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## **Testis development in the absence of *SRY*: chromosomal rearrangements at *SOX9* and *SOX3***

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## ABSTRACT

Duplications in the ~2 Mb desert region upstream of *SOX9* at 17q24.3 may result in familial 46,XX Disorders of Sex Development (DSD) without any effects on the XY background. A balanced translocation with its breakpoint falling within the same region has also been described in one XX DSD subject. We analyzed by conventional and molecular cytogenetics 19 novel *SRY*-negative unrelated 46,XX subjects, both familial and sporadic, with isolated DSD. One of them had a de novo reciprocal t(11;17) translocation. Two cases carried partially overlapping 17q24.3 duplications about 500 kb upstream of *SOX9*, both inherited from their normal fathers. Breakpoints cloning showed that both duplications were in tandem whereas the 17q in the reciprocal translocation was broken at about 800 kb upstream of *SOX9*, close to a previously described 46,XX DSD translocation but also to translocations without any effects on gonadal development. A further XX male, ascertained because of intellectual disability (ID), carried a de novo cryptic duplication at Xq27.1 involving *SOX3*. CNVs involving *SOX3* or its flanking regions have been reported in four XX DSD subjects. Collectively in our cohort of 19 novel cases of *SRY*-negative 46,XX DSD, the duplications upstream of *SOX9* account for about 10.5% of the cases and are responsible for the disease-phenotype even when inherited from a normal father. Translocations interrupting this region may also affect gonadal development, possibly depending on the chromatin context of the recipient chromosome. *SOX3* duplications may substitute *SRY* in some XX subjects.

## INTRODUCTION

46,XX DSDs are congenital conditions in which, in the presence of a female karyotype, the development of gonadal and anatomical sex is atypical, ranging from various degrees of ambiguous genitalia to phenotypic males with azoospermia. These conditions are poorly characterized, at least in subjects whose DNA does not contain *SRY*, the gene triggering testis differentiation in mammals.<sup>1</sup> In fact, in most XX males *SRY* is transposed to the tip of Xp as a consequence of a recurrent Xp;Yp translocation arising predominantly by non-allelic homologous recombination between *PRKX* and *PRKY* on a particular Y haplotypic background.<sup>2,3</sup> These males, usually with small testes, are essentially picked up among men with non-obstructive azoospermia.

A much less well understood category is that of the 46,XX DSDs negative for *SRY*. Recently six of these cases have been reported carrying partially overlapping amplifications of a gene-desert region located about 500 kb upstream of *SOX9*.<sup>4-7</sup> It has been proposed that these CNVs could be responsible for altered expression of *SOX9* in the developing gonad on an XX background. Some of the reported cases represent familial 46,XX DSDs, since normal and fully fertile XY fathers can carry this CNV.

In the XY early gonad, after an initial activation by *SRY*, *SOX9* maintains its expression thanks to a positive feedback loop triggering the pathway of testis differentiation.<sup>8</sup> Actually, *SOX9* transgenic expression in XX gonads is sufficient to induce testis formation in mice.<sup>9</sup> Moreover, a single case of an XX boy is reported with a >11 Mb duplication including *SOX9*.<sup>10</sup> *SOX9* is also involved in several processes during embryo development and defects of this gene are responsible for campomelic dysplasia with/without XY sex reversal (OMIM:114290). This gene has a large upstream desert region of about 2 Mb enriched in tissue- and time-specific regulatory elements, as it can be deduced by different abnormal phenotypes associated with CNVs or reciprocal translocations interrupting the region itself. Among them, isolated Pierre Robin (PRS) sequence (OMIM:261800), congenital heart defects (CHD), and XY sex reversal have been associated mainly with deletions of the region and in few cases with reciprocal translocations,<sup>4,11-13</sup> whereas duplications have been reported both in familial cases of brachydactyly-anonychia<sup>14</sup> and in the

abovementioned XX subjects with DSD. In the latter category, a single case with a reciprocal translocation interrupting the desert region about 800 kb upstream of *SOX9* has also been described.<sup>15</sup>

Recently a role in the developing gonad has also been proposed for *SOX3* (Sry-related HMG box-containing gene 3), a gene closely related to both *SOX9* and *SRY*. Ectopic expression of Sox3 in the mouse bipotential gonad frequently leads to complete XX sex reversal.<sup>16</sup> Moreover, duplications of *SOX3* or its 5' region have been reported in 46,XX DSD patients.<sup>16,17</sup>

Here we present two new subjects with cryptic duplications upstream of *SOX9* that were identified among 19 unrelated novel cases with 46,XX isolated DSDs, either familial (2 cases) or sporadic (17 cases), all *SRY*-negative. In the same cohort we also identified the second case of reciprocal translocation upstream of *SOX9* causing XX DSD. A further 46,XX boy with intellectual disability and a cryptic duplication including *SOX3* is also presented.

Our data confirm that CNVs and structural rearrangements (cases 1-3) involving desert regions are responsible for developmental defects by dysregulation of non-coding *cis*-regulatory elements<sup>18</sup> and that 46,XX *SRY*-negative DSD individuals present a duplication upstream of *SOX9* in at least 10.5% of cases. The role of CNVs in gonadal disorders is further stressed by case 20.

## **SUBJECTS AND METHODS**

### ***Patient samples***

Patients were collected during more than 20 years. The study was approved by the Ethical Committee of the University of Pavia. Written informed consent was obtained from all patients.

### ***Conventional and molecular cytogenetics***



Conventional cytogenetics was done on GTG banded metaphases. Molecular karyotyping was performed for cases 1-3 and for all cases reported in Supplementary Table 1 by using oligonucleotide array-CGH platforms (180K SurePrint G3 Human Kit, Agilent Technologies, Santa Clara, CA). For case 20, the trio analysis was performed using the Genome Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA). See Supplementary Material for the details. FISH experiments were performed on case 3 as reported<sup>19</sup>, by using the following probes: RP11-238F2, RP11-589A10, CTD-2652P12, RP11-879D6, RP11-474K15, RP11-676K3, RP11-13H17, RP11-661B15, RP11-103P20, RP11-36H11. *SRY* analysis was done as reported in Supplementary Material.

The observed CNVs have been submitted to the public database DECIPHER (<http://decipher.sanger.ac.uk>; IDs from case 1 to case 20: 293610, 293615, 293631, 293646).

### ***X-inactivation analysis***

X-inactivation analysis was performed for case 20 according to Allen et al (1992).<sup>20</sup>

### ***Breakpoint mapping***

For cases 1 and 2, we used quantitative PCR (qPCR) to verify and restrict the breakpoint regions characterized by array-CGH, followed by Long-Range PCR.<sup>21</sup> To clone case 3 breakpoint junctions, we used pooled long-range PCR reactions (See Supplementary Material). Target sequences for quantitative PCR (qPCR) analysis were selected using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Long-range PCRs were performed with JumpStart Red ACCUTaq LA DNA polymerase (Sigma-Aldrich, St. Louis, MO). PCR products were analyzed on 0.8% agarose TAE gels. The UCSC Genome Browser (hg19 assembly) maps and sequence were used as reference. Sequencing reactions were performed with a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and run on an ABI Prism 3500DX/XL Genetic Analyzer.

### ***In silico TFBSs evaluation***

For case 3, the presence of potential TFBSs (Transcription Factor Binding Sites) altered by the translocation breakpoints was evaluated by using the TFSEARCH tool (<http://www.rwcp.or.jp/papia/>).<sup>22</sup> In cases 1-3 the region involved by the rearrangement was also evaluated in the light of Integrated Regulation from ENCODE, HMR Conserved Transcription Factor Binding Sites and Human Body Map lincRNAs and TUCP Transcripts tracks embedded in the UCSC Genome Browser (<http://genome.ucsc.edu/>).

## **RESULTS**

### ***Clinical Description***

**Case 1.** The patient, a normal adult male, was investigated because of infertility. Physical examination showed normal male secondary sexual characteristics and bilateral gynecomastia. Laboratory investigations showed azoospermia, low serum testosterone and increased FSH and LH.

**Case 2.** The patient was born at term after two normal female children from non-consanguineous parents. A threatened abortion in the first trimester is documented. External genitalia were ambiguous with hypertrophic clitoris, single meatus, and urogenital sinus. Ecography showed absent uterus, vaginal atresia and two ovoidal gonads detected in the inguinal canal. At 8 months of age hormonal values were the following: LH was below 0.1 mU/ml (normal range <0.1-6 mU/ml), FSH was 0.8 mU/ml (normal range <0.1-18 mU/ml), testosterone was below 10 ng/dL (normal ranges: 12-21 ng/dL males, 6-82 ng/dL females), androstenedione was below 30 ng/dL (normal range 40-260 ng/dL), estradiol was below 25 pg/ml (normal values <25 pg/ml), anti-Müllerian hormone (AMH) was above 21 ng/ml (normal ranges: 84-141 ng/ml

males, < 0,14 ng/ml females). The testosterone response to human chorionic gonadotropin (hHCG) administration was low: 76 ng/dL (normal values >100 ng/dL). After hHCG stimulation the left gonad showed the presence of follicles and assumed the aspect of an ovotestis at echography, whereas the right one appeared as a testicle. Bilateral gonadal biopsy was performed at one year of age. Both gonads were presented with the caudal portions macroscopically compatible with male gonads and the cranial ones with female gonads. At histological examination the caudal segments showed testicular tissue with prepubertal seminiferous tubules, whereas the cranial ones showed ovarian tissue with numerous oocytes (Supplementary Figure 1).

**Case 3.** The patient was ascertained in adult age because of infertility. His height was of 171 cm, between the 10<sup>th</sup> and the 25<sup>th</sup> centile, much shorter than expected on the basis of the mid-parental height (183 cm). He presented with infertility and erectile dysfunction, loss of libido, and asthenia. On general examination the patient had mild dysmorphisms such as micrognathia, hypertelorism, short neck. He had bilateral testicular hypotrophy, normal penis, and absence of gynecomastia. FSH (53.3 mU/mL, normal values 0.7-11.1), as well as LH (19.4 mU/mL, normal values 0.8-8.0) and androstenedione (13.1 nmol/L, normal values 2.1-10.8) levels were elevated, whereas he had low serum testosterone (210 ng/dL, normal values 260-1600). The patient also suffered from osteopenia.

**Cases 4-19.** These cases, all with normal 46,XX karyotype and *SRY* negative were ascertained because of ambiguous genitalia, or hypogonadism, or azoospermia. In all of them genomic arrays did not show any significant CNV. Their clinical details are summarized in Supplementary Table 1. Some of them, previously published, have now been tested by array-CGH.

**Case 20.** The patient was ascertained at eight years of age because of mild developmental and language delay. He was born at term from non-consanguineous parents, after an uncomplicated pregnancy. At birth, he weighed 3240 g (75<sup>th</sup> centile) and his length and head circumference were 49 cm (50<sup>th</sup> centile) and 35 cm (50-75<sup>th</sup> centile), respectively. He started walking and talking after two years of age. No facial or skeletal abnormalities were identified by physical examination and he had normal male genitalia. The parents reported sleep disturbances with some episodes of pavor nocturnus.

### ***Molecular Cytogenetic Investigations***

For all the reported cases, except for case 3, a normal 46,XX karyotype was identified by conventional cytogenetics. Case 3 had a 46,XX,t(11;17)(p13;q24.3) karyotype. The presence of *SRY* was ruled out in all patients by PCR or FISH analysis. Genome-wide copy number analysis was performed by CGH- or SNP-arrays for all reported patients. The results for cases 1-3 and 20 together with their clinical data are summarized in Table 1. In cases 1 and 2, array-CGH analysis identified partially overlapping 17q24.3 duplications of different sizes, involving the gene-desert region upstream of *SOX9* (Figure 1A). For both cases the duplication was also present in paternal DNA. Both sisters of case 2 did not show the *RevSex* duplication.

The breakpoints of the duplications were finely mapped and cloned for both cases (Figure 1B) and were located at 69,513,605 bps (proximally) and 69,692,812 bps (distally) for case 1 and at 69,404,081 bps (proximally) and 69,872,909 bps (distally) for case 2 (hg19). Three of the breakpoints occurred within regions of Long Interspersed Nuclear Elements (LINEs) but there is no homology between the breakpoint sequences. Junction sequencing demonstrated that both duplications are direct. Case 1 junction shows insertion of four additional bases, likely derived by the duplication of adjacent sequence. Case 2 junction has a 1-base overlap.

In case 3, karyotype analysis revealed the presence of a 46,XX karyotype with a *de novo* reciprocal translocation t(11;17)(p13;q24.3). Array-CGH analysis did not detect any imbalance but common CNVs, whereas FISH analysis allowed narrowing the breakpoints on the two derivative chromosomes. The BAC probe RP11-661B15 encompassed the breakpoint on chromosome 11 and the two BAC probes CTD-2652P12 and RP11-879D6 encompassed the one on chromosome 17 (Figure 2B-C). Breakpoint mapping was performed by multiple long-range PCR reactions and sequencing, allowing to locate them at 35,935,981 bp on chromosome 11 and between 69,187,829 and 69,187,844 bp on chromosome 17, with a loss of 15 bp (Figure 2C). The rearrangement did not create or abrogate any predicted transcription factor binding site (TFBS), according to the databases we interrogated.

Cases 4-19 did not show any significant CNV (Supplementary Table 1).

Case 20 showed a 46,XX karyotype. SNP-array analysis detected the presence of a 5.6 Mb duplication of the long arm of a chromosome X involving the *SOX3* gene (Figure 1C); the duplication was *de novo* and had occurred on the paternal allele. X-inactivation analysis showed a random pattern of inactivation.

## DISCUSSION

We present 19 unrelated cases of 46,XX subjects with isolated abnormal gonadal development and male or ambiguous genitalia, all in the absence of the *SRY* gene. A further 46,XX *SRY*-negative subject showed syndromic DSD. A genomic imbalance or a chromosome rearrangement was detected in four. In three of them (cases 1, 3, 20), external genitalia were unquestionably male with testes, whereas in one case (case 2) they were ambiguous with ovotestes.

### *Duplications of the desert region upstream of SOX9*

Cases 1 and 2, an infertile male and a child with ovotestes, had partially overlapping duplications covering the so called *RevSex* critical region on chromosome 17q24; such duplications have already been associated

with 46,XX DSDs.<sup>4-7</sup> Our cases do not further narrow the minimal duplicated interval reported so far (Figure 3) but demonstrate that this genomic alteration is not rare among *SRY*-negative XX males and further establish that the duplication can be inherited by a healthy and fertile father. Although the frequency of genomic alterations involving the *SOX9* coding region in 46,XX testicular or ovotesticular DSDs has been questioned,<sup>23</sup> we identified copy number gains upstream of this gene in two of nineteen novel cases with isolated 46,XX DSDs negative for *SRY*, either sporadic or familial, accounting for the 10.5% of our cohort. Considering that we have previously detected two further cases owing to the same cohort with similar *RevSex* duplications [Vetro et al (2011),<sup>6</sup> and a family that did not give consent to the publication] we may conclude that this frequency is even higher.

In both our cases 1 and 2, the same duplication was present in the proband's father, as for some previously reported cases, suggesting that a copy gain of the region does not affect sex development and fertility in 46,XY subjects, where *SOX9* transcription is anyway activated during gonadal development. In a 46,XX background, in contrast, the presence of the duplication could be responsible of inappropriate expression of *SOX9* in the embryo gonadal ridge.

Hypothetical mechanisms explaining the association between copy number gains at 17q24 and XX DSD are the following:

1. The duplication could alter *SOX9* expression by increasing the dosage of one or more gonadal specific enhancers located within the minimal duplicated interval defined as *RevSex*. This hypothesis is supported by the finding that deletions and duplications of the *RevSex* region have mirror effects, the firsts being associated with sex reversal in XY but not in XX subjects<sup>4,24</sup> and the latter with XX sex reversal but no effect in XY individuals, as shown by familial cases<sup>4-6</sup> (Figure 3). Therefore *RevSex* appears to be dosage sensitive although noteworthy exceptions, namely a duplication in one fertile XX female<sup>4</sup> and a deletion in a fertile XY male,<sup>25</sup> suggest that *SOX9* dysregulation can in some cases be leaky possibly due to genomics modifier(s) of gonadal differentiation.

The minimal overlapping region of *RevSex* CNVs is of about 70 kb (chr17:69,534,400-69,600,000, hg19). To explain why larger duplications containing *RevSex* are associated with brachydactyly-anonychia but not with sex reversal (dark green in Figure 3), we hypothesize that the additional copy of the critical region is placed too far from *SOX9* to be able to influence its expression in the gonads.

2. An alternative model might consider that the amplifications abrogate *SOX9* silencing by moving a hypothetical negative regulatory element located upstream of *RevSex* (*NGRE: Negative Gonadal Regulatory Element*, dashed blue line in Figure 3), too far away to exert any influence on *SOX9* promoter. In fact, *SOX9* repression needs to be maintained on the XX background both in the developing gonad<sup>26</sup> and in the ovary,<sup>27</sup> in order to ensure the differentiation and maintenance of ovarian cell fate. *NGRE* would not be displaced, but rather included in duplications associated with brachydactyly-anonychia but no sex reversal (dark-green in Figure 3). However according to this hypothesis, also proposed by Xiao et al (2013),<sup>7</sup> haploinsufficiency of such *NGRE* element should lead to gonadal overexpression of *SOX9* thus resulting in XX sex reversal. In contrast, a number of deletions, none of them associated with XX DSD, have been reported covering the entire region delimited by the centromeric end of the *RevSex* duplications and the *KCNJ2* gene. These individuals, either XX or XY, were investigated because of Pierre-Robin syndrome or cardiac defects.<sup>11,13,28,29</sup>

The two duplications we describe are both in tandem, as reported for other DSD cases,<sup>4,5</sup> but a specific predisposing genomic architecture has not been highlighted. This duplication, although without effect in the XY background, appears to be very rare, with no cases containing at least the minimal duplicated *RevSex* region among the 14.316 individuals collected in the Database of Genomic Variants.<sup>30</sup> This is in agreement with the extreme rarity of the *SRY*-negative 46,XX DSD condition.

#### *Interruption of the desert region upstream of SOX9*

We also report the second case of a balanced translocation associated with XX sex reversal (case 3). Breakpoint mapping allowed us to precisely define the 17q breakpoint of the t(11;17) translocation, that is located about 115 kb upstream of that reported by Refai et al.<sup>15</sup> The existence of a single gonadal-specific

regulatory element interrupted by both these translocations is contradicted by the presence of translocation breakpoints in the same interval in at least three unrelated 46,XX females with normal sexual development (light green in Figure 3).<sup>31-33</sup> As suggested,<sup>31</sup> the chromatin environment of the recipient region may alter *SOX9* regulation even though in all these cases *RevSex* is translocated to the derivative chromosome together with *SOX9* thus, in theory, retaining the *cis* regulatory elements necessary for its gonadal expression.

Our patient 3 also shows signs of Pierre-Robin sequence (PRS). Several deletions and translocations, mapping in the region from 585 kb to 1.8 Mb upstream of *SOX9* have been reported in patients with isolated PRS.<sup>11,29,31,32,34</sup> These cases point to the existence of *SOX9* regulatory elements driving the expression of this gene in craniofacial structures, although no single element specifically impaired by the reported rearrangements has been yet identified.

### *SOX3 duplication*

Finally, we report a new case of *SOX3* duplication in a 46,XX boy ascertained because of mild developmental delay (case 20). *SOX3*, encoding a protein very similar to *SRY*, might be the ancestral *SOX* gene from which the *SRY* gene was derived.<sup>35</sup>

Duplications involving *SOX3* and deletions of its 5' region (Figure 1D) have been reported in at least 4 cases of XX DSD.<sup>16,17</sup> Moreover, a mouse model in which *SOX3* is ectopically expressed in the developing gonads shows complete XX sex reversal suggesting that gain-of-function mutations of *SOX3* might act as an *SRY* surrogate in sex determination, promoting *SOX9* gonadal expression.<sup>16</sup> Interestingly, *SOX3* duplications have been reported in two unrelated 46,XY individuals with X-linked hypopituitarism, whereas their carrier mothers were unaffected.<sup>36,37</sup> No hypopituitarism was present in our case 20 or in the patient reported by Moalem et al (2012).<sup>17</sup> An X-linked dominant but leaky mutation affecting sex development in a portion of XX subjects might be hypothesized, either as a consequence of the X inactivation pattern in the developing gonad or of specific genomic modifiers.



## Conclusions

We report additional evidences suggesting that, in the absence of *SRY*, altered expression of genes crucial to gonadal development, such as *SOX9* and *SOX3*, may invert the expected embryonic plan.

While for *SOX3* it is easier to envisage a direct link between its duplication and increased gene expression,<sup>16</sup> it is more difficult to understand the true functional link between duplications upstream of *SOX9* and the different abnormal phenotypes including gonadal abnormal differentiation.

Our study reports that the incidence for *RevSex* copy number gains associated with *SRY*-negative isolated 46,XX DSDs is more than 10%.

We can speculate that the *RevSex* duplication causes increased expression of *SOX9* in undifferentiated gonadal cells thus resulting in testis differentiation even in absence of *SRY*. In fact, duplications of *SOX9* are associated with XX sex reversal not only in transgenic mice<sup>9</sup> but also in the recently reported case of a deer<sup>38</sup> and in three cases of dogs<sup>39</sup>. Our case 3 shows that also interruption of the region upstream to the *RevSex* can result in XX sex reversal. Altogether our data reinforce the role of the desert region upstream of *SOX9* in the regulation of this gene as indicated by an altered histone methylation signature demonstrated in one of the *RevSex* duplicated cases.<sup>40</sup> It is noteworthy that *RevSex* includes two lncRNAs, *TCONS\_00025195* and *TCONS\_00025196*, with specific expression in the testis,<sup>41</sup> possibly having a role in *SOX9* transcriptional regulation.

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**Supplementary information is available at the website of the European Journal of Human Genetics.**

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**Table 1** - Clinical and molecular cytogenetics data of cases 1-3 and 20.

| Case N | Age at the diagnosis | Ascertainment                | Genitalia   | Laboratory Findings                           | Others                 | Karyotype | Array results   |
|--------|----------------------|------------------------------|---|---|------------------------|-----------|---|
| 1      | 30                   | infertility, azoospermia     | normal male   | elevated FSH and LH, low serum testosterone   | bilateral gynecomastia | 46,XX     | chr17.hg19:g.(69,401,099_69,458,883)(69,823,311_69,878,197)dup; ISCN nomenclature: arr[hg19]17q24(69,401,099x2,69,458,883-69,823,311x3,69,878,197x2)    |
| 2      | at birth             | ambiguous external genitalia | hypertrophic clitoris, single meatus, and urogenital sinus; ovotestis | low testosterone response to hHCG stimulation | -                      | 46,XX     | chr17.hg19:g.(69,510,367_69,544,737)(69,686,379_69,764,059)dup; ISCN nomenclature: arr[hg19]17q24(69,510,367x2,69,544,737-69,686,379x3,69,764,059x2)pat |

|    |    |                                 |   |  |                          |                             |  |
|----|----|---------------------------------|---|--|--------------------------|-----------------------------|--|
| 3  | 41 | infertility,<br>azoospermia     | normal male with<br>bilateral<br>testicular<br>hypotrophy | elevated FSH, LH<br>and<br>androstenedione,<br>low serum<br>testosterone | microgna<br>thia         | 46,XX,t(11;17)(p13;q24.3)dn | ISCN<br>nomenclature:<br>arr[hg19](1-22,X)x2   |
| 20 | 8  | mild intellectual<br>disability | normal male   | not available  | psychom<br>otor<br>delay | 46,XX                       | chrX.hg19:g.(139,501,182_139,504,721)_(145,120,304_145,126,046)dup; ISCN<br>nomenclature:<br>arr[hg19]Xq27.1q27.3(139,501,182x2,139,504,721-145,120,304x3,145,126,046x2) |

## FIGURE LEGENDS

**Figure 1** - Genomic rearrangements in cases 1, 2 and 20. A) Duplications of partially overlapping 17q24.3 regions in cases 1 (upper panel) and 2 (lower panel): an enlargement of a 2.05 Mb region from chromosome 17 profile is shown, with the duplications highlighted by the shaded areas; B) DNA sequences

spanning the chromosome 17 duplication breakpoints in both cases aligned with the reference sequences. C) SNP-array profile of chromosome X from case 20 showing the 5,6 Mb duplication encompassing *SOX3*; D) Case 20 duplication is compared to the three duplications (blue bars) and the deletion (red bar) involving *SOX3* and its 5' region previously reported in DSD patients,<sup>16,17</sup> based on UCSC Genome Browser (<http://genome.ucsc.edu/>), hg19.

**Figure 2** – Translocation breakpoints mapping in case 3. A) From the left: FISH analysis on patient's lymphocytes with probes RP11-661B15 (chr11:35,875,774-36,048,561), RP11-879D6 (chr17:69,079,298-69,261,570), CTD-2652P12 (chr17:69,142,978-69,339,629) and a schematic representation of the results; arrowheads indicate the signals on the derivative chromosomes while arrows mark normal chromosomes 11 and 17. B) Map positions of the probes on chromosomes 11 and 17 highlighting the breakpoints (red arrowheads). C) DNA sequences spanning the translocation breakpoints on der(11) and der(17) with the reference sequences.

**Figure 3** – Overview of a 2,8 Mb screenshot of chromosome 17q24.3 (chr17:67,429,400-70,288,400, hg19), based on UCSC Genome Browser. Copy-number variations upstream of *SOX9* are shown. **Blue bars**: 46,XX DSD-associated duplications (DSD2 and DSD3 from Benko,<sup>4</sup> and the cases reported by Cox,<sup>5</sup> and Vetro,<sup>6</sup> inherited the duplication from the healthy and fertile father); **dark-green bars**: duplications identified in brachydactyly-anonychia familial cases; **red bars**: 46,XY DSD-associated deletions (cases reported by Lecointre<sup>24</sup> and Benko<sup>4</sup> inherited the deletion from the mother; cases reported by Bhagavath<sup>25</sup> inherited the deletion from the father); **purple bars**: deletions identified in patients with pathological phenotype not including DSD; **light green**: breakpoints of translocations identified in 46,XX subjects. The two cases with 46,XX DSDs (present report, case 3 and Refai,<sup>15</sup>) are indicated by an asterisk. Breakpoints of balanced rearrangements, mainly associated with CD, ACD, or PRS are grouped into proximal (brown) and distal (dark-blue) clusters. The *RevSex* region is highlighted by a vertical light-orange bar. The hypothetical NGRE-

containing region is depicted as a dashed blue line. References of all cases are provided on the left.<sup>4-7,11-</sup>

15,24,25,28,29,31-34,42-46

§: familial cases; PRS: Pierre-Robin Sequence; ACD: Acampomelic Dysplasia CD: Campomelic Dysplasia;

CHD: Congenital Heart Defects

Figure 1

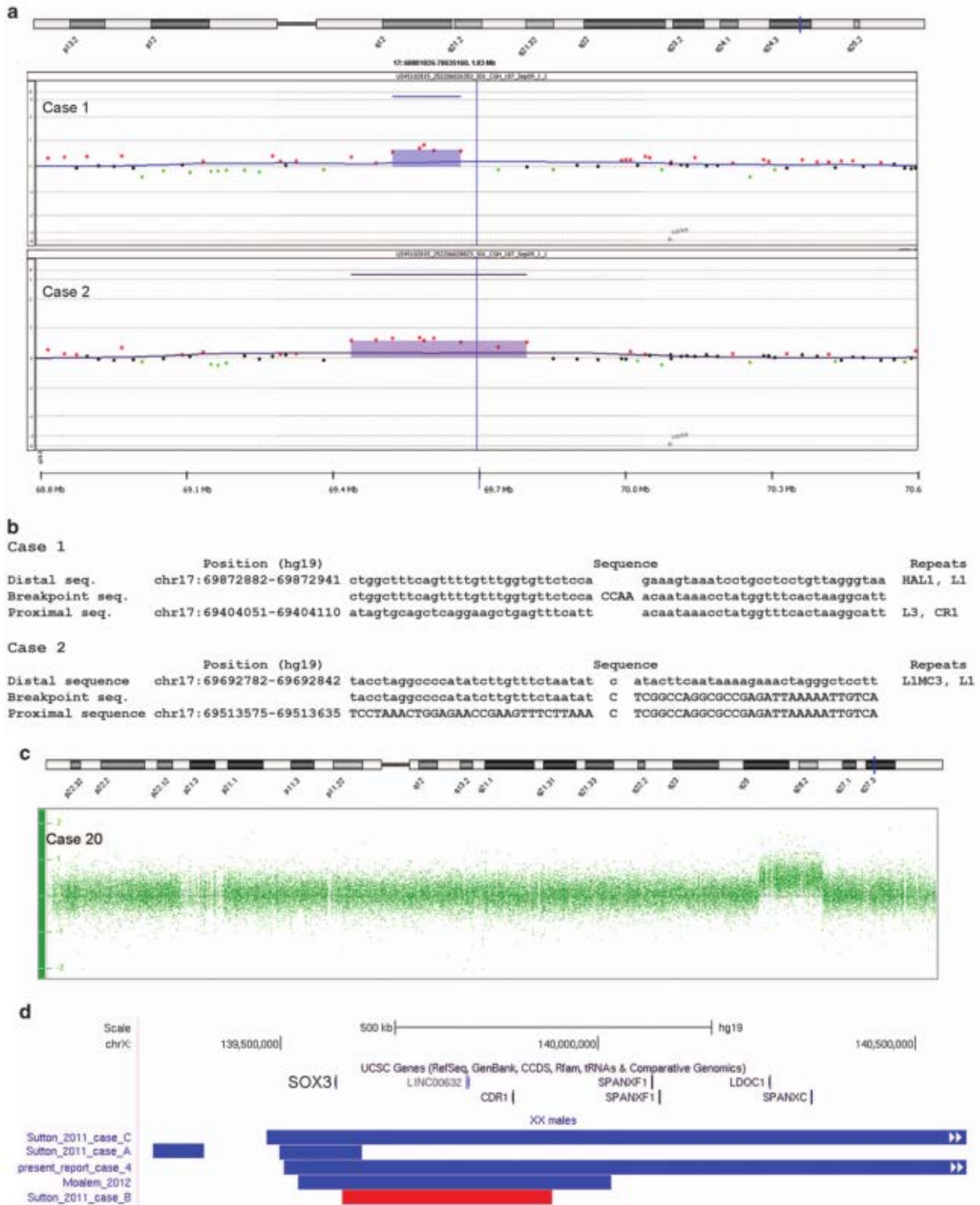


Figure 2

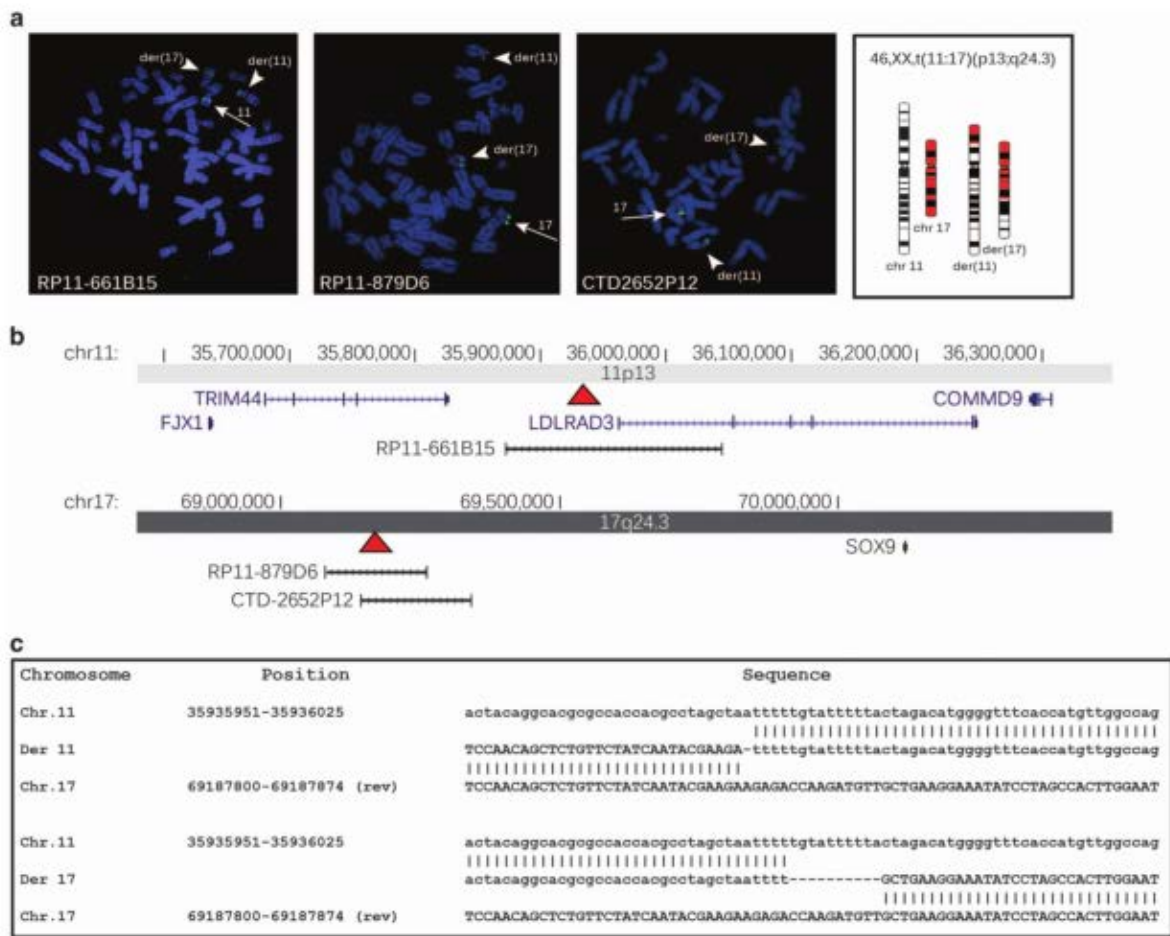




Figure 3

