Genome-wide estrogen receptor β chromatin binding in human colon cancer cells reveals its tumor suppressor activity

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Abstract
Colorectal cancer (CRC) is the third leading cause of cancer death in the western world. In women, menopausal hormone therapy has been shown to reduce CRC incidence by 20%. Studies demonstrate that estrogen activating estrogen receptor beta (ERβ) protects against CRC. ERβ is a nuclear receptor that regulates gene expression through interactions with the chromatin. This molecular mechanism is, however, not well characterized in colon. Here, we present for the first time, the cistrome of ERβ in different colon cancer cell lines. We use cell lines engineered to express ERβ, optimize and validate an ERβ antibody for chromatin-immunoprecipitation (ChIP), and perform ChIP-Seq. We identify key binding motifs, including ERE, AP-1, and TCF sites, and we determine enrichment of binding to cis-regulatory chromatin sites of genes involved in tumor development, cell migration, cell adhesion, apoptosis, and Wnt signaling pathways. We compare the corresponding cistromes of colon and breast cancer and find that they are conserved for about a third of genes, including GREB1, but that ERβ tethering to TCF and KLF family motifs is characteristic for colon. We exemplify upregulation of putative CRC tumor suppressor gene CST5 where ERβ in colon cells binds to cis-regulatory regions nearby (−351 bp) the transcriptional start site. Our work provides a foundation for understanding the mechanism of action of ERβ in CRC prevention.

KEYWORDS
ChIP, colon cancer, ERβ, nuclear receptor

1 | INTRODUCTION

Colorectal cancer (CRC) is the third major cause of cancer death among both women and men in the Western world, but early detection can increase survival by 90%. Patients with ulcerative colitis are at increased risk of developing CRC, and preventive strategies targeting inflammation (e.g., COX-2 inhibitors) can be effective. However, due to increased risk of bleeding or cardiac side effects, the risk-benefit ratio of such preventive approaches is poor. There is a major need to develop better preventive treatments.

It is known that men have a higher risk of developing CRC compared to women, and that menopausal hormonal therapy and long-term use of oral contraception can reduce the incidence of CRC (reviewed in Refs.)
Epidemiological data indicate that estrogen itself has significant preventative effect.\(^{12,13}\) Estrogens act through estrogen receptor alpha (ERα/ESR1), estrogen receptor beta (ERβ/ESR2), and G protein-coupled estrogen receptor 1 (GPER1/GPR30). ERβ has been linked to tumor-repressive effects of CRC, as demonstrated in animal studies.\(^{14,15}\) In our recent study, we showed that deletion of intestinal ERβ increased tumor formation in mice of both sexes.\(^{16}\) These studies suggest that receptor-selective activation of intestinal ERβ could constitute a chemopreventive therapy for colon cancers.

The molecular mechanism of the tumor-suppressive function of ERβ in colon and rectum is, however, not well understood. ERβ is expressed in nontumor colon tissue but lost in CRC.\(^{16,17}\) We and others have previously detailed the antitumor effects of ERβ re-introduced into different colon cancer cell lines, where it reduced cell proliferation, migration, and invasion.\(^{17-20}\) Its homolog, ERα, is upregulated in breast cancer where its genome-wide chromatin-binding pattern is well described.\(^{21-24}\) ERβ chromatin binding in colon cells has, however, not been explored. While the two receptors have a highly conserved DNA-binding domain (DBD), ERβ has a lower affinity to ERE half-sites than ERα,\(^{25,26}\) and their N-terminal domains are less conserved.\(^{27-30}\) These differences can contribute to divergent transcriptional activities and different functionalities.\(^{31}\) In our study, we have used chromatin immunoprecipitation followed by sequencing (ChIP-Seq) with a highly validated antibody to provide the first cistrome of ERβ in colon cells. We can thereby specify its role as the direct regulator of tumor suppressors, cell cycle, and Wnt/b-catenin signaling members in colon cells. Further, we compare the chromatin binding in colon with its cistrome in breast using the same setup, and we identify clear differences. Our data elucidate the tumor-suppressive role of ERβ and contribute to improved understanding of this nuclear receptor in colon.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The cell lines SW480-ERβ and HT29-ERβ and corresponding mock cell lines were previously generated and characterized,\(^{18,20}\) and kindly received from Assoc. Prof. Anders Ström (University of Houston, Houston, Texas), parental SW480 from Professor Sam Okret (Karolinska Institute, Stockholm, Sweden), and MCF7-ERβ from Chunyan Zhao (Karolinska Institute, Stockholm, Sweden). SW480 (RRID: CVCL_0546), HT29 (RRID: CVCL_0320), and MCF7 cells (RRID: CVCL_0031), with or without lentiviral transduction of full-length ERα and ERβ (Karolinska Institute, Stockholm, Sweden). SW480 (RRID: CVCL_0546), and MCF7 cells (RRID: CVCL_0031), with or without lentiviral transduction of full-length ERα (Karolinska Institute, Stockholm, Sweden). SW480 (RRID: CVCL_0546), and MCF7 cells (RRID: CVCL_0031), with or without lentiviral transduction of full-length ERα (Karolinska Institute, Stockholm, Sweden).

What’s new?

Estrogen receptor beta (ERβ) regulates gene expression through interaction with chromatin. ERβ has been shown to protect against colon cancer, and these authors set out to uncover the molecule’s mechanism of action in colon cells. Using chromatin immunoprecipitation (ChIP)-sequencing, they identified binding sites in genes involved in tumor development, cell migration, cell adhesion, apoptosis, and Wnt signaling. They identified clear differences between the chromatin binding pattern of ERβ in colon cells compared with breast cells. The results provide a map of ERβ chromatin binding sites in colon cells and position ERβ as a possible therapeutic target for colorectal cancer.

2.2 | Western blot

Western blot was performed as previously described\(^{25}\) using validated ERβ antibody PPZ0506 (R&D Biosystems, cat no: PP-PPZ0506-00, lot no: A2, mouse monoclonal, RRID:AB_2293861) and GAPDH (ThermoFisher, cat no: MA5-15738, lot no: UH277724, mouse monoclonal, RRID: AB_10977387).

2.3 | Chromatin immunoprecipitation

Cells grown on culture plates (150 mm) were cross-linked with 1% formaldehyde for 10 minutes at room temperature to capture protein-DNA interactions, and quenched by adding glycine (final concentration 0.125 M). Three replicates for each cell line were performed, of which one was performed using four plates. At 4°C, cells were collected by scraping, washed twice (ice-cold PBS), lysed (lysis buffer-LB1 [50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, and 0.25% Triton X-1]), and incubated 10 minutes while shaking. Cells were centrifuged (4500 rpm, 5 minutes), and pellets were resuspended (LB2 buffer: 10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA) for 5 minutes and centrifuged (4500 rpm, 5 minutes). The nuclear chromatin fractions were ruptured in LB3 buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, and 0.5% Na-lauroylsarcosine), and the chromatin was sonicated to an average length of 200-500 bp. The sheared chromatin was centrifuged (13000 rpm, 5 minutes) and supernatants transferred to low-binding DNA tubes and incubated with ERβ antibody (R&D systems, cat no: PPZ0506, lot no: A2, mouse monoclonal, RRID:AB_2293861 and validated in Ref.\(^{33}\)). H3K27Ac antibody (Abcam, cat no: AB4729, lot no: GR321937-1, rabbit polyclonal, RRID: AB_2118291), or IgG (Santa Cruz, cat no: sc-2025, lot no: J1514, mouse polyclonal, RRID:AB_737182), overnight shaking. To each ChIP reaction, 30 μL of Protein G Dynabeads (cat...
no: 10004D, Invitrogen) were added, or sepharose beads (cat no: 17-6002-35 GE health care) for comparison, incubated (3 hours, shaking) and beads were washed for 10 minutes with TSE1 (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, and 0.1% Triton-X), TSE2 (20 mM Tris-HCI, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton-X), LiCl buffer (20 mM Tris-HCI, 1 mM EDTA, 250 mM LiCl, 1% NP-40, and 1% Na-deoxycholate) and TE buffer (10 mM Tris-HCI and 1 mM EDTA). Samples were transferred to new tubes and incubated with elution buffer (0.75% NaHCO3 and 1% SDS) for 30 minutes at 55°C. The elutes were subjected to RNase A treatment for 1 hour at 37°C, followed by Proteinase K digestion overnight at 50°C. DNA was purified using QIAquick PCR purification columns (Qiagen, cat no: 28104).

2.4 | ChIP-Seq analysis

Libraries of the ChIP DNA were prepared and sequenced by the National Genomic Infrastructure (NGI) for Bioinformatics and Expression Analysis (BEA). In short, the NEB Next Ultra II DNA Library Prep Kit for Illumina (p/n NEB #E7645) was used for library preparation, and quality control was performed using TapeStation DNA D1000 ScreenTape (Agilent) and Qubit 2.0 (ThermoFisher Scientific). The libraries were sequenced on NextSeq 550 (Illumina) with V2 reagent kits (Illumina) for 75 cycles single read, with a loading of 1.8 pM end concentration of 1%. ChIP-Seq reads were mapped to the human reference genome assembly hg38 (GRCh38) using STAR with the alignIntronMax flag set to 1 and uniquely mapped reads were used for further analysis. Peak calling was performed by HOMER applying a false discovery rate (FDR) less than 0.001 and 4-fold enrichment over control (IgG ChIP or input) as cut-off. Peaks which overlap within 200 bp were merged to obtain ERβ-binding DNA sequences. Peaks present in at least two replicates were considered for downstream analysis. The data is uploaded to GEO (GSE149979 and GSE166194). Raw tag counts were normalized in R and difference in binding patterns was identified with edger package. Complete heatmap from R was used to cluster and visualize the different peaks. Genomic distribution of binding sites was obtained from HOMER, promoter regions defined as -1 kb to +100 bp from TSS. Differential binding of H3K27AC peaks was performed by Edger, normalized using TMM normalization. Gene Ontology over-representation was performed using Database for Annotation Visualization and Integrated Discovery (DAVID). Pathway analysis was performed in both DAVID and EnrichR database. P < .05 was considered to indicate a statistically significant difference.

2.5 | Motif analysis

De novo motif analysis was performed within 200 bp of identified peaks using HOMER, with randomly generated ±50 kb background sequences used for scoring. Peaks were annotated using selected motif sets (ERE, AP-1, and TCF1) and findMotifsGenome.pl. All ERβ-binding sites were also scanned using annotatePeaks.pl from HOMER. Venn diagrams were used to represent the distribution of these motifs. The motifs sequences used with annotatePeaks were ERE (AACGTCACNGTGACC), AP1 (ATGACTCATC), and TCF (ATCAAGGGRA). In addition, H3K27AC peaks were centered around nucleosome free regions (NFR) to perform de novo motif analysis.

2.6 | ChIP-qPCR

The qPCR primers were designed to align to identified ERβ-binding sites, and a negative ChIP control primer set was designed for a chromosome area where ERβ was not identified to bind in ChIP-Seq (chr21:25509072-25509220, chr8:111624471-111624620). All primers used in our study are listed in Table S1. qPCR was performed on 1 ng DNA, using iTaq universal SYBR Green supermix (Biorad, cat no: 1725120) and CFX96 Touch System (Biorad). Nontemplate negative controls were included in all qPCR experiments to control for contamination. Melting curve analysis was included in each run to control for amplification of one single amplicon. Amplification of ERβ target sites were normalized to input from the same ChIP experiment and to the negative control, using ΔΔCt-method and presented as fold enrichment. GraphPad Prism was used for statistical analysis (GraphPad Software Inc, La Jolla, California). The results are expressed as mean ± SEM. A two-tailed Welch's t-test was used for comparison between two groups. P-value <.05 was considered statistically significant.

2.7 | RNA extraction, cDNA synthesis, and qPCR

RNA was extracted by using 1:5 Trizol (Invitrogen Carlsbad, California): chloroform (Sigma-Aldrich, St. Louis, Missouri) method and purified by miRNeasy or RNeasy spin column (Qiagen, Chatsworth, California). Biorad iScript kit (Bio-Rad, Hercules, California) was used for cDNA synthesis, with 1 μg of RNA, according to the manufacturer’s recommendations. qPCR iTaq universal SYBR Green supermix (Bio-Rad) was used with CFX96 Touch System (Bio-Rad), and GAPDH or ARHGDIA as reference genes for mRNA expression level measurements. Melting curves were analyzed for all amplicons. ΔΔCt-method was used to calculate relative expression. Unpaired two-tailed t-test was used for statistical testing, and results were considered significant if P < .05.

2.8 | Library preparation, RNA sequencing, and data analysis

RNA-Seq was performed in two biological replicates at Sweden’s National Genomics Infrastructure (NGI). The libraries were prepared from polyA-tailed RNA and sequenced using Illumina HiSeq rapid mode. At least 15 million single-reads of 50 bp in length were generated for each sample. Reads were mapped against human genome (GRCh37) using Tophat/2.0.4. Reads with multiple alignments were removed using picard-tools/1.29, htsseq/0.6.1 was used to count reads for each transcript, cufflinks/2.1.1 was used to normalize the reads count to the length of each transcript, and Fragments Per Kilobase per Million (FPKM) were calculated. DEseq2 [94] was used to calculate the differential gene expression and corresponding fold changes, P-values, and FDR. Genes were denoted significantly
differentially expressed when FDR < 0.05, log2FC ≥ 0.4, and FPKM (treated) > 1. log2FC ≥ 0.4 equals a fold change difference of at least 30%, which we consider sufficient as steady state differences. RNA-Seq data is deposited in GEO repository (GSE112569 and GSE112565).

2.9 | ERE and TRE luciferase reporter assay

SW480 seeded at a density of 50,000 cells in 24-well plates were transfected with 1 μg of mock or ERβ plasmids, using XtreemgeneHP transfection reagent (6366236 001, Sigma-Aldrich). Media was replaced 5 hours post transfection. The following day, XtremegeneHP transfection reagent (6366236 001, Sigma-Aldrich). Five hours post transfection, media was replaced with phenol free DMEM media (12676011) containing 10% DCC FBS (12676011, ThermoFisher) and E2 (10 nM), Tamoxifen (TMX, 1 μM), ICI (10 nM), and/or TPA (1 μM), as indicated. After 24 hours, cells were lysed and the luminescence of firefly and Renilla luciferase measured using a Dual-Luciferase reporter assays system (E1910, Promega).

3 | RESULTS

3.1 | ERβ antibody PPZ0506 is specific in ChIP

Widely used ERβ antibodies have been shown to be unspecific, but one (PPZ0506) has been validated for usage in immunohistochemistry, western blotting, and immunoprecipitation. We used this antibody to corroborate protein expression of transduced ERβ in cell lines (previously generated) using western blot, in addition to qPCR (Figure S1A,B). As this antibody has not previously been used in ChIP, we next optimized conditions and evaluated its performance in ChIP assay using sepharose beads or magnetic beads. PPZ0506 appeared highly specific in ChIP using magnetic beads, as evidenced by enriched ERβ binding at the GREB1 promoter (Figure S1C). The same protocol with sepharose beads generated considerably weaker enrichment in ERβ-expressing cells along with a relatively high unspecific background in non-ERβ-expressing cells (Figure S1C). We also evaluated binding in presence and absence of 17β-estradiol (E2) and noted significant binding also without ligand (Figure S1D).

3.2 | ChIP-Seq reveals ERβ binding in colon cancer cell lines

We used the above optimized conditions for ChIP-Seq of two colon cell lines, HT29-ERβ and SW480-ERβ. The analysis was performed in three independent ChIP experiments, with E2 treatment, and normalized to input. The sequencing generated between 67 and 100 M reads per sample, with more than 80% high-quality mapped reads. Details including irreproducible discovery rate (IDR), correlation coefficient, and normalized and relative strand cross correlation coefficient (NSC and RSC) values between replicates are provided in Table S2. The replicated samples show a high correlation (>0.85 correlation coefficient) with one exception (HT29 replicate 1, which used more input and has more binding sites than the others). A heat map illustrating the enrichment of ERβ-bound DNA for each replicate and sample is shown in Figure 1A. The triplicates show a high concordance within the cell lines, with 1499 and 1232 sites detected in all replicates of respective cell line (Figure 1B). We corroborated the sequencing results using ChIP-qPCR for 40 indicated binding sites (detected in replicates), of which 37 were confirmed, demonstrating a high accuracy of the ChIP-Seq data (exemplified in Figure 1F; Figure S1E). For further analysis, we focused on sites present in at least two out of three replicates (2988 in HT29, 1867 in SW480). Thus, ChIP-Seq using our optimized protocol generated conclusive ERβ cistrome data for colon cancer cell lines.

3.3 | The genome-wide chromatin-binding landscape of ERβ in two colon cell lines

To deduce how ERβ controls the regulation of genes, we studied the genomic distribution of ERβ-bound chromatin sites in HT29 and SW480 cells. About 10% of sites in each cell line were located in the promoter area (within −1 kb to +100 bp from the transcription start sites, TSS) of genes, whereas approximately 40% were located within introns or intergenic chromatin regions (Figure 1C). This is in accordance with previous studies of ERα or ERβ in MCF7 cells.

De novo motif analysis was performed to predict which chromatin sites ERβ binds to directly (ERE) and which sites it binds to via interaction with other proteins. As expected, the canonical ERE motif was the most enriched sequence (P < 10−950) and detected in 55% and 77% of all bound sequences in SW480 and HT29, respectively (Figure 1D). Also, tethering between ERβ and AP-1 was evident (HT29: 28%, P < 10−415 and SW480: 10%, P < 10−21, Figure 1D). This supports the accuracy of the antibody and the ChIP-Seq assay. Further, we found motifs not previously linked to ERβ, including enrichment of ELF3, TCF (TCF1, TCF3, TCF4, TCF7, and LEF1) and KLF5 motifs (Figure 1D). We thus here detail the ERβ cistrome in colon cell lines and suggest TCF as a common novel ERβ interactor in colon cells.

3.4 | ERβ binding supports roles in cell migration and tumor suppression

Next, to determine which biological functions ERβ may influence in the colon through its gene regulatory activity, we performed a pathway analysis of the genes located by the ERβ-binding sites. We found that in each cell line, the most enriched biological functions included
cell migration,” “cell-cell adhesion,” and “canonical Wnt signaling” (Figure 1E). Several of these processes have also been indicated in previous transcriptomic analysis of ERβ in colon cells, and its effect on cell migration has been corroborated experimentally. We also investigated the transcriptional levels of these genes in cells with and without ERβ from previous and current studies (microarray [HT29, SW480]).
SW480], bead array [HT29, SW480], and RNA-Seq [SW480, our study], combined, in order to make a comprehensive analysis.

The top up- and downregulated genes for each cell line are shown in Figure 1G. Interestingly, we note a strong upregulation of Cystatin D (CST5) and Cystatin SN (CST1) by ERβ in SW480. ERβ bound chromatin by several CST genes (CST1, 3-5) in HT29, and in close proximity (−351 bp of TSS) of the CST5 promoter in both cell lines (Figure S1F). CST5 is a proposed tumor suppressor in CRC. We confirmed this binding by ChIP-qPCR (Figure 1F), and its increased expression by RT-qPCR (Figure 1F) in both cell lines. We also found that ERβ bound nearby CRC oncogenes, including LDL receptor-related protein 6 (LRP6, −260 of the TSS, Figure S1F). LRP6 is a receptor for Wnt and regulates autophagy and cell migration. We confirmed the binding of ERβ to the LRP6 promoter (Figure 1F) and a corresponding decrease of its mRNA expression (qPCR, Figure 1F). ERβ also bound by other genes related to the oncogenic Wnt/β-catenin signaling pathway, such as in the intron of potassium voltage-gated channel KCNQ1 (+2123 of TSS, see Table 2, confirmed in Figure S1E). KCNQ1 has been identified as a novel regulator of Wnt/β-catenin pathway and proposed as a tumor suppressor gene in CRC. Estrogen has previously been found to regulate the function of KCNQ1, and our data indicate that ERβ is the transcriptional regulator. Also, APC2 (APC regulator of WNT signaling pathway 2), CTNNB1, TCF7L2, TCERG1, and SOX9, all frequently mutated in CRC patients, exhibited ERβ binding in their chromatin regions. APC2 is closely related to the adenomatous polyposis coli (APC) tumor-suppressor protein, has similar tumor-suppressor effects, and interacts with β-catenin. β-catenin activity depends on TCF family proteins for transcriptional activity and subsequent regulation of oncogenic Wnt pathway in colorectal tumors. The TCF family motifs were the second most enriched motifs among ERβ-binding sites in both HT29 (34%) and SW480 (19%) cells. Our data thus provides important new insights into how ERβ can oppose migration and tumorigenesis.

3.5 | The colon ERβ cistrome

To define a general ERβ-binding pattern in cells of colon origin, we characterized the sites common in both cell lines (2566 sites detected in at least one replicate from each cell line, Figure 2A), and denoted this the colon ERβ cistrome. A pathway enrichment analysis of genes nearest to the 2566 binding sites showed that, in addition to generic ER mechanisms (“regulation of transcription,” “intracellular signal transduction,” “steroid hormone-mediated signaling”), “cell migration,” “cell-cell adhesion,” and “Wnt signaling” were common themes (Figure 2B). This is similar to what we found in each respective cell line (Figure 1E). The indicated negative regulation of Wnt signaling is of particular interest because of its critical role in CRC development. Circadian rhythm (including BMAL1, NPAS2), hypoxia, and NFκB signaling were also overrepresented functions that can have an impact on colon carcinogenesis (Figure 2B). These pathways have been linked to ERβ previously, and we here identify the potential mechanisms whereby ERβ could modify these processes. Additional KEGG pathway analysis also yielded results aligning with known or previously proposed functions (MAPK, VEGF, p53 signaling, and insulin resistance), but also less known or unknown functions (Rap1 signaling, choline metabolism, and proteoglycans; Figure S3B). Proteoglycans are involved in colon cancer progression and promote cancer metastasis, and the HT29 cell line is known to synthesize proteoglycans. Further, we note direct ERβ binding to chromatin regions by insulin growth factors genes (IGFBP3, IGFBP4 (Table 2), and IGFBP6), and by multiple nuclear receptors (RARα (Table 2), HNF4α, PPARG, VDR, RXRα, LRH1, TR2, ERRα, and Nur77). In all, we identified that ERβ bound to 240 promoter regions in both cell lines (Table S3). These include a member of the tumor protein D52-like family (TPD52L2), well-characterized breast cancer ERα targets, such as GREB1 (Figure S3A), JUND, and estrogen receptor-related alpha (ESRRA, Table 1). We also note long non-coding RNAs, such as NEAT1 (regulated by ERα in breast and prostate, and KIF9-AS1. Thus, we here characterize the common ERβ colon cistrome and how this may impact tumor suppressive functions in colon cells.

3.6 | ERβ tethering mechanisms in colon

To investigate the motifs in the colon cistrome, we repeated the motif analysis above on the common colon ERβ cistrome (2566 binding sites). Again, significant enrichment of consensus ERE, AP-1, and TCF motifs was found, but also an enrichment of THAP1 motif (37%, P < 10−73, Figure 2C). We also scanned the colon cistrome sequences for ERE, AP-1, and TCF motifs, which were detected in 2095 (76%) of peaks. A density plot illustrates that the ERE motif had the highest probability of TSS occurrence, followed by AP-1 and TCF family motifs (Figure 2D). Venn diagram of their co-occurrence shows that the largest fraction contained ERE motif only (800 peaks), followed by AP-1 and TCF family motifs (188, Figure 2E). This indicates that ERβ may regulate approximately 25% of its target genes only through tethering, that is, independently of its DNA-binding (ERE) functions (Figure 2E).

There have been previous reports that ERα regulates specific biological functions via AP-1 tethering and others via ERE binding. To investigate this in ERβ in colon, we performed gene function enrichment for the genes located by the ERE, AP-1, or TCF sites that ERβ bound to alone. This indicated that genes predicted to be regulated by ERβ through ERE alone are involved in MAPK pathway, circadian rhythm, cell proliferation, and transcriptional regulation (Figure 2F). Through its tethering to AP-1 (only), functions within cell migration, cell adhesion, and protein phosphorylation were enriched. The TCF1 tethering, not previously investigated in relation to ERβ, appeared to primarily regulate apoptosis and extracellular matrix (ECM) organization (Figure 2F).

To further investigate the potential transcriptional impact of the ERβ colon cistrome, we looked at the corresponding transcriptional data generated by RNA-Seq and microarray. We detected gene regulations for 365 of corresponding genes in HT29 and/or SW480 cells. The majority was upregulated (231 genes, 63%; Table S4) and...
**FIGURE 2** Legend on next page.
enriched for functions related to negative regulation of cell-cell proliferation, regulation of focal adhesion, and apoptosis (Figure 2G). The downregulated genes were involved in regulation of transcription and phosphorylation. We performed motif enrichment analysis on these two groups to investigate differences in ERβ-binding mechanisms. Both groups exhibited similar motif distribution (Figure 2H). Overall, this shows that ERβ mediates its main transcriptional regulation, for both upregulated and downregulated genes, via ERE and AP-1 sites. Our analysis also proposes an important contribution through tethering with TCF, especially for its apoptotic function in colon cells.

3.7 | ERβ-tamoxifen does not transactivate standard TRE

The selective estrogen receptor modulator (SERM) tamoxifen is an antagonist to ERα in breast. However, ERβ-tamoxifen (but not ERβ-E2) has been reported to activate AP-1 sites. To investigate this effect in colon, we performed luciferase transactivation assays. We used a standardERE and AP-1 (TRE) element transfected into colon cell line SW480 with and without ERβ, and administered E2, tamoxifen, ICI, or vehicle. TRE transactivation was also evaluated with and without AP-1 activator (TPA). In the presence of ERβ, E2 activated ERE transcription, whereas both ICI and tamoxifen functioned as complete antagonists (Figure 2I). TPA strongly enabled transactivation at the AP-1 site (TRE), but neither ERβ nor its ligands impacted AP-1 transcription (Figure 2J). We conclude that tamoxifen functions as an antagonist to ERβ in colon, and that ERβ does not modify AP-1 transactivation at this standard AP-1 site, regardless of ligand. This indicates that ERβ, in order to tether with AP-1 and impact transcription, may need to bind an adjacent site through either an ERE or another pioneering or activating factor.

3.8 | ERβ modulates acetylation of histone H3 lysine 27

Acetylation of histone H3 lysine 27 (H3K27) is strongly correlated with gene activation. To gain a better understanding of how ERβ impacts the epigenome, we performed H3K27ac ChIP-Seq in HT29 and SW480 cells, in presence and absence of ERβ. We identified that 4159 and 3793 H3K27ac sites were enhanced and another 4835 and 3596 sites were reduced by presence of ERβ in HT29 and SW480 cells, respectively (Figure 3A). ERβ-enhanced H3K27ac sites were located by genes involved in transcriptional regulation, cell migration, apoptotic process, and regulation of GTPase activity, in both HT29 and SW480 cells (Figure 3B). Similarly, ERβ reduced H3K27ac sites by genes involved in cell migration (also), cell proliferation, and intracellular signal transduction in both cell lines (Figure 3C).

Next, to specifically investigate how the acetylation pattern was modified around the ERβ chromatin-binding sites, we integrated the H3K27ac data with the ERβ ChIP-Seq data. Focusing on sites where H3K27ac peaks overlapped within 200 bp of ERβ binding sites, we identified that 1740 (58%) and 904 (48%) of ERβ binding sites also had H3K27ac marks in HT29 and SW480 cells, respectively (Figure S2A). These identified binding sites with H3K27ac marks were involved in cell adhesion, cell proliferation, Wnt signaling, and NFκB signaling (Figure S2B). While ERβ was by far the most enriched motif among ERβ-binding sites in general (Figure 1D), the most enriched motifs within the ERβ-bound DNA that also had H3K27ac marks was AP-1 (followed by ERE, TCF, and KLF; Figure S2C).

Further, we identified that ERβ substantially modulates the H3K27ac status of the chromatin surrounding its binding. Around a third of the H3K27ac sites located around ERβ were either increased or decreased as a consequence of ERβ expression (601/1740 in HT29 and 332/904 in SW480) chromatin sites (Figure 4A). Genes closest to these sites were involved in functions like cell proliferation, NFκB signaling, cell adhesion, and cell migration (Figure 4B). Again, highlighting the biological functions that ERβ impacts through its chromatin binding and subsequent gene activation or repression.

3.9 | ERβ colon versus breast cistrome

Finally, to investigate whether there are tissue-specific differences of ERβ binding, we repeated ChIP-Seq in MCF7 cells engineered to express ERβ,32 using the same antibody (PPZ0506) and protocol as above. We identified 12 376 ERβ-binding sites (Figure S3C), which we refer to as the ERβ breast cistrome. A notable difference is that ERα is highly expressed in MCF7, but not at all in the colon cell lines (per

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**Figure 2** ERβ colon cistrome. A. Venn diagram comparing all ERβ binding sites detected in colon cancer cell lines HT29 and SW480 (in any replicate), identifying a common ERβ colon cistrome of 2566 binding sites. B. Biological functions enriched (P < .05) among genes located nearest to ERβ chromatin-binding sites in colon. C. Top de novo motifs within the colon ERβ cistrome. D. Motif densities for ERβ-binding sites with ERE, AP-1, and TCF motifs. E, Venn diagram comparing distribution and co-occurrence of ERE, AP-1, and TCF motifs. The three motifs together are present in 76% of total ERβ-binding sites in colon cells. F. Biological processes enriched (P < .05) among genes located nearest to ERβ-binding sites with either ERE, AP-1, or TCF (only) motifs. G. Gene functions enriched (P < .05) among genes upregulated or downregulated by ERβ that are also located nearest to ERβ chromatin-binding sites. H. De novo motifs enriched among genes by ERβ-binding sites that are upregulated or downregulated, respectively. I. J, ERE and TRE transactivation by ERβ and corresponding ligands. SW480-mock and SW480-ERβ cells were transfected with (I) ERE or (J) 2xTRE reporter plasmid. After transfection, cells were treated with vehicle, E2 (10 nM), tamoxifen (TMX, 1 μM) and/or ICI (10 nM) for 24 hours and assayed for luciferase activity. Ligands E2, ICI, and TMX were dissolved in DMSO, whereas TPA was dissolved in ethanol. TRE assay was performed with and without TPA (1 μM). Readings were normalized to mock (vehicle). The ERE assay was performed in triplicate and the TRE assay was performed in two replicated experiments, each in triplicates [Color figure can be viewed at wileyonlinelibrary.com]
Expression Atlas, EMBL-EBI; Figure S3E). ERα forms a heterodimer with ERβ which thus increases the number of ER molecules that can bind DNA. Comparing ERβ-bound sites in breast and colon cistromes, we found that 6549 sites were detected in at least one replicate from breast (MCF7) and one from colon (SW480 and/or HT29; Figure S3C). We denote this as the ERβ core cistrome. Sites detected only in colon (at least two replicates, 1599 sites) or only in breast (two replicates, 7552 sites), and not in any replicate of the other tissue, were denoted tissue-specific cistromes. Nuclear receptor (NR) motifs (which include ERE) were the most enriched binding sequences in both general and tissue-specific cistromes. AP-1 was also enriched in all cistromes, whereas FOXA1 and TFAP2B (AP-2 family) motifs were enriched in breast, and not in the core or colon-specific cistromes (Figure 5A; Figure S3D). TCF and KLF5 motifs, on the other hand, were exclusive for ERβ binding in colon. Investigation of the biological functions of corresponding genes showed that cell migration and cell adhesion, regulation of NFκB, canonical Wnt signaling, and apoptosis were enriched in the common core, whereas the colon-specific cistrome were particularly enriched for lipid metabolism, inflammatory response, angiogenesis, and cell proliferation (Figure 5B). To investigate the tissue-specific binding in further detail, we studied specific binding sites. ERβ chromatin-binding sites adjacent to Cathepsin D (CTSD, Figure 5C) and GREB1 (Figure S3A), for example, were found in both breast and colon. Other sites were specific for either colon or breast. HSPB7 (heat shock protein B) and RBM20 (RNA binding motif protein 20) were detected in colon (both cell lines) but not in breast (Figure 5C; Figure S3F). Also, ERβ bound to four sites in the enhancer region of GPER1 of which two were specific for colon and pronounced in HT29 (Figure S3F). Breast-specific ERβ-binding sites included breast carcinoma amplified sequence 4 (BCAS4, Figure 5C), a protein which is overexpressed in breast cancers. Thus, while the ERβ colon and breast cistromes share a significant core, there are substantial differences. This includes variations in genome binding through common motifs (ERE, AP-1), motifs exclusive for either tissue (GATA3 in breast, KLF5 in colon), and gene-specific binding sites (eg, BCAS4 in breast, HSPB7, RBM20 in colon).

| Table 2 | Examples of ERβ-binding site in colon cistrome and distance to nearest gene, based on top-peak score enrichment |
|----------|------------------------------------------------------------------|
| Colon ERβ cistrome (all binding sites) | Distance to TSS (bp) |
| FGF3     | –72 311              |
| E2F6     | –32 558              |
| LRP5L    | –19 402              |
| RALA     | –18 352              |
| CTSD     | –8776                |
| MIR1208  | –8034                |
| KMT5B    | –2923                |
| EPS58L2  | –1161                |
| SUCLA2   | –167                 |
| FUT4     | 445                  |
| KCNQ1    | 2123                 |
| MIR4692  | 3096                 |
| RARA     | 4269                 |
| IGFBP4   | 5425                 |
| PRR5     | 8704                 |
| FOXC1    | 31 038               |
| MMP17    | 32 590               |
| TEMEM120B| 36 297               |
| TEMEM75  | 37 343               |
| KRT80    | 44 410               |

4 | DISCUSSION

The main objective of this work was to explore the DNA binding of ERβ in colon cells to provide a foundation for understanding its CRC prevention mechanism. Work on ERβ has been hampered by unspecific antibodies and we emphasized the validation of antibody performance (PPZ0506) in the ChIP-Seq application for our study. The highly validated antibody used and the pure ChIP-Seq results generated, supports a high reliability of the data and is a strength of the study. We further used two different, previously generated and characterized, colon cancer cell lines engineered to express ERβ (by lentivirus) and, additionally, compared to cells from other tissue origin (breast) using the same antibody and protocol. We found highly enriched motifs, including ERE and AP-1, further supporting the accuracy of our data. In agreement with its known functional properties,
we identified that ERβ directly binds cis-regulatory chromatin of genes involved in cell migration, cell adhesion, and cell proliferation. It is of high interest to understand how ERβ can suppress CRC development, and we reveal several interesting potential mechanisms. We identified that binding to TCF family motifs were predominant in colon and our study reveals targets indicative of ERβ function in colon, such as Wnt signaling and circadian rhythm regulation. We found that ERβ bound to ERE sites that were located in regulatory regions near known or predicted colon tumor suppressor genes, including CST5 and PRR5, and near oncogenic LRP6. Interestingly, it has been shown that vitamin D3 exerts tumor-suppressive activity in CRC, and that CST5 is induced by vitamin D (through VDR) and p53, which suppresses tumor progression and metastasis.37,53 Our finding that ERβ bound an ERE in close proximity of the CST5 promoter, as well as chromatin by VDR, and upregulates CST5, suggests that ERβ has an important tumor preventive role through this mechanism. In a breast cancer cell line, ERβ has been found to transactivate CST5 through ERE, which was inhibited by both ICI and mutated ERE. This supports that the upregulation of CST5 gene is indeed a direct effect by ERβ chromatin binding.54

Additionally, reduction of LRP6 expression has been shown to delay tumor growth in vivo.55,56 Repression by ERβ through promoter

**FIGURE 3** Expression of ERβ modulates H3K27ac marks in colon cell lines. A, MA plot (logFC vs log CPM, counts per million), representing H3K27ac marks modulated by presence of ERβ in the two colon cell lines. Red illustrates marks significantly increased, and blue significantly decreased. B, C, Biological functions enriched (P < .05) among genes with (B) enhanced or (C) reduced H3K27ac upon ERβ expression, for cell lines HT29 and SW480 [Color figure can be viewed at wileyonlinelibrary.com]
binding can thus further explain the antitumorigenic effect of ERβ. Our results link ERβ function to the repression of oncogenic Wnt/β-catenin signaling, and TCF motif binding. This is supported by a previous study linking ERβ activation to repression of Wnt signaling in skin cancer.57 We further found support for multiple regulations related to tumor-suppressive activities, including insulin growth factors, circadian clock proteins, and several nuclear receptors. Recent studies have revealed that IGFBP3 reduces colitis in mice and risk for CRC, or increased survival from CRC, in humans.58,59 We found that IGFBP3 was upregulated by ERβ. Circadian clock genes have also been shown to play a vital role in CRC by regulating genes involved in cell cycle progression, tumor suppressor, and oncogenes. Numerous additional genes, which the ChIP-Seq analysis indicated as regulated by ERβ, correlate to CRC prognosis (eg, BCAR3, TPDS2L2, and SLC17A9).60 We have recently shown that ERβ counteracts obesity-induced proliferation in mice colon, and that treating with ERβ-selective agonist DPN

| Biological Function                        | HT29 ERβ Binding Sites | SW480 ERβ Binding Sites |
|-------------------------------------------|------------------------|-------------------------|
| Regulation of transcription               |                        |                         |
| Steroid hormone signaling                 |                        |                         |
| Cell proliferation                        |                        |                         |
| NF-kappaB signaling                       |                        |                         |
| Negative regulation of cell growth        |                        |                         |
| Cell migration                            |                        |                         |
| Inflammatory response                     |                        |                         |
| Apoptotic process                         |                        |                         |
| Somatic stem cell population maintenance  |                        |                         |

**FIGURE 4** ERβ binding activates a proportion of chromatin. A, Venn diagram representing overlap between ERβ-binding sites at H3K27ac marks and H3K27ac increased or decreased upon ERβ expression. B, Biological functions enriched (P < .05) among genes bound by ERβ with modulated H3K27ac sites [Color figure can be viewed at wileyonlinelibrary.com]
inhibits proliferation, along with inflammatory gene expression (Cxc5, Nos2). Moreover, other studies report that treatment with genistein, a natural phytoestrogen, reduces AOM/DSS-induced colitis and tumors in mice. Our findings reveal the mechanistic underpinnings of these functions. In conclusion, we find strong evidence of ERβ tumor suppressor regulations in colon, but further studies will be needed to dissect these mechanisms and their respective impact in detail.

Several studies describe how ERβ acts as a transcriptional enhancer at AP-1 sites (e.g., in HeLa and MCF7 cells). ERβ has also been reported to increase AP-1 activity in the presence of tamoxifen but not estrogen, and that ERβ DNA binding (to ERE) is not necessary for such nonclassical AP-1 transactivation function. While we found clear evidence that tamoxifen acts as an ERβ antagonist for ERE transactivation in colon, and that ERβ binds to AP-1 sites in the colon cistrome, we could not corroborate that ERβ-tamoxifen, nor ERβ-E2, acts as an agonist for AP-1 transactivation at standard TRE sites.

The ERα cistrome has been shown to be different depending on cell context. We observe that this is also true for the ERβ cistrome, when comparing cells from colon and breast origin. In MCF7, ERα mostly binds through ERE elements and AP1, AP2, FOXA1 motifs, and we identified a similar pattern for ERβ. In colon cells, on the contrary, ERβ did not bind at AP2 or FOXA1 motifs. The breast-specific binding is likely to be influenced by binding through ERα-ERβ heterodimers, and more studies comparing the same type of

**FIGURE 5**  Colon-specific, common, and breast-specific ERβ cistromes. A, Top-5 enriched motifs in breast-specific, common core, or colon-specific ERβ cistromes, in order of significance. B, Biological functions enriched among genes located nearest (−50 kb to +2 kb distance) to ERβ-binding sites in respective breast-specific, common core, and colon-specific cistromes (P < .05). C, Examples of ERβ ChIP enrichment signal for breast-specific (BCAS4), common (CTSD), and colon-specific (HSPB7) binding sites, visualized using the UCSC genome browser [Color figure can be viewed at wileyonlinelibrary.com]
cells with respective receptor homodimers are needed to elucidate further details of tissue and receptor cistrome specificity.

Previous studies have reported ERβ chromatin binding in other types of cells, including breast cancer cell lines,32,36,54,69,70 rat germ cells,71 endometriosis,72 and in U2OS cells.73 However, most ERβ antibodies are not specific towards ERβ protein73,74 and our study is the first to use the validated PPZ0506 antibody in ChiP-Seq. In conclusion, our study provides a map of ERβ-binding sites in cells of colon origin, not previously explored. As a negative regulator of cell proliferation and tumor suppressor mechanisms in colon cancer, ERβ appears as an attractive therapeutic target for the prevention of CRC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The ChIP-Seq and RNA-Seq data generated in our study are available in GEO under accession number GSE149979, GSE166194 (ChIP-Seq) and GSE112565, GSE112569 (RNA-Seq). Other data will be available from the corresponding authors upon request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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