

# Ovarian development in mice requires the GATA4-FOG2 transcription complex

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We have demonstrated previously that mammalian sexual differentiation requires both the GATA4 and FOG2 transcriptional regulators to assemble the functioning testis. Here we have determined that the sexual development of female mice is profoundly affected by the loss of GATA4-FOG2 interaction. We have also identified the *Dkk1* gene, which encodes a secreted inhibitor of canonical  $\beta$ -catenin signaling, as a target of GATA4-FOG2 repression in the developing ovary. The tissue-specific ablation of the  $\beta$ -catenin gene in the gonads disrupts female development. In *Gata4<sup>ki/ki</sup>; Dkk1<sup>-/-</sup>* or *Fog2<sup>-/-</sup>; Dkk1<sup>-/-</sup>* embryos, the normal ovarian gene expression pattern is partially restored. Control of ovarian development by the GATA4-FOG2 complex presents a novel insight into the cross-talk between transcriptional regulation and extracellular signaling that occurs in ovarian development.

**KEY WORDS:** *Fog2* (*Zfpm2*), *Gata4*, *Dkk1*, Ovary,  $\beta$ -catenin, Mouse

## INTRODUCTION

Male sex determination in the majority of mammals is initiated by *Sry*, the Y chromosome-linked testis-determining gene (reviewed by Capel, 2000; Swain and Lovell-Badge, 1999; Wilhelm et al., 2007). By contrast, genetic mechanisms of development remain enigmatic for mammalian females. The idea that ovarian differentiation requires its own set of genes and occurs shortly after (Eicher and Washburn, 1986), or even precedes (McElreavey et al., 1993), that of the testes was put forward a number of years ago. However, sexually dimorphic expression in the developing ovary was not identified until fairly recently. This new evidence clearly demonstrates that the female pathway of development engages a number of dedicated genes. The two alternative sex fates are thought to emerge through the antagonistic activities of sex-specific transcription factors in a restricted number of gonadal cells. This initial cell-fate decision is further expanded by extracellular non-cell-autonomous signals that promote one developmental program, while at the same time suppressing the other (Brennan and Capel, 2004; Capel, 2006; Kim and Capel, 2006; Kim et al., 2006).

We have previously demonstrated an in vivo requirement for the transcription factor GATA4 and its co-factor FOG2 (ZFPM2 – Mouse Genome Informatics) in testis differentiation (Tevosian et al., 2002). *Gata4<sup>ki/ki</sup>* mutants [ki is a V217G mutation in GATA4 that specifically cripples the interaction between GATA4 and FOG proteins (Crispino et al., 2001)], as well as *Fog2*-null embryos (Tevosian et al., 2000), exhibit a profound early block in testis differentiation. Here, we demonstrate that a deficiency in GATA4-FOG2 interaction leads to a block in ovarian development coincidental with a drastic alteration in the female gene expression program.

To avoid referring each time to both FOG2 null and GATA4<sup>ki</sup> mutants we will sometimes refer to them collectively as ‘GATA4/FOG2’ mutants (and to the phenotype as the ‘GATA4-FOG2 interaction/complex loss’). This is justified, as the abrogation

of GATA4-FOG2 interaction by a *Gata4<sup>ki</sup>* mutation (*Gata4<sup>ki/ki</sup>*) or *Fog2* loss (*Fog2<sup>-/-</sup>*) results in equivalent defects in mouse gonadal differentiation in every experiment we have performed so far. It is formally possible, however, that these mutations have non-overlapping roles in gonadogenesis (for example, FOG2 could have a GATA4-independent function; and the ‘ki’ mutation in GATA4 also renders it incapable of interacting with FOG1). Hence, we performed experiments with both mutants to eliminate this possibility. Importantly, the *Gata4<sup>ki</sup>* mutation is not a *Gata4* loss of function; deletion of *Gata4* gene in gonads may have a different outcome that does not necessarily phenocopy *Fog2* gene loss or the *Gata4<sup>ki/ki</sup>* phenotype.

## MATERIALS AND METHODS

### Animals

The generation and genotyping of *Fog2*-null and *Gata4<sup>ki</sup>*-targeted animals have been described previously (Crispino et al., 2001; Tevosian et al., 2000). These strains, as well as *Wnt4<sup>-/-</sup>* mice (Vainio et al., 1999), were maintained on the C57BL/6 background.  $\beta$ -catenin (*Ctnnb1*) mutant mice were obtained from the Jackson Laboratory. The *Flk-1-lacZ* strain has been genotyped, as previously described (Shalaby et al., 1997). The *Dkk1<sup>+/-</sup>* genotype was determined using primers *dkk1* (5'-CTTCCGACACACAAACTCCC-3') and *dkk1rev* (5'-GTAAACCAAACACTCTCGTTCAGC-3'). *Sfl-Cre* mice were genotyped with *Cre*-specific primers as previously described (Bingham et al., 2006). *Axin2<sup>lacZ</sup>* mice (Yu et al., 2005) were obtained from the EMMA repository.

### Affymetrix microarray analysis of gene expression

Gonad-mesonephros complexes were dissected from E12.5 XX wild-type and *Gata4<sup>ki/ki</sup>* mutant embryos and Affymetrix oligonucleotide arrays were used for RNA expression analysis (Chee et al., 1996; Lipshutz et al., 1999). The array experiment was performed by Dartmouth Genomic and Microarray Laboratory according to a standard protocol. The microarray data have been deposited at the GEO database (GSE11314) and were analyzed using Gene Traffic (Iobion Informatics).

### In situ hybridization

In situ hybridization (ISH) analysis was carried out essentially as previously described (Manuylov et al., 2007a). *Sox9*, *Mis* (*Amh*), *Wnt4*, *Cyp17a1*, *Hsd3b1* and *Cyp11a1* RNA probes have been described (Tevosian et al., 2002); the *Irx3*-fragment-containing vector was a gift of Dr Nef (Nef et al., 2005) and the *Gng13*-containing vector was a gift of Dr Arango (Fujino et al., 2007). Other probes were generated with cDNA obtained from the embryonic total or gonadal RNA by RT-PCR (Table 1).

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**Table 1. PCR primers and hybridization probes**

| Gene                 | 5' primer                | 3' primer                 | Product size (bp) |
|----------------------|--------------------------|---------------------------|-------------------|
| <i>Sprr2d</i> *      | ACTTTGGAGAACCCGATCCT     | CAGGAAATGAAACCATGCTG      | 637               |
| <i>Dkk1</i>          | TACCCCTTGCCTGAAGATGAGGAG | ACATCCTTGGGATTGAGCTG      | 657               |
| <i>Fst</i>           | AAAACCTACCGCAACGAATG     | CCGGCTCATCCGACTTACTGTCA   | 440               |
| <i>Foxl2</i>         | CATGTTCCGAGAAGGGCAACTA   | CTCATACTGGGACCACGACA      | 423               |
| <i>Sp5</i>           | AGCGAGCGGCTTCAAGCAGTAG   | TGGGTCATAGGGCACCTGAAGGA   | 208               |
| <i>Inha</i>          | ATGCACAGGACCTCTGAACC     | TATTGAGAGCAGCTCGATGG      | 592               |
| <i>Stra8</i>         | GGAGAAAAAGGCCAGACTCC     | TGGAAGCAGCCTTCTCAAT       | 537               |
| <i>Oct4 (Pou5f1)</i> | AGAGGGAACTCCTCTGAGC      | TGGGAAAGGTGTCCCTGTAG      | 592               |
| <i>Rspo1</i>         | GCTCCAACCTCGGAGACAC      | AAGCGTTTCCCTCTCTCTC       | 506               |
| <i>Bmp2</i>          | TTTGGCCTGAAGCAGAGACCCA   | TTGTGGAAGCTCTCCACTGA      | 443               |
| <i>Lrp6</i>          | GGGTGGGTGCTGGAGATATTGTA  | TTTCCTCGTAAAATTGCTGGATTCC | 442               |

\*Kenn Albrecht, personal communication.

### Real-time PCR

Individual gonad-mesonephros complexes were dissected from E11.5-14.5 embryos and RNA was isolated using the RNeasy Mini Kit (Qiagen). All real-time PCR assays were carried out using the SYBR Green I Kit (Applied Biosystems) as previously described (Manuylov et al., 2007b; Smagulova et al., 2008). The relative expression level for each sample was determined in the same run and was expressed as the ratio of the quantity of RNA of interest to that of a control RNA (*Gapdh*). Gene-specific primers and probes were designed using Primer Express (Perkin Elmer); primer sequences are shown in Table 2 and are also available upon request.

### Immunofluorescence

For protein detection, the following antibodies and dilutions were used: mouse anti-DKK1 (R&D Systems; 1:300); rabbit anti-FOXL2 (a gift of Drs Fellous and Veitia, Université Denis, Diderot, Paris, France; 1:300); rat anti-PECAM1 (BD Biosciences; 1:500); rabbit anti- $\gamma$ H2AX (Upstate; 1:300); rabbit anti- $\beta$ -catenin (Sigma; 1:300); rabbit anti-SYN1 (a gift of Drs Moens and Spyropoulos, York University, Toronto, Canada; 1:300) and rabbit anti-Cre (a gift of Dr Ernst, Dartmouth, Hanover, USA; 1:1000). Secondary antibodies (Invitrogen) were used at 1:500. All antibodies were diluted in Antibody Diluent Solution (Dako). The slides were mounted in Vectashield with DAPI (4',6-diamidino-2-phenylindole, Vector Labs) and photographed. The confocal analysis of anti-PECAM1-stained gonads was performed as described (Manuylov et al., 2007a).

### Germ cell depletion

Germ cell depletion was performed as described (Yao et al., 2003). The depletion was confirmed by *Oct4* ISH and alkaline phosphatase staining. To generate *W/W<sup>v</sup>* embryos depleted of germ cells, male mice carrying the *c-Kit*

mutation, dominant white spotting (*W*), were mated to females carrying viable dominant spotting (*W<sup>v</sup>*) (Mintz and Russell, 1957). Double heterozygotes depleted of germ cells were identified by immunostaining one gonad from each pair with the germ cell marker PECAM1.

## RESULTS

### Normal ovarian differentiation requires GATA4-FOG2 interaction

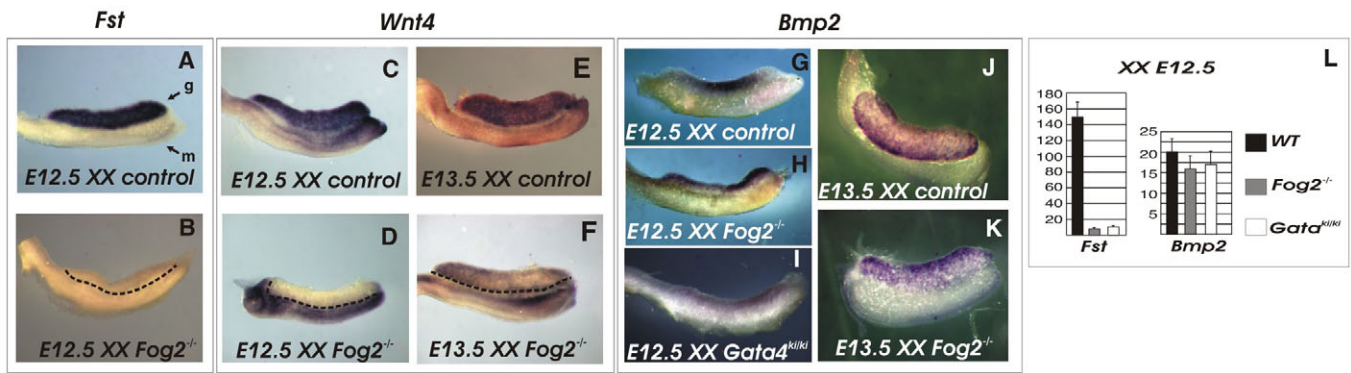
In order to identify the targets of GATA4-FOG2 during ovarian development, we performed an Affymetrix microarray analysis of gene expression, comparing RNA samples from *Gata4<sup>ki/ki</sup>* mutant and wild-type (control) E12.5 ovaries. Two independent probe sets corresponding to the follistatin (*Fst*) gene on the microarray showed the most dramatic downregulation among all the differentially expressed genes (~12-fold). The *Fst* gene encodes a secreted protein that blocks the function of multiple members of the TGF $\beta$  superfamily (Patel, 1998). Although *Fst* expression in embryonic gonads had been reported a number of years ago (Feijen et al., 1994), its sexually dimorphic pattern was not appreciated until recently (Menke and Page, 2002). Both in situ hybridization (ISH) with an antisense *Fst* probe (Fig. 1A,B) and real-time RT-PCR (Fig. 1L) confirmed the loss of *Fst* expression in *Gata4/Fog2* mutants.

*Fst* is a downstream component of *Wnt4* signaling (Yao et al., 2004). *Wnt4* controls *Fst* and *Bmp2* (bone morphogenetic protein 2) expression in embryonic ovaries; in *Wnt4*-null XX E12.5 gonads, expression of both *Fst* and *Bmp2* is lost. The *Wnt4-Fst* pathway

**Table 2. PCR primers for real-time PCR**

| Gene                | 5' primer                  | 3' primer                   |
|---------------------|----------------------------|-----------------------------|
| <i>Sf1 (Nr5a1)</i>  | TCGTGGTGGTAGTCGTCGTA       | CTCCCTCTGGTCTCTTGTCT        |
| <i>Sprr2d</i>       | AAGGGAAAAGAAGAGAATCTATCT   | TTGACACCAAAGCAGGACAACCT     |
| <i>Foxl2</i>        | GCAAGGGAGGCGGGACAACAC      | GAACGGGAACCTGGCTATGATGT     |
| <i>Rspo1</i>        | CAAAGAGACCCGCAAGTGTACCGT   | TGTGCCAGGTAGGTCCTACTGATG    |
| <i>Bmp2</i>         | GCTAGATCTGTACCGCAGGCACCTCA | TCCCACTCATCTCTGGAAGTTCCTC   |
| <i>Sp5</i>          | GCAGGCCTTCTCCAGGACCGCAC    | AGCGCTGGGTATAGGGCACCTGA     |
| <i>Stra8</i>        | GATGCTTTTGACGTGGCAAGTTTCC  | TCATCATCTGGGGGCTCTGGTTC     |
| <i>Fst</i>          | GTCTGTGCCAGTGACAATGCC      | TTCTCCGTTTCTTCCGAGATG       |
| <i>Scp1 (Syce1)</i> | GGAAGATGTGGAAAAGAATAATGATA | AATAACATGGATTGAAGAGACTTTCCG |
| <i>Dmc1</i>         | GGCCAGATGTTGTCACGACTC      | TCAGTTCTCTCTTCCCTTGCG       |
| <i>Irx3*</i>        | CGCCTCAAGAAGGAGAACAAGA     | CGCTCGCTCCCATAAGCAT         |
| <i>Wnt4</i>         | GCGTAGCCTTCTCACAGTCC       | ACGTCTTTACCTCGCAGGAG        |
| <i>Gng13</i>        | GGTAGAGAGCCTCAAGTACCAACTG  | ACACCTTACAGAGAGTGTGGGTCAG   |
| <i>Gapdh</i>        | GCTCACTGGCATGGCCTTCCGTG    | TGGAAGAGTGGGAGTTGCTGTTGA    |
| <i>Dkk1</i>         | GGAAATTGAGGAAAGCATCATTGAA  | CAGATCTTGGACCAGAAGTGTCTTG   |
| <i>Fog2</i>         | CGCCTTTGTGGTGGACTTTGACT    | GCTTCTCGTTGCTCCACTACA       |
| <i>Gata4</i>        | GGCTCCAGAGATTCTTCTCT       | CTCTGCTACGGCCAGTAAGG        |
| <i>Wnt9a</i>        | GGATTTGCGAGCCCGAGTGGACTT   | GGTAGTGTGCCCCACCTTGAGCGA    |

\*(Jorgensen and Gao, 2005).



**Fig. 1. Normal ovarian differentiation requires GATA4-FOG2 Interaction.** (A-K) Whole-mount in situ hybridization (ISH) was performed with the indicated RNA probes on XX gonads from wild-type, *Gata4*<sup>ki/ki</sup> and *Fog2*<sup>-/-</sup> E12.5-13.5 mouse embryos. In all images, samples are oriented with anterior towards the right, and a gonad (g) on top of a mesonephros (m) as indicated in A. In images in which gonadal expression is not apparent, a black dashed line is used to show the gonad-mesonephric border. (L) Real-time PCR analysis of *Fst* and *Bmp2* gene expression in wild-type, *Fog2*<sup>-/-</sup> and *Gata4*<sup>ki/ki</sup> E12.5 XX gonads. The y-axis shows values for both genes normalized to *Gapdh* RNA copy number.

opposes the formation of the male-specific vasculature in the female and ensures the survival of meiotic germ cells; this constitutes the first established signaling pathway important for early development of the mammalian ovary (Yao et al., 2004). *Wnt4* expression was lost in the gonad of E12.5 XX *Fog2*-null mutants (Fig. 1D). Importantly, although E12.5 *Gata4/Fog2* mutant XX gonads did not express *Wnt4*, weak but detectable *Wnt4* expression reappeared in the E13.5 mutant gonads (Fig. 1F). *Wnt4* expression is also observed in XY E13.5-14.5 *Fog2* mutant gonads (Tevosian et al., 2002).

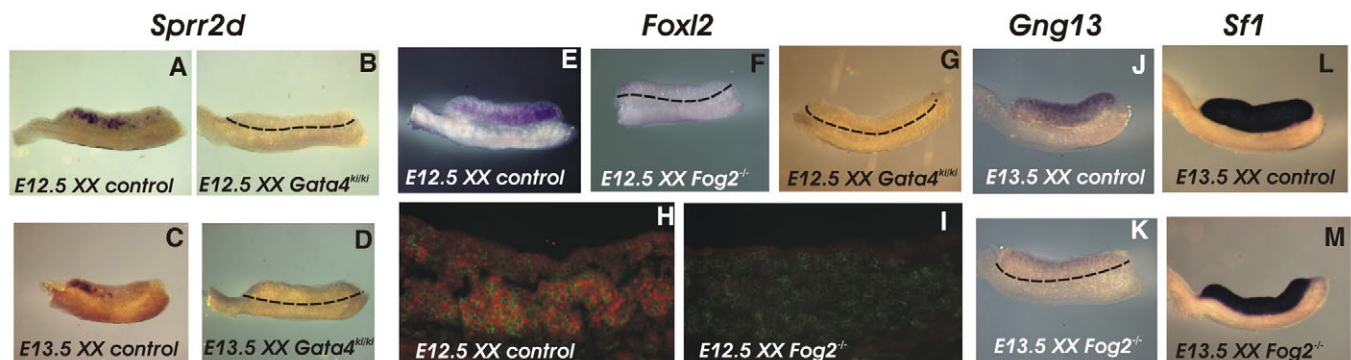
Based on the previously established epistatic relationship between *Wnt4* and *Bmp2*, we expected *Bmp2* expression to be similarly absent from the *Gata4/Fog2* mutant gonads. This, however, was not the case: ISH with a *Bmp2* probe and real-time PCR analysis revealed no change in *Bmp2* expression in either the *Fog2* (Fig. 1H,K,L) or *Gata4*<sup>ki/ki</sup> (Fig. 1I,L) mutant sample and, in agreement, our microarray analysis showed no difference for the *Bmp2* probe set (data not shown). Interestingly, a recently described XX *Rspo1*<sup>-/-</sup> mutation demonstrates a similar relationship between *Wnt4* and *Bmp2*: *Wnt4* expression is temporarily lost in the XX E12.5 gonad (but not mesonephros), whereas *Bmp2* levels remain unchanged (Chassot et al., 2008). In summary, loss of GATA4-FOG2 interaction in the XX gonad results in the loss of *Fst* expression and

a failure to activate *Wnt4* in the gonad at E12.5; however, residual *Wnt4* expression appears to be sufficient for maintaining the normal level of *Bmp2*.

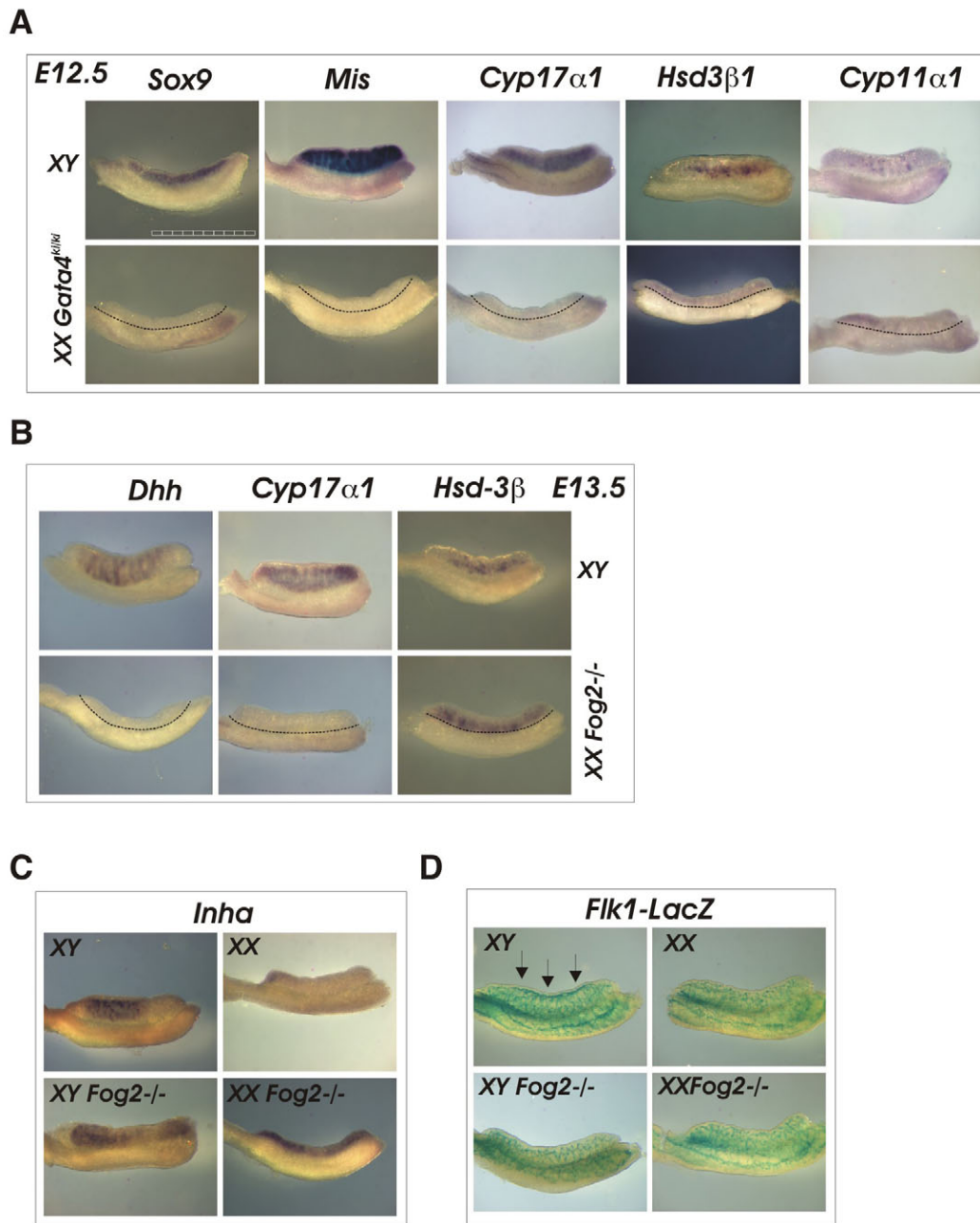
### GATA4/FOG2 loss affects multiple aspects of early ovarian differentiation

In addition to affecting *Wnt4* and *Fst* expression, as described above, loss of the GATA4-FOG2 complex disrupts the expression of numerous other genes that have been implicated in ovarian development. Expression of the dimorphically expressed genes, *Sprr2d* (small proline-rich 2d) (Beverdam and Koopman, 2006) and *Foxl2* (forkhead box L2), was lost in *Gata4/Fog2* mutants (Fig. 2A-I) and expression of *Gng13* (guanine nucleotide binding protein, gamma 13) (Beverdam and Koopman, 2006; Fujino et al., 2007) was strongly downregulated (Fig. 2J,K). By contrast, *Sf1* (*Nr5a1* – Mouse Genome Informatics) expression does not require the GATA4-FOG2 complex (Fig. 2L,M) (see Tevosian et al., 2002).

The current view of mammalian sex determination emphasizes the notion that the two alternative fates, female and male, arise as closely intertwined parities that are determined by antagonistic activities (Kim and Capel, 2006); hence, suppression of one developmental program could result in the emergence of the other.



**Fig. 2. The GATA4-FOG2 complex is essential for the activation of dimorphically expressed genes.** (A-G, J-M) Whole-mount ISH was performed with *Sprr2d* (A-D), *Foxl2* (E-G), *Gng13* (J,K) and *Sf1* (*Nr5a1*; L,M) RNA probes on XX gonads from wild-type, *Gata4*<sup>ki/ki</sup> and *Fog2*<sup>-/-</sup> E12.5-13.5 mouse embryos as indicated. (H,I) Immunofluorescent staining of frozen gonadal sections from *Fog2*<sup>+/-</sup> (control) or *Fog2*<sup>-/-</sup> embryos with an anti-FOXL2 antibody (red). Embryonic germ cells are detected by the anti-PECAM1 antibody (e.g. Yao et al., 2003) (green). Magnification: 200X.



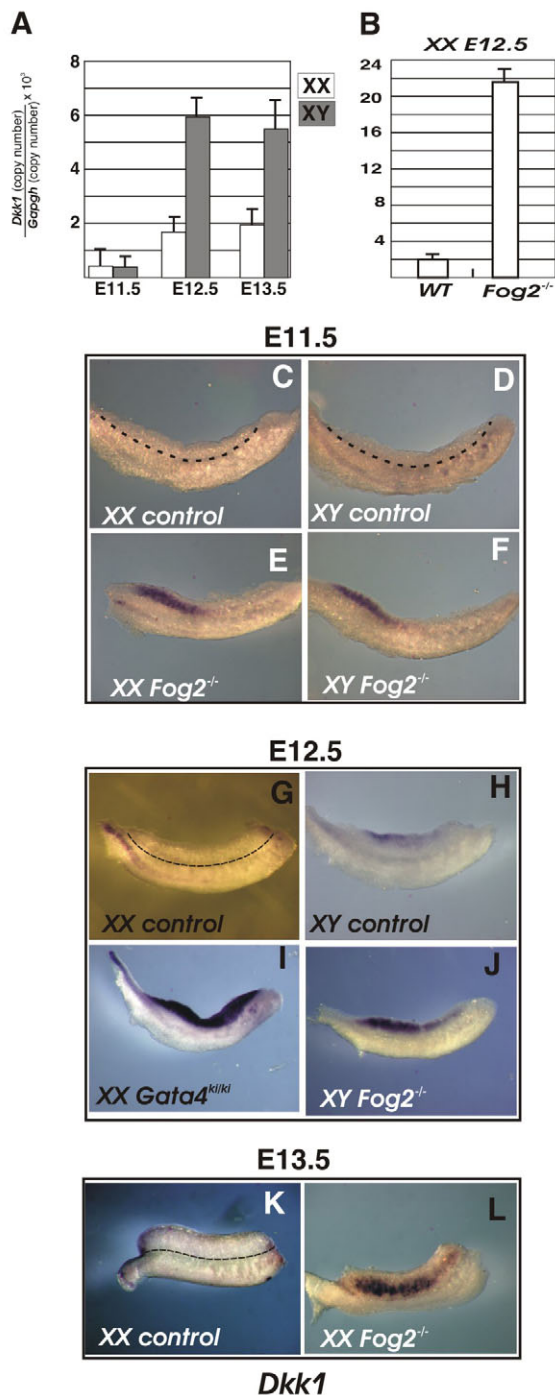
**Fig. 3. GATA4/FOG2 loss affects multiple aspects of early ovarian differentiation without activating Sertoli cell differentiation.**

(A, B) Whole-mount ISH was performed on XX E12.5 control or *Gata4*<sup>kiki</sup> gonads (A) or XY E12.5 control or *Fog2*<sup>-/-</sup> gonads (B) with the indicated probes. Note that the expression of Sertoli cell markers (*Sox9*, *Mis* and *Dhh*) is not increased in E12.5-13.5 XX gonads upon GATA4/FOG2 loss, whereas expression of genes encoding steroidogenic enzymes (e.g. *Hsd3b1*) is relaxed. Scale bar: 1 mm. (C) ISH was performed with an *Inha* probe on XX and XY wild-type and *Fog2*<sup>-/-</sup> gonads. Expression of *Inha* is derepressed in the XX *Fog2*<sup>-/-</sup> gonad. (D) The coelomic blood vessel does not form in *Fog2*<sup>-/-</sup> gonads. XX and XY gonads from E12.5 mouse embryos carrying *Flk1-lacZ* with wild-type or homozygous mutant *Fog2* were stained with X-Gal, which marks the *Flk1-lacZ*-positive endothelial cells. The coelomic blood vessel in the testis is indicated by arrows.

Examination of XX gonads with GATA4/FOG2 loss showed no signs of testis cord formation (see Fig. S1 in the supplementary material) and markers of Sertoli cell differentiation (*Sox9*, *Mis* and *Dhh*) were absent (Fig. 3A,B). The GATA4-FOG2 transcription complex is required for *Sox9* activation and testis differentiation (Manuylov et al., 2007a; Tevosian et al., 2002), so it is not surprising that the loss of GATA4-FOG2 interaction does not result in the activation of Sertoli cell differentiation in the XX mutant gonad. Despite the absence of Sertoli cell differentiation, *Gata4/Fog2* mutants selectively expressed some steroidogenic genes commonly associated with the embryonic testis (Fig. 3A,B and see Fig. S2 in the supplementary material). Similarly, expression of inhibin alpha (*Inha*) has been associated with embryonic testes rather than ovarian development in several vertebrate species (e.g. Majdic et al., 1997; Safi et al., 2001); the regulation of *Inha* expression by GATA factors

has been documented in cultured cells (Robert et al., 2006). The GATA4-FOG2 complex functions to repress *Inha* expression in the developing ovary (Fig. 3C). In the control E12.5 gonads, *Inha* expression was much stronger in the male sample, whereas both XX and XY *Fog2*<sup>-/-</sup> gonads robustly expressed *Inha*. In summary, similar to the recently reported *Rspo1*<sup>-/-</sup> mutation (Chassot et al., 2008; Tomizuka et al., 2008), analysis of the *Gata4/Fog2* mutants revealed a complex blend (rather than exclusive dominance) of female- and male-specific expression in the XX gonads with a compromised female program.

One of the early signs of the active testis-specific program is the development of the male-specific vascular pattern. In mouse mutants with a loss of female-specific genes (*Wnt4*, *Fst* and *Rspo1*), a male-specific coelomic vessel appears in XX gonads (Brennan et al., 2002; Brennan et al., 2003; Chassot et al., 2008; Jeays-Ward et



**Fig. 4. *Dkk1* is a downstream target of the GATA4-FOG2 transcription complex.** (A,B) qRT-PCR analysis of *Dkk1* expression was performed with wild-type (A) or wild-type and *Fog2*<sup>-/-</sup> (B) gonad-mesonephros samples. (C-L) ISH was performed with a *Dkk1* RNA probe on XX and XY gonads from wild-type, *Gata4*<sup>ki/ki</sup> and *Fog2*<sup>-/-</sup> E11.5-13.5 mouse embryos as indicated.

al., 2003; Yao et al., 2004). To examine the vascular development in *Gata4/Fog2* XX mutants, we introduced the *Flk1-lacZ* allele (*Flk* is also known as *Kdr* – Mouse Genome Informatics) (Shalaby et al., 1997) into the crosses and used an X-Gal staining assay to ‘develop’ the vasculature as previously described (Tevosian et al., 2000) (Fig. 3D). We noted that the vascular system in both E12.5 XY and XX

mutants developed similarly to the control XX gonad (ovary) and lacked the coelomic male-specific vessel that is clearly observed in the normal testis (Fig. 3D, arrows).

### ***Dkk1* is a downstream target of the GATA4-FOG2 transcription complex**

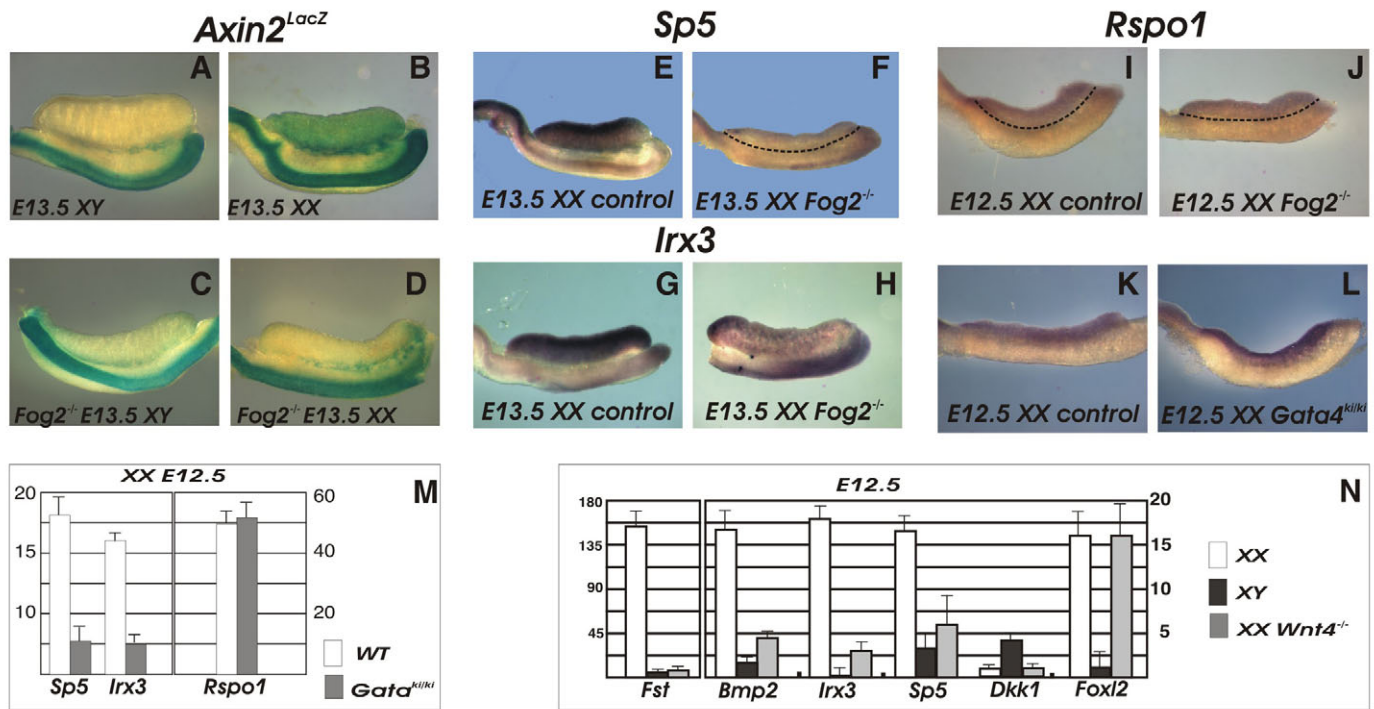
One of the microarray probe sets identified *Dkk1* as a target for GATA4-FOG2 regulation in the developing ovary. Gonadal *Dkk1* expression has not been previously characterized. Quantitative (q) RT-PCR analysis demonstrated that *Dkk1* expression is detectable in both sexes in the developing gonad as early as E11.5, and that the *Dkk1* level increases in the developing testis at E12.5. (Fig. 4A). qRT-PCR also confirmed a strong upregulation of *Dkk1* expression in E12.5 XX *Fog2* mutant gonads (Fig. 4B). ISH with a *Dkk1* antisense RNA probe demonstrated that *Dkk1* is overexpressed in the developing gonad but not mesonephros (Fig. 4C-L). In the control samples, the expression became visible only in the males at E12.5 and was localized to the central region of the gonad (Fig. 3H). By contrast, in the *Gata4/Fog2* mutants strong *Dkk1* expression was already apparent at E11.5 in the posterior region of the gonad (Fig. 4E,F). In summary, this analysis established that *Dkk1* is upregulated in the embryonic gonads as early as E12.5 and that this expression is dramatically increased upon GATA4-FOG2 complex loss.

### **Wnt pathway genes in the *Gata4/Fog2* mutants**

At present, a preponderance of data defines DKK1 function mainly within the context of the antagonism of canonical Wnt/ $\beta$ -catenin signaling (Mukhopadhyay et al., 2001) (reviewed by Kikuchi et al., 2007; Niehrs, 2006). The *Axin2*<sup>lacZ</sup> reporter has proven to be effective in monitoring the activity of the  $\beta$ -catenin signaling pathway in the ovary (Chassot et al., 2008); *Axin2*<sup>lacZ</sup> animals carry the  $\beta$ -galactosidase (*lacZ*) gene in (knocked-in) the *Axin2* locus (Yu et al., 2005). *Axin2* is considered to be one of the two (the other being *Sp5*, see below) candidates for a ‘universal’ Wnt target gene (Clevers, 2006). Although the *Axin2* expression level in embryonic gonads is low (data not shown), a sensitive X-Gal assay in the E13.5 gonads of *Axin2*<sup>lacZ</sup> embryos clearly shows activation of this gene in ovaries but not testes (Fig. 5A,B), as reported previously (Chassot et al., 2008). To examine the *Axin2*<sup>lacZ</sup> expression upon *Fog2* loss, we generated XX *Fog2*-null embryos with an *Axin2*<sup>lacZ</sup> reporter. In the XX *Fog2*-null gonads, *Axin2*<sup>lacZ</sup> expression was lost (Fig. 5A-D).

A list of Wnt/ $\beta$ -catenin target genes is available from the Wnt homepage (<http://www.stanford.edu/~rnusse/wntwindow.html>). We compared this list to the list of genes differentially expressed in *Gata4*<sup>ki/ki</sup> mutants. Although transcriptional outputs of the Wnt pathway are thought to be cell-specific (Clevers, 2006), one of the best ‘universal’ Wnt/ $\beta$ -catenin target genes is considered to be *Sp5* (Clevers, 2006; Weidinger et al., 2005). Microarray analysis detected the downregulation of *Sp5* expression in the *Gata4*<sup>ki/ki</sup> mutant (~3-fold). Likewise, expression of the other Wnt target gene that encodes a transcription factor, *Irx3* (Braun et al., 2003), is reduced. ISH confirmed that *Sp5* and *Irx3* are downregulated in XX E13.5 mutants with GATA4-FOG2 complex loss (Fig. 5E-H). This decrease in the *Sp5* and *Irx3* levels in the mutants was confirmed by qRT-PCR (Fig. 5M). Both *Sp5* and *Irx3* are expressed in an XX-enriched, sexually dimorphic manner (Bouma et al., 2007a; Jorgensen and Gao, 2005; Nef et al., 2005).

Recent work has established that Rspo genes, another gene family that activates the  $\beta$ -catenin pathway, also play a role in female sexual development (Chassot et al., 2008; Parma et al., 2006; Tomizuka et al., 2008). *Rspo1* expression is normal in *Gata4*<sup>ki/ki</sup> and *Fog2* mutants (Fig. 5I-M).



**Fig. 5. Comparative expression of Wnt pathway genes in *Gata4/Fog2* and *Wnt4* mutants.** (A–D) The GATA4-FOG2 complex is essential for canonical  $\beta$ -catenin signaling. Gonads from E12.5 mouse embryos carrying *Axin2<sup>lacZ</sup>* and either wild-type (A,B) or homozygous mutant (C,D) *Fog2* were stained with X-Gal, which labels *Axin2<sup>lacZ</sup>*-positive cells. Only the ovary (B) is positive for *Axin2<sup>lacZ</sup>* expression. (E–L) ISH was performed with the indicated probes on XX gonads from wild-type, *Gata4<sup>kiki</sup>* and *Fog2<sup>-/-</sup>* E12.5–13.5 embryos. (M) qRT-PCR analysis of *Sp5*, *Irx3* and *Rspo1* gene expression in wild-type and *Gata4<sup>kiki</sup>* E12.5 XX gonads. (N) qRT-PCR analysis of gene expression in wild-type and *Wnt4<sup>-/-</sup>* E12.5 gonads.

### GATA4-FOG2 and WNT4 regulate a partially overlapping set of genes

Our data suggest that in *Gata4/Fog2* mutants, gonadal *Wnt4* expression is strongly downregulated during the critical time (E12.5) for ovarian development, as is the WNT4 target gene *Fst*. At the same time, the expression of another WNT4 gonadal target, *Bmp2*, remains unchanged. To examine whether the GATA4-FOG2 and WNT4 pathways overlap with respect to any other gene targets, we performed qRT-PCR in the *Wnt4* XX mutants (Fig. 5N). Both *Fst* and *Bmp2* were severely downregulated in the absence of *Wnt4*, as previously reported (Yao et al., 2004). The targets of canonical  $\beta$ -catenin signaling, *Sp5* and *Irx3*, were also downregulated in the *Wnt4* mutants. However, *Irx3* expression was reduced to a greater extent in the *Wnt4*-null than in the *Gata4/Fog2* XX mutant gonads (Fig. 5, compare *Irx3* in M and N). By contrast, *Dkk1* was not upregulated upon *Wnt4* loss. Similarly, another target of GATA4-FOG2 regulation, *Foxl2*, was expressed normally in the *Wnt4*-null gonads (Fig. 5N).

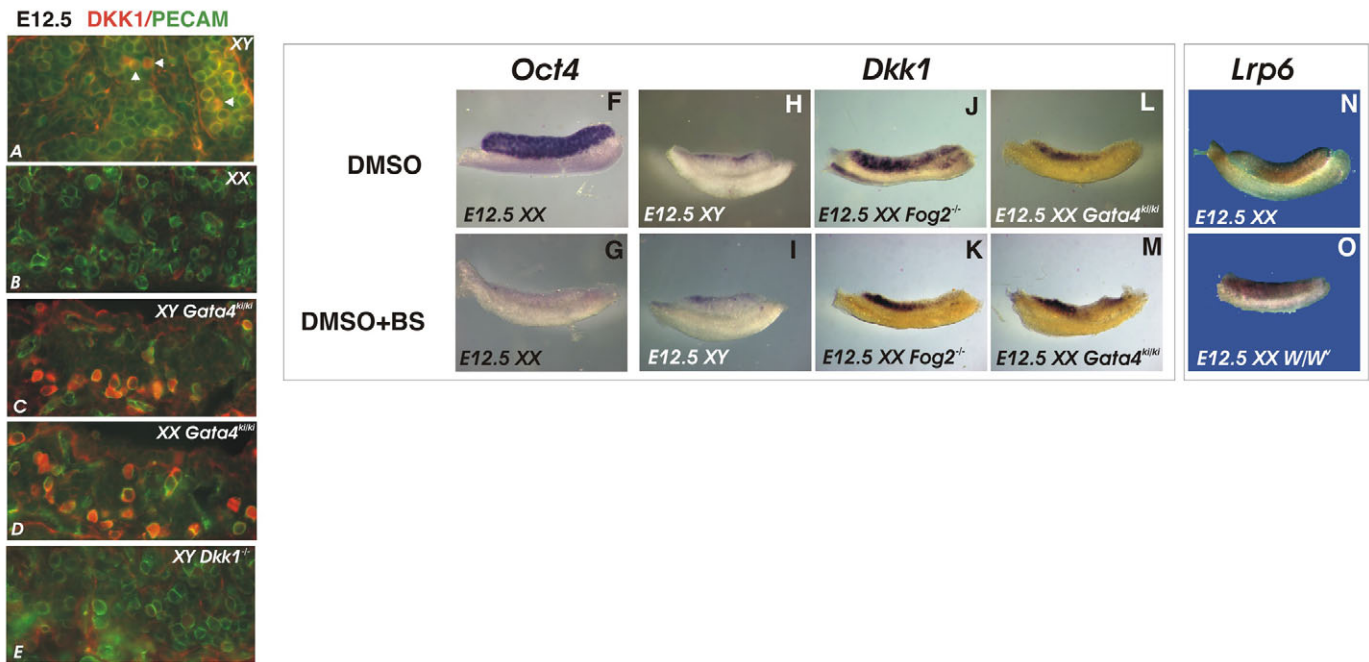
### DKK1 protein accumulates in *Gata4/Fog2* mutant gonads

So far, our data suggest that canonical  $\beta$ -catenin signaling regulates the gene expression program in somatic cells and that ectopic *Dkk1* activation in *Gata4/Fog2* mutants interferes with this signaling (Figs 4 and 5). Although ISH indicated that *Dkk1* is expressed by the somatic cells (data not shown), DKK1 is a secreted protein that acts outside of the cell (Glinka et al., 1998). To investigate the localization of DKK1 protein in the developing gonads, we performed immunofluorescence analysis. In accordance with the

RNA expression data (Fig. 4), DKK1 staining in the E12.5 gonads was faint and appeared marginally enhanced in the testes (Fig. 6A, arrowheads) as compared with the ovaries (Fig. 6B). By contrast, we observed intense DKK1 staining in both XY and XX mutant gonads, as predicted from our ISH experiments (Fig. 6C,D). No staining was observed in the gonads of XY *Dkk1<sup>-/-</sup>* embryos (Fig. 6E).

### DKK1 acts cell-autonomously in the somatic cells of the developing ovary

Immunofluorescence analysis demonstrated that DKK1 accumulates in the vicinity of germ cells (Fig. 6C,D); the localization of DKK1 in other settings, at or near plasma membranes, has been reported previously (Caneparo et al., 2007; Maekawa et al., 2005; Mao et al., 2002). This finding was unexpected, as GATA4 and FOG2 are not expressed in germ cells during embryogenesis. Although this expression pattern could be indicative of germ cell-derived DKK1, DKK1 is a secreted protein and so its accumulation pattern does not necessarily reflect its expression origin. To investigate the origin of gonadal *Dkk1* expression we used busulfan, an alkylating agent that can be used in rodents to deplete embryonic gonads of germ cells (Menke and Page, 2002; Merchant, 1975). Staining for expression of the germ cell-specific POU transcription factor *Oct4* (*Pou5f1* – Mouse Genome Informatics) confirmed that most germ cells were eliminated in the busulfan-treated E12.5 XX gonads (Fig. 6F,G). By contrast, the expression of *Dkk1* was not affected by busulfan in either the control XY sample (Fig. 6H,I) or in XX *Gata4/Fog2* mutants (Fig. 6J–M). Therefore, *Dkk1* expression is not dependent on the presence of germ cells.

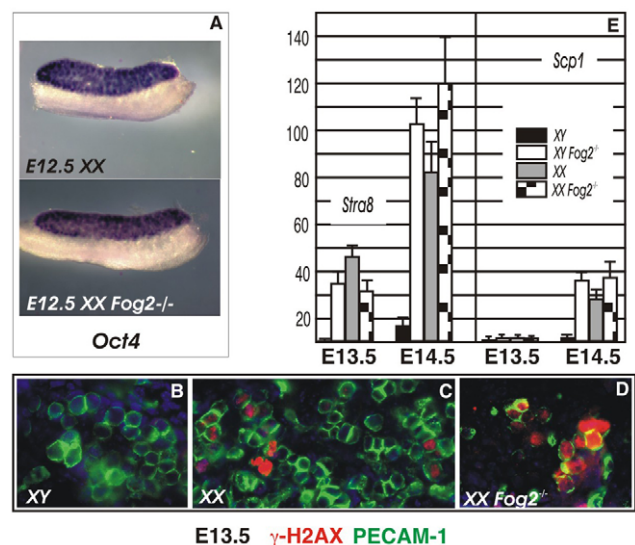


**Fig. 6. *Dkk1* acts cell-autonomously in the somatic cells of the developing ovary.** (A-E) Immunofluorescent staining of frozen sections with an anti-DKK1 antibody (red). Embryonic germ cells are detected by the anti-PECAM1 antibody (green). Note the DKK1 staining in the wild-type testis (A, arrowheads) and mutant gonads (C,D), but not in wild-type ovaries (B) or *Dkk1*-null testis (E). Magnification: 200 $\times$ . (F-O) ISH was performed with *Oct4* (F,G), *Dkk1* (H-M) and *Lrp6* (N,O) RNA probes on gonads from wild-type, *Gata4<sup>ki/ki</sup>*, *Fog2<sup>-/-</sup>* and *W/W<sup>v</sup>* E12.5-13.5 mouse embryos as indicated. (G,I,K,M) Samples were derived from in vivo busulfan-treated embryos.

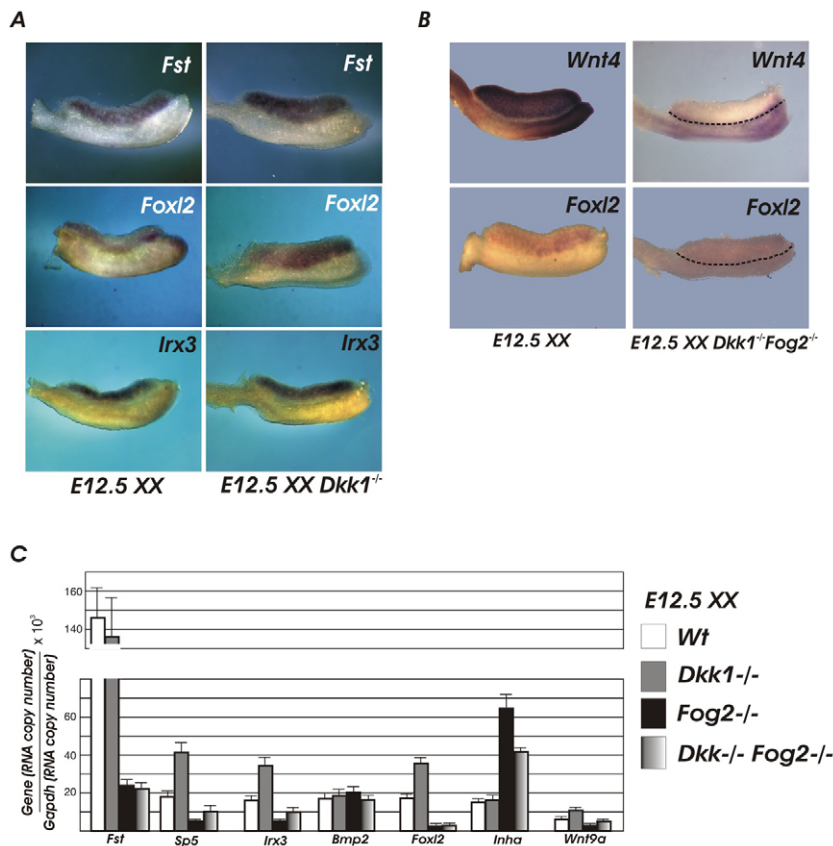
DKK1 acts through binding to the LRP receptors (LRP5 or LRP6) with high affinity (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). *Lrp6* (but not *Lrp5*, data not shown) is expressed in the developing gonad at E13.5 (Fig. 6N) and its expression was unaffected in E13.5 XX *W/W<sup>v</sup>* (*Kit*-mutant) germ cell-deficient gonads (Fig. 6O). In summary, these data suggest that somatic cells in the ovaries are the primary source of *Dkk1* expression as well as being the recipients of canonical  $\beta$ -catenin signaling.

### GATA4/FOG2 loss does not affect the initiation of germ cell sexual differentiation in XX gonads

DKK1 accumulation in the vicinity of germ cells in *Gata4/Fog2* mutants presented the possibility that germ cell differentiation could be affected by loss of GATA4-FOG2 interaction. Germ cell status in *Gata4/Fog2* mutants has not been analyzed previously. Alkaline phosphatase staining of E13.5 gonads detected no change in germ cell number in these mutants. Expression of the germ cell-specific transcription factor *Oct4* also appeared normal (Fig. 7A and data not shown). In XX *Fog2* mutants, germ cells embarked normally on the female differentiation (meiosis) pathway as defined by the upregulation of histone  $\gamma$ H2AX (Fig. 7B-D). Similarly, qRT-PCR analysis of *Stra8* (Baltus et al., 2006) and *Scp1* (*Sycp1* – Mouse Genome Informatics) (Dobson et al., 1994) expression detected no difference between the control and XX *Gata4/Fog2* mutant samples (Fig. 7E). Not surprisingly, XY germ cells in the *Gata4/Fog2* mutants upregulated *Stra8* and *Scp1* expression similarly to their XX counterparts, as male differentiation is blocked in these embryos (Tevosian et al., 2002) (Fig. 7E). As *Gata4/Fog2* mutants are embryonic lethal at E13.5-14.5, further development of the XX germ cells was not analyzed.



**Fig. 7. GATA4/FOG2 loss does not affect the initiation of germ cell sexual differentiation.** (A) ISH was performed with an *Oct4* RNA probe on XX gonads from wild-type or *Fog2<sup>-/-</sup>* mouse embryos as indicated. (B-D) Immunofluorescent staining of frozen sections with an anti- $\gamma$ H2AX antibody (red). Embryonic germ cells are detected by the anti-PECAM1 antibody. Note the anti- $\gamma$ H2AX staining in the normal ovaries (C) and mutant gonads (D), but not in the control testis (B). (E) qRT-PCR analysis of *Stra8* and *Scp1* gene expression in wild-type and *Fog2<sup>-/-</sup>* E13.5-14.5 XX and XY gonads. The y-axis shows values for both genes normalized to *Gapdh* RNA copy number.



**Fig. 8. Analysis of sexual differentiation in *Dkk1* mutant mice.** (A,B) ISH was performed with the indicated RNA probes on XX gonads from wild-type and *Dkk1*<sup>-/-</sup> (A) or *Dkk1*<sup>-/-</sup>; *Fog2*<sup>-/-</sup> (B) E12.5 embryos. (C) qRT-PCR analysis of gene expression in E12.5 wild-type, *Fog2*<sup>-/-</sup>, *Dkk1*<sup>-/-</sup> and *Dkk1*<sup>-/-</sup>; *Fog2*<sup>-/-</sup> XX gonads.

### Analysis of the sexual differentiation phenotype in doubly homozygous mutant mice

We reasoned that if an abnormally high level of DKK1 in *Gata4/Fog2* mutants results in the downregulation of canonical  $\beta$ -catenin pathway targets (Fig. 5), then these same genes could be activated in XX *Dkk1*<sup>-/-</sup> embryonic gonads. Indeed, we observed that several canonical  $\beta$ -catenin pathway targets (*Irx3*, *Sp5* and *Wnt9a*) are upregulated in E12.5 *Dkk1*<sup>-/-</sup> ovaries (Fig. 8A,C). Interestingly, in addition to a ‘ $\beta$ -catenin set’, the expression of *Foxl2* was also increased; this gene has not previously been described as a target for canonical  $\beta$ -catenin signaling (Fig. 8A,C). These data support the previous assertion, by us and others, that the canonical  $\beta$ -catenin pathway functions during ovarian development and, additionally, identify the *Foxl2* gene as a novel target of the canonical  $\beta$ -catenin pathway in the ovary.

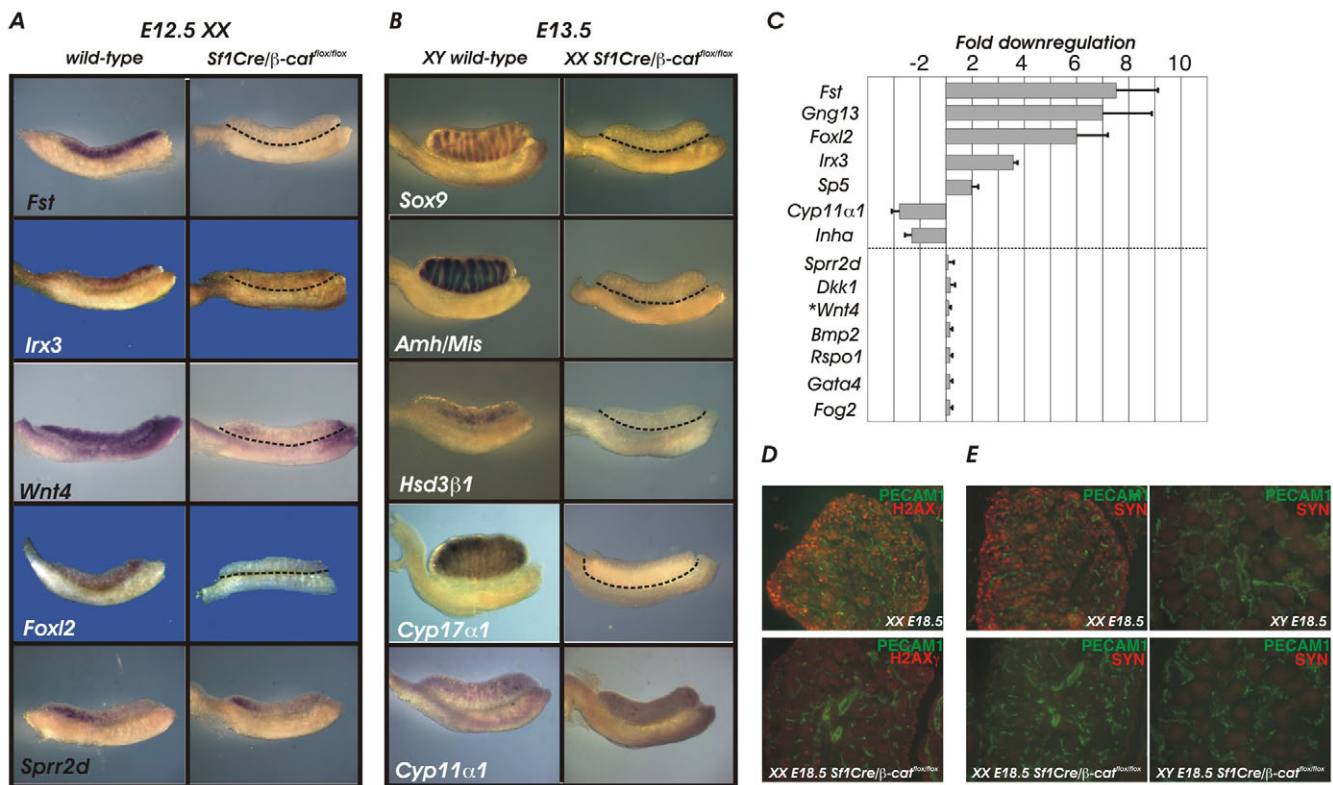
Given the importance of canonical  $\beta$ -catenin signaling for the ovarian developmental fate (Chassot et al., 2008; Maatouk et al., 2008; Tomizuka et al., 2008), a profound block in female development observed upon GATA4/FOG2 loss could be explained solely by a dramatic increase in its secreted inhibitor, DKK1, in *Gata4/Fog2* mutants. Alternatively, an intact GATA4-FOG2 complex could be independently (i.e. regardless of its role in *Dkk1* repression) required for ovarian differentiation and development. To examine XX gonads that are incapable of activating *Dkk1* expression in response to the ablation of the GATA4-FOG2 complex, doubly homozygous *Dkk1*<sup>-/-</sup>; *Fog2*<sup>-/-</sup> and *Dkk1*<sup>-/-</sup>; *Gata4*<sup>ki/ki</sup> embryos were generated. We isolated RNA/cDNA from the E12.5 XX control and mutant gonad samples and examined the expression of genes that we have previously confirmed as targets of GATA4-FOG2 regulation in the ovary. As double mutants are no

longer capable of upregulating *Dkk1*, we predicted that the expression of a subset of GATA4-FOG2-dependent genes, previously inhibited through DKK1, would be (at least partially) restored. The expression of *Sp5*, *Irx3* and *Wnt9a* increased in the double mutants as compared with *Fog2* mutants, whereas *Inha* expression decreased (Fig. 8C). We conclude that the GATA4-FOG2 complex is required to establish the requisite normal level of canonical  $\beta$ -catenin signaling in the ovary by repressing *Dkk1*. By contrast, *Fst* and *Foxl2* expression in the XX *Gata4/Fog2* mutants was not restored by deleting *Dkk1*, and neither was *Wnt4* expression at E12.5 (Fig. 8B,C). These data argue that the GATA4-FOG2 complex regulates these genes independently of its role in maintaining the normal level of canonical  $\beta$ -catenin signaling through repressing *Dkk1*.

### Analysis of the sexual differentiation phenotype in mutants with somatic cell loss of $\beta$ -catenin

The GATA4-FOG2 transcription complex is required for maintaining a normal level of  $\beta$ -catenin signaling; however, it is also essential for maintaining ovary-specific *Wnt4* expression. Either of these GATA4-FOG2-dependent genes ( $\beta$ -catenin or *Wnt4*) could potentially regulate *Fst* transcription. *Fst* levels do not increase in *Dkk1*<sup>-/-</sup> XX gonads and *Fst* expression was not restored in the *Dkk1*<sup>-/-</sup>; *Fog2*<sup>-/-</sup> double mutants (Fig. 8C). To independently assess the contribution of canonical  $\beta$ -catenin signaling to ovarian *Fst* expression, we performed a conditional excision of the  $\beta$ -catenin gene in the ovary. A Cre line of mice based on the BAC harboring the *Sfl* locus has recently been described (Bingham et al., 2006). This *Sfl*-Cre is robustly expressed during early gonadogenesis (see Fig. S3 in the supplementary material) and hence is ideal to excise





**Fig. 9. Analysis of the sexual differentiation phenotype in mutants with somatic cell-restricted loss of  $\beta$ -catenin.** (A,B) Whole-mount ISH with the indicated RNA probes was performed with XX E12.5 gonads from wild-type and *Sf1-Cre/ $\beta$ -cat<sup>flox/flox</sup>* (where  $\beta$ -cat is *Ctnnb1*) mouse embryos (A) or with E13.5 XY gonads from wild-type and XX *Sf1-Cre/ $\beta$ -cat<sup>flox/flox</sup>* embryos (B) to examine the status of the female (A) and the male (B) pathway in XX gonads upon  $\beta$ -catenin loss. (C) qRT-PCR analysis of gene expression in the XX E12.5 wild-type and *Sf1-Cre/ $\beta$ -cat<sup>flox/flox</sup>* gonads. The data are shown as the ratio of normalized expression (gene/*Gapdh* RNA copy number) in wild-type over mutant samples. The combined *\*Wnt4* level in the gonad-mesonephros block does not change; see the corresponding panel in A for gonadal *Wnt4* expression. (D,E) Double immunofluorescent staining of E18.5 gonadal sections for either  $\gamma$ H2AX (D; red) or SYN/COR (E; red) and PECAM1 (green). The wild-type E18.5 ovary contains numerous germ cells with  $\gamma$ H2AX (D, top panel) or SYN1 (synapsin I) staining (E, top left). In the XX *Sf1-Cre/ $\beta$ -cat<sup>flox/flox</sup>* XX gonad, germ cells are largely lost; no SYN1-positive cells are observed in the control or *Sf1-Cre/ $\beta$ -cat<sup>flox/flox</sup>* testis.

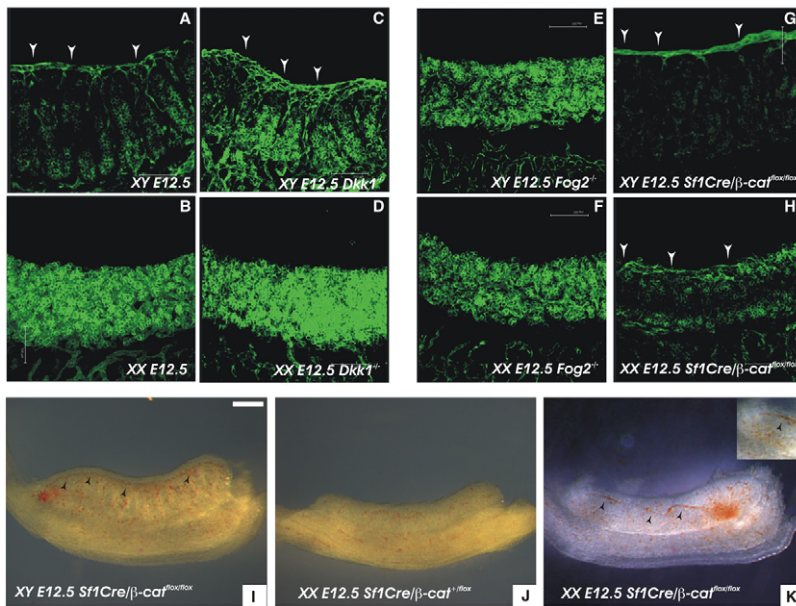
$\beta$ -catenin. *Sf1-Cre* excision led to the loss of somatic  $\beta$ -catenin expression (see Fig. S4 in the supplementary material) and to a ~7-fold reduction in the *Fst* expression level in the XX gonads, whereas *Gata4*, *Fog2* and *Dkk1* were not affected (Fig. 9A,C). This experiment demonstrates that  $\beta$ -catenin regulates *Fst* transcription without affecting the GATA4-FOG2 level. In addition to *Fst*, loss of gonadal  $\beta$ -catenin affected the expression of many other genes. The canonical  $\beta$ -catenin target, *Irx3*, and the essential regulators of ovarian development, *Foxl2* and *Wnt4*, were severely downregulated (Fig. 9A and see Fig. S5 in the supplementary material). Loss of *Wnt4* and *Fst* expression was likely to be responsible for a dramatic reduction in the survival of female germ cells in the XX E18.5  $\beta$ -catenin mutant gonads (Fig. 9D,E) (see Vainio et al., 1999; Yao et al., 2004).

However, the dimorphic gene expression program was not completely extinguished in  $\beta$ -catenin mutants. For example, *Sprr2d* expression was normal, whereas in *Gata4/Fog2* mutants *Sprr2d* expression was completely abolished (compare Fig. 9A, bottom panels, with Fig. 2A-D). Although *Dkk1* activation through the canonical  $\beta$ -catenin feedback loop has been reported in some settings (Chamorro et al., 2005; Gonzalez-Sancho et al., 2005; Niida et al., 2004), *Dkk1* expression was not affected (Fig. 9C).  $\beta$ -catenin deletion in the ovary did not result in the activation of the

alternative male pathway (sex reversal), as shown by the lack of expression of the Sertoli cell-specific genes *Sox9*, *Mis* and *Dhh* at either E13.5 or E18.5 (Fig. 9B; see Fig. S6 in the supplementary material; data not shown). Similar to the *Gata4/Fog2* mutants, the expression of some male-specific genes was derepressed in  $\beta$ -catenin mutants (e.g. *Cyp11a1* and *Inha*, Fig. 9B,C). However, in contrast to the *Gata4/Fog2* mutants (but similar to the XX *Rspo1*-null mutation), XX gonads with  $\beta$ -catenin loss developed a coelomic vessel (Fig. 10H,K). In summary,  $\beta$ -catenin is required for normal ovarian development, whereas testis development in the absence of  $\beta$ -catenin proceeds apparently as normal (Fig. 10G and see Fig. S6 in the supplementary material) (see Chang et al., 2008).

## DISCUSSION

Previously, we determined that the GATA4-FOG2 transcription complex is required for testis differentiation, and visual inspection of the XX *Fog2* mutant gonads at that time suggested that *Fog2* was likely to be needed for female development as well (Tevosian et al., 2002). In the interim period, work by many laboratories has uncovered a number of sexually dimorphic genes in the developing mouse ovary and a functional relationship between them is beginning to emerge (e.g. Bouma et al., 2007a; Chassot et al., 2008;



**Fig. 10. Formation of the male-specific coelomic blood vessel in control and mutant gonads.**

(A-H) Confocal microscopy images of whole-mount anti-PECAM1 immunostaining of E12.5 XY (top row) and E12.5 XX (bottom row) gonads isolated from control mouse embryos (A,B) or *Dkk1*<sup>-/-</sup> (C,D), *Fog2*<sup>-/-</sup> (E,F) and *Sf1-Cre/β-cat*<sup>flx/flx</sup> (G,H) mutants. The male-specific coelomic vessel (white arrowheads) normally develops in the control (A), *Dkk1*<sup>-/-</sup> (C) and *Sf1-Cre/β-cat*<sup>flx/flx</sup> testis (G). An ectopic vessel is visible (especially in the posterior region) in the XX *Sf1-Cre/β-cat*<sup>flx/flx</sup> gonad (H). The vessel lacks the branches normally descending between the testis cords in XY gonads. No coelomic vessel could be seen in the *Fog2*-null samples (E,F; compare with Fig. 3D). (I-K) Light microscopy images of E12.5 gonads. A clear vessel (arrowheads) is seen on the coelomic surface of *Sf1-Cre/β-cat*<sup>flx/flx</sup> XY (I) and *Sf1-Cre/β-cat*<sup>flx/flx</sup> XX gonads (K and the higher magnification inset), but not on the *Sf1-Cre/β-cat*<sup>flx/flx</sup> ovary (J). Scale bars: 100 μm in A-H; 200 μm in I-K.

Jorgensen and Gao, 2005; Menke et al., 2003; Menke and Page, 2002; Nef et al., 2005; Yao et al., 2004). This new body of data has provided a sufficient foundation to integrate the GATA4-FOG2 complex into the transcriptional cascade orchestrating ovarian development in mammals.

### GATA4, FOG2 and the canonical β-catenin pathway

We demonstrate here that in the absence of GATA4-FOG2 interaction, the expression of both *Wnt4* and *Fst* is lost. Although several Wnt family members are expressed in the developing gonads (Cederroth et al., 2007), it is primarily *Wnt4* that has been associated with sexual development in mammals. *Wnt4*-null females are masculinized, as demonstrated by the absence of Müllerian ducts and the retention of Wolffian ducts (Vainio et al., 1999). *Wnt4* is also required to repress steroidogenic and vascular endothelial cell migration into the developing XX gonad; absence of *Wnt4* leads to both ectopic steroid (e.g. testosterone) production and formation of a male-specific coelomic blood vessel (Heikkilä et al., 2005; Jeays-Ward et al., 2003). Four known XY human subjects with duplications of the chromosome 1p35 that includes the *WNT4* locus exhibit symptoms that range from isolated cryptorchidism to severe genital ambiguity (Jordan et al., 2001; Jordan et al., 2003). A recent report demonstrates that in the ovary, *Wnt4* partially acts through canonical β-catenin signaling (Chassot et al., 2008).

Our analysis of *Gata4/Fog2* mutant ovaries revealed a dramatic activation of *Dkk1* expression in the absence of GATA4-FOG2 interaction, concomitant with the downregulation of genes linked to canonical Wnt/β-catenin signaling. DKK1, the founding member of the DKK family (Krupnik et al., 1999; Monaghan et al., 1999), is a secreted protein and a potent Wnt signaling inhibitor (Glinka et al., 1998). It binds to the LRP receptors (LRP5 or LRP6) and prevents interaction between the Wnt ligand and the Fz-LRP receptor complex (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). The mechanism of *Dkk1* activation as a result of GATA4/FOG2 loss is not clear. The c-JUN transcription factor and JNK signaling have been reported to positively regulate *Dkk1* transcription (Colla et al., 2007; Grotewold and Ruther, 2002); however, this is an unlikely

explanation because the *c-Jun* RNA level is unaffected in *Gata4/Fog2* XX mutant gonads, and an active form of JNK is undetectable (data not shown).

### GATA4-FOG2 complex loss affects multiple aspects of ovarian development

One of the genes requiring the GATA4-FOG2 complex is *Foxl2*. FOXL2, a forkhead transcription factor, is essential for reproductive development in females (reviewed by Uhlenhaut and Treier, 2006). In mice [as in humans (Cocquet et al., 2002)] *Foxl2* is one of the earliest genes expressed in a female-specific fashion (Löffler et al., 2003) and *Foxl2* homozygous mutants recapitulate female infertility in humans (Schmidt et al., 2004). Although *Foxl2* is required in granulosa cell function in postnatal ovaries, embryonic ovarian development initiates and proceeds apparently normally in its absence (Ottolenghi et al., 2005; Schmidt et al., 2004). Importantly, in contrast to the loss of *Foxl2* expression in *Gata4/Fog2* XX mutants (Fig. 2) and in β-catenin deficiency (Fig. 9 and see Fig. S5 in the supplementary material), *Foxl2* expression is normal in the *Wnt4*-null (Fig. 5N) and *Rspo1*-null (Chassot et al., 2008) mutants, underscoring the specific requirement for the GATA4-FOG2 complex and β-catenin protein in the control of *Foxl2* transcription.

Although GATA4-FOG2 complex loss affects several key elements of the ovarian gene expression program, some dimorphically expressed ovarian genes, such as *Bmp2*, retain their wild-type levels. Unexpectedly, *Bmp2* levels remain unchanged in the XX E12.5 *Gata4/Fog2* mutants despite the loss of gonadal *Wnt4* expression; *Wnt4* is epistatic to *Bmp2* and in the *Wnt4*-null XX gonads *Bmp2* expression is dramatically reduced (Fig. 5N) (Yao et al., 2004). The mesonephric expression of *Wnt4* that persists in *Gata4/Fog2* mutants (e.g. Fig. 1C-F) could be responsible for maintaining the wild-type level of gonadal *Bmp2*. Alternatively, it is possible that the early (~E11.5) expression of *Wnt4* that is independent of GATA4-FOG2 regulation is sufficient to trigger the activation of *Bmp2* transcription.

Expression of *Rspo1* is also normal in *Gata4/Fog2* mutants (Fig. 5). It was proposed that *Rspo1* functions to relieve the DKK1-imposed inhibition of the β-catenin pathway by antagonizing DKK1-dependent LRP6 receptor internalization (Binnerts et al.,

2007). Our data suggest that in the XX gonads of the *Gata4/Fog2* mutants, excessive DKK1 is no longer adequately antagonized by normal concentrations of the RSP01 protein. This results in an outcome similar to that of *Rspo1* deficiency in that a downregulation of female-specific *Wnt4* expression is observed, whereas *Bmp2* is unaffected (Fig. 1) (Chassot et al., 2008).

### Fst expression requires multiple regulatory inputs

*Gata4/Fog2*, *Wnt4* and *Rspo1* mutations all converge on the  $\beta$ -catenin signaling pathway and *Fst* expression is severely reduced or lost in these mutants. Hence, it was tempting to speculate that *Fst* expression critically depends on the nuclear  $\beta$ -catenin pathway in the ovary. The regulation of an *Fst* promoter by canonical  $\beta$ -catenin signaling has been reported in cell culture (Miyanaaga and Shimasaki, 1993). Mutation of the putative TCF binding site (CTTTGAT) at -223 to -217 relative to the start of *Fst* transcription led to the abrogation of the WNT3A response (Willert et al., 2002).

A conditional knockout of the  $\beta$ -catenin gene in the gonad results in a drastic reduction in *Fst* expression, validating the essential requirement for canonical  $\beta$ -catenin signaling in *Fst* regulation in vivo. A recent report on the *Rspo1* knockout (Chassot et al., 2008) also suggests that RSP01 regulates *Fst* expression through  $\beta$ -catenin; constitutively active  $\beta$ -catenin is sufficient to rescue the ovarian development of the *Rspo1*-null mice, although *Fst* expression was not directly examined in the rescued ovaries. By contrast, *Fst* expression is not upregulated in *Dkk1*<sup>-/-</sup> gonads and is not restored in the *Fog2* mutant by *Dkk1* ablation. This demonstrates that, in addition to its reliance on intact  $\beta$ -catenin signaling, *Fst* also requires a functional GATA4-FOG2 complex for its expression. Whether WNT4 regulates *Fst* expression via (or independently of)  $\beta$ -catenin is currently unclear and will require restoring (or ectopically stabilizing)  $\beta$ -catenin signaling in the *Wnt4*-null gonad.

### A pivotal role for the GATA4-FOG2 complex in sexual differentiation

Previous work demonstrated the importance of the GATA4 and FOG2 proteins in testis development. Loss of GATA4-FOG2 interaction leads to a block in the male pathway because the upregulation of *Sox9* gene expression, which is necessary for testicular development, does not occur in mutant XY gonads (Tevosian et al., 2002). Moreover, a threshold concentration of the functional GATA4-FOG2 complex is required to mount an adequate *Sox9* expression level that will tip the scale towards testis differentiation (Bouma et al., 2007b; Manuylov et al., 2007a). We have now shown that interaction between these protein partners is also required for the ovarian pathway.

As *Gata4* and *Fog2* are expressed in gonads of both sexes, their involvement in both testicular and ovarian development is not entirely surprising. The most parsimonious explanation is that GATA4 and FOG2 control the developmental program or programs common to both fates. The block in the proliferation or survival of the pre-Sertoli/pre-granulosa cells could, in principle, account for the observed loss of gene expression. FOG2-GATA4 function is required to maintain sufficient numbers of SOX9-positive cells in the developing testis (Bouma et al., 2007b; Manuylov et al., 2007a). However, a reduction in cell number is unlikely to play a major role in the ovarian pathway block; in this respect, the dramatic increase in coelomic epithelial proliferation in XY E11.5 gonads is not detected in XX gonads (Schmahl et al., 2000). Correspondingly, we observed no significant reduction in proliferation, as assessed by staining for phosphorylated histone H3 and the proliferation-

associated protein Ki67 (MKI67), in the E11.5-12.5 XX gonads of the *Gata4/Fog2* mutants; the TUNEL assay did not register an increase in apoptosis either. Moreover, whereas *Fog2* haploinsufficiency leads to a measurable decrease in the number of SOX9-positive cells in the testis (Manuylov et al., 2007a), we observe no decrease in the number of FOXL2-positive cells in *Fog2*<sup>+/-</sup> ovaries (data not shown). Finally, the normal expression of the early ovarian markers *Bmp2* (Fig. 1) and *Rspo1* (Fig. 5), which are expressed by the somatic support cells (Bouma et al., 2007a; Chassot et al., 2008), argues strongly against a generalized block in the proliferation or survival of a pre-granulosa cell population.

Loss-of-function mutations in male-specific genes such as *Sox9* (Chaboissier et al., 2004) or *Fgf9* (Colvin et al., 2001; Kim et al., 2006) result in activation of the ovarian-specific expression pattern. Similarly, loss-of-function mutations in ovarian-specific genes (*Wnt4*, *Fst*, *Rspo1* and now  $\beta$ -catenin; Figs 9 and 10) launch the expression of the testis-specific program in the XX gonad (for example, ectopic formation of a male-specific coelomic blood vessel is observed in these knockouts). By contrast, a 'battle of the sexes' that lacks its GATA4 and FOG2 pieces results in an earlier tie, as neither side can win. Loss of *Gata4/Fog2* does not, however, preclude the initiation of female-specific development of germ cells: in *Gata4/Fog2* mutants (XX or XY), germ cells enter meiotic prophase normally, beginning at around E13.5 (Fig. 7).

Many of the crucial events in gonadal (especially ovarian) development can only be realized postnatally, and conditional deletion of *Gata4* and *Fog2* will be required to analyze the mutants after the time of birth. This will be informative for gaining further insight into the function of GATA4-FOG2 in ovarian development, as only once the mutant XX cells have had to march through the major competence test of folliculogenesis can the function of the early ovarian genes be truly exposed.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/22/3731/DC1>

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