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# THÈSE DE DOCTORAT

**Analyse de la voie femelle RSPO/WNT/ $\beta$ -Catenin et de son interaction avec la voie mâle SRY/SOX9 lors de la détermination du sexe chez la souris**

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**Présentée en vue de l'obtention du grade de:**

Docteur en Sciences de la Vie et de la Santé  
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**Analyse de la voie femelle RSPO/WNT/ $\beta$ -Catenin et de son interaction avec la voie mâle SRY/SOX9 lors de la détermination du sexe chez la souris**

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## RESUME

La détermination du sexe est un processus très sophistiqué et ordonné où les gonades mâles et femelles se développent à partir d'une gonade bipotentielle commune en fonction des voies de signalisation activées. Les gonades XY évoluent en testicule favorisé par la voie SRY/SOX9, alors que les gonades XX se développent en ovaire sous l'influence de la voie RSPO1/WNT4/ $\beta$ -Caténine. Les gènes R-spondine (*Rspo*) codent pour un type de protéines sécrétées qui activent la voie canonique WNT/ $\beta$ -caténine en inhibant la dégradation des récepteurs WNT. Après s'être liée à ses récepteurs LGR4/5, RSPO1-LGR4/5 recrute les Ubiquitine-ligases ZNRF3 et RNF43 pour libérer les récepteurs WNT de la dégradation provoquée par le processus d'ubiquitination. Le signal WNT/ $\beta$ -caténine peut donc être activé en continu. *Rspo1* est un régulateur important du développement des ovaires chez diverses espèces. Dans la gonade de souris en développement, *Rspo1* est principalement exprimé dans les cellules de soutien femelle et les gonades mutantes *Rspo1* XX subissent une inversion de sexe en se développant en ovotesticules, des gonades présentant à la fois des caractéristiques mâles et femelles. Les mécanismes moléculaires et cellulaires à l'origine de cette inversion partielle du sexe sont peu connus. Dans ce travail, nous avons développé un nouveau modèle murin permettant une mutation conditionnelle de *Rspo1*. Nous avons montré que *Rspo1* est requis dans la gonade de souris XX autour de E11,5 et que sa fonction n'est plus essentielle pour la différenciation ovarienne à des stades ultérieurs. Nous avons mis en évidence que la stéroïdogénèse ectopique est un événement précoce dans les modifications phénotypiques des gonades XX *Rspo1* mutantes. Grâce à l'inhibition pharmacologique du récepteur aux androgènes, nous avons identifié la voie de signalisation des androgènes comme un acteur majeur de l'inversion sexuelle des gonades XX *Rspo1* mutantes.

Dans la deuxième partie de ce travail, nous avons étudié le phénotype des doubles mutants *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>*, dans lesquels aussi bien les voies mâle que femelle sont altérées. Nous avons constaté que les gonades XX *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>* se développaient sous la forme d'ovotesticules, ce qui indique que la perte de fonction de *Sox9* ne permet pas de sauver l'inversion sexuelle causée par la mutation *Wnt4*. Nous avons également montré que les mutants XY *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>* subissent une phase de développement transitoire de type femelle avant que les gonades ne se développent en ovotesticules. Ce résultat démontre que la perte de *Wnt4* ne peut pas sauver les étapes initiales de l'inversion sexuelle causée par la mutation *Sox9*.

Ces résultats révèlent la fenêtre d'action temporelle de *Rspo1* dans le développement ovarien et mettent en évidence le rôle masculinisant de la signalisation androgénique dans le processus d'inversion sexuelle femelle-mâle. Nos travaux suscitent également de nouvelles réflexions sur les interactions entre les voies mâle et femelle dans la détermination du sexe chez la souris.

**Mots-clés:** Inversion sexuelle, Androge, *Rspo1/Wnt4*, *Sry/Sox9*

## ABSTRACT

Sex determination is a highly sophisticated and ordered process where both male and female gonads develop from a common bipotential gonad depending on different activated signaling pathways. XY gonads develop into a testis promoted by the SRY/SOX9 pathway, whereas XX gonads develop into an ovary through the action of the RSPO1/WNT4/ $\beta$ -Catenin pathway. R-spondin (*Rspo*) genes encode one kind of secreted proteins that activate the canonical WNT/ $\beta$ -Catenin pathway by inhibiting the degradation of WNT receptors. After binding to its receptors LGR4/5, RSPO1-LGR4/5 recruit E3 Ubiquitin-Protein Ligases ZNRF3 and RNF43 to release WNT receptors from being degraded by ubiquitination process, therefore WNT/ $\beta$ -Catenin signaling can be activated continuously. *Rspo1* is a major regulator of ovary development across species. In the developing mouse gonad, *Rspo1* is mainly expressed in the female supporting cells, and *Rspo1* XX mutant gonads undergo female-to-male sex reversal by developing into ovotestis, gonads with both male and female characteristics. The molecular and cellular mechanisms behind this partial sex reversal remain unclear. In this work, we have developed a new mouse model allowing a conditional mutation of *Rspo1*. We have established that the critical window for *Rspo1* requirement in the developing XX mouse gonad is around E11.5, and that *Rspo1* function is dispensable for ovarian differentiation after this time point. We have shown that ectopic steroidogenesis is an early event in the phenotypic changes of XX *Rspo1* mutant gonads. Through pharmacological inhibition of the androgen receptor we have identified androgen signaling pathway as a major player in the female-to-male sex reversal of XX *Rspo1* mutant gonads.

In the second part of this work we have studied the phenotype of *Sox9<sup>CKO</sup>Wnt4<sup>CKO</sup>* double mutants where both the male and female pathway are impaired. We have found that XX *Sox9<sup>CKO</sup>Wnt4<sup>CKO</sup>* gonads develop as ovotestis indicating that the additional deletion of *Sox9* did not rescue the female-to-male sex reversal caused by the *Wnt4* mutation. We have also shown that XY *Sox9<sup>CKO</sup>Wnt4<sup>CKO</sup>* double knockout mutants undergo a transient female-like developmental phase before the gonads develop into ovotestis. This result demonstrates that *Wnt4* deletion cannot rescue the initial steps of the male-to-female sex reversal caused by the *Sox9* mutation.

Together, these results reveal the timing of requirement of *Rspo1* in ovarian development and highlight the pro-male role of androgen signaling in the XX female-to-male sex reversal process. It also rises new thoughts on the interactions between male and female pathway in mouse sex determination.

**Key words:** Female-to-male sex reversal, Androgen, *Rspo1/Wnt4*, *Sry/Sox9*

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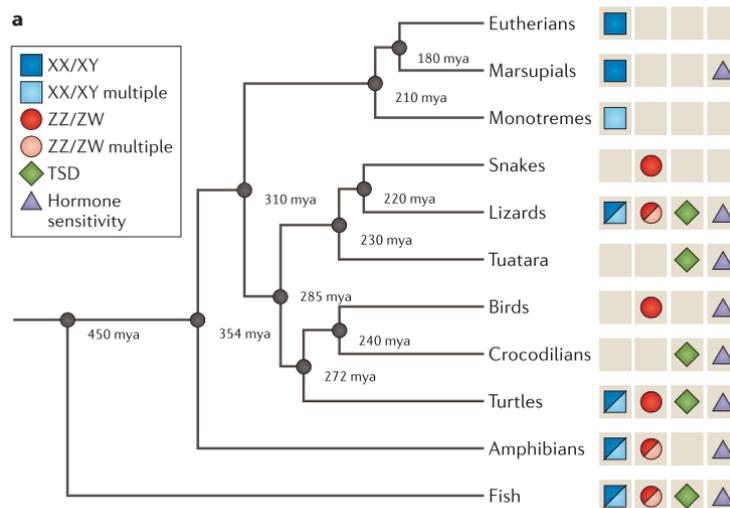
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# CHAPTER 1: INTRODUCTION

## 1. Sex determination in vertebrates

Primary sex determination or gonadal sex determination is the process by which the bipotential gonad makes its developmental decision to become a testis or an ovary. Sex determination across vertebrate species can be divided into genetic sex determination (GSD), environmental sex determination (ESD) and the joint-regulation by GSD and ESD (Ezaz et al., 2006a; Holleley et al., 2015). Specifically, GSD can be further divided into XY or ZW heteromorphic sex chromosomes systems, where XY and ZZ animals can develop into males. In some rare species, who do not have sex chromosomes, a conserved sex determination related gene can be found on an autosome (Capel, 2017; DiTacchio et al., 2012; Otake and Kuroiwa, 2016). ESD mainly includes temperature-dependent sex determination (TSD), hormone susceptibility, and other environmental effects from population density, toxicants, behavioural cues and nutrients. Birds and mammals in endotherms and snakes and amphibians in ectotherms mainly exhibit the GSD-regulated sex determination systems, whereas the sex of turtles, lizards and fish in ectotherm can be regulated by both GSD and ESD systems (Fig. 1) (Capel, 2017; Ezaz et al., 2006b).



**Figure 1.** Sex determination systems across the vertebrate phylogenetic tree: genetic sex determination (GSD), temperature-dependent sex determination (TSD) and hormone sensitivity. (Capel, 2017).

## 1.1 XY sex determination system in most mammals

In mammals, XY heteromorphic sex chromosomes provide a system where XX individuals develop as females, whereas XY individuals develop as males. This system mainly depends on the existence of a Y chromosome in the animal, and this chromosome holds the male sex determining gene *Sry*, which encodes a transcription factor that launches the testis development from the bipotential gonad (Koopman et al., 1991; Sinclair et al., 1990). In the absence of a pro-male trigger from Y chromosome, XX gonads develop as ovaries. The molecular mechanism study of XY heteromorphic sex determination system started from the end of 1950s, when Y chromosome was confirmed to be the key sex chromosome for testis development in the Klinefelter syndrome (47,XXY male) and Turner syndrome (45,X female) patients (Ford et al., 1959; JACOBS and STRONG, 1959). In the next few decades, searching for the testis-determining factor (TDF) on Y chromosome became a focus of sex determination study. In 1990, Goodfellow and Lovell-Badge identified a gene named "sex determining region of the Y" (*Sry*), from a 35 kb region of the Y-chromosome present in 4 XX male patients (Sinclair et al., 1990). *Sry* is conserved in mammals and encodes an HMG-box domain containing protein. *Sry* was confirmed to be necessary and sufficient for testis development by the study of XY females in both humans and mice harboring mutations of *SRY/Sry* and the analysis of XX transgenic male mice where a 14-kb fragment containing *Sry* had been inserted (Berta et al., 1990; Gubbay et al., 1990; Koopman et al., 1991; Lovell-Badge and Robertson, 1990; Vilain et al., 1992).

## 1.2 ZW sex determination system in birds

Sex determination of birds also depends on the constitution of sex chromosomes, but unlike mammals, birds use a ZZ/ZW system. In the ZZ/ZW system, heterogametic ZW individuals develop as females, while ZZ individuals develop as males. Z/W sex chromosomes in birds originate from a different pair of autosomes than X/Y chromosomes in mammals (Graves, 2016). Sex determination in birds thus depends on the dosage of a gene named doublesex and mab-3 related transcription factor 1

(*Dmrt1*) on the Z chromosome. ZZ males develop with two copies of *Dmrt1*, while ZW develop as females with only one copy. Ectopically expressing *Dmrt1* in ZW can induce male development in chicken, whereas knockdown of *Dmrt1* expression by shRNA results in male-to-female sex reversal in ZZ animals (Lambeth et al., 2014; Smith et al., 2009). Interestingly, despite the ZZ/ZW genetic system, the exposure of ZZ/ZW chicken eggs to oestrogen or aromatase inhibitors at the critical period during gonad formation leads to sex reversal (Hudson et al., 2005; Müller et al., 2011). Most of the egg-laying vertebrates are sensitive to oestrogen (Fig 1). Besides, the ZZ/ZW chimaeras can develop as gynandromorphs: the side with a higher percentage of ZZ cell develops into male with male sexual characteristics, while the other side with more ZW cells develops into female (Arnold, 2004; Arnold et al., 2013; Zhao et al., 2010).

### **1.3 Mixed sex determination system in fish**

The sex determination system of fish is more complicated: some fish species use the XX/XY male heterogametic system like the mammals but evolved independently, such as medaka, pejerrey and rainbow trout; some species employ the ZZ/ZW system, like the Chinese tongue sole; and many closely related species use both types of systems, including ricefish, stickleback and tilapia (Cnaani et al., 2008; Mank and Avise, 2009; Ross et al., 2009; Takehana et al., 2008). The male determination gene in the XX/XY system also differs among species: *Oryzias* genus medaka, latipes and curvinotus depend on the somatic sex determination *Dmrt1* related gene (*Dmy*) on the proto-Y chromosome (Matsuda et al., 2002; Nanda et al., 2002), whereas the male development of indian ricefish *Oryzias dancena* depends on *Sox3* (Takehana et al., 2014). There are also some fish species such as *Danio rerio* or zebrafish raised in the laboratory, who do not have sex chromosomes but instead several genes reported to be able to determine the sexual fate (Anderson et al., 2012; Bradley et al., 2011). Notably, the male development of wild zebrafish strains is strongly determined by the ZZ/ZW system, in which the chromosome 4 with a male fate-linked single nucleotide polymorphism (SNP) acts as the sex chromosome (Wilson et al., 2014). The sex

determination of some fish species can also be co-regulated by both GSD and ESD mechanisms. The main environmental sex-modifying elements include temperature, visual cues and population density (see examples in 1.5 section).

#### **1.4 Environmental sex determination (ESD)**

As a classical ESD, temperature-dependent sex determination (TSD) has been mostly studied in reptiles. The gender of their offspring depends on the incubation temperature during the gonad formation period (Bull, 1980). A ~5 °C difference could switch the gender of >90% offspring to male or female. Some species like the red-eared slider turtle gives birth to female offspring at higher incubation temperature, whereas the American alligator gives birth to male offspring at higher incubation temperature. For some other species like the leopard gecko, almost all female offspring are produced by the extreme temperature, and male can be produced with different ratios when incubating at intermediate temperature (Quinn et al., 2011). The mechanism behind the temperature-dependent sex determination is not well known across species, but the sex determination in any species is not likely to be only controlled by TSD. In between incubation temperature control and the determination of sex, there must exist a threshold balancing the two opposite male and female pathways and certain temperature serves as a trigger to break the threshold genetically and tile to male or female development (Ezaz et al., 2009; Holleley et al., 2016; Mork et al., 2014). It has been shown that in the red-eared slider turtle, the histone H3 lysine 27 (H3K27) demethylase (KDM6B) is specifically expressed in the gonads which were incubated at the male-determining temperature. Knocking out *Kdm6b* lead to male-to-female sex reversal, even though the eggs were incubated in the male-determining temperature. KDM6B could eliminate the H3K27 trimethylation near the promoter of sex determination gene *Dmrt1* to promote the transcription of *Dmrt1*. *Dmrt1* overexpression could rescue the male-to-female sex reversal caused by the loss of *Kdm6b* (Ge et al., 2018).

## 1.5 Spontaneous sex reversal

The sex of fish can be regulated by XX/XY or ZZ/ZW and environmental factors, and the sex development of fish shows very high plasticity. The laboratory zebrafish belongs to gonochoristic species, but before final differentiation to male or female, all larval fish show a transient hermaphrodite stage and produce oocytes (Dranow et al., 2013; Orban et al., 2009). More interestingly, some fish species can exchange their sex between male and female during the adult life. The exchange can be protandrous, protogynous and bidirectional, which also depends on the environment, developmental stage and social cues. In a social group, besides the fertile females, there is only one dominant male and other males are all immature by suppression from the dominant male. Removing or blocking the dominant male from view leads to another fish developing into a dominant male. This new dominant male can come from the immature male or from a mature female through sex reversal process, depending on their size and social rank (Fishelson, 1970; Lntnesky, 1994; Warner and Swearer, 1991). This sex exchanges during adult life are usually mediated by visual and neuroendocrine systems (Capel, 2017). No matter the protogynous or protandrous change, it is necessary to switch off the existing transcriptional network to release the suppression of the opposite network (Liu et al., 2015b).

Although there is no functional sex reversal reported so far in mammals, genetically induced sex reversal and some examples of seasonal plasticity show some degree of plasticity in the gonads of mammals. The examples for genetically induced sex reversal are presented in the introduction section 2.2 and 5. Female moles (*Talpa occidentalis*) are one example for seasonal plasticity. Female moles only breed as female, but during the non-breeding season, they become aggressive through the abnormal secretion of sex hormones from the reversible masculinized gonad and their external genitalia also show partially masculinization (Jimenez et al., 1996; Jiménez et al., 1993, 2013). The lack of spontaneous sex reversal in adult mammals is likely due to the more complexed reproductive system and the mutual antagonism between male and female pathways.

## 2. Gonad formation in mouse

Gonads are the primary reproductive organs for male and female. In adulthood, testes and ovaries are responsible for producing male and female gametes, respectively. As endocrine tissues, testes and ovaries also produce sex hormones to maintain the development of reproductive organs and structures and gametes from fetal stage to adulthood. Gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secreted from the pituitary can stimulate the production of sex hormones in the gonads. LH promotes the production of testosterone in testis and progesterone in ovary, and FSH can help the sperm production in males and ovarian follicle maturation in females.

Around mid-gestation in mice, a pair of bipotential long and narrow gonads are formed along the anteroposterior axis by the thickening of the coelomic epithelium of the intermediate mesoderm. Right after the Primordial Germ Cells (PGCs) have reached the gonad around E10.5, the bipotential gonads start sex determination with the up-regulation of *Sry* in the XY supporting cells. At E12.5 the male and female gonads can be morphologically distinguished: the testis exhibits a short rod-shape with a prominent celomic vessel whereas the ovary is long and narrow and does not exhibit a prominent vasculature. Endothelial cells of the testis and ovary originate from the adjacent mesonephros (Martineau et al., 1997), but more endothelial cells migrate into the testis than into the ovary to form the male specific celomic vessel. Supporting cells (Sertoli cells and granulosa cells) can both secret Activin B (Rotgers et al., 2018), which promotes vasculature formation. In testes, the specific vasculature is formed around E12.5, whereas the presence of WNT4 and its down-stream target *Fst* inhibit Activin B activity in the ovary (Yao et al., 2004). XX mutants for *Wnt4* or *Fst* exhibit ectopic vasculature, and additionally deleting the *Inhbb* gene (encoding a subunit of Activin B) could rescue this phenotype (Yao et al., 2006). From E12.5 onwards the testis is organized into testis cords where germ cells are surrounded by one layer of supporting Sertoli cells. The interstitium between the testis cords contains the

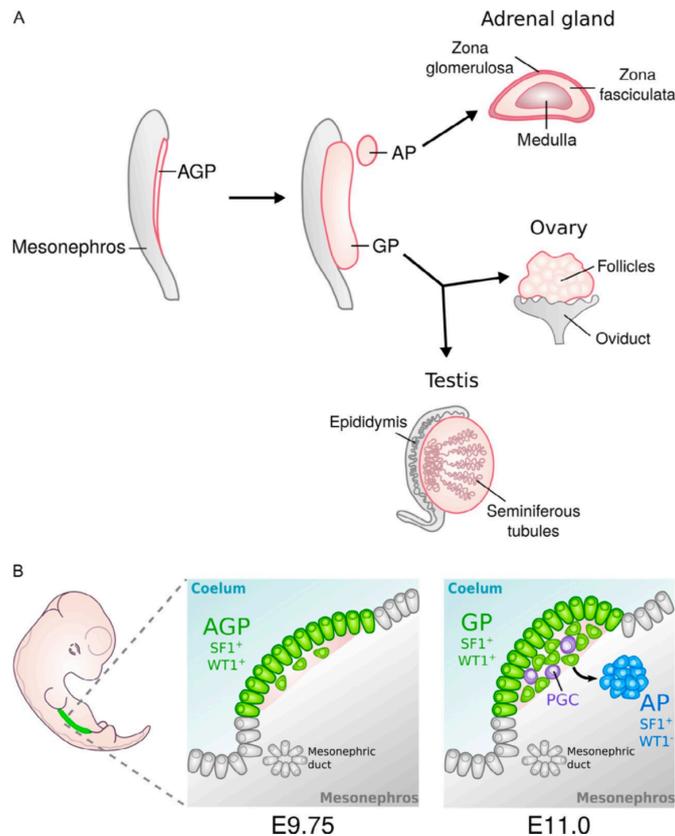
steroidogenic Leydig cells and their progenitors. By contrast, the ovary does not show a particular arrangement of the different cell types. The pre-granulosa cells differentiated from the bipotential supporting cells are mixed with the interstitial cells that will give rise to steroidogenic theca cells after birth, the germ cells and the less abundant endothelial cells.

## **2.1 Gonad formation from genital ridge**

### **2.1.1 Adreno-gonadal primordium formation**

Adreno-gonadal primordium (AGP) is the common primordium for adrenal and gonad, formed from the thickening of the celomic epithelium on the ventral side of the mesonephros around E9.5 (Hatano et al., 1996; Ikeda et al., 2014) (Fig. 2). AGP contains Steroidogenic Factor 1 (SF1) expressing somatic progenitors (Hatano et al., 1996; Morohashi, 1997) and primordial germ cells (PGCs). At E10.5, the somatic progenitors with higher expression of SF1 in the rostral region of the AGP migrate and give rise the adrenal primordium (AP) at the anterior pole of the mesonephros. PGCs together with the rest of somatic progenitors form the long, narrow gonadal primordium (GP) along the anteroposterior axis, also known as genital ridge (Morohashi, 1997). Wilm's tumor 1 (WT1) and SF1 are the only known transcription factors involved in the separation of adrenal and gonadal primordia. WT1/SF1 double positive AGP somatic progenitors switch off WT1 expression to form AP, whereas GP somatic progenitors maintain the expression of both WT1 and SF1 (Kreidberg et al., 1993; Luo et al., 1994; Vidal and Schedl, 2000).

## 2.1.2 Gonad formation



**Figure 2.** Mouse adrenogonadal primordium (AGP) development. (A) Overview of the development of the gonad and the adrenal gland (B) Transverse section views of AGP at E9.75 and the separated gonadal primordium (GP) and adrenal primordium (AP) at E11.0. (Nef et al., 2019)

After separation from the AGP around E10.5, the coelomic epithelium of the gonadal primordium continues to proliferate until the flattened genital ridges become a circle in cross section and separates from the mesonephros gradually to form the long, narrow gonad (Brambell, 2006) (Fig. 2). Single-cell RNA-seq transcriptomic profile of the somatic progenitors at E10.5 in XX and XY individuals did not reveal any sexual dimorphism or cell-lineage-specific fate but rather an epithelial stem cell identity (Stévant et al., 2018a). Around E11.5, the coelomic epithelium cells disconnected from each other and from the basal basement membrane, allowing the cell-oriented division or ingression of coelomic epithelium cells through epithelial-to-mesenchymal transition (EMT) into the gonad to constitute the progenitors of supporting and interstitial cells. The fate of these progenitors differs depending on the sex and stages.

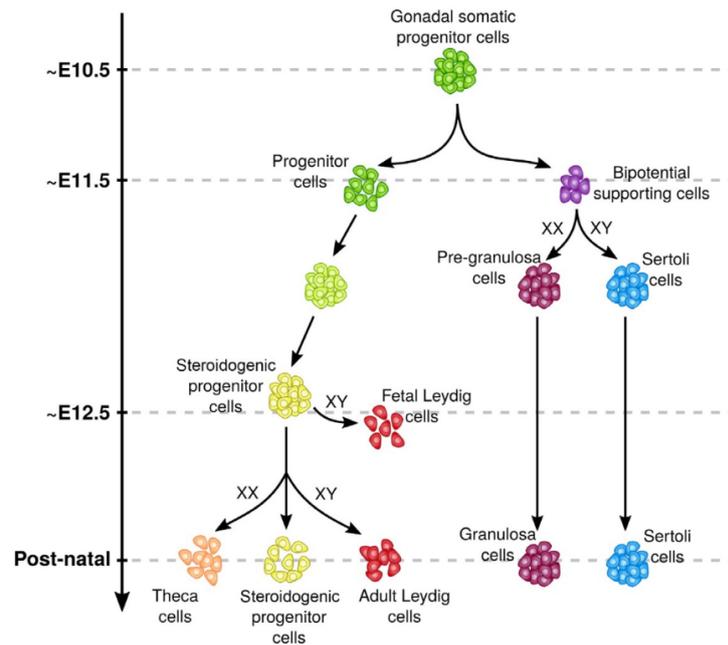
In XY, the proliferation of coelomic epithelium cell is higher than in XX, and cells ingressed before E11.5 (18 ts) can give rise to supporting cells and interstitial progenitor cells (Karl and Capel, 1998). After E11.5, the migrated coelomic epithelium progenitor cells only give rise to interstitial progenitor cells. The ingression is gradually blocked at E12.5 (Karl and Capel, 1998; Parma et al., 2006) with the decrease of LGR5 expression in the coelomic epithelium and the formation of tunica albuginea to separate the gonad from coelomic epithelium. XX coelomic epithelium cells have relatively lower proliferation rates at E11.5 and gradually migrate into the gonad to give rise to both supporting cells and interstitial progenitor cells from around E11.5 until after birth (Karl and Capel, 1998; Mork et al., 2012a). The coelomic epithelium later forms the ovarian surface epithelium (Nef et al., 2019).

### 2.1.3 Regulation of genital ridge formation

Genital ridge formation depends on the thickening and proliferation of the coelomic epithelium. Any regulator affecting the initiation of coelomic epithelium thickening or proliferation leads to smaller gonad or even failure of gonad formation. Coelomic epithelium cells exhibit a molecular signature and express the GATA zinc finger transcription factor GATA4, WT1 and SF1 (Nef et al., 2019). GATA4 deficiency leads to a failure of the initiation of coelomic epithelium thickening so that coelomic epithelium cells stayed in an undifferentiated monolayer without SF1 and LHX9 expression (Hu et al., 2013). SF1 regulates the formation and development of genital ridge in a dose-dependent manner: the loss of one copy of *Sf1* leads to a smaller gonad, whereas complete knock out of *Sf1* results in gonadal degeneration from apoptosis around E12.5 (Nef et al., 2019). *Wt1* (Kreidberg et al., 1993), the polycomb factor M33 (*Cbx2*) (Kato-Fukui et al., 2012), Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (*Cited2*) (Val et al., 2007), the insulin/IGF1R signaling pathway (Pitetti et al., 2013a), the LIM homeobox protein 9 (*Lhx9*) (Birk et al., 2000), the transcription factor Odd-skipped related 1 (*Odd1*) (Wang et al., 2005) and the PBX Homeobox 1 (*Pbx1*) (Schnabel et al., 2003) can affect the development of gonads through regulating *Sf1* expression.

In addition, the transcription factors EMX2 and SIX1/4 can regulate the EMT process and the ingression of coelomic epithelial cells by affecting their cell polarity (Birk et al., 2000; Fujimoto et al., 2013; Kreidberg et al., 1993; Kusaka et al., 2010; Luo et al., 1994; Miyamoto et al., 1997). The deletion of *Numb* and *Numbl*, encoding inhibitors of NOTCH signaling, impairs the ingression and also the differentiation of progenitors in the gonad (Lin et al., 2017). Finally, the pro-female fate genes *Wnt4* and *Rspo1* promote the coelomic epithelium proliferation around E11.5. The double knockout of *Wnt4* and *Rspo1* leads to a reduction of the proliferation in the coelomic epithelium of both XX and XY gonads. As a result, *Wnt4;Rspo1* double mutant testes are much smaller than their control counterparts at E18.5 (Chassot et al., 2012).

## 2.2 Sex determination and supporting cells in XX and XY



**Figure 3.** Gonadal cell lineage specification model during sex determination. Bipotential supporting cells and steroidogenic progenitors are derived from common gonadal somatic progenitor cells, as a result of epithelium ingression from E10.5. Around E11.5, one subset of the progenitors differentiate into supporting cells, then rapidly turn into Sertoli cells in XY, while XX supporting cells differentiate into pre-granulosa cells, a transition-fate before their fully differentiation into granulosa cells after birth. XX and XY supporting cells have similar transcriptional profiles before sex determination. The other set of gonadal progenitors give rise to the steroidogenic precursors around E12.5. In XY, part of the steroidogenic precursors differentiate into fetal Leydig cells right away, whereas theca cells for XX and adult Leydig cells differentiate at postnatal stages. A sub-population of steroidogenic precursors are maintained to support life-time of steroidogenic cell differentiation. XX and XY steroidogenic precursors do not have a strong sexual dimorphism unlike differentiated supporting cells. (Nef et al., 2019).

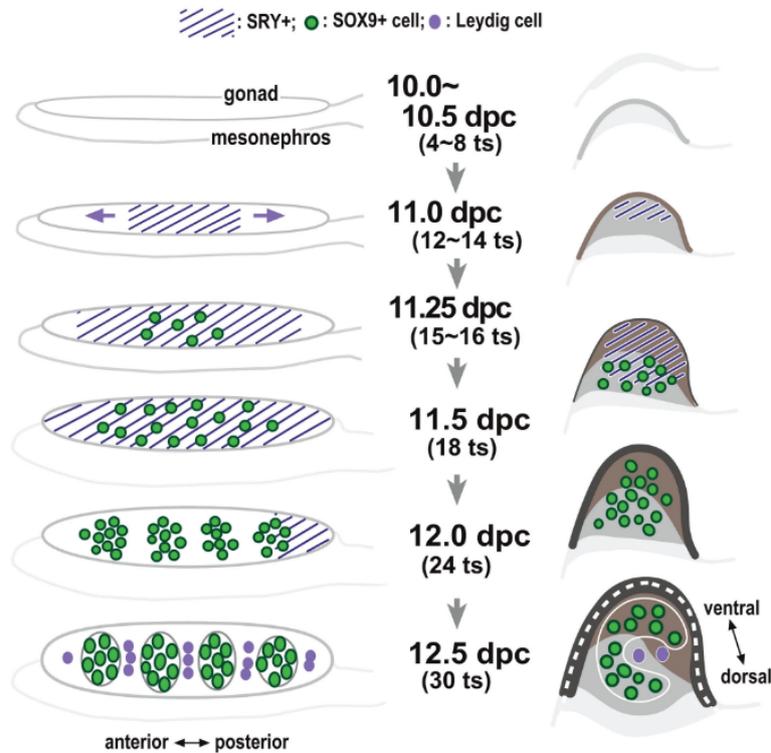
### 2.2.1 Supporting cell progenitor

Around E11.5, XX and XY gonad have three main types of somatic cells. The majority are supporting cells with high expression of WT1 and SF1 (Ikeda et al., 1994; Luo et al., 1994; Vidal and Schedl, 2000). The second largest population is one layer of coelomic epithelial cells covering the gonad and expressing high WT1 and low SF1 (Ikeda et al., 1994; Luo et al., 1994; Vidal and Schedl, 2000). Finally, the interstitial cells are less abundant and express high levels of NR2F2 (Rastetter et al., 2014), and

low levels of WT1 and SF1 (Gregoire et al., 2018). Supporting cells and interstitial cells are derived from a single SF1 positive epithelial stem cell-like progenitor cell population as shown by single-cell RNA-seq analysis. This study also established that XX and XY supporting cell at E11.5 have similar transcriptional profiles (Fig. 3) (Stévant et al., 2018a). ATAC-seq and H3K27ac ChIP-seq analyses of XX and XY supporting cells also showed that XX and XY supporting cells have similar chromatin landscapes before the initiation of sex determination (Garcia-Moreno et al., 2019a). Before sex determination, XX and XY supporting cell are bipotential, meaning that they both have the ability to differentiate into either Sertoli cells or pre-granulosa cells and orchestrate the development of testis or ovary, respectively.

WT1 is essential to maintain the supporting cell lineage identity. Even after differentiation into Sertoli cell or granulosa cell, the expression of WT1 is kept at a high level, whereas SF1 expression is down-regulated after differentiation (Chen et al., 2017). WT1 maintains the identity of differentiated supporting cell by inhibiting SF1 expression, thus blocking the steroidogenic differentiation pathway promoted by SF1 (Chen et al., 2017). Loss of WT1 before sex determination in both XX and XY gonads resulted in failure of supporting cell progenitors to differentiate into Sertoli cells or granulosa cells and most somatic genital ridge cells differentiated into steroidogenic cells with high levels of SF1 expression (Chen et al., 2017). The deletion of WT1 in testis at later stages also triggered the Sertoli cell trans-differentiation into steroidogenic cells (Kashimada et al., 2011; Phillips and De Kretser, 1998; Yao et al., 2006, 2004; Zhang et al., 2015). Therefore, WT1 function is required to maintain supporting cell identity during the whole development of testes.

## 2.2.2 Primary and secondary Sex determination

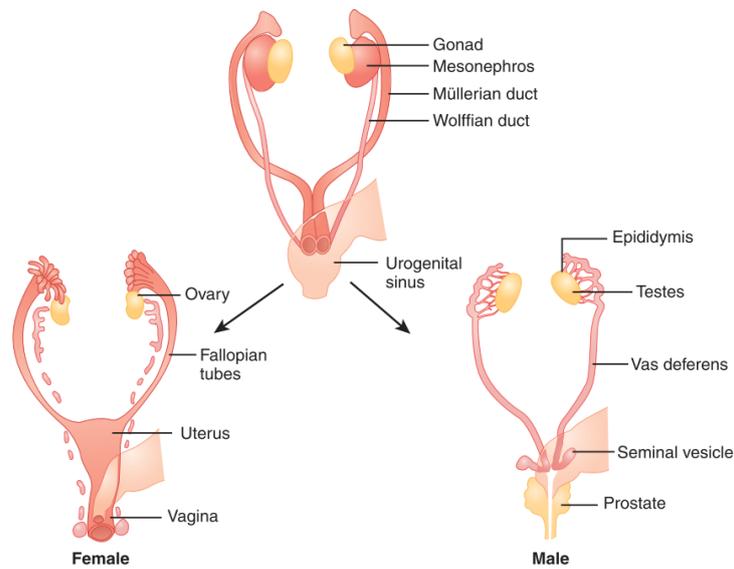


**Figure 4.** SRY and SOX9 spatiotemporal expression pattern during male sex determination. The left panels are lateral views, and right panels are transverse section views. SRY expression (purple broken line) expands in a center-to-pole (left panel) and ventral-to-dorsal pattern (right panel). SOX9 positive cells are depicted by green circles. (Harikae et al., 2013a)

The process by which the bipotential gonad develops into testis or ovary based on its sex chromosomes constitution is known as primary sex determination. In mice, during the sex determination, *Sry* is the earliest differentially expressed gene in somatic supporting cells between male and female gonad. In XY, *Sry* is first detected in the gonadal central region around 12 tail-somite (ts) stage and its expression expands to both pole ends by 18 ts. *Sry* is rapidly down-regulated in differentiated Sertoli cells in the anterior and middle of the gonad until it completely disappears at around 30 ts (Fig. 4) (Harikae et al., 2013a). Expression of SRY in the supporting cells leads to the activation of its down-stream target *Sox9*, an autosomal gene encoding a transcription factor of the SOX family. Once SOX9 expression is activated in the

supporting cells, the Sertoli cell lineage is established, then *Sry* is no longer needed and its expression decreases rapidly in those Sertoli cells. On the other hand, XX gonadal supporting cells differentiate into FOXL2 positive pre-granulosa cells and enter into mitotic arrest during the same period. The single-cell RNA-seq data of XX and XY gonadal SF1-GFP+ cell show that the transition from supporting cell to differentiated Sertoli cell is finished at E12.5, whereas the full differentiation of pre-granulosa cells to granulosa cell is completed after birth around P6 (Stévant et al., 2018a). In XY humans, the bipotential gonad progresses to testis organogenesis with the expression of *SRY* in Sertoli cell precursors around 8 weeks after conception. *SRY* initiates testis determination by activating *SOX9*, which in turn directs Sertoli cell proliferation and organization into tubular cords with SF1/NR5A1 (steroidogenic factor-1). The testis organogenesis can be recognized around week 10 by ultrasound (Délot and Vilain, 2018). Unlike the situation in mouse, human *SRY* expression persists in the sex cords at a low level throughout the embryonic period and beyond (Hanley et al., 2000).

Later, sex hormones secreted by testis or ovary are necessary to establish and maintain the sexual identity and fertility by promoting the development of secondary sexual characteristics. This process is known as secondary sex determination (Nef et al., 2019) and its dependence on gonadal sex was proven by Alfred Jost in 1950s (Délot and Vilain, 2018; Stévant et al., 2018b). In his experiments, XX and XY rabbits developed as females after removal of their undifferentiated gonads during embryonic stages. Conversely, re-implanting testis in XX rabbits lead to the development of male Wolffian ducts and the regression of female Müllerian ducts (Jost, 1948, 1953).



**Figure 5.** XX and XY internal reproductive ducts development Müllerian ducts develop into female internal reproductive ducts including vagina, uterus and fallopian tubes, and Wolffian ducts are degraded in the absence androgen signaling in XX. Wolffian ducts develop into seminal vesicle, vas deferens and epididymis in XY, then Müllerian ducts are degraded following the action of Anti Müllerian Hormone (AMH) secreted by Sertoli cells in the testes. (Délot and Vilain, 2018)

In mouse and human, all XX and XY have two sets of reproductive duct systems: Wolffian ducts for male internal genital tracts and Müllerian ducts for female internal genital tracts. Once the male pathway is established, Sertoli cells are fully differentiated by expressing *Sox9*. The Sertoli cell starts to secrete FGF9, platelet-derived growth factor (PDGF), desert hedgehog (DHH) to recruit interstitial cells and induce Leydig cells differentiation in the gonad. The testosterone secreted by Leydig cells and anti-müllerian hormone (AMH) secreted by Sertoli cells help to establish the male internal and external genitalia and induce the regression of Müllerian duct, respectively. Without those hormones in XX, the Wolffian ducts regress and the Müllerian ducts develop into female genital tracts (Fig. 5). Testosterone could further promote the development of the epididymis, vas deferens and seminal vesicles at later stages. Leydig cell can also secrete insulin-like 3 (INSL3) to mediate the first phase of testicular descent to inguinal ring in mice. Part of

cryptorchid patients also harbor *INSL3* mutations. (Bogatcheva and Agoulnik, 2005). Dihydrotestosterone (DHT), which is the final product from androgen metabolism by the enzyme 5 $\alpha$ -reductase and cannot be transformed into estrogen, promotes the male external genitalia development locally. By week 14 of gestation in human or by E17.5 in mouse, the male differentiation is complete, except the phallic growth and inguinoscrotal descent. In XX, the Müllerian duct will further develop into fallopian tubes, uterus and upper vagina around 12 weeks of gestation for human and E17.5 for mouse (Délot and Vilain, 2018).

### 2.2.2 Sertoli cells function and regulatory pathway

Sertoli cells have different functions at different stages. In the fetal testes, Sertoli cells are the first differentiated somatic cells. In turn, they direct the migration of interstitial cells and differentiation of Leydig cells; they form testis cords by surrounding the germ cells and also prevent the germ cells from entering into meiosis (Mackay, 2000). Besides, Sertoli cells secrete AMH to induce the degradation of Müllerian ducts in XY individuals (Josso et al., 2001). During puberty, Sertoli cells support spermatogenesis including the regulation of self-renewal and differentiation of germ cell, meiosis, spermatozoa transformation and the consumption of residual cytoplasm (Sharpe, RM, 1994). Sertoli cells serve as "nurse" cells for spermatogenesis, therefore the number of the Sertoli cells decides the number of germ cells going through spermatogenesis and the final production of sperm (Orth et al., 1988).

#### **- *Sry* regulation**

Ever since the discovery of the *Sry* gene in 1990, our understanding of sex determination has reached to a new genetic and molecular mechanism level. Any gene affecting the expression of *Sry* leads to failure of the Sertoli cell specification and male-to-female sex reversal. *Sry* expression is regulated by multiple factors. *Wt1* regulates *Sry* expression and mutation of the +KTS WT1 isoform results in complete male-to-female sex reversal in XY (Hammes et al., 2001). MAPK pathway positively

regulates *Sry* expression in both mouse and human. Loss of MAP3K1 and MAP3K4 functions resulted in male-to-female sex reversal following a decrease of SRY expression (Bogani et al., 2009; Granados et al., 2017; Pearlman et al., 2010; Warr et al., 2012). *GATA4*, *SF1* and *CBX2* are also required for the expression of *SRY* and their mutations were found in 46, XY female patients sequencing analysis results (Biason-Lauber et al., 2009; Eggers et al., 2016). *GATA4* mutation leads to a decrease of *SRY* transcriptional level in human, more specifically, *GATA4* cooperates with WT1 (+KTS) to bind *Sry* promoter regulatory elements (Miyamoto et al., 2008). In vitro, *SF1* promotes the expression of *SRY* by directly binding to the *SRY* promoter, which can be blocked by the cAMP-dependent protein kinase (PKA) phosphorylation (de Santa Barbara et al., 2005). Chromobox protein homolog 2 (*CBX2*) mutated 46 XY female patient was found to exhibit the same phenotype as M33 mouse mutants, which had affected *SRY* expression, leading to 50%-75% complete male-to-female sex reversal. In vitro, *CBX2* positively regulate *SRY* expression by directly binding to its promoter (Biason-Lauber et al., 2009; Kato-Fukui et al., 1998). Double knock out of histone/lysine acetyl-transferases CBP/P300 complex led to complete sex reversal in XY mice, owing to low levels of histone acetylation on the *Sry* promoter resulting in reduced transcription (Carré et al., 2018). Another epigenetic regulatory factor for *Sry* expression is the H3K9 demethylase *Jmjd1a*, which could directly de-methylate the H3K9me2 at the *Sry* promoter and open it to be accessible to other transcription factor (Kuroki et al., 2013). The insulin signaling is also very important for male determination by affecting the expression of *Sry*. Triple knock out of *Ir*, *Irr* and *Igf1r* led to a strong decrease of *Sry* expression and male-to-female sex reversal in mice (Nef et al., 2003).

#### **- *Sox9* function in Sertoli cell specification**

A well-known Sertoli cell specification marker is *Sox9*, the direct target of *Sry*. Even though *Sox9* is located on an autosome, its function in Sertoli cell and sex determination can completely replace *Sry*. *Sox9* is necessary and sufficient for male development (Gregoire et al., 2011; Lavery et al., 2011a; Vidal et al., 2001). When

*Sox9* is deleted around E11.5, XY mice develop as complete females with ovaries capable of producing fertile X/Y oocytes (Lavery et al., 2011a). *Wt1-Sox9* transgenic XX mice ectopically expressing *Sox9* in the gonad before sex determination, develop as complete males with testes lacking germ cells in the seminiferous tubules at adult stages (Vidal et al., 2001). During this female-to-male sex reversal process, there is a transient development of ovotestis in the transgenic XX mutant gonads before E16.5 (Gregoire et al., 2011). Mechanistically, mouse SOX9 (E12.5) and SRY (E11.5) ChIP-ChIP experiments showed 907 common target genes can be bound by both SOX9 and SRY (Li et al., 2014b). SRY and SOX9 are transcription factors belonging to the same family, which uses the HMG domain to interact with DNA. It is well known that SRY and SF1 could directly bind the TESCO enhancer of *Sox9* and promote its transcription (Sekido and Lovell-Badge, 2008). However, the deletion of TESCO did not result in severe reduction of *Sox9* expression and male-to-female sex reversal (Gonen et al., 2017). More recently thanks to the next generation of sequencing technique, through comparing the open chromosomal regions of supporting cells in XX and XY gonads at E10.5, E13.5 and E15.5 (Garcia-Moreno et al., 2019a), researchers found a distal enhancer responsible for the transcription of *Sox9* with binding sites for SRY and SOX9. Deletion of this enhancer in XY mice led to complete sex reversal (Gonen and Lovell-Badge, 2019; Gonen et al., 2018).

At the beginning of the sex determination process, *Sry* is first expressed in the center of the long and narrow gonad, hence the central supporting cells differentiate first into Sertoli cells with SOX9 expression (Wilhelm et al., 2005). In order to synchronize the more lateral supporting cells to differentiate, SOX9 activates the expression of a secreted protein, FGF9, which can diffuse rapidly and in turn activates *Sox9* transcription (Hiramatsu et al., 2009a). Therefore, mutations of *Fgf9* and its receptor *Fgfr2c* lead to partial male-to-female sex reversal at the pole regions (Bagheri-Fam et al., 2008, 2017; Colvin et al., 2001). Interestingly, this kind of sex reversal phenotype can be rescued by additionally deleting *Wnt4* (Jameson et al., 2012a) or *Foxl2* (Bagheri-Fam et al., 2017), which give us the hint that in order to develop into testes or ovary, one pathway has to be activated to promote its own

development and also needs to repress the other pathway during the sex determination process (Jameson et al., 2012a). *L-Pgds*/PGD2 is another pathway activated by *Sox9* that can amplify the *Sox9* transcription and also help SOX9 protein translocation into the nucleus (Moniot et al., 2009). The function of *Sox9* amplification by FGF9 and PGD2 is mutually independent. Indeed, chimeric gonads containing XX and XY (at least 90%) mixed supporting cells could develop into testes, indicating that XX supporting cells can differentiate into Sertoli cells under the influence of a non-cell autonomous signal derived from XY cells (Palmer and Burgoyne, 1991).

Besides *Sox9*, other SOX family members are also expressed in Sertoli cells. SOX9 is essential to establish male development, hence when *Sox9* is deleted before sex determination around E11.5 by *Sf1-Cre* transgene, the XY gonad develops as an ovary (Lavery et al., 2011a). However, when *Sox9* was deleted by *Amh-Cre* around E13.5 after the male pathway is well established, sex reversal was not observed (Barrionuevo et al., 2009). Instead, this *Sox9* mutant had seminiferous tubules degeneration starting at the age of 5 months. Additional deletion of *Sox8* leads to a more severe phenotype where seminiferous tubules degenerated at two months old and abundant steroidogenic cells expanded over the testes (Barrionuevo et al., 2009). Surprisingly, in these *Sox9* and *Sox8* double knockout mutant gonads, part of the Sertoli cells started to transdifferentiate into granulosa cells with FOXL2 expression from E15.5, which probably leads to the degeneration of seminiferous tubules at later stages (Georg et al., 2012). In some rare cases, the trans-differentiation resulted in a decrease of AMH production and the retention of Müllerian ducts (Barrionuevo et al., 2009). Single knock out of *Sox8* in XY mice developed testes without any significant defects (Barrionuevo et al., 2009; Georg et al., 2012). Therefore, after the sex determination stage, *Sox9* and *Sox8* have a complementary effect on the maintenance of Sertoli cell identity. It has also been reported that the overexpression of *Sox3* and *Sox10* could result in female-to-male sex reversal in XX gonads (Polanco et al., 2009; Schwarz, 2000). *Sox10* is present in XX and XY bipotential gonads at a low level and up-regulated in XY after the male differentiation starts. *Sox10* can bind

to *Amh* promoter and TESCO element of *Sox9* to promote male development (Polanco et al., 2009). Female-to-male sex reversal was also observed in 46 XX male patient, who had a duplication of *SOX10* at chromosome 22q13 site (Falah et al., 2017). Another reported SOX family gene in gonad is *Sox4*, the deletion of which does not result in sex determination defects but produces abnormally elongated testes and ovary. In XY *Sox4* mutants *Sry* and *Sox9* expression are increased (Zhao et al., 2017b), indicating that SOX family genes in the gonads could also mutually inhibit each other. *Sry* expression is also maintained in the XY *Sox9* mutant gonads (*Sf1-Cre*) until E14.5, compared to the XY control gonads in which *Sry* expression disappears at E12.5 (Lavery et al., 2012; Nicol and Yao, 2015). It suggests that *Sox9* may have a negative feedback on *Sry* expression.

#### **- *Dmrt1* function in Sertoli cell development**

Doublesex and mab-3 related transcription factor 1 (*Dmrt1*) is another Sertoli marker, able to maintain the Sertoli cells identity at adult stages. *Dmrt1* is expressed in XX and XY gonads from E10.5, but it is maintained only in the Sertoli cells in XY after sex determination. XX and XY germ cells also express *Dmrt1* from E12.5 (Lei et al., 2007a). Total knock out *Dmrt1* mutants and Sertoli cell conditional knock out *Dmrt1* mutants from E11.5 show normal male sex determination but exhibit defects at postnatal stages. In the P14 mutant testis (by *Dhh-Cre* or *Sf1-Cre*), Sertoli cells start to transdifferentiate into granulosa cells (FOXL2 positive) inside the seminiferous tubules (Matson et al., 2011). This result indicates that at postnatal and adult stages, *Dmrt1* is essential to maintain the Sertoli cell identity. Furthermore, ChIP-seq studies showed that regions bound by SOX9 in E13.5 fetal testes, also contain binding motifs for GATA4 or DMRT1 (Rahmoun et al., 2017). This suggests that after the sex determination time point, the function of *Sox9* in Sertoli cells can be replaced by other factors, such as *Sox8* and *Dmrt1*. In mice, transgenic overexpression of *Dmrt1* driven by *Wt1* promoter in XX gonads leads to female-to-male sex reversal. The XX transgenic animals exhibited Sertoli-like cell and steroidogenic-like cell differentiation in the gonads and had a masculinized genital tract (Zhao et al., 2015).

### 2.2.3 Granulosa cells function and regulatory pathway

Granulosa cells, the female supporting cells, are an important component of follicles and form the granulosa lutein cells after ovulation. Granulosa cells are able to interact with the developing oocyte (Liu et al., 2015a), to orchestrate the differentiation of theca cells and to produce estrogen from androgens under FSH regulation (Garzo and Dorrington, 1984). Granulosa lutein cells can also produce progesterone essential to maintain the pregnancy (YOSHIMI et al., 1969). In XX gonads, without SRY expression, the supporting cells differentiate into pre-granulosa cells with high level of FOXL2 expression. Pre-granulosa cells enter into mitotic arrest marked by the expression of cyclin-dependent kinase inhibitor CDKN1b/p27 and the down-regulation of Cyclin A1 (Gustin et al., 2016; Nef et al., 2005) after E11.5. This state of pre-granulosa cell is maintained until around P6 (Stévant et al., 2018a).

XX coelomic epithelium exhibits relatively lower proliferation compared to the XY coelomic epithelium and continues to express LGR4/5 (Karl and Capel, 1998; Parma et al., 2006). Cells derived from the XX celomic epithelium can differentiate into granulosa cells and interstitial cells. Two populations of granulosa cells can be distinguished: medulla pre-granulosa cells enter the gonad from the sex determination stage to perinatal stages (Rastetter et al., 2014; Zheng et al., 2014). During the newborn to early postnatal periods (P0-7), the ovarian surface epithelial cells still keep an undifferentiated state with the expression of RSPO1 and LGR4/5, and contribute cells to form the cortical pre-granulosa population (Koizumi et al., 2015; Mork et al., 2012a; Rastetter et al., 2014; Zheng et al., 2014). Medulla granulosa cells are responsible to the first wave of folliculogenesis after puberty, whereas the cortical granulosa cells are involved in the second wave of folliculogenesis during the adult life (Mork et al., 2012a; Zheng et al., 2014).

Without *Sry* expression in the XX gonad during sex determination, the supporting cells will continue to maintain relatively high levels of *Wnt4* and *Rspo1* and differentiate into granulosa expressing FOXL2. During this specification, the

down-regulation of *Sf1* by WT1 directly binding to its promoter is necessary. *Wt1* deletion before or after sex determination results in granulosa cell trans-differentiating into steroidogenic cells (Chen et al., 2017). The loss of the GATA4-FOG2 complex by constitutively knocking out *Dkk1*<sup>-/-</sup> or *Fog2*<sup>-/-</sup>/*Dkk1*<sup>-/-</sup> affects several aspects of early ovarian differentiation such as the decrease of *Wnt4* and *Fst* expression, but masculinization is not induced (Manuylov et al., 2008). Besides having a function on male sex determination (Nef et al., 2003), Insulin and IGF signaling play also important roles for female development. Double knock out the insulin receptor (*Insr*) and the IGF type I receptor (*Igf1r*) lead to a delay of female development such that gonads only start to express FOXL2 at E16.5 (Pitetti et al., 2013b). It has been reported that the XX gonads at E11.0-11.25 stage have the full ability to respond to SRY ectopic expression by activating SOX9 expression and developing into testes; around E11.5, XX gonads still can be induced to express SOX9 but this expression cannot be maintained and sex reversal does not take place. After E12.0-12.5, most XX gonadal cells have lost the SRY-dependent SOX9 inducibility (SDSI). This is the stage when many of the female specific genes start to emerge (Harikae et al., 2013b).

Compared to what is known about the differentiation of Sertoli cells, the study on the female pathway has been delayed for around 10 years. It receives less focus, because female pathway was thought be a pre-programed process after the discovery of male sex determination gene *SRY*. In 1999, *Wnt4* was found to be important for female development. *Wnt4* deletion causes partial female-to-male sex reversal with the formation of an ovotestis instead of an ovary (Vainio et al., 1999). Since then, several studies have established that the WNT signaling enhancer *RSPO1*, the WNT ligand *WNT4* and the effector  $\beta$ -Catenin (encoded by *Ctnnb1*) are essential components of a signaling pathway that promotes female gonadal development (Chassot et al., 2014). *Rspo1* and *Wnt4* are expressed in the gonadal somatic cells (Chassot et al., 2012, 2008a; Parma et al., 2006). Before sex determination, there is no difference in the expression of *Rspo1* and *Wnt4* in mice between XX and XY (Burger, 2002; Chassot et al., 2012; Munger et al., 2013; Parma

et al., 2006; Stévant et al., 2017, 2018a). Right after SRY initiates the male pathway, *Rspo1* and *Wnt4* are down-regulated in XY gonads whereas in XX gonads their expressions are kept to establish the female pathway. In human, *WNT4* expression is found both in XX and XY gonads, whereas *Rspo1* is only up-regulated in XX gonads (Mamsen et al., 2017; Tomaselli et al., 2011). Any mutation of the three factors (*Rspo1/Wnt4/Ctnnb1*) would result in female-to-male sex reversal in XX mice.

#### **- *WNT4* function in ovarian specification**

*Wnt4* is a critical gene for kidney development and *Wnt4* total knock out mouse embryos die several hours after birth because of kidney defects (Stark et al., 1994). In the ovotestes of XX *Wnt4* mutants, the granulosa cells in the anterior part exit mitotic arrest and prematurely differentiate (Maatouk et al., 2013). Indeed, these granulosa cells lose P27 expression and co-express FOXL2 and AMH at E15.5-E17.5, similar to the granulosa cells during folliculogenesis stages after birth (Maatouk et al., 2013). Steroidogenic cell markers such as *Hsd17b1*, *Hsd3b1* and *Cyp17a1* are ectopically expressed in the anterior part of the ovotestis (Heikkilä et al., 2002, 2005). The newborn XX *Wnt4* mutant gonads produce high levels of testosterone, compared to XX controls (Shan et al., 2010). Competitive inhibition of androgen action by flutamide treatment (100 mg/kg) rescues the phenotype of ectopic epididymis development in 80% of XX mutants (Heikkilä et al., 2005). Interestingly, *Cyp21a1*, encoding an adrenal specific steroidogenic enzyme, is also found in the XX *Wnt4* mutants, indicating the potential contribution of adrenal cells to the steroidogenic cell population in XX *Wnt4* mutants (Heikkilä et al., 2002). Germ cell proliferation in this area around E14.5 is not affected but they rapidly die by apoptosis near birth (Chassot et al., 2014; Vainio et al., 1999; Yao et al., 2004). The death of germ cell is related to the activation of Activin signaling by the up-regulation of *Inhbb* expression (Liu et al., 2010). Additional knockout of *Inhbb* rescued the apoptosis of germ cells in XX *Wnt4* mutants (Liu et al., 2010). Around E17.5, testis cords with SOX9/AMH double positive Sertoli-like cells can be observed in the anterior male part of the *Wnt4* mutant ovotestes, whereas the posterior part developed as a relatively normal ovary with

mitotically arrested granulosa cells and meiotic germ cells (Maatouk et al., 2013; Vainio et al., 1999). In addition, *Wnt4* is important for Müllerian duct development. Loss of *Wnt4* led to the complete degeneration of Müllerian ducts, (Heikkilä et al., 2005; Vainio et al., 1999). Instead, the XX *Wnt4* mutants only had the epididymis developed from the Wolffian duct because of the steroidogenic hormones production in the male part (Heikkilä et al., 2005; Vainio et al., 1999). XX *Wnt4* mutants also showed ectopic migration of endothelial cells from the mesonephros to form the celomic vessel around E12.5, similar to the situation in XY wild-type (Jeays-Ward, 2003). The microarray analysis of E12.5 gonads showed that *Fst* is hugely down-regulated in the XX *Wnt4* mutants compared to XX wild-type (Coveney et al., 2008). The additional knockout of *Fst* can rescue the ectopic formation of a celomic vessel in the XX *Wnt4* mutants (Yao et al., 2004). Mechanistically, high levels of FST can repress vasculature formation promoted by Activin B secreted from granulosa cells in ovaries and Sertoli cells in testes (Liu et al., 2010). In human, 46, XX patients of *WNT4* mutation had similar gonadal phenotypes (Biason-Lauber et al., 2004; Philibert et al., 2011).

#### **- *RPSO1* function in ovarian development**

*Rspo1* knockout mouse mutants exhibit partial female-to-male sex reversal phenotypes similar to those observed in *Wnt4* mutants (Chassot et al., 2008a; Tomizuka et al., 2008). In the XX *Rspo1* mutants, WNT/ $\beta$ -Catenin signaling pathway is down-regulated, as shown by the decrease of *Axin2*, *Lef1*, and *Wnt4* expression (Chassot et al., 2008a). Conversely, additional stabilization of  $\beta$ -Catenin is able to rescue the female-to-male sex reversal in XX *Rspo1* mutants, indicating the role of *Rspo1* in activating the WNT/ $\beta$ -catenin signaling (Chassot et al., 2008a). In addition, XX *Rspo1* mutants develop “double ducts”, which means that Wolffian duct and Müllerian duct develop into epididymis and oviduct respectively (Chassot et al., 2008a). Moreover, in *Rspo1* mutants, germ cell proliferation was impaired from E12.5 to E14.5 compared to XX control (Chassot et al., 2011). Some of the germ cells that survived in the anterior part behaved like male germ cells, they did not enter meiosis

and had up-regulated *Nanos2* (Chassot et al., 2011). Interestingly, testosterone was detected in the gonads of newborn XX *Rspo1* mutants (not detectable in XX wildtype), and was found to be responsible for the development of male reproductive tract (Tomizuka et al., 2008). Flutamide treatment also inhibit the formation of epididymis in XX *Rspo1* mutants (Tomizuka et al., 2008). Unlike *WNT4* mutations, 46, XX human patients, who had mutations on *RSPO1*, had a complete sex reversal to male (Parma et al., 2006).

#### **- $\beta$ -Catenin function in ovarian development**

The constitutive *Ctnnb1* knockout leads to embryonic lethality around E8.5, before the gonads start to form (Huelsenken et al., 2000). XX gonads with somatic deletion of *Ctnnb1* by means of the *Sf1-Cre* transgene, develop an ectopic celomic vessel accompanied by decreased expression of *Fst*, exhibit ectopic expression of steroidogenic enzymes followed by development of “double ducts” and show germ cells loss by apoptosis (Liu et al., 2009, 2010; Manuylov et al., 2008). The double duct phenotype in *Ctnnb1* mutants can be rescued by flutamide treatment (100 mg/kg/daily) (Liu et al., 2010). It was initially reported that *Ctnnb1* mutant gonads did not contain transdifferentiated Sertoli-like cells (AMH or SOX9 positive) by E18.5 (Liu et al., 2009, 2010; Manuylov et al., 2008). However, it was later found that AMH and DMRT1 are expressed in XX gonads of the same *Ctnnb1* conditional mouse model (Nicol and Yao, 2015), indicating some variability in the appearance of the granulosa cell pre-maturation and trans-differentiation phenotypes in this mouse model.

#### **- Gain of function studies for the *RSPO1/WNT4*/ $\beta$ -Catenin pathway**

Gain-of-function of  $\beta$ -Catenin in XY mice is sufficient to induce female development (Maatouk et al., 2008). In human, the overexpression of *WNT4* and *RSPO1* by the duplication of chromosome 1p31-p35 led to a male-to-female sex reversal phenotype in 46, XY patients (Jordan et al., 2001). Either ectopically expressing *Wnt4* or *Rspo1* by means of the *Sf1-Cre* transgene in XY mice does not induce female development (De Cian et al., 2017; Jeays-Ward, 2003; Jeays-Ward et

al., 2004). Instead, *Rspo1* overexpression in XX induced tumorigenesis of the granulosa cells (De Cian et al., 2017).

#### **- LGR4/5 function in ovarian development**

LGR4/5 are the receptors of RSPO1 in the ovary. Both genes are expressed in the coelomic epithelial cells that respond to RSPO1 signal to migrate into the gonad and form the medulla fetal pre-granulosa cell (Rastetter et al., 2014). However, once this pre-granulosa cell lineage was established and entered into mitotic arrest, expressions of LGR4/5 in the coelomic epithelial cells decreased (Gustin et al., 2016; Koizumi et al., 2015). At adult stages, LGR5 expression can only be found in the ovarian surface epithelial stem cells where it contributes to the repair of ovarian surface after ovulation (Ng et al., 2014). Deletion of *Lgr5* does not lead to sex reversal. In *Lgr4* XX mutants, the WNT/ $\beta$ -Catenin signaling pathway is inhibited and the gonads develop as ovotestes, indicating that LGR4 and LGR5 have specific roles in the gonads (Koizumi et al., 2015).

#### **- FOXL2 function in ovarian development**

FOXL2, a transcription factor, is specifically expressed in the ovarian granulosa cells. In goats, an 11.7 kb deletion at chromosome 1q43, which is a transcription regulatory region of *FOXL2* led to polled intersex syndrome (PIS) in XX. The loss of *FOXL2* resulted in a complete testis development in XX goat (Boulanger et al., 2014; Pailhoux et al., 2001, 2002; Pannetier et al., 2005). In human, heterozygous mutation of *FOXL2* leads to a defect of ovarian premature failure without any sex reversal phenotype (Crisponi et al., 2001; Harris, 2002). Total knockout XX *Foxl2* mouse mutant gonads developed as normal female during the embryonic stages, but the granulosa cells trans-differentiated to Sertoli-like cells after birth (Ottolenghi et al., 2005; Schmidt, 2004; Uda et al., 2004; Uhlenhaut et al., 2009). Thus, in XX mice *FOXL2* is important for adult ovarian maintenance. The deletion of estrogen receptor *Esr1* and *Esr2* in mice results in the same phenotype as *Foxl2* mutations, and further experiments proved that ESR1/2 receptors could form a transcription factor complex

with FOXL2 and bind to the promoter of *Sox9* to repress its transcription and maintain the granulosa cell identity (Uhlenhaut et al., 2009). These studies suggested that similar to the Sertoli cell, after the establishment of its identity, granulosa cells also need to be maintained by the pro-ovarian signaling during adult life.

Even though FOXL2 is not necessary for the fetal pre-granulosa cell identity, it contributes to maintain this identity in the *Wnt4* or *Rspo1* XX mutants (Auguste et al., 2012; Ottolenghi et al., 2007). Indeed, double knockout of *Wnt4;Foxl2* or *Rspo1;Foxl2* XX mutants had much more transdifferentiated Sertoli-like cells from pre-granulosa cell compared to the *Wnt4* or *Rspo1* single knockout mutants. On the other hand, gain-of-function of *Foxl2* by means of the *Sf1-Cre* transgene in XY mice did induce granulosa cell differentiation and a partial male-to-female sex reversal in developing testes. The XY FOXL2 overexpressing mutant still had testis cords and epididymis development, together with female-like external genitalia (Nicol et al., 2018).

## **2.3 Interstitial cell differentiation and development in XX and XY**

### **2.3.1 Interstitial cell differentiation and function**

Besides producing gametes for reproduction, another important function for testes and ovaries is to produce the sex steroid hormones testosterone and estrogen, respectively. Leydig cells in the testis and theca cells in the ovary are steroidogenic cells. Despite producing different sex hormones, Leydig cells and theca cells are derived from a common source of interstitial progenitors (Stévant et al., 2018a) sharing similar transcriptional profiles before sex determination.

#### **- Leydig cells differentiation and function**

In testes, interstitial cells can derive from the coelomic epithelial progenitor cells during the sex determination period (Karl and Capel, 1998). After their ingression, they become active for NOTCH signaling and decrease WT1 expression (Liu et al., 2016; Tang et al., 2008). Interstitial cells can differentiate into the fetal Leydig cells around E12.5 after the decrease of LHX9 in mouse (DeFalco et al., 2011). This

differentiation process is driven by Desert Hedgehog (DHH) secreted from Sertoli cells under the regulatory control of SOX9 and SRY (Bitgood et al., 1996; Li et al., 2014b; Yao et al., 2002). Another source of interstitial cell is the mesonephros (Cool et al., 2008; DeFalco et al., 2011; Jeays-Ward, 2003; Kumar and DeFalco, 2018; Martineau et al., 1997; Merchant-Larios et al., 1993). These interstitial cells migrate with endothelial cells from the gonad/mesonephric border around E12.5, and contribute to both fetal and adult Leydig cells population (Cool et al., 2008; DeFalco et al., 2011; Kumar and DeFalco, 2018; Merchant-Larios et al., 1993).

In mouse, two kind of populations of Leydig cells exist at different stages. Fetal Leydig cells are formed after male sex determination and acquire high levels of *Sf1* expression and of the genes encoding the steroidogenic enzymes (Jameson et al., 2012a; Mamsen et al., 2017; del Valle et al., 2017). The testosterone produced by fetal Leydig cell can promote the development of internal and external genitalia. In human, the fetal Leydig cell differentiation occurs around the weeks 7 to 8 of gestation and androgen reaches its highest level around weeks 12 to 14 of gestation. In mouse and human, fetal Leydig cells not only produce androgens but also insulin-like 3, which is important for testis descent during the fetal stages (Canto et al., 2003; Marin et al., 2001; Nef and Parada, 1999; Tomboc et al., 2000; Zimmermann et al., 2014). The second population of Leydig cells in mouse is the adult Leydig cells, which emerges after birth. Adult Leydig cells are responsible for androgen production for the whole adult life, because most of the fetal Leydig cells involute after birth. The persisting fetal Leydig cells will gradually change their expression profiles to behave like adult Leydig cells and only represent around 10 percent of all Leydig cells in the adult testis (Miyabayashi et al., 2017; Shima et al., 2015). This expression profile change could be caused by endocrine environment changes after birth.

### **- Theca cells differentiation and function**

In XX gonads, theca cells are the ovarian counterparts of Leydig cells in the testis. Theca cell differentiation starts after birth, when the first wave of follicles starts to assemble. Fully differentiated theca cell surround the developing follicle with several layers. The main function of theca cell is to produce androstenedione, which could be converted into estrogen in granulosa cells by the enzyme CYP19 (Richards and Pangas, 2010). Theca cell also helps to establish the vascular system and provide the nutrients for the development of follicles. Physically, it can also provide the structural support to the follicle as it develops.

The progenitors of the theca cells are the interstitial cells during embryonic development (Liu et al., 2015a). Similar to the situation in the testis, the ovarian interstitial cells also have two different origins: the coelomic epithelium and the gonad/mesonephros border. Most of the interstitial cells derive from the coelomic epithelium, and ingress into the gonad from the sex determination stages until birth (Liu et al., 2015a). Only a small fraction of interstitial cells come from the gonad/mesonephros border and migrate around birth. The progenitors of theca cells in the gonad/mesonephros border, express GLI1 and respond to the DHH and Indian Hedgehog (IHH) signals secreted from granulosa cells, and then migrate into the gonad (Liu et al., 2015a; Yao et al., 2002). At the same time, DHH, IHH and PDGF signals recruit and induce the first theca cell differentiation from the coelomic epithelia-originated interstitial progenitors (Liu et al., 2015a; Schmahl et al., 2008). It has been reported that the mesonephros-originated theca cell has a stronger capacity for steroidogenesis than the ones derived from the coelomic epithelium (Liu et al., 2015a). Whether this difference between the two populations of theca cells results in significantly different functions is unclear.

The majority of interstitial progenitors of theca cells share a common origin with granulosa cells, as both populations derive from gonadal somatic precursors (Fig. 3). Interstitial cells and granulosa cells share similar expression profiles with only few

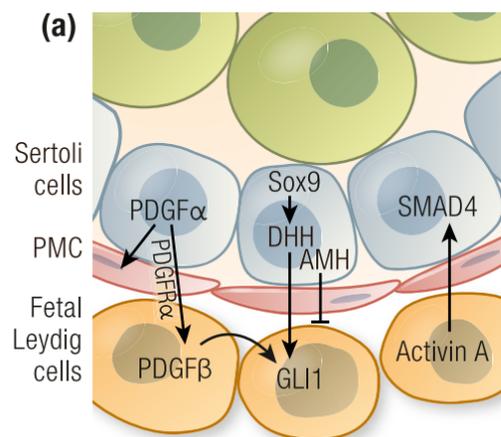
differentially expressed genes (Jameson et al., 2012b) such as *Foxl2* for granulosa cells and *Arx*, *Nr2f2* and *Mafb* (DeFalco et al., 2011; Rastetter et al., 2014) for interstitial cells. In XY gonads, the Sertoli cell lineage starts to separate from the interstitial cell lineage with the initiation of *Sry* expression and the activation of its target *Sox9*. However, in XX gonads, even though after sex determination pre-granulosa cells acquire FOXL2 expression, this specific gene expression is not necessary to establish the granulosa cell identity during the embryonic stages. Hence the mechanisms driving the separation of granulosa and interstitial cells in XX gonads is still largely unknown.

### 2.3.2 Leydig cell regulatory pathway

Interstitial cells are non-steroidogenic, with low expression levels of SF1 and of enzymes for steroidogenesis but high expression levels of progenitor makers such as chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII; also known as NR2F2) and aristaless-related homeobox (ARX) (van den Driesche et al., 2012; Kilcoyne et al., 2014; McClelland et al., 2015). The hedgehog receptor Patched 1 (*Ptch1*) is also highly expressed in interstitial cells, which can differentiate in response to DHH (Wijgerde et al., 2005). The deletion of *Nr2f2* in adult mouse testes resulted in impaired differentiation of adult Leydig cells and the failure of spermatogenesis due to insufficient androgen population (Qin et al., 2008). *ARX* mutations also impair Leydig cell differentiation and testosterone production, in turn affecting the development of secondary reproductive organs in human (Kitamura et al., 2002; Miyabayashi et al., 2013; Sirisena et al., 2014). During the differentiation to Leydig cell, the interstitial progenitors gradually lose the expression of NR2F2 and ARX and up-regulate SF1 to initiate the expression of steroidogenic enzymes (DeFalco et al., 2011; Liu et al., 2016; Miyabayashi et al., 2013). Keeping low expression levels of SF1 is essential to maintain the pool of interstitial cells. TCF21 is involved in the suppression of *Sf1* transcription and its mutants exhibit more steroidogenic cells differentiated in both XX and XY gonads (Cui, 2004). The size of the interstitial progenitors pool determines whether enough differentiated fetal Leydig cells will be formed to support male

development during fetal stages (Rotgers et al., 2018).

In mouse, NOTCH signaling is well known to balance the differentiation between interstitial progenitor cells and fetal Leydig cells. The loss of Notch receptors *Notch2/3* in the gonadal somatic cells before sex determination led to a loss of interstitial progenitor cells and increased numbers of fetal Leydig cells (Liu et al., 2016; Tang et al., 2008). In contrast, over-activated NOTCH signaling resulted in more progenitor cell and less differentiated fetal Leydig cells (Liu et al., 2016; Tang et al., 2008). A transcription cofactor named mastermind-like domain–containing protein 1 (MAMLD1) is an activator of the NOTCH pathway. A complete 46, XY gonadal dysgenesis patient was found to have a mutation on *MAMLD1* (Ruiz-Arana et al., 2015), indicating NOTCH signaling has similar functions in human. The microdeletions of *MAMLD1* also had other under-virilization phenotypes in human including hypospadias, cryptorchidism, micropenis and decreased anogenital distance (Fukami et al., 2006) caused by reduced androgen production following impaired fetal Leydig cell differentiation. *Mamld1* mutants in mouse do not show any defect on the development of male characteristics expect a slight decrease of the expression of genes encoding steroidogenic enzymes (Miyado et al., 2012, 2017).



**Figure 6.** Male steroidogenic cell regulation pathway. Sertoli cell secrete PDGF $\alpha$  and DHH to promote the differentiation and expansion of fetal Leydig cells, whereas AMH, also secreted by Sertoli cells limits the steroidogenic cell population. In testis, germ cells do not have a contribution to the steroidogenic cell differentiation. (Rotgers et al., 2018)

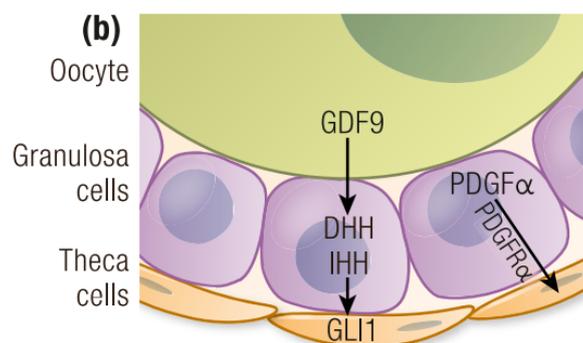
Besides the NOTCH pathway, fetal Leydig cell specification also depends on other paracrine regulations from PDGF and DHH secreted by Sertoli cells (Fig. 6). PDGFs promote the expansion of interstitial progenitor cells, the mutation of its receptor *Pdgfra* leads to the loss of fetal Leydig cells and abnormal testis morphology (Brennan et al., 2003; Karl and Capel, 1998). DHH binds to its receptor PTCH1 on the interstitial progenitor cells to activate the down-stream target GLI transcription factor 1 (GLI1) around E12.5 (Liu et al., 2016). GLI1 in turn promotes the transcription of *Sf1* and *Cyp11a1* (encoding P450 side chain cleavage enzyme) to establish the steroidogenic fate of fetal Leydig cell (Park et al., 2007; Yao et al., 2002). In human, there is an increased expression of *GLI1* before the differentiation of fetal Leydig cells (Mamsen et al., 2017). Mouse *Dhh* mutants still show some degree of steroidogenic cell differentiation, indicating that other pathways are involved in this process (Liu et al., 2016; Yao et al., 2002). In human, the mutations of members of the DHH pathway were identified in several 46, XY gonadal dysgenesis patients (Canto et al., 2004, 2005; Das et al., 2011; Umehara et al., 2000; Werner et al., 2015). One of them is hedgehog acyltransferase (HHAT), which can post-transcriptionally modify the DHH palmitoylation. This patient had gonadal dysgenesis and chondrodysplasia syndrome (Callier et al., 2014). *Hhat* mutants in mice had similar phenotypes and no fetal Leydig cell was differentiated (Callier et al., 2014). Finally, AMH secreted from Sertoli cells seems to have a role in limiting the size of fetal Leydig cell population as the *Amh* mutation in mouse causes a hyperplasia phenotype of fetal Leydig cells (Behringer et al., 1994).

In mouse, fetal Leydig cell mainly products androstenedione, which can be used to promote the development of internal and external genitalia. It needs to be converted into testosterone by the enzyme hydroxysteroid 17b dehydrogenase 3 (HSD17B3), which is expressed in Sertoli cells (Shima et al., 2012a). *HSD17B3* mutations in human leads to female external genitalia development in the 46, XY patients with high levels of androstenedione tested in the blood (Geissler et al., 1994; Lindqvist et al., 2001; Rösler et al., 1996). In adult mice, LH hormone could promote

the androgen production in the adult Leydig cells, but its action is not necessary for fetal Leydig cells (Cattanach et al., 1977; Ma et al., 2004; Zhang et al., 2014). Human patients with luteinizing hormone/choriogonadotropin receptor (*LHCGR*) mutations showed a lack of androgen production and developed 46, XY DSD (Themmen and Huhtaniemi, 2000). This finding suggests that the human fetal Leydig cell has the ability to respond to LH hormone, and that it is necessary to have placental chorionic gonadotropin during the early gestation and LH from the fetal pituitary until the end of gestation to stimulate the production of androgens (Themmen and Huhtaniemi, 2000).

Even though the progenitor of adult Leydig cell migrated from gonad/mesonephros border around E12.5 have the ability to respond to DHH signal, they still stay in a non-steroidogenic state and only start to differentiate after birth through an unknown mechanism. (Kilcoyne et al., 2014; Liu et al., 2016; Yao et al., 2002). The maturation of adult Leydig cells requires an autocrine androgen action for the acquisition of the full steroidogenic capacity, but the differentiation of fetal and adult Leydig cells are independent from this action (O'Hara et al., 2015).

### 2.2.3 Theca cell regulatory pathway



**Figure 7.** Female steroidogenic cells regulation pathway. Theca cell differentiation occurs after birth. PDGF $\alpha$ , secreted by granulosa cells, promotes the recruitment of theca cells. Furthermore, oocytes secrete GDF9, which in turn induces the expression of hedgehog ligands DHH and IHH in granulosa cells and the recruitment of theca cells. (Rotgers et al., 2018)

Similar to its testicular counterpart, the interstitial progenitor of theca cells in mouse also expresses low levels of WT1 and SF1 and high levels of MAFB, ARX and NR2F2 (DeFalco et al., 2011; van den Driesche et al., 2012; Miyabayashi et al., 2013). Around the sex determination stage, ARX expression is found in the coelomic epithelial cells, gonad/mesonephric border and other unknown cell types in the gonadal mesenchyme of the XX gonad. ARX positive cells can be recruited to the medulla part of the ovary, when the first wave of follicle starts to assemble during the perinatal stage (Miyabayashi et al., 2013). NR2F2 is co-expressed with ARX in the interstitial progenitors (Rotgers et al., 2018) but does not co-localize with granulosa cell markers (Rastetter et al., 2014). Haploinsufficiency of NR2F2 expression leads to a reduction of progesterone and a delay of puberty, indicating that NR2F2 is important for steroidogenesis in the postnatal ovary (Takamoto et al., 2005).

Theca cells and Leydig cells differentiate from the same common interstitial progenitor (Fig. 3). Their progenitors show low expressions of WT1 and SF1 and the expression of non-steroidogenic state markers (Stévant et al., 2018a). In addition, when they start to differentiate into theca or Leydig cells, the same enhancer is used to up-regulate *Sf1* transcription (Miyabayashi et al., 2015; Shima et al., 2012b). In mice with a GFP reporter driven by a *Sf1* enhancer, the steroidogenic cells in between the follicles and some theca cells are GFP positive after birth in the ovary, Leydig cells are GFP positive in testes. This observation indicates the existence of a similar set of transcription factors and regulatory elements for the expression of SF1 in theca and Leydig cells. A small subpopulation of theca and Leydig cells were not GFP positive, which might be recruited to differentiate at later stages (Miyabayashi et al., 2015).

In mice, the regulation of theca cell differentiation also depends on DHH/IHH and PDGF mediated paracrine regulation from granulosa cells (Fig. 7). The difference in this regulatory mechanism between male and female is that the secretion of DHH and IHH in granulosa cell only starts around neonatal stages (Liu et al., 2015a; Wijgerde et al., 2005) compared to E12.5, right after the specification of Sertoli cells. Female

interstitial progenitor cells express the receptor PTCH1 around the time of birth, then the expression of GLI1 is initiated after the activation of DHH pathway in the differentiating interstitial progenitor cells (Liu et al., 2015a). Any disruption of DHH and IHH pathway causes the failure of theca cell recruitment and differentiation, impaired steroidogenesis and finally the arrest of follicle growth (Liu et al., 2015a). In addition, oocytes stimulate the surrounding granulosa cells to produce DHH and IHH via the action of growth differentiation factor 9 (GDF9) (Dong et al., 1996; Liu et al., 2015a). Interstitial progenitors failed to differentiate into theca cells in the absence of GDF9, and granulosa cells can try to compensate by activating their own steroidogenesis (Dong et al., 1996). Another paracrine signal that can control the differentiation of theca cells is PDGF. Deletion of *Pdgfra* in somatic cells before sex determination leads to a failure of theca cell development (Schmahl et al., 2008).

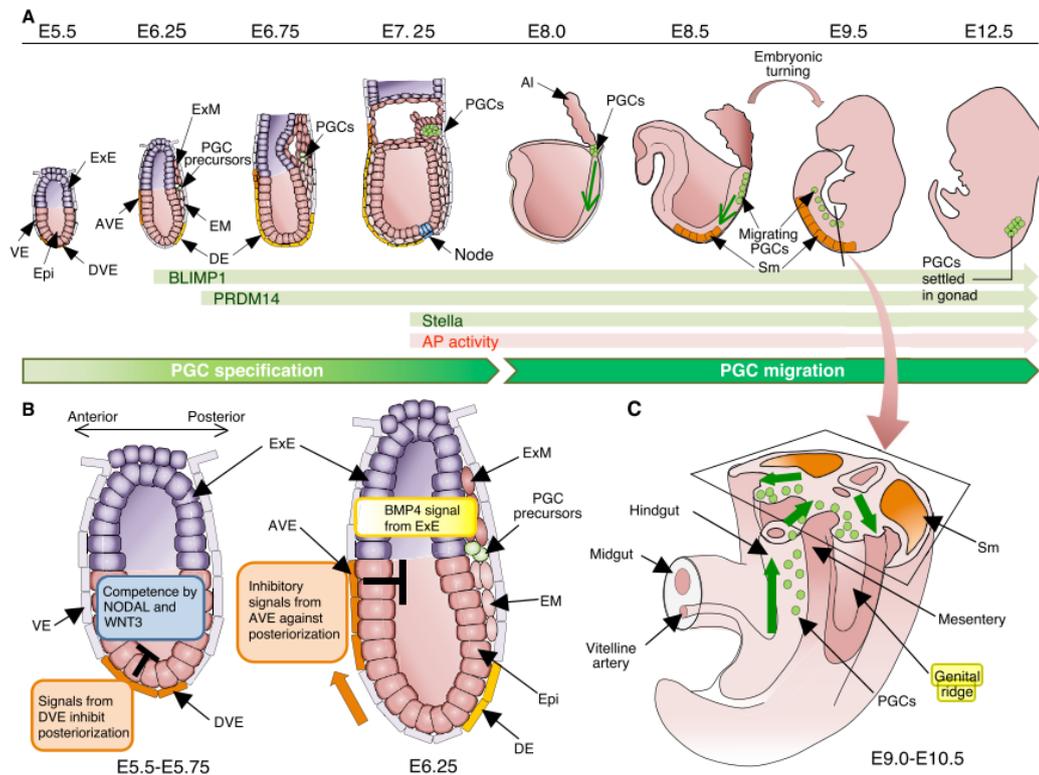
Mouse embryonic granulosa cells do not express DHH until birth, which results in the absence of theca cell differentiation in the fetal ovary. It turns out that silencing of Hedgehog signaling is necessary for the normal differentiation of theca cell (Ren et al., 2012; Schmahl et al., 2008). Ectopic activation of Hedgehog signaling in somatic progenitors by *Sf1-Cre* from the sex determination stage resulted in the appearance of differentiated steroidogenic cells in the embryonic ovary. Part of these steroidogenic cells were more like Leydig cells (Barsoum et al., 2009). However, when activation was induced at later stages and only in the granulosa cells by *Amhr2-Cre*, no steroidogenic cell was formed from granulosa cells (Ren et al., 2012). Taken together, the effects of Hedgehog signal on the plasticity of gonadal somatic cells is dependent on the cell type where it is overexpressed. Up to now, the defects of the components in the DHH pathway are not linked to any ovarian disease or infertility problem in human patients. In contrast, over-activated DHH leads to ovarian carcinoma (Li et al., 2016).

Even though there is no theca cell differentiation in the fetal ovary, the granulosa cells and oocytes express steroidogenic enzymes, such as HSD3b and CYP19, to

produce a small amount of estradiol and progesterone (Dutta et al., 2014; Fowler et al., 2011; Wilson and Joe Jawad, 1979). However, the start of the first wave of folliculogenesis was not affected by the ablation of endogenous estradiol through the mutation of aromatase or by blocking the responsiveness to estrogen (Conte et al., 1994; Couse et al., 1999; Fisher et al., 1998; Krege et al., 2002; Lubahn et al., 1993; Morishima et al., 1995; Shozu et al., 1991). This indicates that even with the low level of steroidogenesis in the fetal ovary, the prenatal ovarian development does not need the presence of steroid hormones. Steroidogenic capacity in the ovaries reaches the maximal peak level on postnatal day 5 in mouse and near birth in human. This occurs after the recruitment and differentiation of theca cells, the follicle development into antral stages, and the gain of responsiveness to gonadotropins (Mannan and O'Shaughnessy, 1991; O'Shaughnessy et al., 1996; Rosenfield and Ehrmann, 2016).

In vitro, isolated mouse ovarian postnatal stromal progenitors can be maintained and induced to differentiate into theca cells depending on added factors in the culture media (Honda et al., 2007). Those theca-like cells can be transplanted into the ovary and integrated into the endogenous theca cell layer.

## 2.4 Germ cell specification, migration and licensing



**Figure 8.** Primordial germ cells (PGCs) specification and migration in developing mouse embryos. **(A)** Sagittal section view of PGCs specification and migration. Embryos prospective anterior is towards the left. The expressions of key factors (BLIMP1, PRDM14 and Stella) and alkaline-phosphatase activity are depicted. **(B)** Signaling activities for PGC specification in the early mouse embryo **(C)** A 3D view of PGC migration tracts. Green circles represent PGC precursors and PGCs, and the green arrows indicate the migration direction of PGCs. (Saitou et al., 2011)

Germ cells are central components of the gonad, giving rise to the sperm in testis and oocytes in ovary. Before gonad formation, both XX and XY germ cells go through the same process: specification to form primordial germ cells (PGCs) and migration into the gonad. During the sex determination period, XX and XY PGCs start the sex specific licensing to become gametogenesis-competent cells (GCCs).

### **- PGCs specification and migration**

In mice, PGCs specification happens around E6.0, at least 3 days earlier than the formation of gonad. The specification occurs in the most proximal epiblast, induced by bone morphogenetic protein (BMP) signals secreted from the extra-embryonic ectoderm (Lawson et al., 1999). In response to BMP signals, the most proximal epiblast cells start to express the transcriptional regulators PR domain zinc finger protein 1 (PRDM1) and PR domain zinc finger protein 14 (PRDM14) around E6.25 and E6.5, respectively. Around E7.25, those PRDM1 and PRDM14 positive cells form the PGCs pool, which comprises 40 cells expressing alkaline phosphatase (AP) and located at the basement of the incipient allantois (Ginsburg et al., 1990; Ohinata et al., 2005, 2009; Vincent, 2005; Yamaji et al., 2008). Starting from E7.5, PGCs move to the hindgut, then the hindgut elongates along with the PGCs, so that the PGCs find themselves in the embryonic region close to genital ridge. Around E9.5-10.0, PGCs migrate away from the hindgut into the genital ridge through the mesentery (Fig. 8) (Dzementsei and Pieler, 2014). It has been shown that several chemotaxis and survival signals such as WNT5a-ROR2 (Chawengsaksophak et al., 2011; Laird et al., 2011), SCF (secreted KitL)-ckit (Gu et al., 2009; Runyan et al., 2006) and SDF1-CXCR4 (Ara et al., 2003; Molyneaux, 2003) can regulate the autonomous migration of PGCs away from the hindgut to the genital ridge region.

### **- PGCs licensing into gametogenesis competent cells**

At E10.5, all the PGCs are settled in the gonad and start the sex specific licensing (Kurimoto et al., 2008; Saitou et al., 2002; Seki et al., 2005). Before licensing, the PGCs exhibit a pluripotent genetic program and express the main pluripotency genes such as *Oct3/4*, *Nanog* and *Sox2*. Indeed, injecting PGCs into the mouse postnatal testis leads to the formation of teratomas (Chuma, 2004; Saitou, 2009; Saitou and Yamaji, 2012). Right after PGCs have reached the genital ridge, their gene expression profiles go through a global change (Molyneaux et al., 2004; Rolland et al., 2011; Seisenberger et al., 2012). PGCs gradually turn off the genes involved in pluripotency maintenance and gain expression of a set of genes, which will allow

them to transit into a state ready for gametogenesis. Once the transition is finished, these germ cells, which are capable of responding to the surrounding environmental signals to enter into meiosis together with male or female differentiation, are referred to as gametogenesis-competent cells (GCCs) (Adams and McLaren, 2002; Gill et al., 2011; McLaren and Southee, 1997). The transformation of PGCs into GCCs is named germ cell licensing, which is a critical step for the transition of germ cells into a sexually competent state. Depending on the result of gonadal sex determination, testis or ovary, GCCs will commit either to spermatogenic or oogenic development based on the signals produced by the supporting cells (Koubova et al., 2006; Suzuki and Saga, 2008).

#### **- Somatic environment dictates germ cell development**

After sex determination, it is the sex-specific behavior of somatic cells, specifically the supporting cells, that decides the fate of germ cells along the male or female pathway (Schmahl et al., 2000), not the sex chromosome constitution of the germ cells. Chimera experiments have shown that no matter what the sex chromosome constitution is, XX or XY, the fate of germ cells is determined by the gonadal environment: XX germ cells could differentiate into the male pathway if surrounded by a testis environment, and XY germ cells could enter the female fate induced by an ovarian environment (Adams and McLaren, 2002). This has also been proven by genetic mouse models. XY *Sox9* mutant gonads develop into ovaries, which are able to produce X oocytes and Y oocytes (Lavery et al., 2011b). Conversely, XX germ cells in XX transgenic animals where *Sox9* is overexpressed in the gonad did not enter into meiosis, unlike the XX control germ cells after sex determination (Gregoire et al., 2011).

Germ cell differentiation depends on proper gonadal formation, but the gonad can be formed in the absence of germ cells, even though testes formation without germ cells is delayed (Merchant, 1975). Fetal ovary without germ cells develop normally until birth (Maatouk et al., 2012). However, folliculogenesis and maintenance

of the ovary require signals from germ cells after birth. Loss of germ cells after birth in ovaries will cause the degeneration of the ovary (Guigon and Magre, 2005; Guigon et al., 2005).

#### **- Female and Male germ cell differentiation**

Before E12.5, germ cells proliferate rapidly and are aggregated in clusters, outlined by E-Cadherin expression (Mork et al., 2012b). During ovarian and testicular development, both XX and XY mesonephros can secrete retinoic acid (RA). In the ovary, the presence of RA up-regulates the expression of *Stra8* in germ cells around E13.5, which marks the entry of meiosis (Bowles et al., 2006; Koubova et al., 2006). Besides, the initiation of meiosis in XX gonads starts from the anterior and then extends to the posterior part (Yao, 2003). In *Rspo1* total knockout mutants, germ cell proliferation is down-regulated and entry into meiosis of germ cells in the anterior region is impaired, indicating the WNT signaling has an impact on germ cell proliferation and meiotic initiation. However, the detailed mechanism of its action and whether it involves a direct effect or not are still unknown. Around birth, primary oocytes are halted at diplotene stage of meiotic prophase I. After puberty upon stimulation by gonadotropins, they resume meiosis to become secondary oocytes during the ovulation and arrest again at the metaphase of meiosis II until fertilization (Picton et al., 1998).

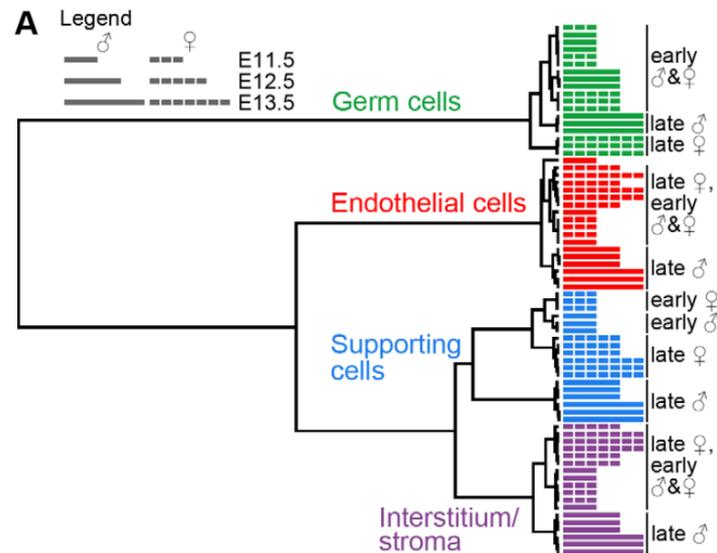
In contrast, in the testis the RA catabolic enzyme CYP26B1 expressed by Sertoli cells blocks the meiosis-inducing effect of RA (Bowles et al., 2006; Koubova et al., 2006). Therefore, the XY germ cells enter in mitotic arrest from around E15.5 until birth (McLaren, 1984). In testis, FGF9 is important for germ cell survival. *Fgf9* mutants showed a significant reduction of germ cell number, which can be rescued by addition of exogenous FGF9 before E11.5. The mechanism behind this is not well known, but FGF9 is able to induce the expression of *Nanos2* and inhibit meiosis (Bowles et al., 2010). After puberty, germ cells start meiosis to produce gametes sequentially under the regulation of follicle-stimulating hormone, testosterone and

retinoic acid, this process is also called spermatogenesis (Hogarth and Griswold, 2010). A lot of RNA-binding proteins are involved in spermatogenesis, such as those encoded by *Nanos2*, *Dazl*, *Vasa*, and their mutations lead the failure of spermatogenesis initiation or spermatogenic arrest (Saga, 2010; Schrans-Stassen et al., 2001; Tanaka et al., 2000). *Lin28a* and *Dnd1* mutations cause significant germ cell number decrease in both XX and XY (Shinoda et al.; Youngren et al., 2005).

### **3. Sex determination regulation**

The molecular mechanisms of sex determination in mammals are mostly still unclear. In XY human, impaired *SRY* expression is the most common cause of 46, XY gonadal dysgenesis, but only 20% of those cases had mutations in the *SRY* coding region (Koopman, 2016). There are still half of 46, XY gonadal dysgenesis cases that cannot be explained at the molecular level (Délot and Vilain, 2018; Rotgers et al., 2018). Compared to XY sex determination, it has long been considered that ovary development is a pre-programmed process in the absence of *SRY/Sry* in XX or when *SRY/Sry* is mutated in XY. Since the discovery of female-to-male sex reversal in XX human patients and XX mouse with mutations of *WNT4/Wnt4* (Jordan et al., 2001; Maatouk et al., 2013; Mandel et al., 2008; Vainio et al., 1999) and *RSPO1/Rspo1* (Chassot et al., 2008a; Parma et al., 2006; Tomizuka et al., 2008) and the development of next generation sequencing technique, a more complicated sex determination network has been built up. The determination of sex is regulated by an antagonistic mechanism such that promoting the development of male (testis) or female (ovary) fate, requires the repression of the alternative pathway during the embryonic and adult stages (Kim et al., 2006b; Matson et al., 2011; McElreavey et al., 1993; Munger et al., 2009, 2013; Uhlenhaut et al., 2009). Interestingly, in this network sexual hormones are not involved in the early sex determination, but they do play roles in the maintenance of sex during the adult stages, at least in female mice (Uhlenhaut et al., 2009).

### 3.1 Genome-wide analyses provides evidence for mutual antagonism between testis and ovary fate

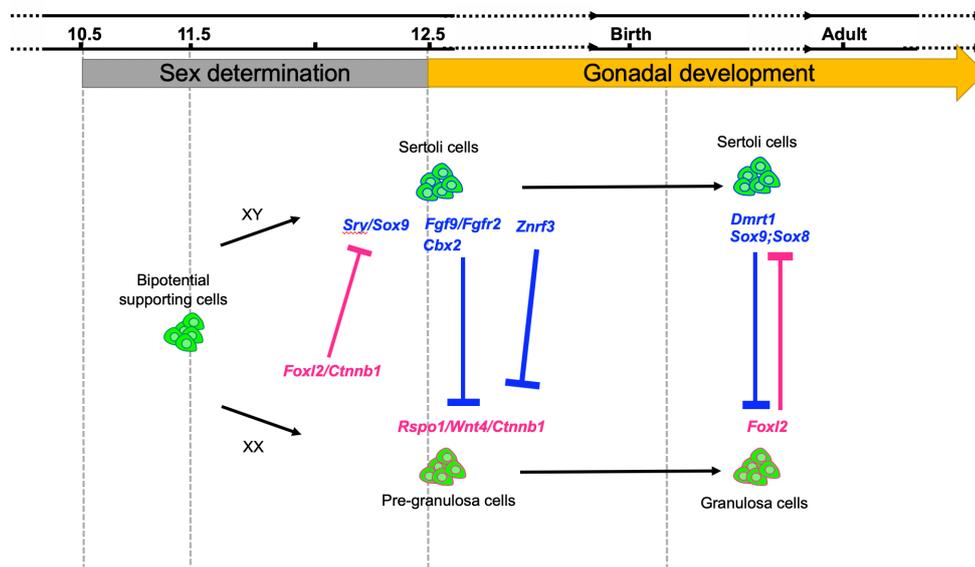


**Figure 9.** Clustering dendrogram of the transcriptional profiles of E11.5-E13.5 XX and XY FACS-sorted gonadal cells by microarray assays. Dashed bars represent XX samples, and solid bars are the XY samples. Short, intermediate and long bars show the different stages from E11.5. E12.5 and E13.5, orderly. (Jameson et al., 2012b)

During the bipotential stages, both XX and XY gonads have the ability to develop into either testis or ovary depending on the pathway activated in the supporting cells around the time of sex determination. The microarray transcriptional analysis of FACS-sorted germ cells, endothelial cells, supporting cells and Interstitium/stroma cells at E11.5 showed the XX and XY supporting cell are similar and biased towards a female fate (Fig. 9) (Jameson et al., 2012b). The single-cell transcriptomic analysis of gonadal SF1-GFP positive cell before and after sex determination also showed that around E11.5, XX and XY supporting cells had similar gene expression profile, despite the presence of *Sry* in XY (Stévant and Nef, 2018). From the epigenetic point of view, *cis*-regulatory element studies in mouse XX and XY supporting cells before and after the sex determination revealed that the XX and XY supporting cell exhibit similar open chromatin regions before the onset of sex determination as shown by

Assay for Transposase-Accessible Chromatin, ATAC-seq, but develop their own open sex-specific chromatin region after they differentiated as shown by ChIP-seq for H3K27ac (Garcia-Moreno et al., 2019a). Taken together, XX and XY supporting cells have similar transcriptomes and chromatin accessibility before sex determination, and then acquire sex-specific gene expression and open chromatin. Interestingly, in E13.5 Sertoli cells the granulosa-promoting genes chromatin region is open but does not contain active enhancers (Garcia-Moreno et al., 2019a). In E15.5 Sertoli cells, DNaseI-seq showed that the promoters of female specific genes are also accessible, but only the promoters of male specific genes contain active enhancers (H3K27ac ChIP) (Maatouk et al., 2017). These observations indicate that in differentiated Sertoli cells the open chromatin sites on the female specific gene promoters are accessible but silenced probably by transcriptional repressors.

### 3.2 Genetic evidence for mutual antagonism between testis and ovary fate



**Figure 10.** Mutual antagonistic network between female and male pathways in mice. *Sry*, *Sox9*, *Fgf9/Fgfr2*, *Cbx2*, *Znr3*, *Dmrt1* and *Sox8* help to establish male pathway, while *Rspo1*, *Wnt4*, *Ctnnb1* and *Foxl2* promote or maintain the female development.

In XY, *Sry* in the Y chromosome triggers male development by directly up-regulating its main autosomal target gene *Sox9*. Both *Sry* and *Sox9* are necessary and sufficient for testis development (Eicher et al., 1995; Gregoire et al., 2011; Lavery et al., 2011b; Vidal et al., 2001). Further, *Sox9* activates the expression of fibroblast growth factor 9 (FGF9), which is a secreted protein. FGF9 can activate *Sox9* to help the quick expansion of Sertoli cell differentiation from the center to the pole regions. The receptor of FGF9 in the gonad is FGFR2, and more specifically the isoform FGFR2c. The loss of *Fgf9* or *Fgfr2/Fgfr2c* leads to partial male-to-female sex reversal at the two poles region (Bagheri-Fam et al., 2008, 2017; Colvin et al., 2001; Kim et al., 2007) with the failed activation of *Sox9*. Ex vivo experiments have showed that FGF9 protein is sufficient to inhibit the expression of *Wnt4* in XX gonads (Kim et al., 2006b). Simultaneous deletion of *Wnt4* and *Fgf9* or *Fgfr2* in XY mice can revert the male-to-female sex reversal phenotypes (Jameson et al., 2012a), indicating that FGF9 signaling pathway represses the WNT pro-female pathway in XY (Fig. 10). *Foxl2* deletion in addition to *Fgfr2* in XY mutants also rescues the male-to-female sex reversal in some individuals but not all (Bagheri-Fam et al., 2017).

Additionally deleting *Wnt4* also can rescue the male-to-female sex reversal in XY *Cbx2* mutants (Garcia-Moreno et al., 2019b), indicating *Cbx2* is necessary for testis development to inhibit the WNT pro-female pathway in XY. ZNRF3 is a negative regulator of RSPO1/WNT4/ $\beta$ -Catenin female signaling pathway. The mutation of *Znrf3* results in the reduction of *Sox9* expression and in turn male-to-female sex reversal in XY mice (Harris et al., 2018). This finding also highlights that it is necessary to inhibit the female pathway during the male sex determination time.

SOX9 is not necessary for establishing the male pathway after sex determination, since XY mutants with deletion of *Sox9* in differentiated Sertoli cells by *Amh-cre* developed normal testes (Barrionuevo et al., 2009). However, *Sox9* and *Sox8* together are needed in embryonic and adult testis to stop the Sertoli cell trans-differentiation into *Foxl2* positive granulosa-like cells (Barrionuevo et al., 2016;

Georg et al., 2012). *Dmrt1* is another important Sertoli cell marker whose ectopic expression in XX mice leads to the development of testis and secondary male characteristics, although the ectopic testis cords were not well formed compared to *Sox9*-expressing transgenic XX testes (Gregoire et al., 2011; Zhao et al., 2015). In the postnatal testis, *Dmrt1* is more important than *Sox9* or *Sox8* in the maintenance of testis fate. The loss of *Dmrt1* leads to massive Sertoli cell trans-differentiation into granulosa-like cells from P14 (Matson et al., 2011). Therefore, in order to develop and maintain the testis fate, the female pathway has to be repressed at the same time.

In XX, RSPO1/WNT4/ $\beta$ -Catenin is the main signaling pathway promoting ovarian development. *Rspo1* and *Wnt4* are two activators of canonical WNT/ $\beta$ -Catenin signaling in the gonad, while  $\beta$ -Catenin executes the activation or repression of the down-stream targets in the nucleus. The loss of the any one of them in the XX gonad during sex determination results in the development of XX ovotestes (Chassot et al., 2008a; Liu et al., 2009; Tomizuka et al., 2008) because of the failed activation of canonical WNT/ $\beta$ -Catenin signaling pathway. As a granulosa cell marker, *Foxl2* is dispensable for the establishment of the female pathway (Ottolenghi et al., 2005; Schmidt, 2004), but it is necessary to maintain the ovarian fate after birth (Ottolenghi et al., 2005; Uhlenhaut et al., 2009). Total knockout of *Foxl2* in XX mice, results in granulosa cells trans-differentiation into Sertoli-like cells around P7 (Ottolenghi et al., 2005), and conditional knockout of *Foxl2* at the adult stage also leads to *Sox9* positive Sertoli-like cell trans-differentiation (Uhlenhaut et al., 2009). Notably, in these adult granulosa cells, estrogen receptor 1/2 (ESR1/2) cooperates with FOXL2 to repress the expression of *Sox9* (Uhlenhaut et al., 2009), indicating that the sexual hormone estrogen possibly plays a role in maintaining the ovarian fate, and that it is necessary to repress the male pathway even in the well-established adult stages.

Male development is triggered by SRY/SOX9 pathway, while RSPO1/WNT4/ $\beta$ -Catenin pathway is indispensable for female development. Since these two pathways are mutually antagonistic to each other in testis and ovary, it was

important to investigate gonad development when both pathways were shut down around the sex determination time. Both XX *Sox9;Rspo1* and *Sox9;Cttnb1* double knockout mutant gonads developed into ovotestis as the XX *Rspo1* or *Cttnb1* mutants, indicating that *Sox9* is not necessary for the induction of female-to-male sex reversal (Lavery et al., 2012; Nicol and Yao, 2015). Surprisingly, XY *Sox9;Rspo1* double knockout mutants developed hypoplastic testis (Lavery et al., 2012), whereas the XY *Sox9;Cttnb1* double knockout mutants developed ovotestis but were more masculinized than XX *Cttnb1* mutants (Nicol and Yao, 2015). When comparing double knockout XY to XX mutants in both cases, XY double knockout mutant gonads always had more masculinized phenotypes, suggesting that in addition to *Sox9* other pro-male genes can act in the XY background, probably the other targets of *Sry*. For instance, *Sox8* and *Sox10* are expressed at relatively higher levels in XY than in XX double mutants (Lavery et al., 2012; Nicol and Yao, 2015).

#### **4. Human disorders of sex development**

Appropriate sex development has a very important role in deciding the human physical attributes, brain structure, the tendency of behavior and self-concept. Our understanding of sex differential development has been improved by the molecular studies on the pathways associated with the disorders of sex development. Disorders of sex development (DSD), is a broad definition for the atypical development of chromosomal, gonadal and anatomical sex in human. It includes all the patients with gonadal dysgenesis and exhibiting the development of ambiguous genitalia but without dysgenetic gonad. Genetically, DSD can be categorized into sex chromosomes DSD, 46, XY DSD and 46, XX DSD.

##### **4.1 Sex chromosomes DSD**

Sex chromosomes DSD, such as the Turner syndrome and Klinefelter syndrome, are caused by sex chromosomes aneuploidies or polyploidies, respectively. For the Turner syndrome, most of which exhibit a 45, X karyotype, it is most likely caused by

the maternal failure of meiotic disjunction in meiosis I. Short stature is the universal feature for almost all the patients, because a short stature controlling genetic locus is located at X chromosome (Reynaud et al., 2004; Stochholm et al., 2006). The gonads of patients are called streak gonads without primary follicles but with primitive connective tissue. The lack of estrogen secretion from the follicular granulosa cells leads to a delayed puberty (Weiss, 1971). In contrast, 47, XXY Klinefelter syndrome patients have hypergonadotropic hypogonadism, and this disorder of polyploidy is caused equally by maternal and paternal errors (Hassold et al., 2007; Thomas and Hassold, 2003). The fetal testes of XXY patients develop normally with germ cells, however the deficient communication between Sertoli cells and germ cells during childhood leads to the degeneration of germ cells and further infertility (Lue et al., 2001).

#### **4.2 46, XY DSD**

46, XY DSD includes disorders of gonadal development and disorders in androgen synthesis and action. Among the 46, XY disorders of gonadal development, *SRY* (Arboleda et al., 2014; Assumpção et al., 2002; McElreavy et al., 1992; Uehara et al., 1999, 2002), *SF1*, *GATA4* and *GATA2* (Ozisik et al., 2002; Tremblay and Viger, 2003) mutations can cause complete gonadal dysgenesis (GD), in which streak gonads develop instead of testis, with no secretion of testosterone. As a consequence, the XY develop into females but with abnormal development of the Müllerian duct. Only 30 percent of XY GD are caused by the deletions or mutations of *SRY* (85%) and *SF1* (15%), suggesting that other genes regulating male development exist and need to be found out. On the other hand, the mutations of *MAP3K1*, *SOX9*, *WT1*, *DHH*, *DMRT1* and the duplication of *WNT4* and *DAX1* lead to XY partial GD, in which the developed dysgenetic testis represents an intermediate state between testis and streak gonads (Domenice et al., 2004; Saylam and Simon, 2003; Vilain, 2008).

Androgen is essential for the male reproductive tract development, so any disruption from the synthesis and action of androgen in XY would lead to the

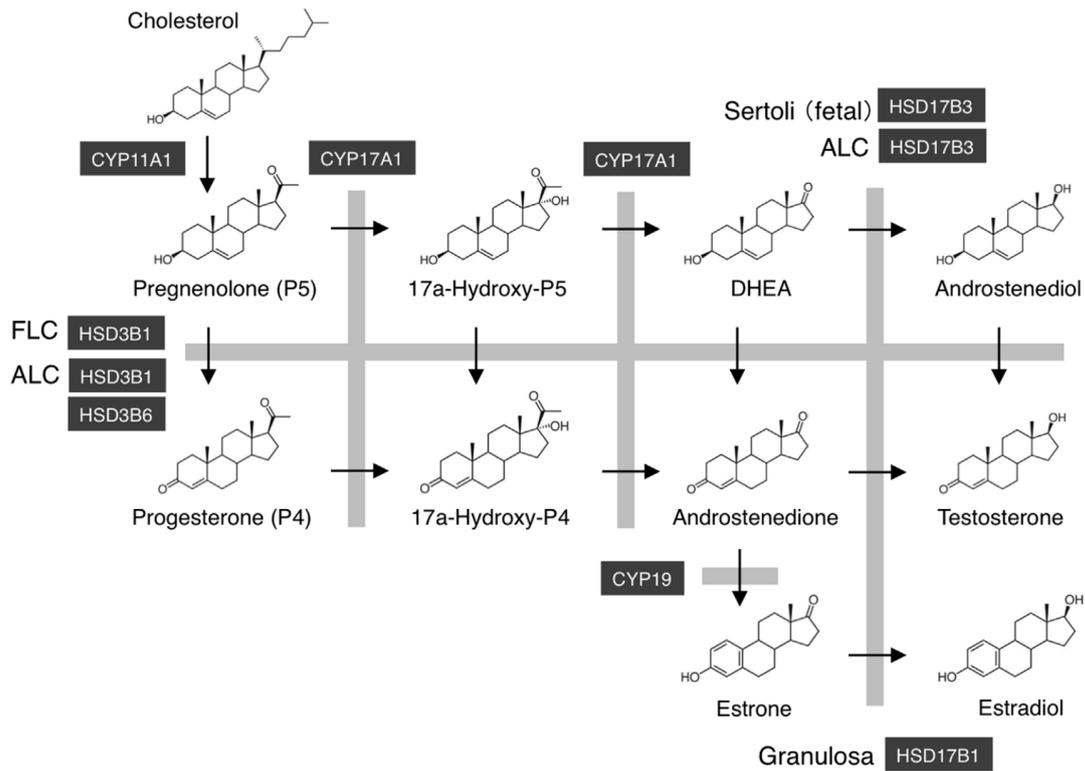
abnormal development of external genitalia. During embryonic stages XX and XY have the same two sets of male and female reproductive ducts, therefore the ectopic presence of androgen leads to the retention of the male reproductive duct. Atypical external genitalia is a type of sex development disease and is always used as the first sign of DSDs. 46, XY disorders of androgen synthesis and action belong to disorders of sex development. The 46, XY DSD patients show different degrees of undervirilized male development or typical female development, since testosterone is required for secondary sex characteristics development in XY individuals.

The mutations of *STAR* and *CYP11A1* can cause global gonadal and adrenal steroidogenesis defects, as these proteins are involved in the initiation of steroidogenesis (Katsumata et al., 2002; Tajima et al., 2001).  $3\beta$ -hydroxysteroid dehydrogenase (*HSD3B2*) mutation blocks the synthesis of cortisol, aldosterone and testosterone in adrenal glands, which further results in ambiguous genital development in XY. *CYP17A1* and *CYP5A* mutations lead to the 17,20-lyase deficiency, which in turn blocks the synthesis of sex steroid and glucocorticoids (Idkowiak et al., 2012; Kok et al., 2010). Since Cytochrome P450 Oxidoreductase (*POR*) is required by multiple enzyme activities, the *POR* deficiency results in a global loss of enzyme activity in the steroidogenesis (Peterson et al., 1985; Scott and Miller, 2008; Shackleton et al., 2004) in XX and XY. The mutations of *HSD17B3* block the testosterone conversion from  $\Delta 4A$  in testis. *SRD5A2* could irreversibly convert testosterone into DHT, which is needed for the development of male external genitalia. Undervirilized external genitalia form in the XY males with *SRD5A2* mutations. Mutations leading to a decreased transactivating activity of *MAMLD1*, lead to a reduced expression of *CYP17A1* and decreased testosterone which will cause hypospadias (Chen et al., 2010; Kalfa et al., 2012; Nakamura et al., 2011) (Fig. 11).

In vertebrates, androgen includes generally testosterone, Dihydrotestosterone (DHT) Dehydroepiandrosterone (DHEA), Androstenedione (A4) and Androstenediol (A5) (Bandyopadhyay and Muthairulan, 2017). Testosterone can be converted into

DHT and estrogen by steroid 5-Alpha-Reductase 1/2 (SRD5A1/2) and aromatase (CYP19A1), respectively (Maccoccia et al., 2017). DHT is the final product of androgen biosynthesis and is not aromatisable to estrogen (Burger, 2002). Partial and complete androgen insensitivity syndrome is caused by the mutations of androgen receptor (*AR*). In the androgen signalling pathway, androgen signals are passed by the nuclear Androgen Receptor (Lee et al., 2016) to regulate the development of male reproductive tracts, male pubertal changes and libido; the anabolism of bone tissues and skeletal muscles and the distribution of subcutaneous fat (Li et al., 2017; Maccoccia et al., 2017). *AR* mutations block all the effects of androgens on the development of internal and external genitalia and result in the unambiguous XY female (Galli-Tsinopoulou et al., 2003; Tut et al., 1997). *AR/Ar* is located on the sexual chromosome X in human and mice (Davey and Grossmann, 2016). Without the binding by androgen, cytoplasmic *AR* is degraded by the Heat Shock Protein (HSP) 90 chaperone complex-mediated ubiquitination (Maccoccia et al., 2017), while in the presence of androgens, *ARs* dissociate from HSP, become hyperphosphorylated and are translocated into the nucleus. Dimerized *ARs* recognise the androgen response element (ARE) of the target genes and initiate transcription with other co-regulatory factors (Banuelos et al., 2014).

Luteinizing hormone/chorionic gonadotropin receptor (LHCGR) mutations lead to Leydig cell hypoplasia and reduction of testosterone during the prenatal stages (Richter-Unruh et al., 2002; Wu and Chan, 1999). The external genitalia of XY patients can be complete female or undervirilized (Martens et al., 2014). Additionally, persistent Müllerian duct syndrome can be caused by the mutations in *AMH* and its receptor *AMHR2*, which is responsible for the regression of Müllerian ducts during fetal development. Patients with this syndrome had normal external male genitalia but male and female internal genitalia, which could lead to infertility. Altogether mutations on the genes involved in the synthesis and action of testosterone and AMH could lead to incomplete masculinization and the retention of the female duct in XY male, even though the development of the testis is normal.



**Figure 11.** The steroidogenesis pathways in gonads. CYP11A1, CYP17A1, HSD17B3 (expressed in fetal Sertoli cells and Leydig cells) and HSD3B1/6 (specific in Leydig cells) are used to produce the testosterone in the testes, while CYP19 and HSD17B1 (only found in granulosa cells) are responsible for catalyzing the transformation from androgen to estrogen in ovaries. FLC: Fetal Leydig Cell, ALC: Adult Leydig Cell. (Inoue et al., 2018)

#### 4.3 46, XX DSD

46, XX DSD includes the disorders of sex determination, which have testicular, ovotesticular phenotypes, and the disorders of sex development with gonadal dysgenesis or excess of androgen during the fetal development. Abnormal activation of the male sex-determining genes, such as *SRY*, *SOX9* and *SOX3* in 46 XX, leads to the testicular development with phenotypic male and genital ambiguity phenotypes (Délot and Vilain, 1993). The loss of female sex-determining genes *WNT4* or *RSPO1* results in ovotestis development with the presence of testicular seminiferous tubules and ovarian follicles in the same gonad (Parma et al., 2006; Tomaselli et al., 2008). The most common 46, XX gonadal dysgenesis is caused by the loss of X chromosomes in the Turner syndrome.

Genes causing 46, XX GD were identified by exome sequencing and include the receptors of LH, FSH and estrogens, transcription factors for oocyte development (*NOBOX*, *SF1*, *FOXL2*, *FIGLA*), growth factors for folliculogenesis (*GDF9*, *BMP15*, *INHA*) and also the genes (*HFM1*, *STAG3*, *MCM8/9*) involved in meiosis, DNA replication and repair and chromosome stability (Chapman et al., 2015). Patients with mutations in those genes had normal female development but ovarian development failure after birth with delayed puberty and infertility in adulthood. High level of androgen exposure of the XX fetus promotes the development of Wolffian duct and varying degrees of masculinized external genitalia (Chassot et al., 2008a; Liu et al., 2009; Shan et al., 2010). Excessive androgen levels can be caused by steroidogenesis deficiencies of the fetus, of the fetal-placental barrier or by high levels of maternal androgen.

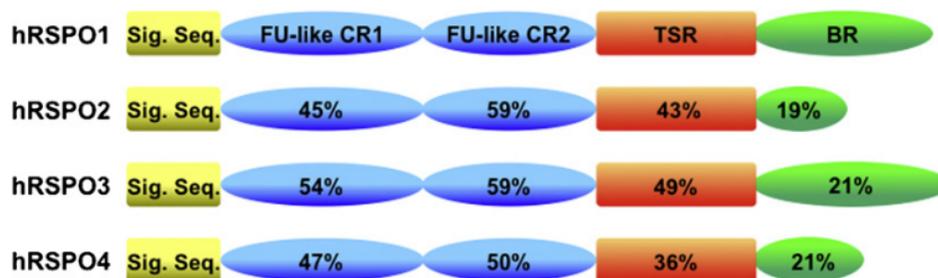
*CYP21A2* mutations block the synthesis of aldosterone and cortisol and causes a shift to the androgen production in the presence of 17 $\alpha$ -hydroxyprogesterone (Clayton et al., 2002; Merke et al., 2002; New, 2003). *CYP11B1* mutations causes increased cortisol precursors for androgen synthesis (New, 2003; Peter, 2002). *CYP19A1* is the responsible for the production of estrogen by catalyzing androgen conversion. Mutations of *CYP19A1* lead to spermatogenesis failure in male and defects in female secondary sex characteristics development. The androgen in the progestin-containing oral contraceptives taken by the pregnant mother and hormone-activated tumors like luteomas in the mother also can cause an excessive androgen to the XX baby (Coenen et al., 1995; Joshi and Dunaif, 2018; Nelson and Goldenberg, 1977; Ziaei et al., 2002) (Fig. 11).

Whether the patients have disorders of sexual differentiation or disorders of sex determination, the level of steroidogenic sex hormones is unbalanced compared to the normally developed men or women. Even though there is no effective cure for DSD, hormonal therapy can be used to some degree to correct the development of the internal genital tract during pregnancy and the external genitalia after birth.

Depending on how the DSD patient will be raised as male or female (Dreger et al., 2005), male patients can be treated with testosterone to help the growth of penile tissue and the induction of puberty (Kühnert et al., 2005), while female patients can be treated with estrogens to help the development of their secondary sexual characteristics and to induce the puberty (Délot and Vilain, 2018). In addition, glucocorticoids and mineralocorticoids can be administered to the patients with congenital adrenal hyperplasia as a life-saving replacement therapy, because the patients usually show a fatal salt-wasting adrenal crisis at birth (Délot and Vilain, 2018). The development of assisted reproductive technologies brings hope for DSD patients to have their own biological children. Depending on the presence of germ cells, competent sperm or oocytes taken from the gonads are fertilized in vitro by intra-cytoplasmic sperm injection. Female patients can cryopreserve their oocytes for future use before the depletion of the oocytes during the premature ovarian failure (Délot and Vilain, 2018).

## 5. The RSPO protein family

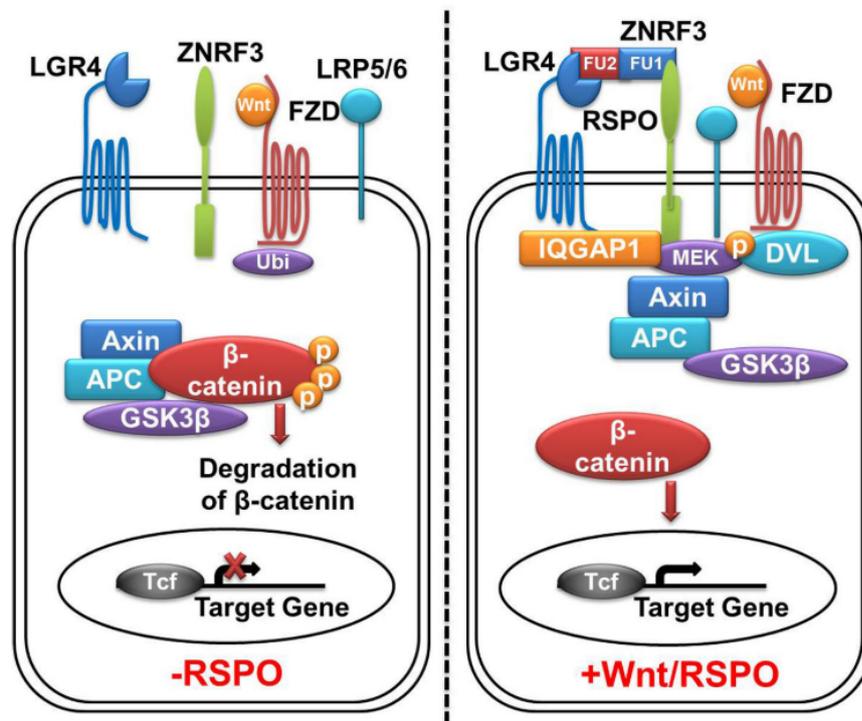
### 5.1 The structure of RSPO proteins



**Figure 12.** The human RSPO proteins. The four human RSPO proteins are RSPO1–4, which have 263, 243, 292 and 234 amino acids, respectively. Sig, Seq: signal sequence; FU-like CR1/2: two cysteine-rich furin-like repeats; TSR: thrombospondin type1 repeat domain; BR: basic amino-acid-rich domain with variable lengths. The percentages of identical amino acids show relative protein sequence conservation. (Jin and Yoon, 2012)

The R-spondin proteins belong to the thrombospondin type 1 repeat (TSR-1) superfamily (de Lau et al., 2012) and were named as *Rspo* (roof plate-specific spondin) after the finding that *Rspo1* was specifically expressed at the roof plate of neural tube in mice (Kamata et al., 2004). In mammals, there are four RSPO proteins: RSPO1, RSPO2, RSPO3 and RSPO4, which are around 35 kDa secreted proteins. The amino acid sequence identity of human and mouse RSPO members is approximately 40–60% (Kazanskaya et al., 2004; Kim et al., 2006a; Nam et al., 2006). RSPO proteins are composed of 4 main domains: N-terminus signal peptide sequence, two amino-terminal furin-like repeats (FU1/2CR) domains, one thrombospondin domain (TSP) and a C-terminus basic amino acid-rich (BR) domain with variable length (Fig. 12). The signal peptide sequence at the N-terminus helps RSPO proteins to be processed and secreted through the canonical secretory pathway by endoplasmic reticulum and Golgi apparatus (Nam et al., 2006). The CR domain is primarily in charge of interacting with RSPO receptors and later the activation of the canonical WNT signaling pathway. In vitro, deletions or mutations of the CR domain resulted in reduced activity of WNT signaling (Jin and Yoon, 2012; Kim et al., 2008; Li et al., 2009). The TSR domain of RSPO protein engages in binding to glycosaminoglycans and/or proteoglycans matrix (Kim et al., 2008; Ohkawara et al., 2011) and is also able to strengthen the activity of RSPO proteins on canonical WNT signaling along with the BR domain. The deletion of TSR and BR domains resulted into less effective activation of canonical WNT signaling (Kim et al., 2008).

## 5.2 RSPO and canonical WNT/ $\beta$ -Catenin signaling



**Figure 13.** The various components involved in canonical RSPO/WNT/ $\beta$ -Catenin signaling pathway. RSPO proteins promote the WNT/ $\beta$ -Catenin signaling pathway by inhibiting the ubiquitination of FZD receptors (Li et al., 2015).

In the WNT/ $\beta$ -Catenin canonical signaling pathway,  $\beta$ -Catenin is the major player in the cascade to turn on the transcription of downstream targets (Yu and Virshup, 2014). In the absence of WNT proteins, a destruction complex, which is mainly composed of the scaffold proteins APC/AXIN and phosphorylase: glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), targets the cytoplasmic  $\beta$ -Catenin to the proteasome via ubiquitination (Niehrs, 2012). As a consequence, only low levels of  $\beta$ -Catenin are kept in the cytoplasm. In the presence of WNT signaling, WNT proteins interacting with Frizzled (FZD) receptors and co-receptors LRP5/6 can phosphorylate and activate the cytoplasmic protein dishevelled (DVL), which further dissociates the “destruction complex” (Bilić et al., 2007; Zeng et al., 2007). Therefore, the accumulation of stabilized  $\beta$ -Catenin in the cytoplasm leads to more  $\beta$ -Catenin being translocated into the nucleus (Janssen et al., 2006; Obrador-Hevia et al., 2010; Phelps et al., 2009).

$\beta$ -Catenin replaces Groucho to bind TCF and/or other tissue-specific activators, acting as transcriptional activator to turn on the common or tissue specific target gene expression (Cadigan and Waterman, 2012; Mosimann et al., 2009).

In order to avoid over-activating WNT/ $\beta$ -Catenin signaling, the abundance of FZD on the membrane is controlled by the ubiquitination process. Ubiquitinated FZD could be degraded by endocytosis, so that less receptor is available to react with WNT ligands leading to less activity of WNT/ $\beta$ -Catenin signaling in the cell (Hao et al., 2012; Koo et al., 2012). The ubiquitination is mediated by transmembrane RING domain-containing E3 ubiquitin ligases ZNFR3 and RNF43, which are up-regulated by WNT signaling as a negative feedback mechanism (Jimenez et al., 1996; Koo et al., 2012). On the other hand, the ubiquitination of FZD is also negatively regulated by RSPO proteins to sensitize cells to WNT signaling. RSPO bridges its receptors LGR4/5/6 with ZNFR3/RNF43 through the two amino-terminal furin-like repeats domains (FU1/2) to decrease the activity of ubiquitination to FZD and hence more FZD receptor are available to receive the WNT signaling (Carmon et al., 2011; Glinka et al., 2011; Hao et al., 2012; Xie et al., 2013). Recently, *Rspo2/3*, but not *Rspo1/4*, have been shown to be able to activate WNT signaling pathway independently from LGR receptors. Instead, their TSP/BR domains binding to the heparan sulfate proteoglycans (HSPGs) together with FU1-ZNFR3/RNF43 interactions mediate the endocytosis and clearance of the ZNRF3/RNF43 ligases from the cell surface (Lebensohn and Rohatgi, 2017, 2018) (Fig. 13).

### **5.3 RSPO function**

#### **5.3.1 *Rspo1***

*Rspo1* is not only essential for the embryonic female development (see “Granulosa cells function and regulatory pathway” chapter) but also important for the maintenance of epithelial stem cells and tissue homeostasis or repair in adults. *Rspo1* and *Lgr5* are highly expressed in the ovarian surface epithelial cells. Overexpression of *Rspo1* mediated by the *Sf1-Cre* transgene in the mouse ovary resulted in

granulosa cell tumors indicating *Rspo1* could act as an oncogene when its expression was elevated ectopically (De Cian et al., 2017). Even though the loss of *Rspo1* leads to female-to-male sex reversal, some of these XX mutant mice remain sub-fertile and give birth. However, the mutant mothers cannot feed their pups, because *Rspo1* mutations also impair mammary gland development affecting duct side-branching and further alveolar formation (Chadi et al., 2009). Overexpression of human *RSPO1* driven by an immunoglobulin k promoter in mouse led to abdominal distention around 8 weeks of age, which is caused by the increase of the small intestine diameter, length, and weight. Ectopic hRSPO1 induced hyper-proliferation of crypt cells caused by stabilization of  $\beta$ -Catenin and activation of the canonical WNT signaling pathway (Kim et al., 2005). Just one damage-induced *Lgr5* positive mouse liver cell (healthy adult liver do not express *Lgr5*) is able to expand and colonize to form organoid in the presence of RSPO1-based culture medium (Huch et al., 2013). Intravenous injection of adenovirus expressing human *RSPO1* (AdRSPO1) could increase the survival rate of irradiated mice. The reactivated WNT/ $\beta$ -Catenin pathway by AdRSPO1 treatment can be observed in the irradiated lung (Guha and Saha, 2016). Transcriptomic analyses showed that *RSPO1* is among the most differentially expressed genes when comparing the lung adenocarcinoma tumor to the normal tissues (Hsu et al., 2015).

*Rspo1* is also expressed in the murine pancreatic islets and  $\beta$ -cell lines MIN6. In vitro, adding RSPO1 in the culture media promoted the proliferation of MIN6 cell line, and induced the transcription of *Insulin* mRNA (Wong et al., 2010). In human, the level of hRSPO1 in the blood is also significantly associated with obesity and insulin resistance as patients in an obese group and an insulin-resistant group both have significantly higher levels of circulating hRSPO1 in their blood compared to a lean group and an insulin-sensitive group, respectively (Kang et al., 2019). The neurons of the ventromedial nucleus of the hypothalamus (VMH) in rat express also *Rspo1*. Insulin is able to up-regulate the transcription of *Rspo1*. Ectopic expression of *Rspo1* by injection into the third brain ventricle repressed food intake of rats (Li et al., 2014a).

In the C2C12 myoblasts or primary satellite cells, overexpression of *Rspo1* enhanced the expression of myogenic determination factor *Myf5* (Han et al., 2011). The overexpression of adult muscle stem cells marker *Pax7* in the primary myoblasts also strongly induces the transcription of *Rspo1* (Soleimani et al., 2012). Later, it was proved that *Rspo1* is an important mediator of skeletal muscle repair. The *Rps1* knock out muscle progenitor cells had less differentiation potential after acute injury. The newly generated myotubes tend to fuse together and form larger myotubes with supernumerary nuclei because of the affected canonical WNT/ $\beta$ -Catenin pathway (Lacour et al., 2017).

### 5.3.2 *Rspo2*

*Rspo2* is important for embryonic limbs development (Nam et al., 2007a). During the development of the embryonic limbs, *Rspo2* is specifically expressed in the apical ectodermal ridge (AER) (Nam et al., 2007b, 2007a). The *Rspo2* knockout by targeted inactivation in mice led to the absence of the claws in the forelimbs and hindlimbs. The formation of fibula, metatarsals and phalanges in the distal hindlimb was severely affected. In the AER, the canonical WNT/ $\beta$ -Catenin pathway target gene expression and Sonic Hedgehog (SHH) signaling were significantly decreased. The expression of AER-specific genes fibroblast growth factor 8 (FGF8) and FGF4 was not maintained (Nam et al., 2007a). During the embryonic lung development, the *Lgr6* and *Rspo2* mediated acceleration of canonical WNT pathway is necessary for the branching of the respiratory tract. Single knockout of *Rspo2* or double knockout of *Rspo2* and *Lgr6* mice all had reduced branching in the lung and limb defects, but double knockout mice showed more severe defects (Bell et al., 2008). *RSPO2* together with *FGF5*, and *KRT71* are also found to control the coat color, growth pattern, length and curl of purebred dog in the United States (Cadieu et al., 2009).

### 5.3.3 *Rspo3*

*Rspo3* is very important for the vasculature. The loss of *Rspo3* is lethal around E10.0 in mice because of severe defects of vasculature in the placenta (Aoki et al., 2007). *Rspo3* deficiency led to a decrease of VEGF signaling, mediated by the canonical WNT/ $\beta$ -Catenin pathway (Kazanskaya et al., 2008). *Nkx2-5* is specifically required for second heart field (SHF) development, and its role in cardiogenesis is achieved by strengthening the WNT signaling through up-regulating *Rspo3* expression. Transgenic overexpression of *Rspo3* can rescue the cardiac growth and proliferation defects in *Nkx2-5* mutants (Cambier et al., 2014). Specific *Rspo3* expression in the endothelial cells of central vein during the perinatal and adult stages is necessary for the onset and maintenance of the liver zonation. *Rspo3* is responsible for activation of the canonical WNT/ $\beta$ -Catenin pathway in the surrounding areas of the central veins (Rocha et al., 2015). In the adrenal glands, *Rspo3* expression is restricted to the capsule, which is important to the onset of adrenal gland zonation during embryonic development. In the adult stage, *Rspo3* is also necessary to induce  $\beta$ -Catenin signaling to maintain the zona glomerulosa and for the replenishment of lost cells (Vidal et al., 2016). The expression of *Rspo3* in angiogenic regions of embryonic heart coronary arteries is essential to promote the arterial-specific proliferation to form the proper arterial tree through the regulation of WNT/ $\beta$ -Catenin signaling (Da Silva et al., 2017).

### 5.3.4 *Rspo4*

In 2006, *RSPO4* homozygous or heterozygous mutations were found in the patients with anonychia and hyponychia congenita, which had no or severely hypoplastic nails developed. In mouse, *Rspo4* is specifically expressed in the mesenchyme beneath the digit tip epithelium from E14.5 (Ishii et al., 2008), indicating the key role of *Rspo4* and associated WNT/ $\beta$ -Catenin signaling in the nail morphogenesis (Blaydon et al., 2006; Ishii et al., 2008). For the moment mouse *Rspo4* mutant phenotype has not been studied yet.

## 6. Thesis project

### 6.1 *Rspo1* function in female sex determination

During sex determination, female development has often been considered as a pre-programmed developmental process in absence of the male sex determination gene *Sry*. The discovery of XX ovotestes harboring *Rspo1/Wnt4/Ctnnb1* mutations challenged this view and built up the idea that female development during sex determination is an active and autonomous process. R-spondin (*Rspo*) genes encode one kind of secreted proteins that activate the canonical WNT/ $\beta$ -Catenin pathway by inhibiting the degradation of WNT receptors. *Rspo1* is a major regulator of ovary development across species. *Rspo1* XX mutant gonads undergo female-to-male sex reversal and develop into ovotestis, gonads with both male and female characteristics. However, the molecular and cellular mechanisms behind this partial sex reversal remain unclear.

*Rspo1* has already been expressed in the lateral plate and intermediate mesodermal cells (where gonads arise from) at E8.5, which is quite earlier than the gonad formation (~E9.5) and sex determination (~E11.5). Although the constitutive *Rspo1* deletion proved *Rspo1* is necessary for female sex determination, the exact spatio-temporal requirement of *Rspo1* is still unknown. In this study, we developed a new mouse model allowing a conditional mutation of *Rspo1* (*Rspo1*<sup>flox/flox</sup>). By taking advantage of *Sf1-Cre*, *Wt1*<sup>GFP-Cre</sup> and *Wt1*<sup>CreERT2</sup> transgenic lines, we can conditionally knock out *Rspo1* at different time points in the somatic cells and determine when *Rspo1* function is required for the normal female development.

XX *Rspo1/Wnt4/Ctnnb1* mutant gonads develop as ovotestes. XX ovotestis developmental process involves the celomic vessel formation at E12.5; the ectopic steroidogenic enzyme expression; the germ cell loss in the gonadal anterior part; the pre-maturation and trans-differentiation of pre-granulosa cells. How these events correlate with the final Sertoli-like cells differentiation is not clear yet. It has been established that ectopic steroidogenic enzyme expression leads to a high level of

testosterone, which in turn promotes the development of epididymis, and also that the coelomic vessel formation is independent from ectopic testosterone action (Heikkilä et al., 2005). However, whether this high level of testosterone plays a role in the germ cell loss, the pre-maturation and trans-differentiation of pre-granulosa cells has not been reported yet. Therefore, by taking advantage of *Rspo1* constitutive mouse model and a pharmaceutical inhibition method, we reevaluate the function of ectopic testosterone action in the testicular development of XX *Rspo1*-deficient ovotestes.

## **6.2 Mutual antagonism between SRY/SOX9 and RSPO1/WNT4/ $\beta$ -Catenin pathway**

Sex determination is a highly sophisticated and ordered process where both male and female gonads develop from a common bipotential gonad depending on different activated signaling pathways. XY gonads develop into testes promoted by the SRY/SOX9 pathway, whereas XX gonads develop into an ovary through the action of the RSPO1/WNT4/ $\beta$ -Catenin pathway. Notably, SRY/SOX9 pathway is essential and sufficient to promote male development, whereas RSPO1/WNT4/ $\beta$ -Catenin pathway is necessary to activate female development. These two pathways antagonize each other in order to initiate and maintain male or female development.

Previous studies have reported that XY *Sox9;Rspo1* and *Sox9;Ctnnb1* double mutant gonads all had testicular differentiation even in the absence of *Sox9*, and XY double mutants were more masculinized than the XX mutants. This could be due to the fact that in the XY other targets of *Sry* promote the masculinization. However, we can still observe granulosa cell marker *Foxl2* expression and even follicles in both XY *Sox9;Rspo1* and *Sox9;Ctnnb1* double mutant gonads. Do those cells represent a female differentiation in XY *Sox9;Rspo1* and *Sox9;Ctnnb1* double mutant gonads? How those granulosa cells were differentiated and why they still persist after testicular differentiation started? Here in our study, we took advantage of *Wnt4;Sox9* double knockout mouse model to study the gonadal sex determination and formation, in order to answer those questions and to understand how *Sox9* and *Wnt4* interact with each other in the male-female mutual antagonistic network.

## **CHAPTER II: RSPO1 FUNCTION IN FEMALE SEX DETERMINATION**

**The WNT potentiator RSPO1 drives ovarian fate at the sex determination stage by controlling androgen signaling in the mouse fetal gonad**

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## **Abstract**

Sex determination is a highly sophisticated and ordered process where both male and female gonads develop from a common bipotential gonad depending on different activated signaling pathways. XY gonad develops into a testis promoted by the SRY/SOX9 pathway, whereas XX gonad develops into an ovary through the action of the RSPO1/WNT4/ $\beta$ -Catenin pathway. R-spondin (RSPO) genes encode one kind of secreted proteins that promote WNT/ $\beta$ -Catenin signaling by inhibiting the degradation of WNT receptors. RSPO1 is a major regulator of ovary development across species, and *Rspo1* XX mutant gonads undergo female-to-male sex reversal by developing into ovotestes, gonads with both male and female characteristics. The molecular and cellular mechanisms behind this partial sex reversal remain unclear. In this study, we have developed a new mouse model allowing a conditional mutation of *Rspo1*. We have shown that the critical window for RSPO1 requirement in the developing XX mouse gonad is around E11.5, and that RSPO1 function is dispensable for ovarian differentiation after this time point. We also found that ectopic expressions of steroidogenic enzymes and androgen receptor are early events before the emergence of trans-differentiated Sertoli-like cells. Through pharmacological inhibition of androgen receptor, we have identified androgen signaling pathway as a major actor promoting granulosa cells premature differentiation and trans-differentiation into Sertoli-like cells in the female-to-male sex reversed XX *Rspo1* mutant gonads. This study brings new thoughts on the mechanisms of primary sex determination in mammals evolving to avoid the effects from sex hormones.

**Key words:** Ovary development, sex determination, RSPO1, WNT signaling, androgen

## Introduction

Primary sex determination or gonadal sex determination is the process by which the bipotential gonad makes the developmental decision to become a testis or an ovary. In mammals, XY heteromorphic sex chromosomes provide a system where XX individuals develop as females, whereas XY individuals develop as males. This system mainly depends on the presence of the male sex determining gene *Sry* on the Y chromosome to launch testis development from the bipotential gonad (Koopman et al., 1991; Sinclair et al., 1990). Sex hormones secreted by testis or ovary promote the development of secondary sexual characteristics to maintain the sexual identity and fertility in a process known as secondary sex determination (Nef et al., 2019). In mouse and human, XX and XY individuals have initially two sets of reproductive duct systems: Wolffian duct for male and Müllerian duct for female. Once the male pathway is established, the androgens produced by Leydig cells promote Wolffian duct development into male genital tract, while anti-Müllerian hormone (AMH) secreted by Sertoli cells induces the regression of Müllerian ducts. Without the inhibition by AMH, Müllerian ducts develop into female genital tract, while degradation of Wolffian ducts in the absence of testosterone requires NR2F2 function (Zhao et al., 2017a).

In mice, *Sry* is expressed in XY gonads from embryonic day 10.5 to 12.5 (E10.5 to E12.5) (Harikae et al., 2013a). SRY directly binds to the distal and TESCO enhancers to promote the expression of *Sox9* (Gonen et al., 2018; Sekido and Lovell-Badge, 2008) thus establishing the Sertoli cell lineage that will further orchestrate testis development. As the top two hierarchical transcription factors, SRY and SOX9 are both necessary and sufficient for testis development in human and mice (Berta et al., 1990; Gregoire et al., 2011; Gubbay et al., 1990; Koopman et al., 1991; Lavery et al., 2011b; Lovell-Badge and Robertson, 1990; Vidal et al., 2001; Vilain et al., 1992). During the same period, XX gonadal supporting cells differentiate into FOXL2 positive pre-granulosa cells and enter into mitotic arrest marked by the expression of cyclin-dependent kinase inhibitor CDKN1B/P27 and the down-regulation of CCNA1/CyclinA1 (Gustin et al., 2016; Nef et al., 2005). Ovarian

development is driven by RSPO1/WNT4/ $\beta$ -Catenin signaling in mouse and human (Biason-Lauber et al., 2004; Chassot et al., 2008a; Liu et al., 2009; Mandel et al., 2008; Parma et al., 2006; Tomizuka et al., 2008; Vainio et al., 1999). Mouse XX gonads harboring mutations in *Wnt4*, *Rspo1* or *Ctnnb1* (encoding  $\beta$ -Catenin) develop into ovotestes (Chassot et al., 2008a; Maatouk et al., 2013; Tomizuka et al., 2008; Vainio et al., 1999), whereas ectopic activation of WNT/ $\beta$ -Catenin signaling in XY gonads disrupts testicular development (Garcia-Moreno et al., 2019a; Harris et al., 2018; Jordan et al., 2001; Maatouk et al., 2008).

The secreted protein RSPO1 acts at the top of this signaling cascade. In *Rspo1* mutants, *Wnt4* expression and  $\beta$ -Catenin signaling are impaired, and stabilization of  $\beta$ -Catenin is able to rescue the female-to-male sex reversal in XX *Rspo1* mutants (Chassot et al., 2008a). In contrast *Rspo1* expression is not modified in *Wnt4* or *Ctnnb1* mutants (Chassot et al., 2008a; Liu et al., 2009). RSPO1 binds to its receptors LGR4/5, and inhibits the action of the transmembrane E3 ubiquitin ligases ZNRF3 and RNF43. These two molecules are involved in targeting the WNT Frizzled receptor for degradation by ubiquitination. Therefore, RSPO1, promotes WNT signaling by decreasing the turnover of Frizzled receptor (Nusse and Clevers, 2017).

In the absence of RSPO1/WNT4/ $\beta$ -Catenin signaling in XX mice, pre-granulosa cells in the anterior part of the gonad (also called male part) exit mitotic arrest and prematurely differentiate (Maatouk et al., 2013). These pre-matured granulosa cells lose P27 expression and co-express FOXL2 and AMH at E17.5, similar to the granulosa cells during folliculogenesis stages after birth (Maatouk et al., 2013; Nicol and Yao, 2015). Subsequently, pre-matured granulosa cells lose FOXL2 expression and trans-differentiate into SOX9/AMH double positive Sertoli-like cells, forming testis cord-like structures around birth (Chassot et al., 2008a; Maatouk et al., 2013; Nicol and Yao, 2015), that will become seminiferous tubules in the adult stages (Chassot et al., 2008a). In addition to the supporting cell lineage defects visible near birth, sex reversal and ovotestis formation in RSPO1/WNT4/ $\beta$ -Catenin signaling-deficient XX gonads are characterized by additional phenotypes during embryonic development.

*Wnt4*, *Rspo1* and *Ctnnb1* XX mutant gonads exhibit a male-like vasculature with the formation of a celomic vessel at E12.5 by ectopic migration of endothelial cells from the adjacent mesonephros (Chassot et al., 2008a; Jeays-Ward, 2003; Liu et al., 2009; Tomizuka et al., 2008). In addition, *Wnt4*, *Rspo1* and *Ctnnb1* XX mutant gonads contain ectopic steroidogenic cells that produce testosterone and masculinize the XX genital tracts (Chassot et al., 2008a; Heikkilä et al., 2002, 2005; Liu et al., 2009, 2010; Tomizuka et al., 2008; Vainio et al., 1999). Finally, germ cells are lost through apoptosis from E16.5 in *Wnt4* and *Ctnnb1* XX mutants (Liu et al., 2010; Tomizuka et al., 2008; Vainio et al., 1999; Yao et al., 2004) or reduced proliferation from E12.5 in *Rspo1* XX mutants (Chassot et al., 2008a).

Despite the prominent role of RSPO1/WNT4/ $\beta$ -Catenin pathway in gonadal development to promote ovarian development, little is known about the molecular and cellular mechanisms underlying this process. In particular, it is currently unknown whether RSPO1/WNT4/ $\beta$ -Catenin signaling is only required at the time of sex determination to launch the ovarian program or whether it functions during the entire gonadal development for the induction and maintenance of ovarian fate. In addition, how the distinct sequential defects arising in *Wnt4*, *Rspo1* and *Ctnnb1* XX mutant gonads relate to each other and contribute to the sex reversal phenotype in the supporting cell lineage is currently unclear (Liu et al., 2010; Maatouk et al., 2013).

In this work, we have generated and validated a new genetic model for conditional deletion of *Rspo1*. We demonstrate that RSPO1 function is required around E11.5, and is dispensable at later stages for ovarian differentiation. We have also addressed for the first time the impact of ectopic steroidogenesis on the development of the supporting cell lineage in *Rspo1* mutants. Our results indicate that androgen signaling acts in the mutant pre-granulosa cells to promote their premature differentiation and trans-differentiation into Sertoli-like cells. This observation provides a link between an early consequence of *Rspo1* mutation, the formation of ectopic steroidogenic cells, and the outcome of sex reversal and ovotestis formation near birth.

## Results

### ***Rspo1* expression and WNT/ $\beta$ -Catenin activity are dynamically regulated in the early mouse gonad**

*Rspo1* is expressed in the lateral and intermediate mesoderm from E8.5, before the formation of the genital ridge (Nam et al., 2007b). *Rspo1* is then expressed in XY and XX early gonads, and is specifically down-regulated in the developing testis (Chassot et al., 2012, 2011; Jameson et al., 2012b; Munger et al., 2013; Stévant et al., 2018a; Zhao et al., 2018). In order to compare the dynamics of *Rspo1* expression and WNT/ $\beta$ -Catenin pathway activity in developing gonads, we have performed RNAscope® in situ hybridization with *Rspo1* and *Axin2* probes. *Axin2*, encodes a negative regulator of WNT/ $\beta$ -Catenin signaling, directly regulated by  $\beta$ -Catenin in a negative feedback loop, and whose expression can be used as a readout of the pathway activity (Lustig et al., 2002).

We have found that at the 18 tail somites stage (18 ts, E11.5), *Rspo1* is expressed in the developing gonad, including the celomic epithelium, of both XY and XX individuals (Fig. 1A, D, (Chassot et al., 2012). *Axin2* is expressed in XY and XX gonads and in the adjacent mesonephros, indicating that WNT/ $\beta$ -Catenin signaling is active in both sexes at this stage (Fig. 1G, J). Around 6 hours later, at the 21ts stage, *Rspo1* is still highly expressed in XX gonads (Fig. 1E). *Rspo1* transcripts are detected in the celomic epithelium and inside the gonad, but seem to be excluded from large cells resembling germ cells. At this time, *Rspo1* expression is dramatically down-regulated in XY gonads, although some transcripts are still present, specially in the celomic epithelium (Fig. 1B). Accordingly, *Axin2* expression is mostly restricted to the celomic epithelium of 21 ts XY gonads (Fig. 1H), whereas it is detected in the entire XX gonads at this stage (Fig. 1K). At E12.5, *Rspo1* and *Axin2* transcripts are detected in the outer layer of the testis (Fig. 1C, I). Inside the gonad, testis morphogenesis is ongoing and NR2F2 positive interstitial cells surround the forming testis cords. *Rspo1* is expressed in scattered NR2F2 negative cells, possibly Sertoli cells (Fig. 1C), whereas *Axin2* transcripts are more widely detected in the NR2F2

positive interstitial cells and inside the forming testis cord structures (Fig. 1I). In E12.5 females, *Rspo1* is strongly expressed in the entire ovary (Fig. 1F). *Rspo1* transcripts are very abundant in NR2F2 negative cells that surround cells with large nuclei. These *Rspo1* positive cells are most likely pre-granulosa cells (Chen et al., 2012). *Axin2* is highly expressed in the E12.5 ovaries, both in NR2F2 positive and negative populations (Fig. 1L), indicating widespread WNT/ $\beta$ -Catenin signaling in the ovary at this stage.

Together, our results demonstrate that *Rspo1* expression and WNT/ $\beta$ -Catenin activity are common features of the XX and XY early gonads, but are specifically maintained in the developing ovary. Moreover, our observations indicate that while *Rspo1* is mainly expressed in pre-granulosa cells, WNT/ $\beta$ -Catenin signaling is active in all ovarian cell types.

### **Generation and validation of a new conditional allele of *Rspo1***

In order to investigate the spatiotemporal dynamics of *Rspo1* requirement in the developing mouse gonad, we have generated a new conditional mutant allele for *Rspo1*, *Rspo1<sup>fllox</sup>*. Exon2 is flanked by LoxP sites and upon CRE-induced recombination both the start codon and the sequences coding for the secretion signal will be deleted giving rise to the mutated *Rspo1 <sup>$\Delta$ Ex2</sup>* allele (Fig. 2A).

We first validated that deletion of Exon2 leads to a loss of function of RSPO1. *Rspo1<sup>fllox</sup>* male mice were crossed with *Sox2-Cre* transgenic females, where CRE is constitutively active in the oocytes (Hayashi et al., 2003), to generate *Rspo1 <sup>$\Delta$ Ex2/+</sup>* animals (Fig. 2A). *Rspo1 <sup>$\Delta$ Ex2/+</sup>* males and females were normal and fertile (data not shown), and when inter-crossed gave rise to *Rspo1 <sup>$\Delta$ Ex2/ $\Delta$ Ex2</sup>* individuals bearing a constitutive deletion of *Rspo1* Exon2. Quantitative RT-PCR analysis of E11.5 *Rspo1 <sup>$\Delta$ Ex2/ $\Delta$ Ex2</sup>* gonads revealed the presence of a mutated *Rspo1* mRNA lacking Exon2 sequences (Fig. 2B).

Histological analysis of the genital system of XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> P30 mice revealed male and female structures in 100% of the animals examined (n=3). Oviduct and uterus were present along with epididymis and vas deferens (Fig. 2C-E). This result demonstrates that Wolffian duct derivatives were abnormally preserved and that the genital tract of XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> animals was masculinized. In addition, XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads developed as ovotestes containing ovarian follicles as well as tubular structures resembling seminiferous tubules with Sertoli-like cells (Fig. 2 F-H). XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> animals display a pseudo-hermaphroditism phenotype similar to the one previously described in other *Rspo1* loss-of-function mouse models (Chassot et al., 2008a; Tomizuka et al., 2008).

Immunofluorescence analysis confirmed that P0 and E17.5 XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads developed as ovotestes (Fig. 2I-T and data not shown). The expression of the granulosa cell marker FOXL2 was reduced when compared to XX control ovaries (Fig. 2I-K). Moreover, in the anterior part of the gonad, FOXL2 was co-expressed with the differentiated granulosa marker AMH (Fig. 2K), and the expression of the quiescence marker P27 was lost (Fig. 2L-N). These observations are consistent with a hallmark process in *Rspo1* mutant gonads where disruption of mitotic arrest and precocious differentiation of granulosa cells, precedes their trans-differentiation into Sertoli-like cells (Maatouk et al., 2013). XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads exhibited tubular structures (similar to the XY control testis cords) containing cells expressing SOX9 and AMH, indicating that Sertoli-like cells develop in the anterior region of XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads (Fig. 2O-Q) (Maatouk et al., 2013).

Reduced proliferation leads to a depletion of germ cells in the anterior region of XX *Rspo1* mutant ovaries (Chassot et al., 2011). A strong reduction of the germ cell population was also observed in the anterior part of XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads (Fig. 2L-N). Previous work has demonstrated that RSPO1 loss-of-function also results in the ectopic formation of steroidogenic cells leading to the production of testosterone by the new-born mutant ovotestis and the abnormal maintenance of Wolffian duct

derivatives (Chassot et al., 2008a; Tomizuka et al., 2008). The anterior region of XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads contained cells expressing the steroidogenic enzyme HSD3β that were never observed in control ovaries at this stage (Fig. 2R-T). This indicates that XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads contain "male-like" testosterone producing cells at the origin of the observed masculinization of the genital tract. Finally, similar to other *Rspo1* loss-of-function models, XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads show the presence of a celomic vessel normally found exclusively in XY control gonads (Chassot et al., 2008a; Tomizuka et al., 2008, data not shown).

Together these observations demonstrate XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> mutants recapitulate all aspects of the *Rspo1* mutant phenotype previously described and strongly indicate the *Rspo1*<sup>ΔEx2</sup> allele is a loss-of-function allele.

### ***Rspo1* deletion in the early gonad leads to XX ovotestis formation**

In order to investigate whether RSPO1 is specifically required in the gonad for ovarian formation, we used two constitutive CRE lines previously employed to target somatic gonadal tissue in mice, *Sf1-Cre* and *Wt1-GFP-Cre* (Bingham et al., 2006; Nicol and Yao, 2015; Zhou et al., 2008). Histological analysis of *Wt1*<sup>GFP-Cre/+</sup>; *Rspo1*<sup>flox/flox</sup> XX individuals revealed the presence of an epididymis as well as tubular structures resembling testis cords in the gonad at P0 (n=8/8, Fig. 3A-C). *Wt1*<sup>GFP-Cre/+</sup>; *Rspo1*<sup>flox/flox</sup> XX gonads displayed all the characteristics of RSPO1-loss of function induced sex reversal in the anterior part of the gonad: reduced FOXL2 and P27 expression (Fig. 3G-I, 3J-L), presence of Sertoli-like cells expressing SOX9, AMH and DMRT1 (Fig. 3D-F, 3G-I), reduced germ cell numbers, ectopic steroidogenic cells and ectopic celomic vessel formation (data not shown). In contrast, *Sf1-Cre*<sup>tg/0</sup>; *Rspo1*<sup>flox/flox</sup> XX individuals did not show any morphological or molecular sign of sex reversal at P0 (n=0/3 Fig. 3A'-C', 3D'-L'). Similar results were obtained at P30: all *Wt1*<sup>GFP-Cre/+</sup>; *Rspo1*<sup>flox/flox</sup> XX gonads were ovotestes with follicles and seminiferous tubules (n=9/9, Fig. 3M-O and Fig. S1-L), whereas only a small fraction of *Sf1-Cre*<sup>tg/0</sup>; *Rspo1*<sup>flox/flox</sup> showed evidence of masculinization (n=4/16, Fig. 3M'-O' and Fig. S1-K).

By using a fluorescent reporter line we have observed that, as previously described, the *Sf1-Cre* transgenic line does not target efficiently the celomic epithelium of the early E11.5 gonad, a tissue with high *Rspo1* expression (Manuylov et al., 2008, 2011, Fig. S1 A-F). Quantitative RT-PCR analysis of *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* gonads recovered at E11.5 and P0 confirmed the incomplete deletion of Exon2 sequences by *Sf1-Cre* at both stages (Fig. S1 G-J). These results indicate that XX female-to-male sex reversal is only induced when *Rspo1* is efficiently deleted in the early gonad.

### **RSPO1 function is required at the time of sex determination but is dispensable at later stages of ovarian differentiation**

CRE expression driven from the *Wt1* locus is sufficient to efficiently delete *Rspo1* and induce XX sex reversal (Fig. 3). To further investigate the temporal window of RSPO1 function requirement in the developing gonad we made use of the inducible *Wt1<sup>CreERT2</sup>* line (Zhou et al., 2008). *Rspo1<sup>flox/flox</sup>* females were crossed with *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* males. Pregnant females were treated with tamoxifen by oral gavage at 9.5, 10.5, 11.5 or 12.5 days of gestation and litters were dissected at E17.5 (Fig. 4A). Quantitative RT-PCR analysis of *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* XX gonads treated with tamoxifen at E10.5 and dissected at E11.5, or treated with tamoxifen at E11.5 and dissected at E12.5, revealed that irrespective of the initial time of tamoxifen treatment, *Rspo1* Exon2 was completely deleted 24 hours after induction of CRE activity (Fig. 4B-C).

CRE activity induced at E9.5 and E10.5 (early deletion) resulted in sex reversal of *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* XX individuals similar to the one observed in *Rspo1<sup>ΔEx2/ΔEx2</sup>* XX gonads at E17.5 (Fig. 4 and data not shown). FOXL2 expression was reduced and prematurely differentiated granulosa cells co-expressing FOXL2 and AMH were observed after CRE induction at E10.5 (Fig. 4G). Trans-differentiated Sertoli-like cells co-expressing SOX9 and AMH were found in the anterior region of *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* XX gonads treated with tamoxifen at E10.5 (Fig. 4L). Moreover, early

deletion of *Rspo1* also resulted in germ cell depletion (Fig. 4Q) and ectopic presence of steroidogenic cells (Fig. 4V). In contrast, CRE activity induced at E11.5 or E12.5 (late deletion) did not interfere with ovarian development of *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* XX individuals (Fig. 4H, M, R, W and data not shown). These observations were confirmed when P10 gonads were examined. Early deletion of *Rspo1* resulted in the formation of ovotestis with testis cords containing SOX9 expressing cells (Fig. 4 Z), whereas late deletion of *Rspo1* did not interfere with the formation of normal ovarian structures (data not shown).

These results demonstrate that deletion of *Rspo1* from E11.5 (tamoxifen induction at E10.5) leads to XX sex reversal, whereas loss-of-function of *Rspo1* after E12.5 (tamoxifen induction at E11.5) does not impair ovarian development. We conclude that RSPO1 function is required for ovarian development at the time of gonadal sex determination around E11.5, but is dispensable after this stage for ovarian differentiation.

### **Ectopic steroidogenesis and Androgen Receptor expression precede granulosa to Sertoli-like cell trans-differentiation in XX *Rspo1* mutants**

We next investigated the early phenotypic changes caused by *Rspo1* loss of function. WNT/ $\beta$ -Catenin pathway activity revealed by the expression of the targets *Wnt4*, *Lef1* and *Axin2* was already significantly down-regulated at the 22-23 tail somite stage in E11.5 XX *Rspo1* mutant gonads (Fig. 5A-C). The expression of the granulosa cell marker *Foxl2* is regulated at least in part by WNT/ $\beta$ -Catenin signaling (Auguste et al., 2012; Nicol and Yao, 2015; Ottolenghi et al., 2007). Accordingly, we found that *Foxl2* expression levels were reduced from E11.5 in XX *Rspo1* mutant gonads (Fig. 5D). The secreted Activin inhibitor Follistatin (FST) is required in XX gonads to prevent the migration of mesonephros derived endothelial cells that form the celomic vessel in XY gonads (Jeays-Ward, 2003; Yao et al., 2004). *Fst* expression is dependent on WNT/ $\beta$ -Catenin and FOXL2 in mouse gonads (Chassot et al., 2008a; Nicol et al., 2018; Yao et al., 2004). We found a strong down-regulation of *Fst* in E11.5

XX *Rspo1* mutant gonads (Fig. 5E). The Sertoli markers *Sox9*, *Sox8* and *Amh* were not up-regulated in XX *Rspo1* mutants at this stage when compared to XX control littermates (Fig. 5F-H). Instead, we found a strong up-regulation of the expression of genes involved in the steroidogenic pathway at E11.5. Genes encoding enzymes involved in sterol biosynthesis from cholesterol such as *Star*, *Cyp11a1*, *Cyp17a1* and *Hsd17b3* were up-regulated in XX *Rspo1* mutant gonads (Fig. 5J-M). In addition, the expression of androgen receptor (*Ar*) involved in androgen binding and signaling was strongly up-regulated in XX *Rspo1* mutant gonads (Fig. 5I).

Despite the up-regulation of steroidogenic enzymes and androgen receptor at the mRNA level in XX E11.5 *Rspo1* mutant gonads, we could only observe ectopic expression of the corresponding proteins from E14.5 onwards (Fig. 6 and data not shown). This discrepancy might be due to the lower sensitivity of the immunofluorescence technique or to post-transcriptional regulation events that delay the appearance of the corresponding proteins.

Steroidogenic cells marked by the expression of HSD3 $\beta$  and CYP11A1 were first observed in XX *Rspo1* mutant gonads at E14.5 (Fig. 6 C), and were very abundant in E17.5 mutant gonads (Fig. 6L). The anterior region of XX *Rspo1* mutant gonads at E14.5 contained AR positive cells that were never observed in the control XX gonads at this stage (Fig. 6F). Interestingly, some AR positive cells also expressed FOXL2, indicating that pre-granulosa cells are competent to respond to androgen signaling in *Rspo1* mutants from E14.5 (inset in Fig. 6F). AR expression extended to a large part of the mutant gonad at E17.5. AR was co-expressed with NR2F2 in interstitial cells that were very abundant in the mutant gonad, resembling the situation in XY testis (Fig. 6M, O). AR was also co-expressed with FOXL2 in granulosa cells (Fig. 6R). At E14.5, the Sertoli cell marker SOX9 was not expressed in *Rspo1* mutants and the germ cell population marked by TRA98 appeared unchanged when compared to control littermates (Fig. 6I). In contrast at E17.5, SOX9 expressing cells were readily observed in the anterior region of *Rspo1* mutants and the reduced germ cell

population was confined to the posterior region (Fig. 4K, 4P).

Together these results demonstrate that ectopic steroidogenesis and ectopic androgen receptor expression are early phenotypic features in the sex reversal process triggered by *Rspo1* loss of function that precede germ cell loss and Sertoli-like cell differentiation.

### **Inhibition of androgen signaling rescues sex reversal in *Rspo1* mutant gonads**

In order to determine the role of androgen signaling in the sex reversal phenotype of *Rspo1* mutants we made use of the non-steroidal anti-androgen flutamide. Flutamide exerts its action through competitive inhibition of the binding of testosterone and its metabolite 5- $\alpha$  dihydrotestosterone (5- $\alpha$ -DHT) to the androgen receptor (Welsh et al., 2006). *Rspo1* <sup>$\Delta$ Ex2/+</sup> females crossed with *Rspo1* <sup>$\Delta$ Ex2/ $\Delta$ Ex2</sup> males were injected with flutamide (200mg/kg body weight) or oil once daily from E10.5 to E18.5. Flutamide treated fetuses recovered at E18.5 appeared morphologically normal and showed similar body weight to those treated with the oil vehicle, indicating that the selected flutamide dose was not toxic for embryonic development (Fig. 7A). Anogenital distance (AD) is a read-out of prenatal androgen signaling with male fetuses displaying a higher AD than their female littermates (Schwartz et al., 2019) (Fig. 7B). We observed a significant reduction of AD in flutamide treated XY fetuses recovered at E18.5, demonstrating that androgen signaling is efficiently inhibited in developing fetuses upon flutamide treatment of pregnant females (Fig. 7B). XX *Rspo1* mutants show a masculinization of the genital tract evidenced by the presence of an epididymis and vas deferens in addition to the oviduct and uterus (Fig. 7E). In flutamide treated XX *Rspo1* mutants, these Wolffian duct derived structures were absent and the genital tract was similar to the one of XX controls (Fig. 7F). These observations are in agreement with a previous report (Tomizuka et al., 2008) and demonstrate that flutamide treatment can efficiently inhibit androgen signaling in XX *Rspo1* mutant fetuses and rescue the masculinization of their genital tract.

We next sought to determine the impact of androgen signaling inhibition on the development of the *Rspo1* mutant gonad by quantitative RT-PCR analysis (n=4 to 6). The expression of the granulosa cell marker *Foxl2* and of the quiescence marker *P27* were significantly up-regulated in flutamide treated XX *Rspo1* mutants, reaching levels comparable to those of XX control gonads (Fig. 7I, J). In addition the expression of the Sertoli or mature granulosa cell marker *Amh* and of the Sertoli marker *Sox9*, were reduced in the gonads of XX *Rspo1* mutants treated with flutamide, when compared to oil treated XX mutants (Fig. 7G, H). These results indicate that inhibition of androgen signaling in XX *Rspo1* mutants inhibits Sertoli-like cell differentiation and restores female supporting cells gene expression. The expression of the germ cell marker *Ddx4*, was also significantly increased in flutamide treated XX *Rspo1* gonads, indicating that inhibition of androgen signaling rescues germ cell loss in *Rspo1* mutants (Fig. 7N). The expression of the interstitial marker *Nr2f2*, of the steroidogenic enzyme coding gene *Cyp11a1*, and of *Ar* were also down-regulated in flutamide treated XX *Rspo1* gonads compared to oil treated mutants (Fig. 7K, L, M). These observations indicate that androgen receptor expression and ectopic steroidogenesis are positively regulated by androgen signaling in *Rspo1* mutant ovaries.

In order to confirm the rescue of *Rspo1* mutant phenotypic features by androgen signaling inhibition, we performed a comparative quantitative immunofluorescence analysis on E18.5 XX *Rspo1* mutant gonads treated with oil or flutamide between E10.5 and E18.5 (Fig. 8). Flutamide treatment reduced the number of cells co-expressing SOX9 and AMH in XX *Rspo1* mutants when compared to oil treated mutants (Fig. 8C-D, M), indicating that Sertoli-like cell differentiation in *Rspo1* mutant ovotestes was dependent on androgen signaling. In addition, the number of cells expressing FOXL2 and P27 was significantly increased in XX *Rspo1* mutant gonads treated with flutamide (Fig. 8C-D, G-H, N). These results confirmed that the granulosa to Sertoli-like cell trans-differentiation was impaired by androgen signaling inhibition in XX *Rspo1* mutant gonads. Immunofluorescence analysis of TRA98 distribution

revealed that germ cell numbers were increased in XX *Rspo1* mutants treated with flutamide compared to oil treated mutants (Fig. 8G-H, N) strongly suggesting that ectopic androgen signaling is deleterious to germ cell development in XX *Rspo1* mutant gonads. The number of interstitial cells co-expressing NR2F2 and AR, and of steroidogenic cells expressing HSD3 $\beta$  was also significantly reduced by flutamide treatment in XX *Rspo1* mutant gonads (Fig. 8K-L, O). This observation indicates that inhibition of androgen signaling can partially rescue the ectopic interstitial and steroidogenic cells phenotypes of XX *Rspo1* mutants.

We have shown that inhibition of androgen signaling during fetal life can rescue the gonadal phenotype of sex reversal in *Rspo1* mutants. These observations indicate that androgen signaling has a positive role in promoting the female-to-male sex reversal of *Rspo1* mutants.

### **Androgen signaling contributes to granulosa cell premature differentiation in *Rspo1* mutants**

In order to investigate the early effects of ectopic androgen signaling in *Rspo1* mutants we used a shorter flutamide treatment, from E10.5 to E15.5. At E15.5, untreated *Rspo1* mutants exhibit ectopic steroidogenesis as shown by the increased expression of *Cyp11a1* and *Cyp17a1* as well as increased *Ar* expression when compared to XX controls (Fig. 9A-C). At this stage, the expression of the germ cell marker *Ddx4* is similar to that of XX controls indicating that germ cell proliferation and survival defects become apparent after E15.5 in XX *Rspo1* mutants (Fig. 9D). Concerning the supporting cell gene expression, oil treated *Rspo1* mutant XX gonads show down-regulation of *Foxl2* but do not exhibit up-regulation of *Sox9* (Fig. 9E, G). Nevertheless, high levels of *Amh* transcripts can already be detected in oil treated XX *Rspo1* mutants at this stage (Fig. 9H). These observations confirm that premature granulosa differentiation has started in *Rspo1* mutants at E15.5 whereas the granulosa to Sertoli-like cell trans-differentiation process will only start around E17.5 (Maatouk et al., 2013). Flutamide treatment reduced the expression of *Cyp11a1*,

*Cyp17a1* and *Ar* in XX *Rspo1* mutants at E15.5 further confirming that androgen signaling sustains ectopic steroidogenesis in *Rspo1* mutants (Fig. 9A-C). Importantly, we found that the expression of *Amh* was significantly reduced in flutamide treated XX *Rspo1* mutants at E15.5 (Fig. 9H). This result indicates that inhibition of androgen signaling can rescue the premature differentiation process of granulosa cells in XX *Rspo1* mutants, the first step in the process of female-to-male sex reversal of the supporting cell population in *Rspo1* mutants.

## Discussion

Female sex determination is an active and autonomous process; whose progression needs the action of RSPO1/WNT4/ $\beta$ -Catenin signaling. By taking advantage of a new conditional knockout mouse model, we have identified the critical window of requirement for RSPO1 in mouse ovarian development around E11.5. RSPO1 dependent signaling is dispensable for later development of the ovary. We have established that ectopic steroidogenesis is an early defect of *Rspo1* mutants preceding germ cell loss and granulosa to Sertoli-like cell trans-differentiation. We have demonstrated that ectopic androgen signaling not only contributes to the masculinization of the genital tract in XX *Rspo1* mutants, but also promotes the progression of the female-to-male sex reversal process in the supporting cells of the mutant gonads.

### ***Rspo1* regulation in the early gonad**

In agreement with previous reports, we have shown that *Rspo1* expression and WNT/ $\beta$ -Catenin signaling are present in early XX and XY gonads (Munger et al., 2013; Stévant et al., 2017, 2018a; Zhao et al., 2018). This observation is consistent with the finding that the gonad is initially a bipotential organ containing undifferentiated precursors with similar expression profiles in both sexes (Stévant and Nef, 2019). SRY expression starting at E10.5 in the XY gonad activates *Sox9* expression and initiates the genetic cascade leading to Sertoli cell specification and testis development (Gonen et al., 2018; Harikae et al., 2013a; Sekido and Lovell-Badge, 2008). *Rspo1* expression and WNT/ $\beta$ -Catenin signaling are down-regulated as testis development proceeds, except in the celomic epithelium where WNT/ $\beta$ -Catenin signaling is required for proliferation of epithelial progenitors (Chassot et al., 2012). Down-regulation of WNT/ $\beta$ -Catenin signaling is essential for testis development as over-activation of this pathway in XY gonads leads to male-to-female sex reversal (Garcia-Moreno et al., 2019a; Harris et al., 2018; Maatouk et al., 2008), however the precise mechanisms driving down-regulation and maintenance of *Rspo1* in the XY and XX gonads respectively are currently unknown. *Rspo1* expression is up-regulated

in E11.75 XY *Fgf9* mutants, despite the presence of SOX9 protein (Jameson et al., 2012a), indicating that *Sox9* activation might not be the only event involved in *Rspo1* down-regulation in the developing testis (Lavery et al., 2011b).

Transcriptomic analyses on isolated gonadal cells have indicated that *Rspo1* is expressed in supporting cells and to a lesser extent in interstitial cells of developing ovaries (Jameson et al., 2012b; Stévant et al., 2018a). Our expression analysis demonstrates that, in the intact whole gonad, *Rspo1* is mainly expressed in the pre-granulosa supporting cell lineage, although *Rspo1* transcripts can also be observed in NR2F2 positive interstitial cells at E12.5.

### **RSPO1 is not required after E12.5 to maintain granulosa cell identity**

*Rspo1* is expressed in pre-granulosa cells of the developing ovary and is down-regulated after birth (De Cian et al., 2017; Stévant et al., 2018a). Gain-of-function of RSPO1 in the post-natal ovary leads to tumor formation, and suggests that RSPO1 regulates important aspects for the maintenance of epithelial cell characteristics in pre-granulosa cells (De Cian et al., 2017). We have shown that deletion of *Rspo1* after E12.5 (tamoxifen induction at E11.5) does not impair ovarian development. This observation indicates that after the critical window of requirement of RSPO1, once ovarian development has been launched, *Rspo1* is dispensable to maintain pre-granulosa cell identity. FOXL2 might co-operate with WNT/ $\beta$ -Catenin signaling for the maintenance of pre-granulosa cells in XX gonads (Auguste et al., 2012; Ottolenghi et al., 2007). However, we cannot exclude that *Rspo1* deletion after E12.5 impacts adult ovarian physiology, as we did not analyze these mutant ovaries after P10.

### **RSPO1 is required in the early gonad for ovarian development**

We have established that *Rspo1* deletion before E11.5 (tamoxifen induction at E9.5 or E10.5) leads to XX ovotestis formation and that WNT/ $\beta$ -Catenin signaling is severely reduced in XX *Rspo1* mutants from E11.5. The phenotype of *Rspo1* mutants

can be rescued by overexpression of active  $\beta$ -Catenin (Chassot et al., 2008a), demonstrating that RSPO1 functions through the activation of the WNT/ $\beta$ -Catenin signaling in the ovary. Therefore, with our *Rspo1* conditional deletion mouse model, we have established the critical window of requirement for WNT/ $\beta$ -Catenin signaling in ovarian development around E11.5. The full development of ovotestes with trans-differentiated Sertoli-like cells only occurs around perinatal stages in *Rspo1*, *Wnt4* and *Ctnnb1* mutants (Chassot et al., 2008a; Liu et al., 2009; Maatouk et al., 2013; Nicol and Yao, 2015; Vainio et al., 1999). Ovotestis formation appears to be a sequential process with different phenotypic features controlled by distinct genetic cascades. How do the events leading to full ovotestis development relate to each other and to the early requirement of RSPO1 function?

One important aspect of the masculinization of XX gonads in *Rspo1*, *Wnt4* and *Ctnnb1* mutants is the presence of a male-like vasculature with the formation of a celomic vessel at E12.5 (Chassot et al., 2008b; Jeays-Ward, 2003; Liu et al., 2009; Tomizuka et al., 2008). Ectopic vasculature formation in *Wnt4* mutants is caused by ectopic endothelial migration from the mesonephros in response to enhanced Activin B signaling occurring upon up-regulation of the Activin B subunit *Inhibin beta B* (*Inhbb*), and the down-regulation of *Fst* expression (Jeays-Ward, 2003; Yao et al., 2006, 2004). *Fst* expression is regulated by WNT/ $\beta$ -Catenin signaling and FOXL2 (Kashimada et al., 2011; Liu et al., 2009; Nicol et al., 2018; Yao et al., 2006, 2004). We show here that *Wnt4*, the WNT/ $\beta$ -Catenin targets *Axin2* and *Lef1*, *Foxl2* and *Fst* are down-regulated as early as E11.5 in *Rspo1* mutants, providing a link between RSPO1 function in the early gonad and the formation of the celomic vessel around E12.5. Nevertheless, abnormal male-like vasculature formation is independent from the appearance of other ovotestis phenotypic features, as *Fst* mutants exhibit XX celomic vessel but do not show ectopic steroidogenesis or Sertoli-like cells formation (Yao et al., 2004), and rescue of celomic vessel formation does not rescue ectopic steroidogenesis in *Wnt4;Inhbb* double mutants (Yao et al., 2006).

Another common feature of XX gonads lacking *Rspo1*, *Wnt4* or *Ctnnb1* is the presence of ectopic steroidogenic cells (Chassot et al., 2008a; Liu et al., 2009; Tomizuka et al., 2008; Vainio et al., 1999). In this work we have shown that ectopic expression of components of the steroidogenic biosynthetic pathway and of the Androgen Receptor are early phenotypic features of XX *Rspo1* mutants that precede the premature differentiation of granulosa cells and their trans-differentiation into Sertoli-like cells. Furthermore, we have demonstrated that inhibition of androgen signaling from E10.5 rescues first the premature differentiation of pre-granulosa cells at E15.5 and then the trans-differentiation into Sertoli-like cells at E18.5. Previous work had demonstrated that testosterone is present in *Wnt4* and *Rspo1* mutant ovotestes at birth, and that inhibition of androgen signaling with flutamide rescued the masculinization of the genital tract in *Wnt4*, *Rspo1* and *Ctnnb1* mutants (Heikkilä et al., 2005; Liu et al., 2010; Tomizuka et al., 2008). However, our report is the first to establish a link between the early appearance of ectopic steroidogenic cells, and the progression of female-to-male sex reversal in the supporting lineage of XX *Rspo1* mutant ovotestes.

### **Origin of ectopic steroidogenic cells in *Rspo1* mutants**

In XY gonads, fetal Leydig cells differentiate and produce androgens as early as E12.5, whereas in XX gonads, steroidogenic theca cells appear only after birth. Single-cell RNA-seq studies, indicate that male and female gonadal steroidogenic cells differentiate from a common interstitial progenitor cell, marked by NR2F2 (Stévant et al., 2017, 2018a). We observed abundant NR2F2 positive interstitial cells surrounding the testis cord-like structures in *Rspo1* mutant ovotestes, and these cells might contain the precursors of the ectopic steroidogenic cells. Hedgehog signaling pathway promotes steroidogenic cell differentiation from interstitial cells. Desert Hedgehog (DHH) secreted from Sertoli cells promotes fetal Leydig cell formation in embryonic testes (Yao et al., 2002), whereas DHH and Indian Hedgehog (IHH) produced by mature granulosa cells promote the differentiation of theca cells in the post-natal ovary (Liu et al., 2015a). Despite these temporal differences, embryonic

ovarian interstitial precursors are competent to differentiate into steroidogenic cells. Ectopic activation of Hedgehog signaling in the developing ovary leads to ectopic formation of fetal Leydig cells (Barsoum et al., 2009). *Dhh* expression is up-regulated in XX *Rspo1* mutants (Tomizuka et al., 2008) and data not shown) and might contribute to the ectopic differentiation of steroidogenic cells from interstitial precursors.

NR2F2 positive interstitial precursors are derived from two sources: the celomic epithelium where they share a common origin with supporting cells, and the gonadal-mesonephric border (Rotgers et al., 2018). Ectopic steroidogenic cells in *Wnt4* and *Ctnnb1* arise at least in part from the aberrant migration of adrenal precursors from the mesonephros (Heikkilä et al., 2002; Jeays-Ward, 2003; Liu et al., 2009). However, steroidogenic cells in XX *Rspo1* mutants do not express adrenal markers (Chassot et al., 2008a). Further work will be required to determine the origin of interstitial precursors and steroidogenic cells in *Rspo1* mutants.

### **Androgen signaling promotes Sertoli-like cell differentiation in *Rspo1* mutants.**

In *Rspo1* mutants, Sertoli-like cells arise through trans-differentiation of pre-matured granulosa cells, but the mechanisms of this trans-differentiation process are poorly understood. Single-cell RNA-seq analyses have provided evidence that Sertoli cells and granulosa cells share a common bipotential supporting cell precursor. Even after their differentiation, Sertoli cells and granulosa cells still share common transcriptional profiles despite the presence of sex specific genes, which makes the trans-differentiation between these two cell types much more likely (Stévant et al., 2017, 2018a). FOXL2 is required to maintain the identity of granulosa cells in the adult ovary but not during fetal stages (Ottolenghi et al., 2005). However, FOXL2 overexpression in somatic cells of the embryonic testis represses Sertoli cell differentiation (Nicol et al., 2018). Moreover, XX *Foxl2;Wnt4* and *Foxl2;Rspo1* double mutant gonads show enhanced Sertoli-like cell differentiation compared to the corresponding *Wnt4* or *Rspo1* single mutants (Auguste et al., 2012; Ottolenghi et al.,

2007). Decreased FOXL2 expression in *Rspo1* mutants might therefore contribute to the trans-differentiation of granulosa cells into Sertoli-like cells.

In this work we have shown that inhibition of androgen signaling in *Rspo1* mutants reduces the premature differentiation of granulosa cells in E15.5 gonads (marked by *Ar* and *Amh* expression) and their trans-differentiation into Sertoli-like cells at E18.5, strongly suggesting that androgens can promote Sertoli-like cell differentiation in *Rspo1* mutant ovotestes. Testosterone has also been shown to promote the progressive appearance of Sertoli-like cells in E13.5 wild-type ovaries grafted into male mice (Harikae et al., 2013b; Miura et al., 2019). Similar to the situation in XX *Rspo1* mutant gonads, Sertoli-like cell differentiation in the ovarian graft only occurs following the up-regulation of AR and AMH expression in granulosa cells (Miura et al., 2019). AR expression is a key event in the process of androgen dependent granulosa to Sertoli-like cell trans-differentiation process. Control XX embryonic gonads show very low AR expression and might not be able to respond to ectopic androgens. Along this line, ovaries containing Leydig cells after over-activation of the Hedgehog pathway or dihydrotestosterone treated control ovaries do not show ectopic Sertoli-like cell formation (Barsoum et al., 2009); Fig. S2). We have found that flutamide treatment inhibits *Ar* expression in E15.5 and E18.5 *Rspo1* mutant gonads, indicating that *Ar* expression is somehow dependent on androgen signaling. Future work will aim at elucidating the molecular mechanisms involved in the initial up-regulation of *Ar* expression in *Rspo1* mutants.

### **Role of androgens in germ cell survival**

Androgen pathway inhibition by flutamide treatment can also rescue the germ cells loss in XX *Rspo1* mutants at E18.5. This might be an indirect result of the rescue of pre-granulosa cells and the decrease in Sertoli-like cell differentiation (Hunt et al., 1998), or might be linked to a rescue of the proliferation defects observed in XX *Rspo1* mutant germ cells (Chassot et al., 2008a). Flutamide treatment did not rescue germ cell loss in *Ctnnb1* mutants (Liu et al., 2010). The different outcomes of

flutamide treatment might be explained by the lower dose of flutamide used in the *Cttnb1* mutant study. Alternatively, germ cell loss might be caused by different mechanisms in *Cttnb1* and *Rspo1* mutants. Indeed, in *Wnt4* and *Cttnb1* mutants, germ cells undergo massive apoptosis from E16.5 and only 10% of germ cells survive at birth (Liu et al., 2010; Vainio et al., 1999; Yao et al., 2004), a phenotype that has not been observed in *Rspo1* mutants (Chassot et al., 2011).

### **Evolutionary perspective and Implications for disease**

In mammals, it is generally assumed that sex hormones do not have major effects on the primary sex determination, since testis development of the XY fetus is not affected by maternal estrogen and ovary development of XX fetus could not be masculinized by ectopic androgens (Capel, 2017). However, in some case like the freemartin syndrome in cattle, circulating factors from a male embryo can reach its female twin through the shared blood supply and cause masculinization of the genital organs including the formation of SOX9 positive Sertoli-like cells (Padula, 2005). In our study, we found the androgen signaling pathway promotes female-to-male sex reversal in the *Rspo1* deficient ovotestes, a process that requires both ectopic steroidogenesis and ectopic AR expression. These findings suggest that a strategy established in mammals to avoid in-utero effects of the outside sex hormone rich environment is the suppression of AR expression and signaling in the XX gonad during embryonic development by canonical WNT signaling.

In our study, we found that it is possible to prevent the process of female-to-male sex reversal in *Rspo1* mutants during embryonic stages by inhibiting androgen signaling pathway, which brings new thoughts and hopes for hormonal treatments in patients with Disorders of Sex Development induced by mutations in *RSPO1*, *WNT4* or *CTNNB1*.

## Materials and Methods

### Mouse strains and genotyping

The experiments described herein were carried out in compliance with the relevant institutional and European animal welfare laws, guidelines and policies. All the experiments were approved by the French Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (APAFIS# 12879-2017120711347631, 2017121515109323, 2018061816265103). All mouse lines were kept on a mixed 129/C57Bl6/J background. The *Rspo1*<sup>flox/+</sup> mouse line where *Rspo1* Exon2 sequences are flanked by LoxP sites was produced by the Institut Clinique de la Souris (Strasbourg, France). *Rspo1*<sup>ΔEx2/+</sup> mouse line was obtained by crossing *Rspo1*<sup>flox/+</sup> males with *Sox2:Cre*<sup>tg/0</sup> females (Hayashi et al., 2003). Deletion by CRE occurred at the one-cell stage, thus generating *Rspo1*<sup>ΔEx2/+</sup> animals. *Rspo1*<sup>ΔEx2/+</sup> or *Rspo1*<sup>ΔEx2/ΔEx2</sup> males were then crossed with *Rspo1*<sup>ΔEx2/+</sup> females to generate XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals used for the mouse line validation, flutamide treatment and qRT-PCR analyses. *Sf1:Cre*<sup>tg/0</sup>; *Rspo1*<sup>flox/flox</sup> mutants were obtained by crossing *Sf1:Cre*<sup>tg/0</sup>; *Rspo1*<sup>flox/+</sup> with *Rspo1*<sup>flox/flox</sup> (Bingham et al., 2006). The CRE was transmitted either from the mother or the father. *Sf1:Cre*<sup>tg/0</sup>; *Rosa26*<sup>mTmG/+</sup> embryos were obtained by crossing *Sf1:Cre*<sup>tg/0</sup> with *Rosa26*<sup>mTmG/+</sup> (Muzumdar et al., 2007). *Wt1*<sup>GFP-Cre/+</sup>; *Rspo1*<sup>flox/flox</sup> mutants were obtained by crossing *Wt1*<sup>GFP-Cre/+</sup>; *Rspo1*<sup>flox/flox</sup> males with *Rspo1*<sup>flox/flox</sup> females (Zhou et al., 2008). *Wt1*<sup>CreERT2/+</sup>; *Rspo1*<sup>flox/flox</sup> mutants were obtained by crossing *Wt1*<sup>CreERT2/+</sup>; *Rspo1*<sup>flox/flox</sup> males with *Rspo1*<sup>flox/flox</sup> females (Zhou et al., 2008). The day when a vaginal plug was found was designated as embryonic day E0.5. The genotype of mice and embryos were determined using genotyping PCR assays on lysates from ear biopsies or tail tips with genotyping primers shown in supplementary table 1. E10.5-E12.5 embryos were staged by counting the number of tail somites (ts) with 8 ts corresponding to E10.5, 18 ts to E11.5 and 30 ts to E12.5 (Hacker et al., 1995).

### **Tamoxifen treatment**

For CreERT2 recombinase activation, tamoxifen (T5648-5G, Sigma-Aldrich) was diluted in corn oil at 55 °C (40mg/ml) and administered to pregnant mice by force feeding at E9.5, E10.5, E11.5 or E12.5 in a single dose of 200mg/kg body weight. Pregnant mice and embryos were sacrificed at E17.5. In order to obtain P10 *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>fllox/fllox</sup>* individuals induced with tamoxifen, the tamoxifen treated dam was sacrificed at E18.5 and the pups were put up for adoption by foster mothers until they were sacrificed at P10.

### **Histological analysis**

Gonads were dissected in Phosphate Buffered Saline (PBS), fixed in Bouin's solution (HT10132, Sigma-Aldrich), Antigen fix (P10016, Diapath) or 4% (w/v) Paraformaldehyde (PFA, 15710-S, EMS) overnight at 4 °C, then dehydrated and embedded in paraffin with a Leica TP1020 tissue processor. Five-micrometer-thick sections were processed for Hematoxylin and Eosin staining using standard procedures. Images were taken on a MZ9.5 microscope (Leica) with a DFC490 camera (Leica) driven by the Application suite software (Leica), and treated with Image Composite Editor and Adobe Photoshop.

### **Immunofluorescence staining**

Five-micrometer-thick sections of samples fixed in Bouin's solution (HT10132, Sigma-Aldrich), Antigen fix (P10016, Diapath) or 4% PFA (15710-S, EMS) were processed for immunofluorescence staining. Paraffin was removed in Xylene baths (2 times, 10min each). Sections were rehydrated in 100%, 90%, 70%, 40% Ethanol (EtOH) and H<sub>2</sub>O baths orderly, 5 min each. Antigen retrieval procedure was obtained by pressure cooking in 10 mM Sodium Citrate pH6, 0.05% Tween-20 for 10 min. Sections were blocked with 3% Bovine serum Albumin (BSA, A2153 Sigma-Aldrich) and 10% inactivated Normal Donkey Serum (NDS) in PBS/0.1% Tween-20 for 1 hour at room temperature. Primary antibodies diluted in 3% BSA, 3% NDS, PBS/0.1% Tween-20 were incubated at 4 °C overnight. After 3 washes in PBS/0.1% Tween-20,

secondary antibodies diluted in PBS were incubated for 1 hour in darkness at room temperature. After 3 washes in PBS/0.1% Tween-20, slides were mounted with Vectashield Hardset solution containing DAPI (H-1500, Vector Laboratories). Images were obtained on a motorized Axio ImagerZ1 microscope (Zeiss) coupled with an Axiocam Mrm camera (Zeiss) and processed with ZEN Blue 2.5 (Zeiss), Fiji and Adobe Photoshop. The dilutions of primary and secondary antibodies are shown in supplementary table 2.

### **Quantification of Immunofluorescence staining**

Quantification was performed using a Fiji Macro on 6 to 8 sections taken 40  $\mu$ m apart and covering the entire gonads of flutamide-treated (n=3) or oil-treated (n=3) E18.5 XX *Rspo1* mutants. For each section and each marker, the area of the gonad showing fluorescent signal was determined using Fiji. The area was used as a proxy for the number of cells expressing a given marker in a gonadal section. The ratio of the area of a given marker to the area of the DAPI signal in the same gonadal section was calculated. Student's t-test was used to compare the ratio values of 6-8 sections per gonad from 3 flutamide-treated gonads to the ones from 3 oil-treated gonads.

### **RNAscope® in situ hybridization**

Gonads were dissected, fixed in 4% (w/v) PFA (15710-S, EMS) overnight at room temperature and embedded in paraffin. 7  $\mu$ m sections were hybridized with *Rspo1*, *Wnt4* and *Axin2* probes purchased from Advanced Cell Diagnostics. The RNAscope® assays combined with immunofluorescence were performed according to the manufacturer's instructions.

### **Quantitative PCR analysis**

Individuals gonads without mesonephros were dissected in PBS, snap-frozen in liquid nitrogen and kept in -80 °C. Total RNAs were extracted by RNeasy Micro Kit (74004, Qiagen) and reverse transcribed by M-MLV reverse transcriptase (M170A, Promega). Q-PCR reactions were prepared in 10  $\mu$ L with LightCycler® 480 SYBR

Green I Master (04887352001, Roche) and run in the LightCycler® 480 System (05015278001, Roche). qRT-PCR primers are shown in the supplementary table 3. All biological replicates of different genotypes and treatments were run in the same plate and repeated at least twice. The number of replicates are indicated in the corresponding figures. Relative gene expression of each gonad was calculated based on a standard curve from each run and normalized to the expression of the housekeeping gene *Shda* for the comparison between different gonads. For each genotype or treatment, the mean of the normalized expression levels was calculated and graphs show mean fold-change +SEM. Student's *t*-test ( $P < 0.05$ ) or ANOVA analysis were performed with JMP9 to compare the normalized gene expression values among samples. For *post hoc* comparisons, Tukey's honestly significant difference (HSD) procedure was used. Values with the same letter are not significantly different from each other (Tukey's HSD,  $P < 0.05$ ).

### **Flutamide treatment**

100mg/mL flutamide (F9397, Sigma-Aldrich) was prepared in corn oil containing 15% ethanol at 60 °C. Pregnant mice were injected subcutaneously at the dose of 200 mg/kg body weight of flutamide or the equivalent volume of oil once daily from E10.5 to E15.5 or from E10.5 to E18.5. Pregnant mice were sacrificed at E15.5 or E18.5. The anogenital distance (from the base of the genital tubercle to the ventral edge of the anus) of E18.5 embryos was measured using a 4" pocket digital caliper under the dissecting microscope.

## References

- Auguste, A., Chassot, A.A., Grégoire, E.P., Renault, L., Pannetier, M., Treier, M., Pailhoux, E., and Chaboissier, M.C. (2012). Loss of R-spondin1 and Foxl2 amplifies female-to-male sex reversal in XX mice. *Sex. Dev.* 5, 304–317.
- Barsoum, I.B., Bingham, N.C., Parker, K.L., Jorgensen, J.S., and Yao, H.H.C. (2009). Activation of the Hedgehog pathway in the mouse fetal ovary leads to ectopic appearance of fetal Leydig cells and female pseudohermaphroditism. *Dev. Biol.* 329, 96–103.
- Berta, P., Hawkins, J.B., Sinclair, A.H., Taylor, A., Griffiths, B.L., Goodfellow, P.N., and Fellous, M. (1990). Genetic evidence equating SRY and the testis-determining factor. *Nature*.
- Biason-Lauber, A., Konrad, D., Navratil, F., and Schoenle, E.J. (2004). A WNT4 Mutation Associated with Müllerian-Duct Regression and Virilization in a 46,XX Woman. *N. Engl. J. Med.*
- Bingham, N.C., Verma-Kurvari, S., Parada, L.F., and Parker, K.L. (2006). Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* 44, 419–424.
- Capel, B. (2017). Vertebrate sex determination: Evolutionary plasticity of a fundamental switch. *Nat. Rev. Genet.* 18, 675–689.
- Chassot, A.-A., Bradford, S.T., Auguste, A., Gregoire, E.P., Pailhoux, E., de Rooij, D.G., Schedl, A., and Chaboissier, M.-C. (2012). WNT4 and RSPO1 together are required for cell proliferation in the early mouse gonad. *Development* 139, 4461–4472.
- Chassot, A.A., Ranc, F., Gregoire, E.P., Roepers-Gajadien, H.L., Taketo, M.M., Camerino, G., de Rooij, D.G., Schedl, A., and Chaboissier, M.C. (2008a). Activation of  $\beta$ -catenin signaling by Rspo1 controls differentiation of the mammalian ovary. *Hum. Mol. Genet.* 17, 1264–1277.
- Chassot, A.A., Gregoire, E.P., Magliano, M., Lavery, R., and Chaboissier, M.C. (2008b). Genetics of ovarian differentiation: Rspo1, a major player. In *Sexual Development*, pp. 219–227.
- Chassot, A.A., Gregoire, E.P., Lavery, R., Taketo, M.M., de Rooij, D.G., Adams, I.R., and Chaboissier, M.C. (2011). RSPO1/ $\beta$ -Catenin signaling pathway regulates oogonia differentiation and entry into meiosis in the mouse fetal ovary. *PLoS One* 6.
- Chen, H., Palmer, J.S., Thiagarajan, R.D., Dinger, M.E., Lesieur, E., Chiu, H., Schulz, A., Spiller, C., Grimmond, S.M., Little, M.H., et al. (2012). Identification of novel markers of mouse fetal ovary development. *PLoS One* 7.
- De Cian, M.-C., Pauper, E., Bandiera, R., Vidal, V.P.I., Sacco, S., Gregoire, E.P., Chassot, A.-A., Panzolini, C., Wilhelm, D., Pailhoux, E., et al. (2017). Amplification of R-spondin1 signaling induces granulosa cell fate defects and cancers in mouse adult ovary. *Oncogene* 36, 208–218.
- Garcia-Moreno, S.A., Futtner, C.R., Salamone, I.M., Gonen, N., Lovell-Badge, R., and Maatouk, D.M. (2019). Gonadal supporting cells acquire sex-specific chromatin landscapes during mammalian sex determination. *Dev. Biol.* 446, 168–179.
- Gonen, N., Futtner, C.R., Wood, S., Alexandra Garcia-Moreno, S., Salamone, I.M., Samson, S.C., Sekido, R., Poulat, F., Maatouk, D.M., and Lovell-Badge, R. (2018). Sex reversal following deletion of a single distal enhancer of Sox9. *Science* (80-. ). 360, 1469–1471.
- Gregoire, E.P., Lavery, R., Chassot, A.A., Akiyama, H., Treier, M., Behringer, R.R., and Chaboissier, M.C. (2011). Transient development of ovotestes in XX Sox9 transgenic mice. *Dev. Biol.* 349, 65–77.
- Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Münsterberg, A., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1990). A gene mapping to the sex-determining

region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature*.

- Gustin, S.E., Hogg, K., Stringer, J.M., Rastetter, R.H., Pelosi, E., Miles, D.C., Sinclair, A.H., Wilhelm, D., and Western, P.S. (2016). WNT/ $\beta$ -catenin and p27/FOXL2 differentially regulate supporting cell proliferation in the developing ovary. *Dev. Biol.* 412, 250–260.
- Hacker, A., Capel, B., Goodfellow, P., and Lovell-Badge, R. (1995). Expression of Sry, the mouse sex determining gene. *Development* 121, 1603–1614.
- Harikae, K., Miura, K., and Kanai, Y. (2013a). Early gonadogenesis in mammals: Significance of long and narrow gonadal structure. *Dev. Dyn. Mamm. Embryog. Gonadal Primordium Arises from Thick. Coelomic Ep. Which Results a Pair Extrem. Long Narrow Gonadal Struct. along Anteroposterior Axis.* 242, 330–338.
- Harikae, K., Miura, K., Shinomura, M., Matoba, S., Hiramatsu, R., Tsunekawa, N., Kanai-Azuma, M., Kurohmaru, M., Morohashi, K. -i., and Kanai, Y. (2013b). Heterogeneity in sexual bipotentiality and plasticity of granulosa cells in developing mouse ovaries. *J. Cell Sci.* 126, 2834–2844.
- Harris, A., Siggers, P., Corrochano, S., Warr, N., Sagar, D., Grimes, D.T., Suzuki, M., Burdine, R.D., Cong, F., Koo, B.-K., et al. (2018). ZNRF3 functions in mammalian sex determination by inhibiting canonical WNT signaling. *Proc. Natl. Acad. Sci. U. S. A.* 115, 5474–5479.
- Hayashi, S., Lewis, P., Pevny, L., and McMahon, A.P. (2003). Erratum: Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain (*Gene Expression Patterns* (2002) vol. 2 (93-97)). *Gene Expr. Patterns* 3, 542–543.
- Heikkilä, M., Peltoketo, H., Leppäluoto, J., Ilves, M., Vuolteenaho, O., and Vainio, S. (2002). Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. *Endocrinology* 143, 4358–4365.
- Heikkilä, M., Prunskaitė, R., Naillat, F., Itäranta, P., Vuoristo, J., Leppäluoto, J., Peltoketo, H., and Vainio, S. (2005). The partial female-to-male sex reversal in Wnt-4 deficient females involves induced expression of testosterone biosynthetic genes and testosterone production and depends on androgen action. *Endocrinology* 146, 4016–4023.
- Hunt, P.A., Worthman, C., Levinson, H., Stallings, J., LeMaire, R., Mroz, K., Park, C., and Handel, M.A. (1998). Germ cell loss in the XXY male mouse: Altered X-chromosome dosage affects prenatal development. *Mol. Reprod. Dev.* 49, 101–111.
- Jameson, S.A., Lin, Y.T., and Capel, B. (2012a). Testis development requires the repression of Wnt4 by Fgf signaling. *Dev. Biol.* 370, 24–32.
- Jameson, S.A., Natarajan, A., Cool, J., DeFalco, T., Maatouk, D.M., Mork, L., Munger, S.C., and Capel, B. (2012b). Temporal transcriptional profiling of somatic and germ cells reveals biased lineage priming of sexual fate in the fetal mouse gonad. *PLoS Genet.* 8.
- Jeays-Ward, K. (2003). Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. *Development* 130, 3663–3670.
- Jordan, B.K., Mohammed, M., Ching, S.T., Délot, E., Chen, X.N., Dewing, P., Swain, A., Rao, P.N., Elejalde, B.R., and Vilain, E. (2001). Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans. *Am. J. Hum. Genet.* 68, 1102–1109.
- Kashimada, K., Pelosi, E., Chen, H., Schlessinger, D., Wilhelm, D., and Koopman, P. (2011). FOXL2 and BMP2 act cooperatively to regulate follistatin gene expression during ovarian development. *Endocrinology* 152, 272–280.
- Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for Sry. *Nature*.

- Lavery, R., Lardenois, A., Ranc-Jianmotamedi, F., Pauper, E., Gregoire, E.P., Vigier, C., Moreilhon, C., Primig, M., and Chaboissier, M.C. (2011). XY Sox9 embryonic loss-of-function mouse mutants show complete sex reversal and produce partially fertile XY oocytes. *Dev. Biol.* 354, 111–122.
- Liu, C., Peng, J., Matzuk, M.M., and Yao, H.H.C. (2015). Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells. *Nat. Commun.*
- Liu, C.F., Bingham, N., Parker, K., and Yao, H.H.C. (2009). Sex-specific roles of  $\beta$ -catenin in mouse gonadal development. *Hum. Mol. Genet.* 18, 405–417.
- Liu, C.F., Parker, K., and Humphrey, Y. (2010). WNT4/ $\beta$ -catenin pathway maintains female germ cell survival by inhibiting activin  $\beta$ B in the mouse fetal ovary. *PLoS One* 5.
- Lovell-Badge, R., and Robertson, E. (1990). XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. *Development* 109.
- Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P.M., Birchmeier, W., et al. (2002). Negative Feedback Loop of Wnt Signaling through Upregulation of Conductin/Axin2 in Colorectal and Liver Tumors. *Mol. Cell. Biol.* 22, 1184–1193.
- Maatouk, D.M., Dinapoli, L., Alvers, A., Parker, K.L., Taketo, M.M., and Capel, B. (2008). Stabilization of  $\beta$ -catenin in XY gonads causes male-to-female sex-reversal. *Hum. Mol. Genet.* 17, 2949–2955.
- Maatouk, D.M., Mork, L., Chassot, A.A., Chaboissier, M.C., and Capel, B. (2013). Disruption of mitotic arrest precedes precocious differentiation and transdifferentiation of pregranulosa cells in the perinatal Wnt4 mutant ovary. *Dev. Biol.* 383, 295–306.
- Mandel, H., Shemer, R., Borochowitz, Z.U., Okopnik, M., Knopf, C., Indelman, M., Drugan, A., Tiosano, D., Gershoni-Baruch, R., Choder, M., et al. (2008). SERKAL Syndrome: An Autosomal-Recessive Disorder Caused by a Loss-of-Function Mutation in WNT4. *Am. J. Hum. Genet.* 82, 39–47.
- Manuylov, N.L., Smagulova, F.O., Leach, L., and Tevosian, S.G. (2008). Ovarian development in mice requires the GATA4-FOG2 transcription complex. *Development* 135, 3731–3743.
- Manuylov, N.L., Zhou, B., Ma, Q., Fox, S.C., Pu, W.T., and Tevosian, S.G. (2011). Conditional ablation of Gata4 and Fog2 genes in mice reveals their distinct roles in mammalian sexual differentiation. *Dev. Biol.* 353, 229–241.
- Miura, K., Harikae, K., Nakaguchi, M., Imaimatsu, K., Hiramatsu, R., Tomita, A., Hirate, Y., Kanai-Azuma, M., Kurohmaru, M., Ogura, A., et al. (2019). Molecular and genetic characterization of partial masculinization in embryonic ovaries grafted into male nude mice. *PLoS One* 14.
- Munger, S.C., Natarajan, A., Looger, L.L., Ohler, U., and Capel, B. (2013). Fine Time Course Expression Analysis Identifies Cascades of Activation and Repression and Maps a Putative Regulator of Mammalian Sex Determination. *PLoS Genet.* 9, e1003630.
- Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* 45, 593–605.
- Nam, J.S., Turcotte, T.J., and Yoon, J.K. (2007). Dynamic expression of R-spondin family genes in mouse development. *Gene Expr. Patterns.*
- Nef, S., Schaad, O., Stallings, N.R., Cederroth, C.R., Pitetti, J.L., Schaer, G., Malki, S., Dubois-Dauphin, M., Boizet-Bonhoure, B., Descombes, P., et al. (2005). Gene expression during sex determination reveals a robust female genetic program at the onset of ovarian development. *Dev. Biol.* 287, 361–377.
- Nef, S., Stévant, I., and Greenfield, A. (2019). Characterizing the bipotential mammalian

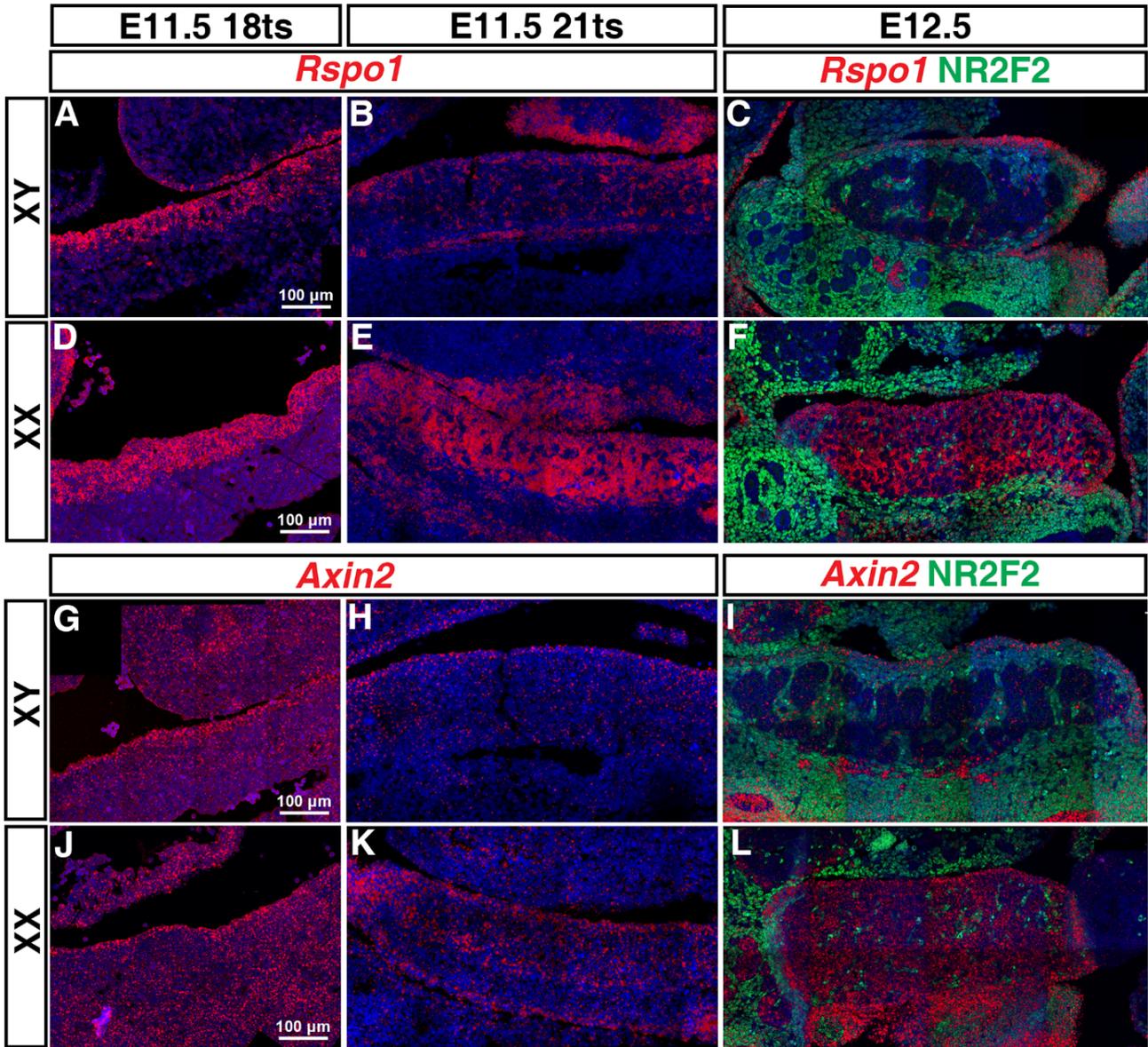
- gonad. In *Current Topics in Developmental Biology*, p.
- Nicol, B., and Yao, H.H.-C. (2015). Gonadal Identity in the Absence of Pro-Testis Factor SOX9 and Pro-Ovary Factor Beta-Catenin in Mice. *Biol. Reprod.* 93.
- Nicol, B., Grimm, S.A., Gruzdev, A., Scott, G.J., Ray, M.K., and Yao, H.H.C. (2018). Genome-wide identification of FOXL2 binding and characterization of FOXL2 feminizing action in the fetal gonads. *Hum. Mol. Genet.* 27, 4273–4287.
- Nusse, R., and Clevers, H. (2017). Wnt/ $\beta$ -Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* 169, 985–999.
- Ottolenghi, C., Omari, S., Garcia-Ortiz, J.E., Uda, M., Crisponi, L., Forabosco, A., Pilia, G., and Schlessinger, D. (2005). Foxl2 is required for commitment to ovary differentiation. *Hum. Mol. Genet.* 14, 2053–2062.
- Ottolenghi, C., Pelosi, E., Tran, J., Colombino, M., Douglass, E., Nedorezov, T., Cao, A., Forabosco, A., and Schlessinger, D. (2007). Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. *Hum. Mol. Genet.* 16, 2795–2804.
- Padula, A.M. (2005). The freemartin syndrome: an update. *Anim. Reprod. Sci.* 87, 93–109.
- Parma, P., Radi, O., Vidal, V., Chaboissier, M.C., Dellambra, E., Valentini, S., Guerra, L., Schedl, A., and Camerino, G. (2006). R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat. Genet.* 38, 1304–1309.
- Rotgers, E., Jørgensen, A., and Yao, H.H.C. (2018). At the crossroads of fate-Somatic cell lineage specification in the fetal gonad. *Endocr. Rev.*
- Schwartz, C.L., Christiansen, S., Vinggaard, A.M., Axelstad, M., Hass, U., and Svingen, T. (2019). Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Arch. Toxicol.* 93, 253–272.
- Sekido, R., and Lovell-Badge, R. (2008). Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature* 453, 930–934.
- Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R., and Goodfellow, P.N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature*.
- Stévant, I., and Nef, S. (2019). Genetic Control of Gonadal Sex Determination and Development. *Trends Genet.* 35, 346–358.
- Stévant, I., Neirijnck, Y., Borel, C., Escoffier, J., Smith, L.B., Antonarakis, S.E., Dermitzakis, E.T., Nef, S., Neirijnck, Y., Borel, C., et al. (2017). Deciphering cell lineage specification during male sex determination with single-cell RNA sequencing. *Cell Rep.* 22, 1589–1599.
- Stévant, I., Kuhne, F., Greenfield, A., Chaboissier, M.-C., Dermitzakis, E.T., and Nef, S. (2018). Single-cell transcriptomics of the mouse gonadal soma reveals the establishment of sexual dimorphism in distinct cell lineages. *BioRxiv*.
- Tomizuka, K., Horikoshi, K., Kitada, R., Sugawara, Y., Iba, Y., Kojima, A., Yoshitome, A., Yamawaki, K., Amagai, M., Inoue, A., et al. (2008). R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Hum. Mol. Genet.* 17, 1278–1291.
- Vainio, S., Heikkilä, M., Kispert, A., Chin, N., and McMahon, A.P. (1999). Female development in mammals is regulated by Wnt-4 signalling. *Nature*.
- Vidal, V.P.I., Chaboissier, M.C., De Rooij, D.G., and Schedl, A. (2001). Sox9 induces testis development in XX transgenic mice. *Nat. Genet.*
- Vilain, E., McElreavey, K., Jaubert, F., Raymond, J.P., Richaud, F., and Fellous, M. (1992).

- Familial case with sequence variant in the testis-determining region associated with two sex phenotypes. *Am. J. Hum. Genet.* 50, 1008–1011.
- Welsh, M., Saunders, P.T.K., Marchetti, N.I., and Sharpe, R.M. (2006). Androgen-Dependent Mechanisms of Wolffian Duct Development and Their Perturbation by Flutamide. *Endocrinology* 147, 4820–4830.
- Yao, H.H.-C., Aardema, J., and Holthusen, K. (2006). Sexually Dimorphic Regulation of Inhibin Beta B in Establishing Gonadal Vasculature in Mice. *Biol. Reprod.*
- Yao, H.H.C., Whoriskey, W., and Capel, B. (2002). Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes Dev.*
- Yao, H.H.C., Matzuk, M.M., Jorgez, C.J., Menke, D.B., Page, D.C., Swain, A., and Capel, B. (2004). Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. *Dev. Dyn.* 230, 210–215.
- Zhao, F., Franco, H.L., Rodriguez, K.F., Brown, P.R., Tsai, M.-J., Tsai, S.Y., H-C Yao, H., and Yao, H.H.-C. (2017). Elimination of the male reproductive tract in the female embryo is promoted by COUP-TFII in mice. *Science* (80-. ). 357, 717–720.
- Zhao, L., Wang, C., Lehman, M.L., He, M., An, J., Svingen, T., Spiller, C.M., Ng, E.T., Nelson, C.C., and Koopman, P. (2018). Transcriptomic analysis of mRNA expression and alternative splicing during mouse sex determination. *Mol. Cell. Endocrinol.* 478, 84 - 96.
- Zhou, B., Ma, Q., Rajagopal, S., Wu, S.M., Domian, I., Rivera-Feliciano, J., Jiang, D., Von Gise, A., Ikeda, S., Chien, K.R., et al. (2008). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature* 454, 109–113.

## Figure and figure legends

### Figure 1. *Rspo1* expression and WNT/ $\beta$ -catenin activity in the early mouse gonad

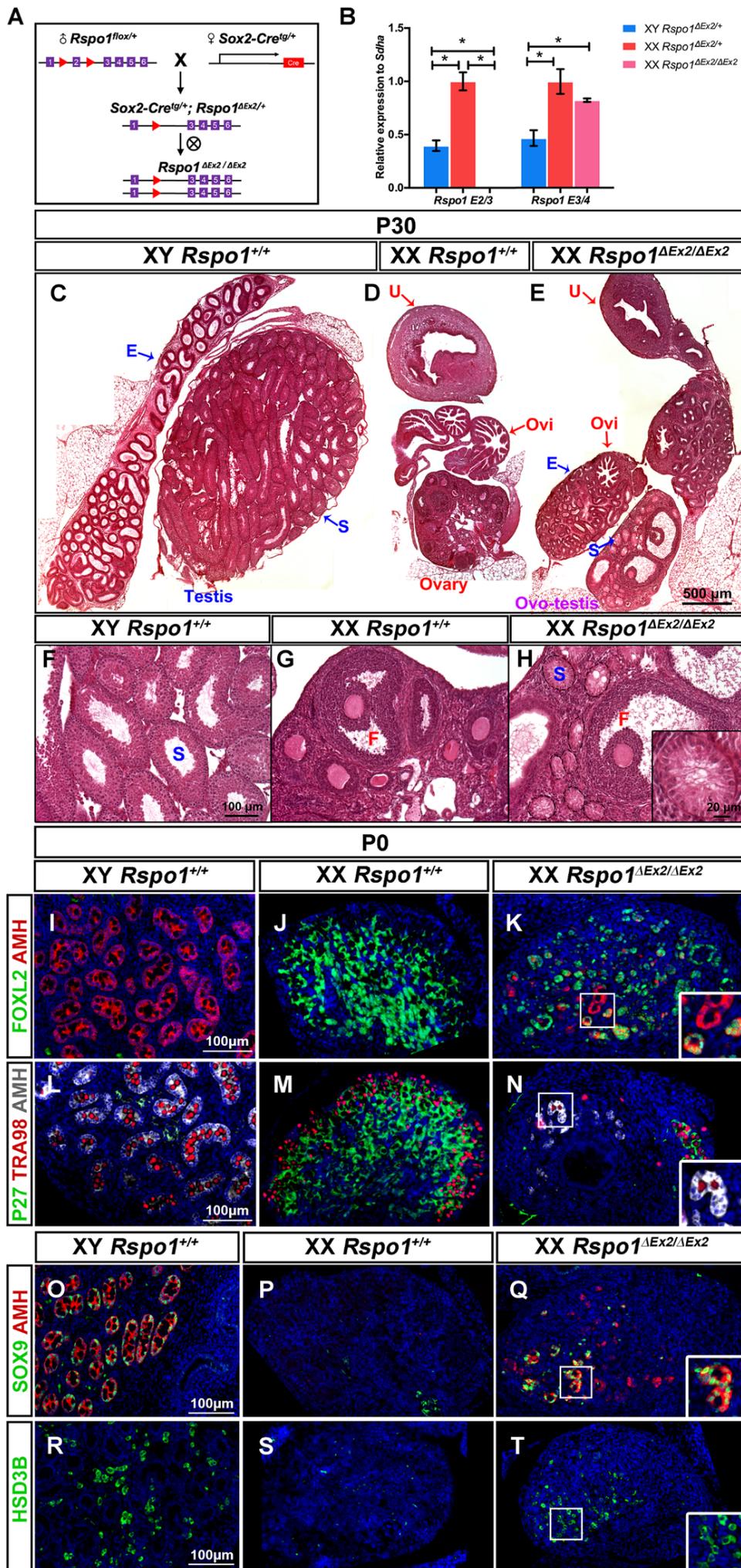
**(A-F)** *Rspo1* mRNA expression (red) in XY and XX wild-type gonads at 18 ts (A and D), 21 ts (B and E) and E12.5 (with immunofluorescence staining for the interstitial marker NR2F2 in green, C and F). **(G-L)** Expression of the WNT/CTNNB1 down-stream target *Aixn2* mRNA (red) in XY and XX wild-type gonads at 18 ts (G and J), 21 ts (H and K) and E12.5 (with immunofluorescence staining for NR2F2 in green, I and L). Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m.



*Rspo1*: Figure 1

## Figure 2. Generation and validation of a new conditional allele of *Rspo1*

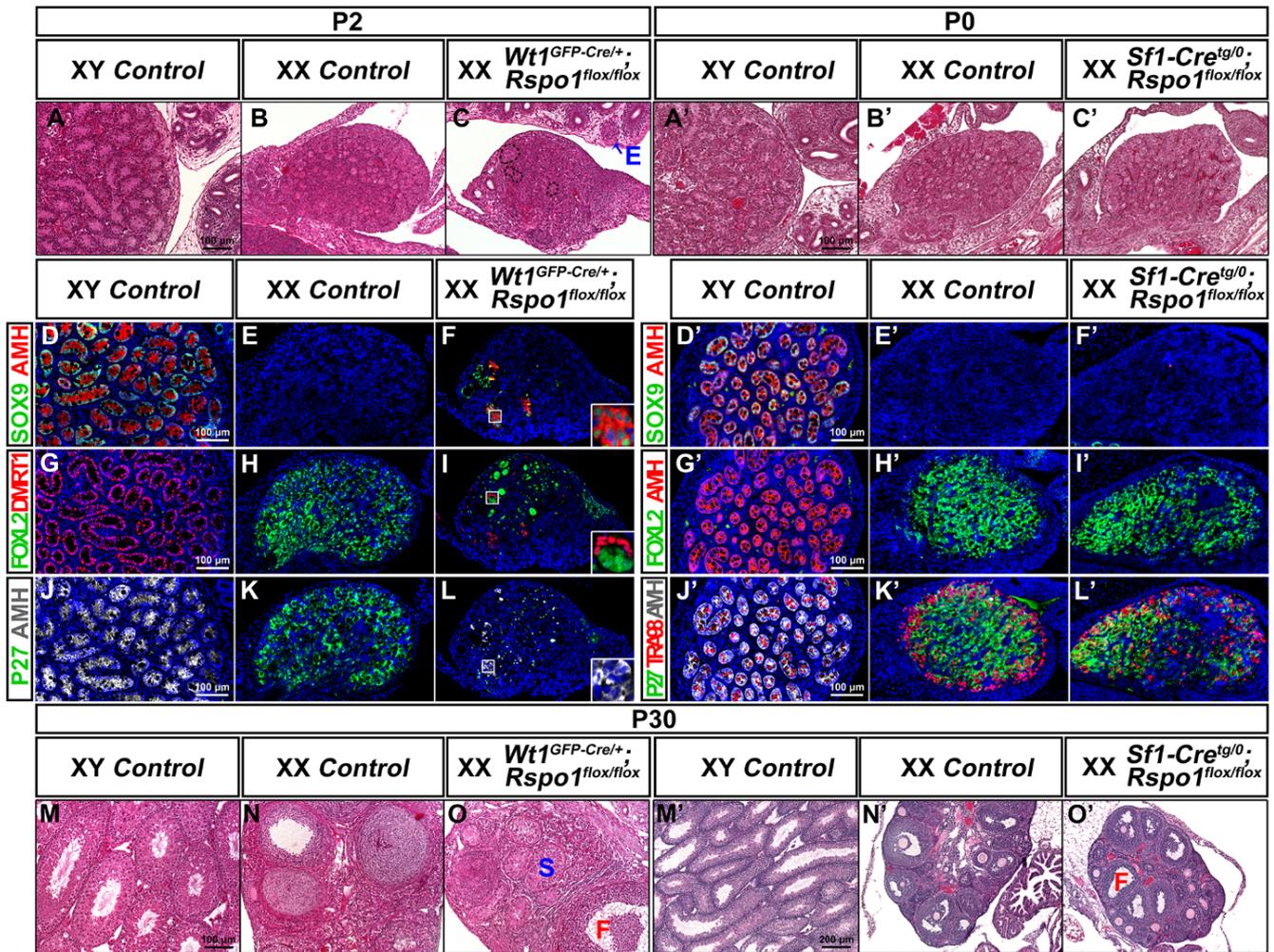
**(A)** Generation of *Rspo1*<sup>ΔEx2/ΔEx2</sup> mice by germ-line deletion of the *Rspo1*<sup>fllox</sup> allele using the Sox2-Cre transgenic line. **(B)** Quantitative PCR analysis of *Rspo1* mRNA expression in XY *Rspo1*<sup>ΔEx2/+</sup>, XX *Rspo1*<sup>ΔEx2/+</sup> and XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads at E11.5. Primers *Rspo1* E2/3 amplify a fragment spanning Exon 2 and Exon 3 allowing the assessment of the deletion efficiency of *Rspo1* Exon 2 in the mutants. Primers *Rspo1* E3/4 amplify a fragment spanning Exon 3 and Exon 4 and allow the detection of wild-type or ΔEx2 *Rspo1* mRNA levels. Results are shown as mean +/- SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test ( $n = 3$  embryos for each genotype). **(C-E)** Histological sections of the genital tracts in XY *Rspo1*<sup>+/+</sup>, XX *Rspo1*<sup>+/+</sup> and XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> at postnatal day 30 (P30). E, epididymis; S, seminiferous tubules; U, uterus; Ovi, oviducts. Scale bar = 500 μm. **(F-H)** Histological sections of the gonads in XY *Rspo1*<sup>+/+</sup>, XX *Rspo1*<sup>+/+</sup> and XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> at P30. Inset in H shows a seminiferous tubule formed in the XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> ovotestis. F, follicles. Scale bar = 100 μm. **(I-T)** Immunofluorescence staining on new born (P0) XY *Rspo1*<sup>+/+</sup>, XX *Rspo1*<sup>+/+</sup> and XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads. **(I-K)** Expression of pre-granulosa cell marker FOXL2 (green) and pre-matured granulosa cell or Sertoli cell marker AMH (red). Inset in K shows pre-matured granulosa cells co-expressing FOXL2 and AMH in XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. **(L-N)** Expression of the mitotic arrest marker P27 (green), germ cell marker TRA98 (red) and AMH (grey). Inset in N shows pre-matured granulosa cells expressing AMH and negative for P27 in XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. **(O-Q)** Expression of the Sertoli cell marker SOX9 (green) and AMH (red). Inset in Q shows Sertoli-like cells co-expressing SOX9 and AMH in XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. **(R-T)** Expression of the steroidogenic enzyme HSD3B (green). Inset in T shows ectopic steroidogenic cells expressing HSD3β in XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. Nuclei labeled with DAPI are shown in blue. Scale bar = 100 μm.



**Rspo1: Figure 2**

### Figure 3. *Rspo1* deletion in the early gonad leads to XX ovotestis formation

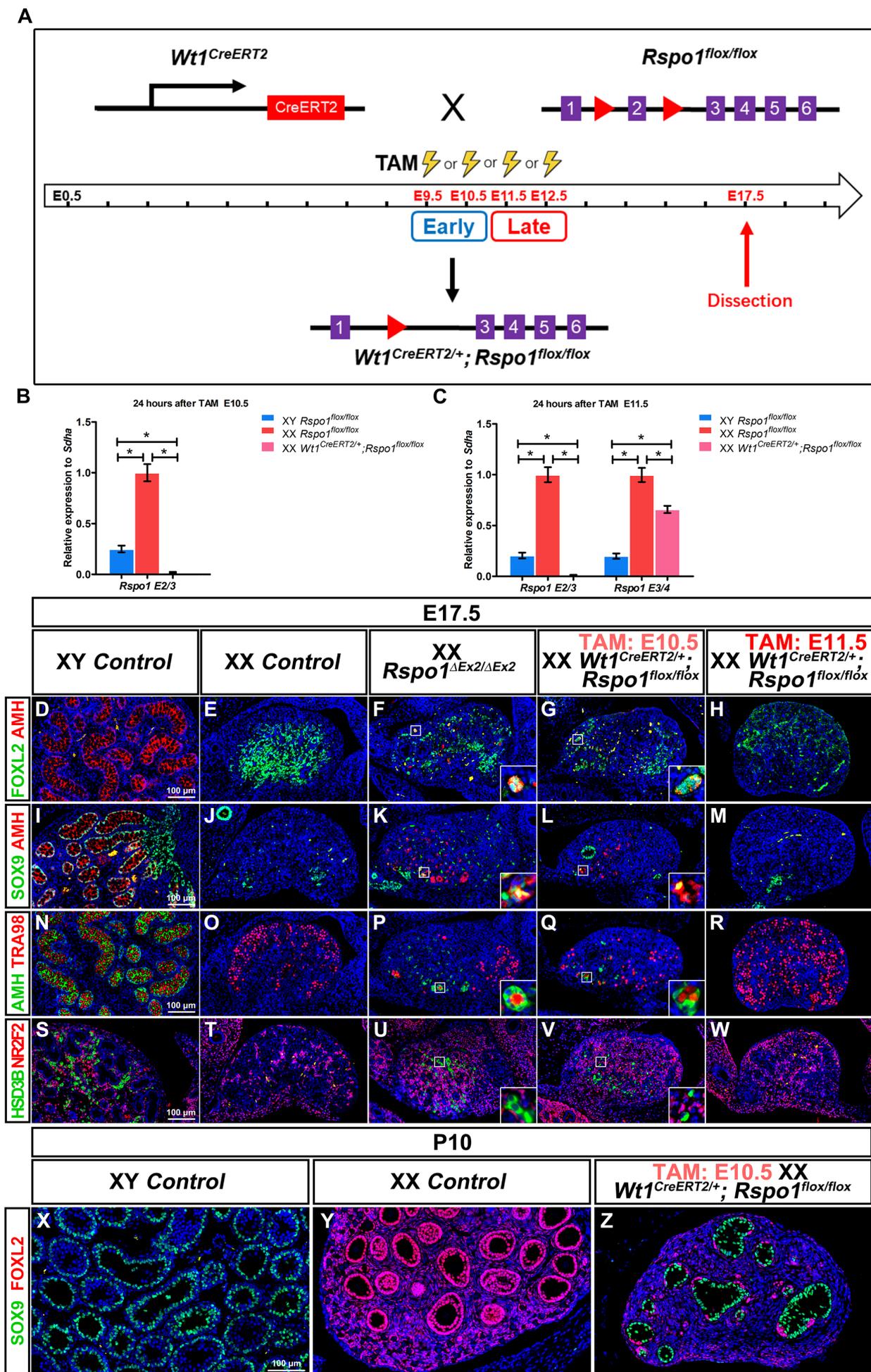
**(A-O)** Phenotype of XY *Control*, XX *control* and XX *Wt1<sup>GFP-Cre/+</sup>; Rspo1<sup>flox/flox</sup>* gonads at postnatal 2 days (P2, A-L) and P30 (M-O). **(A-C)** Histological sections at P2. E, epididymis; Dashed lines in C outline the testis cords in XX *Wt1<sup>GFP-Cre/+</sup>; Rspo1<sup>flox/flox</sup>* ovotestis. Scale bar = 100  $\mu$ m. **(D-L)** Immunofluorescence staining at P2. **(D-F)** Expression of SOX9 (green) and AMH (red). Inset in F shows Sertoli-like cells co-expressing SOX9 and AMH in XX *Wt1<sup>GFP-Cre/+</sup>; Rspo1<sup>flox/flox</sup>* gonad. **(G-I)** Expression of FOXL2 (green) and the Sertoli cell marker DMRT1 (red). Inset in I shows a testis cord-like structure containing Sertoli-like cells expressing DMRT1 and granulosa cells expressing FOXL2 in XX *Wt1<sup>GFP-Cre/+</sup>; Rspo1<sup>flox/flox</sup>* gonad. **(J-L)** Expression of P27 (green) and AMH (grey). Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m. **(M-O)** Histological sections at P30 showing ovotestis formation in XX *Wt1<sup>GFP-Cre/+</sup>; Rspo1<sup>flox/flox</sup>* gonad (O). S, seminiferous tubules, F, follicles. Scale bar = 100  $\mu$ m. **(A'-O')** Phenotype of XY *Control*, XX *control* and XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* gonads at P0 (A'-L') and P30 (M'-O'). **(A'-C')** Histological sections at P0. Scale bar = 100  $\mu$ m. **(D'-L')** Immunofluorescence staining at P0 show normal ovarian development in XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* gonads. **(D'-F')** Expression of SOX9 (green) and AMH (red). **(G'-I')** Expression of FOXL2 (green) and AMH (red). **(J'-L')** Expression of P27 (green), TRA98 (red) and AMH (grey). Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m. **(M'-O')** Histological sections at P30 showing ovarian follicles in XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* gonads, F, follicles. Scale bar = 200  $\mu$ m.



**Rspo1: Figure 3**

**Figure 4. *Rspo1* function is required at the time of sex determination but is dispensable at later stages of ovarian differentiation**

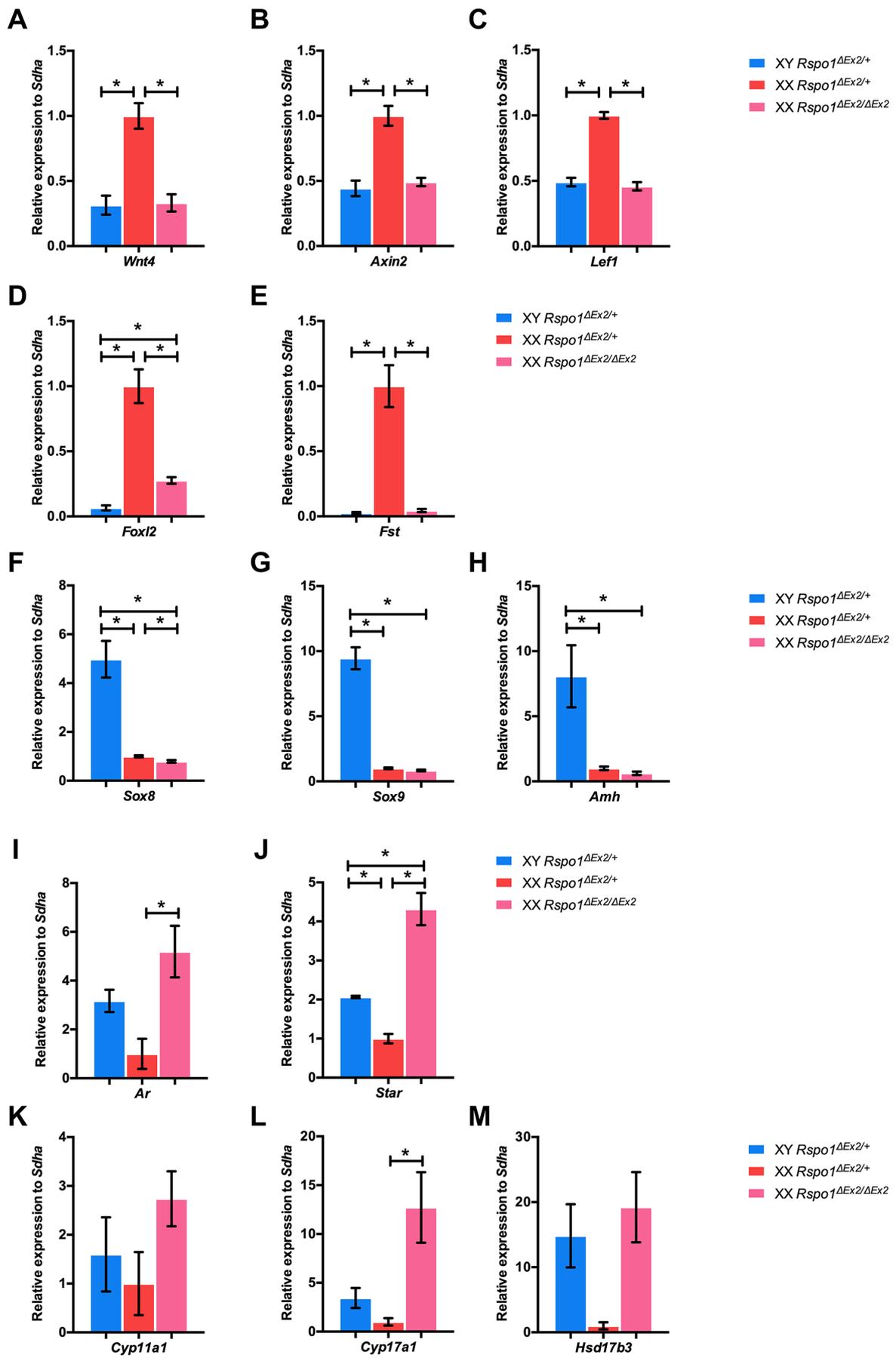
**(A)** Cre mediated deletion of *Rspo1* exon2 in XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* embryos by tamoxifen administration to pregnant mothers at different time points. “Early” induction including E9.5 and E10.5 leads to ovotestes development. “Late” induction at E11.5 and E12.5 does not affect ovarian development. **(B, C)** Quantitative PCR analysis of *Rspo1* mRNA expression in XY *Rspo1<sup>flox/flox</sup>*, XX *Rspo1<sup>flox/flox</sup>* and XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* gonads. **(B)** *Rspo1* mRNA containing Exon 2 sequences is absent in XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* gonads dissected at E11.5, 24 hours after tamoxifen administration at E10.5. **(C)** *Rspo1* mRNA containing Exon 2 sequences is absent in XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* gonads dissected at E12.5, 24 hours after tamoxifen administration at E11.5. A truncated *Rspo1* mRNA detected with Primers *Rspo1 E3/4* is expressed after Cre mediated deletion of Exon 2 sequences. Results are shown as mean +/- SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test ( $n = 3-5$  embryos for each genotype). **(D-W)** Immunofluorescence staining of E17.5 gonads from XY Control, XX Control, XX *Rspo1<sup>ΔEx2/ΔEx2</sup>*, XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* induced with tamoxifen at E10.5 and XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* induced with tamoxifen at E11.5. **(D-H)** Expression of FOXL2 (green) and AMH (red). Insets show pre-matured granulosa cells co-expressing FOXL2 and AMH in XX *Rspo1<sup>ΔEx2/ΔEx2</sup>* (F) and XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* induced at E10.5 (G). **(I-M)** Expression of SOX9 (green) and AMH (red). Insets show Sertoli-like cells co-expressing SOX9 and AMH in XX *Rspo1<sup>ΔEx2/ΔEx2</sup>* (K) and XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* induced at E10.5 (L). **(N-R)** Expression of AMH (green) and TRA98 (red). Insets show AMH positive cells in XX *Rspo1<sup>ΔEx2/ΔEx2</sup>* (P) and XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* induced at E10.5 (Q). **(S-W)** Expression of HSD3β (green) and NR2F2 (red). Insets show ectopic steroidogenic cells expressing HSD3β in XX *Rspo1<sup>ΔEx2/ΔEx2</sup>* (U) and XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* induced at E10.5 (V). **(X-Z)** Immunofluorescence staining of P10 gonads from XY Control, XX Control, and XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* induced with tamoxifen at E10.5. Seminiferous tubules with SOX9 positive Sertoli-like cells and few FOXL2 positive granulosa cells are observed in P10 XX *Wt1<sup>CreERT2/+</sup>; Rspo1<sup>flox/flox</sup>* induced at E10.5. Nuclei labeled with DAPI are shown in blue. Scale bar = 100 μm.



*Rspo1*: Figure 4

**Figure 5. Early gene expression changes in E11.5 XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads.**

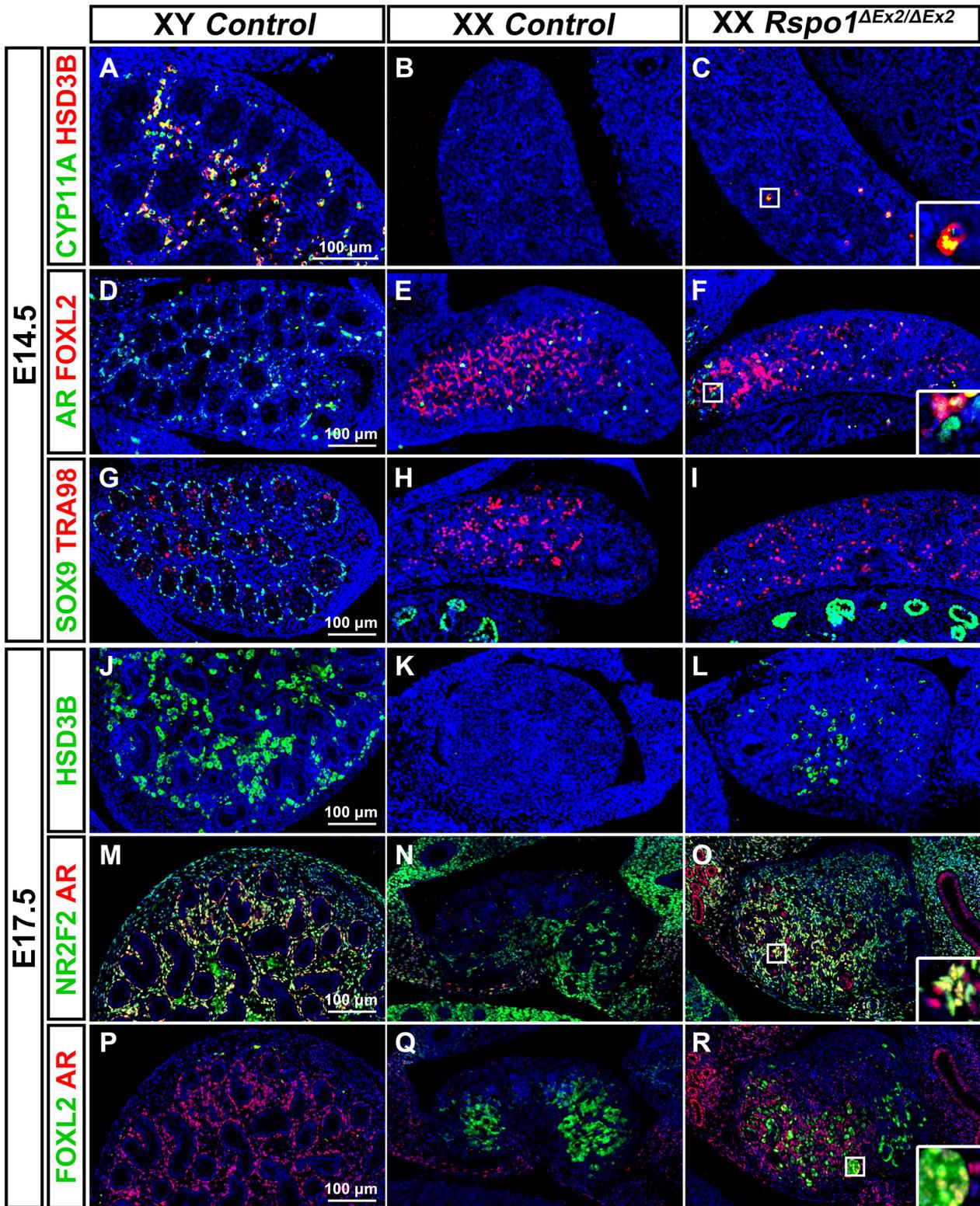
Quantitative PCR analysis of mRNA expression in XY *Rspo1*<sup>ΔEx2/+</sup>, XX *Rspo1*<sup>ΔEx2/+</sup> and XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads at E11.5. **(A-C and E)** The mRNA expression levels of RSPO1/WNT/β-catenin signalling targets: *Wnt4*, *Axin2*, *Lef1* and *Fst* are significantly down-regulated in E11.5 XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads, similar the levels of XY *Rspo1*<sup>ΔEx2/+</sup>. **(D)** Pre-granulosa cell marker *Foxl2* mRNA expression is down-regulated in XX *Rspo1*<sup>ΔEx2/ΔEx2</sup>. **(F-H)** The mRNA expression levels of Sertoli cell or pre-matured granulosa cell markers *Sox8*, *Sox9* and *Amh* is not up-regulated in XX *Rspo1*<sup>ΔEx2/ΔEx2</sup>. **(I)** Androgen receptor (*Ar*) mRNA expression is up-regulated in XX *Rspo1*<sup>ΔEx2/ΔEx2</sup>. **(J-M)** The expression of the mRNAs for the steroidogenic pathway components *Star*, *Cyp11a1*, *Cyp17a1* and *Hsd17b3* is up-regulated in E11.5 XX *Rspo1*<sup>ΔEx2/ΔEx2</sup>. Results are shown as mean +/- SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test (n = 3 embryos for each genotype).



***Rspo1*: Figure 5**

**Figure 6. Steroidogenic enzymes and Androgen Receptor expression pattern in XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads**

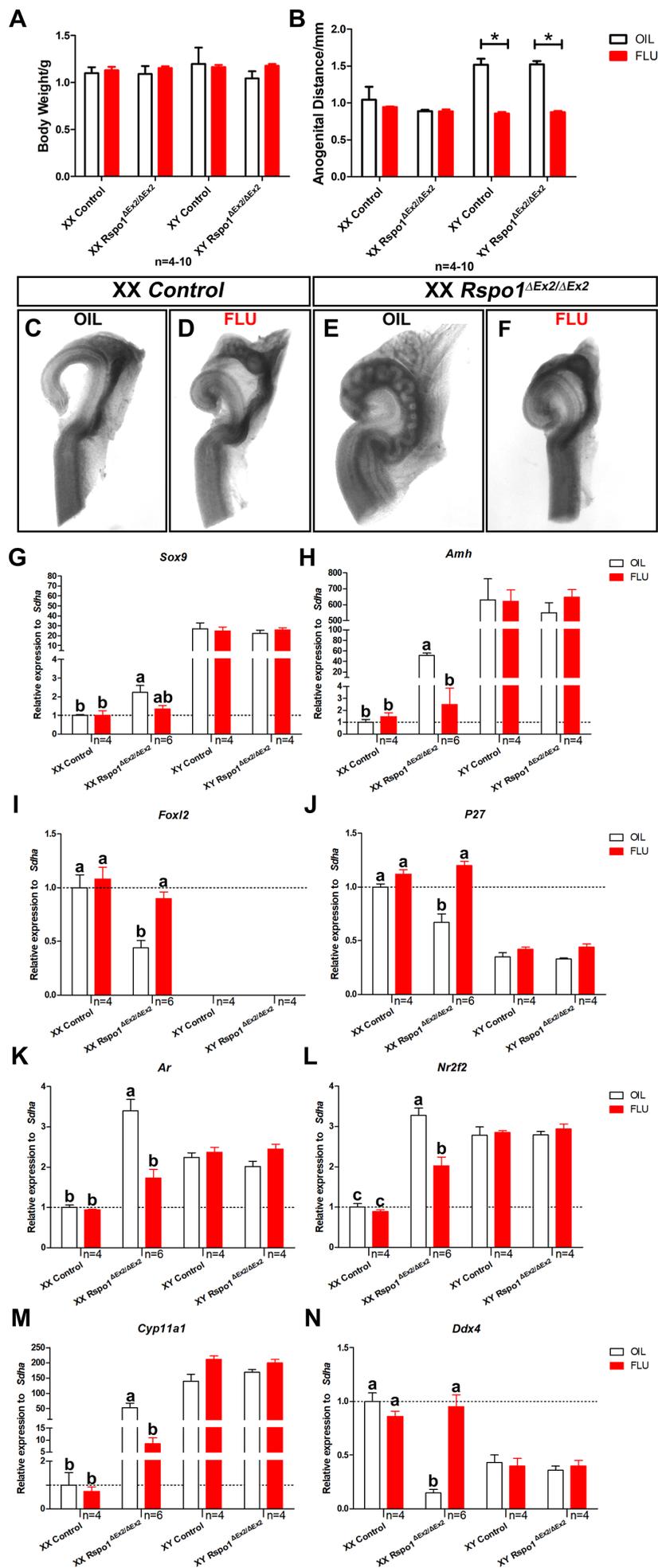
Immunofluorescence staining of E14.5 (A-I) and E17.5 (J-R) gonads from XY Control, XX Control and XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> genotypes. **(A-C)** Expression of steroidogenic enzymes CYP11A1 (green) and HSD3β (red). Inset in C shows a cell co-expressing both enzymes in the XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. **(D-F)** Expression of Androgen receptor (AR, green) and FOXL2 (red). Inset in F shows cells expressing AR and co-expressing AR and FOXL2 in the XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. **(G-I)** Expression of SOX9 (green) and TRA98 (red). SOX9 cells are not detected in the XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. **(J-L)** Expression of HSD3β (green). Ectopic steroidogenic cells are present in the XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. **(M-O)** Expression of NR2F2 (green) and AR (red). Inset in O shows interstitial cells co-expressing NR2F2 and AR in the XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. **(P-R)** Expression of FOXL2 (green) and AR (red). Inset in R shows pre-matured granulosa cells co-expressing FOXL2 and AR in the XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. Nuclei labeled with DAPI are shown in blue. Scale bar = 100 μm.



*Rspo1*: Figure 6

**Figure 7. Flutamide treatment from E10.5-18.5 rescue masculinized genital tract and ovarian gene expression in E18.5 *Rspo1* mutants.**

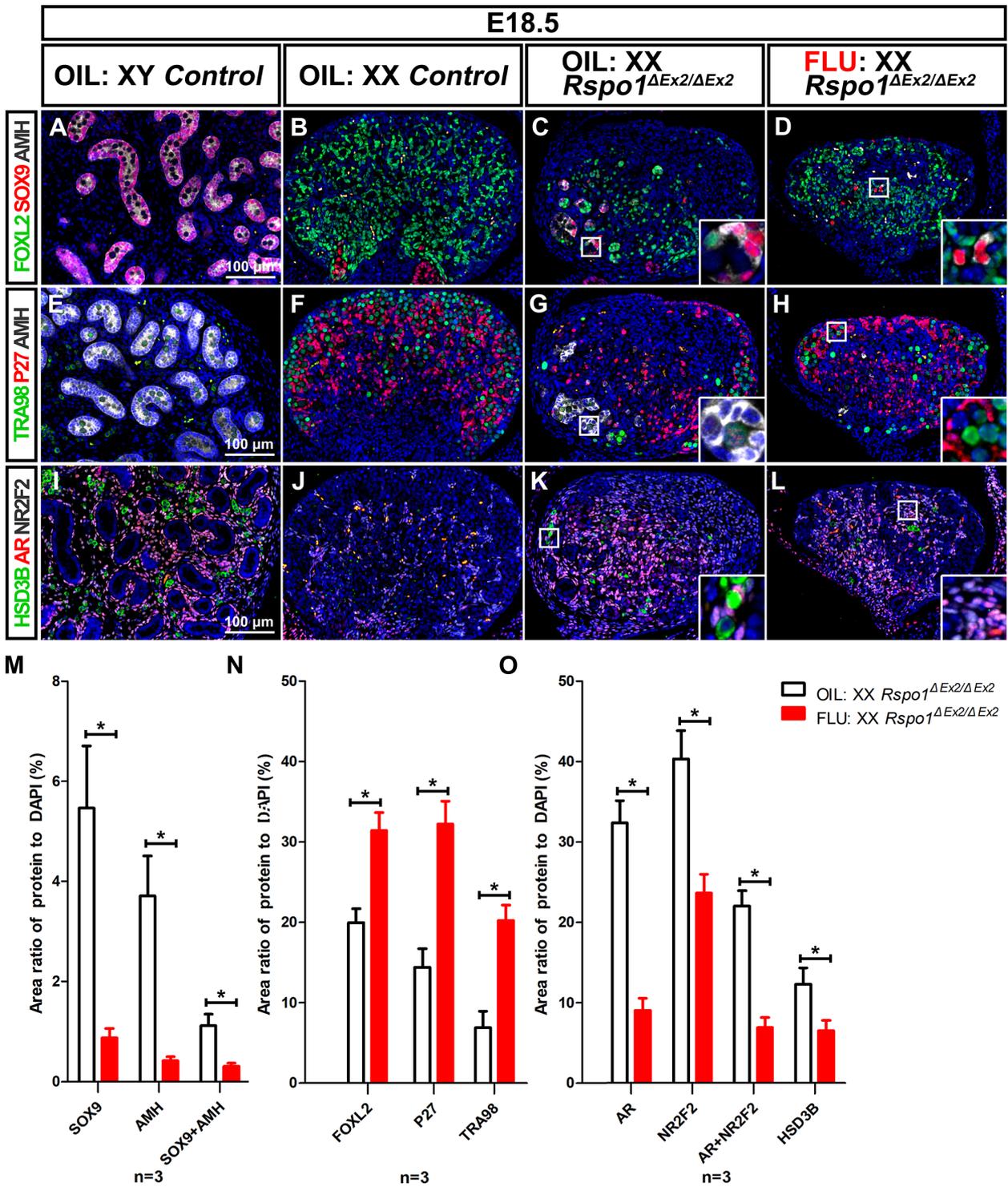
**(A)** Total body weights of E18.5 XX and XY, Control and *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals are not changed by flutamide treatment (red). **(B)** Anogenital distance of E18.5 XX and XY, Control and *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals treated with flutamide (red) or oil (white). Results are shown as mean + SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test (n = 4-10 embryos for each group). Anogenital distance is significantly reduced in XY embryos after treatment with flutamide. **(C-F)** Macroscopic views of the genital tract of E18.5 XX and XY, Control and *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals treated with flutamide or oil. Epididymis development is inhibited in the flutamide treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> (F) compared to the oil treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> (E). **(G-N)** Quantitative PCR analysis of mRNA expression in E18.5 XX and XY, Control and *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals treated with flutamide (red) or oil (white). Results are shown as mean + SEM. Relative expression levels among XX samples were compared by means of ANOVA analysis followed by Tukey's HSD test. Values with the same letter are not significantly different from each other (Tukey's HSD,  $P < 0.05$ ). n = 4-10 embryos for each group).



**Rspo1: Figure 7**

**Figure 8. Flutamide treatments from E10.5-18.5 rescue gonadal sex reversal in E18.5 *Rspo1* mutants.**

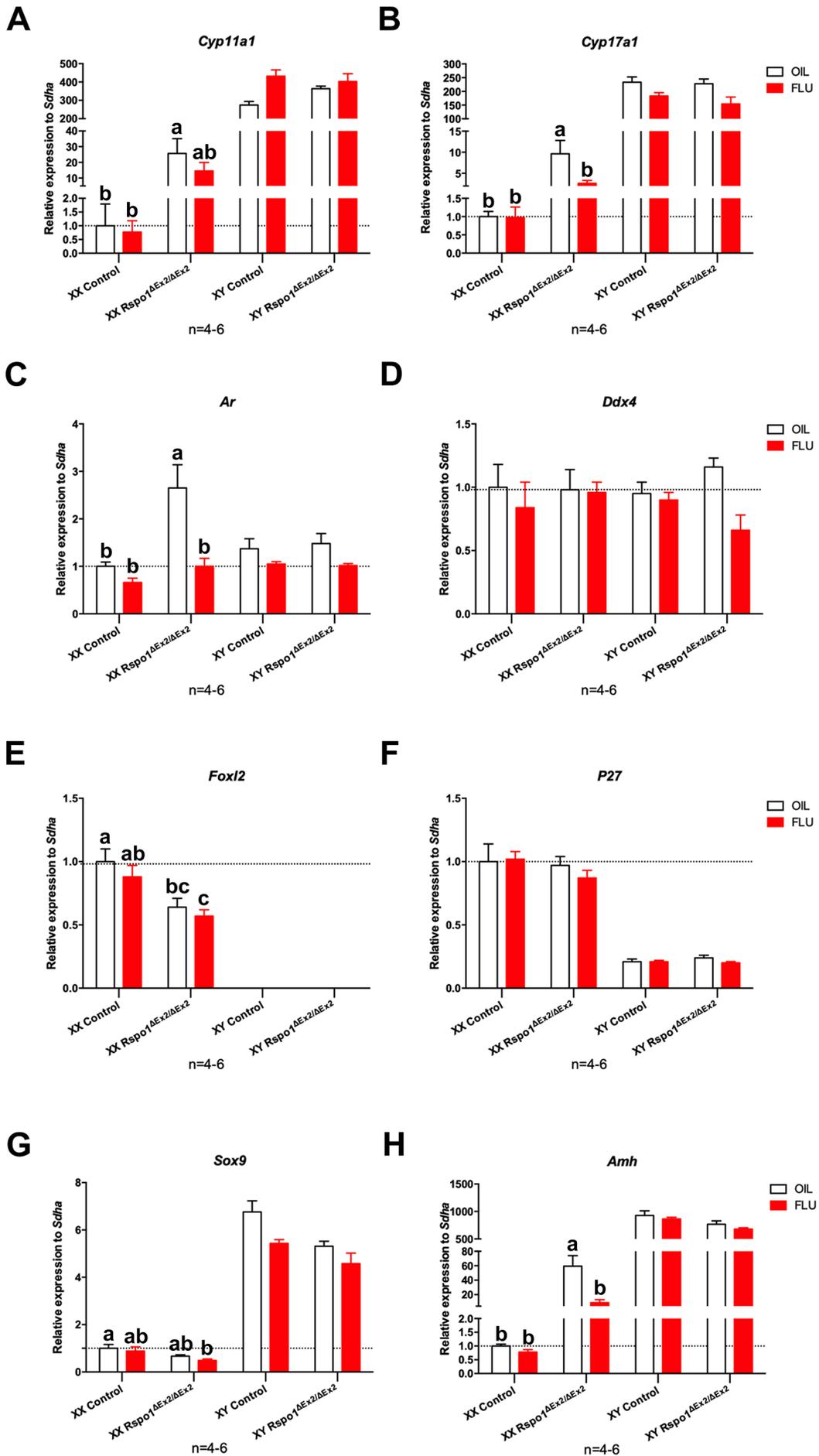
(A-L) Immunofluorescence staining of E18.5 gonads from oil treated XY control, oil treated XX control, oil treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> and flutamide treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads. (A-D) Expression of FOXL2 (green), SOX9 (red) and AMH (white). Insets in C and D show Sertoli-like cells co-expressing SOX9 and AMH. These cells are less abundant in flutamide treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads (D). (E-F) Expression of TRA98 (green), P27 (red) and AMH (white). Insets show AMH positive cells in oil treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad (G) and pre-granulosa cells expressing P27 and surrounding germ cells in flutamide treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad (H). P27 and TRA98 expression are observed in the entire flutamide treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad (H), whereas they are confined to the posterior ovarian part in the oil treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> ovotestis (G). (I-L) Expression of HSD3β (green), AR (red) and NR2F2 (white). Inset in K shows ectopic steroidogenic cells in oil treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad. These cells are less abundant in flutamide treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonad (L). Inset in L shows interstitial cells. Nuclei labeled with DAPI are shown in blue. Scale bar = 100 μm. (M-N) Quantification of protein distribution in immunofluorescence stainings of E18.5 gonads from oil treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> and flutamide treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads. For each marker, the area of detected fluorescence was measured as a proxy for the number of cells expressing the corresponding protein. The fluorescent area measurements were normalized against the area of DAPI staining. Results are shown as mean + SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test (6-8 sections per gonad from 3 flutamide treated gonads and 3 oil treated gonads).



***Rspo1*: Figure 8**

**Figure 9. Flutamide treatments from E10.5-15.5 inhibits granulosa cell premature differentiation in *Rspo1* mutants**

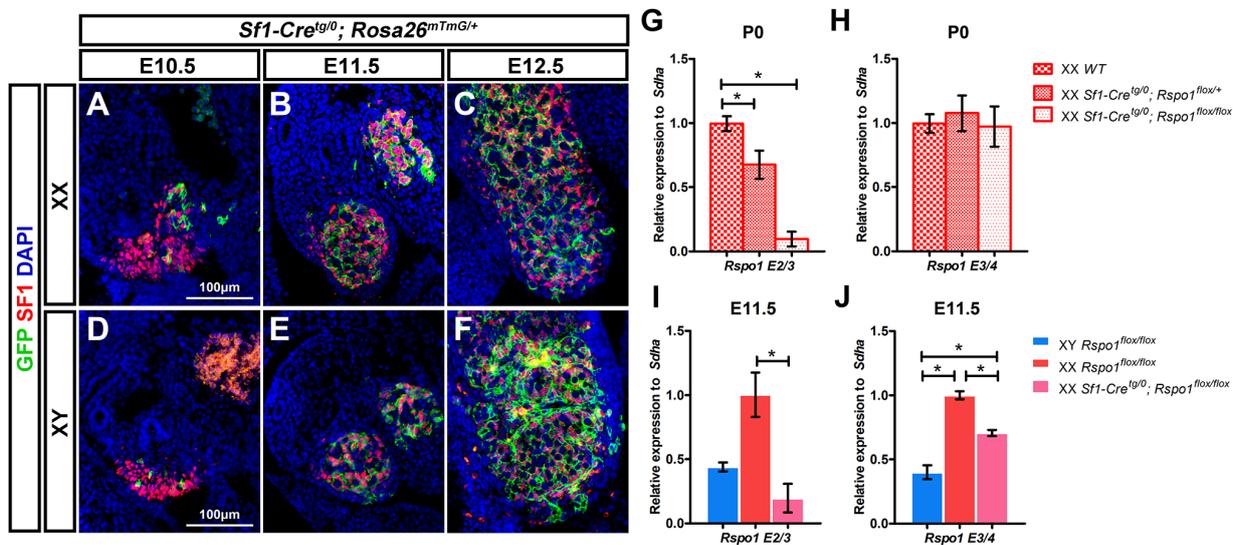
Quantitative PCR analysis of mRNA expression in E15.5 XX and XY, Control and *Rspo1* <sup>$\Delta$ Ex2/ $\Delta$ Ex2</sup> individuals treated with flutamide (red) or oil (white). Results are shown as mean + SEM. Relative expression levels among XX samples were compared by means of ANOVA analysis followed by Tukey's HSD test. Values with the same letter are not significantly different from each other (Tukey's HSD,  $P < 0.05$ ). n = 4-6 embryos for each group).



**Rspo1: Figure 9**

**Figure S1. Deletion efficiency of *Rspo1* by *Sf1-Cre* and sex reversal rates in XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* and XX *Wt1<sup>GFP-Cre/+</sup>; Rspo1<sup>flox/flox</sup>* mutants.**

**(A-F)** Immunofluorescence staining of E10.5 (10 ts), E11.5 (22 ts) and E12.5 XX and XY *Sf1-Cre<sup>tg/0</sup>; Rosa26<sup>mTmG/+</sup>* gonads. Expression of SF1 (red) marks the developing adrenal gland and the developing gonad. GFP expression (green) is detected in cells where *Sf1-Cre<sup>tg/0</sup>* was active. Few cells express GFP in E10.5 XX and XY gonads (A, D). Almost all the gonadal somatic cells express GFP except the coelomic epithelial cells of XX and XY gonads at E11.5 (B, E). At E12.5, most of the coelomic epithelial cells for XX and tunica albuginea for XY are still negative for GFP (C, F). Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m. **(G-J)** Quantitative PCR analysis of mRNA expression in XX control, XY *Rspo1<sup>flox/flox</sup>*, XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/+</sup>* and XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* gonads at P0 (G, H) and E11.5 (I, J). Primers *Rspo1 E2/3* amplify a fragment spanning Exon 2 and Exon 3 allowing the assessment of the deletion efficiency of *Rspo1* Exon 2 in the mutants. Primers *Rspo1 E3/4* amplify a fragment spanning Exon 3 and Exon 4 and allow the detection of wild-type or  $\Delta$ Ex2 *Rspo1* mRNA levels. Results are shown as mean  $\pm$  SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test ( $n = 3$  embryos for each genotype). The deletion efficiency of *Rspo1* Exon 2 in XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* is 79% at P0 (G). The deletion efficiency of *Rspo1* Exon 2 in XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* is 81% at E11.5 (I). **(K, L)** Percentage of female to male sex reversal in XX *Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup>* at P30 (K) and XX *Wt1<sup>GFP-Cre/+</sup>; Rspo1<sup>flox/flox</sup>* from E17.5 to P30 (L) based on the observation of “double ducts”, in which Wolffian duct derivatives (epididymis and vas deferens) develops along with Müllerian ducts (oviduct and uterus).



**K**

Stage	<i>Sf1-Cre<sup>tg/0</sup>; Rspo1<sup>flox/flox</sup></i>	Wolffian duct Retained	Normal oviduct	Female-to-male sex reversal percentage
P30	n=16	n=4	n=12	<25%

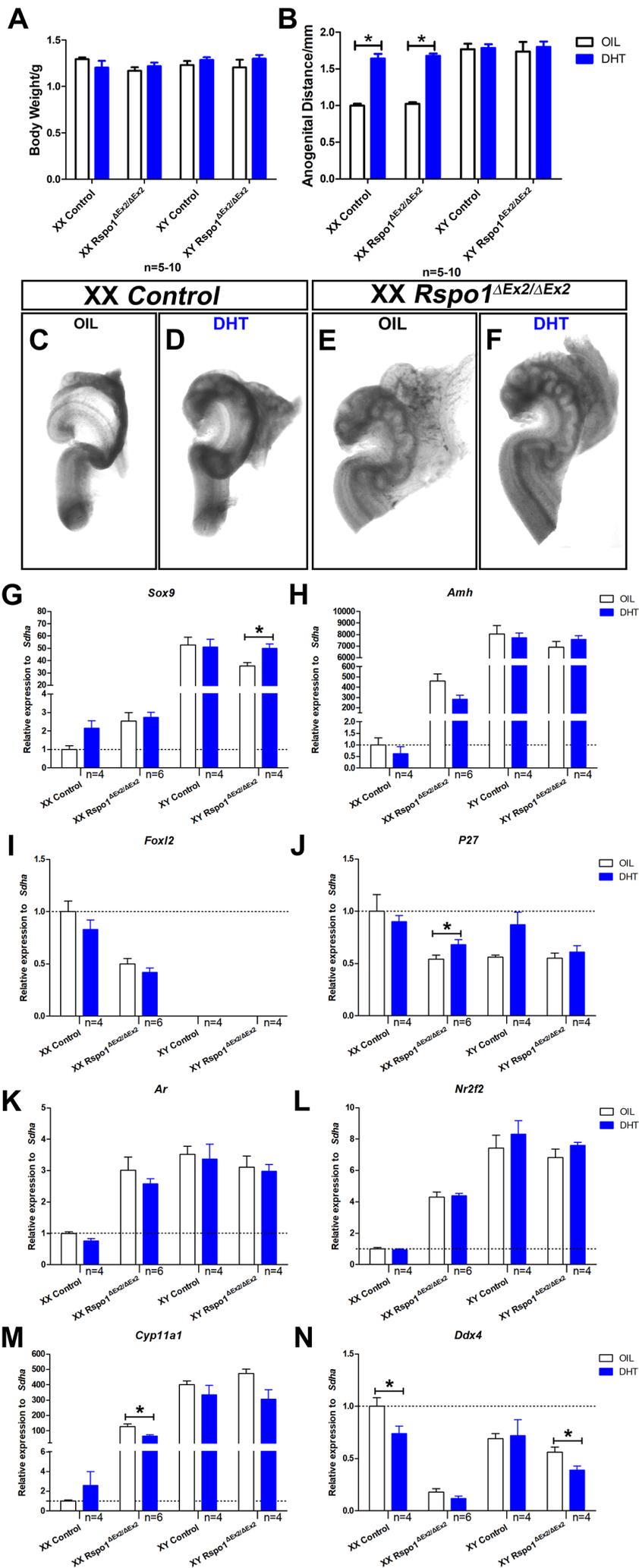
**L**

Stage	<i>Wt1<sup>GFP-Cre/+</sup>; Rspo1<sup>flox/flox</sup></i>	Female-to-male sex reversal	Female-to-male sex reversal percentage
E17.5	n=4	n=4	100%
P0	n=8	n=8	100%
P10	n=3	n=3	100%
P30	n=9	n=9	100%

**Rspo1: Figure S1**

**Figure S2. Dihydrotestosterone (DHT) treatment from E10.5-18.5 results in masculinized genital tract of XX embryos and has no significant effect on gonadal gene expression in XX *Rspo1* mutants.**

**(A)** Total body weights of E18.5 XX and XY, Control and *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals are not changed by DHT treatment (blue). **(B)** Anogenital distance of E18.5 XX and XY, Control and *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals treated with DHT (blue) or oil (white). Results are shown as mean + SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test (n = 5-10 embryos for each group). Anogenital distance is significantly increased in XX embryos after treatment with DHT. **(C- F)** Macroscopic views of the genital tract of E18.5 XX and XY, Control and *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals treated with DHT or oil. Epididymis development is induced by DHT treatment in XX controls (D). **(G-N)** Quantitative PCR analysis of mRNA expression in E18.5 XX and XY, Control and *Rspo1*<sup>ΔEx2/ΔEx2</sup> individuals treated with DHT (red) or oil (white). The expressions of *Sox9* (Sertoli cell marker, G) *Amh* (Sertoli cell or pre-matured granulosa cell marker, H), *Foxl2* (granulosa cell marker, I), *Ar* (androgen receptor, K), *Nr2f2* (interstitial cell marker, L) and *Ddx4* (germ cell marker, N) show no significant difference between oil- and DHT-treated XX *Rspo1*<sup>ΔEx2/ΔEx2</sup> gonads. The mitotic arrest marker *P27* (J) is slightly up-regulated. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test (n = 5-10 embryos for each group).



## Supplementary Material

**Supplementary table 1: Genotyping primer list**

Allele genotyping	Primer name	Forward 5' - 3'	Primer name	Reverse 5' - 3'	Amplicon size
<b>Rspo1<sup>WT</sup></b>	Ef	GCTGTGCAGAGTTTCTGTGGCTGG	Er	GCTATCCTGGTCCTGACTAATATTGGC	303bp
<b>Rspo1<sup>Flox</sup></b>	Ef	GCTGTGCAGAGTTTCTGTGGCTGG	Er	GCTATCCTGGTCCTGACTAATATTGGC	519bp
<b>Rspo1<sup>Flox</sup></b>	Ef	GCTGTGCAGAGTTTCTGTGGCTGG	Lxr	ACTGATGGCGAGCTCAGACCATAAC	1304bp
<b>Rspo1<sup>KO</sup></b>	Ef	GCTGTGCAGAGTTTCTGTGGCTGG	Lxr	ACTGATGGCGAGCTCAGACCATAAC	325bp
<b>Wt1<sup>GFP-Cre</sup></b>	WT1GFP_CRE_Fw	CACTACCAGCAGAACACCCCATC	WT1GFP_CRE_Rv	TTGCGAACCTCATCACTCGTTGC	300bp
<b>Wt1<sup>Cre-ERT2</sup></b>	WCE_FW	CCAAGTCCAGCGCCGAGAAT	WCE_RV	TGTCCATCAGGTTCTTGCGA	150bp
<b>Sf1-Cre<sup>tg</sup></b>	Sf1b	GTGTGCACAGACCAGGGCAA	TNAPCre_R	CAAACGGACAGAAGCATTTTCCAG	300bp
<b>mTmG<sup>WT</sup></b>	OIMR_4349	CTCTGCTGCCTCCTGGCTTCT	OIMR_4350	CGAGGCGGATCACAAGCAATA	330bp
<b>mTmG<sup>KI</sup></b>	OIMR_4349	CTCTGCTGCCTCCTGGCTTCT	OIMR_4351	TCAATGGGCGGGGTCGTT	250bp
<b>Sox8</b>	Sox8 15	GTCCTGCGTGGCAACCTTGG	Sox8 16	GCCCACACCATGAAGGCATTC	431bp

WT: wildtype allele; Flox: Floxed allele; KO: deleted allele; tg: transgenic allele; KI: knock-in allele.

Sox8 was used as internal control.

**Supplementary table 2: Antibody list for Immunofluorescence staining and Whole mount immunostaining**

Antibody	Species	Supplier	Reference	Dilution
TRA98	Rat	Abcam	82527	200X
GFP	Chicken	Abcam	ab13970	400X
AMH	Mouse	Bio-Rad Laboratories	MCA2246	30X
FOXL2	Goat	Novus Biologicals	NB100-1277SS	200X
NR2F2	Mouse	R&D Systems	PP-H7147-00	200X
SOX9	Rabbit	Merck KGaA	HPA001758	250X
P27	Rabbit	Santa Cruz Biotechnology	sc-52	200X
AR	Rabbit	Santa Cruz Biotechnology	sc-816	200X
DMRT1	Mouse	Santa Cruz Biotechnology	SC-377167	200X
AMH	Goat	Santa Cruz Biotechnology	sc6886	200X
HSD3B	Goat	Santa Cruz Biotechnology	sc-30820	200X
CYP11A1	Rabbit	Dagmar Wilhelm's Lab		200X
FOXL2	Rabbit	Dagmar Wilhelm's Lab		200X
CYP17A1	Rabbit	Antoine Martinez's Lab		200X
DαChickenCy3	Donkey	Jackson ImmunoResearch	703-165-155	400X
DαChickenCy5	Donkey	Jackson ImmunoResearch	703-175-155	200X
DαGoat488	Donkey	Thermo Fisher	A11055	500X 200X <sup>WM</sup>
DαGoat555	Donkey	Thermo Fisher	A21432	400X 100X <sup>WM</sup>
DαGoat647	Donkey	Thermo Fisher	A21447	200X 50X <sup>WM</sup>
DαMouse488	Donkey	Thermo Fisher	A21202	500X
DαMouse594	Donkey	Thermo Fisher	A21203	400X
DαMouse647	Donkey	Thermo Fisher	A31571	200X
DαRabbit488	Donkey	Thermo Fisher	A21206	500X
DαRabbit555	Donkey	Thermo Fisher	A31572	400X 100X <sup>WM</sup>
DαRabbit647	Donkey	Thermo Fisher	A31573	200X 50X <sup>WM</sup>
DαRat488	Donkey	Thermo Fisher	A21208	400X 200X <sup>WM</sup>

<sup>WM</sup>: Concentration for whole mount immunostaining, which is different from the one for Immunofluorescence staining

**Supplementary table 3: qRT-PCR primer list**

Gene	Forward 5' - 3'	Reverse 5' - 3'	Annealing temperature	Exon amplification	Amplicon size
<i>Axin2</i>	GCAAGTCCAAGCCCCATA	CGGCTGACTCGTTCTCCT	58°C	E7/8	88bp
<i>Foxl2</i>	ATCGGGGTTCTCTCAACAAC	CATCTGGCAGGAGGCGTA	58°C	E1	73bp
<i>Fst</i>	GCCTATGAGGGAAAGTGTATCAA	TGGAATCCCATAGGCATTTT	58°C	E4/5	82bp
<i>Sox9</i>	GCGGAGCTCAGCAAGACTCTG	ATCGGGGTGGTCTTTCTTGTG	58°C	E1/2	117bp
<i>Sox8</i>	GACCCTAGGCAAGCTGTGG	CTGCACACGGAGCCTCTC	58°C	E1/2	82bp
<i>Dhh</i>	GGACCTCGTACCCAACTACAA	CGATGGCTAGAGCGTTCCACC	58°C	E1/2	113bp
<i>Amh</i>	GGGGAGACTGGAGAACAGC	AGAGCTCGGGCTCCATA	58°C	E1/2	67bp
<i>Ar</i>	GGATTCTGTGCAGCCTATTGC	TCAGGAAAGTCCACGCTCAC	58°C	E7/8	90bp
<i>Nr2f2</i>	AGGCCATAGTCCTGTTCCACC	GGCTCCTAACGTA CTCTCCAA	58°C	E2/3	108bp
<i>Ddx4</i>	GACTCGAAGCAGAGGGTTTTTC	TGCTTCTGAATCGTTTCCATC	58°C	E6/7	61bp
<i>P27</i>	GTTAGCGGAGCAGTGTCCA	TCTGTTCTGTTGGCCCTTTT	58°C	E1/2	84bp
<i>Lef1</i>	CCGTCAGATGTCAACTCCAA	GGGTAGAAGGTGGGATTTTC	58°C	E4/5	71bp
<i>Wnt4</i>	GTCTTTGGGAAGGTGGTGAC	TGCCCTTGCTACTGCAAA	58°C	E2/3	93bp
<i>Rspo1</i>	CGACATGAACAAATGCATCA	CCTCCTGACACTTGGTGCAGA	58°C	E3/4	83bp
<i>Rspo1</i>	CAGTGACTATGCGGCTTGG	AGAGCTCACAGCCCTTGG	58°C	E2/3	147bp
<i>Sdha</i>	TGTTCAAGTCCACCCCA	TCTCCACGACACCCTTCTG	58°C	E7/8	66bp
<i>Hsd17b3</i>	AATATGTCACGATCGGAGCTG	GAAGGGATCCGGTTCAGAAT	58°C	E10/11	79bp
<i>Cyp11a1</i>	TGGCCCCATTTACAGGGAGAA	GGCATCTGAACTCTTAAACAGGA	58°C	E1/2	184bp
<i>Cyp17a1</i>	CAGAGAAGTGCTCGTGAAGAAG	AGGAGCTACTACTATCCGCAAA	58°C	E1/2	116bp
<i>Ptch1</i>	AAAGAACTGCGCAAGTTTTTG	CTTCTCTATCTTCTGACGGGT	58°C	E2/3	164bp
<i>Star</i>	TTGGGCATACTCAACAACCA	ACTTCGTCGCCGTTCTCC	58°C	E3/4	65bp

**CHAPTER III: MUTUAL ANTAGONISM BETWEEN SRY/SOX9  
AND RSPO1/WNT4/ $\beta$ -Catenin PATHWAY**

**Mouse gonad development in the absence of the pro-ovarian factor  
WNT4 and the pro-testis factor SOX9**

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## Abstract

Primary sex determination in mammals involves two antagonistic genetic cascades that drive the differentiation of a testis or an ovary from a common undifferentiated bipotential gonad. The male pathway is controlled by the action of the transcription factors SRY and SOX9, whereas female development requires the activation of RSPO1/WNT4/ $\beta$ -Catenin signaling. In this work we took advantage of a double knockout mouse model to study gonadal sex determination when the pro-male factor *Sox9* and pro-female factor *Wnt4* are both mutated. We showed that phenotypic changes in XX *Wnt4* mutants are independent of SOX9 function, since the additional deletion of *Sox9* did not rescue the female-to-male sex reversal caused by *Wnt4* mutation in XX *Wnt4;Sox9* mutants. Furthermore we found that *Wnt4* deletion could not rescue testis development in XY *Wnt4;Sox9* mutants. Instead, XY double mutant gonads experienced a transient female-like phase before developing into ovotestes. Together, our results reveal independent functions for SOX9 and WNT4. SOX9 is required for early determination of the XY supporting cell identity and this function goes beyond the inhibition of RSPO1/WNT4/ $\beta$ -Catenin signaling. In the XX gonad, RSPO1/WNT4/ $\beta$ -Catenin pathway is dispensable for the initial female supporting cell specification but is required to maintain granulosa cell identity right after the specification.

**Key words:** Gonad development, sex determination, Sox9, Wnt signaling, ovotestis

## Introduction

In mammals, primary sex determination is a highly controlled developmental process leading to the formation of an ovary or a testis from a common bipotential gonad present in both sexes at embryonic stages reviewed by (Nef et al., 2019). Sex hormones subsequently secreted by the testis or the ovary, promote the development of secondary sexual characteristics to maintain the sexual identity and fertility in a process known as secondary sex determination (Nef et al., 2019).

The male sex determining gene *Sry* on the Y chromosome is expressed in mouse XY gonads from embryonic day 10.5 to 12.5 (E10.5 to E12.5, (Bullejos and Koopman, 2001; Gubbay et al., 1990; Hacker et al., 1995; Koopman et al., 1991; Sinclair et al., 1990)). SRY activates the expression of another HMG box-containing transcription factor, SOX9, which in turn up-regulates numerous targets involved in establishing the Sertoli cell lineage that will further orchestrate testis development (Gonen et al., 2018; Li et al., 2014b; Rahmoun et al., 2017; Sekido and Lovell-Badge, 2008). XY *Sox9* mutant mice exhibit complete sex reversal and develop ovaries capable of producing fertile X/Y oocytes (Barrionuevo et al., 2005; Chaboissier, 2004; Lavery et al., 2011b).

In the absence of Y chromosome, XX gonadal supporting cells differentiate into FOXL2 positive pre-granulosa cells and enter into mitotic arrest marked by the expression of cyclin-dependent kinase inhibitor CDKN1B/P27 (Gustin et al., 2016; Nef et al., 2005). FOXL2 is required to maintain granulosa cell identity in post-natal ovaries but is dispensable in the ovary during embryonic stages (Ottolenghi et al., 2005; Uhlenhaut et al., 2009) in mice. In contrast, RSPO1/WNT4/ $\beta$ -Catenin signaling is required for embryonic ovarian development in mouse and human (Biason-Lauber et al., 2004; Chassot et al., 2008a; Liu et al., 2009; Mandel et al., 2008; Parma et al., 2006; Tomizuka et al., 2008; Vainio et al., 1999). Mouse XX gonads harboring mutations in *Wnt4*, *Rspo1* or *Ctnnb1* (encoding  $\beta$ -Catenin) progressively develop into ovotestes, with characteristics of testis and ovaries (Chassot et al., 2008a; Maatouk et al., 2013; Tomizuka et al., 2008; Vainio et al., 1999). Pre-granulosa cells in the

masculinized part of the mutant gonads exit mitotic arrest and prematurely differentiate (Maatouk et al., 2013; Nicol and Yao, 2015). Subsequently, pre-matured granulosa cells lose FOXL2 expression and trans-differentiate into SOX9/AMH double positive Sertoli-like cells, forming testis cord-like structures around birth (Chassot et al., 2008a; Maatouk et al., 2013; Nicol and Yao, 2015) that will become seminiferous tubules in the adult stages (Chassot et al., 2008a). Partial sex reversal in RSPO1/WNT4/ $\beta$ -Catenin deficient XX gonads is characterized by additional phenotypes during embryonic development. *Wnt4*, *Rspo1* and *Ctnnb1* XX mutant gonads exhibit a male-like vasculature with the formation of a celomic vessel at E12.5 by ectopic migration of endothelial cells from the adjacent mesonephros (Chassot et al., 2008a; Jeays-Ward, 2003; Liu et al., 2009; Tomizuka et al., 2008). In addition, *Wnt4*, *Rspo1* and *Ctnnb1* XX mutant gonads contain ectopic steroidogenic cells that produce testosterone and masculinize the XX genital tracts (Chassot et al., 2008a; Heikkilä et al., 2002, 2005; Liu et al., 2009, 2010; Tomizuka et al., 2008; Vainio et al., 1999). Finally, germ cells are lost through apoptosis from E16.5 in *Wnt4* and *Ctnnb1* XX mutants (Liu et al., 2010; Tomizuka et al., 2008; Vainio et al., 1999; Yao et al., 2004) or reduced proliferation from E12.5 in *Rspo1* XX mutants (Chassot et al., 2011).

Single cell RNA-seq analyses of developing gonads have identified an initial supporting cell precursor with similar transcriptional profiles in XX and XY mouse embryos (Stévant et al., 2018a). Differentiation of testicular Sertoli cells and ovarian granulosa cells from this bipotential supporting cell requires the activation of the relevant male or female pathway while repressing the alternate genetic cascade. Ectopic activation of WNT/ $\beta$ -Catenin signaling or FOXL2 in XY gonads is sufficient to induce ovarian development (Garcia-Moreno et al., 2019a; Harris et al., 2018; Maatouk et al., 2008; Nicol et al., 2018). Conversely, XX supporting cells are able to respond to the ectopic expression of SRY by activating SOX9, and both SRY and SOX9 can drive testicular development in XX embryos (Gregoire et al., 2011; Hiramatsu et al., 2009b; Vidal et al., 2001).

The antagonistic relationship between the male and female pathways in gonadal specification and differentiation has been addressed by the study of double mutants. Fibroblast growth factor 9 (FGF9), is a SOX9 target, which binds FGFR2c and in turn activates *Sox9* expression to promote the quick expansion of Sertoli cell differentiation across the developing testis (Kim et al., 2006b). Mutations in *Fgf9* or *Fgfr2/Fgfr2c* lead to reduced SOX9 expression and partial male-to-female sex reversal (Bagheri-Fam et al., 2008, 2017; Colvin et al., 2001; Kim et al., 2006b, 2007). In XY *Fgf9;Wnt4*, *Fgfr2;Wnt4* and *Fgfr2c;Foxl2* double mutants, SOX9 expression and testicular development are rescued indicating that FGF9 also functions to antagonize WNT4- and FOXL2-mediated repression of *Sox9* (Bagheri-Fam et al., 2017; Jameson et al., 2012a). The outcome of mutating *Sox9* together with the female pathway components *Rspo1* or *Cttnb1* has also been studied (Lavery et al., 2012; Nicol and Yao, 2015). The gonads of both XX *Sox9;Rspo1* and XX *Sox9;Cttnb1* double mutants develop as ovotestes, demonstrating that other factors besides SRY and SOX9 can drive Sertoli-like cell trans-differentiation in *Rspo1* and *Cttnb1* mutants (Lavery et al., 2012; Nicol and Yao, 2015). In contrast, conflicting phenotypes have been reported for XY gonads in these two sets of double mutants. XY *Sox9;Rspo1* double mutant gonads were described as hypo-plastic testes after birth, indicating that RSPO1 can rescue testicular development in *Sox9* mutants (Lavery et al., 2012). However, XY *Sox9;Cttnb1* double mutant gonads develop as ovotestes, suggesting that complete testicular development cannot occur without SOX9 even in the absence of WNT/ $\beta$ -Catenin signaling (Nicol and Yao, 2015).

In this study, we report the generation of double mutants for *Sox9* and *Wnt4*, another component of the ovarian WNT/ $\beta$ -Catenin pathway. In order to analyze the dynamics of the antagonistic or independent actions of *Sox9* and *Wnt4*, we have compared the phenotype of XX and XY, single and double mutants at different time points of gonadal development. We show here that, similar to the situation in XX *Sox9;Rspo1* and *Sox9;Cttnb1* mutants, SOX9 function is not required for the masculinization of XX *Wnt4* mutant gonads. In addition, we demonstrate that XY

*Sox9;Wnt4* double-mutated pathological gonads develop as ovotestes in a process involving two sequential sex-reversal events. Loss of *Wnt4* is not sufficient to rescue the initial granulosa cell specification in XY *Sox9* mutant gonads. However, *Wnt4* is required to maintain granulosa cell identity right after, and prevent XY granulosa cells trans-differentiate into Sertoli-like cells.

## Results

### Generation of *Wnt4*;*Sox9* double mutants

SOX9 is transiently up-regulated in XX *Wnt4* mutants between E11.5 and E12.0 (Kim et al., 2006b). Conversely, *Wnt4* expression is up-regulated in XY *Sox9* mutants at E13.5 (Lavery et al., 2011b). In order to investigate the contribution of SOX9 function to the XX *Wnt4* mutant phenotype and of WNT4 function to the *Sox9* mutant phenotype, we generated *Wnt4*;*Sox9* double mutant animals. We made use of a constitutive *Wnt4* mutant line (*Wnt4*<sup>KO</sup>, Vainio et al., 1999), and a conditional *Sox9* mutant line, where *Sox9* deletion occurs in cells expressing the *Sf1-Cre* transgene (*Sox9*<sup>CKO</sup>, Bingham et al., 2006; Lavery et al., 2011b). Previous work has established the efficiency of *Sf1-Cre* for inactivation of *Sox9* in the somatic gonad (Lavery et al., 2011b). Quantitative PCR analysis at E12.5 confirmed that *Sox9* was also efficiently deleted in the gonad of single and double mutant embryos generated for this study (Fig. 2D; Fig. 4C).

### Early phenotypic changes in XX *Wnt4* mutants are independent of SOX9 function

The phenotype of single and double mutants was first examined at E12.5 (the first stage where morphological differences are obvious between XX and XY gonads) and E14.5 (a stage where testis and ovary cellular organization are clearly distinct).

We started our analysis on XX embryos. At E12.5 and E14.5, *Sox9* single mutant XX gonads were indistinguishable from control ovaries (Figs. 1B, 1F, 1J and 3B, 3F, 3J, Lavery et al., 2011b). In XX *Wnt4* single mutants, WNT/ $\beta$ -Catenin pathway activity was already significantly decreased at E12.5 and E14.5 as shown by the reduced expression of the WNT/ $\beta$ -Catenin pathway direct target *Axin2* (Figs. 2A and 4A). At both stages, the morphology of *Wnt4* single mutant XX gonads was similar to the one of control ovaries but some phenotypic features of the *Wnt4* mutation were already apparent. XX *Wnt4* mutant gonads harbored a celomic vessel (Fig. 1G, 1K), a characteristic feature of testis vasculature (Fig. 1A', Jeays-Ward, 2003). The

development of ectopic vasculature in *Wnt4* mutant XX gonads is caused by migration of endothelial cells from the mesonephros, an event normally occurring in control testis and repressed by the action of Follistatin (*Fst*) in control ovaries (Jeays-Ward, 2003; Yao et al., 2004). Accordingly, we found that *Fst* expression was severely down-regulated in *Wnt4* XX mutant gonads at E12.5 (Fig. 2B). Another characteristic phenotype in *Wnt4* mutants is the presence of ectopic steroidogenic cells as revealed by the presence of HSD3 $\beta$  positive cells (Figs. 1K and 3K, (Vainio et al., 1999). XX *Wnt4* mutants expressed FOXL2, indicating the presence of supporting granulosa cells (Figs. 1C and 3C), however *Foxl2* expression levels were reduced when compared to control ovaries (Figs. 2C and 4B). This observation is consistent with *Foxl2* expression being regulated in part by WNT/ $\beta$ -Catenin signaling (Auguste et al., 2012; Nicol and Yao, 2015; Ottolenghi et al., 2007). We did not find any up-regulation of the male supporting cell markers *Sox9*, *Sox8* and *Fgf9* in XX *Wnt4* when compared to control XX gonads at E12.5 and E14.5 (Figs. 2D-F and 4C-D). Indeed, male supporting cells differentiate in XX *Wnt4* mutant gonads only around E16.5 (Maatouk et al., 2013). However, we could detect an increased expression of two genes encoding proteins normally secreted by Sertoli cells, *Dhh* and *Amh* (Figs. 2G-H and 4E-F). This observation suggests that the expression of *Dhh* and *Amh* might be negatively regulated by WNT/ $\beta$ -Catenin signaling pathway in the developing ovary. Nevertheless, AMH protein could not be detected by immunofluorescence at E12.5 or E14.5 (Figs. 1G and 3G) in XX *Wnt4* mutants, possibly owing to the lower sensitivity of the immunofluorescence technique when compared to quantitative PCR.

After an initial proliferative phase, XX germ cells in developing ovaries initiate meiosis between E13.5 and E14.5, whereas XY germ cells in developing testes are arrested in the G0/G1 phase of the cell cycle until spermatogenesis begins after birth. WNT4 regulates XX germ-line development, by controlling meiosis initiation from E14.5 (Naillat et al., 2010) and germ cell survival from E16.5 (Vainio et al., 1999; Yao et al., 2004). We found that XX *Wnt4* mutants exhibit a decreased expression of the

meiosis initiation marker *Stra8* (Baltus et al., 2006) and up-regulation of the male gonocyte marker *Nanos2* (Suzuki and Saga, 2008) (Fig. 5 I-J).

In XX *Wnt4;Sox9* double mutant gonads, *Axin2* expression was down-regulated similar to what was observed in *Wnt4* single mutants (Figs. 2A and 4A). The XX double mutant gonads had a very similar phenotype to XX *Wnt4* single mutants: a celomic vessel was present at E12.5 (Fig. 1 and data not shown) and *Fst* expression was reduced (Fig. 2B), ectopic steroidogenic cells were observed from E12.5 (Figs. 1L and 3L), supporting cells expressed *Foxl2* at reduced levels when compared to control ovaries (Figs. 1D, 2C, 3D and 4B), the expression of *Dhh* and *Amh* was up-regulated in a similar way to XX *Wnt4* single mutants (Figs. 2G-H and 4E-F), and the meiosis initiation marker *Stra8* was down-regulated (Fig. 5I). The only difference detected between XX single and double mutants was a transient up-regulation of *Sox8* at E12.5 and a specific down-regulation of *Fgf9* in the *Wnt4;Sox9* double mutant gonads when compared to XX controls and XX *Wnt4* mutants (Fig. 2E,F).

Together our observations demonstrate that SOX9 function is not required for the appearance of the first phenotypic changes in *Wnt4* XX mutants and suggest that the transient expression of *Sox9* in *Wnt4* mutants is not indispensable for the establishment and progression of the defects associated to WNT4 loss-of-function.

### **WNT4 loss-of-function partially restores testicular development in XY *Sox9* mutants**

We next analyzed XY single and double mutants at E12.5 and E14.5. XY *Wnt4* mutants exhibit an initial delay in Sertoli cell differentiation leading to transient defects in testis cords organization and size that are restored near birth (Chassot et al., 2012; Jeays-Ward et al., 2004; Vainio et al., 1999). Accordingly, we observed that the supporting cell markers *Sox8*, *Dhh* and *Amh* were down-regulated in XY *Wnt4* mutants at E12.5 but reached levels close to those of XY controls at E14.5 (Figs. 2E, 2G, 2H and 4D-F).

Conditional deletion of *Sox9* in the XY somatic gonad leads to complete male-to-female sex reversal (Chaboissier, 2004; Lavery et al., 2011b). XY *Sox9* mutants had the morphological appearance of ovaries, with no signs of testis cords formation and absence of celomic vessel and steroidogenic cells at E12.5 and E14.5 (Figs. 1B', 1F', 1J', 3B', 3F', 3J' and data not shown). XY *Sox9* mutants expressed *Foxl2*/FOXL2, indicating that supporting cells had acquired a granulosa identity (Figs. 1B', 2C, 3B' and 4B). Q-PCR analysis revealed that in addition to *Foxl2*, the Wnt/ $\beta$ -Catenin pathway target genes *Axin2* and *Fst* were also up-regulated in XY *Sox9* mutants (Figs. 2A-B and 4A). In contrast, the expression of male supporting markers such as *Sox8*, *Fgf9*, *Dhh* and *Amh*/AMH was down-regulated compared to control testes (Figs. 1F', 2E-H, 3F', 4D-F,).

In XY gonads, SRY is expressed from E10.5 in pre-Sertoli cells, but is then quickly down-regulated by E12.5 (Fig. 1E', (Bullejos and Koopman, 2001; Wilhelm et al., 2005). SRY was still expressed in XY *Sox9* mutants at E12.5 but was down-regulated by E14.5 (Figs. 1F' and 3B'). This observation suggests that SOX9 might be required for *Sry* down-regulation in the XY developing gonad (Chaboissier, 2004).

Previous work has indicated that unlike the situation in control XY gonads, XY germ cells undergo meiosis in the fetal XY *Sox9* mutant gonads (Chaboissier, 2004; Lavery et al., 2011b). At E14.5, control female germ cells had down-regulated the pluripotency marker OCT3/4 and expressed the meiotic marker SYCP3 (Fig. 5A, 5E). In control testes, OCT3/4 expression was maintained in male germ cells arrested in G0/G1 phase of the cell cycle (Fig. 5B). In E14.5 XY *Sox9* mutant gonads, germ cells had lost OCT3/4 expression and had up-regulated SYCP3 expression (Fig. 5C, 5G). In addition, the male gonocyte marker *Nanos2* was down-regulated whereas the meiosis initiation marker *Stra8* was expressed at levels comparable to those observed

in control ovaries (Fig. 5I, 5J). These results confirm that XY germ cells initiate meiosis in XY *Sox9* fetal gonads, similar to XX germ cells in control fetal ovaries.

XY *Wnt4;Sox9* double mutants had the morphology of an ovary without any signs of testis cord formation at E12.5 and E14.5 (Figs. 1D', 1H', 1L' and 3D', 3H', 3L'), indicating that *Wnt4* deletion cannot rescue testicular development in XY *Sox9* mutants. However, unlike XY *Sox9* mutants, XY *Wnt4;Sox9* double exhibited a celomic vessel and steroidogenic cells (Figs. 1L' and 3L' and data not shown). Celomic vessel formation in XY double mutants could be related to the complete absence of *Fst* expression (Fig. 2B, (Yao et al., 2004). The expression of *Axin2*, another target of the Wnt/ $\beta$ -Catenin pathway was also reduced to levels comparable to those of the control XY gonads at E12.5 and E14.5 in the double mutants (Figs. 2A and 4A). These observations indicate that at least two male specific processes, celomic vessel formation and steroidogenic cells differentiation can be rescued by loss of WNT4 function and down-regulation of WNT/ $\beta$ -catenin pathway activity in XY double mutants compared to *Sox9* single mutants. We conclude that abnormal activation of the WNT/ $\beta$ -catenin pathway is responsible for the absence of celomic vessel formation and steroidogenic cell differentiation in XY *Sox9* mutants.

Despite the presence of some male characteristics, XY *Wnt4;Sox9* double mutants expressed FOXL2, indicating that granulosa cell identity was specified in these gonads (Figs. 1D' and 3D'). However, the levels of *Foxl2* transcripts were reduced when compared to XY *Sox9* mutants (Figs. 2C and 4B). In addition, XY *Wnt4;Sox9* double mutants showed increased expression of the male supporting markers *Sox8*, *Fgf9*, *Dhh* and *Amh*, when compared to XY *Sox9* single mutants (Figs. 2E-H and 4D-F). In particular, AMH protein could be detected from E12.5 in XY double mutants (Figs. 1 H' and 3H'). Similar to XY *Sox9* single mutants, SRY expression was not down-regulated in XY *Wnt4;Sox9* double mutants at E12.5 (Fig. 1H'). SRY expression was even maintained at E14.5, where it was co-expressed with

FOXL2 (Fig. 3D') further demonstrating that XY supporting cells have acquired a granulosa cell fate in XY double mutants.

Unlike *Sox9* single mutants, germ cells in XY *Wnt4;Sox9* mutants maintained OCT3/4 expression (Fig. 5D) and did not show up-regulation of SYCP3 (Fig. 5H), thus resembling male germ cells. Nevertheless, we found that *Stra8* expression was up-regulated, albeit at lower levels when compared to *Sox9* single mutants (Fig. 5I). This observation suggests that a fraction of germ cells in XY double-mutants might initiate meiosis around E14.5. Thus, *Wnt4* loss-of-function in XY double mutants only partially rescues male germ cell development when compared to XY *Sox9* single mutants.

Together these results demonstrate that similar to XY *Sox9* mutant, XY *Wnt4;Sox9* mutant gonads display female characteristics such as the presence of a granulosa cell population revealed by FOXL2 expression, and up-regulation of *Stra8* expression. However, loss of *Wnt4* function in the double mutants rescues specific aspects of the XY *Sox9* mutant male-to-female sex reversal. Celomic vessel formation and steroid cell differentiation are completely rescued, whereas male supporting gene expression and germ cell differentiation are only partially rescued. We conclude that up-regulation of *Wnt4* is only partially responsible for the appearance of ovarian characteristics in XY *Sox9* mutant gonads.

### **XX *Wnt4;Sox9* double mutant gonads develop as ovotestes**

We next examined XX single and double mutant gonads near the end of fetal development, at E17.5. XX *Wnt4* gonads are masculinized (Vainio et al., 1999) and develop as ovotestes with a posterior part containing ovarian structures and an anterior part displaying testicular features (Maatouk et al., 2013). One characteristic of XX *Wnt4* mutants is the premature differentiation of pre-granulosa cells in the anterior part of the gonad. It has been suggested that precocious exit from mitotic arrest and the onset of differentiation of granulosa cells precedes their trans-differentiation into

Sertoli-like cells (Maatouk et al., 2013). We observed the presence of cells co-expressing FOXL2 and AMH (Fig. 6 A-C) and FOXL2 and Androgen Receptor (AR, Fig. 6D-F) in the anterior part of XX *Wnt4* mutant gonads and XX double mutant gonads. AMH and AR are only expressed in granulosa cells during folliculogenesis in the control post-natal ovary (Durlinger et al., 2002; Yang et al., 2015). In addition, the quiescent cells marker P27 (encoded by *Cdkn1b*, (Maatouk et al., 2013) was down-regulated in the anterior region of XX *Wnt4* and *Wnt4;Sox9* mutant gonads (Fig. 6. G-I). These observations indicate that granulosa cells in the anterior region of both mutants have exited mitotic arrest and have started to differentiate. We conclude that SOX9 function is dispensable for premature granulosa cell differentiation in XX *Wnt4* mutants.

In order to assess the trans-differentiation of granulosa into Sertoli-like cells, we analyzed the expression of the Sertoli marker DMRT1 (Lei et al., 2007b). Surprisingly, we could not detect any DMRT1 signal in XX *Wnt4* single mutant at E17.5 or P0 (data not shown), indicating that granulosa to Sertoli-like cell trans-differentiation did not occur efficiently in the XX *Wnt4* mutant gonads generated for this study. DMRT1 expression was also absent in XX *Wnt4;Sox9* mutant gonads, and therefore we could not conclude on the role of SOX9 in Sertoli-like cell formation in XX *Wnt4* mutants.

Another characteristic phenotype of the XX *Wnt4* mutant gonad is the strong reduction of germ cell numbers in the anterior region, following apoptosis (Yao et al., 2004). Accordingly, a reduction in the germ cells marked by the TRA98 antigen was observed both in XX *Wnt4* single and *Wnt4;Sox9* double mutants (Fig. 6H, 6I). Finally, the testicular region of the XX *Wnt4* mutant ovotestis contained abundant interstitial cells expressing AR and NR2F2 (Fig. 6E, 6K), as well as ectopic steroidogenic cells marked by the expression of HSD3 $\beta$  (Fig. 6K). This phenotype was identical in the XX *Wnt4;Sox9* double mutants (Fig. 6F, 6L). Together our results demonstrate that ovotestis formation in XX *Wnt4* mutant gonads occurs in absence of SOX9 function.

### **XY *Wnt4*;*Sox9* double mutant gonads develop as ovotestes**

At E17.5 XY *Sox9* mutant gonads had all the characteristics of an ovary: FOXL2 and P27 were expressed in the quiescent pre-granulosa cells across the entire gonad (Fig. 7B, 7E, 7H), meiotic germ cells were found in the cortical region of the ovary surrounded by pre-granulosa cells (Fig. 7H, 7N), and steroidogenic cells were absent (Fig. 7K).

By contrast, two distinct regions could be clearly identified in the XY *Wnt4*;*Sox9* double mutant gonads that resembled ovotestes. The “ovary-like” region exhibited abundant FOXL2 expressing cells (Fig. 7C, 7F) and less NR2F2 expressing interstitial cells (Fig. 7L). In this region, germ cells expressed the meiotic marker  $\gamma$ H2AX (Fig. 7O) and were surrounded by quiescent granulosa cells expressing P27 (Fig. 7I). This observation demonstrates that XY *Wnt4*;*Sox9* germ cells initiate meiosis in the ovarian region of the double mutant ovotestis. In contrast, in the masculinized region of XY double mutant gonads, cells co-expressing FOXL2 and AMH or FOXL2 and AR (Fig. 7C, 7F) were organized in tubular structures surrounded by abundant interstitial cells expressing NR2F2 and AR and by steroidogenic cells (Fig. 7F, 7L). P27 expression was down-regulated (Fig. 7I) and germ cells were negative for the meiotic marker  $\gamma$ H2AX (Fig. 7O). These observations suggest that, in the masculinized region of XY *Wnt4*;*Sox9* ovotestes, FOXL2 expressing granulosa cells initially formed by the process of male-to-female sex reversal caused by *Sox9* mutation, undergo premature differentiation upon *Wnt4* deletion. We next investigated Sertoli cell formation in XY double mutants. DMRT1 was expressed in germ cells present inside the testis cords and co-expressed with AMH in Sertoli cells of control testes at P0 (Fig. 7P, (Lei et al., 2007b)). XY *Sox9* mutant gonads were devoid of DMRT1 and AMH expression, but expressed FOXL2 (Fig. 7Q). In XY double mutants, cells co-expressing DMRT1 and AMH were observed, indicating that Sertoli-like cell differentiation had occurred (Fig. 7R). The fact that some tubular structures contained FOXL2 positive cells adjacent to cells co-expressing AMH and DMRT1 strongly suggests that Sertoli-like cells transdifferentiate from granulosa cells in XY double mutants (Fig. 7R).

Together our results strongly indicate that XY double mutant gonads develop through a sequence of two phenotypic sex-reversal processes. Initially *Sox9* deletion causes a male-to-female sex reversal revealed by the presence of female supporting cells expressing FOXL2 in XY double mutant gonads. However, these gonads also exhibit a celomic vessel and steroidogenic cells, two male structures normally inhibited by WNT4 function. As development proceeds, a sub-population of granulosa cells in the sex-reversed double mutant gonad prematurely differentiate and eventually trans-differentiate into Sertoli-like cells, thus transitioning from a female to a male fate as they do in XX *Wnt4* mutant gonads.

## Discussion

We have shown that deletion of the pro-testis factor *Sox9* and of the pro-ovarian factor *Wnt4* in the mouse gonad leads to formation of ovotestes both in XX and XY individuals.

### **SOX9 function is not required for ovotestes development in *Wnt4* mutants**

In absence of *Wnt4*, SOX9 is transiently up-regulated at E11.5, raising the question of its role during the initial steps of female to male sex reversal in XX *Wnt4* mutants (Kim et al., 2006b). We show here that XX *Sox9;Wnt4* mutants ovotestes are similar to those formed in XX *Wnt4* single mutants, indicating that SOX9 function is not required for the different aspects of partial sex reversal observed in the absence of WNT4 function. In particular, ectopic celomic vessel formation, ectopic steroidogenic cell differentiation and pre-mature granulosa cell differentiation are SOX9 independent processes. This finding is in agreement with previous reports describing ovotestes development in *Sox9;Rspo1* and *Sox9;Ctnnb1* double mutants (Lavery et al., 2012; Nicol and Yao, 2015). We did not detect DMRT1 expression in XX *Wnt4* or XX *Sox9;Wnt4* mutants, and therefore we could not conclude on the presence of trans-differentiated Sertoli-like cells in the XX single and double mutants. It has been previously noted that Sertoli-like cells expressing SOX9 but not DMRT1 appear only sporadically in newborn XX *Wnt4* mutants, and *Wnt4* gonads cannot be analyzed at later stages owing to the perinatal lethality caused by *Wnt4* deficiency in the kidney (Maatouk et al., 2013; Stark et al., 1994). The less efficient granulosa to Sertoli-like cell trans-differentiation in XX *Wnt4* mutant gonads compared to *Rspo1* or *Ctnnb1* mutants might be explained by the compensatory action of other WNT proteins (Chassot et al., 2008b; Maatouk et al., 2013; Nicol and Yao, 2015). Alternatively, WNT4 function might be specifically involved in some aspects of Sertoli cell differentiation (Jeays-Ward et al., 2004).

## **WNT4 function is not required for initial granulosa cell specification in Sox9-mutated pathological gonads**

XY *Sox9;Wnt4* mutant gonads also develop as ovotestes demonstrating that *Wnt4* deletion cannot completely rescue testicular development in XY *Sox9* mutants. We have shown that XY double mutant gonads express FOXL2 from E12.5, although at reduced levels compared to XY *Sox9* single mutants. Moreover, we have observed that FOXL2 is up-regulated in SRY expressing cells. Our results demonstrate that in the absence of SOX9, and despite the presence of SRY and reduced  $\beta$ -Catenin signaling, bipotential supporting cell precursors initially adopt a female pre-granulosa fate, possibly through the action of FOXL2. Activation of  $\beta$ -Catenin signaling or FOXL2 in XY gonads can trigger ovarian development, indicating that both pathways can drive granulosa cell differentiation (Maatouk et al., 2008; Nicol et al., 2018). Although, pre-granulosa cell specification occurs in XX *Rspo1/Wnt4/Ctnnb1* and XX *Foxl2* single mutants (Chassot et al., 2008a; Liu et al., 2009; Maatouk et al., 2013; Nicol and Yao, 2015; Tomizuka et al., 2008; Vainio et al., 1999), *Rspo1;Foxl2* and *Wnt4;Foxl2* double mutants exhibit enhanced Sertoli-like cell formation (Auguste et al., 2012; Ottolenghi et al., 2007), indicating that RSPO1/WNT/ $\beta$ -Catenin and FOXL2 can compensate for each other but also co-operate in granulosa cell specification and maintenance.

We show here that FOXL2 is expressed in XY *Sox9;Wnt4* demonstrating that *Wnt4* up-regulation is not the only event involved in *Foxl2* induction in XY *Sox9* mutants. Similar results were obtained in *Sox9;Ctnnb1* mutants, indicating that *Foxl2* induction is independent from  $\beta$ -Catenin signaling activation in XY *Sox9* mutants. One possibility is that SOX9 is involved in the repression of *Foxl2* expression in differentiating XY supporting cells either directly, or through the action of its target FGF9 (Gustin et al., 2016; Wilhelm et al., 2009). Interestingly, CHIP-seq experiments have identified FOXL2 binding peaks in a region containing an enhancer involved in *Sox9* repression in granulosa cells (Nicol et al., 2018). Thus FOXL2 and SOX9 might

repress each other expression to ensure the appropriate differentiation of bipotential supporting precursors along the male or female pathway.

### **SRY regulation in XY *Sox9;Wnt4* double mutants**

We have observed that SRY expression is maintained in E12.5 XY *Sox9* and *Sox9;Wnt4* mutants, indicating that without the downstream activation of SOX9, SRY cannot antagonize granulosa cell formation to initiate Sertoli cell differentiation. SRY expression is down-regulated in control testes around E12.5 (Bullejos and Koopman, 2001; Wilhelm et al., 2005). We found that SRY was widely detected in XY *Sox9* mutants at E12.5 and was further maintained in E14.5 *Sox9;Wnt4* mutants. It has been previously suggested that SOX9 might be involved in *Sry* down-regulation in the XY developing gonad (Chaboissier, 2004). However, additional factors are at play as demonstrated by the correct down-regulation of SRY expression at the poles of B6-XY<sup>POS</sup> ovotestes devoid of SOX9 expression (Wilhelm et al., 2009). Interestingly, SRY expression is rescued in XY *Cbx2;Wnt4* mutant gonads (Garcia-Moreno et al., 2019b), indicating that WNT4 function, possibly through the activation of  $\beta$ -catenin signaling, might contribute to the down-regulation of SRY in differentiating supporting cells.

### **Vasculature formation and steroidogenic cell differentiation are WNT-dependent processes that do not require SOX9 function**

Despite the initial specification of pre-granulosa cells, XY *Sox9;Wnt4* double mutant gonads exhibit some male specific traits that are not present in XY *Sox9* mutants. In particular, deletion of *Wnt4* rescues the male vasculature in XY *Sox9* mutants. Celomic vessel forms in developing testes through the migration of endothelial cells of mesonephric origin (Jeays-Ward, 2003). This process is stimulated by Activin B, whose subunit *Inhbb* is expressed in Sertoli and granulosa cells (Yao et al., 2006). In XX gonads, RSPO1/WNT/ $\beta$ -Catenin and FOXL2 promote the expression of the Activin antagonist FST, thus inhibiting endothelial cell migration (Chassot et al., 2008a; Kashimada et al., 2011; Liu et al., 2009, 2010; Nicol et al.,

2018; Tomizuka et al., 2008; Yao et al., 2004). Reduced *Fst* expression in the absence of *Wnt4* in XY *Sox9;Wnt4* double mutants is the most likely cause of the celomic vessel rescue in these gonads.

In addition, XY *Sox9;Wnt4* double mutant gonads exhibit steroidogenic cell differentiation, similar to the XY control gonads. Deletion of *Rspo1*, *Wnt4* or *Ctnnb1* induces ectopic steroidogenic cell formation in XX gonads before the overt differentiation of Sertoli-like cells (Chassot et al., 2008a; Liu et al., 2009; Tomizuka et al., 2008; Vainio et al., 1999). Hedgehog signaling, and in particular Desert Hedgehog, is required for Fetal Leydig cell differentiation from undifferentiated interstitial precursors in developing testes (Yao et al., 2002). *Dhh* expression is rescued in XY *Sox9;Wnt4* double mutants, suggesting that Hedgehog signaling might be involved in steroidogenic cell formation in these gonads.

Altogether our observations indicate that at least two male specific processes, celomic vasculature and fetal steroidogenic cell formation, do not depend on SOX9 itself but require the inhibition of the ovarian WNT signaling.

### **Premature granulosa cell differentiation and Sertoli-like trans-differentiation in XY *Sox9;Wnt4* double mutants**

We have shown that at E17.5 a fraction of the pre-granulosa cells in XY *Sox9;Wnt4* double mutants have exited mitotic arrest and have started to express AMH and AR, suggesting that they have prematurely differentiated. Therefore, maintenance of pre-granulosa cell identity in XY *Sox9* mutant ovaries requires WNT4 function. Premature granulosa cell differentiation is a common feature of XX gonads harboring mutations in *Rspo1*, *Wnt4* or *Ctnnb1* (Maatouk et al., 2013; Nicol and Yao, 2015). This process is independent of *Sox9* function as shown here in XX *Sox9;Wnt4* double mutants, but also in *Sox9;Rspo1* and *Sox9;Ctnnb1* double mutant XX gonads (Lavery et al., 2012; Nicol and Yao, 2015). It is currently unknown whether premature granulosa cell differentiation in absence of *Rspo1/Wnt4/Ctnnb1* is a cell autonomous

process triggered by the decrease of  $\beta$ -Catenin signaling in pre-granulosa cells or is linked to other phenotypic features such as the presence of ectopic steroidogenic cells.

We have shown that Sertoli-like cells marked by the expression of DMRT1 and AMH are formed in newborn XY *Sox9;Wnt4* mutant gonads. These cells can be found in tubular structures that also contain FOXL2 positive cells, suggesting that Sertoli-like cell differentiation in XY *Sox9;Wnt4* mutants is the result of a trans-differentiation process from prematurely differentiated granulosa cells. Thus in XY *Sox9;Wnt4* mutants, supporting cells go through a sequence of two sex reversal processes: first they differentiate as XY pre-granulosa cells in absence of SOX9, then they trans-differentiate into Sertoli-like cells owing to the lack of WNT4 function. We did not observe Sertoli-like cells expressing DMRT1 in XX double mutants, indicating that the Y chromosome, and possibly the target genes of SRY (Nicol and Yao, 2015), favor Sertoli-like cell development in XY *Sox9;Wnt4* ovotestes.

### **Antagonism of male and female pathways in gonadal development**

SOX9 function is required for the initial choice that drives Sertoli cell specification from a bipotential supporting cell precursor. This function cannot be rescued by mutations in *Wnt4* or *Ctnnb1* (this work and (Nicol and Yao, 2015)). It was previously reported that testicular development is rescued in XY *Sox9;Rspo1* double mutants (Lavery et al., 2012). These gonads develop as hypoplastic testes at post-natal stages. However, the embryonic XY *Sox9;Rspo1* double mutant gonads have an ovarian morphology and express the meiosis initiation marker *Stra8* at E14.5. Moreover, the authors noted some rare FOXL2 expressing follicles at P21. These observations are compatible with the interpretation that similar to XY *Sox9;Wnt4* and *Sox9,Ctnnb1* mutants, XY *Sox9;Rspo1* double mutant gonads develop as ovotestes, with an ovarian part that fails to be maintained at late post-natal stages.

Together these studies suggest that SOX9 and WNT/ $\beta$ -Catenin signaling act at different levels in the mutual antagonistic network that drives normal supporting cell differentiation and gonadal development. SOX9 function is essential for the initial decision in the supporting cell lineage to drive Sertoli cell differentiation. WNT/ $\beta$ -Catenin co-operates with FOXL2 for the initial granulosa cell specification, inhibits male vascular and steroidogenic development and is essential to maintain pre-granulosa cells in an undifferentiated quiescent state during embryonic ovarian development.

## **Materials and Methods**

### **Mouse strains and genotyping**

The experiments described herein were carried out in compliance with the relevant institutional and European animal welfare laws, guidelines and policies. All the experiments were approved by the French Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche. All mouse lines were kept on a mixed 129/C57Bl6/J background. *Sf1:Cre<sup>tg/0</sup>*; *Sox9<sup>flox/flox</sup>*; *Wnt4<sup>+/+</sup>* females were crossed with *Sf1:Cre<sup>tg/0</sup>*; *Sox9<sup>flox/+</sup>*; *Wnt4<sup>+/-</sup>* males to obtain mutant embryos at different stages. *Sf1-Cre<sup>tg</sup>*, *Sox9<sup>flox</sup>* and *Wnt4* knockout mouse lines were genotyped as previously described (Bingham et al., 2006; Chaboissier, 2004; Lavery et al., 2011b; Vainio et al., 1999). The day when a vaginal plug was found was designated as embryonic day E0.5. The genotype of mice and embryos were determined using genotyping PCR assays on lysates from ear biopsies or tail tips with genotyping primers shown in supplementary table 1. E10.5-E12.5 embryos were staged by counting the number of tail somites (ts) with 8 ts corresponding to E10.5, 18 ts to E11.5 and 30 ts to E12.5 (Hacker et al., 1995).

### **Histological analysis**

Gonads were dissected in Phosphate Buffered Saline (PBS), fixed in Bouin's solution (HT10132, Sigma-Aldrich) then dehydrated and embedded in paraffin with a Leica TP1020 tissue processor. Five-micrometer-thick sections were processed for Hematoxylin and Eosin staining using standard procedures. Images were taken on a MZ9.5 microscope (Leica) with a DFC490 camera (Leica) driven by the Application suite software (Leica), and treated with Image Composite Editor and Adobe Photoshop.

### **Immunofluorescence staining**

Five-micrometer-thick sections of samples fixed in Bouin's solution (HT10132, Sigma-Aldrich), Antigen fix (P10016, Diapath) or 4% PFA (15710-S, EMS) were processed for immunofluorescence staining. Paraffin was removed in Xylene baths (2

times, 10min each). Sections were rehydrated in 100%, 90%, 70%, 40% Ethanol (EtOH) and H<sub>2</sub>O baths orderly, 5min each. Antigen retrieval procedure was obtained by pressure cooking in 10 mM Sodium Citrate pH6, 0.05% Tween-20 for 10min. Sections were blocked with 3% Bovine serum Albumin (BSA, A2153 Sigma-Aldrich) and 10% inactivated Normal Donkey Serum (NDS) in PBS/0.1% Tween-20 for 1 hour at room temperature. Primary antibodies diluted in 3% BSA, 3% NDS, PBS/0.1% Tween-20 were incubated at 4 °C overnight. After 3 washes in PBS/0.1% Tween-20, secondary antibodies diluted in PBS were incubated for 1 hour in darkness at room temperature. After 3 washes in PBS/0.1% Tween-20, slides were mounted with Vectashield Hardset solution containing DAPI (H-1500, Vector Laboratories). Images were obtained on a motorized Axio ImagerZ1 microscope (Zeiss) coupled with an Axiocam Mrm camera (Zeiss) and processed with ZEN Blue 2.5 (Zeiss), Fiji and Adobe Photoshop. The dilutions of primary and secondary antibodies are shown in supplementary table 2.

### **Quantitative PCR analysis**

Individuals gonads without mesonephros were dissected in PBS, snap-frozen in liquid nitrogen and kept in -80 °C. Total RNAs were extracted by RNeasy Micro Kit (74004, Qiagen) and reverse transcribed by M-MLV reverse transcriptase (M170A, Promega). Q-PCR reactions were prepared in 10 µL with LightCycler® 480 SYBR Green I Master (04887352001, Roche) and run in the LightCycler® 480 System (05015278001, Roche). qRT-PCR primers are shown in the supplementary table 3. All biological replicates of different genotypes were run in the same plate and repeated at least twice. The number of replicates are indicated in the corresponding figures. Relative gene expression of each gonad was calculated based on a standard curve from each run and normalized to the expression of the housekeeping gene *Shda* for the comparison between different gonads. For each genotype, the mean of the normalized expression levels was calculated and graphs show mean fold-change +SEM. Unpaired Student *t*-test ( $p < 0.05$ ) was performed to compare the normalized gene expression values among samples.

## References

- Auguste, A., Chassot, A.A., Grégoire, E.P., Renault, L., Pannetier, M., Treier, M., Pailhoux, E., and Chaboissier, M.C. (2012). Loss of R-spondin1 and Foxl2 amplifies female-to-male sex reversal in XX mice. *Sex. Dev.* 5, 304–317.
- Bagheri-Fam, S., Sim, H., Bernard, P., Jayakody, I., Taketo, M.M., Scherer, G., and Harley, V.R. (2008). Loss of Fgfr2 leads to partial XY sex reversal. *Dev. Biol.* 314, 71–83.
- Bagheri-Fam, S., Bird, A.D., Zhao, L., Ryan, J.M., Yong, M., Wilhelm, D., Koopman, P., Eswarakumar, V.P., and Harley, V.R. (2017). Testis determination requires a specific FGFR2 isoform to repress FOXL2. *Endocrinology* 158, 3832–3843.
- Baltus, A.E., Menke, D.B., Hu, Y.-C., Goodheart, M.L., Carpenter, A.E., de Rooij, D.G., and Page, D.C. (2006). In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat. Genet.* 38, 1430–1434.
- Barrionuevo, F., Bagheri-Fam, S., Klattig, J., Kist, R., Taketo, M.M., Englert, C., and Scherer, G. (2005). Homozygous Inactivation of Sox9 Causes Complete XY Sex Reversal in Mice. *Biol. Reprod.* 74, 195–201.
- Biason-Lauber, A., Konrad, D., Navratil, F., and Schoenle, E.J. (2004). A WNT4 Mutation Associated with Müllerian-Duct Regression and Virilization in a 46,XX Woman. *N. Engl. J. Med.*
- Bingham, N.C., Verma-Kurvari, S., Parada, L.F., and Parker, K.L. (2006). Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* 44, 419–424.
- Bullejos, M., and Koopman, P. (2001). Spatially Dynamic Expression of Sry in Mouse Genital Ridges.
- Chaboissier, M.-C. (2004). Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development* 131, 1891–1901.
- Chassot, A.-A., Bradford, S.T., Auguste, A., Gregoire, E.P., Pailhoux, E., de Rooij, D.G., Schedl, A., and Chaboissier, M.-C. (2012). WNT4 and RSPO1 together are required for cell proliferation in the early mouse gonad. *Development* 139, 4461–4472.
- Chassot, A.A., Ranc, F., Gregoire, E.P., Roepers-Gajadien, H.L., Taketo, M.M., Camerino, G., de Rooij, D.G., Schedl, A., and Chaboissier, M.C. (2008a). Activation of  $\beta$ -catenin signaling by Rspo1 controls differentiation of the mammalian ovary. *Hum. Mol. Genet.* 17, 1264–1277.
- Chassot, A.A., Gregoire, E.P., Magliano, M., Lavery, R., and Chaboissier, M.C. (2008b). Genetics of ovarian differentiation: Rspo1, a major player. In *Sexual Development*, pp. 219–227.
- Chassot, A.A., Gregoire, E.P., Lavery, R., Taketo, M.M., de Rooij, D.G., Adams, I.R., and Chaboissier, M.C. (2011). RSPO1/ $\beta$ -Catenin signaling pathway regulates oogonia differentiation and entry into meiosis in the mouse fetal ovary. *PLoS One* 6.
- Colvin, J.S., Green, R.P., Schmahl, J., Capel, B., and Ornitz, D.M. (2001). Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell*.
- Durlinger, A.L.L., Gruijters, M.J.G., Kramer, P., Karels, B., Ingraham, H.A., Nachtigal, M.W., Uilenbroek, J.T.J., Grootegoed, J.A., and Themmen, A.P.N. (2002). Anti-Müllerian Hormone Inhibits Initiation of Primordial Follicle Growth in the Mouse Ovary. *Endocrinology* 143, 1076–1084.
- Garcia-Moreno, S.A., Futtner, C.R., Salamone, I.M., Gonen, N., Lovell-Badge, R., and Maatouk, D.M. (2019a). Gonadal supporting cells acquire sex-specific chromatin landscapes during mammalian sex determination. *Dev. Biol.* 446, 168–179.

- Garcia-Moreno, S.A., Lin, Y.-T., Futtner, C.R., Salamone, I.M., Capel, B., and Maatouk, D.M. (2019b). CBX2 is required to stabilize the testis pathway by repressing Wnt signaling. *PLOS Genet.* 15, e1007895.
- Gonen, N., Futtner, C.R., Wood, S., Alexandra Garcia-Moreno, S., Salamone, I.M., Samson, S.C., Sekido, R., Poulat, F., Maatouk, D.M., and Lovell-Badge, R. (2018). Sex reversal following deletion of a single distal enhancer of Sox9. *Science* (80-. ). 360, 1469–1471.
- Gregoire, E.P., Lavery, R., Chassot, A.A., Akiyama, H., Treier, M., Behringer, R.R., and Chaboissier, M.C. (2011). Transient development of ovotestes in XX Sox9 transgenic mice. *Dev. Biol.* 349, 65–77.
- Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Münsterberg, A., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature.*
- Gustin, S.E., Hogg, K., Stringer, J.M., Rastetter, R.H., Pelosi, E., Miles, D.C., Sinclair, A.H., Wilhelm, D., and Western, P.S. (2016). WNT/ $\beta$ -catenin and p27/FOXL2 differentially regulate supporting cell proliferation in the developing ovary. *Dev. Biol.* 412, 250–260.
- Hacker, A., Capel, B., Goodfellow, P., and Lovell-Badge, R. (1995). Expression of Sry, the mouse sex determining gene. *Development* 121, 1603–1614.
- Harris, A., Siggers, P., Corrochano, S., Warr, N., Sagar, D., Grimes, D.T., Suzuki, M., Burdine, R.D., Cong, F., Koo, B.-K., et al. (2018). ZNRF3 functions in mammalian sex determination by inhibiting canonical WNT signaling. *Proc. Natl. Acad. Sci. U. S. A.* 115, 5474–5479.
- Heikkilä, M., Peltoketo, H., Leppäluoto, J., Ilves, M., Vuolteenaho, O., and Vainio, S. (2002). Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. *Endocrinology* 143, 4358–4365.
- Heikkilä, M., Prunskaitė, R., Naillat, F., Itäranta, P., Vuoristo, J., Leppäluoto, J., Peltoketo, H., and Vainio, S. (2005). The partial female-to-male sex reversal in Wnt-4 deficient females involves induced expression of testosterone biosynthetic genes and testosterone production and depends on androgen action. *Endocrinology* 146, 4016–4023.
- Hiramatsu, R., Matoba, S., Kanai-Azuma, M., Tsunekawa, N., Katoh-Fukui, Y., Kurohmaru, M., Morohashi, K.I., Wilhelm, D., Koopman, P., and Kanai, Y. (2009). A critical time window of Sry action in gonadal sex determination in mice. *Development.*
- Jameson, S.A., Lin, Y.T., and Capel, B. (2012). Testis development requires the repression of Wnt4 by Fgf signaling. *Dev. Biol.* 370, 24–32.
- Jeays-Ward, K. (2003). Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. *Development* 130, 3663–3670.
- Jeays-Ward, K., Dandonneau, M., and Swain, A. (2004). Wnt4 is required for proper male as well as female sexual development. *Dev. Biol.* 276, 431–440.
- Kashimada, K., Pelosi, E., Chen, H., Schlessinger, D., Wilhelm, D., and Koopman, P. (2011). FOXL2 and BMP2 act cooperatively to regulate follistatin gene expression during ovarian development. *Endocrinology* 152, 272–280.
- Kim, Y., Kobayashi, A., Sekido, R., DiNapoli, L., Brennan, J., Chaboissier, M.C., Poulat, F., Behringer, R.R., Lovell-Badge, R., and Capel, B. (2006). Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. *PLoS Biol.* 4, 1000–1009.
- Kim, Y., Bingham, N., Sekido, R., Parker, K.L., Lovell-Badge, R., and Capel, B. (2007). Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. *Proc. Natl. Acad. Sci.* 104, 16558–16563.

- Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for Sry. *Nature*.
- Lavery, R., Lardenois, A., Ranc-Jianmotamedi, F., Pauper, E., Gregoire, E.P., Vigier, C., Moreilhon, C., Primig, M., and Chaboissier, M.C. (2011). XY Sox9 embryonic loss-of-function mouse mutants show complete sex reversal and produce partially fertile XY oocytes. *Dev. Biol.* 354, 111–122.
- Lavery, R., Chassot, A.A., Pauper, E., Gregoire, E.P., Klopfenstein, M., de Rooij, D.G., Mark, M., Schedl, A., Ghyselinck, N.B., and Chaboissier, M.C. (2012). Testicular Differentiation Occurs in Absence of R-spondin1 and Sox9 in Mouse Sex Reversals. *PLoS Genet.* 8.
- Lei, N., Hornbaker, K.I., Rice, D.A., Karpova, T., Agbor, V.A., and Heckert, L.L. (2007). Sex-Specific Differences in Mouse DMRT1 Expression Are Both Cell Type- and Stage-Dependent During Gonad Development<sup>1</sup>. *Biol. Reprod.* 77, 466–475.
- Li, Y., Zheng, M., and Lau, Y.F.C. (2014). The sex-determining factors SRY and SOX9 regulate similar target genes and promote testis cord formation during testicular differentiation. *Cell Rep.* 8, 723–733.
- Liu, C.F., Bingham, N., Parker, K., and Yao, H.H.C. (2009). Sex-specific roles of  $\beta$ -catenin in mouse gonadal development. *Hum. Mol. Genet.* 18, 405–417.
- Liu, C.F., Parker, K., and Humphrey, Y. (2010). WNT4/ $\beta$ -catenin pathway maintains female germ cell survival by inhibiting activin  $\beta$ B in the mouse fetal ovary. *PLoS One* 5.
- Maatouk, D.M., Dinapoli, L., Alvers, A., Parker, K.L., Taketo, M.M., and Capel, B. (2008). Stabilization of  $\beta$ -catenin in XY gonads causes male-to-female sex-reversal. *Hum. Mol. Genet.* 17, 2949–2955.
- Maatouk, D.M., Mork, L., Chassot, A.A., Chaboissier, M.C., and Capel, B. (2013). Disruption of mitotic arrest precedes precocious differentiation and transdifferentiation of pregranulosa cells in the perinatal Wnt4 mutant ovary. *Dev. Biol.* 383, 295–306.
- Mandel, H., Shemer, R., Borochoowitz, Z.U., Okopnik, M., Knopf, C., Indelman, M., Drugan, A., Tiosano, D., Gershoni-Baruch, R., Choder, M., et al. (2008). SERKAL Syndrome: An Autosomal-Recessive Disorder Caused by a Loss-of-Function Mutation in WNT4. *Am. J. Hum. Genet.* 82, 39–47.
- Naillat, F., Prunskaitė-Hyyryläinen, R., Pietilä, I., Sormunen, R., Jokela, T., Shan, J., and Vainio, S.J. (2010). Wnt4/5a signalling coordinates cell adhesion and entry into meiosis during presumptive ovarian follicle development. *Hum. Mol. Genet.* 19, 1539–1550.
- Nef, S., Schaad, O., Stallings, N.R., Cederroth, C.R., Pitetti, J.L., Schaer, G., Malki, S., Dubois-Dauphin, M., Boizet-Bonhoure, B., Descombes, P., et al. (2005). Gene expression during sex determination reveals a robust female genetic program at the onset of ovarian development. *Dev. Biol.* 287, 361–377.
- Nef, S., Stévant, I., and Greenfield, A. (2019). Characterizing the bipotential mammalian gonad. In *Current Topics in Developmental Biology*, p.
- Nicol, B., and Yao, H.H.-C. (2015). Gonadal Identity in the Absence of Pro-Testis Factor SOX9 and Pro-Ovary Factor Beta-Catenin in Mice<sup>1</sup>. *Biol. Reprod.* 93.
- Nicol, B., Grimm, S.A., Gruzdev, A., Scott, G.J., Ray, M.K., and Yao, H.H.C. (2018). Genome-wide identification of FOXL2 binding and characterization of FOXL2 feminizing action in the fetal gonads. *Hum. Mol. Genet.* 27, 4273–4287.
- Ottolenghi, C., Omari, S., Garcia-Ortiz, J.E., Uda, M., Crisponi, L., Forabosco, A., Pilia, G., and Schlessinger, D. (2005). Foxl2 is required for commitment to ovary differentiation. *Hum. Mol. Genet.* 14, 2053–2062.

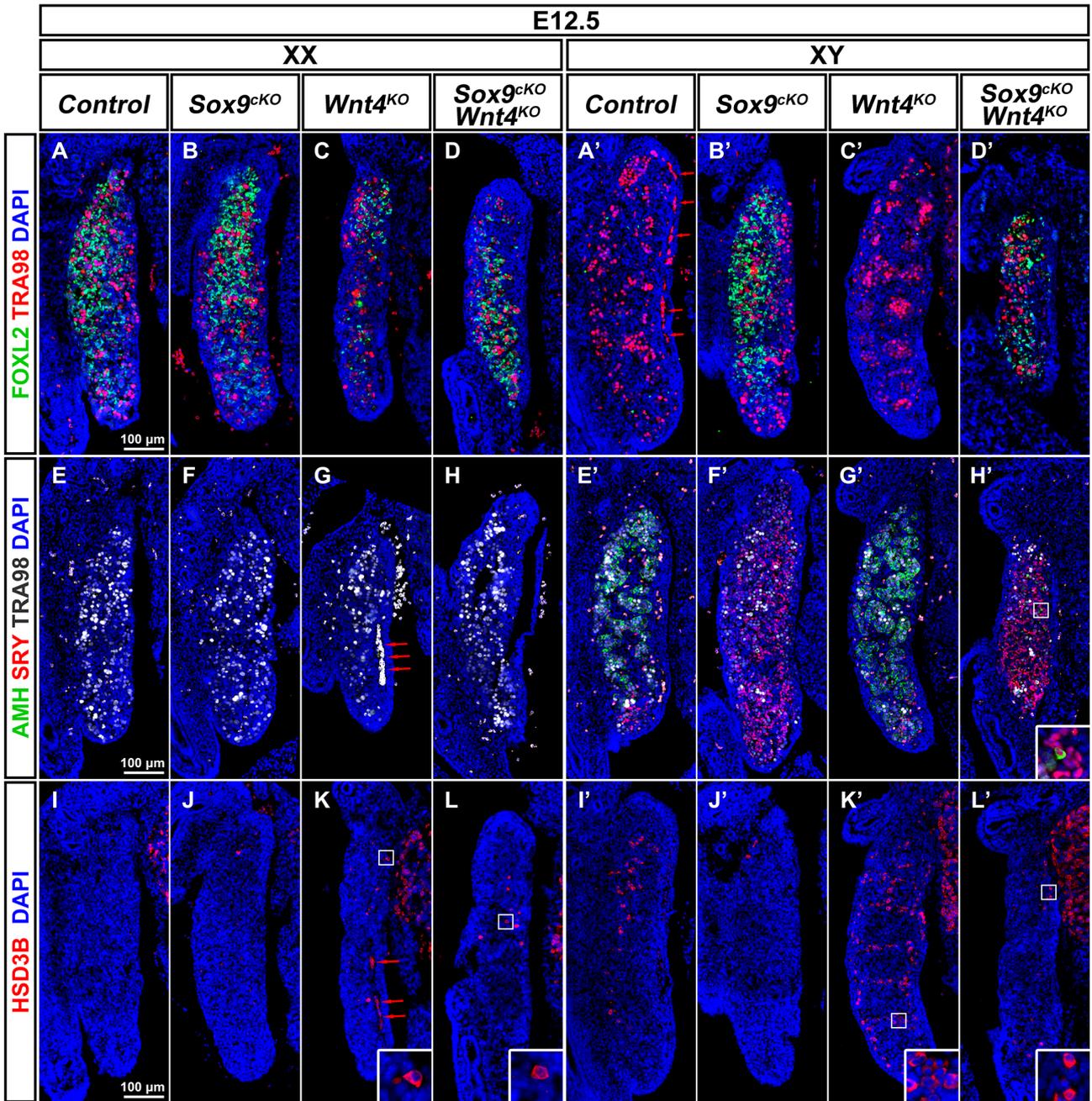
- Ottolenghi, C., Pelosi, E., Tran, J., Colombino, M., Douglass, E., Nedorezov, T., Cao, A., Forabosco, A., and Schlessinger, D. (2007). Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells. *Hum. Mol. Genet.* 16, 2795–2804.
- Parma, P., Radi, O., Vidal, V., Chaboissier, M.C., Dellambra, E., Valentini, S., Guerra, L., Schedl, A., and Camerino, G. (2006). R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat. Genet.* 38, 1304–1309.
- Rahmoun, M., Lavery, R., Laurent-Chaballier, S., Bellora, N., Philip, G.K., Rossitto, M., Symon, A., Pailhoux, E., Cammas, F., Chung, J., et al. (2017). In mammalian foetal testes, SOX9 regulates expression of its target genes by binding to genomic regions with conserved signatures. *Nucleic Acids Res.* 45, 7191–7211.
- Sekido, R., and Lovell-Badge, R. (2008). Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature* 453, 930–934.
- Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R., and Goodfellow, P.N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature.*
- Stark, K., Vainio, S., Vassileva, G., and McMahon, A.P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature.*
- Stévant, I., Kuhne, F., Greenfield, A., Chaboissier, M.-C., Dermitzakis, E.T., and Nef, S. (2018). Single-cell transcriptomics of the mouse gonadal soma reveals the establishment of sexual dimorphism in distinct cell lineages. *BioRxiv.*
- Suzuki, A., and Saga, Y. (2008). Nanos2 suppresses meiosis and promotes male germ cell differentiation. *Genes Dev.*
- Tomizuka, K., Horikoshi, K., Kitada, R., Sugawara, Y., Iba, Y., Kojima, A., Yoshitome, A., Yamawaki, K., Amagai, M., Inoue, A., et al. (2008). R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Hum. Mol. Genet.* 17, 1278–1291.
- Uhlenhaut, N.H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., Kress, J., Treier, A.C., Klugmann, C., Klasen, C., Holter, N.I., et al. (2009). Somatic Sex Reprogramming of Adult Ovaries to Testes by FOXL2 Ablation. *Cell* 139, 1130–1142.
- Vainio, S., Heikkilä, M., Kispert, A., Chin, N., and McMahon, A.P. (1999). Female development in mammals is regulated by Wnt-4 signalling. *Nature.*
- Vidal, V.P.I., Chaboissier, M.C., De Rooij, D.G., and Schedl, A. (2001). Sox9 induces testis development in XX transgenic mice. *Nat. Genet.*
- Wilhelm, D., Martinson, F., Bradford, S., Wilson, M.J., Combes, A.N., Beverdam, A., Bowles, J., Mizusaki, H., and Koopman, P. (2005). Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Dev. Biol.*
- Wilhelm, D., Washburn, L.L., Truong, V., Fellous, M., Eicher, E.M., and Koopman, P. (2009). Antagonism of the testis- and ovary-determining pathways during ovotestis development in mice. *Mech. Dev.* 126, 324–336.
- Yang, M., Li, J., An, Y., and Zhang, S. (2015). Effects of androgen on immunohistochemical localization of androgen receptor and Connexin 43 in mouse ovary. *Tissue Cell* 47, 526–532.
- Yao, H.H.-C., Aardema, J., and Holthussen, K. (2006). Sexually Dimorphic Regulation of Inhibin Beta B in Establishing Gonadal Vasculature in Mice<sup>1</sup>. *Biol. Reprod.*

- Yao, H.H.C., Whoriskey, W., and Capel, B. (2002). Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes Dev.*
- Yao, H.H.C., Matzuk, M.M., Jorgez, C.J., Menke, D.B., Page, D.C., Swain, A., and Capel, B. (2004). Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. *Dev. Dyn.* 230, 210–215.

## Figure and figure legends

### Figure 1. Consequences of inactivation of *Sox9* and *Wnt4* in XX and XY gonads at E12.5.

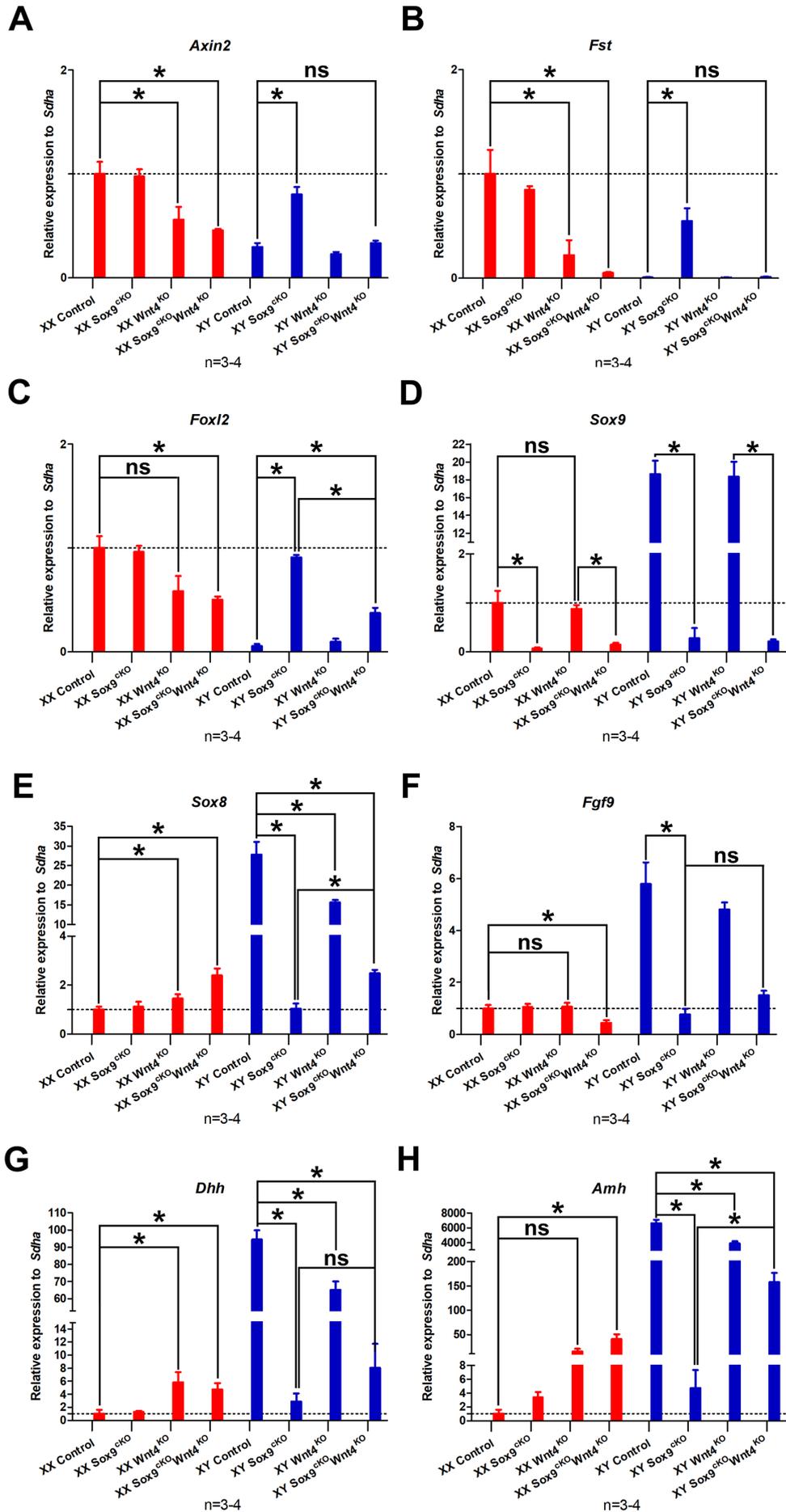
Immunofluorescence staining on E12.5 XX (A-L) and XY (A'-L') gonads of *Control*, *Sox9<sup>CKO</sup>*, *Wnt4<sup>KO</sup>*, *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* genotypes. **(A-D and A'-D')** Expression of the granulosa cell marker FOXL2 (green) and of the germ cell marker TRA98 (red). **(E- H and E'-H')** Expression of the mature granulosa cell or Sertoli cell marker AMH (green), of the male sex determination protein SRY (red) and TRA98 (white). XY *Sox9<sup>CKO</sup>* and XY *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* gonads maintain high levels of SRY expression in the entire gonad. Inset in H' is a higher magnification of the XY *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* double mutant gonad showing a cell expressing SRY and AMH. **(I-L and I'-L')** Expression of the steroidogenic enzyme HSD3B (red). Insets show HSD3B positive cells in XX *Wnt4<sup>KO</sup>* mutant (K), XX *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* double mutant (L), XY *Wnt4<sup>KO</sup>* mutant (K'), and XY *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* double mutant (L'). Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m. Red arrows in G, K and A' indicate the coelomic vessel. Bright yellow spots are auto-fluorescent blood cells.



*Wnt4*;*Sox9*: Figure 1

## Figure 2. Quantitative PCR analysis at E12.5

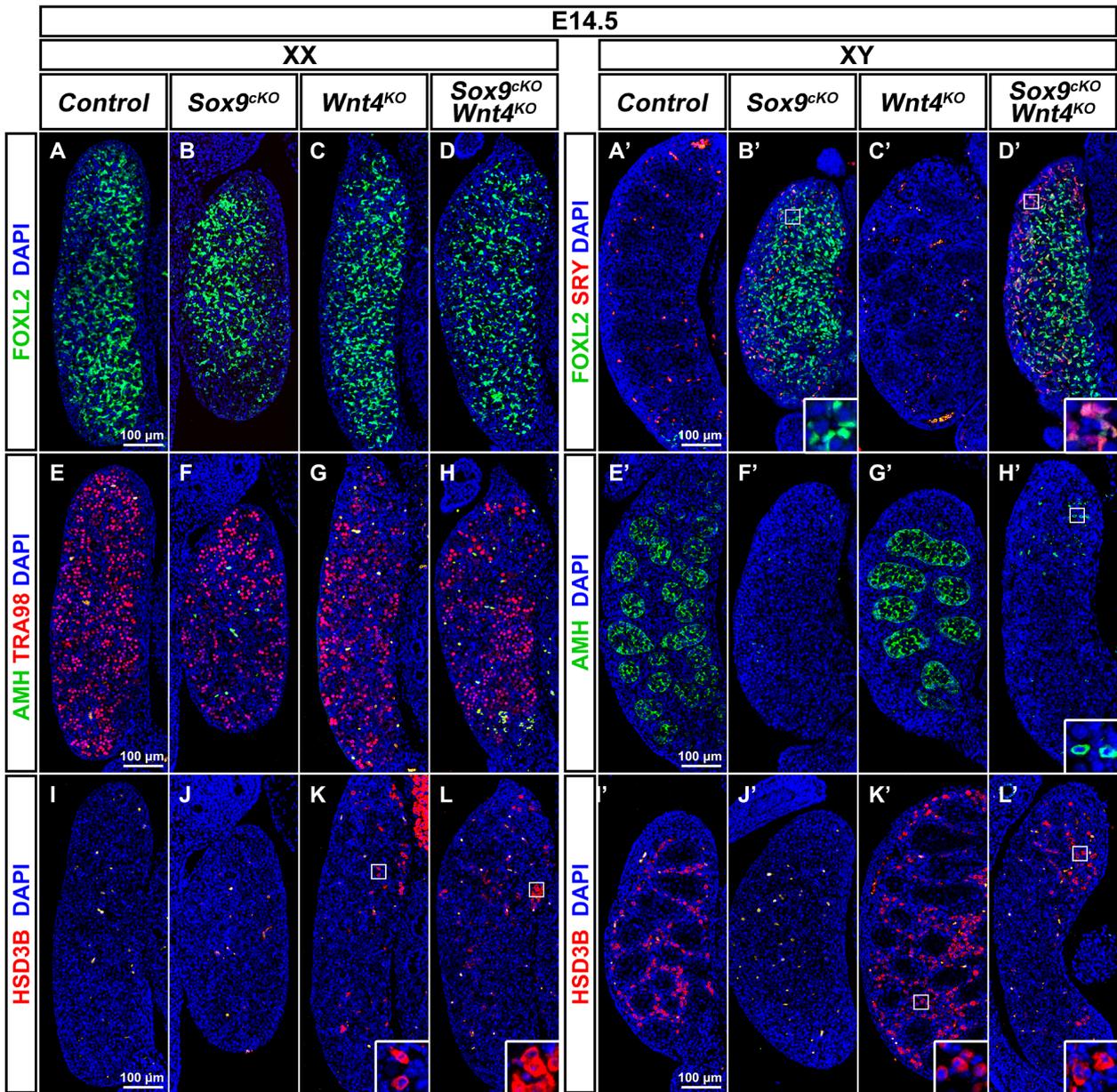
Quantitative PCR analysis of mRNA expression in E12.5 XX or XY gonads of *Control*, *Sox9<sup>cKO</sup>*, *Wnt4<sup>KO</sup>*, *Sox9<sup>cKO</sup>Wnt4<sup>KO</sup>* genotypes. Results are shown as mean + SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test (n = 4 embryos for each genotype). ns: no significant difference ( $P > 0.05$ ).



**Wnt4;Sox9: Figure 2**

**Figure 3. Consequences of inactivation of Sox9 and Wnt4 in XX and XY gonads at E14.5.**

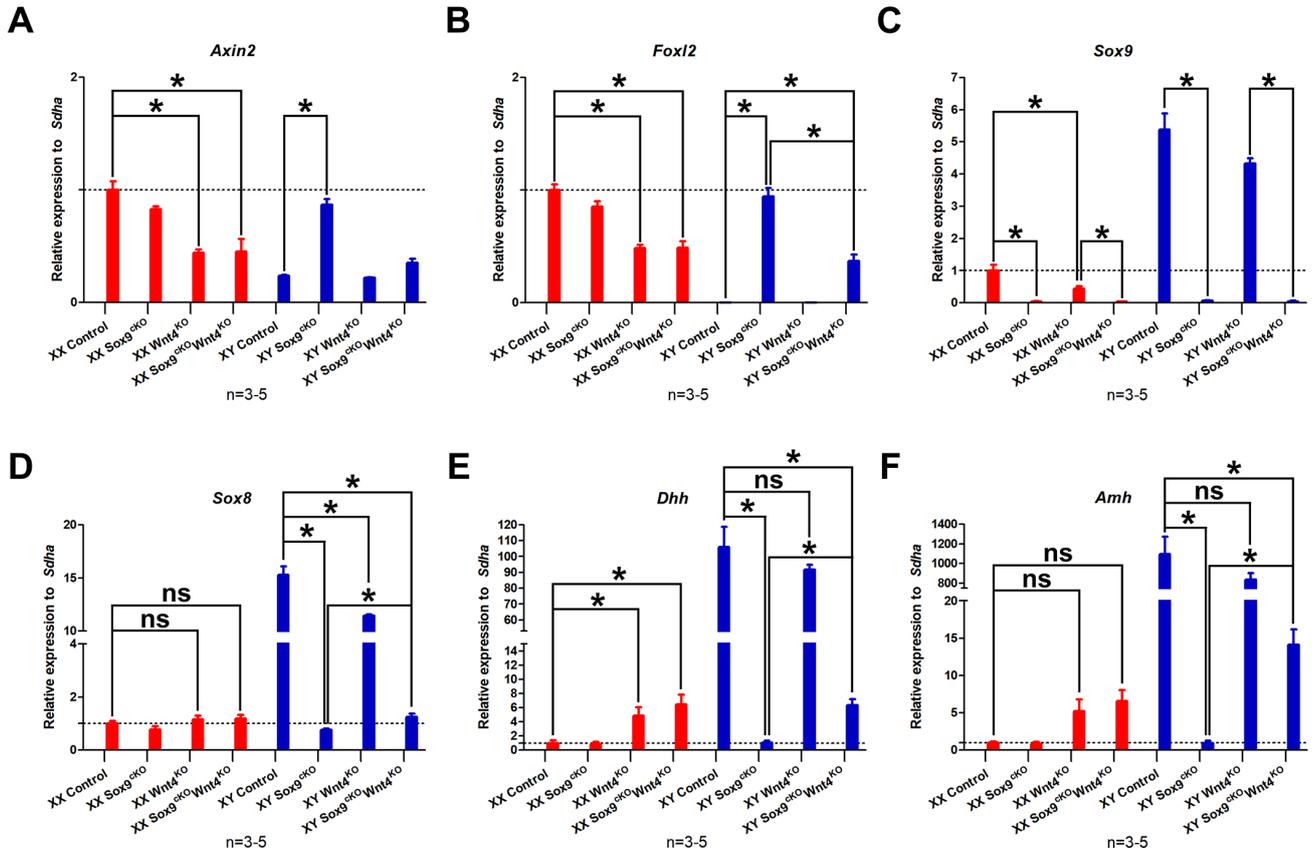
Immunofluorescence staining on E14.5 XX (A-L) and XY (A'-L') gonads of *Control*, *Sox9<sup>ckO</sup>*, *Wnt4<sup>KO</sup>*, *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>* genotypes. **(A-D and A'-D')** Expression of FOXL2 (green) and SRY (red, A'-D'). FOXL2 expression is abundant in XY *Sox9<sup>ckO</sup>* (B') and in XY *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>* (D'). Inset in D' is a higher magnification of the XY *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>* double mutant gonad showing cells co-expressing SRY and FOXL2. **(E-H and E'-H')** Expression of AMH (green) and TRA98 (red, E-H). Inset in H' is a higher magnification of the XY *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>* double mutant gonad showing AMH positive cells in the anterior region. **(I-L and I'-L')** Expression of HSD3B (red). Insets show HSD3B positive cells in XX *Wnt4<sup>KO</sup>* mutant (K), XX *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>* double mutant (L), XY *Wnt4<sup>KO</sup>* mutant (K'), and XY *Sox9<sup>ckO</sup>Wnt4<sup>KO</sup>* double mutant (L'). Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m. Bright yellow spots are auto-fluorescent blood cells.



*Wnt4*; *Sox9*: Figure 3

#### **Figure 4. Quantitative PCR analysis at E14.5**

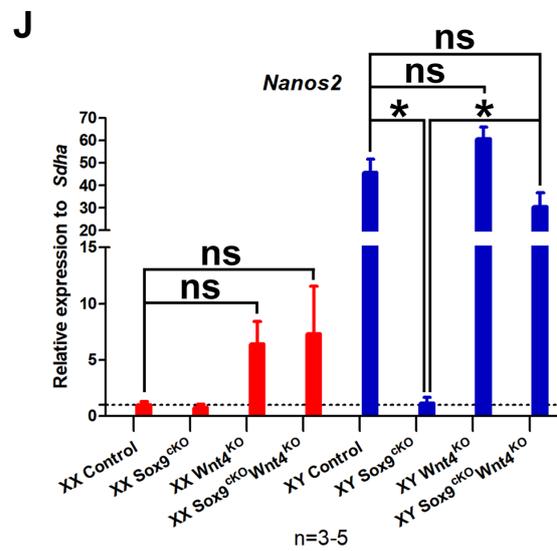
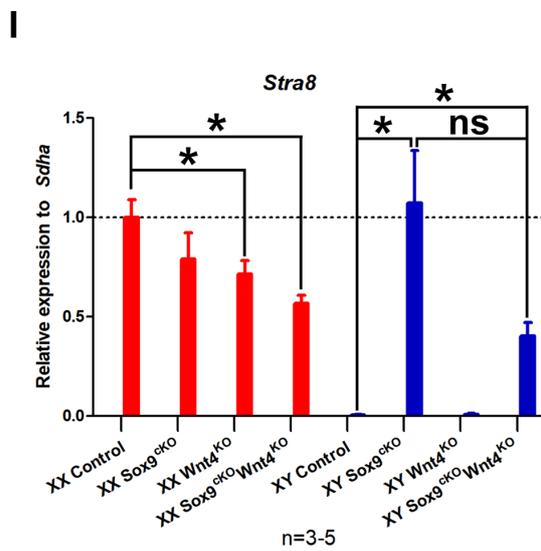
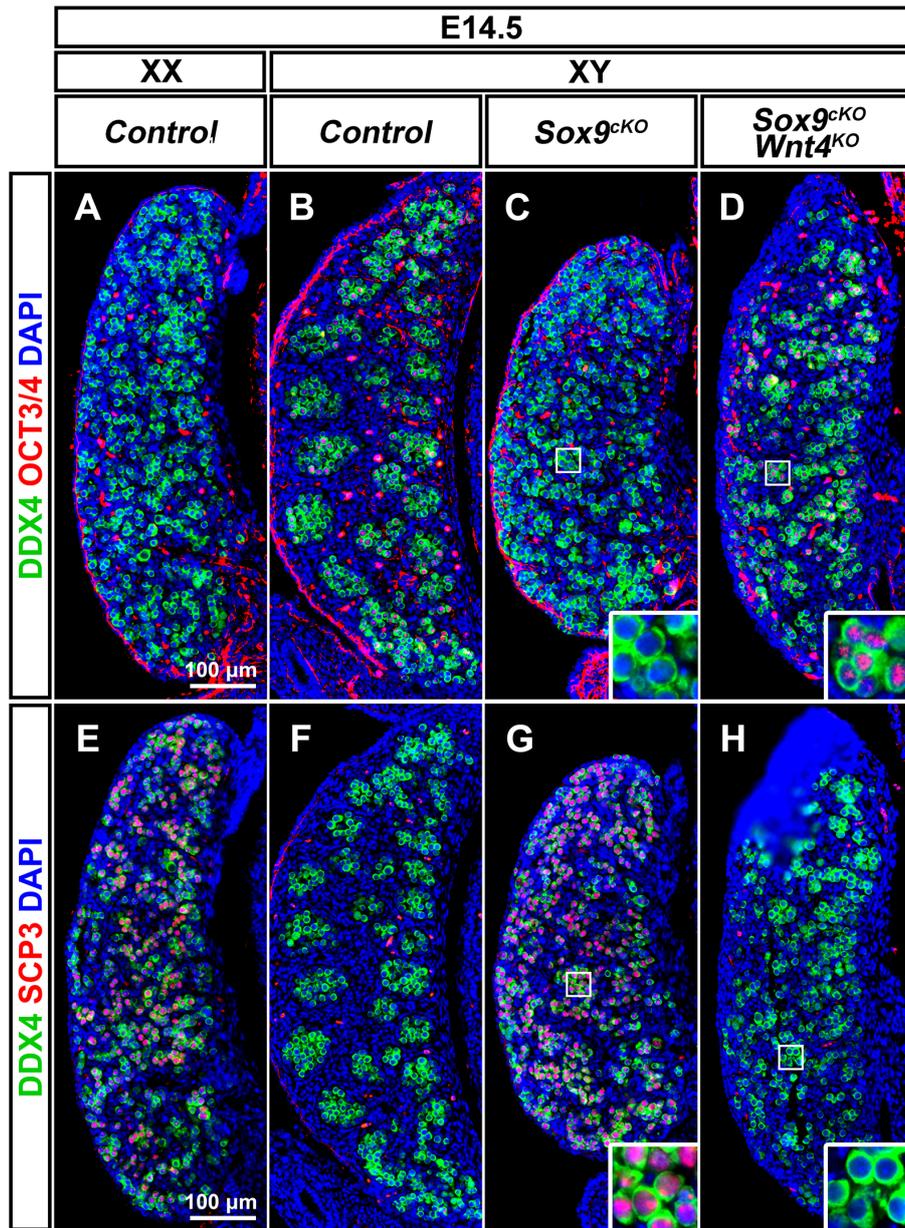
Quantitative PCR analysis of mRNA expression in E14.5 XX or XY gonads of *Control*, *Sox9<sup>CKO</sup>*, *Wnt4<sup>KO</sup>*, *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* genotypes. Results are shown as mean + SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test (n = 4 embryos for each genotype). ns: no significant difference ( $P > 0.05$ ).



**Wnt4;Sox9: Figure 4**

**Figure 5. Germ cells of XY *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* double mutants maintain pluripotency but have ectopic *Stra8* mRNA expression.**

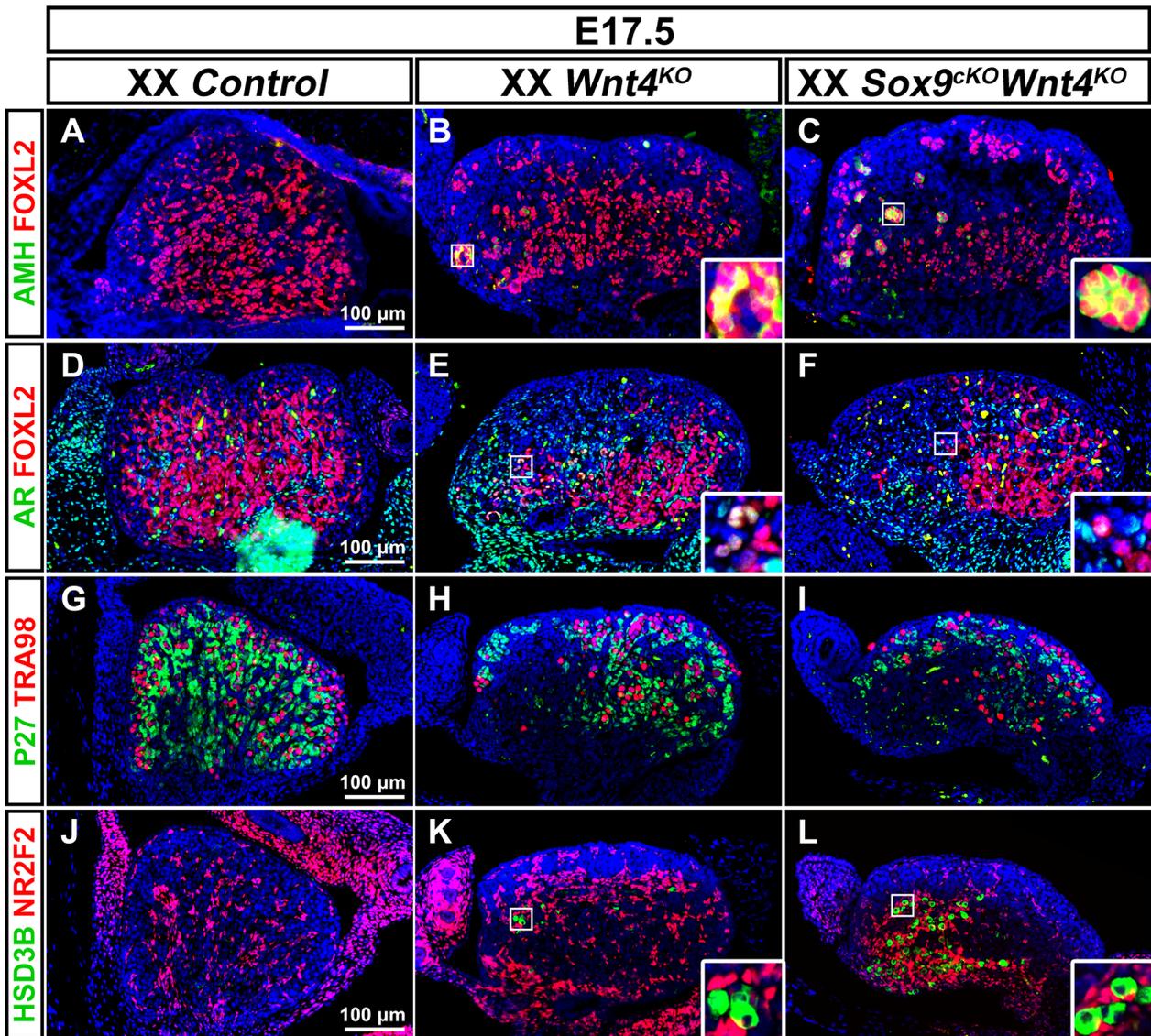
**(A-H)** Immunofluorescence staining on E14.5 XX control gonads (A, E) and E14.5 XY (B-D, F-H) gonads of *Control*, *Sox9<sup>CKO</sup>*, *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* genotypes. **(A-D)** Expression of the germ cell marker DDX4 (green) and of the pluripotency marker OCT3/4 (red). Inset in C shows germ cells that have lost pluripotency in XY *Sox9<sup>CKO</sup>*. Inset in D shows pluripotent germ cells expressing OCT3/4 in *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>*. **(E-H)** Expression of DDX4 (green) and of the meiotic marker SCP3 (red). Inset in G shows meiotic germ cells in XY *Sox9<sup>CKO</sup>*. Inset in H shows germ cells negative for SCP3 in *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>*. Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m. **(I-J)** Quantitative PCR analysis of *Stra8* and *Nanos2* mRNA expression in E14.5 XX or XY gonads of *Control*, *Sox9<sup>CKO</sup>*, *Wnt4<sup>KO</sup>*, *Sox9<sup>CKO</sup>Wnt4<sup>KO</sup>* genotypes. Results are shown as mean + SEM. Asterisks represent statistical significance of  $P < 0.05$  by using Student's *t*-test ( $n = 4$  embryos for each genotype). ns: no significant difference ( $P > 0.05$ ).



**Wnt4;Sox9: Figure 5**

**Figure 6. XX *Sox9<sup>ckO</sup>Wnt4<sup>ko</sup>* double mutants develop ovotestes as XX *Wnt4<sup>ko</sup>* mutants.**

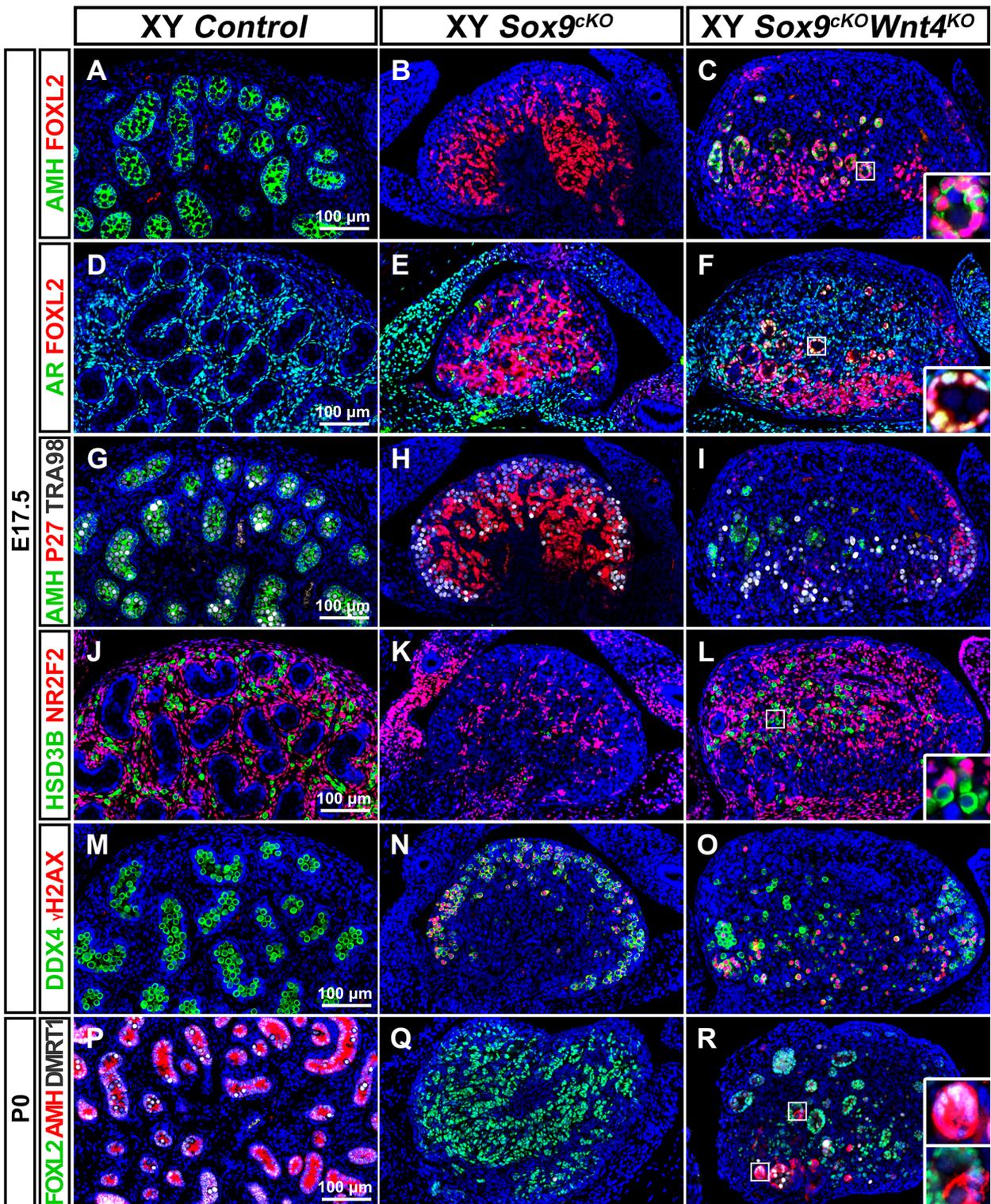
Immunofluorescence staining on E17.5 XX gonads of *Control*, *Wnt4<sup>ko</sup>* and *Sox9<sup>ckO</sup>Wnt4<sup>ko</sup>* genotypes. **(A-C)** Expression of AMH (green) and FOXL2 (red). Insets show AMH and FOXL2 double positive pre-matured granulosa observed in the anterior region of XX *Wnt4<sup>ko</sup>* (B) and XX *Sox9<sup>ckO</sup>Wnt4<sup>ko</sup>* (C) mutant gonads. **(D-F)** Expression of androgen receptor (AR, green) and FOXL2 (red). Insets show pre-matured granulosa cells co-expressing AR and FOXL2 in the anterior region of XX *Wnt4<sup>ko</sup>* (E) and XX *Sox9<sup>ckO</sup>Wnt4<sup>ko</sup>* (F) mutant gonads. **(G-I)** Expression of the mitotic arrest marker P27 (green) and TRA98 (red). Germ cells and P27 expression are reduced in the anterior region in the XX *Wnt4<sup>ko</sup>* and XX *Sox9<sup>ckO</sup>Wnt4<sup>ko</sup>* mutant gonads. **(J-L)** Expression of HSD3B (green) and of the interstitial cell marker NR2F2 (red). Ectopic steroidogenic cells (insets in K and L) and interstitial cells are abundant in XX *Wnt4<sup>ko</sup>* and XX *Sox9<sup>ckO</sup>Wnt4<sup>ko</sup>* mutant gonads. Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m.



*Wnt4*;*Sox9*: Figure 6

**Figure 7. XY Sox9<sup>cko</sup>Wnt4<sup>ko</sup> double mutants develop ovotestes**

Immunofluorescence staining on E17.5 (A-O) and P0 (P-R) XY gonads of *Control*, *Sox9<sup>ko</sup>* and *Sox9<sup>cko</sup>Wnt4<sup>ko</sup>* genotypes. **(A-C)** Expression of AMH (green) and FOXL2 (red). Inset shows AMH and FOXL2 double positive pre-matured granulosa observed in the anterior region of XY *Sox9<sup>cko</sup>Wnt4<sup>ko</sup>* mutant gonads (C). **(D-F)** Expression of androgen receptor (AR, green) and FOXL2 (red). Inset shows pre-matured granulosa cells co-expressing AR and FOXL2 in XY *Sox9<sup>cko</sup>Wnt4<sup>ko</sup>* mutant gonads (F). **(G-I)** Expression of the mitotic arrest marker P27 (red), AMH (green) and TRA98 (white). Germ cells and P27 expression are reduced in the AMH positive region of XY *Sox9<sup>cko</sup>Wnt4<sup>ko</sup>* mutant gonads. **(J-L)** Expression of HSD3B (green) and NR2F2 (red). Inset in L shows steroidogenic cells in XY *Sox9<sup>cko</sup>Wnt4<sup>ko</sup>* mutant gonads. **(M-O)** Expression of the germ cell marker DDX4 (green) and the DNA double strands break marker or meiotic marker  $\gamma$ H2AX (red). The germ cell close to posterior region of E17.5 XY *Sox9<sup>cko</sup>Wnt4<sup>ko</sup>* (O) enter meiosis (DDX4 and  $\gamma$ H2AX double positive), whereas germ cells close to the anterior region do not show  $\gamma$ H2AX expression as the XY *Control* (M). **(P-R)** Expression of AMH (red), FOXL2 (green) and of the Sertoli cell maker DMRT1 (white). The insets in R show trans-differentiated Sertoli-like cells (DMRT1 and AMH double positive) and pre-matured granulosa cells (AMH and FOXL2 double positive) observed in the P0 XY *Sox9<sup>cko</sup>Wnt4<sup>ko</sup>* (R). Nuclei labeled with DAPI are shown in blue. Scale bar = 100  $\mu$ m.



Wnt4;Sox9: Figure 7

## Supplementary Material

**Supplementary table 1: Genotyping primer list**

Allele genotyping	Primer name	Forward 5' - 3'	Primer name	Reverse 5' - 3'	Amplicon size
<b>Sox8</b>	Sox8 15	GTCCTGCGTGGCAACCTTGG	Sox8 16	GCCCACACCATGAAGGCATTC	431bp
<b>Sry</b>	SRY 5'	GAGAGCATGGAGGGCCATG	SRY 3'	CTGTGTAGGATCTTCAATC	203bp
<b>Sox9<sup>WT</sup></b>	Sox9 R3'158_	AACTAGGAGTAGTTAAGCTG	Sox9 F2135	GGGGCTTGTCTCTCCTTCAGAG	250bp
<b>Sox9<sup>Flox</sup></b>	Sox9 R3'158_	AACTAGGAGTAGTTAAGCTG	Sox9 F034 NEO2	ACACAGCATAGGCTACCTG	250bp
<b>Sf1-Cre<sup>tg</sup></b>	Sf1b	GTGTGCACAGACCAGGGCAA	TNAPCre_R	CAAACGGACAGAAGCATTTCAG	300bp
<b>Wnt4<sup>WT</sup></b>	WNT4_Exon3	TTCACAACAACGAGGCTGGCAGG	WNT4_4ex4R	ACCCGCATGTGTGTCAGATGG	700bp
<b>Wnt4<sup>KO</sup></b>	WNT4PgkNeo	GCATTGTCTGAGTAGGTGTCATTC	WNT4_4ex4R	ACCCGCATGTGTGTCAGATGG	400bp

WT: wildtype allele; Flox: Floxed allele; KO: deleted allele; Tg: transgenic allele;

Sox8 was used as internal control.

**Supplementary table 2:** Antibody list for Immunofluorescence staining.

<b>Antibody</b>	<b>Species</b>	<b>Supplier</b>	<b>Reference</b>	<b>Dilution</b>
<b>DDX4</b>	Rabbit	Abcam	ab13840	200X
<b>TRA98</b>	Rat	Abcam	82527	200X
<b>AMH</b>	Mouse	Bio-Rad Laboratories	MCA2246	30X
<b>OCT3/4</b>	Mouse	BD Transduction Laboratories	611202	200X
<b>FOXL2</b>	Goat	Novus Biologicals	NB100-1277SS	200X
<b>NR2F2</b>	Mouse	R&D Systems	PP-H7147-00	200X
<b>γH2AX</b>	Mouse	Merck KGaA	16-193	400X
<b>SOX9</b>	Rabbit	Merck KGaA	HPA001758	250X
<b>P27</b>	Rabbit	Santa Cruz Biotechnology	sc-52	200X
<b>AR</b>	Rabbit	Santa Cruz Biotechnology	sc-816	200X
<b>DMRT1</b>	Mouse	Santa Cruz Biotechnology	SC-377167	200X
<b>AMH</b>	Goat	Santa Cruz Biotechnology	sc6886	200X
<b>HSD3B</b>	Goat	Santa Cruz Biotechnology	sc-30820	200X
<b>SCP3</b>	Mouse	Santa Cruz Biotechnology	Sc-74569	200X
<b>SRY</b>	Rabbit	Dagmar Wilhelm's Lab		200X
<b>SDMG1</b>	Rabbit	Ian Adams's Lab		600X
<b>DαGoat488</b>	Donkey	Thermo Fisher	A11055	500X
<b>DαGoat555</b>	Donkey	Thermo Fisher	A21432	200X
<b>DαGoat647</b>	Donkey	Thermo Fisher	A21447	200X
<b>DαMouse488</b>	Donkey	Thermo Fisher	A21202	500X
<b>DαMouse594</b>	Donkey	Thermo Fisher	A21203	500X
<b>DαMouse647</b>	Donkey	Thermo Fisher	A31571	200X
<b>DαRabbit488</b>	Donkey	Thermo Fisher	A21206	500X
<b>DαRabbit555</b>	Donkey	Thermo Fisher	A31572	200X
<b>DαRabbit647</b>	Donkey	Thermo Fisher	A31573	200X
<b>DαRat488 old</b>	Donkey	Thermo Fisher	A21208	400X

**Supplementary table 3: qRT-PCR primer list**

<b>Gene</b>	<b>Forward 5' - 3'</b>	<b>Reverse 5' - 3'</b>	<b>Annealing temperature</b>	<b>Exon amplification</b>	<b>Amplicon size</b>
<i>Axin2</i>	GCAAGTCCAAGCCCCATA	CGGCTGACTCGTTCTCCT	58°C	E7/8	88bp
<i>Foxl2</i>	ATCGGGGTTCTCAACAAC	CATCTGGCAGGAGGCGTA	58°C	E1	73bp
<i>Fst</i>	GCCTATGAGGGAAAGTGTATCAA	TGGAATCCCATAGGCATTTT	58°C	E4/5	82bp
<i>Sox9</i>	GCGGAGCTCAGCAAGACTCTG	ATCGGGGTGGTCTTTCTTGTG	58°C	E1/2	117bp
<i>Sox8</i>	GACCCTAGGCAAGCTGTGG	CTGCACACGGAGCCTCTC	58°C	E1/2	82bp
<i>Fgf9</i>	TGCAGGACTGGATTTCAATTTAG	CCAGGCCCACTGCTATACTG	58°C	E1/2	112bp
<i>Dhh</i>	GGACCTCGTACCCAACTACAA	CGATGGCTAGAGCGTTCACC	58°C	E1/2	113bp
<i>Amh</i>	GGGGAGACTGGAGAACAGC	AGAGCTCGGGCTCCCATA	58°C	E1/2	67bp
<i>Nanos2</i>	AGGTCCCCCGATCTCAAC	CAGCATTCCCAGTGTTCAG	58°C	E1	60bp
<i>Stra8</i>	TGACGTGGCAAGTTTCCTG	GTTGCAGGTGGCAAACATAG	58°C	E7/8	76bp

## CHAPTER IV: CONCLUSIONS AND PERSPECTIVES

### Conclusions

Female sex determination in XX is an active and autonomous process, whose progression needs the activation of canonical RSPO1/WNT4/ $\beta$ -Catenin signaling. In this study, by taking advantage of a new conditional knockout mouse model, we determined that RSPO1/WNT4/ $\beta$ -Catenin signaling is specifically critical for female development during the sex determination window. After female development is established around E11.5, *Rspo1* is not necessary for the later development of the ovary. During ovotestes development in *Rspo1* mutants, up-regulation of the expression of Androgen receptor and of components of the steroidogenic pathway precede the pre-maturation of pre-granulosa cells. Furthermore, we discovered that androgen signaling supports ovotestis development in XX *Rspo1* mutants by promoting the pre-maturation of pre-granulosa cells and their trans-differentiation into Sertoli-like cells.

Our findings highlight the spatio-temporal requirement of the pro-female RSPO1/WNT4/ $\beta$ -Catenin pathway during the time of sex determination in XX and provide the first direct evidence of how sex hormones affect the primary gonadal development in the ovotestis context. Our findings suggest that mammals evolved to avoid the effects from a sex hormone rich environment in the uterus in part through the inhibition of AR expression in the XX embryonic gonad by canonical WNT signaling. Our findings also prove that it is possible to prevent the process of female-to-male sex reversal in mouse *Rspo1* mutants during the embryonic stages by inhibiting the androgen signaling pathway, which brings new thoughts and hopes to fight *RSPO1/WNT4/CTNNB1* mutations-led DSD.

Mutual antagonistic mechanisms between male and female development start from the sex determination and last the whole lifetime (Bagheri-Fam et al., 2017; Kim et al., 2006b; Matson et al., 2011; Uhlentaut et al., 2009). Along with the stage

progression, the main factors in pro-male and pre-female pathway used for this antagonism also change. Here in our studies, we identified that *Sox9* is dispensable for the XX *Wnt4* deficient ovotestes development at the early stages, since both XX single *Wnt4* and *Wnt4;Sox9* mutants had similar female-to-male sex reversal phenotypes: coelomic vessel, ectopic expression of steroidogenic enzymes, pre-maturation of pre-granulosa cells. Furthermore, we discovered that even though the additional deletion of *Wnt4* rescues the coelomic vessel and steroidogenic cells of XY *Sox9* mutants, the XY double mutant gonads develop through a sequence of two phenotypic sex-reversal processes. Initially *Sox9* deficiency leads to male-to-female sex reversal shown by the supporting cells gaining granulosa cell identity and the presence of meiotic germ cells in the posterior region of the gonad; later the granulosa cells in the anterior region experience pre-maturation and trans-differentiation to become Sertoli-like cells, similar to the female-to-male sex reversal of XX *Wnt4* mutants.

By using *Wnt4;Sox9* double knockout mouse model and rethinking on the studies of *Sox9;Rspo1* and *Sox9;Ctnnb1* double mutants, we conclude that *Rspo1/Wnt4/Ctnnb1* and *Sox9* functions are relatively independent in the mutual antagonistic network: *Sox9* is able to directly determine the specification of supporting cells (the presence of *Sox9* determines Sertoli cell identity and the absence of *Sox9* determines granulosa cell identity); *Rspo1/Wnt4/Ctnnb1* are partially responsible for the expression of *Foxl2* but not the main regulators to determine the granulosa cell identity, instead they act through an indirect way to maintain the granulosa cell identity. This finding helps to depict the independent interaction relationship between *Sox9* and *Rspo1/Wnt4/Ctnnb1* in the mutual antagonistic network and also urges to find out the up-stream regulators of *Foxl2*, which will allow us to understand more deeply female sex determination and mutual antagonism between male and female during sex determination.

## Perspectives

### ***Search for the direct targets of RSPO1/WNT4/ $\beta$ -Catenin pathway***

Down-regulation of the well-known direct target of RSPO1/WNT4/ $\beta$ -Catenin pathway *Fst* is responsible for the coelomic vessel formation and germ cell apoptosis, but not for testis cord formation and differentiated Sertoli-like cells (Yao et al., 2004). Along with the development of sequencing technology, sequencing data in the developing gonads around sex determination have been gathered (Stévant and Nef, 2019). Even though the microarray analysis of E12.0, E12.5 and E14.5 XX *Wnt4* mutants and E14.5 XX *Ctnnb1* mutants helped to identify some differentially expressed genes between mutants and control (Coveney et al., 2008; Naillat et al., 2015; Nicol and Yao, 2015), the down-stream effectors of RSPO1/WNT4/ $\beta$ -Catenin pathway for female sex determination are largely unknown. This could be due to the fact that female-to-male sex reversal is a complicated sequential process, which includes multiple steps to achieve the final Sertoli-like cell differentiation. Therefore, it is very important to find a stage when the direct down-stream target changes can be distinguished from the later defects caused by them. We have confirmed that *Rspo1* is required for the female sex determination around E11.5, suggesting that the defect caused by *Rspo1* deletion resulting into female-to-male sex reversal occurs right at this stage. We have chosen to perform RNA-seq analyses on XX E11.5 *Rspo1* mutants to try to find out the direct targets of RSPO1/WNT4/ $\beta$ -Catenin pathway. High quality sequencing data has been obtained for 4 biological replicates of E11.5 XX and XY gonads of control and *Rspo1* mutant genotypes. The bio-informatics analysis is currently ongoing.

### ***Confirm the role of androgens in XX *Ar*;*Rspo1* double mutants***

In our study, we have revealed a major role of androgen signaling pathway in the XX *Rspo1* ovotestis development by pharmacological inhibition of androgen receptor. However, few SOX9/AMH double positive Sertoli-like cells are still observed in the flutamide-treated XX mutants, although the flutamide treatments indeed inhibit the

pre-maturation of granulosa cells and their further trans-differentiation. This could be due to incomplete inhibition by the pharmacological treatment. Indeed, flutamide and more generally the non-steroidal anti-androgens, have a very high specificity for AR but a relatively low affinity (Gauthier, Martel, Labrie 2012; Kolvenbag, Furr, Blackledge, 1998). In order to establish the exact role of androgen signaling in ovotestis development we will adopt a genetic approach. We will build a *Ar;Rspo1* double knockout mouse model, in which the deletion of Androgen receptor will completely block androgen signaling in *Rspo1* mutants (De Gendt et al., 2004).

### ***Sequence of two phenotypic sex-reversal processes in XY Rspo1;Sox9 mutants***

XY *Sox9;Rspo1* double mutant gonads develop into testes at postnatal stages and have well-preserved germ cells entering meiosis in the seminiferous tubules (Lavery et al., 2012). These double mutants gonads are more masculinized than the XY *Sox9;Ctnnb1* and *Wnt4;Sox9* double mutants, however the embryonic XY *Sox9;Rspo1* gonads still look like female gonads morphologically and exhibit *Stra8* expression at E14.5 (Nicol and Yao, 2015). This observation indicates that embryonic XY *Sox9;Rspo1* double mutant gonads probably have a female development interphase. The observation of 1 follicle in rare adult XY *Sox9;Rspo1* testes is consistent with this hypothesis. Therefore, it will be important to confirm whether the sequence of two phenotypic sex-reversal processes also exist in XY *Rspo1;Sox9* double mutants by re-analyzing these double mutants at different embryonic stages.

## CHAPTER V: BIBLIOGRAPHY

Adams, I.R., and McLaren, A. (2002). Sexually dimorphic development of mouse primordial germ cells: Switching from oogenesis to spermatogenesis. *Development*.

Anderson, J.L., Marí, A., Braasch, I., Amores, A., Hohenlohe, P., Batzel, P., and Postlethwait, J.H. (2012). Multiple sex-associated regions and a putative sex chromosome in zebrafish revealed by RAD mapping and population genomics. *PLoS One*.

Aoki, M., Mieda, M., Ikeda, T., Hamada, Y., Nakamura, H., and Okamoto, H. (2007). R-spondin3 is required for mouse placental development. *Dev. Biol.*

Ara, T., Nakamura, Y., Egawa, T., Sugiyama, T., Abe, K., Kishimoto, T., Matsui, Y., and Nagasawa, T. (2003). Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proc. Natl. Acad. Sci.*

Arboleda, V.A., Fleming, A., Barseghyan, H., Délot, E., Sinsheimer, J.S., and Vilain, E. (2014). Regulation of sex determination in mice by a non-coding genomic region. *Genetics*.

Arnold, A.P. (2004). Sex chromosomes and brain gender. *Nat. Rev. Neurosci.*

Arnold, A.P., Chen, X., Link, J.C., Itoh, Y., and Reue, K. (2013). Cell-autonomous sex determination outside of the gonad. *Dev. Dyn.*

Assumpção, J.G., Benedetti, C.E., Maciel-Guerra, A.T., Guerra, G., Baptista, M.T.M., Scolfaro, M.R., and De Mello, M.P. (2002). Novel mutations affecting SRY DNA-binding activity: The HMG box N65H associated with 46,XY pure gonadal dysgenesis and the familial non-HMG box R30I associated with variable phenotypes. *J. Mol. Med.*

Auguste, A., Chassot, A.A., Grégoire, E.P., Renault, L., Pannetier, M., Treier, M., Pailhoux, E., and Chaboissier, M.C. (2012). Loss of R-spondin1 and Foxl2 amplifies female-to-male sex reversal in XX mice. *Sex. Dev.* 5, 304–317.

Bagheri-Fam, S., Sim, H., Bernard, P., Jayakody, I., Taketo, M.M., Scherer, G., and Harley, V.R. (2008). Loss of Fgfr2 leads to partial XY sex reversal. *Dev. Biol.* 314, 71–83.

Bagheri-Fam, S., Bird, A.D., Zhao, L., Ryan, J.M., Yong, M., Wilhelm, D., Koopman, P., Eswarakumar, V.P., and Harley, V.R. (2017). Testis determination requires a specific FGFR2 isoform to repress FOXL2. *Endocrinology* 158, 3832–3843.

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

Bandyopadhyay, M., and Muthuirulan, P. (2017). Androgen Signaling: Promising Target for Prospective Cancer Therapy. *J. Drug Des. Dev.* 1, 1–10.

Banuelos, C.A., Lal, A., Tien, A.H., Shah, N., Yang, Y.C., Mawji, N.R., Meimetis, L.G., Park, J., Kunzhong, J., Andersen, R.J., et al. (2014). Characterization of niphatenones that inhibit androgen receptor n-terminal domain. *PLoS One*.

Barrionuevo, F., Bagheri-Fam, S., Klattig, J., Kist, R., Taketo, M.M., Englert, C., and Scherer, G. (2005). Homozygous Inactivation of Sox9 Causes Complete XY Sex Reversal in Mice1. *Biol. Reprod.* 74, 195–201.

Barrionuevo, F., Georg, I., Scherthan, H., Lécureuil, C., Guillou, F., Wegner, M., and Scherer, G. (2009). Testis cord differentiation after the sex determination stage is independent of Sox9 but fails in the combined absence of Sox9 and Sox8. *Dev. Biol.* 327, 301–312.

Barrionuevo, F.J., Hurtado, A., Kim, G.-J., Real, F.M., Bakkali, M., Kopp, J.L., Sander, M., Scherer, G., Burgos, M., and Jiménez, R. (2016). Sox9 and Sox8 protect the adult testis from male-to-female genetic reprogramming and complete degeneration. *Elife* 5.

Barsoum, I.B., Bingham, N.C., Parker, K.L., Jorgensen, J.S., and Yao, H.H.C. (2009). Activation of the Hedgehog pathway in the mouse fetal ovary leads to ectopic appearance of fetal Leydig cells and female pseudohermaphroditism. *Dev. Biol.* 329, 96–103.

Behringer, R.R., Finegold, M.J., and Cate, R.L. (1994). Müllerian-inhibiting substance function during mammalian sexual development. *Cell*.

Bell, S.M., Schreiner, C.M., Wert, S.E., Mucenski, M.L., Scott, W.J., and Whitsett, J.A. (2008). R-spondin 2 is required for normal laryngeal-tracheal, lung and limb morphogenesis. *Development*.

Berta, P., Hawkins, J.B., Sinclair, A.H., Taylor, A., Griffiths, B.L., Goodfellow, P.N., and Fellous, M. (1990). Genetic evidence equating SRY and the testis-determining factor. *Nature*.

Biason-Lauber, A., Konrad, D., Navratil, F., and Schoenle, E.J. (2004). A WNT4 Mutation Associated with Müllerian-Duct Regression and Virilization in a 46,XX Woman. *N. Engl. J. Med.*

Biason-Lauber, A., Konrad, D., Meyer, M., deBeaufort, C., and Schoenle, E.J. (2009). Ovaries and Female Phenotype in a Girl with 46,XY Karyotype and Mutations in the CBX2 Gene. *Am. J. Hum. Genet.*

Bilić, J., Huang, Y.L., Davidson, G., Zimmermann, T., Cruciat, C.M., Bienz, M., and Niehrs, C. (2007). Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science* (80- ).

Bingham, N.C., Verma-Kurvari, S., Parada, L.F., and Parker, K.L. (2006). Development of a steroidogenic factor 1/Cre transgenic mouse line. *Genesis* 44, 419–424.

Birk, O.S., Caslano, D.E., Wassif, C.A., Cogilat, T., Zhaos, L., Zhao, Y., Grinberg, A., Huang, S.P., Kreidberg, J.A., Parker, K.L., et al. (2000). The LIM homeobox gene *Lhx9* is essential for mouse gonad formation. *Nature*.

Bitgood, M.J., Shen, L., and McMahon, A.P. (1996). Sertoli cell signaling by Desert hedgehog regulates the male germline. *Curr. Biol.*

Blaydon, D.C., Ishii, Y., O'Toole, E.A., Unsworth, H.C., Teh, M.T., Rüschen-dorf, F., Sinclair, C., Hopsu-Havu, V.K., Tidman, N., Moss, C., et al. (2006). The gene encoding R-spondin 4 (RSPO4), a secreted protein implicated in Wnt signaling, is mutated in inherited anonychia. *Nat. Genet.*

Bogani, D., Siggers, P., Brixey, R., Warr, N., Beddow, S., Edwards, J., Williams, D., Wilhelm, D., Koopman, P., Flavell, R.A., et al. (2009). Loss of mitogen-activated protein kinase kinase kinase 4 (MAP3K4) reveals a requirement for MAPK signalling in mouse sex determination. *PLoS Biol.*

Bogatcheva, N. V., and Agoul-nik, A.I. (2005). INSL3/LGR8 role in testicular descent and cryptorchidism. *Reprod. Biomed. Online*.

Boulanger, L., Pannetier, M., Gall, L., Allais-Bonnet, A., Elzaïat, M., Le Bourhis, D., Daniel, N., Richard, C., Cotinot, C., Ghyselinck, N.B., et al. (2014). FOXL2 Is a Female Sex-Determining Gene in the Goat. *Curr. Biol.* 24, 404–408.

Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsak-sophak, K., Wilson, M.J., Rossant, J., et al. (2006). Retinoid signaling determines germ cell fate in mice. *Science* (80- ).

Bowles, J., Feng, C.W., Spiller, C., Davidson, T.L., Jackson, A., and Koopman, P. (2010). FGF9 suppresses meiosis and promotes male germ cell fate in mice. *Dev. Cell*.

Bradley, K.M., Breyer, J.P., Melville, D.B., Broman, K.W., Knapik, E.W., and Smith, J.R. (2011). An SNP-Based Linkage Map for Zebrafish Reveals Sex Determination Loci. *G3&#amp;#58; Genes|Genomes|Genetics*.

Brambell, F.W.R. (2006). The Development and Morphology of the Gonads of the Mouse.--Part I. The Morphogenesis of the Indifferent Gonad and of the Ovary. *Proc. R. Soc. B Biol. Sci.*

Brennan, J., Tilmann, C., and Capel, B. (2003). Pdgfr- $\alpha$  mediates testis cord organization and fetal Leydig cell development in the XY gonad. *Genes Dev.* 17, 800–810.

Bull, J.J. (1980). Sex Determination in Reptiles. *Q. Rev. Biol.* 55, 3–21.

Bullejos, M., and Koopman, P. (2001). Spatially Dynamic Expression of Sry in Mouse Genital Ridges.

Burger, H.G. (2002). Androgen Production in Women. 77, 4–6.

Cadiou, E., Neff, M.W., Quignon, P., Walsh, K., Chase, K., Parker, H.G., Vonholdt, B.M., Rhue, A., Boyko, A.B., Byers, A., et al. (2009). Coat variation in the domestic dog is governed by variants in three genes. *Science* (80- ).

Cadigan, K.M., and Waterman, M.L. (2012). TCF/LEFs and Wnt signaling in the nucleus. *Cold Spring Harb. Perspect. Biol.*

Callier, P., Calvel, P., Matevossian, A., Makrythanasis, P., Bernard, P., Kurosaka, H., Vannier, A., Thauvin-Robinet, C., Borel, C., Mazaud-Guittot, S., et al. (2014). Loss of Function Mutation in the Palmitoyl-Transferase HHAT Leads to Syndromic 46,XY Disorder of Sex Development by Impeding Hedgehog Protein Palmitoylation and Signaling. *PLoS Genet.*

Cambier, L., Plate, M., Sucov, H.M., and Pashmforoush, M. (2014). Nkx2-5 regulates cardiac growth through modulation of Wnt signaling by R-spondin3. *Development.*

Canto, P., Escudero, I., Söderlund, D., Nishimura, E., Carranza-Lira, S., Gutierrez, J., Nava, A., and Mendez, J.P. (2003). A novel mutation of the insulin-like 3 gene in patients with cryptorchidism. *J. Hum. Genet.*

Canto, P., Söderlund, D., Reyes, E., and Méndez, J.P. (2004). Mutations in the Desert hedgehog (DHH) gene in patients with 46,XY complete pure gonadal dysgenesis. *J. Clin. Endocrinol. Metab.*

Canto, P., Vilchis, F., Söderlund, D., Reyes, E., and Méndez, J.P. (2005). A heterozygous mutation in the desert hedgehog gene in patients with mixed gonadal dysgenesis. *Mol. Hum. Reprod.*

Capel, B. (2017). Vertebrate sex determination: Evolutionary plasticity of a fundamental switch. *Nat. Rev. Genet.* 18, 675–689.

Carmon, K.S., Gong, X., Lin, Q., Thomas, A., and Liu, Q. (2011). R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/ -catenin signaling. *Proc. Natl. Acad. Sci.*

Carré, G.A., Siggers, P., Xipolita, M., Brindle, P., Lutz, B., Wells, S., and Greenfield, A. (2018). Loss of p300 and CBP disrupts histone acetylation at the mouse Sry promoter and causes XY gonadal sex reversal. *Hum. Mol. Genet.* 27, 190–198.

Cattanach, B.M., Iddon, C.A., Charlton, H.M., Chiappa, S.A., and Fink, G. (1977). Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature.*

Chaboissier, M.-C. (2004). Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development* 131, 1891–1901.

Chadi, S., Buscara, L., Pechoux, C., Costa, J., Laubier, J., Chaboissier, M.C., Pailhoux, E., Vilotte, J.L., Chanat, E., and Le Provost, F. (2009). R-spondin1 is required for normal epithelial morphogenesis during mammary gland development. *Biochem. Biophys. Res. Commun.*

Chapman, C., Cree, L., and Shelling, A.N. (2015). The genetics of premature ovarian failure: Current perspectives. *Int. J. Womens. Health.*

Chassot, A.-A., Bradford, S.T., Auguste, A., Gregoire, E.P., Pailhoux, E., de Rooij, D.G., Schedl, A., and Chaboissier, M.-C. (2012). WNT4 and RSPO1 together are required for cell proliferation in the early mouse gonad. *Development* 139, 4461–4472.

Chassot, A.A., Ranc, F., Gregoire, E.P., Roepers-Gajadien, H.L., Taketo, M.M., Camerino, G., de Rooij, D.G., Schedl, A., and Chaboissier, M.C. (2008a). Activation of  $\beta$ -catenin signaling by *Rspo1* controls differentiation of the mammalian ovary. *Hum. Mol. Genet.* 17, 1264–1277.

Chassot, A.A., Gregoire, E.P., Magliano, M., Lavery, R., and Chaboissier, M.C. (2008b). Genetics of ovarian differentiation: *Rspo1*, a major player. In *Sexual Development*, pp. 219–227.

Chassot, A.A., Gregoire, E.P., Lavery, R., Taketo, M.M., de Rooij, D.G., Adams, I.R., and Chaboissier, M.C. (2011). RSPO1/ $\beta$ -Catenin signaling pathway regulates oogonia differentiation and entry into meiosis in the mouse fetal ovary. *PLoS One* 6.

Chassot, A.A., Gillot, I., and Chaboissier, M.C. (2014). R-spondin1, WNT4, and the *ctnbn1* signaling pathway: Strict control over ovarian differentiation. *Reproduction* 148, R97–R110.

Chawengsaksophak, K., Svingen, T., Ng, E.T., Epp, T., Spiller, C.M., Clark, C., Cooper, H., and Koopman, P. (2011). Loss of *Wnt5a* Disrupts Primordial Germ Cell Migration and Male Sexual Development in Mice. *Biol. Reprod.*

Chen, H., Palmer, J.S., Thiagarajan, R.D., Dinger, M.E., Lesieur, E., Chiu, H., Schulz, A., Spiller, C., Grimmond, S.M., Little, M.H., et al. (2012). Identification of novel markers of mouse fetal ovary development. *PLoS One* 7.

Chen, M., Zhang, L., Cui, X., Lin, X., Li, Y., Wang, Y., Wang, Y., Qin, Y., Chen, D., Han, C., et al. (2017). *Wt1* directs the lineage specification of sertoli and granulosa cells by repressing *Sf1* expression. *Development*.

Chen, Y., Thai, H.T.T., Lundin, J., Lagerstedt-Robinson, K., Zhao, S., Markljung, E., and Nordenskjöld, A. (2010). Mutational study of the MAMLD1-gene in hypospadias. *Eur. J. Med. Genet.*

Chuma, S. (2004). Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. *Development*.

De Cian, M.-C., Pauper, E., Bandiera, R., Vidal, V.P.I., Sacco, S., Gregoire, E.P., Chassot, A.-A., Panzolini, C., Wilhelm, D., Pailhoux, E., et al. (2017). Amplification of R-spondin1 signaling induces granulosa cell fate defects and cancers in mouse adult ovary. *Oncogene* 36, 208–218.

Clayton, P.E., Miller, W.L., Oberfield, S.E., Ritzén, E.M., Sippell, W.G., Speiser, P.W., Hintz, R.L., and Savage, M.O. (2002). Consensus statement on 21-hydroxylase deficiency from the European Society for Paediatric Endocrinology and the Lawson Wilkins Pediatric Endocrine Society. *Horm. Res.*

Cnaani, A., Lee, B.Y., Zilberman, N., Ozouf-Costaz, C., Hulata, G., Ron, M., D'Hont, A., Baroiller, J.F., D'Cotta, H., Penman, D.J., et al. (2008). Genetics of sex determination in tilapiine species. *Sex. Dev.*

Coenen, C.M., Thomas, C.M., Borm, G.F., and Rolland, R. (1995). Comparative evaluation of the androgenicity of four low-dose, fixed-combination oral contraceptives. *Int. J. Fertil. Menopausal Stud.*

Colvin, J.S., Green, R.P., Schmahl, J., Capel, B., and Ornitz, D.M. (2001). Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell*.

Conte, F.A., Grumbach, M.M., Ito, Y., Fisher, C.R., and Simpson, E.R. (1994). A syndrome of female pseudohermaphroditism, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase

(P450arom). *J. Clin. Endocrinol. Metab.*

Cool, J., Carmona, F.D., Szucsik, J.C., and Capel, B. (2008). Peritubular myoid cells are not the migrating population required for testis cord formation in the XY gonad. *Sex. Dev.*

Couse, J.F., Curtis Hewitt, S., Bunch, D.O., Sar, M., Walker, V.R., Davis, B.J., and Korach, K.S. (1999). Postnatal sex reversal of the ovaries in mice lacking estrogen receptors  $\alpha$  and  $\beta$ . *Science* (80- ).

Coveney, D., Ross, A.J., Slone, J.D., and Capel, B. (2008). A microarray analysis of the XX Wnt4 mutant gonad targeted at the identification of genes involved in testis vascular differentiation (DOI:10.1016/j.modgep.2006.05.012). *Gene Expr. Patterns* 8, 529–537.

Crisponi, L., Deiana, M., Loi, A., Chiappe, F., Uda, M., Amati, P., Bisceglia, L., Zelante, L., Nagaraja, R., Porcu, S., et al. (2001). The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nat. Genet.*

Cui, S. (2004). Disrupted gonadogenesis and male-to-female sex reversal in Pod1 knockout mice. *Development.*

Das, D.K., Sanghavi, D., Gawde, H., Idicula-Thomas, S., and Vasudevan, L. (2011). Novel homozygous mutations in Desert Hedgehog gene in patients with 46,XY complete gonadal dysgenesis and prediction of its structural and functional implications by computational methods. *Eur. J. Med. Genet.*

Davey, R.A., and Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *Clin. Biochem. Rev.*

DeFalco, T., Takahashi, S., and Capel, B. (2011). Two distinct origins for Leydig cell progenitors in the fetal testis. *Dev. Biol.* 352, 14–26.

Délot, E.C., and Vilain, E. (2018). Disorders of Sex Development. In Yen & Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management: Eighth Edition, (Elsevier Inc.), pp. 365-393.e5.

Délot, E.C., and Vilain, E.J. (1993). Nonsyndromic 46,XX Testicular Disorders of Sex Development.

DiTacchio, L., Bowles, J., Shin, S., Lim, D.S., Koopman, P., and Janknecht, R. (2012). Transcription factors ER71/ETV2 and SOX9 participate in a positive feedback loop in fetal and adult mouse testis. *J. Biol. Chem.*

Domenice, S., Correa, R. V., Costa, E.M.F., Nishi, M.Y., Vilain, E., Arnhold, I.J.P., and Mendonca, B.B. (2004). Mutations in the SRY, DAX1, SF1 and WNT4 genes in Brazilian sex-reversed patients. *Brazilian J. Med. Biol. Res.*

Dong, J., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu, N., and Matzuk, M.M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature.*

Dranow, D.B., Tucker, R.P., and Draper, B.W. (2013). Germ cells are required to maintain a stable sexual phenotype in adult zebrafish. *Dev. Biol.*

Dreger, A.D., Chase, C., Sousa, A., Gruppuso, P.A., and Frader, J. (2005). Changing the nomenclature/taxonomy for intersex: A scientific and clinical rationale. *J. Pediatr. Endocrinol. Metab.*

van den Driesche, S., Walker, M., McKinnell, C., Scott, H.M., Eddie, S.L., Mitchell, R.T., Seckl, J.R., Drake, A.J., Smith, L.B., Anderson, R.A., et al. (2012). Proposed role for COUP-TFII in regulating fetal leydig cell steroidogenesis, perturbation of which leads to masculinization disorders in rodents. *PLoS One.*

Durlinger, A.L.L., Grijters, M.J.G., Kramer, P., Karels, B., Ingraham, H.A., Nachtigal, M.W., Uilenbroek, J.T.J., Grootegoed, J.A., and Themmen, A.P.N. (2002). Anti-Müllerian

Hormone Inhibits Initiation of Primordial Follicle Growth in the Mouse Ovary. *Endocrinology* 143, 1076–1084.

Dutta, S., Mark-Kappeler, C.J., Hoyer, P.B., and Pepling, M.E. (2014). The Steroid Hormone Environment During Primordial Follicle Formation in Perinatal Mouse Ovaries. *Biol. Reprod.*

Dzementsei, A., and Pieler, T. (2014). Primordial Germ Cell Migration. In *Xenopus Development*, p.

Eggers, S., Sadedin, S., van den Bergen, J.A., Robevska, G., Ohnesorg, T., Hewitt, J., Lambeth, L., Bouty, A., Knarston, I.M., Tan, T.Y., et al. (2016). Disorders of sex development: Insights from targeted gene sequencing of a large international patient cohort. *Genome Biol.*

Eicher, E.M., Shown, E.P., and Washburn, L.L. (1995). Sex reversal in C57BL/6J-YPOS mice corrected by a Sry transgene. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 350.

Ezaz, T., Stiglec, R., Veyrunes, F., and Marshall Graves, J.A. (2006a). Relationships between Vertebrate ZW and XY Sex Chromosome Systems. *Curr. Biol.*

Ezaz, T., Stiglec, R., Veyrunes, F., and Marshall Graves, J.A. (2006b). Relationships between Vertebrate ZW and XY Sex Chromosome Systems. *Curr. Biol.* 16, R736–R743.

Ezaz, T., Quinn, A.E., Sarre, S.D., O’Meally, D., Georges, A., and Marshall Graves, J.A. (2009). Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards. *Chromosom. Res.*

Falah, N., Posey, J.E., Thorson, W., Benke, P., Tekin, M., Tarshish, B., Lupski, J.R., and Harel, T. (2017). 22q11.2q13 duplication including SOX10 causes sex-reversal and peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and Hirschsprung disease. *Am. J. Med. Genet. Part A.*

Fishelson, L. (1970). Protogynous sex reversal in the fish anthias squamipinnis (Teleostei, Anthiidae) regulated by the presence or absence of a male fish. *Nature.*

Fisher, C.R., Graves, K.H., Parlow, A.F., and Simpson, E.R. (1998). Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proc. Natl. Acad. Sci.*

Ford, C.E., Jones, K.W., Polani, P.E., De Almeida, J.C., and Briggs, J.H. (1959). A SEX-CHROMOSOME ANOMALY IN A CASE OF GONADAL DYSGENESIS (TURNER’S SYNDROME). *Lancet.*

Fowler, P.A., Anderson, R.A., Saunders, P.T., Kinnell, H., Mason, J.I., Evans, D.B., Bhattacharya, S., Flannigan, S., Franks, S., Monteiro, A., et al. (2011). Development of steroid signaling pathways during primordial follicle formation in the human fetal ovary. *J. Clin. Endocrinol. Metab.*

Fujimoto, Y., Tanaka, S.S., Yamaguchi, Y.L., Kobayashi, H., Kuroki, S., Tachibana, M., Shinomura, M., Kanai, Y., Morohashi, K. ichirou, Kawakami, K., et al. (2013). Homeoproteins Six1 and Six4 regulate male sex determination and mouse gonadal development. *Dev. Cell.*

Fukami, M., Wada, Y., Miyabayashi, K., Nishino, I., Hasegawa, T., Camerino, G., Kretz, C., Buj-Bello, A., Laporte, J., Yamada, G., et al. (2006). CXorf6 is a causative gene for hypospadias. *Nat. Genet.*

Galli-Tsinopoulou, A., Hiort, O., Schuster, T., Messer, G., and Kuhnle, U. (2003). A novel point mutation in the hormone binding domain of the androgen receptor associated with partial and minimal androgen insensitivity syndrome. *J. Pediatr. Endocrinol. Metab.*

Garcia-Moreno, S.A., Futtner, C.R., Salamone, I.M., Gonen, N., Lovell-Badge, R., and Maatouk, D.M. (2019a). Gonadal supporting cells acquire sex-specific chromatin landscapes during mammalian sex determination. *Dev. Biol.* 446, 168–179.

Garcia-Moreno, S.A., Lin, Y.-T., Futtner, C.R., Salamone, I.M., Capel, B., and Maatouk, D.M. (2019b). CBX2 is required to stabilize the testis pathway by repressing Wnt signaling. *PLOS Genet.* *15*, e1007895.

Garzo, V.G., and Dorrington, J.H. (1984). Aromatase activity in human granulosa cells during follicular development and the modulation by follicle-stimulating hormone and insulin. *Am. J. Obstet. Gynecol.*

Ge, C., Ye, J., Weber, C., Sun, W., Zhang, H., Zhou, Y., Cai, C., Qian, G., and Capel, B. (2018). The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species. *Science* (80-. ). *360*, 645–648.

Geissler, W.M., Davis, D.L., Wu, L., Bradshaw, K.D., Patel, S., Mendonca, B.B., Elliston, K.O., Wilson, J.D., Russell, D.W., and Andersson, S. (1994). Male pseudohermaphroditism caused by mutations of testicular 17 $\beta$ -hydroxysteroid dehydrogenase 3. *Nat. Genet.*

De Gendt, K., Swinnen, J. V., Saunders, P.T.K., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lecureuil, C., et al. (2004). A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc. Natl. Acad. Sci.* *101*, 1327–1332.

Georg, I., Barrionuevo, F., Wiech, T., and Scherer, G. (2012). Sox9 and Sox8 Are Required for Basal Lamina Integrity of Testis Cords and for Suppression of FOXL2 During Embryonic Testis Development in Mice<sup>1</sup>. *Biol. Reprod.* *87*.

Gill, M.E., Hu, Y.-C., Lin, Y., and Page, D.C. (2011). Licensing of gametogenesis, dependent on RNA binding protein DAZL, as a gateway to sexual differentiation of fetal germ cells. *Proc. Natl. Acad. Sci.* *108*, 7443–7448.

Ginsburg, M., Snow, M.H., and McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development.*

Glinka, A., Dolde, C., Kirsch, N., Huang, Y.L., Kazanskaya, O., Ingelfinger, D., Boutros, M., Cruciat, C.M., and Niehrs, C. (2011). LGR4 and LGR5 are R-spondin receptors mediating Wnt/ $\beta$ -catenin and Wnt/PCP signalling. *EMBO Rep.*

Gonen, N., and Lovell-Badge, R. (2019). The regulation of Sox9 expression in the gonad. *Curr. Top. Dev. Biol.* *134*, 223–252.

Gonen, N., Quinn, A., O'Neill, H.C., Koopman, P., and Lovell-Badge, R. (2017). Normal Levels of Sox9 Expression in the Developing Mouse Testis Depend on the TES/TESCO Enhancer, but This Does Not Act Alone. *PLoS Genet.*

Gonen, N., Futtner, C.R., Wood, S., Alexandra Garcia-Moreno, S., Salamone, I.M., Samson, S.C., Sekido, R., Poulat, F., Maatouk, D.M., and Lovell-Badge, R. (2018). Sex reversal following deletion of a single distal enhancer of Sox9. *Science* (80-. ). *360*, 1469–1471.

Granados, A., Alaniz, V.I., Mohnach, L., Barseghyan, H., Vilain, E., Ostrer, H., Quint, E.H., Chen, M., and Keegan, C.E. (2017). MAP3K1-related gonadal dysgenesis: Six new cases and review of the literature. *Am. J. Med. Genet. Part C Semin. Med. Genet.*

Graves, J.A.M. (2016). Evolution of vertebrate sex chromosomes and dosage compensation. *Nat. Rev. Genet.*

Gregoire, E.P., Lavery, R., Chassot, A.A., Akiyama, H., Treier, M., Behringer, R.R., and Chaboissier, M.C. (2011). Transient development of ovotestes in XX Sox9 transgenic mice. *Dev. Biol.* *349*, 65–77.

Gregoire, E.P., Stevant, I., Chassot, A.A., Martin, L., Lachambre, S., Mondin, M., de Rooij, D.G., Nef, S., and Chaboissier, M.C. (2018). NRG1 signalling regulates the establishment of Sertoli cell stock in the mouse testis. *Mol. Cell. Endocrinol.* *478*, 17–31.

Gu, Y., Runyan, C., Shoemaker, A., Surani, A., and Wylie, C. (2009). Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. *Development*.

Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Münsterberg, A., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature*.

Guha, C., and Saha, S. (2016). Therapy for radiation-induced lung injury (U.S.).

Guigon, C.J., and Magre, S. (2005). Contribution of Germ Cells to the Differentiation and Maturation of the Ovary: Insights from Models of Germ Cell Depletion. *Biol. Reprod*.

Guigon, C.J., Coudouel, N., Mazaud-Guittot, S., Forest, M.G., and Magre, S. (2005). Follicular cells acquire sertoli cell characteristics after oocyte loss. *Endocrinology*.

Gustin, S.E., Hogg, K., Stringer, J.M., Rastetter, R.H., Pelosi, E., Miles, D.C., Sinclair, A.H., Wilhelm, D., and Western, P.S. (2016). WNT/ $\beta$ -catenin and p27/FOXL2 differentially regulate supporting cell proliferation in the developing ovary. *Dev. Biol.* 412, 250–260.

Hacker, A., Capel, B., Goodfellow, P., and Lovell-Badge, R. (1995). Expression of Sry, the mouse sex determining gene. *Development* 121, 1603–1614.

Hammes, A., Guo, J.-K., Lutsch, G., Leheste, J.-R., Landrock, D., Ziegler, U., Gubler, M.-C., and Schedl, A. (2001). Two Splice Variants of the Wilms' Tumor 1 Gene Have Distinct Functions during Sex Determination and Nephron Formation.

Han, X.H., Jin, Y.R., Seto, M., and Yoon, J.K. (2011). A WNT/ $\beta$ -catenin signaling activator, R-spondin, plays positive regulatory roles during skeletal myogenesis. *J. Biol. Chem.*

Hanley, N., Hagan, D., Clement-Jones, M., Ball, S., Strachan, T., Salas-Cortés, L., McElreavey, K., Lindsay, S., Robson, S., Bullen, P., et al. (2000). SRY, SOX9, and DAX1 expression patterns during human sex determination and gonadal development. *Mech. Dev.* 91, 403–407.

Hao, H.X., Xie, Y., Zhang, Y., Zhang, O., Oster, E., Avello, M., Lei, H., Mickanin, C., Liu, D., Ruffner, H., et al. (2012). ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature*.

Harikae, K., Miura, K., and Kanai, Y. (2013a). Early gonadogenesis in mammals: Significance of long and narrow gonadal structure. *Dev. Dyn. Mamm. Embryog. Gonadal Primordium Arises from Thick. Coelomic Ep. Which Results a Pair Extrem. Long Narrow Gonadal Struct. along Anteroposterior Axis.* 242, 330–338.

Harikae, K., Miura, K., Shinomura, M., Matoba, S., Hiramatsu, R., Tsunekawa, N., Kanai-Azuma, M., Kurohmaru, M., Morohashi, K. -i., and Kanai, Y. (2013b). Heterogeneity in sexual bipotentiality and plasticity of granulosa cells in developing mouse ovaries. *J. Cell Sci.* 126, 2834–2844.

Harris, S.E. (2002). Identification of novel mutations in FOXL2 associated with premature ovarian failure. *Mol. Hum. Reprod*.

Harris, A., Siggers, P., Corrochano, S., Warr, N., Sagar, D., Grimes, D.T., Suzuki, M., Burdine, R.D., Cong, F., Koo, B.-K., et al. (2018). ZNRF3 functions in mammalian sex determination by inhibiting canonical WNT signaling. *Proc. Natl. Acad. Sci. U. S. A.* 115, 5474–5479.

Hassold, T., Hall, H., and Hunt, P. (2007). The origin of human aneuploidy: Where we have been, where we are going. *Hum. Mol. Genet.*

Hatano, O., Takakusu, A., Nomura, M., and Morohashi, K.I. (1996). Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. *Genes to Cells*.

Hayashi, S., Lewis, P., Pevny, L., and McMahon, A.P. (2003). Erratum: Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain (*Gene Expression Patterns* (2002) vol. 2 (93-97)). *Gene Expr. Patterns* 3, 542–543.

Heikkilä, M., Peltoketo, H., Leppäluoto, J., Ilves, M., Vuolteenaho, O., and Vainio, S. (2002). Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. *Endocrinology* 143, 4358–4365.

Heikkilä, M., Prunskaitė, R., Naillat, F., Itäranta, P., Vuoristo, J., Leppäluoto, J., Peltoketo, H., and Vainio, S. (2005). The partial female-to-male sex reversal in Wnt-4 deficient females involves induced expression of testosterone biosynthetic genes and testosterone production and depends on androgen action. *Endocrinology* 146, 4016–4023.

Hiramatsu, R., Harikae, K., Tsunekawa, N., Kurohmaru, M., Matsuo, I., and Kanai, Y. (2009a). FGF signaling directs a center-to-pole expansion of tubulogenesis in mouse testis differentiation. *Development* 137, 303–312.

Hiramatsu, R., Matoba, S., Kanai-Azuma, M., Tsunekawa, N., Katoh-Fukui, Y., Kurohmaru, M., Morohashi, K.I., Wilhelm, D., Koopman, P., and Kanai, Y. (2009b). A critical time window of Sry action in gonadal sex determination in mice. *Development*.

Hogarth, C.A., and Griswold, M.D. (2010). The key role of vitamin A in spermatogenesis. *J. Clin. Invest.* 120, 956–962.

Holleley, C.E., O’Meally, D., Sarre, S.D., Marshall Graves, J.A., Ezaz, T., Matsubara, K., Azad, B., Zhang, X., and Georges, A. (2015). Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. *Nature*.

Holleley, C.E., Sarre, S.D., O’Meally, D., and Georges, A. (2016). Sex Reversal in Reptiles: Reproductive Oddity or Powerful Driver of Evolutionary Change? *Sex. Dev.*

Honda, A., Hirose, M., Hara, K., Matoba, S., Inoue, K., Miki, H., Hiura, H., Kanatsu-Shinohara, M., Kanai, Y., Kono, T., et al. (2007). Isolation, characterization, and in vitro and in vivo differentiation of putative thecal stem cells. *Proc. Natl. Acad. Sci.*

Hsu, M.-K., Wu, I.-C., Cheng, C.-C., Su, J.-L., Hsieh, C.-H., Lin, Y.-S., and Chen, F.-C. (2015). Triple-layer dissection of the lung adenocarcinoma transcriptome; regulation at the gene, transcript, and exon levels. *Oncotarget*.

Hu, Y.C., Okumura, L.M., and Page, D.C. (2013). Gata4 Is Required for Formation of the Genital Ridge in Mice. *PLoS Genet*.

Huch, M., Dorrell, C., Boj, S.F., Van Es, J.H., Li, V.S.W., Van De Wetering, M., Sato, T., Hamer, K., Sasaki, N., Finegold, M.J., et al. (2013). In vitro expansion of single Lgr5 + liver stem cells induced by Wnt-driven regeneration. *Nature*.

Hudson, Q.J., Smith, C.A., and Sinclair, A.H. (2005). Aromatase inhibition reduces expression of FOXL2 in the embryonic chicken ovary. *Dev. Dyn.*

Huelsken, J., Vogel, R., Brinkmann, V., Erdmann, B., Birchmeier, C., and Birchmeier, W. (2000). Requirement for  $\beta$ -Catenin in Anterior-Posterior Axis Formation in Mice. *J. Cell Biol.* 148, 567.

Hunt, P.A., Worthman, C., Levinson, H., Stallings, J., LeMaire, R., Mroz, K., Park, C., and Handel, M.A. (1998). Germ cell loss in the XXY male mouse: Altered X-chromosome dosage affects prenatal development. *Mol. Reprod. Dev.* 49, 101–111.

Idkowiak, J., Randell, T., Dhir, V., Patel, P., Shackleton, C.H.L., Taylor, N.F., Krone, N., and Arlt, W. (2012). A Missense mutation in the human cytochrome b5 gene causes 46,XY disorder of sex development due to true isolated 17,20 lyase deficiency. *J. Clin. Endocrinol. Metab.*

Ikeda, Y., Shen, W.H., Ingraham, H.A., and Parker, K.L. (1994). Developmental

expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. *Mol. Endocrinol.*

Ikedo, Y., Shen, W.H., Ingraham, H.A., and Parker, K.L. (2014). Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. *Mol. Endocrinol.*

Inoue, M., Baba, T., and Morohashi, K. (2018). Recent progress in understanding the mechanisms of Leydig cell differentiation. *Mol. Cell. Endocrinol.* 468, 39–46.

Ishii, Y., Wajid, M., Bazzi, H., Fantauzzo, K.A., Barber, A.G., Blaydon, D.C., Nam, J.S., Yoon, J.K., Kelsell, D.P., and Christiano, A.M. (2008). Mutations in R-spondin 4 (RSPO4) underlie inherited anonychia. *J. Invest. Dermatol.*

JACOBS, P.A., and STRONG, J.A. (1959). A case of human intersexuality having a possible XXY sex-determining mechanism. *Nature* 183, 302–303.

Jameson, S.A., Lin, Y.T., and Capel, B. (2012a). Testis development requires the repression of Wnt4 by Fgf signaling. *Dev. Biol.* 370, 24–32.

Jameson, S.A., Natarajan, A., Cool, J., DeFalco, T., Maatouk, D.M., Mork, L., Munger, S.C., and Capel, B. (2012b). Temporal transcriptional profiling of somatic and germ cells reveals biased lineage priming of sexual fate in the fetal mouse gonad. *PLoS Genet.* 8.

Janssen, K.P., Alberici, P., Fsihi, H., Gaspar, C., Breukel, C., Franken, P., Rosty, C., Abal, M., El Marjou, F., Smits, R., et al. (2006). APC and Oncogenic KRAS Are Synergistic in Enhancing Wnt Signaling in Intestinal Tumor Formation and Progression. *Gastroenterology*.

Jeays-Ward, K. (2003). Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. *Development* 130, 3663–3670.

Jeays-Ward, K., Dandonneau, M., and Swain, A. (2004). Wnt4 is required for proper male as well as female sexual development. *Dev. Biol.* 276, 431–440.

Jimenez, R., Sanchez, A., Burgos, M., and Diaz De La Guardia, R. (1996). Puzzling out the genetics of mammalian sex determination. *Trends Genet.*

Jiménez, R., Burgos, M., Sánchez, A., Sinclair, A., Alarcón, F., Marín, J., Ortega, E., and Díaz de la Guardia, R. (1993). Fertile females of the mole *Talpa occidentalis* are phenotypic intersexes with ovotestes. *Development*.

Jiménez, R., Barrionuevo, F.J., and Burgos, M. (2013). Natural exceptions to normal gonad development in mammals. *Sex. Dev.*

Jin, Y.R., and Yoon, J.K. (2012). The R-spondin family of proteins: Emerging regulators of WNT signaling. *Int. J. Biochem. Cell Biol.*

Jordan, B.K., Mohammed, M., Ching, S.T., Délot, E., Chen, X.N., Dewing, P., Swain, A., Rao, P.N., Elejalde, B.R., and Vilain, E. (2001). Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans. *Am. J. Hum. Genet.* 68, 1102–1109.

Joshi, R., and Dunaif, A. (2018). Ovarian Disorders of Pregnancy. *Endocrinol. Metab. Clin. North Am.*

Josso, N., Di Clemente, N., and Gouédard, L. (2001). Anti-Müllerian hormone and its receptors. *Mol. Cell. Endocrinol.*

Jost, P.A. (1948). Le controle hormonal de la différenciation du sexe. *Biol. Rev.*

Jost, P.A. (1953). Problems of fetal endocrinology: The gonadal and hypophyseal hormones. *Recent Progr Horm. Res* 8, 379–413.

Kalfa, N., Fukami, M., Philibert, P., Audran, F., Pienkowski, C., Weill, J., Pinto, G., Manouvrier, S., Polak, M., Ogata, T., et al. (2012). Screening of *mamld1* mutations in 70

children with 46,xy dsd: Identification and functional analysis of two new mutations. *PLoS One*.

Kamata, T., Katsube, K.I., Michikawa, M., Yamada, M., Takada, S., and Mizusawa, H. (2004). R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. *Biochim. Biophys. Acta - Gene Struct. Expr.*

Kang, Y.E., Kim, J.M., Yi, H.-S., Joung, K.H., Lee, J.H., Kim, H.J., and Ku, B.J. (2019). Serum R-Spondin 1 Is a New Surrogate Marker for Obesity and Insulin Resistance. *Diabetes Metab. J.*

Karl, J., and Capel, B. (1998). Sertoli cells of the mouse testis originate from the coelomic epithelium. *Dev. Biol.* 203, 323–333.

Kashimada, K., Pelosi, E., Chen, H., Schlessinger, D., Wilhelm, D., and Koopman, P. (2011). FOXL2 and BMP2 act cooperatively to regulate follistatin gene expression during ovarian development. *Endocrinology* 152, 272–280.

Katoh-Fukui, Y., Miyabayashi, K., Komatsu, T., Owaki, A., Baba, T., Shima, Y., Kidokoro, T., Kanai, Y., Schedl, A., Wilhelm, D., et al. (2012). Cbx2, a polycomb group gene, is required for Sry gene expression in mice. *Endocrinology*.

Katoh-Fukui, Y., Tsuchiya, R., Shiroishi, T., Nakahara, Y., Hashimoto, N., Noguchi, K., and Higashinakagawa, T. (1998). Male-to-female sex reversal in M33 mutant mice. *Nature*.

Katsumata, N., Ohtake, M., Hojo, T., Ogawa, E., Hara, T., Sato, N., and Tanaka, T. (2002). Compound heterozygous mutations in the cholesterol side-chain cleavage enzyme gene (CYP11A) cause congenital adrenal insufficiency in humans. *J. Clin. Endocrinol. Metab.*

Kazanskaya, O., Glinka, A., del Barco Barrantes, I., Stanek, P., Niehrs, C., and Wu, W. (2004). R-Spondin2 is a secreted activator of Wnt/ $\beta$ -catenin signaling and is required for *Xenopus* myogenesis. *Dev. Cell*.

Kazanskaya, O., Ohkawara, B., Heroult, M., Wu, W., Maltry, N., Augustin, H.G., and Niehrs, C. (2008). The Wnt signaling regulator R-spondin 3 promotes angioblast and vascular development. *Development*.

Kilcoyne, K.R., Smith, L.B., Atanassova, N., Macpherson, S., McKinnell, C., van den Driesche, S., Jobling, M.S., Chambers, T.J.G., De Gendt, K., Verhoeven, G., et al. (2014). Fetal programming of adult Leydig cell function by androgenic effects on stem/progenitor cells. *Proc. Natl. Acad. Sci.*

Kim, K.-A., Wagle, M., Tran, K., Zhan, X., Dixon, M.A., Liu, S., Gros, D., Korver, W., Yonkovich, S., Tomasevic, N., et al. (2008). R-Spondin Family Members Regulate the Wnt Pathway by a Common Mechanism. *Mol. Biol. Cell*.

Kim, K.A., Kakitani, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., Liu, Y., Boyle, B., Park, E., Emtage, P., et al. (2005). Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* (80- ).

Kim, K.A., Zhao, J., Andarmani, S., Kakitani, M., Oshima, T., Binnerts, M.E., Abo, A., Tomizuka, K., and Funk, W.D. (2006a). R-spondin proteins: A novel link to  $\beta$ -catenin activation. *Cell Cycle*.

Kim, Y., Kobayashi, A., Sekido, R., DiNapoli, L., Brennan, J., Chaboissier, M.C., Poulat, F., Behringer, R.R., Lovell-Badge, R., and Capel, B. (2006b). Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. *PLoS Biol.* 4, 1000–1009.

Kim, Y., Bingham, N., Sekido, R., Parker, K.L., Lovell-Badge, R., and Capel, B. (2007). Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. *Proc. Natl. Acad. Sci.* 104, 16558–16563.

Kitamura, K., Yanazawa, M., Sugiyama, N., Miura, H., Iizuka-Kogo, A., Kusaka, M., Omichi, K., Suzuki, R., Kato-Fukui, Y., Kamiirisa, K., et al. (2002). Mutation of ARX causes

abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans. *Nat. Genet.*

Koizumi, M., Oyama, K., Yamakami, Y., Kida, T., Satoh, R., Kato, S., Hidema, S., Oe, T., Goto, T., Clevers, H., et al. (2015). *Lgr4* Controls Specialization of Female Gonads in Mice. *Biol. Reprod.*

Kok, R.C., Timmerman, M.A., Wolffenbuttel, K.P., Drop, S.L.S., and De Jong, F.H. (2010). Isolated 17,20-lyase deficiency due to the cytochrome b5 mutation W27X. *J. Clin. Endocrinol. Metab.*

Koo, B.K., Spit, M., Jordens, I., Low, T.Y., Stange, D.E., Van De Wetering, M., Van Es, J.H., Mohammed, S., Heck, A.J.R., Maurice, M.M., et al. (2012). Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature.*

Koopman, P. (2016). The Curious World of Gonadal Development in Mammals. In *Current Topics in Developmental Biology*, p.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. *Nature.*

Koubova, J., Menke, D.B., Zhou, Q., Capel, B., Griswold, M.D., and Page, D.C. (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc. Natl. Acad. Sci.*

Krege, J.H., Hodgkin, J.B., Couse, J.F., Enmark, E., Warner, M., Mahler, J.F., Sar, M., Korach, K.S., Gustafsson, J.-A., and Smithies, O. (2002). Generation and reproductive phenotypes of mice lacking estrogen receptor . *Proc. Natl. Acad. Sci.*

Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D., and Jaenisch, R. (1993). *WT-1* is required for early kidney development. *Cell.*

Kühnert, B., Byrne, M., Simoni, M., Köpcke, W., Gerss, J., Lemnitz, G., Nieschlag, E., Eickenberg, H.U., Brüning, J., Haidl, G., et al. (2005). Testosterone substitution with a new transdermal, hydroalcoholic gel applied to scrotal or non-scrotal skin: A multicentre trial. *Eur. J. Endocrinol.*

Kumar, D.L., and DeFalco, T. (2018). A perivascular niche for multipotent progenitors in the fetal testis. *Nat. Commun.* 9.

Kurimoto, K., Yabuta, Y., Ohinata, Y., Shigeta, M., Yamanaka, K., and Saitou, M. (2008). Complex genome-wide transcription dynamics orchestrated by *Blimp1* for the specification of the germ cell lineage in mice. *Genes Dev.*

Kuroki, S., Matoba, S., Akiyoshi, M., Matsumura, Y., Miyachi, H., Mise, N., Abe, K., Ogura, A., Wilhelm, D., Koopman, P., et al. (2013). Epigenetic regulation of mouse sex determination by the histone demethylase *Jmjd1a*. *Science* (80- ).

Kusaka, M., Katoh-Fukui, Y., Ogawa, H., Miyabayashi, K., Baba, T., Shima, Y., Sugiyama, N., Sugimoto, Y., Okuno, Y., Kodama, R., et al. (2010). Abnormal epithelial cell polarity and ectopic Epidermal Growth Factor Receptor (EGFR) expression induced in *Emx2* KO Embryonic Gonads. *Endocrinology.*

Lacour, F., Vezin, E., Bentzinger, C.F., Sincennes, M.C., Giordani, L., Ferry, A., Mitchell, R., Patel, K., Rudnicki, M.A., Chaboissier, M.C., et al. (2017). *R-spondin1* Controls Muscle Cell Fusion through Dual Regulation of Antagonistic Wnt Signaling Pathways. *Cell Rep.* 18, 2320–2330.

Laird, D.J., Altshuler-Keylin, S., Kissner, M.D., Zhou, X., and Anderson, K. V. (2011). *Ror2* enhances polarity and directional migration of primordial germ cells. *PLoS Genet.*

Lambeth, L.S., Raymond, C.S., Roeszler, K.N., Kuroiwa, A., Nakata, T., Zarkower, D., and Smith, C.A. (2014). Over-expression of *DMRT1* induces the male pathway in embryonic chicken gonads. *Dev. Biol.*

de Lau, W.B.M., Snel, B., and Clevers, H.C. (2012). The R-spondin protein family. *Genome Biol.* 13, 242.

Lavery, R., Lardenois, A., Ranc-Jianmotamedi, F., Pauper, E., Gregoire, E.P., Vigier, C., Moreilhon, C., Primig, M., and Chaboissier, M.C. (2011a). XY Sox9 embryonic loss-of-function mouse mutants show complete sex reversal and produce partially fertile XY oocytes. *Dev. Biol.*

Lavery, R., Lardenois, A., Ranc-Jianmotamedi, F., Pauper, E., Gregoire, E.P., Vigier, C., Moreilhon, C., Primig, M., and Chaboissier, M.C. (2011b). XY Sox9 embryonic loss-of-function mouse mutants show complete sex reversal and produce partially fertile XY oocytes. *Dev. Biol.* 354, 111–122.

Lavery, R., Chassot, A.A., Pauper, E., Gregoire, E.P., Klopfenstein, M., de Rooij, D.G., Mark, M., Schedl, A., Ghyselinck, N.B., and Chaboissier, M.C. (2012). Testicular Differentiation Occurs in Absence of R-spondin1 and Sox9 in Mouse Sex Reversals. *PLoS Genet.* 8.

Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C. V, Korving, J.P., and Hogan, B.L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev.* 13, 424–436.

Lebensohn, A.M., and Rohatgi, R. (2017). R-spondins can potentiate WNT signaling without LGR receptors. *BioRxiv* 198572.

Lebensohn, A.M., and Rohatgi, R. (2018). R-spondins can potentiate WNT signaling without LGRs. *Elife* 7.

Lee, M.S., Lee, S.O., Kim, S.H., Lee, E.O., and Lee, H.J. (2016). Anti-cancer effect of lambertianic acid by inhibiting the AR in LNCaP cells. *Int. J. Mol. Sci.* 17.

Lei, N., Hornbaker, K.I., Rice, D.A., Karpova, T., Agbor, V.A., and Heckert, L.L. (2007a). Sex-Specific Differences in Mouse DMRT1 Expression Are Both Cell Type- and Stage-Dependent During Gonad Development. *Biol. Reprod.* 77, 466–475.

Lei, N., Hornbaker, K.I., Rice, D.A., Karpova, T., Agbor, V.A., and Heckert, L.L. (2007b). Sex-Specific Differences in Mouse DMRT1 Expression Are Both Cell Type- and Stage-Dependent During Gonad Development1. *Biol. Reprod.* 77, 466–475.

Li, H., Li, J., and Feng, L. (2016). Hedgehog signaling pathway as a therapeutic target for ovarian cancer. *Cancer Epidemiol.*

Li, J.Y., Chai, B., Zhang, W., Fritze, D.M., Zhang, C., and Mulholland, M.W. (2014a). LGR4 and its ligands, R-spondin 1 and R-spondin 3, regulate food intake in the hypothalamus of male rats. *Endocrinology.*

Li, P., Chen, J., and Miyamoto, H. (2017). Androgen receptor signaling in bladder cancer. *Cancers (Basel).*

Li, S.J., Yen, T.Y., Endo, Y., Klauzinska, M., Baljinnyam, B., Macher, B., Callahan, R., and Rubin, J.S. (2009). Loss-of-function point mutations and two-furin domain derivatives provide insights about R-spondin2 structure and function. *Cell. Signal.*

Li, Y., Zheng, M., and Lau, Y.F.C. (2014b). The sex-determining factors SRY and SOX9 regulate similar target genes and promote testis cord formation during testicular differentiation. *Cell Rep.* 8, 723–733.

Li, Z., Zhang, W., and Mulholland, M.W. (2015). LGR4 and Its Role in Intestinal Protection and Energy Metabolism. *Front. Endocrinol. (Lausanne).*

Lin, Y.-T., Barske, L., DeFalco, T., and Capel, B. (2017). Numb regulates somatic cell lineage commitment during early gonadogenesis in mice. *Development.*

Lindqvist, A., Hughes, I.A., and Andersson, S. (2001). Substitution mutation C268Y causes 17 $\beta$ -hydroxysteroid dehydrogenase 3 deficiency. *J. Clin. Endocrinol. Metab.*

Liu, C., Peng, J., Matzuk, M.M., and Yao, H.H.C. (2015a). Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells. *Nat. Commun.*

Liu, C., Rodriguez, K., and Yao, H.H.-C. (2016). Mapping lineage progression of somatic progenitor cells in the mouse fetal testis. *Development.*

Liu, C.F., Bingham, N., Parker, K., and Yao, H.H.C. (2009). Sex-specific roles of  $\beta$ -catenin in mouse gonadal development. *Hum. Mol. Genet.* 18, 405–417.

Liu, C.F., Parker, K., and Humphrey, Y. (2010). WNT4/ $\beta$ -catenin pathway maintains female germ cell survival by inhibiting activin  $\beta$ B in the mouse fetal ovary. *PLoS One* 5.

Liu, H., Lamm, M.S., Rutherford, K., Black, M.A., Godwin, J.R., and Gemmell, N.J. (2015b). Large-scale transcriptome sequencing reveals novel expression patterns for key sex-related genes in a sex-changing fish. *Biol. Sex Differ.*

Lntnesky, M.M.F. (1994). Density-dependent protogynous sex change in territorial-haremic fishes: Models and evidence. *Behav. Ecol.*

Lovell-Badge, R., and Robertson, E. (1990). XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy. *Development* 109.

Lubahn, D.B., Moyer, J.S., Golding, T.S., Couse, J.F., Korach, K.S., and Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci.*

Lue, Y., Rao, P.N., Sinha Hikim, A.P., Im, M., Salameh, W.A., Yen, P.H., Wang, C., and Swerdloff, R.S. (2001). XXY male mice: An experimental model for Klinefelter syndrome. *Endocrinology.*

Luo, X., Ikeda, Y., and Parker, K.L. (1994). A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell.*

Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P.M., Birchmeier, W., et al. (2002). Negative Feedback Loop of Wnt Signaling through Upregulation of Conductin/Axin2 in Colorectal and Liver Tumors. *Mol. Cell. Biol.* 22, 1184–1193.

Ma, X., Dong, Y., Matzuk, M.M., and Kumar, T.R. (2004). Targeted disruption of luteinizing hormone  $\beta$ -subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proc. Natl. Acad. Sci.*

Maatouk, D.M., Dinapoli, L., Alvers, A., Parker, K.L., Taketo, M.M., and Capel, B. (2008). Stabilization of  $\beta$ -catenin in XY gonads causes male-to-female sex-reversal. *Hum. Mol. Genet.* 17, 2949–2955.

Maatouk, D.M., Mork, L., Hinson, A., Kobayashi, A., McMahon, A.P., and Capel, B. (2012). Germ Cells Are Not Required to Establish the Female Pathway in Mouse Fetal Gonads. *PLoS One.*

Maatouk, D.M., Mork, L., Chassot, A.A., Chaboissier, M.C., and Capel, B. (2013). Disruption of mitotic arrest precedes precocious differentiation and transdifferentiation of pregranulosa cells in the perinatal Wnt4 mutant ovary. *Dev. Biol.* 383, 295–306.

Maatouk, D.M., Natarajan, A., Shibata, Y., Song, L., Crawford, G.E., Ohler, U., and Capel, B. (2017). Genome-wide identification of regulatory elements in Sertoli cells. *Development* 144, 720–730.

Mackay, S. (2000). Gonadal development in mammals at the cellular and molecular levels. *Int. Rev. Cytol.*

Mamsen, L.S., Ernst, E.H., Borup, R., Larsen, A., Olesen, R.H., Ernst, E., Anderson, R.A., Kristensen, S.G., and Andersen, C.Y. (2017). Temporal expression pattern of genes during the period of sex differentiation in human embryonic gonads. *Sci. Rep.*

Mandel, H., Shemer, R., Borochoy, Z.U., Okopnik, M., Knopf, C., Indelman, M., Drugan, A., Tiosano, D., Gershoni-Baruch, R., Choder, M., et al. (2008). SERKAL Syndrome: An Autosomal-Recessive Disorder Caused by a Loss-of-Function Mutation in WNT4. *Am. J. Hum. Genet.* 82, 39–47.

Mank, J.E., and Avise, J.C. (2009). Evolutionary diversity and turn-over of sex determination in teleost fishes. *Sex. Dev.*

Mannan, M.A., and O'Shaughnessy, P.J. (1991). Steroidogenesis during postnatal development in the mouse ovary. *J. Endocrinol.*

Manuylov, N.L., Smagulova, F.O., Leach, L., and Tevosian, S.G. (2008). Ovarian development in mice requires the GATA4-FOG2 transcription complex. *Development* 135, 3731–3743.

Manuylov, N.L., Zhou, B., Ma, Q., Fox, S.C., Pu, W.T., and Tevosian, S.G. (2011). Conditional ablation of Gata4 and Fog2 genes in mice reveals their distinct roles in mammalian sexual differentiation. *Dev. Biol.* 353, 229–241.

Marcoccia, D., Pellegrini, M., Fiocchetti, M., Lorenzetti, S., and Marino, M. (2017). Food components and contaminants as (anti)androgenic molecules. *Genes Nutr.*

Marin, P., Ferlin, A., Moro, E., Rossi, A., Bartoloni, L., Rossato, M., and Foresta, C. (2001). Novel insulin-like 3 (INSL3) gene mutation associated with human cryptorchidism [4]. *Am. J. Med. Genet.*

Martens, J.W.M., Verhoef-Post, M., Abelin, N., Ezabella, M., Toledo, S.P.A., Brunner, H.G., and Themmen, A.P.N. (2014). A Homozygous Mutation in the Luteinizing Hormone Receptor Causes Partial Leydig Cell Hypoplasia: Correlation between Receptor Activity and Phenotype. *Mol. Endocrinol.*

Martineau, J., Nordqvist, K., Tilmann, C., Lovell-Badge, R., and Capel, B. (1997). Male-specific cell migration into the developing gonad. *Curr. Biol.*

Matson, C.K., Murphy, M.W., Sarver, A.L., Griswold, M.D., Bardwell, V.J., and Zarkower, D. (2011). DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature* 476, 101–105.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C.E., Shibata, N., Asakawa, S., Shimizu, N., et al. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature.*

McClelland, K.S., Bell, K., Larney, C., Harley, V.R., Sinclair, A.H., Oshlack, A., Koopman, P., and Bowles, J. (2015). Purification and Transcriptomic Analysis of Mouse Fetal Leydig Cells Reveals Candidate Genes for Specification of Gonadal Steroidogenic Cells1. *Biol. Reprod.*

McElreavey, K., Vilain, E., Abbas, N., Herskowitz, I., and Fellous, M. (1993). A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development. *Proc. Natl. Acad. Sci. U. S. A.* 90, 3368–3372.

McElreavy, K., Vilain, E., Abbas, N., Costa, J.M., Souleyreau, N., Kucheria, K., Boucekkine, C., Thibaud, E., Brauner, R., and Flamant, F. (1992). XY sex reversal associated with a deletion 5' to the SRY "HMG box" in the testis-determining region. *Proc. Natl. Acad. Sci.*

McLaren, A. (1984). Meiosis and differentiation of mouse germ cells. *Symp. Soc. Exp. Biol.*

McLaren, A., and Southee, D. (1997). Entry of mouse embryonic germ cells into meiosis.

Dev. Biol.

Merchant-Larios, H., Moreno-Mendoza, N., and Buehr, M. (1993). The role of the mesonephros in cell differentiation and morphogenesis of the mouse fetal testis. *Int. J. Dev. Biol.*

Merchant, H. (1975). Rat gonadal and ovarian organogenesis with and without germ cells. An ultrastructural study. *Dev. Biol.*

Merke, D.P., Bornstein, S.R., Avila, N.A., and Chrousos, G.P. (2002). Future directions in the study and management of congenital adrenal hyperplasia due to 21-hydroxylase deficiency. In *Annals of Internal Medicine*, p.

Miura, K., Harikae, K., Nakaguchi, M., Imaimatsu, K., Hiramatsu, R., Tomita, A., Hirate, Y., Kanai-Azuma, M., Kurohmaru, M., Ogura, A., et al. (2019). Molecular and genetic characterization of partial masculinization in embryonic ovaries grafted into male nude mice. *PLoS One* 14.

Miyabayashi, K., Katoh-Fukui, Y., Ogawa, H., Baba, T., Shima, Y., Sugiyama, N., Kitamura, K., and Morohashi, K. (2013). Aristaless Related Homeobox Gene, *Arx*, Is Implicated in Mouse Fetal Leydig Cell Differentiation Possibly through Expressing in the Progenitor Cells. *PLoS One*.

Miyabayashi, K., Tokunaga, K., Otake, H., Baba, T., Shima, Y., and Morohashi, K. (2015). Heterogeneity of ovarian theca and interstitial gland cells in Mice. *PLoS One*.

Miyabayashi, K., Shima, Y., Inoue, M., Sato, T., Baba, T., Ohkawa, Y., Suyama, M., and Morohashi, K.I. (2017). Alterations in Fetal Leydig Cell Gene Expression during Fetal and Adult Development. *Sex. Dev.*

Miyado, M., Nakamura, M., Miyado, K., Morohashi, K.I., Sano, S., Nagata, E., Fukami, M., and Ogata, T. (2012). *Mamld1* deficiency significantly reduces mRNA expression levels of multiple genes expressed in mouse fetal leydig cells but permits normal genital and reproductive development. *Endocrinology*.

Miyado, M., Yoshida, K., Miyado, K., Katsumi, M., Saito, K., Nakamura, S., Ogata, T., and Fukami, M. (2017). Knockout of murine *Mamld1* impairs testicular growth and daily sperm production but permits normal postnatal androgen production and fertility. *Int. J. Mol. Sci.*

Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I., and Aizawa, S. (1997). Defects of urogenital development in mice lacking *Emx2*. *Development*.

Miyamoto, Y., Taniguchi, H., Hamel, F., Silversides, D.W., and Viger, R.S. (2008). A GATA4/WT1 cooperation regulates transcription of genes required for mammalian sex determination and differentiation. *BMC Mol. Biol.*

Molyneaux, K.A. (2003). The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development*.

Molyneaux, K.A., Wang, Y., Schaible, K., and Wylie, C. (2004). Transcriptional profiling identifies genes differentially expressed during and after migration in murine primordial germ cells. *Gene Expr. Patterns*.

Moniot, B., Declosmenil, F., Barrionuevo, F., Scherer, G., Aritake, K., Malki, S., Marzi, L., Cohen-Solal, A., Georg, I., Klattig, J., et al. (2009). The PGD2 pathway, independently of FGF9, amplifies SOX9 activity in Sertoli cells during male sexual differentiation. *Development*.

Morishima, A., Grumbach, M.M., Simpson, E.R., Fisher, C., and Qin, K. (1995). Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. *J. Clin. Endocrinol. Metab.*

Mork, L., Maatouk, D.M., McMahon, J.A., Guo, J.J., Zhang, P., McMahon, A.P., McMahon, J.A., Capel, B., McMahon, J.A., and Capel, B. (2012a). Temporal Differences in Granulosa

- Cell Specification in the Ovary Reflect Distinct Follicle Fates in Mice<sup>1</sup>. *Biol. Reprod.* 86, 1–9.
- Mork, L., Tang, H., Batchvarov, I., and Capel, B. (2012b). Mouse germ cell clusters form by aggregation as well as clonal divisions. *Mech. Dev.*
- Mork, L., Czerwinski, M., and Capel, B. (2014). Predetermination of sexual fate in a turtle with temperature-dependent sex determination. *Dev. Biol.*
- Morohashi, K.I. (1997). The ontogenesis of the steroidogenic tissues. *Genes to Cells.*
- Mosimann, C., Hausmann, G., and Basler, K. (2009).  $\beta$ -Catenin hits chromatin: Regulation of Wnt target gene activation. *Nat. Rev. Mol. Cell Biol.*
- Müller, U., Franke, W.W., and Scheib, D. (2011). Effects and Role of Estrogens in Avian Gonadal Differentiation. In *Mechanisms of Gonadal Differentiation in Vertebrates*, p.
- Munger, S.C., Aylor, D.L., Syed, H.A., Magwene, P.M., Threadgill, D.W., and Capel, B. (2009). Elucidation of the transcription network governing mammalian sex determination by exploiting strain-specific susceptibility to sex reversal. *Genes Dev.*
- Munger, S.C., Natarajan, A., Looger, L.L., Ohler, U., and Capel, B. (2013). Fine Time Course Expression Analysis Identifies Cascades of Activation and Repression and Maps a Putative Regulator of Mammalian Sex Determination. *PLoS Genet.* 9, e1003630.
- Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. *Genesis* 45, 593–605.
- Naillat, F., Prunskaitė-Hyyryläinen, R., Pietilä, I., Sormunen, R., Jokela, T., Shan, J., and Vainio, S.J. (2010). Wnt4/5a signalling coordinates cell adhesion and entry into meiosis during presumptive ovarian follicle development. *Hum. Mol. Genet.* 19, 1539–1550.
- Naillat, F., Yan, W., Karjalainen, R., Liakhovitskaia, A., Samoylenko, A., Xu, Q., Sun, Z., Shen, B., Medvinsky, A., Quaggin, S., et al. (2015). Identification of the genes regulated by Wnt-4, a critical signal for commitment of the ovary. *Exp. Cell Res.* 332, 163–178.
- Nakamura, M., Fukami, M., Sugawa, F., Miyado, M., Nonomura, K., and Ogata, T. (2011). Maml1 knockdown reduces testosterone production and Cyp17a1 expression in mouse Leydig tumor cells. *PLoS One.*
- Nam, J.S., Turcotte, T.J., Smith, P.F., Choi, S., and Jeong, K.Y. (2006). Mouse cristin/R-spondin family proteins are novel ligands for the frizzled 8 and LRP6 receptors and activate  $\beta$ -catenin-dependent gene expression. *J. Biol. Chem.*
- Nam, J.S., Park, E., Turcotte, T.J., Palencia, S., Zhan, X., Lee, J., Yun, K., Funk, W.D., and Yoon, J.K. (2007a). Mouse R-spondin2 is required for apical ectodermal ridge maintenance in the hindlimb. *Dev. Biol.*
- Nam, J.S., Turcotte, T.J., and Yoon, J.K. (2007b). Dynamic expression of R-spondin family genes in mouse development. *Gene Expr. Patterns.*
- Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., et al. (2002). A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proc. Natl. Acad. Sci.*
- Nef, S., and Parada, L.F. (1999). Cryptorchidism in mice mutant for *Insl3*. *Nat. Genet.*
- Nef, S., Verma-Kurvari, S., Merenmies, J., Vassalli, J.D., Efstratiadis, A., Accili, D., and Parada, L.F. (2003). Testis determination requires insulin receptor family function in mice. *Nature* 426, 291–295.
- Nef, S., Schaad, O., Stallings, N.R., Cederroth, C.R., Pitetti, J.L., Schaer, G., Malki, S., Dubois-Dauphin, M., Boizet-Bonhoure, B., Descombes, P., et al. (2005). Gene expression during sex determination reveals a robust female genetic program at the onset of ovarian development. *Dev. Biol.* 287, 361–377.

Nef, S., Stévant, I., and Greenfield, A. (2019). Characterizing the bipotential mammalian gonad. In *Current Topics in Developmental Biology*, p.

Nelson, K.G., and Goldenberg, R.L. (1977). Sex hormones and congenital malformations: a review. *J. Med. Assoc. State Ala.*

New, M.I. (2003). Inborn errors of adrenal steroidogenesis. In *Molecular and Cellular Endocrinology*, p.

Ng, A., Tan, S., Singh, G., Rizk, P., Swathi, Y., Tan, T.Z., Huang, R.Y.J., Leushacke, M., and Barker, N. (2014). Lgr5 marks stem/progenitor cells in ovary and tubal epithelia. *Nat. Cell Biol.*

Nicol, B., and Yao, H.H.-C. (2015). Gonadal Identity in the Absence of Pro-Testis Factor SOX9 and Pro-Ovary Factor Beta-Catenin in Mice<sup>1</sup>. *Biol. Reprod.* 93.

Nicol, B., Grimm, S.A., Gruzdev, A., Scott, G.J., Ray, M.K., and Yao, H.H.C. (2018). Genome-wide identification of FOXL2 binding and characterization of FOXL2 feminizing action in the fetal gonads. *Hum. Mol. Genet.* 27, 4273–4287.

Niehrs, C. (2012). The complex world of WNT receptor signalling. *Nat. Rev. Mol. Cell Biol.*

Nusse, R., and Clevers, H. (2017). Wnt/ $\beta$ -Catenin Signaling, Disease, and Emerging Therapeutic Modalities. *Cell* 169, 985–999.

O'Hara, L., McInnes, K., Simitsidellis, I., Morgan, S., Atanassova, N., Slowikowska-Hilczer, J., Kula, K., Szarras-Czapnik, M., Milne, L., Mitchell, R.T., et al. (2015). Autocrine androgen action is essential for Leydig cell maturation and function, and protects against late-onset Leydig cell apoptosis in both mice and men. *FASEB J.*

O'Shaughnessy, P.J., Dudley, K., and Rajapaksha, W.R.A.K.J.S. (1996). Expression of follicle stimulating hormone-receptor mRNA during gonadal development. In *Molecular and Cellular Endocrinology*, p.

Obrador-Hevia, A., Chin, S.F., González, S., Rees, J., Vilardell, F., Greenson, J.K., Cordero, D., Moreno, V., Caldas, C., and Capellá, G. (2010). Oncogenic KRAS is not necessary for Wnt signalling activation in APC-associated FAP adenomas. *J. Pathol.*

Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S.C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., et al. (2005). Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature.*

Ohinata, Y., Ohta, H., Shigeta, M., Yamanaka, K., Wakayama, T., and Saitou, M. (2009). A Signaling Principle for the Specification of the Germ Cell Lineage in Mice. *Cell.*

Ohkawara, B., Glinka, A., and Niehrs, C. (2011). Rspo3 Binds Syndecan 4 and Induces Wnt/PCP Signaling via Clathrin-Mediated Endocytosis to Promote Morphogenesis. *Dev. Cell.*

Orban, L., Sreenivasan, R., and Olsson, P.E. (2009). Long and winding roads: Testis differentiation in zebrafish. *Mol. Cell. Endocrinol.*

Orth, J.M., Gunsalus, G.L., and Lamperti, A.A. (1988). Evidence from sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of sertoli cells produced during perinatal development. *Endocrinology.*

Otake, T., and Kuroiwa, A. (2016). Molecular mechanism of male differentiation is conserved in the SRY-absent mammal, *Tokudaia osimensis*. *Sci. Rep.*

Ottolenghi, C., Omari, S., Garcia-Ortiz, J.E., Uda, M., Crisponi, L., Forabosco, A., Pilia, G., and Schlessinger, D. (2005). Foxl2 is required for commitment to ovary differentiation. *Hum. Mol. Genet.* 14, 2053–2062.

Ottolenghi, C., Pelosi, E., Tran, J., Colombino, M., Douglass, E., Nedorezov, T., Cao, A., Forabosco, A., and Schlessinger, D. (2007). Loss of Wnt4 and Foxl2 leads to female-to-male

sex reversal extending to germ cells. *Hum. Mol. Genet.* 16, 2795–2804.

Ozisk, G., Achermann, J.C., and Jameson, J.L. (2002). The role of SF1 in adrenal and reproductive function: Insight from naturally occurring mutations in humans. *Mol. Genet. Metab.*

Padula, A.M. (2005). The freemartin syndrome: an update. *Anim. Reprod. Sci.* 87, 93–109.

Pailhoux, E., Vigier, B., Chaffaux, S., Servel, N., Taourit, S., Furet, J.P., Fellous, M., Grosclaude, F., Crihiu, E.P., Cotinot, C., et al. (2001). A 11.7-kb deletion triggers intersexuality and polledness in goats. *Nat. Genet.*

Pailhoux, E., Vigier, B., Vaiman, D., Servel, N., Chaffaux, S., Crihiu, E.P., and Cotinot, C. (2002). Ontogenesis of female-to-male sex-reversal in XX polled goats. *Dev. Dyn.*

Palmer, S.J., and Burgoyne, P.S. (1991). In situ analysis of fetal, prepuberal and adult XX---XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. *Development.*

Pannetier, M., Renault, L., Jolivet, G., Cotinot, C., and Pailhoux, E. (2005). Ovarian-specific expression of a new gene regulated by the goat PIS region and transcribed by a FOXL2 bidirectional promoter. *Genomics* 85, 715–726.

Park, S.Y., Tong, M., and Jameson, J.L. (2007). Distinct roles for Steroidogenic factor 1 and Desert hedgehog pathways in fetal and adult leydig cell development. *Endocrinology.*

Parma, P., Radi, O., Vidal, V., Chaboissier, M.C., Dellambra, E., Valentini, S., Guerra, L., Schedl, A., and Camerino, G. (2006). R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat. Genet.* 38, 1304–1309.

Pearlman, A., Loke, J., Le Caignec, C., White, S., Chin, L., Friedman, A., Warr, N., Willan, J., Brauer, D., Farmer, C., et al. (2010). Mutations in MAP3K1 cause 46,XY disorders of sex development and implicate a common signal transduction pathway in human testis determination. *Am. J. Hum. Genet.* 87, 898–904.

Peter, M. (2002). Congenital adrenal hyperplasia: 11 $\beta$ -Hydroxylase deficiency. *Semin. Reprod. Med.*

Peterson, R.E., Imperato-McGinley, J., Gautier, T., and Shackleton, C. (1985). Male Pseudohermaphroditism Due to Multiple Defects in Steroid-Biosynthetic Microsomal Mixed-Function Oxidases. *N. Engl. J. Med.*

Phelps, R.A., Chidester, S., Dehghanizadeh, S., Phelps, J., Sandoval, I.T., Rai, K., Broadbent, T., Sarkar, S., Burt, R.W., and Jones, D.A. (2009). A Two-Step Model for Colon Adenoma Initiation and Progression Caused by APC Loss. *Cell.*

Philibert, P., Biason-Lauber, A., Gueorguieva, I., Stuckens, C., Pienkowski, C., Lebon-Labich, B., Paris, F., and Sultan, C. (2011). Molecular analysis of WNT4 gene in four adolescent girls with mullerian duct abnormality and hyperandrogenism (atypical Mayer-Rokitansky-Küster-Hauser syndrome). *Fertil. Steril.*

Phillips, D.J., and De Kretser, D.M. (1998). Follistatin: A multifunctional regulatory protein. *Front. Neuroendocrinol.*

Picton, H., Briggs, D., and Gosden, R. (1998). The molecular basis of oocyte growth and development. *Mol. Cell. Endocrinol.* 145, 27–37.

Pitetti, J.L., Calvel, P., Romero, Y., Conne, B., Truong, V., Papaioannou, M.D., Schaad, O., Docquier, M., Herrera, P.L., Wilhelm, D., et al. (2013a). Insulin and IGF1 Receptors Are Essential for XX and XY Gonadal Differentiation and Adrenal Development in Mice. *PLoS Genet.* 9.

Pitetti, J.L., Calvel, P., Romero, Y., Conne, B., Truong, V., Papaioannou, M.D., Schaad, O., Docquier, M., Herrera, P.L., Wilhelm, D., et al. (2013b). Insulin and IGF1 Receptors Are Essential for XX and XY Gonadal Differentiation and Adrenal Development in Mice. *PLoS Genet.* 9, e1003160.

Polanco, J.C., Wilhelm, D., Davidson, T.L., Knight, D., and Koopman, P. (2009). Sox10 gain-of-function causes XX sex reversal in mice: Implications for human 22q-linked disorders of sex development. *Hum. Mol. Genet.*

Qin, J., Tsai, M.-J., and Tsai, S.Y. (2008). Essential roles of COUP-TFII in Leydig cell differentiation and male fertility. *PLoS One* 3, e3285.

Quinn, A.E., Sarre, S.D., Ezaz, T., Graves, J.A.M., and Georges, A. (2011). Evolutionary transitions between mechanisms of sex determination in vertebrates. *Biol. Lett.*

Rahmoun, M., Lavery, R., Laurent-Chaballier, S., Bellora, N., Philip, G.K., Rossitto, M., Symon, A., Pailhoux, E., Cammas, F., Chung, J., et al. (2017). In mammalian foetal testes, SOX9 regulates expression of its target genes by binding to genomic regions with conserved signatures. *Nucleic Acids Res.* 45, 7191–7211.

Rastetter, R.H., Bernard, P., Palmer, J.S., Chassot, A.A., Chen, H., Western, P.S., Ramsay, R.G., Chaboissier, M.C., and Wilhelm, D. (2014). Marker genes identify three somatic cell types in the fetal mouse ovary. *Dev. Biol.* 394, 242–252.

Ren, Y., Cowan, R.G., Migone, F.F., and Quirk, S.M. (2012). Overactivation of Hedgehog Signaling Alters Development of the Ovarian Vasculature in Mice<sup>1</sup>. *Biol. Reprod.*

Reynaud, K., Cortvrint, R., Verlinde, F., De Schepper, J., Bourgain, C., and Smitz, J. (2004). Number of ovarian follicles in human fetuses with the 45,X karyotype. *Fertil. Steril.*

Richards, J.S., and Pangas, S.A. (2010). The ovary: Basic biology and clinical implications. *J. Clin. Invest.*

Richter-Unruh, A., Martens, J.W.M., Verhoef-Post, M., Wessels, H.T., Kors, W.A., Sinnecker, G.H.G., Boehmer, A., Drop, S.L.S., Toledo, S.P.A., Brunner, H.G., et al. (2002). Leydig cell hypoplasia: Cases with new mutations, new polymorphisms and cases without mutations in the luteinizing hormone receptor gene. *Clin. Endocrinol. (Oxf).*

Rocha, A.S., Vidal, V., Mertz, M., Kendall, T.J., Charlet, A., Okamoto, H., and Schedl, A. (2015). The Angiocrine Factor Rspondin3 Is a Key Determinant of Liver Zonation. *Cell Rep.*

Rolland, A.D., Lehmann, K.P., Johnson, K.J., Gaido, K.W., and Koopman, P. (2011). Uncovering Gene Regulatory Networks During Mouse Fetal Germ Cell Development. *Biol. Reprod.*

Rosenfield, R.L., and Ehrmann, D.A. (2016). The Pathogenesis of Polycystic Ovary Syndrome (PCOS): The hypothesis of PCOS as functional ovarian hyperandrogenism revisited. *Endocr. Rev.*

Rösler, A., Silverstein, S., and Abeliovich, D. (1996). A (R80Q) mutation in 17 $\beta$ -hydroxysteroid dehydrogenase type 3 gene among Arabs of Israel is associated with pseudohermaphroditism in males and normal asymptomatic females. *J. Clin. Endocrinol. Metab.*

Ross, J.A., Urton, J.R., Boland, J., Shapiro, M.D., and Peichel, C.L. (2009). Turnover of sex chromosomes in the stickleback fishes (Gasterosteidae). *PLoS Genet.*

Rotgers, E., Jørgensen, A., and Yao, H.H.C. (2018). At the crossroads of fate-Somatic cell lineage specification in the fetal gonad. *Endocr. Rev.*

Ruiz-Arana, I.L., Hübner, A., Cetingdag, C., Krude, H., Grüters, A., Fukami, M., Biebermann, H., and Köhler, B. (2015). A novel hemizygous mutation of MAMLD1 in a patient with 46,XY complete gonadal dysgenesis. *Sex. Dev.*

Runyan, C., Schaible, K., Molyneaux, K., Wang, Z., Levin, L., and Wylie, C. (2006). Steel factor controls midline cell death of primordial germ cells and is essential for their normal proliferation and migration. *Development*.

Saga, Y. (2010). Function of Nanos2 in the male germ cell lineage in mice. *Cell. Mol. Life Sci.* 67, 3815–3822.

Saitou, M. (2009). Germ cell specification in mice. *Curr. Opin. Genet. Dev.*

Saitou, M., and Yamaji, M. (2012). Primordial germ cells in mice. *Cold Spring Harb. Perspect. Biol.*

Saitou, M., Barton, S.C., and Surani, M.A. (2002). A molecular programme for the specification of germ cell fate in mice. *Nature*.

Saitou, M., Kagiwada, S., and Kurimoto, K. (2011). Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. *Development*.

de Santa Barbara, P., Méjean, C., Moniot, B., Malclès, M.-H., Berta, P., and Boizet-Bonhoure, B. (2005). Steroidogenic Factor-1 Contributes to the Cyclic-Adenosine Monophosphate Down-Regulation of Human SRY Gene Expression<sup>1</sup>. *Biol. Reprod.*

Saylam, K., and Simon, P. (2003). WT1 gene mutation responsible for male sex reversal and renal failure: The Frasier syndrome. *Eur. J. Obstet. Gynecol. Reprod. Biol.*

Schmahl, J., Eicher, E.M., Washburn, L.L., and Capel, B. (2000). Sry induces cell proliferation in the mouse gonad. *Development*.

Schmahl, J., Rizzolo, K., and Soriano, P. (2008). The PDGF signaling pathway controls multiple steroid-producing lineages. *Genes Dev.* 22, 3255–3267.

Schmidt, D. (2004). The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development*.

Schnabel, C.A., Selleri, L., and Cleary, M.L. (2003). Pbx1 is Essential for Adrenal Development and Urogenital Differentiation. *Genesis*.

Schrans-Stassen, B.H.G.J., Saunders, P.T.K., Cooke, H.J., and de Rooij, D.G. (2001). Nature of the Spermatogenic Arrest in *Dazl* <sup>-/-</sup> Mice. *Biol. Reprod.* 65, 771–776.

Schwartz, C.L., Christiansen, S., Vinggaard, A.M., Axelstad, M., Hass, U., and Svingen, T. (2019). Anogenital distance as a toxicological or clinical marker for fetal androgen action and risk for reproductive disorders. *Arch. Toxicol.* 93, 253–272.

Schwarz, D.G. (2000). Related function of mouse SOX3, SOX9, and SRY HMG domains assayed by male sex determination. *Genesis*.

Scott, R.R., and Miller, W.L. (2008). Genetic and clinical features of P450 oxidoreductase deficiency. *Horm. Res.*

Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W., and Reik, W. (2012). The Dynamics of Genome-wide DNA Methylation Reprogramming in Mouse Primordial Germ Cells. *Mol. Cell*.

Seki, Y., Hayashi, K., Itoh, K., Mizugaki, M., Saitou, M., and Matsui, Y. (2005). Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. *Dev. Biol.*

Sekido, R., and Lovell-Badge, R. (2008). Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature* 453, 930–934.

Shackleton, C., Marcos, J., Arlt, W., and Hauffa, B.P. (2004). Prenatal diagnosis of P450 oxidoreductase deficiency (ORD): A disorder causing low pregnancy estriol, maternal and fetal virilization, and the Antley-Bixler syndrome phenotype. *Am. J. Med. Genet.*

Shan, J., Jokela, T., Skovorodkin, I., and Vainio, S. (2010). Mapping of the fate of cell lineages generated from cells that express the *Wnt4* gene by time-lapse during kidney development. *Differentiation* 79, 57–64.

Sharpe, RM (1994). Regulation of spermatogenesis. In *The Physiology of Reproduction* 2nd Edn, (New York), pp. 1363–1434.

Shima, Y., Miyabayashi, K., Haraguchi, S., Arakawa, T., Otake, H., Baba, T., Matsuzaki, S., Shishido, Y., Akiyama, H., Tachibana, T., et al. (2012a). Contribution of Leydig and Sertoli Cells to Testosterone Production in Mouse Fetal Testes. *Mol. Endocrinol.*

Shima, Y., Miyabayashi, K., Baba, T., Otake, H., Oka, S., Zubair, M., and Morohashi, K.I. (2012b). Identification of an enhancer in the *Ad4BP/SF-1* gene specific for fetal leydig cells. *Endocrinology*.

Shima, Y., Matsuzaki, S., Miyabayashi, K., Otake, H., Baba, T., Kato, S., Huhtaniemi, I., and Morohashi, K. (2015). Fetal Leydig Cells Persist as an Androgen-Independent Subpopulation in the Postnatal Testis. *Mol. Endocrinol.*

Shinoda, G., Yvanka De Soysa, T., Seligson, M.T., Yabuuchi, A., Fujiwara, Y., Huang, P.Y., Hagan, J.P., Gregory, R.I., Moss, E.G., and Daley, G.Q. *Lin28a* Regulates Germ Cell Pool Size and Fertility.

Shozu, M., Akasofu, K., Harada, T., and Kubota, Y. (1991). A new cause of female pseudohermaphroditism: Placental aromatase deficiency. *J. Clin. Endocrinol. Metab.*

Da Silva, F., Rocha, A.S., Motamedi, F.J., Massa, F., Basboga, C., Morrison, H., Wagner, K.D., and Schedl, A. (2017). Coronary Artery Formation Is Driven by Localized Expression of *R-spondin3*. *Cell Rep.*

Sinclair, A.H., Berta, P., Palmer, M.S., Hawkins, J.R., Griffiths, B.L., Smith, M.J., Foster, J.W., Frischauf, A.M., Lovell-Badge, R., and Goodfellow, P.N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature*.

Sirisena, N.D., McElreavey, K., Bashamboo, A., De Silva, K.S.H., Jayasekara, R.W., and Dissanayake, V.H.W. (2014). A Child with a Novel de novo Mutation in the Aristaless Domain of the Aristaless-Related Homeobox (*ARX*) Gene Presenting with Ambiguous Genitalia and Psychomotor Delay. *Sex. Dev.*

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

Soleimani, V.D., Punch, V.G., Kawabe, Y. ichi, Jones, A.E., Palidwor, G.A., Porter, C.J., Cross, J.W., Carvajal, J.J., Kockx, C.E.M., van IJcken, W.F.J., et al. (2012). Transcriptional Dominance of *Pax7* in Adult Myogenesis Is Due to High-Affinity Recognition of Homeodomain Motifs. *Dev. Cell*.

Stark, K., Vainio, S., Vassileva, G., and McMahon, A.P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by *Wnt-4*. *Nature*.

Stévant, I., and Nef, S. (2018). Single cell transcriptome sequencing: A new approach for the study of mammalian sex determination. *Mol. Cell. Endocrinol.*

Stévant, I., and Nef, S. (2019). Genetic Control of Gonadal Sex Determination and Development. *Trends Genet.* 35, 346–358.

Stévant, I., Neirijnck, Y., Borel, C., Escoffier, J., Smith, L.B., Antonarakis, S.E., Dermitzakis, E.T., Nef, S., Neirjinck, Y., Borel, C., et al. (2017). Deciphering cell lineage specification during male sex determination with single-cell RNA sequencing. *Cell Rep.* 22, 1589–1599.

Stévant, I., Kuhne, F., Greenfield, A., Chaboissier, M.-C., Dermitzakis, E.T., and Nef, S. (2018a). Single-cell transcriptomics of the mouse gonadal soma reveals the establishment of sexual dimorphism in distinct cell lineages. *BioRxiv*.

Stévant, I., Papaioannou, M.D., and Nef, S. (2018b). A brief history of sex determination. *Mol. Cell. Endocrinol.* 468, 3–10.

Stochholm, K., Juul, S., Juel, K., Naeraa, R.W., and Gravholt, C.H. (2006). Prevalence, incidence, diagnostic delay, and mortality in Turner syndrome. *J. Clin. Endocrinol. Metab.*

Suzuki, A., and Saga, Y. (2008). *Nanos2* suppresses meiosis and promotes male germ cell differentiation. *Genes Dev.*

Tajima, T., Fujieda, K., Kouda, N., Nakae, J., and Miller, W.L. (2001). Heterozygous mutation in the cholesterol side chain cleavage enzyme (*P450scc*) gene in a patient with 46,XY sex reversal and adrenal insufficiency. *J. Clin. Endocrinol. Metab.*

Takamoto, N., Kurihara, I., Lee, K., DeMayo, F.J., Tsai, M.-J., and Tsai, S.Y. (2005). Haploinsufficiency of Chicken Ovalbumin Upstream Promoter Transcription Factor II in Female Reproduction. *Mol. Endocrinol.*

Takehana, Y., Hamaguchi, S., and Sakaizumi, M. (2008). Different origins of ZZ/ZW sex chromosomes in closely related medaka fishes, *Oryzias javanicus* and *O. hubbsi*. *Chromosom. Res.*

Takehana, Y., Matsuda, M., Myosho, T., Suster, M.L., Kawakami, K., Shin-I, T., Kohara, Y., Kuroki, Y., Toyoda, A., Fujiyama, A., et al. (2014). Co-option of *Sox3* as the male-determining factor on the y chromosome in the fish *Oryzias dancena*. *Nat. Commun.*

Tanaka, S.S., Toyooka, Y., Akasu, R., Katoh-Fukui, Y., Nakahara, Y., Suzuki, R., Yokoyama, M., and Noce, T. (2000). The mouse homolog of *Drosophila Vasa* is required for the development of male germ cells. *Genes Dev.* 14, 841–853.

Tang, H., Brennan, J., Karl, J., Hamada, Y., Raetzman, L., and Capel, B. (2008). Notch signaling maintains Leydig progenitor cells in the mouse testis. *Development.*

Themmen, A.P.N., and Huhtaniemi, I.T. (2000). Mutations of gonadotropins and gonadotropin receptors: Elucidating the physiology and pathophysiology of pituitary-gonadal function. *Endocr. Rev.*

Thomas, N.S., and Hassold, T.J. (2003). Aberrant recombination and the origin of Klinefelter syndrome. *Hum. Reprod. Update.*

Tomaselli, S., Megiorni, F., De Bernardo, C., Felici, A., Marrocco, G., Maggiulli, G., Grammatico, B., Remotti, D., Saccucci, P., Valentini, F., et al. (2008). Syndromic true hermaphroditism due to an *R-spondin1* (*RSPO1*) homozygous mutation. *Hum. Mutat.* 29, 220–226.

Tomaselli, S., Megiorni, F., Lin, L., Mazzilli, M.C., Gerrelli, D., Majore, S., Grammatico, P., and Achermann, J.C. (2011). Human *RSPO1/R-spondin1* is expressed during early ovary development and augments  $\beta$ -catenin signaling. *PLoS One.*

Tomboc, M., Lee, P.A., Mitwally, M.F., Schneck, F.X., Bellinger, M., and Witchel, S.F. (2000). Insulin-like 3/relaxin-like factor gene mutations are associated with cryptorchidism. *J. Clin. Endocrinol. Metab.*

Tomizuka, K., Horikoshi, K., Kitada, R., Sugawara, Y., Iba, Y., Kojima, A., Yoshitome, A., Yamawaki, K., Amagai, M., Inoue, A., et al. (2008). *R-spondin1* plays an essential role in ovarian development through positively regulating *Wnt-4* signaling. *Hum. Mol. Genet.* 17, 1278–1291.

Tremblay, J.J., and Viger, R.S. (2003). A Mutated Form of Steroidogenic Factor 1 (SF-1 G35E) That Causes Sex Reversal in Humans Fails to Synergize with Transcription Factor

GATA-4. *J. Biol. Chem.*

Tut, T.G., Ghadessy, F.J., Trifiro, M.A., Pinsky, L., and Yong, E.L. (1997). Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility. *J. Clin. Endocrinol. Metab.*

Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J.E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., and Pilia, G. (2004). *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Hum. Mol. Genet.*

Uehara, S., Funato, T., Yaegashi, N., Suzuki, H., Sato, J., Sasaki, T., and Yajima, A. (1999). SRY mutation and tumor formation on the gonads of XY pure gonadal dysgenesis patients. *Cancer Genet. Cytogenet.*

Uehara, S., Hashiyada, M., Sato, K., Nata, M., Funato, T., and Okamura, K. (2002). Complete XY gonadal dysgenesis and aspects of the SRY genotype and gonadal tumor formation. *J. Hum. Genet.*

Uhlenhaut, N.H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., Kress, J., Treier, A.C., Klugmann, C., Klasen, C., Holter, N.I., et al. (2009). Somatic Sex Reprogramming of Adult Ovaries to Testes by FOXL2 Ablation. *Cell* 139, 1130–1142.

Umehara, F., Tate, G., Itoh, K., Yamaguchi, N., Douchi, T., Mitsuya, T., and Osame, M. (2000). A novel mutation of desert hedgehog in a patient with 46, XY partial gonadal dysgenesis accompanied by minifascicular neuropathy. *Am. J. Hum. Genet.*

Vainio, S., Heikkilä, M., Kispert, A., Chin, N., and McMahon, A.P. (1999). Female development in mammals is regulated by Wnt-4 signalling. *Nature.*

Val, P., Martinez-Barbera, J.-P., and Swain, A. (2007). Adrenal development is initiated by *Cited2* and *Wt1* through modulation of *Sf-1* dosage. *Development.*

del Valle, I., Buonocore, F., Duncan, A.J., Lin, L., Barenco, M., Parnaik, R., Shah, S., Hubank, M., Gerrelli, D., and Achermann, J.C. (2017). A genomic atlas of human adrenal and gonad development. *Wellcome Open Res.*

Vidal, V., and Schedl, A. (2000). Requirement of WT1 for gonad and adrenal development: Insights from transgenic animals. In *Endocrine Research*, p.

Vidal, V., Sacco, S., Rocha, A.S., Da Silva, F., Panzolini, C., Dumontet, T., Doan, T.M.P., Shan, J., Rak-Raszewska, A., Bird, T., et al. (2016). The adrenal capsule is a signaling center controlling cell renewal and zonation through *Rspo3*. *Genes Dev.*

Vidal, V.P.I., Chaboissier, M.C., De Rooij, D.G., and Schedl, A. (2001). *Sox9* induces testis development in XX transgenic mice. *Nat. Genet.*

Vilain, E.J. (2008). X-Linked Adrenal Hypoplasia Congenita. In *GeneReviews at GeneTests Medical Genetics Information Resource*, p.

Vilain, E., McElreavey, K., Jaubert, F., Raymond, J.P., Richaud, F., and Fellous, M. (1992). Familial case with sequence variant in the testis-determining region associated with two sex phenotypes. *Am. J. Hum. Genet.* 50, 1008–1011.

Vincent, S.D. (2005). The zinc finger transcriptional repressor *Blimp1/Prdm1* is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. *Development.*

Wang, Q., Lan, Y., Cho, E.S., Maltby, K.M., and Jiang, R. (2005). Odd-skipped related 1 (*Odd1*) is an essential regulator of heart and urogenital development. *Dev. Biol.*

Warner, R.R., and Swearer, S.E. (1991). Social control of sex change in the bluehead wrasse, *Thalassoma bifasciatum* (Pisces: Labridae). *Biol. Bull.*

Warr, N., Carre, G.A., Siggers, P., Faleato, J.V., Brixey, R., Pope, M., Bogani, D., Childers,

M., Wells, S., Scudamore, C.L., et al. (2012). Gadd45γ and Map3k4 Interactions Regulate Mouse Testis Determination via p38 MAPK-Mediated Control of Sry Expression. *Dev. Cell*.

Weiss, L. (1971). Additional evidence of gradual loss of germ cells in the pathogenesis of streak ovaries in Turner's syndrome. *J. Med. Genet.* 8, 540–544.

Welsh, M., Saunders, P.T.K., Marchetti, N.I., and Sharpe, R.M. (2006). Androgen-Dependent Mechanisms of Wolffian Duct Development and Their Perturbation by Flutamide. *Endocrinology* 147, 4820–4830.

Werner, R., Merz, H., Birnbaum, W., Marshall, L., Schröder, T., Reiz, B., Kavran, J.M., Bäumer, T., Capetian, P., and Hiort, O. (2015). 46,XY gonadal dysgenesis due to a homozygous mutation in desert hedgehog (DHH) identified by exome sequencing. *J. Clin. Endocrinol. Metab.*

Wijgerde, M., Ooms, M., Hoogerbrugge, J.W., and Grootegoed, J.A. (2005). Hedgehog signaling in mouse ovary: Indian hedgehog and desert hedgehog from granulosa cells induce target gene expression in developing theca cells. *Endocrinology*.

Wilhelm, D., Martinson, F., Bradford, S., Wilson, M.J., Combes, A.N., Beverdam, A., Bowles, J., Mizusaki, H., and Koopman, P. (2005). Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Dev. Biol.*

Wilhelm, D., Washburn, L.L., Truong, V., Fellous, M., Eicher, E.M., and Koopman, P. (2009). Antagonism of the testis- and ovary-determining pathways during ovotestis development in mice. *Mech. Dev.* 126, 324–336.

Wilson, E.A., and Joe Jawad, M. (1979). The Effect of Trophic Agents on Fetal Ovarian Steroidogenesis in Organ Culture. *Fertil. Steril.*

Wilson, C.A., High, S.K., McCluskey, B.M., Amores, A., Yan, Y.L., Titus, T.A., Anderson, J.L., Batzel, P., Carvan, M.J., Scharl, M., et al. (2014). Wild sex in zebrafish: Loss of the natural sex determinant in domesticated strains. *Genetics*.

Wong, V.S.C., Yeung, A., Schultz, W., and Brubaker, P.L. (2010). R-spondin-1 is a novel β-cell growth factor and insulin secretagogue. *J. Biol. Chem.*

Wu, S.M., and Chan, W.Y. (1999). Male pseudohermaphroditism due to inactivating luteinizing hormone receptor mutations. *Arch. Med. Res.*

Xie, Y., Zamponi, R., Charlat, O., Ramones, M., Swalley, S., Jiang, X., Rivera, D., Tschantz, W., Lu, B., Quinn, L., et al. (2013). Interaction with both ZNRF3 and LGR4 is required for the signalling activity of R-spondin. *EMBO Rep.*

Yamaji, M., Seki, Y., Kurimoto, K., Yabuta, Y., Yuasa, M., Shigeta, M., Yamanaka, K., Ohinata, Y., and Saitou, M. (2008). Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nat. Genet.*

Yang, M., Li, J., An, Y., and Zhang, S. (2015). Effects of androgen on immunohistochemical localization of androgen receptor and Connexin 43 in mouse ovary. *Tissue Cell* 47, 526–532.

Yao, H.H.-C. (2003). Meiotic germ cells antagonize mesonephric cell migration and testis cord formation in mouse gonads. *Development*.

Yao, H.H.-C., Aardema, J., and Holthusen, K. (2006). Sexually Dimorphic Regulation of Inhibin Beta B in Establishing Gonadal Vasculature in Mice<sup>1</sup>. *Biol. Reprod.*

Yao, H.H.C., Whoriskey, W., and Capel, B. (2002). Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes Dev.*

Yao, H.H.C., Matzuk, M.M., Jorgez, C.J., Menke, D.B., Page, D.C., Swain, A., and Capel,

B. (2004). Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. *Dev. Dyn.* 230, 210–215.

YOSHIMI, T., STROTT, C.A., MARSHALL, J.R., and LIPSETT, M.B. (1969). Corpus Luteum Function in Early Pregnancy. *J. Clin. Endocrinol. Metab.* 29, 225–230.

Youngren, K.K., Coveney, D., Peng, X., Bhattacharya, C., Schmidt, L.S., Nickerson, M.L., Lamb, B.T., Deng, J.M., Behringer, R.R., Capel, B., et al. (2005). The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. *Nature*.

Yu, J., and Virshup, D.M. (2014). Updating the Wnt pathways. *Biosci. Rep.*

Zeng, X., Huang, H., Tamai, K., Zhang, X., Harada, Y., Yokota, C., Almeida, K., Wang, J., Doble, B., Woodgett, J., et al. (2007). Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. *Development*.

Zhang, F.-P., Poutanen, M., Wilbertz, J., and Huhtaniemi, I. (2014). Normal Prenatal but Arrested Postnatal Sexual Development of Luteinizing Hormone Receptor Knockout (LuRKO) Mice. *Mol. Endocrinol.*

Zhang, L., Chen, M., Wen, Q., Li, Y., Wang, Y., Wang, Y., Qin, Y., Cui, X., Yang, L., Huff, V., et al. (2015). Reprogramming of Sertoli cells to fetal-like Leydig cells by Wt1 ablation. *Proc. Natl. Acad. Sci.* 112, 4003–4008.

Zhao, D., McBride, D., Nandi, S., McQueen, H.A., McGrew, M.J., Hocking, P.M., Lewis, P.D., Sang, H.M., and Clinton, M. (2010). Somatic sex identity is cell autonomous in the chicken. *Nature*.

Zhao, F., Franco, H.L., Rodriguez, K.F., Brown, P.R., Tsai, M.-J., Tsai, S.Y., H-C Yao, H., and Yao, H.H.-C. (2017a). Elimination of the male reproductive tract in the female embryo is promoted by COUP-TFII in mice. *Science* (80- ). 357, 717–720.

Zhao, L., Svingen, T., Ng, E.T., and Koopman, P. (2015). Female-to-male sex reversal in mice caused by transgenic overexpression of Dmrt1. *Development* 142, 1083–1088.

Zhao, L., Arsenault, M., Ng, E.T., Longmuss, E., Chau, T.C.Y., Hartwig, S., and Koopman, P. (2017b). SOX4 regulates gonad morphogenesis and promotes male germ cell differentiation in mice. *Dev. Biol.*

Zhao, L., Wang, C., Lehman, M.L., He, M., An, J., Svingen, T., Spiller, C.M., Ng, E.T., Nelson, C.C., and Koopman, P. (2018). Transcriptomic analysis of mRNA expression and alternative splicing during mouse sex determination. *Mol. Cell. Endocrinol.* 478, 84–96.

Zheng, W., Zhang, H., Gorre, N., Risal, S., Shen, Y., and Liu, K. (2014). Two classes of ovarian primordial follicles exhibit distinct developmental dynamics and physiological functions. *Hum. Mol. Genet.* 23, 920–928.

Zhou, B., Ma, Q., Rajagopal, S., Wu, S.M., Domian, I., Rivera-Feliciano, J., Jiang, D., Von Gise, A., Ikeda, S., Chien, K.R., et al. (2008). Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. *Nature* 454, 109–113.

Ziaei, S., Rajaei, L., Faghihzadeh, S., and Lamyian, M. (2002). Comparative study and evaluation of side effects of low-dose contraceptive pills administered by the oral and vaginal route. *Contraception*.

Zimmermann, S., Steding, G., Emmen, J.M.A., Brinkmann, A.O., Nayernia, K., Holstein, A.F., Engel, W., and Adham, I.M. (2014). Targeted Disruption of the Ins13 Gene Causes Bilateral Cryptorchidism. *Mol. Endocrinol.*