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

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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. SF1 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, ovarian insufficiency, and adrenal cortical 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.

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 tests, 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 adrenocorticotropic 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
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 SF1 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 SF1 (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 SF1–regulated genes important for spleen development. We therefore searched for SF1 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 SF1 binding sites was not found in the promoters of other spleen-development genes (PBX-1, NKX2-5, NKX3-2, WT-1, and POD-1). TLX1 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 SF1 regulates TLX1, 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 activity 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 SF1 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 SF1 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 SF1 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 SF1 transactivation of the TLX1 promoter provides a mechanism for the observed asplenia and suggests a role for SF1 as a facilitator of normal spleen development in humans.

SF1 plays a critical role in many aspects of gonadal development and testicular differentiation, including steroidogenesis (17). SF1 is thought to induce SRY expression in the early gonad, leading to synergistic activation (by SRY and SF1) 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 SF1 to help maintain its own expression. Failure of this SF1–induced SOX9 activation may lead to sex reversal (3, 18). In the next stage of testicular differentiation, SF1 in Leydig cell progenitors also

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 pro-bond’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 μm (C); 200 μm (D and E); 30 μm (F).
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 inhibit 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
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. Activation 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 SFI mutations may be explained by their different effects on SRY and SOX9 activation and testicular steroidogenesis. The recessive SFI 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 phenotype sex reversal. Severe mutations such as G35E may act earlier in development to curtail SOX9 expression altogether.

In conclusion, our present study describes a unique clinical phenotype of severe 46,XY-DSD with asplenia, caused by a novel homozygous SFI mutation, R103Q. Transactivation studies of TLX1 demonstrated for the first time that SF-1 is required for spleen development in humans, which shows that SFI-DSD and asplenia are not simply coincidental. We also found that SFI mutations can lead to sex reversal due to a defect in testosterone synthesis without disrupting the SFI-SRY-SOX9 activation cascade. Because asplenia has therapeutic consequences, the splenic status of individuals with DSD harboring SFI 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 SFI and STAR (Supplemental Table 2). The SFI mutation identified was tested in ethnically matched controls by PCR and Apal digestion (Supplemental Figure 1).

Antibody staining: SOX9 and inhibin. Paraffin-embedded sections of the patient’s gonads were stained with mouse anti–gonads and monoclonal mouse anti-inhibin, as previously described (23), and visualized by fluorescence and DAB staining, respectively.

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

Statistics. For transfection experiments, 2-tailed paired t test was used to compare WT and mutant SFI 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).

Acknowledgments

This study was supported by the Legacy Heritage Biomedical Program of the Israel Science Foundation (grant 1531/2009; to D. Zangen) and by the US Agency for International Development (USAID) program for Middle East Regional Cooperation (grant TA-MOU-10-M30-021; to E. Levy-Lahad and M. Kanaan). We thank Ryohei Sekido for providing the SRY-MYC, SOX9, and SFI expression vectors as well as the TESCO-luc reporter vector.

Received for publication February 22, 2013, and accepted in revised form February 20, 2014.

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