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DMRT1 promotes oogenesis by transcriptional activation of *Stra8* in the mammalian fetal ovary

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ABSTRACT

Dmrt1 belongs to the DM domain gene family of conserved sexual regulators. In the mouse *Dmrt1* is expressed in the genital ridge (the gonadal primordium) in both sexes and then becomes testis-specific shortly after sex determination. The essential role of DMRT1 in testicular differentiation is well established, and includes transcriptional repression of the meiotic inducer *Stra8*. However *Dmrt1* mutant females are fertile and the role of *Dmrt1* in the ovary has not been studied. Here we show in the mouse that most *Dmrt1* mutant germ cells in the fetal ovary have greatly reduced expression of STRA8, and fail to properly localize SYCP3 and γH2AX during meiotic prophase. Lack of DMRT1 in the fetal ovary results in the formation of many fewer primordial follicles in the juvenile ovary, although these are sufficient for fertility. Genome-wide chromatin immunoprecipitiation (ChIP-chip) and quantitative ChIP (qChIP) combined with mRNA expression profiling suggests that transcriptional activation of *Stra8* in fetal germ cells is the main function of DMRT1 in females, and that this regulation likely is direct. Thus DMRT1 controls *Stra8* sex-specifically, activating it in the fetal ovary and repressing it in the adult testis.

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Introduction

In mammals sex is genetically determined in the genital ridge by the presence or absence of the Y chromosome gene *Sry*. Transient expression of *Sry* in the somatic supporting cells of the gonad between embryonic day 10.5 (E10.5) and E12.5 activates expression of the related gene *Sox9* (Sekido et al., 2004). *Sox9* expression is sufficient to induce the differentiation of bipotential somatic precursor cells into male Sertoli cells, whereas the absence of *Sry* in XX animals allows these cells to undergo an alternative differentiation program and form female granulosa cells (Foster et al., 1994; Koopman et al., 1990, 1991).

The sexual fate of mammalian germ cells is determined by the sex of the surrounding somatic cells rather than by the sex chromosome complement of the germ cells themselves (McLaren, 1975, 1981). The genital ridge is colonized around E10.5 by germ cells migrating from their site of origin in the proximal epiblast. Upon their arrival at the genital ridge, XX and XY germ cells are indistinguishable, but their developmental paths soon diverge (Hilscher et al., 1974). In males, fetal germ cells arrest in G_0 of mitosis around E13.5, resume active mitosis perinatally, and initiate meiosis at puberty. Female germ cells instead enter prophase I of meiosis around E13.5, and remain arrested in the diplotene stage of prophase I until after puberty. The timing of meiotic initiation therefore differs dramatically in the two sexes: female germ cells begin meiosis during embryonic development whereas male germ cells do so only postnatally.

Retinoic acid (RA) is believed to trigger meiotic initiation in both sexes (Bowles et al., 2006; Ghyselinck et al., 2006; Koubova et al., 2006; Li and Clagett-Dame, 2009; Livera et al., 2000), although recently it has been suggested that another molecule, perhaps related to RA, may act as an inducer in the fetal ovary (Kumar et al., 2011). In the fetal ovary the meiotic initiation signal activates transcription of *Stra8* (*Stimulated by retinoic acid 8*) (Anderson et al., 2008; Baltus et al., 2006). *Stra8* is required for premeiotic DNA replication and for the subsequent events of prophase I in germ cells of both sexes (Anderson et al., 2008; Baltus et al., 2006; Koubova et al., 2006; Oulad-Abdelghani et al., 1996). In fetal males the P450 enzyme CYP26B1 prevents *Stra8* expression and meiotic initiation (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007), likely by oxidation of RA to an inactive form.

Despite the importance of *Stra8* for meiotic initiation, relatively little is known about how transcription of *Stra8* is regulated in fetal germ cells. Here we examine the role in female gonadal development of the transcription factor *Dmrt1*, a member of the DM domain gene

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family, which appears to play a deeply conserved role in sexual differentiation among metazoan phyla (Baker and Ridge, 1980; Raymond et al., 1998, 1999b). *Dmrt1* is a critical regulator of male gonadal development in mammals, with essential functions in both germ line and soma. These functions include regulation of fetal germ cell proliferation and pluripotency, control of the mitosis versus meiosis decision in adult spermatogonia, and differentiation of Sertoli cells in neonates (Kim et al., 2007; Krentz et al., 2009; Matson et al., 2010; Raymond et al., 2000). *Dmrt1* and its orthologs control sex determination and gonadal differentiation in diverse vertebrates, participating in a variety of primary sex-determining mechanisms (Matsuda et al., 2002; Nanda et al., 2002; Smith et al., 2009; Yoshimoto et al., 2008). Although DMRT1 is transiently expressed in fetal ovaries in birds, reptiles, and mammals, null mutant female mice are fertile, and hence it has been unclear whether DMRT1 has any role in ovarian development or function. Here we show in mice that *Dmrt1* mutant females have severely reduced *Stra8* expression and abnormal meiotic prophase, and they form reduced numbers of ovarian follicles. Chromatin immunoprecipitation and mRNA expression profiling suggest that the primary function of DMRT1 in females is to directly activate transcription of *Stra8* in the fetal ovary.

Materials and methods

Mouse breeding

Experiments involving *Dmrt1*$^{+/-}$ or *Dmrt1*$^{-/-}$ mice (Raymond et al., 2000) were performed on mice outbred to 129S6/SvEvTac (Taconic Labs) at least seven times. Presence of a copulation plug in the morning was recorded as day E0.5. Swiss Webster outbred mice (Charles River) were used for ChIP experiments. Genotyping of *Dmrt1* was as described (Kim et al., 2007).

Immunofluorescence

Ovaries were dissected and fixed by immersion in 4% paraformaldyhyde (PFA) overnight at 4 °C. Embryonic ovaries were prepared for cryosectioning as described (Krentz et al., 2009). Postnatal ovaries were dehydrated in an ethanol series and embedded in paraffin, and 7 μm sections were cut. Antigen retrieval was performed on postnatal tissue by boiling in citric acid buffer for 10 min. Both embryonic and postnatal tissue was permeabilized in 1xPBS +0.1% Tween-20 (PBSTw) followed by blocking in 10% heat inactivated goat or donkey serum with 3% BSA in PBSTw for at least 1 h at RT. Primary antibodies were diluted in blocking media and incubated overnight at 4 °C in humidity chamber. Primary antibodies were TRA98 (Bio Academia 73-003; 1:500), MVH (Abcam ab13840; 1:500), DMRT1 ((Raymond et al., 2000) 1:100), Ki67 (Thermo Scientific RM-9106-S1; 1:250) STRA8 (gift of Pierre Chambon; 1:200), γH2AX (Upstate 05-636; 1:250), MSY2 (Santa Cruz sc-21314; 1:200), H1FOO (gift of Jon Hennebold and Eli Adashi; 1:200), GCNA (gift of George Enders) and SYCP3 (Abcam ab15092; 1:250). Sections were washed followed by incubation with secondary antibodies (Molecular Probes) diluted 1:500 in PBSTw plus 3% BSA. Sections were mounted with Permafluor (Lab Vision).

mRNA expression profiling

Ovaries were dissected from E13.5 embryos and placed in RNAlater (Qiagen) until all genotypes were collected. RNA was prepared using the RNeasy Micro Kit (Qiagen). 100 ng of total RNA was amplified and labeled using NuGen Ovation labeling kit and hybridized to Affymetrix 430 2.0 microarrays. CEL files were normalized using the GC-RMA algorithm (Wu et al., 2004) from the Genedata Analyst Software Package and analyzed as previously described (Krentz et al., 2009).

qRT-PCR

Ovaries were stored in RNAlater and RNA was prepared using the RNeasy Micro kit (Qiagen). RNA was reverse-transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen) and cDNA was amplified using FastStart SYBR green (Roche). Primer sequences: *Stra8* (CKM94/95) (Matson et al., 2010); *Nanos2* (5′-ATGGACCATTCACGTTCAGC-3′ and 5′-ACCCGAGGCAGTCTGAAGTT-3′).

ChIP

Embryos were collected at E13.5 and sexed by gonadal morphology. Ovaries from 190 to 197 females were dissected and pooled for each of three biological replicates. Chromatin was prepared as described (Krentz et al., 2009). DNA from three independent DMRT1 ChIP samples was amplified by ligation-mediated PCR (Oberley et al., 2004) modified to use DNATerminator (Lucigen Corp.). Amplified ChIP and input material was labeled by MOgene and hybridized to Mouse ChIP 385 K RefSeq Promoter Arrays (Nimblegen). Promoters with enrichment were identified as described (Murphy et al., 2010). qPCR was performed on unamplified immunoprecipitated DNA using gene specific primers that directly flank the ChIP peak for validation of array data (Table S2). Testes from 70 males were dissected and pooled and chromatin was prepared as described (Krentz et al., 2009) and analyzed by qChIP.

Discovery of polyC tracts

The nucleotide sequence under the area of the DMRT1 binding peak was used for motif scanning using Multiple Em for Motif Elicitation (MEME) (Bailey and Elkan, 1994).

Quantifying the number of follicles

Ovary sections were stained for MVH and every fifth section was counted to determine the number of primordial, primary and secondary follicles and the number of germ cell nests. Germ cells were counted as being in a nest if MVH-positive cytoplasm was continuous between two cells. Follicles were scored as primordial if they were MVH-positive and contained a single layer of spindle-shaped granulosa cells and primary/secondary if the oocyte was surrounded by one or more layers of cuboidal granulosa cells. Total number of germ cells counted was multiplied by five to take into account that every fifth section was counted (Flaws et al., 2001; Pepling et al., 2010).

Fertility assay

Eight-week old virgin *Dmrt1*$^{+/+}$ and *Dmrt1*$^{-/-}$ females were crossed to wild type males for 9 months (n=4 females for each genotype, 4 males). Two females were housed together with one male, and every 2 weeks the males were rotated to different cages. The total number of litters produced and pups per litter were recorded for each female.

Results

DMRT1 is required for STRA8 expression and normal meiotic prophase in the fetal ovary

Prior to sex determination, *Dmrt1* mRNA and DMRT1 protein are expressed in somatic cells and germ cells of the bipotential gonad, with similar levels in XX and XY animals (Lei et al., 2007; Raymond et al., 1999a). In the ovary DMRT1 disappears from somatic cells by E13.5 and it is nearly absent from germ cells by E15.5 (Lei et al., 2007). To delimit the possible functions of DMRT1 in the ovary we more closely examined the timing of its down-regulation. At E13.5, when meiosis is

initiating, we observed nuclear DMRT1 expression in most female germ cells (Figs. 1A–C), as previously reported (Lei et al., 2007). At E14.5 DMRT1 localization had shifted to the cytoplasm (Figs. 1D–F). Thus DMRT1 is expressed in nuclei of germ cells prior to and during female sex determination and meiotic initiation and then it is rapidly excluded from the nucleus and eliminated. If DMRT1 plays a role in the ovary, it therefore is likely to function prior to or during meiotic initiation.

Meiotic initiation in the fetal ovary requires the transcriptional activation of *Stra8* (Baltus et al., 2006). In wild type females, STRA8 was expressed in >80% of germ cells by E13.5, in about 95% of germ cells by E14.5, and it was nearly absent by E15.5 (Figs. 1G–L, S). In *Dmrt1*$^{-/-}$ females the timing of STRA8 accumulation was unaffected, but STRA8 was only detected in up to 25% of germ cells during the period from E13.5 to E15.5 (Figs. 1M–S). Because meiosis initiates asynchronously in the ovary (Bullejos and Koopman, 2004), this represents a minimum estimate of the number of STRA8-expressing germ cells. At E17.5 STRA8 expression was undetectable in nuclei of both wild type and *Dmrt1*$^{-/-}$ germ cells (data not shown). We conclude that *Dmrt1* is required in the fetal ovary for robust activation of STRA8 expression.

Because most *Dmrt1*$^{-/-}$ female germ cells fail to express detectable STRA8, they would be expected to display phenotypes similar to those of *Stra8* mutants. *Stra8* is required for germ cells to enter meiotic prophase I (Anderson et al., 2008; Baltus et al., 2006; Mark et al., 2008). Hallmarks of prophase I include chromosome pairing (synaptonemal complex

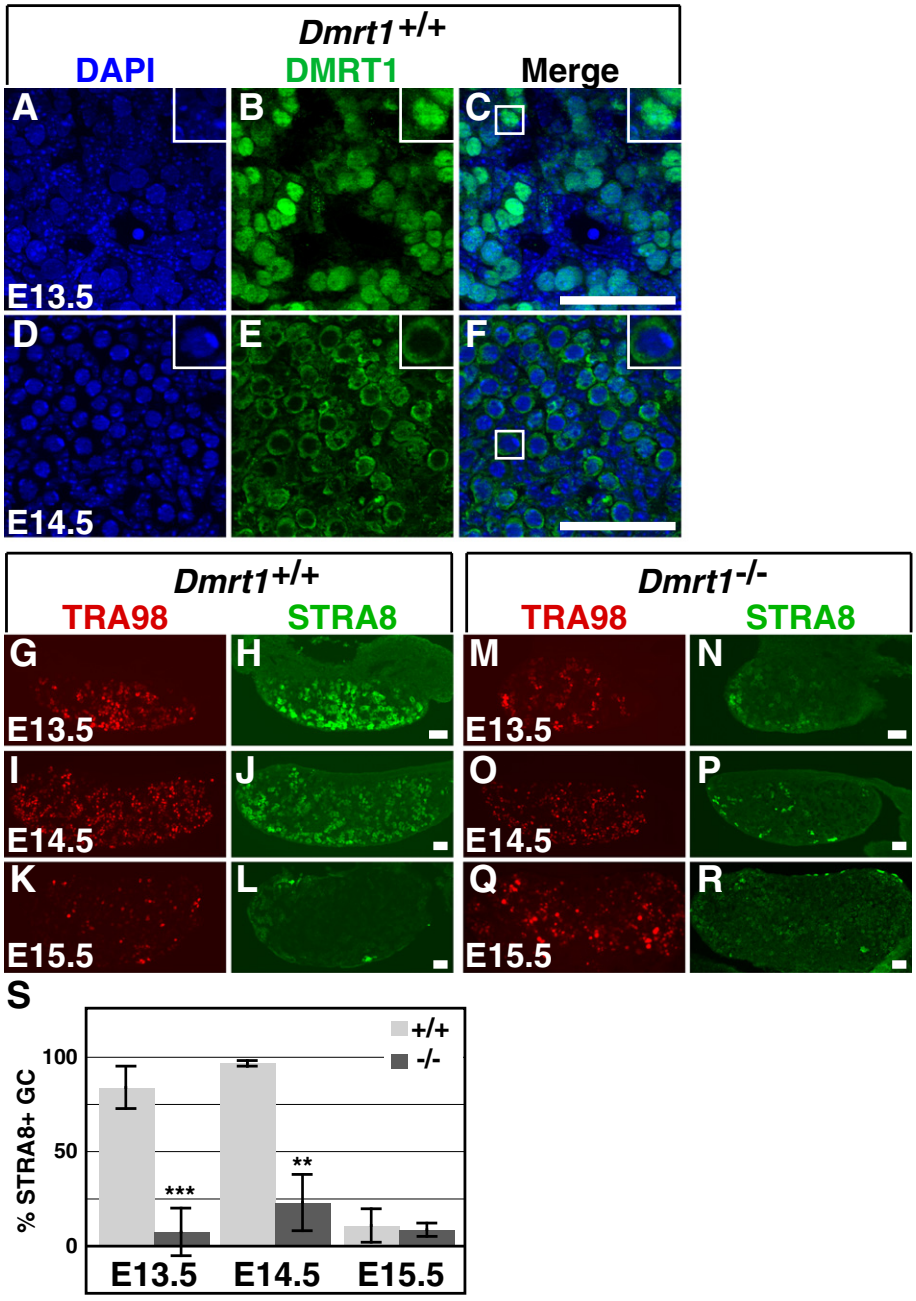

Fig. 1. DMRT1 is required to activate STRA8 in fetal ovarian germ cells. (A–F) Immunofluorescence (IF) staining for DMRT1 and DNA staining with DAPI. (A–C) DMRT1 is expressed in the nucleus of germ cells at E13.5. (D–F) DMRT1 is expressed in germ cell cytoplasm at E14.5. (G–R) Double staining for germ cell marker TRA98 and meiotic inducer STRA8 in (G–L) wild type and (M–R) *Dmrt1*$^{-/-}$ ovary sections at E13.5 (G–H, M–N), E14.5 (I–J, O–P) and E15.5 (K–L, Q–R). Scale bars: 50 microns. (S) Percentage of STRA8-positive germ cells in wild type versus *Dmrt1*$^{-/-}$ ovaries at E13.5–E15.5 ($n \geq 3$ animals, $^{**}P<0.005$, $^{***}P<0.0005$).

formation) and homologous recombination. To evaluate these processes we examined expression and localization of SYCP3, a component of the synaptonemal complex, and γH2AX, a phosphorylated form of histone H2AX that accumulates at double-strand breaks associated with homologous recombination. In control ovaries SYCP3 was strongly expressed and localized to nuclei of germ cells by E13.5 (Figs. 2A–C) and by E15.5 it was loaded onto paired chromosomes (Figs. 2G–I). In *Dmrt1* mutant germ cells SYCP3 was expressed at E13.5, but it was primarily cytoplasmic (Figs. 2D–F). At E15.5, only a small proportion of germ cells in mutant ovaries had nuclear SYCP3 and most of these did not have proper association of SYCP3 with paired chromosomes (Figs. 2J–L). Similarly, nearly all germ cells in wild type females at E15.5 had foci of γH2AX (Figs. 2M–O), whereas very few germ cells in mutant ovaries had γH2AX foci (Figs. 2P–R). Based on the expression and localization of SYCP3 and γH2AX, we conclude that meiotic prophase is abnormal in the majority of germ cells in the mutant fetal ovary. To determine whether these defects result in failure to progress through meiotic prophase, we examined the expression of the diplotene-specific protein MSY2 (Gu et al., 1998). At postnatal day 4 (P4) MSY2 was expressed in all germ cells of wild type and *Dmrt1* $^{-/-}$ ovaries (Figs. 2S–X). We also observed similar timing of MSY2 accumulation in wild type and *Dmrt1* mutant fetal oocytes (data not shown). The normal expression of MSY2 indicates that mutant germ cells can initiate meiosis and proceed to diplonema with normal timing. We also examined the expression of the oocyte-specific H1 linker histone H1FOO, which is expressed in growing oocytes (Tanaka et al., 2001, 2005) and found that mutant oocytes had normal H1F00 localization (Fig. S1). We conclude that *Dmrt1* mutant oocytes are able to initiate and complete meiotic prophase through diplonema and undergo postnatal growth, despite their failure to accumulate SYCP3 and γH2AX during early prophase.

DMRT1 plays a role establishing G_0 arrest in mitotic germ cells of the fetal testis (Krentz et al., 2009). We therefore asked whether exit from the mitotic program might also be abnormal in female fetal germ cells. We assayed mitosis using the active cell cycle marker Ki67 (Gerdes et al., 1983). About 50% of *Dmrt1* $^{-/-}$ germ cells still expressed Ki67 at E13.5, compared with 20% in wild type (Fig. S2) but Ki67 was absent from wild type and mutants at E14.5 (not shown). This result suggests that exit from mitosis may be delayed in mutant germ cells but germ cells do not persist in mitosis in significant numbers. Because DMRT1 is expressed during germ line sex determination, we also asked whether *Dmrt1* $^{-/-}$ ovarian germ cells might be masculinized. We examined two sex-specific germ cell markers and found no evidence for masculinization. E-CADHERIN (CDH1), is strongly expressed in male gonocytes at E13.5, but only weakly expressed in females (Mackay et al., 1999), and *Dmrt1* $^{-/-}$ ovarian germ cells expressed CDH1 at normal female levels (data not shown). *Nanos2* is expressed specifically in male germ cells upon entrance into the genital ridge (Tsuda et al., 2003) and also showed no change in

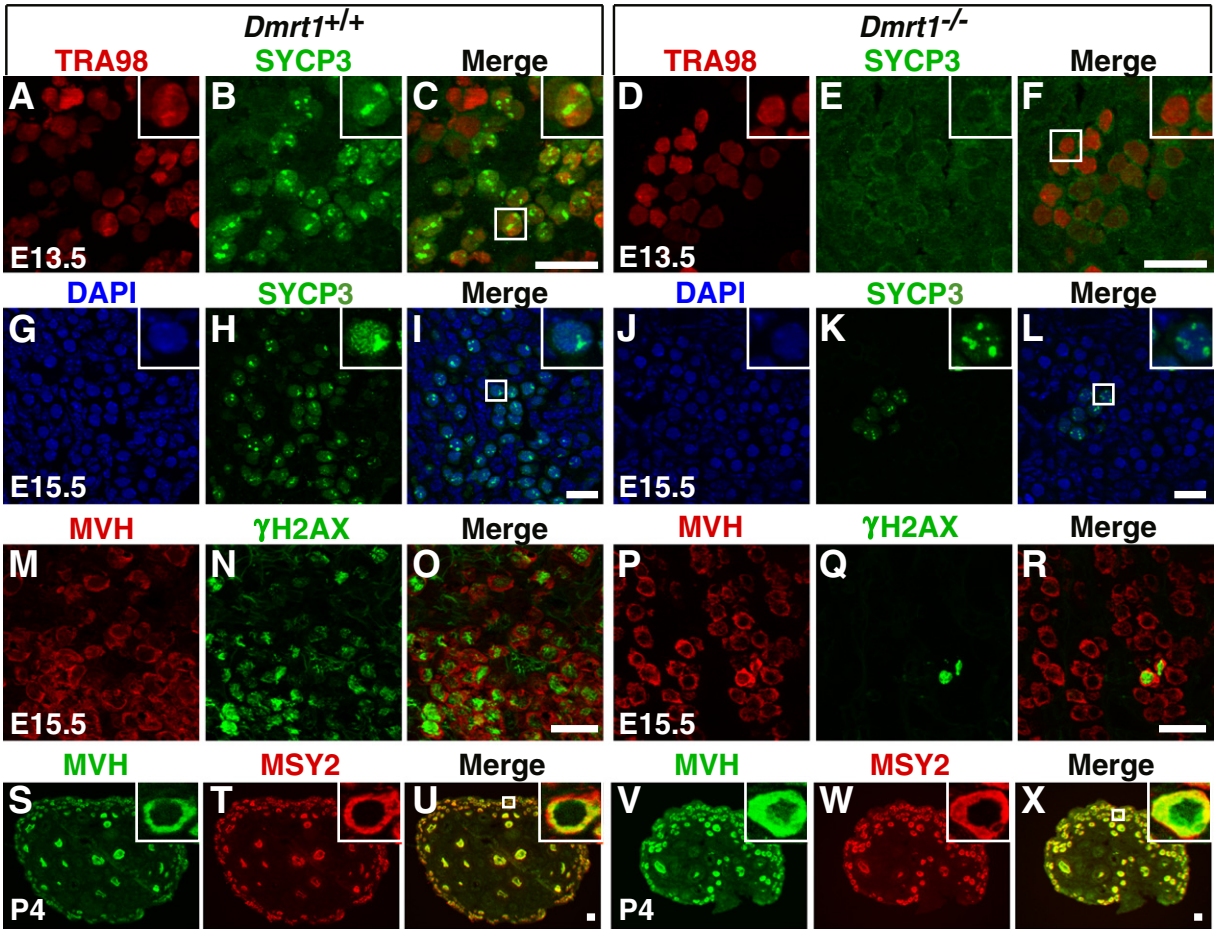

Fig. 2. Abnormal prophase I in *Dmrt1* mutant ovaries. (A–F) Double staining for SYCP3 and TRA98 by IF in wild type (A–C) and *Dmrt1* $^{-/-}$ (D–F) ovaries. (A–F) SYCP3 is expressed in the nucleus of wild type germ cells at E13.5 and in (D–F) the cytoplasm of *Dmrt1* $^{-/-}$ germ cells. (G–L) SYCP3 is properly loaded onto chromosomes in wild type (G–I), but not in *Dmrt1* $^{-/-}$ (J–L) germ cells at E15.5. DNA is marked with DAPI. (M–R) Double staining for the germ cell marker MVH and γH2AX in wild type (M–O) and *Dmrt1* $^{-/-}$ (P–R) ovaries at E15.5. Most germ cells in the wild type ovary (M–R) are γH2AX positive, whereas *Dmrt1* $^{-/-}$ germ cells (P–R) are only rarely γH2AX positive. (S–X) Double staining for MVH and the diplotene marker MSY2 in wild type (S–U) and *Dmrt1* $^{-/-}$ (V–X) ovaries at P4. Scale bars: 25 micron.

expression in *Dmrt1* $^{-/-}$ ovaries based on qRT-PCR ($p = 0.99$; $n = 3$). Finally, we examined expression of FOXL2, a female-specific transcription factor that is expressed in ovarian granulosa cells (Crisponi et al., 2001) and found that FOXL2 was expressed normally in *Dmrt1* $^{-/-}$ ovaries (data not shown). Together these data suggest that *Dmrt1* $^{-/-}$ female germ cells have a slight delay in the transition from mitosis to meiosis but neither mutant germ cells nor granulosa cells are masculinized.

Genome-wide assessment of DMRT1 promoter association in the fetal ovary

To help elucidate how DMRT1 regulates the mitosis to meiosis transition in the fetal ovary, we sought promoter regions bound by DMRT1 using chromatin immunoprecipitation and microarray hybridization (ChIP-chip). At E13.5 ChIP-chip identified 89 promoter regions bound by DMRT1 in at least two of three experiments (Fig. 3A, Table S1). Taking into account bidirectional promoters, 136 genes were associated with promoter regions bound by DMRT1 in the fetal ovary. We used quantitative ChIP (qChIP) to validate the ChIP-chip data with an independent pool of ovarian chromatin, and this confirmed binding of DMRT1 to 11/12 promoters tested (Fig. 3C). Twenty-seven of the 89 promoter regions also were bound by DMRT1 in E13.5 and P9 testes ((Murphy et al., 2010) and unpublished data). Of these 27 promoter regions, 9 had a close match to the DMRT1 DNA recognition motif near the center of the DMRT1 binding peak (Murphy et al., 2007), 10 had long polyC tracts closely coincident with the DMRT1 binding peak, and 8 had both (Fig. 3A, Table S1). ChIP-chip also identified 30 peaks bound in the fetal ovary that were also bound in the testis at E13.5 but not at P9 ((Murphy et al., 2010) and unpublished data). These 30 targets lacked a close match to the DMRT1 in vitro defined recognition motif but all of them contained long polyC tracts underneath the peaks (Fig. 3B, Table S1). The presence of polyC tracts closely associated with DMRT1 binding and the absence of nearby canonical DMRT1 DNA recognition motifs in many target promoters suggests the possibility that DMRT1 associates with DNA at some sites by an alternative mechanism.

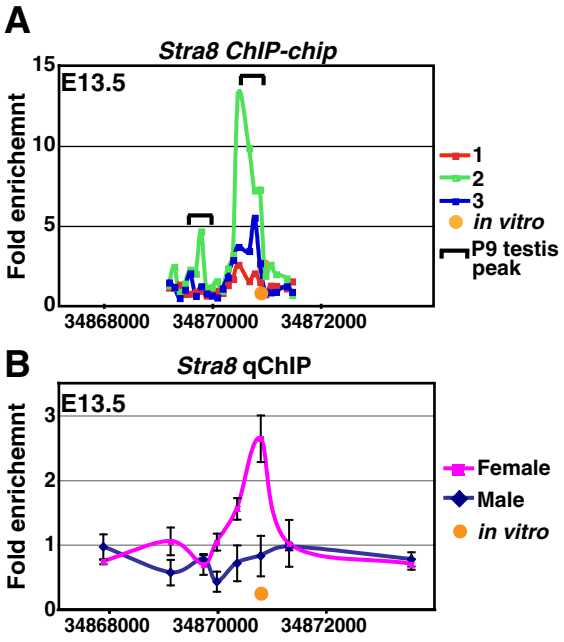

Fig. 4. In vivo binding of DMRT1 to the proximal promoter region of *Stra8*. (A) ChIP-chip showing enrichment in *Stra8* proximal promoter with DMRT1 in vitro defined motif (Murphy et al., 2007) in fetal ovaries in three separate experiments. (B). qChIP validating enrichment for *Stra8* promoter in fetal ovaries (pink), but not fetal testes (blue). Error bars: SD from technical replicates. Gold dot indicates position of in vitro defined DMRT1 consensus binding motif (Murphy et al., 2007) and peak from P9 testis.

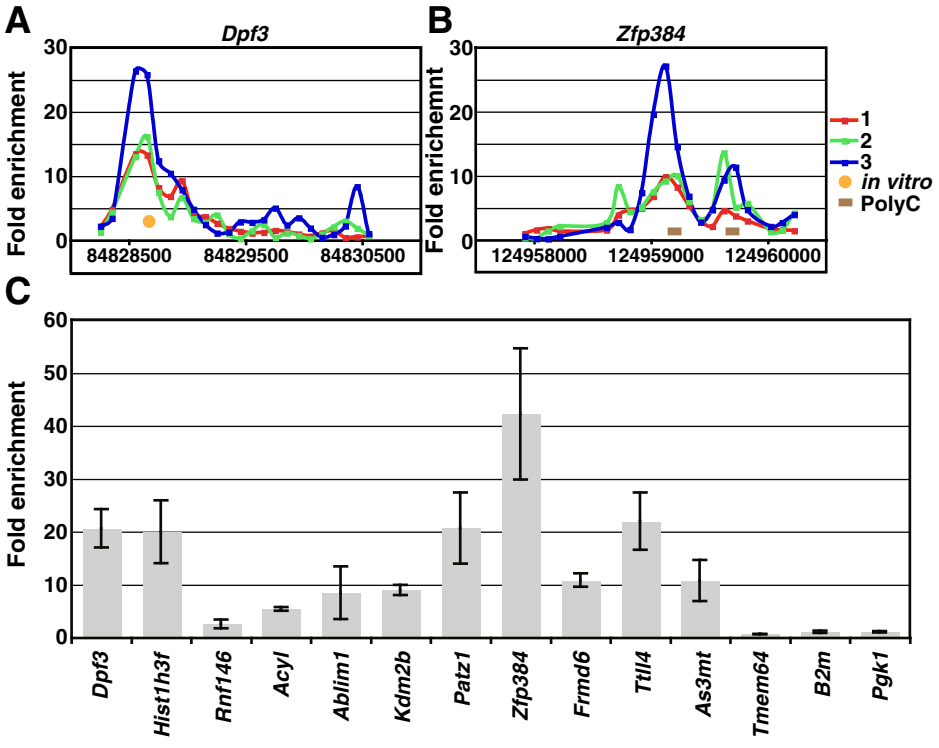

Fig. 3. Identification of DMRT1 direct targets in the E13.5 ovary. DMRT1 binding to the proximal promoter region of (A) *Dpf3* and (B) *Zfp384* detected by ChIP-chip in three independent experiments. Gold dot indicates position of *in vitro* defined DMRT1 consensus binding motif (Murphy et al., 2007) and brown bar indicates position of polyC tract. (C) Validation of promoter binding by qChIP for 12 genes detected by ChIP-chip, using an independent chromatin sample. Eleven of twelve promoter regions show greater than 2.5 fold enrichment. *B2m* and *Pgk1* are negative controls. Error bars: SD from technical replicates.

DMRT1 as an activator of Stra8 transcription

Of particular note among promoters bound by DMRT1 in the fetal ovary was that of *Stra8* (Fig. 4A). qChIP confirmed that DMRT1 in the fetal ovary associates with the same pair of promoter proximal sites in *Stra8* that it binds in the postnatal testis (Fig. 4B) (Matson et al., 2010; Murphy et al., 2010). By contrast, qChIP did not detect binding of DMRT1 to the *Stra8* promoter in fetal testes (Fig. 4B).

To ask which genes bound by DMRT1 are most likely to mediate its regulation of ovarian development, we compared mRNA expression in wild type versus *Dmrt1*$^{-/-}$ ovaries at E13.5. Microarray expression profiling at E13.5 identified only eight mRNAs other than *Dmrt1* that were misexpressed two-fold or more in *Dmrt1*$^{-/-}$ ovaries ($P<0.05$; Fig. 5A). Among these, only *Stra8* had detectable binding of DMRT1 to its promoter region in the fetal ovary (Figs. 4A and B, Table S1). The reduction in *Stra8* levels was confirmed in three independent samples by qRT-PCR (Fig. 5B). We cannot exclude binding of DMRT1 to non-promoter regions of other misexpressed genes, but these data suggest that regulation of *Stra8* may be the primary function of DMRT1 in the fetal ovary.

Reduced oocyte numbers in mutant ovaries

Because meiotic prophase appeared abnormal in most *Dmrt1*$^{-/-}$ germ cells in the mutant fetal ovary, we asked whether there were consequences in the postnatal ovary. At birth (P0) there was no obvious reduction in total germ cell numbers assessed by hematoxylin and eosin staining and MVH positive germ cells (data not shown). However at postnatal day eight (P8), *Dmrt1*$^{-/-}$ ovaries were smaller than wild type and had a clear deficit in the number of follicles (Figs. 6A–C). To quantify this defect we counted MVH-positive germ cells in serial-sectioned P8 ovaries (Figs. 6A–D; Materials and methods). Primordial follicles were reduced approximately 50% (Fig. 6C) and primary and secondary follicles were reduced 25% (Fig. 6D).

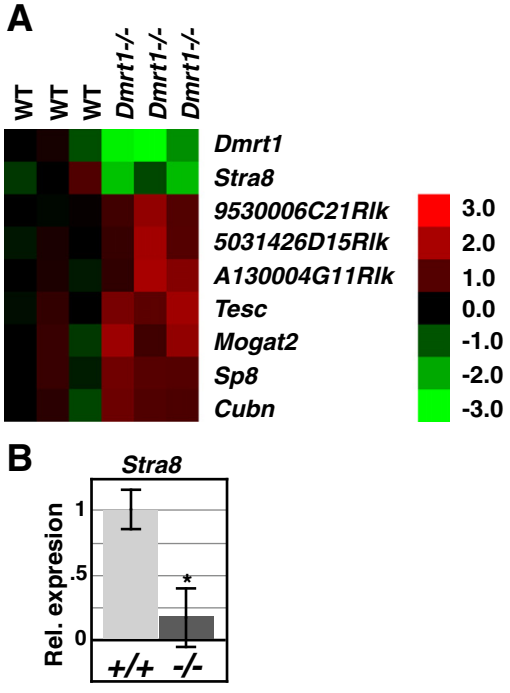

Fig. 5. mRNA expression profiling in *Dmrt1* mutant ovaries. (A) Heat map showing seven mRNAs elevated greater than two-fold and two mRNAs reduced greater than two-fold in *Dmrt1*$^{-/-}$ ovaries at E13.5 ($P<0.05$). (B) qRT-PCR of *Stra8* at E13.5 normalized to *HPRT*. Error bars: SD from 3 animals of each genotype. (*$P<0.05$).

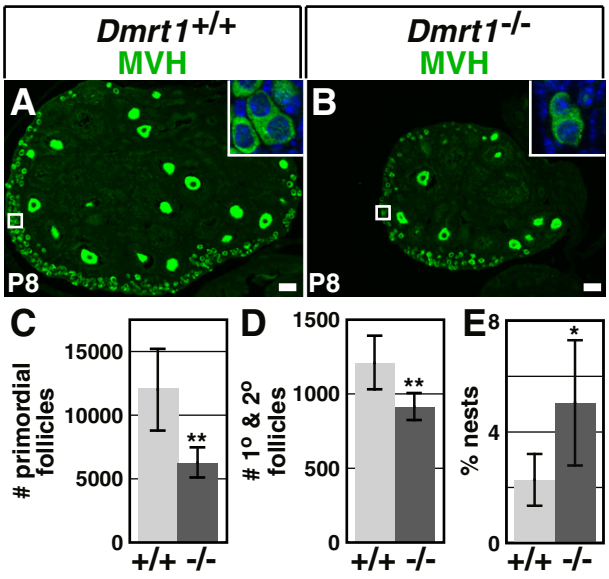

Fig. 6. Reduction in number of follicles at P8. (A,B) IF staining for germ cell marker MVH in wild type (A) and *Dmrt1*$^{-/-}$ (B) ovaries at P8. DNA is stained with DAPI. Scale bars: 50 microns. (C) Number of primordial and (D) primary and secondary follicles per ovary at P8. (E) Percentage of oocytes found in nests at P8. Error bars: SD from 3 animals of each genotype (*$P<0.05$, **$P<0.005$).

An important step during postnatal oocyte development is the breakdown of germline cysts, or nests of germ cells joined by intercellular bridges (Gondos, 1973; Pepling et al., 1999). These bridges are severed postnatally between P2 and P4 in a process termed nest breakdown, allowing each oocyte to be enclosed by granulosa cells to form a primordial follicle, the basic functional unit for female reproduction. Up to two-thirds of oocytes are eliminated during nest breakdown and mutations that cause recombination failure can increase germ cell loss during this process, suggesting that nest breakdown may serve a quality control function by eliminating defective oocytes (Baudat et al., 2000; Hunt and Hassold, 2002; McClellan et al., 2003; Pepling and Spradling, 2001). The reduction in oocytes in *Dmrt1*$^{-/-}$ ovaries at P8 may therefore reflect an increase in cell death during nest breakdown. In addition, nest breakdown may be compromised in *Dmrt1*$^{-/-}$ ovaries, as they had a two-fold increase in the proportion of oocytes still in nests at P8 (Figs. 6A–B, E). However despite the severe reduction in follicle numbers, mutant females had apparently normal fertility under continuous breeding conditions (Fig. S3).

Discussion

Because *Dmrt1* is expressed only transiently in the fetal ovary and mutant females are fertile, the role, if any of *Dmrt1* in females has not previously been examined. We have found that *Dmrt1* does indeed function in the fetal ovary. In the absence of *Dmrt1* germ cells can initiate meiosis but they do so abnormally: they have severely reduced STRA8 expression, fail to accumulate SYCP3 and γH2AX, and contain only half the normal number of follicles in the juvenile ovary. However, despite these defects, mutant females have enough functional follicles for a normal fertile lifespan under conditions of continuous breeding.

The majority of *Dmrt1* mutant oocytes failed to express detectable STRA8 and even fewer expressed γH2AX and loaded SYCP3 onto chromosomes, yet mutant females were fertile. SYCP3 and γH2AX are dispensable for female fertility (Baltus et al., 2006; Celeste et al., 2002; Yuan et al., 2002). *Stra8*, by contrast, is essential for fertility in both sexes. *Stra8* mRNA levels were reduced five-fold in mutant ovaries and only about 25% of mutant germ cells had detectable STRA8 protein, yet many cells initiated meiosis. There are at least two possible explanations, which are not mutually exclusive. First, a small

amount of STRA8 may be sufficient for meiosis. Second, since fetal germ cells undergo incomplete cytokinesis and are found in syncytia (Gondos, 1973), strongly STRA8-positive cells may be able to rescue those with low STRA8. In either event, it is clear that meiotic prophase is not normal in *Dmrt1* mutant females and it may be informative to more closely examine recombination and other meiotic events.

Up to two-thirds of oogonia die during nest breakdown in the first postnatal week (Bristol-Gould et al., 2006; Pepling and Spradling, 2001). The reduction in follicles in *Dmrt1*$^{-/-}$ ovaries at P8 may therefore reflect an increase in cell death during this period. Nest breakdown occurs many days after DMRT1 expression ceases so any defect must be a consequence of loss of *Dmrt1* in the fetal ovary. We speculate that the defects responsible for the failure to localize SYCP3 and γH2AX may later lead to mutant germ cells being culled at a higher frequency during nest breakdown.

Previous work suggested that accumulation of RA in the fetal ovary activates *Stra8* and causes meiotic initiation, implicating the RA receptor as a presumptive transcriptional activator of *Stra8* (Anderson et al., 2008; Bowles et al., 2006; Koubova et al., 2006; Li and Clagett-Dame, 2009). Our results indicate that DMRT1 also plays a significant role in activation of *Stra8* in the fetal ovary, binding the *Stra8* promoter near a pair of RA response elements and activating *Stra8* transcription. DMRT1 is not essential for *Stra8* transcription but in its absence *Stra8* is greatly reduced. We suggest therefore that DMRT1 is likely to act as an important competence factor that is required for appropriate sensitivity of germ cells to another temporally controlled inducer, possibly RA. In addition it was striking that DMRT1 had a stochastic effect on STRA8 expression in the fetal ovary: some mutant germ cells expressed apparently normal levels of STRA8, whereas in others STRA8 was undetectable. It therefore appears that DMRT1 serves a "gating" function: DMRT1 helps determine whether a cell can activate *Stra8*, but is not essential for full *Stra8* activation. Another competence factor for female *Stra8* expression is the RNA binding protein *Dazl* (Lin et al., 2008). *Dazl* expression was unaffected in *Dmrt1* mutant ovaries, so the connection between these regulators will require further investigation.

Expression profiling at E13.5 identified only eight mRNAs other than *Dmrt1* that were misexpressed more than two-fold in *Dmrt1* mutant ovaries compared to wild type. Among these the only one known to be important for meiotic progression was *Stra8*. The small number of misexpressed mRNAs suggests a very specific transcriptional function for DMRT1, controlling activation of *Stra8*. The defects in chromosomal loading of SYCP3 and γH2AX were not directly attributable to DMRT1 transcriptional regulation. In *Stra8*$^{-/-}$ ovaries, *Sycp3* and *Rec8* are transcribed and translated, but fail to load onto meiotic chromosomes (Baltus et al., 2006). Thus unlocalized SYCP3 and possibly other meiotic defects are likely to stem from reduced STRA8 expression.

In the adult testis DMRT1 represses *Stra8* transcription (Matson et al., 2010), whereas here we show that DMRT1 activates *Stra8* in the fetal ovary, apparently via the same regulatory element. This implies either a difference in DMRT1 itself, for example a post-translational modification, or a difference in coregulators that act with DMRT1. We did not detect binding of DMRT1 to the *Stra8* promoter in E13.5 fetal testes, suggesting that the activity of DMRT1 can be controlled at the level of DNA association. Because male and female fetal gonads appear to differ in RA levels (Koubova et al., 2006) it is possible that an essential coregulator or modifier of DMRT1 is induced by RA. In postnatal males DMRT1 not only represses *Stra8* transcription but also restricts RA-dependent transcription generally (Matson et al., 2010). We found no evidence that this occurs in the fetal ovary, as mRNA expression profiling detected no misexpression of RA-responsive mRNAs in mutant ovaries.

In summary, we find that DMRT1 plays a significant role in the ovary. It is required for normal meiotic prophase in the fetal ovary and for formation of normal numbers of ovarian follicles postnatally. DMRT1 directly regulates *Stra8* transcription in both sexes but it does so sex-specifically, activating *Stra8* in females and repressing it in males. These findings highlight *DMRT1* as a candidate to be involved in female infertility or premature ovarian failure in humans. It also will be important to ask whether *Dmrt1* homologs regulate female meiosis in non-mammalian vertebrates or in other phyla.

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References

Anderson, E.L., Baltus, A.E., Roepers-Gajadien, H.L., Hassold, T.J., de Rooij, D.G., van Pelt, A.M., Page, D.C., 2008. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc. Natl Acad. Sci. U. S. A. 105, 14976–14980.

Bailey, T.L., Elkan, C., 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc. Int. Conf. Intell. Syst. Mol. Biol. 2, 28–36.

Baker, B.S., Ridge, K.A., 1980. Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster*. Genetics 94, 383–423.

Baltus, A.E., Menke, D.B., Hu, Y.C., Goodheart, M.L., Carpenter, A.E., de Rooij, D.G., Page, D.C., 2006. In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. Nat. Genet. 38, 1430–1434.

Baudat, F., Manova, K., Yuen, J.P., Jasin, M., Keeney, S., 2000. Chromosome synapsis defects and sexually dimorphic meiotic progression in mice lacking Spo11. Mol. Cell 6, 989–998.

Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M.J., Rossant, J., Hamada, H., Koopman, P., 2006. Retinoid signaling determines germ cell fate in mice. Science 312, 596–600.

Bristol-Gould, S.K., Kreeger, P.K., Selkirk, C.G., Kilen, S.M., Cook, R.W., Kipp, J.L., Shea, L.D., Mayo, K.E., Woodruff, T.K., 2006. Postnatal regulation of germ cells by activin: the establishment of the initial follicle pool. Dev. Biol. 298, 132–148.

Bullejos, M., Koopman, P., 2004. Germ cells enter meiosis in a rostro-caudal wave during development of the mouse ovary. Mol. Reprod. Dev. 68, 422–428.

Celeste, A., Petersen, S., Romanienko, P.J., Fernandez-Capetillo, O., Chen, H.T., Sedelnikova, O.A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M.J., Redon, C., Pilch, D.R., Olaru, A., Eckhaus, M., Camerini-Otero, R.D., Tessarollo, L., Livak, F., Manova, K., Bonner, W.M., Nussenzweig, M.C., Nussenzweig, A., 2002. Genomic instability in mice lacking histone H2AX. Science 296, 922–927.

Crisponi, L., Deiana, M., Loi, A., Chiappe, F., Uda, M., Amati, P., Bisceglia, L., Zelante, L., Nagaraja, R., Porcu, S., Ristaldi, M.S., Marzella, R., Rocchi, M., Nicolino, M., Lienhardt-Roussie, A., Nivelon, A., Verloes, A., Schlessinger, D., Gasparini, P., Bonneau, D., Cao, A., Pilia, G., 2001. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. Nat. Genet. 27, 159–166.

Flaws, J.A., Hirshfield, A.N., Hewitt, J.A., Babus, J.K., Furth, P.A., 2001. Effect of bcl-2 on the primordial follicle endowment in the mouse ovary. Biol. Reprod. 64, 1153–1159.

Foster, J.W., Dominguez-Steglich, M.A., Guioli, S., Kwok, C., Weller, P.A., Stevanovic, M., Weissenbach, J., Mansour, S., Young, I.D., Goodfellow, P.N., Brooks, J.D., Schafer, A.J., 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 372, 525–530.

Gerdes, J., Schwab, U., Lemke, H., Stein, H., 1983. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer 31, 13–20.

Ghyselinck, N.B., Vernet, N., Dennefeld, C., Giese, N., Nau, H., Chambon, P., Viville, S., Mark, M., 2006. Retinoids and spermatogenesis: lessons from mutant mice lacking the plasma retinol binding protein. Dev. Dyn. 235, 1608–1622.

Gondos, B., 1973. Germ cell degeneration and intercellular bridges in the human fetal ovary. Z. Zellforsch. Mikrosk. Anat. 138, 23–30.

Gu, W., Tekur, S., Reinbold, R., Eppig, J.J., Choi, Y.C., Zheng, J.Z., Murray, M.T., Hecht, N.B., 1998. Mammalian male and female germ cells express a germ cell-specific Y-Box protein, MSY2. Biol. Reprod. 59, 1266–1274.

Hilscher, B., Hilscher, W., Bulthoff-Ohnolz, B., Kramer, U., Birke, A., Pelzer, H., Gauss, G., 1974. Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis. Cell Tissue Res. 154, 443–470.

Hunt, P.A., Hassold, T.J., 2002. Sex matters in meiosis. Science 296, 2181–2183.

Kim, S., Bardwell, V.J., Zarkower, D., 2007. Cell type-autonomous and non-autonomous requirements for Dmrt1 in postnatal testis differentiation. Dev. Biol. 307, 314–327.

Koopman, P., Munsterberg, A., Capel, B., Vivian, N., Lovell-Badge, R., 1990. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 348, 450–452.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., Lovell-Badge, R., 1991. Male development of chromosomally female mice transgenic for Sry. Nature 351, 117–121.

Koubova, J., Menke, D.B., Zhou, Q., Capel, B., Griswold, M.D., Page, D.C., 2006. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc. Natl Acad. Sci. U. S. A. 103, 2474–2479.

Krentz, A.D., Murphy, M.W., Kim, S., Cook, M.S., Capel, B., Zhu, R., Matin, A., Sarver, A.L., Parker, K.L., Griswold, M.D., Looijenga, L.H., Bardwell, V.J., Zarkower, D., 2009. The DM domain protein DMRT1 is a dose-sensitive regulator of fetal germ cell proliferation and pluripotency. Proc. Natl Acad. Sci. U. S. A. 106, 22323–22328.

Kumar, S., Chatzi, C., Brade, T., Cunningham, T.J., Zhao, X., Duester, G., 2011. Sex-specific timing of meiotic initiation is regulated by Cyp26b1 independent of retinoic acid signalling. Nat. Commun. 2, 151.

Lei, N., Hornbaker, K.I., Rice, D.A., Karpova, T., Agbor, V.A., Heckert, L.L., 2007. Sex-specific differences in mouse DMRT1 expression are both cell type- and stage-dependent during gonad development. Biol. Reprod. 77, 466–475.

Li, H., Clagett-Dame, M., 2009. Vitamin A deficiency blocks the initiation of meiosis of germ cells in the developing rat ovary in vivo. Biol. Reprod. 81, 996–1001.

Lin, Y., Gill, M.E., Koubova, J., Page, D.C., 2008. Germ cell-intrinsic and -extrinsic factors govern meiotic initiation in mouse embryos. Science 322, 1685–1687.

Livera, G., Rouiller-Fabre, V., Valla, J., Habert, R., 2000. Effects of retinoids on the meiosis in the fetal rat ovary in culture. Mol. Cell. Endocrinol. 165, 225–231.

Mackay, S., Nicholson, C.L., Lewis, S.P., Brittan, M., 1999. E-cadherin in the developing mouse gonad. Anat. Embryol. (Berl.) 200, 91–102.

MacLean, G., Li, H., Metzger, D., Chambon, P., Petkovich, M., 2007. Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. Endocrinology 148, 4560–4567.

Mark, M., Jacobs, H., Oulad-Abdelghani, M., Dennefeld, C., Feret, B., Vernet, N., Codreanu, C.A., Chambon, P., Ghyselinck, N.B., 2008. STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature chromosome condensation. J. Cell Sci. 121, 3233–3242.

Matson, C.K., Murphy, M.W., Griswold, M.D., Yoshida, S., Bardwell, V.J., Zarkower, D., 2010. The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. Dev. Cell 19, 612–624.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C.E., Shibata, N., Asakawa, S., Shimizu, N., Hori, H., Hamaguchi, S., Sakaizumi, M., 2002. DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417, 559–563.

McClellan, K.A., Gosden, R., Taketo, T., 2003. Continuous loss of oocytes throughout meiotic prophase in the normal mouse ovary. Dev. Biol. 258, 334–348.

McLaren, A., 1975. The independence of germ-cell genotype from somatic influence in chimaeric mice. Genet. Res. 25, 83–87.

McLaren, A., 1981. The fate of germ cells in the testis of fetal sex-reversed mice. J. Reprod. Fertil. 61, 461–467.

Murphy, M.W., Zarkower, D., Bardwell, V.J., 2007. Vertebrate DM domain proteins bind similar DNA sequences and can heterodimerize on DNA. BMC Mol. Biol. 8, 58.

Murphy, M.W., Sarver, A.L., Rice, D., Hatzi, K., Ye, K., Melnick, A., Heckert, L.L., Zarkower, D., Bardwell, V.J., 2010. Genome-wide analysis of DNA binding and transcriptional regulation by the mammalian Doublesex homolog DMRT1 in the juvenile testis. Proc. Natl Acad. Sci. U. S. A. 107, 13360–13365.

Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., Schartl, M., 2002. A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. Proc. Natl Acad. Sci. U. S. A. 99, 11778–11783.

Oberley, M.J., Tsao, J., Yau, P., Farnham, P.J., 2004. High-throughput screening of chromatin immunoprecipitates using CpG-island microarrays. Methods Enzymol. 376, 315–334.

Oulad-Abdelghani, M., Bouillet, P., Decimo, D., Gansmuller, A., Heyberger, S., Dolle, P., Bronner, S., Lutz, Y., Chambon, P., 1996. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. J. Cell Biol. 135, 469–477.

Pepling, M.E., Spradling, A.C., 2001. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Dev. Biol. 234, 339–351.

Pepling, M.E., de Cuevas, M., Spradling, A.C., 1999. Germline cysts: a conserved phase of germ cell development? Trends Cell Biol. 9, 257–262.

Pepling, M.E., Sundman, E.A., Patterson, N.L., Gephardt, G.W., Medico Jr., L., Wilson, K.I., 2010. Differences in oocyte development and estradiol sensitivity among mouse strains. Reproduction 139, 349–357.

Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J., Zarkower, D., 1998. Evidence for evolutionary conservation of sex-determining genes. Nature 391, 691–695.

Raymond, C.S., Kettlewell, J.R., Hirsch, B., Bardwell, V.J., Zarkower, D., 1999a. Expression of Dmrt1 in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. Dev. Biol. 215, 208–220.

Raymond, C.S., Parker, E.D., Kettlewell, J.R., Brown, L.G., Page, D.C., Kusz, K., Jaruzelska, J., Reinberg, Y., Flejter, W.L., Bardwell, V.J., Hirsch, B., Zarkower, D., 1999b. A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. Hum. Mol. Genet. 8, 989–996.

Raymond, C.S., Murphy, M.W., O'Sullivan, M.G., Bardwell, V.J., Zarkower, D., 2000. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes Dev. 14, 2587–2595.

Sekido, R., Bar, I., Narvaez, V., Penny, G., Lovell-Badge, R., 2004. SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors. Dev. Biol. 274, 271–279.

Smith, C.A., Roeszler, K.N., Ohnesorg, T., Cummins, D.M., Farlie, P.G., Doran, T.J., Sinclair, A.H., 2009. The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature 461, 267–271.

Tanaka, M., Hennebold, J.D., Macfarlane, J., Adashi, E.Y., 2001. A mammalian oocyte-specific linker histone gene H1oo: homology with the genes for the oocyte-specific cleavage stage histone (cs-H1) of sea urchin and the B4/H1M histone of the frog. Development 128, 655–664.

Tanaka, M., Kihara, M., Hennebold, J.D., Eppig, J.J., Viveiros, M.M., Emery, B.R., Carrell, D.T., Kirkman, N.J., Meczekalski, B., Zhou, J., Bondy, C.A., Becker, M., Schultz, R.M., Misteli, T., De La Fuente, R., King, G.J., Adashi, E.Y., 2005. H1FOO is coupled to the initiation of oocytic growth. Biol. Reprod. 72, 135–142.

Tsuda, M., Sasaoka, Y., Kiso, M., Abe, K., Haraguchi, S., Kobayashi, S., Saga, Y., 2003. Conserved role of nanos proteins in germ cell development. Science 301, 1239–1241.

Wu, Z., Irizarry, R.A., Gentleman, R., Martinez Murillo, F., Spencer, F., 2004. A model based background adjustment for oligonucleotide expression arrays. J. Am. Stat. Assoc. 99, 909–917.

Yoshimoto, S., Okada, E., Umemoto, H., Tamura, K., Uno, Y., Nishida-Umehara, C., Matsuda, Y., Takamatsu, N., Shiba, T., Ito, M., 2008. A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. Proc. Natl Acad. Sci. U. S. A. 105, 2469–2474.

Yuan, L., Liu, J.G., Hoja, M.R., Wilbertz, J., Nordqvist, K., Hoog, C., 2002. Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3. Science 296, 1115–1118.