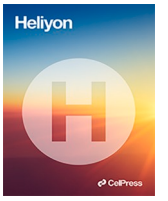
Heliyon 10 (2024) e33173

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Epigenetic control of *SOX9* gene by the histone acetyltransferase P300 in human Sertoli cells

Daniel González [a], María José Peña [a], Camila Bernal [a], Mary García-Acero [a], Maria Carolina Manotas [a], Fernando Suarez-Obando [a], Adriana Rojas [a,b,c,d,*]

[a] *Institute of Human Genetics, Faculty of Medicine, Pontificia Universidad Javeriana. Carrera 7 No. 40-62, 110231, Bogotá, Colombia*
[b] *Department of Genetics, University of Córdoba, 14071, Córdoba, Spain*
[c] *Maimónides Biomedical Research Institute of Córdoba (IMIBIC), 14071, Córdoba, Spain*
[d] *Reina Sofía University Hospital, 14071 Córdoba, Spain*

ARTICLE INFO

Keywords:
SOX9
Histone modifications
P300
Epigenetics
Transcriptional control
Sertoli
Disorders of sexual development (DSD)

ABSTRACT

Background: The transcription factor SOX9 is a key regulator of male sexual development and Sertoli cell differentiation. Altered *SOX9* expression has been implicated in the pathogenesis of disorders of sexual development (DSD) in mammals. However, limited information exists regarding the epigenetic mechanisms governing its transcriptional control during sexual development.

Methods: This study employed real-time PCR (qPCR), immunofluorescence (IIF), and chromatin immunoprecipitation (ChIP) assays to investigate the epigenetic mechanisms associated with *SOX9* gene transcriptional control in human and mouse Sertoli cell lines. To identify the specific epigenetic enzymes involved in SOX9 epigenetic control, functional assays using siRNAs for P300, GCN5, and WDR5 were performed.

Results: The transcriptional activation of SOX9 was associated with selective deposition of active histone modifications, such as H3K4me3 and H3K27ac, at its enhancer and promoter regions. Importantly, the histone acetyltransferase P300 was found to be significantly enriched at the SOX9 enhancers, co-localizing with the H3K27ac and the SOX9 transcription factor. Silencing of P300 led to decreased SOX9 expression and reduced H3K27ac levels at the eSR-A and e-ALDI enhancers, demonstrating the crucial role of P300-mediated histone acetylation in SOX9 transcriptional activation. Interestingly, another histone lysine acetyltransferases like GNC5 and methyltransferases as the Trithorax/COMPASS-like may also have a relevant role in male sexual differentiation.

Conclusions: Histone acetylation by P300 at SOX9 enhancers, is a key mechanism governing the transcriptional control of this essential regulator of male sexual development. These findings provide important insights into the epigenetic basis of sexual differentiation and the potential pathogenesis of DSDs.

* Corresponding author. Institute of Human Genetics, Faculty of Medicine, Pontificia Universidad Javeriana, Carrera 7 No. 40-62, 110231, Bogotá, Colombia; Department of Genetics, University of Córdoba, 14071, Córdoba, Spain; Maimónides Biomedical Research Institute of Córdoba (IMIBIC), 14071, Córdoba, Spain; Reina Sofía University Hospital,14071, Córdoba, Spain.
E-mail addresses: ge2romoa@uco.es, rojas-adriana@javeriana.edu.co (A. Rojas).

https://doi.org/10.1016/j.heliyon.2024.e33173
Received 1 January 2024; Received in revised form 11 June 2024; Accepted 14 June 2024
Available online 18 June 2024

Plain language summary

Human sexual determination is the result of molecular events that allow the differentiation of bipotential gonads in the testis or ovary. The bipotential gonad is developed between weeks 6–7 of fetal life and in this stage, several genes are expressed in the gonadal crest XY and XX. Testicular differentiation depends on SRY, a gene that encodes a sufficient and necessary transcription factor. SRY transcription factor translocates to the nucleus to induce transcriptional activation of *S*OX9. After translation, The SOX9 transcription factor controls male sexual development and Sertoli cell differentiation by regulating the expression of a significant number of male sexual-related target genes. Alterations in *S*OX9 expression have previously been described in the pathogenesis of disorders of sexual development (DSD) in mammals. However, few data are available about the epigenetic mechanisms responsible for its transcriptional control during sexual development. Here we report that transcriptional activation and repression of the *S*OX9 gene via its enhancer regions and promoter is accompanied by selective deposition and remotion of histone marks during male sexual differentiation. These epigenetic profiles are mediated by histone lysine acetyltransferases like P300 and GNC5 and the Trithorax/COMPASS-like complex.

1. Introduction

Male sexual differentiation is a two-step process, determined by 1) testis formation from primitive gonad through various transcription factors known as a sex determination, and 2) differentiation of internal and external genitalia by the action of hormones secreted by the fetal testicle such as anti-Müllerian hormone (AMH) secreted by Sertoli cells. The testicular differentiation depends on *SRY*, a gene that encodes a sufficient and necessary transcription factor (TF) to induce testicular development [1]. SRY TF binds to the enhancer region of *SOX9*, to intervene in the differentiation of Sertoli cells [1].

Activation of the *SOX9* gene, located in chromosome 17, is produced by the joint action of SRY and SF1 TFs on the TESCO region (testicular enhancer specific to Sox9 core). Although *SOX9* expression depends on SRY, testicular differentiation can occur in its absence, which has been observed in XX men with duplications and/or translocations of chromosome 17 [1,2]. Other studies related to genetic expression showed a relation between *SOX9* over-expression and sexual reversion (DSD) in patients with karyotype 46,XX without identified genetic alterations [3–5]. Disorders of sexual development (DSD) are conditions with an atypical chromosomal, gonadal, or phenotypic sex, leading to differences in urogenital tract development and distinct clinical phenotypes. Key genes involved in sexual development include *SRY, SOX9,* and *DAX1* which play pivotal roles during gonadal and functional differentiation. These genes contribute to maintaining the gonadal somatic sex as either male or female by suppressing the alternative pathway [1,2]. In addition to the genetic landscape, it has been established that in the development of DSD, epigenetic factors can play a pivotal role in regulating the expression of important genes in the normal context of sexual differentiation [2].

Epigenetics is the study of changes in gene expression unrelated to changes in DNA sequence. The principal epigenetic mechanisms described are DNA methylation, covalent histone modification, non-coding RNA, histone variants, and chromatin remodeling complexes [6–8]. Specifically, for *SOX9*, it has been proposed that epigenetic alterations may be involved in the development of DSD [9]; However, the molecular mechanisms involved in the transcriptional control of the *SOX9* gene have not been fully elucidated. Here, we identify histone modifications and enzymes responsible for transcriptional activation of the *SOX9* gene in Sertoli cells.

2. Materials and methods

2.1. Cell culture

Three testicular cell lines were used for this study: "HSerC" (Adult Human Sertoli Cells) (ScienCell #4520); Hs1.Tes (embryonic human testis cells) (ATCC® CRL7002™); and "TM4" (mouse Sertoli cells of 11–13 days) (RRID:CVCL_4327) (ATCC® CRL-1715™). Additionally, primary cultures of normal ovary, placenta, and a human ovarian dysgerminoma case (46,XY) (a malignant tumor composed of germ cells histogenetically derived from the embryonic gonads) were included (clinical characteristics summarized in Suppl. Table 1). These tissues were obtained by biopsy from voluntary donors who signed informed consent. Cells were cultured using the culture medium described in their manual, supplemented with 5 % fetal bovine serum (Cat #0025, ScienCell) and 1 % penicillin-streptomycin (#0503, ScienCell), and incubated at 37 °C with 5 % CO2.

2.2. Reverse transcriptase and quantitative real-time PCR (qRT-PCR)

To evaluate the expression of *SOX9, SRY, NR5A1, GCN5, P300,* and *WDR5* messenger RNA (mRNA) in the biological models studied, the extraction of total RNA and synthesis of cDNA were performed. Total RNA was extracted from 40 to 100 mm culture dishes at 90 % confluence, using TRIzol (Cat #15596, Ambion life technologies). 2 μg of total RNA was used for cDNA synthesis using ProtoScript First Strand cDNA Synthesis (Cat #E6300, New England Biolabs). Results were quantified by qPCR using the gene-specific primers detailed in Suppl. Table 2 using FastStart SYBR Green Master (Cat # 04707516001, Roche) on LightCycler Nano and COBAS z 480 (Roche) devices. The results were analyzed using the method of 2ˆ(-ΔΔCt) for relative quantification using GAPDH and β-actin genes as housekeeping.

2.3. Indirect immunofluorescence (IIF) assays

Immunofluorescence assays were performed to evaluate SOX9, SRY, and P300 expression proteins. The specific antibodies

employed are listed in the Suppl. Table 3. Cells were fixed with paraformaldehyde and permeabilized with 0,2 % Triton X-100. Goat Anti-Mouse IgG HyL (Alexa Fluor® 488) (ab150113, Abcam) and Donkey Anti-Rabbit IgG HyL (Alexa Fluor® 647) (ab150075, abcam) secondary antibodies were used. ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, REF. P36931) was used for contrast core staining. For fluorescence evaluation, images of 640 × 640 pixels resolution were obtained with an FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan) using the UPLSAPO 60 × 1.35 NA oil immersion objective. The images were processed using the ImageJ 1.53t Fiji distribution (National Institutes of Health, USA) [10].

2.4. Chromatin immunoprecipitation (ChIP-qPCR)

ChIP assays were performed on cross-linked chromatin samples as described earlier [11], with the following modifications: Sertoli cells (100-mm diameter tissue culture dishes) were incubated for 10 min with 1 % formaldehyde with gentle agitation at room temperature. The cells were then washed with 1X PBS three times, resuspended in 1 ml of cell lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl, Triton X-100, and proteinase inhibitors), and homogenized with a Dounce homogenizer (approximately 60 strokes with a tight pestle). The cell extract was collected by centrifugation at 3000×*g* for 5 min, resuspended in 0.5 ml of sonication buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % deoxycholate acid, 0.1 % SDS, and a mixture of proteinase inhibitors), and incubated for 10 min on ice. Chromatin was sheared in a water bath sonicator Bioruptor (Diagenode Inc.) to obtain fragments of 500 bp or smaller. The extracts were sonicated at high power for 10–15 pulses of 30 s each and centrifuged at 16,000×*g* for 15 min at 4 °C. The supernatant was collected, aliquoted, frozen in liquid nitrogen, and stored at −80 °C; one aliquot was used for A260 measurements. Chromatin size was confirmed by electrophoretic analysis.

Cross-linked chromatin extracts (2 A260 units) were resuspended in a sonication buffer to a final volume of 500 μl. The samples were precleared by incubating with 2–4 μg of normal IgG and 50 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 h at 4 °C with agitation. The chromatin was centrifuged at 4000×*g* for 5 min, and the supernatant was collected and immunoprecipitated with the specific antibodies listed in Supplementary Table 3 for 12–16 h at 4 °C. The immune complexes were recovered with the addition of 50 μl of protein A/G-agarose beads, followed by incubation for 1 h at 4 °C with gentle agitation. Immunoprecipitated complexes were washed once with sonication buffer, twice with LiCl buffer (100 mM Tris-HCl, pH 8.0, 500 mM LiCl, 0.1 % Nonidet P-40, and 0.1 % deoxycholic acid), and once with Tris-EDTA buffer, pH 8.0 (2 mM EDTA and 50 mM Tris-HCl, pH 8.0), each time for 5 min at 4 °C; this was followed by centrifugation at 4000×*g* for 5 min.

The protein-DNA complexes were eluted by incubation with 100 μl of elution buffer (50 mM NaHCO3 and 1 % SDS) for 15 min at 65 °C. Extracts were centrifuged at 10,000×*g* for 5 min, and the supernatant was collected and incubated for 12–16 h at 65 °C to reverse the cross-linking. Proteins were digested with 100 μg/ml proteinase K for 2 h at 50 °C, and the DNA was recovered using the kit ChIP DNA Clean and Concentrator (Zymo Research, D5201). The qPCR primers used to evaluate the SOX9 promoter region, and their enhancer are described in Supplementary Table 2. For all ChIP assays, an absolute quantification method was employed, generating a standard curve using serial dilutions of all input samples to accurately quantify the input percentage (% input) of the target proteins across the analyzed samples.

2.5. Small interfering RNA (siRNA) knockdown

Human Sertoli Cells (HSerC, ATCC® CRL-1715™) were plated on 6-well plates at 70 % confluence and incubated overnight. The cells were then transfected with 50 μM siRNA oligonucleotides targeting P300 (sc-2943, Santa Cruz Biotechnology), GCN5 (sc-37946, Santa Cruz Biotechnology), and WDR5 (sc-61798, Santa Cruz Biotechnology). A non-targeting 20–25 nt siRNA (sc-37007, Santa Cruz Biotechnology), designated as control siRNA (siRNA), was used as a negative control.

The transfection was performed using a transfection reagent (sc-29528, Santa Cruz Biotechnology) according to the manufacturer's protocol. The transfection reagent mixture was overlaid onto the washed cells and incubated for 6 h at 37°CC in a CO2 incubator. Subsequent experiments were performed 48 h after the transfection.

2.6. Nuclear extracts and protein expression analyses

Nuclear extracts were prepared from the HSerC Sertoli cell cultures using the Dignam method [12]. The buffer used contained 420.0 mM NaCl, 25.0 % glycerol, 0.2 mM EDTA, 1.0 mM DTT, 20.0 mM HEPES (pH 7.9) and 1.5 mM MgCl2. The total protein concentration was quantified using the Bradford technique [13].

A total of 15 μg protein/lane was separated using 4–15 % Mini-PROTEAN® TGX™ Precast Protein Gels (Cat #4561086). The separated proteins were then transferred to nitrocellulose membranes. The membranes were blocked with 5 % milk solution in TBS-Tween (0.1 %) for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies (see Suppl. Table 3). Goat anti-Rabbit IgG Poly-HRP (32260 Thermo Fisher Scientific) was used as the secondary antibody at a dilution of 1/5000, with an incubation time of 2 h, at room temperature. The immunoblots were visualized in CL-Xposure Film using SuperSignal West Femto Maximum Sensitivity Substrate (Cat #34095, Thermo Scientific, Inc.).

2.7. Statistical analyses

To determine statistically significant differences in the gene expression analyses, the non-parametric Wilcoxon rank-sum test and Kruskal-Wallis test were employed. The significance threshold was set at $p < 0.05$.

For the ChIP-qPCR assay analysis, Wilcoxon rank-sum tests were used to determine the significance of the results between the protein of interest and its respective control IgG. To assess the biological significance of these enrichment/binding results, Kruskal-Wallis and Dunn's multiple comparison tests were employed to contrast them with control regions. These results are presented in Supplementary Table 4. For the human cells, the Exon 1 region of the GAPDH gene was used as the control, as it has high levels of histone marks associated with active gene transcription and shows a lack of enrichment for repressive histone marks or SOX9 binding [14]. For the mouse cells, the control region corresponds to an intergenic region located upstream of *Sox9*, which was included as a negative control (NC) for Sox9 binding [15].

All analyses and results presented in this study are derived from the calculation of median and 95 % confidence intervals obtained from three independent experimental replicates. The statistical analyses were carried out in R v4.3.1 using the RStudio IDE [16–18]. The figures were created using GraphPad Prism software version 8.0.1 for Windows (GraphPad Software, Boston, Massachusetts USA).

3. Results

3.1. SOX9 expression in sertoli cells involves changes in epigenetic histone marks

To validate the expression of *SRY* and *SOX9* genes, qPCR and IIF assays were performed on HSerC and Hs 1.Tes cell lines, with ovarian and placental tissues as controls.

Under our experimental conditions, the HSerC and Hs 1.Tes cells exhibited higher levels of *SRY* (Fig. 1A) and *SOX9* (Fig. 1B) gene expression compared to the control tissues (placenta and ovary), as assessed by mRNA levels. Concordantly, the IIF assays showed robust protein expression of SRY (Fig. 1C) and SOX9 (Fig. 1D), with nuclear localization in human testicular cell lines. In contrast, the control tissues showed low expression of SRY and SOX9, as expected. Based on these gene expression findings, the HSerC cell line was considered a suitable model for investigating the epigenetic control of the *SOX9* gene activation.

To assess the impact of covalent histone modifications on the transcriptional regulation of the *SOX9* gene in humans, ChIP assays

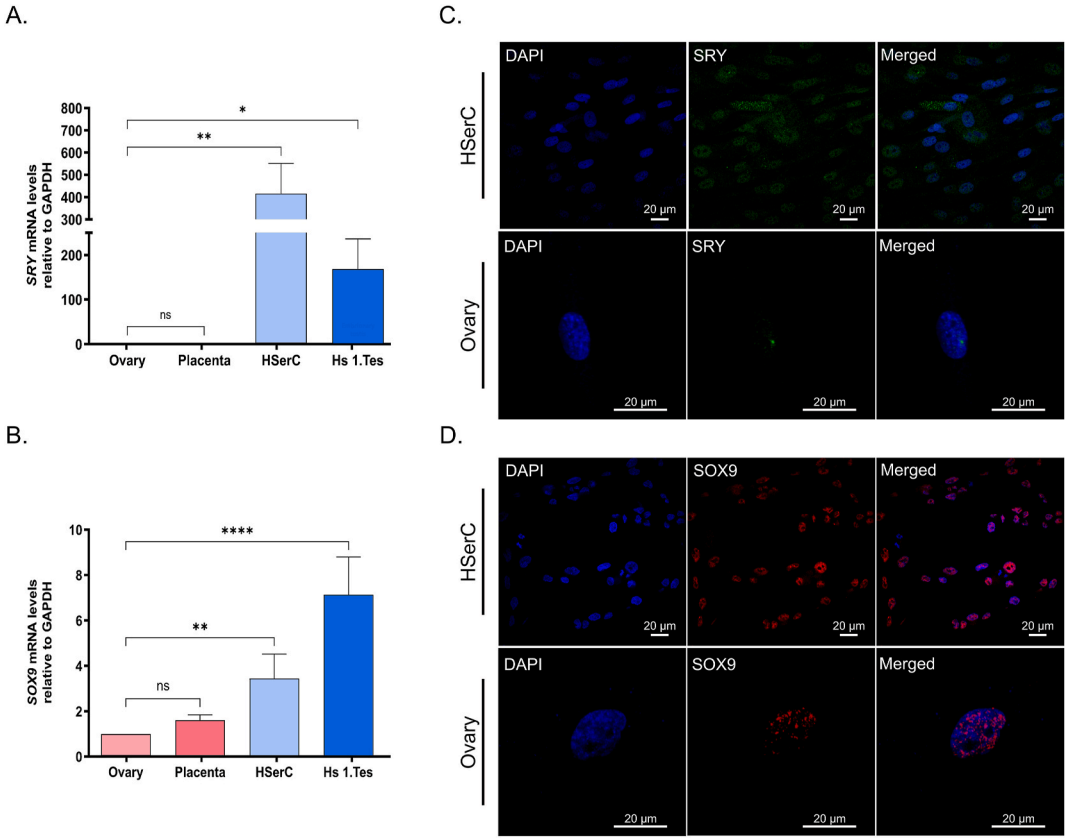


Fig. 1. Expression of *SRY* and *SOX9* in human Sertoli cells. mRNA levels comparison of *SRY* (**A.**) and *SOX9* (**B.**) normalized to *GAPDH* between the human testis cell lines (HSerC, Hs 1.Tes) and the ovarian and placenta control cells. Confocal microscopy IIF assay images for SRY (green, **C.**) and SOX9 (red, **D.**) protein expression in the HSerC Sertoli cells versus human ovarian tissue cells. Nuclei were labeled with DAPI (blue). Merged images are shown at the bottom of each row. Imaging was performed at 60× magnification using Alexa Fluor 488 and 647 fluorophores and processed using Fiji. Statistical significance is denoted as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were performed using specific antibodies to determine the enrichment degree of the histone marks H3K4me3, H3K9ac, H3K27ac, H3K9me3, and H3K27me3; and the SOX9 transcription factor binding [19]. qPCR analyses were conducted with specific primers that recognized four *SOX9* regulatory regions. Croft et al. (2018) described three enhancer regions, designated as *eSR-B, eSR-A*, and *eALDI*, which were found *in silico* and *in vitro* to be important gonadal enhancers for *SOX9*, as their deletion induced 46,XY sex reversal [5]. Additionally, the promoter region of the *SOX9* was analyzed (Suppl. Fig. 1A). A control region corresponding to the Exon 1 of the *GAPDH* gene, a constitutively expressed gene, was also evaluated and used as the reference threshold to determine the biologically significant enrichments in active regions (Suppl. Table 4).

In agreement with the results described earlier, the HSerC cells exhibited post-translational modifications in histone H3 that are typical of active regions, such as H3K4me3 and H3K9ac, in the promoter region (Fig. 2A). However, it is important to note that in this region, the analyses also identified the presence of H3K27me3, modifications that are characteristic of bivalent promoters (Fig. 2B).

On the other hand, the enhancer regions *eSR-A, eSR-B,* and *e-ALDI* showed enrichment of H3K27ac and H3K9ac, except *eSR-B* enhancer, which exhibited a loss of this mark in this region (Fig. 2A). The results also showed low levels of enrichment of repressive post-translational modifications in histone H3 such as H3K9me3 and H3K27me3, in the enhancer regions (Fig. 2B–Suppl. Table 4).

Since it has been described that once the SOX9 TF is activated, it binds to its enhancers, forming a positive autoregulatory loop [14, 20], the binding of SOX9 TF to these regions was evaluated. The results showed binding of SOX9 to the *eSR-A* and *eALDI* enhancers (Fig. 2C). These findings suggest that histone acetylation of Histone H3 in the enhancer regions is an important post-translational modification for the *SOX9* gene activation in human Sertoli cells (Fig. 2D).

To assess the presence of active histone modifications in the *SOX9* regulatory regions when it is aberrantly expressed, ChIP assays were performed on gonadal tissue samples from a woman diagnosed with ovarian dysgerminoma (Suppl. Table 1). The results of the qPCR analysis showed a significant increase in the *SOX9* gene expression levels in the gonadal tissue of the ovarian dysgerminoma case compared to the mRNA levels detected in the HSerC and HS.1.Tes cells (Suppl. Fig. 2A). The results of ChIP assays on the gonadal tissue of this case showed enrichment of H3K4me3, H3K9ac, and H3K27ac on regulatory regions, with concomitantly low levels of enrichment of the repressive histone marks H3K9me3 and H3K27me3 (Suppl. Table 4). Additionally, the binding of the SOX9 TF was detected in the *eSR-A* and *eALDI* enhancer regions (Suppl. Figs. 2B–E). Taken together, these results allow us to conclude that covalent

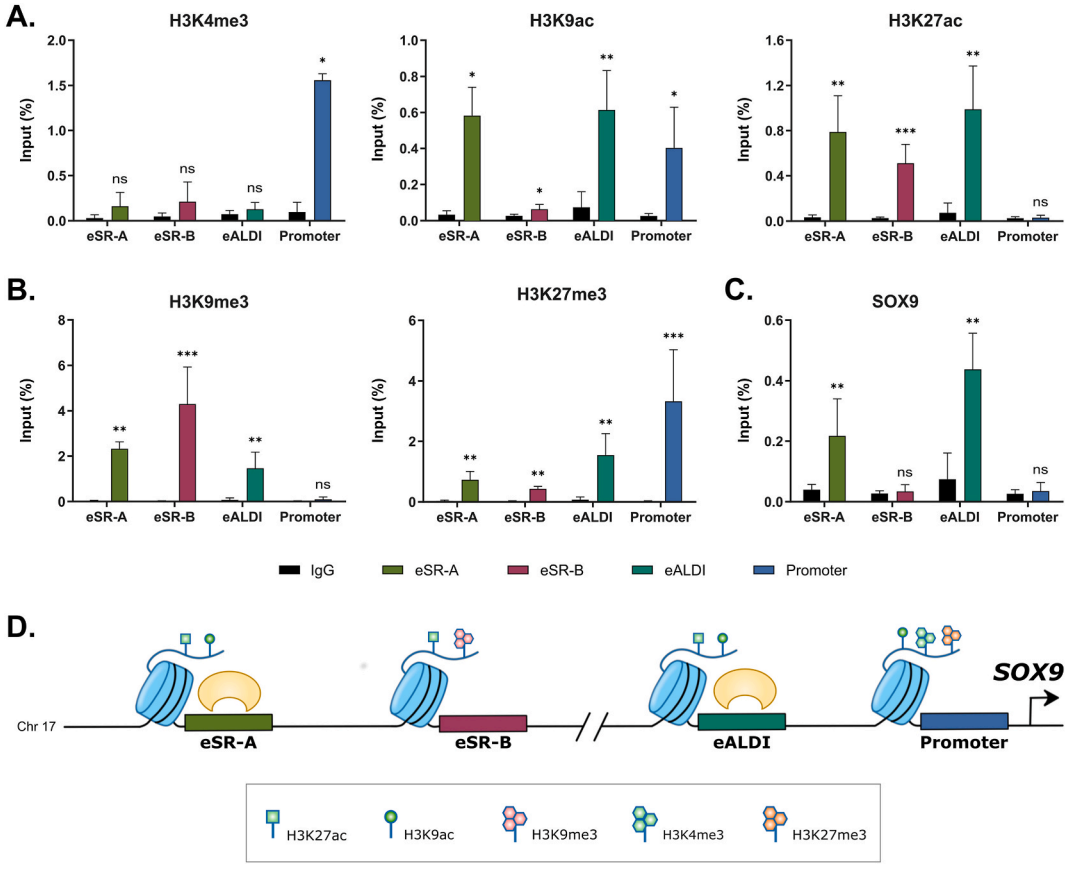


Fig. 2. Epigenetic regulation of *SOX9* in human Sertoli cells. Analysis of activating (**A.**) and repressive (**B.**) histone marks enrichment and SOX9 transcription factor binding (**C.**) across the regulatory regions of the *SOX9* gene in HSerC. (**D.**) Schematic summary of the significant enrichment and binding patterns observed in transcriptionally active *SOX9* state. Nonspecific IgG was used as a control (black bars), and statistical significance is denoted as: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

histone modifications are present during the *SOX9* transcriptional activation in humans, and the enrichment levels of these marks are directly related to gene transcription.

On the other hand, we sought to characterize the epigenetic state of regulatory regions when *SOX9* is not expressed, for which placental tissue was used (Fig. 1B). In these analyses, low levels of enrichment of the activating histone marks H3K4me3, H3K9ac, and H3K27ac were found in the regulatory regions analyzed (Fig. 3A). Concomitantly, the analyzes of the repressor marks revealed statistically significant enrichments of H3K27me3 in the *SOX9* promoter, eSR-A, eSR-B and eALDI enhancers (Suppl. Table 4). Additionally, enrichment of the H3K9me3 repression mark was detected in the eSR-B enhancer and the promoter region (Fig. 3B–Suppl. Table 4). Finally, the ChIP assays for SOX9 did not show binding of this transcription factor to the regulatory regions (Fig. 3C and D). All results demonstrate the existence of a specific pattern of histone modifications for the activation or repression of the *SOX9* gene, especially in its enhancer regions, according to the tissue expression [21].

Subsequently, we analyzed the epigenetic control of the *Sox9* gene in a murine model. For this, Sertoli cell line TM4 was used, where it was confirmed that, as seen in humans, there are high levels of expression of the *Sox9* gene (Suppl. Figs. 3A–B). To evaluate the covalent histone modifications mediating this activation, we examined three regulatory regions of the *Sox9* gene: a region close to the transcription start site (mTSS), as described by Chen et al. in 2015 [22]; and a region spanning the Sox9 binding site in the TESCO and enhancer 13 enhancers, as described by Gonen et al., in 2018 [23] (Suppl. Fig. 1B). Additionally, a negative control region (NC) for Sox9 binding was used, which corresponds to a intergenic region upstream the chromosome 11 of the mouse.

The ChIP results showed significant enrichments of H3K4me3 and H3K27ac in the mTSS region, indicating that this promoter is transcriptionally active. Interestingly, in the TESCO enhancer region, significant enrichments of H3K4me3, H3K9ac, and Sox9 binding were found (Suppl. Figs. 3C and E). In the evaluated region of enhancer 13, no significant enrichment was detected for the histone modifications assessed (Suppl. Figs. 3C and F).

All these results demonstrate that epigenetic control by histone modifications, especially on enhancer regions, represents an important mechanism for the *Sox9* gene transcriptional activation in the male gonad, as it is dynamic between repressed and activated states.

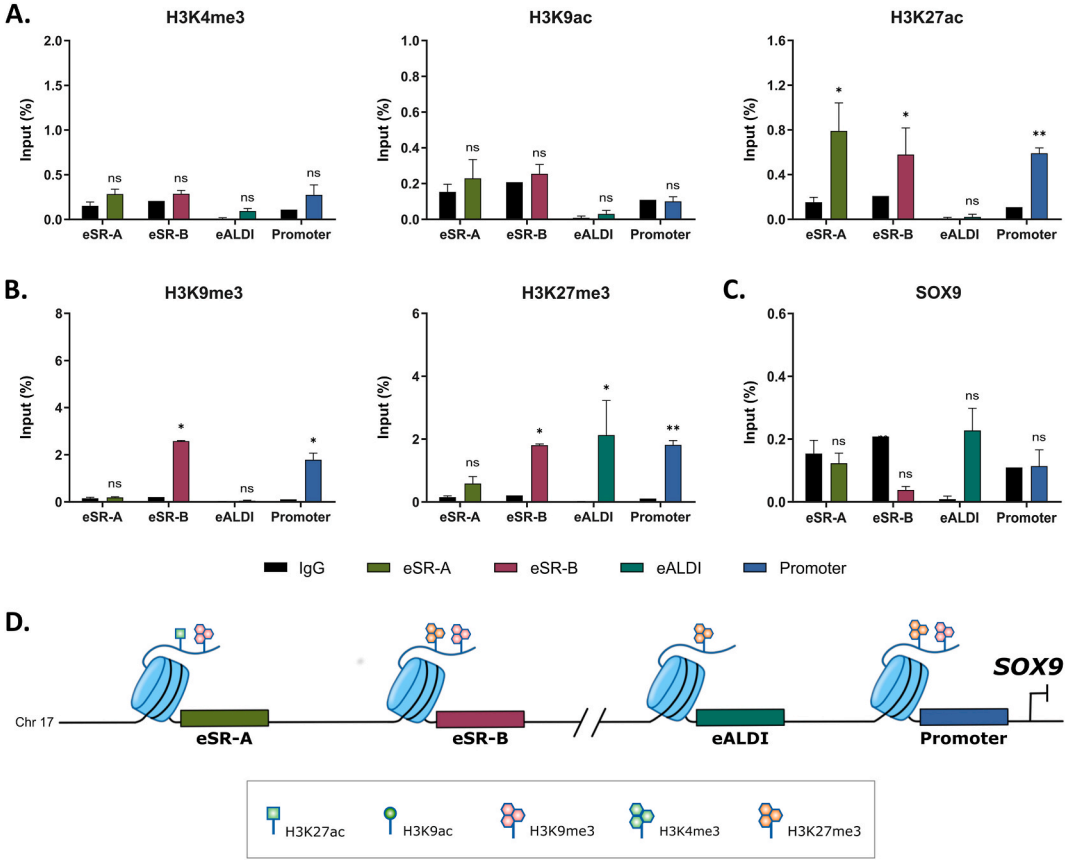


Fig. 3. *SOX9* repression is mediated by histone modifications in human placental cells. Analysis of activating (**A.**) and repressive (**B.**) histone marks enrichment and SOX9 transcription factor binding (**C.**) across the regulatory regions of the *SOX9* gene in placental tissue cells. (**D.**) Schematic summary of the significant enrichment and binding patterns observed in transcriptionally inactive *SOX9* state. Nonspecific IgG was used as a control (black bars), and statistical significance is denoted as: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.2. Epigenetic regulators mediate SOX9 transcriptional activation in sertoli cells

To identify the epigenetic enzymes responsible for the transcriptional control of the *SOX9* gene in Sertoli cells, we conducted ChIP analysis against specific epigenetic regulators. We used specific antibodies against P300 (H3K27 HAT), GCN5/PCAF (H3K9 HAT), and WDR5 (part of COMPASS H3K4 HMT).

We found that in Sertoli cells, *SOX9* transcriptional activation is associated with a significant enrichment of the P300 histone acetyltransferase at the *eALDI* and *eSR-A* enhancers (Fig. 4A). These regions also exhibited the H3K27ac activating histone mark, suggesting that P300 may be responsible for depositing this mark in the enhancer regions of the *SOX9* gene. In contrast, the GCN5 epigenetic enzyme showed a low percentage of interaction in all regions evaluated (Fig. 4B). Lastly, WDR5 binding was observed in the eSR-A and eSR-B enhancers (Fig. 4C). Given the detection of both P300 enzyme and SOX9 transcription factor binding in *eSR-A* and *eALDI* enhancer regions, re-ChIP assays were conducted in Sertoli cells (Fig. 4D–F). These results revealed the interaction between P300 and SOX9 in the eSR-A enhancer region (Fig. 4D). Additionally, qPCR and IIF assays confirmed active transcription and translation of the epigenetic regulators P300, GCN5, and WDR5 in HSerC cells (Suppl. Fig. 4).

On the other hand, in placenta tissue where the *SOX9* gene is transcriptionally repressed, we found that P300, GCN5, and WDR5 did not bind to the enhancer or promoter regions (Fig. 4G–I). These findings suggest that P300 and WDR5 are involved in the epigenetic regulation of the *SOX9 gene* through their action on regulatory regions when the gene is active.

3.3. The SOX9 expression requires the presence of H3K27ac and P300 at the eSR-A and eALDI enhancer regions

To investigate whether SOX9 transcription in Sertoli cells is modulated by the epigenetic regulator P300 identified as bound to *eSR-A* and *eALDI* enhancer regions, we conducted specific siRNA-mediated knockdown of P300 in Sertoli cells. Knockdown with siRNA led to a significant decrease in the *P3*00 mRNA expression levels compared with a control of nonspecific siRNA (siCTRL) (Fig. 5A). Likewise, IIF and Western blot confirmed a decrease in P300 protein expression, validating the effectiveness of gene silencing (Fig. 5B, 5E-F)(Suppl. Fig. 5). To examine the impact of P300 on *SOX9* gene activation, we assessed its expression after the silencing. The results showed a significant decrease in *SOX9* mRNA and protein levels in human Sertoli cells (Fig. 5C–F), confirming that the epigenetic

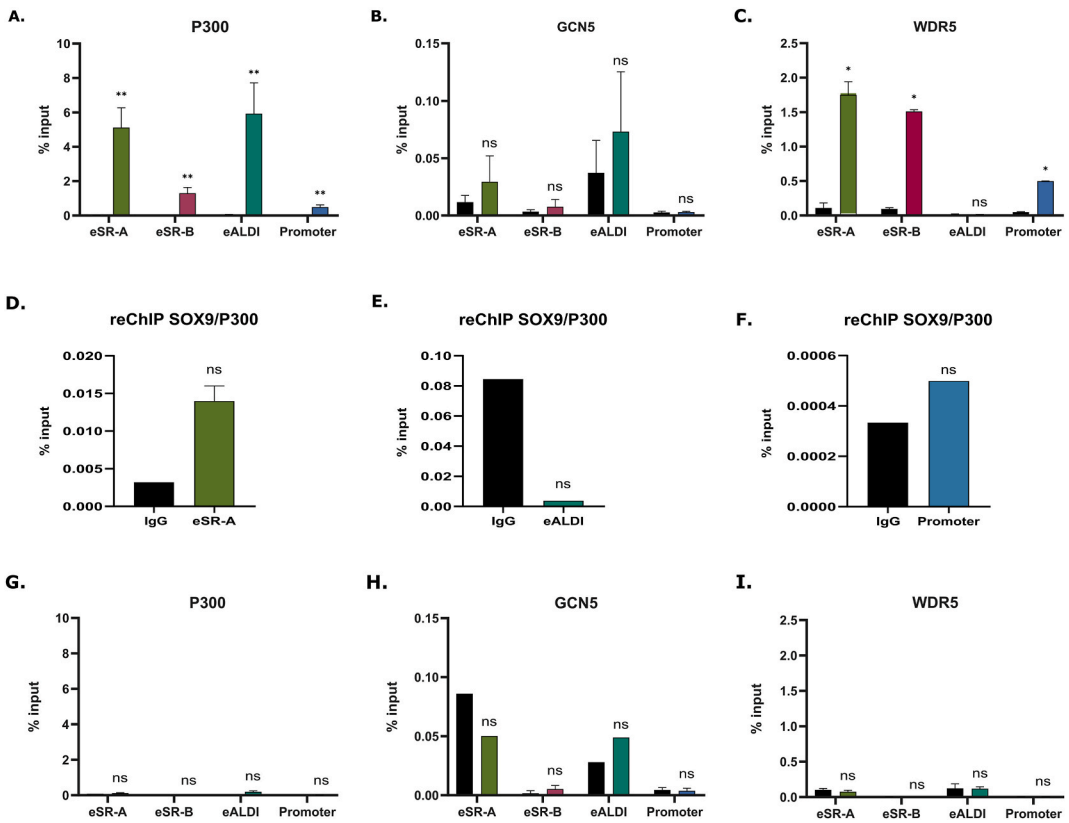
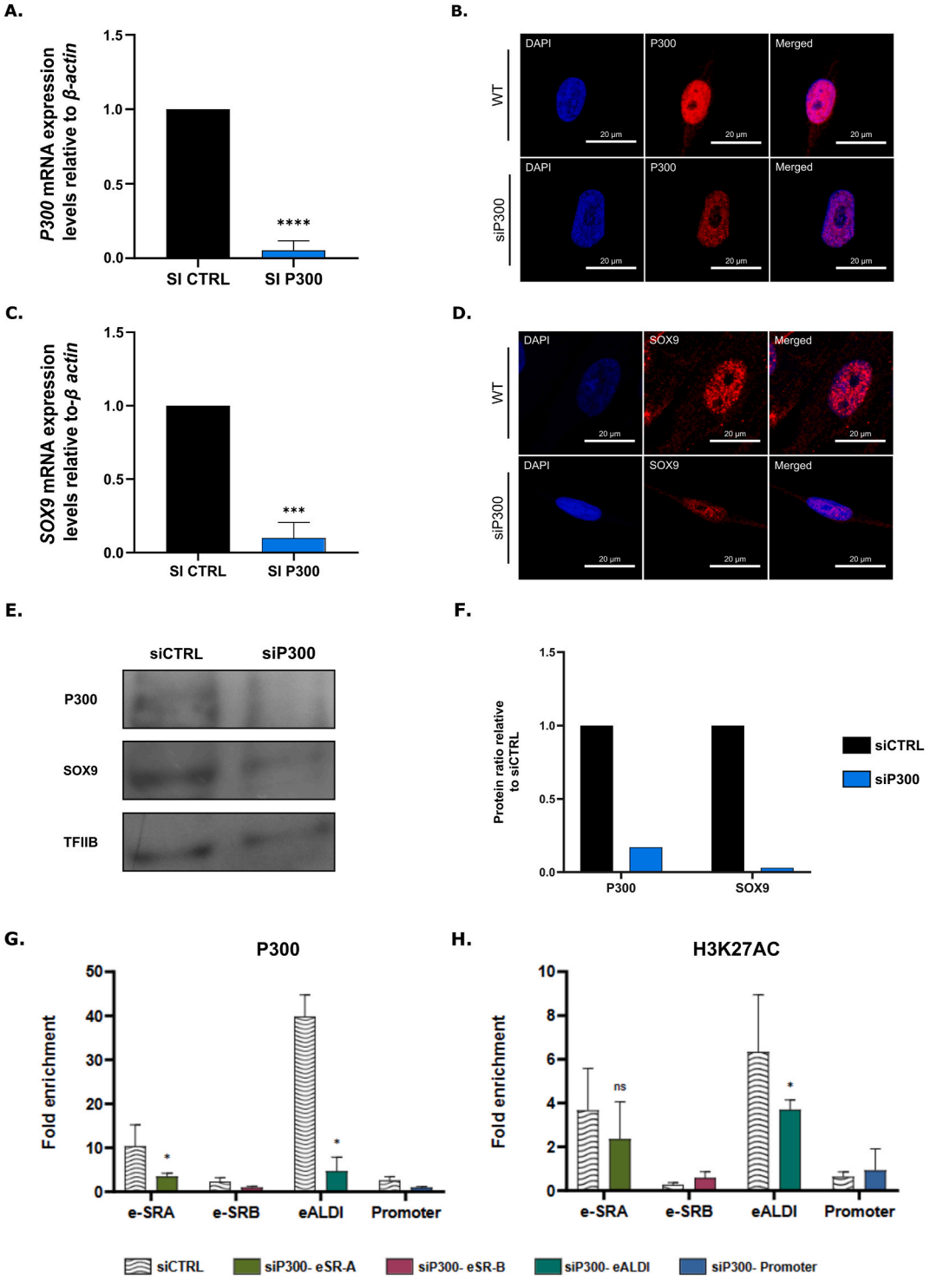


Fig. 4. P300 and WDR5 epigenetic enzymes interact at SOX9 regulatory regions in human Sertoli cells. Binding analysis of the epigenetic enzymes P300 (**A.**), GCN5 (**B.**), and WDR5 (**C.**) at regulatory regions of the SOX9 gene. re-ChIP analysis to assess the interaction between SOX9 and P300 at the *eSR-A* (**D.**), *e-ALDI* (**E.**), and promoter regions (**F.**) Additional ChIP results are provided for the binding of P300 (**G.**), GCN5 (**H.**), and WDR5 (**I.**) in human placental tissue cells at regulatory regions of the SOX9 gene. Nonspecific IgG was used as a control (black bars), and statistical significance is denoted as: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.



(caption on next page)

Fig. 5. *P300* silencing decreases *SOX9* expression through H3K27ac loss in human Sertoli cells. This figure explores the impact of P300 silencing on SOX9 expression in human Sertoli cells. (**A.**) mRNA levels of *P300* and (**C.**) *SOX9* relative to *β-actin* after P300 knockdown. (**B.**, **D.**) Confocal IIF images showing P300 and SOX9 protein expression, respectively, upon P300 silencing. Nuclei were labeled with DAPI (blue), and merged images are shown at the bottom of each row. Imaging was performed at 60× magnification using Alexa Fluor 488 and 647 fluorophores and processed using Fiji. (**E.**) Western blot assay showing the protein expression of P300 and SOX9 after knockdown, and (**F.**) its quantification presented as the protein ratio of siP300/siCTRL after background subtraction and TFIIB normalization. (**G.**) ChIP analysis of P300 binding and (**H.**) H3K27ac enrichment at SOX9 regulatory regions following P300 silencing. Data are presented as fold enrichment over a non-specific IgG control. A scramble siRNA was used as a control (siCTRL, striped bars). Statistical significance is denoted as: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

enzyme P300 plays a key role in the *SOX9* gene transcriptional activation.

To determine whether P300's transcriptional regulation of the *SOX9* gene involves its function as an epigenetic enzyme depositing H3K27ac on the enhancer regions, we performed ChIP assays following *P300* knockdown. Silencing of *P300* resulted in the loss of its interaction at the *eSR-A* and *eALDI* enhancer regions compared to the siCTRL condition (Fig. 5G). Moreover, there was a significant decrease in the epigenetic mark H3K27ac in the eSR-A and eALDI regions compared to the siCTRL condition (Fig. 5H).

Since the loss of P300 function leads to a drastic decrease in *SOX9* gene expression, accompanied by a significant reduction of H3K27ac in the eSR-A and eALDI enhancer regions, it was confirmed that epigenetic enzyme P300 plays a direct role in the transcriptional activation of the *SOX9* gene catalyzing the acetylation of lysine 27 of histone 3 (H3K27ac) and thus contributing to proper human sexual differentiation.

Additionally, we evaluated the mRNA expression of *DHH*, a downstream target of the SOX9 transcription factor, in cells transfected with siP300. The results revealed a significant decrease in its expression in cells with P300 knockdown, where we had previously detected a loss of *SOX9* gene expression (Suppl. Fig. 6A). *SRY* and *WNT4* genes also showed a significant change in cells transfected with siP300 compared to the control condition (siCTRL) (Suppl. Figs. 6B–C).

Finally, although the interaction of GCN5 and WDR5 enzymes with *SOX9* regulatory regions was not observed, we explored the possibility of their indirect regulation of the transcriptional activity of the *SOX9* gene or other genes from the sexual differentiation cascade. Concerning GCN5 enzyme, knockdown assays (Suppl. Fig. 7A) led to a significant decrease in *SOX9* gene expression (Suppl. Fig. 7B) and other genes key genes involved in the male sexual differentiation upstream of SOX9 such as *SRY* and *SF1* (Suppl. Figs. 7C–D). Similarly, WDR5 silencing (Suppl. Fig. 8A) resulted in a significant decrease in *SOX9* (Suppl. Fig. 8B) and *SRY* (Suppl. Fig. 8C) gene expression levels.

4. Discussion

The SOX9 transcription factor plays a crucial role during embryogenesis in the formation of different tissues. In the testes, it contributes to the differentiation of Sertoli cells, which compose part of the seminiferous tubules and provides support to the Leydig cells in the production of testosterone [24,25].

One of the most important epigenetic mechanisms for gene expression control, owing to its plasticity, is the covalent modification of histones, which is linked to the transcriptional activation or inhibition of genes [26]. In the transcriptional process, the binding of transcription factors to the enhancer and promoter regions of a gene depends on the accessibility to these regions, which in turn depends on the existence of covalent histone modifications, DNA methylation, presence of non-coding RNAs, among other epigenetic mechanisms. Currently, between 12 and 51 different chromatin states are described, each characterized by specific patterns of covalent modifications of histone activators and/or repressors on the regulatory regions of a gene [27]. In general, regulatory regions can be divided into three major states: active, bivalent, and repressed [30–32].

Interestingly, our results from ChIP assays in human Sertoli cells (HSerC), showed enrichments of H3K9ac and H3K27ac in the *eSR-A* and *eALDI* enhancers regions, where we detected the binding of the SOX9 transcription factor, thereby associating with a transcriptional activation state. Additionally, the *SOX9* promoter in the adult human testicle showed enrichment of H3K4me3 and H3K27Me3, classifying it as bivalent [27,28]. Enhancer regions play a central role in the control of tissue-specific gene expression and can enhance the transcription of their target genes over long distances, functioning as a binding platform for transcription factors [29]. Our results showed that the *eSR-A* and *eALDI* enhancers, which are active in the adult testis, could play a leading role in maintaining *SOX9* expression and in the formation of the continuous loop of positive autoregulation, thus triggering its expression in this tissue despite the bivalence of its promoter.

After comparing the epigenetic patterns of HSerC cells with abnormal gonadal tissue samples (ovarian dysgerminoma), patterns of histone modifications associated with transcriptional activity and more open chromatin were detected on the *eALDI* enhancer. *eALDI* is the only enhancer that has a binding sequence for SRY [5], which suggests that its activation in these tissues allows the initial activation of the gene and contributes to its maintenance through the formation of a positive autoregulation loop [29,33].

Studies in TM4 mouse Sertoli cells demonstrated the crucial role played by the enhancer *TESCO*, which stands out as a strong distal enhancer based on the simultaneous enrichments of H3K4me3, H3K9ac, and the binding of the SOX9 transcription factor. This suggests its role in mediating the positive autoregulation of *SOX9* expression once it reaches a critical threshold mediated by SRY [20]. Additionally, it is important to highlight that by sequence, the *eALDI* enhancer is considered homologous to the *TESCO* enhancer [5]. Unlike the human model, the region near mTSS was classified as highly active due to its enrichments of H3K4me3 and H3K27ac, as these mediate chromatin decompaction in the promoter region and allow the recruitment of RNA polymerase II. Despite the mouse enhancer 13 being described as a critical regulatory region of *Sox9* expression, on which transcription factors such as FOXL2, RSPO1,

and WNT4 bind, and being near to the homologous region of the *eSR-A* enhancer [20], no significant enrichments of the studied post-translational modifications were found. This suggest that the enhancer region could be regulated by other epigenetic mechanisms. In summary, these findings reinforce and highlight the role of histone modifications in the promoter region and *TESCO* enhancer in the activation of gene expression, especially at early ages, since the TM4 cell line corresponds to the Sertoli cell of 11–13 days-old mice.

Covalent modifications of histones are catalyzed and reversed by epigenetic enzymes that fulfill various functions in diverse biological contexts. Based on the literature background, the role of three specific histone-modifying enzymes important in gene transcriptional activation was evaluated. GCN5 and P300 are histone acetyltransferases responsible for catalyzing H3K9ac and H3K27ac, respectively. In contrast, WDR5, through the *COMPASS* complex, is involved with the H3K4me1, H3K4me2, and H3K4me3 [19,30].

Our results show that the HAT epigenetic enzyme P300 interacts with the enhancer regions *eSR-A* and *eALDI,* depositing the H3K27ac mark, which distinguishes these enhancers as truly active and contributing to the transcriptional activation of the *SOX9* gene. In this way, these findings confirm and complement those found in a previous study conducted by *Carre* et al. in 2018, referring to the role of P300 in mouse testicular determination through *SRY* acetylation [31]. Our ChIP results showed that P300 interacts with the enhancer regions of the *SOX9* gene in humans, confirming the important role of this enzyme in the testicular gene cascade beyond its role in *SRY* activation. These findings allow us to propose that P300 enzyme also has a direct role in the transcriptional activation of *SOX9* gene through the histone acetylation mechanism by interacting with the regulatory regions of *eSR-A* and *eALDI*, in which P300 catalyzes the H3K27ac. Remarkably, P300 enzyme interacted with the transcription factor SOX9 in *eSR-A* enhancer region, which suggests that P300 may contribute to the autoregulatory loop for SOX9 sustained transcription [20]. Previous reports in chondrocyte differentiation from mesenchymal cells showed that the transcriptional regulatory property of P300 is exerted through several mechanisms. P300 acts as a protein scaffold and bridging factor for forming multicomponent complexes and connecting DNA-binding

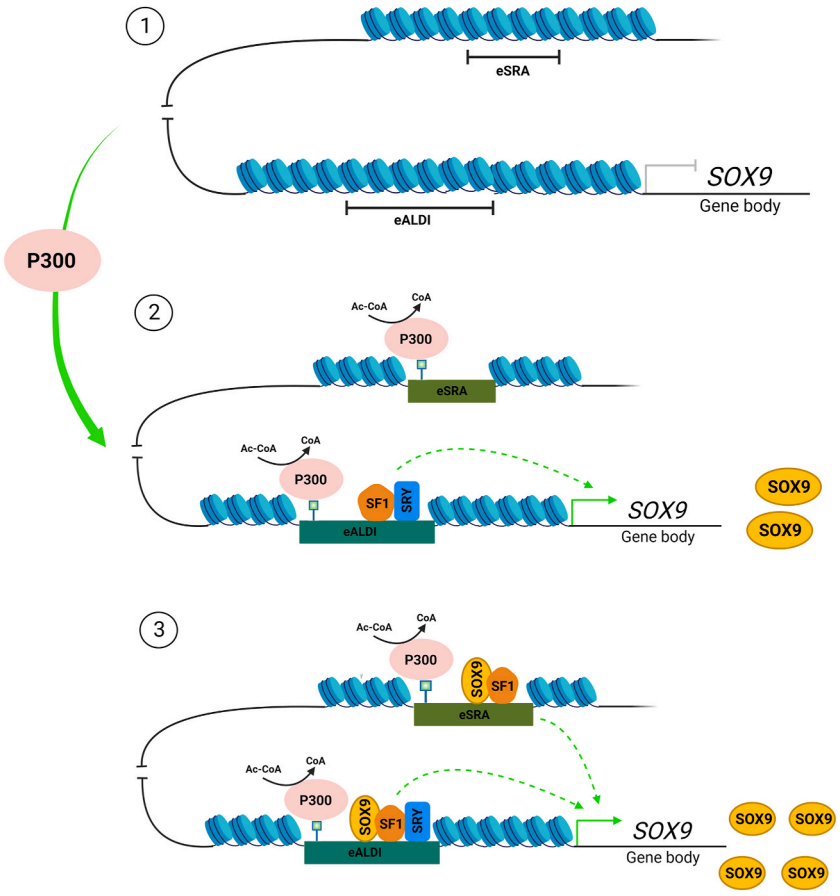


Fig. 6. Proposed model of P300 regulating *SOX9* gene transcriptional activation. This schematic representation illustrates the proposed mechanism for transcriptional activation of the SOX9 gene in human Sertoli cells. (**1**) In the bipotential gonad, the SOX9 gene is inactive and its chromatin is in a compact state. (**2**) After stimulation of the male differentiation pathway, the epigenetic enzyme P300 is recruited to the *eSR-A* and *eALDI* enhancers, depositing the H3K27ac histone mark. This mark facilitates chromatin decompaction, enabling the binding of transcription factors SF1 and SRY at the *eALDI* enhancer, which initiates SOX9 transcription. (**3**) The translated SOX9 protein then interacts with its own enhancer regions, forming a positive autoregulatory loop that maintains SOX9 expression and drives sexual differentiation towards the male gonad. This illustration was created using Biorender.com.

transcription factors, like SOX9, to the transcription apparatus [32].

The intrinsic HAT activity of P300 has the potential to facilitate transcriptional activation by histone modification [32]. This action regulates chromatin accessibility by altering electrostatic interaction between DNA and the histone proteins that compose the nucleosome core. Acetylation of histone lysine residues reduces the histone affinity for DNA facilitating transcription factor access, whereas histone deacetylation is characteristic of transcriptionally repressed chromatin. This could explain the loss of expression of the *SRY*, *DHH* and *WNT4* genes in cells with P300 silencing. This would suggest that affecting an epigenetic enzyme responsible for gene activation can lead to changes in the expression of other genes [32]. In addition, Inhibition of the P300 enzyme has been described to abolish enhancer activation altering the recruitment of transcription factors such as TFIID, thereby affecting transcription assembly and initiation. The recruitment mechanism of histone modifying enzymes such as P300 is known to have interaction domains with other coactivator protein complexes and in some cases may even interact with lncRNAs [32–34]. In the present study, we evaluated whether the enzymes GCN5, P300, and WDR5 are responsible for the transcriptional regulation of *SOX9* in the male gonad. Although, in the ChIP assays, an interaction of the enzymes GCN5 and WDR5 with some of the regulatory regions of the gene was not found. It was evident that in the loss of function assays of GCN5 and WDR5, the expression *SOX9* gene decreased significantly. Interestingly, this loss of function also affected the *SRY* gene expression. These results suggest a role of GCN5 and WDR5 in the transcriptional regulation of two of the central genes in the testicular differentiation way and in maintaining the biological balance between gonadal determination pathways.

It has been shown *in vitro* that GCN5 enzyme has a direct role in the acetylation of *SF1* gene, acting as an important coactivator in its transcriptional activation [35]. SF1 in the male sexual differentiation pathway has been described as an initiating factor by targeting the *SRY* gene through its interaction with enhancer regions. Additionally, it has been determined that SF1, through its interaction with SRY TF in the *TESCO* and *eALDI* enhancer regions, regulates the expression of the *SOX9* gene. Consequently, SF1 TF together with SOX9 TF interacts with the enhancer regions *eSR-A, eSR-B,* and *eALDI,* to maintain the loop of *SOX9* expression in testicular tissue [4,5]. According to the above, our results indicate that the transcriptional regulation of GCN5 enzyme on the *SOX9* gene could be indirect by affecting the upstream expression of *SF-1* and consequently affecting *SRY* and *SOX9* expression levels.

Instead, regarding the *COMPASS* complex (WDR5) it has been described as important during embryonic development [36]. Our findings showed the significant binding of WDR5 in the *eSR-A* enhancer region of *SOX9* in the activation condition. However, H3K4Me3 was not detected in this region. Despite this, the COMPASS complex has been involved with a characteristic mark of active enhancers, such as H3K4me1 [37]. This epigenetic mark was not evaluated in this work and could explain the binding of WDR5 in *eSR-A* enhancer region. According to a study conducted in mouse embryonic stem cells by *Tang* et al., the interaction of the *COMPASS* complex and P300 in enhancer regions is sufficient and necessary to catalyze the H3K4me1 mark [38]. In this way, our results showed that in the transcriptional regulation of the *SOX9* gene, the interaction of the COMPASS complex with P300 on the *eSR-A* enhancer region allows the presence of H3K4me1. Therefore, it would explain that when silencing WDR5 enzyme, *SOX9* gene expression levels are significantly decreased. However, additional studies are recommended to evaluate this hypothesis.

The findings reported in the present work allowed us to propose a biological model of the role of P300 enzyme in the transcriptional activation of *SOX9* gene mediated by the *eSR-A* and *eALDI* enhancers**.** In the context of the male differentiation pathway, the P300 enzyme recognizes and binds to the *eSR-A* and *eALDI* enhancer regions, generating a state of chromatin decompaction by altering the DNA-Protein interaction through the deposition of H3K27ac. Consequently, the *eALDI* region is recognized by the SRY TF and SF1 TF, and finally stimulates the expression of *SOX9* gene. Once translated into protein, SOX9 TF and SF1 TF interact and bind to the *eSR-A* and *eALDI* enhancer regions to promote gene expression of *SOX9* gene (Fig. 6) [5,39].

During the diagnostic approach to disorders of sexual development (DSD), a genetic approach is commonly performed, which typically includes cytogenetic and molecular analyses. The latter considers sequence and copy number variants without any transcriptomic or epigenetic research [4]. The likelihood of finding a specific genetic diagnosis with the current approach has ranged between 7 and 60 % [40,41]. Our results highlight the importance of considering mechanisms beyond specific DNA changes involved in expression anomalies of *SOX9*, such as epigenetic markers, which are not currently included in the diagnostic evaluation. Additionally, it allows us to confirm that epigenetic regulation by histone modifications plays a key role in the process of human sexual differentiation, and therefore, how alterations of these could be potentially involved in the development of DSD.

5. Conclusion

To date, the molecular networks that direct the sexual determination process in humans remain unclear. This lack of knowledge impacts the efficiency of diagnosis in patients with DSD, as currently, only 7–60 % of patients receive a specific genetic diagnosis. Our results prove the important role of epigenetic regulation through covalent histone modifications in the sexual differentiation process by dynamically modulating the expression of the *SOX9* gene.

Specifically, our results demonstrate the existence of a specific pattern of covalent histone modifications associated with the activation and repression of *SOX9* in Sertoli cells of both biological models. In the murine model, transcriptional activation of *Sox9* is mediated by the enrichment of the activating marks H3K4me3, H3K9ac, and H3K27ac in the TESCO enhancer and TSS. In humans, transcriptional activation is mediated by the enrichment of the activating marks H3K9ac and H3K27ac in the *eALDI* and *eSR-A* enhancers, with the latter modification being specifically mediated by the P300 enzyme. This allows the expression of the *SOX9* gene and the maintenance of its positive self-regulation loop. In contrast, the repression of *SOX9* gene is mediated by the inactivation of the promoter region with the repressive marks H3K9me3 and H3K27me3. On the other hand, the findings related to the enzymes GCN5 and WDR5 suggest a probable indirect regulatory role in the expression of the *SOX9* gene. This statement must be studied in greater depth for a better understanding of its role in the molecular pathway of sexual differentiation.

Perspectives and significance

This work constitutes a substantial contribution to the field of sexual development, which is currently understudied and specific etiology remains unclear in most cases, so it is necessary to explore it to achieve a broad approach and understanding of the epigenetic mechanisms linked to gonadal differentiation processes, framing the importance of epigenetics in the determination of the gonads.

Although this research focuses on the mechanisms controlling SOX9 expression in the gonad for the study of human sexual differentiation, the gene plays a role in various other biological processes in both normal and abnormal contexts. Therefore, these findings may be applicable to the study of other diseases, and further research should evaluate the epigenetic regulation of SOX9 in these specific tissues and processes.

Funding

This work was supported by grant from the Fundación para la promoción de la Investigación y la tecnología of Banco de la República de Colombia (grant number: P.T.I 4554). DG was supported by Grant Jóvenes Investigadores-MINCIENCIAS-Beca/Pasantía Concurso Nacional Otto de Greiff 2021.

Data availability statement

Data related to this study have not been deposited in a publicly available repository. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The studies were performed in accordance with the Declaration of Helsinki and approved by the ethical committee of Hospital Universitario San Ignacio (FM-CIE-0169-20) and the School of Medicine of the Pontificia Universidad Javeriana. Written informed consent to participate in this study was provided by the participants. All methods were carried out following relevant guidelines and regulations.

CRediT authorship contribution statement

Daniel González: Methodology, Investigation, Formal analysis. **María José Peña:** Validation, Investigation. **Camila Bernal:** Investigation. **Mary García-Acero:** Investigation. **Maria Carolina Manotas:** Investigation. **Fernando Suarez-Obando:** Writing – review & editing, Conceptualization. **Adriana Rojas:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Not applicable.

Abbreviations

HSerC - Human Sertoli Cells
DSD - Disorders of sexual development
AMH - anti-Müllerian hormone
TF - Transcription factor
HAT – Histone acetyltransferase enzyme
HMT – Histone methyltransferase enzyme
IIF Indirect immunofluorescence
siRNA Small interfering RNA knockdown
siCTRL Control scramble siRNA
qRT-PCR Reverse Transcriptase and Quantitative Polymerase Chain Reaction
ChIP-qPCR Chromatin Immunoprecipitation followed by quantitative PCR

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33173.

References

[1] M. García-Acero, O. Moreno, F. Suárez, A. Rojas, Disorders of sexual development: current status and progress in the diagnostic approach, Curr. Urol. 13 (2019) 169–178, https://doi.org/10.1159/000499274.

[2] R. Sreenivasan, N. Gonen, A. Sinclair, SOX genes and their role in disorders of sex development, Sex. Dev. 16 (2022) 80–91, https://doi.org/10.1159/000524453.

[3] S.C. Munger, B. Capel, Sex and the circuitry: progress toward a systems-level understanding of vertebrate sex determination, Wiley Interdiscip Rev Syst Biol Med 4 (2012) 401–412, https://doi.org/10.1002/wsbm.1172.

[4] M. Ono, V.R. Harley, Disorders of sex development: New genes, new concepts, Nat. Rev. Endocrinol. 9 (2013) 79–91, https://doi.org/10.1038/nrendo.2012.235.

[5] B. Croft, T. Ohnesorg, J. Hewitt, J. Bowles, A. Quinn, J. Tan, V. Corbin, E. Pelosi, J. van den Bergen, R. Sreenivasan, I. Knarston, G. Robevska, D.C. Vu, J. Hutson, V. Harley, K. Ayers, P. Koopman, A. Sinclair, Human sex reversal is caused by duplication or deletion of core enhancers upstream of SOX9, Nat. Commun. 9 (2018) 1–10, https://doi.org/10.1038/s41467-018-07784-9.

[6] E. Bártová, J. Kreǰcí, A. Harničarová, G. Galiová, S. Kozubek, Histone modifications and nuclear architecture: a review, J. Histochem. Cytochem. 56 (2008) 711–721, https://doi.org/10.1369/jhc.2008.951251.

[7] A.J. Bannister, T. Kouzarides, Regulation of Chromatin by Histone Modifications, Nature Publishing Group, 2011, pp. 381–395, https://doi.org/10.1038/cr.2011.22.

[8] R. Marmorstein, M.-M. Zhou, Writers and readers of histone acetylation: structure, mechanism, and inhibition, Cold Spring Harb Perspect Biol 6 (2014), https://doi.org/10.1101/cshperspect.a018762.

[9] S. Salamon, K. Flisikowski, M. Switonski, Methylation patterns of SOX3, SOX9, and WNT4 genes in gonads of dogs with XX (SRY -negative) disorder of sexual development, Sex. Dev. 11 (2017) 86–93, https://doi.org/10.1159/000466712.

[10] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biological-image analysis, Nat. Methods 9 (2012) 676–682, https://doi.org/10.1038/nmeth.2019.

[11] A. Rojas, R. Aguilar, B. Henriquez, J.B. Lian, J.L. Stein, G.S. Stein, A.J. Van Wijnen, B. Van Zundert, M.L. Allende, M. Montecino, Epigenetic control of the bone-master Runx2 gene during osteoblast-lineage commitment by the histone demethylase JARID1B/KDM5B, J. Biol. Chem. 290 (2015) 28329–28342, https://doi.org/10.1074/jbc.M115.657825.

[12] M.F. Carey, C.L. Peterson, S.T. Smale, Dignam and roeder nuclear extract preparation, Cold Spring Harb. Protoc. 2009 (2009), https://doi.org/10.1101/pdb.prot5330 pdb.prot5330.

[13] F. He, Bradford protein assay, Bio Protoc 1 (2011), https://doi.org/10.21769/BioProtoc.45.

[14] B. Croft, T. Ohnesorg, J. Hewitt, J. Bowles, A. Quinn, J. Tan, V. Corbin, E. Pelosi, J. van den Bergen, R. Sreenivasan, I. Knarston, G. Robevska, D.C. Vu, J. Hutson, V. Harley, K. Ayers, P. Koopman, A. Sinclair, Human sex reversal is caused by duplication or deletion of core enhancers upstream of SOX9, Nat. Commun. 9 (2018) 1–10, https://doi.org/10.1038/s41467-018-07784-9.

[15] N. Gonen, C.R. Futtner, S. Wood, S.A. Garcia-Moreno, I.M. Salamone, S.C. Samson, R. Sekido, F. Poulat, D.M. Maatouk, R. Lovell-Badge, Sex reversal following deletion of a single distal enhancer of *Sox9*, Science 360 (2018) 1469–1473, https://doi.org/10.1126/science.aas9408, 1979.

[16] O.J. Dunn, Multiple comparisons using rank sums, Technometrics 6 (1964) 241–252, https://doi.org/10.1080/00401706.1964.10490181.

[17] P. Mair, R. Wilcox, Robust statistical methods in R using the WRS2 package, Behav. Res. Methods 52 (2020) 464–488, https://doi.org/10.3758/s13428-019-01246-w.

[18] N.J. Horton, K. Kleinman, Using R and RStudio for Data Management, Statistical Analysis, and Graphics, Chapman and Hall/CRC, Vienna, Austria, 2015, https://doi.org/10.1201/b18151.

[19] T. Kouzarides, Chromatin modifications and their function, Cell 128 (2007) 693–705, https://doi.org/10.1016/j.cell.2007.02.005.

[20] N. Gonen, R. Lovell-Badge, The regulation of Sox9 expression in the gonad, in: Curr Top Dev Biol, first ed., Elsevier Inc., 2019, pp. 223–252, https://doi.org/10.1016/bs.ctdb.2019.01.004.

[21] E. Sjöstedt, W. Zhong, L. Fagerberg, M. Karlsson, N. Mitsios, C. Adori, P. Oksvold, F. Edfors, A. Limiszewska, F. Hikmet, J. Huang, Y. Du, L. Lin, Z. Dong, L. Yang, X. Liu, H. Jiang, X. Xu, J. Wang, H. Yang, L. Bolund, A. Mardinoglu, C. Zhang, K. von Feilitzen, C. Lindskog, F. Pontén, Y. Luo, T. Hökfelt, M. Uhlén, J. Mulder, An atlas of the protein-coding genes in the human, pig, and mouse brain, Science 367 (2020), https://doi.org/10.1126/science.aay5947, 1979.

[22] N.-M. Chen, G. Singh, A. Koenig, G.-Y. Liou, P. Storz, J.-S. Zhang, L. Regul, S. Nagarajan, B. Kühnemuth, S.A. Johnsen, M. Hebrok, J. Siveke, D.D. Billadeau, V. Ellenrieder, E. Hessmann, NFATc1 links EGFR signaling to induction of Sox9 transcription and acinar–ductal transdifferentiation in the pancreas, Gastroenterology 148 (2015) 1024–1034.e9, https://doi.org/10.1053/j.gastro.2015.01.033.

[23] N. Gonen, C.R. Futtner, S. Wood, S. Alexandra Garcia-Moreno, I.M. Salamone, S.C. Samson, R. Sekido, F. Poulat, D.M. Maatouk, R. Lovell-Badge, Sex reversal following deletion of a single distal enhancer of Sox9, Science 360 (2018) 1469–1471, https://doi.org/10.1126/science.aas9408, 1979.

[24] A. Jost, Hormonal factors in the sex differentiation of the mammalian foetus, Philos. Trans. R. Soc. Lond. B Biol. Sci. 259 (1970) 119–130, https://doi.org/10.1098/rstb.1970.0052.

[25] K. McClelland, J. Bowles, P. Koopman, Male sex determination: insights into molecular mechanisms, Asian J. Androl. 14 (2012) 164–171, https://doi.org/10.1038/aja.2011.169.

[26] S. Kuroki, S. Matoba, M. Akiyoshi, Y. Matsumura, H. Miyachi, N. Mise, K. Abe, A. Ogura, D. Wilhelm, P. Koopman, M. Nozaki, Y. Kanai, Y. Shinkai, M. Tachibana, Epigenetic regulation of mouse sex determination by the histone demethylase Jmjd1a, Science 341 (2013) 1106–1109, https://doi.org/10.1126/science.1239864, 1979.

[27] J. Ernst, M. Kellis, Discovery and characterization of chromatin states for systematic annotation of the human genome, Nat. Biotechnol. 28 (2010) 817–825, https://doi.org/10.1038/nbt.1662.

[28] J. Ernst, M. Kellis, Chromatin-state discovery and genome annotation with ChromHMM, Nat. Protoc. 12 (2017) 2478–2492, https://doi.org/10.1038/nprot.2017.124.

[29] E. Calo, J. Wysocka, Modification of enhancer chromatin: what, how, and why? Mol Cell 49 (2013) 825–837, https://doi.org/10.1016/j.molcel.2013.01.038.

[30] L. Wang, C.K. Collings, Z. Zhao, K.A. Cozzolino, Q. Ma, K. Liang, S.A. Marshall, C.C. Sze, R. Hashizume, J.N. Savas, A. Shilatifard, A cytoplasmic COMPASS is necessary for cell survival and triple-negative breast cancer pathogenesis by regulating metabolism, Genes Dev. 31 (2017) 2056–2066, https://doi.org/10.1101/gad.306092.117.

[31] G.A. Carré, P. Siggers, M. Xipolita, P. Brindle, B. Lutz, S. Wells, A. Greenfield, Loss of p300 and CBP disrupts histone acetylation at the mouse Sry promoter and causes XY gonadal sex reversal, Hum. Mol. Genet. 27 (2018) 190–198, https://doi.org/10.1093/hmg/ddx398.

[32] T. Narita, S. Ito, Y. Higashijima, W.K. Chu, K. Neumann, J. Walter, S. Satpathy, T. Liebner, W.B. Hamilton, E. Maskey, G. Prus, M. Shibata, V. Iesmantavicius, J. M. Brickman, K. Anastassiadis, H. Koseki, C. Choudhary, Enhancers are activated by p300/CBP activity-dependent PIC assembly, RNAPII recruitment, and pause release, Mol Cell 81 (2021) 2166–2182.e6, https://doi.org/10.1016/j.molcel.2021.03.008.

[33] T. Furumatsu, M. Tsuda, K. Yoshida, N. Taniguchi, T. Ito, M. Hashimoto, T. Ito, H. Asahara, Sox9 and p300 cooperatively regulate chromatin-mediated transcription, J. Biol. Chem. 280 (2005) 35203–35208, https://doi.org/10.1074/jbc.M502409200.

[34] S.-P. Wang, Z. Tang, C.-W. Chen, M. Shimada, R.P. Koche, L.-H. Wang, T. Nakadai, A. Chramiec, A.V. Krivtsov, S.A. Armstrong, R.G. Roeder, A UTX-MLL4-p300 transcriptional regulatory network coordinately shapes active enhancer landscapes for eliciting transcription, Mol Cell 67 (2017) 308–321.e6, https://doi.org/10.1016/j.molcel.2017.06.028.

[35] A.L. Jacob, J. Lund, P. Martinez, L. Hedin, Acetylation of steroidogenic factor 1 protein regulates its transcriptional activity and recruits the coactivator GCN5, J. Biol. Chem. 276 (2001) 37659–37664, https://doi.org/10.1074/jbc.M104427200.

[36] B.K. Cenik, C.C. Sze, C.A. Ryan, S. Das, K. Cao, D. Douillet, E.J. Rendleman, D. Zha, N.H. Khan, E. Bartom, A. Shilatifard, A synthetic lethality screen reveals ING5 as a genetic dependency of catalytically dead Set1A/COMPASS in mouse embryonic stem cells, Proc Natl Acad Sci U S A 119 (2022) 1–12, https://doi.org/10.1073/pnas.2118385119.

[37] R. Rickels, H.M. Herz, C.C. Sze, K. Cao, M.A. Morgan, C.K. Collings, M. Gause, Y.H. Takahashi, L. Wang, E.J. Rendleman, S.A. Marshall, A. Krueger, E.T. Bartom, A. Piunti, E.R. Smith, N.A. Abshiru, N.L. Kelleher, D. Dorsett, A. Shilatifard, Histone H3K4 monomethylation catalyzed by Trr and mammalian COMPASS-like proteins at enhancers is dispensable for development and viability, Nat. Genet. 49 (2017) 1647–1653, https://doi.org/10.1038/ng.3965.

[38] Z. Tang, W.Y. Chen, M. Shimada, U.T.T. Nguyen, J. Kim, X.J. Sun, T. Sengoku, R.K. McGinty, J.P. Fernandez, T.W. Muir, R.G. Roeder, SET1 and p300 act synergistically, through coupled histone modifications, in transcriptional activation by p53, Cell 154 (2013) 297, https://doi.org/10.1016/j.cell.2013.06.027.

[39] S.C. Munger, B. Capel, Sex and the circuitry: progress toward a systems-level understanding of vertebrate sex determination, Wiley Interdiscip Rev Syst Biol Med 4 (2012) 401–412, https://doi.org/10.1002/wsbm.1172.

[40] M.C. Manotas, M. García-Acero, A.P. Rojas Moreno, O. Moreno, J. Pérez, C. Céspedes, C. Forero, N. Fernández, F. Suárez-Obando, Perfiles clínicos de 28 pacientes con trastornos del desarrollo sexual en un centro de referencia, Pediatria (Bucur) 55 (2022) 60–69, https://doi.org/10.14295/rp.v55i2.317.

[41] L. Persani, M. Cools, S. Ioakim, S. Faisal Ahmed, S. Andonova, M. Avbelj-Stefanija, F. Baronio, J. Bouligand, H.T. Bruggenwirth, J.H. Davies, E. De Baere, I. Dzivite-Krisane, P. Fernandez-Alvarez, A. Gheldof, C. Giavoli, C.H. Gravholt, O. Hiort, P.-M. Holterhus, A. Juul, C. Krausz, K. Lagerstedt-Robinson, R. McGowan, U. Neumann, A. Novelli, X. Peyrassol, L.A. Phylactou, J. Rohayem, P. Touraine, D. Westra, V. Vezzoli, R. Rossetti, The genetic diagnosis of rare endocrine disorders of sex development and maturation: a survey among Endo-ERN centres, Endocr Connect 11 (2022), https://doi.org/10.1530/EC-22-0367.