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Activation of β-catenin signaling by Rspo1 controls differentiation of the mammalian ovary

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The sex of an individual is determined by the fate of the gonad. While the expression of *Sry* and *Sox9* is sufficient to induce male development, we here show that female differentiation requires activation of the canonical β-catenin signaling pathway. β-catenin activation is controlled by Rspo1 in XX gonads and *Rspo1* knockout mice show masculinized gonads. Molecular analyses demonstrate an absence of female-specific activation of *Wnt4* and as a consequence XY-like vascularization and steroidogenesis. Moreover, germ cells of XX knockout embryos show changes in cellular adhesions and a failure to enter XX specific meiosis. Sex cords develop around birth, when *Sox9* becomes strongly activated. Thus, a balance between Sox9 and β-catenin activation determines the fate of the gonad, with Rspo1 acting as a crucial regulator of canonical β-catenin signaling required for female development.

INTRODUCTION

The sex of an individual is determined during early development by the fate of the gonad. Transgenic analysis has demonstrated that the expression of the sex determining gene *Sry* within the initially bipotential gonad is sufficient to induce the male developmental program (1). In mice, *Sry* is expressed for only 2 days starting from E10.5 (2,3). Shortly after the onset of *Sry* expression, the transcription factor *Sox9* becomes upregulated, which in turn induces the differentiation of Sertoli cells (4). Sox9 stimulates expression of *Pgds* (5), which—together with Fgf9—is required for maintaining *Sox9* expression in the XY gonad (2,6). Sertoli cells surround primordial germ cells to form the sex cords (7). The addition of further cell types completes the formation of seminiferous tubules. *Sox9* activates Amh/Mis, which in turn induces the regression of the Mullerian duct, the precursor of the uterus, oviduct and part of the vagina. Testosterone secreted by Leydig cells induces Wolffian duct development into epididymis, vas deferens and seminal vesicles.

In contrast to male sex determination, very little is known about the molecular pathways governing ovary differentiation. Histologically, the first ovarian feature appears at E13.5 when oogonia enter meiosis (8). Around birth, oocytes are blocked in diplotene of prophase I and separated by cytoplasmic extensions of epithelial pregranulosa cells to form primordial follicles (9). Meiosis requires signaling through retinoic acid (RA), which stimulates the expression of *Stra8* (10,11). In the XY gonad, the degradation of RA by Cyp26b1 prevents gonocytes from entering meiosis (12). Germ cells are not required for Sertoli cell differentiation, but are necessary for follicle formation. Their absence in XX gonads results in streak gonads or structures resembling sex cords (13). Adams *et al*. (14) showed that gonocytes can induce masculinisation of somatic cells via the paracrine signal PGD2. Moreover, after initiation of meiosis, XX germ cells antagonize sex

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cord formation (15) indicating the importance of germ cells for ovarian development.

Wnt4 is expressed specifically in XX gonads from E11.5 onwards and is required to suppress endothelial and steroidogenic cell migration from the mesonephros and the adrenal, respectively (16). Thus, XX *Wnt4*$^{-/-}$ mice develop ovotestes with the presence of sex cords and functional steroidogenic Leydig cells (17,18). Strikingly, oocytes degenerate after E14.5 with less than 10% remaining at birth when sex cords develop. In goats, XX sex reversal has been associated with PIS mutations (19) that result in the downregulation of *FOXL2*. XX *FoxL2*$^{-/-}$ mice show premature ovarian failure (20,21) and postnatal expression of male specific genes (22). Recently, female to male sex reversal in XX patients has been shown to be due to homozygous mutations in *RSPO1* (23). Rspondins are secreted activators of the transcriptional activity of β-catenin (24,25) by modifying the levels of LRP6 protein available on the cell surface (26–28). In mouse gonads, *Rspo1* is specifically up-regulated in XX somatic cells from E11.5 onwards (23).

RESULTS

The transcriptional function of β-catenin is specifically activated in female gonads

R-spondin genes encode secreted proteins that activate the canonical β-catenin pathway. Because of the importance of *RSPO1* and *Wnt4* for ovarian differentiation, it has been proposed that β-catenin may also be activated in ovary development (29,30), but until now no direct experimental evidence has been reported.

To determine whether β-catenin is involved in mammalian sex determination, we employed the *Axin2*$^{+/LacZ}$ reporter line, which carries *LacZ* fused to *Axin2* (31). Since *Axin2* is a target of β-catenin, this strain provides a readout of the canonical β-catenin signaling. While XY gonads were devoid of blue signal at all stages analyzed (E12.5, E13.5, E14.5, E16.5, E18.5), whole-mount β-galactosidase staining revealed a robust staining of XX gonads from E12.5 onwards. Additional staining was detected in the Wolffian and Mullerian ducts of both sexes (Fig. 1A).

To identify the cellular lineage in which β-catenin becomes activated, sections were examined for β-galactosidase coloration in combination with immunostaining against the germ cell marker Mvh. Overlay of the resulting images from XX gonads clearly demonstrated strong, but mutually exclusive signals indicating that *Axin2* activation occurs predominantly in somatic cells (Fig. 1B). These results strongly suggest a sex-specific role for the nuclear translocation of β-catenin during ovarian development.

To confirm this female-specific activation of the canonical signaling pathway, we measured the expression of *Axin2* and *Lef1*, two known β-catenin target genes (http://www.stanford.edu/~rnusse/wntwindow.html). Expression of *Dax1*, *Emx2* and *Wnt4* has been shown to be regulated by Wnt/β-catenin signaling (29,32,33) and were all up-regulated in E12.5 XX gonads, when compared to their XY littermates (Fig. 1C).

To test whether Wnt4 is required for activation of the canonical β-catenin pathway *in vivo*, we analyzed *Lef1* expression levels. From E12.5, *Lef1* was clearly down-regulated in XX *Wnt4*$^{-/-}$ compared to XX wild-type gonads (Fig. 1D). Moreover, X-gal staining at E13.5 showed down-regulation of the *Axin2*$^{+/LacZ}$ allele in XX *Wnt4*$^{-/-}$ gonads. However, in contrast to XY gonads weak staining persisted in XX *Wnt4*$^{-/-}$ tissues, indicating that ablation of *Wnt4* is not sufficient to completely prevent β-catenin signaling. This suggests that while *Wnt4* is involved in β-catenin activation, additional *Wnt4* independent pathway(s) also exist.

Sox9 is a key regulator of male development and ectopic expression of this gene in XX transgenic gonads (XX *Wt1:Sox9*$^{Tr/+}$) triggers female to male sex reversal (34). To test whether ectopic activation of *Sox9* would also interfere with β-catenin signaling *in vivo*, we performed X-Gal staining on mice double transgenic for *Axin2*$^{+/LacZ}$ and *Wt1:Sox9*. Strikingly, XX gonads carrying the *Wt1:Sox9* transgene remained unstained (Fig. 1E).

Taken together, these results demonstrate that the canonical β-catenin signaling pathway is activated in the somatic cells during ovarian embryonic development and is antagonized by ectopic expression of *Sox9*.

Targeted disruption of *Rspo1*

To address whether *Rspo1* is a critical activator of the β-catenin pathway during normal development, we generated a knockout allele by inserting the *LacZ* reporter gene into exon 3 (Fig. 2A). Germ-line transmission was achieved for two targeted clones (Fig. 2B), both of which exhibited an identical phenotype in the homozygous state.

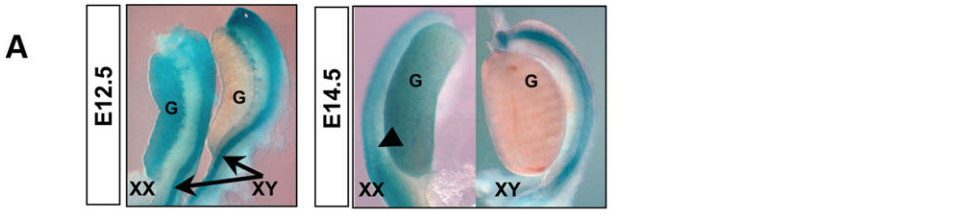
Rspo1$^{+/-}$ mice appeared phenotypically normal and fertile. RT–PCR experiments using primers located in exon 4 or 5 and a reverse primer in the 3′-UTR of *Rspo1* revealed expression in *Rspo1*$^{+/-}$ but not in *Rspo1*$^{-/-}$ gonads (Fig. 2C) indicating that the insertion resulted in a complete termination of transcripts and thus loss of *Rspo1* function.

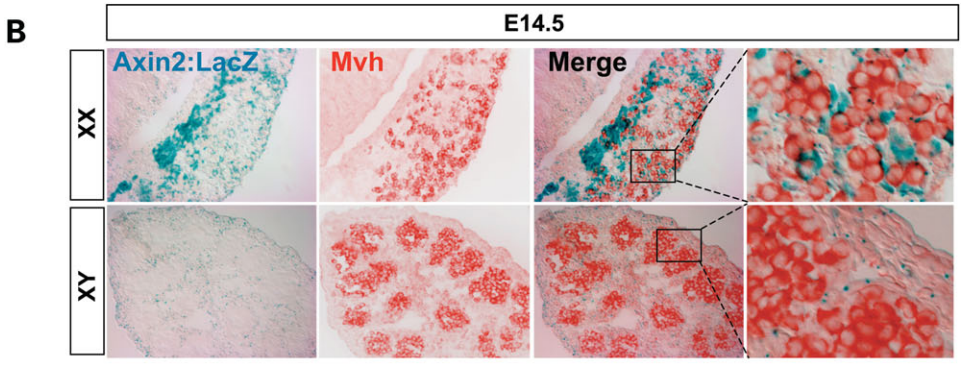
RSPO1 mutations in human patients lead to palmoplantar hyperkeratosis and a predisposition to develop squamous cell carcinoma. Initial analysis of adult *Rspo1*$^{-/-}$ mice (10 weeks old) did not reveal skin defects of the footpads.

Development of ovotestes in XX *Rspo1*$^{-/-}$ mice

We next analyzed the phenotype of the genital system in *Rspo1*$^{-/-}$ mice. A comparison of the phenotype on a mixed versus pure (SV129) genetic background showed no differences. Hence, all further analyses were performed on a mixed C57Bl6/SV129 genetic background. The sex ratio, litters size and number of adult *Rspo1*$^{-/-}$ mice were normal. XY *Rspo1*$^{-/-}$ mice appeared phenotypically normal and fertile.

To determine at what stage masculinization occurs, we examined both the morphology and the histology of the XX *Rspo1*$^{-/-}$ reproductive system at various stages of development. In normal XY gonads, a coelomic vessel forms at E12.5 through endothelial cell migration from the mesonephros (16). This blood vessel also formed on XX *Rspo1*$^{-/-}$ gonads at E12.5 and became prominent at E13.5 (Fig. 3A).





C

Gene symbol	Assigned function	E12.5 XX/XY
Axin2	Axin2 (conductin)	↑ 2
Dax1	Adrenal hypoplasia congenita-like protein	↑ 1,5
Emx2	Homeodomain transcription factor 2	↑ 2,6
Fst	Follistatin	↑ 79
Lef1	Lymphoid enhancer-binding factor 1	↑ 2
Wnt4	wingless-related MMTV integration site 4	↑ 3,25
Rspo1	R-spondin 1	↑ 2,5
Sox9	SRY-box containing gene 9	↓ 3,3

D

Gene symbol	E12.5 KO/Wt	E14.5 KO/Wt
Genes involved in β-catenin signalling		
Lef1	↓ 4	↓ 1,7
Rspo1	NC	NC

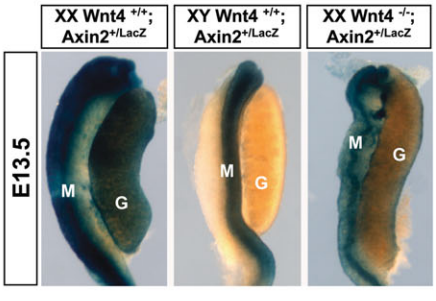


Figure 1. Canonical β-catenin signaling is specifically activated in female gonads. (**A**) X-Gal whole-mount staining of *Axin2*$^{+/LacZ}$ urogenital ridges (magnification ×20). Blue staining reflecting β-galactosidase activity was observed in XX gonads, whereas XY gonads remained unstained. Arrows and arrowhead show the Wolffian ducts and Mullerian duct staining, respectively (G: gonad). (**B**) X-Gal staining and immunostaining on cryosections of XX and XY *Axin2*$^{+/LacZ}$ gonads at E14.5 (magnification ×40). Blue staining localized to somatic cells and did not overlap with the germ cell marker Mvh (red). (**C**) Quantitative RT–PCR expression analysis of E12.5 XX and XY gonads. For each genotype, $n = 6$. Gene expression levels were normalized against expression levels of *Hprt1*. β-catenin target genes were up-regulated in XX gonads. (**D**) Quantitative analysis of *Lef1* and *Rspo1* in XX *Wnt4*$^{-/-}$ gonads compared to XX wild-type littermates at E12.5 and E14.5 (XX *Wnt4*$^{-/-}$, $n = 8$; wild type $n = 4$). Gene expression levels were normalized against expression levels of *Hprt1*. *Lef1* was down-regulated in XX *Wnt4*$^{-/-}$ gonads. KO: *Wnt4* knock-out; Wt: Wild type. NC: no change in gene expression between KO and Wt samples. X-Gal whole-mount staining of urogenital ridges at E13.5. XX *Wnt4*$^{-/-}$ gonads showed decreased β-galactosidase activity when compared to XX gonads. (**E**) X-Gal staining of gonads from XY and XX wild type, as well as *Wt1:Sox9* transgenic mice (XX sex reversal) carrying the *Axin2*$^{+/LacZ}$ allele. Note the complete absence of signal in XX, *Wt1:Sox9* sex-reversed gonads. Arrowheads indicate sex cords of the XY or XX *Wt1:Sox9*$^{Tr/+}$ gonads.

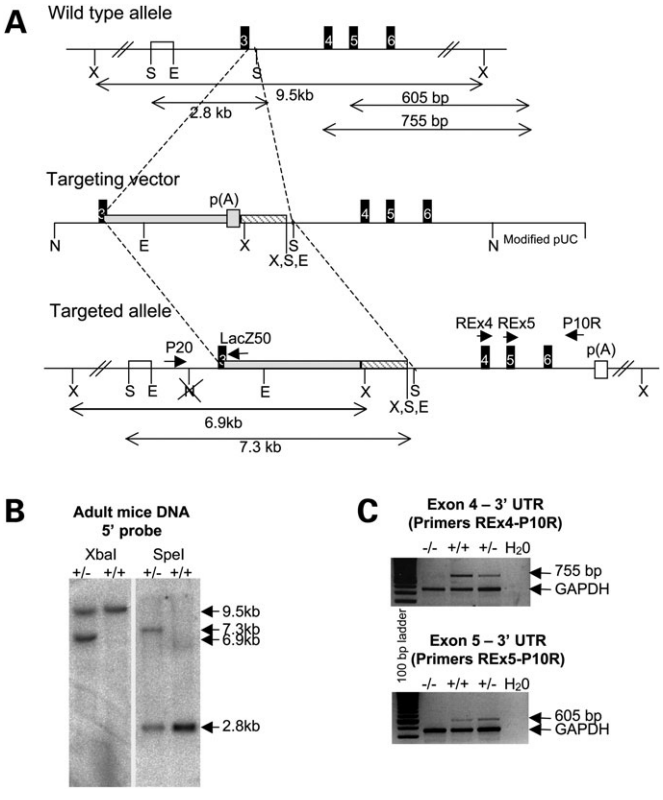


Figure 2. Targeted disruption of *Rspo1*. (**A**) Schematic representation of the *Rspo1* genomic locus, targeting vector and mutant allele. Black boxes represent protein-coding regions of exons, gray boxes represent the *LacZ* gene and shaded boxes the *neomycine* selection marker. X: XbaI; S: SpeI; E: EcoRI; N: NotI restriction enzyme recognition sites. Southern probe (white boxes) and PCR primers (P20, LacZ50, REx4, REx5, P10R) are shown. *p*(A): polyadenylation site. (**B**) Genotyping of adult mice by southern blot using a 5′ probe detecting a 9.5 kb XbaI fragment and a 2.8 kb SpeI fragment in the wild-type genomic locus, and a 6.9 kb XbaI fragment and a 7.3 kb SpeI fragment in the mutant allele. (**C**) RT–PCR analysis demonstrating termination of the transcript in the targeted allele. Seven hundred and fifty-five base pairs transcript joining exon 4 to the 3′-UTR (primers REx4 5′-AAGATCGAGCACTGTGAGGC/P10R 5′-TATACAGCTGGGCCACAGAA) and 605 bp transcript joining exon 5 to the 3′-UTR (primers REx5 5′-AATGTGAAATGAGCGAGTGG/P10R) were readily detected in RNA isolated from E13.5 wild-type and heterozygote embryos, but absent in RNA from *Rspo1*$^{-/-}$ embryos. GAPDH primers were used as a positive control.

XX *Rspo1*$^{-/-}$ gonads were smaller than XY testes of the same age and resembled XX gonadal tissue in shape. Histological analysis revealed an absence of sex cords at this early age (data not shown).

At E18.5, normal seminiferous cords that contained gonocytes were present in XY *Rspo1*$^{-/-}$ gonads (Fig. 3B). XX *Rspo1*$^{-/-}$ gonads were smaller, but contained several clear seminiferous tubules in addition to some less-developed cord structures albeit with few gonocytes within. Interestingly, some gonocytes were also detected outside of cord structures and resembled quiescent G1 gonocytes that are typical for XY gonads of this age. However, sex reversal was incomplete and part of the gonads showed nests with pachytene oocytes usually found in E18.5 XX gonads.

Adult XX *Rspo1*$^{-/-}$ showed external masculinized genitalia with an increased distance from vagina to anus (data not shown). Macroscopic inspection of 10 week old XX *Rspo1*$^{-/-}$ animals demonstrated persistent Wolffian and Mullerian ducts with epididymis, vas deferens, small seminal vesicles and prostate being associated with oviduct, uterus and vaginal tissues, respectively (Fig. 3C). Strikingly, the gonads resembled small testes. Histological analyses revealed the presence of few ovarian follicles, as well as seminiferous tubules with Sertoli and Leydig cells (Fig. 3C). As expected, tubules were devoid of germ cells due to the incompatibility of XX germ cells with a male somatic environment (35).

These data demonstrate that *Rspo1* is required for female development. Its ablation impairs the differentiation into ovaries and triggers the development of ovotestes in XX mice.

Rspo1 activates β-catenin signaling during ovarian development

As shown above, β-catenin signaling is specifically activated during female sex determination. To confirm that *Rspo1* mediates this activation, we measured the levels of expression of *Lef1* by QPCR and *in situ* hybridization experiments (Fig. 4A and B). While high levels of *Lef1* were found in XX gonads at E12.5 and E14.5 (Figs 1C and 4A), expression was downregulated in the knockout tissue (Fig. 4A and B). Strikingly, using the *Axin2*$^{+/LacZ}$ reporter, we observed a complete absence of staining in E13.5 XX *Rspo1*$^{-/-}$; *Axin2*$^{+/LacZ}$ gonads (Fig. 4A). This downregulation of *Axin2* was confirmed by QPCR at E14.5 (Fig. 4A). Interestingly, in XX *Wnt4*$^{-/-}$; *Axin2*$^{+/LacZ}$ gonads some blue staining persisted in contrast to XX *Rspo1*$^{-/-}$; *Axin2*$^{+/LacZ}$ gonads (compare Figs 1D and 4A). These data suggest that while *Rspo1* is essential for ovarian β-catenin activation, Wnt4 is only contributing to this pathway. *Wnt9a* has been shown to be up-regulated during ovarian differentiation and this gene may partially complement loss of Wnt4 action (36). Using QPCR, we measured the level of expression of *Wnt9a* in XX *Rspo1*$^{-/-}$ and wild-type gonads (Fig. 4A). *Wnt9a* expression was down-regulated like *Wnt4* at E12.5.

To confirm that Rspo1 acts through the β-catenin signaling pathway, we next tested whether ectopic activation of β-catenin was able to rescue the abnormal masculinization in XX *Rspo1*$^{-/-}$ gonads. A conditional allele that allows stabilization of β-catenin by ablation of exon 3 (*Catnb*$^{ex3/+}$) has been described previously (37). To activate the floxed allele, we made use of an *Sf1:cre* line that in addition to strong gonad specific expression, shows widespread expression in the developing embryo (4). The majority of *Catnb*$^{ex3/+}$; *Sf1:cre*^{Tr} embryos died during early embryonic development due to extensive activation of the canonical β-catenin signaling pathway. However, two XX *Rspo1*$^{-/-}$; *Catnb*$^{ex3/+}$; *Sf1:cre*^{Tr} were obtained at E18.5 and adult. Macroscopic and histological analyses of the genital system of the 11-week-old female showed the presence of primordial, primary, secondary and antral follicles and an absence of sex cords, as expected for normal ovaries (Fig. 4C). Finally, nine embryos were implanted into the uterus thus further confirming the normal function of the rescued ovaries. Taken together these data show that *Rspo1* is essential for the activation of the canonical β-catenin signaling pathway.

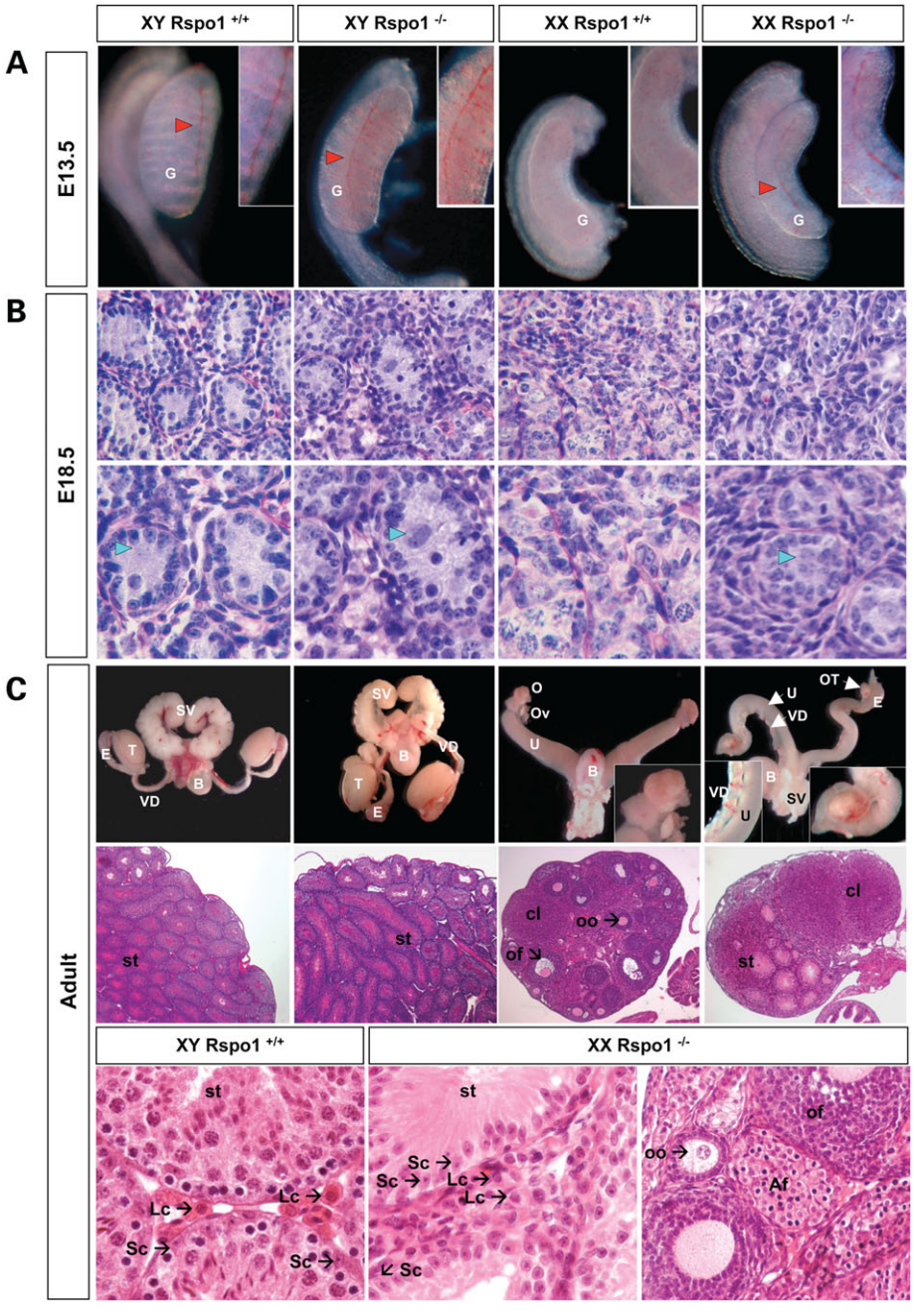
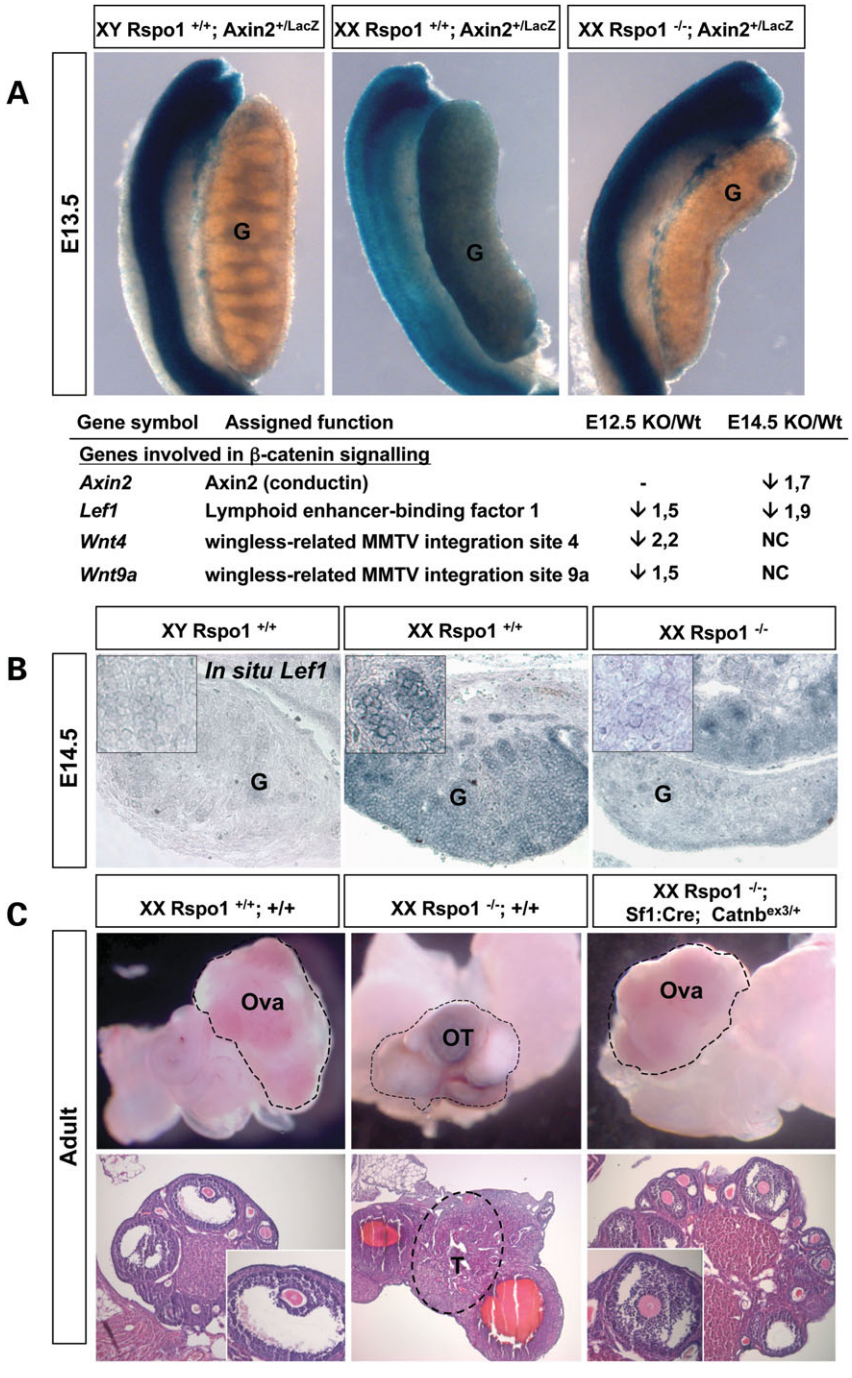


Figure 3. Masculinization of XX *Rspo1*$^{-/-}$ urogenital systems. (**A**) Mesonephroi and gonads dissected from E13.5 embryos (magnification ×20; insets ×63). In XX *Rspo1*$^{-/-}$ gonads, an ectopic coelomic vessel formed as in wild-type XY gonads (red arrowheads and insets) (G = Gonad). (**B**) PAS staining of sections from E18.5 gonads. Sex cords containing G1-quiescent gonocytes (blue arrowheads) were detected in XY *Rspo1*$^{+/+}$, XY *Rspo1*$^{-/-}$ and XX *Rspo1*$^{-/-}$ gonads and absent in XX *Rspo1*$^{+/+}$ gonads (magnification ×40 and ×63). (**C**) *Upper panels*: Macroscopic view of XX and XY urogenital systems of wild-type and *Rspo1*$^{-/-}$ mice (magnification ×5; insets at ×20). Note the persistence of Wolffian and Mullerian ducts and the presence of an ovotestis surrounded by an epididymis in XX *Rspo1*$^{-/-}$ animals (Insets and Fig. 5D). (B: bladder, E: epididymis, SV: seminal vesicles, O: ovary, Ov: oviduct T: testis, U: uterus, VD: vas deferens). *Lower panels*: Hematoxylin and eosin staining of the gonads from the same animals (magnification ×10 and ×100). St: seminiferous tubules; of: ovarian follicle; cl: corpus luteum; Sc: Sertoli cells; Lc: Leydig cells; oo: oocyte; af: atretic follicle.

Rspo1 controls *Wnt4* expression in XX gonad

To understand the relationship between *Rspo1* and *Wnt4*, we examined the level of *Wnt4* expression in our knockout model. In XX gonads *Wnt4* is activated from E11.5. At this stage, *Rspo1* is also expressed in the XX gonad whereas its expression is restricted to the coelomic epithelium of the XY gonad (Fig. 5A). *In situ* hybridizations revealed that *Wnt4* expression is down-regulated at E11.5 and E12.5 in XX *Rspo1*$^{-/-}$ gonads (Fig. 5B). In contrast, *Wnt4* expression



Gene symbol	Assigned function	E12.5 KO/Wt	E14.5 KO/Wt
Genes involved in β-catenin signalling			
Axin2	Axin2 (conductin)	-	↓ 1,7
Lef1	Lymphoid enhancer-binding factor 1	↓ 1,5	↓ 1,9
Wnt4	wingless-related MMTV integration site 4	↓ 2,2	NC
Wnt9a	wingless-related MMTV integration site 9a	↓ 1,5	NC

Figure 4. *Rspo1* activates β-catenin signaling during ovarian development. (**A**) X-Gal whole-mount staining of urogenital ridges at E13.5. While strong blue staining was detected in XX gonads, XY and XX *Rspo1*$^{-/-}$ gonads remained unstained. QPCR analysis of genes involved in the β-catenin signaling pathway in XX *Rspo1*$^{-/-}$ and wild-type gonads at E12.5 (wild type, $n = 6$; *Rspo1*$^{-/-}$, $n = 8$) and E14.5 (wild type, $n = 4$; *Rspo1*$^{-/-}$, $n = 10$). NC: no change in gene expression between KO and Wt samples. Gene expression levels were normalized against expression levels of *Hprt1*. β-catenin target genes were down-regulated in XX *Rspo1*$^{-/-}$ gonads. (**B**) *Lef1 In situ* hybridization at E14.5 (magnification ×20, inset ×40). XX gonads showed strong *Lef1* expression, whereas no signal was detected in both XY control and XX mutant gonads. (**C**) *Upper panels*: Macroscopic view of adult XX urogenital systems of wild type, *Rspo1*$^{-/-}$ and *Rspo1*$^{-/-}$; *Sf1:Cre*^{TR}; *Catnb*$^{ex3/+}$ mice (magnification ×5). The gonads are circled. Ova: Ovary; OT; Ovotestis. *Lower panels*: H&E staining of the gonads from the same animals (magnification ×10, insets ×20). The dotted line circles the region containing seminiferous tubules. No sex cords were present in the XX *Rspo1*$^{-/-}$; *Sf1:Cre*^{TR}; *Catnb*$^{ex3/+}$ ovaries whereas they exhibit ovarian follicles (antral follicles in insets) demonstrating rescue of the *Rspo1* knockout phenotype by ectopic activation of β-catenin. T: testicular part.

persisted in *Rspo1*$^{-/-}$ mesonephroi suggesting that activation in this tissue occurs by an Rspo1-independent pathway. At E14.5, expression differences between XX and XY gonads were less pronounced, but levels in XX *Rspo1*$^{-/-}$ gonads remained lower than in XX wild-type littermates (Fig. 5B and data not shown).

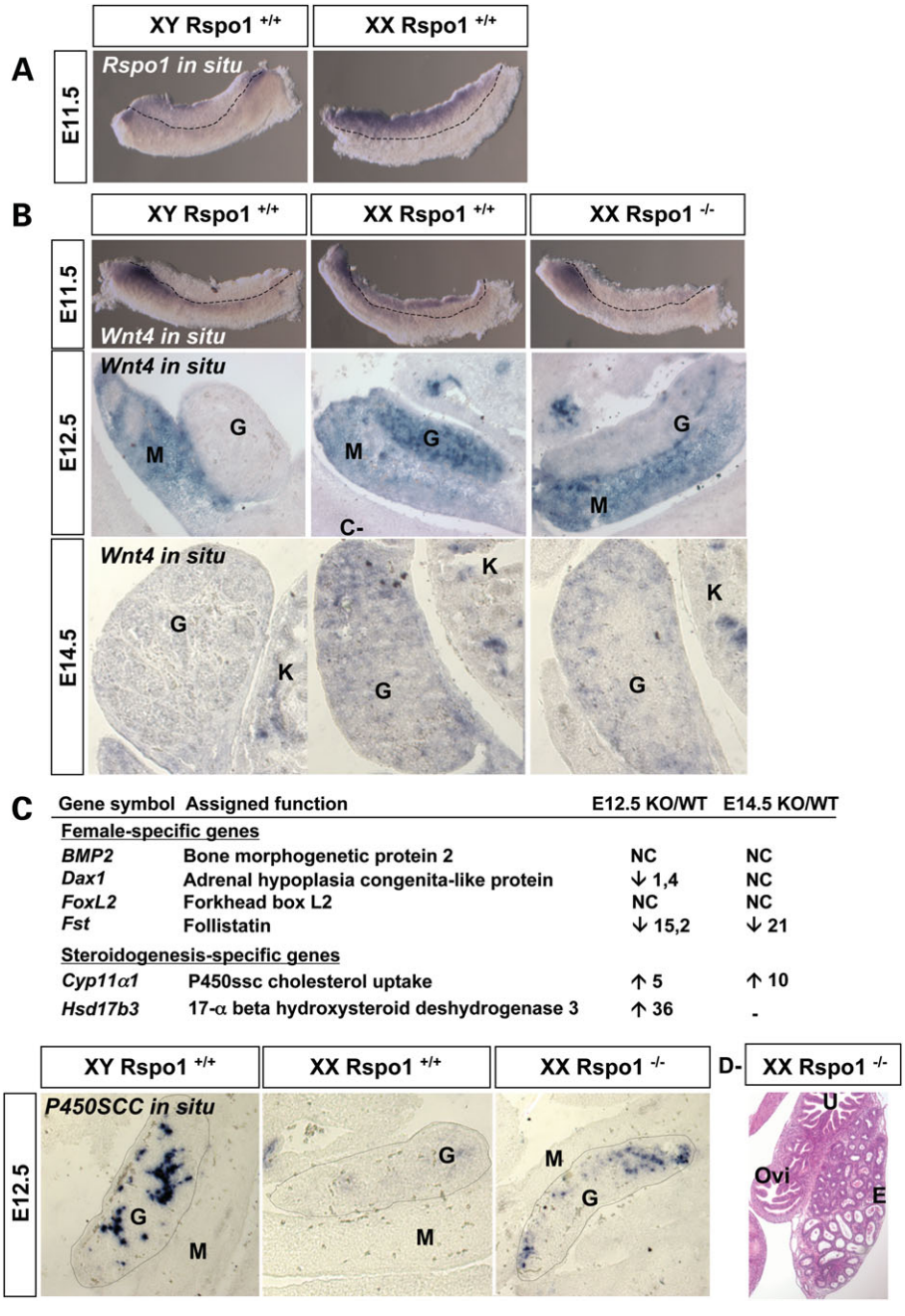


Figure 5. *Rspo1* regulates female somatic differentiation. (**A**) Whole-mount *in situ* hybridizations of *Rspo1* at E11.5 (19 tail somites (ts)). *Rspo1* was expressed throughout the XX gonads, whereas it was restricted to the coelomic epithelium in XY gonads. (**B**) Whole-mount and section *in situ* hybridization using *Wnt4* as a probe. At E11.5 (ts19) and E12.5, XX $Rspo1^{-/-}$ gonads exhibited a complete absence of staining. At E14.5, *Wnt4* expression was down-regulated in XX $Rspo1^{-/-}$ gonads. (**C**) QPCR analysis of female-specific and male steroidogenic-specific genes in E12.5 gonads (wild type, $n = 6$; $Rspo1^{-/-}$, $n = 8$) and E14.5 (wild type, $n = 4$; $Rspo1^{-/-}$, $n = 10$). NC: no change in gene expression between KO and Wt samples. Gene expression levels were normalized against expression levels of *Hprt1*. Note the down-regulation of *Fst*, a target of *Wnt4*, in $Rspo1^{-/-}$ gonads and the up-regulation of *Cyp11α1* and *Hsd17β3*, two enzymes of the male steroidogenesis pathway. *In situ* hybridizations of *P450Scc*, a steroidogenic cell marker, demonstrated ectopic expression in XX $Rspo1^{-/-}$ gonads. (**D**) Hematoxylin and eosin staining of XX $Rspo1^{-/-}$ showing the presence of epididymis and oviduct and uterus. E: Epididymis; G: Gonad; K; Kidney; M: Mesonephros; Ovi: Oviduct; U: Uterus.

As levels of *Rspo1* expression at E12.5 and E14.5 were unchanged in XX $Wnt4^{-/-}$ gonads (Fig. 1D), *Rspo1* appears to be activated independently and upstream of *Wnt4*. Taken together, these data indicate that female specific expression of *Wnt4* in gonads depends on *Rspo1*.

Rspo1 prevents XY-like steroidogenesis and vascularization

Since Rspo1 appears to act upstream of *Wnt4*, it was important to test whether pathways down-regulated in the *Wnt4* mutant

were also affected in the *Rspo1* mutant. XX *Wnt4*$^{-/-}$ gonads contain steroidogenic cells that have migrated from the mesonephros indicating that Wnt4 is required to prevent this migration in XX gonads (16). These cells synthesize steroids required for the development of the Wolffian derivatives.

In *XX Rspo1*$^{-/-}$ embryos, the development of the epididymis, vas deferens and seminal vesicles (Figs 3C and 5D) suggests the presence of steroidogenic cells within the gonad at an early stage of development. Indeed, levels of *Cyp11α1* and *Hsd17β3*, two enzymes of the steroidogenic pathway which are up-regulated in the XX *Wnt4*$^{-/-}$ gonads (18), were also increased in XX *Rspo1*$^{-/-}$ gonads at E12.5 (Fig. 5C). Similarly, *in situ* hybridizations with *P450Scc*, another marker of the steroidogenic pathway (Fig. 5C) revealed positive cells in XX *Rspo1*$^{-/-}$ and XY littermates, but not in XX gonads.

Expression of follistatin (*Fst*) is induced during early ovarian differentiation and is required to prevent coelomic vessel formation in the XX gonad (38). As expected from the male-specific vascularization observed in knockout mice (Fig. 3A), XX *Rspo1*$^{-/-}$ gonads showed a dramatic down-regulation of *Fst* as early as E12.5 (Fig. 5C).

Thus, both steroidogenic and endothelial cells show ectopic migration into XX *Rspo1* knockout gonads, a phenotype similar to that found in XX *Wnt4*$^{-/-}$ embryos (16,18).

Rspo1 regulates female germ-cell differentiation

To understand the cellular changes in *Rspo1* mutant gonads, we next analyzed the cellular localization of the dephosphorylated (active) form of β-catenin (nuclear, cytoplasmic and membrane-bound forms) within gonads using immunofluorescence. While in XY gonads, the strongest staining was found at the interface of germ cells (plasma membrane), in XX gonads it appeared mostly cytosolic and to some extent nuclear at E12.5 and 14.5 (Fig. 6A). This is consistent with the robust transcriptional activation of *Axin2* in XX somatic cells reported in Figure 1B. In XX *Rspo1*$^{-/-}$ gonads β-catenin localized at the surface of germ cells with a proportion showing a very strong and sharp signal in an XY specific manner. Expression of E-cadherin, a previously identified germ-cell marker, decreases once XX germ cells have entered meiosis at E15.5 (39). Immunostaining for E-cadherin demonstrated a marked difference between male and female gonads at E14.5 but not at E12.5 (Fig. 6A). This result suggests that β-catenin and E-cadherin are involved in maintaining adhesion between germ cells before commitment to meiosis. Taken together these data indicate that Rspo1 participates to the loss of adherent junctions in female germ cells that normally precedes meiosis.

An important feature of ovarian differentiation is the onset of germ cell meiosis at E13.5 (8,15). Histological examination revealed that the majority of germ cells did not enter meiosis in XX *Rspo1*$^{-/-}$ gonads (Fig. 3B). Indeed, expression of *Oct4*, a pre-meiotic germ-cell marker, was increased in XX *Rspo1*$^{-/-}$ compared to XX wild-type gonads at E14.5 (Fig. 6B). However, *Oct4* expression levels were lower than in XY controls confirming that only a proportion of germ cells in XX *Rspo1*$^{-/-}$ gonads failed to enter meiosis.

RA is required for the induction of meiosis. In XY gonads, induction is delayed by the expression of Cyp26b1, an enzyme that reduces local RA levels (12). In XX *Rspo1*$^{-/-}$ gonads, *Cyp26b1* showed a 2.5-fold increase compared to XX gonads, but was more than 20 times weaker than in XY gonads, which is unlikely to be sufficient to diminish the RA concentration. Moreover, QPCR and *in situ* hybridization experiments indicated that expression of *Stra8*, a downstream target of RA signaling (10,11), was not significantly changed between XX and XX *Rspo1*$^{-/-}$ (Fig. 6B). These results suggest that the RA pathway is not the main cause of the blockage of meiosis in the XX *Rspo1*$^{-/-}$ gonads.

Rspo1 is a suppressor of male sex determination

To decipher the molecular pathways involved in somatic differentiation in *Rspo1*$^{-/-}$ mice, we examined expression levels of genes with a known function during female and male sex determination.

Levels of *Bmp2* and *FoxL2*, two markers of the XX gonad, were similar between XX *Rspo1*$^{-/-}$ and wild-type gonads at E12.5 and E14.5 (Fig. 5C) suggesting that the somatic lineage has partially kept its female identity in the absence of *Rspo1*. This was expected since a part of the XX *Rspo1*$^{-/-}$ gonad exhibited ovarian characteristics with follicles in adults (Fig. 3C).

In XY gonads, activation of *Sox9* involves a combination of nuclear import of its protein and an increase of its transcription (2,40). Both of these mechanisms seem to be enhanced by prostaglandin D2, a hormone synthesized by Pgds (2). At E12.5, *Pgds*, *Sox9* and *Amh/Mis* expression were slightly increased in XX *Rspo1*$^{-/-}$ gonads (Fig. 7A and B), but insufficient to allow sex cord formation and early testis development. Insufficient levels of *Amh/Mis* were expected given the persistence of the Mullerian ducts (Figs 3C and 5D). *Fgf9* is involved in testis-specific proliferation and the maintenance of *Sox9* expression (6). At E12.5, *Fgf9* was undetectable in XX wild-type and XX *Rspo1*$^{-/-}$ gonads, which is consistent with the lack of male-specific proliferation (Fig. 7A). However, at E14.5 and E18.5 both *Fgf9* and *Pgds* levels were increased (Fig. 7A and C). Since both of these factors are able to activate Sox9, the expression of these genes in *Rspo1*$^{-/-}$ gonads is likely to contribute to the expression of *Sox9* and *Amh/Mis* expression in the absence of *Sry* (Fig. 7C).

DISCUSSION

Sex determination is a unique process that determines the differentiation of a bipotential organ, which in turn triggers the full sexual development of an individual. Here we have shown that *Rspo1* is required for *Wnt4* expression in XX gonads and acts as a key regulator of β-catenin activation in female sex determination. The ablation of these genes induces differentiation of seminiferous tubules in XX gonads [present study and (17)], thus demonstrating their requirement to suppress male sexual determination. Mechanistically, RSPO1 appears to interact with Kremen, a receptor that is involved in DKK mediated internalization of LRP6.

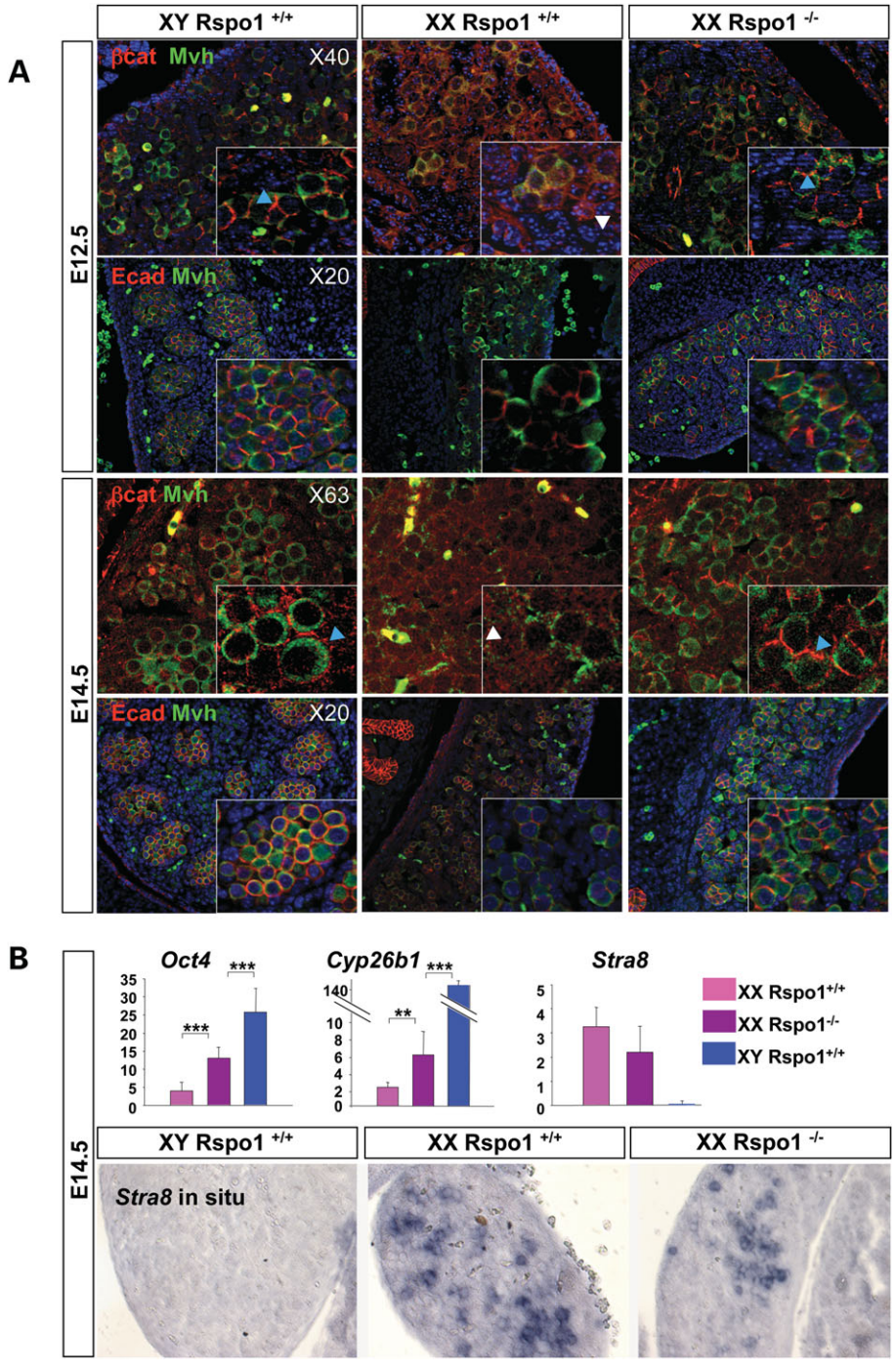


Figure 6. *Rspo1* ablation interferes with female germ cell differentiation. (**A**) Immunodetection of the dephosphorylated active forms of β-catenin (red) (*upper panel*), and Mvh (green) (*upper panel*) in gonads at E12.5 (magnification ×40). Active β-catenin was predominantly detected at the plasma membrane of XY and XX *Rspo1*$^{-/-}$ germ cells (blue arrowheads) and in the cytoplasm and nucleus of somatic XX cells (white arrowhead). Immunodetection of E-cadherin (red) and Mvh (green) in gonads at E12.5 (magnification ×20). Dapi (blue) was used to detect nuclei. Immunodetection of β-catenin (red) and Mvh (green) (*upper panel*) (magnification ×63), and E-cadherin (red) and Mvh (green) (*lower panel*) (magnification ×20) in gonads at E14.5. β-catenin was predominantly expressed at the membrane of germ cells in XY *Rspo1*$^{+/+}$ and XX *Rspo1*$^{-/-}$ (blue arrowheads), whereas it was more cytoplasmic and nuclear in XX *Rspo1*$^{+/+}$ gonads (white arrowhead). Similar as in XY gonads, germ cells of XX *Rspo1*$^{-/-}$ gonads showed strong β-catenin and E-cadherin staining at the membranes between germ cells. (**B**) Quantitative RT–PCR expression analysis of meiotic markers *Oct4*, *Cyp26b1* and *Stra8* at E14.5 (wild type, $n = 4$; *Rspo1*$^{-/-}$, $n = 10$). Gene expression levels were normalized against expression levels of *Mvh* (for *Oct4* and *Stra8*) and *Hprt1* (for *Cyp26b1*). *Oct4* and *Cyp26b1* expression levels were significantly increased in XX *Rspo1*$^{-/-}$ gonads compared to their XX wild-type littermates, but did not reach those observed in XY control gonads. *Stra8* expression levels were not significantly changed in *Rspo1*$^{-/-}$ gonads. Asterisks indicate levels of statistical significance: two asterisks, $P < 0.01$; three asterisks, $P < 0.0001$. *In situ* hybridization analysis confirmed unchanged expression levels of *Stra8* in XX *Rspo1*$^{-/-}$ gonads.

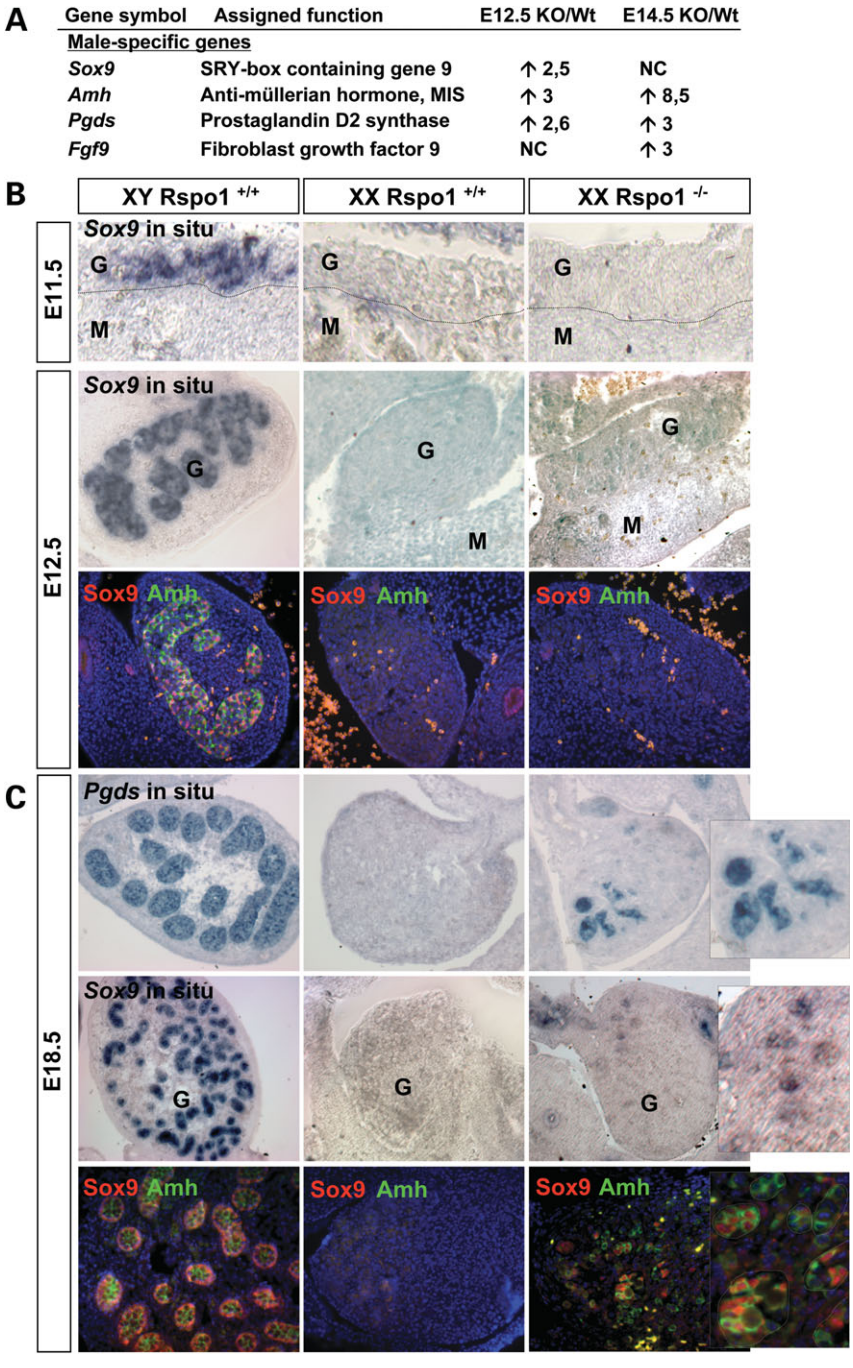


Figure 7. *Rspo1* is a suppressor of male sex determination. (**A**) QPCR expression analysis of male-specific genes in E12.5 and E14.5 gonads (wild type, $n = 4$; *Rspo1*$^{-/-}$, $n = 10$). Gene expression levels were normalized against expression levels of *Hprt1*. Note the up-regulation of genes in XX *Rspo1*$^{-/-}$ gonads that are normally specifically expressed in XY gonads. NC: no change in gene expression between KO and Wt samples. (**B**) *In situ* hybridization of *Sox9* and immunofluorescence analysis of Sox9 and Amh/Mis at E11.5 and 12.5 (magnification ×20, insets at ×40). Sox9 and Amh/Mis were undetectable in XX *Rspo1*$^{+/+}$ and XX *Rspo1*$^{-/-}$ gonads. (**C**) *In situ* hybridization of *Pgds*, *Sox9* and immunofluorescence analysis of Sox9 and Amh/Mis at E18.5 (magnification ×20, insets at ×40). XX *Rspo1*$^{-/-}$ gonads exhibited *Pgds*, Sox9 and Amh expression in sex cords. G: gonad, M: mesonephros; K: kidney.

This interaction inhibits LRP6 internalization thus providing increased receptor levels at the cell surface (26). Ablation of *Rspo1* has a more dramatic effect on β-catenin activation than *Wnt4*, which suggests the existence of functional redundancy between Wnt ligands. Alternatively, *Rspo1* may activate β-catenin signaling in a Wnt-independent manner. *In vitro* RSPO1 binding induces LRP6 phosphorylation, which in turn favors stabilization of cytosolic β-catenin (41). Interestingly, LRP6 ablation has been described to trigger abnormal ovary development (42). To our knowledge, the gonadal

phenotype in *Lrp6*$^{-/-}$ animals has not been analyzed in detail and it will be interesting to address whether aspects of the Rspo1 knockout phenotype is recapitulated in this mutant.

Once in the nucleus, β-catenin can interact with transcription factors, such as Lef/TCF, which trigger the transcriptional regulation of target genes. While *Lef1* is clearly up-regulated in female gonads (this study), so far no gonadal phenotype has been reported in *Lef1* knockout mice and it is likely that at least part of the transcriptional control of β-catenin is mediated through its interaction with other, yet unidentified cofactors.

We have previously shown that *Rspo1* is expressed in XX somatic cells of the gonad (23). Here we show that this secreted protein activates the canonical β-catenin signaling pathway in the somatic cells. Absence of *Rspo1* in XX gonads prevents up-regulation of *Wnt4* and results in the presence of steroidogenic cells and the formation of a coelomic vessel. As XX *Wnt4*$^{-/-}$ gonads also show this phenotype, it is likely that ectopic migration of steroidogenic and endothelial cells is the result of *Wnt4* down-regulation in the *Rspo1*$^{-/-}$ embryos.

The second striking observation is that some germ cells fail to enter meiosis. Commitment to meiosis starts at E13.5 in females and is stimulated by the RA pathway and its regulator *Stra8* (10,12). In XX *Rspo1*$^{-/-}$ gonads, the RA signaling pathway does not seem to be dramatically affected. In addition, as XX *Stra8*$^{-/-}$ mice do not exhibit sex reversal (11) the partial sex reversal phenotype in *Rspo1*$^{-/-}$ mice is unlikely to be due to a simple block of meiosis.

At E12.5 immunofluorescent staining for β-catenin and at E14.5 β-catenin and E-cadherin show that cell–cell adhesion complexes between germ cells in XX *Rspo1*$^{-/-}$ animals are more abundant than in XX controls and are organized in a similar manner as in the XY gonad. These changes of adherens junctions are likely to be involved in the commitment to meiosis.

Indeed, E-cadherin has been shown to be down-regulated in XX meiotic embryonic germ cells (39). Down-regulation of cell–cell adhesions can be orchestrated by different factors. One pathway involves tyrosine kinase receptors (43). Interestingly, the receptors ErbB2 and ErbB3 are both expressed in germ cells and are down-regulated at E14.5 suggesting that they might be involved in meiosis commitment (44). Whatever the pathway, our data suggest that *Rspo1* is regulating germ-cell fate. To know whether this regulation is direct, or whether it involves a secondary signal that is released from somatic cells after activation of β-catenin, warrants further investigation. Misregulation of the XX germ-cell fate in XX *Rspo1*$^{-/-}$ gonads results in the presence of G1 gonocytes that are specific to the embryonic testis.

XX meiotic germ cells have been shown to prevent sex cord formation (15) suggesting that the identity of germ cells as meiotic oogonia is required to block masculinisation of the somatic lineage. The lack of such an identity of some germ cells in the *Rspo1*$^{-/-}$ gonads can allow partial sex reversal to occur. Thus, the occurrence of these gonocytes is followed by masculinization of somatic supporting cells and formation of sex cords around birth. Gonocytes release signals that induce supporting cells to differentiate into Sertoli cells (14). The effector appears to be prostaglandin D2 that is synthetized by *Pgds* and is able to activate *Sox9* (5). We have shown that *Pgds* is upregulated in early XX *Rspo1*$^{-/-}$ gonads and a strong expression is detected at E18.5. High levels of expression of *Sox9* and the formation of sex cords in XX *Rspo1*$^{-/-}$ gonads appear to be secondary events, which could be triggered by the ectopic expression of *Pgds. Sox9* and *Pgds* regulate each other in a positive feed-back loop (5,6) and the expression of these genes has to reach a critical threshold to allow sex cord formation. However, we cannot exclude that other signals such as steroids are responsible for masculinization of the somatic cells.

As shown above, Rspo1 is involved in the female specific up-regulation of *Wnt4*. Rspo1 knock-out animals partially recapitulate the phenotype of the *Wnt4* mutants. However, *Wnt4* is also required for the formation of the Mullerian duct, a process that is not under the control of Rspo1. As a result *Rspo1*$^{-/-}$ animals show hermaphroditism of the ducts. It is noteworthy that 90% of XX *Wnt4*$^{-/-}$ germ cells die before birth, whereas their number is grossly normal in XX *Rspo1*$^{-/-}$ gonads. While sexually dimorphic expression of *Wnt4* in the early embryo is under control of *Rspo1*, levels are normal from E14.5 onwards. *Wnt4* expression at this age is therefore likely to be involved in germ-cell survival.

In this study, we show that *Rspo1* acts at the top of the female sex determination pathway. However, ablation of *Rspo1* results in formation of an ovotestis. This is likely due to the partial and late differentiation of the Sertoli lineage. At the molecular level, this could be due to the involvement of an *Rspo1*-independent pathway. *FoxL2* is also involved in ovarian differentiation. Although *Foxl2* expression is independent of *Rspo1*, XX *Wnt4*$^{-/-}$ *FoxL2*$^{-/-}$ show a gonadal phenotype at birth (45) that is comparable to *Rspo1*$^{-/-}$ mutants. This raises the question whether mutations of the regulator of *Rspo1* and/or *FoxL2* would have a more dramatic effect. The future identification of this regulator will address this question.

Taken together, our results indicate that while the male pathway is directed through somatic events, female differentiation requires a crosstalk between somatic and germinal lineages. Moreover, our data support a model in which sex determination is controlled by a balance between two very different pathways (6). *Sry* is clearly a major weight that induces a cascade resulting in male differentiation. Here we have shown that Rspo1 induces β-catenin signaling to tip the balance to the female side.

MATERIALS AND METHODS

Generation of *Rspo1*$^{-/-}$ mice

A targeting vector was constructed by fusing a *LacZ* gene from pCH110 (Promega) followed by a neomycine resistance in frame into exon3 of *Rspo1* (details on request). After electroporation clones were selected on G418 (400 μg/ml), and homologous recombination events identified by PCR P20 (5′-TTTGATGCCTGACCCCTGAG) and LacZ50 (5′-AATATCGCGGCTCATTCGAGG-3′) and confirmed by Southern blotting. Blastocyst injections allowed the generation of two independent lines using C57/Bl6J donors.

Mouse strains

Wnt4 knock-out mice were generated by (46), $Axin2^{+/LacZ}$ transgenic mice by (31). Mice carrying the *β-catenin* exon 3 floxed allele ($Catnb^{ex3/+}$) generated by (37) were mated with $Rspo1^{+/-}$ mice. $Sf1{:}Cre^{TR}$ mice previously described by (4) were mated with $Rspo1^{+/-}$; $Catnb^{ex3/+}$ mice to obtain $Rspo1^{-/-}$; $Sf1{:}Cre^{TR}$; $Catnb^{ex3/+}$ embryos.

Genotyping of embryos and mice

The *Rspo1* alleles were determined using primers P2 (5′-ATCCAGGGTCCCTCTTGATC-3′) and P12 (5′-TTGA GGCAACCGTTGACTTC-3′) for the wild type, and primers P2 and LacZ50 (see above) for the mutant. $Axin2^{+/LacZ}$ mice were genotyped using forward *Axin2* primer 5′-AAGC TGCGTCGGATACTTGAGA-3′, reverse *Axin2* primer 5′-AG TCCATCTTCATTCCGCCTAGC-3′ and reverse *LacZ* primer 5′-TGGTAATGCTGCAGTGGCTTG-3′ (sequences kindly provided by Michel V. Hadjihannas). *Wnt4* and $Catnb^{ex3/+}$ alleles were identified as published (37,46). The presence of the *Sf1:Cre* transgene and the presence of the Y chromosome (Sry PCR) were determined as described in (4).

X-gal staining, histological and immunological analyses

X-Gal staining was performed according to (47).

Urogenital organs were dissected and fixed either in Bouin's solution or in 4% paraformaldehyde overnight at 4°C, then dehydrated, placed in xylene and paraffin embedded. Sections of 7 μm thickness were performed and HE staining, or PAS staining performed as published (34). Immunohistochemical experiments were performed as described (4). The following dilutions of primary antibodies were used: Sox9 (provided by Michael Wegner), 1:1000, Amh (C-20, cat sc6886, Santa Cruz), 1:100, E-cadherin (cat 610182, BD Transduction Laboratory), 1:100, DDX4/Mvh (cat 13840, Abcam), 1:200, β-catenin (anti-ABC clone 8E7, cat 05-665, Upstate).

In situ hybridization

In situ hybridizations on paraformaldehyde fixed/paraffin embedded sections or on wholemount embryos were carried out essentially as described (4). *Sox9* riboprobes were synthesized according to da Silva *et al.* (48). The plasmids used to synthetize *Wnt4*, *Lef1, Stra8* and *Pgds* riboprobes were from Seppo Vainio, Jörg Hülsken, David Page and Peter Koopman, respectively.

Quantitative PCR analysis

Individual gonads without mesonephros were dissected in PBS from E12.5 and 14.5 embryos and immediately frozen at −80°C. RNA was extracted using the RNeasy Qiagen kit, and reverse transcribed using the RNA RT−PCR kit (Stratagene). Primers and probes were designed by the Roche Assay design center (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). *Amh*: primers 5′-ggggagactggagaacag c-3′ and 5′-agagctcgggctcccata-3′ (probe 41), *Axin2*: 5′-gcag gagcctcacccttc-3′ and 5′-tgccagtttctttggctctt-3′ (probe 50), *Bmp2*: 5′-cggactgcggtctcctaa-3′ and 5′-ggggaagcagcaacacta ga-3′ (probe 49), *Cyp11α1*: 5′-aagtatggccccatttacagg-3′ and 5′-tggggtccacgatgtaaact-3′ (probe 104), *Cyp26b1*: 5′-acatcca ccgcaacaagc-3′ and 5′-gggcaggtagctctcaagtg-3′ (probe 41), *Dax1*: 5′-cgtgctctttaacccagacc-3′ and 5′-ccggatgtgctcagtaa gg-3′ (probe 3), *Emx2*: 5′-cacgcttttgagaagaacca-3′ and 5′-gttct ccggttctgaaacca-3′ (probe 38), *Fgf9*: 5′-tgcaggactggatttc atttag-3′ and 5′-ccaggcccactgctatactg-3′ (probe 60), *Foxl2*: 5′-ggcgtcgtgaactcctaca-3′ and 5′-tgcagatgatgtgcgtga g-3′ (probe 51), *Fst*: 5′-tggattagcctatgagggaaag-3′ and 5′-tggaa tcccataggcatttt-3′ (probe 47), *Hprt1*: 5′-tcctcctcagaccg ctttt-3′ and 5′-cctggttcatcatcgctaatc-3′ (probe 95), *Hsd17β3*: 5′-aatatgtcacgatcggagctg-3′ and 5′-gaagggatccggttcagaat-3′ (probe 5), *Lef1*: 5′-tcctgaaatccccaccttc-3′ and 5′-acccgtgatggga taaacag-3′ (probe 94), *Mvh*: 5′-ccaagatcaggggacacagt-3′ and 5′-ctttggtaagtgtcaccattgc-3′ (probe 77), *Oct4*: 5′-gttggag aaggtggaaccaa-3′ and 5′-ctccttctgcagggctttc-3′ (probe 95), *Pgds*: 5′-ggctcctggacactacacct-3′ and 5′-atagttggcctccac cactg-3′ (probe 89), *Rspo1*: 5′-cgacatgaacaaatgcatca-3′ and 5′-ctcctgacacttggtgcaga-3′ (probe 5), *Sox9*: 5′-cagcaagactctgg gcaag-3′ and 5′-tccacgaagggtctcttctc-3′ (probe 66), *Stra8*: 5′-accgtggtggccttaaaga-3′ and 5′-atcatcactgggttggttgc-3′ (probe 80), *Wnt4*: 5′-ctggactccctccctgtctt-3′ and 5′-atgcccttgt cactgcaaa-3′ (probe 62), *Wnt9a*: 5′-acctcgtgggtgtgaaggt-3′ and 5′-acctcgtggaagggtgcta-3′ (probe 62). All real-time PCR assays were carried out using the LC-Faststart DNA Master kit Roche. QPCR was performed on cDNA from one gonad and compared to a standard curve. The number of gonads per genotype is given in the Figure legends. QPCR were repeated at least twice. Relative expression levels of each sample were determined in the same run and normalized by measuring the amount of *Hprt1* cDNA or of *Mvh* cDNA (to normalize germ-cell specific genes). The results were analyzed using Sigma plot and Graphpad for statistical relevance.

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Conflict of Interest statement. None declared.

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