The chromatin landscape of tamoxifen-associated endometrial tumors is reshaped toward estrogen receptor alpha sites independent of progesterone signaling

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Tamoxifen is an effective steroid estrogen receptor modulator (SERM) widely used in breast cancer treatment. It acts through competitive inhibition of natural ligand estrogen (E2) at the estrogen receptor (ER), affecting ERα interactions with other nuclear receptors, chromatin modulators, genomic structural proteins and co-regulators. Although tamoxifen inhibits the progression of breast cancer, it increases the risk of endometrial cancer, particularly in postmenopausal women. Progesterone receptor (PR) and ERα cistromes of endometrial adenocarcinoma Ishikawa cells treated with ovarian hormones showed that ERα binding sites are shared by PR at open sites of chromatin. This could explain, in part, progestin regulation of estrogen effects on ER/PR positive endometrial cancer cells. Here, we compared ERα cistromes from endometrial tumors of tamoxifen users and non-users with ERα and PR cistromes from Ishikawal model to evaluate how SERM exposure affects ER-binding signature. Non-user ER cistrome is closer to R5020-treated Ishikawa PR cistrome, while tamoxifen users ER cistrome is similar to estradiol-treated Ishikawa ER cistrome. The subset of ERbs of non-users regulates inflammatory signaling pathway genes. In contrast, the subset of ERbs of tamoxifen users modulates early estrogen response pathway genes, such as the oncogene NRIP1. This suggests that exposure to tamoxifen redirects ERα binding toward genomic regions typically responsive to estrogen, whereas ERα binding in non-users retains features associated with PR-occupied regulatory sites.
These results provide an example of how SERM treatment may remodel endometrial tumor epigenetic landscape, highlighting context-dependent genomic responses that should be considered to prevent deregulation of endometrial tissue under tamoxifen exposure.

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Introduction

Endometrial cancer (EC) is the sixth most common malignancy worldwide (1). Epidemiological trends indicate a rapid rise in EC incidence (1, 2). Notably, mortality rates have increased by 1.8% annually since 2010 (1, 2). Historically, EC has been classified in two categories: Type I or Type II (3). Type I EC, which are more prevalent and generally associated with a favorable prognosis, are linked to unopposed estrogen exposure and typically consist of grade I or II endometrioid adenocarcinomas. In contrast, Type II ECs are considered hormone-independent and tend to be more aggressive, encompassing grade III endometrioid adenocarcinomas, serous, clear cell, undifferentiated carcinomas, and carcinosarcomas (4, 5).

In hormone-dependent Type I EC, the interplay between estrogen and progesterone signaling -mediated by estrogen receptor alpha (ERα) and progesterone receptor (PR), respectively- plays a crucial role in tumor development and progression. Estrogens, particularly 17β-estradiol (E2), are essential for the growth and differentiation of various tissues, including the endometrium, where they promote proliferation through ERα and ERβ. ERα modulates gene expression via several mechanisms, the most classical of which involves direct binding to estrogen response elements (EREs) within chromatin, regulating target genes such as the progesterone receptor gene (PGR) (6). In addition, ERs can influence transcription through ERE-independent pathways, wherein DNA binding is facilitated by interactions with other transcription factors, such as Fos and Jun (6).

Estrogen promotes the proliferation of the endometrial glandular epithelium -the primary cell type involved in EC- whereas progesterone antagonizes these estrogenic effects (7). A disruption in the equilibrium between ERα and PR signaling has been associated with endometrial carcinogenesis (8). Specifically, prolonged exposure to unopposed estrogen can result in endometrial hyperplasia and overgrowth, a recognized precursor to malignancy (9, 10). Progesterone exerts a protective effect by inhibiting estrogen-mediated proliferation and promoting cellular differentiation (7). Notably, the loss of PR expression has been correlated with increased proliferation in both ER-positive and ER-negative endometrial tumors (11).

Progesterone mediates its biological effects through two main isoforms of the progesterone receptors, PRA and PRB, which are transcribed from distinct promoters of the PGR gene (12). In the endometrium, the relative expression of these isoforms is cell-type specific:, PRA predominates in

stromal cells, whereas PRB is more abundant in epithelial cells. Upon ligand binding, progesterone receptors modulate gene expression by interacting with progesterone response elements (PREs) in the genome, thereby regulating the transcription of downstream target genes.

Selective estrogen receptor modulators (SERMs), such as tamoxifen, are commonly used in treatment of estrogen receptor-positive breast cancer. Tamoxifen exerts its effects by binding to the ligand-binding domain of ERα. While it functions predominantly as an ER antagonist in breast tissue, inhibiting estrogen-driven proliferation (13, 14), its activity is highly tissue-specific. In the endometrium, tamoxifen paradoxically acts as an ER agonist, promoting epithelial proliferation and increasing the risk of EC (15–17). Studies using the Ishikawa EC cell line have demonstrated that tamoxifen modulates gene expression through ERα-dependent mechanisms (18). Notably, genome-wide analyses revealed significant overlap between the ERα cistromes of tamoxifen-associated endometrial tumors and those observed in breast cancer (19). Moreover, tamoxifen-associated endometrial tumors exhibit a distinct ERα DNA-binding profile compared to spontaneous cases, with these altered binding patterns correlating with differential gene expression (20).

In breast cancer, PR modulates ERα activity by influencing its chromatin binding dynamics and downstream gene expression (21). Studies in T47D mammary cancer cells have identified hormone control regions (HCRs), which harbor both ERα and PR binding sites and physically interact with promoters of hormone-regulated genes within hormone-responsive topologically associating domains (TADs) (22, 23). In contrast, the interplay between ERα and PR in EC remains poorly characterized. Recent work by (24) demonstrated that estrogen priming induces the formation of progestin-dependent PR binding sites in Ishikawa EC cells. These sites are frequently located within 'progestin control regions' (PgCRs), a subset of HCRs situated in TADs, involving coordinated binding of ERα , PR, and the transcription factor PAX2 to regulate gene expression. Notably, PgCRs are characterized by an open chromatin conformation and are associated with the regulation of genes implicated in endometrial carcinogenesis.

While it is established that prolonged tamoxifen treatment is associated with a distinct ERα binding profile in endometrial tumors compared to those not associated with tamoxifen, the relationship between these altered ERα binding sites and the regulatory landscape of the PR remains uncharacterized. The interplay between ERα and PR is critical in hormone-dependent EC, with PR having the capacity to modulate ERα's effects. Previous work has highlighted that ERα's positioning is a key determinant in the development of hormone-dependent EC. Therefore, this study aims to investigate how tamoxifen-induced changes in the ERα cistrome relate to PR binding sites in EC. We hypothesize that tamoxifen disrupts the co-regulatory relationship between ERα and PR, leading to a distinct oncogenic program. To test this, we conducted a comparative genomic analysis integrating publicly available ERα ChIP-seq data from endometrial tumors of tamoxifen users and non-users with our previously published PR and ERα cistromes from the Ishikawa EC cell line. This approach allowed us to delineate the differential ERα and PR genomic interplay in tamoxifen-associated versus non-associated ECs, providing novel insights into the specific molecular pathways dysregulated by tamoxifen exposure.

Methods and Materials

ChIP-seq Data Processing.

High-quality sequencing reads were aligned to the human reference genome (hg38, UCSC) using STAR v3.0.1 (25), with alignment parameters set to a maximum intron size of 1 and end-to-end alignment enabled. For initial quality control and sample clustering, reads for each sample were aligned independently. Subsequently, samples were stratified based on tamoxifen exposure (users vs. non-users) and reads within each group were merged into a single BAM file for downstream analysis. Peak calling was performed using MACS2 v2.2.6 (26) with default parameters, employing the corresponding input controls to identify regions of significant signal enrichment. Venn diagrams illustrating peak set overlaps were generated using the venn function from the Intervene package (27). Principal Component Analysis (PCA) and heatmap visualizations were generated using deepTools (28). Specifically, computeMatrix was used to calculate signal intensities, plotHeatmap for heatmap generation, multiBigwigSummary for region-wise signal quantification, and plotPCA for dimensionality reduction. Genomic overlap and distance between ER and PR binding sites in Ishikawa cells were assessed using the intersectBed and closest utilities from bedTools (29).

RNA-seq Data Processing.

Differential gene expression analysis was conducted using the DESeq2 package v1.44.0. (30). Genes exhibiting an absolute log fold change ($|\log2FC| \geq 1$ and a Benjamini-Hochberg adjusted p-value (Padj) < 0.05 were considered differentially expressed. Expression levels, quantified as FPKM (Fragment Per Kilobase of transcript, per Million mapped reads) and TPM (Transcripts Per Million) were visualized using custom Python scripts employing the seaborn library (31).

Gene Set Enrichment Analysis (GSEA) and Gene Ontology Enrichment analysis.

The association between binding sites with genes was done through two different approaches. The first one was done using GREAT algorithm (32, 33). This algorithm first assigns every gene a regulatory domain and then associates each genomic region with all genes whose regulatory domain it overlaps. The association was done using the Basal plus extension setting, where each gene is assigned a basal regulatory domain of a minimum distance upstream and downstream of the TSS (regardless of other nearby genes). The gene regulatory domain is extended in both directions to the nearest gene's basal domain but no more than the maximum extension in one direction. The algorithm was runned with 5kb upstream

and 1kb downstream from TSS as the proximal distance and up to 1000kb as the maximum extension. The second approach to find genes associated with binding sites was performed by selecting the TADs obtained from Ishikawa Hi-C that comprised binding sites of interest and retrieving all the genes contained in that TAD.

Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO) enrichment were performed using the GSEApy package (34), a Python-based implementation of GSEA and a wrapper for the Enrichr database. Gene expression data obtained from GEO was analyzed using the GSEA function in GSEApy with the following parameters: permutation type set to "phenotype", 1000 permutations, and "difference of class means" as the scoring method. For GO and pathway enrichment, the enrichr function was employed to query the following libraries: "BioCarta_2016" , "KEGG_2021_Human", "Reactome_2022", "MSigDB_Hallmark_2020", and "Elsevier_Pathway_Collection". Enriched terms with an adjusted p-value (Padj) < 0.05 were considered significant.

Hi-C Data Processing.

Previously filtered Hi-C datasets from (24), excluding low-quality reads, were further processed using the Juicer pipeline (35) with the human reference genome hg38. After individual sample contact maps were generated, contact matrices from biological replicates were merged by summing the contact counts between corresponding genomic loci, resulting in a combined contact map. Topological Associated Domains (TADs) were identified using the Arrowhead algorithm, and chromatin loops (i.e., locally enriched contacts) were detected using the HiCCUPS algorithm, both implemented within the Juicer framework.

Virtual 4C.

Virtual 4C profiles were generated through custom Python scripts using Hi-C matrices of Observed/expected contacts with a 5kb resolution and normalized via Knight-Ruiz (KR) algorithm as input (accessible at Figshare under reference number 28840463). The matrices for the region containing NRIP1 gene were obtained from hic files through Straw (35).

Endometrial Cancer Samples (TCGA).

RNA-seq data (FPKM values) for human endometrial cancer samples (n = 394) were obtained from The Cancer Genome Atlas (TCGA) project. Each sample was annotated with its corresponding FIGO stage. Differential expression analysis of NRIP1 across FIGO stages was performed using pairwise comparisons with Tukey's Honestly Significant Difference (HSD) post-hoc test, implemented via the statsmodels Python package (36). An adjusted p-value (p-adj) < 0.09 was considered statistically significant.

Results

Tamoxifen-driven shifts in ER binding sites reflect estrogen-responsive, PR-independent regulation.

To investigate how tamoxifen alters the estrogen receptor (ER) chromatin landscape in relation to progesterone receptor (PR) signaling, we conducted a comparative analysis of ER binding sites from endometrial tumors of tamoxifen users and non-users. Due to the absence of publicly available PR binding data from endometrial tumors, we employed our previously characterized ER and PR cistromes from the Ishikawa EC cell line as a reference framework for interpreting the changes in ER cistrome of users and non-users. This comparative analysis supported the hypothesis that tamoxifen treatment leads to an ER binding profile less influenced by PR in tumors from tamoxifen users.

ER landscape of tamoxifen user and non-user endometrial tumors (19, 20) were compared to our previously published ER and PR cistromes generated from Ishikawa EC cell line treated with 17β-estradiol (E2) or the synthetic progestin R5020 (24) (Figure 1a). In our previous work, we observed that estrogen pre-treatment leads to an increase in the total PR binding sites (PR bs), while retaining 97% of PR bs identified without E2 pre-treatment (Figure 1—Figure supplement 1a). Accordingly, we focused on the PR cistrome in estrogen-primed Ishikawa cells to characterize PR binding within the identified estrogen-responsive context (Figure 1—Figure supplement 1a).

For this comparison, raw data of ER ChIP-seqs from 6 non-users tumor and 9 tamoxifen users tumors were reprocessed under the same conditions as the ChIP-seq of Ishikawa cell line (Figure 1—Figure supplement 1b). Principal Component Analysis (PCA) of the reprocessed samples is shown in Figure 1—Figure supplement 2a. PC1 vs PC2 graph revealed two distinct clusters corresponding to tamoxifen users and non-user (Figure 1—Figure supplement 2b).

To characterize the ER binding site population in the endometrial tumor samples, we classified sites into three categories: unique to tamoxifen users, unique to non-users, and common to both (Figure 1—Figure supplement 3a). In tamoxifen users, the ER ChIP-seq signal showed a loss of 2167 binding sites compared to non-users, while tamoxifen treatment contributed to the acquisition of additional 2259 binding sites in tumors (Figure 1—Figure supplement 3a). To associate these sites with enhancer activity, K3K27ac levels were analyzed, revealing that tamoxifen-treated tumors exhibited increased activity at the binding sites gained after treatment (Figure 1—Figure supplement 3b).

To understand the functional ER binding landscape in tumor samples, we quantified the overlap with the ER bs and PR bs identified in Ishikawa cells. This revealed a distinct pattern between non-users and tamoxifen users (Figure 1b). Ishikawa ER bs shared 3-fold (249/84) 165 more binding sites with ER bs from tamoxifen users than with those from non-users (Figure 1b). Conversely, Ishikawa PR bs exhibited 4.7-fold (419/89) greater overlap with ER bs from non-users compared to tamoxifen users (Figure 1b). This asymmetry was also evident considering in the percentage of binding sites uniquely shared: 5.8% of all ER bs in tamoxifen users were uniquely shared with Ishikawa ER bs (202 bs), while only 1.2% were uniquely shared with Ishikawa PR bs (20 bs). In contrast, non-users shared 0.59% of their ER bs exclu-

sively with Ishikawa ER bs (42 bs) and 10.4% with Ishikawa PR bs (355 bs) (Figure 1c). Furthermore, the ChIP-seq signal intensity for ER at the binding sites shared with Ishikawa ER was higher in tamoxifen users than in non-users, whereas non-users showed a stronger ER signal at sites overlapping with Ishikawa PR bs (Figure 1d).

These results suggest that tamoxifen exposure in endometrial tumors is associated with an ER chromatin binding profile that more closely resembles the ER binding landscape observed in estrogen-treated Ishikawa cells. In contrast, the ER binding profile of non-tamoxifen users exhibits greater similarity to the PR binding pattern in the same cell line.

ER/PR binding in non-user links to inflammatory gene expression .

To understand the potential regulatory roles of the 355 unique non-user ER bs that overlap with Ishikawa PR binding, we employed two complementary approaches to associate these regions with nearby genes.

First, we used the GREAT algorithm (32), which defines both a basal and a distal domain for each gene. The basal regulatory domain extends 5 kb upstream and 1 kb downstream of the transcription start site (TSS). Briefly, the distal regulatory domain extends in both directions from the basal domain until it reaches the basal domain of the nearest gene, but no more than 1Mb in any direction. The genes whose regulatory domains contained one of the 355 unique non-user ER bs overlapping Ishikawa PR bs were selected. Gene ontology (GO) analysis of the associated gene set revealed significant enrichment in the Mucyn type O-glycan biosynthesis pathway, Basal transcription factors, and two NOTCH-related pathways: NOTCH receptor signaling and NOTCH signaling deregulation in cancer (Figure 2a and supplementary material). Given the established involvement of the NOTCH pathway in various cancers -including breast, ovarian, and cervical (37)- we assessed potential differential expression of these pathways in the publicly available microarray dataset of endometrial tumors from tamoxifen users and non-users (20). DEseq analysis of this dataset for individual differentially expressed genes failed to reveal any significant differences (p-value < 0.05)(Figure 2—Figure supplement 1a). GSEA of "NOTCH signaling deregulation in cancer" did not reveal statistically significant enrichment (p-value: 0.2) (Figure 2—Figure supplement 1b), possibly due to the high variability among the microarray samples. On the other hand, gene expression of NOTCH-related genes in Ishikawa cells treated with ethanol (vehicle), estradiol (E2), or tamoxifen revealed differential expression of several (CCND1, CDKN1A, FURIN, HES1, JAG1, JAG2, MYC, NCOR2, NCSTN, NOTCH1, NOTCH3 and TWIST1) genes within both the "NOTCH receptor signaling" and "NOTCH signaling deregulation in cancer" pathways (Figure 2—Figure supplement 1c) (38).

These findings suggest that overlapping ER and PR binding regions may contribute to the regulation of NOTCH signaling genes; however, validation through more precise gene expression analyses in tumor samples will be required.

In a second approach, we associated the shared ER bs with genes located within the same topologically associated domains (TADs), based on published Hi-C data for Ishikawa cells (24). Ontology analysis of genes located within TADs containing these ER bs revealed a significant enrichment in several pathways involved in inflammation processes, including the Chemokines Receptors Bind Chemokines (R-HSA-380108), Inflammatory Response, TNF-alpha Signaling via NF-kB and IL-6/JAK/STAT3 Signaling pathways (Figure 2b). Genes from the Chemokines Receptors Bind Chemokines and Inflammatory Response pathways were pooled and GSEA on the endometrial tumor microarray dataset was conducted. This analysis revealed a significant enrichment of the "Chemokines Receptors Bind Chemokines" pathway in non-users compared to tamoxifen users (Figure 2c), suggesting higher expression of inflammatory genes in the absence of tamoxifen. This subset of genes is distributed across different TADs, with the exception of the families of chemokines (C-X-C subfamily chemokines) and receptors (CCR) that cluster as expected (see supplementary material).

Among these, the proto-oncogene MYC, a key regulator of inflammation, emerged as a notable candidate de-regulated in EC (39–41). The epigenetic landscape of the MYC locus (Figure 3) showed a cluster of PR bs upstream of the MYC transcription start site (TSS) with high contact frequency. This subset overlaps more with ER bs from non-users than with those from tamoxifen users. We observed increased H3K27ac ChIP-seq signal at the MYC locus in tamoxifen users (Figure 3, Tamoxifen users H3K27ac signal). Consistent with this, reanalysis of RNA-seq data from Ishikawa cells (24) showed significantly increased MYC expression following E2 treatment compared to control (adjusted p-value < 0.05). Interestingly, MYC expression after 1-hour tamoxifen treatment did not significantly differ from E2 treatment (adjusted p-value < 0.05)(Figure 3—Figure supplement 1). Together, these data suggest a complex regulatory landscape involving inflammatory genes and proto-oncoges such as MYC, with divergent regulatory mechanisms in tamoxifen users and non-users.

ER occupancy at PR-independent sites in tamoxifen treated tumors.

Next, we investigated the genomic proximity of Ishikawa ER bs to the nearest PR bs (Figure 4—Figure supplement 1). Most ER bs were located within 5 kb of a PR bs (Figure 4—Figure supplement 1). To better dissect their potential interactions, we categorized ER bs into two groups: those overlapping PR bs by at least one base pair (ER overlapping PR) and those not overlapping PR bs (1 bp from the nearest PR bs) (ER not overlapping PR) (Figure 4a). The former group likely represents loci potentially co-regulated by both ER and PR, whereas the latter are probably independent of direct PR influence. Our analysis revealed that 732 out of the 1591 Ishikawa ER bs overlapped with PR bs (Figure 4b). To explore whether the regulatory behavior of these two categories differs between tamoxifen users and non-users, we focused on Ishikawa ER bs (both overlapping and not overlapping PR bs) that were also detected in tumors from ei-

ther of the tamoxifen users or non-users. When examining the ER ChIP-seq signal in tamoxifen users and non-users, we observed higher ER occupancy at ER bs not co-occupied by PR in tamoxifen users than non-users (Figure 4c). In contrast, at ER bs co-occupied by PR, the ER signal was higher in non-users compared to tamoxifen users (Figure 4c). Interestingly, ER bs overlapping with PR bs showed elevated H3K27ac levels in tumors from tamoxifen users (Figure 4d), suggesting that enhancers capable of binding both ER and PR exhibit increased activity in the context of tamoxifen.

To identify potential target genes associated with these binding sites, we applied the two approaches described previously. Ontology analysis of genes located within the same topologically associating domains (TADs) as ER bs overlapping PR bs revealed significant enrichment for the Estrogen Response Early pathway (Figure 4e). When we instead considered genes within TADs harboring binding sites shared by both Ishikawa ER bs and PR bs, the Estrogen Response Early pathway remained the most enriched, albeit with lower significance compared to ER bs not shared with PR (Figure 4—Figure supplement 2a). Similar trends were observed with the second approach, which focused on genes proximal to the binding sites: the Estrogen Response Early pathway showed stronger enrichment for ER bs not shared with PR bs (Figure 4—Figure supplement 2b) compared to those shared with PR bs (Figure 4—Figure supplement 2c). Finally, we analyzed the ER ChIP-seq signal near the transcription start sites (TSSs) of Estrogen Response Early genes in tamoxifen users and non-users (Figure 4f). Notably, the ER signal around the TSSs of these genes was significantly higher in tamoxifen users compared to non-users.

Tamoxifen-associated Chromatin Reorganization Influences NRIP1 Expression and Estrogen Response Gene Activity.

To determine whether ER binding near genes within the Estrogen Response Early pathway leads to corresponding changes in gene expression, we first performed an ontology analysis of genes differentially upregulated in Ishikawa cells treated with E2 compared to R5020 (adjusted p-value < 0.05, log2 fold change ≥ 0), using data from our previous study (24). As expected, genes from the Estrogen Response Early pathway were upregulated when treated with estrogen (Figure 5a). We next performed a GSEA analysis of this pathway using microarray expression profiles from tamoxifen users and non-users (20) (Figure 5b). This revealed higher expression of Estrogen Response Early pathway in tamoxifen users compared to non-users.

Within this pathway, the Nuclear Receptor Interacting Protein 1 (NRIP1) gene emerged as a key component (Figure 5). NRIP1 encodes a nuclear co-regulator that modulates the transcriptional activity of various transcription factors, including ER. Its differential epigenetic landscape between tamoxifen users and non-users suggests altered regulation of endometrial tumors (Figure 5c). Hi-C chromatin conformation analysis revealed a differential loop (highlighted by blue dashed lines) present in R5020-treated, but not in E2-treated, Ishikawa cells. This loop connects two CTCF binding sites. In addition, a virtual 4C analysis centered on the NRIP1 TSS indicated a higher contact frequency between the promoter and ER/PR binding sites in Ishikawa cells (5th track).

Expression profiling of NRIP1 in Ishikawa cells showed downregulation under R5020 treatment compared to E2, and upregulation when comparing E2 to untreated conditions (adjusted p-value < 0.05) (Figure 5c, 3 last tracks). Similar epigenetic features were also observed for the genes: Growth Regulated by Estrogen in Breast Cancer 1 (GREB1), Cyclin A1 (CCNA1) and Dehydrogenase/reductase 3 (DHRS3) from the Estrogen Response Early pathway (Figure 5—Figure supplement 1). To assess NRIP1 expression in clinical samples, we analyzed RNA-seq data from 394 endometrial adenomas and adenocarcinomas obtained from The Cancer Genome Atlas Program (Figure 5d). Tumors at stage IV showed significantly lower NRIP1 expression compared to stage I (adjusted p-value < 0.05). Since NRIP1 expression correlates with ER levels (42), this downregulation may reflect the reduced ER expression typically observed in advanced-stage endometrial cancer. Finally, NRIP1 expression was significantly higher in Ishikawa cells treated with E2 (adjusted p-value < 0.05) compared to tamoxifen or control (Figure 5e).

Collectively, these results suggest that the Estrogen Response Early Pathway plays a pivotal role in shaping endometrial response to tamoxifen. Prolonged tamoxifen treatment may enhance ER binding at sites limited PRco-regulation, leading to upregulation of the Estrogen Response Early pathway, influencing key genes such as NRIP1, and contributing to a distinct genomic and transcriptional landscape in tamoxifen-associated endometrial cancer compared to tumors from non-users.

The findings suggest that endometrial cancer development in tamoxifen users may involve different molecular mechanisms compared to non-users, potentially reflecting a loss of PR-mediated regulation in non-users and the deregulation of PR-independent pathways in tamoxifen users.

Discussion

Our analysis revealed that the ERα binding profile in tamoxifen users closely mirrors the ER landscape observed in estrogen-treated Ishikawa cells, whereas the ER profile of non-users more closely aligns with the PR binding pattern in the same cell line. This shift is consistent with previous observations by (19, 20), which demonstrated a distinct ERα binding profile in tamoxifen-associated endometrial tumors. Prior studies have established that PR can modulate ERα activity and downstream gene expression in breast cancer (21) and that the coordinated binding of ERα, PR, and PAX2 regulates transcriptional programs implicated in endometrial carcinogenesis (24). In this context, our findings suggest that tamoxifen disrupts PR-mediated regulation of ERα, resulting in alternative transcriptional programs that may contribute to cancer progression.

Further characterization of the differential binding patterns revealed that ER binding sites uniquely shared with Ishikawa PR sites in non-users were enriched for pathways related to inflammatory responses, which correlated with increased

expression of the associated genes. Among inflammatory genes, overexpression of c-Myc has been reported in over 70% of endometrial tumors (40) and its expression correlates with disease prognosis (41). Prior studies indicate that PR can act as a negative regulator of MYC transcription in endometrial cancer (39). The PR enhancers in non-users is consistent with the possibility of PR regulation of tumors without tamoxifen treatment. In Ishikawa cells, MYC expression was significantly upregulated following both estradiol (E2) and tamoxifen treatment, although it is important to note that short-term in vitro exposure to tamoxifen does not fully replicate the chronic treatment experienced by patients.

The anti-inflammatory effects of progesterone, mediated through PR, are well documented in the endometrium, including in endometrial adenocarcinomas (43). Our results are consistent with a model in which PR, acting in concert with ER, regulates inflammatory pathways in non-users, and loss of this regulatory axis may contribute to tumor development (Figure 6). By contrast, tamoxifen-associated EC may engage alternative pathways not typically modulated by PR, such as PI3K signaling (44). These observations underscore the need for further investigation to delineate how tamoxifen-induced shifts in ER cistromes reprogram transcriptional networks, particularly those governing inflammatory signaling, and how these changes influence endometrial tumor biology.

In tamoxifen users ER binding shifts toward PR-independent ERbs, and it is linked with an upregulation of the Estrogen Response Early pathway. The ER co-regulator NRIP1 is a key component of this pathway, and its expression is known to inhibit ERα activity in mammary cells (45). NRIP1 silencing has also been linked to EC, and it has been reported as the most frequently mutated gene in microsatellite-unstable EC (46). Recently, epigenomic analysis on the human endometrial stromal cell line tHESC identified NRIP1 as one of the genes upregulated by E2, presenting three ERbs interacting with its promoter (approximately 60kb, 200kb and 270kb away from the TSS) (47). Only one (+60kb) of these ERbs was identified in the epithelial adenocarcinoma cell line Ishikawa treated with E2, suggesting that these ERbs are specific from stromal cells. Non-users endometrial tumors have not presented these ERbs at all, while tamoxifen users share all of them. This observation indicates that tamoxifen tumor samples could include stromal cells with this signal or that these tumors changed their phenotype closely to stromal signature.

A major limitation is the lack of in vivo PR cistrome data in endometrial tumors, and particularly in tamoxifen-associated cases. Thus, our inference relies on Ishikawa-derived PR cistromes, which may not fully capture the complexity of primary tumors. Specifically, Ishikawa cells cannot model the heterogeneity of tumor subtypes or the stromal–epithelial interactions that shape receptor activity in vivo. Nevertheless, the convergence of our findings with prior mechanistic studies supports the hypothesis that tamoxifen reshapes the ER–PR regulatory axis, highlighting the need for future studies using primary tumor material to define PR-dependent modulation of ERα under tamoxifen exposure.

These findings support a model in which prolonged tamoxifen exposure reshapes the ERα–PR regulatory axis, leading to reduced PR-mediated control of inflammatory pathways and enhanced ER-driven activation of the Estrogen Response Early program (Figure 6). These alternative transcriptional states may influence tumor behavior and progression. Our work provides a chromatin-level framework for understanding how long-term SERM treatment reprograms hormone receptor signaling in the endometrium, with potential implications for prognosis and targeted therapy development.

Data Availability

ERα and H3K27ac ChIP-seq data and microarray data from endometrial cancers from tamoxifen users and non-users, as reported by (19, 20) are accessible in Gene Expression Omnibus (GEO) under accession number GSE94524. ERα and PR ChIP-seq data along with RNA-seq data from Ishikawa cells treated with E2 and R5020 (24) are available under accession number GSE139398. RNA-seq data from Ishikawa cells treated with tamoxifen and E2 (38) can be accessed via GEO accession number GSE115608. CTCF binding sites in Ishikawa cells, used in omics track figures, are available from ENCODE under accession number ENCSR000BQE. RNAseq data for human endometrial cancer samples were obtained from The Cancer Genome Atlas (TCGA) under project TCGA. Processed data and custom scripts for virtual 4C, TCGA samples analysis and RNAseq differential expression analysis are accessible at Figshare under reference number 28840463 (https://doi.org/10.6084/m9.figshare.28840463). For information about previously published datasets used see Table 1

Competing interests

The authors declare no competing interests.

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Author contributions

Luciana Ant: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – original draft, Writing – review and editing; **Nicolás Bellora:** Data curation, Formal analysis, Methodology, Supervision; **Alejandro La Greca:** Formal analysis, Writing – review and editing; **François Le Dily:** Formal analysis; **Patricia Saragüeta:** Conceptualization, Formal analysis, Funding acquisition, Investigation,

Author(s)	Year	Dataset title	Dataset URL	Database / ID
Droog M, Nevedomskaya E, Dackus GM, Fles R, Kim Y, Hollema H, Mourits MJ, Nederlof PM, Van Boven HH, Linn SC, Van Leeuwen FE, Wessels LFA, Zwart W	2017	Tamoxifen-associated endometrial tumors expose differential enhancer activity for Estrogen Receptor alpha	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94524	GEO, GSE94524
La Greca A, Bellora N, Le Dily F, Jara R, Nacht AS, Quilez Oliete J, Villanueva JL, Vidal E, Merino G, Fresno C, Tarifa Reischle I, Vallejo G, Vicent G, Fernández E, Beato M, Saragüeta P	2022	Higher-order chromatin organization defines PR and PAX2 binding to regulate endometrial cancer cell gene expression	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139398	GEO, GSE139398
Puyang X, Furman C, Wu ZJ	2018	Transcriptional profiling of Ishikawa cells treated with H3B-5942, E2, or standard of care compounds	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE115608	GEO, GSE115608
Korch C, Spillman MA, Jackson TA, Jacobsen BM, Murphy SK, Lessey BA, Jordan VC, Bradford AP	2012	DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination	https://www.encodeproject.org/experiments/ENCSR000BQE/	ENCODE, ENCSR000BQE

Table 1. Previously published datasets used in this study.

Project administration, Supervision, Writing – original draft, Writing – review and editing.

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Ethics Statement

The present study relies exclusively on publicly available, open-source data. As no new experiments were conducted on human subjects or animals, and no identifiable data was used, this work did not require ethical approval or review by any committee.

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Generative AI was used to enhance the clarity, grammar, and style of the text. All scientific content, data interpretation, and conclusions remain the sole responsibility of the authors.
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Figures

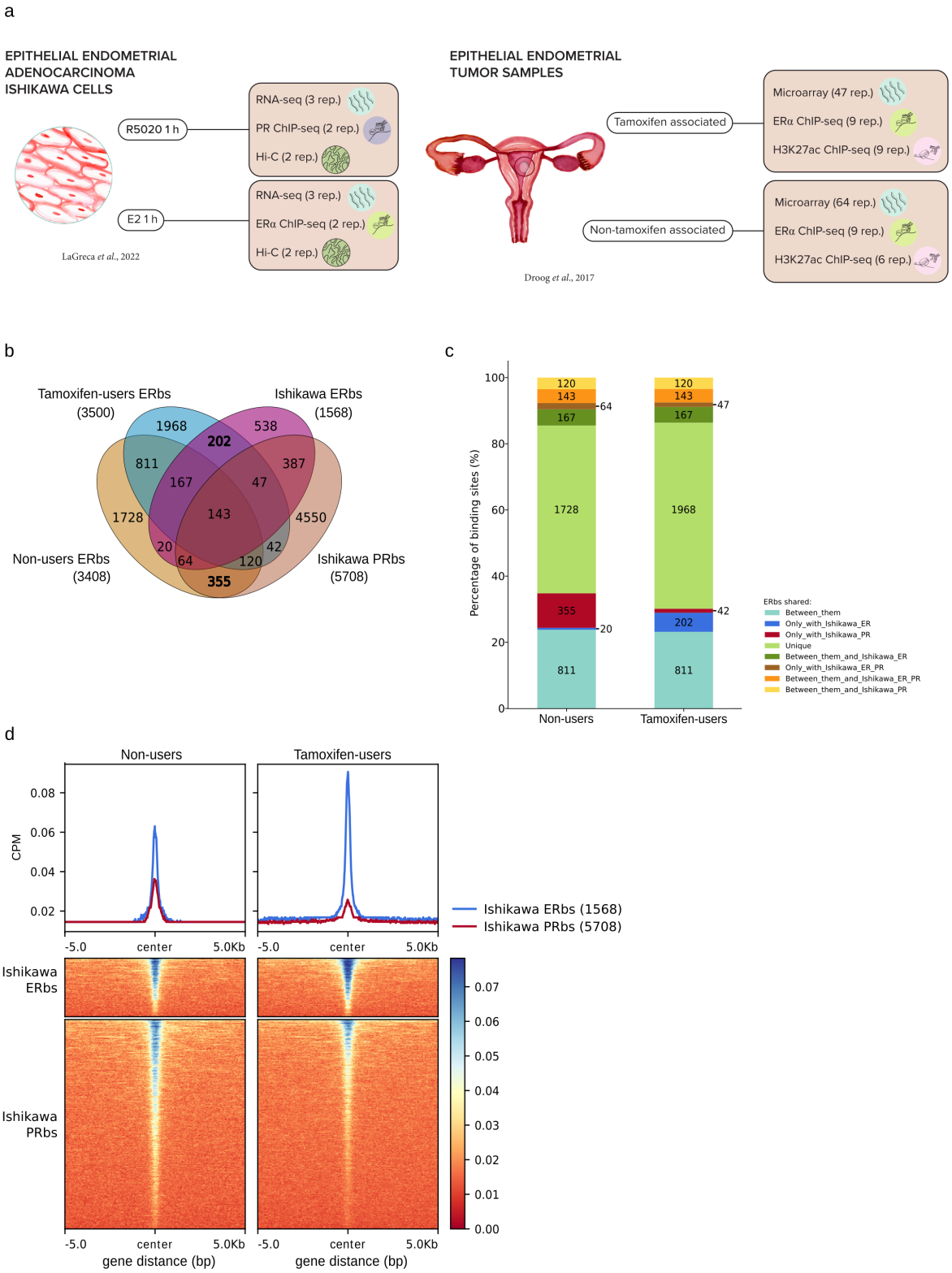


Figure 1. Comparative analysis of ER and PR binding profiles in endometrial tumors and Ishikawa cells. **a.** Schematic overview of the experimental set up involving endometrial tumors from tamoxifen users and non-users, as well as Ishikawa endometrial adenocarcinoma cells. **b.** Venn diagram showing the overlap between PR binding sites (PR bs) and ER binding sites (ER bs) in Ishikawa cells, along with ER bs identified in tamoxifen users and non-users. The total number of binding sites (bs) for each group is indicated in parentheses. **c.** Percentage of ER bs in tamoxifen users and non-users, categorized by their sharing pattern: shared between users and non-users (Between_them); shared only with Ishikawa ER (Only_with_Ishikawa_ER) or Ishikawa PR (Only_with_Ishikawa_PR); unique to each group (Unique); shared between users, non-users, and Ishikawa ER (Between_them_and_Ishikawa_ER); shared only with both Ishikawa ER and PR (Only_with_Ishikawa_ER_PR); shared across all groups (Between_them_and_Ishikawa_ER_PR); and shared between users, non-users, and Ishikawa PR (Between_them_and_Ishikawa_PR). **d.** ERα ChIP-seq signal (CPM) in tumors from tamoxifen users and non-users. Signal was quantified within a 10 kb window centered on Ishikawa ER bs and PR bs ($\pm$5 kb). Top panel: Median CPM signal across all Ishikawa ER and PR bs. Bottom panels: Signal intensity for each individual region.

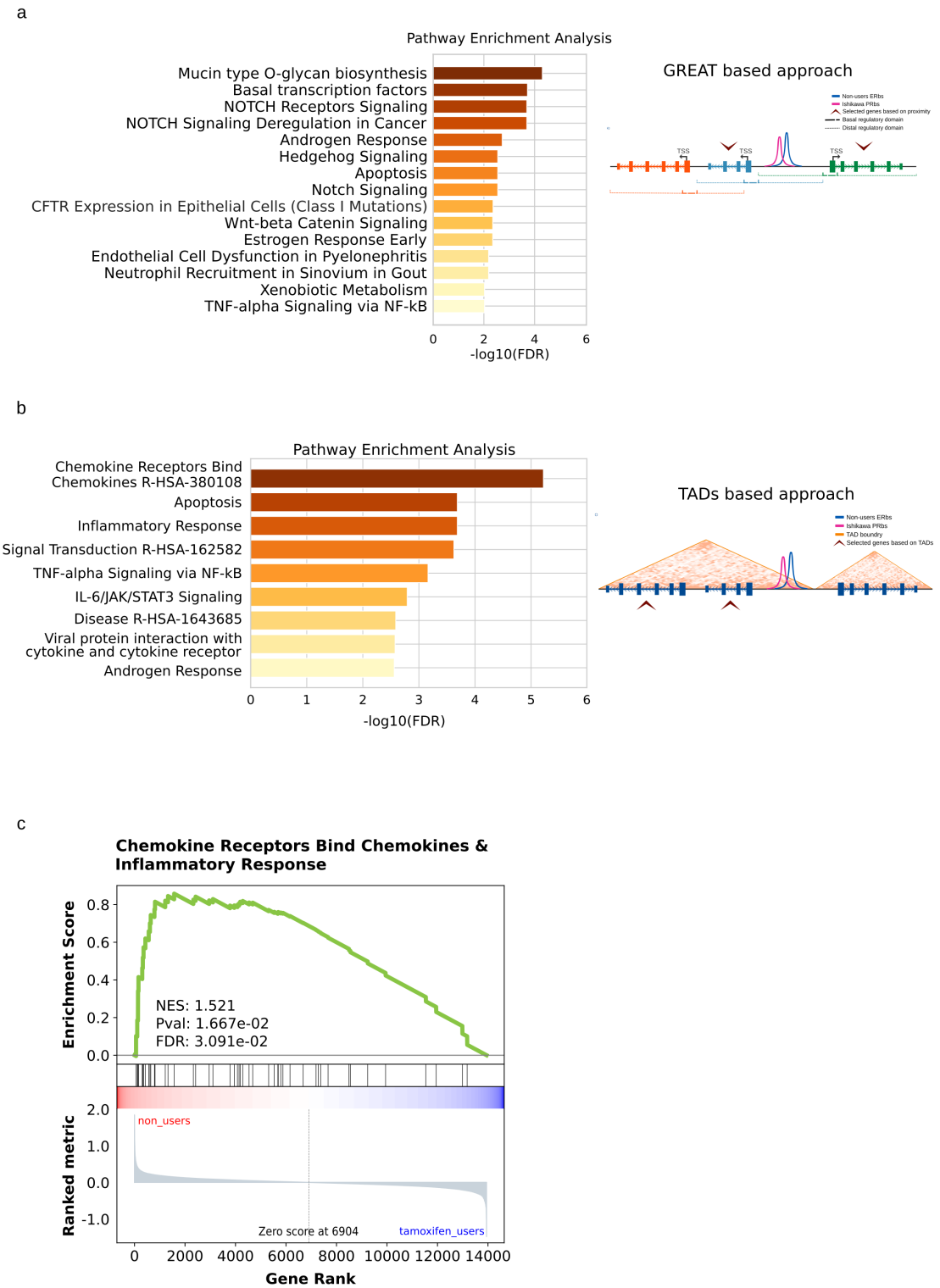


Figure 2. Pathway enrichment analysis of genes associated with PR and non-user ER co-bound regions. a. Top enriched pathways among genes located near Ishikawa PR bs that overlap with ER bs from non-user (±5 kb from the transcription start site, TSS (left). Representation of the GREAT-based approach used to associate binding sites to nearby genes (right). The basal regulatory domain of each gene extends 5 kb upstream and 1 kb downstream of the transcription start site (TSS) and the distal regulatory domain extends in both directions from the basal domain until it reaches the basal domain of the nearest gene, but no more than 1Mb in any direction. The genes whose regulatory domains contained one of the 355 unique non-user ER bs overlapping Ishikawa PR bs were selected. **b.** Top enriched pathways in genes located within topologically associating domains (TADs) that contain at least one Ishikawa PR bs overlapping with ER bs from non-users (left). Representation of the TADs-based approach used to associate binding sites to nearby genes (right) **c.** Gene set enrichment analysis (GSEA) of gene expression profiles from tamoxifen users and non-users. The analyzed gene set includes Chemokine Receptors Bind Chemokines and Inflammatory Response genes located in TADs containing at least one Ishikawa PR bs overlapping with non-users ER bs. The enrichment profile (green) indicates the correlation of gene expression with the ranked expression dataset. Normalized enrichment score (NES) and nominal p-value (nom.p) are reported.

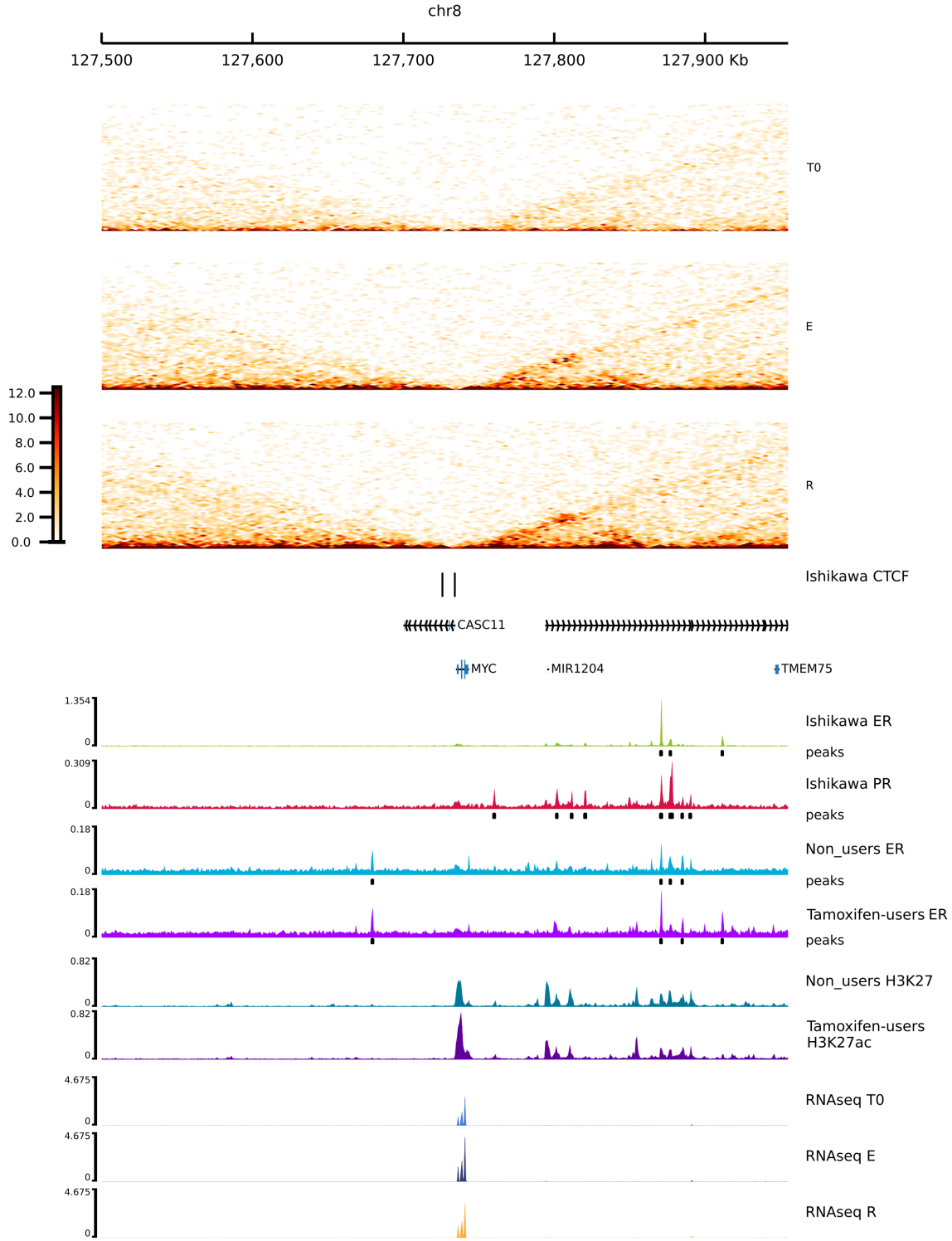


Figure 3. Genomic architecture, epigenetic landscape, and gene expression at MYC locus. From top to bottom, the tracks display: genome scale; contact matrices from Ishikawa cells treated with T0, E2 (E), or R5020 (R); ENCODE CTCF binding sites in Ishikawa cells; UCSC gene annotations; ER and PR ChIP-seq signal in Ishikawa cells (black lines indicate peak regions); ER ChIP-seq signal in endometrial tumors from non-users and tamoxifen users (black lines indicate peaks); H3K27ac ChIP-seq signal in non-users and tamoxifen users; and RNA-seq RPKM values in Ishikawa cells treated with T0, E2 (E), or R5020 (R).

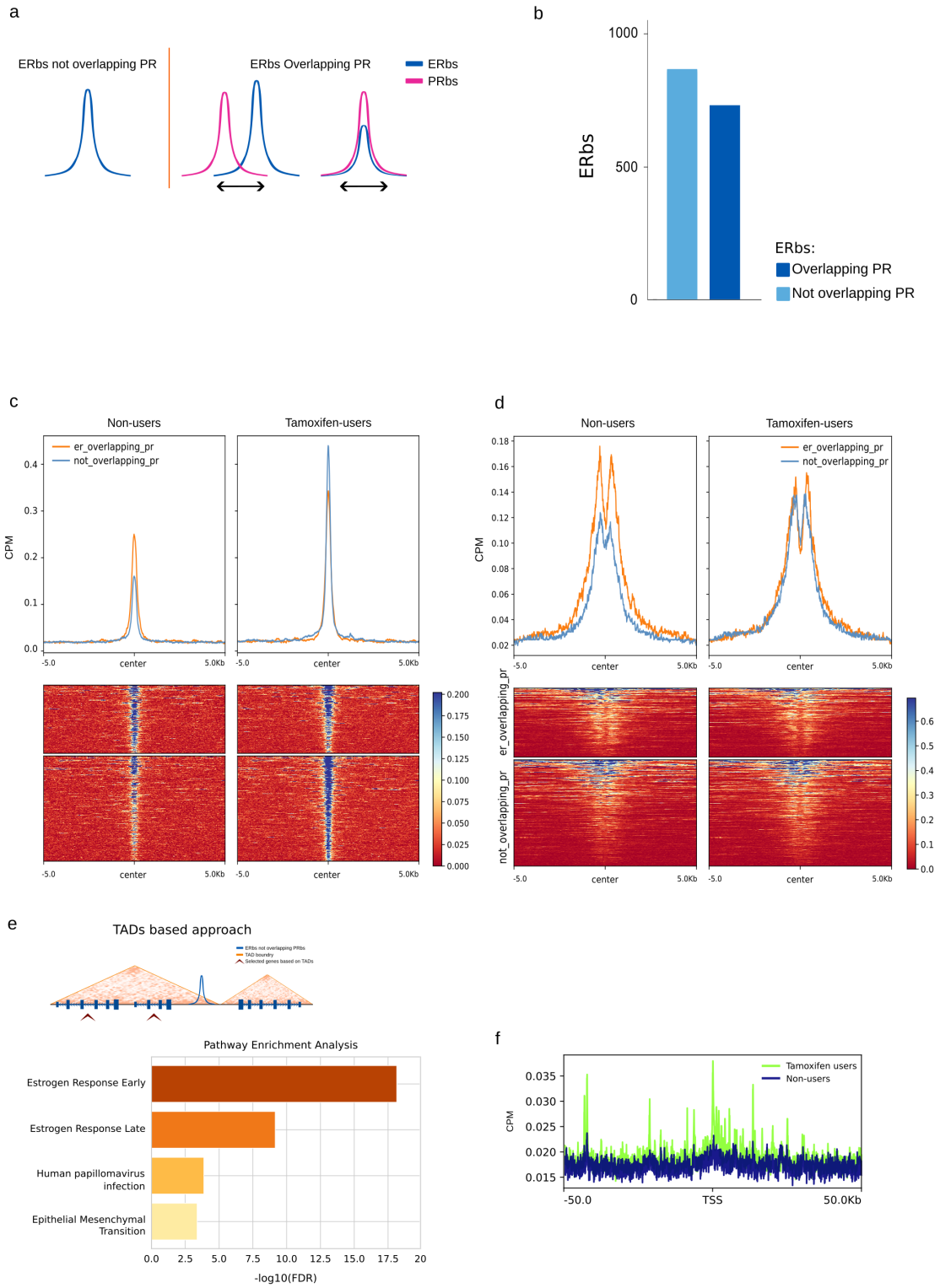


Figure 4. ER bs overlapping PR bs and regulation of Hallmark Estrogen Response Early Pathway. a. Ishikawa ER bs were defined as overlapping PR bs if their peaks shared at least 1 bp. **b.** Number of Ishikawa ER bs that overlap or do not overlap with Ishikawa PR bs. **c.** ER signal distribution in tamoxifen users and non-users. Regions were defined within a 10kb window centered on Ishikawa ER bs ($\pm$5 kb) that were also identified as ER bs in either group (tamoxifen users and non-users). Signal intensity represents the median of accumulated reads per region. ER bs are categorized into two mutually exclusive groups: those overlapping PR bs (er_overlapping_pr) and those that do not (not_overlapping_pr). **d.** H3K27ac ChIP-seq signal distribution in tamoxifen users and non-users. Regions were defined as in (a), with signal intensity representing the median of accumulated reads per region. ER bs are categorized as in (a) **e.** Representation of the TADs-based approach used to associate binding sites to nearby genes (top). Top pathways enriched in genes located within TADs containing at least one Ishikawa ER bs that does not overlap a PR bs (bottom). **f.** Median of ER ChIP-seq signal in tamoxifen users and non-users around the transcription start sites (TSS, $\pm$50 kb) of the 200 genes in the Hallmark Estrogen Response Early Pathway.

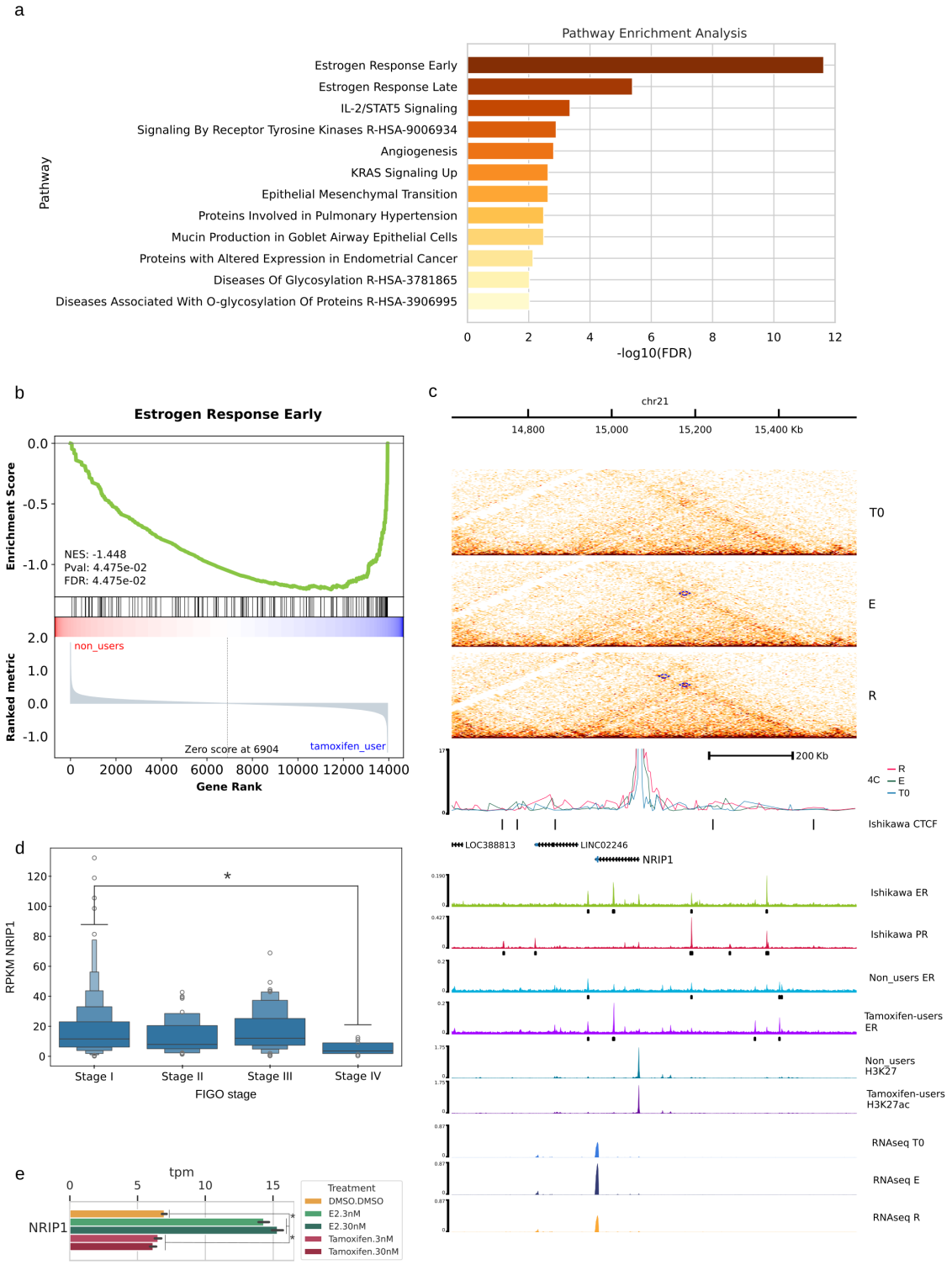


Figure 5. Pathway enrichment and regulatory features associated with NRIP1 and estrogen signaling. a. Top pathways enriched among genes upregulated in Ishikawa cells treated with E2 compared to R5020 (adjusted p-value < 0.05, log_2 fold change $\geq$ 0). **b.** Gene Set Enrichment Analysis (GSEA) of the Hallmark Estrogen Response Early pathway using gene expression profiles from tamoxifen users and non-users. The enrichment profile (green) indicates the correlation with the ranked gene list. The Normalized Enrichment Score (NES) and nominal p-values (nom.p) are shown. **c.** Genomic and Epigenetic landscape, and gene expression at NRIP1 locus. Tracks, from top to bottom, display: genome scale; contact matrices from Ishikawa cells treated with R5020 or E2 (blue dashed lines indicate chromatin loops); virtual 4C centered at the NRIP1 transcription start site (TSS); ENCODE CTCF binding sites in Ishikawa cells; UCSC gene annotations; ER and PR ChIP-seq signals in Ishikawa cells (black lines indicate peaks); ER ChIP-seq signals from non-users and tamoxifen users (black lines indicate peaks); H3K27ac ChIP-seq signals from non-users and tamoxifen users; and RNA-seq RPKM values from Ishikawa cells treated with T0, E2 (E), and R5020 (R). **d.** NRIP1 expression (FPKM) from RNA-seq data in The Cancer Genome Atlas (TCGA), including 394 endometrial adenomas and adenocarcinomas. Tumors are grouped by FIGO stage. Asterisks (*) indicate statistically significant differences in expression (p $\pm$ 0.05). **e.** NRIP1 expression (TPM) in Ishikawa cells treated with DMSO, 3nM, and 30nM of estrogen or tamoxifen. Asterisks (*) indicate statistically significant differential expression between treatment groups (p $\pm$ 0.05).

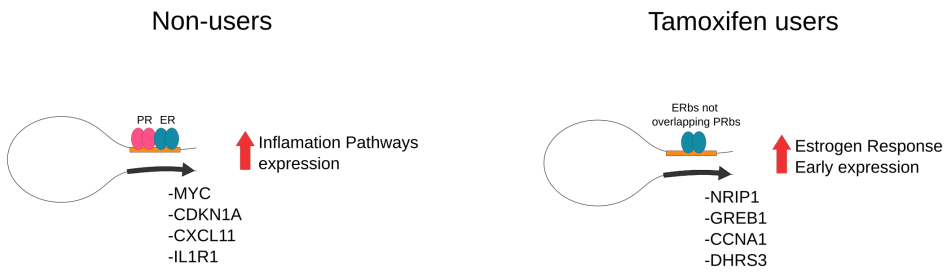


Figure 6. Tamoxifen Disrupts ER–PR Crosstalk in Endometrial Cancer. Model of tamoxifen effects on ER (blue)–PR (pink) crosstalk. In non-users (Left), ER binding overlaps with PR, mediating inflammatory pathway expression (red arrow height). In tamoxifen users (Right), PR regulation is lost and ER redistributes to PR-independent sites, enhancing the Estrogen Response Early pathway.

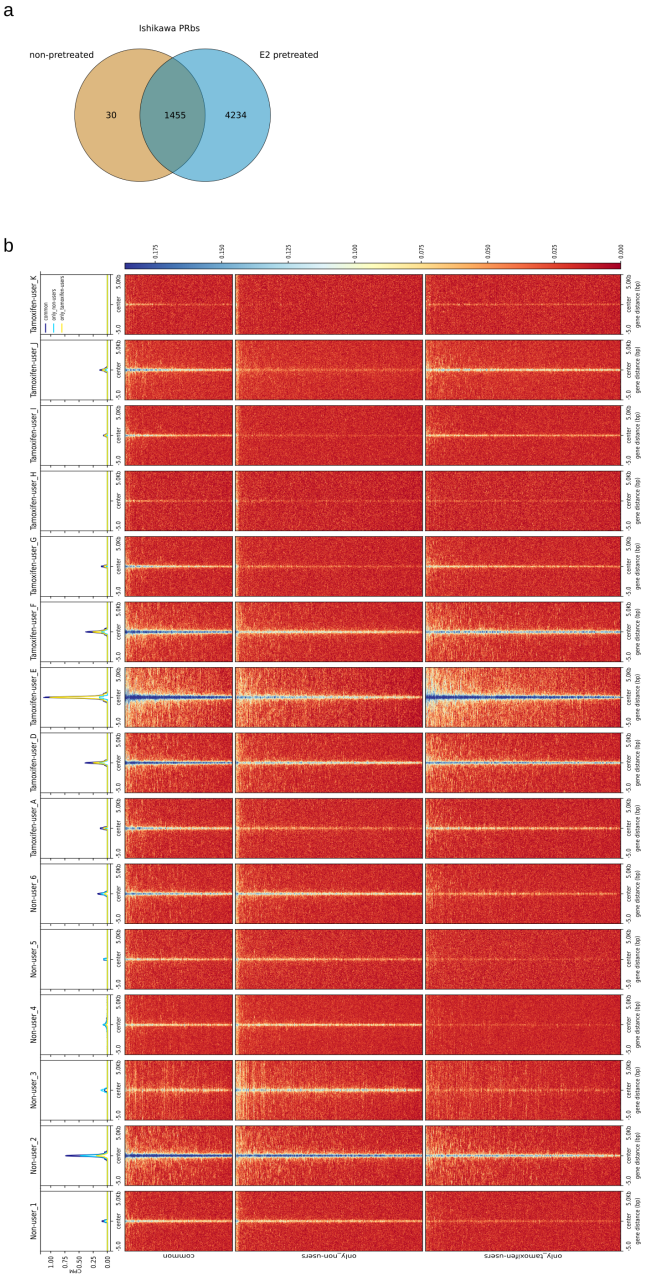


Figure 1—Figure supplement 1. a. Venn diagram showing the overlap of PR binding sites (PR bs) in Ishikawa cells pretreated with E2 for 12 hours and those without pretreatment. The intersection represents PR bs shared between both conditions. **b.** Distribution of ERα ChIP-seq signal in tumor samples from tamoxifen users and non-users. Regions were defined within a 10 kb window ($\pm$5 kb) centered on ER binding sites (ER bs) identified in tamoxifen users, non-users, or in both groups (common sites). Top panel: Median accumulated ERα ChIP-seq signal (CPM) across each ER bs group for tamoxifen users and non-users. Bottom panel: Heatmap showing ERα ChIP-seq signal intensity across all tamoxifen-user and non-user samples.

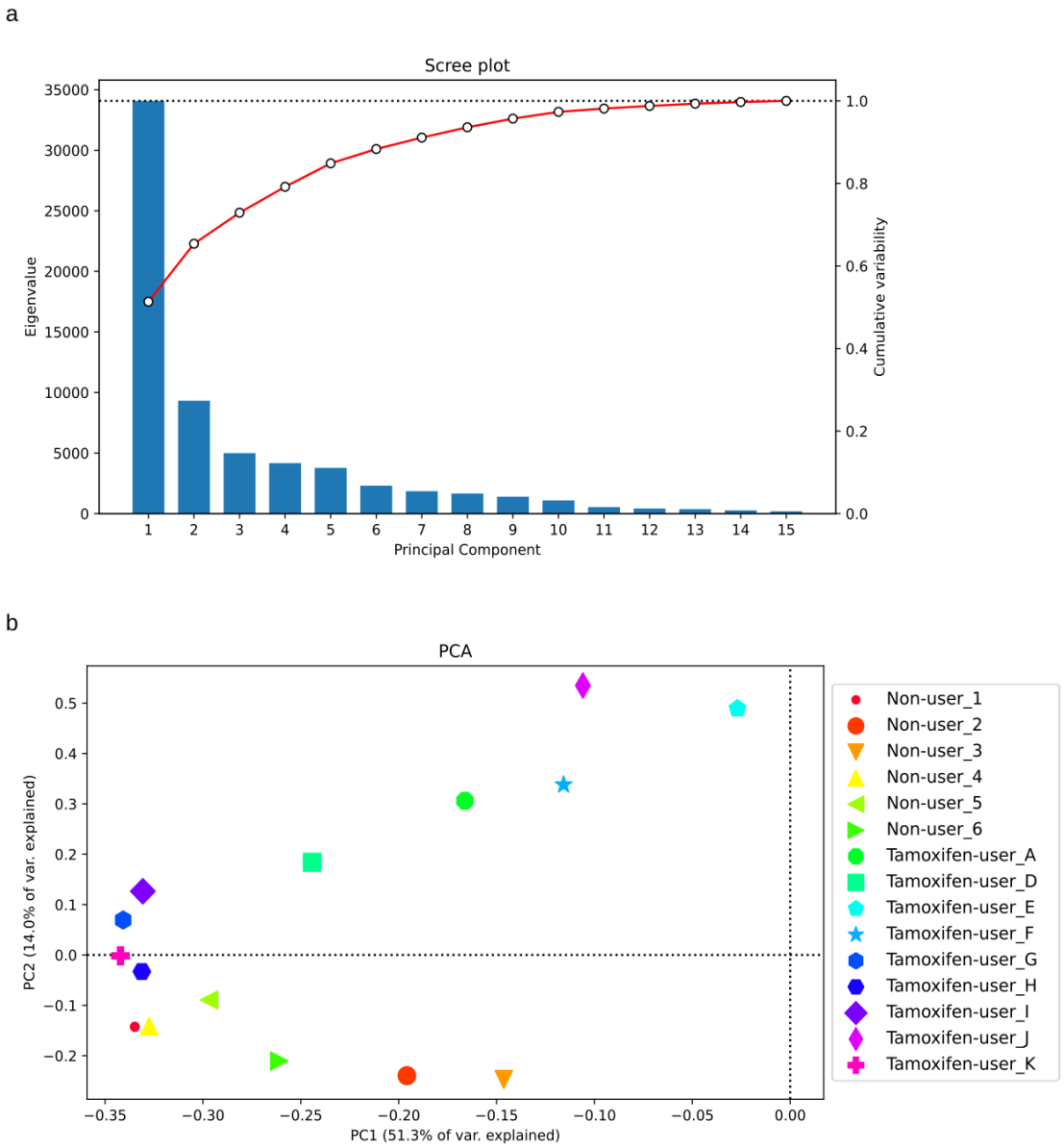
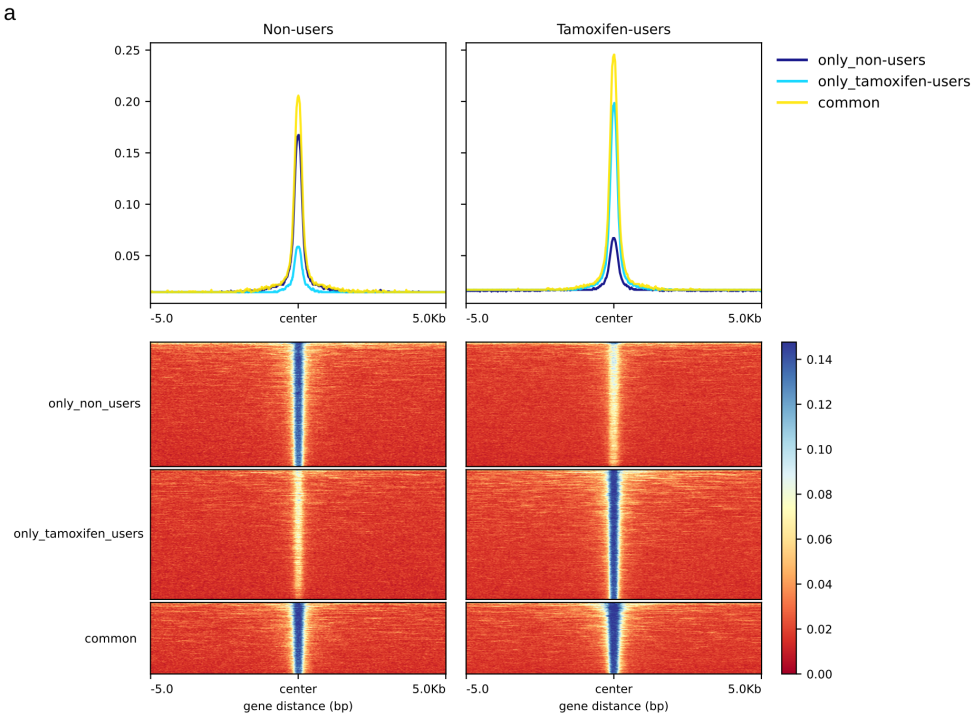


Figure 1—Figure supplement 2. Principal Component Analysis of ER ChIP-seq signal in tamoxifen user and non-user samples. a. Scree plot illustrating the proportion of variance in ER ChIP-seq signal explained by each principal component. **b.** Principal Component Analysis (PCA) plot showing PC1 versus PC2 based on ERα ChIP-seq signal across all tamoxifen user and non-user samples.



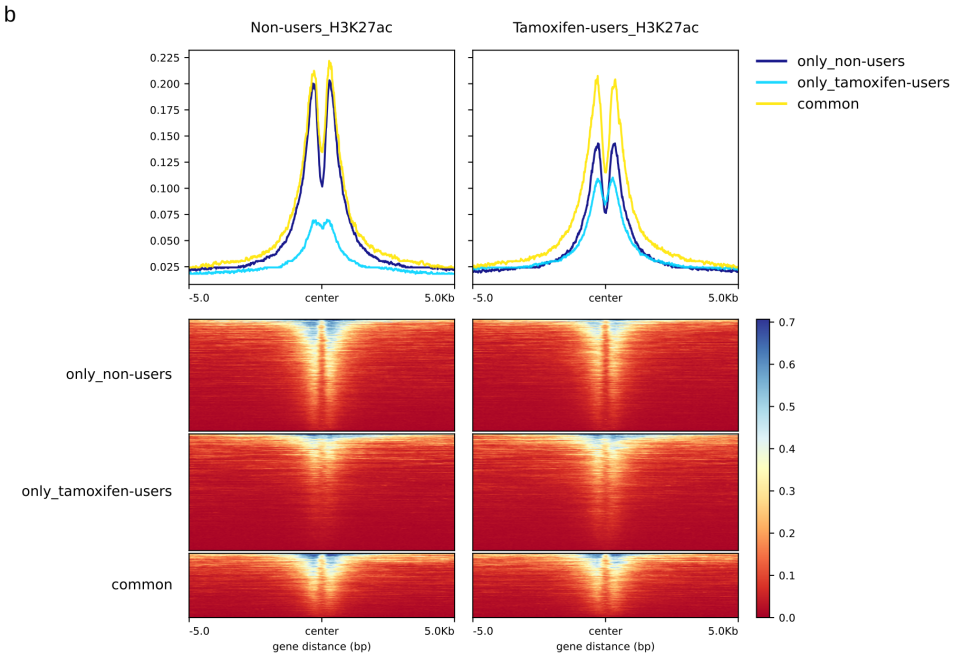


Figure 1—Figure supplement 3. ER$\alpha and H3K27ac signal distribution in tumors from tamoxifen users and non-users. a. Regions were defined within a 10kb window (\pm$5 kb) centered on ER binding sites (ER bs) identified in tamoxifen users, non-users, or in both groups (common sites). Top panel: Median accumulated ERα ChIP-seq signal for each binding site group (non-users, tamoxifen users, and common). Bottom panel: Heatmap showing ERα ChIP-seq signal intensity across individual regions, grouped by ER bs category: non-users, tamoxifen users, and common sites b. Same analysis as in (a), showing H3K27ac ChIP-seq signal in tumors from tamoxifen users and non-users centered on ER binding sites (ER bs) identified in tamoxifen users, non-users, or in both groups (common sites). Regions were defined as in (a).

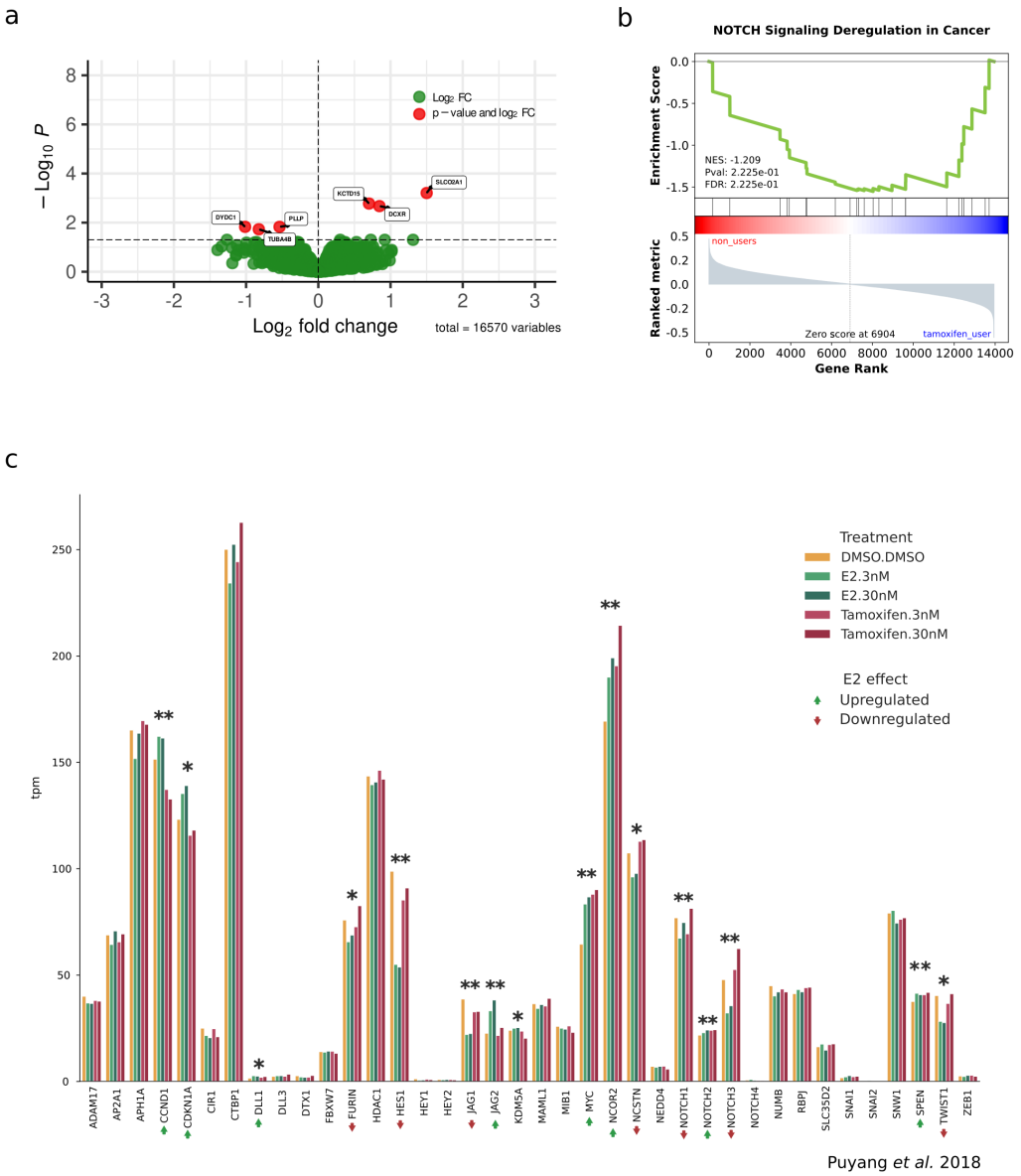


Figure 2—Figure supplement 1. Notch signaling pathway analysis in tamoxifen-treated samples and Ishikawa cells. a. Volcano plot depicting the results of differential expression analysis comparing gene expression profiles between tamoxifen users and non-users (N=16,570 variables/genes analyzed). The dashed horizontal line indicates a p-value threshold of 0.05, with genes above this line considered statistically significant. Genes highlighted in red show a p-value less than 0.05. **b.** Gene set enrichment analysis (GSEA) of the Notch Signaling Deregulation in Cancer pathway using gene expression profiles from tamoxifen users and non-users. The enrichment profile (green) illustrates the correlation of gene expression with the ranked gene list. Normalized enrichment scores (NES) and nominal p-values (nom. p) are shown. **c.** Expression levels (TPM) of genes from the Notch Receptor Signaling and Notch Signaling Deregulation in Cancer pathways in Ishikawa cells treated with control (DMSO), tamoxifen (3nM and 30nM), and estrogen (3nM and 30nM) (E2.3nM and E2.30nM). One asterisk (*) indicate significant differential expression between E2 (30nM) and DMSO (p 0.05). Two asterisks (**) indicate significant differential expression between E2 (30nM) and DMSO and tamoxifen (30nM) and DMSO ($p < 0.05$). KDM5A is the only gene that presents a differential expression between tamoxifen (30nM) and DMSO but not between E2 (30nM) and DMSO ($p < 0.05$).

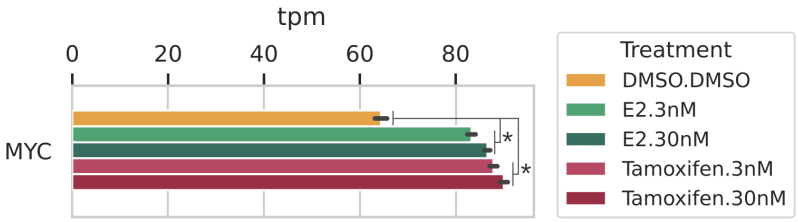


Figure 3—Figure supplement 1. MYC expression in Ishikawa cells. MYC expression (TPM) in Ishikawa cells treated with DMSO, 3nM, and 30nM of estrogen or tamoxifen. Asterisks (*) indicate statistically significant differential expression between treatment groups (p-value < 0.05).

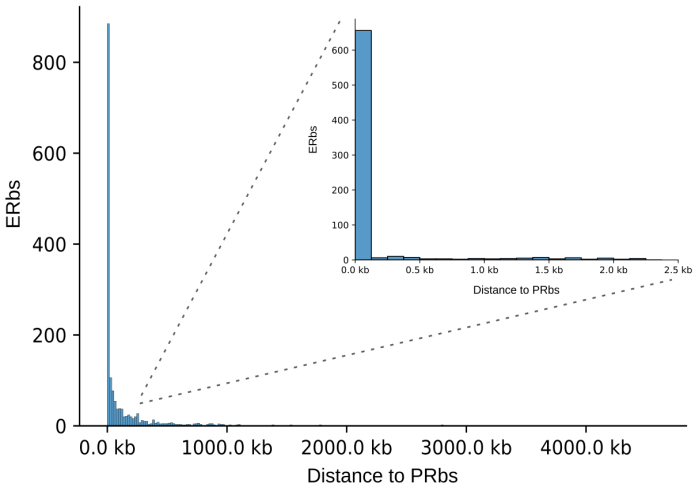


Figure 4—Figure supplement 1. Relationship between ER and PR binding sites in Ishikawa cells. Histogram displaying the distribution of ER binding sites (ER bs) based on their distance to the nearest PR bs in Ishikawa cells. The inset highlights the subset of ER bs located within 2.5 kb of a PR bs.

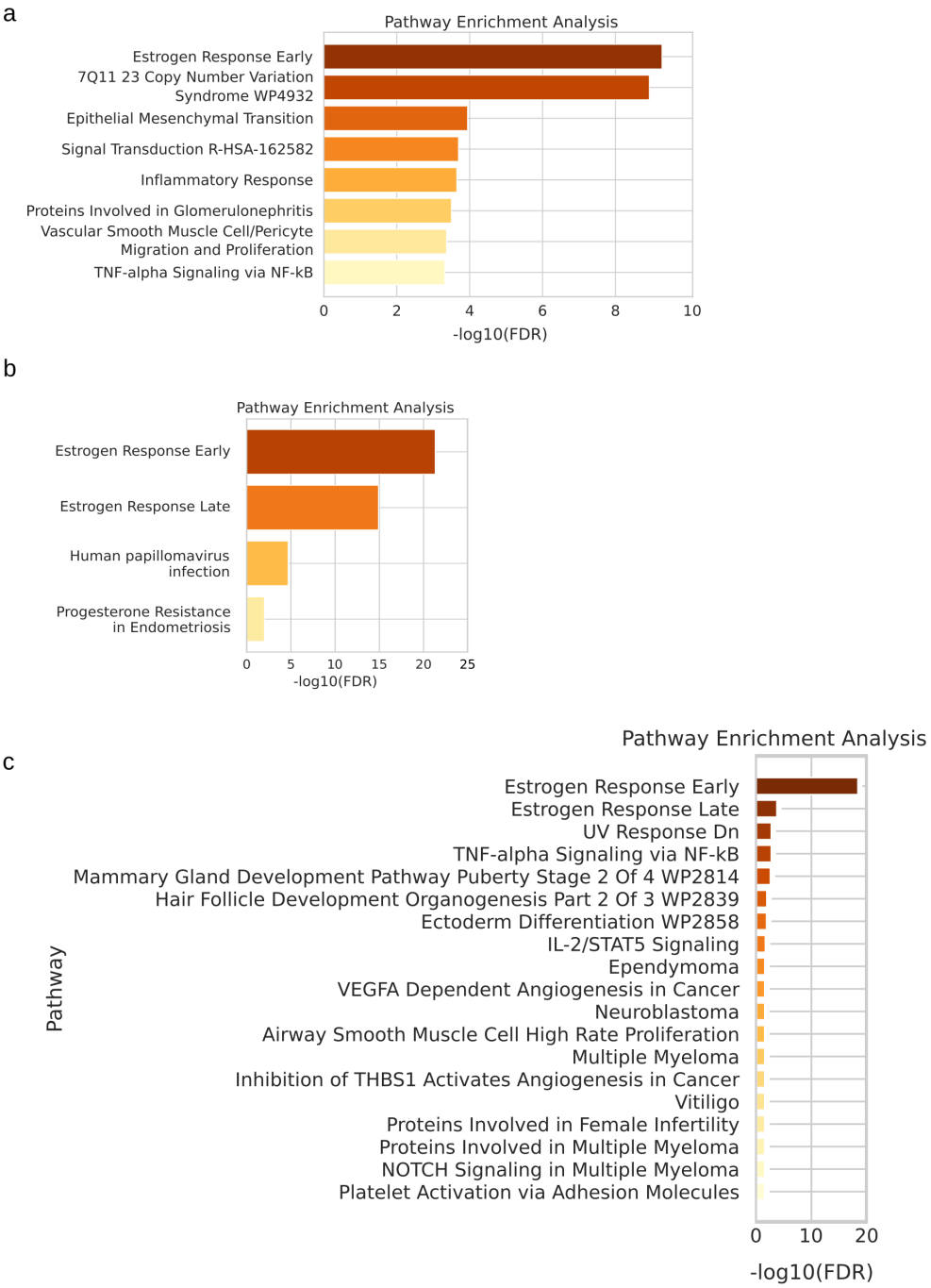


Figure 4—Figure supplement 2. Pathway enrichment associated with ER and PR binding site co-occupancy in Ishikawa cells and tumor samples. a. Top enriched pathways for genes located in TADs containing at least one Ishikawa ER binding site (ER bs) that overlaps with a PR binding site (PR bs) and is also present as an ER bs in either tamoxifen users or non-users. **b.** Top enriched pathways for genes located near Ishikawa ER bs that do not overlap with PR bs but are also present as ER bs in either tamoxifen users or non-users. **c.** Top enriched pathways for genes located near Ishikawa ER bs that overlap with PR bs and are also present as ER bs in either tamoxifen users or non-users.

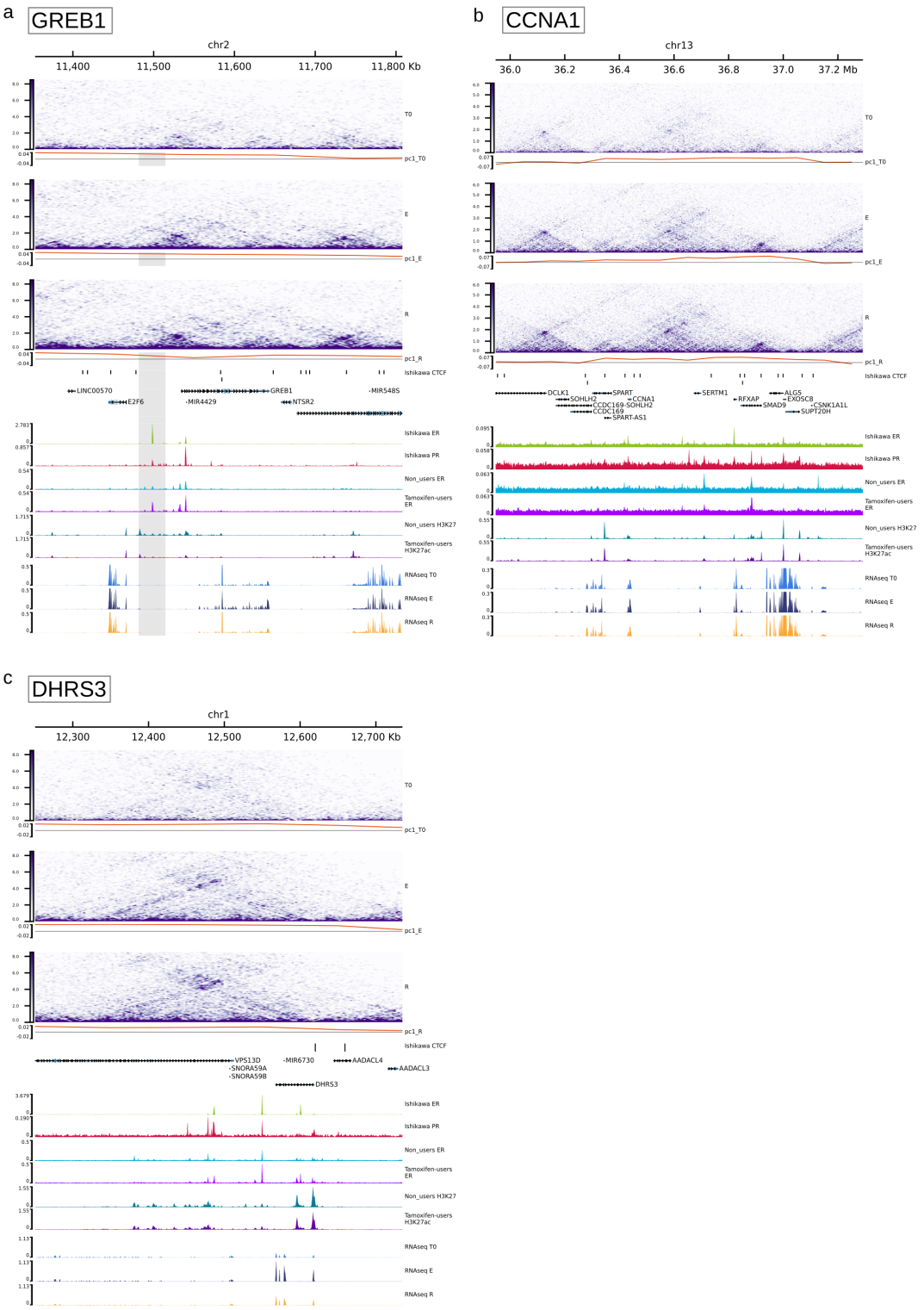


Figure 5—Figure supplement 1. Epigenetic landscape and gene expression of GREB1 (a), CCNA1 (b), and DHRS3 (c), key genes in the Estrogen Early Response pathway. From top to bottom, tracks display: genome scale; contact matrices of Ishikawa cells treated with T0, E2, and R5020, along with their corresponding eigenvalues; ENCODE CTCF binding sites in Ishikawa cells (ENCSR000BQE); UCSC gene annotations; ER and PR ChIP-seq signals from Ishikawa cells; ERα ChIP-seq signals from tamoxifen users and non-users; H3K27ac ChIP-seq signals from tamoxifen users and non-users; and RNA-seq RPKM values for Ishikawa cells of T0, E2, and R5020.