Mutations in the **FOG2/ZFPM2** gene are associated with anomalies of human testis determination

Anu Bashamboo1,∗, Raja Brauner2, Joelle Bignon-Topalovic1, Stephen Lortat-Jacob3, Vasiliki Karageorgou1, Diana Lourenco1, Alessandro Guffanti4 and Ken McElreavey1

1Human Developmental Genetics, Institut Pasteur, Paris, France, 2Université Paris Descartes and Pediatric Endocrinology Unit, Fondation Ophtalmologique Adolphe de Rothschild, Paris 75940, France, 3Assistance Publique-Hôpitaux de Paris, Hôpital Necker-Enfants Malades, Service de chirurgie viscérale pédiatrique, Paris 75743, France and 4Genomina srl, via Nerviano, 31-20020 Lainate, Milano, Italy

Received January 7, 2014; Revised and Accepted February 13, 2014

In recent years, considerable advances have been made in our understanding of genetics of mammalian gonad development; however, the underlying genetic aetiology in the majority of patients with 46,XY disorders of sex development (DSD) still remains unknown. Based on mouse models, it has been hypothesized that haploinsufficiency of the Friend of GATA 2 (**FOG2**) gene could lead to 46,XY gonadal dysgenesis on specific inbred genetic backgrounds. Using whole exome sequencing, we identified independent missense mutations in **FOG2** in two patients with 46,XY gonadal dysgenesis. One patient carried a non-synonymous heterozygous mutation (p.S402R), while the other patient carried a heterozygous p.R260Q mutation and a homozygous p.M544I mutation. Functional studies indicated that the failure of testis development in these cases could be explained by the impaired ability of the mutant FOG2 proteins to interact with a known regulator of early testis development, GATA4. This is the first example of mutations in the coding sequence of **FOG2** associated with 46,XY DSD in human and adds to the list of genes in the human known to be associated with DSD.

INTRODUCTION

Human sex determination is a tightly controlled and highly complex process where the bi-potential gonad anlage develops to form either a testis or an ovary (1). The choice of somatic cell fate occurs following the up-regulation of **Sox9** expression by **Sry** on the Y chromosome, and the subsequent maintenance of gonadal fate can be viewed as a battle for dominance between male (**Dmrt1, Sox9**) and female (**Foxl2 and Wnt/b-catenin**) regulatory gene networks (1). Thus, the development and maintenance of the mammalian gonad is regulated by a double repressive system where equilibrium of mutually antagonistic pathways must be attained for normal development of either the testis or ovaries. Changes in this delicate balance may result in disorders of sex development (DSD) in the human (1,2). DSD are defined as congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical and they encompass a range of gonadal phenotypes that include a failure of testis determination - 46,XY complete or partial gonadal dysgenesis (2,3) Although mutations in a number of testis determining genes such as **SRY, NR5A1, WT1, GATA4, MAP3K1** and **SOX9** are associated with both syndromic and non-syndromic forms of human 46,XY gonadal dysgenesis, in the majority of cases of 46,XY DSD the molecular aetiology remains unknown (3,4). It is likely that mutations in other genes and their cofactors, which are known to be involved in somatic sex determination in mammals, could be responsible for some of these unexplained cases of DSD.

GATA4 is an evolutionarily conserved transcription factor that is essential for early development of multiple organs including the testis (5). Gata4, together with Wt1, synergistically activates the transcription of Sry, which triggers the testicular differentiation of the genital ridge (6,7). Gata4 together with the cofactors **Nr5a1** (Sf-1) and **Fog2** (Friend of Gata, 2; also known as **Zfpm2**) has also been demonstrated to regulate the expression of other key genes involved in sex determination or differentiation such as **Sox9** and **Amh** (8,9). In XY mouse embryos, which are homozygous for a **Gata4** knock-in allele (**Gata4**), that abrogates GATA4 binding to the cofactor FOG2, testis differentiation is blocked (6). The direct physical interaction

∗ To whom correspondence should be addressed. Tel: +33 145688920; Fax: +33 145688639; Email: anu.bashamboo@pasteur.fr

© The Author 2014. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oup.com
between Gata4 and Fog2 proteins may be necessary for appropriate levels of Sry expression and the block in testis development may be due to a reduction in Sry expression and other downstream effectors of testis formation such as Sox9. We have recently described a human familial case of 46,XY DSD associated with a missense mutation in the N-terminal zinc-finger domain of GATA4, which impaired the physical interaction between the GATA4 protein and FOG2 (10). The FOG2 protein is a multi-type zinc-finger cofactor that binds to the N-terminal zinc finger of the GATA4 protein, and depending on the cellular context and target gene, it can act as a transcriptional coactivator or corepressor (11,12,13). Fog2 is coexpressed with Gata4 in the heart, brain and gonads during mouse development, with high expression in the XY mouse gonad at the moment of Sertoli cell formation (14). The Fog2<sup>−/−</sup> mice die between E12.5 and 15.5 due to a complex congenital cardiac defect; they also show a failure of testis differentiation and the expression of key genes involved in testis formation (Sry, Sox9 and Amh) is reduced (6). Although the exact molecular mechanism remains unknown, a physical interaction between Gata4 and Fog2 proteins is required for normal testis development (6). Haploinsufficiency of either Gata4 or Fog2 is sufficient to induce murine gonadal sex-reversal on specific genetic backgrounds (15). Here, we for the first time present a heterozygous c.1206T>C that is predicted to result in the substitution of a serine residue by an alanine.

### RESULTS

#### Clinical phenotypes

Patient 1 of French ethnic origin was diagnosed at birth with 46,XY DSD (Table 1). Histology of the gonads indicated 46,XY complete gonadal dysgenesis. Clinodactyly of the 5th finger was observed. The mother had menopause at 50 years. A great uncle of the patient’s mother was described as being undervirilized and neither married or had children (Fig. 1A). Patient 2 was born at term to second-degree cousins from Casablanca, Morocco (Fig. 1A). At birth, the external genitalia was ambiguous and the karyotype was 46,XY (Table 1). Gonadectomy performed at 16 months revealed a testis-like structure and the gonad histology indicated partial gonadal dysgenesis. There was no family history of DSD. The father was evaluated at 52 years. Levels of inhibin B (300 pg/ml), AMH (130 pmol/l) and FSH (1.7 U/l) indicated normal testicular function. The mother had onset of menopause at 50 years. The patient also presented with severe learning and language difficulties and was diagnosed with autistic spectrum disorder.

#### FOG2/ZFPM2 mutations associated with 46,XY DSD

We performed high-throughput sequencing of DNA samples from the patients after exome capture (Methods section in the Supplementary Material, Appendix). Bioinformatics analysis of sequence data identified novel non-synonymous variants that were linked to the patients’ phenotype. Exome sequencing of patient 1 revealed a heterozygous c.1206T>C that is predicted to result in the substitution of a serine residue by an alanine.

### Table 1. Summary of the clinical presentation of two cases of 46,XY DSD carrying FOG2 mutations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Ancestry</th>
<th>Karyotype/ assigned sex</th>
<th>Genital anatomy and hormonal profile</th>
<th>Gonad histology</th>
<th>Other somatic anomalies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 French</td>
<td>46,XY/ female</td>
<td>At birth, genital tubercle 5 × 5 mm. Striated genital folds, gonads not palpable. At 18 m basal T &lt;0.05 ng/ml; T&lt;0.05 ng/ml after 7 × 1500 U hCG stimulation. DHT &lt;0.05 ng/ml. Uterus present (14 mm × 3.5 mm)</td>
<td>At 2 years, gonadectomy revealed bilateral streak gonads. Streak gonads consisted of wavy ovarian-like stroma with the absence of tubules and germ cells</td>
<td>Bilateral clinodactyly of the 5th finger</td>
<td></td>
</tr>
<tr>
<td>2 Moroccan</td>
<td>46,XY/ female</td>
<td>Ambiguous external genitalia. Single perineal opening at base of genital bud (10 × 2 mm). Striated genital folds with hypertrophy of labia majora and fusion of labia minora. Right gonad in genital fold with hydromecele, left gonad in inguinal canal. At day 2, T 0.06 ng/ml, DHT 0.06 ng/ml, 17OHP 0.4 ng/ml, Δ4-androstenedione 0.6 ng/ml, DHA 0.3 ng/ml, 11-desoxycortisol 0.7 ng/ml with normal response to ACTH test. LHRH stimulation—basal LH &lt;0.2 rising to 2.4 IU/l and basal FSH 2.7 rising to 18.3 IU/l. Stimulation by hCG (7 × 1500 U) showed T at 0.45 ng/ml, DHT at 0.24 ng/ml and Δ4-androstenedione at 0.15 ng/ml. At 2 m genitography indicated a vaginal cavity (3 cm) opening in the lower part of the urethra, and the absence of a uterus</td>
<td>Gonadectomy at 16 months revealed a testis-like structure with deferens on left (1.5 × 0.8 cm) and right sides (1.5 × 0.5 cm). Histology—rare Sertoli cells, absence of germ cells and an interstitium consisting of rare wavy stroma-like cells. An atrophic small rete testis. Leydig cells were not observed and Müllerian structures were absent</td>
<td>At 16.5 years (height 165 cm, weight 51 kg)—head circumference (59.5 cm), major language, reading and learning difficulties. MRI revealed sus tentorial ventricular dilatation, without hydrocephalus signs and normal olfactive bulbs</td>
<td></td>
</tr>
</tbody>
</table>
arginine amino acid at position 402 (Fig. 1B). Direct sequencing indicated that this mutation was present in her mother and maternal grandmother. This variant was not observed in 200 in-house and healthy ancestry-matched control individuals (400 alleles). Exome sequencing revealed that the patient carried a heterozygous FOG2 mutation (c.779G>A) in the first zinc finger. This mutation is predicted to result in the substitution of an arginine at position 260 by a glutamine residue within the evolutionary conserved N-terminal zinc-finger motif of the protein (Fig. 1B). Furthermore, the exome sequencing of the same patient revealed a mutation (c.1631G>A) immediately upstream from the fifth zinc finger of FOG2. This homozygous mutation results in the substitution of another evolutionary conserved amino acid—a methionine in position 544 is replaced by an isoleucine (Fig. 1B). The presence of these mutations was confirmed by Sanger sequencing. Both parents carried the c.1631G>A allele, while the c.779G>A variant is a de novo mutation. Neither of these two non-synonymous variants was observed in over 400 healthy ancestry-matched and normospermic control individuals (800 alleles). In silico analysis indicated that both mutations are possibly damaging (PSIC score difference of 1.595 and 1.617, respectively). Exome sequencing in each of the two patients did not reveal mutations in other genes known to be involved in either 46,XY DSD or testis development (Supplementary Material, Appendix).

To further assess the degree of genetic variability in the FOG2 gene, we sequenced the open reading frame of the FOG2 gene in 200 subjects with normal sperm levels (>20×10⁶ sperm per millilitre), of whom 55 had fathered at least one child. In this panel, we detected only two known non-synonymous variants: p.A403G (rs11993776, allelic frequencies, C 0.83 and G 0.17) and p.E782D (rs2920048, allelic frequencies G 0.76 and C 0.24).

Figure 1. (A) Pedigrees of the sporadic and familial case of 46,XY DSD. Squares represent male family members, and circles represent female family members. Solid circles within squares represent affected 46,XY DSD subjects who were raised as girls. Symbols containing a black dot represent apparently unaffected carriers of the mutation. (B) Schematic representation of the zinc-finger domains (red) of the FOG2 protein are shown with an arrow indicating the localization of the FOG2 p.R260Q mutation in relation to the N-terminal zinc-finger domain of the protein. Representative chromatograms are shown of the heterozygous and homozygous mutations found in both 46,XY DSD cases. Sequence alignment of the mutated residues of the human FOG2 protein with other species shows a high degree of amino acid conservation. In each example, the position of the mutation is highlighted in green.
In silico analysis predicts the disruptive effect of the mutations on three-dimensional structure of the FOG2 protein

The three-dimensional (3D) structure of the wild-type and mutant FOG2 proteins were created using the 3D-JIGSAW (version 3.0) Protein Comparative Modelling server, where structure templates are identified using the hidden-Markov model and the returned alignments are used to generate the structure models. The 3D-JIGSAW returned the predicted structure for the FOG2 protein between AA205 and AA410. The secondary structures were visualized using FirstGlance in Jmol (Fig. 2). The protein chain is shown as a smooth backbone with the random coils shown in white and the turns in blue. The alpha helices are shown as pink rockets with the arrow-head pointing towards the C-terminus and the beta strands are shown as yellow planks. The WT and p.M544I are predicted to have 11.6% alpha helices and 5.8% beta strands. The p.R260Q, p.R260Q/M544I are predicted to have 12% of alpha helices and no beta strands at all. The p.S402R is predicted to have no change in the percentage of beta helices but the proportion of alpha helices is increased to 14.5%. Thus, the in silico structure prediction analyses predict the non-synonymous mutations to have a disruptive or relevant effect on the secondary structure of the FOG2 protein.

Missense mutations in FOG2 alter the biological activity of the protein

To investigate the functional consequences of the observed mutations on the biological activity of the FOG2 protein, we performed a series of transactivation assays using the AMH promoter and Tesco enhancer region of Sox9, in HEK293-T cells and mouse embryonic stem cell line, E14. Among other genes, Gata4 and Nr5a1 are known involved in the regulation of AMH expression in developing Sertoli cells at the moment of sex determination (16). Previously, transactivation assays using reporter constructs containing the minimal responsive AMH promoter showed that wild-type FOG2 represses the GATA4-dependent transactivation of AMH in CV-1 and Sertoli cell lines (9,17,18), whereas it does not have any effect on GATA4-dependent transactivation of AMH in HEK-293 cells (19).

In the present study, cotransfection of the ?260 AMH promoter with either GATA4, FOG2 or NR5A1 alone showed a significant activation of the AMH promoter in both the HEK-293 (Fig. 3A and B) and E-14 cell lines. Wild-type FOG2 does not have any effect on GATA4-dependent transactivation of AMH (Fig. 3A). Three of the four mutant proteins (p.R260Q; p.R260Q/M544I; p.S402R) lost the FOG2 transactivation activity on the AMH promoter and acquire a repression effect on the GATA4-dependent transactivation of AMH (Fig. 3A). In contrast, the p.M544I mutant protein shows an activation of AMH at levels similar to that of wild-type FOG2 protein and this mutation does not show any effect on GATA4-dependent transactivation of AMH promoter (Fig. 3A).

Similar to previously published results (9,17,18), in this study also, we observe that the wild-type FOG2 can repress the synergistic transactivation of AMH by GATA4 and NR5A1 (Fig. 3B). All the mutated FOG2 proteins, including p.M544I, showed a
variable but significant reduction in their capacity to repress GATA4/NR5A1-dependent transactivation of the AMH promoter when compared with the wild-type protein (Fig. 3B). Since both patients have gonadal dysgenesis, we then examined the effects of each FOG2 mutations on SOX9 gene expression using the luciferase reporter system designed by Sekido and

Figure 3. (A) Transcriptional regulation of the AMH promoter by FOG2 and FOG2+GATA4. The transcriptional activities of WT FOG2 and the mutant FOG2 proteins (p.R260Q, p.M544I, p.R260Q+p.M544I, p.S402R) with (red bars) or without (blue bars) WT GATA4 were studied using the human AMH promoter fused to luciferase as a reporter following transfection in HEK293-T cells. The data shown here represent the mean ± SEM of at least three independent experiments, each of which was performed in quadruplicate. The results are expressed as fold change in the relative activity of transfected proteins on the AMH promoter, above the background. (B) Transcriptional regulation of the AMH promoter by FOG2+GATA4+NR5A1. The transcriptional activities of WT FOG2 and the mutant FOG2 proteins (p.R260Q, p.M544I, p.R260Q+p.M544I, p.S402R) with WT GATA4 and WT NR5A1 were studied using the human AMH promoter fused to luciferase as a reporter following transfection in HEK293-T cells. The data shown here represent the mean ± SEM of at least three independent experiments, each of which was performed in quadruplicate. The results are expressed as fold change in the relative activity of transfected proteins on AMH promoter, above the background.
Figure 4. (A) Transcriptional regulation of the Tesco enhancer element by FOG2 and FOG2 + GATA4. The transcriptional activities of WT FOG2 and the mutant FOG2 proteins (p.R260Q, p.M544I, p.R260Q + p.M544I, p.S402R) with (red bars) or without (blue bars) WT GATA4 were studied using the mouse Tesco enhancer region fused to luciferase as a reporter following transfection in HEK293-T cells. The data shown here represent the mean ± SEM of at least three independent experiments, each of which was performed in quadruplicate. The results are expressed as fold change in the relative activity of transfected proteins on Tesco reporter, above the background. (B) Transcriptional regulation of the Tesco enhancer element by FOG2 + GATA4 + NR5A1. The transcriptional activities of WT FOG2 and the mutant FOG2 proteins (p.R260Q, p.M544I, p.R260Q + p.M544I, p.S402R) with WT GATA4 and WT NR5A1 was studied using the mouse Tesco enhancer region fused to luciferase as the reporter following transfection in HEK293-T cells. The data shown here represent the mean ± SEM of at least three independent experiments, each of which was performed in quadruplicate. The results are expressed as fold change in the relative activity of transfected proteins on the Tesco reporter, above the background.
showed a very strong activation of the Tesco reporter (Fig. 4A). Lovell-Badge (20), which assays transactivation of the core mouse Sox9 enhancer Tesco. Similar to the data of the AMH transfection results, both GATA4 and FOG2 alone activated Tesco reporter to different degrees, whereas NR5A1 alone showed a very strong activation of the Tesco reporter (Fig. 4A and B). However, each of the mutant FOG2 proteins, except p.M544I, was unable to activate the Tesco reporter (Fig. 4A). Interestingly, the p.M544I mutation alone considerably enhanced the transcription from the Tesco element when compared with the wild-type FOG2 (Fig. 4A). A combination of wild-type FOG2 and GATA4 proteins showed no difference in transactivation of Tesco when compared with GATA4 alone, although the level of activity was significantly reduced in comparison with wild-type FOG2 alone. When cotransfected with GATA4, the p.R260Q and p.S402R mutants supressed the GATA4-dependent transactivation of the Tesco, which remained unchanged in the case of p.R260Q/M544I. The p.M544I mutant, on the other hand, exhibited significantly increased activation of Tesco reporter when compared with FOG2 WT and all the mutated FOG2 proteins (Fig. 4B).

To explore further whether GATA4 and NR5A1 can synergistically activate the Tesco reporter and if FOG2 can suppress this transactivation, we cotransfected these factors together with the Tesco reporter. In contrast to the activation of AMH, GATA4 and NR5A1 together do not activate the Tesco reporter more than NR5A1 alone. Although the addition of FOG2 represses the activity of NR5A1 alone or NR5A1+GATA4, this repression of transactivation was significantly weaker than that seen in AMH assays (Fig. 4B). All the four mutated FOG2 proteins lack to varying degrees the ability to repress the activation of the Tesco reporter by NR5A1 (Fig. 4B).

All the data for luciferase assays was analysed statistically using Student’s t-test. A P-value of <0.05 was considered statistically significant. The transactivation assays using the AMH, GATA4 and NR5A1 together do not activate the Tesco reporter more than NR5A1 alone. Although the addition of FOG2 represses the activity of NR5A1 alone or NR5A1+GATA4, this repression of transactivation was significantly weaker than that seen in AMH assays (Fig. 4B). All the four mutated FOG2 proteins lack to varying degrees the ability to repress the activation of the Tesco reporter by NR5A1 (Fig. 4B).

FOG2 mutant proteins showed reduced or no direct physical interaction with GATA4

It has been postulated that the physical interaction between GATA4 and FOG2 proteins is indispensable for correct testis determination in mouse (6,15). To further investigate the functional consequences of the p.R260Q, p.M544I, p.R260Q/M544I and p.S402R mutations on FOG2 protein, we analysed the ability of the WT and mutant FOG2 proteins to physically interact with the protein partner GATA4. The plasmids encoding either FOG2 WT or mutant genes were cotransfected with plasmid encoding WT-GATA4 in HEK-293 cells. The whole cell lysate was prepared and the FOG2-GATA4 protein complex was immunoprecipitated using anti-GATA4 antibody. The complex was purified on protein A/G agarose and fractionated on a 5% SDS–PAGE gel and transferred to nitrocellulose membrane. The binding was then detected with anti-FOG2 antibody, followed by secondary antibody conjugated with HRP and visualized by chemiluminescence. Multiple coimmunoprecipitation experiments revealed that the WT and p.R260Q proteins retained their ability to interact with GATA4 (Fig. 5); however, the interaction between GATA4 and the other mutated FOG2 proteins was either significantly reduced (p.M544I, p.R260Q/M544I) or abolished (p.S402R). The protein–protein interaction was quantified using ImageJ freeware available from NIH. The band in each lane was enclosed in a rectangular box of same size and the average intensity of pixels from the top of the rectangle to the bottom of the rectangle was measured. The dark band appears as a peak that rises above the background. The area under each peak was measured and the relative band intensity between lanes was calculated when compared with the interaction between the WT- GATA4 and FOG2 proteins, which was considered 100% (denoted by 1). The respective band intensities are written as numbers above the bands in top panel.

DISCUSSION

Mutations involving the human FOG2 gene have been associated with congenital heart disease and antipsychotic-induced Parkinsonism in schizophrenia, with no reported gonadal anomalies (21,22). However, recently, a boy with a balanced t(8;10)(q23.1;q21.1) translocation was reported with congenital secundum-type atrial septal defect and 46,XY gonadal dysgenesis (23). The breakpoint on chromosome 8 mapped within the FOG2 gene. Quantitative analysis of FOG2 expression revealed the presence of a truncated transcript but there was no detectable change in the expression of the genes flanking the breakpoint, thus making it possible to assign the observed clinical phenotype to altered FOG2 expression. Furthermore, Tan et al. (24) described an individual with mental retardation, congenital heart disease, multiple cancellous exostoses of long tubular
bones, cleft lip and palate, crowding of teeth, umbilical hernia, pectus carinatum, scoliosis, cone-shaped epiphyses of middle phalanges and gonadal dysgenesis. This phenotype was in association with a de novo chromosomal translocation: 46, XY t(8;18)(q22; q21). The region comprised of 16-Mb de novo deletion within chromosomal region 8q23.1-8q24.1 that includes the FOG2 gene. However, to date, no point mutations in FOG2 have been described in association with 46,XY DSD.

Bouma et al. (15) had hypothesized that the presence of a single normal FOG2 gene could lead to 46,XY gonadal dysgenesis. Here, for the first time, using the exome sequencing approach, we have identified two patients with different missense mutations in the FOG2 gene that are associated with 46,XY gonadal dysgenesis and somatic anomalies. In both the families, there was no reported history of cardiac anomalies. In patient 1, the p.S402R mutation was carried by her unaffected mother and maternal grandmother (Fig. 1A II.2, III.2). It is possible that this mutation may have contributed to the reported undervirilization of the maternal uncle; however DNA was not available to test this hypothesis. FOG2 with p.S402R mutation failed to bind to GATA4 and exhibited an altered biological activity on gonadal promoters when compared with WT FOG2.

The second patient carried an inherited homozygous p.M544I mutation and a de novo heterozygous p.R260Q mutation. The p.M544I variant was previously reported in an individual of Italian ancestry in association with tetralogy of Fallot with no reported gonadal anomalies (25). Our data suggest that p.M544I may be a rare variant as we did not detect it in 400 normospermic individuals from the Casablanca region. The p.M544I variant by itself has little effect on the biological activity of FOG2 protein in transactivation of the gonadal promoters, but it shows reduced binding with GATA4. However, both the father and mother of the parent, who carry the mutation, have apparently normal functioning gonads. In the in vitro assays, a combination of both the p.R260Q and the p.M544I variants altered the biological activity of the FOG2 protein on specific downstream targets, as well as obliterated its interaction with GATA4. In the patient, the two mutations together may result in an imbalance of the delicate equilibrium between antagonistic male and female pathways leading ultimately to gonadal dysgenesis.

It is difficult to directly correlate the changes in biological activity of the mutant FOG2 proteins identified in both patients with a precise mechanism whereby these mutations lead to a failure of testis determination. Indeed, in the mouse, although Fog2 is necessary for testis determination via an interaction with Gata4, the mechanism of how this leads to testis formation is unclear (6). We used a series of biological assays to explore the change in biological activity of the mutant FOG2 proteins. These assays show clear differences in activities of the mutant proteins compared with the wild-type protein. The genetic data, as well as the observed changes in the functional activity, demonstrate a proof of principal that mutations of FOG2 cause gonadal dysgenesis. The experiments do not reveal the precise mechanism involved. It is possible that the GATA4-FOG2 complex may be involved in the active repression of the female WNT4/FOXL2 pathways or, as we have demonstrated, the FOG2 protein by itself has transcriptional activation properties and may be a direct positive regulator of testis determination. Until, we have an appropriate cellular system for addressing these issues, the precise mechanism involved may remain unresolved.

Although FOG2 is expressed in the granulosa cells of the growing follicle (19), the mother of both patient 1 and patient 2 reported menopause at 50 years, suggesting that ovarian development and function is less susceptible to altered FOG2 activity than the testis. This is similar to the previously reported mutation in GATA4, where disruption of the FOG2/GATA interaction is associated with 46,XY DSD, but has no apparent effect on ovarian development or function (10).

Both the patients presented with somatic anomalies associated with the gonadal phenotype. Patient 1 had bilateral clinodactyly of the 5th finger. Interestingly, the patient described by Finelli et al. with a balanced translocation disrupting FOG2 also presented with clinodactyly of the 5th finger of both hands. Patient 2 presented with language, reading and learning difficulties and was diagnosed with autistic spectrum disorder. The relationship between FOG2 and autistic spectrum disorder is unclear. In the developing mouse brain, Fog2 is expressed in the corticothalamic axons that convey sensory and motor information essential for cortical functioning and its expression becomes detectable in neurons within the brain and neural tube beginning at about E10.5 (11,26). As research develops, the role of FOG2 in human brain development and function may become clearer.

In conclusion, our data show an association between nonsynonymous mutations in FOG2 and 46,XY DSD indicating a requirement for FOG2 in human testicular development and that the mutations in FOG2 are a novel cause of human 46,XY DSD.

**MATERIALS AND METHODS**

Complete methods are described in detail in Supplementary Material, Materials and Methods.

A sporadic and a familial case of 46,XY DSD were studied as well as 200 ancestry-matched control individuals. The methods are summarized in the Supplementary Material, Appendix. Written informed consent was obtained from all individuals who participated in this study in agreement with the recommendations of the local French Ethics Committee.

**Functional analyses of FOG2 mutant proteins**

After generation of expression vectors containing either the p.S402R, p.M544I, p.R260Q or p.M544I+R260Q variants by site-directed mutagenesis, we performed transient gene expression assays and co-IP assays as described elsewhere (Supplementary Material, Appendix).

**SUPPLEMENTARY MATERIAL**

Supplementary material is available at HMG online.

**ACKNOWLEDGEMENTS**

We thank Dr J. D. Molkentin for GATA4-GST plasmid and Dr Francis Poulat for Tesco reporter construct.
Conflict of Interest statement. None declared.

FUNDING
This work was supported by Laboratoire Lilly France, the Agence Nationale de la Recherche-GIS Institut des Maladies Rares (K.M.), March of Dimes Foundation Research Grant 1-FY07-490 (K.M.), EuroDSD in the European Community’s Seventh Framework Programme FP7/2007–2013 under Grant 201444 (to K.M. and A.B.), Project Blanc Institut Pasteur/Assistance Publique-Hôpitaux de Paris 2011 (K.M. and R.B.), grant N°295097, GM_NCD_in_Co-Reinforcing IPT capacities in Genomic Medicine, Non Communicable Diseases Investigation and international cooperation as part of the EU call FP7-INCO-2011-6 (K.M. and A.B.) and grant ACIP A16-2013 from Actions concertees Inter-Pasteuriennes (A.B.). The work is also partially funded by an Franco-Egyptian AIRD-STDF grant (K.M.). We also wish to thank Dr Pauchet 95480 Pierrelaye who managed patient 1.

REFERENCES