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DMRT1 prevents female reprogramming in the postnatal mammalian testis

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Abstract

Sex in mammals is determined in the foetal gonad by the presence or absence of the Y chromosome gene *Sry*, which controls whether bipotential precursor cells differentiate into testicular Sertoli cells or ovarian granulosa cells[1]. This pivotal decision in a single gonadal cell type ultimately controls sexual differentiation throughout the body. Sex determination can be viewed as a battle for primacy in the foetal gonad between a male regulatory gene network in which *Sry* activates *Sox9* and a female network involving Wnt/β-catenin signaling (Supplemental Fig. 1)[2]. In females the primary sex-determining decision is not final: loss of the FOXL2 transcription factor in adult granulosa cells can reprogramme granulosa cells into Sertoli cells[2]. Here we show that sexual fate is also surprisingly labile in the testis: loss of the DMRT1 transcription factor[3] in mouse Sertoli cells, even in adults, activates *Foxl2* and reprogrammes Sertoli cells into granulosa cells. In this environment, theca cells form, oestrogen is produced, and germ cells appear feminized. Thus *Dmrt1* is essential to maintain mammalian testis determination, and competing regulatory networks maintain gonadal sex long after the foetal choice between male and female. *Dmrt1* and *Foxl2* are conserved throughout vertebrates[4,5] and *Dmrt1*-related sexual regulators are conserved throughout metazoans[3]. Antagonism between *Dmrt1* and *Foxl2* for control of gonadal sex may therefore extend beyond mammals. Reprogramming due to loss of

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Author Contributions C.K.M. performed mouse breeding and analysis of protein and mRNA expression; M.W.M. performed ChIP analysis; A.S. performed bioinformatic analysis; C.K.M., D.Z. and V.J.B. designed the study, analyzed data, and wrote the paper; M.D.G. provided mRNA profiling expertise; all authors discussed the results and edited the paper.

Author Information mRNA expression profiling data have been deposited at GEO (GSE27261) and can be reviewed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zlctbiugsamymtc&acc=GSE27261.

Dmrt1 also may help explain the etiology of human syndromes linked to *DMRT1*, including disorders of sexual differentiation[6] and testicular cancer[7].

Human chromosome 9p deletions removing *DMRT1* are associated with XY male-to-female sex reversal, and *Dmrt1* homologues determine sex in several non-mammalian vertebrates[8,9,10]. In mice, *Dmrt1* is expressed and required in both germ cells and Sertoli cells of the testis[11,12,13]. XY *Dmrt1* null mutant mice are born as males with testes, although these gonads later undergo abnormal differentiation[14]; hence the role of *Dmrt1* in mammalian sex determination has been unclear. Here we examine *Dmrt1* mutant testes during postnatal development, asking whether loss of *Dmrt1* causes postnatal feminization in mice.

We first examined gonads of *Dmrt1* null mutant males ($Dmrt1^{-/-}$) for the presence of FOXL2, a female-specific transcription factor expressed in granulosa cells and theca cells[15,16], the two somatic cell types of the ovarian follicle (Fig. 1a). Four weeks after birth, abundant FOXL2-positive cells were present within mutant seminiferous tubules (Fig. 1b), which in control testes contain only germ cells and Sertoli cells (Fig 1c). To establish the origin of the FOXL2-positive cells, we deleted *Dmrt1* either in germ cells (using *Nanos3-cre*) or in Sertoli cells (using *Dhh-cre* or *Sf1*-cre) (Supplemental Fig. 2a–l; Supplemental Table 1). Loss of *Dmrt1* in foetal Sertoli cells (*SCDmrt1KO*) but not in foetal germ cells (*GCDmrt1KO*) induced FOXL2 expression (Fig. 1d–f). *SCDmrt1KO* gonads retained small numbers of germ cells, which appeared to arrest in meiotic prophase based on SYCP3 localization (Supplemental Fig. 3). These results demonstrate that DMRT1 expression in Sertoli cells prevents FOXL2 expression and suggest that *Dmrt1* mutant testes become feminized during the first postnatal month.

Next we examined the timing of FOXL2 induction. At postnatal day 7 (P7), *SCDmrt1KO* testes had seminiferous tubules in which all Sertoli cells expressed SOX9 normally (Supplemental Fig. 2m–r), but at P14 some intratubular cells co-expressed SOX9 and FOXL2 or lacked SOX9 and strongly expressed FOXL2 (Fig. 1g–l). By P28 few SOX9-positive cells remained and most intratubular cells strongly expressed FOXL2 (Fig. 1m–o). Histologic analysis of mutant gonads is shown in Supplemental Fig. 4. These results show that foetal loss of *Dmrt1* causes postnatal Sertoli cells to lose the male-promoting SOX9 and instead express the female-promoting FOXL2.

Loss of *Foxl2* in the adult ovary can lead to transdifferentiation of granulosa cells to Sertoli cells[2], so we asked whether loss of *Dmrt1* in the adult testis activates *Foxl2* and causes the reciprocal sex transformation, from Sertoli to granulosa. Indeed, one month after deletion of *Dmrt1* in adult males (using a tamoxifen-inducible *cre* transgene), we observed cells with typical Sertoli cell features including tripartite nucleoli but expressing both SOX9 and FOXL2 (Fig. 2a–d), as well as cells with typical granulosa cell nuclear morphology that lacked SOX9 and strongly expressed FOXL2 (Fig. 2e–h). Thus antagonism between DMRT1 and FOXL2 continues into adulthood and Sertoli cell fate remains plastic even after terminal differentiation.

To further evaluate the transformation of mutant gonads, we compared the mRNA profile of control and mutant P28 testes; 5030 mRNAs were expressed >8-fold differently across this dataset or a dataset comparing testis to 21 other tissues including ovary; (Supplemental Fig. 5a). We calculated Pearson correlation coefficients for expression of these 5030 mRNAs in mutant gonads relative to each tissue and found that the mutant gonad most closely resembled ovary (Supplemental Fig. 5b; average R=0.75). Many mRNAs with decreased expression in mutant gonads also were low in other tissues, likely reflecting a lack of male germ cells, which comprise much of the testis mass. Also, some mRNAs elevated in mutant gonads were elevated in other tissues. Therefore, to specifically evaluate ovary-enriched mRNAs, we used bioGPS (biogps.gnf.org; SI) to identify 65 mRNAs with expression closely correlated to *Foxl2* and then compared their expression in ovary relative to the other 21 tissues (Fig. 3a; Supplemental Fig. 6). This comparison confirmed that these mRNAs are highly ovary-enriched. About 40% were elevated in mutant gonads relative to control testes; about 80% of the remainder were oocyte-enriched. Thus loss of *Dmrt1* causes large changes in mRNA expression, including induction of multiple ovary-enriched mRNAs. mRNA profiling of *Dmrt1* mutant gonads perinatally and at P9 did not reveal apparent feminization[17,18], consistent with the observation that FOXL2 expression starts at ~P14.

Further analysis of the mRNA profiling data identified highly elevated expression (>5-fold, p<0.001) of many mRNAs expressed in granulosa cells and required for ovarian development or function. These included *Foxl2*, *Nr5a2/Lrh1*, *Wnt4,* LH receptor (*Lhcgr*), prolactin receptor (*Prlr*), FSH receptor (*Fshr*), follistatin (*Fst*), *Sfrp4*, *Igfbp5*, *Inhbb*, *Inha*, and *Lnfg* (Supplemental Table 2). *Foxl2os*, a noncoding RNA transcribed from the opposite strand of the *Foxl2* coding region, also was highly over-expressed and has been suggested as a positive regulator of *Foxl2*[19]. We confirmed elevated expression in mutant gonads of LRH1, a transcription factor expressed only in granulosa cells within the ovary[20] and absent from the testis (Supplemental Fig. 7a–f). *Nr5a2*/*Lrh1* is likely a direct target of DMRT1 regulation, based on binding of DMRT1 to its promoter proximal sequences in vivo (Supplemental Fig. 7g). Based on mRNA and protein expression data and changes in cellular morphology, we conclude that loss of *Dmrt1* in testes reprogrammes Sertoli cells into granulosa cells.

Granulosa cells produce oestrogens, which are essential for ovarian development in many vertebrates; in mammals oestrogen signaling also acts with FOXL2 to repress *Sox9* transcription in adult granulosa cells[2]. HSD17β1 and CYP19A1/Aromatase are enzymes critical for oestrogen synthesis, and mRNAs for both enzymes were elevated in mutant gonads (Supplemental Fig. 8). Aromatase protein is robustly expressed in granulosa cells and was strongly expressed in mutant gonads (Fig. 3b–d). Consistent with these enzyme changes, oestradiol was elevated in serum of adult mutants relative to control adult males (SI). Although expression of the androgenic enzyme *Hsd17β3* was not affected in mutant gonads (Supplemental Fig. 8), androgen levels were reduced based on severely decreased seminal vesicle weight, a sensitive indicator of androgen activity (350 +/− 52 mg vs 182 +/− 36 mg; n = 3, P=0.01).

Theca cells are induced during follicle growth in the ovary, likely in response to granulosa cell signals[21], and together with granulosa cells and oocytes they comprise the functional unit of the ovary. Because mutant gonads contained apparently functional granulosa cells, we asked whether theca cells also formed. Theca cells have spindle-shaped nuclei and express both FOXL2 and smooth muscle actin (SMA) (Fig. 3e). Adult mutant gonads contained cells closely resembling theca cells and expressing both proteins (Fig. 3f). The theca-like cells likely derive either from granulosa cells or peritubular myoid cells (which also are elongated and express SMA; Figure 3g). However, since seminiferous tubule integrity was lost prior to formation of these cells (Fig. 3f; Supplemental Fig. 9) they could potentially derive from interstitial cells that invaded the tubule remnants. We also observed intratubular cells strongly expressing the steroidogenic enzyme SCC (Fig. 3h–j); these cells resembled luteinized granulosa cells of the ovary (Fig. 3h), suggesting that granulosa cells in the mutant gonad are responsive to gonadotropins. We therefore tested the effect of exogenous gonadotropin stimulation; treated mutants, but not controls, had additional luteinized granulosa cells and germ cells with oocyte-like nuclear morphology that expressed the oocyte-specific proteins MATER and ZP2 (Supplemental Fig. 10). This result suggests that both somatic cells and germ cells are feminized in mutant gonads.

The preceding results indicate that DMRT1 is essential for postnatal sex maintenance. DMRT1 is a sequence-specific transcriptional regulator, capable of activating or repressing transcription of target genes[18,22]. To help find targets of DMRT1 regulation with potential roles in sex maintenance we examined expression of known foetal sex-determining genes in mutant gonads at P28 by qRT-PCR (Fig. 4a). Among masculinizing genes, *Ptgdr, Sox9*, and *Sox8*, which acts redundantly with *Sox9*[23,24], were reduced. Among feminizing genes, *Foxl2*, *Esr1*, *Esr2*, *Wnt4* and *Rspo1* were elevated. We assayed binding of DMRT1 to DNA of P28 testes by quantitative chromatin immunoprecipitation (qChIP), guided by genome-wide ChIP data from P9 testes (ChIP-chip[18] and ChIP-seq [unpublished]). DMRT1 bound both upstream and downstream of *Sox9* and upstream of *Sox8*, and bound weakly near *Ptgdr*. DMRT1 bound strongly near *Foxl2*, *Esr1*, *Esr2*, *Wnt4* and *Rspo1* (Fig. 4b). All of the DMRT1-associated regions contained at least one close match to the DMRT1 DNA binding consensus[18,22].

Based on mRNA and protein expression data and ChIP analysis, we propose a model for postnatal sex maintenance (Fig. 4b) in which DMRT1 maintains male fates by repressing multiple female-promoting genes and activating male-promoting genes. *Sox9* is dispensable for testis differentiation after sex determination[24,25], suggesting that other critical male regulators remain to be found; *Sox8* is a clear candidate based on its redundancy with *Sox9*[23,24]. We find that DMRT1 represses *Foxl2*, which is known to maintain postnatal ovarian fate. FOXL2 also represses *Dmrt1*[2]; thus antagonism between these sex-specific transcriptional regulators may be central to sex maintenance in both sexes throughout reproductive life. *Wnt4* and *Rspo1* also are prime candidates for postnatal sex maintenance based on their requirement in ovarian determination in the foetus[26,27]. Indeed, P28 mutant gonads had elevated nuclear β-Catenin in somatic cells, as in ovaries, but control testes did not, indicating active Wnt/β-catenin signaling in the mutant gonads (Supplemental Fig. 11). Functional analysis of *Wnt4*, *Rspo1* and other known foetal sex regulators will be important to establish their roles in sex maintenance. The analysis presented here demonstrates that deletion of *Dmrt1* during foetal development induces postnatal feminization of the testis, causing male-to-female primary sex reversal. Moreover, deletion of *Dmrt1* in adults can reprogramme differentiated Sertoli cells into apparent granulosa cells. Why are *Dmrt1* mutants feminized only after birth? Another male-promoting gene may act redundantly with *Dmrt1* prior to P14, masking its function; alternatively, the testis may lack potential feminizing activity from genes such as *Foxl2* prior to P14. Why are *Dmrt1* mutant mice born male, whereas human 9p deletions removing *DMRT1* can cause XY feminization at birth? The human sex reversal may reflect failure to maintain male sex determination, and the longer human gestation may permit testis-to-ovary reprogramming before birth. Alternatively, human testes may have potential feminizing activity earlier or may lack masculinizing genes redundant with *DMRT1*. Our results may provide insights into the aetiology of human gonadal disorders, including gonadoblastoma and granulosa cell tumors of the testis. Moreover, because many genes implicated in this study are evolutionarily conserved, similar mechanisms may control adult sex-switching in fish and may maintain sexual fate in the adult gonads of other vertebrates or even in other phyla.

Methods Summary

Mouse breeding

Dmrt1 mutant and control males were generated as described[12]; tissue-specific Cre recombinase strains are in Supplemental Table 1. Adult wild type or *Dmrt1*$^{flox/flox}$ females were used as controls. Mice were mixed C57BL/6J, 129S1, and FVB genetic background. Protocols were approved by the Institutional Animal Care and Use Committee.

Immunofluorescence (IF) and immunohistochemistry (IHC)

IF and IHC were performed as described[12]. Antibodies are listed in Supplemental Table 3. Analyses included at least two biological replicates.

Tamoxifen treatment

Tamoxifen-inducible deletion of *Dmrt1* in adult males was as described[12]. Testes were harvested one to two months post-treatment.

mRNA expression analysis

mRNA expression profiling and data analysis were as described[13] except total testis RNA was isolated from 4-week-old animals using TRIzol reagent (Invitrogen #15596-026). Additional detail is in Supplemental Methods.

qRT-PCR

qRT-PCR was as described[12]. qRT-PCR primers are listed in Supplemental Table 4.

Chromatin immunoprecipitation

ChIP followed by either microarray (ChIP-chip) or qPCR analysis (qChIP) were as described[18]. Gene-specific primers used for qChIP are in Supplemental Table 4.

Methods

Mouse breeding

Conditional *Dmrt1* mutant and control males were generated as described[12]; tissue-specific Cre recombinase strains are in Supplemental Table 1. Adult wild type or *Dmrt1*$^{flox/flox}$ females were used as controls. Mice were of mixed C57BL/6J, 129S1, and FVB genetic background. Protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Immunofluorescence (IF) and immunohistochemistry (IHC)

Both IF and IHC were performed as described[12]. Antibodies are listed in Supplemental Table 3. Analyses included a minimum of two biological replicates.

Tamoxifen treatment

Tamoxifen-inducible deletion of *Dmrt1* in adult males was performed as previously described[12]. Testes were harvested one to two months following treatment.

mRNA expression analysis

mRNA expression profiling and data analysis were performed as described[13] except total testis RNA was isolated from 4-week-old animals using TRIzol reagent (Invitrogen #15596-026). Affymetrix Mouse Genome 439 2.0 arrays were normalized by GC-RMA normalization[28] using GeneData Refiner. The Raw .cel files and the normalized data are deposited in GEO[29] as GSE27261. GSE9954 was obtained from the GEO database. The arrays with the highest sample IDs were removed from the tissue dataset to select 22 tissue types, each with three experimental replicates. When multiple probe sets were mapped to the same gene symbol, these values were averaged to obtain one value for each gene symbol. Direct Pearson Correlation R-values were calculated using all array data following reduction to gene symbols, and these values are shown in Figure 2b.

Each experiment in our dataset was divided by the average expression value from control testis tissue. GSE9954 data were separately divided by the average signal obtained from the GSE9954 testis samples. This was done separately for each dataset to determine how samples from each dataset differed from a baseline “testis” expression state. Cluster 3.0 software[30] was used to: i) log base 2 transform the data; ii) filter the dataset for genes that showed at least three observations with abs(val) >= 3 (8-fold) which resulted in 5030 genes passing the filter using both datasets combined; and iii) cluster the data on the gene-axis using average linkage hierarchial clustering. The experimental axis was defined by order of decreasing correlation to the mutant testes calculated as described above. Javatreeview Software[31] was used to generate heatmap images.

qRT-PCR

qRT-PCR was performed as described[12]. qRT-PCR primers are listed in Supplemental Table 4.

Chromatin immunoprecipitation

ChIP followed by either microarray (ChIP-chip) or qPCR analysis (qChIP) were performed as described[18]. Gene specific primers used for qChIP are in Supplemental Table 4.

Oestadiol assays

Serum oestradiol was assayed using a clinical electrochemiluminescence immunoassay (Roche Estradiol II, 03000079 122) according to manufacturer's instructions. Three of three males assayed had levels below the detection limit, whereas two of three females had measurable oestradiol (5.0 and 19.7 pg/dl). Two of three *SCDmrt1KO(Dhh)* mutant males had measurable oestradiol (5.6 and 21.2 pg/dl).

Gonadotropin treatment

6–8 week old mutant males, control males, and control females were treated with 5 units of pregnant mare serum by intraperitoneal injection and gonads were harvested 48 hours later.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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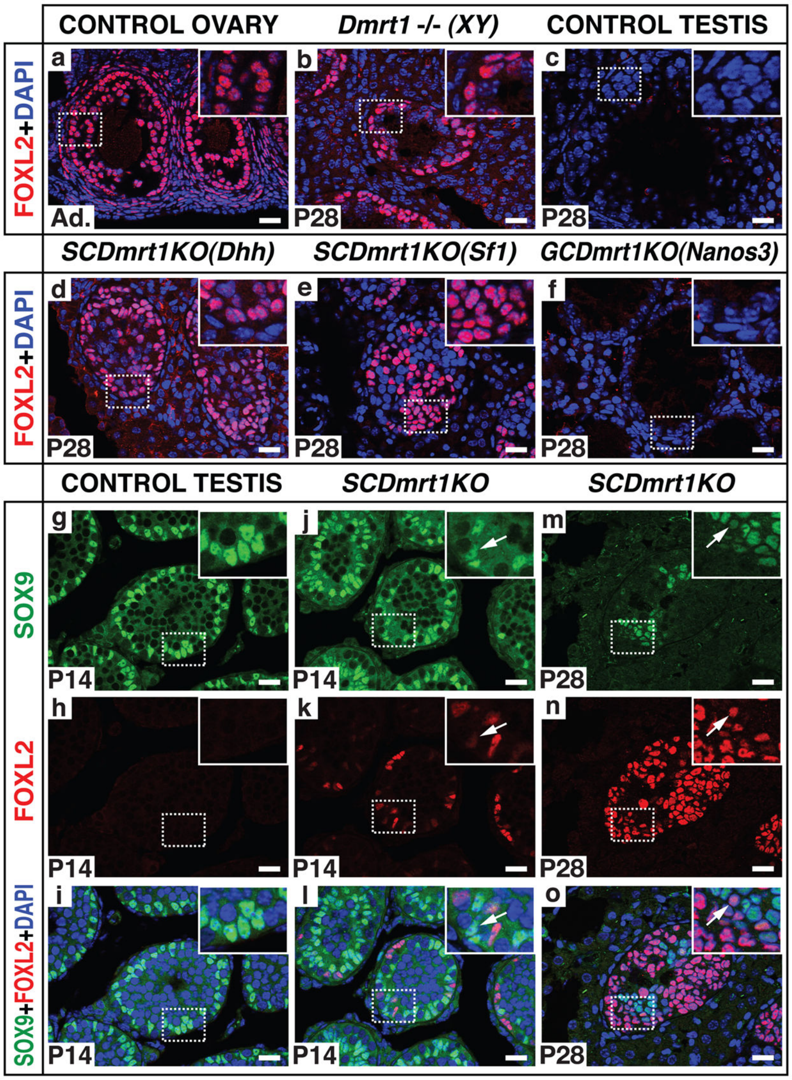

Figure 1. DMRT1 maintains SOX9 and suppresses FOXL2 expression in postnatal Sertoli cells

(a–c) FOXL2 expression detected by immunofluorescence (IF) in adult granulosa and theca cells of control ovary (a) and intratubular cells of *Dmrt1* null testis at postnatal day 28 (P28) (b) but not in control testis (c). **(d–f)** FOXL2 is robustly expressed when *Dmrt1* is mutated in foetal Sertoli cells with *Dhh-cre* (d) or *Sf1-cre* (e) but not when *Dmrt1* is mutated in foetal germ cells with *Nanos3-cre* (f). **(g–o)** Timing of FOXL2 expression. FOXL2 is absent from control testis at P14 (g–i). Cells expressing FOXL2 or FOXL2 and SOX9 (arrowheads) are present in *SCDmrt1KO* testis at P14 (j–l). FOXL2-positive cells are abundant in *SCDmrt1KO* testis at P28 and most cells no longer express SOX9 (m–o). Scale bars: 20 mm.

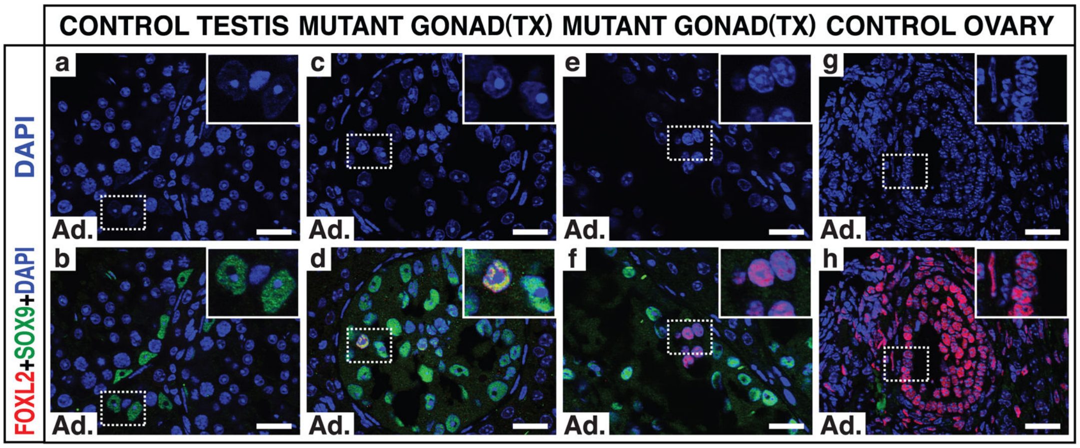

Figure 2. Sertoli-to-granulosa transdifferentiation in the adult testis

Expression of FOXL2 and SOX9 one month after tamoxifen injection into *Dmrt1*$^{flox/flox}$ adult males (8 weeks and older) carrying inducible ubiquitous cre transgene UBC-cre/ERT2. (**a,b**) Sertoli cells in control testis express SOX9 but not FOXL2. (**c–f**) Mutant testis has Sertoli-like cells expressing SOX9 or SOX9 and FOXL2 (inset, d) and granulosa-like cells expressing only FOXL2 (inset, f). (**g,h**) FOXL2-positive cells in control ovary have DAPI morphology similar to FOXL2 single-positive cells of mutant testis. FOXL2-positive cells in mutant testis resemble granulosa cells: they lack the tripartite nucleoli of Sertoli cells, have smaller and more rounded nuclei, and have more punctate DAPI staining. UBC-cre/ERT2 also deletes *Dmrt1* in germ cells, causing precocious meiosis[12]; after one month germ cells are nearly absent. Scale bars: 20 μm.

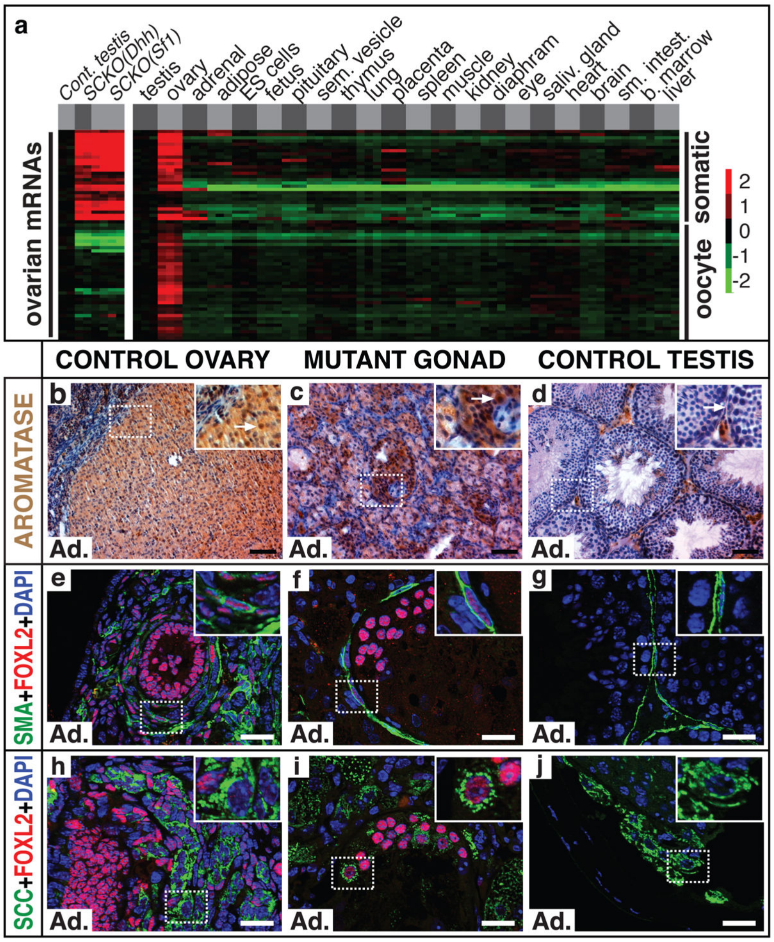

Figure 3. Feminization of *SCDmrt1KO* XY gonads

(a) Expression of ovary-enriched mRNAs with expression profiles similar to *Foxl2* (see SI). mRNAs labeled “somatic” were enriched in ovarian somatic cells; those labeled “oocyte” were enriched in female germ cells. See Supplemental Fig. 6 for higher resolution image. **(b–d)** IHC detection of CYP19A1/Aromatase expression in follicles of control adult ovary (a) and in adult XY *SCDmrt1KO* gonad (b) but only in interstitial Leydig cells of control testis (c). Arrows indicate Aromatase-positive granulosa cells in ovary and mutant gonad and negative Sertoli cell in control testis. Scale bars: 50 μm. (**e–g**) IF detection of smooth muscle actin (SMA) and FOXL2. Ovarian theca cells (inset, e) are elongated cells expressing both proteins; similar cells are present in mutant gonads (f); peritubular myoid cells in control testes express SMA and not FOXL2 (g). Scale bars: 20 μm. **(h–j)** IF detection of cells coexpressing FOXL2 in the nucleus and steroidogenic enzyme CYP11A1/SCC at high levels in the cytoplasm in control ovary (h) and XY *Dmrt1KO* gonads (i). SCC-positive cells in control testis (j) are interstitial Leydig cells. Mutant gonads were *SCDmrt1KO(Dhh).* Ad.=adult. Scale bars: 20μm.

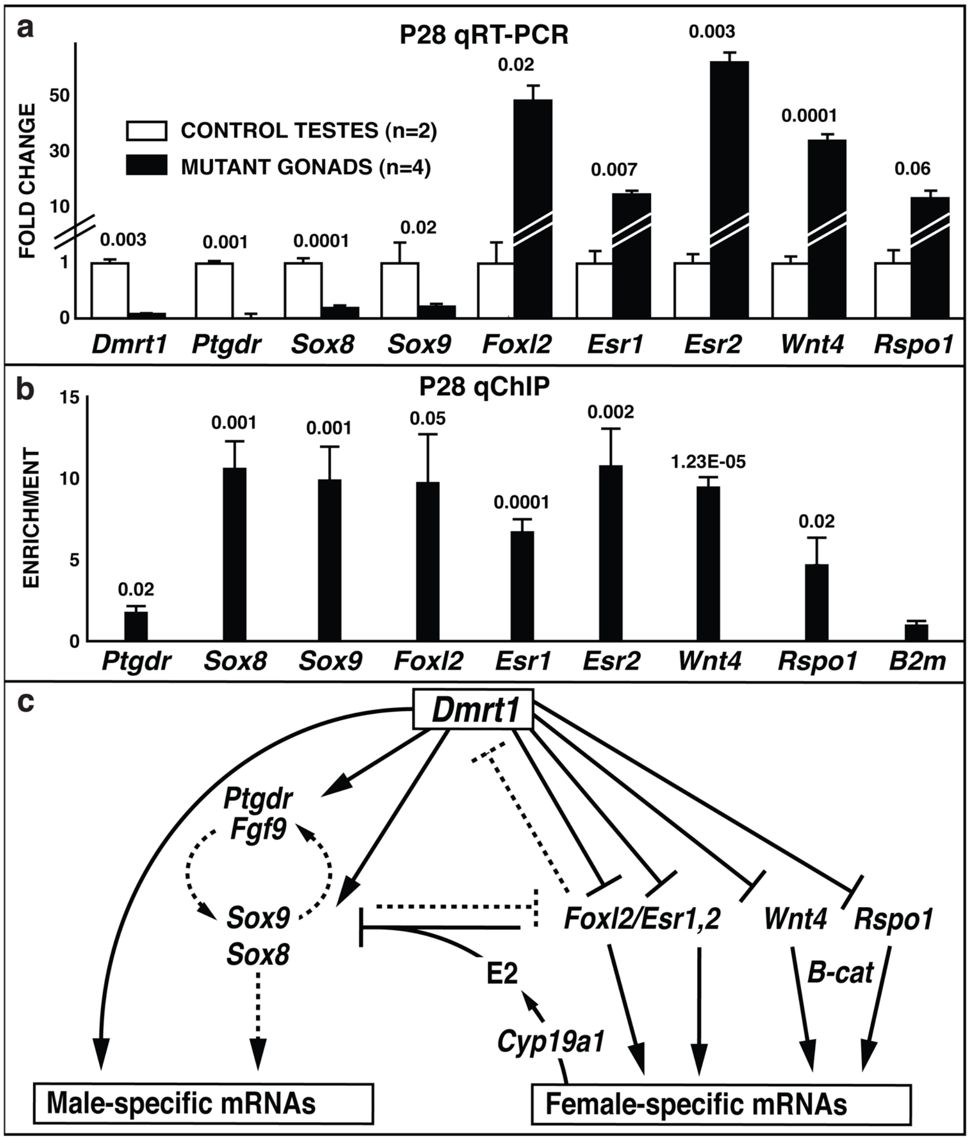

Figure 4. DMRT1 regulation of postnatal gene expression

(a) qRT-PCR analysis of sex-determining genes at P28. Significance of expression changes is indicated (Students t-test). Mutant gonads were *SCDmrt1KO(Sf1); SCDmrt1KO(Dhh)* mutant gonads and equivalent expression changes. (b) qChIP analysis of DMRT1 DNA binding in P28 testes. Significance of enrichment relative to *B2m* (Students t-test) is shown. (c) Model for regulation by postnatal sex maintenance by DMRT1. Proposed direct regulation based on ChIP and mRNA expression data is indicated by solid lines; indirect or potential regulation is indicated by dashed lines. (Model adapted from Veitia[2]).