in peritubular myoid cells at all stages, and is up-regulated in Sertoli cells prior to their maturation (Bremner et al., 1994). This up-regulation occurred between P5 and P9 in Sertoli cells of control testes but not in those of *SCDmrt1KO* testes (Figure 6). However, at P21 some Sertoli cell expression of AR was present, indicating that Sertoli cell maturation was delayed. Consistent with this conclusion, GATA4 expression was elevated in Sertoli cells at P21 and P28 in *SCDmrt1KO* testes (not shown). GATA1 was activated in Sertoli cells of *SCDmrt1KO* mutants, but later than in controls, and GATA1 expression was lost in

adulthood (14 weeks) in the mutants (Figure 7). GATA1 staining at P21 also clearly revealed the abnormal arrangement of Sertoli cell nuclei in *SCDmrt1KO* tubules described earlier.

Both the histological analysis and antibody staining indicated that Sertoli cell defects observed in *SCDmrt1KO* mutants closely resemble those we previously observed in *Dmrt1* null mutants. We conclude that *Dmrt1* activity is required autonomously in the Sertoli cell lineage, and that *Dmrt1* activity in germ cells has little overt effect on Sertoli cell differentiation.

Dmrt1 is required cell-autonomously for germ cell radial migration

The results described so far demonstrate that *Dmrt1* activity in Sertoli cells is required for their postnatal differentiation and also for meiotic progression and survival of germ cells. In principle, *Dmrt1* activity in Sertoli cells could explain much of the defective germ cell development we previously observed in *Dmrt1* null mutants. However, germ cells in *Dmrt1* null mutants are more severely affected than those in *SCDmrt1KO* mutants, strongly suggesting that germ cell development requires *Dmrt1* activity in the germ cells as well as in the Sertoli cells.

To test the requirement for *Dmrt1* in germ cells directly, we conditionally targeted *Dmrt1* to generate germ cell-specific *Dmrt1* knockout (*GCDmrt1KO*) mice. We used a *TNAP-Cre* (Tissue Non-specific Alkaline Phosphatase-Cre) transgene, which is active in primordial germ cells prior to their colonization of the gonad (Lomeli et al., 2000). Although *TNAP-Cre* activity is less efficient and cell type-specific than that of *Dhh-Cre*, comparison of germ cell phenotypes between *SCDmrt1KO* and *GCDmrt1KO* mutants should nevertheless reveal germ line-autonomous requirements for *Dmrt1*.

GCDmrt1KO animals and littermate controls were generated by a breeding scheme analogous to that for *SCDmrt1KO* mice. To confirm the selective deletion of *Dmrt1* in *GCDmrt1KO* mutant germ cells, we first double-stained with antibodies to DMRT1 and GATA4 (Figure 8A). At P7, this revealed a number of tubule sections in *GCDmrt1KO* testes in which no *Dmrt1*-positive germ cells were present and nearly all Sertoli cells retained DMRT1 expression. Thus *TNAP-Cre* was able to selectively eliminate *Dmrt1* from germ cells. Next we stained P7 *GCDmrt1KO* testes for DMRT1 and TRA98 (Figure 8B). As expected, we observed tubule sections lacking DMRT1-positive germ cells. In contrast to the germ cells in control testes, many germ cells lacking DMRT1 had failed to migrate to the periphery (mutant: 63% failed to migrate, 265 cells counted in 60 tubule sections; control: 16% failed to migrate, 1003 cells counted in 68 tubule sections). We conclude from these results that *Dmrt1* is required in germ cells but not in Sertoli cells for germ cell radial migration, as suggested by the *SCDmrt1KO* results described earlier. In addition, the reduced number of germ cells in *GCDmrt1KO* testes is consistent with the expected failure of mutant germ cells to reactivate mitosis.

Dmrt1 is required germ line-autonomously for germ cell survival

In *Dmrt1* null mutants, germ cells are virtually absent by P14 and do not enter meiosis. This contrasts with *SCDmrt1KO* mutants, in which many germ cells were still present at P14 and had efficiently entered meiotic prophase. The likely explanation is that *Dmrt1* is required cell-autonomously in germ cells for survival beyond P10 and for meiotic initiation. However, we could not exclude an alternative possibility: the more severe germ cell defects in *Dmrt1* null mutations might result exclusively from an embryonic requirement for *Dmrt1* in pre-Sertoli cells, prior to the time of *Dhh-Cre* action. We therefore examined *GCDmrt1KO* mutants at P14, asking whether the germ cell phenotype more closely resembles that of *Dmrt1* null mutants or *SCDmrt1KO* mutants (Figure 9). Histological analysis at P14 revealed that *GCDmrt1KO* mutants had some tubules completely lacking germ cells, and these contained normally

polarized Sertoli cells (Figure 9A, arrowheads). This was confirmed by double-staining with antibodies to DMRT1 and TRA98 (Figure 9B): in control testes all tubule sections contained many TRA98-positive germ cells; but some tubule sections of *GCDmrt1KO* had no germ cells (arrowheads). The identity of the surviving cells was confirmed by staining for DMRT1 and GATA1 (Figure 9C), which revealed tubule sections in which all cells were GATA1-positive Sertoli cells. These data demonstrate that *Dmrt1* loss in the germ line causes germ cells to die much earlier than does *Dmrt1* loss in Sertoli cells.

Based on analysis of *GCDmrt1KO* mutants, we conclude that *Dmrt1* is required cell-autonomously in germ cells for radial migration and survival to P14, but *Dmrt1* expression in germ cells is not required for Sertoli cell polarization.

DISCUSSION

In this study we have used conditional gene targeting to dissect the functions of *Dmrt1* in Sertoli cells and germ cells, comparing the phenotypes of animals lacking *Dmrt1* function in each of these cell lineages to each other and to null mutant animals lacking *Dmrt1* in all cells. This approach has uncovered cell type-autonomous requirements for *Dmrt1* during early postnatal testis development in both Sertoli cells and germ cells, as well as a cell non-autonomous requirement for *Dmrt1* in Sertoli cells, as summarized in Table 1 and discussed below.

Deletion of *Dmrt1* in Sertoli cells was particularly informative. We found that *Dmrt1* mutant Sertoli cells in *SCDmrt1KO* animals failed to become closely associated with the tubule periphery. Instead they were distributed throughout the tubules, eventually filling the entire tubule. This closely resembles the Sertoli cell phenotype of *Dmrt1* null mutants. Mutant Sertoli cells in *SCDmrt1KO* animals also failed to up-regulate the androgen receptor and polarize, indicating a developmental block at an immature stage, again very similar to the phenotype of *Dmrt1* null mutants. We conclude that these defects are indicative of lineage-autonomous functions for *Dmrt1* in Sertoli cells.

Although Sertoli cells in *SCDmrt1KO* mice initially activated GATA1 expression by P21, suggesting that they had undergone partial maturation, this expression was lost by 14 weeks. We speculate that this might indicate “de-differentiation” of mutant Sertoli cells, which may help explain the disintegration of seminiferous tubules and changes in Sertoli cell morphology that occur in *Dmrt1* null mutants between 6 weeks and about four months (Raymond et al., 2000) (DZ, unpublished data). The basement membrane surrounding the semiferous tubules is produced jointly by peritubular myoid (PTM) cells and Sertoli cells (Skinner et al., 1985; Tung and Fritz, 1987). De-differentiation of mutant Sertoli cells in adults may result in failure to maintain the basement membrane and eventual loss of tubule integrity.

Sertoli cell-specific targeting also revealed that *Dmrt1* is required in Sertoli cells for germ cell development. In *SCDmrt1KO* mice, germ cells underwent radial migration and mitotic reactivation, and initiated meiosis, but they failed to complete meiosis and eventually died. This demonstrates that *Dmrt1* activity in Sertoli cells is required for proper support of the germ line during meiosis, but apparently not for the events leading up to meiotic initiation. The eventual complete loss of germ cells, including spermatogonia, in *SCDmrt1KO* mutants suggests that *Dmrt1* activity in Sertoli cells is required to establish a functional niche capable of supporting spermatogenesis. Germ cell failure in *SCDmrt1KO* mutants might result directly from the mutant Sertoli cells failing to produce a specific signal(s), such as a secreted signaling molecule, or from a more general failure of Sertoli cells to functionally associate with and nutritionally support germ cells, or both. Several candidate signaling pathways have been identified by mRNA expression profiling at P1 and P2, which suggested possible disruption

in *Dmrt1* null mutants of several signaling pathways, including GDNF, FSH, and retinoic acid (Fahrioglu et al., 2007).

Deletion of the androgen receptor (AR) in Sertoli cells (in "*SCARKO*" mutant mice), like deletion of *Dmrt1* in Sertoli cells, has been shown to affect Sertoli cell maturation and germ cell development (Chang et al., 2004; De Gendt et al., 2004; Holdcraft and Braun, 2004; Tan et al., 2005). Thus the delayed activation of AR in *Dmrt1* mutant Sertoli cells may partially account for the Sertoli cell and germ cell phenotypes of *SCDmrt1KO* mutants. However, we observed additional defects in *SCDmrt1KO* testes, including the failure of Sertoli cells to localize properly and to polarize, and a more severe disruption of germ cell development in *SCDmrt1KO* mutants versus *SCARKO* mutants. These phenotypic differences indicate that *Dmrt1* cannot function exclusively via the AR pathway.

The germ cell defects in *SCDmrt1KO* mutants were less severe than in *Dmrt1* null mutants, suggesting that *Dmrt1* also is required autonomously in germ cells, and we confirmed this interpretation using *GCDmrt1KO* animals. *Dmrt1* mutant germ cells in *GCDmrt1KO* mice were defective in radial migration and died between P7 and P14, just as in *Dmrt1* null mutants. This contrasts with the wild type germ cells in *SCDmrt1KO* animals, which migrated and were still present at P14. The relatively low targeting efficiency of the *TNAP-Cre* transgene limited the analysis possible in *GCDmrt1KO* mutants. However, comparison of germ cells in *Dmrt1* null mutants and *GCDmrt1KO* animals versus *SCDmrt1KO* animals clearly showed that *Dmrt1* is autonomously required in germ cells for radial migration and survival. To our knowledge this is the first demonstration of a genetic requirement in germ cells for postnatal migration.

It is clear from *SCDmrt1KO* mutants that *Dmrt1* activity is required in Sertoli cells for germ cell survival and differentiation, as discussed above. We did not observe any evidence of the converse: that *Dmrt1* activity in germ cells is required for Sertoli cell differentiation. This conclusion is somewhat limited by the caveat that tubule sections in *GCDmrt1KO* mutants with all germ cells negative for *Dmrt1* were uncommon, but in such tubules the wild type Sertoli cells were properly organized at the periphery and were polarized.

As mentioned earlier, *Dmrt1* is unusual in being expressed in germ cells and Sertoli cells. These cell types are derived from unrelated progenitor populations in different parts of the early embryo and only come together later as a result of long-range migration by the germ cells. Thus it is perhaps unsurprising that most other testis regulators are expressed only in one cell type or the other. It is unknown in which cells the ancestral function of *Dmrt1* likely arose, or even whether the expression pattern of *Dmrt1* in the mouse is typical, because a variety of *Dmrt1* expression patterns have been reported in vertebrates. In humans, fetal *DMRT1* mRNA has been described only in Sertoli cells (Moniot et al., 2000), while DMRT1 protein is expressed only in germ cells in the adult testis (Looijenga et al., 2006). Similarly, in a lizard, *Dmrt1* mRNA is expressed initially in pre-Sertoli cells and then in germ cells (Sreenivasulu et al., 2002). In fish, *Dmrt1* mRNA has been reported to be expressed only in Sertoli cells, or only in germ cells, or in both (Kobayashi et al., 2004; Marchand et al., 2000; Xia et al., 2007). However, most of these studies examined limited developmental stages, so expression in both Sertoli cells and germ cells cannot be excluded in any group of vertebrates. Although it is unclear how common combined Sertoli and germ cell expression of *Dmrt1* may be among vertebrates, our results demonstrate that *Dmrt1* function is essential in both cell types for testicular differentiation and fertility in the mouse.

In conclusion, the work described here demonstrates that *Dmrt1* function is required in Sertoli cells and germ cells for their respective postnatal development, and also has a function in Sertoli cells that is required for germ cell differentiation and survival. These data better define what

this essential and highly conserved testicular regulator does in each cell type, which is important for eventually understanding how the remodeling and differentiation of the juvenile testis is controlled.

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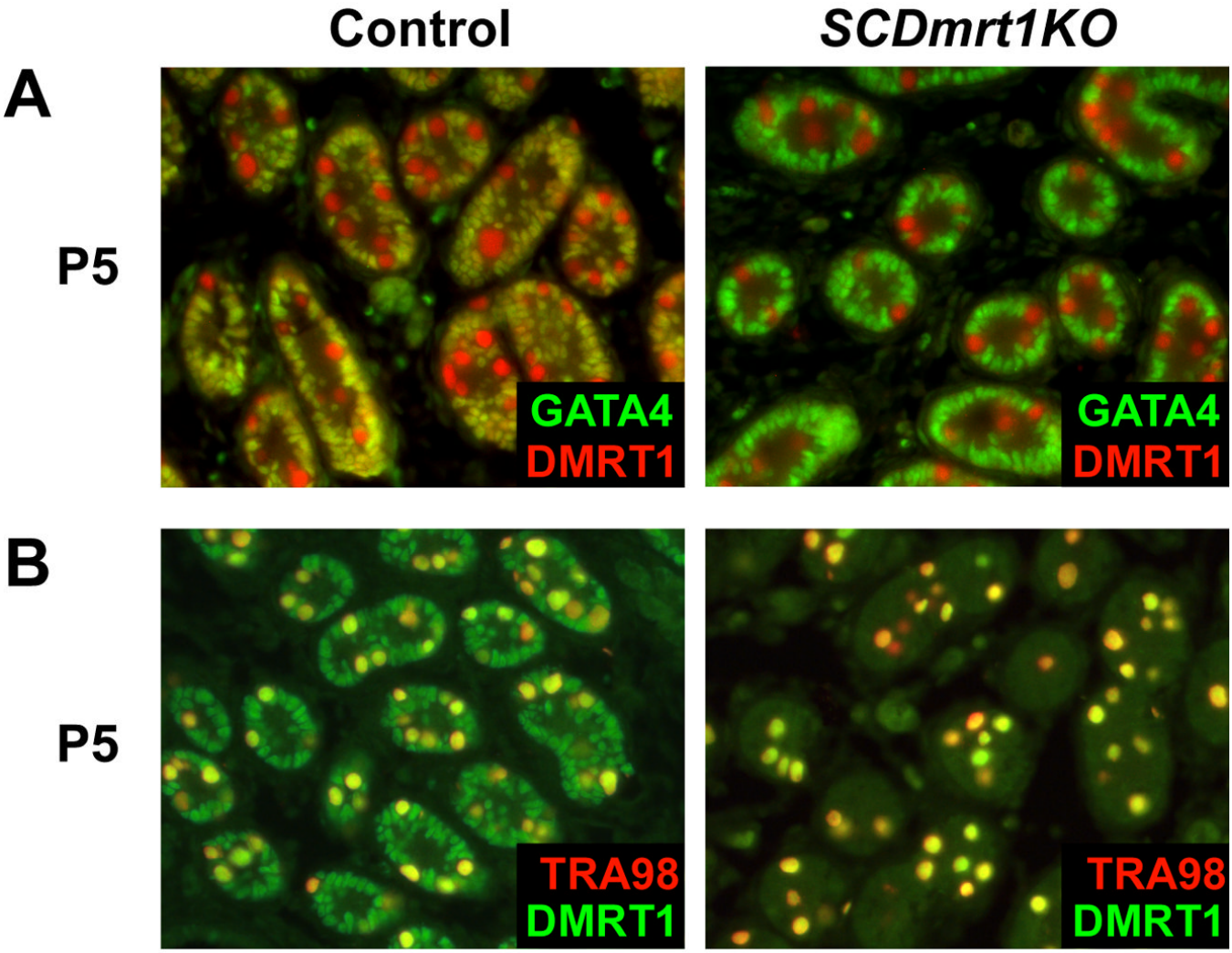

Figure 1. Specific and efficient deletion of *Dmrt1* in Sertoli cells of *SCDmrt1KO* mutants

(A) DMRT1 expression in Sertoli cells of control and *SCDmrt1KO* testes at P5. Double staining with antibodies to DMRT1 and the juvenile Sertoli cell marker GATA4. Merged image shows that DMRT1 is ablated from virtually all Sertoli cells in *SCDmrt1KO* testis.

(B) DMRT1 expression in germ cells of control and *SCDmrt1KO* testes at P5. Double staining with antibodies to DMRT1 and the gonocyte and spermatogonial marker TRA98. Merged image shows that almost all germ cells express DMRT1 in *SCDmrt1KO* testis. The rare germ cells lacking DMRT1 may be entering meiosis and down-regulating DMRT1 expression, rather than mutant for *Dmrt1*, as they also were present in control animals (not shown).

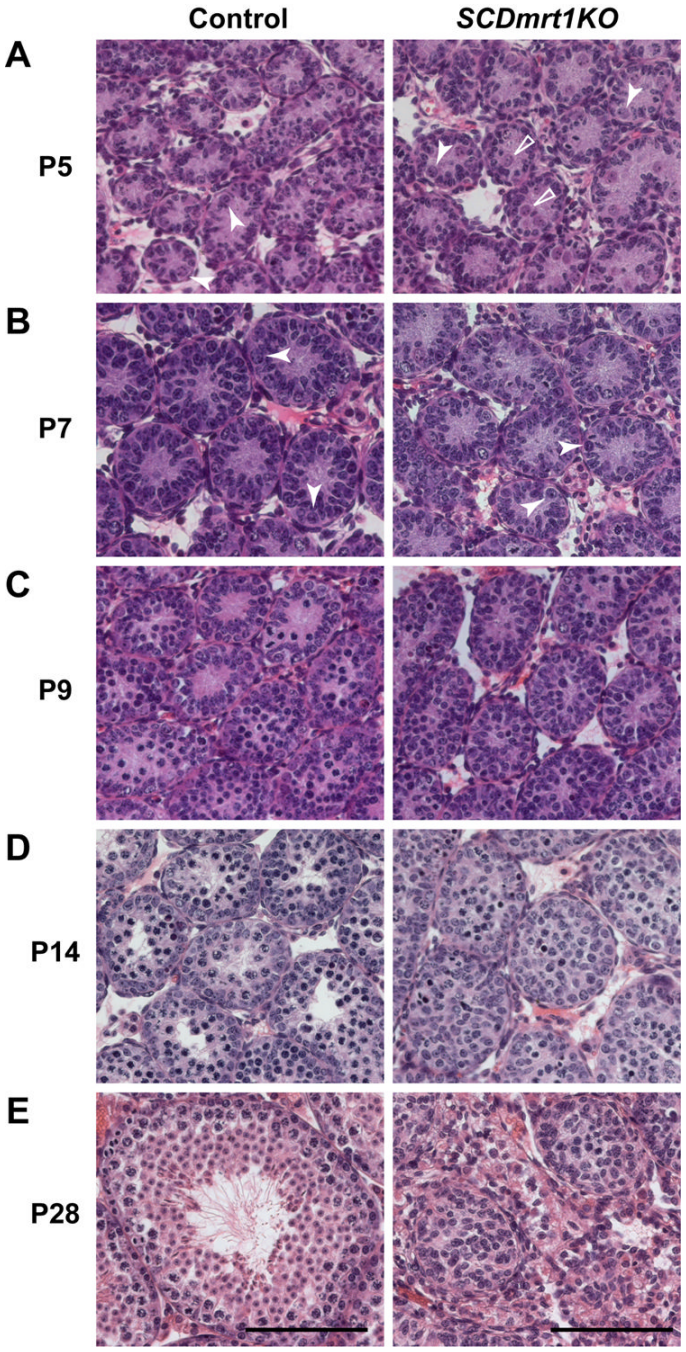

Figure 2. Abnormal seminiferous tubule morphology in *SCDmrt1KO* testes

Hematoxylin/eosin staining of testis sections.

(A) At P5 in both control and *SCDmrt1KO* testes most germ cells have migrated to the basal lamina at the periphery of the seminiferous tubule (closed arrowheads), although some germ cells remain in the center of the tubules (open arrowheads).

(B) At P7, germ cell migration is complete in control and *SCDmrt1KO* testes (closed arrowheads) and cellular organization is normal in both.

(C) At P9, control testes have Sertoli cells and spermatogonia at the periphery, meiotic cells in the adluminal compartment, and a developing central lumen. In *SCDmrt1KO* tubules Sertoli

cells and spermatogonia are present at the periphery, but Sertoli cells also are found away toward the center of the tubules and minimal lumen formation is apparent.
(D) At P14, control tubules are normally organized, with Sertoli cells and spermatogonia at the periphery, meiotic cells in the center, and an enlarging lumen forming. *SCDmrt1KO* tubules are disorganized with Sertoli cells and germ cells not properly localized and no lumen evident.
(E) At P28, control tubule has abundant spermatogenesis, with spermatogonia established at the periphery, meiotic germ cells up to elongated spermatid stage, mature Sertoli cells and fully formed lumen. In *SCDmrt1KO* tubules germ cells are absent, and Sertoli cells retain immature morphology and fill the tubules.
Scale bar: 100 μM. All images are at same magnification.

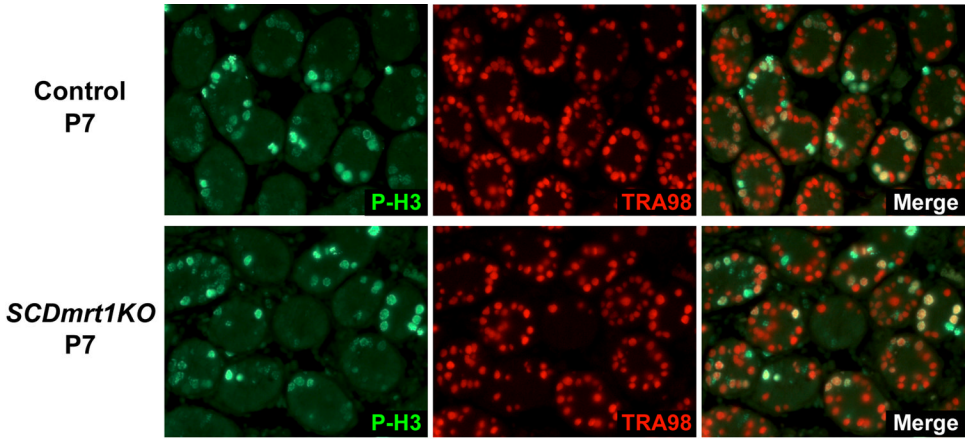

Figure 3. Germ cell mitotic reactivation does not require *Dmrt1* in Sertoli cells

Double staining for the mitotic marker phospho-histone H3 (P-H3) and germ cell marker TRA98 reveals similar numbers of mitotic germ cells in control and *SCDmrt1KO* testes. In merged panels at far right, yellow cells are mitotic germ cells, and green cells are mitotic Sertoli cells. In these experiments, staining with the early meiotic prophase marker BC7 was used to confirm that the P-H3 positive germ cells were not meiotic (not shown).

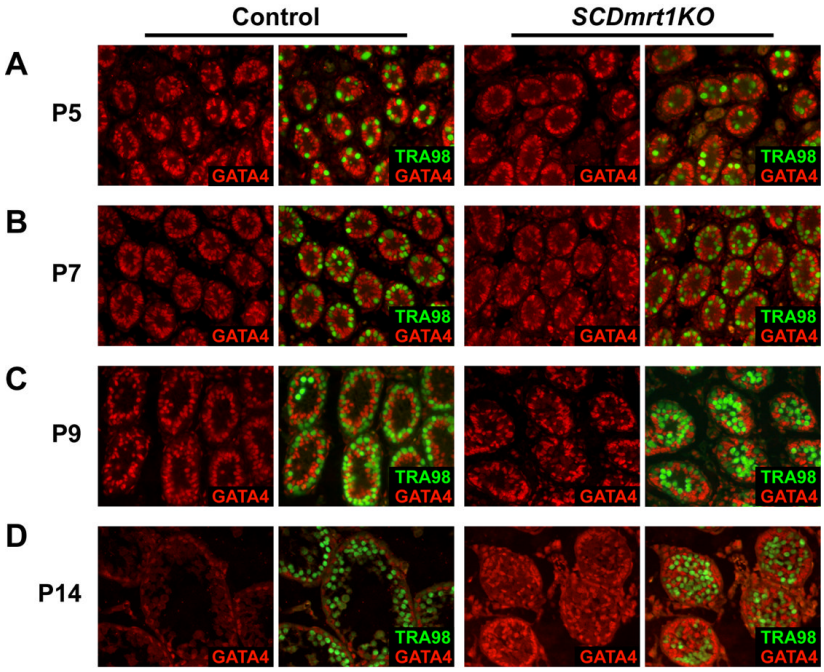

Figure 4. *Dmrt1* is not required in Sertoli cells for germ cell radial migration and meiotic initiation

(A) At P5, most germ cells (TRA98-positive) are at the periphery in both control and *SCDmrt1KO* tubules.

(B) At P7 tubules in *SCDmrt1KO* testes appear normal, with most germ cells interspersed with Sertoli cells (GATA4-positive) at the periphery.

(C) At P9, abundant germ cells are present in control and *SCDmrt1KO* tubules, and brightly staining TRA98-positive meiotic cells are present in both, but germ cells and Sertoli cells are beginning to become abnormally organized.

(D) At P14, *SCDmrt1KO* testes still have abundant meiotic germ cells, but germ cells and Sertoli cell organization is severely abnormal in many tubules compared with controls.

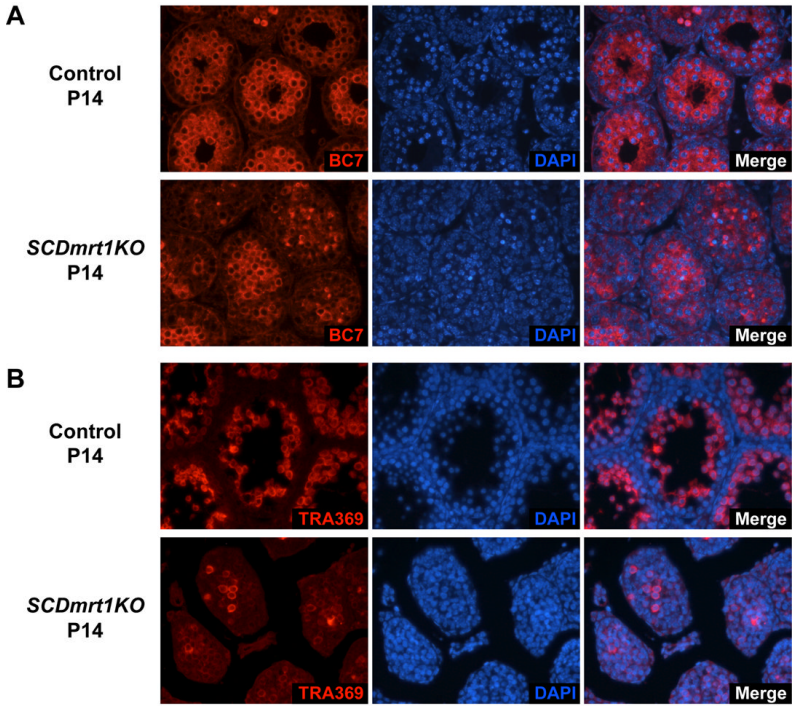

Figure 5. Progression through meiotic prophase requires *Dmrt1* in Sertoli cells

(A) Leptotene and zygotene marker BC7 at P14, showing many BC7-positive cells in both control and *SCDmrt1KO* testes.

(B) Pachytene to elongated spermatid marker TRA369, showing very few positive cells in *SCDmrt1KO* testis, indicating a disruption in meiosis between leptonema and pachynema. All images are at same magnification; differences in tubule morphology between panels A and B are due to different fixation protocols used for the two antibodies.

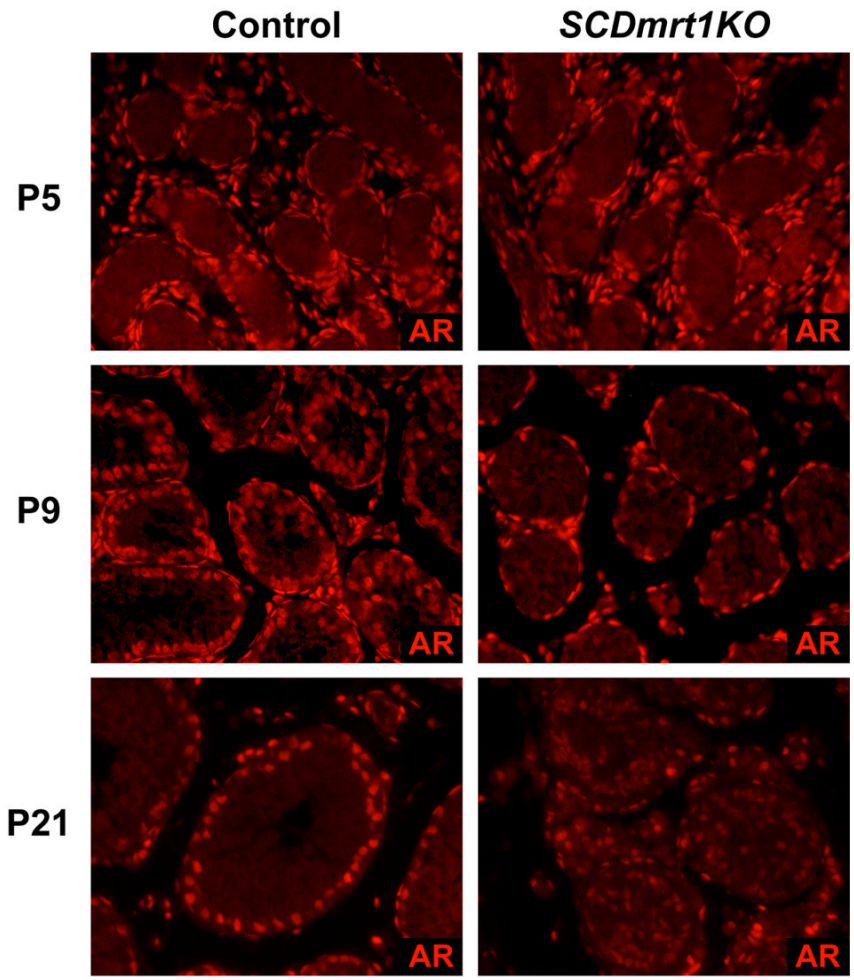

Figure 6. *Dmrt1* is required for normal activation of androgen receptor in Sertoli cells

At P5, androgen receptor (AR) is expressed in peritubular myoid cells (at the tubule margins) and interstitial cells in control and *SCDmrt1KO* testes. At P9, AR has been activated in Sertoli cells of control testes but not in *SCDmrt1KO*. At P21, AR expression is present in Sertoli cells of *SCDmrt1KO*, but at lower level than in control.

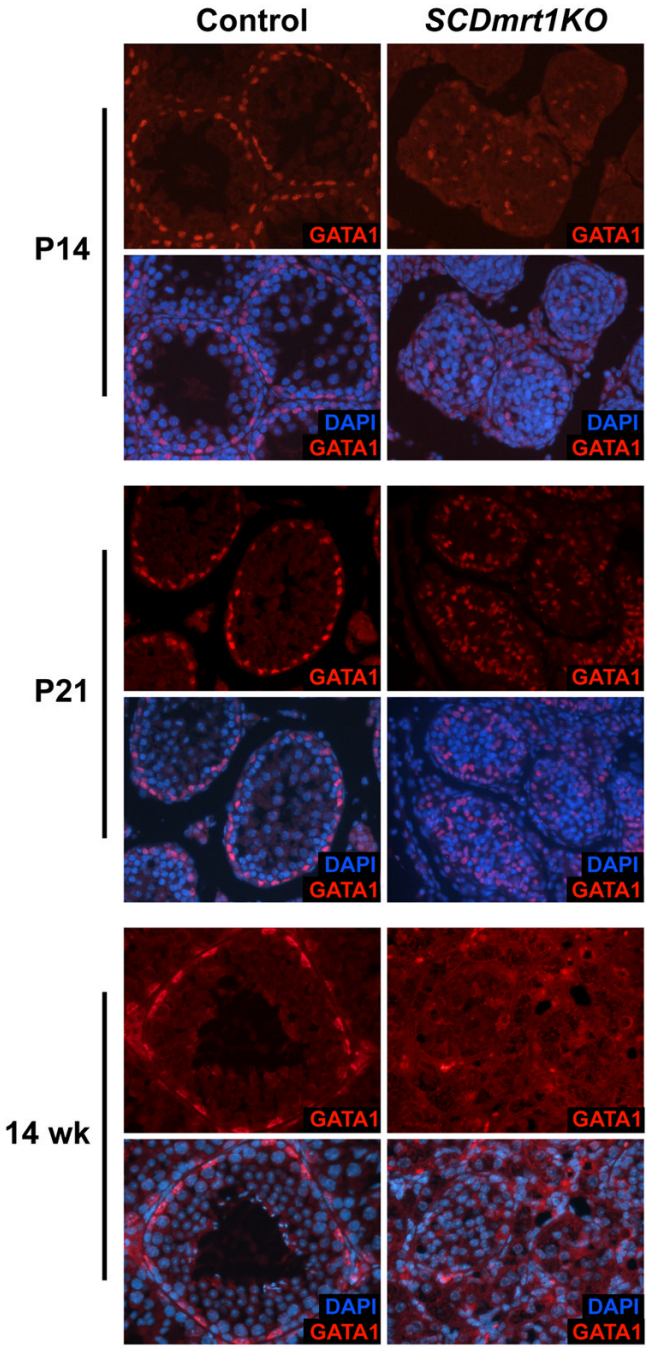

Figure 7. *Dmrt1* is required for normal activation and for maintenance of GATA1 expression in Sertoli cells

At P14, GATA1 is expressed at reduced level in *SCDmrt1KO* testes relative to control (top panels) and reaches near-normal levels in Sertoli cells of *SCDmrt1KO* testes by P21 (middle). At 14 weeks (bottom), GATA1 expression is maintained in Sertoli cells of control but not *SCDmrt1KO* testes.

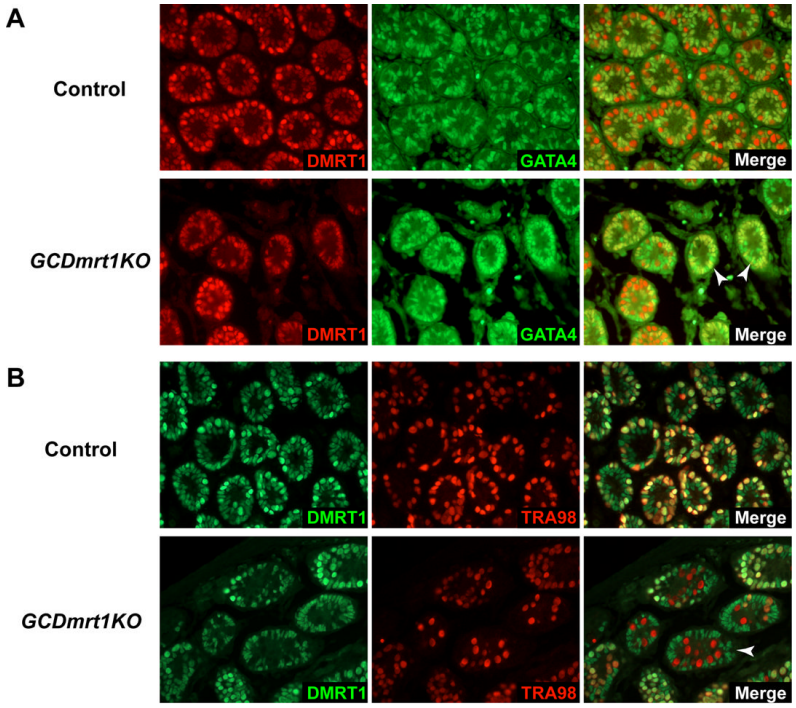

Figure 8. *Dmrt1* is required in germ cells for their radial migration

(A) Double staining with antibodies to DMRT1 and GATA4 showing tubules in *GCDmrt1KO* testes at P7 in which all germ cells lack DMRT1 (arrowheads).

(B) Double staining with antibodies to DMRT1 and TRA98 at P7 showing that many *Dmrt1* mutant germ cells in *GCDmrt1KO* testis have failed to migrate to the periphery (arrowhead), whereas the great majority of germ cells in control testis have done so.

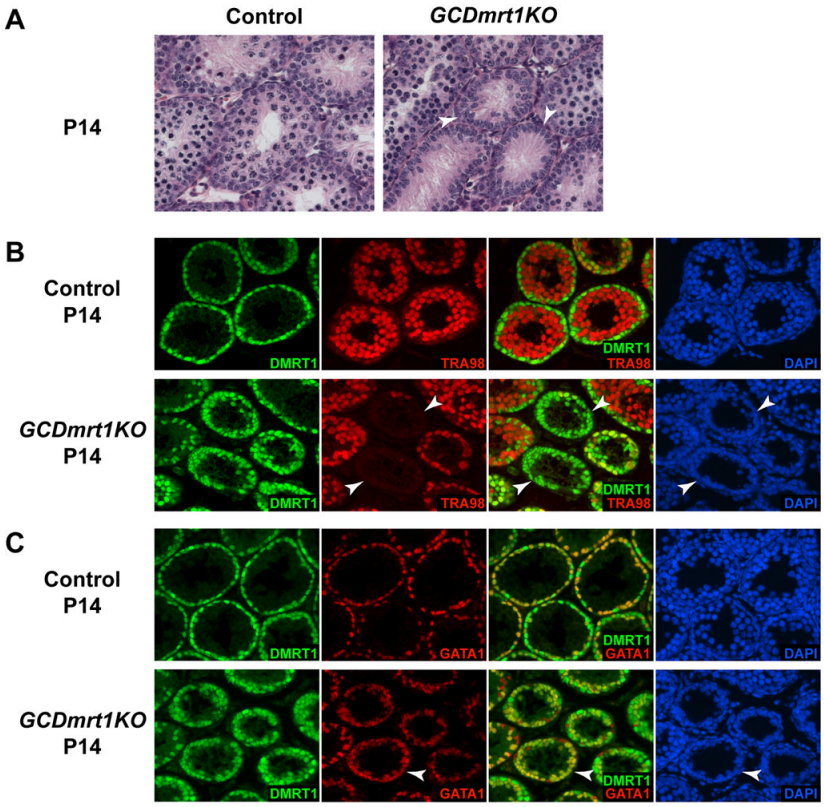

Figure 9. Germ cell survival and meiosis require *Dmrt1* in germ cells

(A) Histology of control and *GCDmrt1KO* testes at P14. In *GCDmrt1KO* some tubule sections lack germ cells and have Sertoli cells of normal polarized morphology (arrowheads).

(B) Double-staining with DMRT1 and TRA98 antibodies at P14. Some tubules in *GCDmrt1KO* testis have few or no germ cells (arrowheads). Surviving germ cells all are DMRT1-positive. Tubules in which wild type spermatogonia (double-positive cells) are present have abundant meiotic germ cells.

(C) Double-staining with DMRT1 and GATA1 antibodies at P14, showing *GCDmrt1KO* tubule in which germ cells are absent and DMRT1 is expressed in almost all Sertoli cells (arrowhead). Red cells in merged image of *GCDmrt1KO* mutant are Sertoli cells (GATA4 positive) in which *Dmrt1* was deleted by *TNAP-Cre*.

Table 1

Genotype	Cell type	Processes affected
Dmrt1$^{-/-}$	Sertoli	maturation
" "	Germ	radial migration, mitosis, meiotic initiation, survival
SCDmrt1KO	Sertoli	maturation
" "	Germ	meiotic progression, survival
GCDmrt1KO	Sertoli	none observed
" "	Germ	radial migration, mitosis, meiotic initiation, survival