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Comprehensive genomic analysis reveals that the pioneering function of FOXA1 is independent of hormonal signalling

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Abstract:	Considerable work has linked hormone receptors, such as estrogen receptor-alpha (ER), with the pioneer factor FOXA1 in breast cancer. FOXA1 has been shown to function as the link between ER and chromatin at cis-regulatory elements. Altered FOXA1 levels contribute to endocrine-resistant breast cancer, where it maintains ER-chromatin interactions, even in contexts where cells are refractory to ER-targeted drugs. As such, FOXA1 has generated interest as a therapeutic target for endocrine-resistant cancer. A recent study published by Swinstead et al, controversially suggests that FOXA1 binding can be induced by hormonal pathways, including the Estrogen-ER complex. These findings imply that FOXA1 could theoretically be modulated by existing ER antagonists and that the paradigm of the pioneer factor mediating hormonal receptors is not entirely accurate. We now show that the previously observed estrogen-induced FOXA1 binding sites are not technically reproducible, a result of highly variable and insufficient replicates and instead, the vast majority (>99%) of FOXA1 binding events are unaffected by steroid activation. Under antibody-specific experimental conditions, a small number (<1%) of FOXA1 binding sites appear to be induced by estrogen, but these are created from chromatin interactions between ER binding sites and adjacent FOXA1 binding sites within clusters of estrogen-regulated cis-regulatory elements and do not represent genuine new FOXA1-pioneering elements or new cis-regulatory elements. FOXA1 is therefore not regulated by estrogen and remains a bone fide pioneer factor that is entirely upstream of the ER complex.
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Dear Ruth,

Thank you for the pre-acceptance of the paper. We have revised the paper according to the requested suggestions. Specifically, we have now:

- Uploaded a word document for editing
- Shortened the summary to less than 150 words
- Included information about the Lead contact on the front page
- Included the Key Resource table as requested after the STAR methods
- Included high resolution figures
- All supplementary text has been changed as suggested and the reference to the related figure is included
- Declaration of interest form is submitted
- Have now included the highlights and eTOC blurb as a separate document
- Graphical abstract has been changed according to recommendations.

Sincerely

Jason S. Carroll

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Comprehensive genomic analysis reveals that the pioneering function of FOXA1 is independent of hormonal signalling

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Summary

Considerable work has linked hormone receptors, such as estrogen receptor-alpha (ER), with the pioneer factor FOXA1. Altered FOXA1 levels contribute to endocrine-resistant breast cancer, where it maintains ER-chromatin interactions, even in contexts where cells are refractory to ER-targeted drugs. A recent study, controversially suggests that FOXA1 binding can be induced by hormonal pathways, including the Estrogen-ER complex. We now show that the previously observed estrogen-induced FOXA1 binding sites are not technically reproducible and instead, the vast majority (>99%) of FOXA1 binding events are unaffected by steroid activation. A small number (<1%) of FOXA1 binding sites appear to be induced by estrogen, but these are created from chromatin interactions between ER binding sites and adjacent FOXA1 binding sites and do not represent genuine new FOXA1-pioneering elements. FOXA1 is therefore not regulated by estrogen and remains a *bone fide* pioneer factor that is entirely upstream of the ER complex.

Introduction

Whilst the term 'pioneer factor' has been used recently for any transcription factor that can mediate binding of another transcription factor to chromatin, a *bone fide* pioneer can associate with condensed chromatin, independently of other factors, to initiate chromatin opening and creation of a *cis*-regulatory element (Zaret and Carroll, 2011). FOXA1 is the archetypal pioneer factor, capable of binding to compact chromatin independently of other proteins and creating a localized euchromatic environment (Cirillo et al., 2002; Cirillo et al., 1998). It can mediate Estrogen Receptor (ER) binding events in breast cancer cell lines (Carroll et al., 2005; Hurtado et al., 2011; Laganiere et al., 2005), it is required for growth of drug-resistant cancer models (Hurtado et al., 2011), and it has been shown to directly contribute to endocrine resistance (Fu et al., 2016).

FOXA1 has been shown to be important for other nuclear receptors (NR), such as Androgen Receptor (AR) in prostate cancer (Lupien et al., 2008), where elevated levels can contribute to disease outcome (Jain et al., 2011; Robinson et al., 2014). A role for FOXA1 in Castrate-Resistant Prostate Cancer (CRPC) is exemplified by the fact that

models of CRPC, driven by AR splice variants, are still dependent on FOXA1 for cell growth (He et al., 2018; Jones et al., 2015).

FOXA1 binding has been consistently implicated as an event that happens upstream of NR association with *cis*-regulatory elements and experimental data to date, show no change in FOXA1 binding when ER is modulated (Hurtado et al., 2011) and FOXA1 chromatin interaction does not require ER when exogenously expressed (Serandour et al., 2011). The dependence on a single catalytic transcription factor for hormone receptor signalling represents an attractive therapeutic target (Jozwik and Carroll, 2012; Nakshatri and Badve, 2007). Importantly, an inhibitor targeting FOXA1 would circumvent many of the known mechanisms of resistance, including changes in NR fidelity, growth factor activation, changes in the occupancy of co-factors and additional mechanisms that alter the binding potential or ligand dependency of the NR.

The above-mentioned paradigms have recently been challenged, with a study suggesting that FOXA1 binding can be influenced by steroid activation of the cognate NR (Swinstead et al., 2016). This suggests that FOXA1 binding potential can be partly dictated by hormones, including estrogen and glucocorticoids. This questions the concept of transcription factor hierarchies, where specialized transcription factors can function as biological pathway-determining catalysts. We have repeated the key genomic transcription factor mapping experiments that lead to the paradigm-challenging conclusions. We find that the estrogen-induced FOXA1 binding sites, which were described before (Swinstead et al., 2016), result from a lack of robust replicates and are not observable when additional, technically similar, ChIP-seq biological replicates are conducted. Any altered FOXA1 binding sites represent a tiny fraction of the overall FOXA1 binding sites (less than 1%) that result from chromatin loops that occur between *cis*-regulatory elements at estrogen-regulated gene regions, creating shadow binding events that do not represent new *cis*-regulatory elements.

Results

By mapping FOXA1 binding using ChIP-seq in ER+ breast cancer cells, Swinstead *et al* conclude that FOXA1 binding could be substantially altered by hormonal steroid treatment. The primary conclusion that FOXA1 binding was hormonally regulated was based largely on the results from their ChIP-seq experiments. We downloaded their FOXA1 ChIP-seq data conducted in breast cancer cell lines, but could not reproduce

the binding numbers described in the publication, due to insufficient information about peak calling and how input DNA was integrated into the analyses. We used the peak co-ordinates described in Swinstead *et al* and compared read densities of their duplicate libraries mapped to those coordinates using both Principal Component Analysis (PCA) and hierarchical clustering. Their samples did not cluster by treatment condition when assessed using PCA and samples from the same treatment condition showed substantial variability (Figure 1A), suggesting that the replicate samples were not similar. This lack of consistency between duplicates is a potential source of false positive 'differential' binding sites. As expected, differential peak patterns showed little consistency between replicates (Figure 1B) implying that any differential binding sites might be due to technical variability between replicates. Given this replicate-to-replicate variability (even between samples of the same treatment conditions), the lack of any ChIP-qPCR validation and the significant implication of the conclusions, we sought to repeat the key ChIP-seq experiments to determine if FOXA1 binding was in fact modulated by hormonal stimulation, as claimed (Swinstead et al., 2016).

We hormone deprived MCF-7 and ZR-75-1 breast cancer cells and treated with vehicle or estrogen for 45 minutes, a time point shown to result in maximal ER binding and enhancer activity (Shang et al., 2000). ER ChIP-qPCR was conducted at known binding loci (Figure S1, Table S1), in order to confirm the estrogen-response. We subsequently conducted FOXA1 ChIP-seq experiments using two different antibodies, in both cell line models with three biological replicates from independent passages. Importantly, these were collected from matched experiments used to confirm estrogen responsiveness (Figure S1). One of the antibodies used in our study was the same antibody (ab23738) used by Swinstead *et al*. Matched Input samples were included for each experiment. Peaks were called using MACS2 (Ross-Innes et al., 2012; Stark and Brown). In MCF-7 cells, this resulted in 64,823 FOXA1 peaks in vehicle-treated and 62,000 peaks in estrogen-treated conditions using the same antibody as Swinstead et al., and 37,318 vehicle and 35,925 estrogen FOXA1 peaks with the second independent antibody ab5089 (Table S2). PCA analysis of our samples showed that the samples clustered tightly based on replicates (Figure 1C), providing confidence when comparing peaks (Figure 1D). The samples clustered based on the antibody used for ChIP-seq and showed minimal difference between vehicle or estrogen conditions. In

ZR-75-1 cells, the ab23738 antibody generated 70,602 FOXA1 peaks in vehicle conditions and 66,604 peaks in estrogen conditions. The second antibody (ab5089) generated 35,763 FOXA1 peaks in vehicle conditions and 31,361 peaks in estrogen conditions (Table S2). As such, estrogen treatment did not result in a global increase in FOXA1 binding events, with either antibody or in either cell line assessed.

One possibility is that FOXA1 binding could be redistributed, resulting in similar binding numbers, but at different locations in the genome. We therefore performed DiffBind analyses (Ross-Innes et al., 2012) (Table S3) and observed no FOXA1 redistribution. In MCF-7 cells, there were 14 estrogen-induced peaks with the ab23738 antibody and 2 peaks enriched in vehicle conditions, representing 0.02% of all FOXA1 peaks that are estrogen induced (Figure 2A and C). This is in contrast to the results obtained using the exact same antibody and cell line in the Swinstead study, attesting to the potential problems that result from lack of sufficient replicates. The biggest change observed in any of the ChIP-seq experiments we undertook was in MCF-7 cells using the ab5089 FOXA1 antibody (which was not used in the Swinstead study) (Figure 2A-C), which revealed a total of 357 FOXA1 peaks enriched in estrogen conditions (representing less than 1% of all peaks called) and 5 peaks enriched in vehicle conditions (Figure 2B).

To establish the degree of variability in this ChIP-seq experiment conducted with sufficient biological replicates, we purposely mixed up the samples from the ab23738 antibody-based ChIP-seq in different combinations and subsequently called peaks. Following DiffBind analysis, we found between 121 and 180 peaks that were considered differential, even in samples that were randomly mixed up with the incorrect treatment samples, representing ~0.5% of all peaks.

In the ZR-75-1 cell line, we observed 23 estrogen-enriched and 2 vehicle-enriched FOXA1 binding sites using the same FOXA1 antibody used by Swinstead et al., (Figure 2D and F). This small number of estrogen-induced FOXA1 binding sites represents less than 0.03% of all peaks. When using the second FOXA1 antibody (ab5089) in ZR-75-1 cells, we found 109 estrogen-induced FOXA1 binding sites (0.03% of total FOXA1 binding sites) and 1 vehicle-enriched site (Figure 2E).

Our ChIP-seq data with two different FOXA1 antibodies, conducted in two independent cell line models, revealed that 0.02-1% of the FOXA1 binding sites were induced by estrogen. This is in contrast to Swinstead *et al*., which claim that there is an appreciable number of FOXA1 binding events that can be hormonally regulated. Importantly, the same antibody that was used in the Swinstead study revealed no significant changes in FOXA1 binding in either cell line model in our ChIP-seq analysis.

The second FOXA1 antibody (ab5089) that we utilized, produced a small number of estrogen-induced FOXA1 binding sites (357 sites), although it is important to note that these differential binding events constitute less than 1% of total FOXA1 binding events in the ChIP-seq dataset. Only 28 common FOXA1 binding events were identified in both MCF-7 and ZR-75-1 cell lines, implying that these differential sites are not reproducible between different cancer models (Figure 2G).

Further analysis of the estrogen-induced FOXA1 binding sites in MCF-7 and ZR-75-1 revealed the Estrogen Responsive Element (EREs) motif ($p=1x10^{-42}$), but no Forkhead motifs (Figure 2H), suggesting that FOXA1 is not directly interacting with the chromatin at these regions. Based on the motif analysis, we hypothesized that the small number of estrogen-induced FOXA1 binding sites might be indirect FOXA1 binding events, potentially mediated via chromatin loops connecting estrogen-induced genes and their enhancers.

Given the wealth of genomic, transcriptomic and chromatin looping data in the MCF-7 cell line model, we investigated the underlying properties of the 357 estrogen-induced FOXA1 binding sites. We utilized published RNA-seq data following estrogen-treatment of MCF-7 cells (Figure 3A) and observed that the 357 estrogen-induced FOXA1 binding sites were significantly biased towards the most estrogen-regulated genes (Figure 3B) with almost all of the binding sites within *cis*-regulatory domains adjacent to ER target genes.

It is well established that lineage specific genes tend to be regulated by clusters of transcription factor binding sites (Hnisz et al., 2013; Whyte et al., 2013). This is true for estrogen-regulated genes, where the classic estrogen-induced genes (i.e. those with the greatest estrogen response) are regulated by clusters of closely associated *cis*-regulatory domains (Carroll et al., 2006). Several well-characterized ER target genes

are shown in Figure 3C as examples. As typified by the examples shown, there are FOXA1 and ER co-bound regions, but importantly, there are sites where one transcription factor binds but the other one does not. The 357 estrogen-induced FOXA1 binding sites are all adjacent to an independent ER binding event and other FOXA1 binding sites (Figure 3D and 3E), indicating their presence in regions of enriched transcription factor binding.

Following estrogen-mediated stimulation, physical associations between *cis*-regulatory elements occur (Fullwood et al., 2009; Pan et al., 2008) and we postulated that FOXA1 could associate with adjacent ER binding sites through chromatin looping. Due to the cross-linking in the ChIP-seq protocol, these indirect chromatin loops create FOXA1 binding sites that are not direct *cis*-regulatory elements and therefore represent 'shadow peaks'. At these regions, FOXA1 does not function as a pioneer factor and new regulatory elements are not created. Our hypothesis is that the small fraction (<1%) of FOXA1 binding events that appear to be induced by estrogen, are in fact simply indirect peaks mediated via ER at those genomic regions. To assess this possibility, we utilized the ChIA-PET data that provides an unbiased map of the ER-mediated chromatin interactions that occur, in the presence of estrogen, in MCF-7 cells (Fullwood et al., 2009). Of the 357 estrogen induced FOXA1 peaks in MCF-7 cells, 89% of these were detected in experimentally identified ER ChIA-PET chromatin loops (Figure 4A). Examples of estrogen-induced FOXA1 binding sites existing within ChIA-PET chromatin loops, are shown in Figure 4B. This finding confirms that the limited number of estrogen-induced FOXA1 binding events are in fact created by clusters of *cis*-regulatory elements brought into proximity during gene expression. Therefore, FOXA1 is a *bone fide* pioneer factor that binds upstream of nuclear receptors and direct FOXA1-chromatin binding is not influenced by steroid hormones.

Discussion

It is well established that many nuclear receptors and other transcription factors regulate genes from significant distances (Carroll et al., 2005) (Lin et al., 2007). However, additional factors are required for NR to work (Glass and Rosenfeld, 2000; Shang et al., 2000). Recent observations have revealed that cells containing mutations in ER (ESR1) can be enriched due to selective pressure imposed by specific ER targeted drugs (Merenbakh-Lamin et al., 2013) (Robinson et al., 2013) (Toy et al., 2013), resulting in

ligand-independent ER activity. As such, there is a significant interest in defining critical components of the ER complex that might constitute potential drug targets. One such protein is FOXA1, a pioneer factor, shown to facilitate chromatin 'opening' independently of additional proteins, enabling binding and activity of other transcription factors. Importantly, this includes ER in breast cancer and Androgen Receptor (AR) in prostate cancer. Whilst additional modes of nuclear receptor binding can occur, such as assisted loading, involving complexes of multiple ATP-dependent chromatin factors (Voss et al., 2011), an absolute dependence on a single functionally catalytic protein, such as FOXA1, holds promise for therapeutic exploitation.

FOXA1 has been shown to be required for growth of resistant cancers (Hurtado et al., 2011), it contributes to endocrine resistance (Fu et al., 2016), and importantly, it is essential for ER binding and activity, even in endocrine resistant contexts (Hurtado et al., 2011). This places FOXA1 as a key driver of resistance and reveals a vulnerability in the ER pathway, where absolute dependence on a single upstream pioneer factor, creates an opportunity for therapeutic intervention, potentially overcoming known mechanisms of resistance. Interest in FOXA1 as a therapeutic target for ER+ breast cancer (Jozwik and Carroll, 2012; Nakshatri and Badve, 2007, 2009) was compromised by recent claims that FOXA1 binding is estrogen regulated (Swinstead et al., 2016). The significance of this conclusion means that ER targeted agents should, in theory, show effectiveness in inhibiting FOXA1 binding and transcriptional potential, reducing the need for developing direct FOXA1 inhibitors. Our comprehensive analysis of FOXA1 binding following estrogen stimulation reveals no appreciable estrogen regulation of FOXA1 binding. Different antibodies and different ER+ breast cancer cell line models show that >99% of FOXA1 binding sites are impervious to hormonal context. The residual FOXA1 changes represent less than 1% of FOXA1 binding events and result from peaks formed within clusters of ER/FOXA1 binding sites at genes that are estrogen regulated. As such, these lack the hallmarks of genuine FOXA1 binding sites, they do not result in the creation of new regulatory elements and they do not result in new gene expression events. The lack of robust, reproducible FOXA1 binding sites confirms that FOXA1 binding is not estrogen regulated and functions upstream of ER activity. In support to this conclusion, previous experimental data showed that the breast cancer treatment, Fulvestrant (ICI 182780), an ER degrader, does not alter FOXA1 binding (Hurtado et al., 2011).

The major distinction in conclusions between the Swinstead *et al* work and the current dataset, result from technical differences that can be attributed to insufficient replicates in the previous study (Figure 1). A lack of biological and/or technical replicates are a source of problems in the reproducibility of ChIP-seq datasets, particularly when claiming treatment or condition-specific binding events. We conclude that recent claims of estrogen-mediated FOXA1 binding events are influenced, in large part, by a lack of independent biological ChIP-seq replicates and duplicate samples that show unacceptable variability between purportedly replicate samples (Figure 1A and 1B).

Swinstead et al., identified similar steroid hormone changes in FOXA1 binding in two distinct systems, namely estrogen responsiveness and dexamethasone-activation of GR (Swinstead et al., 2016). Whilst we have only focused on the estrogen-treated conditions, it is reasonable to assume that the majority of dex-mediated changes in FOXA1 are also false positives that result from a lack of independent biological replicates. This is based on the fact that the experimental approach was comparable and the same degree of differential FOXA1 binding was observed in both hormonal systems. The conclusion that steroids could change FOXA1 binding was suggested in large part by ChIP-seq analyses. In addition to these assays, Swinstead *et al*, also assessed FOXA1 chromatin dwell time using an exogenous, tagged-FOXA1 based approach (Swinstead et al., 2016). Despite the caveat that exogenous FOXA1 alters levels and potentially the function of endogenous FOXA1 and the tagged protein might not faithfully recapitulate endogenous FOXA1, there was a minimal change in FOXA1 dwell time comparing the presence or absence of estrogen, suggesting that this non-ChIP-based method supports the conclusion that FOXA1 binding is not altered in an appreciable way by hormone status.

Understanding what enables FOXA1 binding is of importance and recent suggestions that steroid hormones could function in this capacity to modulate FOXA1-DNA binding potential (Swinstead et al., 2016) present an attractive hypothesis. We show that the vast majority (>99%) of FOXA1 binding is not regulated by estrogen and the small fraction of altered FOXA1 binding events are created via chromatin interactions during the course of estrogen-receptor mediated gene expression. FOXA1 therefore exists entirely upstream of the nuclear receptor, its chromatin binding capacity is not

influenced by estrogen signalling and it remains a relevant and important drug target in hormone-dependent cancers.

Author Contributions

Experiments were designed by S-E.G and J.S.C and all work was conducted by S-E.G. All bioinformatic work was conducted by I.C. All authors wrote the paper.

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Conflict of interest

Jason Carroll is the Founder and CSO of Azeria Therapeutics.

Figure legends

Figure 1: PCA analysis and unbiased clustering of the different ChIP-seq datasets. Read densities from aligned libraries of equal size of 20 million reads were measured on corresponding FOXA1 binding sites from Swinstead *et al* (GEO identifier: GSE72249). **A.** The peaks for all treatments were merged in a single set prior to the measurement for each study and obtained data was subjected to PCA. The PCA plots illustrate degree of similarity between the replicates. **B.** Hierarchical clustering of the Swinstead *et al* binding sites. For hierarchical clustering of the Swinstead *et al* binding sites, the yielded read densities were normalized using median absolute deviation and clustered in MATLAB framework using "ward" method with the linkage function. The duplicate samples from Swinstead *et al*, did not cluster based on treatment condition. **C.** PCA analysis of our FOXA1 ChIP-seq generated with two different FOXA1 antibodies (ab23738 and ab5089). **D**. Hierarchical clustering of our FOXA1 binding sites, showing clustering based on replicates.

Figure 2: Analysis of FOXA1 ChIP-seq binding with two separate antibodies in response to estrogen treatment in MCF-7 and ZR-75-1 cells. ChIP-seq tag densities visualized at FOXA1-occupied genomic locations in control and estrogen-treated MCF-7 (**A** and **C**) and ZR-75-1 (**D** and **F**) cells, using antibodies ab23738 and ab5089. Zoomed heatmap show differential binding of FOXA1 specific to ab5089 in MCF-7 cells (**B**) and ZR-75-1 (**E**), respectively; **G.** Overlap of estrogen-enriched FOXA1 binding sites between MCF-7 and ZR-75-1 cells; **H.** Transcription factor motifs found overrepresented in the common and estrogen induced FOXA1 sites.

Figure 3: Integration of the estrogen-enriched FOXA1 binding events with estrogen-mediated gene expression events. A. RNA-seq expression profile following short-term (3hr) estrogen treatment of MCF-7, shown as a dispersion plot. **B**. GSEA Pre-ranked test correlating estrogen-induced genes with the 357 estrogen-induced FOXA1 binding sites. **C.** Examples of sites co-bound by FOXA1 and ER, as well as sites unique to each of the two transcription factors. Proximity of estrogen-induced FOXA1 peaks and the closest ER (**D**) or FOXA1 (**E**) site. Heat map represents FOXA1-gained sites in red.

Figure 4: **ER binding mediates indirect FOXA1 binding via chromatin looping at *cis*-regulatory elements. A.** Correlation between ER-mediated chromatin interactions (ChIA-PET) and the 357 estrogen-induced FOXA1 binding sites (ab5089). The table shows the correlation values between ChIA-PET interactions and the 357 estrogen-induced FOXA1 binding sites. **B.** Examples of ER and FOXA1 peaks at regions that are involved in chromatin loops, as detected by ChIA-PET. The images of the ChIA-PET loops are taken from (Fullwood et al., 2009).

STAR methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be direct to and will be fulfilled by the lead contact, Jason Carroll (Jason.carroll@cruk.cam.ac.uk)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture

MCF-7 and ZR-75-1 cell lines were obtained from ATCC (Middlesex, UK) and represent female breast cancer cell line models. MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium DMEM (Gibco, Thermo Scientific, Leicestershire, UK, ref. 41966). ZR-75-1 cells were grown in RPMI-1640 medium (Gibco, Thermo Scientific, Leicestershire, UK, ref. 21875-034). Both media were supplemented with foetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine.

MCF-7 and ZR-75-1 cells were seeded and treated either with ethanol or with 10nM Estrogen (Sigma) for 45 minutes previously described (Schmidt et al., 2009). All cell lines were regularly genotyped to ensure they were the correct cell lines.

METHOD DETAILS

Chromatin Immunoprecipitation

To validate the Estrogen induction, ER ChIP-qPCR was performed using the rabbit polyclonal sc-543 (Santa Cruz) antibody. FOXA1 ChIP-seq was performed using the goat polyclonal ab5089 (Abcam), and rabbit polyclonal ab23738 (Abcam) antibodies. Chromatin was prepared as previously described (Schmidt et al., 2009). DNA was isolated and purified using the phenol-chloroform-isoamyl DNA extraction method. ChIP-seq and the input libraries were prepared using the ThruPlex® DNA-seq kit (Rubicon Genomics, ref. R400407).

Integration of RNA-seq and ChIP-seq data

Genes located around +/- 50kb from the peak regions were selected. –log10 transformed p-values from DESeq2 analyses of the RNA-Seq data were subsequently

used for ranking and weighting of genes. GSEAPreranked (18) analysis tool from Gene Set Enrichment Analysis (GSEA) software, version 2.2.3, was used for the evaluation of statistically significant genes.

QUANTIFICATION AND STATISTICAL ANALYSIS

ChIP Sequencing Analysis

ER ChIP-qPCR and FOXA1 ChIP-seq were performed in biological triplicates, using cells from independent passages.

ChIP-seq reads were mapped to hg38 genome using bowtie2 2.2.6 (Langmead and Salzberg, 2012). Aligned reads with the mapping quality less than 5 were filtered out. The read alignments from three replicates were combined into a single library and peaks were called using MACS2 version 2.0.10.20131216 (Zhang et al., 2008) with sequences from MCF7 chromatin extracts as a background input control. The peaks yielded with MACS2 q value <= 1e-3 were selected for downstream analysis. MEME tool FIMO version 4.9.1 (Bailey et al., 2009) was used for searching all known TF motifs from JASPAR database (JASPAR CORE 2016 vertebrates) in the tag-enriched sequences. As a background control, peak size - matching sequences corresponding to known open chromatin regions in MCF7 cells were randomly selected from hg38. Motif frequency for both tag-enriched and control sequences calculated as sum of motif occurrences adjusted with MEM q-value. Motif enrichment analysis was performed by calculating odds of finding an overrepresented motif among MACS2-defined peaks by fitting Student's t-cumulative distribution to the ratios of motif frequencies between tag-enriched and background sequences. Yielded p-values were further adjusted using Benjamini-Hochberg correction.

For visualizing tag density and signal distribution, heatmaps were generated with the read coverage in a window of +/- 2.5 or 5 kb region flanking the tag midpoint using the bin size of 1/100 of the window length. Differential binding analysis (Diffbind) was performed as described previously (Stark and Brown).

DATA AND SOFTWARE AVAILABILITY

All ChIP-seq data is deposited in GEO under the accession number: GSE112969. Data can be accessed using the password: gzmtegactlqtxwp

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Anti- ERα (HC-20) polyclonal antibody	Santa Cruz	Cat# sc-543, RRID; AB_631471
Goat Anti-FOXA1 polyclonal antibody – ChIP grade	Abcam	Cat# ab5089, RRID; AB_304744
Rabbit Anti-FOXA1 polyclonal antibody – ChIP grade	Abcam	Cat# ab23738, RRID; AB_2104842
Bacterial and Virus Strains		
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Dynabeads® Protein A	Invitrogen	Cat#10001D
Dynabeads® Protein G	Invitrogen	Cat#10003D
Pierce™ 16% Formaldehyde (w/v), Methanol-free	Thermo Scientific	Cat# 28908
β-Estradiol	Sigma-Aldrich	Cat# E8875
Dulbecco's Modified Eagle Medium (DMEM)	GIBCO	Cat# 41966029
RPMI 1640 Medium	GIBCO	Cat# 21875034
Fetal Bovine Serum, qualified, heat inactivated	GIBCO	Cat# 16140071
Fetal Bovine Serum, charcoal stripped	GIBCO	Cat# 12676029
Penicillin-Streptomycin	GIBCO	Cat#15070063
L-Glutamine (200 mM)	GIBCO	Cat# 25030081
Trypsin-EDTA (0.5%), no phenol red	GIBCO	Cat# 15400054
cOmplete EDTA-free Protease inhibitor cocktail	Sigma-Aldrich	Cat# 05056489 001
Phosphatase Inhibitor cocktail	Thermo Scientific	Cat#78427
Critical Commercial Assays		
ThruPlex® DNA-seq kit	Rubicon Genomics	Cat# R400407
Deposited Data		
Gene Expression Omnibus (GEO)	https://www.ncbi.nlm.nih.gov/geo/	GSE112969; RRID:SCR_005012
Experimental Models: Cell Lines		
MCF-7	ATCC	Cat# HTB-22, RRID:CVCL_0031; ATCC HTB-22
ZR-75-1	ATCC	Cat# CRL-1500, RRID:CVCL_0588; ATCC CRL-1500
Experimental Models: Organisms/Strains		
Oligonucleotides		
Primer for ChIP Forward: ER3 negative site (5'-GCCACCAGCCTGCTTTCTGT-3')	This study	n/a

Primer for ChIP Reverse: ER3 negative site (5'-CGTGGATGGGTCCGAGAAAC-3')	This study	n/a
Primer for ChIP Forward: XBP1 negative site (5'-ACCCTCCAAAATTCTTCTGC-3')	This study	n/a
Primer for ChIP Reverse: XBP1 negative site (5'-ATGAGCATCTGAGAGCAAGC-3')	This study	n/a
Primer for ChIP Forward: XBP1 target site (5'-ATACTTGGCAGCCTGTGACC-3')	This study	n/a
Primer for ChIP Reverse: XBP1 target site (5'-GGTCCACAAAGCAGGAAAAA-3')	This study	n/a
Primer for ChIP Forward: GREB1 target site (5'-GAAGGGCAGAGCTGATAACG-3')	This study	n/a
Primer for ChIP Reverse: GREB1 target site (5'-GACCCAGTTGCCACACTTTT-3')	This study	n/a
Primer for ChIP Forward: MYC target site (5'-GCTCTGGGCACACACATTGG-3')	This study	n/a
Primer for ChIP Reverse: MYC target site (5'-GGCTCACCCTTGCTGATGCT-3')	This study	n/a
Recombinant DNA		
Software and Algorithms		
Bowtie 2 v2.2.6	Langmead and Salzberg, 2012	https://sourceforge.net/projects/bowtie-bio/files/bowtie2/2.2.6/ ; RRID:SCR_016368
MEME tool FIMO v4.9.1	Bailey et al., 2009	http://meme-suite.org/doc/install.html?man_type=web ; RRID:SCR_001783
JASPAR CORE 2016 vertebrates	JASPAR	http://jaspar.genereg.net/matrix-clusters/vertebrates/ ; RRID:SCR_003030
MACS2 version 2.0.10.20131216	Zhang et al., 2008	https://pypi.org/project/MACS2/2.0.10.20131216/ ; RRID:SCR_013291
GSEAPreranked (18) analysis tool Gene Set Enrichment Analysis (GSEA) v2.2.3	Broad Institute, Inc., Massachusetts Institute of Technology	http://www.broadinstitute.org/gsea/ ; RRID:SCR_003199
Diffbind	Stark and Brown	https://bioconductor.org/packages/release/bioc/html/DiffBind.html ; RRID:SCR_012918

DESeq2	Love et al., 2014	https://bioconductor.org/packages/release/bioc/html/DESeq2.html ; RRID:SCR_015687
Other		
Bioruptor Plus sonicator	Diagenode	NA

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Figure 1

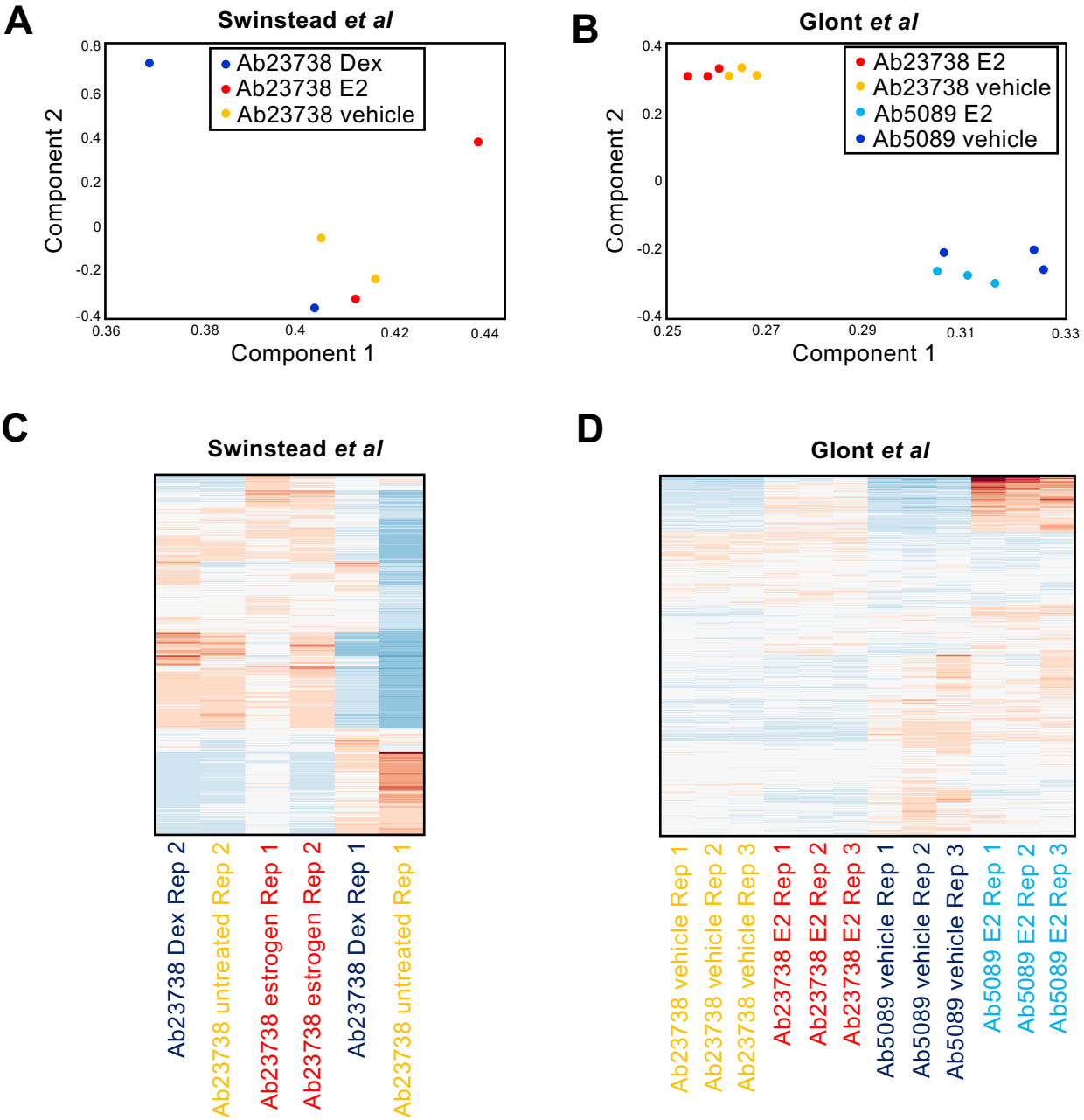
A
Swinstead et al
Ab23738 Dex
Ab23738 E2
Ab23738 vehicle
Component 2
Component 1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
0.36
0.38
0.4
0.42
0.44
B
Glont et al
Ab23738 E2
Ab23738 vehicle
Ab5089 E2
Ab5089 vehicle
Component 2
Component 1
0.4
0.2
0
-0.2
-0.4
0.25
0.27
0.29
0.31
0.33
C
Swinstead et al
Ab23738 Dex Rep 2
Ab23738 untreated Rep 2
Ab23738 estrogen Rep 1
Ab23738 estrogen Rep 2
Ab23738 Dex Rep 1
Ab23738 untreated Rep 1
D
Glont et al
Ab23738 vehicle Rep 1
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Figure 2

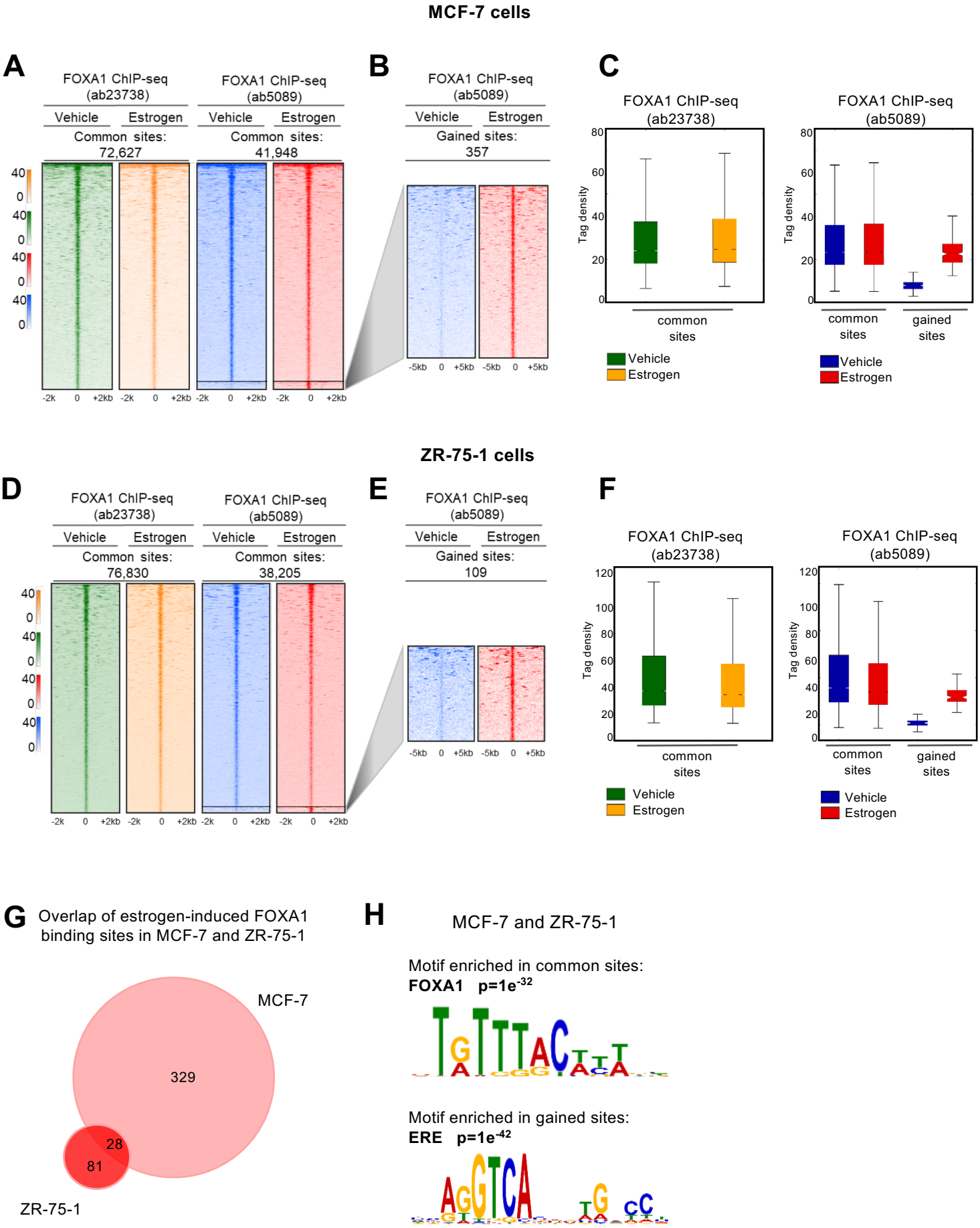
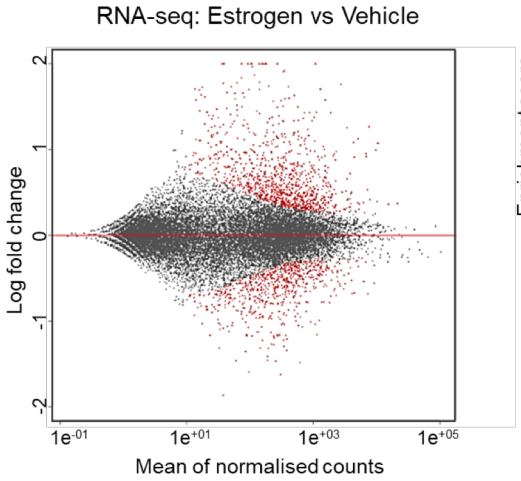
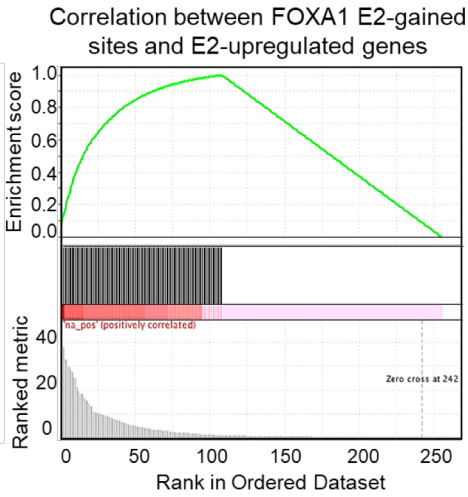

MCF-7 cells
A
FOXA1 ChIP-seq (ab23738)
Vehicle
Estrogen
Common sites: 72,627
FOXA1 ChIP-seq (ab5089)
Vehicle
Estrogen
Common sites: 41,948
B
FOXA1 ChIP-seq (ab5089)
Vehicle
Estrogen
Gained sites: 357
C
FOXA1 ChIP-seq (ab23738)
Tag density
common sites
Vehicle
Estrogen
FOXA1 ChIP-seq (ab5089)
Tag density
common sites
gained sites
Vehicle
Estrogen
ZR-75-1 cells
D
FOXA1 ChIP-seq (ab23738)
Vehicle
Estrogen
Common sites: 76,830
FOXA1 ChIP-seq (ab5089)
Vehicle
Estrogen
Common sites: 38,205
E
FOXA1 ChIP-seq (ab5089)
Vehicle
Estrogen
Gained sites: 109
F
FOXA1 ChIP-seq (ab23738)
Tag density
common sites
Vehicle
Estrogen
FOXA1 ChIP-seq (ab5089)
Tag density
common sites
gained sites
Vehicle
Estrogen
G
Overlap of estrogen-induced FOXA1 binding sites in MCF-7 and ZR-75-1
MCF-7
329
28
81
ZR-75-1
H
MCF-7 and ZR-75-1
Motif enriched in common sites:
FOXA1 p=1e-32
Motif enriched in gained sites:
ERE p=1e-42

Figure 3

A



B



FOXA1 ChIP-seq: E2 gained sites

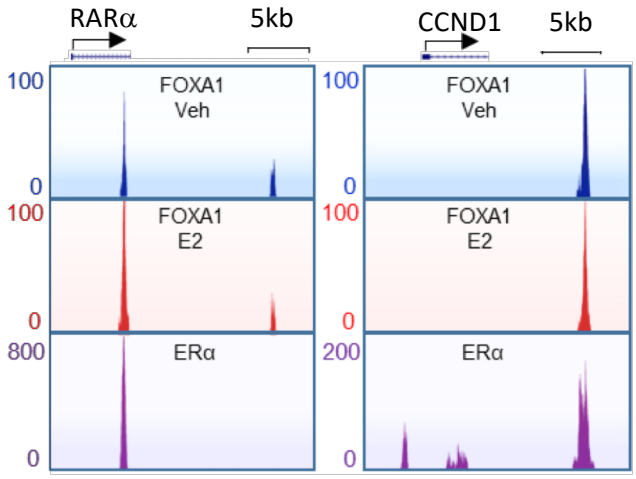
RNA-seq: E2 upregulated genes

E2 induced genes: Ranking metric score

GSEA Results Summary	
Upregulated genes vs FOXA1 gained sites	Positively correlated
Enrichment Score (ES)	1
Nominal p value	<0.001
FDR q value	<0.001

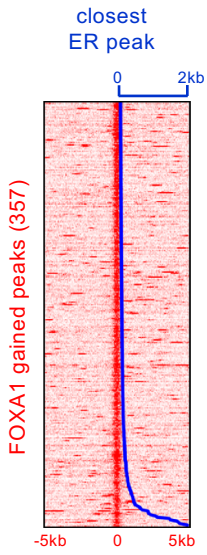
C

Examples of sites co-bound by FOXA1 and ER or specific to each of the two factors



Proximity of E2- induced FOXA1 peaks to the closest ER and FOXA1 site

D



E

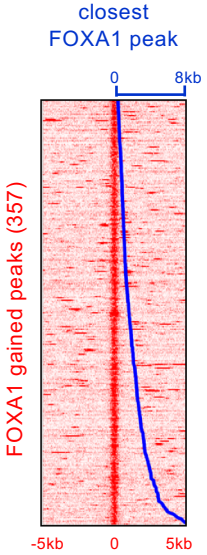
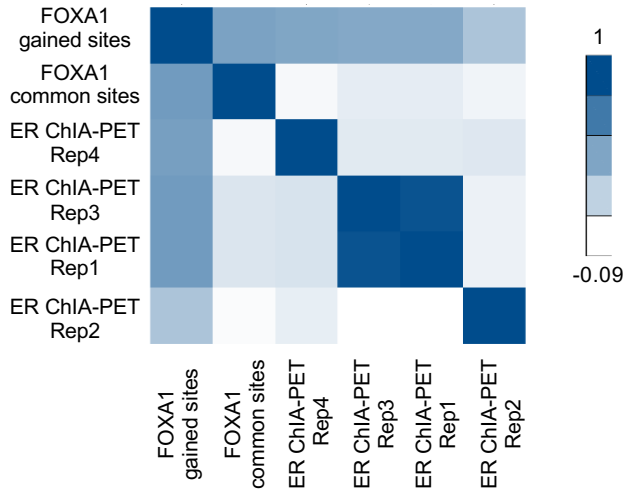


Figure 4

A

Correlation between ER-mediated chromatin interactions and E2- induced FOXA1 sites



ChIA-PET Results Summary		
sample	**no. sites**	**no. of sites overlapped with FOXA1 gained peaks**
ER ChIA-PET Rep1	14393	319
ER ChIA-PET Rep2 (technical rep)	14393	319
ER ChIA-PET Rep3	2710	200
ER ChIA-PET Rep4	6671	257

B

Examples of peaks and their proximity to ER-mediated chromatin loops

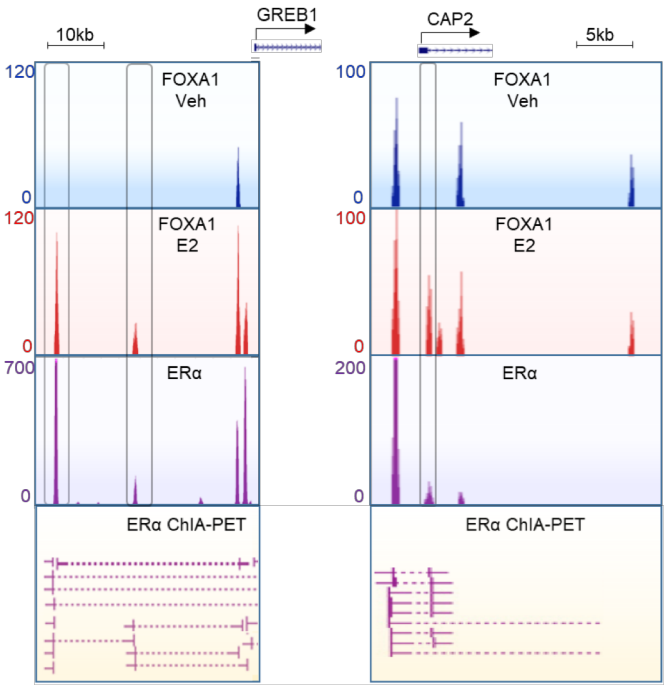


Figure S1: Related to Figure 1. Validation of estrogen activity in MCF-7 (A) and ZR-75-1 (B) cells. ER ChIP-qPCR was conducted in biological triplicates and these samples were matched with the FOXA1 ChIP-seq used for the study. The data is related to the samples used in Figure 1.

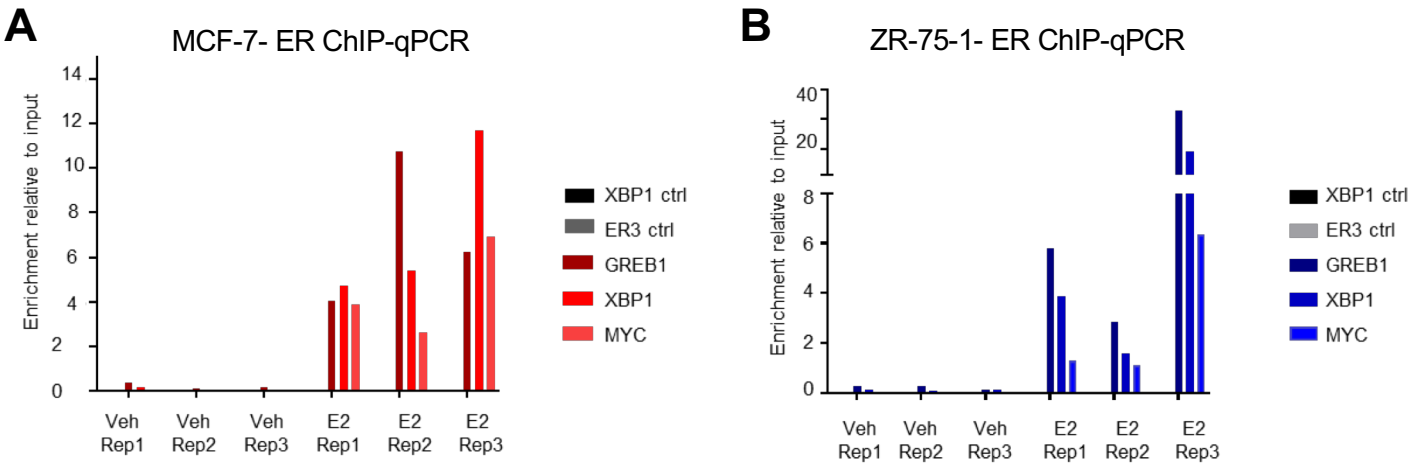


Table S1: Related to data used in Supplemetary Figure 1 and Figure 1. Primer sets used for ER ChIP-qPCR. Two control regions and three previously validated ER target regions were included.

Gene name	Forward primer	Reverse primer
ER3 negative site	GCCACCAGCCTGCTTTCTGT	CGTGGATGGGTCCGAGAAAC
XBP1 negative site	ACCCTCCAAAATTCTTCTGC	ATGAGCATCTGAGAGCAAGC
XBP1 target site	ATACTTGGCAGCCTGTGACC	GGTCCACAAAGCAGGAAAAA
GREB1 target site	GAAGGGCAGAGCTGATAACG	GACCCAGTTGCCACACTTTT
MYC target site	GCTCTGGGCACACACATTGG	GGCTCACCCTTGCTGATGCT

Table S2: Related to data in Figure 1. Total number of peaks called in each condition.

Cell line	Antibody	Condition	Total no. of peaks
MCF-7	ab23738	Vehicle	64823
		Estrogen	62000
	ab5089	Vehicle	37318
		Estrogen	35925
ZR-75-1	ab23738	Vehicle	70602
		Estrogen	66604
	ab5089	Vehicle	35763
		Estrogen	31361

Table S3: Related to Figure 2. Differential binding comparison between vehicle and estrogen treatment.

Cell line	Antibody	Conditions	Differential binding comparison		
			gained	lost	common
MCF-7	ab23738	Estrogen vs Vehicle	14	2	72627
	ab5089	Estrogen vs Vehicle	357	5	41948
ZR-75-1	ab23738	Estrogen vs Vehicle	23	2	76830
	ab5089	Estrogen vs Vehicle	109	1	38205