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

Supplementary Appendix:

Investigators:

David Zangen, Yotam Kaufman, Ehud Banne, Ariella Weinberg-Shukron, Abdulsalam Abulibdeh, Benjamin P. Garfinkel, Dima Dweik, Moein Kanaan, Núria Camats, Christa Flück, Paul Renbaum and Ephrat Levy-Lahad.

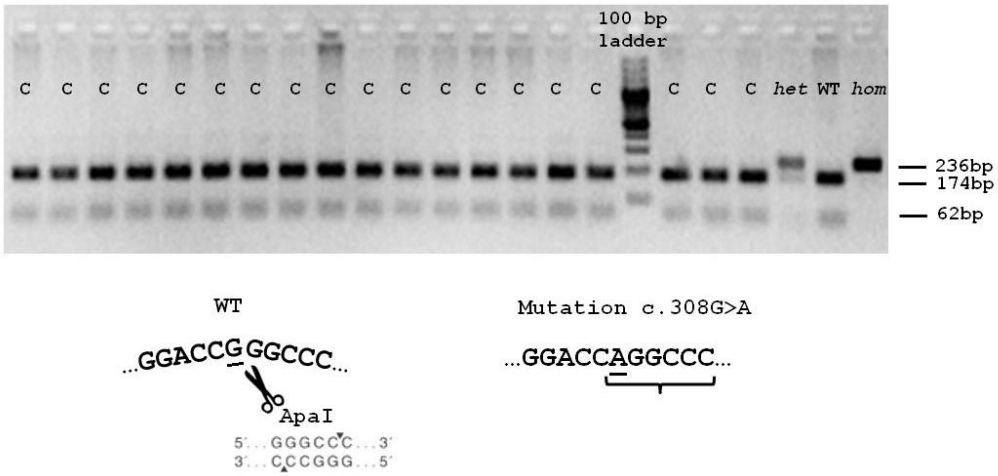
SUPPLEMENTARY METHODS

Antibody staining:

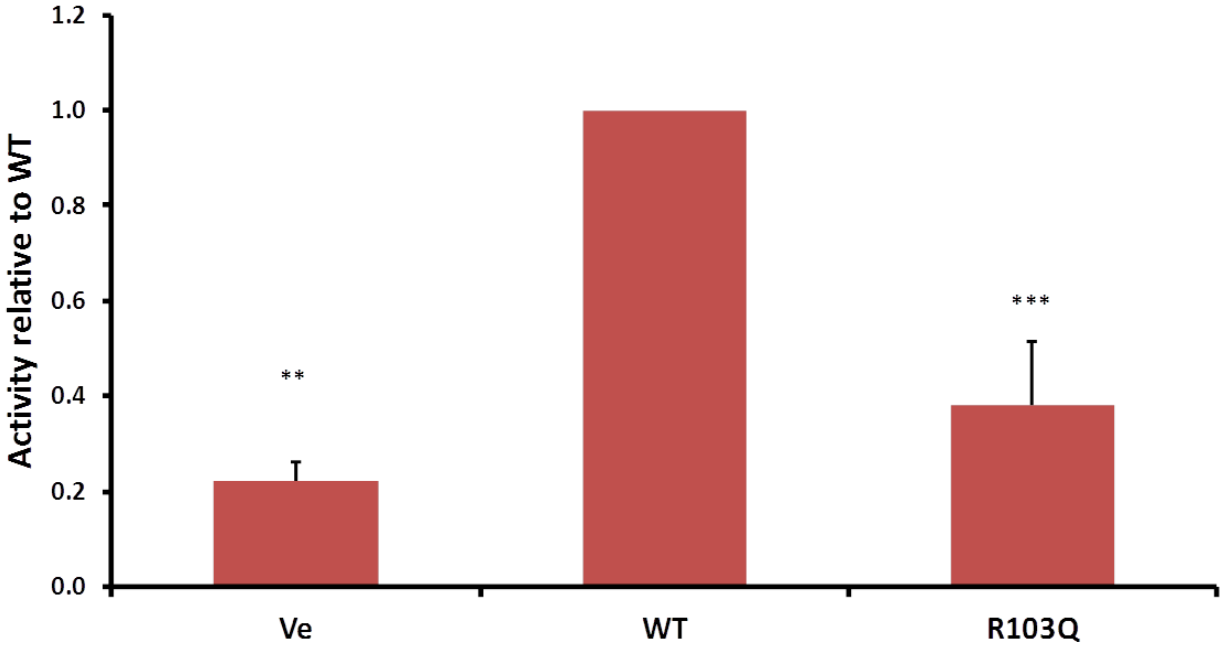
Tissues were fixed with 4% paraformaldehyde (24h/4^{0}C) and embedded in paraffin for sectioning. Hematoxylin-eosin staining was performed according to standard protocol. Inhibin staining using monoclonal mouse anti-inhibin (1:40, AbD Serotech #MCA951S) was performed by using BenchMark ULTRA (Ventana Medical Systems, Inc.). For immunofluorescence, following rehydration and heat mediated antigen retrieval, the sections were incubated overnight at 4°C with monoclonal mouse anti-SOX-9 (1:100, Abcam #76997), diluted in blocking buffer (PBS with 0.05% Tween-20, 0.3% Triton-X100 and 4% normal donkey serum). Slides were next washed twice with PBS-T and incubated for 2h with 1:200 dilution of biotin conjugated secondary antibody (Jackson immunoresearch, West Grove, PA) in blocking buffer. After two washes with PBS the sections were incubated for 40 min with a 1:100 dilution of Cy2-conjugated avidin (Jackson immunoresearch, West Grove, PA) followed by DAPI and mounting with Immuno-mount (Thermo Scientific, Pittsburgh, PA). Fluorescent labeling was examined using an FV-1000 confocal work station (Olympus, Japan), based on an IX81 inverted microscope.

Functional analysis of R103Q mutant *SF-1* protein

Promoter activity assays were performed in non-steroidogenic, human embryonic kidney HEK293, monkey COS-7, and CHO cells. Luciferase reporter vectors – for the genes *CYP17A1* (227*CYP17A1*_Δluc), *CYP11A1*(152*CYP11A1*_pGL3), and *HSD3B2* (301*HSD3B2*_pGL3), empty control vectors (Δluc, pGL3, pcDNA3) and *SF-1* expression vectors were available from previous studies (1-3). The TESCO (testis-specific enhancer of SOX-9)-luc reporter and Sry and Sox-9 expression vectors were kindly provided by Ryohei Sekido (4). The *TLX-1*-luc promoter was generated by PCR and ligated into pGL4.70[hRluc] (Promega). Mutant *SF-1* expression vectors were generated by PCR-based site-directed mutagenesis (Quikchange, Agilent technologies, Inc.). Cells were transiently co-transfected with wild-type (WT) or mutant *SF-1* together with luciferase reporters and/or the SRY and SOX-9 expression vectors using jetPEI® reagent (Polyplus Transfection). Forty-eight hours after transfection, cells were lysed and assayed for firefly and Renilla luciferase activities (Dual-Luciferase® Reporter Assay System, Promega). For the TESCO and steroidal genes, Firefly luciferase was used to measure reporters' activity, with Renilla luciferase used to control for transfection-efficiency; for the *TLX-1* reporter, vice versa.

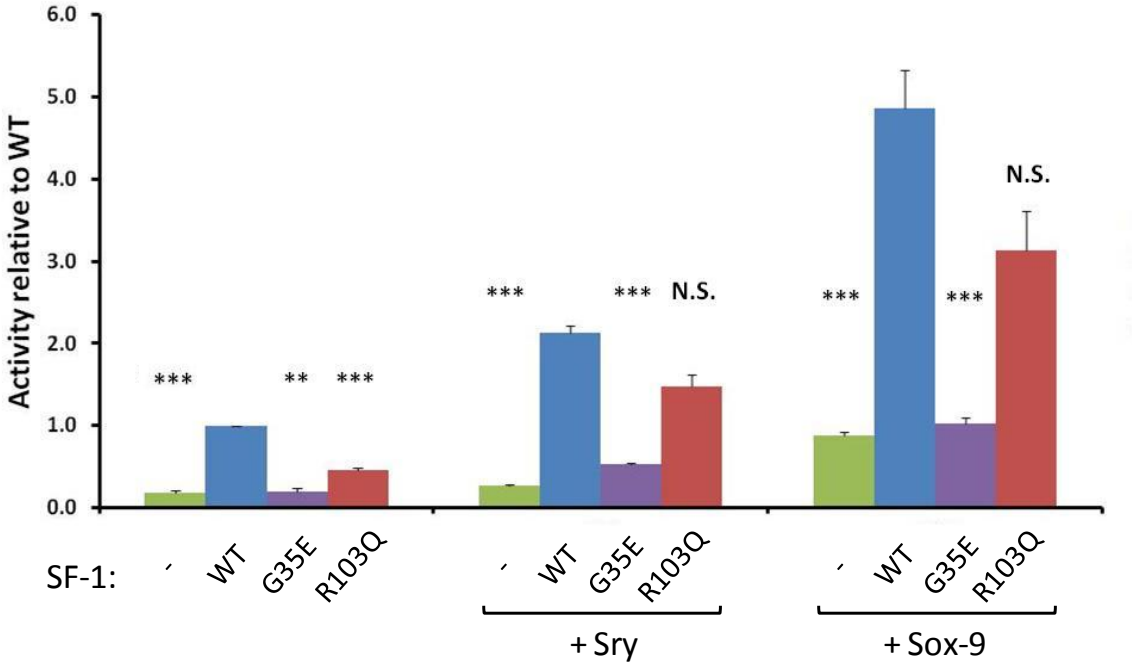


Supplementary Figure 1: Description of restriction assay to test for *SF-1* c.308G>A mutation (R103Q): DNA was isolated by standard procedures from blood taken from ~190 individuals of the same ethnic background as the patient. Using standard PCR, we amplified a 236bp product spanning parts of intron3 and exon4 of the gene, using primers 5`-GTGTTGAGCAGGGGAGAGAG-3` (Forward) and 5`-GCCCTGTCTCCAGCTTGAA-3` (Reverse). Following amplification, the ApaI restriction enzyme (NEB) was used to distinguish products harboring the mutation, which were not cut and remained at 236 bp, from WT products, which were cut into two separate fragments (174 + 62 bp). Following restriction, PCR products were electrophoresed on 3% agarose gel and visualized under UV radiation. The picture shows a sample gel with 19 individuals tested (C), as well as a heterozygote (het), a homozygote (hom) and WT controls for the mutation. The Apa I restriction site is illustrated below the picture, together with the WT and c.308G>A *SF-1* sequences relevant to the assay.



Supplementary Figure 2: *TLX-1* transactivation by SF-1 in CHO cells.

Transcriptional activation of the spleen-development-specific *TLX-1* promoter by the indicated SF-1 WT and mutant constructs was studied in CHO cells by transient co-transfection of SF-1 expression vectors with Renilla-luciferase reporters and measured with the Promega Dual Luciferase assay system. Ve, empty vector. Results were normalized against a Fire-fly luciferase vector (pGL2-basic, Promega) and represent the mean ± SEM relative luciferase activity of 3 independent experiments performed, each in duplicate. **: $p<0.01$; ***: $p<0.001$.



Supplementary Figure 3: TESCO transactivation by SF-1 in CHO cells.

Transcriptional activation of the Sox-9 enhancer TESCO by the indicated *SF-1* WT and mutant constructs (G35E, R103Q), together with Sry-myc or Sox-9 was studied in CHO cells by transient co-transfection of SF-1 expression vectors with Fire-fly reporters and measured with the Promega Dual Luciferase assay system. (-) , empty vector. Results were normalized against a basic Renilla-luciferase vector (pRL-TK, promega) and represent the mean ± SEM relative luciferase activity of 3 independent experiments, each performed in duplicate. **: $p<0.01$; ***: $p<0.001$; N.S.: non-significant.

ACTH stimulation- test		Hormones tested:	DHEAS	Cortisol	Testosterone	17OH-Progesterone	Androstene-dione	Estradiol
Hormone levels following ACTH injection:		Baseline	<0.41 mmol/l	231 nmol/l	<0.9 nmol/l	0.49 nmol/l	<0.3 nmol/l	<70 pmol/l
		60 min.	<0.41 mmol/l	589 nmol/l	<0.9 nmol/l	4.48 nmol/l	0.34 nmol/l	<70 pmol/l
Normal values (after 60 min. of ACTH injection):			0.9-11.7	138-690	0.9-2.6	0.9-7.6	1-11.5	<70 (pre-pubertal)
Steady-state hormones levels:	ACTH	TSH	FT4	PRL	LH	FSH	PRA	ALDO
	12.1 pmol/l	1.72 mu/l	12.9 pmol/l	403 mu/l	7.7 u/l	54.5 u/l	14.66 ng/nl/h	717 pmol/l
Normal values:	1.9-10.2	0.5-4.5	11-24	45-475	0.1-1 (pre-pubertal)	3-14	10-15	27-440

Supplementary Table 1: *SF-1*_R103Q DSD Patient's hormonal profile. Hormone levels in ACTH-stimulation test (above double horizontal line) and steady-state (below double horizontal line) are listed, with normal values below each measurement. Abbreviations: ACTH (Adrenocorticotropic Hormone); TSH (Thyroid Stimulating Hormone); FT4 (Free Thyroxine 4); PRL (Prolactin); LH (Luteinizing Hormone); FSH (Follicle Stimulating Hormone); PRA (Plasma Renin Activity); ALDO (Aldosterone).

Gene	Exon	Forward primer (5`→3`)	Reverse Primer (5`→3`)
StAR	1	CTGCTTCTCTCCCCTCCATC	CAGAATTGGGTGGCCTGAG
	1a	AGGGGCACACAGAGTAGCTT	CAGAGCCTGCTGGAGTTTCT
	2	CACTTCCCTCTCCAAACCAA	AAGCCACATGCACCACATC
	3	CTTTAGTCCGGGCTCTTCAG	TTGCTACCCAGTGACTGCTG
	4-5	ATAGGCGTGAACCACCATGC	CTTGGAGCTGGGGATGCAGTC
	6	AACGTGTTATCTATGGTACTGGTGT	ATCACAGGCTTTGGAGATGG
	7	TGGCAGCCTGTTTGTGATAG	ACTTGCAGGCTTCCAGTAGG
NR5A1	2-3	CAGAGAGGGGATTACGCGAC	CGGTTCTCTTGCAGCGACTG
	4	GTGTTGAGCAGGGGAGAGAG	CAAAGGACAGTCGGGCTAAG
	5	CTTTGATAGCCTAGACATCTG	CAACTCCAGTGTGTTATTTCC
	6	CTGTGCAGATGTTACCCGTG	CCCTAGATCCAGGGATTAGAG
	7	TGTCTTTGATGGTCATAGGGAA	CAAACACACAGTGTCAGAACTCAG

Supplementary Table 2: Primers used to amplify and sequence the *StAR* (OMIM#600617) and *SF-1* (OMIM#184757) genes in the DSD patient and family.

Gene	SF1-Binding sequences	Position relative to start codon	Position relative to TSS
CYP17A1	GGGAGGTCA	-260/-268	-88/-96
	AGGAGGCCT	-209/-201	-37/-29
	TCAAGGTGA	-107/-100	+65/+73
CYP11A1	TCAAGGCCA	-100/-108	+56/+48
HSD3B2	TCAAGGTAA	-334/-326	-234/-226
TLX-1	CCAAGGCC	+143/+136	+381/+374
	CCAAGGCCA	+322/+314	+560/+552
Sox-9 (TESCO)	ACAAGGAAG		
	GCAAGGCCT		
	CCAAGGATC		
	GCAAGGGCA		
	ACAAGGCCT		
	ACAAGGTCA		

Supplementary Table 3: SF-1 binding sites in luciferase reporters.

For each promoter analyzed, *SF-1* binding sites are noted, with positions relative to the Transcription Start Site (TSS) and start codon. Positions for the human sequences *CYP17A1*, *CYP11A1*, *HSD3B2*, and *TLX-1* are according to hg19 in the UCSC human genome browser. Binding sites for *CYP17A1*, *CYP11A1*, *and HSD3B2* were published previously (2, 5-8) For positions of the mouse TESCO sites, see Sekido et al (4). Nucleotides identical to known *SF-1* consensus sequences are underlined (4, 9).

References:

1. Huang, N., and Miller, W.L. 2000. Cloning of factors related to HIV-inducible LBP proteins that regulate steroidogenic factor-1-independent human placental transcription of the cholesterol side-chain cleavage enzyme, P450scc. The Journal of biological chemistry 275:2852-2858.
2. Fluck, C.E., and Miller, W.L. 2004. GATA-4 and GATA-6 modulate tissue-specific transcription of the human gene for P450c17 by direct interaction with Sp1. Molecular endocrinology 18:1144-1157.
3. Camats, N., Pandey, A.V., Fernandez-Cancio, M., Andaluz, P., Janner, M., Toran, N., Moreno, F., Bereket, A., Akcay, T., Garcia-Garcia, E., et al. 2012. Ten Novel Mutations in the NR5A1 Gene Cause Disordered Sex Development in 46,XY and Ovarian Insufficiency in 46,XX Individuals. The Journal of clinical endocrinology and metabolism 97:E1294-1306.
4. Sekido, R., and Lovell-Badge, R. 2008. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature 453:930-934.
5. Hanley, N.A., Rainey, W.E., Wilson, D.I., Ball, S.G., and Parker, K.L. 2001. Expression profiles of SF-1, DAX1, and CYP17 in the human fetal adrenal gland: potential interactions in gene regulation. Mol Endocrinol 15:57-68.
6. Sewer, M.B., Nguyen, V.Q., Huang, C.J., Tucker, P.W., Kagawa, N ,.and Waterman, M.R. 2002. Transcriptional activation of human CYP17 in H295R adrenocortical cells depends on complex formation among p54(nrb)/NonO, protein-associated splicing factor, and SF-1, a complex that also participates in repression of transcription. Endocrinology 143:1280-1290.
7. Morohashi, K., Honda, S., Inomata, Y., Handa, H., and Omura, T. 1992. A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. J Biol Chem 267:17913-17919.
8. Leers-Sucheta, S., Morohashi, K., Mason, J.I., and Melner, M.H. 1997. Synergistic activation of the human type II 3beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase promoter by the transcription factor steroidogenic factor-1/adrenal 4-binding protein and phorbol ester. J Biol Chem 272:7960-7967.
9. Zhang, P., and Mellon, S.H. 1997. Multiple orphan nuclear receptors converge to regulate rat P450c17 gene transcription: novel mechanisms for orphan nuclear receptor action. Mol Endocrinol 11:891-904.