



# Testicular differentiation factor SF-1 is required for human spleen development

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**The transcription factor steroidogenic factor 1 (SF-1; also known as NR5A1) is a crucial mediator of both steroidogenic and nonsteroidogenic tissue differentiation. Mutations within SF1 underlie different disorders of sexual development (DSD), including sex reversal, spermatogenic failure, ovarian insufficiency, and adrenocortical deficiency. Here, we identified a recessive mutation within SF1 that resulted in a substitution of arginine to glutamine at codon 103 (R103Q) in a child with both severe 46,XY-DSD and asplenia. The R103Q mutation decreased SF-1 transactivation of TLX1, a transcription factor that has been shown to be essential for murine spleen development. Additionally, the SF1 R103Q mutation impaired activation of steroidogenic genes, without affecting synergistic SF-1 and sex-determining region Y (SRY) coactivation of the testis development gene SOX9. Together, our data provide evidence that SF-1 is required for spleen development in humans via transactivation of TLX1 and that mutations that only impair steroidogenesis, without altering the SF1/SRY transactivation of SOX9, can lead to 46,XY-DSD.**

## Introduction

Steroidogenic factor 1 (SF-1; also known as NR5A1) is a transcription factor involved in steroidogenesis, reproduction, and sexual differentiation (1). In mice, it is expressed in all primary steroidogenic tissues and in the embryonic urogenital ridge. SF-1 participates with sex-determining region Y (SRY) and SOX-9 in mammalian sex determination and regulates Müllerian-inhibiting substance (*AMH*) (2, 3).

*SF1* mutations lead to several phenotypes, including disorders of sexual development (DSD) with sex reversal, spermatogenic failure, premature ovarian failure, and adrenocortical insufficiency (AI) (OMIM 184757). The only 2 recessive *SF1* mutations described to date, D293N and R92Q (4, 5), caused severe 46,XY-DSD as well as adrenal failure in the R92Q homozygote. Here, we report a novel homozygous *SF1* mutation, R103Q, presenting with 46,XY complete sex reversal and asplenia, but without AI. This mutation impaired SF-1 activation of the spleen development gene *TLX1*. It also impaired activation of steroid synthesis, but did not affect synergistic SF-1 and SRY activation of *SOX9*, a gene considered to be crucial for testicular differentiation.

## Results and Discussion

**Clinical features and laboratory studies.** A 13.5-year-old girl of consanguineous Palestinian parents presented with abdominal pain ultimately ascribed to fecal impaction. History was notable only for delayed puberty and pneumococcal sepsis at 9 months of age. Ultrasound and CT imaging revealed asplenia, bilateral inguinal testes, and absence of the uterus, ovaries, and fallopian tubes (Figure 1, A and B). Peripheral blood smear revealed typical asplenia-

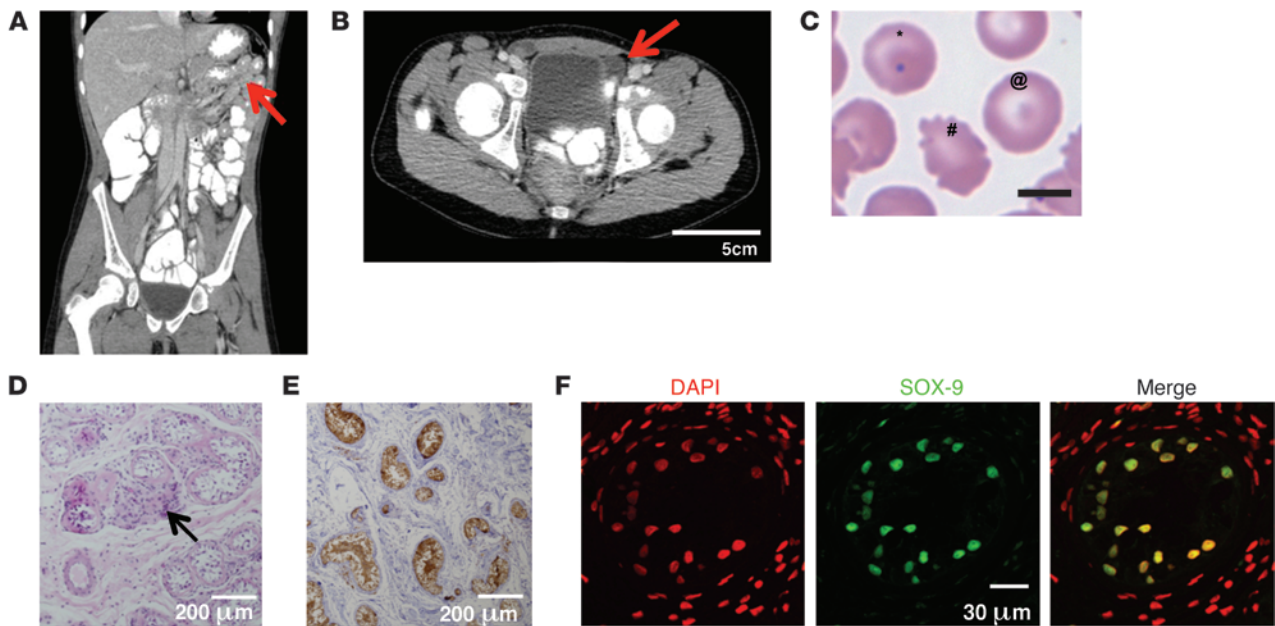
associated Howell Jolly bodies and poikilocytosis (Figure 1C). Karyotype was 46,XY. Blood tests showed normal electrolytes, aldosterone, basal and adrenocorticotrophic hormone-stimulated (ACTH-stimulated) cortisol, and 17-hydroxylase–progesterone. Dehydroepiandrosterone, androstenedione, and testosterone levels remained low to undetectable despite ACTH stimulation, while ACTH levels were slightly elevated (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI73186DS1). Estrogen and progesterone levels were prepubertal. These results excluded a precursory major defect in steroid biosynthesis. 17-hydroxylase deficiency was thought to be an unlikely cause for the phenotype, given the patient's normal serum electrolytes and normal blood pressure at 13.5 years of age. The severe sex reversal (46,XY-DSD) was therefore postulated to result from a genetic defect affecting testicular differentiation and gonadal function. The patient was reared as a female, given lack of male genitalia, and her gonads were surgically removed and studied for premalignant changes. Gonadal histopathology revealed normal and abundant Sertoli cells, few Leydig cells (consistent with low serum testosterone levels), absence of germinal or pregerminal cells, absence of staining for premalignant markers (e.g., placental-like alkaline phosphatase [PLAP]), and positive inhibin and SOX-9 staining in Sertoli cells (Figure 1, D–F).

**Genetic analysis.** Analysis for XY-DSD included testing for *SRY* (by PCR) and sequencing of *STAR* and *SF1* as candidates for nonclassic steroidogenic failure or gonadal dysgenesis. *SRY* was present, and no mutations were identified in *STAR*, consistent with the patient's normal mineralocorticoid and glucocorticoid levels (Supplemental Table 1). However, in *SF1*, we identified a homozygous G-to-A transition (c.308G>A) resulting in a substitution of arginine to glutamine at codon 103 (p.R103Q). Both parents and all 5 unaffected brothers of the patient were heterozygous for this mutation (Figure 2, A–C), which was not detected in 190 ethnically matched controls (Supplemental Figure 1). This novel amino acid substitution, the

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**Figure 1**

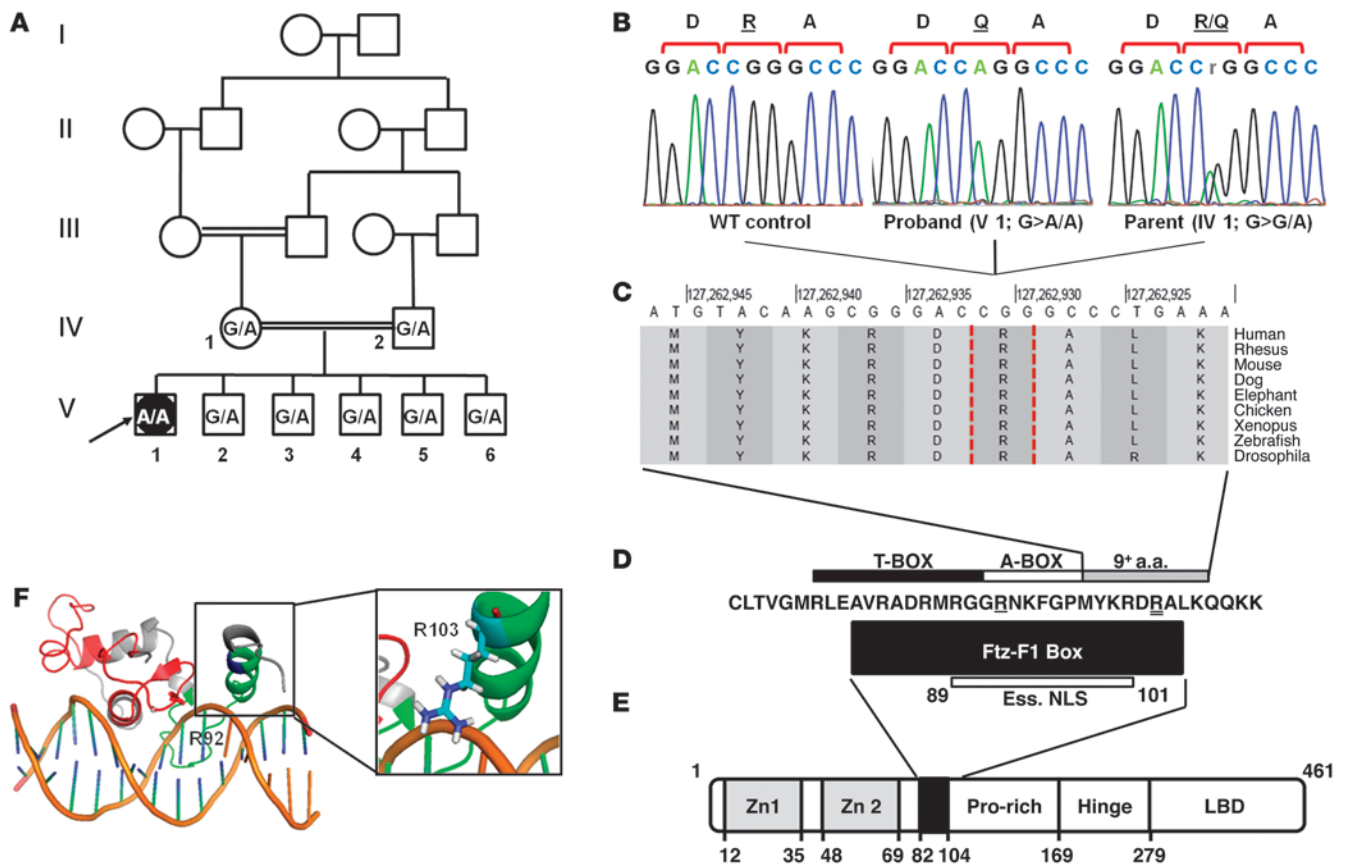
Phenotypic features of the patient. **(A)** CT scan of the patient's abdomen, revealing asplenia (arrow) and lack of uterus. **(B)** CT scan of the inguinal region, revealing inguinal testes (arrow). **(C)** Patient's peripheral blood smear, with Howell-Jolly bodies (\*), target cells (@), and poikilocytosis (#) indicative of asplenia. **(D)** H&E staining showed paucity of Leydig cells (arrow), lack of germinal cells, and abundance of Sertoli cells. **(E)** Paraffin-embedded sections of the patient's testes immunostained with anti-inhibin antibody (brown). **(F)** Immunofluorescent staining of the proband's testis with anti-SOX-9 antibody (green) and general nuclear staining with DAPI (red). Positive SOX-9 staining in Sertoli cells was similar in strength in the patient's testes and in the control testes, which had a higher total number of cells, consisting mostly of germinal and pregerminal cells (not shown). Scale bars: 5  $\mu\text{m}$  (**C**); 200  $\mu\text{m}$  (**D** and **E**); 30  $\mu\text{m}$  (**F**).

third recessive *SF1* mutation reported to date, results in loss of positive charge in the otherwise basic sub-domain of the cross-species conserved Fushi-tarazu (Ftz-F1) box of *SF1* (Figure 2, D and E, and ref. 6). In a 3D model derived from SF-1 solution structure, the positively charged R103 appears to be in close proximity to the negatively charged sugar-phosphate backbone of the DNA site bound by SF-1 (Figure 2F and ref. 7). Replacement with a noncharged residue is likely to affect DNA binding, as previously shown for similar substitutions in the Ftz-F1 box (8).

The unique finding of asplenia in this patient (Figure 1A), together with impaired spleen development reported in *Sf1* knockout mice (9), raised the hypothesis that the R103Q mutation may alter expression of SF-1-regulated genes important for spleen development. We therefore searched for SF-1 recognition elements in genes previously implicated in spleen development, and identified 2 such bona fide elements in the first exon of *TLX1* (OMIM 186770). A similar cluster of SF-1 binding sites was not found in the promoters of other spleen-development genes (*PBX-1*, *NKX2-5*, *NKX3-2*, *WT-1*, and *POD-1*). *TLX-1* is a homeodomain-containing transcription factor critical for embryonic spleen development, as *Tlx1* knockout mice have isolated asplenia (10, 11); in contrast, the other above-described spleen development genes also affect the development of other organs. To determine whether SF-1 regulates *TLX1*, and whether this effect is altered by the *SF1* R103Q mutation, we engineered and tested the activity of a luciferase reporter construct controlled by the minimal promoter and first exon of *TLX1* (Figure 3A). Whereas WT *SF1* promoted *TLX1* transcription, the *SF1* R103Q mutation dramatically decreased this transcriptional activ-

ity by 2.7-fold in COS-7 cells and similarly in CHO cells (Figure 3A and Supplemental Figure 2). Interestingly, previously reported *SF1* mutations, G35E and R92Q, also decreased SF-1 activation of the *TLX1* promoter, whereas the D293N mutation, associated with a milder phenotype, had no significant effect (Figure 3A and refs. 5, 12, 13). This may explain the early demise of a homozygous R92Q patient, who died of sepsis, a well-known complication of hyposplenism, at 4–5 months of age (14). While SF-1 is characterized as a transcription factor involved in human gonadal and adrenal development, it was not previously known to be important for human spleen development. Such a role for SF-1 was surmised from tissue expression studies (15) and from the splenic phenotype of *Sf1* knockout mice, which have small and maldeveloped spleens, but not complete asplenia (9). Interestingly, another case of asplenia in a patient with a *SF1* mutation was presented after our study was completed (16). Our finding that the *SF1* R103Q mutation impaired SF-1 transactivation of the *TLX1* promoter provides a mechanism for the observed asplenia and suggests a role for SF-1 as a facilitator of normal spleen development in humans.

SF-1 plays a critical role in many aspects of gonadal development and testicular differentiation, including steroidogenesis (17). SF-1 is thought to induce *SRY* expression in the early gonad, leading to synergistic activation (by *SRY* and SF-1) of *SOX9* transcription through its testis-specific enhancer, TES, and when sufficient levels of *SOX9* are achieved, *SOX9* replaces *SRY* and binds its own enhancer together with SF-1 to help maintain its own expression. Failure of this SF-1-induced *SOX9* activation may lead to sex reversal (3, 18). In the next stage of testicular differentiation, SF-1 in Leydig cell progenitors also



**Figure 2**

Characterization of the *SF1* mutation in the patient's family. (A) Pedigree of the patient's consanguineous family. The proband (V1, arrow) a 46,XY female, has healthy first-cousin parents (IV1 and IV2) and 5 siblings (V2–V6). *SF1* c.308 genotype (G, WT; A, mutant) is shown for each. (B) DNA sequencing chromatograms of the c.308G>A mutation (p.R103Q): homozygous in the proband, heterozygous in the mother, absent in a control (subject numbering as in A). (C) Cross-species conservation of the residues adjacent to R103 (dashed vertical outlines). Data were obtained from the UCSC human genome browser (24). (D) Conserved sequence of the Ftz-F1 box, the T-box and A-box subdomains, and the essential nuclear localization signal (Ess. NLS) of *SF1* (6, 25). Residues mutated in the R92Q and R103Q mutations are denoted by single and double underline, respectively. (E) *SF1* protein domains, including 2 zinc finger motifs, Ftz-F1 box (black box), proline-rich domain, hinge domain, and ligand-binding domain (LBD). (F) 3D model (generated using PyMol software; ref. 26) based on the solution structure of *SF1* (7), showing the DNA-binding domain bound to its target sequence in the inhibin  $\alpha$  subunit promoter. The 2 zinc finger motifs are shown in red, Ftz-F1 box is green, and R103 is blue. Inset shows the orientation of the R103 side chain, with the positive charge adjacent to the negatively charged DNA backbone.

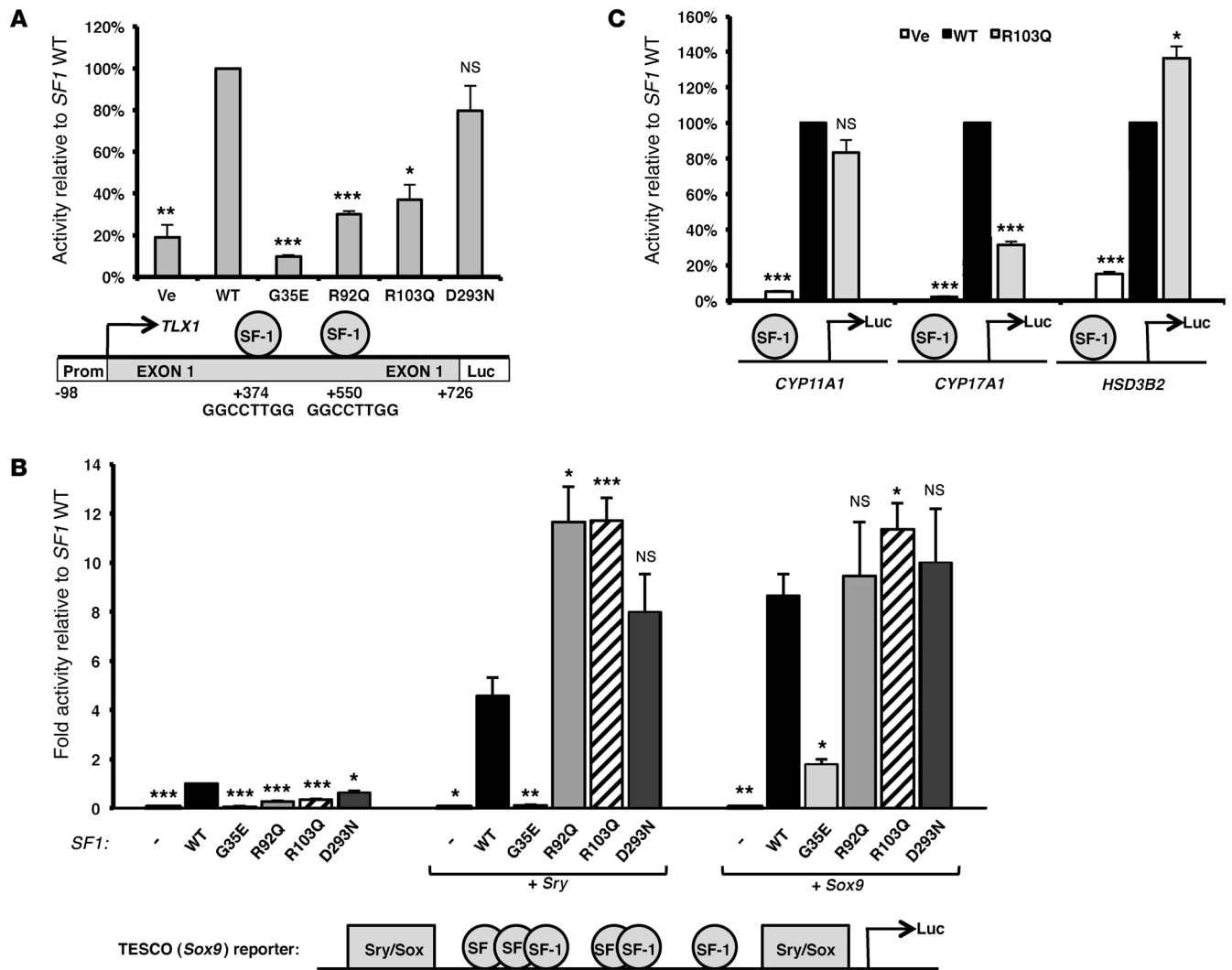
regulates expression of key testosterone synthesis genes, including *STAR* and *CYP17A1*. *SF1* mutations can lead to XY sex reversal by impairing any of these functions, but most reported mutations have not been evaluated for their effects on *SOX9* expression.

Based on the developmental roles of SF-1, we examined the effects of *SF1* mutations R103Q, G35E, R92Q, and D293N on *SOX9* gene expression using the luciferase reporter system designed by Sekido et al. (3), which assays transactivation of the core mouse *SOX9* enhancer, *TESCO*. In the absence of SF-1, neither SRY nor SOX-9 overexpression stimulated *TESCO*-dependent reporter activity (Figure 3B). Recapitulating the known sequence of testicular development requiring a synergistic effect of SF-1 and SRY on the *SOX9* promoter, expression of SRY or SOX-9 together with WT *SF1* stimulated *TESCO* reporter activity ~4.5- and ~8.5-fold, respectively (Figure 3B).

*SF1* mutations, in the absence of SRY or SOX-9, reduced *TESCO* activation: R103Q reduced activation compared with WT *SF1* (by 65%), as did the R92Q and D293N mutations (by 72% and 39%,

respectively), and G35E drastically decreased *TESCO* activity by 93% (Figure 3B). However, the synergistic effect of SF-1 with either SRY or *SOX9* was not impaired by the novel R103Q mutation or by the 2 previously clinically reported recessive mutations, R92Q and D293N (Figure 3B).

These findings were consistent with the normal histological appearance and inhibin staining of Sertoli cells in the gonads removed from the patient. Furthermore, staining of these gonadal sections with antibodies against SOX-9, the critical protein for male gonadal sexual development (19), demonstrated, for the first time, SOX-9 protein expression in gonadal Sertoli cells of a 46,XY DSD patient (Figure 1F). In contrast to the recessive *SF1* mutations, impairment of *TESCO* activation by G35E (which is known to cause autosomal-dominant DSD; ref. 20) remained even with coexpression of SRY or SOX-9, and similar results were obtained in CHO cells (Figure 3B and Supplemental Figure 3). This qualitative difference between recessive mutations and a severe dominant *SF1* mutation was not



**Figure 3**

Functional studies of the *SF1* R103Q mutant. Transcriptional activation of spleen-specific (A), testes-specific (B), and steroidogenic (C) promoters by WT or mutant *SF1* expression vectors were assayed by transient cotransfection of the expression vectors using the Promega Dual Luciferase assay system. For SF-1 binding elements in the reporters, see Supplemental Table 3. (A) Transcriptional activation of the spleen development-specific *TLX1* promoter by WT and mutant *SF1* constructs was studied in COS-7 cells. Ve, empty vector control. The *TLX1* promoter–luciferase reporter construct is shown below, with the *TLX1* promoter, transcription start site (arrow), exon 1 harboring 2 SF-1 binding sequences (spheres), and the luciferase reporter gene (Luc). Numbering is relative to the *TLX1* transcription start site, at position +1. (B) Activity of the *SOX9* testis-specific TESCO-luciferase enhancer, harboring both SF-1 and SRY/SOX binding sites, was measured in COS-7 cells. Transfections were performed using empty vector control (–) or WT or mutant *SF1* expression vectors, either alone or together with *Sry-myc* or *Sox9* expression vectors (3). A schematic illustration of the reporter construct is shown below. (C) Activity of the steroidogenic *CYP11A1* (left), *CYP17A1* (middle), and *HSD3B2* (right) promoter reporter constructs (27) transfected with the *SF1* constructs into nonsteroidogenic HEK293 cells. Results represent mean ± SEM relative luciferase activity of 4–5 independent experiments performed in duplicate. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs. WT.

distinguishable in a previous study conducted with the human TES (hTES) element, most probably because SRY alone is sufficient for hTES activation (21).

Finally, to understand the XY sex reversal phenotype and the patient’s lack of blood testosterone, we studied the effect of the *SF1* R103Q mutation on transcriptional activation of 3 steroidogenic gene promoters: *CYP11A1*, *CYP17A1*, and *HSD3B2*. Compared with WT *SF1*, R103Q reduced by 3-fold activation of the *CYP17A1* promoter, which lacks SRY/SOX-9 binding sites. Activa-

tion of *CYP11A1* was not significantly affected by the mutation, and *HSD3B2* promoter activity was slightly elevated (1.3-fold; Figure 3C). It therefore appeared that XY sex reversal in this patient was due to a defect in steroidogenesis, rather than impaired *SOX9* activation. This is consistent with the general observation that the degree of hypoandrogenism in *SF1* mutant patients is greater than the degree of testicular dysgenesis, as well as with examples of sex-limited dominant inheritance of *SF1* mutations from unaffected mothers to affected sons (22).



We hypothesize that the different phenotypes observed with *SF1* mutations may be explained by their different effects on *SRY* and *SOX9* activation and testicular steroidogenesis. The recessive *SF1* mutations R92Q and D293N may be similar to R103Q in enabling sufficient activation of *SRY* to thus activate *SOX9* (Figure 1E and Figure 3B), leading to early gonadal and testicular differentiation and inhibin expression (Figure 1, E and F). However, the defect in testosterone synthesis caused by the R103Q mutation was apparently sufficiently severe to lead to undescended testes and phenotypic sex reversal. Severe mutations such as G35E may act earlier in development to curtail *SOX9* activation altogether.

In conclusion, our present study describes a unique clinical phenotype of severe 46,XY-DSD with asplenia, caused by a novel homozygous *SF1* mutation, R103Q. Transactivation studies of *TLX1* demonstrated for the first time that SF-1 is required for spleen development in humans, which shows that *SF1*-DSD and asplenia are not simply coincidental. We also found that *SF1* mutations can lead to sex reversal due to a defect in testosterone synthesis without disrupting the *SF1*-*SRY*-*SOX9* activation cascade. Because asplenia has therapeutic consequences, the splenic status of individuals with DSD harboring *SF1* mutations should be actively examined.

## Methods

Further information can be found in Supplemental Methods.

**Genetic studies.** Standard Sanger sequencing was performed on genomic DNA extracted from the proband and her nuclear family. We sequenced the coding regions of both *SF1* and *STAR* (Supplemental Table 2). The *SF1* mutation identified was tested in ethnically matched controls by PCR and *ApaI* digestion (Supplemental Figure 1).

**Antibody staining: *SOX9* and *inhibin*.** Paraffin-embedded sections of the patient's gonads were stained with mouse anti-SOX-9 and monoclonal

mouse anti-inhibin, as previously described (23), and visualized by fluorescence and DAB staining, respectively.

**Functional analysis of the *SF1* R103Q mutation.** Promoter activity assays were performed by transfecting luciferase reporter vectors controlled by various gene promoters into cells, and used to assay for *SF1* activity as a transcription factor.

**Statistics.** For transfection experiments, 2-tailed paired *t* test was used to compare WT and mutant *SF1* vectors. A *P* value less than 0.05 was considered significant.

**Study approval.** Histological and molecular analysis of the patient's tissues and blood, as well as genetic analysis of the patient's family, were under informed written consent of the subjects themselves and/or the legal guardians thereof. The study was approved by the institutional Helsinki board of Shaare Zedek Medical Center (approval no. 20/10).

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