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RESEARCH ARTICLE

The mammalian Doublesex homolog DMRT6 coordinates the transition between mitotic and meiotic developmental programs during spermatogenesis

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ABSTRACT

In mammals, a key transition in spermatogenesis is the exit from spermatogonial differentiation and mitotic proliferation and the entry into spermatocyte differentiation and meiosis. Although several genes that regulate this transition have been identified, how it is controlled and coordinated remains poorly understood. Here, we examine the role in male gametogenesis of the *Doublesex*-related gene *Dmrt6* (*Dmrtb1*) in mice and find that *Dmrt6* plays a crucial role in directing germ cells through the mitotic-to-meiotic germ cell transition. DMRT6 protein is expressed in late mitotic spermatogonia. In mice of the C57BL/6J strain, a null mutation in *Dmrt6* disrupts spermatogonial differentiation, causing inappropriate expression of spermatogonial differentiation factors, including SOHLH1, SOHLH2 and DMRT1 as well as the meiotic initiation factor STRA8, and causing most late spermatogonia to undergo apoptosis. In mice of the 129Sv background, most *Dmrt6* mutant germ cells can complete spermatogonial differentiation and enter meiosis, but they show defects in meiotic chromosome pairing, establishment of the XY body and processing of recombination foci, and they mainly arrest in mid-pachynema. mRNA profiling of *Dmrt6* mutant testes together with DMRT6 chromatin immunoprecipitation sequencing suggest that DMRT6 represses genes involved in spermatogonial differentiation and activates genes required for meiotic prophase. Our results indicate that *Dmrt6* plays a key role in coordinating the transition in gametogenic programs from spermatogonial differentiation and mitosis to spermatocyte development and meiosis.

KEY WORDS: DMRT6, DMRT1, Spermatogenesis, Meiosis, Testis, Mouse

INTRODUCTION

Male mammals continuously produce large numbers of sperm from puberty onwards, in some cases for decades. This sustained gametogenesis is supported by a population of undifferentiated type A spermatogonia that includes spermatogonial stem cells, or SSCs (Nakagawa et al., 2010), and is embedded within a seminiferous epithelium formed by Sertoli cells (Griswold, 1998). In the course of developing from single undifferentiated A_s spermatogonia to mature spermatozoa, male germ cells in the mouse undergo a series of nine mitotic and two meiotic cell divisions, accompanied by a number of differentiation steps. This extended series of cell divisions allows an individual A_s cell that undergoes differentiation to produce more than 4000 spermatozoa and involves dramatic changes, that include epigenetic reprogramming, reduction of ploidy from 2N to 1N, repackaging of chromatin by histone-to-protamine replacement and morphological transformation to allow survival and motility in the female reproductive tract (Dada et al., 2012; de Rooij and Russell, 2000; Jan et al., 2012).

Spermatogenesis involves several crucial transitions between distinct developmental programs. During steady-state spermatogenesis, division of A_s spermatogonia produces, by incomplete cytokinesis, chains of two to 16 undifferentiated spermatogonia (A_{pr} and A_{al}) linked by cytoplasmic bridges. Spermatogonial differentiation starts when these undifferentiated spermatogonia form A_1 spermatogonia. This transition occurs in waves that sweep the seminiferous tubules with a species-specific period, which is 8.6 days in mice. A_1 spermatogonia then undergo five additional rounds of mitotic division, coupled with differentiation, to generate $A_{2\text{-}4}$, intermediate (Int) and type B spermatogonia (de Rooij, 1998, 2001; de Rooij and Grootegoed, 1998; de Rooij and Russell, 2000). A_1-B spermatogonia are collectively called differentiating spermatogonia, but they have discrete morphologies and gene expression profiles and are found at distinct stages of the seminiferous epithelial cycle.

When spermatogonial differentiation is complete, germ cells need to make a further transition into meiosis. B spermatogonia divide mitotically to form preleptotene spermatocytes, which undergo a final mitotic division before entering meiosis to eventually form haploid spermatids. Spermatids do not divide further after meiosis but undergo extensive postmeiotic differentiation to form spermatozoa. Because spermatid differentiation requires 35 days but new waves of differentiation initiate every 8.6 days, differentiating germ cells accumulate in layers above the undifferentiated spermatogonia. The cellular composition of these layers varies during the cycle, allowing the cycle to be divided into morphologically distinct stages (de Rooij, 1998, 2001; de Rooij and Grootegoed, 1998; de Rooij and Russell, 2000). Continuously producing large numbers of gametes without depleting the intermediate cell types requires tight and well-integrated control of proliferation and differentiation at many points in the process, all in the context of major transitions in the genome, epigenome and cell biology of the male germ line.

Spermatogonial development is under the control of two prominent signaling pathways. GDNF signaling plays a crucial role in maintenance of SSCs. Mutations in *Gdnf* or its co-receptors *Ret* and *Gfra1* cause progressive germ cell loss indicative of SSC depletion, whereas overexpression of GDNF causes accumulation of undifferentiated A_s cells (Buageaw et al., 2005; Meng et al., 2000; Naughton et al., 2006). Retinoic acid (RA) is required for the initiation of spermatogonial differentiation in the juvenile testis (Mark et al., 2008), for entry of undifferentiated spermatogonia into

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differentiation (the A_{al} to A_1 transition) during steady-state adult spermatogenesis and likely for the initiation of meiosis by preleptotene spermatocytes (Griswold et al., 1989; Hogarth and Griswold, 2010; McCarthy and Cerecedo, 1952; Snyder et al., 2010; Thompson et al., 1964; Van Pelt and de Rooij, 1990). The latter two functions of RA are thought to be mediated by the RA-inducible gene *Stra8* (Anderson et al., 2008; Mark et al., 2008; Oulad-Abdelghani et al., 1996).

A number of transcriptional regulators also have been shown to play essential roles in controlling spermatogonial differentiation, including the basic helix-loop-helix (bHLH) proteins SOHLH1 and SOHLH2, and the DMRT protein DMRT1 (Ballow et al., 2006; Hao et al., 2008; Matson et al., 2010; Suzuki et al., 2012). DMRT proteins are transcription factors that bind DNA via the DM domain, a structurally distinct class of zinc-finger motif (Erdman and Burtis, 1993; Zhu et al., 2000). DMRT proteins occur in virtually all metazoan animals and regulate sexual development in a wide variety of species, ranging from planaria to insects to nematodes to vertebrates (Matson and Zarkower, 2012). In mice, DMRT1 is required in germ cells at several stages of their development, and another DMRT protein, DMRT7 (DMRTC2 – Mouse Genome Informatics), associates with the sex chromosomes of spermatocytes during meiosis and is required for sex chromatin modification (Fahrioglu et al., 2007; Kim et al., 2007b; Krentz et al., 2009; Matson et al., 2010; Raymond et al., 2000).

Here, we examine the role of the DMRT protein DMRT6 (DMRTB1 – Mouse Genome Informatics) in gametogenesis. Previous analysis showed that *Dmrt6* is widely conserved among vertebrates and is expressed strongly in the gonad in mice (Kim et al., 2003; Ottolenghi et al., 2002). We show that DMRT6 protein is expressed in the postnatal mouse testis in differentiating spermatogonia, disappearing as B spermatogonia become preleptotene spermatocytes. Using a *Dmrt6* null allele, we found that *Dmrt6* is crucial for spermatogenesis: loss of *Dmrt6* in C57BL/6J (B6) mice disrupted the transition from A_4 to In and B spermatogonia and caused the extended expression of spermatogonial differentiation factors, such as SOHLH1, SOHLH2 and DMRT1 and the meiotic initiation factor STRA8, into inappropriate cell types. Analysis of *Dmrt6* in mice of the 129Sv genetic background revealed an additional requirement for spermatogonial expression of *Dmrt6*: in these mice most spermatogonia completed differentiation and enter meiosis but showed defects in chromosome pairing, establishment of the XY body and processing of recombination foci, with very few cells progressing beyond mid-pachynema. mRNA profiling of *Dmrt6* mutant testes and DMRT6 chromatin immunoprecipitation sequencing (ChIP-seq) analysis suggested that DMRT6 helps to coordinate the transition from spermatogonial development to meiosis by repressing genes involved in spermatogonial differentiation and by activating genes required for meiotic prophase. Relatively little is known about how differentiation of late-stage spermatogonia is controlled or how spermatogonia make the transition to spermatocytic development. Our results reveal that *Dmrt6* plays a key role in coordinating an orderly transition between gametogenic programs from spermatogonial differentiation and mitosis to spermatocyte development and meiosis, and allow identification of a number of new candidates to mediate this process.

RESULTS

DMRT6 is expressed in intermediate and B spermatogonia

Dmrt6 is one of seven vertebrate DM domain genes (Kim et al., 2003; Ottolenghi et al., 2002). Some analysis of *Dmrt6* expression has previously been reported: in the fetal mouse *Dmrt6* mRNA was detected primarily in the brain (Kim et al., 2003). *Dmrt6* is strongly expressed in adult testis in mouse and human and weakly in human adult ovary and pancreas (Ottolenghi et al., 2002). mRNA expression profiling in the postnatal mouse testis detected *Dmrt6* from P5 onward (www.mrgd.org) (Shima et al., 2004; Su et al., 2004). In the adult mouse, using RT-PCR we detected *Dmrt6* strongly in testis and more weakly in ovary and brain but not in pancreas (supplementary material Fig. S1).

To examine expression of DMRT6 protein we generated a polyclonal antibody directed against a region of DMRT6 C-terminal to the DM domain and confirmed that it specifically recognizes DMRT6 in immunofluorescence (IF) and western blots (Fig. 1; supplementary material Fig. S2A-C). ENSEMBL predicts DMRT6 to have two potential protein isoforms of about 22 kDa and 38 kDa. We detected just one strongly expressed protein of about 47 kDa that was specific to wild-type testes (supplementary material Fig. S2C). The slower than predicted gel mobility is typical of DMRT proteins and may reflect secondary structure or protein modification.

We first compared expression of DMRT6 with that of DMRT1, which is expressed in Sertoli cells and spermatogonia (Matson et al., 2010; Raymond et al., 2000). Double staining of adult testes for DMRT6 and DMRT1 showed that DMRT6 is expressed in a subset of spermatogonia and not in Sertoli cells or in meiotic and postmeiotic germ cells (Fig. 1A). Double staining for DMRT6 and the meiotic marker SYCP3 (Yuan et al., 2000) showed mutually exclusive expression, confirming that DMRT6 is not expressed in germ cells that have initiated meiotic prophase (Fig. 1B).

To determine which spermatogonial cell types express DMRT6 we examined its expression relative to proteins expressed during different stages of spermatogonial development and also assessed during which stages of the cycle of the seminiferous epithelium DMRT6 is expressed. Double staining showed no overlap of DMRT6 expression with the undifferentiated spermatogonial marker PLZF (ZBTB16 – Mouse Genome Informatics) (Ballow et al., 2006; Costoya et al., 2004), indicating that DMRT6 is expressed only in differentiating spermatogonia (Fig. 1C,D). SOHLH1 and SOHLH2 proteins appear together in a subset of A_{al} spermatogonia; SOHLH2 expression then disappears before formation of Int spermatogonia, whereas SOHLH1 disappears later, before formation of type B spermatogonia (Suzuki et al., 2012). In the seminiferous epithelial cycle we found that DMRT6 expression begins during stage I and encompasses SOHLH2-positive differentiating A_4 spermatogonia (Fig. 1E,F) as well as SOHLH1-positive Int and SOHLH1-negative B spermatogonia (Fig. 1G-J) (Ballow et al., 2006; Hao et al., 2008; Suzuki et al., 2012). DMRT6 was absent by stage VII, when preleptotene spermatocytes are formed, indicating that its disappearance is coincident with the completion of spermatogonial differentiation (Fig. 1K,L). A diagram summarizing the dynamics of DMRT6 expression based on these data is shown in Fig. 1M.

Dmrt6 regulates the transition from early to late spermatogonial differentiation

To investigate the role of *Dmrt6* in spermatogonial development, we generated a conditional mutant allele, $Dmrt6^{flox}$, in which the proximal promoter and first exon are flanked by Cre recombinase recognition sites (*loxP* sites; 'floxed') (supplementary material Fig. S2E). Breeding $Dmrt6^{flox}$ to *beta-actin-Cre* mice generated the putative null allele $Dmrt6^{-}$, which was used for all experiments. $Dmrt6^{-}$ lacks both the proximal promoter and sequences encoding the DNA-binding DM domain, which is essential for DMRT protein

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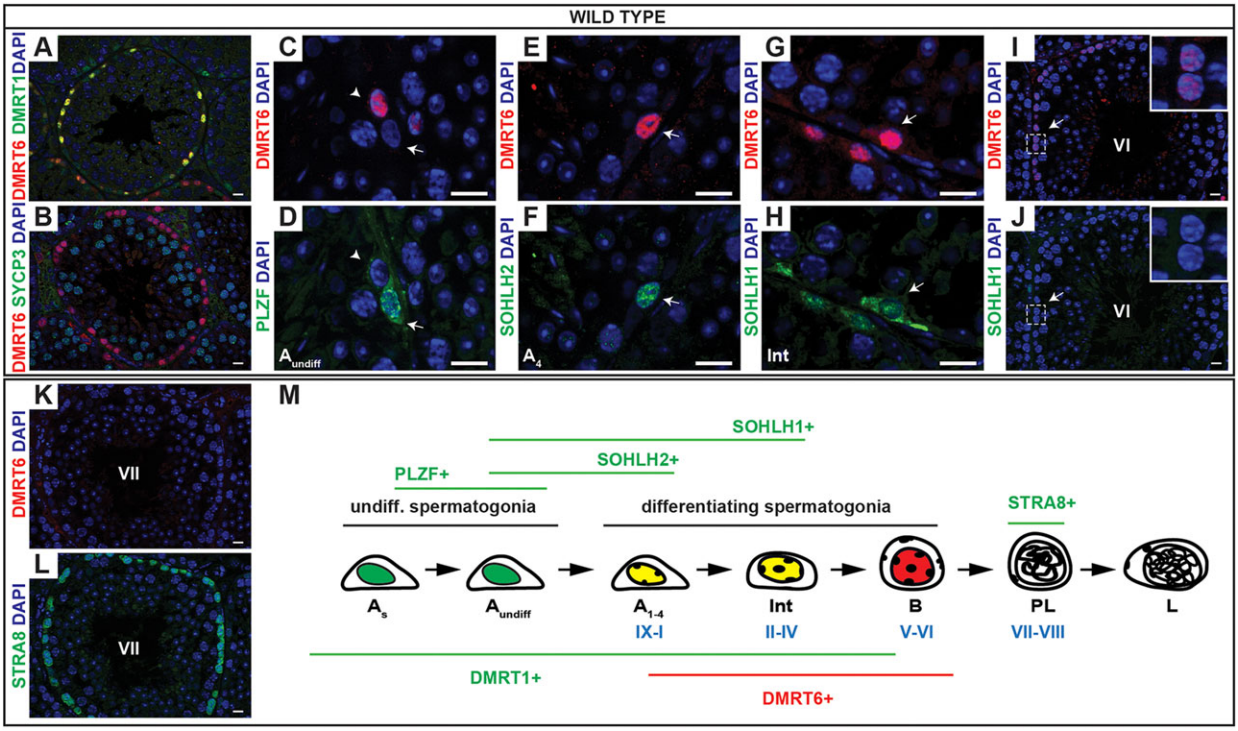

Fig. 1. DMRT6 is expressed in differentiating spermatogonia in the adult mouse testis. IF of adult wild-type testis sections. (A) DMRT6 is expressed in DMRT1-positive spermatogonia but not in DMRT1-positive Sertoli cells. (B) DMRT6 is not expressed in SYCP3-positive primary spermatocytes. (C-L) Paired co-stainings of DMRT6 with other germ cell proteins. (C,D) DMRT6 expression does not overlap with that of the undifferentiated spermatogonial marker PLZF. (E,F) DMRT6 expression overlaps with that of the spermatogonial differentiation marker SOHLH2 in A4 spermatogonia. (G,H) DMRT6 expression overlaps with that of the spermatogonial differentiation marker SOHLH1 in Int spermatogonia. (I,J) DMRT6 is expressed in B spermatogonia at stage VI, whereas SOHLH1 is not. (K,L) Preleptotene spermatocytes at stage VII are negative for DMRT6 and strongly express STRA8. (M) Graphical summary of expression data from A-L. Scale bars: 10 µm.

function, and does not express detectable DMRT6 protein (supplementary material Fig. S2A-C). *Dmrt6*$^{-/-}$ males had severe spermatogenesis defects (described below), but were otherwise normal and healthy. *Dmrt6*$^{-/-}$ females were normal and fertile with no apparent ovarian defects (not shown).

Testes of *Dmrt6*$^{-/-}$ mutants on the C57BL/6J (B6) genetic background were small and deficient in TRA98-positive differentiating germ cells as early as postnatal day 10 (supplementary material Fig. S2D; Fig. 2A-F) but had apparently normal SOX9-positive Sertoli cells (Fig. 2B,D,F). No mature spermatozoa were detected either in the seminiferous tubules or the epididymis in 8-week-old adult mutant males (Fig. 2E-H). Mutant animals were also aged to 8 months and we did not observe any striking progression of the phenotype, suggesting that the spermatogonial stem cell population was not compromised. To further evaluate the basis of the germ cell deficiency we examined expression of markers of major germ cell types. In adult mutant testes, undifferentiated spermatogonia appeared normal and expressed PLZF (not shown), but differentiating spermatogonia had altered expression of the differentiation proteins SOHLH1 and SOHLH2 (Fig. 3A-D). The number of spermatogonia strongly expressing these proteins was greatly increased in *Dmrt6* mutants, suggesting either an accumulation of differentiating spermatogonia or misexpression of these proteins outside their normal cell types. By contrast, the number of strongly STRA8-positive preleptotene spermatocytes was greatly reduced (Fig. 3E,F). Likewise, the number of BC7-positive primary spermatocytes (stages IX-XII) was greatly reduced and SUMO-1 positive XY bodies, which normally form during mid-pachynema (Koshimizu et al., 1995; La Salle et al., 2008), were absent from DMRT6 mutant testes (Fig. 3G,H). Together, these results indicate that loss of *Dmrt6* disrupts spermatogonial differentiation starting at or before the formation of preleptotene spermatocytes and also prevents progression of meiotic prophase beyond early pachynema.

Cell-type-inappropriate gene expression in *Dmrt6* mutant germ cells

As mentioned above, the increased number of cells expressing SOHLH1 and SOHLH2 could reflect either an accumulation of differentiating spermatogonia or the expression of these proteins outside their normal cell types. Consistent with the latter possibility, we observed many SOHLH1- and SOHLH2-positive germ cells in mutant tubules at stages II-VII, when Int and B spermatogonia and preleptotene spermatocytes normally are present and SOHLH2 should not be expressed. To confirm that key regulators were expressed outside their normal cell types, we performed double-staining experiments in adult mutant testes. These revealed that both SOHLH1 and SOHLH2 were ectopically expressed with STRA8 in mutant preleptotene spermatocytes (Fig. 4A-D), and that mutant stage VII preleptotene spermatocytes also ectopically expressed DMRT1 (Fig. 4E,F), which normally is silenced in late B spermatogonia (Matson et al., 2010). STRA8 was also misexpressed: it normally disappears before meiotic entry (Oulad-Abdelghani et al., 1996), but in *Dmrt6* mutants STRA8 persisted into meiotic prophase in stage IX SYCP3-positive leptotene spermatocytes (Fig. 4G,H). Based on these results, we conclude that *Dmrt6* mutant spermatogonia can progress to preleptonema. However, they fail to appropriately downregulate expression of key regulatory genes at several stages of differentiation:

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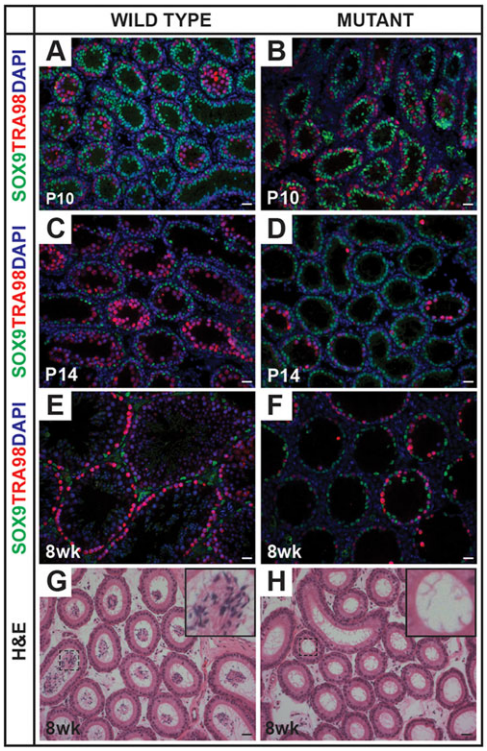

Fig. 2. Germ cell depletion in *Dmrt6* mutant testes. (A,B) By P10, *Dmrt6* mutants are deficient in TRA98-positive differentiating germ cells (primarily differentiating spermatogonia and primary spermatocytes) but have normal expression of the Sertoli cell marker SOX9. (C,D) At P14, a severe deficit in meiotic germ cells, which accumulate in the interior of seminiferous tubules in wild type, is clearly apparent in *Dmrt6* mutants. (E,F) In wild-type adults a ring of differentiating spermatogonia and primary spermatocytes, which have very strong TRA98 expression, is apparent; *Dmrt6* mutants have many fewer cells with strong TRA98 expression and lack spermatids based on DAPI staining. (G,H) Hematoxylin and eosin (H&E) staining detects abundant epididymal sperm in wild type but none in *Dmrt6* mutants. Scale bars: 20 µm.

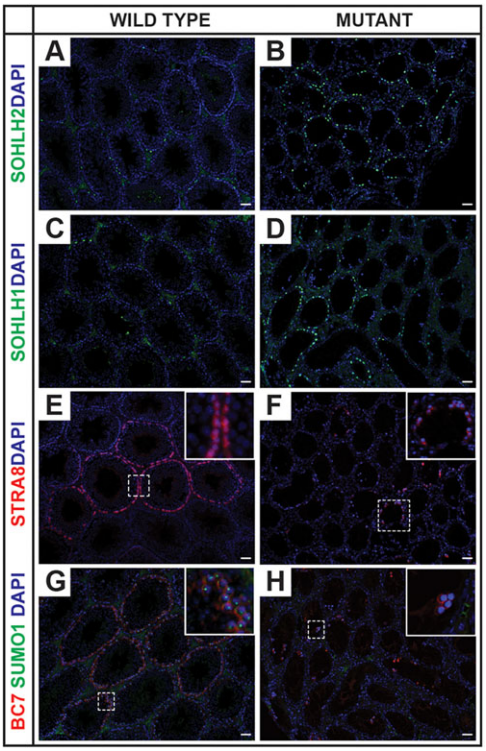

Fig. 3. Disrupted spermatogonial development in *Dmrt6* mutants. IF of adult testes. (A,B) *Dmrt6* mutants accumulate spermatogonia strongly expressing SOHLH2, which is normally expressed mainly in undifferentiated spermatogonia through A_4 spermatogonia. (C,D) *Dmrt6* mutants accumulate spermatogonia strongly expressing SOHLH1, which is normally expressed in undifferentiated spermatogonia through Int spermatogonia. (E,F) *Dmrt6* mutants are severely deficient in strongly STRA8-positive preleptotene spermatocytes and appear to have reduced nuclear STRA8 relative to wild type (insets). (G,H) *Dmrt6* mutants are severely deficient in germ cells expressing the primary spermatocyte marker BC7 and completely lack SUMO1-positive XY bodies that normally form in mid-pachynema (insets). Scale bars: 40 µm.

SOHLH2 when they progress from A4 to Int; SOHLH1 when they progress from Int to B; DMRT1 when they progress from B to preleptotene; and STRA8 when they enter meiotic prophase. The inappropriate combinations of germ cell regulators that result are likely to disrupt the transition from mitosis to meiosis and cause the observed failure of spermatogenesis in *Dmrt6* mutants.

The cell type analysis described above showed that *Dmrt6* mutants have abundant A_4 spermatogonia but become severely deficient in germ cells from late spermatogonial stages onward. Presumably this germ cell deficiency represents reduced proliferation or increased cell death of late spermatogonia, or possibly both. We used bromodeoxyuridine (BrdU) incorporation to assess proliferation and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) to assess apoptosis in juveniles at 10 and 14 days and in adults. *Dmrt6* mutant germ cells from all three time points were positive for BrdU in a stage-specific manner as in wild type, but the percentage of BrdU-positive A_4 cells was slightly higher in *Dmrt6* mutant tubules, consistent with our observation of increased A_4 cells in *Dmrt6* mutants and suggesting that their proliferation was not reduced and may indeed have been increased (not shown). Mutants at each time point had TUNEL-positive cells in tubules containing late spermatogonia, unlike the control, where apoptotic cells were rare in these stages (Fig. 4I,J). Together, these results suggest that the reduced abundance of late spermatogonia in *Dmrt6* mutants is largely due to elevated apoptosis.

DMRT6 activity in mitotic spermatogonia is required for progression through meiotic prophase

Dmrt6 mutant germ cells have at least two apparent defects, a disruption of spermatogonial differentiation and a failure to complete meiotic prophase. We were able to partially separate these phenotypes by breeding the *Dmrt6* mutation onto a different genetic background. We found that loss of *Dmrt6* in 129Sv strain mice caused minimal loss of spermatogonia, with greater numbers of germ cells entering meiosis, but mutant cells still arrested in meiotic prophase as in B6 mutants (supplementary material Fig. S3A-D). The genetic separation of these phenotypes suggests that DMRT6 performs two distinct functions during late spermatogonial differentiation, one required at the time of DMRT6 expression for completion of spermatogonial development and one that becomes important later for successful execution of meiotic prophase.

We used the 129Sv mutant strain to more closely examine the meiotic defect in *Dmrt6* mutants by assessing several landmarks of normal meiotic prophase. First, we stained meiotic chromosome spreads with SYCP1 and SYCP3 to examine synaptonemal complex (SC) formation, which normally begins during leptonema and is completed by pachynema (Cohen et al., 2006; Fraune et al., 2012; Yang and Wang, 2009). The SC formed in mutant spermatocytes but frequently was incomplete: 37/50 mutant pachytene spermatocytes had incomplete synapsis compared with 4/50 in wild type (Fig. 5A-F).

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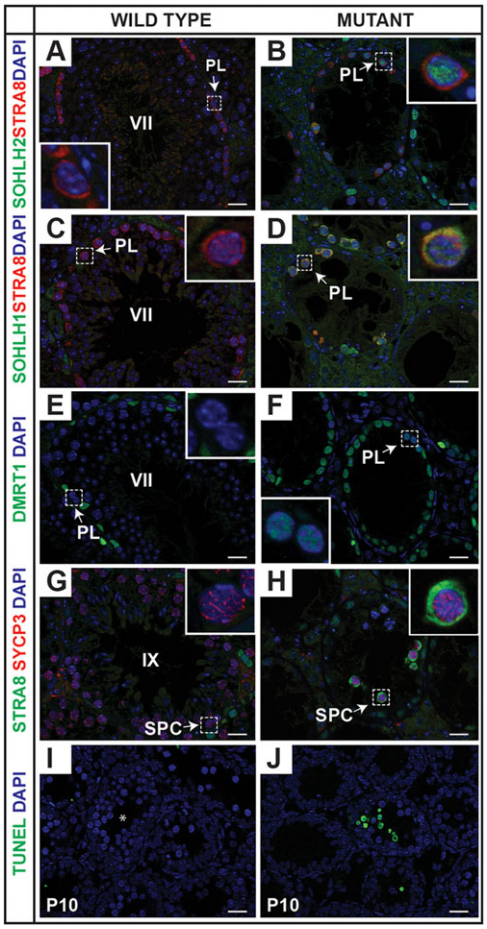

Fig. 4. Inappropriate overlapping expression of germ cell differentiation regulators. (A,B) Double staining for the differentiating A spermatogonial marker SOHLH2 and preleptotene spermatocyte marker STRA8 shows no overlap in wild type at stage VII but preleptotene spermatocytes in *Dmrt6* mutant express both markers (insets). (C,D) Wild-type preleptotene spermatocytes express STRA8 but not SOHLH1, whereas mutant preleptotene spermatocytes express both proteins (insets). (E,F) Preleptotene spermatocytes in wild type do not express DMRT1 but many *Dmrt6* mutants do (insets). (G,H) Wild-type stage IX primary spermatocytes express SYCP3 but not STRA8, whereas *Dmrt6* mutant primary spermatocytes frequently express both (insets). (I,J) TUNEL labeling at P10 detects elevated apoptosis in mutant spermatogonia. Scale bars: 20 µm. PL, preleptotene spermatocyte; SPC, primary spermatocyte.

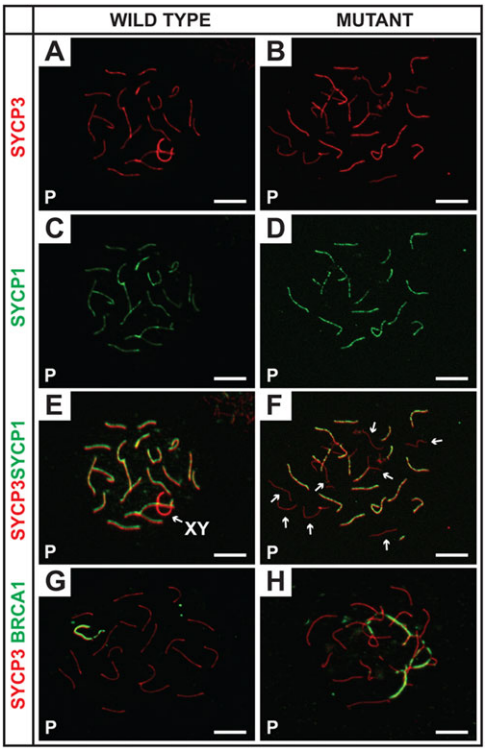

Fig. 5. Incomplete chromosomal synapsis in *Dmrt6* primary spermatocytes. (A-H) IF of chromosome spreads from wild-type or *Dmrt6* adult pachytene spermatocytes. (A,B) The synaptonemal complex lateral element protein SYCP3 accumulates in wild-type and *Dmrt6* spermatocytes, indicating that meiotic pairing does occur in mutants. (C,D) The synaptonemal complex transverse element protein SYCP1 also accumulates in wild-type and *Dmrt6* spermatocytes, indicating that synaptonemal complexes in mutant cells can contain key structural components. (E,F) Merge of SYCP3 and SYCP3 staining shows that whereas synaptonemal complex formation is complete in wild type (except for the non-homologous regions of the sex chromosomes labeled 'XY'), synaptonemal complex formation is incomplete in *Dmrt6* mutant spermatocytes not only on the sex chromosomes but also on a number of autosomes. White arrows indicate unpaired chromosomes. (G,H) In wild-type germ cells, BRCA1 accumulates on the unpaired sex chromosomes, but in mutant spermatocytes there is more extensive BRCA1 accumulation, further illustrating the synapsis defect and confirming that surveillance for unpaired chromosomes is active in mutant cells. Scale bars: 10 µm.

The unpaired chromosomes and chromosome segments accumulated BRCA1 (Fig. 5G,H), indicating that surveillance for unpaired chromosomes was intact in the mutant spermatocytes (Turner et al., 2004).

We next examined meiotic crossing over, which normally occurs during pachynema. Double-strand breaks (DSBs) were initiated and repaired in *Dmrt6* mutants, with RAD51 foci normal in number and distribution except on unpaired regions (supplementary material Fig. S4A-D) (Cohen et al., 2006). Transitional nodules made up of MSH4, MSH5 and other associated proteins normally form at DSBs during zygonema (Baudat and de Massy, 2007; Cohen et al., 2006; Hoffmann and Borts, 2004). In *Dmrt6* mutants we observed a severe decrease in the number of MSH4 foci (supplementary material Fig. S4E,F). During pachynema, a subpopulation of transitional nodules should convert to MLH1-positive recombination nodules, at which crossovers occur (Baker et al., 1996; Cohen et al., 2006). In *Dmrt6* mutant testes no MLH1-positive nodules were detectable (supplementary material Fig. S4G,H), indicating a lack of meiotic recombination, and we did not observe spermatocytes progressing beyond pachynema. Together, these results indicate that loss of *Dmrt6* causes defects in meiotic pairing that lead to arrest and apoptosis during pachynema, before recombination. These defects are present both in B6 mutants, which have reduced numbers of late spermatogonia and early spermatocytes, and in 129Sv and mixed background mice, which have normal numbers of differentiated spermatogonia and early primary spermatocytes. Because DMRT6 is expressed only during spermatogonial differentiation, the meiotic defects must be consequences of improper gene regulation before the initiation of the meiotic program.

DMRT6 activates and represses spermatogonial gene expression

Phenotypic analysis indicated that DMRT6 in differentiating spermatogonia is required both for the completion of the spermatogonial program and transition into preleptonema, as well as to properly preconfigure spermatogonia for subsequent success in meiotic prophase. For more insight into how DMRT6 performs

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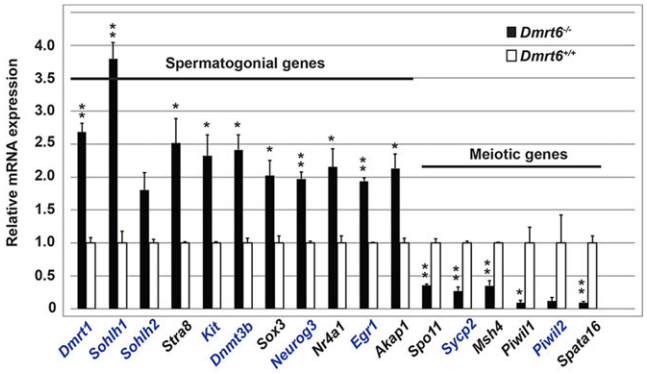

Fig. 6. Misregulation of spermatogonial and spermatocytic mRNAs in *Dmrt6* mutant testes. RT-qPCR comparing mRNA expression in P14 wild-type (white) and *Dmrt6* mutant (black) mice. Expression was normalized to *Hprt* expression. Error bars indicate s.d. **P*<0.05 (Student's *t*-test). ***P*<0.005. Blue font indicates genes that are potential direct target of DMRT6 based on ChIP-seq.

these functions, we profiled mRNA expression in *Dmrt6* mutant testes at P8 on the B6 background. In wild-type testes at this stage the first wave of differentiating DMRT6-positive spermatogonia has formed. In P8 mutants germ cells have not yet arrested and undergone apoptosis, and thus a normal mix of germ cell types is still present, increasing the chance of detecting the proximal changes in gene expression that lead to later phenotypic defects. Because P8 is before the overt spermatogonial phenotype in *Dmrt6* mutants, we should avoid false positives caused by the depletion of late spermatogonia and spermatocytes; however, we would also expect to miss some of the mRNAs misexpressed in those cell types.

We performed RNA sequencing to compare wild-type and *Dmrt6* mutant testes, each in triplicate (data analysis is described in Materials and Methods). This analysis identified 1595 misexpressed genes ($P<0.05$; supplementary material Table S1). As expected, based on the early time point examined, most expression differences between mutant and wild type were modest. We therefore selected a number of genes for validation based on expression in type A and type B spermatagonia and Ingenuity-defined functions in meiosis and testis development (supplementary material Table S2) and assayed expression of 20 of these genes at P14 by RT-qPCR. We also assayed expression of *Dmrt1*, *Sohlh2* and *Stra8* mRNAs at this stage. Finally, because mRNA profiling at P8 was expected to miss meiotic mRNAs activated by DMRT6, we also assayed a number of key meiotic genes by RT-qPCR at P14.

RT-qPCR confirmed the misexpression of many of these mRNAs and also revealed a consistent pattern: genes involved in spermatogonial differentiation or expressed in spermatogonia generally had elevated expression in mutant testes, whereas genes involved in meiosis were reduced in expression (Fig. 6). Among the overexpressed genes were the known regulators of spermatogonial differentiation *Sohlh1* and *Sohlh2*, *Dmrt1*, *Stra8*, *Kit*, *Sox3* and *Neurog3* (Raverot et al., 2005; Schrans-Stassen et al., 1999; Shirakawa et al., 2013; Yoshida et al., 2004), as well as several genes (*Nr4a1*, *Egr1* and *Akap1*) expressed in spermatogonial cells but of unknown function (www.mrgd.org) (McCarrey and Skinner, 1999; Shima et al., 2004). Among the underexpressed genes were meiotic genes, including *spo11*, *sycp2* and *msh4*. Expression of genes involved in epigenetic modification was also altered in *Dmrt6* mutants: *Dnmt3b* is a DNA methyltransferase required for spermatogonial differentiation (Shirakawa et al., 2013), and *Piwil1* (*miwi*) and *Piwil2* (*mili*) are essential regulators of retrotransposon silencing (Di Giacomo et al., 2013; Grivna et al., 2006; Kuramochi-Miyagawa et al., 2004; Vourekas et al., 2012). Collectively, the mRNA profiling and RT-qPCR results suggest that DMRT6 acts in late spermatogonia to coordinate the transition from the mitotic program to the meiotic program by repressing genes that promote spermatogonial differentiation and proliferation and by activating genes that will later be required for meiosis.

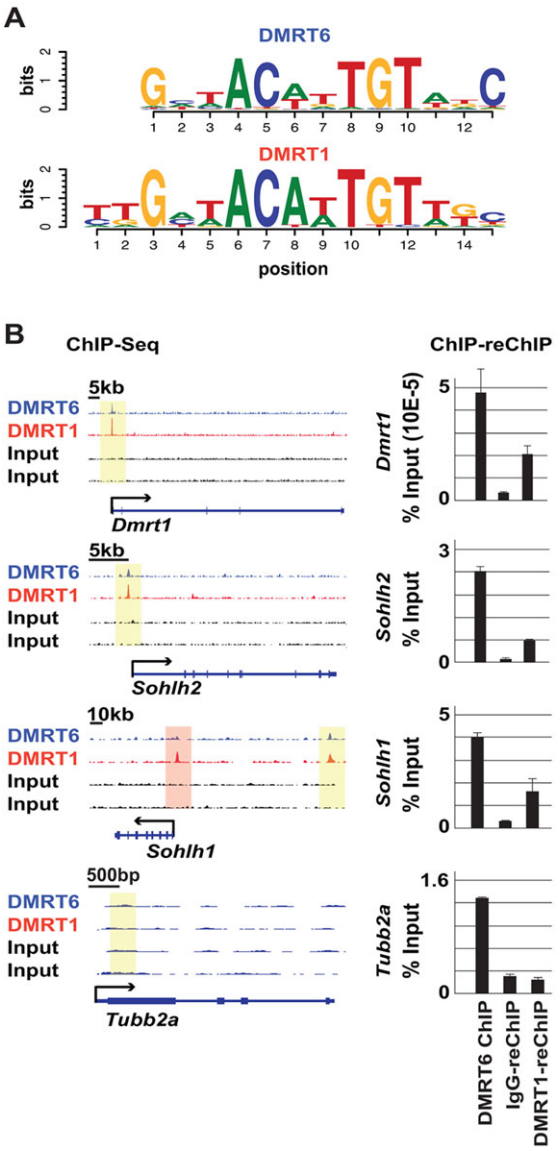

Fig. 7. DMRT6 and DMRT1 bind similar DNA sequences and associate with shared sites *in vivo*. ChIP-Seq was performed on adult testis with antibodies specific to DMRT6 (blue profile) and DMRT1 (red profile). (A) An enriched DNA motif was identified within regions bound by DMRT6 that is similar to the previously described DNA sequence bound by DMRT1 *in vitro*. (B) Left column: binding profiles for both antibodies are shown for the *Dmrt1*, *Sohlh2*, *Sohlh1* and *Tubb2a* (negative control) loci. Yellow filled boxes indicate sites bound comparably by DMRT6 and DMRT1. The red filled box represents preferential association with DMRT1. The data range shown for each binding profile is between 0 and 2 counts per million. Right column: re-ChIP experiments were also conducted for *Dmrt1*, *Sohlh2* and *Sohlh1* loci on sites bound by both DMRT6 and DMRT1 (yellow boxes). ChIP was performed using anti-DMRT6 antibody and then re-ChIP was performed using either protein A purified pre-immune serum (negative control) or anti-DMRT1 antibody. Re-ChIP products were evaluated by qPCR and enrichment reported as percent total input recovered.

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To help identify potential direct targets of DMRT6 transcriptional regulation we profiled DMRT6 DNA association in the adult testis using ChIP-Seq. It identified 14,862 peaks associated with 10,363 unique genes (supplementary material Table S3; see Materials and Methods). Binding of DMRT6 at these sites was correlated with binding by DMRT1 (Spearman correlation=0.642). Motif enrichment analysis of the 1724 strongest peaks bound by DMRT6 identified a motif that closely resembled the site bound by DMRT1 (Fig. 7A). Sites bound by both DMRT6 and DMRT1 were more likely to have DMRT1 binding motif than sites specifically enriched for DMRT6 (chi-squared test, P=2.2e-16). In some cases (e.g. *Dmrt1* and *Sohlh2*; Fig. 7B, left column), DMRT1 and DMRT6 binding appeared equivalent, whereas in others (e.g. *Sohlh1*; Fig. 7B, left column), the relative strength of binding of the two proteins appeared to differ at some sites. To determine whether DMRT1 and DMRT6 bind concurrently to the same chromatin interval, we performed re-ChIP analysis, precipitating chromatin first with the DMRT6 antibody and then with the DMRT1 antibody or non-specific pre-immune IgG. Re-ChIP indicated that a number of chromatin sites are associated with both DMRT1 and DMRT6 (Fig. 7B, right column).

In addition to *Dmrt1*, *Sohlh1* and *Sohlh2*, other germ cell regulatory genes that were bound by DMRT6 and misexpressed in *Dmrt6* mutant testes included the spermatogonial regulators *Kit*, *Dnmt3b* and *Neurog3*, and the meiotic genes *Sycp2* and *Piwil2*. The ChIP and RT-qPCR results together suggest that DMRT6 acts as a bifunctional transcription factor to control late spermatogonial development and early meiosis, repressing spermatogonial genes and activating meiotic genes (Fig. 8).

DISCUSSION

Here, we have found that the DM domain protein DMRT6 is expressed in differentiating mammalian spermatogonia and has two distinct functions in male germ cells: first, it helps spermatogonia complete their differentiation program; and second, it is required for spermatocytes to undergo proper meiotic prophase. Based on protein expression data, RNA-seq and ChIP-seq, we suggest DMRT6 helps coordinate the shift from the mitotic spermatogonial program to the meiotic spermatocyte program, one of the major transitions in male gametogenesis, by controlling the transcription of suites of key regulatory genes. DMRT6 ensures that genes promoting spermatogonial proliferation and differentiation are downregulated appropriately during several steps of late spermatogonial development and also activates genes that will be required once meiosis initiates. In this manner, DMRT6 helps bring spermatogonial development to an orderly conclusion, sets the stage for meiosis and ensures the separation of the mitotic and meiotic programs. In *Dmrt6* mutants the timing of gene expression is disrupted, and we suggest that the resulting simultaneous expression of genes normally active during different stages of germ cell differentiation (e.g. Fig. 4) is likely to cause the observed developmental arrest and apoptosis.

Dmrt6, together with *Dmrt1* and *Dmrt7*, is one of three Dmrt genes that have been shown to play essential roles in mammalian spermatogenesis. The three genes act at different steps of germ cell development and have distinct functions. *Dmrt1* regulates proliferation and pluripotency of fetal germ cells, stimulates resumption of mitosis and differentiation in neonatal germ cells, and acts both to stimulate spermatogonial proliferation and differentiation and to inhibit meiotic initiation in adult spermatogonia (Fahrioglu et al., 2007; Krentz et al., 2009; Matson et al., 2010). *Dmrt7* is expressed in primary spermatocytes, preferentially associates with the XY body and is required for formation of postmeiotic sex chromatin and passage through diplonema (Kim et al., 2007a). Here, we have found that *Dmrt6* acts between *Dmrt1* and *Dmrt7*, controlling the transition between the phases of gametogenesis that the other two genes regulate. Thus, *Dmrt* genes participate in a regulatory 'relay' that helps guide germ cells through much of their development. Our observation that DMRT6 acts in spermatogonia to activate transcription of genes that will later be required in meiosis suggests that DMRT6 serves not only to prevent the inappropriate mixed expression of genes from different stages of germ cell development (e.g. STRA8 with SYCP3; Fig. 4), but also helps establish, before meiosis, a 'dowry' of meiotic regulators that will allow successful synapsis and meiotic recombination after meiotic initiation.

DMRT6 is co-expressed with DMRT1 in differentiating spermatogonia, and several factors indicate an antagonistic relationship between the two: each gene affects expression of the other (this study and Murphy et al., 2010); DMRT6 binds *Dmrt1* (Fig. 7); most of the genomic sites bound by DMRT6 also are bound by DMRT1; and we found that DMRT1 and DMRT6 both regulate expression of several key spermatogonial regulators, including *Sohlh1* and *Sohlh2* (this study and Matson et al., 2010).

Examination of target gene expression suggests two ways in which DMRT1 and DMRT6 may functionally intersect. First, by repressing *Dmrt1* expression, DMRT6 can indirectly regulate some of its targets. This might be the case for *Sohlh1*, which shows strong promoter-proximal binding only for DMRT1 but the expression of which is regulated by both genes (Fig. 4). Second, the association of DMRT1 and DMRT6 at many shared sites suggests that they compete for binding or heterodimerize on DNA to somehow modify each other's activity (Murphy et al., 2010). Re-ChIP analysis showed that binding of DMRT1 and DMRT6 is not mutually exclusive; an attractive model is that DMRT1/DMRT6 heterodimers in late spermatogonia may repress genes such as *Sohlh1* that are activated by DMRT1 in early spermatogonia.

The transition from spermatogonial differentiation to meiosis is a crucial step in male gametogenesis and is incompletely

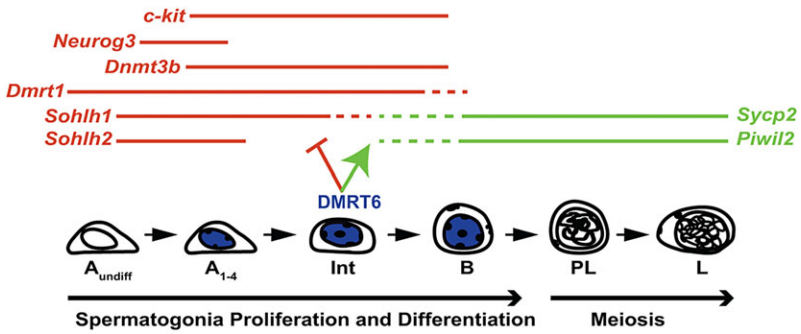

Fig. 8. Model of DMRT6 regulation of mammalian spermatogenesis. Based on mRNA and protein expression together with ChIP-seq analysis, a parsimonious working model is that DMRT6 acts in late spermatogonial cells to shut off spermatogonial differentiation through direct transcriptional repression of key spermatogonial differentiation factors that include *Dmrt1*, *Sohlh1*, *Sohlh2*, *Kit*, *Neurog3* and *Dnmt3b* (red). DMRT6 also promotes meiosis by directly activating transcription of genes including *Sycp2* and *Piwil2* (green). DMRT6-positive cells are indicated in blue.

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understood. It has been apparent for some time that RA and its target *Stra8* are likely to play key roles in stimulating the shift to meiosis but is less clear how the spermatogonial program is brought to a conclusion; indeed, DMRT6 is the first transcriptional regulator shown to be essential in B spermatogonia. We have found that DMRT6 helps to 'draw the curtain' on spermatogonial development and set the stage for meiosis. Important future directions will include testing whether DMRT6 also plays this role in other vertebrates, elucidating how the regulatory networks anchored by DMRT1 and DMRT6 and possibly other DMRT proteins functionally intersect, and determining how DMRT proteins activate and repress transcription in a context-dependent manner via the same consensus DNA elements to achieve different outcomes in germline gene regulation.

MATERIALS AND METHODS

Dmrt6 targeting vector construct

We isolated BAC clones containing *Dmrt6* from a 129/SvJ strain BAC library (Source Bioscience) and used these to construct the *Dmrt6* targeting vector by bacterial recombineering (Liu et al., 2003). The left and right homology arms were cloned into the backbone vector PL253 (Liu et al., 2003). The final targeting construct, DM6-TV4, is shown in supplementary material Fig. S2D. To generate *Dmrt6* mutant animals, the targeting vector DM6-TV4 was linearized with *Not*I and transfected into CJ7 ES cells by electroporation (Liu et al., 2003). Three homologous recombinant colonies were identified from 300 G418-resistant colonies by Southern blot hybridization using a DNA probe containing sequences upstream of exon 1 to screen genomic DNA digested with *Eco*RV. Homologous recombination was confirmed on both ends of the targeted region by Southern blot hybridization using probes generated by PCR using primers LKB016/LKB017 (5′ probe) and LKB053/LKB054 (3′ probe), listed below. Two targeted embryonic stem cell clones containing the floxed allele $Dmrt6^{neo}$ were injected into C57BL/6J blastocysts to generate chimeras. Chimeric males were bred with C57BL/6J females to generate heterozygotes carrying $Dmrt6^{neo}$. $Dmrt6^{+/neo}$ females were then bred with male *beta-actin-Cre* transgenic mice (Lewandoski et al., 1997) to delete the floxed sequences and to generate heterozygous $Dmrt6^{+/-}$ animals, which were interbred to generate homozygous $Dmrt6^{-/-}$ mutants. The $Dmrt6^{-}$ allele was used for all experiments in this study. Experimental protocols involving animals were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Genotyping

For genotyping, tail-clip DNA was amplified for 35 cycles with an annealing temperature of 56°C. The *Dmrt6* wild-type and floxed alleles were detected by PCR with DM6F/DM6R1, resulting in 480 bp or 424 bp amplicons, respectively. The *Dmrt6* null allele was detected by PCR with DM6F/DM6R2, resulting in a 309 bp amplicon.

Primers

Primers used for genotyping of ES cells and mice were: LKB016, 5′-CACACACACACGTGTTCA-3′; LKB017, 5′-ATTCCAAGCAGGGATGTGAC-3′; LKA053, 5′-TAACGCTCTGCTAGGGGAAA-3′; LKA054, 5′-GT-GGTGCGTAAGATCCCTGT-3′; DM6F, 5′-TTCATTGGGAGTTTTAAAAC-3′; DM6R1, 5′-CCATGGTTCCGGCACCTAGA-3′; DM6R2, 5′-AC-CATCAGAGCCAGCAAGATGGCTAAAGGCA-3′. Primers used for construction of *Dmrt6* targeting vector by BAC recombineering (Liu et al., 2003) were: A, 5′-GCGGCCGCTAACTTCACTTAGATTCTAC-3′; B, 5′-AAGCTTGGCAAGATCACGGTCTTTTA-3′; C, 5′-AAGCTTTGGTTTCTGCAATGCCTGTA-3′; D, 5′-GAATTCGATATCGTTAACTGTTCCCGCCAAATTGTAAC-3′; E, 5′-GGATCCATTGCAGTTCTCCGGACATT-3′; F, 5′-GCGGCCGCCACAGATGCACTGCTTCCAG-3′; G, 5′-CTCGAGGAGTCTGAGTCGAGGGGATG-3′; H, 5′-GAATTCGAGGCAGGTGGATTTCTGAG-3′; I, 5′-GGATCCGATATCGGAAACCGGTTCTTTCCTTC-3′; J, 5′-GCGGCCGCTAACCCCACTGTCCCTGAAC-3′; Y, 5′-AAGCTTTTTTTAACTTTTAAAAACCA-3′; Z, 5′-ACTAGTCAAAAGAAAGCCCAAGAATT-3′.

DMRT6 antibody

Rabbit polyclonal antibodies to DMRT6 were raised against a purified DMRT6 fusion protein containing glutathione-S-transferase (GST) fused to the C-terminal 148 amino acids of DMRT6. Antibodies to GST were removed by GST-affigel 10 chromatography and the antiserum was then purified by GST-DMRT6-affigel 10 chromatography. For IF, DMRT6 antibody was used at 1:200 dilution with a goat anti-rabbit secondary antibody (Abcam) at 1:500 dilution.

Histological analysis

Dissected testes were fixed 4% PFA overnight at 4°C, progressively dehydrated in graded ethanol series and embedded in paraffin wax. Sections of 5 µm were deparaffinized, rehydrated and stained with hematoxylin and eosin.

Tissue immunofluorescent staining

Slides with paraffin sections were rehydrated and boiled with 10 mM of citric acid (pH 6.0). Slides were blocked with 10% serum (goat or donkey depending on the secondary antibody used) in PBS with 0.1% Triton X-100 at room temperature for 1 h and incubated with primary antibody overnight at room temperature followed by 2 h incubation with secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). All images were captured with a Zeiss Imager Z1 microscope using a Zeiss MRm camera. Antibodies, suppliers, catalog numbers and dilutions used are listed in supplementary material Table S4.

BrdU incorporation

BrdU (Sigma) was dissolved in phosphate-buffered saline (PBS) and injected intraperitoneally at 50 mg per kg of body weight. Testes were harvested from 4% PFA perfusion-fixed animals at 2 h following BrdU injection. To detect BrdU incorporation, testis sections were stained with an anti-BrdU antibody listed in supplementary material Table S1.

TUNEL staining

Slides with paraffin sections were rehydrated and apoptotic cells were detected using DeadEnd Fluorometric TUNEL System (Promega).

Meiotic spreads

Meiotic chromosome spreads were prepared from testes of 4-week-old mice as described previously (Peters et al., 1997).

RNA-Seq

RNA was isolated from testes of three independent wild-type and three $Dmrt6^{-/-}$ P8 animals using TRIzol reagent (Life Technologies), further purified with the RNeasy MinElute cleanup kit (74204, Qiagen), and RNA quality was verified with Qubit 2.0 (Invitrogen). TruSeq RNA libraries (Illumina) were prepared for sequencing on the Illumina HiSeq 2000 platform.

ChIP-Seq

Chromatin from testes of three adult wild-type B6 and 129Sv mixed genetic background mice was cross-linked with formaldehyde, sheared and immunoprecipitated with anti-DMRT6 antibody as described previously (Krentz et al., 2013).

Re-ChIP analysis

First-round ChIP was performed as for ChIP-Seq. After elution from the first round using 1% sodium dodecyl sulfate (SDS), 100 mM $NaHCO_3$, complexes were diluted 1:11 with DOC RIPA (Murphy et al., 2010). Diluted complexes were subjected to a second round of ChIP using magnetic beads (Dynabeads M-280 Tosylactivated, Invitrogen) covalently attached to either affinity purified anti-DMRT1 antibody or protein A purified pre-immune serum. Re-ChIP products were evaluated by qPCR and enrichment reported as percent total input.

Bioinformatics analysis

Briefly, RNA-Seq reads were mapped to the mm9 mouse genome assembly and differential gene expression was determined by counting reads within

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genes defined by the Ensembl release 67 using published protocols (Anders et al., 2013). To identify potentially relevant genes we annotated the gene list based on expression in published microarray data [GSE4193 (Namekawa et al., 2006)]. We also annotated this list to identify genes associated with Ingenuity functional terms related to testis development and meiosis (Development of Genital Organ, Gamet*, Germ Cell, Gonad, Meiosis, Seminiferous, Seminal, Sperm* and Testis). The annotated gene list (supplementary material Table S1) was filtered based on expression in type A or type B spermatagonia and a match to one or more Ingenuity function terms. This filtered gene list (supplementary material Table S2) was further annotated with the number of PubMed publications for each gene and the keyword 'testis' and whether that gene was associated with a peak in the DMRT1 and DMRT6 ChIP-Seq data. ChIP-Seq data were mapped to mm9 and peaks were identified using MACS (Feng et al., 2012) using a *P*-value cutoff of 10^{-5} as described previously (Krentz et al., 2013). ChIP-Seq peaks were annotated with overlapping and nearest start features (supplementary material Table S3). Details can be found in the DMRT6 integrative analysis in the supplementary material. Our analysis data have been deposited at the NCBI Gene Expression Omnibus (GEO) under accession number GSE60440.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.Z, M.W.M. and M.D.G. performed the experiments; all authors helped design experiments and interpret data; T.Z., V.J.B. and D.Z. wrote the paper.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.113936/-/DC1

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