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Conditional Ablation of *Gata4* and *Fog2* Genes in Mice Reveals their Distinct Roles in Mammalian Sexual Differentiation

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Abstract

Assembly of functioning testis and ovary requires a GATA4-FOG2 transcriptional complex. To define the separate roles for GATA4 and FOG2 proteins in sexual development of the testis we have ablated the corresponding genes in somatic gonadal cells. We have established that GATA4 is required for testis differentiation, for the expression of *Dmrt1* gene, and for testis cord morphogenesis. While Sf1Cre-mediated excision of *Gata4* permitted normal expression of most genes associated with embryonic testis development, gonadal loss of *Fog2* resulted in an early partial block in male pathway and sex reversal. We have also determined that testis sexual differentiation is sensitive to the timing of GATA4 loss during embryogenesis. Our results now demonstrate that these two genes also have non-overlapping essential functions in testis development.

INTRODUCTION

Unlike other organs that can either develop normally or become malformed, the gonadal primordia are fully competent to embark on two natural developmental paths. This bipotential nature of the gonadal anlagen provides an unparalleled system to compare the two rival developmental mechanisms that culminate in outcomes that are dramatically divergent and, at the same time, completely predictable.

Transformation of the indifferent gonad into a testis is a prerequisite for male sex determination. The *Sry* gene is the initiator of testis development in eutherians (Swain and Lovell-Badge, 1999; Wilhelm et al., 2007) and many of the cellular and morphological events that occur downstream of *Sry* have been well characterized in mammals, particularly in mice. The critical morphogenetic event in embryonic testis development is testis cord formation (Combes et al., 2009; Coveney et al., 2008; Nel-Themaat et al., 2009). This

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profound reorganization of gonadal cells gives an embryonic testis its characteristic appearance and is required for normal male development. Shortly after the initiation of Sry expression at ~E10.5, there is a marked increase in the proliferation of coelomic epithelial cells in XY gonads. A fraction of the gonadal somatic cells differentiates to become Sertoli cells – the specialized cells that surround germ cells and form testis cords between E11.2 and E12.5. In the interstitial space between the cords reside the Leydig cells, which are responsible for testosterone production (Cool and Capel, 2009). SRY and subsequently the transcriptional regulator SOX9 are two key proteins required to initiate this distinctive structural arrangement. In contrast to the dramatic restructuring of embryonic testis, the mammalian ovary undergoes major morphological changes only after birth. Despite appearing almost dormant, embryonic ovaries initiate and maintain an active gene expression program that acts to suppress the male pathway of development and to promote meiosis (Brennan and Capel, 2004; Sekido and Lovell-Badge, 2009; Tevosian and Manuylov, 2008).

The GATA zinc-finger transcription factors (designated GATA1 to GATA6) bind the consensus target sequence WGATAR. These proteins play critical roles in various developmental processes, including hematopoietic and T cell differentiation, cardiac and coronary vasculature development, and liver, lung and gut morphogenesis (reviewed in (LaVoie, 2003; Patient and McGhee, 2002; Viger et al., 2008)). *Gata4* appears to be the sole GATA family member active in somatic (and not germ) cells in the early developing gonad in mice (Anttonen et al., 2003; Heikinheimo et al., 1997; Lavoie et al., 2004; Viger et al., 1998). At E11.5, *Gata4* is expressed in somatic cells of both XX and XY genital ridges (Heikinheimo et al., 1997; Ketola et al., 2000; Viger et al., 1998). At E13.5, *Gata4* expression becomes sexually dimorphic: in XY gonads expression is upregulated in Sertoli cells and to some extent reduced in interstitial cells, whereas in XX gonads a strong-to-moderate expression is observed in all somatic cells (Anttonen et al., 2003). A similar pattern of ovarian *Gata4* expression has been reported in the rat (Lavoie et al., 2004). *Gata4* expression persists in the somatic cells of postnatal testes and in adult ovaries with predominant expression in granulosa cells (Anttonen et al., 2003; Heikinheimo et al., 1997; Viger et al., 1998).

The normal function of GATA proteins in vertebrates requires a physical interaction with multitype zinc-finger co-factors of the FOG (Friend of GATA) family (for reviews, see (Cantor and Orkin, 2005; Fossett et al., 2001)). The gonadal *Fog2* expression generally parallels that of *Gata4* between E11.0–E13.5 albeit *Fog2* expression is skewed even stronger towards Sertoli cells; after E13.5 *Fog2* becomes notably reduced in the testis and remains low during subsequent embryonic development ((Anttonen et al., 2003; Lu et al., 1999; Manuylov et al., 2007a; Svensson et al., 1999; Tevosian et al., 1999); Tevosian, unpublished).

Mouse fetuses homozygous for a null allele of *Fog2* die at mid-gestation from cardiac defects (Tevosian et al., 2000). Because $Fog2^{-/-}$ embryos survive until ~E14.0, analysis of early gonad development in the absence of FOG2 was possible (Manuylov et al., 2008; Tevosian et al., 2002). In contrast, *Gata4* null embryos die at E7.0–9.5 (Kuo et al., 1997; Molkentin et al., 1997), which precluded analysis of their gonadal differentiation. This problem was partially overcome by using a *Gata4* knock-in allele ($Gata4^{ki}$, a V217G amino acid substitution) that abrogates the interaction between GATA4 and FOG2 (or FOG1) (Crispino et al., 2001). Homozygous $Gata4^{ki}$ embryos survive to E13.0 when they die from cardiac abnormalities similar to those noted in $Fog2^{-/-}$ embryos (Crispino et al., 2001).

We demonstrated previously that GATA4 and FOG2 and their physical interaction are required for normal testis development (Tevosian et al., 2002). *Fog2* null and $Gata4^{ki/ki}$

mutant XY gonads are able to initiate the expression of *Sry* (albeit at a substantially lower level compared to the wild-type controls), but not of several other genes crucial for normal Sertoli cell function (e.g., *Sox9*, *Mis* (*Amh*) and *Dhh*) (Tevosian et al., 2002). We have also reported that *Fog2* haploinsufficiency prevents (suppresses) the dominant sex-reversal in *Ods* and *Wt1-Sox9* XX mice (Manuylov et al., 2007a). Subsequently we have determined that ovarian development is profoundly affected by the loss of GATA4-FOG2 interaction (Manuylov et al., 2008). We have also identified the *Dkk1* gene, which encodes a secreted inhibitor of canonical β-catenin signaling, as a target (direct or indirect) of GATA4-FOG2 repression in the gonad (Manuylov et al., 2008).

The *Gata4^{ki}* allele allows unique insight into the importance of GATA4-FOG2 interaction in mammalian development. However, the specific function of GATA4 in gonadal development has not been examined separately from its FOG2 protein partner. To determine the distinct role of GATA4 in gonadal sex differentiation, we now used a conditional GATA4 loss-of-function approach. This circumvented early embryonic lethality of conventional *Gata4* knockouts and permitted temporally and spatially restricted GATA4 inactivation. Outcomes of *Gata4* gene loss in gonads were compared to conditional inactivation of *Fog2*. Our results demonstrated that the two proteins also have non-overlapping essential functions in the development of the testis.

MATERIALS and METHODS

Animals

Floxed strains—$Gata4^{flox/flox}$ mice and $Gata4^{flap/flap}$ were described previously (Ma et al., 2008; Zeisberg et al., 2005) and were maintained on a mixed 129xC56BL/6 genetic background. Briefly, the $Gata4^{flox}$ allele, which contains loxP sites flanking the translational start site and the region encoding the GATA4 N-terminal activation domains, expresses wild-type *Gata4* (Zeisberg et al., 2005). Exposure of $Gata4^{flox}$ allele to a Cre recombinase removes exon 2. Due to aberrant splicing and an internal in frame start codon, the recombined allele synthesizes a truncated, transcriptionally inactive form of GATA4 (Bosse et al., 2006; Rivera-Feliciano et al., 2006). Mice were genotyped with primers *Gata4for* 5'-ccttgctttctgcctgctaacacac-3' and *Gata4rev* 5'-tgtcattcttcgctggagccgc-3' (all primers are shown 5'-to-3'). To ensure that the GATA4 loss phenotype is not influenced by the way *Gata4* allele is modified (floxed), we have also used two other targeted alleles of *Gata4*, $Gata4^{flap}$ (Ma et al., 2008) (as described below) and $Gata4^{loxP}$ ($Gata4^{tm1.1Sad/J}$ (Watt et al., 2004)) (data not shown) with similar results. $Gata4^{flap}$ contains a loxP-Gata4 cDNA-transcriptional stop-loxP cassette followed by an alkaline phosphatase (AP) cDNA at the endogenous *Gata4* start codon. In these animals, *Gata4* cDNA completely replaced *Gata4* expression from the endogenous gene. Cre recombinase deletes the *Gata4* cDNA and the transcriptional stop signal with concomitant AP expression (Ma et al., 2008). In $Gata4^{loxP}$ animals Cre recombinase deletes exons 3–5 of the *Gata4* gene that encode for both zinc finger DNA-binding domains and the nuclear localization signal that are essential for GATA4 function (Watt et al., 2004)). $Fog2^{flox/flox}$ mice have been previously described (Manuylov et al., 2007b) and were on pure C57BL/6 strain genetic background (a kind gift of Eva Eicher). Mice were genotyped with primers *Fog2for* ggtcttcgacatccatgtttcacagc and *Fog2rev* tgcgagaacaggtgttggtggaagtt (Manuylov et al., 2007b).

Cre strains—To produce mouse strains with gonad-specific deletions, targeted mice were crossed with a *Sf1Cre* line of animals (a kind gift of Dr. Keith Parker;(Bingham et al., 2006)) to obtain *Sf1cre*/+;*Fog2^{flox/+}* and *Sf1cre*/+;*Gata4^{flox/+}* males. *Sf1Cre* transgenic mice, in which a bacterial artificial chromosome (BAC) harboring *Sf1* regulatory elements directs Cre expression to the somatic cells of the gonads (as well as to a limited number of extra-

gonadal tissues), have been previously described (Bingham et al., 2006). *Sf1Cre* line was maintained on a mixed 129xC56BL/6 background. These males were mated to *Gata4^{flox/flox}* females or *Fog2^{flox/flox}* females to generate mutant animals with gonad-specific deletions, GATA4^{SF} and FOG2^{SF}. A *Wt1^{CreERT2}* line was previously described and maintained on a mixed 129xC56BL/6 background (Zhou et al., 2008). *Wt1^{CreERT2}* animals were used to obtain *Gata4^{flox}*, *Gata4^{flap}* and *Gata4^{loxP}* mutants in a fashion similar to the one described above for *Sf1Cre* animals. Tamoxifen (Sigma T5648 emulsified in sesame oil) was injected intraperitoneally at 0.1 mg/g into pregnant females at E10.5, E11.5 or into adult mice to induce Cre activity. Animal sex was determined with Y chromosome specific primers: zfy5 gactagacatgtcttaacatctgtcc and zfy3 cctattgcatggactgcagcttatg. All animal work was approved by the Institutional Animal Care and Use Committees.

In Situ Hybridization

Whole mount in situ hybridization (WISH) was performed essentially as previously described (Manuylov et al., 2008). Embryos of various stages were dissected from the uterus and fixed with 4% paraformaldehyde in 1xPBS at 4°C overnight. To generate RNA in situ probes, cDNA fragments were produced from the embryonic total or embryonic gonadal RNA by RT-PCR. RNA in situ probes were generated by in vitro transcription of subcloned fragments. The following primers were used to generate *Cst9* and *Clu* RNA probes: *Cst9f*, gcagtagctgtggagttctg and *Cst9r*, ttcagaccatggctctcctg; *Cluf*, actgttcaaccaacaatcctg and *Clur*, cttttcctgcggtattcctgt.

Immunofluorescence

Frozen sections were cut on the cryostat (Leica) and mounted on Vectabond (Vector Laboratories)-covered glass slides. Primary antibodies used for protein detection and their dilutions are described in Supp_Table 1. All primary antibodies were diluted in the antibody diluent solution (Dako); fluorescent secondary antibodies (all Alexa Fluor from Invitrogen) were used at 1:500 dilution. The slides were mounted in Vectashield with DAPI (40,6-Diamidino-2-phenylindole, Vector) and photographed. The confocal analysis was performed as previously described (Manuylov et al., 2007a).

TUNEL Assay

Testes and ovaries were dissected at different stages and apoptotic cells were detected by terminal deoxynucleotide UTP nick-end labeling (TUNEL) using an In Situ Cell Death Detection kit (Roche, Indianapolis, IN, USA). Nuclei were counterstained with Vectashield with DAPI (Vector, Burlingame, CA, USA). TUNEL-positive nuclei were counted in 10 random microscopic fields; three mice of each genotype (*Gata4^{flox/flox}*, *GATA4^{SF}*, *Fog2^{flox/flox}* and *FOG2^{SF}*) were analyzed.

Histology

Whole embryos or dissected gonads were fixed in Bouin's solution, dehydrated through an ethanol series, and transferred to paraffin. Paraffin blocks were sectioned on an Olympus Cut 4060; the 7μ sections were collected onto Superfrost slides (Fisher) and dried overnight. Sections were stained with hematoxylin and eosin (H&E) and photographed.

RESULTS

Loss of GATA4 is Incompatible with Normal Testis Development

The crosses of *Sf1Cre; Gata4^{flox/wt}* males with *Gata4^{flox/flox}* females to generate experimental animals with the *Sf1Cre; Gata4^{flox/flox}* genotype (abbreviated GATA4^{SF}) yielded approximately equal numbers of XX and XY mice (52 and 48%, respectively; n = 6

litters). The genotype of all animals corresponded to the phenotype of the external genitalia. All (N=75) GATA4[SF] XY mice (Fig.1B) appeared homogenously as undervirilized males with a hypoplastic penis, mammary teats, and a relatively shorter ano-genital distance compared to that in the control males (Fig.1A). GATA4[SF] males (n=10) housed for 2–6 months with fertility-proven wild-type C57BL/6 females did not sire any progeny. The mean paired testis weight (±SEM) in heterozygous animals at 6 weeks of age was significantly greater than that of GATA4[SF] males of this age (84.7 ± 6.8 mg vs. 2.21 ± 0.43 mg, n = 6, P < 0.001; Fig.1D, GATA4[SF]). The testicle germinal epithelium in the mutants was disorganized and no germ (VASA+) cells or mature sperm were observed (Fig.1F; also Supp. Fig.1A–C). The abnormalities were already apparent at two weeks of age in live animals when GATA4[SF] testes appeared only partially descended and very small (Fig.1I; also Supp_Fig.1D–F); however, no female genital structures were ever observed.

Gonad-specific Loss of *Fog2* Partially Blocks Testis Development and Leads to Sex Reversal

Conditionally targeted *Fog2* animals have been previously used to evaluate the consequences of this gene loss in mammary gland and cardiac development (Manuylov et al., 2007b; Zhou et al., 2009). To directly compare the outcome of GATA4 loss to FOG2 loss we performed the gonad-specific deletion of *Fog2* with the same *Sf1Cre* driver used above for GATA4[SF] mutants. Although external appearance of the XY *Sf1Cre;Fog2[flox/flox]* (abbreviated XY FOG2[SF]) mice (Fig.1C) was quite similar to that of GATA4[SF] mutants (Fig.1B), all XY FOG2[SF] animals presented as true hermaphrodites. The mutant gonads were in partially descended position and externally appeared either as small testes or ovotestes (Fig.1D, FOG2[SF] and data not shown). The majority of tubules in XY FOG2[SF] ovotestes appeared disintegrating and had a dramatically reduced epithelium width due to a severely decreased cellularity of spermatogenic epithelium; these tubules contained no spermatogenic cells (Fig.1G and Supp_Fig.2A,C). Upon close examination some tubules contained spermatogonia and rare mature sperm (Supp._Fig.2D). In approximately half of the cases the FOG2[SF] gonad morphologically resembled an ovary (Supp.Fig.2B; also described in more detail later). The reproductive tracts of these animals contained persistent Müllerian duct-derived structures (oviduct, uterus and vaginal tissues) in addition to the expected Wolffian-derived male organs (Fig.1K and Supp. Fig.2E). Similar to GATA4[SF] males, the XY FOG2[SF] animals were tested and found to never sired any progeny.

Efficient Inactivation of *Gata4* and *Fog2* in the SPCs of GATA4[SF] and FOG2[SF] Gonads

As has been shown previously by others and us, *Sf1Cre* excision is highly efficient in a subset of somatic progenitor cells (SPCs) within the gonad while most coelomic epithelial cells generally do not undergo efficient recombination in this setting (Bingham et al., 2006; Kim et al., 2007b; Manuylov et al., 2008). Recombination of the *Gata4[flox]* allele was highly effective during embryogenesis. The reduction in the amount of the GATA4-positive cells was already noticeable as early as E11.5 in both sexes, and at E12.5, GATA4 staining was practically absent from all the cells in gonad proper (Fig.2A–B; see also Fig.3A). In accordance with previous reports, *Sf1Cre* recombined inefficiently in coelomic epithelial cells, and many coelomic cells retained GATA4 expression even at E12.5 (e.g. arrowheads, Fig2B; Fig.3A). *Sf1Cre*-mediated excision of *Fog2* was not apparent in gonads of either sex at E11.5 (Fig.2C). However at E12.5, FOG2 expression in SPCs was already notably diminished and it was greatly reduced by E13.5 (Fig.2D, E). Importantly, both proteins remain expressed as normal upon the partner protein loss (Fig.2E and Supp_Fig.3).

Analysis of Male Gene Expression in Gonads with GATA4 Gene Loss

To evaluate the sex differentiation status of supporting cells in the GATA4[SF] testis we examined *Sox9* gene expression. SOX9 is a transcription factor that is up-regulated by SRY

in Sertoli cell precursors between E10.5 and E11.5 in the mouse (Barrionuevo et al., 2006; Chaboissier et al., 2004; Kent et al., 1996; Morais da Silva et al., 1996; Sekido and Lovell-Badge, 2008). *Sox9* plays a central role in testis differentiation; it is sufficient for testis development (Qin and Bishop, 2005; Vidal et al., 2001) and, unlike SRY, SOX9 expression is easily detectable. Therefore, SOX9 is an informative early indicator of male development and Sertoli cell differentiation. Surprisingly, SOX9 expression appeared intact in GATA4^{SF} E12.5 testis (Fig.3A).

This was unexpected, given that we have previously shown that the GATA4-FOG2 complex is required for normal development of Sertoli cells. Disruption of the interaction between these two protein partners led to an early block in testis development with concomitant loss of *Sox9* expression (Tevosian et al., 2002). Testis development is traditionally broken into three stages: formation of the bipotential gonadal anlagen followed by a commitment to testis rather than ovary fate (sexual determination) and subsequent differentiation into a functional organ. To identify the stage at which testis development in the GATA4^{SF} mutants became abnormal, we analyzed histology and gene expression patterns of mutant gonads. Before and at E13.5–E14.5 GATA4^{SF} testes appeared grossly normal relative to testes from control littermates (Fig.3B) although the size of the mutant organ was consistently smaller (e.g. Fig.3F). Evaluation of gene expression associated with male development also did not identify any gross abnormalities (Fig.3C–J). We found no difference in the expression of genes normally associated with Sertoli-, Leydig- or germ cell development either at E14.5 (Fig.3C, F–J) or at E18.5 (Fig.4). The dramatic decrease in testis size in GATA4^{SF} mutants (Fig.1D) cannot be attributed to excessive apoptosis, as the TUNEL assay revealed no increase in cell death (Fig.3D and data not shown).

However, in contrast to the homogenous appearance of the testis cords in E18.5 control males (Fig.4A–C), cord structures in the GATA4^{SF} mutants were clearly asymmetrical (Fig. 4D–F). Upon closer examination, the irregularity in cord formation was already apparent at E14.5 (Fig.3C). An additional atypical feature of mutant E18.5 cords was the absence of an anuclear central space (pseudolumen); instead the mutant cords appeared to be packed with cell nuclei (Fig.4F). This last feature of the phenotype resembled that in *Dmrt1* null testis as described in more detail below.

Dmrt1 Gene Expression in Sertoli Cells requires GATA4

DMRT1 (Doublesex and Mab-3 Related Transcription factor-1) is an evolutionarily conserved transcriptional regulator that harbors a DNA-binding domain (the DM domain) highly homologous to the DNA binding module in transcription factors that regulate sexual differentiation in flies (DOUBLESEX) and worms (MAB-3) (for a recent review, see (Graves, 2009; Koopman, 2009)). It has been proposed that DMRT1 performs a fundamental conserved function in testis development of all vertebrates (Raymond et al., 1998). In mice (as well as in other vertebrates) *Dmrt1* expression is restricted to the developing indifferent gonads in both sexes (De Grandi et al., 2000; Raymond et al., 1999) and both somatic and germ cells express this protein shortly before sex determination at E10.5–E11.5 (Lei et al., 2007). A clear dimorphism for DMRT1 in males becomes apparent by E12.5, with robust expression of DMRT1 in both Sertoli and germ cells. In contrast, in females at E12.5 DMRT1 expression is markedly lower in somatic cells than in germ cells and somatic expression becomes extinct at E13.5 (Lei et al., 2007). Studies of *Dmrt1* null males revealed this gene's role in testis differentiation (Raymond et al., 2000). Sex determination in these animals initially proceeded as normal, but after birth (beginning ~at P14) the seminiferous tubules of *Dmrt1*$^{-/-}$ testis became crowded (similar to Fig.4F) with immature Sertoli cells that failed to exit the cell cycle and differentiate (Raymond et al., 2000).

The perceived similarity between $Dmrt1^{-/-}$ and GATA4^{SF} testis phenotypes led us to ask if GATA4 is required for DMRT1 expression. We found that unlike other proteins (e.g. SOX9 and AMH (MIS), Figs.3, 4) DMRT1 expression was dramatically downregulated in Sertoli cells of GATA4^{SF} males as early as E13.5, while DMRT1 expression in germ cells appeared unabated (Fig.5B). DMRT1 staining remains depleted in GATA4^{SF} mutants throughout embryogenesis (Fig.5D). Consistent with this result, *Gata4* was previously described to regulate *Dmrt1* expression in vitro (Lei and Heckert, 2004). Loss of DMRT1 expression in GATA4^{SF} testis lends further support to the notion that GATA4 regulates *Dmrt1*.

In contrast to GATA4^{SF} males, XY FOG2^{SF} mice continued expressing DMRT1 in the remaining Sertoli cells in early and late embryogenesis (Fig.6A,B and D–G). We concluded that in differentiated Sertoli cells FOG2 is not required to support DMRT1 expression. The previous study also proposed a role for FOG2 in *Dmrt1* regulation based on the qRT-PCR analysis of XY *Fog2* null gonadal samples (Lei and Heckert, 2004). Hence we also examined DMRT1 expression in XY *Fog2* null gonads, which never expressed FOG2. Unlike FOG2^{SF} testes, XY $Fog2^{-/-}$ gonads did not express somatic DMRT1 (Fig.6C). This was expected as XY *Fog2* null gonads (and previously described GATA4^{ki} gonads) had a complete early block in Sertoli cell development and did not express any Sertoli-specific genes (e.g. *Sox9* or *Amh/Mis*) besides *Sry* (Tevosian et al., 2002). In summary, both proteins (i.e. GATA4-FOG2 interaction) are required for the differentiation of pre-Sertoli cells (Tevosian et al., 2002); however, once the cells are differentiated, GATA4 but not FOG2 is necessary to retain somatic *Dmrt1* expression in the testis.

DMRT1 Expression in the Sertoli Cells does not Require Continuous GATA4 Presence

Our data suggests that SOX9 expression in Sertoli cells is maintained in the absence of GATA4 (e.g., Figs. 3 and 4). In contrast, GATA4 is required for somatic DMRT1 expression (Fig.5). It was therefore possible that, unlike that of *Sox9* or *Amh*, maintenance of *Dmrt1* expression requires a continuous presence of GATA4 throughout the life of the animal. To evaluate this possibility we took advantage of tamoxifen-inducible CreERT2 expressed from the Wt1 locus ($Wt1^{CreERT2}$) (Zhou et al., 2008). Upon induction with tamoxifen, Wt1-driven CreERT2 was active in Sertoli cells and germ cells of the adult testis (Supp_Fig.4E,F).

To induce *Gata4* loss in the adult testis we used $Gata4^{flap}$ animals (Ma et al., 2008). $Gata4^{flap/flap};Wt1^{CreERT2}$ males were injected with tamoxifen at 4 weeks of age and their testes examined two weeks later. As was expected, in the control testis both GATA4 and DMRT1 were robustly expressed (Fig.7A, C and (Raymond et al., 2000; Viger et al., 1998)). Wt1-driven Cre efficiently abolished GATA4 expression in adult Sertoli cells. Despite GATA4 loss, DMRT1 expression remained unscathed (Fig.7B); similarly, adult-stage testicular inactivation of *Gata4* had no observable consequences on testis appearance and spermatogenesis (Fig.7B, D). This contrasted with embryonic loss of GATA4 in GATA4^{SF} mutants that led to the dramatic reduction of somatic DMRT1 staining (Fig.5). SOX9 expression was similarly unperturbed upon GATA4 loss (Fig.7D). This was not entirely surprising given that SOX9 expression was not diminished in GATA4^{SF} mutants as well (Figs.3 and 4). We conclude that, once initiated, DMRT1 expression in Sertoli cells does not require a continuous presence of GATA4.

The Early Loss of Somatic GATA4 Expression Leads to a Block in Male Development and Sex Reversal

Our previous work convincingly showed that constitutive loss of interaction between GATA4 and FOG2 is incompatible with normal sex determination and expression of most sexually dimorphic genes (Tevosian et al., 2002; Tevosian and Manuylov, 2008). In

contrast, *Sf1Cre*-mediated deletion of the *Gata4* gene at E12.5 resulted in definitive embryonic testes and markers of male development were expressed (Figs.1–4). The milder phenotype of conditional GATA4^{SF} gonads compared to the constitutive disruption of GATA4-FOG2 interaction (in *Fog2* null or *Gata4$^{ki/ki}$* mutants (Tevosian et al., 2002)) led us to hypothesize that gonadal GATA4 activity prior to excision by Sf1Cre at E12.5 was sufficient for normal sex determination and expression of sexually dimorphic genes. To test this hypothesis we used inducible *WT1^{CreERT2}* described above to delete *Gata4* early in gonadal development. Using a reporter strain, we determined that tamoxifen injection at E10.5 induced broad *WT1^{CreERT2}*–mediated recombination in the developing genital ridge (Supp_Fig.4A–D). To delete *Gata4*, *Gata4$^{flap/flap}$* females were crossed to *Wt1$^{CreERT2/+}$*;Gata4$^{flap/flap}$ males and injected with tamoxifen at E10.5. Analysis of E15.5 XY embryos that underwent *Wt1^{CreERT2}*-mediated deletion of *Gata4* showed an early block of male sexual differentiation and sex reversal (Fig.7E, F). The gonads in these embryos had a dramatically diminished expression of Sertoli (e.g., SOX9) and Leydig (e.g., 3bHSD) cell markers (Fig.7E). Instead, expression of the early ovary-specific marker FOXL2 was observed (Fig.7F).

This result clearly differs from that in GATA4^{SF} mutants where definitive testes developed and sex reversal was not observed (Fig.3), or from that in the adult where GATA4 loss appeared to have no deleterious effect in the testis (Fig.7A–D). This result is also in agreement with our previous assertion that GATA4-FOG2 complex is essential in testis differentiation during the initial sex determination stage (Tevosian et al., 2002). In summary, these observations imply that GATA4 performs different functions at different stages of gonadal development.

Timing of Gene Deletion is Critical for the Outcome of Gata4 Loss

In this work we aimed to determine the function of GATA4 protein during testis development. To examine embryonic gonads deficient in *Gata4* we used two different Cre recombinase drivers that led to different phenotypes, with embryonically-induced *Wt1Cre*-mediated excision clearly being more severe (e.g., compare Figs. 3,4 and Fig.7E, F). We reasoned that the difference in outcomes could be due to an earlier activation of Wt1-driven Cre in the gonad leading to an earlier loss of *Gata4*. We also noted that while Wt1Cre is active in the coelomic epithelium (e.g., Supp_Fig.4D), Sf1Cre excises inefficiently in these cells. To determine whether a phenotype observed with Sf1Cre could be recapitulated using Wt1_CreERT2, we induced Wt1_CreERT2 one day (24hours) later, at E11.5. As expected, in contrast to GATA4^{SF} testis (e.g., Fig.8C), GATA4 expression is lost from Wt1Cre-excised coelomic cells, regardless of later injection (Fig.8A–B). However, unlike an E10.5-induced excision (Fig.7E,F), E11.5-induced embryos expressed SOX9, AMH and 3bHSD (Fig.8D,E) and their irregular testis cord structure strongly resembled that in GATA4^{SF} testis (compare to Figs.3–5). Most importantly, *Wt1_CreERT2; Gata4$^{flox/flox}$* animals similarly cease DMRT1 expression in their Sertoli cells (compare Fig.8G to Fig.5). We conclude that the requirement for GATA4 in embryonic testis development is highly dynamic: early on GATA4 is required for Sertoli cell differentiation and later plays a more restricted but essential role in controlling particular genes (e.g. *Dmrt1*; see also Supp_Fig.6). We also reason that continuous GATA4 expression in coelomic cells (at least past E11.5) is dispensable for testis development.

XY FOG2^{SF} Gonads Undergo an Early Block in Male Development and Sex Reversal

Sf1-driven Cre recombinase allows for efficient and early deletion of either *Gata4* or *Fog2* genes in the differentiating gonads (Fig.2). While the loss of GATA4 staining was already prominent at E11.5 and practically complete by E12.5 in GATA4^{SF} testis, we did not observe a similar loss of FOG2 expression in FOG2^{SF} mutants until E13.5 (Fig.2 and data

not shown). Despite this lingering expression, the male specific program was severely perturbed in E13.5–E14.5 XY FOG2^{SF} gonads (Fig.9). Specifically, we observed a dramatic down-regulation of the Sertoli-associated gene expression (*Sox9*, *Amh*, *Dhh*; Fig.9A–C). This was in sharp contrast with contemporaneous GATA4^{SF} testis (Fig.3). Additionally the coelomic vessel appeared underdeveloped (data not shown). However, in contrast to *Fog2* null embryos that exhibited a complete failure in testis differentiation (Tevosian et al., 2002), *Sf1Cre*-mediated *Fog2* excision resulted in a limited expansion of the male program and was compatible with expression of genes associated with differentiated Sertoli cells (i.e. *Sox9*; Fig.9A). Despite gross abnormalities in the Sertoli cell differentiation and testis cord development, the steroidogenic program in XY FOG2^{SF} gonads appeared grossly normal, suggesting that the residual function of Sertoli cells in XY FOG2^{SF} embryos was adequately supporting Leydig cells and steroidogenesis (Fig.9G–I).

Our data demonstrate that the male differentiation program was severely compromised in XY gonads of FOG2^{SF} mice. To examine whether this collapse of male differentiation leads to the resurgence of female pathway, we examined expression of female-specific genes in FOG2^{SF} XY gonads. Consistent with the hermaphrodite phenotype of the FOG2^{SF} males, we observed that *Fog2* deletion was sufficient to cause partial sex reversal, with ectopic expression of the FOXL2 gene in the XY gonads (Fig.10A–C, Supp. Fig.5A–G). The gonads (but not individual cells) in XY FOG2^{SF} animals co-express SOX9 and FOXL2 genes (Fig.10D; Supp_Fig.5D–F). Consistent with sex-reversal of the somatic cells, most gonocytes in these XY mutants express the marker of meiotic cells G-H2AX during embryogenesis (Fig.10E, F). These cells are lost prenatally and in the newborn XY FOG2^{SF} animals the remaining germ cells are associated with the residual testis cords (Sup_Fig.5H,I; also Fig.6D). By 3 weeks of age the gonads in the XY FOG2^{SF} mutants strongly resembled ovaries except for the presence of rare SOX9 positive cells (Supp_Fig.5J). While we observed robust FOXL2 expression in the somatic cells, no early stage (primordial or primary) follicles were detected in the cortex of the XY FOG2^{SF} ovary (Sup_Fig.5J–L). In contrast to FOG2 mutants, ovarian-associated gene expression was not observed in GATA4^{SF} testes (data not shown), consistent with largely preserved male gene expression in XY GATA4^{SF} mice.

DISCUSSION

The Genetic Analysis in Mice Highlights the Complexity of GATA4:FOG2-dependent Transcriptional Regulation

The promoter studies in cultured cells provided important inroads into understanding GATA4 function in the testis. It had been suggested that GATA4 participates in the regulation of many testicular genes (e.g., *Mis/Amh* (Viger et al., 1998; Watanabe et al., 2000), *Sf1* and aromatase (*Cyp19a1*) (Tremblay and Viger, 1999), *Inha* (Ketola et al., 1999), *StAR* (Nishida et al., 2008; Silverman et al., 2006) and *Rhox5* (Bhardwaj et al., 2008); see (Lavoie and King, 2009; Viger et al., 2008) for review). Nevertheless, direct analysis of gene regulation in the absence of GATA4 previously has not been possible due to early embryonic lethality of *Gata4*$^{−/−}$ mice (Kuo et al., 1997; Molkentin et al., 1997). Now our results reveal the complexity of the GATA4- and FOG2-dependent regulation of gene expression and gonadal development, which could have only been exposed by genetically manipulating gene function in mice (Table 1).

GATA4 is required for Normal Gonadal Development of Both Sexes in Mice

We have shown previously that loss of GATA4-FOG2 interaction (e.g. GATA4^{ki} mutation as well as *Fog2* gene deletion) leads to a comprehensive block in gonadal development in mice. The phenotype of GATA4^{ki} animals firmly implicated GATA4 as an indispensable

component in both sex differentiation programs. Importantly, *Gata4^{ki}* mutation is not equivalent to *Gata4* loss of function: the *Gata4^{ki}* mutation results in the production of GATA4 protein that is impaired solely in its ability to interact with FOG co-factors (Crispino et al., 1999). To establish the specific requirements for GATA4 protein in sexual development we have now generated its loss of function mutation in gonads using two different Cre recombinase carrier strains.

Despite the loss of GATA4 expression by E12.5, GATA4^{SF} mice did not recapitulate the early acute arrest of gonadogenesis that is characteristic of the GATA4^{ki} phenotype. While gonads in the GATA4^{SF} males eventually succumbed to a prominent block in their development (Fig.1D, F and Supp_Fig.1), testis differentiation in these animals appeared to initiate normally. As one expects a more dramatic defect in a complete rather than partial loss-of-function, the most likely explanation for the milder phenotype is the delayed timing of *Gata4* gene loss in GATA4^{SF} animals. Specifically, while the excision is practically complete in the gonad proper at E12.5 (Fig.2B and Fig.3A), at E11.5 (during the critical window for sex determination) many GATA4-positive cells were still present among the gonadal somatic cells (Fig.2A).

Inducible Wt1CreERT2 allowed us to incorporate a time-dependent deletion series that was informative. It showed that when induced at E10.5 *Wt1^{CreERT2}* excision of *Gata4* leads to an early and broad failure of Sertoli cell differentiation and male development with concurrent sex reversal (Fig.7E, F). In contrast, by using *Sf1Cre* we have derived an insight for dedicated GATA4 function in the formation of testis cords and defined genes that critically depend on GATA4 presence as early as E12.5. With the majority of Sertoli-specific genes expressed as normal (e.g., *Sox9*, *Amh/Mis*; Figs.3, 4) our results unequivocally show that GATA4 is required for *Dmrt1* expression in the context of otherwise normally developing Sertoli cells (Figs.5 and 8). The requirement for GATA4 in regulating *Dmrt1* expression is not constant. Specifically, though GATA4 is obligatory during embryogenesis for retaining *Dmrt1* expression in Sertoli cells, persistent *Dmrt1* expression in the adult does not require GATA4 (likely due to GATA1 compensation).

Sf1Cre recombines inefficiently in coelomic epithelium (Bingham et al., 2006; Kim et al., 2007b; Manuylov et al., 2008) and GATA4 expression in these cells remained largely intact even at E12.5 (Fig.2B and 3A). Coelomic epithelium harbors the progenitor population for the somatic gonadal cells (Karl and Capel, 1998) and although these GATA4-positive cells do not express Sertoli cell markers (e.g. SOX9, Fig.3A), their continuous impact on gonadal development remained possible. In this respect, equivalent results were obtained with Wt1CreERT2 induced at E11.5 (with efficient deletion of *Gata4* in coelomic cells) further strengthening the conclusion that the phenotype is determined by the timing rather than residual expression of GATA4 in coelomic cells (compare Fig.8 to Figs.3–5)

Dmrt1 is an Integral Transcriptional Target of GATA4, but not FOG2 in Embryonic Sertoli Cells

Among several gene targets of GATA4 in the developing testis (Supp_Fig.6 and Manuylov et al., unpublished), *Dmrt1* is the most informative. DMRT1 expression is limited to the developing gonads (where both Sertoli and germ cells express the protein) and postnatal testis. In humans DMRT1 is implicated in embryonic testis development and sex determination, while in mice it appears to be required only after the time of birth. Specifically, murine DMRT1 is required autonomously for Sertoli cell postnatal differentiation, while germ cell DMRT1 promotes their radial migration to the tubule periphery and supports mitotic reactivation and viability (Kim et al., 2007a).

The regulatory cis-elements required for *Dmrt1* expression were characterized in primary cultures of rat Sertoli cells (Lei and Heckert, 2004; Lei et al., 2009). The control region contributing to Sertoli cell-specific transcriptional activity was located between −3.2kb and −2.8kb and contained several critical regulatory elements capable of binding GATA4. Furthermore, reduced *Dmrt1* mRNA levels were reported for the gonads from E13.5 *Fog2*$^{-/-}$ XY embryos, but not *Fog2*$^{-/-}$ XX embryos, providing additional support for the role of the GATA4-FOG2 complex in sex-specific *Dmrt1* regulation (Lei and Heckert, 2004).

We have now shown that *Dmrt1* expression is lost from the Sertoli cells of GATA4^{SF} (Fig. 5) and *Wt1_CreERT2; Gata4*$^{fl/fl}$ (Fig.8) embryonic testis, and that the expansion of Sertoli cells in GATA4 mutants partially resembles that in postnatal *Dmrt1*$^{-/-}$ mutants (e.g. compare Fig.4F in this work to Fig.5F in (Raymond et al., 2000)). Importantly, the testis cord defect in the GATA4 mutants is manifested considerably earlier; hence it cannot be attributed solely to the loss of *Dmrt1* expression. While it is possible that a more severe phenotype of GATA4^{SF} mutants results from down-regulation of other *Dmrt* family genes, these are not expressed markedly in the developing testis. We favor a hypothesis that deregulated gene expression in addition to *Dmrt1* contributes to an earlier phenotype in GATA4^{SF} mutants. In this respect, the WISH assay confirmed that, in addition to *Dmrt1*, at least two other genes, *Cst9* (cystatin 9) and Clu/ApoJ (clusterin), are dramatically down regulated in GATA4^{SF} testis as early as E14.5 (Supp_Fig.6). Analysis of clusterin (Bailey et al., 2002) and *Cts9* (testatin) (Hasegawa et al., 2006; Tohonen et al., 2005) mutants did not reveal these genes' definitive role in testis development. However, a combined loss of expression for these (and possibly other, yet unidentified) genes in addition to *Dmrt1* could be responsible for the observed phenotype of the GATA4^{SF} mutants.

While the complete roster of genes under GATA4 control remains to be elucidated, this data highlights a previously unappreciated role for GATA4 in Sertoli-specific *Dmrt1* expression and testis cord development. In contrast to GATA4^{SF} testis, DMRT1 staining remains strong in the Sertoli cells of the XY FOG2^{SF} mutants (Fig.6) thus underscoring a specific role for GATA4 but not FOG2 in regulating somatic *Dmrt1* expression in the testis.

Divergent Outcomes of *Sf1Cre*-induced GATA4 versus FOG2 Loss

Our previous work has demonstrated that FOG2 is strictly required for testis development (Tevosian et al., 2002). Here we specifically ablated *Fog2* in gonadal somatic cells. In contrast to *Fog2* null gonads where early sexual development is blocked completely (Tevosian et al., 2002), FOG2^{SF} animals initiate Sertoli cell differentiation and the male pathway. Interestingly, the Leydig cell markers in the XY FOG2^{SF} gonads are expressed at the levels comparable to that in the control testis indicating that residual Sertoli cell function is sufficient to adequately support the male steroidogenic program (Fig.9). Nevertheless, testis cord development in FOG2^{SF} mutants was disrupted and other markers of Sertoli cell differentiation besides SOX9 were either not expressed or dramatically diminished (Fig.9). Moreover, XY FOG2^{SF} gonads underwent sex reversal and expressed markers of early ovarian development (Fig.10; Supp_Figs.2, 5). This contrasts with GATA4^{SF} testis where initial testis development proceeded with relatively little disruption (Figs.3, 4) and progressively deteriorated after birth. The parsimonious explanation for a different phenotype of FOG2^{SF} gonads is a loss of GATA4-independent function upon *Fog2* deletion; a GATA-independent function for FOG2 was recently suggested by others (Hyun et al., 2009).

We conclude here that our results support the notion that both proteins (GATA4-FOG2 complex) are required for somatic cell sex specification. In the developing testis, supporting cell progenitors (SCPs) rely on GATA4-FOG2 complex to successfully transition out of

their undifferentiated state into Sertoli cells (Tevosian et al., 2002). In this respect, *Gata4ki/ki* or *Fog2* null gonads express neither male (Sox9, Dhh, Mis, Dmrt1somatic) (Tevosian et al., 2002) nor female (*Foxl2*, *Fst*, *Sprr2d*) (Manuylov et al., 2008) markers. In contrast, in the conditional setting described here, gene deletions occur past the point of SCP specification and reveal separate functions for the two proteins. Shortly after differentiation, the bulk of Sertoli-specific genes becomes GATA4-independent (Fig.3). Sertoli cells still rely on GATA4 for the expression of essential genes (*Dmrt1* being one important example, Fig.5); not surprisingly, GATA4-deficient Sertoli cells fail to mature and do not develop functional testis or support spermatogenesis. In contrast, in differentiated Sertoli cells a global requirement for *Fog2* lingers on (Fig.9), but is likely becoming dispensable shortly after.

While compensation by another GATA protein able to associate with FOG2 in the Sertoli cells of GATA4SF mutants cannot be completely excluded, this possibility is remote since prior to sexual differentiation the phenotype of the GATA4ki mutation is identical to FOG2 null both in XY and XX animals (Manuylov et al., 2008; Tevosian et al., 2002). Moreover, thorough examination failed to reveal expression of other GATA family members in the somatic cells of developing testis at E13.5 (data not shown).

Research Highlights

We have established that GATA4 is required for:

- testis differentiation

- for the expression of *Dmrt1* gene,

- and for testis cord morphogenesis.

Gonadal loss of *Fog2* resulted in

- an early partial block in male pathway and sex reversal.

We have determined that

- testis sexual differentiation is sensitive to the timing of GATA4 loss

Our results now demonstrate that

- Gata4 and Fog2 have non-overlapping essential functions in testis development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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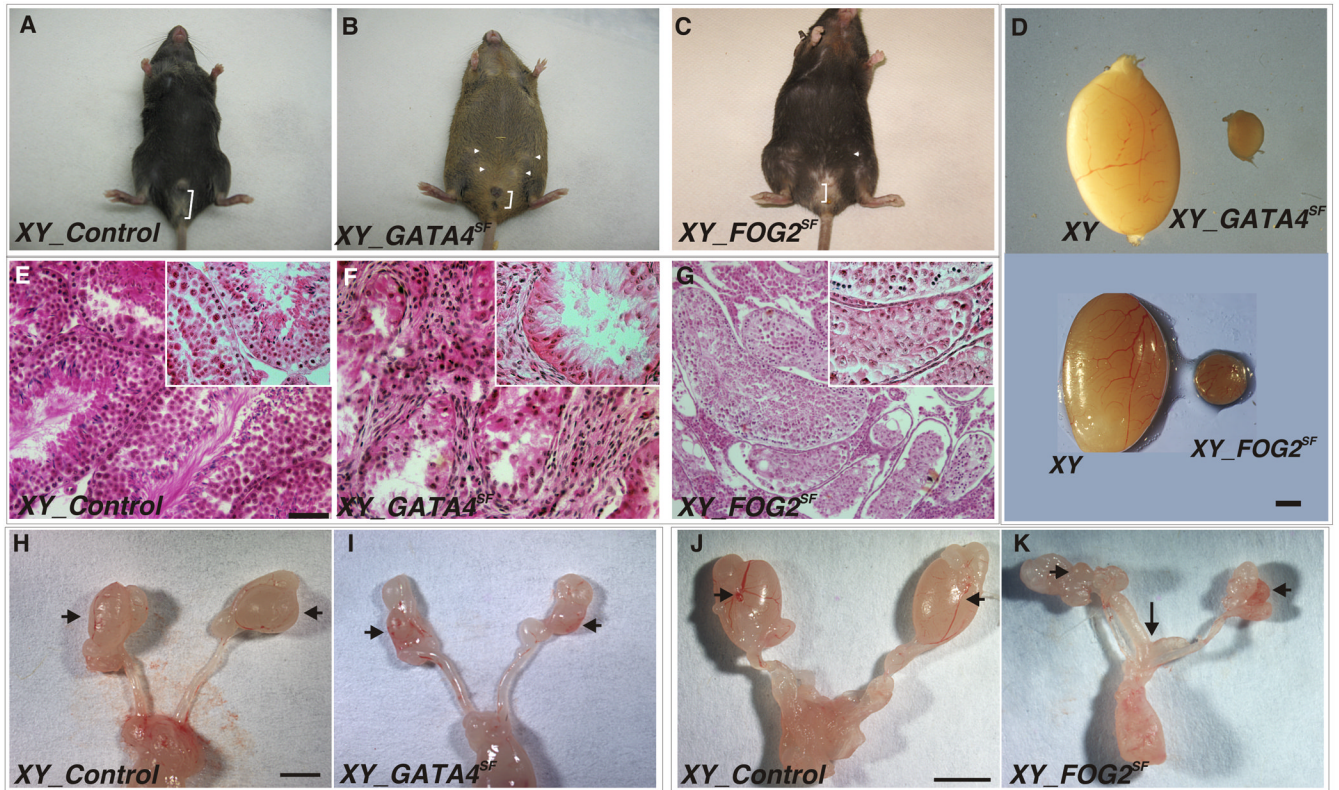
Fig. 1.
A–C. Male development is arrested upon gonad-specific loss of GATA4 or FOG2. Ventral view of 6 week-old mice: normal male (A), XY GATA4^{SF} (B) and XY FOG2^{SF}(C). Mammary teats are evident in GATA4^{SF} and FOG2^{SF} animals (white arrowheads); the ano-genital distance (a white bracket) is shorter in both mutants. D. Isolated gonads from control males (left) and either GATA^{SF} (top) or FOG2^{SF} (bottom) mice. Scale bar 1mm. E–G. Normal tubules contain mature sperm in control testis at 1.5 months (E) while the tubules in contemporaneous GATA4^{SF} (F) and FOG2^{SF} (G) gonads are malformed. No mature sperm is present in GATA4^{SF} mutants at this stage (see also Supp_Fig.1) while rare mature sperm is present in FOG2^{SF} mutants (Supp_Fig.2). Scale bar 100µ. Insets in (E–G) are at 400×. H–K. Isolated testes and associated reproductive organs from control (H, J) and either GATA4^{SF} (I) or FOG2^{SF} (K) XY mice. Scale bar 1mm. Note the reduced size of the gonads in both mutants (arrowheads) and presence of the uterus in the FOG2^{SF} (arrow in K, see also Supp_Fig.2), but not GATA4^{SF} animal.
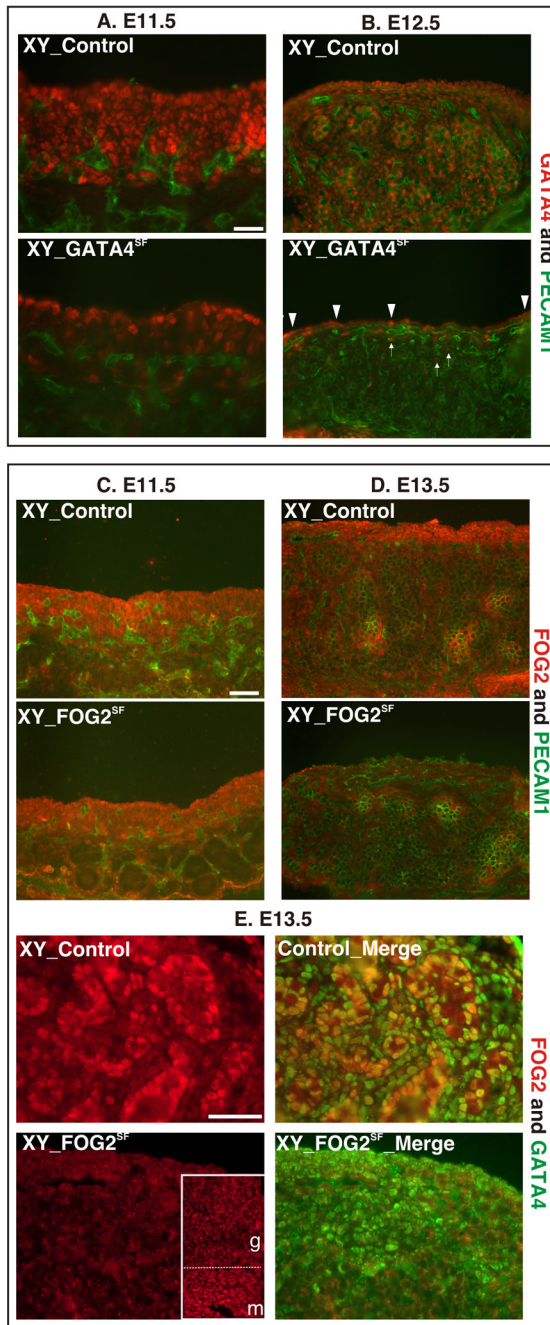
Fig. 2.
Efficient excision of *Gata4/Fog2* genes in GATA4[SF]/FOG2[SF] gonads. Immunofluorescent staining of frozen gonadal sections from the control and GATA4[SF] (A–B) or FOG2[SF] (C–E) mice with anti-GATA4 (red, A and B; green, E), anti-FOG2 (red, C–E) antibodies. Embryonic germ cells and vasculature were stained with the anti-PECAM1 antibody (green) in A–D. Note that residual GATA4 staining in the E12.5 GATA4[SF] samples is confined to the coelomic epithelial (CE) area (arrowheads in B); a few rare GATA4 positive cells below CE are also indicated (arrows). Also note that GATA4 is expressed as normal upon *Fog2* excision (E). Inset in (E) is a lower magnification of the same section which shows that

while FOG2 staining is lost from the FOG2^{SF} gonad (g), FOG2 staining in the mesonephros (m) appears intact as Sf1Cre is not active there. Scale bars 100μ.
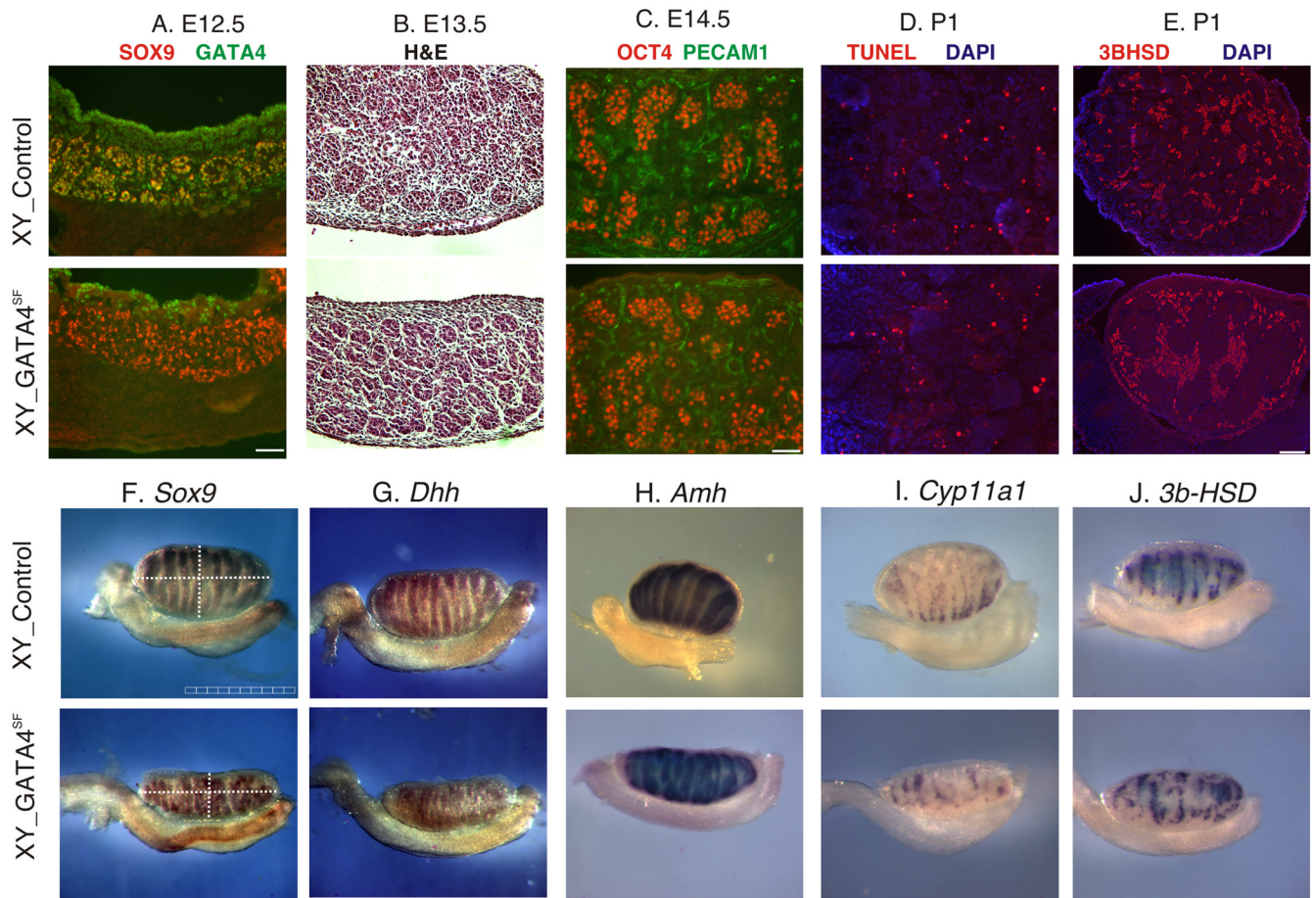
Fig.3.

Normal expression of testis-associated genes in the GATA4^{SF} males. A. Immunofluorescent staining of frozen gonadal sections from the control (*top*) and GATA4^{SF} (*bottom*) E12.5 embryos with anti-SOX9 (red) and anti-GATA4 (green) antibodies. Note that, despite the loss of GATA4 expression, SOX9 was expressed normally in the mutant sample; also note residual GATA4 staining in the CE cells in the mutant. Scale bar 100μ. B. Histological staining of the control (top) and GATA4^{SF} E13.5 testis sections. C. Immunofluorescent staining of gonadal sections from the control (*top*) and GATA4^{SF} (*bottom*) E14.5 embryos with anti-OCT4 (red) and anti-PECAM1 (green) antibodies. Scale bar 100μ. D. TUNEL staining (*red*) of the frozen testis sections from the neonatal (P1) control (*top*) and GATA4^{SF} mutant (*bottom*) animals. E. Immunofluorescent staining of frozen gonadal sections from the control (*top*) and GATA4^{SF} (*bottom*) neonatal embryos with anti-3BHSD (red) antibody. Scale bar 200μ. F–J. Whole-mount ISH was performed on XY E14.5 control (top) or GATA4^{SF} gonads (bottom) with indicated probes. Expression of Sertoli cell (A, F–H), germ cell (OCT4, C) and Leydig cell (E, I, J) markers did not change upon GATA4 loss; note that gonad thickness was slightly reduced in the mutant (e.g., F). The scale bar is 1mm.
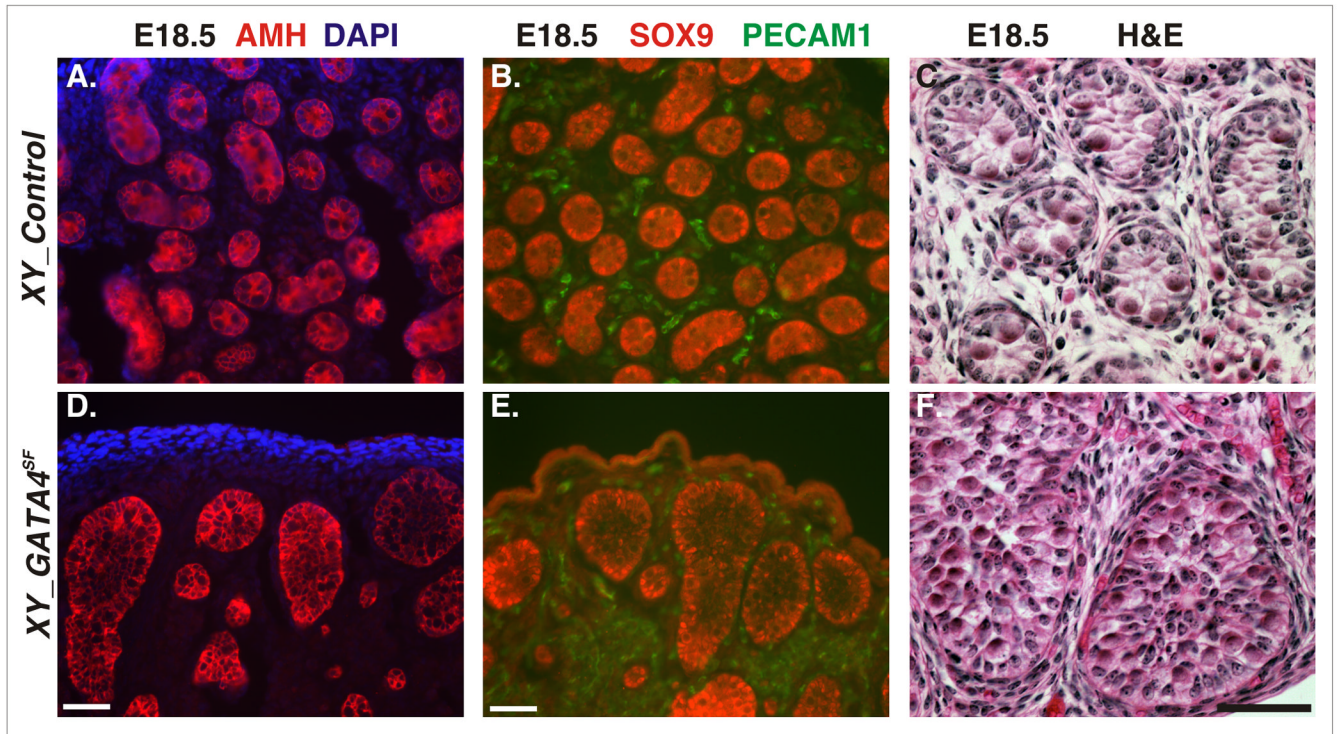
Fig.4.
Testis cord development in GATA4SF testis. Frozen testis sections from the control (A–C) and GATA4SF E18.5 testis (D–F) were stained with anti-AMH (red in A,D); co-stained with anti-SOX9 (red in B,E) and PECAM-1 (green in B,E) or histological stains (C,F). Note that, despite normal expression of the Sertoli cell markers (AMH and SOX9), testis cords in the mutant appeared highly irregular. Scale bars 100μ.
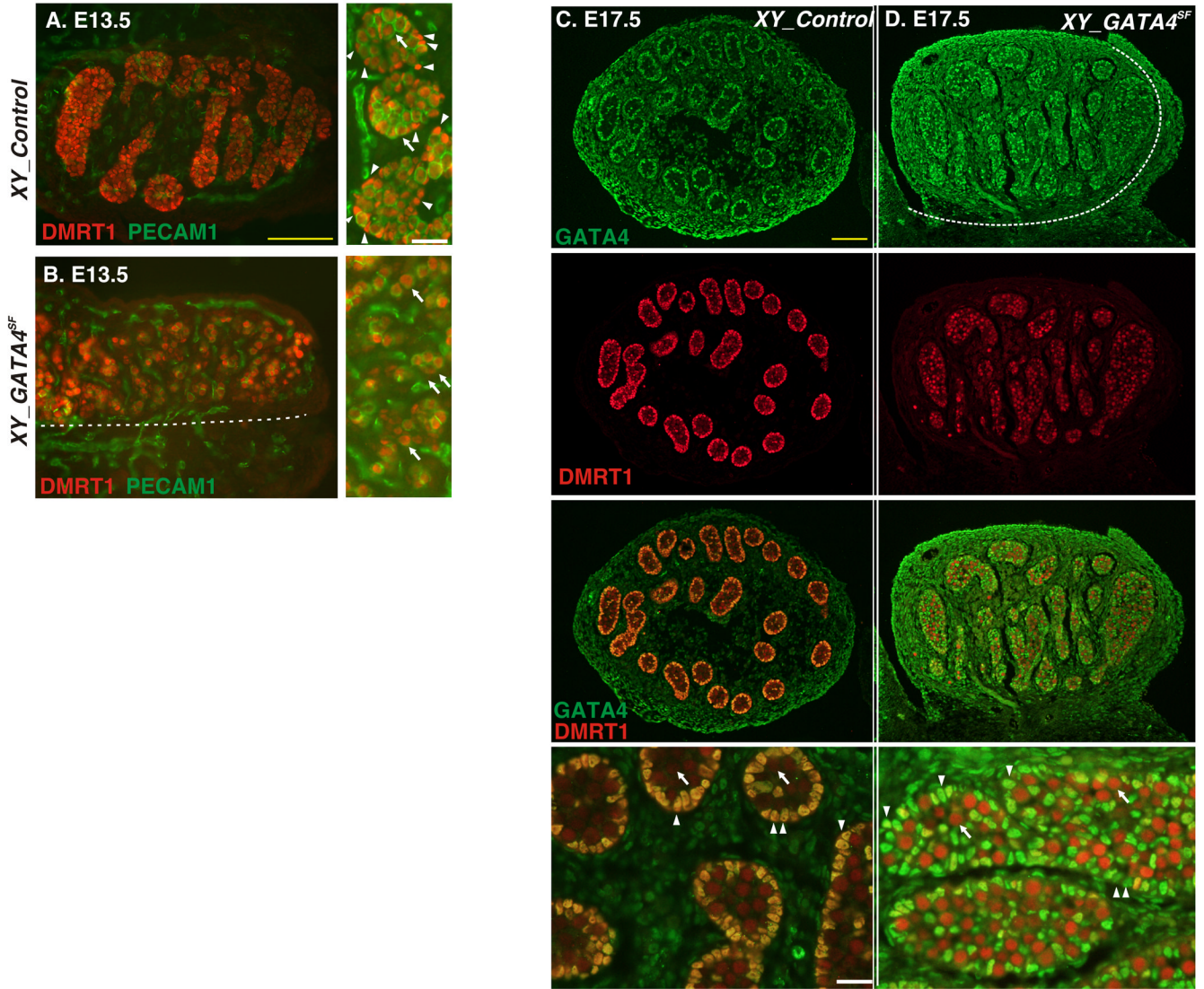
Fig.5.
Loss of *Dmrt1* Gene Expression in Sertoli Cells of GATA4[SF] mutants. A–D. Frozen sections of E13.5 (A and B) or E17.5 (C and D) XY gonads from the control (A and C) or GATA4[SF] mutants (B and D) were stained with anti-DMRT1 (red) and anti-PECAM1 (green) antibodies (A and B), or anti-DMRT1(red) and anti-GATA4[S] (green) antibody (C and D). PECAM-1 identifies germ cells in (A, B) and GATA4[S] labels somatic cells in (C and D). Dashed line (B,D) indicates a gonad-mesonephros border. Notice that DMRT1 staining is virtually absent in Sertoli (arrowheads) but not germ cells (arrows) in the GATA4[SF] mutants. In the GATA4[SF] mutant sample anti-GATA4[S] antibody (green) recognizes inactive partial GATA4 peptide. Scale bars: 100μ (yellow) and 25μ (white).
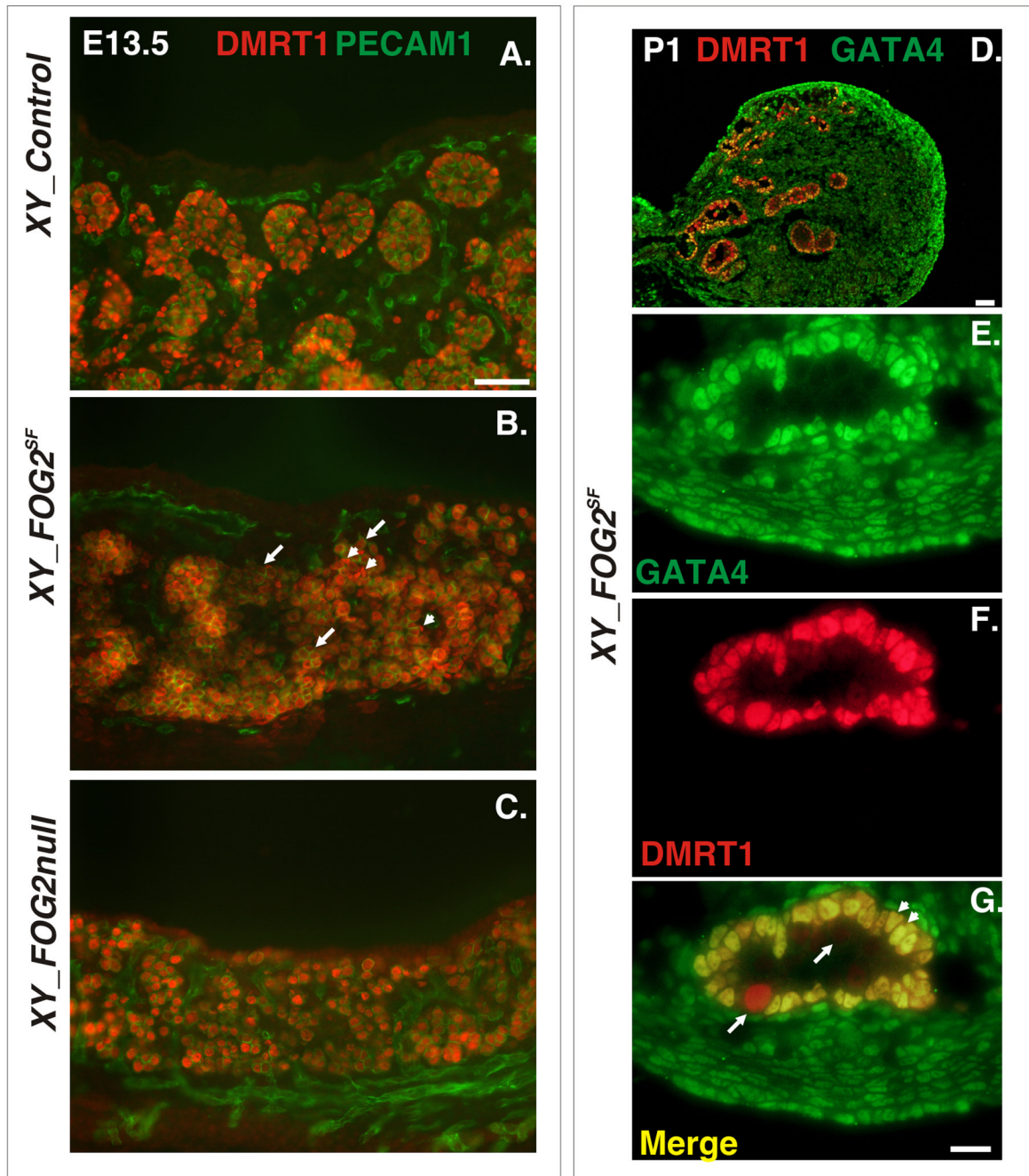
Fig.6.
The Residual Sertoli Cells in FOG2SF Mutants Retain DMRT1 Expression. A–G. Frozen sections of E13.5 (A–C) or P1 (D–G) XY gonads from the control (A), FOG2SF (B, D–G) or FOG2 null mutants (C) were co-stained with anti-DMRT1 (red) and anti-PECAM1 (green) antibodies (A–C), or anti-DMRT1 (red) and anti-GATA4 (green) antibody (D–G). Notice that DMRT1 staining is normal both in the Sertoli (arrowheads) and germ (arrows) cells in the FOG2SF mutants (B, G); notice also that DMRT1 expression is lost in the somatic cells of the XY *Fog2* null gonadal sample (C). Scale bars: 100μ (A and D) and 25μ (G); E–G are higher magnification of a section adjacent to (D).
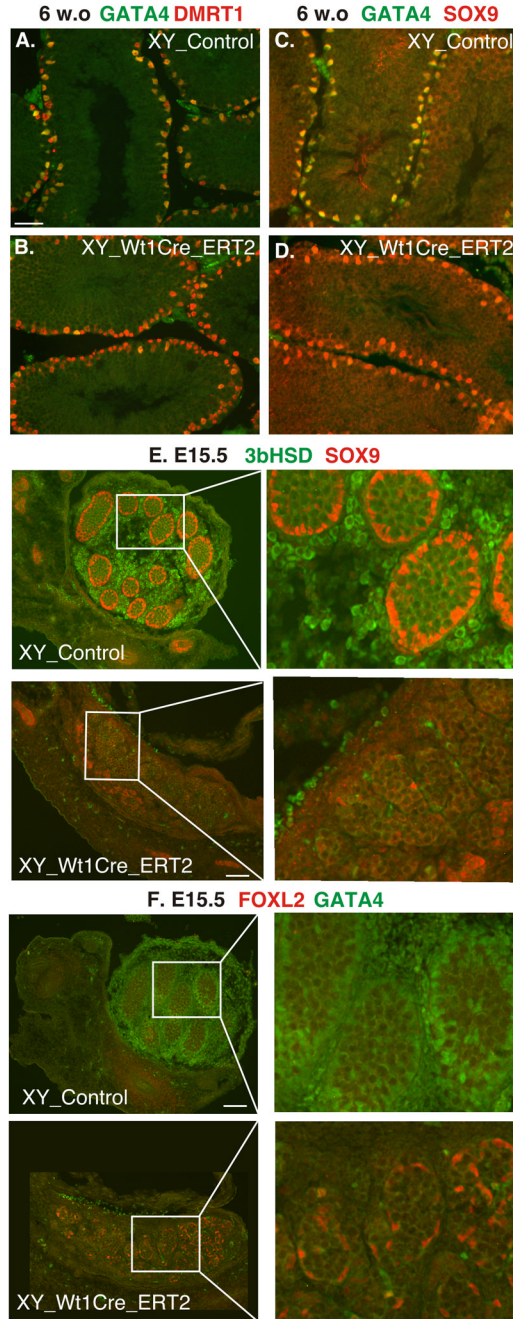
Fig.7.
The Specific Timing of GATA4 Loss Ascertains Divergent Phenotypes in XY gonads. A, B. Immunofluorescent staining of gonadal sections from the control (*top*) and *Wt1^{CreERT2}; Gata4^{flap/flap}* (*bottom*) 6 week-old male mice with anti-GATA4 (green) and anti-DMRT1 (red, A) or anti-SOX9 (red, B) antibodies. Cre function was induced at 4 weeks of age. Note that while GATA4 expression (green) was absent in the somatic cells in the mutant, DMRT1 and SOX9 expression were unscathed. C, D. Immunofluorescent staining of gonadal sections from the control (*top*) and *Wt1^{CreERT2}; Gata4^{flap/flap}* (*bottom*) E15.5 XY embryos either with anti-3bHSD (green) and anti-SOX9 (red) in (C) or anti-FOXL2 (red) and anti-GATA4 (green) antibodies in (D). Note the dramatic reduction of both Sertoli and Leydig

cell marker expression upon *Gata4* excision in the mutant. Cre function was induced by tamoxifen injection at E10.5. Scale bars: 50μ (A,B) and 200μ (C,D); right panels in C and D are magnifications of the areas delimited on the left.
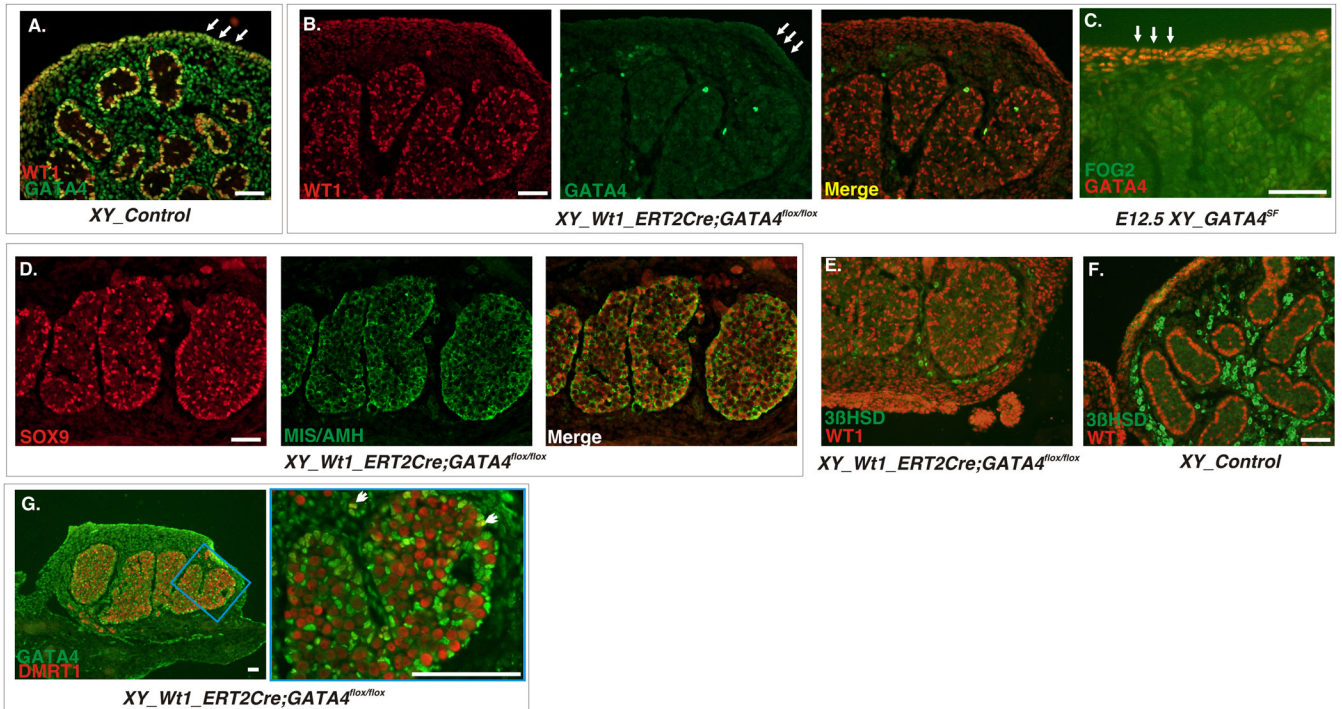
Fig. 8.
Timing of Gene Deletion is Critical for the Outcome of the *Gata4* Gene Loss. A–B. Frozen sections of E15.5 XY gonads from the control (A) or *Wt1^CreERT2; Gata4^flox/flox* mutants (B) were stained with anti-GATA4 (green) and anti-WT1 (red) antibodies. Cre function was induced by tamoxifen injection at E11.5. Note virtual absence of GATA4 staining in the mutant sample (B), including cells of the coelomic domain (arrows in A–C). C. A frozen section of E12.5 XY gonads from the GATA4^SF mutant stained with anti-GATA4 (red) and anti-FOG2 (green) antibodies is shown. Note that most cells in the coelomic domain retain GATA4 protein (arrows). D and E. Sections adjacent to those in (B) stained with anti-SOX9 (red) and anti-AMH (green) antibodies (D) or stained with anti-WT1 (red) and anti-3βHSD antibodies (green) (E) show ample presence of testis-associated proteins despite WT1-Cre mediated GATA4 loss. A section from the control E15.5 testis stained for 3βHSD and WT1 is shown for comparison in (F). G. Same section as in (B) stained with anti-DMRT1 (red) and anti-GATA4^S (green) antibody. Notice the abnormal cord structure similar to that in GATA4^SF samples (e.g., Fig.4 and Fig.5D). Notice also that, similar to the GATA4^SF mutant (Fig.5), the majority of Sertoli cells are no longer positive for DMRT1 (arrows indicate two rare DMRT1- positive cells). In the GATA4^SF sample anti-GATA4^S antibody (G, green) recognizes inactive partial GATA4 peptide. Scale bars: 100μ.
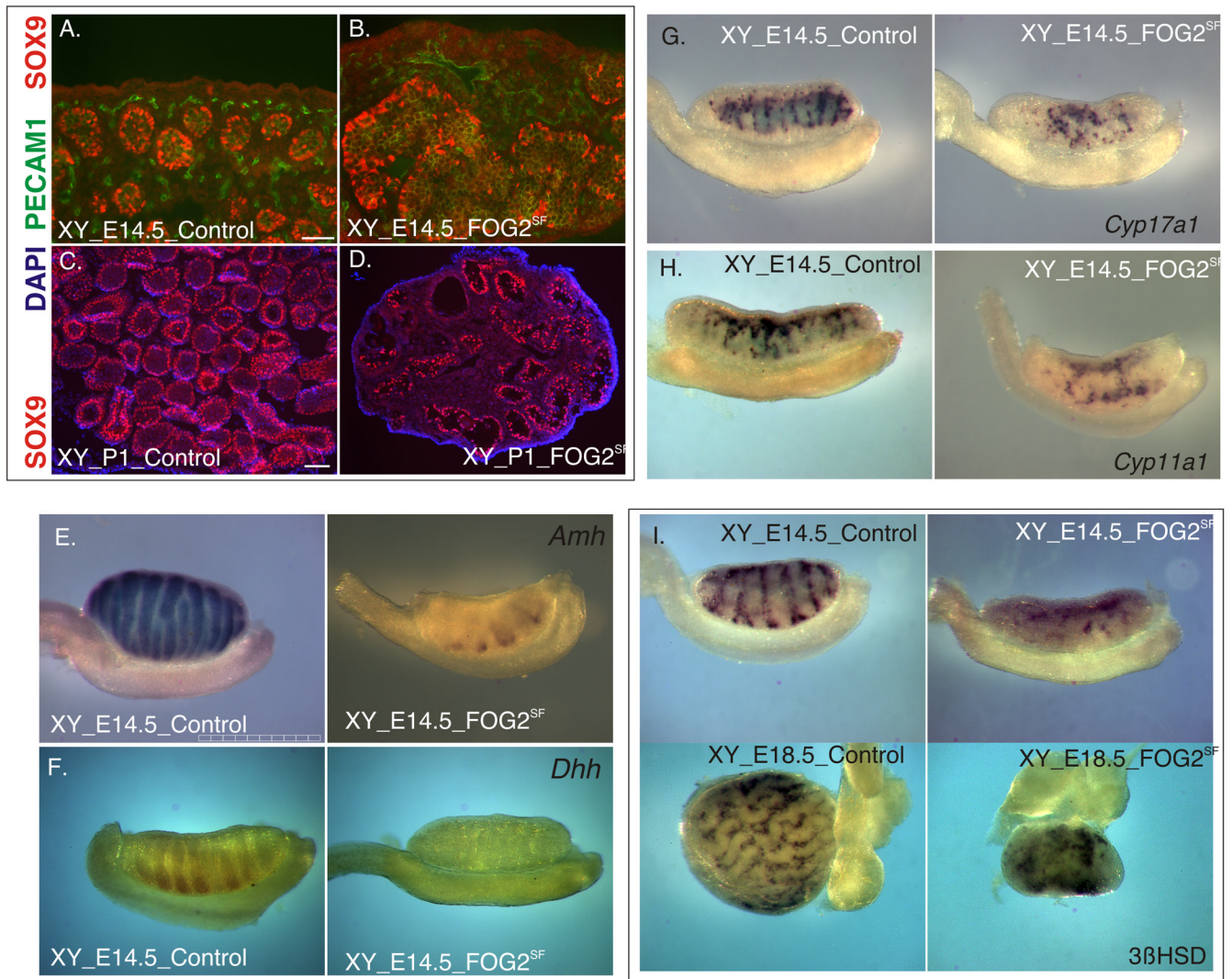
Fig.9.
Testis differentiation was compromised in XY FOG2^{SF} fetuses. A. Immunofluorescent staining of gonadal sections from the control (A, C) and FOG2^{SF} (B, D) XY E14.5 embryos (A, B) or neonatal animals (C, D) with anti-SOX9 (red) antibody and either anti-PECAM1 (green) antibody or DAPI nuclear stain. E–I. Whole-mount ISH was performed on XY control or FOG2^{SF} gonads with indicated probes. Note that while Sertoli cell marker expression was dramatically reduced in the FOG2^{SF} samples (A–F), Leydig cell expression proceeded unabated (G–I). Scale bars 100µ (A,B) and 200µ (C,D) and 1mm (E).
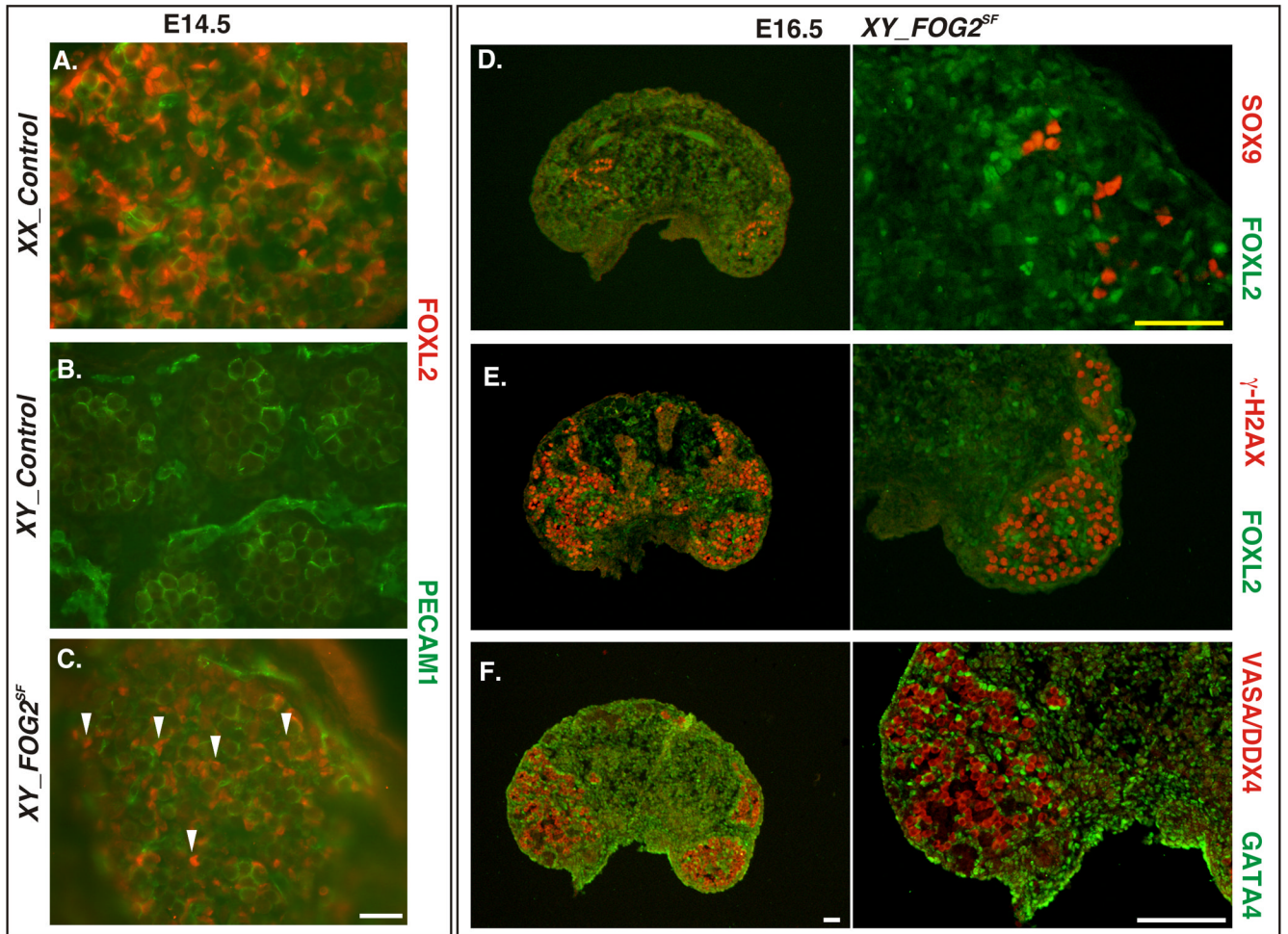
Fig.10.
Sex Reversal in XY FOG2^{SF} gonads. A–C. Immunofluorescent staining of gonadal sections from control female (A), male (B) and FOG2^{SF} XY (C) fetuses with anti-FOXL2 (red) antibody. Germ cells and vasculature are stained with anti-PECAM1 (green). Note that female-associated FOXL2 protein was expressed in the somatic cells in the ovary (A) and XY FOG2 mutant (C, arrowheads), but not in the control testis (B). D–F. Adjacent XY FOG2^{SF} gonadal sections were stained with anti-SOX9 (red) and anti-FOXL2 antibodies (green, D); anti-γ-H2AX (red) and anti-FOXL2 antibodies (green, E), and anti-VASA/DDX4 (red) and anti-GATA4 antibodies (green, F). Right panels in D–F are higher magnifications of adjacent sections. Note that while SOX9 and FOXL2 expression is not segregated spatially, the proteins are not expressed in the same cell (D). Also note that the majority of the germ cells in the XY FOG2^{SF} mutant also express the meiosis-associated marker, γ-H2AX (E,F). Scale bars: 100μ (white) and 50μ (yellow).

Table 2

Summary of the *Gata4/Fog2* Loss-of-function Models and the Resulting Phenotypes.

Genetic Sex	Targeted Gene	Modification	Time of Gene Loss	Phenotype	References
XY	*Fog2*	Deletion *Fog2−/−*	Constitutive	Embryonic lethal ~ E13.0–E14.0 Complete block in testis development No sex reversal	Tevosian et al., 2002
		Excision with *Sf1Cre*	Conditional; E13.5	Hermaphrodites Incomplete early block in testis differentiation Sex reversal	This study
	Gata4	Point mutation, *Gata4ki/ki*	Constitutive	Embryonic lethal ~ E13.0–E14.0 Complete block in testis development No sex reversal	Tevosian et al., 2002
		Excision with *Sf1Cre*	Conditional; E12.5	Undervirilized males Definitive testis development Testis cords defect Loss of *Dmrt1* gene expression in Sertoli cells	This study
		Excision with *Wt1CreERT2*	Conditional; Induced at E10.5	Early block in testis differentiation Sex reversal	
		Excision with *Wt1CreERT2*	Conditional; Induced at E11.5	Definitive testis development Testis cords defect Loss of Dmrt1 gene expression in Sertoli cells	
		Excision with *Wt1CreERT2*	Conditional; Induced at 4 w.o.	Normal gene expression	