SHORT REPORT

**SOX9 chromatin folding domains correlate with its real and putative distant cis-regulatory elements**

Marta Smyka, Kadir Caner Akdemir, and Paweł Stankiewicz

*Department of Medical Genetics, Institute of Mother and Child, Warsaw, Poland; bGenomic Medicine Department, MD Anderson Cancer Center, Houston, TX, USA; cDepartment of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

**ABSTRACT**

Evolutionary conserved transcription factor SOX9, encoded by the dosage sensitive SOX9 gene on chromosome 17q24.3, plays an important role in development of multiple organs, including bones and testes. Heterozygous point mutations and genomic copy-number variant (CNV) deletions involving SOX9 have been reported in patients with campomelic dysplasia (CD), a skeletal malformation syndrome often associated with male-to-female sex reversal. Balanced and unbalanced structural genomic variants with breakpoints mapping up to 1.3 Mb up- and downstream to SOX9 have been described in patients with milder phenotypes, including acampomelic campomelic dysplasia, sex reversal, and Pierre Robin sequence. Based on the localization of breakpoints of genomic rearrangements causing different phenotypes, 5 genomic intervals mapping upstream to SOX9 have been defined. We have analyzed the publicly available database of high-throughput chromosome conformation capture (Hi-C) in multiple cell lines in the genomic regions flanking SOX9. Consistent with the literature data, chromatin domain boundaries in the SOX9 locus exhibit conservation across species and remain largely constant across multiple cell types. Interestingly, we have found that chromatin folding domains in the SOX9 locus associate with the genomic intervals harboring real and putative regulatory elements of SOX9, implicating that variation in intra-domain interactions may be critical for dynamic regulation of SOX9 expression in a cell type-specific fashion. We propose that tissue-specific enhancers for other transcription factor genes may similarly utilize chromatin folding sub-domains in gene regulation.

**KEYWORDS**

chromatin looping; long distance gene regulation; non-coding variants; structural variants; tissue-specific enhancers

**CONTACT**

Dr. Paweł Stankiewicz pawels@bcm.edu Dept. of Molecular & Human Genetics, Baylor College of Medicine, One Baylor Plaza, Rm. R809, Houston, TX 77030, USA.

*Equal contribution

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**Introduction**

The dosage sensitive SOX9 (sex-determining region Y-related high-mobility group box 9) gene at 17q24.3 encodes a transcription factor essential for chondrocyte differentiation and testis development.1 Haploinsufficiency of SOX9 due to inactivating single nucleotide variants (SNVs) or genomic copy-number variant (CNV) deletions has been described in patients with a rare skeletal malformation campomelic dysplasia (CD, MIM 114290) that is frequently lethal and associated with male-to-female sex reversal in two-thirds of XY patients.2-13

Structural genomic variants, e.g. balanced translocations and inversions and unbalanced CNV deletions with breakpoints mapping up to 1.3 Mb up- and downstream to SOX9 have been identified in patients with milder phenotypes, including acampomelic campomelic dysplasia (ACD) and Pierre Robin sequence (PRS), suggesting that distant cis-regulatory elements of SOX9 may be compromised.1,4,6-11,14-16 Alignment of genomic breakpoint clusters in the protein-gene desert region 5’ to SOX9 enabled delineation of 5 genomic intervals associated with different phenotypes: moderate to severe CD (50–375 kb), ACD (789–932 kb),10,17 female-to-male (XXSR, 516–584 kb) and male-to-female (XYSR, 607–640 kb) sex reversals,18 and PRS (1.06–1.23 Mb to SOX9).15,19 More recently, CNV deletions mapping as far as 1.15–2.3 Mb upstream to SOX9 have been reported in patients with isolated congenital cardiac defects and/or PRS.16,20 Interestingly, CNV duplications mapping ~0.78–1.99 Mb 5’ to SOX9 have been reported in...
patients with Cooks syndrome (brachydactyly-anonychia) (OMIM 106995). Another male-to-female sex reversal genomic region was mapped 1.3–1.6 Mb downstream of SOX9.9,12

SOX9 expression has been shown to be regulated by several tissue-specific long distance enhancers/cis-regulatory elements, whose disruption causes bone anomalies and disorders of sex development (DSD). Reporter assay, epigenetic, and bioinformatic analyses have revealed the presence of real and putative distant non-genic evolutionarily conserved cis-regulatory elements of SOX9.7,9,10,15,22,25 Using chromatin conformation capture-on-chip (4C) analyses in Sertoli cells and lymphoblasts, Smyk et al.26 reported several novel cis-interacting regions both up- and downstream to SOX9.

Recent studies using high-throughput chromosome conformation capture (Hi-C) revealed that mammalian genomic nuclear DNA is highly organized and folded in chromosomal topologically associated domains (TADs)27 or CTCF-anchored chromatin loops.28 Genomic regions within a particular domain are interacting more frequently with each other, compared with those located in other domains. Moreover, genes and enhancers inside a domain show a high expression correlation.29 Thus, organization of these chromatin folding domains is important for proper gene regulation. Recently, Lupiáñez et al.30 described patients with limb anomalies due to disruption of TADs by structural genomic variations and referred this phenomenon as “enhancer adoption.”

Here, we report association of SOX9 TADs with its reported regulatory elements, implicating that variation in intra-domain interactions, such as chromatin looping, may be critical for dynamic regulation of gene expression in a cell type-specific fashion.

Results

In silico Hi-C analyses of chromosome 17q24.3 have revealed that this region is organized into several chromatin domains (Fig. 1). SOX9 is the only protein coding gene located inside the ~1.87 Mb TAD spanning through 68.67 to 70.45 Mb on chromosome

Figure 1. Schematic representation of Hi-C in cis genomic interactions within an ~3 Mb genomic region flanking SOX9 at 17q24.3. (A) Hi-C profiles at 25 kb resolution around SOX9 in HMEC, HUVEC, NHEK, and IMR90 cell lines. An ~1.87 Mb SOX9 topologically associated domain (TAD) extending between 68.67 to 70.54 Mb (hg19) is designated by the black dashed lines. (B) Hi-C Juicebox view profiles at 5 kb resolution around SOX9 in HMEC, HUVEC, NHEK, and IMR90 cell lines. (C) Location of the protein coding genes KCNJ2, SOX9 (red), and SLC39A11 is shown. (D, E) Histograms depict CTCF ChIP-seq enrichment levels and chromatin states (ChromHMM output) (active TSS - red, transcribed - green, enhancer - yellow, low – gray, and heterochromatin - purple). (F) Arcs represent “arrowhead” chromatin folding domains reported by Rao et al.28
17q24.3. This TAD is flanked by an upstream TAD containing the KCNJ2 gene and a down-stream TAD encompassing the SLC39A11 gene.\footnote{31} Interestingly, SOX9 domain is further sub-compartmentalized based on the transcriptional and epigenetic status. For example, in umbilical vein endothelia cells (HUVEC), this domain is silenced with the heterochromatic histone modification (Fig. 1a,b). Thus, there is a sole domain formation without any major inter-domain folding. However, in the cell types in which the SOX9 region is transcriptionally more active, such as mammalian epithelial cells (HMEC), epidermal keratinocyte cells (NHEK) cells, or lung fibroblast cells (IMR90), SOX9 is confined into smaller folding domain structures interacting with the several other adjacent domains\footnote{32} (Fig. 1). Interestingly, we have found that distribution of smaller domains observed in the SOX9 genomic region correlates with location of enhancers and genomic intervals associated with different phenotypes (Fig. 2). Therefore, structural alterations impacting the boundary regions between the SOX9 domain and its flanking domains could result in different phenotypic consequences based on alterations effect on chromatin looping domains.\footnote{31}

\begin{figure}[h]
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\caption{(A) Hi-C contact map around the SOX9 locus in NHEK cells at 25 kb resolution. Black dashed lines depict the SOX9 TAD. Vertical arrows show borders of TADs present in NHEK cells (black) and other cells (dotted). (B) The structural variants breakpoint cluster intervals mapping 5' to SOX9 and related to campomelic dysplasia (CD, violet box), Pierre Robin sequence (PRS, blue box), acampomelic campomelic dysplasia (ACD, navy box), and sex reversal (XXSR, red box; XYSR, gray box) are shown. (C) Regulatory elements shown as ellipses found to be active in: mandibular mesenchyme (blue),\footnote{15} testes (red),\footnote{24} node, notochord, gut, bronchial epithelium, and pancreas E1, migrating cranial neural crest cells - E3, fore- and midbrain E7 (brown),\footnote{22} chondrosarcoma cells (navy),\footnote{23} heart (green),\footnote{16} craniofacial and palatal tissue (gray),\footnote{25} somatic tissues (orange),\footnote{37} and cartilage (black).\footnote{38} Note the correlation of these regulatory elements and breakpoint cluster intervals with the SOX9 TADs. (D) Hi-C Juicebox view\footnote{28} contact map around the SOX9 locus in GM12878 cells at 5 kb resolution. Black dashed lines depict the SOX9 TAD. Please note that the SOX9 gene maps inside a CTCF/cohesin loop and that there are several enhancers contained within this loop that interact with the SOX9 promoter.}
\end{figure}
**Discussion**

Thus far, several regulatory elements and putative tissue-specific enhancers have been characterized in the genomic region upstream to SOX9. E1, located 28 kb 5' to SOX9, has been shown to drive SOX9 expression in node, notochord, gut, bronchial epithelium, and pancreas; E3 (251 kb 5' to SOX9) has been found to direct expression in migrating cranial neural crest cells and the inner ear; and E7 (95 kb 3' to SOX9) has been demonstrated to regulate expression in fore- and midbrain.22 Another putative cis-acting regulatory element called SOX9cre1 has revealed enhancer activity in the reporter constructs in a dose-dependent and tissue-specific manner.9,23 The other 2 highly conserved non-coding elements have been described by Benko et al.15 to have enhancer activity within mandibular mesenchyme of transgenic mice; human orthologs of these elements map 1.25 Mb and 1.44 Mb upstream to SOX9 and centromeric to the clustered breakpoints in patients with nonsyndromic PRS. Furthermore, other regulatory elements have been identified in the mouse models of the syntenic genomic region.32,33

Using a chromosome conformation capture-on-chip (4C) analysis, we have reported that the SOX9 promoter interacts with the upstream enhancer sequences in a tissue specific manner.26 In this study, we show that the Hi-C-defined SOX9 chromatin domain also exhibits the tissue-specific folding pattern. Interactions between the both up- and downstream cis-regulatory regions and SOX9 are dependent on the expression and histone modification status in a given tissue type. For example, when SOX9 domain is silenced with heterochromatic histone modification H3K9me3, such as in HUVEC cells, it loses its internal looping formations and generates only one large folding domain (Fig. 1D). In cells in which SOX9 is transcriptionally more active, there are several folding sub-domains inside the same domain, and alterations interfering with different anchoring points of the each folding sub-domains can result in various SOX9-related misregulations. Supporting this notion, TAD boundaries exhibit conservation across species and remain largely constant in multiple cell types.34

Importantly, we have found that SOX9 sub-domains associate with the genomic intervals harboring its real and putative regulatory elements, implicating that variation in intra-domain interactions, such as chromatin looping, in this genomic region may be critical for dynamic regulation of SOX9 expression in a cell type-specific fashion. These observations suggest that studying effects of variation on chromatin folding domains, it’s crucial taking dynamic regulation of SOX9 gene expression into account as this locus exhibits various chromatin folding patterns in a cell type-specific fashion. Supporting this notion, very recently, Franke et al.31 reported changes of genotype-phenotype correlation in mice due to genomic duplications forming new TADs (neo-TADs) in the Sox9 region. We propose that tissue-specific enhancers for other transcription factor genes may similarly associate with their chromatin domains.

**Material and methods**

Hi-C interaction data sets for Human Umbilical Vein Endothelial Cells (HUVEC), Normal Human Epidermal Keratinocytes (NHEK), and Human Mammary Epithelial Cells (HMEC), and human fetal lung fibroblasts (IMR90) cell lines were downloaded from the GEO database (GSE63525). Normalized 25 kb resolution Hi-C interaction matrices of chromosome 17 for the aforementioned 4 cell lines were generated by multiplying Knight and Ruiz normalization scores for 2 contacting loci and dividing raw observed values (MAPQGE30 filtered reads) at the interacting positions with this calculated normalization-score.31 IMR90 cell line Pol2 ChIP-Seq was downloaded from UCSC Genome Browser ENCODE portal. Chromatin states calls (ChromHMM) were downloaded from http://compbio.mit.edu/roadmap/. HiCPlotter (https://github.com/ckakdemir/HiCPlotter/) was used to plot Hi-C data with chromatin states.36

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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