

RevSex duplication-induced and sex-related differences in the *SOX9* regulatory region chromatin landscape in human fibroblasts

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Abbreviations: ChIP, chromatin immunoprecipitation; CTCF, CCCTC-binding Factor; DMR, differentially methylated region; DSD, disorder of sex development; ENCODE, Encyclopedia of DNA Elements; ESR, estrogen receptor; H3, histone 3; H3K4me3, Histone 3 trimethylated at lysine-4; H3K27me3, Histone 3 trimethylated at lysine-27; H3K9me3, Histone 3 trimethylated at lysine-9; H3K9ac, histone 3 acetylated at lysine-9; HMGB, high-mobility group box; LEF, lymphoid enhancer-binding Factor; MLPA, multiple ligation-dependent probe amplification; RSPO1, R-Spondin-1; SF1, Stereogenic Factor-1; *SOX9*, SRY box containing gene 9; SRY, sex determining region on the Y chromosome; TCF, T-Cell Factor; TESCO, testis enhancer sequence core element; TFBS, transcription factor binding site; UCSC, University of California Santa Cruz; UCSF, University of California San Francisco; WNT, from *Drosophila*-wingless

It was recently shown that duplications of the RevSex element, located 0.5 Mb upstream of *SOX9*, cause XX-disorder of sex development (DSD), and that deletions cause XY-DSD. To explore how a 148 kb RevSex duplication could have turned on gonadal *SOX9* expression in the absence of SRY in an XX-male, we examined the chromatin landscape in primary skin fibroblast cultures from the index, his RevSex duplication-carrier father and six controls. The ENCODE project supports the notion that chromatin state maps show overlap between different cell types, i.e., that our study of fibroblasts could be of biological relevance. We examined the *SOX9* regulatory region by high-resolution ChIP-on-chip experiments (a kind of “chromatin-CGH”) and DNA methylation investigations. The RevSex duplication was associated with chromatin changes predicting better accessibility of the SRY-responsive TESCO enhancer region 14–15 kb upstream of *SOX9*. Four kb downstream of the TESCO evolutionary conserved region, a peak of the enhancer/promoter-associated H3K4me3 mark was found together with a major dip of the repressive H3K9me3 chromatin mark. Similar differences were also found when three control males were compared with three control females. A marked male/female difference was a more open chromatin signature in males starting ~400 kb upstream of *SOX9* and increasing toward the *SOX9* promoter. In the RevSex duplication-carrier father, two positions of DNA hypomethylation were also found, one corresponding to the H3K4me3 peak mentioned above. Our results suggest that the RevSex duplication could operate by inducing long-range epigenetic changes. Furthermore, the differences in chromatin state maps between males and females suggest that the Y chromosome or X chromosome dosage may affect chromatin conformation, i.e., that sex-dependent gene regulation may take place by chromatin modification.

Introduction

SRY and *SOX9* are master regulators of male sexual development. SRY-stimulated *SOX9* expression in the undifferentiated fetal gonad causes Sertoli cell formation, and these cells orchestrate further testicular development, ultimately leading to fetal testosterone production and male differentiation of the external genitalia and probably also male-specific patterning of the brain.^{1–5} The most important, but not the only, downstream

effect of SRY is enhancement of *SOX9* transcription in the genital ridge.⁶ Both SRY and *SOX9* are high mobility group box (HMGB) domain transcription factors with partly overlapping DNA binding profiles.^{7,8} *SOX9* plays an important role in, for example, branchial arch, skeletal, and brain development, in addition to its sex-determining function in the gonads.^{9,10} The regulation of *SOX9* gene expression is complex, governed by a ~2 Mb genomic region upstream of the gene itself, which is quite small and occupies only 5–6 kb of genomic sequence.¹¹

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Translocations and copy number changes in the large *SOX9* regulatory region can cause a Pierre-Robin sequence type of cleft palate, and disturbed chondrocyte differentiation with skeletal dysplasia and/or sex reversal, all dependent on the position and size of the genomic aberrations.^{9,12-15} This variability suggests that different domains of the upstream “gene desert” regulate *SOX9* expression in a time- and tissue-specific manner, and that the genomic architecture is a critical factor for proper regulation.

The HMGB domain group of transcription factors, including SRY and *SOX9*, are known to cause DNA bending and to interact with other transcription factors (e.g., members of the OCT family) and probably also chromatin modifying proteins.^{7,16,17} SRY acts synergistically with steroidogenic factor-1 (SF1, encoded by *NR5A1*) at an enhancer element 14–15 kb upstream of the *SOX9* transcription start site.⁶ This ~1.4 kb enhancer element is called TESCO (testis enhancer sequence core element) and it contains a 176 nt highly conserved motif in its 5' half; the TESCO evolutionarily conserved region (TESCO-ECR, 70 103 197–70 103 373 bp from 17pter, hg19).¹⁸ Mutations in TESCO were not observed in 66 patients with XY-gonadal dysgenesis without an *SRY* mutation (i.e., *SRY*-mutation negative Swyer syndrome patients).¹⁹ This indirectly suggests that if malfunction of TESCO should be an important cause of XY-gonadal dysgenesis, it is probably due to other factors than SRY binding-site mutations.

It was therefore of interest to investigate if the genomic architecture of the *SOX9* regulatory region could represent an additional and superior level of *SOX9* regulation. For this, a family with a 46,XX ovotesticular DSD (DSD; disorder of sex development) male with a 148 kb duplication of the so-called RevSex element 0.5 Mb upstream of *SOX9* was well suited. This family and three other RevSex duplication/deletion families was published by Benko et al. in 2011, and the family was labeled DSD2 in this article.¹² In this family, the RevSex duplication was inherited from a normal grandmother through the XX-DSD male's father (Fig. S1). This gave us a chance to investigate the epigenetic profile of the duplicated strand on 46,XX and 46,XY genetic backgrounds. The results led us to explore if there also could be epigenetic differences between three control males and three control females. We found clear indications that the presence of a Y chromosome and/or only one X chromosome affects the conformation of the regulatory region from *SOX9* and approximately 0.7 Mb upstream, and in particular the 15 kb region from the TESCO enhancer to the *SOX9* transcription start site. Since these results are based on one XX and one XY RevSex duplication carrier and 3 female + 3 male controls, they should be considered as preliminary, especially since chromatin investigations were done in skin fibroblast primary cultures. In support of our study of skin fibroblasts as a proxy for the chromatin state in biologically more relevant but also inaccessible human tissues, it has recently been shown that the chromatin landscape of human primary fibroblasts have qualitative similarities to human embryonic stem cells, the major difference being an expansion of repressive chromatin blocks in the differentiated cell type.²⁰ Whether chromatin data from skin fibroblasts also have relevance for Sertoli cells in early embryonic development is unknown but conceivable, especially since high-order heterochromatin formation and

epigenetic remodeling of the genome can be discrete events, as was recently shown in senescent fibroblast.¹¹ Another indication of architectural conservation on a large-scale chromatin level is the preservation of topology-associated domains (TADs) across different cell types.²¹

If the chromatin differences between males and females in the *SOX9* regulatory region are Y chromosome or X-dosage related, a potential epigenetic mechanism for disturbed sex development has been found, with relevance for the large number of unexplained DSD-cases.^{5,22-24} Our findings also give reason to explore whether SRY may regulate gene expression through regional epigenetic modification.

Results

Since RevSex duplications apparently can bypass the need for SRY to induce testicular *SOX9* expression (i.e., cause XX-DSD) and RevSex deletions are associated with insufficient *SOX9* response to SRY stimulation (i.e., cause XY-DSD), normal males can be carriers of duplications and normal females can be carriers of deletions. It was therefore unexpected to find a RevSex duplication inherited from a normal female in our previously reported family, i.e., that this was not a de novo or grandpaternal duplication as had been expected (Fig. S1).¹² Potential causes of such non-penetrance could be mosaicism for the RevSex duplication in the carrier grandmother, stochastic differences during embryonic development, e.g., random epigenetic differences between grandmother and XX-DSD grandson, or the inheritance of different modifier alleles in grandmother and grandson. A SNP-array profile of blood-DNA did not show signs of mosaicism in the grandmother, but such mosaicism could still have been present in tissues types other than blood.

To explore if the duplication affected the chromatin architecture of the *SOX9* regulatory region, we performed chromatin immunoprecipitation studies on cultured dermal fibroblasts from the index patient (an XX-DSD male with the RevSex duplication on his paternal chromosome 17), his normal XY brother, his father (a carrier of the RevSex duplication on his maternal chromosome 17) and his mother (Fig. S2 show all the raw signal-to-input data). Unfortunately, dermal fibroblasts from the duplication-carrier grandmother could not be obtained. The antibodies used were against histone H3 trimethylated at lysine 4 (H3K4me3), histone H3 trimethylated at lysine 27 (H3K27me3), histone H3 trimethylated at lysine 9 (H3K9me3) and histone H3 acetylated at lysine 9 (H3K9ac); the latter two modifications cannot coexist, as they target the same residue (lysine 9) of histone H3. H3K4me3 and H3K9ac are euchromatic marks, i.e., associated with open chromatin, while H3K27me3 and H3K9me3 are heterochromatic marks, associated with two different types of closed chromatin.²⁵ Sometimes the H3K4me3 and H3K27me3 mark coexist on a promoter (bivalent promoters), which may be a sign of dynamic and developmental regulation.²⁶

The chromatin pattern of immunoprecipitated DNA was determined by comparative hybridization to input (non-immunoprecipitated) DNA on custom-designed high-density oligonucleotide arrays, i.e., a kind of “chromatin-CGH.” To supplement



Figure 1. An UCSC genome browser panel with custom tracks showing the differences in H3K4me3 (top), H3K27me3 (middle) and H3K9me3 (bottom) chromatin profiles between the 46,XX ovotesticular DSD male and his father in the area containing the RevSex duplication (marked with a black line). The curve displays the CHIP-on-chip signal of the index case after subtraction of the signal of his XY-father. The red lines mark the position of predicted SRY and SOX9 binding sites (“HMR Conserved Transcription Factor Binding Sites”), all with high Z-scores for SRY binding: 3.29 for SRY-1 and 3.13 for SRY-2. SRY-2 had in addition a high degree of genomic sequence conservation beyond the human/mouse/rat comparison that the Z-score calculations are based on; See the UCSC browser “Vertebrate Multiz Alignment & Conservation (46 Species)” track for details. A position of difference commented in the text is marked with “A.”

the chromatin data, whole genome CpG methylation was determined using Infinium HumanMethylation450 BeadChips. In our main region of interest (69.2–70.2 Mb from 17pter, hg19), the BeadChip interrogates 69 CpG positions, a small subset of the total number in this region. In addition, 4 CpG positions were investigated by methylation-sensitive MLPA.

The first region of interest was the RevSex duplication itself. This was fine-mapped and the breakpoints were sequenced after long-PCR. It was found to be a direct duplication of the segment from 69 521 863 to 69 670 036 nucleotides from 17pter (hg19), i.e., a 148 kb direct duplication 0.5 Mb upstream of *SOX9* without loss of genomic sequence, as previously published.¹² The raw CHIP-on-chip data suggested that this duplication had a more repressive chromatin signature in the XX-DSD male than in his duplication-carrier father (Fig. 1). To ease data visualization and to attempt to eliminate the confounding effect of the other (normal) *SOX9* locus, the XY father’s and the XX-son’s chromatin profiles were compared after subtraction of the profile of the XY-brother of the index male. This brother had not inherited the RevSex duplication from his father, and he shared the normal *SOX9* locus with his father and the maternal *SOX9* locus with his XX-DSD brother. The latter was determined by haplotyping using a selection of small tandem repeat PCR primers (data not shown). Both before and after such correction, it could be seen that both the repressive chromatin marks (H3K27me3 and H3K9me3) of the RevSex duplication region were enriched in

the XX-DSD case compared with his XY-father (Fig. 1). This did not correspond to differences in the degree of DNA methylation (Table 1), but one should note that the Infinium BeadChip array contains oligonucleotides against only two CpG positions within the duplicated segment. However, a similar result was obtained with custom designed methylation-sensitive MLPA testing of two other CpG positions within the RevSex duplication (Table 1).

When focusing on a region 158 kb downstream of the duplication (0.3 Mb upstream of *SOX9*) that contains conserved transcription factor and CTCF binding sites (annotated in the “Transcription Factor ChIP-seq from ENCODE” and “HMR Conserved Transcription Factor Binding Sites” tracks in the UCSC genome browser), a similar but milder tendency of increased heterochromatinization was found in the index compared with his father (Fig. 2). Close to the CTCF binding site itself the difference was striking: a H3K9me3 peak in the index had the same position as an H3K27me3 peak in the father (marked B in Fig. 2). That suggests different chromatin dynamics at this position in father and XX-DSD son. These peaks corresponded to a site of differential methylation (DMR2) that was hypomethylated in the father (Table 1, $P < 0.01$), in line with the chromatin immunoprecipitation results.

To explore how these chromatin differences between father and index could affect *SOX9* regulation, we looked for chromatin profile differences in a 120 kb region upstream of *SOX9*, including a known *SOX9* enhancer called TESCO (70 103 kb from

Table 1. Selected methylation values of the *SOX9* regulatory region in RevSex duplication carriers (the XX-DSD index case and his father) compared with an average of male (n = 5) and female (n = 4) controls

	Index	Father	Males	Females
Mean <i>SOX9</i> 69.03 - 70.20 Mb region	50%	50%	52%	52%
Mean RevSex duplication (148 kb)	83%	83%	83%	85%
DMR1: 69199815 lincRNA promoter	58%	52%	66%	63%
RevSex 69531725 MLPA	100%	100%	100%	100%
RevSex 69666757 MLPA	8%	6%	13%	10%
TFBS 69824353 MLPA	11%	11%	9%	8%
DMR2: 69826617 TF/CTCF-BS	65%	47%**	60%	60%
DNaseI 69972353 MLPA	34%	29%*	36%	38%
DMR3: 70107185 <i>SOX9</i> promoter	42%	24%*	41%	36%
DMR4: 70120411 <i>SOX9</i> gene body	42%*	80%**	60%	56%

The few positions of methylation differences are numbered as differential methylated regions (DMRs) 1–4. DMR4 shows the average value of two CpG positions. The values of most interest are shown in bold. * $P < 0.05$; ** $P < 0.01$. Please note that only 69 CpG positions in this interval were investigated by the Illumina BeadChip array, and about 1/3 of these positions were in the *SOX9* promoter and gene itself. In addition, 4 CpG positions were investigated by custom designed methylation-sensitive MLPA.

17pter) and a position of hypomethylation in the father (70 107 kb from 17pter, called DMR3 in Table 1). In addition to this point of paternal hypomethylation (DMR3, $P < 0.05$), the only striking difference between father and son was a stronger H3K27me3 profile in the XX-DSD son from the TESCO element and about 100 kb upstream (Fig. 3).

Conceivably, these differences could be due to the presence of a Y chromosome in the father, or different X chromosome dosage. To investigate this possibility, dermal fibroblast chromatin profiles of three unrelated males and three unrelated females were compared (Fig. 4). In the 736 kb region stretching from *SOX9* and upstream and including the RevSex duplication, interesting male/female differences were found. Corresponding to a potential regulatory element of ~10 kb at position 69 630 000 (hg19), i.e., in the RevSex duplication region, the females had a stronger H3K4me3 signal flanked by stronger H3K9me3 signals than males (marked A in Fig. 4), i.e., a similar tendency as in the XX-DSD case when compared with his father (marked A in Fig. 1). This site corresponds to a “regulatory” H3K4me1/H3K27ac peak in the ENCODE data, indicating a putative enhancer element (Figs. 1 and 4, bottom). Of interest was also a clearly more pronounced decrease in H3K9me3 signal toward the *SOX9* gene in males compared with females (marked D in Fig. 4). The H3K4me3 “TESCO-peak” found in males was almost invisible in females (marked C in Fig. 4), while the DSD-case had a peak of intermediate size (marked C in Fig. 3). A direct XX-DSD-case/female and RevSex-carrier-male/male comparison can be seen in Figure S3. Of note, the XX-DSD case had a stronger H3K4me3 signal than control females corresponding to the known *SOX9* enhancer/promoter region, suggesting more open chromatin and increased potential for *SOX9* activation. In addition, hypomethylation of two CpG positions in the *SOX9* gene body was found (Table 1, $P < 0.05$). These positions were hypermethylated in his RevSex duplication carrier father ($P < 0.01$). Unfortunately, using highly sensitive RT-PCR we did not detect any *SOX9* expression in leukocytes or fibroblasts from any of the investigated individuals,

and correlating our findings with mRNA expression levels was therefore not possible.

These chromatin profile differences between females and males were not reflected in differences in DNA methylation (Table 1). There were only four positions of differential methylation (DMRs) among the 50 CpG positions interrogated by the Illumina BeadChip upstream of *SOX9*. In addition, 4 positions of potential differential methylation were assayed using methylation-sensitive MLPA; these positions were chosen based on data from the “UCSF Brain DNA methylation” table of the UCSC genome browser. In none of these did males and females have different degrees of methylation (Table 1). For comparison, the chromatin profiles of the whole region and of the DMR positions described in Table 1 were quantitated, and, in this case, marked differences between females and males were detected. Overall, the female *SOX9* locus had a higher level of heterochromatin protein 1 (HP1)-associated repressive chromatin marks (H3K9me3) than males ($P < 0.01$, Table 2). The DMR1 region, close to a lincRNA start site, appeared more “active” in females (stronger H3K4me3 and H3K9ac profiles). DMR2 appeared more polycomb repressive complex 2 (PRC2)-protein associated in males (more H3K27me3) than females, which had a stronger signal of the HP1-associated repressive mark H3K9me3. DMR4, corresponding to two CpGs in the *SOX9* gene body, showed no significant differences in chromatin profiles, while DMR3, close to the *SOX9* promoter and 4 kb downstream the TESCO element, displayed the marked differences described above (Table 2). The differences in male and female chromatin profiles in the 120 kb region upstream of *SOX9* can be seen more clearly in Figure 5, where the average female profile has been subtracted from the average male profile. The opposing H3K4me3 (high in males) and H3K9me3 (high in females) peaks at DMR3 just downstream of the TESCO enhancer are striking. Of note, the female/male differences correspond to the XX-DSD-case/XY-father differences described above, the main exception being a more open chromatin state of the TESCO enhancer region in the RevSex duplication carriers.

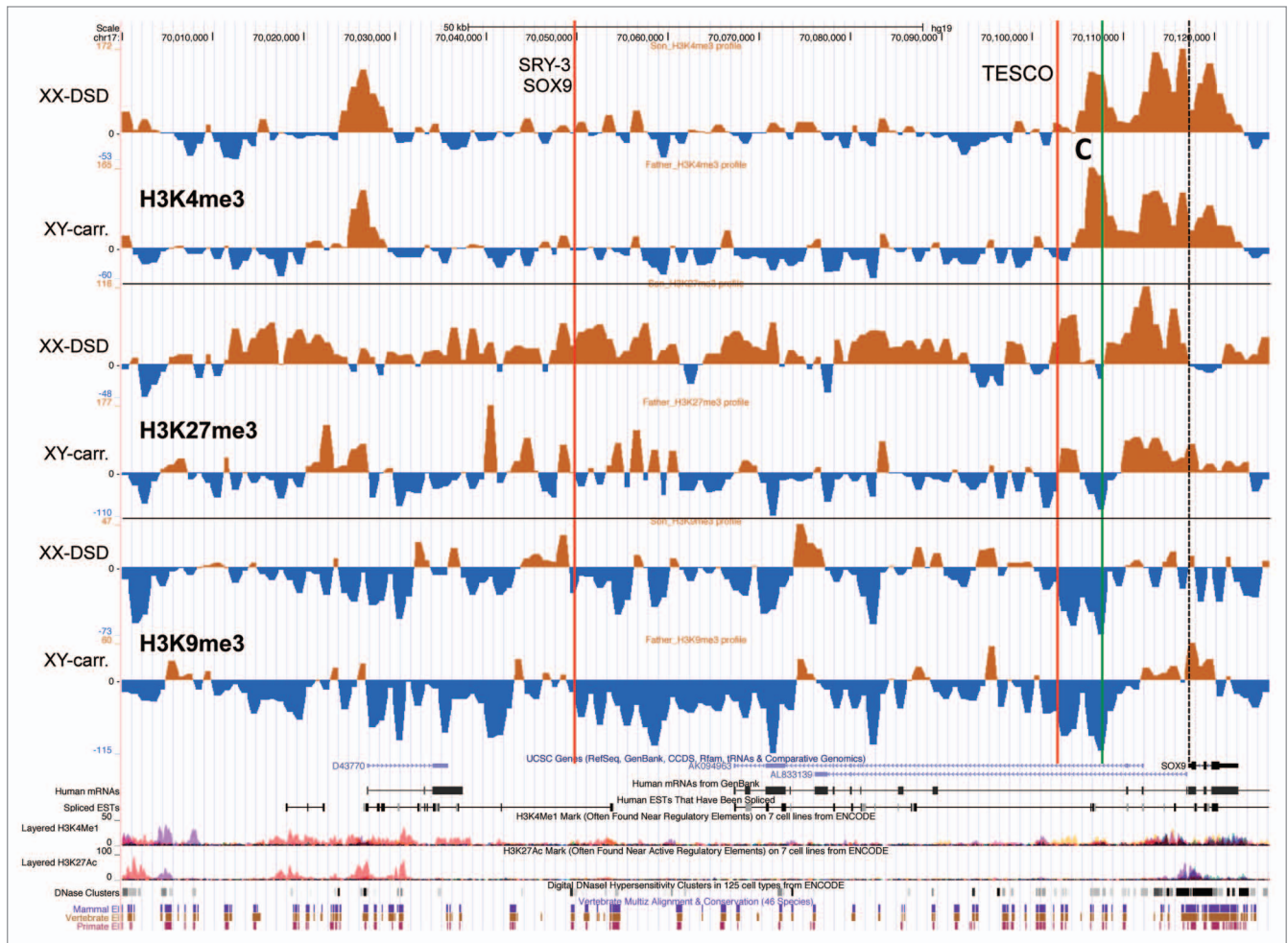


Figure 3. A UCSC genome browser panel with custom tracks displaying the signal-to-input chromatin profiles (H3K4me3, H3K27me3, and H3K9me3) of the XX-DSD case (upper profiles) compared with his RevSex duplication-carrier father (lower profiles) in the area from *SOX9* and 120 kb upstream (70 000–70 126 kb from 17pter, hg19). The red line called TESCO corresponds to the position of the TESCO-ECR (70 103 197–70 103 373 bp from 17pter, hg19), the green line marked C to DMR3 in Table 1, and the dashed line the start of the *SOX9* gene. The same position marks and more detailed explanations can be found in Figure 5.

between father and son can also be seen for H3K27me3 in the 100 kb region upstream of TESCO, with a stronger signal in the XX-DSD son than his XY father (Fig. 3). The most notable difference between the XX-DSD case and three female controls was a stronger H3K4me3 signal just downstream of TESCO in the RevSex duplication carrier (marked C in Fig. S3).

In addition to different sex chromosome complements, there is another potentially significant difference between the father and son: the father's RevSex duplication is on the maternal chromosome 17, while the son's duplication is on the paternal chromosome. An alternative explanation for non-penetrance in the grandmother could be imprinting, i.e., that only the maternal *SOX9* locus is responsive to SRY in the undifferentiated gonads. There are, however, reported instances of a paternal translocation/deletion or just a deletion that caused XY-DSD,^{15,27} showing that also the paternal allele is needed for appropriate *SOX9* transcription in the gonads. Therefore, the hypothesis that imprinting renders the maternal allele responsive and the paternal allele irresponsive to SRY seems unlikely.

Our data support a model where the RevSex duplication facilitates *SOX9* activation in the primordial gonads by inducing a conformational change to the *SOX9* regulatory region that may or may not lead to *SOX9* transcription in the genital ridge in the absence of SRY, establishing a self-stimulatory *SOX9* feedback loop.⁶ Stochastic or genetic background-based epigenetic changes may be a superimposed regulatory element explaining expression differences. This could be the reason for non-penetrance in the grandmother and partly non-penetrance in the XX-DSD case (only one of his gonads developed into a testis). Conceivably, the tiny ~500 nt spliced lincRNA in the RevSex region (Fig. 1, Ensembl ENSG00000225818) could also play a role. This lincRNA is expressed only in the testes (UCSC genome browser track “Human Body Map lincRNAs and TUCP Transcripts Tracks”) with expression quantified by Cufflinks to be 237–413 FPKM (out of a maximum of 1000) in the two analyzed testicular libraries.

Conceivably, the difference in chromatin profiles between XX and XY RevSex duplication carriers could be due to the Y

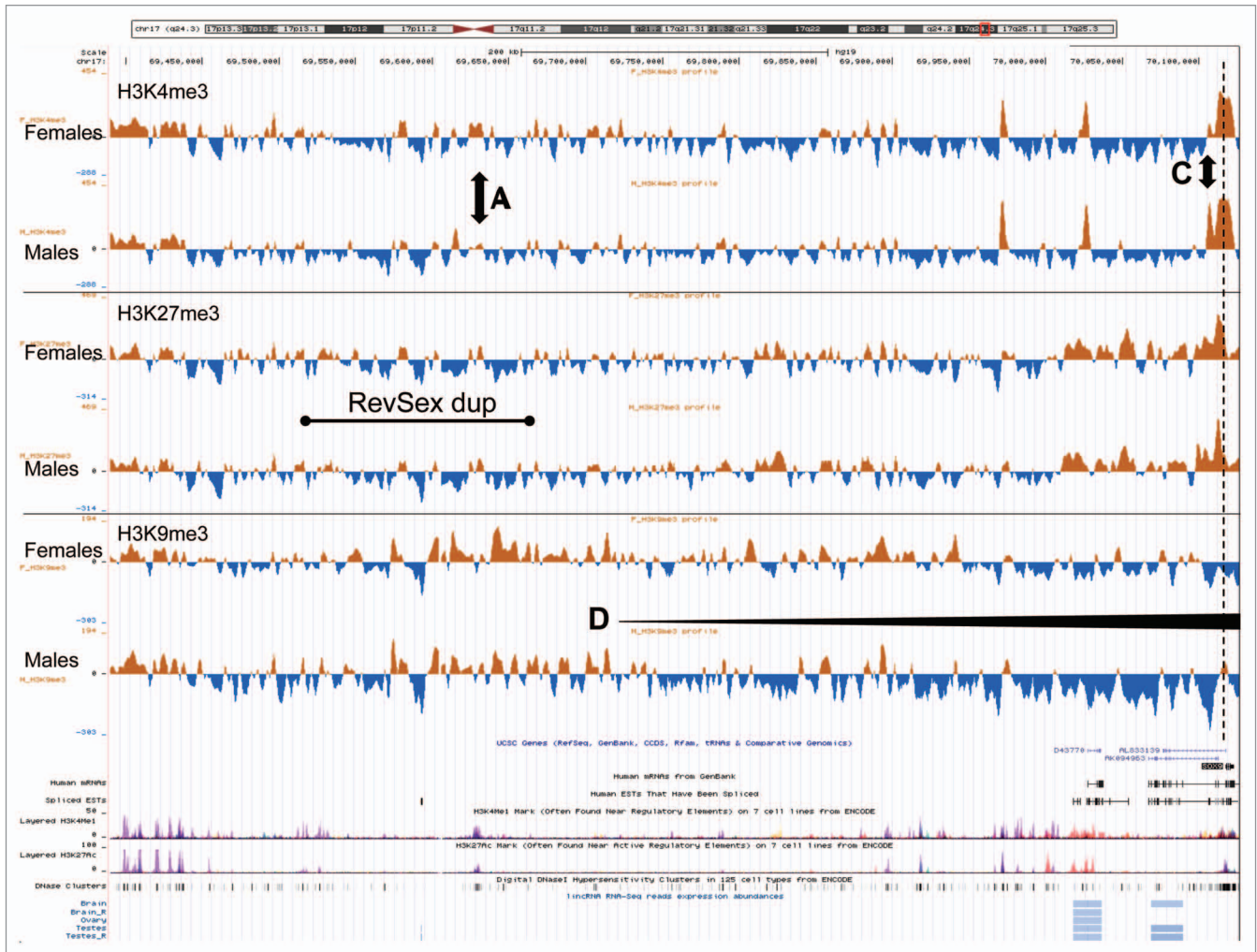


Figure 4. An UCSC genome browser panel with custom tracks showing the average signal-to-input chromatin profiles (H3K4me3, H3K27me3, and H3K9me3) of control females (upper profiles, n = 3) and control males (lower profiles, n = 3) in the 736 kb region from 69,390,000 to 70,126,000 (hg19) after 1 kb binning of data. A, C, and D mark positions of differences commented in the text. The dashed line marks the start of the *SOX9* gene.

chromosome and/or X chromosome dosage. In a mouse model, X chromosome dosage has been found to be at least as important as SRY for sex-dependent modulation of general gene expression.²⁸ However, the fact that *SOX9* is the major SRY-responsive gene in the primordial gonads, and the existence of three strongly predicted and one verified SRY binding sites upstream of *SOX9* (Figs. 1 and 3), makes it more likely that it is the presence of a Y chromosome and not different X chromosome complements that causes the father-son difference. To investigate this further, the average chromatin profiles of three unrelated males were compared with three unrelated females (Fig. 4). In the 120 kb region upstream of *SOX9* the clearest differences were found: females tended to have a more HP1-associated repressive chromatin profile of this region than males (Fig. 4). A similar and statistically highly significant tendency was found when the chromatin profile of the whole *SOX9* regulatory region was quantified (Table 2). This suggests that females and males have different chromatin state maps of the *SOX9* regulatory region. We do not know how this affects tissue specific *SOX9* expression as patient

tissues or cells in which this gene is normally expressed were not available. Our study was conducted on skin fibroblast primary cultures, which we assume to recapitulate aspects of the *SOX9* locus chromatin conformation in more relevant tissues (see Introduction for supportive argumentation), but we do not know if this is really true. Maybe the difference in chromatin states between males and females would have been even greater if a relevant tissue for sex-dependent *SOX9* expression had been investigated.

A summary of our findings is displayed in Figure 6. Between TESCO and *SOX9*, males had a more open chromatin profile than females, and the XX-DSD case had a profile that was in-between. The clearest marker for this RevSex influence and sex difference is the DMR3 locus (Tables 1 and 2), marked “C” in Figures 3, 4, 6 and Figure S3. In Figure 6 the H3K4me3 peak signal from this DMR site has been compared with an internal control, the peak signal from the *SOX9* promoter. The female H3K4me3 DMR3/*SOX9* ratio was 3:10 and the male ratio was 1:1. In the XX-DSD case, the ratio was 7:10, while his RevSex

Table 2. Male/female difference: Average male H3 chromatin profiles (n = 3) subtracted by average female H3 chromatin profiles (n = 3)

	K4me3	K27me3	K9me3	K9acet	n
SOX9 regulatory region 2.5 Mb: 68 160–70 660 kb	1	0	-7 ± 1	1	~37000
RevSex duplication 148 kb: 69 522–69 670 kb	-4 ± 2	-1	-5 ± 2	1	690
DMR1: lincRNA promoter 20 kb: 69 190–69 210 kb	-9 ± 2	-1	0	-7 ± 2	300
DMR2: TF/CTCF-binding site 10 kb: 69 821–69 831 kb	7 ± 3	13 ± 3	-18 ± 4	0	150
DMR3: Upstream SOX9 promoter 10 kb: 70 102–70 112 kb	29 ± 6	-6 ± 4	-20 ± 4	12 ± 2	150
DMR4: SOX9 gene body 5 kb: 70 118–70 123 kb	3	2	3	-14 ± 11	75

The values shown are signal-to-input differences (raw data comparisons). The regions of differential methylation (DMRs) correspond to the regions shown in **Table 1**. Values were the 99% confidence intervals (CI's) do not include zero are shown with the 99% CI range indicated. Major differences most likely to be also of biological significance are shown in bold. n = the approximate number of oligonucleotide probes on the custom array covering each region.

duplication carrier father had a ratio of 10:7. This indicates that the RevSex duplication and Y chromosome might have additive effects on the *SOX9* activation potential.

As far as we know, the question of whether the presence or absence of a Y chromosome can affect the chromatin landscape in the regulatory region of the main SRY target gene (*SOX9*) has not been addressed before. Since both SRY and *SOX9* are HMGB domain group transcription factors known to cause DNA bending,¹⁶ and since the DNA bending property of SRY is essential for its sex-determining function,¹⁷ modification of chromatin architecture is likely to be a SRY function. This adds to the complexity of sex determination, where a large number of genes and pathways have been found to be of importance, and variability between closely related species is the rule rather than an exception.²⁴ Foremost, among these genes are the *SRY/SOX9* and *FOXL2/ESR* pairs encoding transcription factors that promote male or female differentiation of the gonads, respectively. *FOXL2* ablation in female mice lead to Sertoli cell-like differentiation of ovarian granulosa cells, despite absence of *SRY*.²⁹ It appears that *FOXL2*, together with the estrogen receptor (ESR), is needed to keep *SOX9* in an inactive state in the mouse ovaries.²⁹ Notably, the *SRY/SOX9/SF1* responsive TESCO element was also able to bind *FOXL2/ESR*.²⁹ Since *FOXL2* mutations cause the autosomal dominant blepharophimosis-ptosis-epicanthus-inversus syndrome (BPES) in humans, a condition associated with premature ovarian failure in females, it is likely that *FOXL2/ESR* and *SRY/SOX9* act antagonistically also on the human TESCO enhancer. In our XX-DSD index case, the RevSex duplication must be able to overcome or bypass *SOX9* inhibition by *FOXL2/ESR*, maybe through a conformational change that makes the TESCO element more accessible to *SOX9*, potentially creating a positive feedback-loop for *SOX9* transcription.⁶ It is also noteworthy that another female differentiation-associated pathway, namely canonical WNT signaling stimulated by *WNT4* and R-spondin (RSPO1), may cause chromatin modification via β -catenin's interaction with another type of high-mobility group (HMG) transcription factors; the TCF/LEF family.^{7,30} Taken together, the shaping of the chromatin landscape of the *SOX9*

locus may be a common pathway for important sex determination factors, i.e., *SRY/SOX9*, *FOXL2/ESR*, and *WNT4/RSPO1*. This is also in line with the DSD-susceptibility (hypogonadism and ambiguous genitalia) found in boys with mutations in the X-linked genes *ARX* or *ATRX*, encoding a homeobox-containing transcription factor and a chromatin remodeling protein, respectively.²⁴ *SRY* might just be a facilitator for establishment of a *SOX9* positive feedback loop in the male gonads through an influence on chromatin conformation, and a similar feedback loop may be switched on by the RevSex duplication¹² or a lack *FOXL2*.²⁹

Taken together, we have shown that the RevSex duplication 0.5 Mb upstream of *SOX9* is associated with a more open chromatin landscape at the TESCO enhancer and further downstream. This may be a cause of testicular differentiation of primordial gonads despite the absence of *SRY*. We have also found indications that the sex chromosome complement, most likely the presence of a Y chromosome, influences the chromatin state of the *SOX9* regulatory region. If reproduced, our data provides a new angle for understanding sexual differentiation and unexplained DSD cases. Such epigenetic investigations may also lead to a better understanding of how the Y chromosome or *SRY* may affect the regulation of other genes, e.g., genes involved in autism, which for unexplained reasons is a male-predominant trait.

Patients and Methods

Family and case controls

The family with an XX-DSD male was recently described together with three other families with XX- or XY-DSD individuals due to duplications or a deletion of the RevSex element.¹² Of note, the index patient was born with hypospadias, rudimentary anlagen for vagina and uterus, and a scrotum containing a testis on one side and an ovarian remnant on the other side, all findings confirmed histologically. For the ChIP-on-chip experiments, dermal fibroblast samples from three unrelated males and three unrelated females were used as controls (see **Table S1** for an overview of all individuals investigated by ChIP-on-chip). For

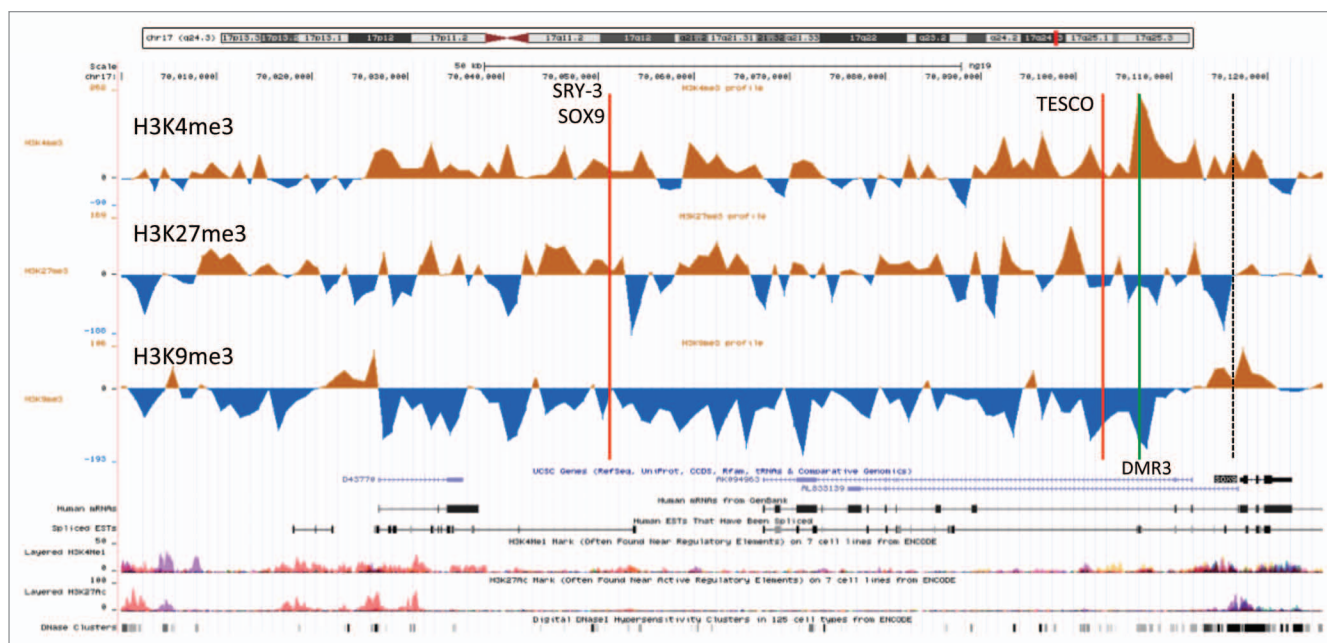


Figure 5. An UCSC genome browser panel with custom tracks displaying the difference between control male ($n = 3$) and control female ($n = 3$) chromatin profiles of the part *SOX9* locus from the gene itself and 120 kb upstream (70 000–70 126 kb from 17pter, hg19). The profiles shown are H3K4me3 (top), H3K27me3 (middle), and H3K9me3 (bottom). The red line called TESCO marks the position of the TESCO-ECR (70,103,197–70,103,373 bp from 17pter, hg19) and the green line the position of DMR3 in Table 1. The predicted SRY-3/SOX9 binding site had a Z-score of 3.45 for SOX9 and 2.41 for SRY, while the TESCO enhancer had a Z-score of 2.58 for SOX9 and 2.66 for SRY (UCSC, “HMR Conserved Transcription Factor Binding Sites”). In addition, the TESCO enhancer had a high degree of genomic sequence conservation beyond the human/mouse/rat comparison used to calculate the Z-score (UCSC, “Vertebrate Multiz Alignment & Conservation [46 Species]”). The TESCO SRY binding site has been experimentally verified.³¹

the methylation testing, the number of controls was extended to five males and four females.

All procedures were in accordance with the Helsinki declaration of 1975. After genetic counseling, written informed consent from family members and control individuals were obtained before the experiments were undertaken. Since this started 15 y ago as a routine diagnostic genetic investigation to find the cause of XX-DSD and determine recurrence risks, there is no Regional Ethics Committee file number associated with this investigation. The family has been informed of all results obtained.

Cell cultures

Dermal fibroblasts from family members and controls were grown from skin biopsies under standard conditions, frozen in batches in liquid nitrogen, and later used for short-term fibroblast cultures. T-lymphocyte cultures were also grown under standard conditions and used for genomic mapping of the RevSex duplication and studying mRNA expression.

Molecular characterization of the RevSex duplication

After fine-mapping of the 17q24.3 duplication on the Cytogenetics Whole-Genome 2.7 M array (Affymetrix), following the manufacturer’s instructions, the junction fragment of the duplicated region was PCR amplified using primers between markers rs11871027 and rs11871770 in the 5’ end, and rs2429978 and rs2430557 and in the 3’ end, followed by sequencing of the PCR product.

SOX9 haplotyping

To determine *SOX9* haplotypes and allele segregation, simple-tandem repeat markers were PCR amplified and size determined,

and there were two informative markers upstream and two downstream of the *SOX9* gene. Primer sequences can be provided upon request.

SOX9 mRNA expression

The *SOX9* mRNA expression levels were investigated by qRT-PCR on cDNA from patient and control blood T-lymphocytes and skin fibroblasts. Primers and probes were from an AssayOnDemand Hs00165814_m1 kit from Applied Biosystems (Life technologies). Assessments of *GAPDH* and *ACTB* mRNAs (Applied Biosystems, Part number 4333764F and 4352935E) served as endogenous controls.

Chromatin DNA crosslinking and isolation

For each sample, a total of 4×10^7 dermal fibroblasts were cultured to about 90% confluence before crosslinking DNA with 1% formaldehyde at 37 °C for 15 min. Thereafter, glycine was added to a final concentration of 125 mM followed by 5 min incubation at room temperature. Next, cells were washed 3 times with ice-cold PBS before harvesting by scraping and centrifugation. To isolate the chromatin-associated DNA, the cell pellet was resuspended in 4 ml cell lysis buffer (5 mM PIPES pH8; 85 mM KCL; 0.5% NP-40; 1× protease inhibitors) followed by 10 min incubation on ice, centrifugation and resuspension in 1 ml of nuclear lysis buffer (50 mM Tris, pH8; 10 mM EDTA; 1% SDS; 1× protease inhibitors). After incubation on ice for 30 min, the homogenate was fragmented in a S220 Focused-ultrasonicator (Covaris) according to the manufacturer’s recommendations. The level of DNA fragmentation was examined on a 1% agarose gel, and fragmentation was continued until the major

part of the DNA fragments had sizes of approximately 0.6 kb. After centrifugation, 50 μ l of the supernatant was removed to determine the chromatin-DNA concentration (for input DNA determination), and the rest of the supernatant was stored in 100 μ l batches at -80°C .

Chromatin Immunoprecipitation (ChIP)

Per ChIP experiment, 50 μ l lysate (on average containing 13.9 μ g DNA), 25 μ l protein A/G PLUS beads (Santa Cruz Biotechnology), and 425 μ l incubation buffer (0.2% SDS, 1% Triton, 150 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 8.5, 1 \times protease inhibitors) were mixed and incubated with rotation for 1 h at 4°C . Protein-DNA complexes were immunoprecipitated with a ChIP-validated antibody over night at 4°C . The antibodies used were against histone H3 (histone H3, #4620; Cell signaling), histone H3 trimethylated at lysine 4 (H3K4me3, ab8580; Abcam), histone H3 trimethylated at lysine 27 (H3K27me3, ab6002; Abcam), histone H3 trimethylated at lysine 9 (H3K9me3, ab8898; Abcam), histone H3 acetylated at lysine 9 (H3K9ac, ab4441; Abcam), and rabbit IgG (#2729; Cell signaling). The amount of antibody added was according to the manufacturers' recommendations. Next day, 25 μ l protein A/G PLUS beads were added to each ChIP and incubated for 2 h at 4°C . Thereafter the protein-DNA complexes were subjected to a series of washes in four different buffers: 2 \times (0.1% SDS, 0.1% DOC, 1% triton, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 8.5), 1 \times (0.1% SDS, 0.1% DOC, 1% triton, 500 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 8.5), 1 \times (0.25% LiCl, 0.5% DOC, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 8.5), and 2 \times (1 mM EDTA, 0.5 mM EGTA, 10 mM Tris pH 8.5). The formaldehyde-induced crosslinks of the ChIPs were reversed by incubating the samples for 30 min at room temperature in alkaline solution (0.1 M NaHCO_3 , 1% SDS), and the DNA was recovered by standard procedures. The ChIP yield was controlled by PCR amplification of two genes (*FBXO33* and *LRFN5*) in the final samples. In all ChIP-PCR verifications, the amount of immunoprecipitated DNA in input lysate was compared with a positive control (a histone H3 precipitate) and a non-immune control (rabbit IgG precipitate) for determination of the immunoprecipitation efficiency and specificity.

High Resolution ChIP-on-Chip assay

For ChIP-on-ChIP analysis, 60% of each of 2 replicate ChIP samples (from H3K9me3, H3K4me3, H3K27me3, and H3K9ac ChIPs) were concentrated using Microcon YM-30 spin columns (Merck Millipore) and amplified using the GenomePlex

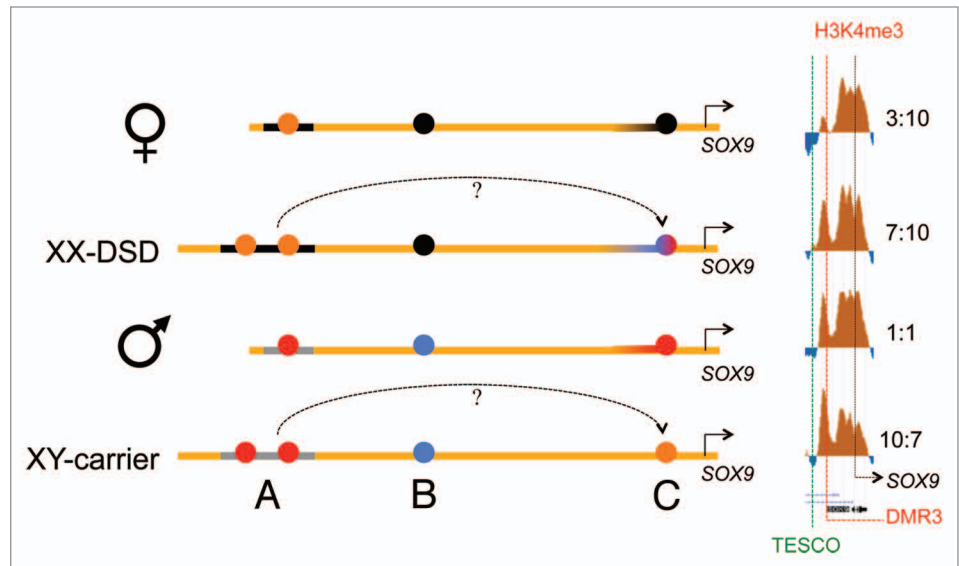


Figure 6. A schematic drawing of our major findings: In females the RevSex duplication (black line, region A) has more repressive chromatin with a more “active” putative enhancer element (orange dot) than in males. In the XX-DSD case similar findings are done with the exception of a more “open” *SOX9* enhancer/promoter (region C). In males a downstream CTCF binding site is enriched in H3K27me3 (PRC2-related) chromatin compared with females (region B), and the *SOX9* enhancer/promoter region (region C) is more open (red dot). This region is even more open in the XY RevSex carrier (orange dot). On the right side a comparison of the DMR3 H3K4me3 peaks (region C) can be seen with internal comparisons to the H3K4me3 peaks of the *SOX9* promoter with peak ratios shown on the right. Chromatin color codes are follows: black, H3K9me3; blue, H3K27me3; red, H3K4me3; orange, stronger H3K4me3.

Complete Whole Genome Amplification kit (Sigma-Aldrich). The ChIP-on-Chip hybridization was done on a custom-designed high-resolution NimbleGen $3 \times 720\text{K}$ arrays (Roche NimbleGen). The array probes (50-mers; positions according to GRCh37/hg19) were designed for uniform “tile-path” coverage of a 10 Mb regions of chromosome 2 (168.5–178.5 Mb from 2pter), an 18 Mb region of chromosome 13 (94–113 Mb from 13pter), a 41.5 Mb region of chromosome 14 (21 475–62 975 kb from 14pter), and a nearly 40 Mb region of chromosome 17 (49 370–90 870 kb from 17pter), with a median probe spacing of 0.1 kb. DNA labeling, array hybridization, post-hybridization washes and scanning were performed according to the manufacturer’s protocol for ChIP-chip assays, version 6.2 (Roche NimbleGen). In short, the ChIP and non-precipitated (input) DNA were labeled with Cy5- and Cy3-conjugated random nonamers, respectively. The labeled samples were purified, combined, denatured and hybridized for 16 h at 42°C to the array. After stringent washing the array was scanned using an Axon 4200AL Scanner (Molecular Devices) at 5- μm resolution. The acquired images were analyzed using DEVA v1.2 software (Roche NimbleGen) creating pair reports, including raw intensities for each probe and per image. From these data, ratio files were generated. For data visualization, the average ratios of two replicate experiments were binned per kb, adjusted for the number of probes per bin, and plotted against their chromosomal position.

ChIP-on-chip data analysis and presentation

As mentioned, 1 kb binning of the oligonucleotide signal-to-input-DNA ratios was performed, each 1 kb bin representing on

average 8 oligonucleotide signals (range 6–9) from two replicate experiments (i.e., the signal from 12–18 oligonucleotides). After such binning, there were 97 374 data points per ChIP experiment per individual (from selected regions of chromosomes 2, 13, 14, and 17). Thereafter, the average ratio of these 97 374 data points was calculated, and a minor adjustment of all ratio values was done to make an average signal equal to the value of zero (Table S1). The H3K4me3 ratios ranged from –1.22 to +2.65, the H3K27me3 ratios ranged from –1.98 to +2.28, the H3K9me3 ratios ranged from –1.51 to +1.15, and the H3K9ac ratios ranged from –1.54 to +2.00. To ease visualization of this large amount of ratios on Excel spread sheets, all values were multiplied by 100 and thereafter viewed as whole numbers. These data were used to make signal-to-input profiles (as in Fig. S2), i.e., a kind of comparative genomic hybridization (CGH) comparing immunoprecipitated DNA to input DNA from the same volume of cell lysate. A few chromatin profiles figures were made after subtracting such signal-to-input ratios from one another, e.g., for comparing males to females (Fig. 5) or the XX-son to his XY-father (Figs. 1 and 2).

Methylation chip assay

Methylation studies of DNA from blood samples were done using the Infinium Human Methylation 450K BeadChip assay (Illumina) according to the manufacturer's instructions. The work was provided as a service of the Oslo-node of the Norwegian Microarray Consortium (see www.mikromatrise.no). Briefly, from each sample, 500 ng genomic DNA was bisulphite modified using the EZ DNA Methylation Kit (Zumo). Thereafter, the bisulphite converted sample was whole-genome amplified, fragmented, precipitated, and hybridized onto the BeadChip for 16 h at 48 °C. After post-hybridization washing, single-base extension of the probes on the BeadChip, using the captured DNA as a template, was used to identifying the methylation level of the query CpG sites. Labeling was detected by the Illumina iScan System, and signal intensities were estimated by the GenomeStudio Methylation Module v1.9 software (Illumina).

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Methylation chip data analysis

The methylation level of each CpG site was calculated as a β value using Illumina's formula: $\beta = [\text{methylated signal intensity} / (\text{methylated signal intensity} + \text{unmethylated signal intensity})]$. Sample independent and sample dependent control probes on the BeadChip were used for data normalization and background subtraction. The degree of methylation of each queried CpG position was calculated as a percentage (from 0–100% methylation), and used for calculation of methylation values in Table 1.

MLPA-based methylation analysis

Methylation-sensitive MLPA analysis of four CpG positions in the *SOX9* regulatory region was done using the SALSA MLPA P200 Human DNA reference-1 kit (MRC-Holland) following the manufacturer's instructions. The CpG positions were at 69 531 725, 69 666 757, 69 824 353, and 69 972 353 (hg19). Primer sequences can be provided upon request.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

Lybæk H designed the experiments and did the experimental work with the help of de Bruijn D, den Engelsman-van Dijk AHA and Brendehaug A. Vanichkina D and Nepal C helped with computational analysis of the huge amounts of data. Houge G analyzed the data, made the figures, and wrote the manuscript.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/epigenetics/article/27474

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