

## RESEARCH REPORT

# Female-to-male sex reversal in mice caused by transgenic overexpression of *Dmrt1*

Liang Zhao, Terje Svingen\*, Ee Ting Ng and Peter Koopman<sup>‡</sup>**ABSTRACT**

Genes related to *Dmrt1*, which encodes a DNA-binding DM domain transcription factor, act as triggers for primary sex determination in a broad range of metazoan species. However, this role is fulfilled in mammals by *Sry*, a newly evolved gene on the Y chromosome, such that *Dmrt1* has become dispensable for primary sex determination and instead maintains Sertoli cell phenotype in postnatal testes. Here, we report that enforced expression of *Dmrt1* in XX mouse fetal gonads using a *Wt1*-BAC transgene system is sufficient to drive testicular differentiation and male secondary sex development. XX transgenic fetal gonads showed typical testicular size and vasculature. Key ovarian markers, including *Wnt4* and *Foxl2*, were repressed. Sertoli cells expressing the hallmark testis-determining gene *Sox9* were formed, although they did not assemble into normal testis cords. Other bipotential lineages differentiated into testicular cell types, including steroidogenic fetal Leydig cells and non-meiotic germ cells. As a consequence, male internal and external reproductive organs developed postnatally, with an absence of female reproductive tissues. These results reveal that *Dmrt1* has retained its ability to act as the primary testis-determining trigger in mammals, even though this function is no longer normally required. Thus, *Dmrt1* provides a common thread in the evolution of sex determination mechanisms in metazoans.

**KEY WORDS:** Sex determination, DM domain genes, *Sox9*, Evolution, Mouse

**INTRODUCTION**

DM domain genes encode transcription factors that contain a conserved DM-type DNA-binding domain that was originally identified in the sex regulators *doublesex* in *Drosophila melanogaster* and *male abnormal-3* in *Caenorhabditis elegans* (Raymond et al., 1998). In several non-mammalian vertebrate species, they act as triggers for primary sex determination. Examples include *DMY* in the medaka fish *Oryzias latipes* (Matsuda et al., 2002; Nanda et al., 2002; Otake et al., 2010), *DM-W* and *DMRT1* in the amphibian *Xenopus laevis* (Yoshimoto et al., 2008, 2010) and *DMRT1* in chicken (Smith et al., 2009; Lambeth et al., 2014). However, *Dmrt1* has become dispensable for testis determination in mammals, as evidenced by the experimental observation that XY *Dmrt1* null mutant mice are born as males with testes (Raymond et al., 2000). Nevertheless, *Dmrt1* plays essential roles in maintaining Sertoli cell phenotype in postnatal mouse testes (Matson et al., 2011; Minkina et al., 2014).

Most eutherian mammals, including mice, use *Sry*, a newly evolved gene on the Y chromosome, as the trigger for primary male sex determination (Gubbay et al., 1990; Sinclair et al., 1990; Koopman et al., 1991). The expression of SRY in the pre-supporting cells in the developing fetal gonads (Albrecht and Eicher, 2001; Wilhelm et al., 2005) directs these cells to differentiate into Sertoli cells, which orchestrate the differentiation of other testis-specific cell lineages and their assembly into functional testes (Svingen and Koopman, 2013). *Sry* encodes a transcription factor that upregulates expression of the related HMG box transcription factor gene *Sox9* (Sekido and Lovell-Badge, 2008). Unlike *Sry*, the involvement of *Sox9* as a pivotal, early-acting effector of male sex determination has been documented in all vertebrates studied, leading to the prevailing view that the different switch mechanisms that have evolved in different vertebrate taxa all converge on *Sox9* (Cutting et al., 2013).

In the current study, we investigated the ability of *Dmrt1* to initiate male development in mammals using a transgenic mouse model. We overexpressed *Dmrt1* in XX mouse fetal gonads using a *Wt1*-BAC transgene system (Polanco et al., 2010; Zhao et al., 2014a). Surprisingly, ectopic expression of *Dmrt1* was sufficient to drive testicular differentiation at the fetal stage and male secondary sex development postnatally. Our results suggest that, despite the diversity and plasticity of sex determination mechanisms (Graves, 2008; Cutting et al., 2013), DM domain genes provide a common thread in the evolution of sex determination mechanisms in metazoans, with *Sry* likely having replaced *Dmrt1* in this role in mammals.

**RESULTS AND DISCUSSION****Transgenic overexpression of *Dmrt1* causes female-to-male sex reversal of the fetal gonads**

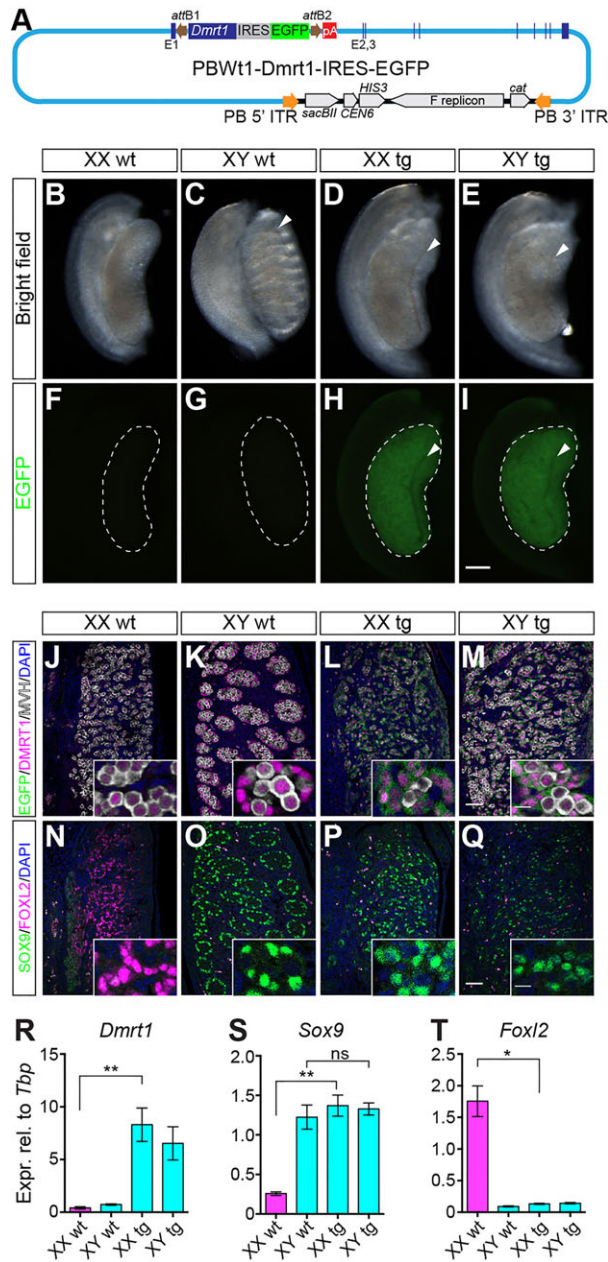
We set out to investigate whether overexpression of *Dmrt1* might be sufficient to cause testis development in XX mice, and used a *Wt1* enhancer in a *piggyBac*-based BAC vector to drive *Dmrt1* expression in transgenic mice (Fig. 1A) (Zhao et al., 2014a). Using this enhancer, we have previously demonstrated that the transgene of interest is specifically expressed in XX and XY gonadal somatic pre-supporting cells, and not in the mesonephros, beginning at 10.5 days post coitum (dpc) (Polanco et al., 2010), before sex determination takes place.

We first confirmed that the *Wt1:Dmrt1*-IRES-EGFP transgene was expressed in developing fetal gonads. Strong EGFP fluorescence was observed in XX and XY transgenic gonads at 14.5 dpc (Fig. 1F-I). Furthermore, using immunofluorescence staining, we confirmed that DMRT1 and EGFP proteins were co-expressed in somatic cells of transgenic fetal gonads (both XX and XY; Fig. 1L,M), as expected, whereas in wild-type ovaries endogenous DMRT1 was only detectable in germ cells (Fig. 1J). Quantitative RT-PCR (qRT-PCR) analysis showed that *Dmrt1* expression levels were increased 21-fold in XX and ninefold in XY transgenic fetal gonads, relative to wild type (Fig. 1R).

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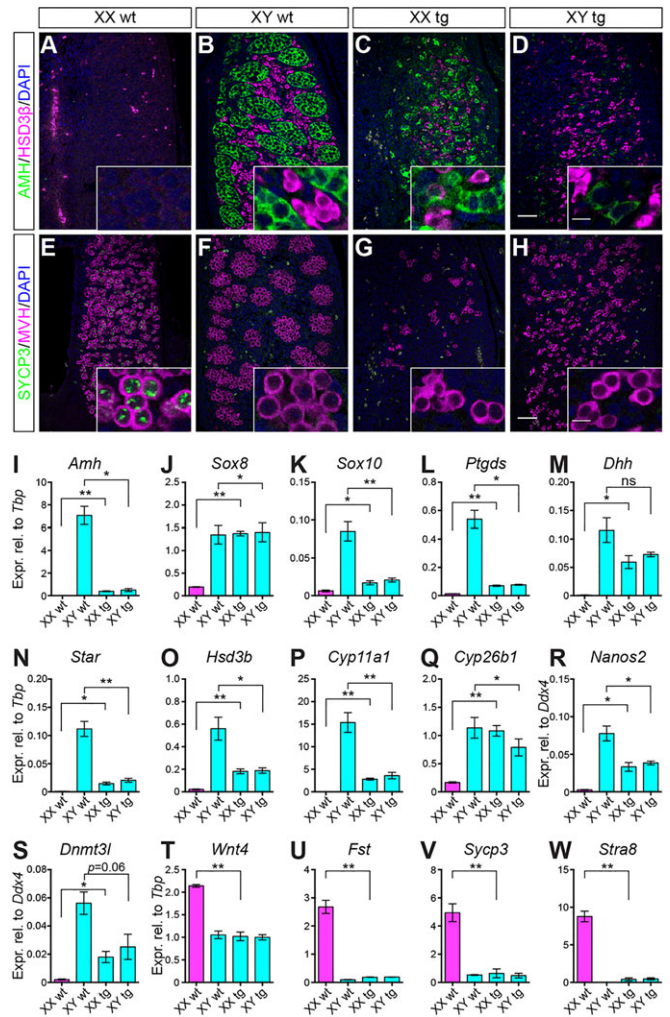
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**Fig. 1. Overexpression of *Dmrt1* in XX mouse fetal gonads induces testis development.** (A) The PBWt1-*Dmrt1*-IRES-EGFP BAC vector. pA, polyadenylation signal; *attB1/2*, Gateway recombination sites; PB 5'/3' ITR, *piggyBac* 5'/3' inverted terminal repeats; E1-3, exons 1-3 of *Wt1*. Not to scale. (B-I) Bright-field (B-E) and fluorescent (F-I) images of 14.5 dpc gonads from wild-type (wt) and transgenic (tg) embryos. Testis-specific blood vessels are indicated by arrowheads. Dashed lines delineate gonad perimeters. Scale bar: 200  $\mu$ m. (J-Q) Immunofluorescence analyses of gonad sagittal sections at 14.5 dpc. (J-M) Transgenic DMRT1 (magenta) and EGFP (green) were co-expressed in somatic cells only in XX transgenic fetal gonads (L). Germ cells were marked by MVH (grey). (N-Q) XX transgenic gonads showed strong expression of SOX9 (green) but no detectable expression of FOXL2 (magenta). Nuclei were counterstained with DAPI (blue). Scale bars: 50  $\mu$ m; 10  $\mu$ m in insets. (R-T) qRT-PCR analyses of *Dmrt1*, *Sox9* and *Foxl2*. Pink bars represent phenotypic females and blue bars phenotypic males. Mean $\pm$ s.e.m.,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$  (Student's *t*-test); ns, not significant.

XX transgenic fetal gonads resembled testes in their gross morphology, with a volume larger than that of typical ovaries (Fig. 1B-E) and a prominent blood vessel on the coelomic surface, a



**Fig. 2. Marker analysis of XX transgenic fetal testes.** (A-H) Immunofluorescence analyses on sagittal sections at 14.5 dpc. (A-D) Sertoli cells (marked by AMH, green) and fetal Leydig cells (marked by HSD3 $\beta$ , magenta) were present in XX transgenic (tg) gonads (C). (E-H) Germ cells (marked by MVH, magenta) in XX transgenic testes did not express SYCP3 (green). Nuclei were counterstained with DAPI (blue). Scale bars: 50  $\mu$ m; 10  $\mu$ m in insets. (I-W) qRT-PCR analyses of lineage markers. Compared with XX wild-type ovaries, markers of testicular cell types (I-S) were upregulated and those of ovarian cell types (T-W) were downregulated in XX transgenic testes. Pink bars represent phenotypic females and blue bars phenotypic males. Mean $\pm$ s.e.m.,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$  (Student's *t*-test); ns, not significant.

hallmark of testis development (arrowheads, Fig. 1C-E,H,I). Double immunofluorescence analysis of XX transgenic gonads for the differentiation markers SOX9 and FOXL2 revealed the presence of Sertoli cells and absence of granulosa cells, indicating female-to-male gonadal sex reversal caused by transgenic overexpression of *Dmrt1* (Fig. 1N-P). Quantitation of *Sox9* and *Foxl2* expression by qRT-PCR confirmed these findings (Fig. 1S,T).

As a sequence-specific transcription factor, DMRT1 is capable of binding to DNA and activating or repressing the expression of putative target genes (Murphy et al., 2010). Previously, it has been shown that DMRT1 binds near *Sox9*, *Sox8*, *Wnt4* and *Foxl2* and activates (*Sox9*, *Sox8*) or represses (*Wnt4*, *Foxl2*) their expression in postnatal testes (Matson et al., 2011). *Wnt4* and *Foxl2* are known to repress *Sox9* expression (Ottolenghi et al., 2007). Therefore, activation of *Sox9* in XX *Dmrt1* transgenic fetal gonads might be

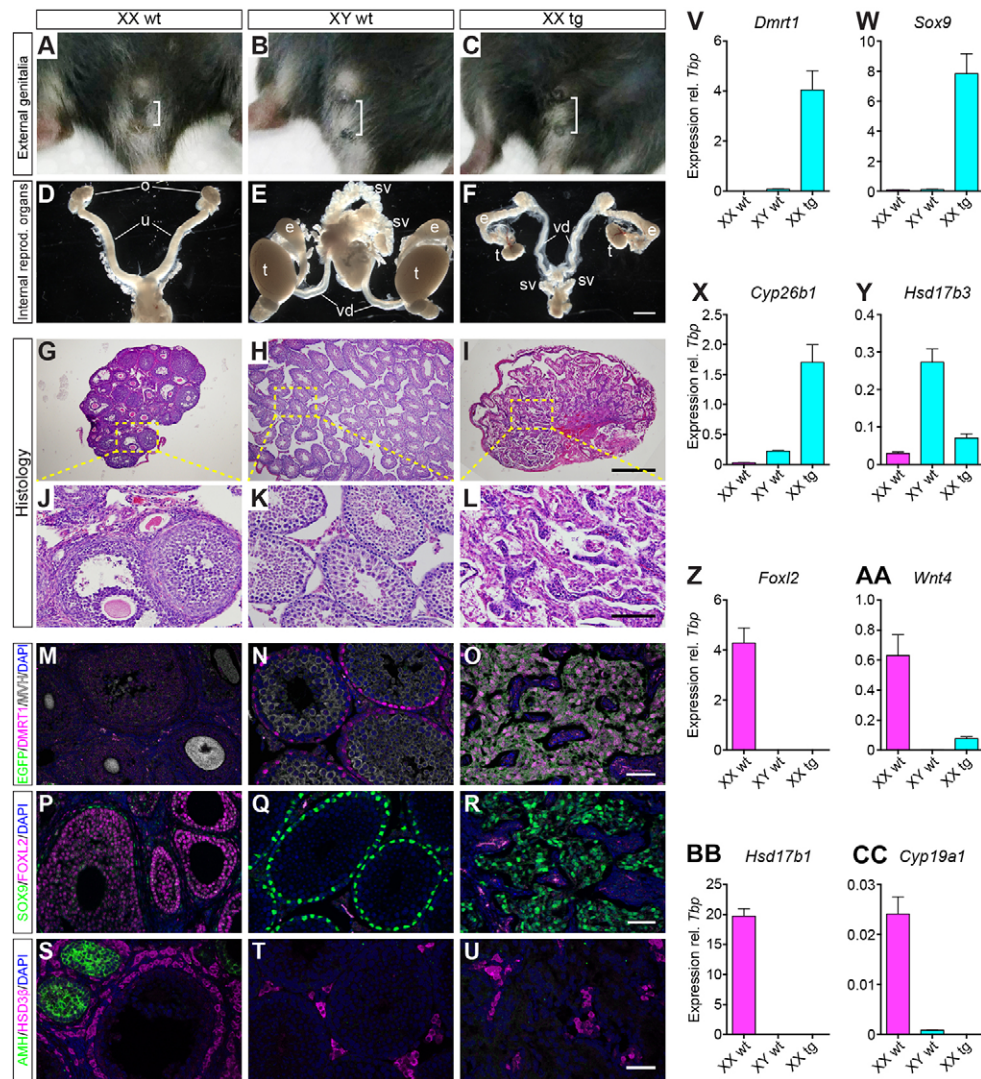
a result of direct transactivation by DMRT1, or repression of *Wnt4* (Fig. 2T; see below) and *Foxl2* (Fig. 1T), or a combination of both.

Unlike wild-type testes, which had well-defined cords at this stage (Fig. 1C,O), no cord structures were observed in XX or XY transgenic fetal testes (Fig. 1C-E,K-M,O-Q and Fig. 2A-F). The lack of distinct testis cords was confirmed by anti-laminin immunofluorescence (supplementary material Fig. S1). Failure of cord formation in XY transgenic testes suggests active repression of this process resulting from *Dmrt1* overexpression. The expression of *Pdgfra*, an essential mediator of testis cord formation (Brennan et al., 2003), was unaltered in the transgenic fetal gonads (data not shown). Moreover, flattened,

smooth muscle actin (SMA)-positive peritubular myoid cells were present in adult XX transgenic gonads (supplementary material Fig. S2F; see below), suggesting that a lack of myoid cells cannot account for the perturbed cord formation. These results uncouple the processes of Sertoli cell differentiation and testis cord formation, which were previously understood to be tightly linked.

### Differentiation of testicular cell lineages in XX *Dmrt1* transgenic fetal gonads

We examined in detail the differentiation of Sertoli, fetal Leydig and germ cells in XX transgenic fetal gonads at 14.5 dpc. We first



**Fig. 3. Sex reversal in XX adult *Dmrt1* transgenic mice.** (A-C) External genitalia of XX wild-type (wt), XY wild-type and sex-reversed XX *Dmrt1* transgenic (tg) mice at 4 weeks of age. Male development of an XX transgenic mouse is indicated by the development of a penis and increased ano-genital distance (bracket). (D-F) Male internal reproductive structures in an XX transgenic mouse. o, ovary; u, uterine horn; t, testis; e, epididymis; vd, vas deferens; sv, seminal vesicle. (G-L) Histological analysis of gonadal sections at 4 weeks using Hematoxylin and Eosin staining. XX transgenic testes did not show conspicuous seminiferous tubules (I,L), in contrast to wild-type testes (H,K). (M-U) Immunofluorescence analyses of gonadal sections at 4 weeks. (M-O) DMRT1 (magenta) and EGFP (green) were co-expressed in Sertoli cells in an XX transgenic testis (O). These double-positive Sertoli cells formed clusters but not intact seminiferous tubules, in contrast to wild-type testes (N). Germ cells (marked by MVH, grey) were absent from these Sertoli cell clusters. (P-R) SOX9-positive Sertoli cells (green) formed in XX transgenic testes, and no FOXL2-positive granulosa cells (magenta) were present (R; some non-specific staining of vasculature is seen using this antibody). (S-U) Adult Leydig cells in wild-type testes (T) and theca cells in the wild-type ovary (S) were positively stained for the steroidogenic marker HSD3 $\beta$  (magenta). The distribution pattern of HSD3 $\beta$ -positive cells in XX transgenic testes (U) resembled that of Leydig cells in wild-type testes (T). AMH (green) marks granulosa cells in wild-type ovaries (S). Nuclei were counterstained with DAPI (blue). (V-CC) qRT-PCR analyses of cell type marker genes. Compared with XX wild-type ovaries, genes expressed in Sertoli and/or Leydig cells (V-Y) were upregulated and those expressed in granulosa or theca cells (Z-CC) were downregulated in XX transgenic testes. Pink bars represent phenotypic females and blue bars phenotypic males. Mean  $\pm$  s.e.m.,  $n=3$ . Scale bars: 2 mm in D-F; 0.5 mm in G-L; 100  $\mu$ m in J-L; 50  $\mu$ m in M-U.

performed immunofluorescence for AMH, a marker of Sertoli cell differentiation and a direct transcriptional target of SOX9 (De Santa Barbara et al., 1998), and for HSD3 $\beta$ , a steroidogenic enzyme expressed by fetal Leydig cells. The presence of cells expressing AMH or HSD3 $\beta$  confirmed that both Sertoli and fetal Leydig cells had formed in the sex-reversed XX *Dmrt1* transgenic testes, as in wild-type XY testes but not XX ovaries (Fig. 2A-D). These results were confirmed by qRT-PCR (Fig. 2I,O).

We next assayed the expression of additional Sertoli and fetal Leydig cell marker genes using qRT-PCR. Genes involved in the differentiation of Sertoli (*Sox8*, *Sox10*, *Ptgds* and *Dhh*) and fetal Leydig (*Star*, *Cyp11a1*, *Insl3* and *Cyp17a1*) cells were consistently upregulated in XX transgenic testes, as compared with wild-type ovaries (Fig. 2J-N,P; supplementary material Fig. S2A,B). By contrast, genes characteristic of ovarian development, such as *Wnt4* and *Fst*, were markedly downregulated in XX transgenic testes, similar to the situation in wild-type testes (Fig. 2T,U). Similar results were obtained from samples at 13.5 dpc (data not shown).

Germ cells in fetal ovaries begin to enter meiosis around 13.5 dpc in response to retinoic acid (Bowles et al., 2006; Koubova et al., 2006). In fetal testes, CYP26B1 expressed by Sertoli and fetal Leydig cells (Bowles et al., 2006; Koubova et al., 2006) degrades retinoic acid so that male germ cells avoid meiosis and are instead mitotically arrested. Consistent with the presence of Sertoli and fetal Leydig cells, expression of *Cyp26b1* was upregulated in XX transgenic testes to levels similar to wild-type XY testes, and well above those seen in wild-type ovaries (Fig. 2Q). Accordingly,

SYCP3, a meiotic marker, labelled most germ cells in wild-type ovaries but none in XX transgenic testes (Fig. 2E-H). qRT-PCR analyses of *Sycp3* and *Stra8* (a target gene of retinoic acid signalling) further confirmed the absence of meiosis in XX transgenic testes (Fig. 2V,W). We also analysed the expression of *Nanos2* (Suzuki and Saga, 2008) and *Dnmt3l* (Bourc'his and Bestor, 2004), markers of male fetal germ cell fate, and found that both were upregulated in XX transgenic gonads relative to wild-type ovaries (Fig. 2R,S). Together, these results demonstrate that germ cells in XX *Dmrt1* transgenic gonads had adopted a male fate.

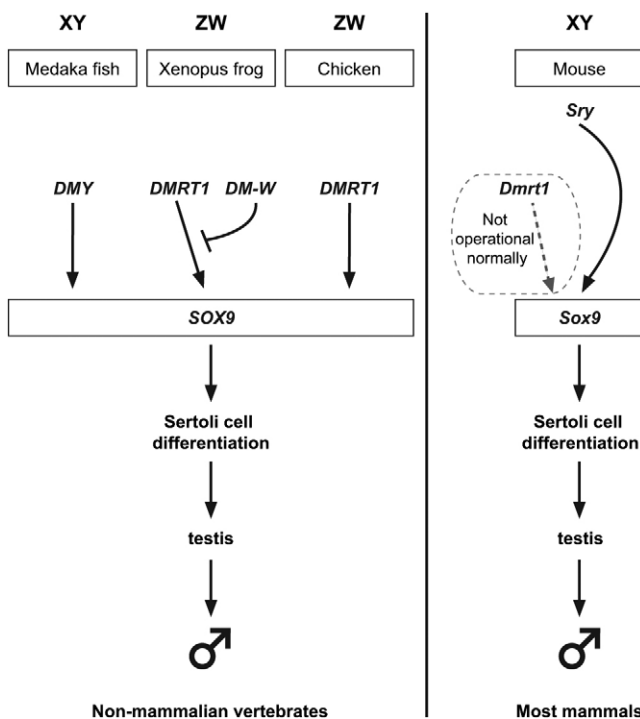
Unexpectedly, the expression levels of several marker genes were lower in XY transgenic gonads than in XY wild-type testes (Fig. 2I,K,L,N-P,R,S; supplementary material Fig. S2). In addition, transgenic gonads were disorganised, without well-defined testis cords. By contrast, *Sox9* was expressed at similar levels in XY wild-type and both XX and XY transgenic gonads (Fig. 1S). Based on these data, we hypothesize that high levels of *Dmrt1* in XX and XY transgenic gonads may act as a double-edged sword, activating *Sox9* expression (directly or indirectly) to induce fetal testis development but, at the same time, repressing the expression of other downstream genes important for proper testis morphogenesis.

#### Female-to-male sex reversal in an adult XX mouse transgenic for *Dmrt1*

In mammals, once the fetal gonads develop as testes, androgens produced by fetal and adult Leydig cells stimulate the differentiation of male external and internal reproductive organs. We next analysed transgenic gonads and the secondary sex phenotype at 4 weeks of age. Again, we observed female-to-male sex reversal, as characterised by male external genitalia similar to those of wild-type male littermates (penis and increased ano-genital distance; Fig. 3A-C) and the presence of a male reproductive tract (epididymides, vasa deferentia and seminal vesicles; Fig. 3D-F), albeit hypoplastic. Testes were small and did not descend (Fig. 3E,F). No female internal genitalia developed. As with transgenic fetuses, no well-structured seminiferous tubules formed in XX transgenic testes (Fig. 3G-L; supplementary material Fig. S3A-F).

Persistent expression of the *Dmrt1* transgene was confirmed by immunofluorescence and qRT-PCR (Fig. 3M-O,V; data not shown). Accordingly, several known DMRT1 targets, such as *Espn*, *Cst9*, *Etd* and *Vsig1* (Murphy et al., 2010; Agbor et al., 2013), were massively upregulated in XX transgenic testes (supplementary material Fig. S3G-J). Genes and proteins expressed in Sertoli cells, such as SOX9 and *Cyp26b1*, were strongly expressed in XX transgenic testes (Fig. 3P-R,W,X), whereas markers of postnatal ovaries, including FOXL2, AMH, *Wnt4*, *Cyp19a1* (aromatase) and *Hsd17b1*, were absent or greatly reduced (Fig. 3P-U,Z-CC; supplementary material Fig. S3L).

Adult Leydig cells expressing HSD3 $\beta$ , CYP11A1 (SCC), *Hsd17b3* and *Insl3* were present in XX transgenic testes (Fig. 3S-U,Y; supplementary material Fig. S3D-F,K). However, androgen production might have been compromised, since expression of *Hsd17b3* and *Cyp17a1*, which both encode steroidogenic enzymes, was suppressed in XX transgenic testes (Fig. 3Y; supplementary material Fig. S3M), perhaps accounting for the underdevelopment of male reproductive organs observed (Fig. 3F). As expected, germ cells were absent from XX transgenic testes (supplementary material Fig. S3A-C,N), similar to the situation in XX testes transgenic for *Sry* (Koopman et al., 1991), *Sox9* (Vidal et al., 2001) or *Sox10* (Polanco et al., 2010).



**Fig. 4. Common themes in sex-determining mechanisms among vertebrates.** In this model, vertebrates have evolved diverse switch mechanisms for sex determination that may converge on a common pathway of testis development, which is likely to involve SOX9. DM domain genes have evolved as the trigger for primary sex determination in several non-mammalian vertebrate taxa, but in most eutherian mammals, including mice, male sex determination is instead initiated by *Sry*. Our findings indicate that DMRT1 gene and protein are able to activate the mouse testis-determining pathway under experimental conditions, implicating changes in *Dmrt1* gene regulation in the loss of this role in mammals.

## Conclusions and perspectives

DM domain genes act as the trigger for primary sex determination in several non-mammalian vertebrate species, acting upstream of *Sox9*, which appears to be the common point of convergence of the various switch mechanisms used among vertebrates (Cutting et al., 2013) (Fig. 4). The emergence of *SRY* in the common progenitor of marsupial and placental mammals has rendered *Dmrt1* redundant, such that the regulatory elements required for sufficient expression in the supporting cell lineage (Lei et al., 2007; Agbor et al., 2013) were lost or degraded.

Our results here indicate that DMRT1 remains capable of driving testicular differentiation in mammals, provided that it is expressed at sufficient levels at the right place and time. Our marker analyses further suggest that it acts in this role upstream of *Sox9*, a conclusion that is supported by the recent observation that upregulation of *DMRT1* precedes *SOX9* expression during female-to-male sex reversal of polled intersex goats (Elzaiat et al., 2014). Thus, despite the existence of a variety of sex-determining mechanisms in the animal kingdom, our results support a model in which *Dmrt1* provides a common genetic thread among metazoans (Fig. 4).

We suggest that a *Dmrt1*-related gene might have resumed (or retained) the role of primary sex determinant in mammalian species that have lost *Sry* (Graves, 2002; Jiménez et al., 2013). Furthermore, the ability of members of both the DM and Sox families of transcription factors to prime the male sex-determining pathway might have provided resilience to mutation, a mechanism for replacement of one sex-determining switch gene with another on an evolutionary time-scale, and a high degree of plasticity that has resulted in the variety of sex-determining mechanisms seen in metazoans today.

## MATERIALS AND METHODS

### Mouse transgenesis

A sequence containing the mouse *Dmrt1* coding region followed by IRES-EGFP (*Dmrt1*-IRES-EGFP) was cloned into the PBWt1-Dest vector via Gateway LR recombination as described (Zhao et al., 2014a). Two transgenic founder mice (an infertile XX female and an XY male) were produced by pronuclear injection of PBWt1-*Dmrt1*-IRES-EGFP vector DNA and hyperactive *piggyBac* transposase mRNA as described (Zhao et al., 2014a). The XY male founder transmitted the transgene through the germ line and was mated to C57BL/6J females to generate embryos and pups for analyses. All animal procedures were approved by the University of Queensland Animal Ethics Committee. Genotyping protocols are described in the supplementary materials and methods.

### mRNA and protein expression analyses

RNA extraction, cDNA synthesis, qRT-PCR and immunofluorescence were performed as described (Zhao et al., 2014b). Further details including PCR primers and antibodies are provided in the supplementary materials and methods and Tables S1 and S2. Bright-field and EGFP fluorescence images of freshly dissected organs were captured on a Leica M165 FC stereomicroscope. Confocal images were captured on a Zeiss LSM710 confocal microscope.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

P.K., L.Z. and T.S. designed experiments; L.Z. and E.T.N. performed the research; L.Z. and P.K. analysed data and wrote the paper.

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### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.122184/-/DC1>

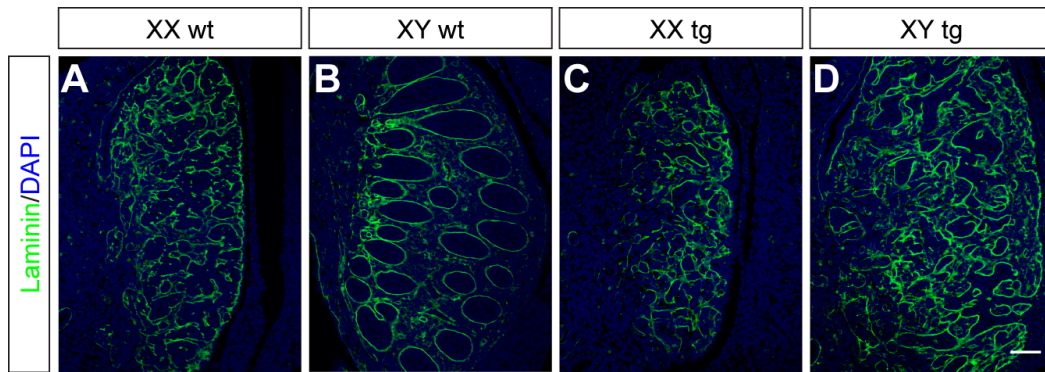
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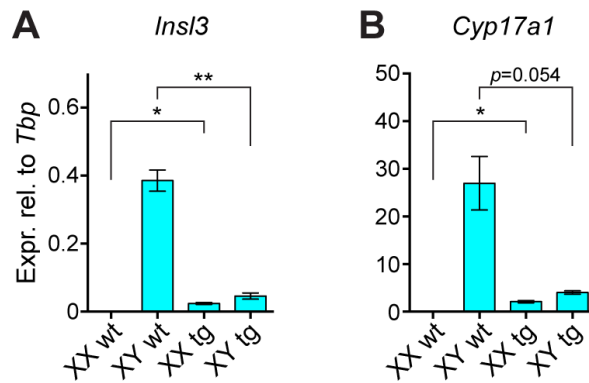
## Supplementary Material

### Supplementary Figures



**Fig. S1. Lack of cord formation in XX/XY fetal gonads expressing the *Dmrt1* transgene.**

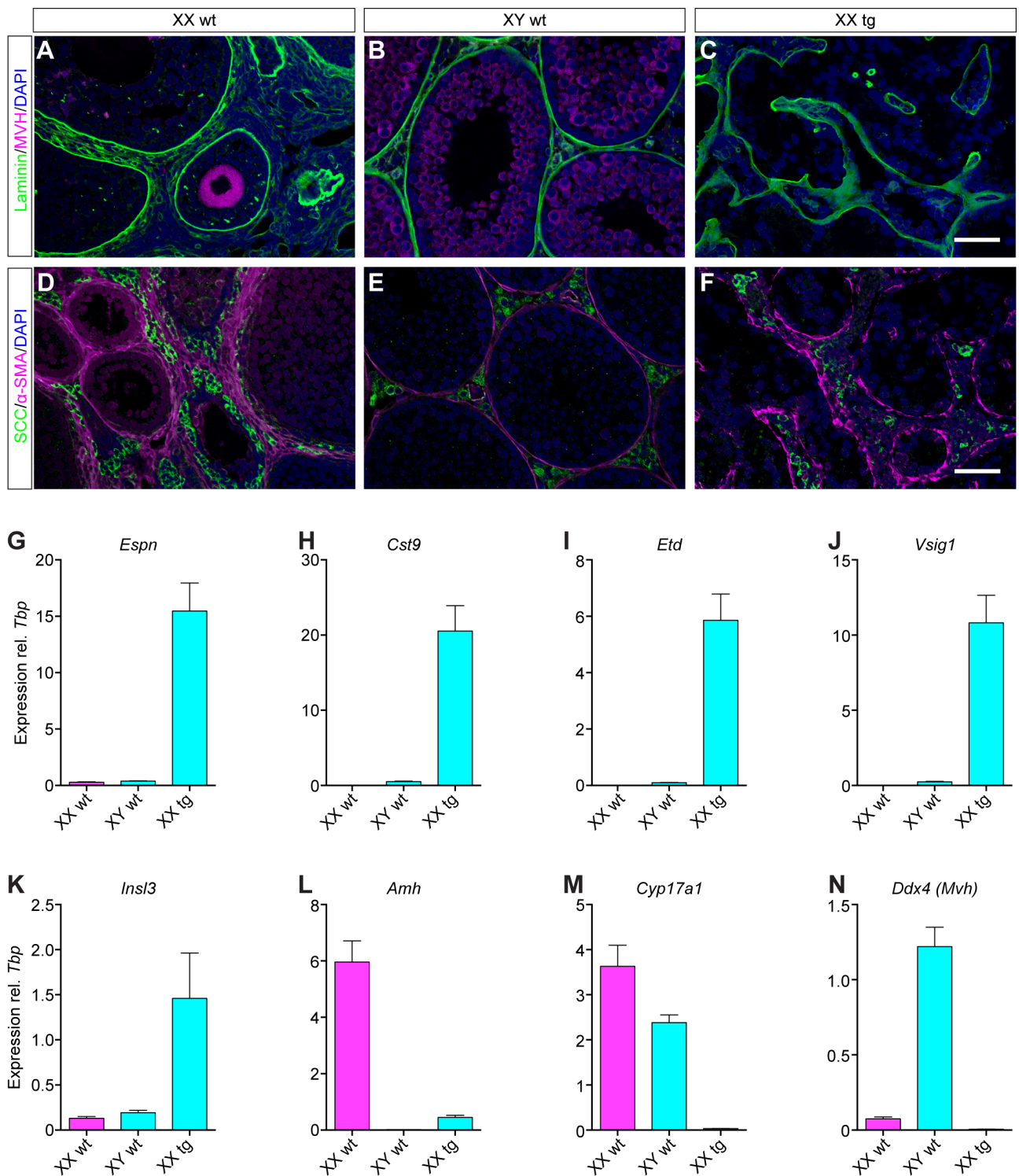
(A-D) Immunofluorescence staining for laminin (green) on sagittal sections of fetal gonads at 14.5 dpc. Testis cords were observed in wild type testes but not in XX or XY testes transgenic for *Dmrt1*. Nuclei were counterstained with DAPI (blue). Scale bar, 50  $\mu$ m.



**Fig. S2. Quantitative gene expression analyses of steroidogenic marker genes in fetal mouse gonads at 14.5 dpc using qRT-PCR.**

(A-B) Both *Insl3* and *Cyp17a1* were up-regulated in XX transgenic gonads, compared with XX wild-type ovaries. Mean  $\pm$  s.e.m.,  $n=3$ . Statistical significance was determined for two-way comparisons indicated by brackets. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ : Student's *t* test.





**Fig. S3. Expression analyses of marker genes in adult mouse gonads.**

(A-F) Immunofluorescence analyses of sections of adult mouse gonads at 4 weeks. Sertoli cell clusters in sex-reversed transgenic testes (C) were outlined by laminin (green), a marker for basal

membrane. These resembled neither ovarian follicles (A) nor testis cords (B). Germ cells (marked by MVH, magenta) were absent in XX transgenic testes. (D-F) In the wild type (E) and transgenic testes (F), interstitial Leydig cells stained positive for SCC, whereas in wild type ovaries, theca cells surrounding the follicles were positive for SCC (D). Sections were also analysed for alpha-smooth muscle actin ( $\alpha$ -SMA); staining for  $\alpha$ -SMA in combination with flattened cell morphology marks peritubular myoid cells. Scale bar, 50  $\mu$ m. (G-N) Quantitative gene expression analyses in adult gonads at 4 weeks using qRT-PCR. Pink bars represent phenotypic female samples and blue bars phenotypic male samples, mean  $\pm$  s.e.m. of three technical replicates.

## Supplementary Materials and Methods

### Genotyping

Genomic DNA was extracted from mouse tissue using QuickExtract solution (Epicentre). Genotyping PCRs were performed using GoTaq (Promega) to determine genetic sex and presence of the transgene. Sexing primers were as described (McFarlane et al., 2013). Primers (5'-3') for the *Wt1:Dmrt1-IRES-EGFP* transgene were 5'Wt1-F (GTGCGTCCAGCAGCCGGAGCAA) and Dmrt1-R (ATCAGGCTGCACTTCTTGCCTG).

### qRT-PCR

qRT-PCR analyses were performed as described (Zhao et al., 2014). Expression was normalized to *Tbp* (Svingen et al., 2009) or *Ddx4* in the cases of *Nanos2* and *Dnmt3l*. Statistical analyses were performed using a two-tailed Student's *t* test. Primers or TaqMan probes (Life Tech) used are listed in Table S1.

**Table S1. Primers and probes**

| Primer  | Sequence or TaqMan probe ID |
|---------|-----------------------------|
| Tbp.F   | ACGGACAACCTGCGTTGATTTT      |
| Tbp.R   | GAAGGGAGGAGGGGTGAG          |
| Sox9.F  | AGTACCCGCATCTGCACAAC        |
| Sox9.R  | TACTTGTAATCGGGGTGGTCT       |
| Dmrt1.F | CCTACTCAGAAGCCAAAGCCAGTG    |
| Dmrt1.R | CCGAGGACGCAGACTCACATTC      |
| Sox8.F  | GCAAGACCCTAGGCAAGCTGT       |
| Sox8.R  | TCTGGGTGGTCTTTCTTGTGC       |
| Sox10.F | CAAGGAGGGGCTGCTGCTAT        |
| Sox10.R | ATGGCTCTGGCCTGAGGGGT        |
| Amh.F   | CGAGCTCTTGCTGAAGTTCCA       |
| Amh.R   | GAAGTCCACGGTTAGCACCAA       |
| Wt1.F   | CGGTCCGACCATCTGAAGAC        |
| Wt1.R   | GTTGTGATGGCGGACCAATT        |
| Ptdgs.F | GCTCTTCGCATGCTGTGGAT        |

|           |                             |
|-----------|-----------------------------|
| Ptdgs.R   | GCCCCAGGAACTTGTCTTGTT       |
| Dhh.F     | CCCGACATAATCTTCAAGGATGA     |
| Dhh.R     | GCGATGGCTAGAGCGTTCAC        |
| Cyp26b1.F | TGGACTGTGTCATCAAGGAGGT      |
| Cyp26b1.R | GTCGTGAGTGTCTCGGATGCTA      |
| Star.F    | AAACTCACTTGGCTGCTCAGTATTGAC |
| Star.R    | CAGGTGGTTGGCGAACTCTATCTG    |
| Insl3.F   | TCCTCGGCAGGCTTCTCAGC        |
| Insl3.R   | GCTCCTTCAGTGGGGACACAGAC     |
| Cyp11a1.F | CAGTGATGACCTATTCCGCTTTTCC   |
| Cyp11a1.R | TCTGGTAGACAGCATTGATGAACCG   |
| Hsd3b.F   | GGACAAAGTATTCCGACCAGAAACC   |
| Hsd3b.R   | CAGCAGTGTGGATGACAACAGAGATG  |
| Cyp17a1.F | CATCCCACACAAGGCTAACA        |
| Cyp17a1.R | CAGTGCCCAGAGATTGATGA        |
| Hsd17b1.F | TGTTGCGCTAGCTTCTGACC        |
| Hsd17b1.R | AGCAGCCACAGATTTGGAGT        |
| Hsd17b3.F | ATGGCATCGGGAAAGCCTAT        |
| Hsd17b3.R | CTCTTCTGCAATGGTCTGTAGC      |
| Foxl2.F   | AGGGAGAGAATAAAACATTCATGG    |
| Foxl2.R   | GCAAACCTCCAAGGCCATTAC       |
| Wnt4.F    | CTGGACTCCCTCCCTGTCTTT       |
| Wnt4.R    | CATGCCCTTGTCACTGCAA         |
| Rspo1.F   | CGACATGAACAAATGCATCA        |
| Rspo1.R   | CTCCTGACACTTGGTGCAGA        |
| Fst.F     | GCAGCCGGAAGTAGAAGTACA       |
| Fst.R     | ACACAGTAGGCATTATTGGTCTG     |
| Stra8.F   | TTCAGCTCTACATACAGATCATTGAG  |
| Stra8.R   | ATCTGGGGGCTCTGGTTC          |
| Sycp3.F   | AAATCTGGGAAGCCACCTTTGG      |
| Sycp3.R   | TGGAGCCTTTTCATCAGCAACATC    |
| Cyp19a1.F | CCTGGACGAAAGTGCTATTGTGAAG   |
| Cyp19a1.R | GAAGATGTTTGGTTTGATGAGGAGAGC |
| Ddx4.F    | CAGGAATGCCATCAAAGGAACAAC    |
| Ddx4.R    | CCCAACAGCGACAAACAAGTAACTG   |
| Espn.F    | TTACATGCAGACCAAGAACAAGCT    |
| Espn.R    | CCACCTTGGGCTCCTTGAG         |
| Cst9.F    | TGAATATGCCTACAGGATGGAA      |
| Cst9.R    | CATGGAATACACGGTTGGAA        |

|         |                            |
|---------|----------------------------|
| Etd.F   | CTTCTCTCACCGCACAAACAA      |
| Etd.R   | CTCAAGGCAATGGAGAAAGC       |
| Vsig1.F | GGTGTTCATTTTGGGAAGG        |
| Vsig1.R | TGAAAGCTTTTCCAGGGACT       |
| Ddx4    | TaqMan probe Mm00802445_m1 |
| Nanos2  | TaqMan probe Mm02525720_s1 |
| Dnmt3l  | TaqMan probe Mm00457635_m1 |

## Immunofluorescence

Immunofluorescence staining was performed on paraffin sections of mouse embryos or adult gonads. Antibodies and dilutions are listed in Table S2.

**Table S2. Antibodies**

| Antibody                  | Source/Reference                      | Dilution |
|---------------------------|---------------------------------------|----------|
| Goat anti-GFP             | Abcam (Ab5450)                        | 1:500    |
| Mouse anti-SOX9           | Abnova (H00006662-M01)                | 1:200    |
| Rabbit anti-FOXL2         | Polanco et al. (Polanco et al., 2010) | 1:500    |
| Rabbit anti-DMRT1         | Santa Cruz (H-240; SC-98341)          | 1:100    |
| Rabbit anti-MVH           | Abcam (Ab13840)                       | 1:800    |
| Mouse anti-MVH            | Abcam (ab27591)                       | 1:500    |
| Goat anti-AMH             | Santa Cruz (C-20; SC-6886)            | 1:300    |
| Rabbit anti-HSD3 $\beta$  | Transgenic Inc (KAL-KO607)            | 1:500    |
| Mouse anti-SCP3           | Abcam (ab97672)                       | 1:200    |
| Rabbit anti-Laminin       | Sigma (L9393)                         | 1:200    |
| Mouse anti- $\alpha$ -SMA | Sigma (A2547)                         | 1:200    |
| Rabbit anti-SCC           | Svingen et al. (Svingen et al., 2012) | 1:300    |

## Supplementary References

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