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Mutational analysis of steroidogenic factor 1 (*NR5a1*) in 24 boys with bilateral anorchia: a French collaborative study†

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Abstract

BACKGROUND—Steroidogenic factor 1 (SF1/AdBP4/FTZF1, *NR5A1*) is a nuclear receptor transcription factor that plays a key role in regulating adrenal and gonadal development, steroidogenesis and reproduction. Recently, haploin-sufficiency of SF1 has been described in several 46,XY individuals with mild gonadal dysgenesis and impaired androgenization, but normal adrenal function, suggesting that dosage-sensitive or domain-specific effects of SF1 action are important in human testicular development and function. Our objective was to investigate whether partial defects in SF1 function might be associated with milder male reproductive phenotypes, such as bilateral anorchia ('vanishing testis syndrome') and micropenis.

METHODS—This study involved mutational analysis of *NR5A1* in 24 individuals with bilateral anorchia and micropenis from the French Collaborative Anorchia study, as well as *in vitro* functional studies of SF1-dependent transcriptional activation and computer modeling.

RESULTS—A novel heterozygous missense mutation (V355M) in SF1 was found in one boy with a micropenis and testicular regression syndrome. This non-synonymous change was found to affect a highly conserved amino acid within helix 7 of the ligand-binding domain of SF1. This V355M mutation did not affect stability or nuclear localization, but did result in an ~50% reduction in SF1 activity in several different assay systems.

CONCLUSIONS—In conclusion, heterozygous partial loss of function mutations in SF1 may be associated with bilateral anorchia ('vanishing testis syndrome') and micropenis in humans.

Keywords

SF1; gonadal dysgenesis; vanishing testis syndrome; testicular regression; disorders of sexual development

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Introduction

Bilateral anorchia ('vanishing testis syndrome' or 'testicular regression syndrome') is defined as the absence of testicular tissue in 46,XY individuals with a male phenotype (Abeyaratne *et al.*, 1969; Borrow and Gough, 1970; Aynsley-Green *et al.*, 1976). The prevalence of this condition is approximately 1 in 20 000 males, but may be under-estimated due to its possible clinical misdiagnosis as bilateral cryptorchidism (Frey and Rajfer, 1982). Appropriate diagnosis therefore requires surgical exploration for testicular tissue, together with extremely low or undetectable anti-Müllerian hormone (AMH) levels and complete absence of plasma testosterone in response to human choriogonadotropin (hCG) stimulation (Davenport *et al.*, 1995; Lee *et al.*, 1997; Kirsch *et al.*, 1998).

Boys with bilateral anorchia have a normal male phenotype. Therefore, fetal testes must have been present during early development (8–16 weeks gestation), producing sufficient androgen for normal or moderate abnormal development of the external male genitalia and adequate AMH (Müllerian inhibiting substance, MIS) to permit the uterus, fallopian tubes and upper vagina to disappear (Aynsley-Green *et al.*, 1976; Zenaty *et al.*, 2006). It is therefore likely that testicular regression syndrome occurs during late fetal life in this condition, although regression of the testes in early infancy has also been described in a number of cases. In a recent study, micropenis was associated with bilateral anorchia in almost half of the cases, suggesting that gonads may have been functionally abnormal before they disappeared (Zenaty *et al.*, 2006).

Various mechanical causes have been proposed as the etiology of anorchia, such as testicular torsion, trauma or vascular occlusion of spermatic structures (Huff *et al.*, 1991). However, a number of familial cases of bilateral anorchia have now been reported, suggesting that a genetic cause may be responsible in some situations (Hall *et al.*, 1975; Garcia Centenera *et al.*, 1980; Josso and Briard, 1980; Martinon Sanchez *et al.*, 1984; de Grouchy *et al.*, 1985; Naffah, 1989; Rai *et al.*, 1994). Indeed, the occurrence of anorchia with 46,XY genital ambiguity within families supports the hypothesis that some forms of this condition may be a part of the clinical spectrum of 46,XY gonadal dysgenesis or testicular maldescent syndromes (Josso and Briard, 1980; Mantonarcio *et al.*, 1994). Although limited molecular studies of *SRY, INSL3* or *LGR8*, genes involved in testicular determination or descent, have failed to identify any mutation in association with anorchia to date (Lobaccaro *et al.*; 1993; Vinci *et al.*, 2004), advances in our understanding of the molecular mechanisms of testis determination and development have generated a number of other candidate genes that could be involved in maintaining testicular integrity and mediating testis migration [e.g. *WT1*, SF1 (*NR5A1*), *HOXA10*].

One of these candidate genes is steroidogenic factor 1 (*NR5A1*, SF1/Ad4BP/FTZF1). SF1 is a member of the nuclear receptor superfamily, which plays a crucial role in the development of the adrenal gland and testes as well as in regulating many aspects of steroidogenesis and reproduction (Parker *et al.*, 2002; Ferraz-de-Souza *et al.*, 2006). In XY mice, complete deletion of the gene encoding SF1 causes agenesis or apoptosis of the adrenal glands and gonads early in development, resulting in a post-natal phenotype of adrenal agenesis associated with gonadal dysgenesis, undervirilization and persistence of Müllerian ducts (Luo *et al.*, 1994). In humans, heterozygous (or rare homozygous) SF1 mutations have now been reported in 46,XY patients with a spectrum of phenotypes ranging from adrenal and gonadal failure at the most severe end of the spectrum, through to mild gonadal dysgenesis and impaired androgenization with normal adrenal function associated with SF1 haploinsufficiency (Achermann *et al.*, 1999, 2002; Correa *et al.*, 2004; Hasegawa *et al.*, 2004; Mallet *et al.*, 2004; Lin *et al.*, 2007). Moreover, non-synonymous polymorphisms in

SF1 causing a mild partial loss of activity have been reported to be associated with cryptorchidism (Wada *et al.*, 2005) and micropenis (Wada *et al.*, 2006) in population studies, suggesting that dosage-sensitive or domain-specific changes in SF1 can result in a spectrum of male reproductive phenotypes (Jameson, 2004).

This collaborative project aimed to investigate whether SF1 mutations could be associated with bilateral anorchia/micropenis. In a cohort of 24 children, we have identified one heterozygous SF1 mutation, which resulted in reduced transcription activation of several SF1 target genes. Thus, we suggest that partial loss of SF1 function may be an important contributory factor to late fetal or post-natal testicular regression in certain individuals.

Patients and Methods

Cohort

The cohort studied consisted of 24 boys with bilateral anorchia recruited from several French Pediatric Endocrine centers as part of a French Collaborative Study of Anorchidism. Surgical exploration was performed in 80% of the children to confirm the absence of testes. The chronological age at diagnosis was between 0 and 4 years. Penile length was −1.4 ± 1.3 SDS. Further clinical details of this cohort have been reported recently (Zenaty *et al.*, 2006).

Mutational analysis

With Ethical Committee approval (University of Paris, Saint Louis) and written informed consent from patients or parents, DNA was extracted from peripheral blood lymphocytes of all 24 patients. The entire coding region and splice sites of *NR5A1* (SF1) were PCR amplified using primers and conditions described previously (Achermann *et al.*, 2002), and sequenced directly using BigDye terminator v1.1 (Applied Biosystems, Courtaboeuf, France) and an ABI Prism 310 DNA sequencer (Applera, Courtaboeuf, France).

Mutant SF1 expression vectors

Three different mutant SF1 expression vectors containing the V355M mutation (GTG→ATG) were created by site-directed mutagenesis (Quik change Site-Directed Mutagenesis Kit, Stratagene, Amsterdam, The Nederlands) using wild-type (WT) human SF1 cDNA as a template. For transient gene expression studies of target promoters, mutations were introduced into full-length SF1 (1–461) in a pCMX expression vector (Ito *et al.*, 2000; Lin *et al.*, 2007). For additional analysis, the V355M change was generated in a pBINDGAL4-SF1 (133–461) fusion protein (Promega, Southampton, UK) for co-transfection with a UASTKLuc reporter (Achermann *et al.*, 2001). Finally, a mutant green fluorescent protein (GFP)–SF1 construct was generated in a pAcGFP-C1 vector (Clontech, Oxford, UK), to produce a fusion protein of SF1 with a GFP tag at its amino-terminal end (Lin *et al.*, 2007). The entire sequence of all mutant plasmids was verified prior to investigation.

Transient gene expression assays

All transient gene expression assays were performed in 96-well plates (TPP) using lipofectamine 2000 (Invitrogen, Paisley, UK) and a Dual-Luciferase reporter assay system (Promega, Southampton, UK) (Lin *et al.*, 2007). Co-transfection of pRLSV40 *Renilla* luciferase (Promega) was used as a marker of transfection efficiency. For analysis of target gene activation, WT or mutant V355M SF1 expression vectors (2 ng/well) were transfected into tsa201 human embryonic kidney cells with reporters containing SF1 responsive promoters [AMH (MIS), Insl3, Scc (Cyp11a), Cyp19, 100 ng/well], as reported previously (Ito *et al.*, 2000; Lin *et al.*, 2007). The effect of the V355M ligand-binding domain mutant was also assessed: (i) in a native testicular cell line by transfecting SF1 vectors (20 ng/well)

together with the Scc (Cyp11a) promoter (150 ng/well) into a TM3 mouse Leydig cells (Lin *et al.*, 2007); (ii) by transfecting SF1 constructs (2 ng/well) with Scc (Cyp11a) into CHO cells (Lin *et al.*, 2007) and (iii) in a modified mammalian two-hybrid system by transfecting 5 ng/well pBINDGAL4-SF1 WT or mutant cDNA with 100 ng/well UASTKLuc reporter into tsa201 cells (Achermann *et al.*, 2001). SF1 cDNA vectors containing the G35E P-box mutation associated with severe gonadal dysgenesis and adrenal dysfunction were used a controls (Achermann *et al.*, 1999; Lin *et al.*, 2007). Luciferase assays were performed 24 h after transfection using a FLUOstar Optima fluorescence microplate reader (BMG Labtech, Aylesbury, UK) and data were standardized for *Renilla* expression. Results are shown as the mean ± SEM of at least three independent experiments, each performed in triplicate.

GFP studies of SF1 expression and nuclear localization

WT and mutant pAcGFP-C1 SF1 expression vectors (0.8 μg) were transfected into tsa cells using lipofectamine 2000 (Invitrogen, Paisley, UK). After 24 h, media were removed and nuclear counter staining was performed with Vectashield containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Peterborough, UK). Cells were visualized on a Zeiss Axioskop microscope, and images captured using a ZeissAxiocam camera.

Results

Mutational analysis

Among the 24 patients studied, we have been able to identify a heterozygous V355M substitution in SF1 in one individual (Fig. 1A–C). This valine to methionine substitution affects a highly conserved amino acid in helix 7 of the putative ligand-binding domain of SF1 (Fig. 1D), and was not found in over 200 racially-matched control alleles. The proband's mother and dizygotic twin brother were also heterozygous for the V355M change, but the father had normal SF1 sequence.

Case report

The proband is a dizygotic, diamniotic twin boy who was born at 37 gestational weeks to non-consanguineous parents following ovarian stimulation therapy. The mother had undergone left ovariectomy and homolateral fallopian tube ablation for ovarian cysts at 22 years of age, and had experienced two spontaneous miscarriages prior to this pregnancy.

The proband was well at birth (weight 2.6 kg, length 47 cm), but was assessed by a pediatrician in the neonatal period on account of an ectopic testis and a small penis (1.8 cm stretched length, 0.8 cm circumference; normal length, 3.4 ± 0.3 cm). At four months of age, a detailed examination was performed by a pediatric endocrinologist. The left testis could not be palpated, whereas a very small testis (the size of a 'rice grain') was reported in the right inguinal region. Penile length was 1.8 cm and circumference was 1.0 cm. No other anomalies were found. Endocrine investigation at this time revealed markedly elevated levels of FSH [552 IU/l (normal range: 1–12)] and LH [142 IU/l (normal range: 1–5)], and AMH was undetectable [<1 ng/ml(<7 pmol/l) (normal range: 29–57)]. Basal testosterone was low [15 ng/dl (0.5 nmol/l); (normal range: 30–100)] and showed no increase following hCG stimulation [1500 IU/day, 6 days; post-stimulation testosterone, 16 ng/dl (0.55 nmol/l)].

Testosterone supplementation was given, which resulted in a good response in penile growth. At two years of age, surgical exploration of the left inguinal region revealed an atrophic deferent duct placed against the bladder wall without any evidence of a testis. At four years of age, exploration of the right inguinal region revealed an atrophic testis with

apparently normal duct. At 13 years of age, the right testicular vestigium was removed. Histopathology revealed no testicular structures but only fibrous tissue. Penile length was 5 cm. Müllerian structures were not present. As expected, the proband failed to enter puberty. Basal testosterone at 13 years of age was extremely low [4 ng/dl (0.1 nmol/l)], AMH was undetectable [<1 ng/ml (<7 pmol/l) (normal range: 35–95)], and gonadotropins were elevated [FSH, 130 IU/l (normal range: 1–12); LH, 33 IU/l (normal range: 1–5)]. Adrenal function was normal and he was normotensive. Puberty was induced with testosterone. His twin brother is reported to have had normal development. Birth weight was 2.7 kg. He underwent puberty at an appropriate age and had Tanner ratings of P5, G5 at 15 years of age.

Nuclear localization and function

The V355M mutation did not alter expression, stability or nuclear localization of SF1 in an *in vitro* system using GFP-tagged SF1 (Fig. 2) and, as expected, this ligand-binding domain change did not alter binding to the DNA of SF1 target genes (data not shown). However, the V355M mutant was found to have partial function in several different native promoter assay systems and cell lines (Fig. 3A–G, mean activity 55% of WT) as well as in a modified two-hybrid system with GAL4-SF1 fusion proteins (Fig. 3H). No dominant negative effect was seen in SF1 expressing cell lines (Fig. 3E and F), or when considerably greater ratios (10:1) of V355M mutant SF1 cDNA to WT SF1 cDNA were transfected into tsa201 cells (data not shown).

Structural analysis

Modeling of the ligand-binding domain of SF1 (PDB # 1ZDT) shows that the valine residue at position 355 lies within helix 7 of this receptor (Fig. 4). Replacing the valine with a bulkier methionine residue would be expected to alter ligand or co-factor binding capacity of SF1. This modification in helix orientation may be responsible for the partial reduction in SF1 activation observed in transient gene expression studies (Fig. 3) (Krylova *et al.*, 2005; Li *et al.*, 2005;Wang *et al.*, 2005).

Discussion

SF1 clearly plays an important role in many aspects of testicular development and function as it regulates a number of critical genes involved in these processes (Parker *et al.*, 2002; Ferraz-de-Souza *et al.*, 2006). Consequently, complete deletion of the gene encoding *Sf1* in the mouse results in apoptosis of the developing testicular progenitor cells at around embryonic Day 11.5–12.0, and tissue-specific deletion of SF1 following targeting by *Amh*-driven Cre recombinase results in impaired testicular descent and marked structural abnormalities (Luo *et al.*, 1994; Parker *et al.*, 2002; Jeyasuria *et al.*, 2004). However, it is the recent description of several 46,XY patients with mild testicular dysgenesis and impaired androgenization due to heterozygous loss-of-function mutations in SF1 that has revealed the sensitivity of human testis development to gene-dosage effects of SF1 (Achermann *et al.*, 1999, 2002; Correa *et al.*, 2004; Hasegawa *et al.*, 2004; Jameson, 2004; Mallet *et al.*, 2004; Lin *et al.*, 2007). In some of these cases, a progressive form of testicular degeneration may be evident, consistent with the proposal that SF1 plays an important role in maintaining testicular integrity.

A potential genetic basis to some forms of bilateral anorchia has been proposed, as a number of familial cases of this condition have now been reported and it is unlikely that bilateral mechanical events (e.g. torsion) occur in multiple affected individuals within one family (Hall *et al.*, 1975; Garcia Centenera *et al.*, 1980; Josso and Briard, 1980; Martinon Sanchez *et al.*, 1984; de Grouchy *et al.*, 1985; Naffah, 1989; Rai *et al.*, 1994). Given the role of SF1

in multiple aspects of testicular development, integrity and function, we hypothesized that partial defects in SF1 could account for cases of bilateral anorchia, especially when micropenis is present. Furthermore, the inheritance of SF1 heterozygous mutations as de-novo alterations or sex-limited dominant changes from the mother would be consistent with the distribution of individual and familial cases of bilateral anorchia reported to date (Lin *et al.*, 2007).

Here, we describe a novel heterozygous V355M mutation in SF1 in one of a cohort of 24 boys with bilateral anorchia. This non-synonymous change affects a highly conserved amino acid residue in helix 7 of the ligand-binding domain of SF1 (Krylova *et al.*, 2005; Li *et al.*, 2005; Wang *et al.*, 2005). The V355M conversion did not interfere significantly with receptor expression, nuclear localization or DNA-binding, but did reduce SF1-dependent transcriptional activation by ~50%, based on data from several *in vitro* promoter assays systems and different cell lines. Although a number of missense mutations in the DNA-binding regions of SF1 have been reported in association with mild testicular dysgenesis and marked underandrogenization, the only other reported heterozygous ligand-binding mutation in SF1 (L437Q) was associated with hypospadias, small fibrotic testes and possible progressive partial hypogonadotropic hypogonadism (Lin *et al.*, 2007). Thus, it is possible that ligand-binding domain changes in SF1 may affect testicular integrity more significantly than fetal Leydig cell function. Although cases are limited at present, this finding may reflect differences in phospholipid ligand signaling and co-factor interaction, rather than more global effects on DNA-binding associated with most heterozygous SF1 changes reported to date. Furthermore, the partial loss of function observed with the V355M change would be consistent with the milder phenotype (micropenis, progressive testicular degeneration) reported here. It is interesting to note that the SF1 G146A anomaly has been reported in patients with cryptorchidism and micropenis, and causes a 20% reduction of *in vitro* transcriptional activity (Wada *et al.*, 2005, 2006). Since this SF1 gene anomaly was found also in control individuals, this suggests that SF1 activity appears to be modulated by some cofactors and/or endogenous or exogenous ligands.

Although the presence of the heterozygous SF1 mutation in the mother is consistent with a sex-limited dominant mode of transmission seen in several other individuals with heterozygous SF1 changes, the detection of a similar heterozygous V355M mutation in the dizygotic twin brother is more challenging to explain. Furthermore, although the brother is reported to have progressed though puberty, it has not been possible to obtain further detailed physical and endocrinological re-evaluation in his case. Nevertheless, it is quite possible that genetic and environmental modifying factors or epigenetic variability influenced the expression of phenotype in these two boys. For example, a spectrum of DSD/anorchia phenotypes has been previously reported within families (Josso and Briard, 1980); monozygotic 46,XY twins can show differences in sexual development (Mendonca *et al.*, 2002); and variability in the expression of developmental or syndromic defects between monozygotic twins has been reported for a number of endocrine and non-endocrine conditions (Perry *et al.*, 2002; Weksberg *et al.*, 2002; Gicquel *et al.*, 2005; Toyoshima *et al.*, 2006). Very recently, Coutant *et al.* (2007) reported that the same SF1 mutation within two siblings is associated with discordant phenotypes, suggesting too that SF1 activity may be modulated by various others factors such as co-factors, bioactive lipids or exogenous ligands. As the heterozygous V355M mutation likely represents a partial defect in SF1 function (75% normal), epigenetic variability, modifying factors or environmental influences could account for the differences in phenotypes seen. It also possible however that the 50% reduction in transcriptional activity, as observed for the mutated SF1 in this study, is within the bounds of variation in normal individuals. Further studies will be needed to clarify this.

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In conclusion, this report strengthens the hypothesis for dosage-dependent effects of SF1 in male reproductive development and function, and documents the first possible monogenic cause of bilateral anorchia in humans.

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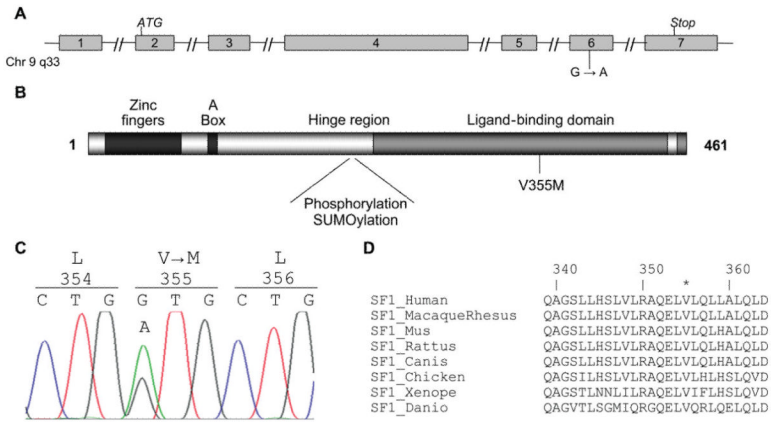

Figure 1.

(**A**) Genomic arrangement of the gene encoding SF1 (*NR5A1*) showing the G to A transversion in exon 6

(**B**) Cartoon of the structure of SF1 with the V355M mutation in the ligand-binding domain.

(**C**) Chromatograph showing the heterozygous G to A transversion in the proband. (**D**) The mutated valine at position 355 lies within helix 7 and is highly conserved in homologues of SF1 from all species studied to date

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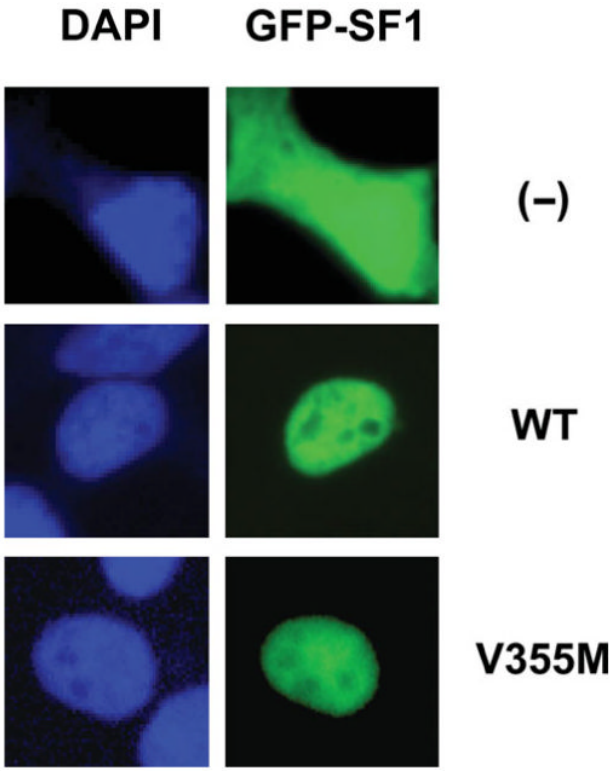

Figure 2.
Studies of SF1 expression and cellular localization
GFP-SF1 fusion proteins (green) were created and expressed in tsa201 cells using a pAcGFP-C1 vector. Nuclear counterstaining was performed with DAPI (blue). Empty vector (−) showed diffuse localization throughout the cell cytoplasm and nucleus (upper panels). WT SF1 showed strong nuclear localization (center panels). A similar pattern of expression and localization to WT was seen following introduction of the V355M mutation (lower panels)

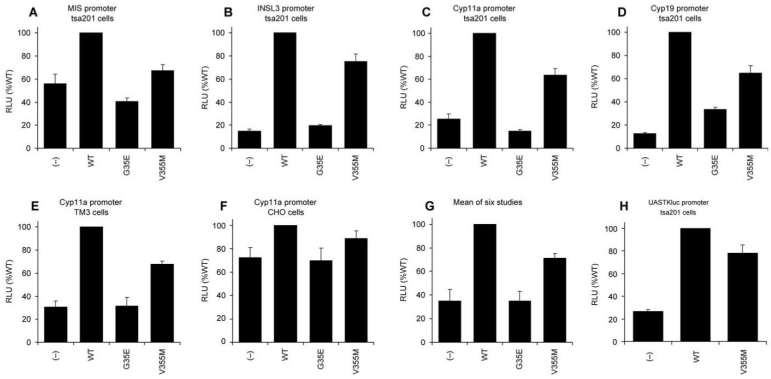

Figure 3.

Transcriptional activation of SF1 target gene promoters by WT and mutant V355M SF1 The activity of the G35E DNA-binding domain mutant associated with a more severe phenotype is shown for comparison. (**A**–**D**) Data for the *MIS, Insl3, Cyp11a (scc)* and *Cyp19* minimal promoters in tsa 201 cells expressed as percentage of WT activity. (**E** and **F**) Data for the *Cyp11a (scc)* promoter in the TM3 Leydig cell line and CHO cells, respectively. (**G**) Mean transcriptional activation of WT and mutant SF1 for all six studies (A–F). (**H**) Activation of a UASTKluc promoter by GAL4-WT or V355M fusion proteins in tsa201 cells. Data represent the mean ± SEM of three independent experiments, each performed in triplicate

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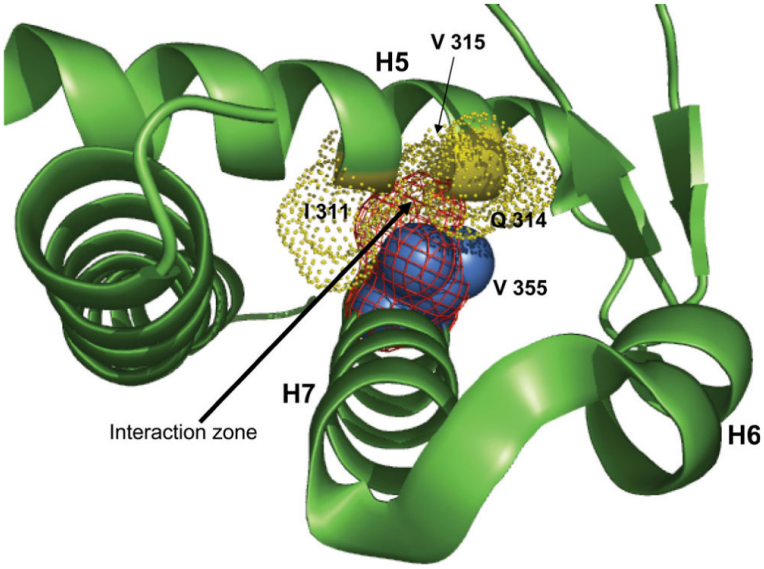

Figure 4.
Predicted position of valine 355 within helix 7 of SF1 using recently published coordinates following crystallization of the SF1 ligand-binding domain (PDB; 1ZDT)
Replacing this valine with a bulkier methionine residue would be expected to alter co-factor binding capacity of SF1

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