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

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.[1] 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.[2,3] 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.*[4-7] 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.[8] Actually, *SOX9* transgenic expression in XX gonads is sufficient to induce testis formation in mice.[9] Moreover, a single case of an XX boy is reported with a >11 Mb duplication including *SOX9*.[10] *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,[4,11-13] whereas duplications have been reported both in familial cases of brachydactyly-anonychia[14] 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.[15]

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.[16] Moreover, duplications of *SOX3* or its 5' region have been reported in 46,XX DSD patients.[16,17]

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[18] 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[19], 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).[20]

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.[21] 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/).[22] 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 10th and the 25th 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 (75th centile) and his length and head circumference were 49 cm (50th centile) and 35 cm (50-75th 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.[4-7] 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,[23] 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),[6] 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[4,24] and the latter with XX sex reversal but no effect in XY individuals, as shown by familial cases[4-6] (Figure 3). Therefore *RevSex* appears to be dosage sensitive although noteworthy exceptions, namely a duplication in one fertile XX female[4] and a deletion in a fertile XY male,[25] 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[26] and in the ovary,[27] 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),[7] 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.[11,13,28,29]

The two duplications we describe are both in tandem, as reported for other DSD cases,[4,5] 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. [30] 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.[15] 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).[31-33] As suggested,[31] 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.[11,29,31,32,34] 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.[35]

Duplications involving *SOX3* and deletions of its 5' region (Figure 1D) have been reported in at least 4 cases of XX DSD.[16,17] 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.[16] Interestingly, *SOX3* duplications have been reported in two unrelated 46,XY individuals with X-linked hypopituitarism, whereas their carrier mothers were unaffected.[36,37] No hypopituitarism was present in our case 20 or in the patient reported by Moalem et al (2012).[17] 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,[16] 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[9] but also in the recently reported case of a deer[38] and in three cases of dogs[39]. 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.[40] It is noteworthy that *RevSex* includes two lncRNAs, *TCONS_00025195* and *TCONS_00025196*, with specific expression in the testis,[41] possibly having a role in *SOX9* transcriptional regulation.

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References

1. Berta P, Hawkins JR, Sinclair AH *et al*: Genetic evidence equating SRY and the testis-determining factor. *Nature* 1990; **348:** 448-450.

2. Jobling MA: A selective difference between human Y-chromosomal DNA haplotypes. *Current Biology* 1998; **8:** 4.

3. Rosser ZH, Balaresque P, Jobling MA: Gene conversion between the X chromosome and the male-specific region of the Y chromosome at a translocation hotspot. *Am J Hum Genet* 2009; **85:** 130-134.

4. Benko S, Gordon CT, Mallet D *et al*: Disruption of a long distance regulatory region upstream of SOX9 in isolated disorders of sex development. *J Med Genet* 2011; **48:** 825-830.

5. Cox JJ, Willatt L, Homfray T, Woods CG: A SOX9 duplication and familial 46,XX developmental testicular disorder. *N Engl J Med* 2011; **364:** 91-93.

6. Vetro A, Ciccone R, Giorda R *et al*: XX males SRY negative: a confirmed cause of infertility. *J Med Genet* 2011; **48:** 710-712.

7. Xiao B, Ji X, Xing Y, Chen YW, Tao J: A rare case of 46, XX SRY-negative male with approximately 74-kb duplication in a region upstream of SOX9. *Eur J Med Genet* 2013; **56:** 695-698.

8. Sekido R, Lovell-Badge R: Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature* 2008; **453:** 930-934.

9. Vidal VP, Chaboissier MC, de Rooij DG, Schedl A: Sox9 induces testis development in XX transgenic mice. *Nat Genet* 2001; **28:** 216-217.

10. Huang B, Wang S, Ning Y, Lamb AN, Bartley J: Autosomal XX sex reversal caused by duplication of SOX9. *Am J Med Genet* 1999; **87:** 349-353.

11. Benko S, Fantes JA, Amiel J *et al*: Highly conserved non-coding elements on either side of SOX9 associated with Pierre Robin sequence. *Nat Genet* 2009; **41:** 359-364.

12. White S, Ohnesorg T, Notini A *et al*: Copy number variation in patients with disorders of sex development due to 46,XY gonadal dysgenesis. *PLoS One* 2011; **6:** e17793.

13. Sanchez-Castro M, Gordon CT, Petit F *et al*: Congenital Heart Defects in Patients with Deletions Upstream of SOX9. *Hum Mutat* 2013; **34:** 1628-1631.

14. Kurth I, Klopocki E, Stricker S *et al*: Duplications of noncoding elements 5' of SOX9 are associated with brachydactyly-anonychia. *Nat Genet* 2009; **41:** 862-863.

15. Refai O, Friedman A, Terry L *et al*: De novo 12;17 translocation upstream of SOX9 resulting in 46,XX testicular disorder of sex development. *Am J Med Genet A* 2010; **152A:** 422-426.

16. Sutton E, Hughes J, White S *et al*: Identification of SOX3 as an XX male sex reversal gene in mice and humans. *J Clin Invest* 2011; **121:** 328-341.

17. Moalem S, Babul-Hirji R, Stavropolous DJ *et al*: XX male sex reversal with genital abnormalities associated with a de novo SOX3 gene duplication. *Am J Med Genet A* 2012; **158A:** 1759-1764.

18. Spielmann M, Klopocki E: CNVs of noncoding cis-regulatory elements in human disease. *Curr Opin Genet Dev* 2013; **23:** 249-256.

19. Bonaglia MC, Giorda R, Beri S *et al*: Molecular mechanisms generating and stabilizing terminal 22q13 deletions in 44 subjects with Phelan/McDermid syndrome. *PLoS Genet* 2011; **7:** e1002173.

20. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW: Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am J Hum Genet* 1992; **51:** 1229-1239.

21. Rossi E, Giorda R, Bonaglia MC *et al*: De novo unbalanced translocations in Prader-Willi and Angelman syndrome might be the reciprocal product of inv dup(15)s. *PLoS One* 2012; **7:** e39180.

22. Heinemeyer T, Wingender E, Reuter I *et al*: Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Res* 1998; **26:** 362-367.

23. Seeherunvong T, Ukarapong S, McElreavey K, Berkovitz GD, Perera EM: Duplication of SOX9 is not a common cause of 46,XX testicular or 46,XX ovotesticular DSD. *J Pediatr Endocrinol Metab* 2012; **25:** 121-123.

24. Lecointre C, Pichon O, Hamel A *et al*: Familial acampomelic form of campomelic dysplasia caused by a 960 kb deletion upstream ofSOX9. *American Journal of Medical Genetics Part A* 2009; **149A:** 1183-1189.

25. Bhagavath B, Layman LC, Ullmann R *et al*: Familial 46,XY sex reversal without campomelic dysplasia caused by a deletion upstream of the SOX9 gene. *Mol Cell Endocrinol* 2014; **393:** 1-7.

26. Maatouk DM, DiNapoli L, Alvers A, Parker KL, Taketo MM, Capel B: Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal. *Hum Mol Genet* 2008; **17:** 2949-2955.

27. Uhlenhaut NH, Jakob S, Anlag K *et al*: Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell* 2009; **139:** 1130-1142.

28. Fukami M, Tsuchiya T, Takada S *et al*: Complex genomic rearrangement in the SOX9 5' region in a patient with Pierre Robin sequence and hypoplastic left scapula. *Am J Med Genet A* 2012; **158A:** 1529-1534.

29. Gordon CT, Attanasio C, Bhatia S *et al*: Identification of novel craniofacial regulatory domains located far upstream of SOX9 and disrupted in Pierre Robin sequence. *Hum Mutat* 2014; **35:** 1011-1020.

30. Macdonald JR, Ziman R, Yuen RK, Feuk L, Scherer SW: The Database of Genomic Variants: a curated collection of structural variation in the human genome. *Nucleic Acids Res* 2014; **42:** D986-992.

31. Fonseca AC, Bonaldi A, Bertola DR, Kim CA, Otto PA, Vianna-Morgante AM: The clinical impact of chromosomal rearrangements with breakpoints upstream of the SOX9 gene: two novel de novo balanced translocations associated with acampomelic campomelic dysplasia. *BMC Med Genet* 2013; **14:** 50.

32. Hill-Harfe KL, Kaplan L, Stalker HJ *et al*: Fine mapping of chromosome 17 translocation breakpoints ≥ 900 Kb upstream of SOX9 in acampomelic campomelic dysplasia and a mild, familial skeletal dysplasia. *Am J Hum Genet* 2005; **76:** 663-671.

33. Velagaleti GV, Bien-Willner GA, Northup JK *et al*: Position effects due to chromosome breakpoints that map approximately 900 Kb upstream and approximately 1.3 Mb downstream of SOX9 in two patients with campomelic dysplasia. *Am J Hum Genet* 2005; **76:** 652-662.

34. Amarillo IE, Dipple KM, Quintero-Rivera F: Familial microdeletion of 17q24.3 upstream of SOX9 is associated with isolated Pierre Robin sequence due to position effect. *Am J Med Genet A* 2013; **161A:** 1167-1172.

35. Foster JW, Graves JA: An SRY-related sequence on the marsupial X chromosome: implications for the evolution of the mammalian testis-determining gene. *Proc Natl Acad Sci U S A* 1994; **91:** 1927-1931.

36. Solomon NM, Nouri S, Warne GL, Lagerstrom-Fermer M, Forrest SM, Thomas PQ: Increased gene dosage at Xq26-q27 is associated with X-linked hypopituitarism. *Genomics* 2002; **79:** 553-559.

37. Woods KS, Cundall M, Turton J *et al*: Over- and underdosage of SOX3 is associated with infundibular hypoplasia and hypopituitarism. *Am J Hum Genet* 2005; **76:** 833-849.

38. Kropatsch R, Dekomien G, Akkad DA *et al*: SOX9 Duplication Linked to Intersex in Deer. *PLoS One* 2013; **8:** e73734.

39. Rossi E, Radi O, De Lorenzi L *et al*: Sox9 duplications are a relevant cause of Sry-negative XX sex reversal dogs 2014; **9:** e101244.

40. Lybaek H, de Bruijn D, den Engelsman-van Dijk AH *et al*: RevSex duplication-induced and sex-related differences in the SOX9 regulatory region chromatin landscape in human fibroblasts. *Epigenetics* 2014; **9:** 416-427.

41. Smyk M, Szafranski P, Startek M, Gambin A, Stankiewicz P: Chromosome conformation capture-on-chip analysis of long-range cis-interactions of the SOX9 promoter. *Chromosome Res* 2013; **21:** 781-788.

42. Blyth M, Huang S, Maloney V, Crolla JA, Karen Temple I: A 2.3Mb deletion of 17q24.2-q24.3 associated with 'Carney Complex plus'. *Eur J Med Genet* 2008; **51:** 672-678.

43. De Gregori M, Ciccone R, Magini P *et al*: Cryptic deletions are a common finding in "balanced" reciprocal and complex chromosome rearrangements: a study of 59 patients. *J Med Genet* 2007; **44:** 750-762.

44. Jakubiczka S, Schroder C, Ullmann R *et al*: Translocation and deletion around SOX9 in a patient with acampomelic campomelic dysplasia and sex reversal. *Sex Dev* 2010; **4:** 143-149.

45. Lestner JM, Ellis R, Canham N: Delineating the 17q24.2-q24.3 microdeletion syndrome phenotype. *Eur J Med Genet* 2012; **55:** 700-704.

46. Pop R: Screening of the 1 Mb SOX9 5' control region by array CGH identifies a large deletion in a case of campomelic dysplasia with XY sex reversal. *Journal of Medical Genetics* 2004; **41:** e47-e47.

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, azoospermia	normal male with bilateral testicular hypotrophy	elevated FSH, LH and androstenedione, low serum testosterone	micrognathia	46,XX,t(11;17)(p13;q24.3)dn	ISCN nomenclature: arr[hg19](1-22,X)x2
20	8	mild intellectual disability	normal male	not available	psychomotor delay	46,XX	chrX.hg19:g.(139,501,182_139,504,721)_(145,120,304_145,126,046)dup; ISCN nomenclature: 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,[16,17] 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,[4] and the cases reported by Cox,[5] and Vetro,[6] 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[24] and Benko[4] inherited the deletion from the mother; cases reported by Bhagavath[25] 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,[15]) 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.[4-7,11-15,24,25,28,29,31-34,42-46]

§: familial cases; PRS: Pierre-Robin Sequence; ACD: Acampomelic Dysplasia CD: Campomelic Dysplasia; CHD: Congenital Heart Defects

Figure 1

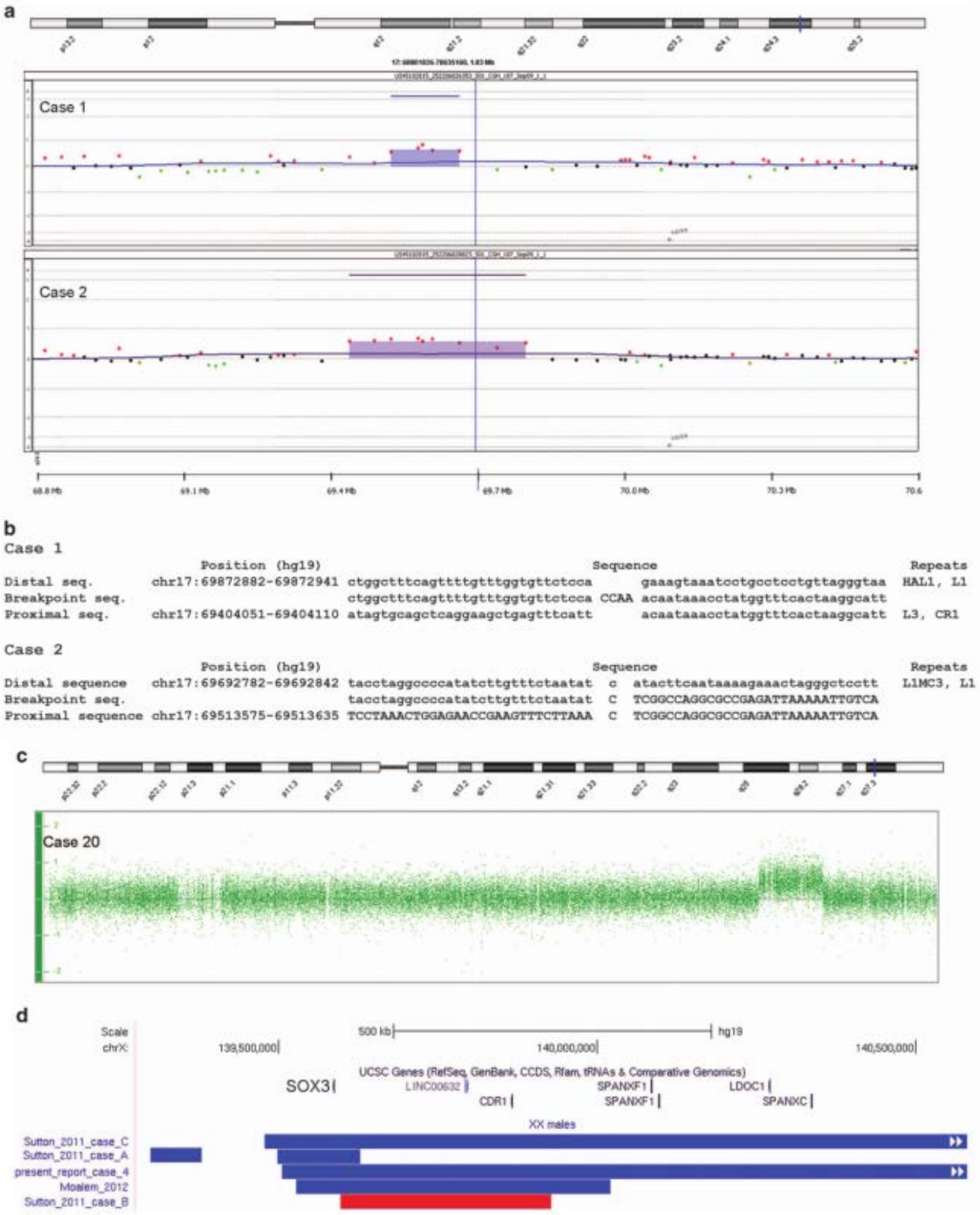

a
Case 1
Case 2
68.8 Mb
69.1 Mb
69.4 Mb
69.7 Mb
70.0 Mb
70.3 Mb
70.6
b
Case 1
Position (hg19) Sequence Repeats
Distal seq. chr17:69872882-69872941 ctggctttcagttttgtttggtgttctcca gaaagtaaatcctgcctcctgttagggtaa HAL1, L1
Breakpoint seq. ctggctttcagttttgtttggtgttctcca CCAA acaataaacctatggtttcactaaggcatt
Proximal seq. chr17:69404051-69404110 atagtgcagctcaggaagctgagtttcatt acaataaacctatggtttcactaaggcatt L3, CR1
Case 2
Position (hg19) Sequence Repeats
Distal sequence chr17:69692782-69692842 tacctaggccccatatcttgtttctaatat c atacttcaataaaagaaactagggctcctt L1MC3, L1
Breakpoint seq. tacctaggccccatatcttgtttctaatat C TCGGCCAGGCGCCGAGATTAAAAATTGTCA
Proximal sequence chr17:69513575-69513635 TCCTAAACTGGAGAACCGAAGTTTCTTAAA C TCGGCCAGGCGCCGAGATTAAAAATTGTCA
c
Case 20
d
Scale
chrX
139,500,000
500 kb
hg19
140,000,000
140,500,000
UCSC Genes (RefSeq, GenBank, CCDS, Rfam, tRNAs & Comparative Genomics)
SOX3
LINC00632
SPANXF1
LDOC1
CDR1
SPANXF1
SPANXC
XX males
Sutton_2011_case_C
Sutton_2011_case_A
present_report_case_4
Moalem_2012
Sutton_2011_case_B

Figure 2

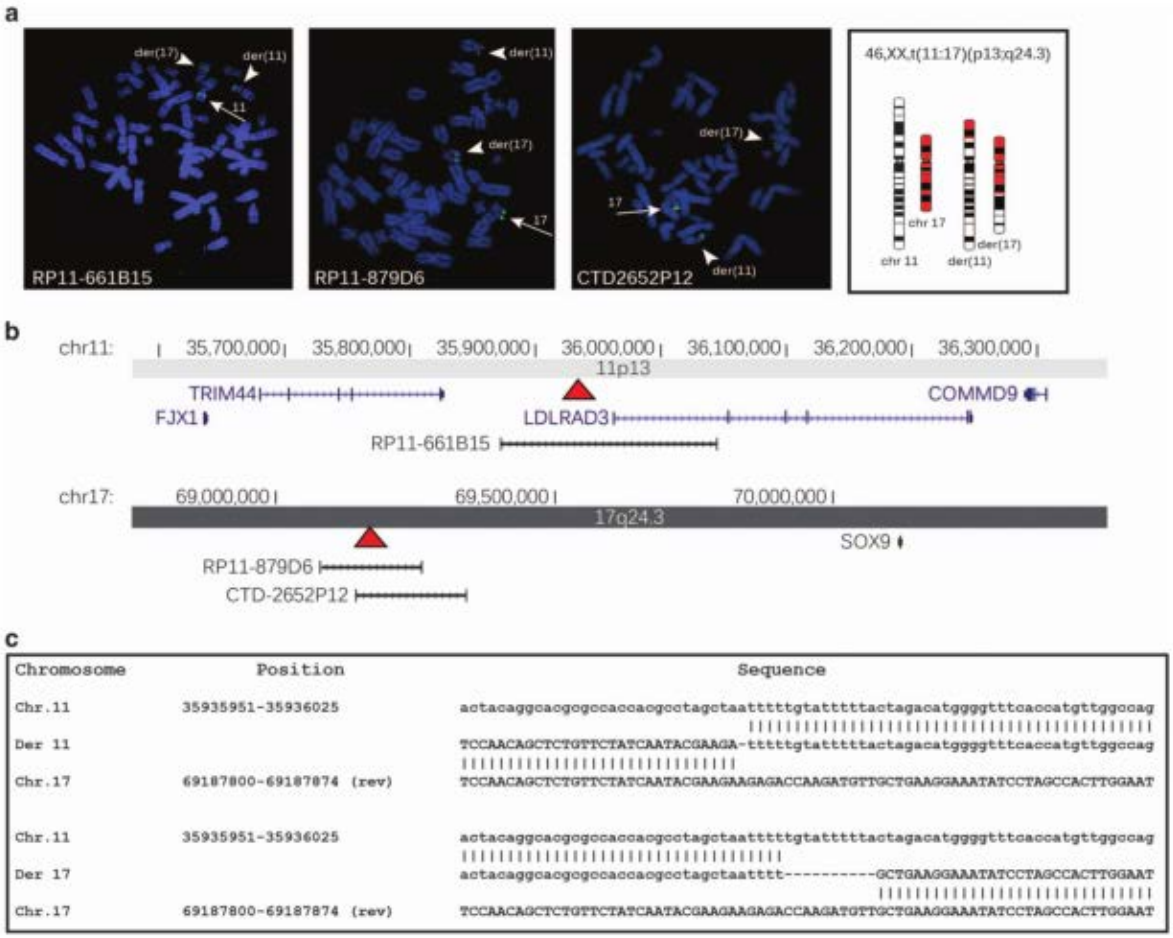
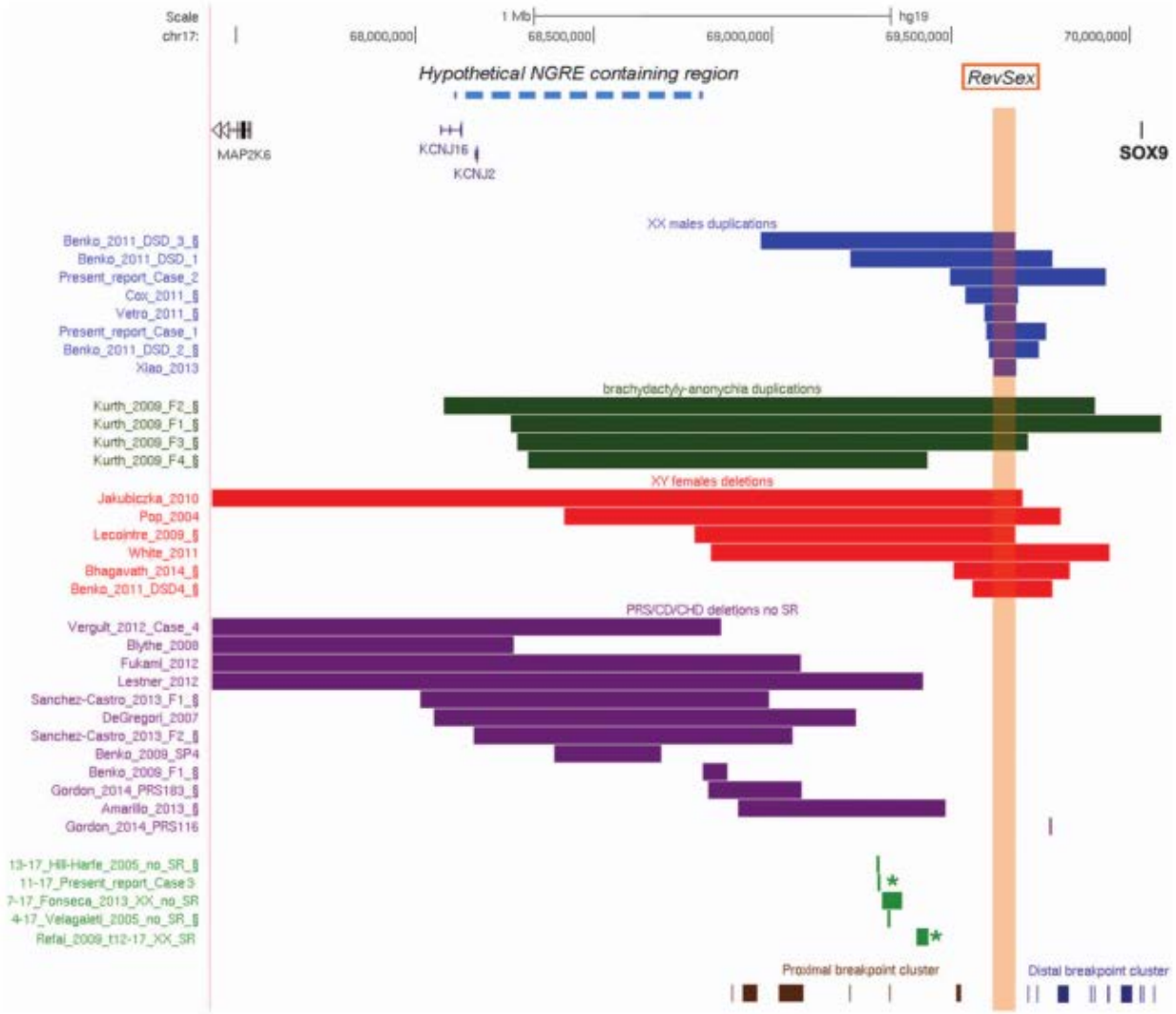

a
der(17)
der(11)
11
RP11-661B15
der(11)
der(17)
17
RP11-879D6
der(17)
17
der(11)
CTD2652P12
46,XX,t(11;17)(p13;q24.3)
chr 17
der(17)
chr 11
der(11)
b
chr11:
35,700,000
35,800,000
35,900,000
36,000,000
36,100,000
36,200,000
36,300,000
11p13
TRIM44
FJX1
LDLRAD3
COMMD9
RP11-661B15
chr17:
69,000,000
69,500,000
70,000,000
17q24.3
SOX9
RP11-879D6
CTD-2652P12
c
Chromosome Position Sequence
Chr.11 35935951-35936025 actacaggcacgcgccaccacgcctagctaatttttgtatttttactagacatggggtttcaccatgttggccag
Der 11 TCCAACAGCTCTGTTCTATCAATACGAAGA-tttttgtatttttactagacatggggtttcaccatgttggccag
Chr.17 69187800-69187874 (rev) TCCAACAGCTCTGTTCTATCAATACGAAGAAGAGACCAAGATGTTGCTGAAGGAAATATCCTAGCCACTTGGAAT
Chr.11 35935951-35936025 actacaggcacgcgccaccacgcctagctaatttttgtatttttactagacatggggtttcaccatgttggccag
Der 17 actacaggcacgcgccaccacgcctagctaatttt----------GCTGAAGGAAATATCCTAGCCACTTGGAAT
Chr.17 69187800-69187874 (rev) TCCAACAGCTCTGTTCTATCAATACGAAGAAGAGACCAAGATGTTGCTGAAGGAAATATCCTAGCCACTTGGAAT

Figure 3


Scale
chr17:
1 Mb
hg19
68,000,000
68,500,000
69,000,000
69,500,000
70,000,000
Hypothetical NGRE containing region
RevSex
MAP2K6
KCNJ16
KCNJ2
SOX9
XX males duplications
Benko_2011_DSD_3_§
Benko_2011_DSD_1
Present_report_Case_2
Cox_2011_§
Vetro_2011_§
Present_report_Case_1
Benko_2011_DSD_2_§
Xiao_2013
brachydactyly-anonychia duplications
Kurth_2009_F2_§
Kurth_2009_F1_§
Kurth_2009_F3_§
Kurth_2009_F4_§
XY females deletions
Jakubiczka_2010
Pop_2004
Lecointre_2009_§
White_2011
Bhagavath_2014_§
Benko_2011_DSD4_§
PRS/CD/CHD deletions no SR
Vergult_2012_Case_4
Blythe_2008
Fukami_2012
Lestner_2012
Sanchez-Castro_2013_F1_§
DeGregori_2007
Sanchez-Castro_2013_F2_§
Benko_2009_SP4
Benko_2009_F1_§
Gordon_2014_PRS183_§
Amarillo_2013_§
Gordon_2014_PRS116
13-17_Hill-Harfe_2005_no_SR_§
11-17_Present_report_Case 3
7-17_Fonseca_2013_XX_no_SR
4-17_Velagaleti_2005_no_SR_§
Refai_2009_t12-17_XX_SR
Proximal breakpoint cluster
Distal breakpoint cluster