

Male-to-female sex reversal associated with an ~250 kb deletion upstream of *NROB1* (*DAX1*)

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Abstract Deletion of the dosage sensitive gene *NROB1* encoding *DAX1* on chromosome Xp21.2 results in congenital adrenal hypoplasia (AHC), whereas *NROB1* duplication in 46,XY individuals leads to gonadal dysgenesis and a female phenotype. We describe a 21-year-old 46,XY female manifesting primary amenorrhea, a small immature uterus, gonadal dysgenesis, and notably absent adrenal insufficiency with a submicroscopic (257 kb) deletion upstream of *NROB1*. We hypothesize that loss of regulatory sequences may have resulted in position effect up-regulation of *DAX1* expression, consistent with phenotypic consequences of *NROB1* duplication. We propose that this genomic region and by extension those surrounding the dosage sensitive *SRY*, *SOX9*, *SFI*, and *WNT-4* genes,

should be examined for copy-number variation in patients with sex reversal.

Introduction

Rare individuals with discordance between reproductive system phenotypes and chromosome complements (“inter-sex” conditions) have provided insights into the basic mechanisms of reproductive system development. 46,XX sex reversal is rare (~1:20,000 newborns) whereas male-to-female (46,XY) sex reversal is more common (~1:3000 newborns) (Camerino et al. 2006). Loss of function for *SRY* in 46,XY individuals leads to a female phenotype (Berta et al. 1990). Similarly, XY sex reversal is found in two thirds of subjects with mutations of *SOX9* and campomelic dysplasia (CD) (Mansour et al. 1995). In contrast, gain of *SRY* (Sinclair et al. 1990) or *SOX9* (Huang et al. 1999) in XX individuals leads to development of a male reproductive system. Duplication of a 160 kb genomic region in Xp21.2 containing the *NROB1* gene (which encodes *DAX1*) was found to cause dosage-sensitive sex reversal (DSS) in 46,XY individuals, raising the possibility that *DAX1* has an “anti-testis” function that could override male reproductive system development when present in excess copy-number (Bardoni et al. 1994). Furthermore, XY sex reversal in humans was also shown to result from mutations and deletion in steroidogenic factor-1 *SFI* (*NR5A1*) (Achermann et al. 1999; Schlaubitz et al. 2007) and duplication of *WNT-4* (Jordan et al. 2001). Recently, Parma et al. (2006) described a complete female-to-male sex reversal in the absence of *SRY* resulting from mutation in *R-spondin1* (*RSPO1*). However, in approximately 75% of patients with sex reversal, the cause remains unknown (Vilain et al. 1998).

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Phenotypic females with 46,XY chromosome complements come to medical attention in a variety of ways, sometimes as teenagers or young adults for primary amenorrhea or an infertility evaluation. Establishing a diagnosis in these cases is important, as some XY sex reversal patients are at increased risk for gonadoblastoma and require gonadectomy. Given the importance of gene dosage in reproductive system development, those individuals in whom an initial workup is unrevealing may benefit from additional diagnostic studies including microarray-based comparative genome hybridization (array-CGH) to evaluate for submicroscopic genomic deletions or duplications.

Material and methods

Patients

The proband, a 21-year-old female with primary amenorrhea, was referred to genetics clinic after she was found to have a 46,XY chromosome complement. Fluorescence in situ hybridization (FISH) analysis with a probe for *SRY* ruled out deletion of *SRY* or any significant degree of mosaicism. Complete bidirectional sequencing of the coding region of *SRY* did not identify any mutations.

Her gestational history, growth and developmental milestones, and childhood medical history were essentially normal, but she had delayed onset of puberty. She began to see axillary and pubic hair development at age 15, followed shortly thereafter by breast development. She was evaluated by an endocrinologist for primary amenorrhea and was found to have elevated follicle stimulating hormone (51.3 U/L—range for ovarian failure 16.6–115) and leuteinizing hormone (12.5 U/L—range for ovarian failure 11.3–55.9), consistent with ovarian failure. Serum estradiol was unmeasurable (<50 pmol/L). DHEA sulphate was 3.7 µmol/L (normal 1–12) and free testosterone was 2.3 pmol/L (normal 0.5–13.2) both normal values for pubertal females. The patient had a normal ACTH (Cortrosyn) test. Cortisol baseline was 626.52 nmol/L (AM cortisol 185–624, PM < 280), 551.97–30 min post Cortrosyn and 585.40–60 min post Cortrosyn. Suspicion for primary ovarian failure prompted a pelvic ultrasound that revealed a small immature uterus and reportedly normal right ovary with several tiny follicular structures. The left ovary was not visualized.

Physical examination revealed height and weight greater than 95th percentile, nondysmorphic facial features, and normal examination of the heart, lungs, abdomen, and extremities. She was found to have appropriate secondary sexual characteristics (axillary and pubic hair, normal breast development). Her vaginal introitus was too small to permit a speculum examination.

A pelvic MRI confirmed the previous ultrasound results, but the right gonad was characterized as somewhat ovoid and suspicious for testicular tissue. Because of the concern for possible malignancy (gonadoblastoma), the patient was offered gonadectomy and underwent an exam under anesthesia at the time of surgery. The vaginal introitus was small but the vagina was otherwise of normal dimensions and led to an immature cervix. At laparotomy, she was noted to have thin round ligaments and rudimentary fallopian tubes that did not end in fimbriae. A streak gonad was identified in the usual position of the left ovary and a gonad with the appearance of a testis was identified on the right.

The proband is now a 21-year-old and will soon graduate from college. For 3 years she has undergone estrogen replacement and had regular menstruation. An extended pedigree was obtained during the course of her evaluation and was significant for a 16-year-old sister who began menses at 14 years of age and for the presence of ovarian cysts in her mother. Her mother has a large number of brothers and sisters, all of whom have children. Of note, the proband also has a female cousin (the daughter of one of the maternal aunts) with ovarian cysts. Unfortunately, the proband's mother declined further genetic testing in other family members, including her younger daughter.

We obtained samples from the proband and her mother after acquiring informed consent approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine and appropriate institutions.

High-resolution human genome analysis

To further evaluate the etiology of the patient's sex reversal, her DNA was examined by array-CGH, a new technology that permits simultaneous evaluation of large numbers of chromosome loci for copy number imbalance. The clinical microarray (Cheung et al. 2005; Lu et al. 2007) contains 853 BAC and PAC clones designed to cover genomic regions of 75 known genomic disorders (Lupski and Stankiewicz 2006), all 41 subtelomeric regions, and 43 pericentromeric regions, including 13 clones spanning the genomic region of chromosome Xp21.1-p21.2, encompassing the *NR0B1*, glycerol kinase (*GKI*), and Duchene muscular dystrophy (*DMD*) genes (Baylor College of Medicine, Chromosome Microarray Analysis, V.5, <http://www.bcm.edu/cma/assets/abnormalities.pdf>).

FISH analysis

FISH was performed on PHA-stimulated peripheral blood lymphocytes obtained from the patient and her mother using a standard protocol. BAC clone RP11-420P1 specific for the *NR0B1* (*DAX1*) region was identified from the existing physical map (UCSC genome browser, <http://www.genome.ucsc.edu>).

PCR and sequence analysis

Genomic and BAC clone (RP11-662D2) DNAs were extracted (Qiagen), PCR was performed using several primer sets (sequences available upon request), and the deletion rearrangement junction was sequenced. Genomic sequence information was downloaded from the UCSC genome browser (May 2004 freeze), and assembled using Sequencher (Gene Codes) and NCBI BLAST 2 (<http://www.ncbi.nlm.nih.gov/blast/>).

The genomic region upstream to *NROB1* was analyzed for copy-number variation (CNV) using the Database of Genomic Variants <http://www.projects.tcag.ca/variation/>.

Searches for potential regulatory elements and evolutionarily conserved sequences in the deleted fragment were performed using the UCSC database with a seven-way regulatory potential (RP) analysis. By comparing human, chimp, macaque, dog, cow, mouse, and rat syntenic DNA sequences, the RP score predicts DNA elements with significantly reduced background, as compared with the results of other similar programs (Kolbe et al. 2004).

Results

Pathological examination of the left streak gonad revealed an irregular mass of fibromembranous tissue that resembled neither testes nor ovaries, with lack of seminiferous tubules or ovarian follicles. In some areas there were loosely associated collections of tubules with a fibrovascular stroma consistent with a rudimentary epididymis (Fig. 1).

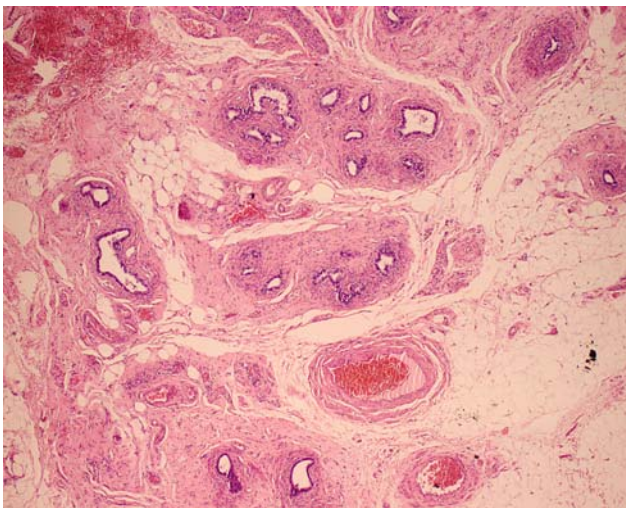


Fig. 1 A histopathology picture of the left streak gonad. Note the irregular mass of fibromembranous tissue that resembles neither testes nor ovaries, with lack of seminiferous tubules or ovarian follicles. There is a loosely associated collection of tubules with a fibrovascular stroma consistent with a rudimentary epididymis

Identification of a deletion in the genomic region surrounding *NROB1*

Surprisingly, initial examination of the array-CGH in the patient showed a loss of one BAC clone RP11-662D2 that harbors *NROB1* (Fig. 2a, d) whereas FISH with this clone showed a fluorescent signal on Xp21 (Fig. 2a, e). To clarify this discrepancy, we analyzed several genetic markers from BAC RP11-662D2 by PCR. The *NROB1* gene was intact; however, we found a loss of chromosome material proximal to *NROB1*. Deletion breakpoints were mapped using a standard PCR walking approach. DNA sequencing of a 306 bp PCR product that spans the junction revealed that the distal deletion breakpoint mapped within a unique sequence 11,320 bp upstream (proximal) to *NROB1*, ~2.5 kb from a large ~3.9 kb repetitive LINE/L1 element LIPA3 (Figs. 2b, 3). The deletion truncated 53% of the BAC clone RP11-662D2, which explains the apparent contradictory results of array-CGH and FISH and highlights the sensitivity of array-CGH to detect copy-number changes. The signal intensity of the BAC clone RP11-662D2 was likely decreased; however, it was overlooked because it could not have been compared with the signal on the normal X chromosome. The higher sensitivity of BAC array CGH than FISH has been demonstrated recently by Johnston et al. (2007). To increase the sensitivity and thus the detection rate of array CGH, a greater number of shorter overlapping probes such as oligonucleotides instead of BAC clones should be applied (Ylstra et al. 2006; Hehir-Kwa et al. 2007).

The second deletion breakpoint mapped within a repetitive *AluY* sequence, 257,782 bp from the distal breakpoint, leaving the *GKI* gene ~75 kb upstream of *NROB1* intact. Parenthetically, as anticipated, triglyceride levels (glycerol) were normal. No additional nucleotides were found at the junction (Fig. 2b). The presence of the deletion was then confirmed by FISH with BAC clone RP11-420P1, which completely maps within the deleted fragment. To test whether the deletion in the proband was a de novo event, samples from the mother were examined by PCR for the identified junction fragment and interphase FISH using clone RP11-420P1 and she was found to carry the deletion (Fig. 2c, f).

To address whether the identified deletion was a benign CNV, we added the BAC clone RP11-420P1 to the next version (V.6.0) of the clinical microarray. None of the 1,184 individuals analyzed demonstrated abnormal copy-number. In addition, no CNV in the region deleted in the described family was found in the Database of Genomic Variants. These data indicate that this genomic region is not polymorphic and further confirm its potential causative role in the phenotype in the described family.

To further investigate the consequences of the deletion of noncoding sequences adjacent to *NROB1*, we performed

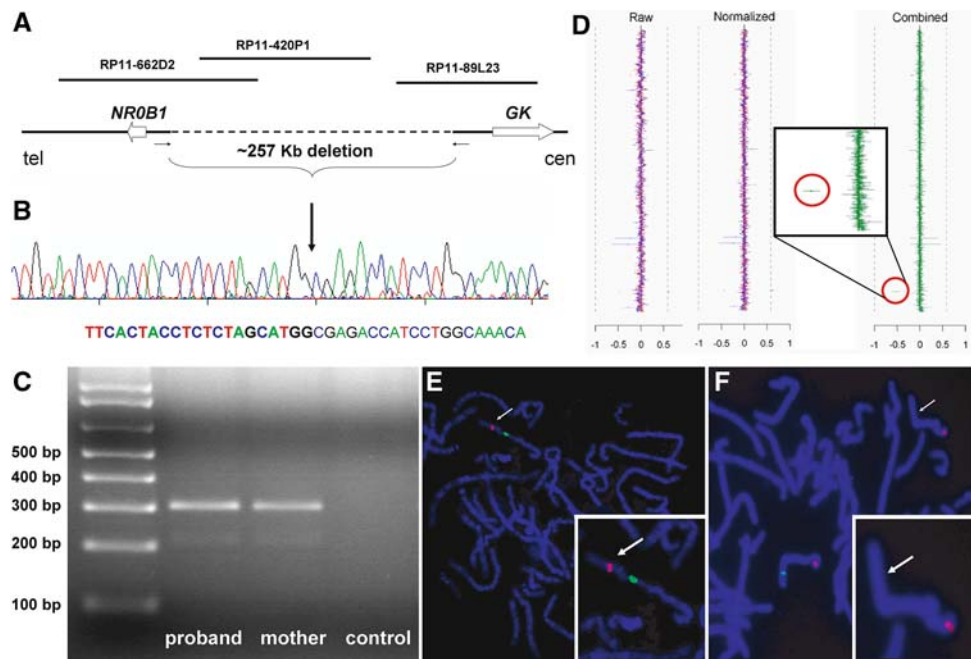


Fig. 2 Genetic analyses. **a** Schematic representation of the analyzed genomic region between the *DAX1* and *GK* genes on Xp21.2. *Open horizontal arrows* depict the genes with the *arrowhead* in the direction of transcription. *Horizontal arrows* show the positions of primers used to amplify the breakpoint junction. The BAC clones used in array CGH, FISH, and PCR analyses are shown above. **b** Chromatogram of the DNA sequence of the junction fragment. The *vertical black arrow* indicates the junction point. **c** The 1.5% agarose gel with the 306 bp PCR products that span the junction fragment found in the proband and her mother. **d** Array based comparative genomic hybridization. This profile represents two hybridizations performed simultaneously with dye reversal using reference DNA. In the column marked “raw” for raw data, the mean values of the test/reference ratio and *error bars* in a hybridization experiment are shown in *blue* and dye reversal is shown in *red*. The effect of normalization is shown by comparing the middle set of data marked “normalized” with the “raw” data. There is one

clone from Xp21.2 that shows displacement to the left in *red* and to the right in the dye reversal, both indicating a loss of Xp material in the patient versus the reference DNA. In the “combined” column, the sign of one of the two reversed hybridizations is changed and the data are averaged with gains shown to the *right* and losses to the *left*. For the combined data, there is a strong indication of a loss detected with the clone RP11-662D2. **e** Proband’s metaphase chromosomes after FISH with BAC clone RP11-662D2, specific for *DAX1*, showed the presence of the fluorescence signal (*white arrow*). The *DXZI* centromeric probe (*green*) was used as a control. The abnormal X chromosome is shown enlarged in a separate inset. **f** FISH on maternal lymphocytes with BAC clone RP11-420P1 (*green*) mapping within the deleted fragment confirmed that the mother is a carrier of the deletion (*white arrow*). The clone RP11-25E18 (*red*) located at chromosome Xq27.3 was used as a control probe. The abnormal X chromosome is shown enlarged in a separate *inset*

extensive *in silico* analysis of this region using the UCSC Genome Browser and Sequencer. In the deleted fragment, we identified 27 potential SF1 consensus-binding sites (TGAAATCA; CCAAGGTCA); however, these are known to be promiscuous. Using seven-way RP and comparative genomics in vertebrate organisms, we found several evolutionarily conserved segments and potential *cis*-acting regulatory elements. Interestingly, the conserved and potential regulatory elements, and SF1 binding sites overlapped in four different regions (Table 1, Fig. 3).

Discussion

NROB1 encodes DAX1, a member of the orphan nuclear receptor family whose functions include roles in hypothalamic, pituitary, adrenal and gonadal development, as well as transcriptional regulation in early embryo maintenance

(Niakan and McCabe 2005). DAX1 is expressed in the segment of the adrenogenital primordium from which the gonadal ridge forms, where it clearly plays an important role in sex determination. However, its expression becomes sexually dimorphic and seems to disappear concurrently with differentiation of the testes and to persist in the ovaries (Swain et al. 1996). Expression of DAX1 is strongly dependent on SF1, a master regulator of the adrenal and gonadal systems, likely through binding of SF1 to a highly conserved consensus site ~4 kb upstream of *NROB1* (Hoyle et al. 2002). WT1 has been reported also to activate promoter sequences near the *NROB1* TATA box (Kim et al. 1999). However, the factors that bring about down-regulation of DAX1 during development have yet to be identified.

DAX1 is generally postulated to have a repressive effect on transcription through its interactions with multiple network partners (reviewed in Clipsham and McCabe 2003; McCabe 2007). Among its numerous target genes is Mülle-

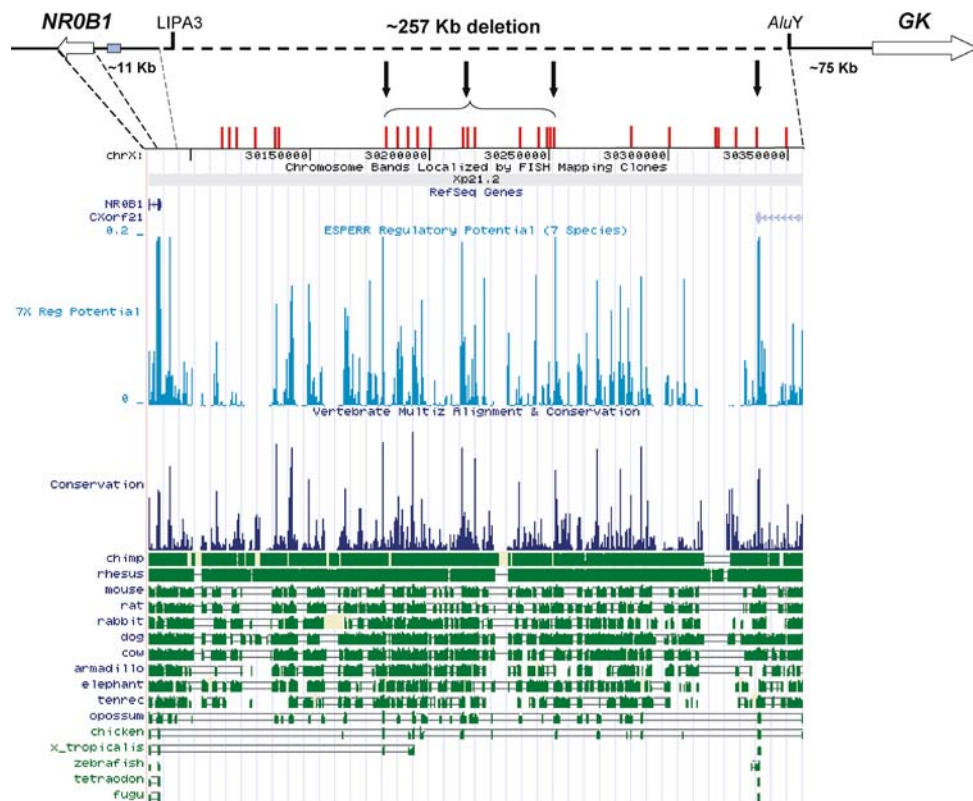


Fig. 3 Computational analysis of the potential regulatory elements, conserved sequences, and SF1 consensus binding sites in the region analyzed on Xp21.2. UCSC graphical representation (May 2004 freeze) of the genomic region between *DAX1* and *GK* genes with the identified ~257 kb deletion in between. The evolutionary conserved sequences regions are depicted in *black* and high seven-way RP potential regulatory elements (human, chimp, macaque, dog, cow, mouse, and rat) values are shown as *blue*. Consensus binding sites for SF1 pro-

tein are shown with *red lines*. SF1 binding site identified by Hoyle et al. (2002) 4 kb upstream of *DAX1* as a strong promoter of *DAX1* expression is depicted as a *dashed box*. Note clustering of binding sites in the middle of the deleted fragment. The repetitive sequences *LIPA3* and *AluY* at the breakpoints are shown as *vertical bars*. Map not to scale. *Four vertical arrows* depict the regions where the conserved, RP elements, and SF1-consensus binding sites overlap (Table 1)

rian Inhibiting Substance (MIS), which *DAX1* down-regulates by inhibiting the synergistic interactions between SF1 and WT1 (Nachtigal et al. 1998) and between SF1 and GATA-4 (Tremblay and Viger 2001). Antagonism of MIS promotes the development of female internal reproductive system structures and thus one role of *NROB1* seems to be that of an “anti-testis” gene. Consistent with this hypothesis, increased gene dosage of *NROB1* (and presumably therefore increased *DAX1* protein expression) causes gonadal dysgenesis and female phenotype in XY individuals (Bardoni et al. 1994; Swain et al. 1996; Sanlaville et al. 2004).

Conversely, loss of *DAX1* protein function leads to a completely different phenotype, adrenal hypoplasia congenita (AHC) characterized by small adrenal glands and adrenal insufficiency that can be lethal in early infancy if untreated (Muscatelli et al. 1994; Zanaria et al. 1994). Males with *DAX1* mutations also have hypogonadotropic hypogonadism and disorganized testis cords. *DAX1* is critical for normal testis development in mouse (Meeks et al. 2003) and SF1 and *DAX1* play a cooperative role in mouse

testis differentiation (Park et al. 2005). Thus, *NROB1* also has a pro-testis function, suggesting that the temporal and spatial context of *DAX1* function results in a narrow window of activity necessary for proper testis development (Meeks et al. 2003; Ludbrook and Hurley 2004).

How could a deletion upstream of the *NROB1* gene result in a female phenotype? We propose that the deletion removes negative *cis*-regulatory elements that control the spatial or temporal expression of the *DAX1* protein, resulting in an increase in *DAX1* expression, either globally or restricted to the tissues giving rise to the reproductive system. The unavailability of such tissues from this patient precludes molecular documentation, but the absence of adrenal insufficiency for 21 years certainly argues against *DAX1* loss-of-function. Interestingly, in the deleted fragment, we found a cluster of SF1 consensus-binding sites, several evolutionarily conserved segments in vertebrates and potential regulatory elements (Fig. 3). In support of this notion, using the 3C (Capture Chromosome Conformation) technique (Dekker et al. 2002), we have found an indication for a physical proximity between the SF1 consensus binding

Table 1 Summary of the regulatory potential (longer than 120 bp) and conserved elements (longer than 280 bp), and SF1 binding sites identified in the deleted region. Only the overlapping regulatory potential and conserved elements are shown. Nucleotide numbering is based on human genome, build 35 (UCSC genome browser, March 2004). In bold are loci in which these elements overlap with SF1 binding sites

Conserved elements	Regulatory potential elements	SF1 binding sites
		30,112,226–30,112,233
		30,116,799–30,116,806
		30,118,154–30,118,162
		30,126,492–30,126,499
30,135,429–30,136,035	30,135,729–30,135,870	30,135,187–30,135,194
		30,136,720–30,136,727
30,141,920–30,142,500	30,142,150–30,142,270	
30,180,175–30,180,500	30,180,110–30,180,410	
		30,182,857–30,182,864
		30,187,183–30,187,190
		30,189,382–30,189,389
		30,194,836–30,194,843
		30,201,108–30,201,115
		30,215,362–30,215,369
		30,216,853–30,216,861
		30,217,766–30,217,773
30,218,745–30,219,220	30,219,050–30,219,230	30,219,169–30,219,176
		30,237,617–30,237,624
		30,245,987–30,245,995
		30,250,306–30,250,314
		30,250,507–30,250,515
30,252,700–30,252,980	30,252,490–30,252,930	30,252,876–30,252,884
30,269,855–30,270,140	30,269,865–30,270,130	
30,279,668–30,279,946	30,279,645–30,279,767	
		30,284,311–30,284,318
30,288,290–30,288,715	30,288,350–30,288,575	
		30,300,405–30,300,412
		30,320,912–30,320,919
		30,321,701–30,321,708
		30,328,749–30,328,756
30,337,000–30,338,200 (<i>CXorf21</i>)	30,337,350–30,337,896 (<i>CXorf21</i>)	30,337,011–30,337,018 (<i>CXorf21</i>)
		30,349,655–30,349,662

sites clusters and *DAX1* (Smyk et al., manuscript in preparation). However, the centromeric side of the deleted region contains a predicted gene *CXorf21*. Nevertheless, *CXorf21* is not expressed in testes, ovaries, adrenal cortex, pituitary gland, nor uterus, thus its causative role in our case is unlikely.

It should be noted that the SF1 binding site identified by Hoyle et al. (2002) as a strong promoter of *DAX1* expression is not deleted in our proband. It is therefore possible that binding of SF1 and other factors to the proximal regulatory elements could promote *DAX1* expression early in gonadal development, while binding to the distal conserved region described above could down-regulate *DAX1* expression. Alternatively, the deletion could have removed a boundary element (insulator) and/or brought a genital ridge-specific long-range enhancer element in proximity to

NROB1 causing loss of *DAX1* expression with no effect upon the adrenal.

Of note, dosage sensitive genes are particularly likely to be susceptible to position effects (Kleinjan and van Heyningen 2005). A similar phenomenon was reported recently in a patient with male-to-female sex reversal and CD in whom an ~1.5 Mb microdeletion was identified ~380 kb upstream of dosage-sensitive *SOX9* (Pop et al. 2004). XY sex reversal has been described also in CD patients with apparently balanced chromosome translocations and breakpoints scattered up to 1 Mb upstream and even >1 Mb downstream of *SOX9*, leaving the gene itself intact (Velagaleti et al. 2005; Lupski and Stankiewicz 2006).

Given what is known about the dosage-sensitive nature of this region, excess *DAX1* expression (in this case due to

a transcriptional regulatory mechanism rather than gene CNV; duplication of *NROB1* causes male-to-female sex reversal in 46,XY individuals) is the most parsimonious explanation for the cause of the gonadal dysgenesis and sex reversal seen in our proband. The presence of the deletion in the proband's mother is intriguing given that DAX1 has suspected functions in adult ovary (Tajima et al. 2003) and pituitary gland (Dorn et al. 1999); it is tempting to speculate that DAX1 overexpression in these tissues could contribute to the mild phenotype of ovarian cysts.

In this post-genomic era it is important to recognize that submicroscopic DNA rearrangements outside the coding region of a dosage sensitive gene can be responsible for clinical phenotypes (Lee et al. 2006). High-resolution genome analysis by array CGH provides a rapid and effective means to identify copy-number imbalance. We propose that in patients with sex reversal, in addition to standard karyotype analysis and DNA mutation screening, one should search for submicroscopic genomic deletions or duplications within the sex determination/differentiation dosage-sensitive genes as well as their flanking genomic regions.

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