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A mutation inactivating the distal SF1 binding site on the human anti-Müllerian hormone promoter causes persistent Müllerian duct syndrome

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## Abstract

The persistent Müllerian duct syndrome (PMDS) is a 46,XY disorder of sexual development characterized by the persistence of Müllerian duct derivatives, uterus and tubes, in otherwise normally masculinized males. The condition, transmitted as a recessive autosomal trait, is usually due to mutations in either the anti-Müllerian hormone (AMH) gene or its main receptor. Many variants of these genes have been described, all targeting the coding sequences. We report the first case of PMDS due to a regulatory mutation. The AMH promoter contains two binding sites for steroidogenic factor 1 (SF1), one at -102 and the other at -228. Our patient carries a single base deletion at -225, significantly decreasing its capacity for binding SF1, as measured by electrophoresis mobility shift assay. Furthermore, by linking the AMH promoter to the luciferase gene, we show that the transactivation capacity of the promoter is significantly decreased by the mutation, in contrast to disruption of the -102 binding site. To explain the difference in impact we hypothesize that SF1 could partially overcome the lack of binding to the -102 binding site by interacting with a GATA4 molecule linked to a nearby response element. We show that disruption of both the -102 SF1 and the -84 GATA response elements significantly decreases the transactivation capacity of the promoter. In conclusion, we suggest that the distance between mutated SF1 sites and potentially rescuing GATA-binding motifs might play a role in the development of PMDS.

## Introduction

Anti-Müllerian hormone (AMH), a member of the TGF $\beta$  superfamily, is produced by fetal Sertoli cells at a very early stage of fetal development, 12.5 dpc in the mouse (1) and approximately 7 to 8 weeks post-conception in humans (2-4). Transcription factors SOX9, SF1 (also known as Ad4bp, encoded by *NR5A1*) and GATA4 have response elements on the proximal *AMH* promoter (5-10) thus driving AMH expression before the window of Müllerian duct responsiveness to AMH closes (11, 12). This occurs when the Müllerian duct loses its mesoepithelial character and acquires epithelial cell markers (13). AMH-induced Müllerian regression has been actively studied in the mouse, namely by Behringer's group (13-15) and others (16, 17). For obvious reasons, human studies have lagged behind, although the main components of the human AMH signaling pathway have been cloned a long time ago (18, 19).

To produce Müllerian duct regression, the translated full-length AMH molecule must be proteolytically cleaved into an inactive N-terminus and a biologically active C-terminus with homology to the other members of the TGF- $\beta$  superfamily (20, 21). The cleaved AMH molecule can then bind to AMHR2 expressed in the Müllerian mesenchyme under the influence of Wnt7a (reviewed in ref. (15)). Once activated by ligand binding, AMHR2 forms a complex with the type 1 receptors ALK2 or ALK3 and phosphorylates Smads 1, 5 or 8, the first step towards intracellular signaling (reviewed in ref. (15)). Genetic studies in the mouse indicate that *Amhr2* is the sole primary receptor required for *Amh* signaling and is likely dedicated to the AMH signaling pathway. Mutation of the *Amhr2* gene rescues the phenotype of female mice overexpressing the *Amh* gene (22). Male *Amh* and *Amhr2* mutant mice have identical phenotypes, they are normally virilized but retain Müllerian derivatives, uterus and tubes (23).

Persistence of Müllerian derivatives in normally virilized human males has been described under various names: internal male pseudohermaphroditism, "homme à utérus", *hernia uteri inguinalis*. The condition, a 46,XY disorder of sex development, is now known as Persistent Müllerian Duct Syndrome (PMDS). Nilson (24) is usually credited with the first description but earlier reports have been published (25, 26). In 2016, approximately 300 cases had been published worldwide and more

than 30 have appeared since, suggesting that the condition, an autosomal recessive disorder, is not as rare as formerly thought. There is no anatomical difference between patients with either *AMH* or *AMHR2* mutations. They are all externally completely virilized, appearing normal males at birth and are brought to medical attention because of the abnormal position of the testes. Slightly more than half present with bilateral cryptorchidism, followed by 20% with unilateral cryptorchidism and contralateral hernia, and the rest with transverse testicular ectopia defined by the descent of both testes into the same hemiscrotum (reviewed in ref. (27)). The clinical picture is not genetically determined and may vary between siblings. Patients with *AMHR2* gene mutations have normal AMH serum levels while they are very low or even undetectable in patients bearing a mutation of the *AMH* gene.

A recent study (27) referenced 64 different mutations of the *AMH* gene and 80 different mutations of the *AMHR2* gene. The most common mutation is a 27-bp deletion in the kinase domain of the receptor gene, found in patients of Northern European extraction. All the reported base changes target the coding sequences of the gene. We now describe the first regulatory mutation detected in a PMDS patient, a single base deletion in a steroidogenic factor 1 (SF1) response element of the *AMH* promoter.

## Results

### Case report

The patient was born at 39 weeks gestation by normal vaginal delivery with a birth weight of 3.54 kg and length of 51 cm. On examination, he was non dysmorphic, with a normal sized penis measuring 2 x 1.2 cm and with empty underdeveloped scrotum bilaterally. The family, who was originally from Mexico, denied consanguinity. There was no family history to suggest any possible disorder of sexual development. A sonogram showed a neonatal uterus measuring 5 x 1.4 x 1.9 cm, and without gonads visualized in the scrotum or pelvis. A voiding cystourethrogram showed a normal male urethra without a urogenital sinus, magnetic resonance imaging did not identify gonads, and confirmed the existence of a neonatal uterus with a fluid filled, blind ending vagina. Genetic testing included a 46,XY karyotype and fluorescence *in situ* hybridization that revealed the existence of the *SRY* gene on

the short arm of Y chromosome in 200 cells. Hormonal testing on day of life 4 showed normal baseline 17-hydroxy progesterone at 61 ng/dL, FSH of 4.2 mIU/mL and LH of 14 mIU/mL (Esoterix Laboratory Services, Inc., Calabasas, CA). Human chorionic gonadotropin (hCG) stimulation testing performed on day 11 consisting of 3 daily injections of 1500 IU/m<sup>2</sup> hCG showed baseline testosterone of 358 ng/dL that increased to 783 ng/dL one day after the 3<sup>rd</sup> injection; dihydrotestosterone (DHT) increased from 50 to 146 ng/dL. AMH was low at 1.1 ng/mL compared with the normal range for the first two weeks of life at 15.5-48.7 ng/mL (Esoterix Laboratory Services, Inc., Calabasas, CA). The persistence of a uterus in an otherwise normally virilized 46,XY male, associated with a very low level of circulating AMH, suggested the existence of a mutation in the *AMH* gene and molecular genetic studies were initiated.

### ***AMH* and *AMHR2* gene sequencing**

Sanger sequencing of the *AMH* coding sequence, exon-intron boundaries and proximal promoter revealed a homozygous deletion of an adenine base 225 bp upstream of the translation start site (Figure 1). *AMH* promoter site notation, counted from the translation initiation site ATG, is used according to Picard et al (27) for homogeneity with AMH coding mutations in PMDS, instead of counting from the transcription initiation site, 10 base pairs upstream of ATG, used in previous publications (28-30). The mutation, c.-225delA, lies within the canonical response element for SF1 at -228, changing *tcaaggacag* into *tcaggacag*. The parents were heterozygous for the mutation. An apparently healthy 8-year-old sister was homozygous, and there were also other three phenotypically normal siblings: one heterozygous sister, one heterozygous brother and one homozygous wild-type sister.

### **SF1 binding to the human *AMH* promoter**

By electrophoresis mobility shift assay (EMSA) we assessed the effect of the deletion of adenine -225 on the binding capacity of the SF1 binding site (*tcaaggacag*) occupying positions -228 to 219 of the human *AMH* promoter. We first confirmed that radiolabeled SF1 binds to the -228 site on the normal *AMH* promoter, and that it is displaced by increasing amounts of cold SF1, supporting SF1 binding

specificity (Figure 2 A). Binding was lost when a -225delA probe was used instead of the normal sequence (Figure 2 B). As a positive control we used an artificial probe containing an extensively mutated -228 SF1 binding site (*tcaaggacag --> tggtaccag*) with proven inactivating effect (30). Taken together, these results show that SF1 binds to the -228 site on the human *AMH* promoter, and that the binding is abolished by the c.-225delA mutation present in our patient.

### **Transactivation studies: comparison of proximal and distal SF1 response elements**

The human *AMH* promoter carries two SF1 sites, a distal one at -228 and a proximal one at -102 (Figure 1). We tested the functional relevance of these sites for human *AMH* transcriptional activity in luciferase reporter assays by transfecting a previously validated 3078-bp human *AMH* promoter (28, 30) in the prepubertal Sertoli cell line SMAT1 (31). An artificial mutation of the -102 SF1 binding site did not significantly impair *AMH* promoter activity but a similar alteration of the -228 site resulted in a significant ( $65 \pm 6\%$ ,  $P < 0.05$ ) decrease in *AMH* promoter activity (Figure 3A). Deletion of a single base at -225, like the one described in our patient, also significantly decreased *AMH* promoter activity ( $42 \pm 4\%$ ,  $P < 0.05$ ). Taken together these observations confirm that an intact -228 SF1 response element is crucial to *AMH* human promoter activity, while the -102 one is dispensable.

### **Combined mutation of the proximal SF1 site and the adjacent GATA-binding motif**

To understand the difference between the two SF1 response elements, we examined the position of binding sites for other transcription factors on the human *AMH* promoter. We noticed the presence of a GATA-binding motif at -84, only 18 bases downstream of the proximal SF1 site (Figure 1 A). We proceeded to inactivate the -84 GATA and the -102 SF1 sites separately or together. Human *AMH* promoter activity was not significantly affected by the independent mutation of either the -102 SF1 ( $11 \pm 27\%$ ) or the -84 GATA ( $24 \pm 22\%$ ) binding sites, but a significant decrease was observed when both sites were mutated at the same time ( $54 \pm 9\%$ ,  $P < 0.05$ ) (Figure 3B). Thus, an intact -84 GATA-binding motif is able to compensate for a mutation of the -102 SF1 response element. In contrast, it does not rescue a mutation of the -228 SF1 binding site (Figure 3 A).

## Discussion

We report the first case of PMDS due to a regulatory mutation on the *AMH* promoter. Regulatory mechanisms of *AMH* transcription by Sertoli cells change in the course of development. After birth, testosterone inhibits and FSH stimulates AMH production, hCG or LH have no direct effect.

Androgen action is transduced through the androgen receptor in the Sertoli cells and requires intact SF1 binding sites on the *AMH* proximal promoter (32). SF1- and SOX9- response elements are also involved in *AMH* upregulation by FSH via cyclic AMP (see ref. (30) for details). However, because the fate of Müllerian derivatives is determined very early in fetal life, endocrine factors cannot explain the occurrence of PMDS. To block the regression of Müllerian ducts, *AMH* transcription in differentiating Sertoli cells should not be initiated at all.

The first stages of *AMH* transcription have been studied in Sertoli cell models and in transgenic mice. The human (33) and mouse (34) *AMH* promoters are very similar. Initially the proximal 180 bp were considered sufficient for a correct initiation of AMH expression by cultured mouse Sertoli cells (34) or by transgenic mice (35) though it was recognized later that additional upstream sequences are required to confer the correct temporal expression of the gene (36). The -151 SOX9 response element (Figure 1A) plays a fundamental role: its mutation in transgenic male mice blocks Müllerian regression (7) in keeping with the key role of SOX9 in Sertoli cell differentiation (37) and the initiation of *AMH* expression (7). SF1 is another important regulator of *AMH* transcription. In the mouse, mutation of the proximal -102 SF1 binding site decreases *AMH* transcription but still allows Müllerian regression to proceed (7, 10). Some promoters contain several binding sites for SF1, not necessarily of equal importance (38). As shown by Watanabe et al (9), the *AMH* promoter contains a second one, slightly upstream at -228. In their hands, mutation of either the -102 or the -228 sites yielded similar results, transcription efficiency was decreased but not abolished in cultured neonatal rat Sertoli cells.

However, the experiments were performed under non-optimal conditions: because of a very low transfection efficiency, adenoviral constructs were required to drive luciferase expression and a short 269-bp promoter was used. We ourselves performed preliminary experiments with a 435 bp promoter and obtained erratic results (not shown). Using the SMAT1 cell line, derived from neonatal mice, transfected with a natural human 3078-bp *AMH* promoter, we show that mutation of the distal -228,



but not of the proximal -102, site significantly reduces transcription efficiency (Figure 3 A). We also show, by EMSA, that binding of SF1 to the -228 site is abolished by the c.-225delA mutation present in our patient (Figure 2).

We then took a closer look at the environment of the SF1 response elements relative to binding sites for other transcription factors on the *AMH* promoter. Transcription factors often cooperate to enhance their activity (6, 39-42). Tremblay and Viger (8) have shown that GATA4 enhances *AMH* gene transcription through a direct protein-protein interaction with SF1, mediated through the zinc fingers of GATA4, an interaction which does not absolutely require binding to DNA. GATA4 mutants which lose their capacity to activate different promoters retain their ability to interact and cooperate with SF1, thereby compensating in part for the loss of transcriptional activity (43). Similarly, functional cooperation between GATA4 and cJUN on the mouse *Star* promoter does not require that both transcription factors simultaneously bind to response elements on DNA. On the *STAR* promoter, the GATA and cJUN binding sites are only 15 bases apart and this proximity is conserved across species, suggesting that cooperation is facilitated by the proximity of binding sites (41). The proximal *AMH* promoter contains 2 conserved GATA-binding motifs, at -418 and -84 and another one at -178 (Figure 1A) which exists on the human, bovine and porcine but not the rodent promoters.

We hypothesized that perhaps SF1 could activate *AMH* transcription even in the absence of a functional -102 binding site through interaction with GATA4 bound to its own specific response element. Human *AMH* promoter activity was not significantly affected by the independent mutation of either the -102 SF1 or the -84 GATA binding sites, but when both sites were mutated at the same time, the decrease was similar to that elicited by the mutation of the -228 SF1 response element (Figure 3B). In other words, in transactivation studies, an intact -84 GATA-binding motif is able to rescue a disrupted -102 SF1 response element. In contrast, disruption of the -228 SF1 response element cannot be compensated.

Other explanations cannot be ruled out. Experimentally, the damage inflicted by the simultaneous mutation of the -102 SF1 and the -84 GATA sites (Figure 3 B) could simply be due to an additive effect. Bouchard et al (43) have described GATA4 mutants that retained their ability to physically interact with SF1 thereby compensating for their loss of intrinsic transcriptional activity but

unfortunately the reverse has not been reported. On the other hand, the discrepancy between the our PMDS patient and transgenic mice (7, 10) could be due to species differences, not to the position of the SF1 element involved. For instance, SF1 null mice lack gonads and adrenal glands (44) while heterozygotes are phenotypically normal (45). In humans, however, heterozygotes are sexually abnormal, but their adrenal function is usually not affected (46). The p.R92W variant of the *NR5A1* gene coding for SF1 induces testicular development in 46,XX women but not female mice (47). Timing may be crucial. We do not know how much AMH is actually required to elicit human Müllerian regression. In transgenic mice with mutated GATA and SF1 binding sites, young fetal Sertoli cells still produced significant amounts of the hormone (10).

In conclusion, we describe the first case of PMDS due to a regulatory mutation. We show that there is a hierarchy of importance between the two binding sites for SF1 on the human *AMH* promoter and suggest, on the basis of transactivation studies, that it could be related to cooperation between adjacent SF1 and GATA response elements.

## Material and Methods

### *AMH* and *AMHR2* gene sequencing

DNA extraction from peripheral blood lymphocytes and PCR amplification of exons and intron-exon boundaries of *AMH* and *AMHR2* genes were performed as previously described (19). PCR products, purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, Calif., USA), were sequenced by Eurofins MWG (Ebersberg, Germany).

### Cell culture

SMAT1 cells, an immortalized immature Sertoli cell line of mouse origin (31), were cultured in Dulbecco Modified Eagle Medium (Gibco 11995-065, Invitrogen, Carlsbad, Calif., USA) supplemented with 10% fetal bovine serum (Natocor, Córdoba, Argentina), 2% MEM amino acids solution 50x (Gibco 11130051), 2% Amphotericin B (0.125 mg/ml, Sigma A-4888) and 1% Penicillin-

Streptomycin (Gibco 15070-063, 5000 U/ml) and maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### **Electrophoresis mobility shift assays (EMSA)**

SMAT1 cells were plated at 10<sup>7</sup> cells in 75cm<sup>2</sup> flasks and transiently transfected with 1 µg of the SF1 expression plasmid pCMV-SF1 (48) using the Lipofectamine 3000 (Invitrogen) method, following manufacturer's instructions. Briefly, one day after initial plating, DMEM with fetal calf serum was changed for DMEM without serum, and 24 hours later transfections were performed. Nuclear extracts from SF1-transfected SMAT1 cells were used to assess the functional relevance of SF1 sites present in the human *AMH* promoter.

Three oligonucleotides, respectively designed to contain the c.-225delA (patient), the wild type (normal control SF1 site) or the -228 (previously validated control for non-functional SF1 site, (30)) variants (Supplementary Table 1), were labeled with <sup>32</sup>P. SF1-transfected nuclear extracts (1, 5 or 10 µg) were incubated with the binding mixture containing a γ-<sup>32</sup>P-labeled probe (20,000 cpm) for 30 min. Competitive binding was performed under the same conditions, with the addition of 50- to 250-fold molar excess of unlabeled oligonucleotides. To test the specificity of the bands, EMSA supershift assays were carried out with 1 µg of KAL-KO611 anti-SF1 antibody (Cosmo Bio Company Limited, Tokyo, Japan) (49) or of nonimmune IgG. Electrophoresis was performed on a 5% polyacrylamide gel, and the gel then dried and subjected to autoradiography.

### **Targeted mutagenesis**

Plasmids with mutations in binding sites for SF1 and GATA4 of the human *AMH* promoter were generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). SF1 binding sites were mutated (CAAGG → GGTAC) at positions -102 and -228, and GATA binding site was mutated (AGATAG → GGTACC) at positions -84. Mutagenic oligonucleotide primers (Supplementary Table 2) were synthesized by Eurogentec (Liège, Belgium). Sequence changes were verified by direct sequencing.

### **Luciferase assays**

SMAT1 cells were plated at  $2 \times 10^5$  cells/well in 24 multi well plates and transiently transfected with 0.5  $\mu\text{g}$ /well of luciferase reporter plasmids under control of 3078 bp of the human *AMH* promoter, either wild-type or with mutations in the SF1 or GATA sites (Supplementary Table 3) by using the Lipofectamine 3000 (Invitrogen) method, following manufacturer's instructions. The construct pRL-TK (0.15  $\mu\text{g}$ /well) was used as transfection control, as previously described (30, 32). Briefly, 1 day after initial plating, DMEM with fetal calf serum was changed for DMEM without serum. Twenty-four hours later, transfections were performed. Luciferase activity was measured with the Luciferase Assay System (Promega, Madison, Wisconsin, USA) using the Synergy HTX multimode reader with the Gen5 software version 3.02 (BioTek Instruments Inc, Winooski, Vermont, USA.). Relative Luciferase Units (RLU) were defined as the normalization of firefly against renilla luciferase readings.

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## Conflicts of interest

The authors report no conflicts of interest.

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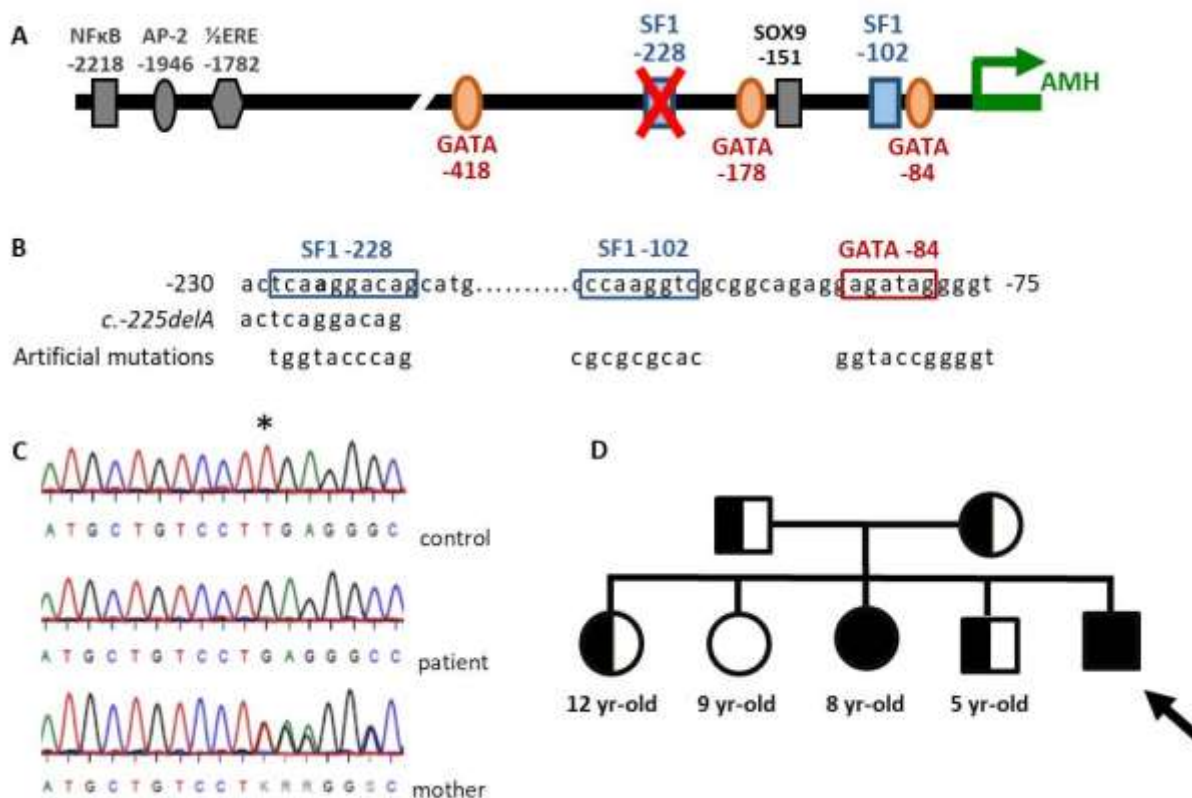
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**Figure 1. A:** Cartoon of the human *AMH* promoter showing binding sites for various transcription factors. The SF1 binding site mutated in our patient is crossed out.

**B:** Normal and mutated sequences of the canonical SF1 and GATA binding sites in the human *AMH* promoter. Upper line: wild-type sequence; middle line: c.-225delA mutation found in the PMDS patient bottom line: sequences artificially created carrying previously proven inactivating mutations of the -228 SF1 and -84 GATA sites.

**C:** Reverse sequencing results of the -228 SF1 site in control DNA, index case and his mother. Pattern in mother and in father (strictly identical to mother, not shown) corresponds to superposition of bases of control and deleted sequences after the deletion of the T marked by an asterisk in the control sequence.

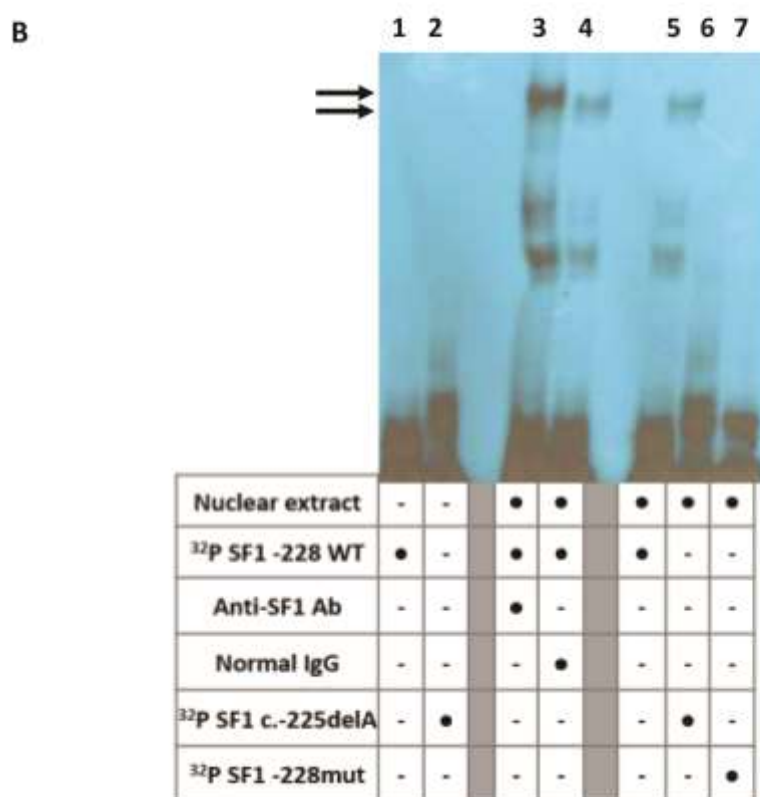
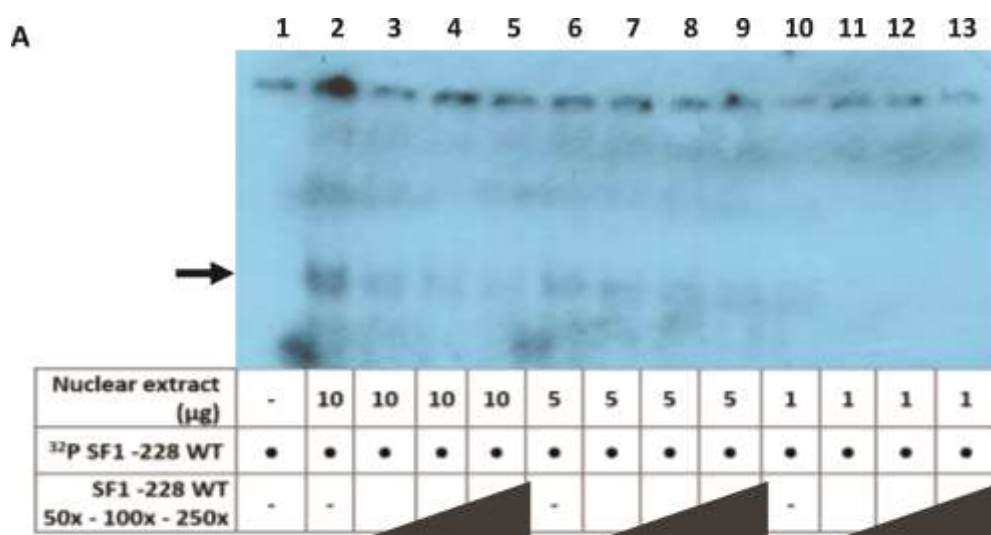
**D:** Pedigree of the family reported in this study. The index case is indicated by the arrow.



**Figure 2.** Electrophoresis mobility shift assays to test SF1 binding to the canonical SF1 site *tcaaggacag* sequence present at -228 of the human *AMH* promoter.

**A:** Nuclear extracts of SMAT1 cells (10, 5 or 1  $\mu\text{g}$ ) were incubated with a  $^{32}\text{P}$ -labeled DNA 27-mer probe encompassing the -228 wild-type SF1 site sequence and an excess (50x, 100x or 250x) of cold wild-type SF1 probe. The arrow indicates the band corresponding to the radiolabeled wild-type SF1 probe. Lane 1 contains no nuclear extract, and lanes 1, 2, 6 and 10 contain no excess SF1 probe.

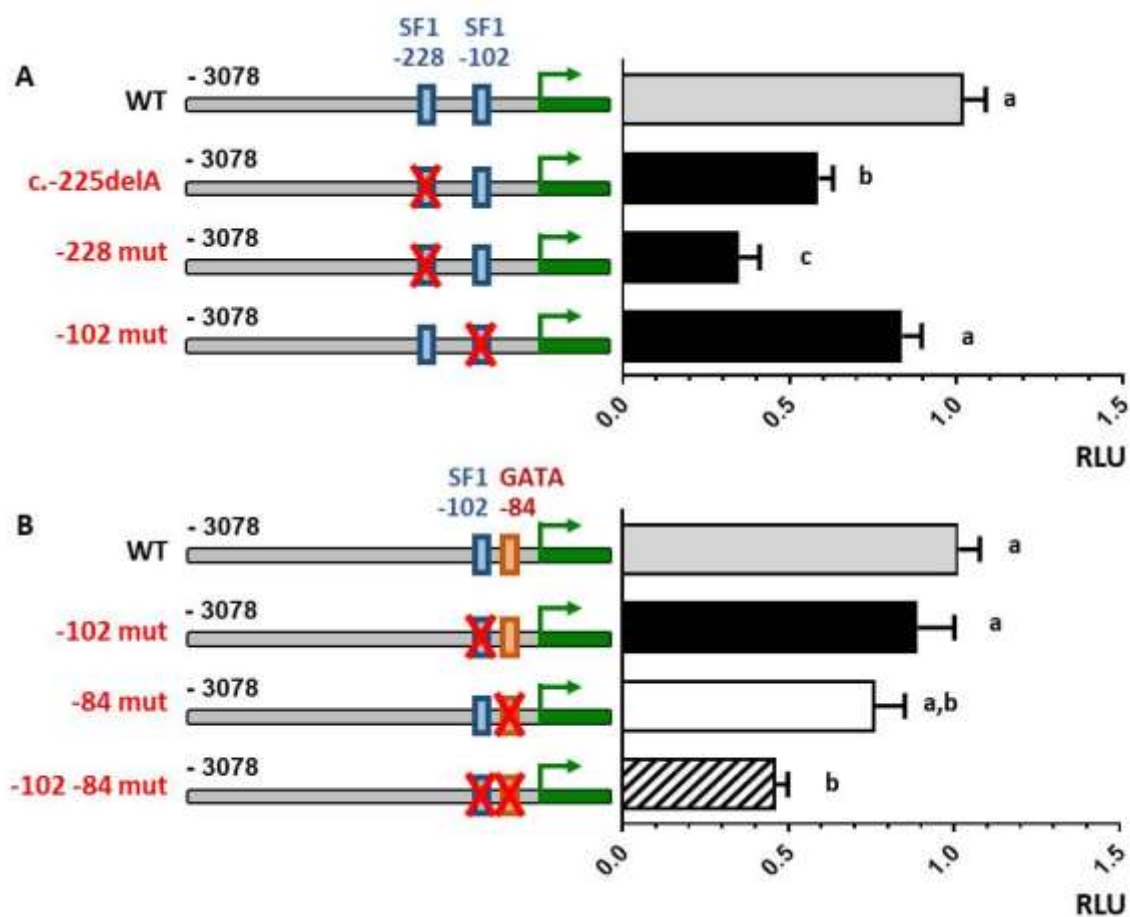
**B:** Nuclear extracts of SMAT1 cells (10  $\mu\text{g}$ ) were incubated with a  $^{32}\text{P}$ -labeled DNA 27-mer probe encompassing the -228 wild-type canonical SF1 site sequence or with  $^{32}\text{P}$ -labeled probes carrying the c.-225delA or a previously proven inactivated SF1 site (-228 mut). The arrows indicate the band corresponding to the radiolabeled wild-type SF1 probe (lane 3 corresponds to incubations containing an anti-SF1 antibody showing a super-shift, and lane 4 to incubations with a non-immune IgG). Lanes 1 and 2 contain no nuclear extracts.



**Figure 3.** Luciferase reporter assays to assess the effect of SF1 and GATA site mutations on a 3078-bp human *AMH* promoter.

**A:** Comparative effects of mutations on the distal (-228) or the proximal (-102) SF1 sites.

**B:** Comparative effects of mutations on the proximal (-102) SF1 site, its neighboring GATA site (-84) and both. Values as expressed as mean  $\pm$  SD of relative luciferase units (RLU). Different letters indicate statistically significant differences between columns ( $P < 0.05$ ), as analyzed by ANOVA followed by Tukey's multiple comparisons test.



## Abbreviations

AMH: anti-Müllerian hormone

AMHR2: AMH receptor type 2

bp: base pairs

DHT: dihydrotestosterone

EMSA: electrophoresis mobility shift assay

hCG: human Chorionic Gonadotropin

PCR: polymerase chain reaction

PMDS: Persistent Müllerian Duct Syndrome

StAR: Steroidogenic Acute Regulatory protein

SF1: Steroidogenic Factor 1

TGF $\beta$ : Transforming-Growth Factor  $\beta$