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Gonadal Identity in the Absence of Pro-Testis Factor SOX9 and Pro-Ovary Factor Beta-Catenin in Mice[1]

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ABSTRACT

Sex-reversal cases in humans and genetic models in mice have revealed that the fate of the bipotential gonad hinges upon the balance between pro-testis SOX9 and pro-ovary beta-catenin pathways. Our central query was: if SOX9 and beta-catenin define the gonad's identity, then what do the gonads become when both factors are absent? To answer this question, we developed mouse models that lack either *Sox9*, beta-catenin, or both in the somatic cells of the fetal gonads and examined the morphological outcomes and transcriptome profiles. In the absence of *Sox9* and beta-catenin, both XX and XY gonads progressively lean toward the testis fate, indicating that expression of certain pro-testis genes requires the repression of the beta-catenin pathway, rather than a direct activation by SOX9. We also observed that XY double knockout gonads were more masculinized than their XX counterpart. To identify the genes responsible for the initial events of masculinization and to determine how the genetic context (XX vs. XY) affects this process, we compared the transcriptomes of *Sox9*/beta-catenin mutant gonads and found that early molecular changes underlying the XY-specific masculinization involve the expression of *Sry* and 21 SRY direct target genes, such as *Sox8* and *Cyp26b1*. These results imply that when both *Sox9* and beta-catenin are absent, *Sry* is capable of activating other pro-testis genes and drive testis differentiation. Our findings not only provide insight into the mechanism of sex determination, but also identify candidate genes that are potentially involved in disorders of sex development.

beta-catenin, sex determination, Sox9, Sry, *testis*

INTRODUCTION

In mammals, both ovary and testis derive from a common embryonic structure called the gonadal primordium or bipotential gonads. The developmental path of bipotential gonads toward an ovary or a testis is orchestrated by activation or repression of signaling pathways in the supporting cell lineage (for a review, see [1]). In male or XY individual, *SRY*, the testis-determining gene on the Y-chromosome, induces differentiation of the supporting cell lineage into the testis-specific Sertoli cells and consequent transformation of the bipotential gonad into a testis [2–4]. During its short period of expression in the mouse fetal testis, from 10.5 to 12.5 days postcoitum (dpc) [5, 6], SRY induces the expression of *Sox9* [7], which promotes testis organogenesis through the activation of pro-testis genes and the repression of pro-ovarian genes. Loss of *Sox9* in XY gonads leads to complete testis-to-ovary sex reversal [8–10], whereas a gain of *Sox9* expression in XX gonads results in complete ovary-to-testis sex reversal [11, 12]. Therefore, SOX9, acting as a downstream effector of SRY, is necessary and sufficient to drive testis differentiation.

In the absence of the Y chromosome (XX genetic background) or *Sry* gene (XY genetic background with *Sry* mutations), the supporting cell linages of the bipotential gonad commit to the ovary-specific granulosa cell fate (for a review, see [13]). In XX mouse embryos, global loss of *Wnt4* [14, 15], *Rspo1* [16, 17], or targeted loss of their downstream effector β-catenin in the somatic cells of the fetal gonad [18, 19] results in a partial ovary-to-testis sex-reversal characterized by the presence of a testis-specific coelomic vessel and steroidogenic cells. Close to birth in $Wnt4^{-/-}$ and $Rspo1^{-/-}$ ovaries, supporting cells start to differentiate into testis-specific Sertoli cells that express *Sox9* [16, 20]. On the other hand, ectopic activation of β-catenin in the somatic cells of XY fetal gonads leads to repression of *Sox9* expression and consequent testis-to-ovary sex reversal [21]. These studies implicate an antagonism between the pro-testis SOX9 and pro-ovary RSPO1/WNT4/β-catenin pathways in shaping the fate of the bipotential gonad. The characterization of global double knockout (DKO) models such as pro-ovary *Wnt4*/pro-testis *Fgf9* DKO [22] and pro-ovary *Rspo1*/pro-testis *Sox9* DKO [23] suggests that this antagonism operates in a sex chromosome-specific manner and that fate determination of the testis may involve factors other than *Sox9*.

The main goal of this study is to identify the early molecular changes that determine the gonadal identity when pro-testis *Sox9* and pro-ovary β-catenin are inactivated in the somatic cells of bipotential gonads. We compared the outcome of sex determination in embryos lacking Sox9, β-catenin, or both, with a particular interest in the supporting cell lineage. To identify the candidate genes responsible for the fate determination in the absence of *Sox9* and β-catenin, we characterized the transcriptomes of fetal gonads during early events of sex-reversal and made comparisons between XX and XY genetic backgrounds.

MATERIALS AND METHODS

Experimental Animals

All the embryos used in this study resulted from the crossing between $Ctnnb1^{f/f}$; $Sox9^{f/f}$ females with *Sf1*-$cre^{+/Tg}$; $Ctnnb1^{+/f}$; $Sox9^{+/f}$ males. These $Ctnnb1^{f/f}$; $Sox9^{f/f}$ females were generated by crossing B6.129-$Ctnnb1^{tm2Kem}$/J (004152; Jackson Laboratory) to B6.129S7-$Sox9^{tm1Crm}$/J (013106; Jackson Laboratory) mice, and the resulting $Ctnnb1^{+/f}$; $Sox9^{+/f}$ mice were then

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intercrossed to generate double homozygotes. Males *Sf1*-cre$^{+/Tg}$; *Ctnnb1*$^{+/f}$; *Sox9*$^{+/f}$ were generated by crossing *Ctnnb1*$^{f/f}$; *Sox9*$^{f/f}$ to *Sf1*-cre$^{Tg/Tg}$ transgenic mice [24]. The Cre recombinase expression under the control of steroidogenic factor 1 (*Sf1*) regulatory elements allowed the conditional KO of *Ctnnb1* and *Sox9* in the somatic cells of the gonads (Supplemental Fig. S1; Supplemental Data are available online at www.biolreprod.org). The *Ctnnb1*$^{f/f}$; *Sox9*$^{f/f}$ females were mated overnight with *Sf1*-cre$^{+/Tg}$; *Ctnnb1*$^{+/f}$; *Sox9*$^{+/f}$ males, and checked for vaginal plug the next morning. Embryonic Day 0.5 corresponds to noon of the day of the vaginal plug detection. The genotype of the gonads used in this study is as follows: *Sf1*-cre$^{+/Tg}$; *Ctnnb1*$^{+/f}$; *Sox9*$^{+/f}$ as control, *Sf1*-cre$^{+/Tg}$; *Ctnnb1*$^{f/f}$; *Sox9*$^{+/f}$ as *Ctnnb1* KO, *Sf1*-cre$^{+/Tg}$; *Ctnnb1*$^{+/f}$; *Sox9*$^{f/f}$ as *Sox9* KO, and *Sf1*-cre$^{+/Tg}$; *Ctnnb1*$^{f/f}$; *Sox9*$^{f/f}$ as *Ctnnb1*/*Sox9* DKO. All the animal procedures were approved by the National Institutes of Health Animals Care and Use Committee and were performed in accordance with an approved National Institute of Environmental Health Sciences animal study proposal.

Immunohistochemistry

Fetal gonads were collected and fixed overnight at 4°C in 4% paraformaldehyde. The 14.5 dpc gonads were immunostained as whole mounts, and 17.5 dpc gonads were cryosectioned to 8–10 μm sections before staining. Immunofluorescence analyses were performed as previously described [19]. The antibodies used in this study are listed in Supplemental Table S1.

Real-Time PCR Analyses

A single pair of gonads (14.5 or 17.5 dpc) was separated from the mesonephros and was used as one biological replicate (five biological replicates were included for each genotype). Total RNA was isolated using Picopure extraction kit (Arcturus). RNA quality and concentration were determined using the Nanodrop 2000c. Complementary DNA was synthetized using the Superscript II cDNA synthesis kit (Invitrogen); 250 ng of RNA was used for each biological replicate. Gene expression was analyzed by real-time PCR using Bio-Rad CFX96TM Real-Time PCR Detection system. Taqman probes (Bio-Rad) and primers used are listed in Supplemental Table S1. Relative expression was calculated as the percentage of the highest expression level recorded for each gene. All the values were expressed as mean ± SEM, and statistical analysis was performed using unpaired Student *t*-test ($P < 0.05$).

Microarray Analysis

Three to four biological replicas (one single pair of 14.5 dpc gonads) were used for each genotype. RNA quality and concentration were determined using N Agilent 2100 BioAnalyzer (Agilent Technologies). Gene expression analysis was conducted using Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix). A total of 40 ng of total RNA was amplified as directed in the Ovation Pico RNA Amplification System protocol and labeled with biotin following the Encore Biotin Module. Biotin-amplified RNAs (4.6 μg) were fragmented and hybridized to each array for 18 h at 45°C in a rotating hybridization. Array slides were stained with streptavidin/phycoerythrin utilizing a double-antibody staining procedure and then washed for antibody amplification according to the GeneChip Hybridization, Wash and Stain Kit and user manual following protocol FS450-0004. Arrays were scanned in an Affymetrix Scanner 3000 and data (GEO accession no. GSE67463, http://www.ncbi.nlm.nih.gov/geo/) was obtained using the GeneChip Command Console Software (AGCC Version 1.1) using the MAS5 algorithm to generate .CHP files. Partek software was used for data analysis. Principal component analysis (PCA) was performed on all biological replicates plotted in a three-dimensional manner using the three principal components that captures most of the variance in the dataset. The hierarchical clustering was created using a one-way ANOVA comparing the robust multiarray average normalized log-2 intensities of 2215 probes that were significantly different between control XX and XY (fold change ≥ 2, $P < 0.05$). The gene lists comparing two genotypes were filtered with fold-change cut-off of 1.5 with $P < 0.05$. To identify the genes normally expressed in Sertoli cells, a published cell-type-specific microarray dataset performed on fetal gonads was used [25]. This dataset permitted the identification of genes that are more highly expressed in fetal Sertoli cells than in fetal granulosa cells (from 11.5 to 13.5 dpc, fold change ≥ 1.5, $P < 0.05$). In addition, genes that were more highly expressed in Leydig cells than Sertoli cells (fold change ≥ 1.5, $P < 0.05$) were removed from this list. This leads to the creation of a list of 796 genes representative of the Sertoli cell identity (see green circle in Fig. 6A). To compare our data with this gene list based on Jameson et al. microarray data [25], a Venn diagram was generated using the gene-symbol as the identifier because the probes identification from these two microarrays are different.

RESULTS

Phenotypes in the XX Gonads: Consequences of Inactivation of Both β-Catenin and Sox9 at 14.5 dpc

To determine what the bipotential gonad becomes in the absence of β-catenin (or *Ctnnb1*) and *Sox9*, we inactivated *Sox9* and *Ctnnb1* genes using the *Sf1*-Cre transgenic mouse [24]. Expression of the Sf1-Cre transgene in the somatic compartment of the gonadal ridge around 9.5/10.5 dpc [24] allowed the specific inactivation of Sox9 and Ctnnb1 genes in the somatic cells of the bipotential gonads (Supplemental Fig. S1). We first characterized the mutant gonads in the XX genetic context at 14.5 dpc, when dimorphic development of the gonads is apparent (Fig. 1, A–I). Consistent with previous observations by our group and others [18, 19], XX *Ctnnb1* single KO gonads developed morphologically similar to the control ovaries (Fig. 1B), with the exception of ectopic testis-specific vasculature (Fig. 1E, arrow) and steroidogenic cells (see Supplemental Fig. S2). The supporting cells of XX *Ctnnb1* KO gonads (Fig. 1H) had an ovarian identity with the presence of FOXL2 (granulosa cell marker) and absence of AMH (Sertoli cell marker). However, *Foxl2* mRNA level was significantly reduced compared to the control ovaries (Fig. 1S). XX *Ctnnb1*/*Sox9* DKO gonads were indistinguishable from XX *Ctnnb1* KO gonads with the same vasculature and steroidogenic phenotypes (Fig. 1F and Supplemental Fig. S2). The supporting cells of XX DKO gonads maintained their FOXL2-positive ovarian identity (Fig. 1I) similar to XX *Ctnnb1* KO gonads. These results indicate that *Sox9* is not required for the appearance of testis characteristics observed in the XX *Ctnnb1* KO gonads at 14.5 dpc.

Phenotypes in the XY Gonads: Consequences of Inactivation of Both β-Catenin and Sox9 at 14.5 dpc

Next we examined the effects of loss of both *Ctnnb1* and *Sox9* in the male or XY genetic context, and we compared these gonads to control and XY *Sox9* KO gonads at 14.5 dpc (Fig. 1, J–R). Consistent with the findings by others [10, 26], XY *Sox9* single KO gonads underwent complete testis-to-ovary sex reversal, characterized by its ovarian shape and size (Fig. 1K), presence of AMH-negative/FOXL2-positive granulosa cells (Fig 1Q), absence of the testis-specific vessel (Fig. 1N) and steroidogenic cells (Supplemental Fig. S2). In the XY *Ctnnb1*/*Sox9* DKO gonads, the supporting cells were mostly FOXL2-positive, resembling the XY *Sox9* KO gonads (Fig. 1, Q and R). However, XY DKO gonads developed a testis-specific coelomic vessel (Fig. 1O), steroidogenic cells (Supplemental Fig. S2), and a few AMH-positive Sertoli cells (Fig. 1R, inset). Real-time PCR analyzes confirmed the expression of *Amh* in XY DKO gonads and its absence in XY *Sox9* KO gonads (Fig. 1T). Moreover, *Foxl2* expression in the XY *Sox9* KO gonads was sex-reversed to the level of control XX gonads (Fig. 1S) whereas *Foxl2* expression in the XY DKO gonad was significantly reduced (Fig. 1S). The restoration of some testicular characteristics by additional inactivation of *Ctnnb1* in the *Sox9* KO XY gonads indicates that *Ctnnb1* is partially responsible for the appearance of ovarian phenotypes in the absence of *Sox9*. In addition, masculinization phenotypes in the XX and XY DKO further support that certain testicular characteristics such as formation of the coelomic vessels do not require *Sox9*.

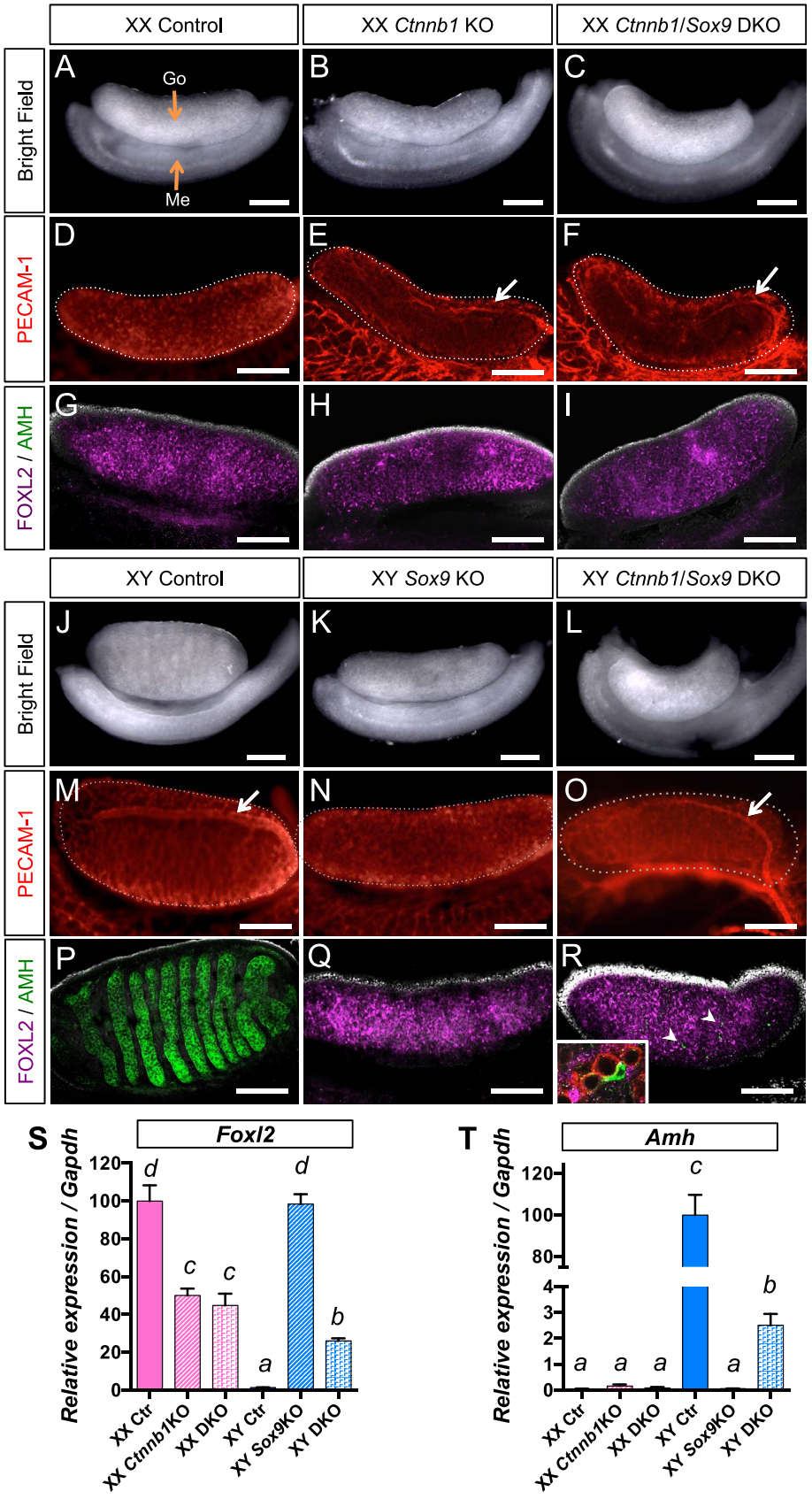


FIG. 1. Consequences of inactivation of both β-catenin and *Sox9* in the gonads at 14.5 dpc. The 14.5 dpc XX (**A**–**I**) and XY (**J**–**R**) gonads of various genotypes (control, *Ctnnb1* KO, *Sox9* KO, and *Ctnnb1/Sox9* DKO) were analyzed by bright field microscopy (**A**–**C**, **J**–**L**), immunofluorescence for germ cell/vasculature marker PECAM-1 (**D**–**F**, **M**–**O**) and supporting cell markers FOXL2/AMH (**G**–**I**, **P**–**R**). White arrows show the testis-specific vessel at the surface of the gonad (**E**, **F**, **M**, **O**). Nuclei are labeled with 4′,6-diamidino-2-phenylindole ([DAPI] grey) in **G**–**I** and **P**–**R**. **R**) Arrowheads show the AMH$^+$ cells, and the inset is a higher magnification of a part of the XY *Ctnnb1/Sox9* DKO gonads that contain PECAM$^+$ germ cells (red) surrounded by FOXL2$^+$

Y Chromosome-Specific Effects of Loss of β-Catenin and Sox9 *on Gonadal Differentiation*

When comparing the XX and XY *Ctnnb1*/*Sox9* DKO phenotypes at 14.5 dpc (Fig. 1, C, F, and I vs. L, O, and R), we noticed several subtle differences: a few AMH-positive cells were detected in XY DKO gonads (Fig. 1R) but not in XX DKO gonads (Fig. 1I). Moreover, *Foxl2* expression was significantly reduced in XY DKO gonads compared to XX DKO gonads (Fig. 1S). These results imply that loss of both *Ctnnb1* and *Sox9* has a sex chromosome-specific effect (XX/XY) on the supporting cells, similar to *Wnt4*/*Fgf9* DKO [22] and *Rspo1*/*Sox9* DKO [23]. To test this hypothesis, we allowed the gonads of various genotypes to develop to 17.5 dpc and investigated whether the differences between XX and XY *Ctnnb1*/*Sox9* DKO gonads become more pronounced (Fig. 2). At 17.5 dpc, the XX DKO gonads expressed the Sertoli-cell markers AMH and DMRT1 in a few cells, similarly to XX *Ctnnb1* KO (Fig. 2, B, C, E, F, and N). In contrast, the XY DKO gonads contained many more AMH^+/$DMRT1^+$ Sertoli cells that clustered and formed cordlike structures in the center of the gonads (Fig. 2, I and L). FOXL2 and DMRT1 cells in the XY DKO gonads were predominantly mutually exclusive with the exception of a few double-positive cells (inset in Fig. 2L). This observation correlated with a lower expression of the granulosa cell-specific marker *Foxl2* (Fig. 2M) and a higher expression of the Sertoli cell-specific marker *Amh* (Fig. 2N) in the XY DKO gonads compared to the XX DKO gonads. The impact of loss of both β-catenin and *Sox9* on supporting cell fate determination is clearly dependent upon the genetic context (XX vs. XY) of the gonadal environment.

Sex-Specific Masculinization of Gonadal Transcriptome in the Absence of β-Catenin and Sox9

To obtain a global picture of the early molecular changes that lead to the different phenotypes between *Ctnnb1*/*Sox9* DKO XX and XY fetal gonads, we compared the transcriptomes of control, *Ctnnb1* KO, *Sox9* KO, and *Ctnnb1*/*Sox9* DKO gonads of both sexes at 14.5 dpc, the onset of the sex-specific differences. Gene expression analysis was conducted using Affymetrix Mouse Genome 430 2.0 GeneChip arrays with three to four biological replicas for each genotype. The raw microarray data are available in GEO (accession no. GSE67463, http://www.ncbi.nlm.nih.gov/geo/). A full dataset containing the normalized log-2 intensity of all probes for each genotype and a graphic view of their expression are provided in Supplemental Dataset S1. Data for XX *Sox9* KO and XY *Ctnnb1* KO gonads are shown in Supplemental Dataset S1 but are not included in the following analysis because they do not differ from the control XX and XY, respectively.

A heat map based on the expression of sexually dimorphic genes (Fig. 3A) revealed three clusters that were correlated to the morphological phenotypes (Fig. 1): ovary, intersex (defined as gonads containing features of both testis and ovary), and testis. The ovary group included XX control and XY *Sox9* KO gonads, and the testis group contained only XY control gonads. The intersex group consisted of XX *Ctnnb1* KO, XX DKO, and XY DKO gonads. Within the intersex group, transcriptomes of XX *Ctnnb1* KO and XX DKO clustered together while XY DKO belonged to its own distinct group. PCA provided a simplified visualization of the dataset distribution (Fig. 3B). Transcriptomes of control XX gonads (in pink) and control XY gonads (in blue) clustered in opposed positions as expected. Both XX *Ctnnb1* KO (in green) and XX DKO (in lilac) clusters were located close to XX control cluster, with a slight shift toward the XY control cluster. This slight shift toward a testis profile was due to the upregulation of testis specific genes such as *Hsd17b3, Cbln1, Nrg1,* or *Ptgds*, and the downregulation of ovarian genes such as *Fst, Wnt4,* or *Bmp2* (Supplemental Fig. S3 and Supplemental Datasets S2 and S3). XX *Ctnnb1* KO and XX DKO transcriptomes were not significantly different, consistent with the identical morphological phenotypes observed at 14.5 and 17.5 dpc (Figs. 1 and 2). These results further support that *Sox9* is not responsible for the testis characteristics that appear in XX *Ctnnb1* KO gonads, including the appearance of Sertoli-like cells.

PCA also illustrated that while XY *Sox9* KO (in yellow) transcriptome was almost identical to that of the XX control (Supplemental Dataset S4), XY DKO gonads (in purple) clearly shifted toward a testis profile. Compared to XY *Sox9* KO gonads, XY DKO gonads had 1783 genes differentially expressed, with higher expression of testis-enriched genes such as *Cyp11a1, Cbln1,* or *Ptgds* and lower expression of ovary-enriched genes such as *Fst, Wnt4,* or *Irx3* (Supplemental Dataset S5 and Supplemental Fig. S3). However, XY DKO remained significantly different from the XY control, with 1745 genes differentially expressed (Supplemental Dataset S6).

*Identification of Candidate Genes Underlying the Y Chromosome-Specific Masculinization of β-Catenin/*Sox9 *DKO gonads*

While only little morphological differences were observed between XX and XY *Ctnnb1*/*Sox9* DKO gonads at 14.5 dpc (Fig. 1), XY DKO transcriptome was clearly more masculinized than XX DKO gonads (Fig. 3; Supplemental Dataset S7). To determine what genes are potentially involved in these sex-specific differences, we performed extensive transcriptome comparisons. We first identified the genes expressed higher in XY DKO than in XX DKO (314 genes in the purple circle, Fig. 4A; fold-change ≥ 1.5, $P < 0.05$). Next, in order to focus on the molecular changes that occur in the supporting cells, we took advantage of a cell-type specific microarray performed in fetal gonads [25] to create a list of 796 genes representatives of fetal Sertoli cell identity (green circle, Fig. 4A; see *Materials and Methods*). Comparison of our data with this gene list led to the identification of 64 genes (42 + 22 in Fig. 4A) specific for Sertoli cells. Interestingly, out of these 64 Sertoli-specific genes that are more highly expressed in XY DKO gonads than their XX counterpart, 21 genes are potential direct targets of SRY according to SRY chromatin immunoprecipitation (ChIP) sequencing data [27, 28], such as *Sox8, Cyp26b1, Dhh,* or *Cbln4* (Supplemental Dataset S8 and Supplemental Fig. S3). We then hypothesized that the genes responsible for the more pronounced masculinization in XY DKO gonads must be upregulated only in XY DKO and not in XX DKO (pink circle in Fig. 4A). This analysis led to the identification of 42 Sertoli-cell genes that are specifically upregulated in XY DKO (the red dotted area in Fig. 4A and Supplemental Dataset S8). Several

granulosa cells (magenta) and one AMH^+ Sertoli-like cell (green). Go, gonad; Me, mesonephros. The white dotted lines outline the gonads. Bars = 200 μm. **S** and **T**) represent quantitative PCR analysis of *Foxl2* (**S**) and *Amh* (**T**) mRNA expression in 14.5 dpc gonads. Error bars represent SEM of five biological replicates (Student *t*-test, $P < 0.05$; a < b < c < d).

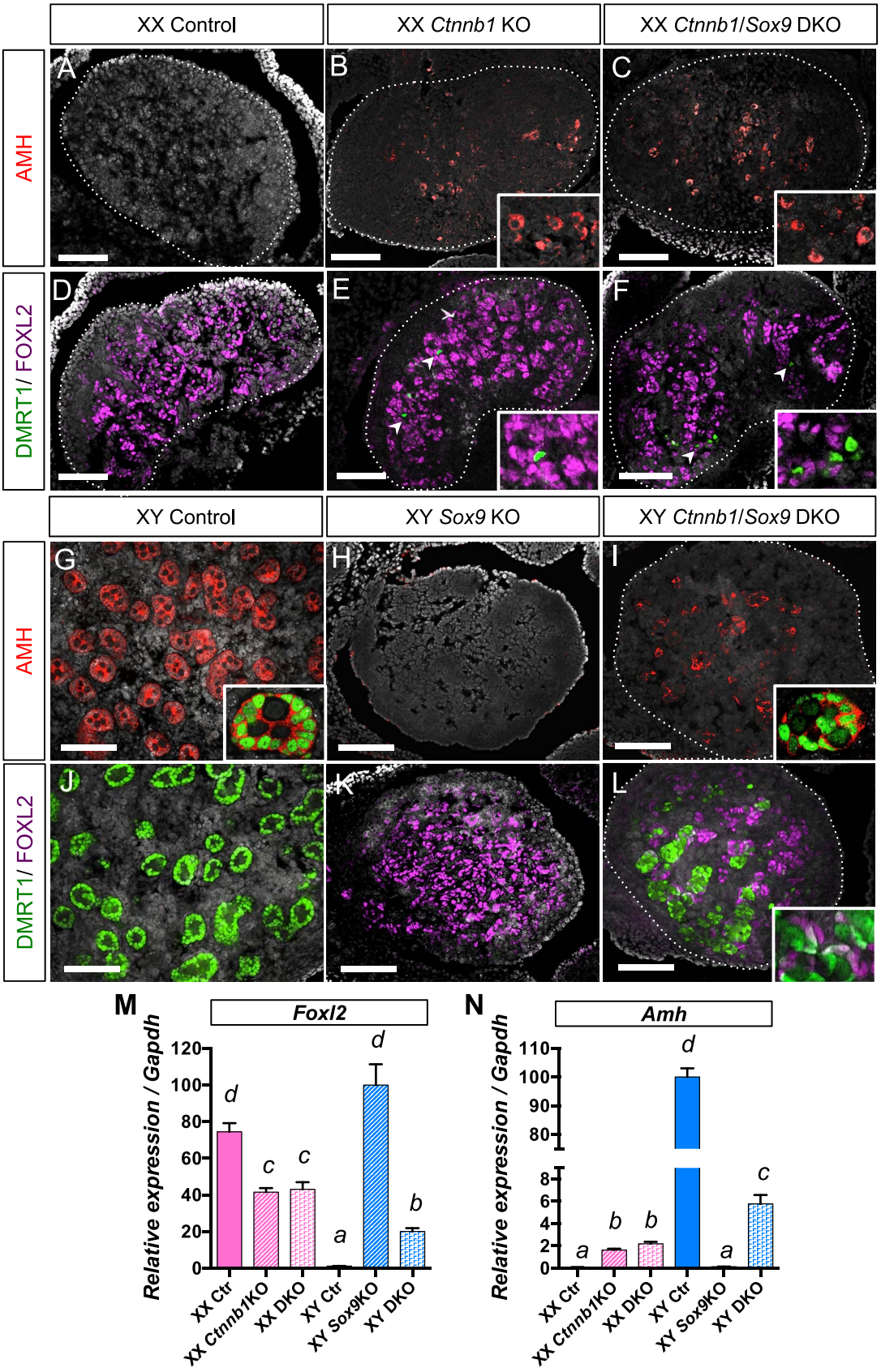


FIG. 2. Consequences of inactivation of both β-catenin and *Sox9* in the gonads at 17.5 dpc. The supporting cell identity of 17.5 dpc XX (**A**–**F**) and XY (**G**–**L**) gonads of various genotypes (control, *Ctnnb1* KO, *Sox9* KO, and *Ctnnb1/Sox9* DKO) was analyzed by immunofluorescence for Sertoli cell markers AMH (red) and DMRT1 (green) and the granulosa cell marker FOXL2 (magenta). Nuclei are labeled with 4′,6-diamidino-2-phenylindole (grey). Arrowheads in **E** and **F** show the DMRT1$^+$ cells. Insets in **G** and **I** show testis-cord structures formed by AMH$^+$ (red, cytoplasmic)/DMRT1$^+$ (green, nuclear) Sertoli cells. Bars = 100 µm. **M** and **N**) Quantitative PCR analysis of *Foxl2* (**M**) and *Amh* (**N**) mRNA expression in 17.5 dpc gonads. Error bars represent SEM of five biological replicates (Student *t*-test, $P < 0.05$; a < b < c < d).

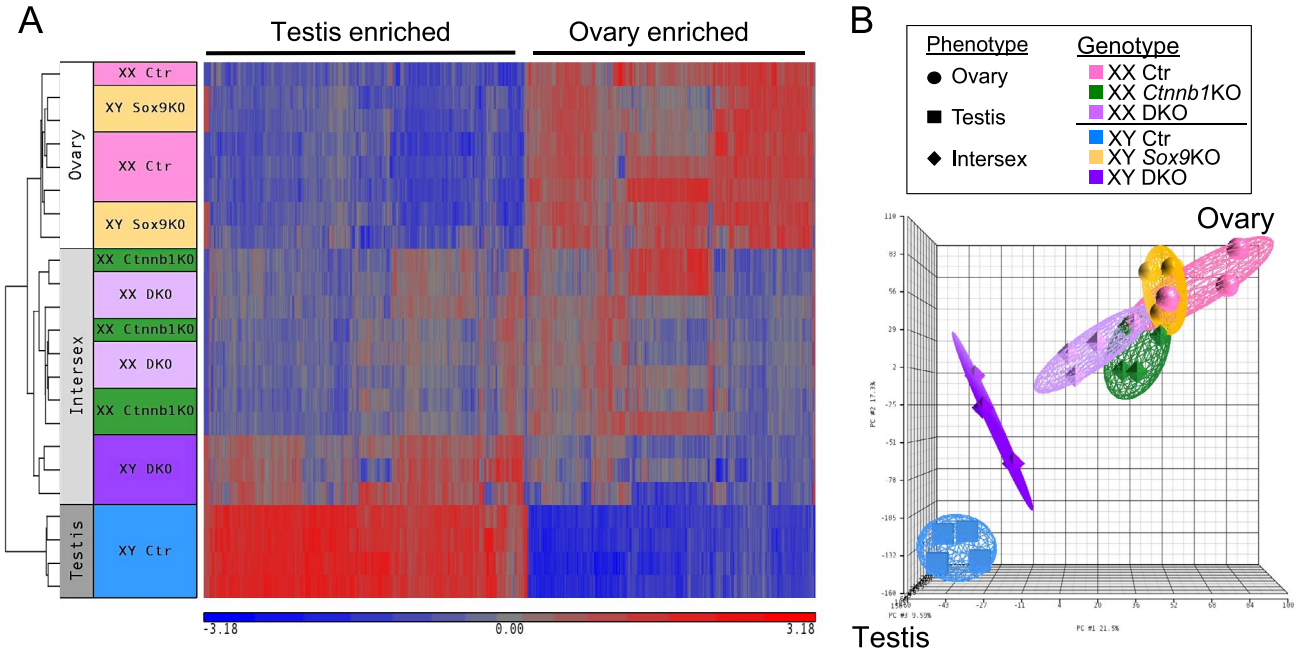


FIG. 3. Transcriptome analyses of 14.5 dpc control, *Ctnnb1* single KO, *Sox9* single KO, and *Ctnnb1/Sox9* DKO XX and XY gonads. **A**) The heat map of the sexually dimorphic genes at 14.5 dpc (fold change ≥ 2 and $P < 0.05$) consists of three clusters that follow the gonadal phenotypes: ovary (XX control and XY *Sox9* KO), intersex (XX *Ctnnb1* KO, XX *Ctnnb1/Sox9* DKO, and XY *Ctnnb1/Sox9* DKO), and testis (XY control). Red indicates high expression and blue for low expression. **B**) Principal component analysis (PCA) shows that XX *Ctnnb1* KO and XX *Ctnnb1/Sox9* DKO transcriptomes show a shift toward a testis profile, whereas XY *Ctnnb1/Sox9* DKO transcriptome is even more masculinized.

Y-linked genes were present at the top of the list, such as *Eif2s3y, Ddx3y,* or *Kdm5d* (Fig. 4B), but these genes are not expected to play a role in the early differentiation of Sertoli cells [25, 29].

Among the genes that were only upregulated in XY *Ctnnb1/Sox9* DKO at 14.5 dpc, *Sry* and *Sox8* were the most promising candidates for the sex-specific difference (Fig. 4B and Supplemental Dataset S8) due to the fact that these two genes are known to be involved in acquisition of Sertoli cell identity [2–4, 9, 30]. In the control XY gonads, *Sry* expression was not detected at 14.5 and 17.5 dpc as described previously [5, 31]. However in the XY DKO gonads, *Sry* expression was maintained at 14.5 dpc, and still detected at low levels at 17.5 dpc (Figs. 4C and 5, A–C and Supplemental Dataset S6). Expression of *Sry* mRNA and protein was also detected in XY *Sox9* KO gonads similar to other observations [9, 10], albeit at a level much lower than in the XY DKO gonads (Figs. 4C and 5B). *Sox8*, a close homologue of *Sox9* and target of SRY, was also upregulated in XY DKO gonads at 14.5 dpc (Figs. 4D and 5, D–G). In 14.5 dpc XY DKO gonads, most of the SOX8-positive cells start to express AMH, a potential direct target gene of SOX8 [32] (Fig. 5F).

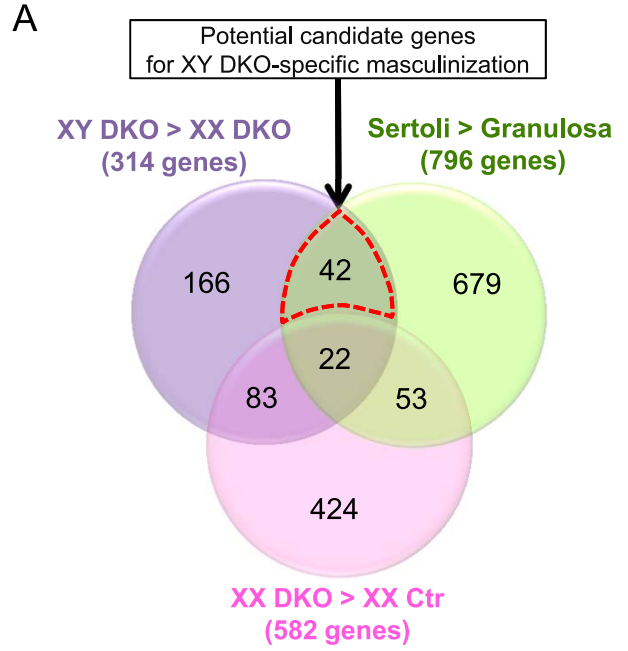
In addition to *Sox8,* the list of 42 Sertoli-specific genes that were upregulated only in XY DKO gonads contained nine other potential SRY target genes, such as *Cyp26b1* [27] (Fig. 4B). *Cyp26b1* is involved in the male germ cells fate by repressing germ cells entry into meiosis during fetal life [33]. Interestingly, this expression of *Cyp26b1* in XY DKO was correlated with a reduced expression of meiosis markers compared to XX DKO (Supplemental Fig. S3 and Supplemental Dataset S1). Finally, this list also contained *Map7*, a gene whose function in the testis is unknown but its mutation (*Map7*^{mshi}) leads to reduced testis size and sterility [34]. Most of the other genes in this list are of unknown functions in testis differentiation.

Our analysis of the early events of the sex-chromosome specific differences in the masculinization of *Ctnnb1/Sox9* DKO gonads demonstrate that XY DKO gonads develop much more pronounced testicular characteristics than the XX DKO gonads, potentially due to the expression of *Sry* and the activation of several of its target genes recently identified by ChiP-sequencing [27], including *Sox8* and *Cyp26b1*.

Identification of Candidate Genes Commonly Involved in the Masculinization of Ctnnb1/Sox9 *DKO XX and XY Gonads*

Despite the sex chromosome-specific differences in phenotypes and transcriptomes between XX and XY DKO gonads, gonads of these two genotypes share similar features such as the appearance of a testis-specific coelomic vessel and steroidogenic cells. We suspected that some pro-testis genes commonly responsible for these phenotypes were activated in *Ctnnb1/Sox9* DKO gonads regardless of their genetic contexts (XX vs. XY). To identify these common pro-testis genes, we first identified the genes upregulated in XX DKO by comparing its transcriptome to XX control transcriptome (582 genes with a fold-change ≥ 1.5, $P < 0.05$; pink circle in Fig. 6A and Supplemental Dataset S3). Second, we identified the genes upregulated in XY DKO in comparison to XY *Sox9* KO gonads (990 genes with a fold-change ≥ 1.5, $P < 0.05$; blue circle in Fig. 6A and Supplemental Dataset S5). We cross-referenced these two lists of genes and identified 451 (387 + 64; Fig. 6A, purple shaded area with black outline) genes that commonly associate with the masculinization of both XX and XY DKO gonads. Finally, we narrowed down our search to the genes known to be expressed in Sertoli cells by comparing our data with the list of genes representative of fetal Sertoli cell identity based on Jameson et al. microarray data [25] (Fig. 6A, green circle, see *Materials and Methods*). This comparison removed 387 genes that were not Sertoli cell

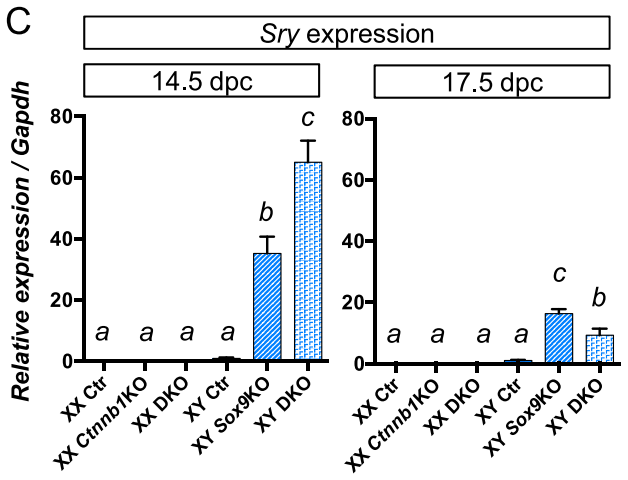
A



B

Subset of Sertoli cells-enriched genes upregulated in XY DKO but not XX DKO		
Gene Symbol	**Probeset ID**	**Fold-Change XY DKO vs. XX DKO**
Eif2s3y	1417210_at	107.873
Ddx3y	1426438_at	96.720
Kdm5d	1452562_at	45.831
Cyp26b1*	1460011_at	2.616
Sel1l3*	1452227_at	2.438
Pak3	1435486_at	2.426
Prkar2b*	1438664_at	2.288
Iqgap2	1433885_at	2.265
Efhd1	1448507_at	2.258
Mmd2*	1424534_at	2.064
Sry	1450578_at	1.964
Svil*	1460694_s_at	1.671
Spry4*	1445669_at	1.594
Nav1*	1455849_at	1.542
Cebpa*	1418982_at	1.532
Map7	1421836_at	1.763
Sox8*	1435438_at	1.518

C



D

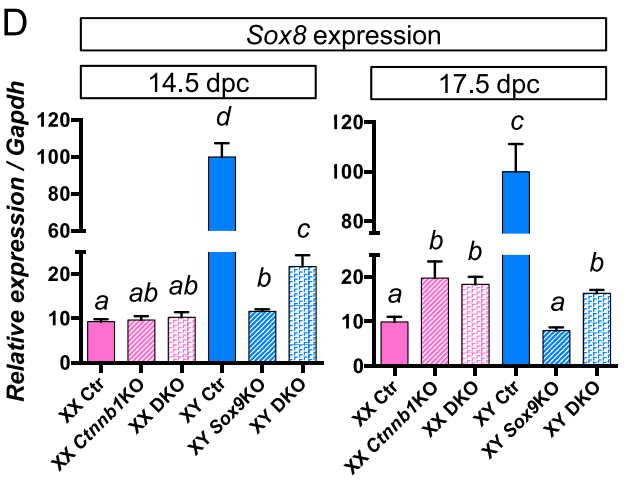


FIG. 4. Identification of the potential mechanisms underlying the differences between XX and XY *Ctnnb1/Sox9* DKO gonads. **A**) Venn diagram comparing three groups of genes: 1) purple circle: genes upregulated in XY *Ctnnb1/Sox9* DKO versus XX *Ctnnb1/Sox9* DKO gonads, 2) green circle: genes enriched in fetal Sertoli cells versus granulosa cells from a published dataset [25], and 3) pink circle: genes upregulated in XX *Ctnnb1/Sox9* DKO versus XX control gonads. The red dotted area represents the 42 genes upregulated in XY but not XX *Ctnnb1/Sox9* DKO gonads. A subset of genes from this 42 genes list is shown in **B**; asterisk (*) represents putative SRY target genes. **C** and **D**) Quantitative PCR analysis for *Sry* and *Sox8* at 14.5 and 17.5 dpc. Error bars represent SEM of five biological replicates (Student *t*-test, $P < 0.05$; a < b < c < d).

specific, such as genes involved in steroidogenesis, including *Cyp11a1*, *Hsd17b,* or *Cyp17a1* (Fig. 6C and Supplemental Dataset S9). This series of comparisons led to the identification of 64 Sertoli cell-specific genes that were potential components of the common pathway responsible for the masculinization of both XX and XY *Ctnnb1/Sox9* DKO gonads (Fig. 6 and Supplemental Dataset S9). Desert hedgehog or *Dhh*, a gene expressed in Sertoli cells and involved in specification of the Leydig cells lineage [35], was upregulated in both XX and XY DKO gonads (Fig. 6B). In addition, several genes involved in the acquisition of Sertoli cell fate were upregulated in both XX and XY DKO gonads. These genes included *Ptgds*, a paracrine signaling factor involved in the recruitment of somatic cells into the Sertoli fate [36, 37], and the transcription cofactor *Lmo4*, a novel regulator of early testis differentiation [38] (Fig. 6B and Supplemental Dataset S9). Inhibin beta B or *Inhbb*, a gene involved in the testis-specific vasculature [39], was also a part of this candidate list (Fig. 6D). This list also contains many genes whose function during gonads differentiation remains undetermined (Supplemental Dataset S9).

DISCUSSION

The fate determination of the fetal gonads hinges upon the balance between pro-testis SOX9 and pro-ovary β-catenin pathways. By analyzing the gonadal identity of *Sox9* and β-catenin DKO embryos, we discovered that in the absence of *Sox9* and β-catenin in the somatic cells that 1) both XX and XY gonads progressively lean toward a male fate, with appearance of testis-specific vasculature, steroidogenic cells, and Sertoli cells and 2) XY gonads become more masculinized than their XX counterparts. This Y-chromosome specific masculinization is associated with the expression of Sertoli-specific genes such as *Sry* and several putative SRY target genes.

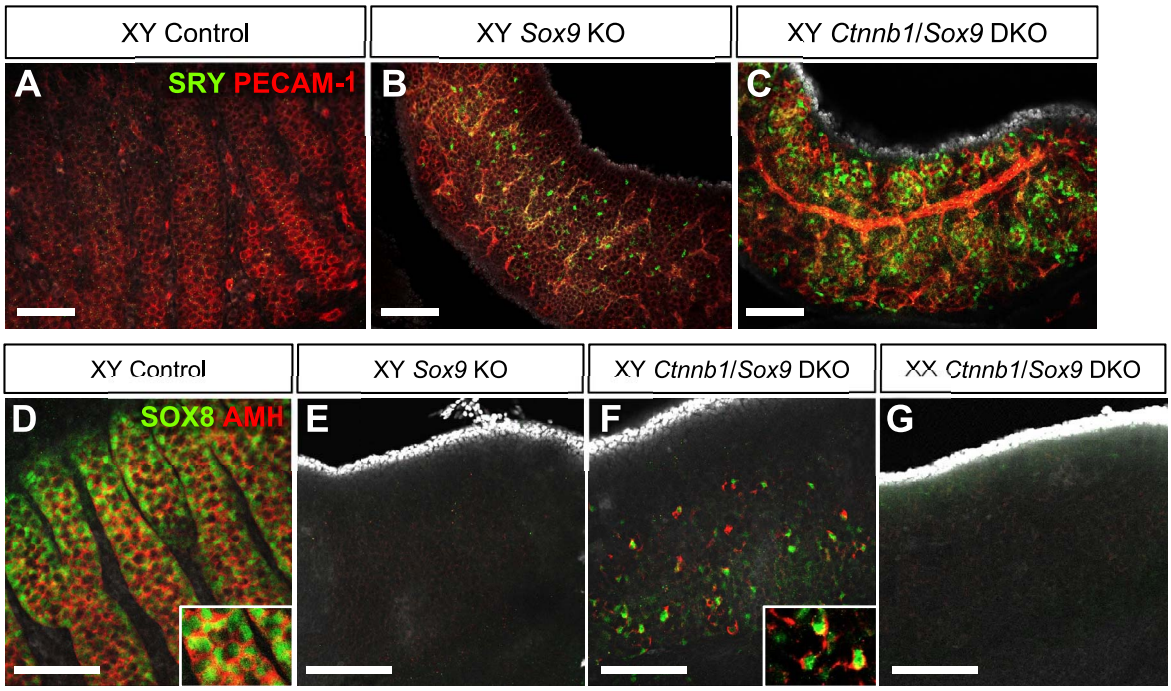


FIG. 5. Immunostaining for SRY and SOX8 in control and different KO gonads at 14.5 dpc. **A**–**C**) Whole mount immunofluorescence for SRY and PECAM-1 showed the presence of SRY in XY *Sox9* KO (**B**) and XY *Ctnnb1/Sox9* DKO (**C**) gonads. **D**–**G**) Whole mount immunofluorescence for SOX8 and AMH at 14.5 dpc showed the presence of $SOX8^{+}$ cells in XY *Ctnnb1/Sox9* DKO (**F**), and most of these cells coexpressed AMH (inset). SOX8 was absent in 14.5 dpc XY *Sox9* KO (**E**) and XX *Ctnnb1/Sox9* DKO (**G**) gonads. Nuclei are labeled with 4′,6-diamidino-2-phenylindole (grey). Bars = 100 μm.

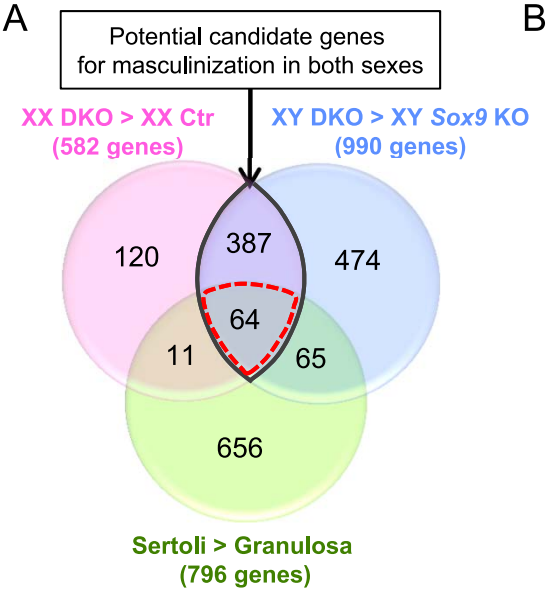
β-Catenin in the XX Gonad Promotes Ovarian Fate by Suppressing Pro-Testis Genes Other than Sox9

Based on the observation that WNT/β-catenin pathway represses the key effector of testis differentiation *Sox9* in gonads and other tissues [21, 40, 41], it was therefore hypothesized that when β-catenin is absent in the ovary, *Sox9* is upregulated and responsible for the partial ovary-to-testis sex-reversal. However, we found that loss of *Sox9* does not rescue any aspects of the masculinization phenotypes in β-catenin KO XX gonads. Similarly, loss of *Sox9* did not restore the partial ovary-to-testis sex reversal of XX *Rspo1* KO gonads [23]. Lavery et al. [23] characterized the postnatal phenotypes of *Rspo1*/*Sox9* DKO gonads and found that newborn *Rspo1* KO and *Rspo1*/*Sox9* DKO ovaries developed phenotypes similar to our observations at 17.5 dpc, characterized by the presence of a few Sertoli-like cells. The analyses of *Rspo1*/*Sox9* DKO ovaries mainly focused on postnatal stages, and the early molecular changes that contribute to their masculinization were not known. Regardless of the presence or absence of a functional *Sox9* gene, both *Ctnnb1* KO and *Rspo1* KO ovaries underwent a shift toward the testis fate. These observations imply that pro-testis genes, other than *Sox9,* induce the appearance of Sertoli-like cells and the other testis characteristics observed in the absence β-catenin or *Rspo1*. We identified a list of candidate genes associated with the fetal appearance of testis features in the *Ctnnb1* KO ovary independent of *Sox9*, including *Inhbb*, a Sertoli-specific gene responsible for the formation of the coelomic vessel [39]. We speculate that in the wild-type fetal ovary, *Inhbb* expression and formation of testis-specific coelomic vessel are absent as a result of repression by the β-catenin pathway, rather than a lack of direct activation by SOX9. Other Sertoli cell-enriched genes induced in fetal *Ctnnb1*/*Sox9* DKO ovaries, including *Ptgds* [36], *Lmo4* [38], *Dhh* [42], *Cbln4* [28], and later, close to birth, *Sox8* [9] and *Amh* [32], might be responsible for the differentiation of supporting cells into Sertoli-like cells. Interestingly, *Ptgds, Amh,* and *Cbln4* are known to be direct target genes of SOX9 [28, 43, 44]; however, our results indicate that these genes can be induced in a *Sox9*-independent manner. We conclude that in the supporting cells of the ovary, β-catenin suppresses the testis fate by inhibiting not only *Sox9*, but also other *Sox9*-independent pro-testis genes (Fig. 7). It remains unclear whether β-catenin acts directly to repress these pro-testis genes, or if other pro-ovarian genes such as *Foxl2* are involved in this process. The antagonism between testis and ovary pathways is clearly more complex than previously thought.

Testis Differentiation in XY Embryos Requires Sox9 *in the Supporting Cell Lineage in Order to Repress the Pro-Ovary β-Catenin Pathway*

Absence of β-catenin partially reverses the complete testis-to-ovary sex-reversal phenotype observed in XY *Sox9* KO gonads. In XY gonads lacking both *Sox9* and β-catenin, some supporting cells transdifferentiate from FOXL2-positive granulosa cells to a DMRT1-positive Sertoli cell identity, resulting in the formation of ovotestes. A similar restoration of testis differentiation was observed by other groups in *Wnt4*/*Fgf9* DKO [22] and *Rspo1*/*Sox9* DKO models [23]. Our findings along with these observations suggest that during normal testis differentiation, SOX9 promotes testis differentiation directly, via the transcriptional activation of pro-testis genes, and indirectly, via repression of the β-catenin pathway (Fig. 7). The repression of β-catenin by SOX9 is necessary for not only preventing the expression pro-ovary genes downstream of β-catenin, but also promoting the expression of pro-testis genes that are negatively regulated by β-catenin.

A



B

Subset of Sertoli cells-enriched genes upregulated in both XY DKO and XX DKO			
Gene Symbol	**Probeset ID**	**Fold-Change XX DKO vs. XX Ctr**	**Fold-Change XY DKO vs. XY Sox9KO**
Hsd17b3	1421671_at	16.73	32.04
Lect1	1460258_at	6.95	21.50
Cbln4	1433607_at	5.35	10.20
Ptgds	1423859_a_at	3.26	4.42
Dhh	1434959_at	3.20	7.24
Inha	1422728_at	2.78	3.61
Fgf18	1449545_at	2.59	2.36
Lmo4	1420981_a_at	2.13	1.55
Fbln2	1423407_a_at	1.76	1.99
Inhbb	1426858_at	1.50	2.08

C D

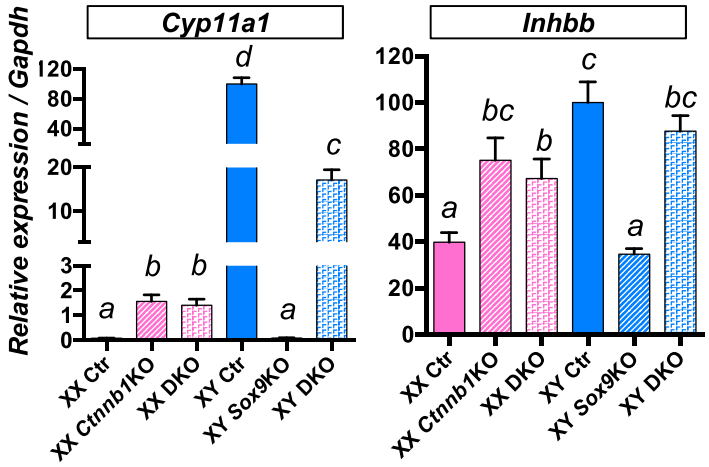


FIG. 6. Identification of candidate genes commonly involved in the masculinization of both XX and XY *Ctnnb1/Sox9* DKO gonads. **A**) Venn diagram comparing three groups of genes: 1) pink circle: genes upregulated in XX *Ctnnb1/Sox9* DKO versus XX control gonads, 2) blue circle: genes upregulated in XY *Ctnnb1/Sox9* DKO versus XY *Sox9* KO gonads, and 3) green circle: genes enriched in fetal Sertoli cells versus granulosa cells from a published dataset [25]. A subset of these Sertoli-specific genes is shown in **B**. Quantitative PCR analyses of *Cyp11a1* (**C**) and *Inhbb* (**D**), two genes that were upregulated in both XX and XY *Ctnnb1/Sox9* DKO gonads in non-Sertoli cells (387 genes in the purple area) and Sertoli cells (64 genes in the red-dotted area), respectively. Error bars represent SEM of five biological replicates (Student *t*-test, $P < 0.05$; a < b < c < d).

A Potential Involvement of SRY in the Y Chromosome-Specific Masculinization of the Gonads in the Absence of SOX9 and β-Catenin

We observed a Y-chromosome dependent appearance of Sertoli cells in *Ctnnb1/Sox9* DKO gonads. Among the Y-linked genes, *Sry* is an obvious candidate because of its role in directing supporting cell progenitors toward the male fate [45]. SRY ChIP analyses in normal fetal testes have revealed that *Sox9* is not the only target of SRY [7, 27, 28, 46]. Interestingly, we found that among the Sertoli-specific genes upregulated in XY DKO gonads, 21 genes were recently identified as potential SRY target genes [27]. We hypothesize that SRY is able to activate these genes and therefore drive the Y-chromosome dependent masculinization in the absence of SOX9 and β-catenin. However, *Sry* expression alone cannot explain the activation of these genes and consequent appearance of Sertoli cells in XY *Ctnnb1/Sox9* DKO gonads. Indeed, *Sry* is also expressed in XY *Sox9* KO gonads, in which a complete testis-to-ovary sex reversal still occurs [9, 10]. In vitro studies have shown that SRY requires the cooperation of cofactors to activate its target genes and fulfill its testis-inducing functions [7, 27]. SRY acts in synergy with SF1 to activate many target genes, including *Sox8* and *Mmd2* [27]. Interestingly, β-catenin is able to prevent SF1 binding to the SRY target promoter [7, 40]. In XY control and XY *Ctnnb1/Sox9* DKO, the conditions are met to allow the expression of SRY target genes: SRY is expressed and SF1 cannot be repressed by β-catenin. On the other hand in XY *Sox9* KO, SRY is expressed, but the presence of β-catenin may prevent SF1 binding, consequently blocking the expression of SRY target genes. We propose that SRY, in association with SF1 activates pro-testis genes other than *Sox9* to initiate the appearance of Sertoli cells in the XY *Ctnnb1/Sox9* DKO gonads (Fig. 7).

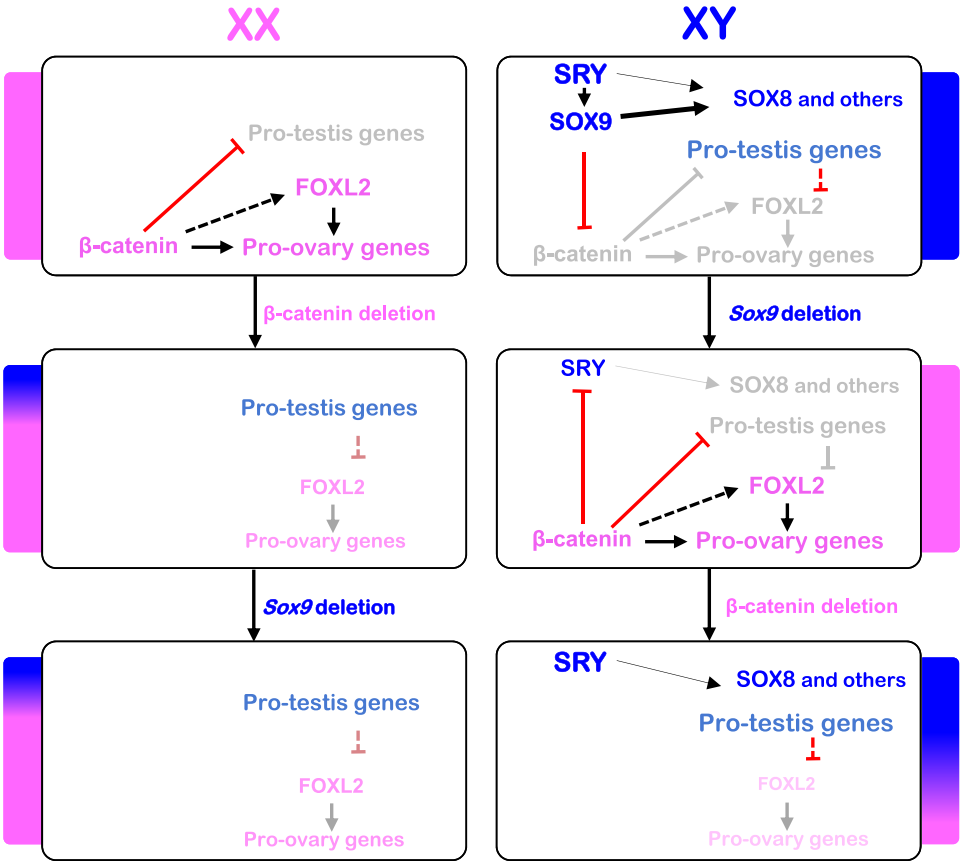


FIG. 7. Model for the molecular pathways controlling supporting cell fate in the gonads. In the XX gonad, β-catenin is involved in expression of pro-ovary genes, including *Foxl2,* and blocks Sertoli cell fate through repression of some pro-testis genes. In the absence of β-catenin, some pro-testis genes such as *Inhbb, Dhh,* and *Ptgds* are activated, and expression of pro-ovarian genes is reduced, leading to a partial ovary-to-testis sex-reversal. Additional deletion of *Sox9* results to an identical phenotype, indicating that *Sox9* is not required for the activation of the pro-testis genes in the absence of β-catenin. In the XY gonad, *Sox9* expression is induced by SRY to drive Sertoli cell differentiation. SOX9 activates the expression of pro-testis genes directly and indirectly through repression of β-catenin. SRY may also be involved in the expression of pro-testis genes other than *Sox9*, such as *Sox8*. When *Sox9* is deleted in the supporting cell lineages, β-catenin becomes activated, subsequently leading to repression of the pro-testis genes, the expression of pro-ovary genes, and the acquisition of granulosa cell identity. In the absence of both *Sox9* and β-catenin, SRY activates pro-testis genes other than *Sox9* (such as *Sox8*) to drive testis differentiation.

A certain degree of redundancy appears to exist between SOX transcription factors. For instance, SRY and SOX9 are able to activate common target genes such as *Sox9*, *Sox8*, or *Cyp26b1* [7, 27]. This redundancy is probably due to the conserved DNA-binding high-mobility group box among members of the SOX family. Lavery et al. [23] showed that *Sox8* and *Sox10* were expressed in $Rspo1^{KO}$ $Sox9^{KO}$ mutants at Postnatal Day 12 and 0, respectively. In our XY *Ctnnb1*/*Sox9* DKO model, in addition to *Sry*, *Sox8* is the only *Sox* gene expressed during the initial events of masculinization. While *Sox8* inactivation does not affect testis determination [47], analyses of *Sox8*/*Sox9* double mutants showed that SOX8 is able to compensate for the loss of *Sox9* after sex determination [9, 30, 48]. Moreover, SOX8 and SOX9 activate common target genes such as *Amh* and *Cbln4* [32, 48]. Therefore, we propose that SRY and its target gene SOX8 partially compensate for the absence of SOX9 in XY *Ctnnb1*/*Sox9* DKO supporting cells and promote Sertoli cell differentiation (Fig. 7).

Altogether, our findings shed light onto the complex antagonisms between pro-ovary and pro-testis pathways. Testis differentiation, which was thought to be the result of a direct action of SOX9, requires both SOX9-dependent and -independent morphogenetic changes along with the repression of the β-catenin pathway. Moreover, the Y-chromosome dependent masculinization in the absence of SOX9 and β-catenin indicates that SRY, along with its downstream target genes, is capable of guiding Sertoli cell differentiation in a SOX9-independent manner. In humans, the majority of the congenital disorders of sex development are of unknown genetic origin [49]. The microarray analyses we provide in this mouse sex-reversal study are useful tools to identify new genes potentially associated with disorders of sex development. Many of the genes associated with testis differentiation in our mouse models, such as *Lmo4*, *Cbln4*, *or Nrg1*, and several potential SRY targets genes have an unknown function in the fetal gonads. It would be interesting to determine the role of these genes in testis differentiation and investigate if these genes are associated with disorders of sex development in humans.

ACKNOWLEDGMENT

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