Mutant NR5A1/SF-1 in patients with disorders of sex development shows defective activation of the *SOX9* TESCO enhancer

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Abstract

Nuclear receptor subfamily 5 group A member 1/Steroidogenic factor 1 (*NR5A1*; *SF-1*; *Ad4BP*) mutations cause 46,XY disorders of sex development (DSD), with phenotypes ranging from developmentally mild (e.g. hypospadias) to severe (e.g. complete gonadal dysgenesis). The molecular mechanism underlying this spectrum is unclear. During sex determination, SF-1 regulates *SOX9* [*SRY* (sex determining region Y)-box 9] expression. We hypothesized that *SF-1* mutations in 46,XY DSD patients affect *SOX9* expression via the Testis-specific Enhancer of *Sox9* core element, TESCO. Our objective was to assess the ability of twenty *SF-1* mutants found in 46,XY DSD patients to activate TESCO. Patient DNA was sequenced for *SF-1* mutations and mutant SF-1 proteins were examined for transcriptional activity, protein expression, sub-cellular localization and *in silico* structural defects. Fifteen of the twenty mutants showed reduced SF-1 activation on TESCO, 11 with atypical sub-cellular localization. Fourteen SF-1 mutants were predicted *in silico* to alter DNA, ligand or cofactor interactions. Our study may implicate aberrant SF-1-mediated transcriptional regulation of *SOX9* in 46,XY DSDs.

Introduction

Nuclear receptor subfamily 5, group A, member 1 [NR5A1 (MIM# 184757), also known as Steroidogenic factor 1 (SF-1) or Adrenal 4 binding protein (Ad4BP)] is an orphan nuclear hormone receptor that plays key roles in regulating adrenal and reproductive development and function (Ferraz-de-Souza, Lin, & Achermann, 2011). As a transcription factor, it binds directly to DNA in a sequence-specific manner and recruits cofactors and/or ligands, enabling transcriptional activation or repression of downstream target genes. *SF-1* is expressed in the adrenal glands and in the bipotential gonads of the developing embryo (Hanley et al., 1999; Ramayya et al., 1997). During male sex determination (embryonic day 10.5 in mice and 6 weeks in human), *SF-1* is expressed in the Sertoli cells of the testis (Sekido & Lovell-Badge, 2008) and later in the Leydig cells where it directly controls expression of a number of steroidogenic target genes (Lala, Rice, & Parker, 1992; Morohashi, Honda, Inomata, Handa, & Omura, 1992; Rice, Mouw, Bogerd, & Parker, 1991).

Homozygous deletion of *Sf-1* in mice leads to complete adrenal and gonadal agenesis, and reproductive phenotypes include female external genitalia and persistent Müllerian structures in XY mice (Luo, Ikeda, & Parker, 1994; Sadovsky et al., 1995). *SF-1* mutations described in humans to date are predominantly heterozygous and can lead to adrenal failure, 46,XX primary ovarian insufficiency (POI) or 46,XY disorders of sex development (DSD) (Ferraz-de-Souza et al., 2011). A wide phenotypic spectrum exists among 46,XY DSD patients with *SF-1* mutations, ranging from developmentally mild forms such as hypospadias and cryptorchidism, to more pronounced developmental disruptions such as clitoromegaly, micropenis, bilateral anorchia, complete gonadal dysgenesis and female genitalia (Achermann, Ito, Hindmarsh, & Jameson, 1999; Achermann et al., 2002; Allali et al., 2011; Correa et al., 2004; Kohler et al., 2008; Lin et al., 2007; Philibert et al., 2007; Wada, Okada, Fukami, Sasagawa,

& Ogata, 2006; Wada, Okada, Hasegawa, & Ogata, 2005). A link between *SF-1* mutations and the occurrence of male infertility was found recently (Bashamboo et al., 2010).

The precise mechanism of SF-1 action that fails in DSDs is not fully understood, nor has an explanation for the wide-ranging phenotypes associated with *SF-1* mutations been determined. Attempts to explain this phenotypic variability have focused on analyzing defective SF-1 function at target promoters of either steroidogenic genes such as *CYP11A1*, *CYP17A1* and *CYP19A1*, or sex differentiation genes such as *AMH* and *INSL3* [see Supplementary Data for full gene names; (Allali et al., 2011; Camats et al., 2012; Fabbri-Scallet et al., 2018; Ito, Achermann, & Jameson, 2000; Kohler et al., 2008; Lin et al., 2007; Philibert et al., 2007; WuQiang et al., 2003)]. However, no correlation between SF-1 function and patient phenotype has been identified.

The role of SF-1 in sex determination involves activation of *SRY* (Sex determining region Y)-box 9 (*Sox9*), a gene essential for testicular development. Mutations in and around *SOX9* result in campomelic dysplasia, a skeletal malformation syndrome with associated DSD in 75% of XY cases (Foster, 1996). SF-1 initiates *Sox9* expression by directly binding and activating a testis-specific enhancer called TES, the core element of which is termed TESCO (Knower et al., 2011; Sekido & Lovell-Badge, 2008). *Sox9* expression is further upregulated and maintained through synergistic activation of TES by SF-1-SRY and SF-1-SOX9 respectively (Sekido & Lovell-Badge, 2008). The importance of the TESCO enhancer in regulating SOX9 levels has been demonstrated through deletion of TESCO in mice, which resulted in reduced expression of *Sox9* as well as its target *Amh* (Gonen, Quinn, O'Neill, Koopman, & Lovell-Badge, 2017). DSD-associated mutations in *SF-1*, *SRY* and *SOX9* cause defective activation of TES *in vitro* (Knower et al., 2011), highlighting the possibility that TES function could be implicated in 46,XY DSDs. In addition, variable *SOX9* expression have been

associated with variable DSD phenotypes in familial *SRY* mutations where mutant SRY activity is at a threshold of SOX9 activation likely affected by genetic background differences (ie. modifiers) between the fertile father and his affected XY DSD daughter (Knower et al., 2011). This led us to hypothesize that defective TESCO activation in 46,XY DSD patients bearing *SF-1* mutations accounts for the high variability of phenotypes observed.

An *in vitro* assay of the sex determining activity of SF-1 was developed and twenty SF-1 clinical mutants identified in 46,XY DSD patients were analyzed. Fifteen SF-1 mutants showed markedly reduced TESCO activation, of which eleven showed nuclear localization defects. *In silico* analysis predicted SF-1 structural defects in 14 cases, possibly affecting DNA, ligand or cofactor interactions. We conclude that a possible sex determining function of human SF-1 is the activation of *SOX9* transcription.

Materials and methods

Patient information

DNA was obtained from 46,XY DSD patients and sequenced for *NR5A1*/*SF-1* mutations after institutional review committee approval and with informed patient consent. Nucleotide variations described are based on the Genbank NM_004959.4 cDNA reference sequence with the A of the ATG translation initiation site regarded as nucleotide 1.

In vitro luciferase assays to assess wild-type and mutant SF-1 activity

To test for wild-type and mutant SF-1 activation of TESCO, *in vitro* luciferase assays were performed in a human embryonic kidney carcinoma cell line (HEK293T; see Supplementary Data).

Western blot analysis of wild-type and mutant SF-1 protein expression

Expression of wild-type and mutant SF-1 proteins in HEK293T cells was assessed by Western blot using an anti-SF-1 antibody (see Supplementary Data).

Immunofluorescence

Immunofluorescence using an anti-SF-1 antibody was performed to determine the sub-cellular localization of wild-type and mutant SF-1 proteins in HEK293T cells (see Supplementary Data).

In silico protein structural analysis

The positions of the substituted amino acids were visualized using PyMOL (DeLano, 2010). For DNA binding domain (DBD) mutants, the 3-dimensional (3D) nuclear magnetic resonance (NMR) structure of mouse SF-1 DBD (100% homology to human SF-1) bound to the inhibin α-subunit promoter [Protein Data Bank no. PDB 2FF0; (Little et al., 2006)] was used. The X-ray crystal structure of human SF-1 ligand binding domain (LBD) bound to a putative phospholipid ligand (PL) and a cofactor peptide [nuclear receptor coactivator 2 (NCOA2), PDB 1ZDT; (Wang et al., 2005)] was used to visualize amino acid substitutions in the LBD.

Results

Classification of patient phenotypes and SF-1 mutations

The clinical phenotypes of the twenty 46,XY DSD patients and their *SF-1* mutations are summarized in Table 1. Patient phenotypes ranged from 'developmentally mild' (hypospadias, cryptorchidism and suspected male infertility) to 'developmentally severe' forms (micropenis, clitoromegaly,

persistent Müllerian structures, reduced germ cell number, female external genitalia and complete gonadal dysgenesis). Two of the twenty patients analyzed, bearing *NR5A1/SF-1* mutations c.493G>C (p.G165R) and c.23A>G (p.D8G), were identified in this study. These variants have been submitted to the Leiden Open Variation Database (LOVD; http://www.lovd.nl/3.0/home; c.493G>C variant ID: 0000369707; c.23A>G variant ID: 0000369708). The former case of Indian ancestry had oligozoospermia; the latter case of Moroccan ancestry is a 46,XY female with complete gonadal dysgenesis. In both cases, parental DNA was unavailable for study. Sequencing of genes known to cause non-syndromic forms of 46,XY DSD, including *SRY*, *GATA4*, *DMRT1*, *FOG2* and *MAMLD1*, revealed no pathogenic mutations. These two variants were not observed in >100 control samples from ancestry-matched populations. Of the remaining variants, 17 were not found in 100 - 358 control individuals (Supp. Table S1). p.G146A is a common variant with an allele frequency of 0.1183 [Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org/)].

Sixteen patients have heterozygous missense mutations in *SF-1* (p.D8G, p.V15M, p.C33S, p.G35E, p.R39P, p.M78I, p.R84H, p.G91S, p.G146A, p.G165R, p.R281P, p.R313C, p.V355M, p.L361P, p.D380Y, p.L437Q; Table 1). Three patients have heterozygous nonsense *SF-1* mutations (p.Y138X, p.Q316X and p.Y404X), while one patient has a homozygous missense *SF-1* mutation (p.R92Q) inherited from unaffected consanguineous parents carrying heterozygous mutations. While the homozygous p.R92Q mutation is associated with a severe phenotype, this mutation has been categorized as 'mild' in our study since the mutation in a heterozygous state is associated with a developmentally normal phenotype.

The human SF-1 protein spans 461 amino acids and the position of the substituted amino acid or nonsense mutation in the 20 patients is shown in Figure 1. Typical of nuclear receptors, the SF-1

protein contains an amino terminal core DBD with a primary proximal box (P-box), an accessory DBD (Ftz-F1 box/A-box) and a carboxy terminal LBD (Figure 1). The DBD and LBD flank a hinge region. The LBD and its carboxy terminal activation function 2 (AF2) domain are sites of interaction for cofactors (Ito, Yu, & Jameson, 1998; Park et al., 2007) and putative phospholipid ligands (Krylova et al., 2005; Li et al., 2005; Wang et al., 2005). The clinical mutations occur in all domains of the SF-1 protein (Figure 1). Many of the mutations categorized as developmentally severe map to key functional domains of the SF-1 protein, namely the DBD and LBD (Figure 1).

SF-1 mutants show impaired activation of TESCO

The TESCO luciferase assay is an accepted proxy measure of *SOX9* regulation, as it was recently shown in knock out experiments that TESCO is responsible for 50% of *Sox9* expression in the developing testis (Gonen et al., 2017). Therefore, human wild-type SF-1 activity on TESCO was assessed as a measure of sex determining activity. HEK293T cells were used as they lack endogenous SF-1 expression. Relative to SF-1 alone, addition of human SRY led to a 6-fold increase in TESCO activation, while addition of human SOX9 increased TESCO activation by 9-fold (Figure 2A), consistent with results previously described for mouse SF-1, SRY and SOX9 (Sekido & Lovell-Badge, 2008).

Protein expression of wild-type and mutant SF-1 in HEK293T cells was analyzed by Western blot and densitometry (Supp. Figure S1). All SF-1 missense mutant proteins with the exception of p.G91S were expressed at levels comparable to wild type SF-1 and migrated according to their predicted molecular weight of 53 kDa. Truncated protein p.Y138X was weakly expressed, while p.Q316X and p.Y404X were expressed but showed additional degradation products. Expression levels of the

p.G91S and p.Y138X mutants were significantly reduced compared to WT SF-1 (Supp. Figure S1B), indicating decreased protein stability. Additional artifactual bands specific to each vector type were observed (~60 kDa for pCMX-based mutants and ~45 kDa for pcDNA3-based mutants).

The ability of the 20 SF-1 mutants to activate TESCO in HEK293T cells was determined. SF-1 from five patients (p.R92Q, p.G146A, p.G165R, p.V355M and p.D8G) showed wild-type SF-1 activity (Figure 2B). Fifteen of the twenty SF-1 clinical mutants showed markedly reduced ability to activate TESCO *in vitro*, compared to wild-type SF-1 (Figure 2B). When tested with SRY (Figure 2C) or SOX9 (Figure 2D), the same fifteen mutants also showed reduced activation of TESCO.

Sub-cellular localization of mutant SF-1 proteins

The function of SF-1 as a transcriptional activator is dependent upon correct nuclear localization of the translated protein. To examine the sub-cellular localization of mutant SF-1, we performed immunofluorescence using an anti-SF-1 antibody on HEK293T cells transfected with either wild-type or mutant SF-1 (Figure 3; Supp. Table S1). The SF-1 antibody was specific to an epitope at the N-terminal, permitting detection of all clinical variants including the three nonsense mutants. Wild-type SF-1 was localized almost exclusively in the nucleus (~2% cells showing atypical localization), consistent with its function as a transcription factor. Nine of the 20 mutants (p.R92Q, p.G146A, p.G165R, p.R281P, p.R313C, p.V355M, p.L437Q, p.D8G and p.G35E) showed predominant and uniform protein localization within the nucleus (0.8-5% cells with atypical localization), similar to the pattern observed for wild-type SF-1. Eleven mutants (p.C33S, p.R39P, p.G91S, p.R84H, p.Y404X, p.Y138X, p.V15M, p.M78I, p.L361P, p.D380Y and p.Q316X) showed atypical sub-cellular localization (19.1-93.6% cells with atypical localization). The atypical staining patterns included SF-1-

immunoreactive aggregates within sub-nuclear foci (p.C33S, p.R39P, p.G91S, p.V15M and p.M78I) and cytoplasmic retention of the protein (p.R84H, p.Y404X and p.L361P). The p.Y138X mutant showed a weak SF-1 expression pattern. Mutants p.D380Y and p.Q316X showed intense staining within large foci on the outer periphery of nuclei which appeared to be pyknotic. For mutant p.Q316X, some cells also showed protein localization in aggregates within the nucleus.

In silico protein structure analysis

To predict the possible structural and functional effects of SF-1 mutations, we inspected the position of the substituted amino acids of the SF-1 clinical mutants within the 3D NMR and X-ray structures of wild-type SF-1 DBD and LBD domains respectively (Krylova et al., 2005; Li et al., 2005; Wang et al., 2005). Amino acid substitutions not previously analyzed *in silico* are shown in Figure 4. 3D models for eight SF-1 mutants have been published elsewhere and their interpretations are summarized in Supp. Table S1. Mutants p.G146A, p.G165R and p.D8G could not be analyzed on a structural basis as there is currently no crystal structure of full length SF-1 protein solved.

The core DBD of SF-1, comprising a short β-hairpin and two α-helices, spans from amino acids 10 – 85 and contains two Cys4-zinc-finger motifs within its P-box (Figure 4A; Figure 1). The first helix within the first zinc finger binds to the major groove of target DNA, facilitating sequence-specific DNA binding (Little et al., 2006; Ueda & Hirose, 1991). The SF-1 DNA binding site comprises an 8 bp consensus sequence 5'-YCAAGGYY-3' in target gene regulatory regions (Morohashi et al., 1992). The TESCO enhancer of *Sox9* contains six consensus SF-1 binding sites postulated to be critical for mediating SF-1 activation of *SOX9* expression (Sekido & Lovell-Badge, 2008). The A-box contains a nuclear localization signal (NLS; amino acids 89 - 101) and binds to the minor groove of target DNA,

stabilizing SF-1-DNA binding (Ito et al., 2000; Little et al., 2006; Ueda, Sun, Murata, & Hirose, 1992). The SF-1 LBD comprises 12 α-helices and two β-sheets (Figure 4B). Its AF2 domain facilitates cofactor recruitment, thereby mediating SF-1 transcriptional activity [Figure 1; (Ito et al., 1998; Park et al., 2007)]. Phospholipid ligands also bind to the LBD and modulate SF-1 activity *in vitro* (Krylova et al., 2005; Li et al., 2005; Wang et al., 2005).

Glycine 91 lies within a highly conserved RGGR motif in the A-box that may be required for DNA interaction. Glycine 91 interacts with the ribose ring of guanine 7 within the SF-1-DNA binding site of 5'- $G_1G_2C_3T_4C_5A_6G_7G_8G_9C_{10}C_{11}A_{12}C_{13}A_{14}G_{15}$ -3' [Figure 4A; (Little et al., 2006)]. A substitution of glycine to a larger, hydrophilic serine residue (p.G91S, severe phenotype, 3% TESCO activation) is predicted to disrupt DNA binding. Furthermore, this substitution lies within the NLS, which correlates with the disrupted nuclear localization pattern observed (Figure 3). Cysteine 33 contacts the zinc ion in the first Cys4-zinc-finger motif (Figure 4A). The cysteine to serine substitution (p.C33S, severe phenotype, 0% TESCO activation) is predicted to weaken interactions with zinc, thereby affecting DNA binding. Arginine 84 projects away from the DNA and into the solvent (Figure 4A). Hence, a substitution to histidine (p.R84H, severe phenotype, 20% TESCO activation), another positively-charged residue, is not expected to have a major effect on DNA binding, but may disrupt interaction with cofactors required for transcriptional activation. Arginine 39 lies within the first helix of the core DBD and interacts with guanine 20 of the SF-1-DNA binding site of 3'- $C_{30}C_{29}G_{28}A_{27}G_{26}T_{25}C_{24}C_{23}C_{22}G_{21}G_{20}T_{19}G_{18}T_{17}C_{16}$ -5' [Figure 4A; (Little et al., 2006)]. A substitution of arginine to proline at this location (p.R39P, severe phenotype; 0% TESCO activation) is predicted to disrupt DNA binding as prolines induce sharp bends in protein helices.

Arginine 313 lies at the bottom of the ligand binding pocket (Figure 4B). A substitution to cysteine (p.R313C, mild phenotype, 0% TESCO activation) is predicted to affect ligand binding. Leucine 437 lies within the ligand binding pocket close to the putative ligand (Figure 4B). A substitution of the hydrophobic leucine residue to a hydrophilic glutamine (p.L437Q, severe phenotype, 0% TESCO activation) is expected to impair interactions with the ligand. An arginine 281 (Figure 4B) to proline substitution (p.R281P, mild phenotype, 0% TESCO activation) is predicted to break helix 3, affecting activity of the nearby AF2 domain. Leucine 361 substitution for proline (p.L361P, severe phenotype, 0% TESCO activation) is expected to induce a bend at the end of helix 7 (Figure 4B), possibly affecting helix alignment. However, the large distance of leucine 361 from the ligand binding pocket and AF2 domain indicate that it may not be required for ligand and/or cofactor binding. Similarly, aspartic acid 380 (Figure 4B) faces away from both the ligand binding pocket and AF2 domain. Therefore, a substitution to tyrosine (p.D380Y, severe phenotype, 0% TESCO activation) is expected to have a minor effect on protein activity.

Fourteen cases that had missense SF-1 mutations and for which 3D protein structural information was available were subjected to PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) analyses (Supp. Table S1). The mutations were predicted to be damaging in 12/14 cases. In 9/14 cases, analyses of protein function using PolyPhen-2 and SIFT correlated with the *in silico* 3D protein structural analysis.

Discussion

In this study, we examined whether the transcriptional activity of clinical SF-1 mutants on the *SOX9* testis-specific enhancer TESCO could account for the spectrum of developmental features of SF-1-

associated DSD. We analyzed 20 SF-1 mutations from 46,XY DSD patients located across all functional domains of SF-1, the majority of which are missense. We found that 15 out of the 20 SF-1 mutants showed defective activation of TESCO both alone and synergistically with SRY and SOX9. A failure of initiation of *SOX9* expression by SF-1 may underlie the defective synergistic activation. SF-1 sex determining activity is poorly understood at the phenotypic level. This study has shed light on the consequences of *SF-1* mutations in DSDs, and how they relate to phenotypic features and clinical severity.

A key property of a transcription factor is that it is localized to the nucleus, its site of action. Disruption to the nuclear localization signals of the SRY and SOX9 proteins has been proposed as an explanation for reduced transcriptional activity and clinical phenotype in DSDs. Analysis of the effects of *SF-1* mutations on the sub-cellular localization of SF-1 protein revealed atypical localization in eleven cases. Five mutants localized in aggregates within the nucleus. This observation is novel for mutant p.R39P and previously described for mutants p.C33S (Kohler et al., 2008), p.V15M (Lin et al., 2007) and p.M78I (Lin et al., 2007). While mutant p.G91S was previously described as having a wild-type pattern of nuclear localization (Lin et al., 2007), we observed localization within subnuclear aggregates. Mutants p.L361P and p.R84H had cytoplasmic retention of the protein in some cells, in contrast to a previous report showing no effect of p.R84H on nuclear localization (Kohler et al., 2008). Cytoplasmic retention of the p.Y404X mutant is likely caused by protein instability due to the truncation as revealed by Western blot. Similarly, the instability of the truncated p.Y138X protein may explain the weak SF-1 immunostaining. For mutants, p.D380Y and p.Q316X, pyknotic, irregularly-shaped nuclei were observed in cells with large extranuclear foci, suggesting apoptosis. The large foci could be a result of protein aggregates being expunged out of the nuclei during

apoptosis. Similarly, apoptotic cells with pyknotic nuclei and chromatin condensation occur in the gonadal primordium of E12.0 SF-1 null mouse embryos, leading to gonadal regression (Luo et al., 1994). Thus, certain human *SF-1* mutations might induce cell death, possibly resulting in gonadal regression.

In 16 out of 20 mutants, sub-cellular localization correlated with TESCO activation: mutants p.R92Q, p.G146A, p.G165R, p.V355M and p.D8G showed normal nuclear localization and normal TESCO activation, while mutants p.C33S, p.R39P, p.R84H, p.G91S, p.Y138X, p.Y404X, p.V15M, p.M78I, p.Q316X, p.L361P and p.D380Y had atypical sub-cellular localization and reduced TESCO activation (Figure 5, Supp. Table S1). The latter observations suggest that nuclear localization of SF-1 is critical for proper SF-1 function. The TESCO assay and sub-cellular localization data therefore largely complement each other. Despite the normal nuclear localization of mutants p.R281P, p.R313C, p.L437Q and p.G35E, reduced TESCO activation was observed. In these cases, impaired transactivation may have been due to defects in SF-1 ligand (p.R313C, p.L437Q) or cofactor interactions (p.R281P), as predicted *in silico*, or loss of DNA binding (p.G35E) as previously reported (Achermann et al., 1999).

The *in silico* analysis was performed to inspect the positions of the substituted amino acids on SF-1 protein structure and assess their likely importance, as well as to speculate upon the possible structural and functional consequences of the SF-1 mutations. Inspection of the 3D structures of the SF-1 DBD and LBD in this (Figure 4) and previous studies (Achermann et al., 1999; Achermann et al., 2002; Lin et al., 2007; Philibert et al., 2007) predicted that 14 out of 17 *SF-1* mutations substitute amino acids in regions critical for DNA or ligand/cofactor interactions (Figure 5, Supp. Table S1). Of these, 12 SF-1 mutants showed reduced TESCO activation. Therefore, apart from atypical sub-

cellular localization, defective SF-1-DNA binding or ligand/cofactor interactions may underlie impaired TESCO activation. In five cases (p.R92Q, p.V355M, p.R84H, p.L361P, p.D380Y), *in silico* structural analysis did not reflect levels of TESCO activation (Figure 5, Supp. Table S1). In all these cases, sub-cellular localization correlated with TESCO activation.

SF-1 nuclear localization and SF-1 activation of TESCO correlate with the presence of testes in 13 out of 17 cases (in 2 out of 3 cases where the testes were normal, and in 11 out of 14 cases where testes were abnormal), a moderately strong correlation (Supp. Table S1). In contrast, TESCO activation and nuclear localization of SF-1 correlate with the absence of Müllerian structures in only 7 out of 19 cases (in 4 out of 14 cases where Müllerian ducts were absent and 3 out of 5 cases where Müllerian ducts were present). For the remaining cases, information regarding Müllerian structures and gonadal histology was unavailable. Overall, TESCO activation correlated with phenotypic severity for 17 out of the 20 SF-1 mutants in our study (Figure 5). However, a definitive genotype-phenotype correlation cannot be drawn from this study due to the lack of gonadal histological information for some of the patients. This highlights a limitation of our study wherein the lack of gonadal histology limits our assessment of phenotype. Ideally, it would be best to undertake SOX9 immunostaining on gonads of DSD patients. This would more directly address SF-1 activation of endogenous *SOX9* expression and shed light upon the legitimacy of the use of TESCO as a proxy marker of *SOX9* expression. Recent studies have suggested that the large variation in phenotypes in SF-1-associated DSDs may be attributed to digenic inheritance since rare variants were identified in additional DSD genes in several patients with SF-1 mutations (Mazen et al., 2016; Robevska et al., 2018).

The p.R281P and p.R313C mutants were associated with mild phenotypes despite being unable to activate TESCO. It is possible that these mutants may not have abolished activation of other SF-1

targets that are required for male reproductive development. Variable effects of mutant SF-1 activation of different targets have been observed in 46,XY DSD. For example, the SF-1 p.R103Q homozygous mutant in an XY patient with female external genitalia, normal and abundant Sertoli cells, few Leydig cells and absence of germinal or pregerminal cells, showed defective activation of the *CYP17A1* promoter but normal activation of the TESCO enhancer (Zangen et al., 2014). The sex reversal in this patient was therefore due to a defect in steroidogenesis, rather than impaired *SOX9* expression. It is therefore important to note that not all cases of SF-1 associated 46,XY DSD display aberrant SOX9 expression levels, particularly in cases where SF-1 function on steroidogenic promoters have been compromised. While TESCO activation is a proxy measure of *SOX9* transcription, it may not be representative of *SOX9* transcription in all cases due to the contribution of other unidentified *SOX9* enhancers, such as in the RevSex region (Benko et al., 2011) and XYSR region (Kim et al., 2015). Conversely, the p.D8G mutant was associated with a severe clinical phenotype despite having normal TESCO activation. Since the p.D8G substitution lies outside the SF-1 DBD, a mutation in that region may not affect binding to TESCO. The SF-1-p.D8G mutation could be a rare polymorphism and may not be causative for the phenotype. We have ruled out the possibility that mutant SF-1-p.D8G protein may be suppressing wild-type SF-1 activity through a dominant negative effect (data not shown). The p.G165R mutant identified in this study occurs at a low frequency in the general population and did not affect TESCO activation and SF-1 nuclear localization. While this mutant could possibly be linked to the observed mild oligozoospermia phenotype, further studies such as knock-in of the mutation in mice and subsequent analysis of spermatogenesis and fertility need to be performed.

Analysis of this large cohort of SF-1 mutants allowed us to identify protein domains important for the sex determining functions of SF-1. All six DBD mutants and seven out of eight LBD mutants severely reduced TESCO activation. Our data highlights the importance of the LBD and suggests that ligand binding and/or cofactor recruitment mediate SF-1 transcriptional activity during sex development. In contrast, a recent study on steroidogenic promoters showed that activity of 46,XY DSD SF-1 DBD mutants was severely reduced, while LBD mutants showed variable activity (Camats et al., 2012). Testing the ability of SF-1 LBD mutants to bind to a putative phospholipid ligand (Krylova et al., 2005; Li et al., 2005; Wang et al., 2005) in the context of TESCO activation may help determine whether SF-1 is a true orphan nuclear receptor. In addition, only a few SF-1 cofactors involved in sex determination (e.g. DAX1 and GATA4) have been identified (Ludbrook et al., 2012; Tremblay & Viger, 1999). Future studies utilizing the TESCO assay may uncover more SF-1 co-activators and co-repressors involved in its sex determining role and possibly associated with DSD.

In summary, the TESCO activation assay provided in conjunction with an analysis of SF-1 subcellular localization is an effective means of assessing SF-1 sex determining function. The data presented here supports the hypothesis that the conserved TESCO element plays an important role in human sex determination despite the absence of an *in vivo* mutation in TESCO (Georg et al., 2010).

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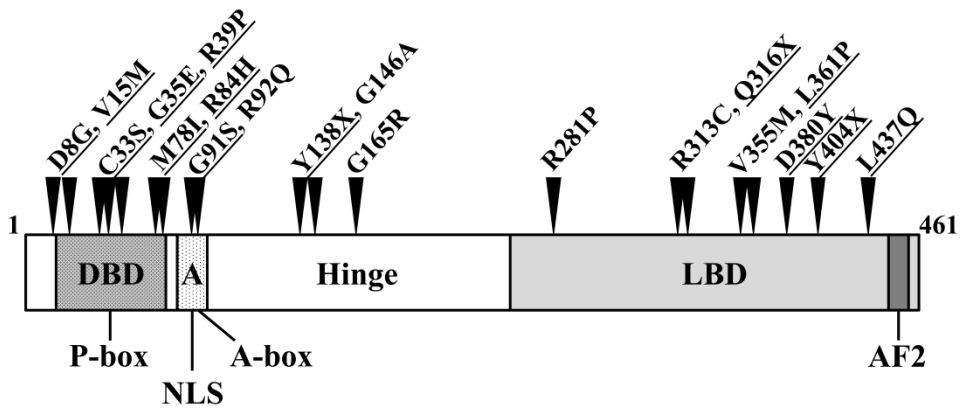
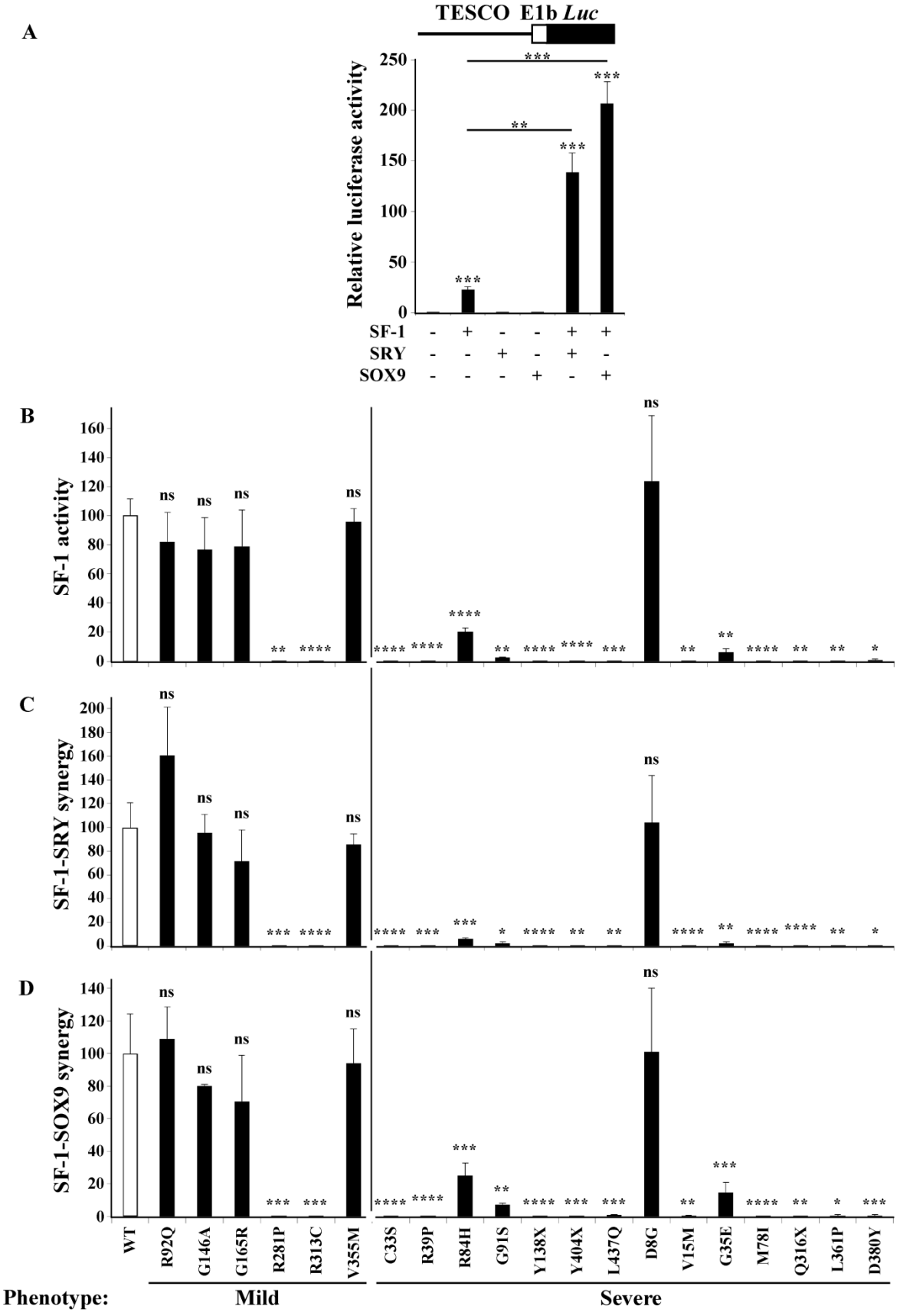
Figure legends

FIGURE 1: Structure of wild-type SF-1 protein showing location of 20 mutants found in 46,XY DSD patients. Each amino acid substitution is coded according to severity of developmental phenotype (black for developmentally mild phenotypes with under-masculinized male genitalia; underlined for severe phenotypes with ambiguous or female genitalia). Numbers represent the first and last amino acid of the SF-1 protein. Functional domains of SF-1 are labelled as follows: DBD: DNA binding domain, P-box: proximal box, NLS: nuclear localization signal, A-box: accessory box, Hinge: hinge domain, LBD: ligand binding domain, AF2: activation function 2



Sreenivasan *et al.*, Fig. 1

FIGURE 2: Transcriptional activation of TESCO by wild-type and mutant SF-1. (A) *In vitro* activation of TESCO in HEK293T cells by wild-type SF-1, SRY and SOX9 and by the synergistic activation of SF-1 with SRY and SF-1 with SOX9. (B) Activity of mutant SF-1 on TESCO in HEK293T cells. Cells were co-transfected with TESCO-E1b-*Luc* and WT or mutant SF-1 to assess for activation of TESCO via SF-1 alone; (C) Co-transfection of TESCO-E1b-*Luc* with WT or mutant SF-1 and SRY, to test for activation of TESCO via SF-1-SRY synergy; (D) Co-transfection of TESCO-E1b-*Luc* with WT or mutant SF-1 and SOX9, to test for activation of TESCO via SF-1-SOX9 synergy. The mean percentages of relative luciferase activity relative to WT levels from three to four independent assays (each performed in triplicate) are shown. Error bars represent standard error of the mean (SEM). Mutants are grouped according to their clinical phenotype (developmentally mild or severe). WT: wild-type SF-1. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)



Sreenivasan *et al.*, Fig. 2

FIGURE 3: Analysis of the sub-cellular localization of 20 SF-1 mutant proteins. Immunofluorescence with an anti-SF-1 antibody was performed on HEK293T cells transfected with WT or mutant SF-1. DNA was labelled with 4,6-diamidino-2-phenylindole (DAPI). Scale bar represents: 5 µm. Mutants are grouped according to their clinical phenotype (developmentally mild or severe). WT: wild-type SF-1.

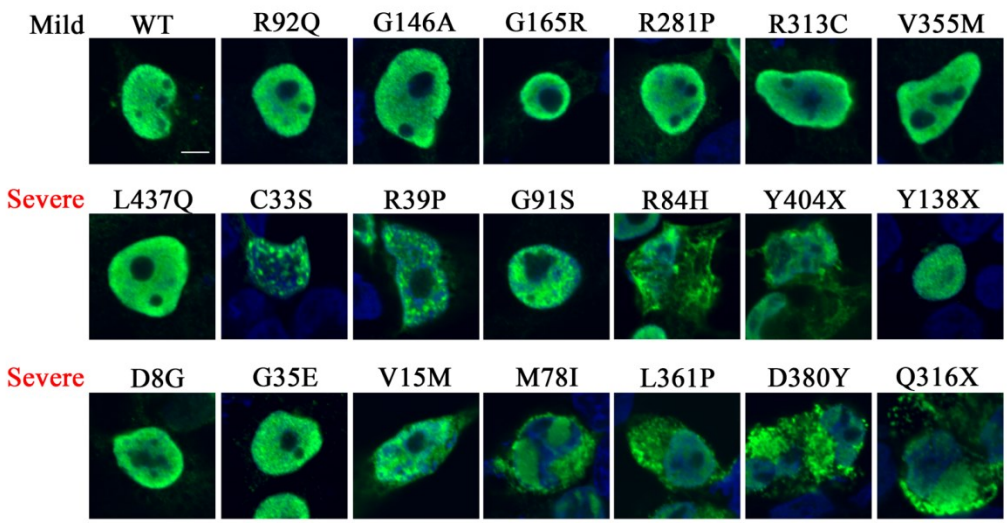
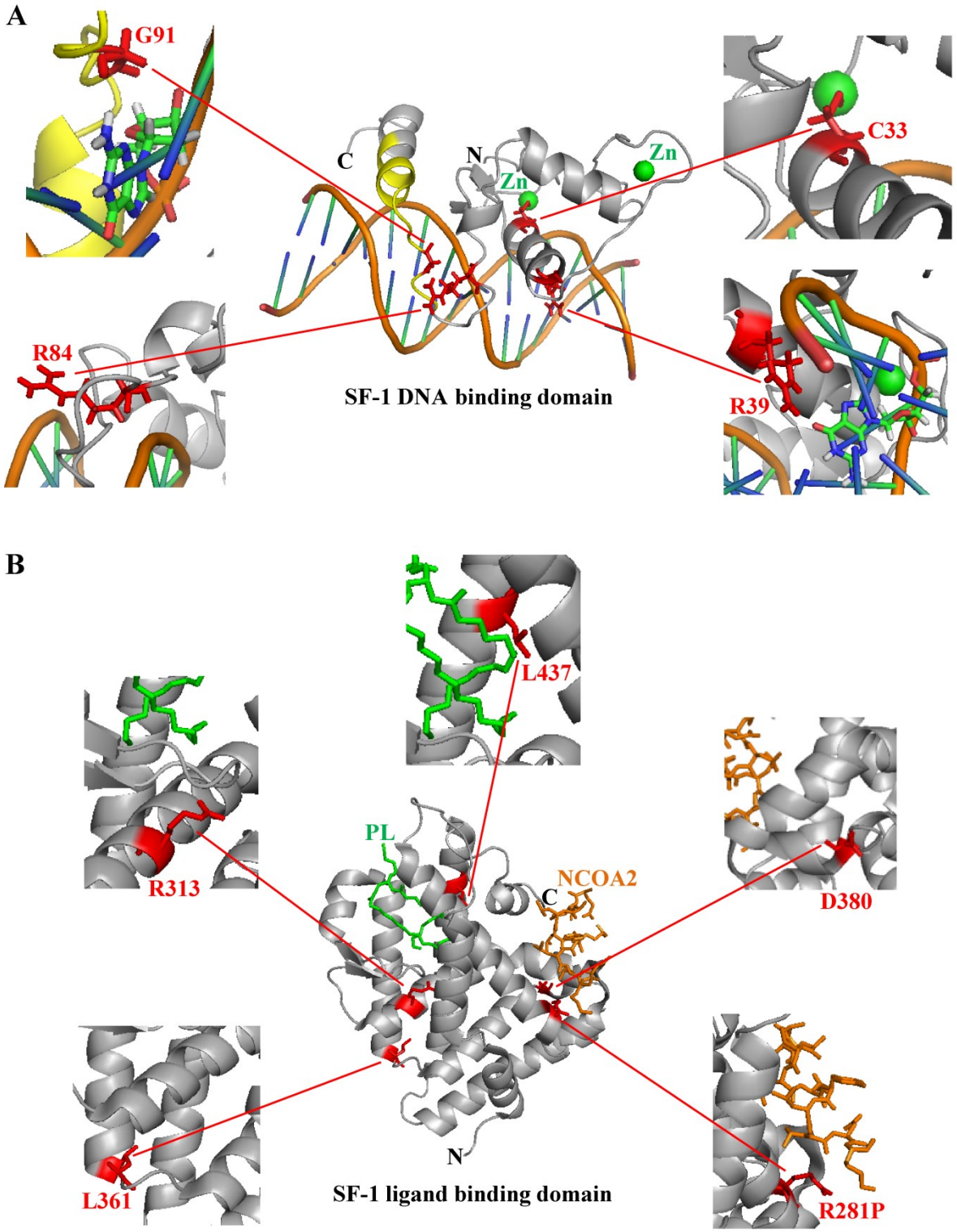
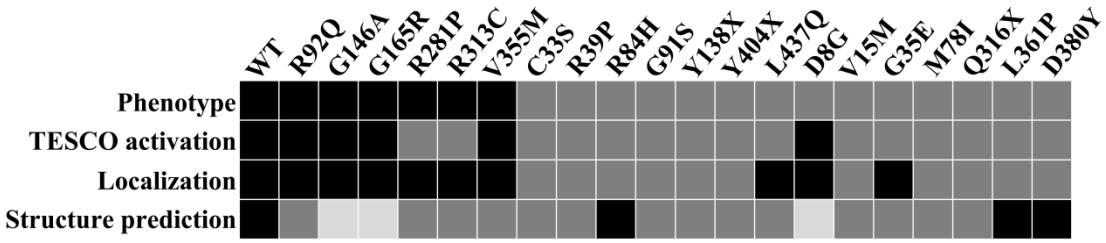


FIGURE 4: *In silico* protein structural analysis of SF-1 mutants. (A) Model of the mouse SF-1 DNA binding domain bound to the inhibin α-subunit promoter obtained from the NMR structure in PDB 2FF0 (Little et al., 2006). (B) Model of the human SF-1 ligand binding domain bound to a putative phospholipid ligand (PL) and a cofactor peptide (NCOA2), obtained from the crystal structure in PDB 1ZDT (Wang et al., 2005). Both domains are displayed as ribbons. Amino acids that are substituted in 46,XY DSD patients are shown enlarged and are displayed as red stick models. DNA bases in contact with substituted amino acids are shown as sticks colored according to elements (C: green, H: white, N: blue, O: red). The zinc (Zn) ions of the Cys4-zinc-finger motifs are labelled as green spheres. The nuclear localization signal is labelled in yellow. N: N-terminal, C: C-terminal



Sreenivasan *et al.*, Fig. 4

FIGURE 5: Pictorial summary of patient genotype-phenotype profile. The four properties assessed for the 20 SF-1 mutants (developmental phenotype, TESCO activation, sub-cellular localization and predicted protein structural effects) were color-coded as black for 'WT', dark grey for 'atypical' and light grey for 'not determined'.



Sreenivasan *et al.*, Fig. 5

Table 1: Genetic information and clinical phenotypes of 20 46,XY DSD patients with SF-1 mutations

Patient SF-1 Mutation	Declared sex	Age	Inheritance mode	Adrenal Phenotype	Reproductive phenotype			Hormonal assessment*					Diagnosis	Assigned DSD category	Ref
					External genitalia	Müllerian	Gonads	T (ng/mL)	FSH (IU/L)	LH (IU/L)	AMH (ng/mL)	Inhibin B (pg/mL)			
p.R92Q	F	36D	homozygous, autosomal recessive	adrenal failure	female	uterus	ND	Basal: 0.1 (0.1 – 3.5)	1.4 (1.5 – 3.5)	0.01 (1.5 – 4.5)	ND	ND	Primary adrenal failure; Complete sex reversal; Gonadotropin deficiency	severe	(11, 18)
	M, F		heterozygous (consanguineous parents of above patient)	normal	normal	absent	normal	ND	ND	ND	ND	ND	Normal	mild	
p.G146A	M	0 - 13Y	polymorphism	normal	normal male or micropenis	absent	normal testes or cryptorchidism	Basal T normal	All normal except for 2 patients with <0.2	Normal	ND	ND	Undervirilization	mild	(13, 14, 21)
p.G165R	M	32Y	heterozygous (parent DNA unavailable)	normal	male	absent	normal testes	Basal T normal	ND	ND	ND	ND	Oligozoospermia	mild	This stu

														dy
p.R281P	M	14 Y, 5 M	heterozygous (father normal mosaic carrier)	normal	hypospadias, bifid scrotum, micropenis, chordee	absent	palpable gonads (33 mm left, 35 mm right; progressive Sertoli cell defect	hCG stimulation at birth: Peak: 2 (>3)	20.8 (<6)	6.6 (<4.5)	ND	18 (>80)	Partial gonadal dysgenesis; Progressive Sertoli cell deficiency; T and AMH deficiency; Elevated gonadotropins	mild	(38,39)
p.R313C	M	4 D	heterozygous *de novo*	normal	male (phallus length normal: 3.3 X 1.5 cm, glandular hypospadias, bifid scrotum)	absent	normal testes (18 mm in scrotum)	Basal: 1.2 (0.1 – 3.5)	0.5 (1.5 – 3.5)	1.6 (1.5 – 4.5)	24.5 (3 3.26 ± 8.29)	99 (94–383)	Undervirilization; FSH and AMH deficiency	mild	(10)
p.V335M	M	13 Y	heterozygous, sex-limited (brother a normal carrier)	normal	male (micropenis: neonatal phallus length 1.8 X 0.8 cm; normal: 3.4 ±0.3 cm)	absent	only one inguinal atrophied testis	Basal: 0.04 (0.35 - 0.9)	130 (1–12)	33 (1–5)	<1 (35–95)	ND	Testicular regression syndrome; T and AMH deficiency; Elevated gonadotropins	mild	(16)
p.C33S	F	4 Y	heterozygous *de novo*	normal	ambiguous (clitoromegaly, urogenital sinus, fused	absent	bilateral inguinal testes (Sertoli rich, few	hCG stimulation: Basal:	ND	ND	ND	ND	Partial gonadal dysgenesis; T defici	severe	(15)

					labioscrotal folds)		sperma togonia l and Leydig cells); epididymis and vas deferens present	0.08, Peak: 0.07					ency		
p.R 39P	F	6 Y	heteroz ygous (parent DNA unavail able)	nor mal	ambiguous (Prader III; clitoromegaly, phallus 2 cm, penoscrotal hypospadias)	abs ent	testes in labia (20 mm, Sertoli and Leydig cells, no germ cells); epididymis present	hCG stim ulati on: Bas al: 0.05, Peak: 0.85	ND	ND	N D	N D	Partial gonadal dysgenesis; T defici ency	sev ere	(1 0)
p.R 84H	F	17 Y	heteroz ygous *de novo*	nor mal	ambiguous (clitoromegaly, urogenital sinus, fused labioscrotal folds)	abs ent	bilateral inguinal testes; epididymis and vas deferens present	hCG stim ulati on: Bas al: <0.01, Peak: 2.5	ND	ND	N D	N D	Partial sex revers al; T defici ency	sev ere	(1 5)
p.G 91S	F	4 M	heteroz ygous, sex-limited domina nt	nor mal	ambiguous (clitoromegaly 1 cm, single perineal opening, labioscrotal folds)	rem nan t	labiosc rotal testes (abund ant tubules, Sertoli cells, germ cells; tubule density reduced in some areas); epididymis and vas deferen	hCG stim ulati on: Peak: 0.43 (>15)	16 (2 - 4)	13 (0.5 - 6.9)	N D	N D	Partial gonad al dysge nesis; T defici ency; Elevat ed gonad otropi ns	sev ere	(1 1)

						s present								
p.Y138X	F	10 Y	heterozygous *de novo*	normal	ambiguous (clitoromegaly, urogenital sinus, fused labioscrotal folds)	absent	bilateral inguinal testes (Sertoli cells only); epididymis and vas deferens present	Basal T at birth: 0.25	ND	ND	ND	ND	Partial gonadal dysgenesis; T deficiency	severe	(15)
p.Y404X	M	7 Y	heterozygous (parent DNA unavailable)	normal	ambiguous (bifid scrotum, hypospadias)	absent	testes (few seminiferous tubules, no spermatogonia or Leydig cells)	hCG stimulation: Basal: 0.29 ; Peak: 1.12	ND	ND	ND	ND	Partial gonadal dysgenesis; T deficiency	severe	(38)
p.L437Q	M	6 Y	heterozygous *de novo*	normal	ambiguous (micropenis, severe penoscrotal hypospadias, chordee)	absent	high scrotal bilateral testes (hyalinized tubule structures, predominantly Sertoli cell-only, very few germ cells)	hCG stimulation: Peak: <0.2 (>15)	7.4 (2.9 ± 4.3)	21.4 (2.3 ± 2.2)	ND	ND	Partial gonadal dysgenesis; T deficiency; Elevated gonadotropins	severe	(11)
p.D8G	F	16 Y	heterozygous (parent DNA unavailable)	normal	female	present	streak gonads (no evidence of tubules)	ND	ND	ND	ND	ND	Complete gonadal dysgenesis	severe	This study

p.V15M	F	4 M	heterozygous *de novo*	normal	female (blind vagina, rugose labia)	absent	small bilateral labial testes (8 mm; well-formed tubules, multiple germ cells); epididymis and vas deferens present	hCG stimulation: Peak: 0.09 (>3.65)	24 (2 - 4)	10 (0.5 - 6.9)	7.3 (15 - 48)	ND	Partial gonadal dysgenesis; T and AMH deficiency; Elevated gonadotropins	severe	(11)
p.G35E	F	10 Y	heterozygous *de novo*	adrenal failure	female	present	streak gonads (poorly differentiated tubules)	hCG stimulation: no T response	GnRH stimulation: 17.8 to 38	GnRH stimulation: 1.2 to 8.6	ND	ND	Primary adrenal failure; Complete gonadal dysgenesis; T deficiency	severe	(17)
p.M78I	F	5 M	heterozygous, sex-limited dominant	normal	female	remnant	small bilateral inguinal testes (tubules present, germ cells scarce, vacuolated interstitial cells); epididymis and vas deferens present	hCG stimulation: Peak: 0.62	81 (2 - 4)	28 (0.5 - 6.9)	3.2 (29 - 57)	ND	Partial gonadal dysgenesis; T and AMH deficiency; Elevated gonadotropins	severe	(11)

p.Q316X	F	4 Y	heterozygous (parent DNA unavailable)	normal	ambiguous (fused labia, clitoromegaly)	absent	inguinal testes	hCG stimulation: Basal: 0.24, Peak: 1.6	3 (1-12)	0.5 (1-5)	42 (29-57)	70 (16-224)	Partial sex reversal; T deficiency	severe	(38)
p.L361P	F	4 Y	heterozygous (parent DNA unavailable)	normal	female	ND	small inguinal testes	Basal T: ≤ 0.1	7.2 (2.5 - 4.5)	<0.3 (2 - 5)	28 (29-57)	41 (16-224)	Partial gonadal dysgenesis; T, LH and AMH deficiency; Elevated FSH	severe	(38,40)
p.D380Y	F	Adolescent	heterozygous (mother and sister carriers)	normal	female	uterus	small homogeneous gonads	Basal T: 0.3 (0.35 - 0.9)	40 (1 - 12)	18 (1 - 5)	ND	ND	Partial gonadal dysgenesis; T deficiency; Elevated gonadotropins	severe	(38,41)

Y: year; M: month; D: day; ND: not determined; T: testosterone; Ref: reference
*Normal hormonal levels in parentheses; Hormonal levels higher or lower than normal are double- or single-underlined respectively