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Epigenetic Basis for the Regulation of Estrogen Receptor Alpha Activity in Breast Cancer Cells

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14. ABSTRACT

A key factor involved in breast cancer development and progression is the estrogen receptor alpha (ER). Genome-wide computational studies on ER have identified over 70,000 putative Estrogen Response Elements (EREs) in the human genome. However, a genome-wide functional study using ChIP-Chip, has indicated that less than 1/10 of all putative ER binding sites are recognized by the receptor following estrogen stimulation in breast cancer cells. Through genome-wide positional analyses, we demonstrate that ER recruitment is dependent on a specific epigenetic signature characterized by mono and dimethylation of lysine 4 on histone 3 (H3K4me1/me2). Furthermore the pioneer factor FoxA1 translates this epigenetic signature into changes in chromatin structure in a cell type-specific manner for transcription factors, such as ER. Hence, this allows for the establishment of lineage-specific transcriptional enhancers and programs.

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A key factor involved in breast cancer development and progression is the estrogen receptor alpha (ER). Genome-wide computational studies on ER have identified over 70,000 putative Estrogen Response Elements (EREs) in the human genome. However, a genome-wide functional study using ChIP-Chip, has indicated that less than 1/10 of all putative ER binding sites are recognized by the receptor following estrogen stimulation in breast cancer cells. These sites are predominantly distant from promoters but lie in close proximity to genes actively transcribed following estrogen (E2) stimulation.

Chromatin condensation state directly impacts gene expression level. In accordance, specific histone modifications, such as trimethylation of histone H3 on lysine 4 (H3K4me3), are present at the promoter of actively expressed genes. In addition, monomethylation of this same residue (H3K4me1) appears to define putative enhancer regions. Whether epigenetic modifications, such as H3K4me1 or me3, can determine functional binding sites for ER and therefore restrict its activity to a small subset of its putative binding sites in breast cancer cells remains ill defined.

Body……..

Aim1) Screen multiple epigenetic modifications for their specific occurrence on functional estrogen receptor alpha (ER) prior to its recruitment.

This was accomplished and identified the presence of the mono and dimethylation of lysine 4 on histone 3 (H3K4me1/me2) on functional ER binding sites (Lupien et al., 2008). The dimethylation of lysine 9 on histone 3 (H3K9me2) was found enriched on non-functional regulatory elements containing the estrogen responsive element (ERE). We extended our research to demonstrate that this association between ER binding and the presence of H3K4me1/me2 also applied to other transcription factors, namely FoxA1 and AP1 (Lupien et al., 2008). Furthermore, we demonstrated that this epigenetic signature defines the cell type-specific recruitment pattern of transcription factors (Lupien et al., 2008).

Aim2) Test the requirement for the epigenetic modifications identified under the first objective.

This was accomplished by over-expressing the lysine demethylase KDM1 (LSD1) which lead to a significant reduction in H3K4 methylation state. In doing so, we revealed the requirement for the methylation of H3K4 at distant regulatory elements for transcription factor recruitment (Lupien et al., 2008).

Aim3) Does ER over-expression alter the distribution of the epigenetic signature.

We have demonstrated that ER activation leads to the induction of specific epigenetic modifications, such as dimethylation of arginine 17 on histone 3 (H3R17me2), acetylation of lysine 18 on histone 3 (H3K18ac) and of lysine 12 on histone 4 (H4K12ac), on a limited number of ER binding site across the genome of breast cancer cells (Lupien et al., 2009). More importantly, only ER binding sites undergoing epigenetic changes following ER binding associate with regulation of gene expression (Lupien et al., 2009). This suggest that antagonizing these ER sites alone should be sufficient to block breast cancer growth

We also published a review article presenting all of these results in context with the field of breast and prostate cancer (Lupien and Brown, 2009).

Key Research Accomplishments……..……..

We identified the epigenetic signature that defines functional and non-functional regulatory elements

We have demonstrated for the first time the cell-type specific distribution of this epigenetic signature

We have demonstrated that this epigenetic signature is required for transcription factor recruitment to the genome

We have identified a specific novel therapeutic target for breast cancer as removal of the H3K4me1/me2 epigenetic signature can block transcription factor recruitment, namely ER.

We identified the genome wide distribution of the dimethylation of arginine 17 on histone 3 (H3R17me2) epigenetic modification following ER activation

We identified that a subset of ER binding sites associate with changes in epigenetic modifications such as dimethylation of arginine 17 on histone 3, acetylation of lysine 18 on histone 3 and of lysine 12 on histone 4 (H3R17me2, H3K18ac and H4K12ac, respectively).

We revealed that only ER sites driving the estrogenic response associate with these epigenetic modifications following activation.

Reportable Outcomes……

Mono and dimethylation of lysine 4 on histone 3 (H3K4me1 and me2) is a novel therapeutic target against breast cancer as this epigenetic signature defines the functional regulatory elements driving transcription in breast cancers (Lupien et al., 2008).

ER coativation and associated epigenetic remodeling (H3R17me2, H3K18ac, H4K12ac) selectively occurs on a restricted subset of its binding sites that drive the estrogenic response (Lupien et al., 2009).

Conclusion……

Methylation of histone H3 lysine 4 is part of the epigenetic signature that defines lineage-specific recruitment sites in chromatin for transcription factors such as ER. Furthermore, the pioneer factor FoxA1 translates this epigenetic signature into changes in chromatin structure thereby establishing lineage-specific transcriptional enhancers and programs.

Transcriptional response to E2 in breast cancer cells is dependent on the subset of the ERα cistrome associated with coactivation and epigenetic remodeling.

References…….

Lupien, M., and Brown, M. (2009). Cistrom ics of horm one-dependent cancer. Endocr Relat Cancer.

Lupien, M., Eeckhoute, J., Meyer, C.A., Krum , S.A., Rhodes, D.R., Liu, X.S., and Brown, M. (2009). Coactiv ator F unction Defines the Active Estrogen Receptor-{alpha} Cistrom e. Molecular and cellular biology *29*, 3413-3423.

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Appendices……

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Cell

FoxA1 Translates Epigenetic Signatures into Enhancer-Driven Lineage-Specific Transcription

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SUMMARY

Complex organisms require tissue-specific transcriptional programs, yet little is known about how these are established. The transcription factor FoxA1 is thought to contribute to gene regulation through its ability to act as a pioneer factor binding to nucleosomal DNA. Through genome-wide positional analyses, we demonstrate that FoxA1 cell type-specific functions rely primarily on differential recruitment to chromatin predominantly at distant enhancers rather than proximal promoters. This differential recruitment leads to cell type-specific changes in chromatin structure and functional collaboration with lineage-specific transcription factors. Despite the ability of FoxA1 to bind nucleosomes, its differential binding to chromatin sites is dependent on the distribution of histone H3 lysine 4 dimethylation. Together, our results suggest that methylation of histone H3 lysine 4 is part of the epigenetic signature that defines lineage-specific FoxA1 recruitment sites in chromatin. FoxA1 translates this epigenetic signature into changes in chromatin structure thereby establishing lineage-specific transcriptional enhancers and programs.

INTRODUCTION

Over the course of development, cells transit from a pluripotent state to one of many committed cell lineages. During this process, transcription factor networks are activated in order to establish cell type-specific transcriptional programs (Son et al., 2005). FoxA1 (Hepatocyte Nuclear Factor 3α), a member of the Forkhead family of winged-helix transcription factors, is involved in the development and differentiation of several organs including liver, kidney, pancreas, lung, prostate, and mammary gland (Friedman and Kaestner, 2006; Kouros-Mehr et al., 2006; Spear et al., 2006). In addition, high expression of FoxA1 is commonly observed in tumors arising from these organs, including prostate and estrogen receptor α (ERα)-positive breast tumors (Lacroix and Leclercq, 2004; Lin et al., 2002; Mirosevich et al., 2006). Interestingly, FoxA1 expression is a positive prognostic factor among patients with ERα-positive breast tumors and correlates with sensitivity to endocrine therapy (Badve et al., 2007). Consistent with its originally reported role as a pioneer factor involved in liver-specific gene expression (Bossard and Zaret, 2000; Cirillo et al., 1998; Gualdi et al., 1996), FoxA1 acts as a pioneer factor in the recruitment of ERα to several *cis*-regulatory elements in the genome and subsequent transcriptional induction of target genes such as *Cyclin D1* (*CCND1*) in breast cancer cells (Carroll et al., 2005; Eeckhoute et al., 2006; Laganiere et al., 2005). This is mediated in part through the chromatin remodeling activity of FoxA1 (Cirillo et al., 2002; Eeckhoute et al., 2006), reminiscent of its role in the induction of liver-specific gene expression (Friedman and Kaestner, 2006). FoxA1 also interacts with the androgen receptor (AR) in prostate cancer cells where it is thought to impact the regulation of AR target genes (Gao et al., 2003). Hence, FoxA1 appears capable of regulating distinct transcriptional programs in cells of different lineages. However, the molecular bases for the differential transcriptional activities of FoxA1 remain to be established. In the present study, we have investigated FoxA1 differential transcriptional activities in breast and prostate cancer cells and their functional relation with the epigenome of these cells.

RESULTS

Dual Regulatory Role of FoxA1 in E2 Signaling Revealed by Genome-wide ChIP-chip

Estrogen (E2) stimulation leads to the establishment of specific transcriptional programs in ERα-positive breast cancer cells. To address how FoxA1 participates in this process we initially performed an unbiased genome-wide chromatin immunoprecipitation study using tiling microarrays (ChIP-chip) to define the repertoire of FoxA1-binding sites, which we define as its "cistrome," in

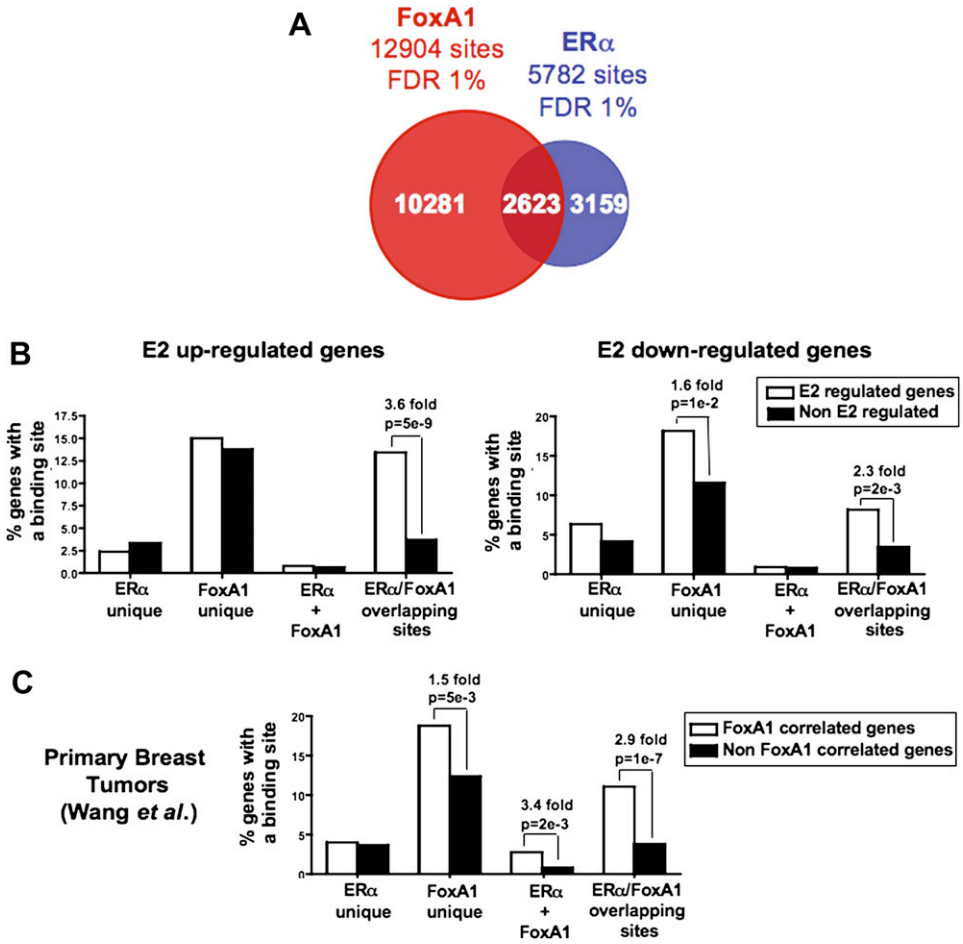

Figure 1. Genome-wide Identification of FoxA1-Binding Sites Reveals Its Global Role in Control of E2 Signaling in Breast Cancer Cells

(A) Overlap analysis at FDR1% showing the number of binding sites specific to FoxA1 or ERα or shared between the two factors in MCF7 cells.

(B) Correlation between E2 upregulated (left panel) or downregulated (right panel) genes and binding of either ERα only (ERα unique), FoxA1 only (FoxA1 unique), both factors at different sites (ERα+FoxA1), or both factors at a shared site (ERα/FoxA1 overlapping sites) within 20 kb of the TSS of genes. Fold change is presented for instances where significant differences are observed between regulated (t test p value $\leq 10^{-3}$) and nonregulated genes (t test p value $\geq 10^{-3}$).

(C) Correlation between ERα- and FoxA1-binding sites and genes coexpressed with FoxA1 in primary breast tumors (Wang et al., 2005) were analyzed as in (B). Fold change is presented for instances where significant differences are observed.

the MCF7 breast cancer cell line. A total of 12904 high-confidence FoxA1 recruitment sites were identified in these cells (using a stringent statistical false discovery rate [FDR] of 1%) (Figures S1 and S2 available online). In comparison, the ERα cistrome in MCF7 cells (Carroll et al., 2006) reanalyzed using the MAT algorithm (Johnson et al., 2006) and updated to the most recent human genome sequence (Hg18) revealed 5782 high-confidence sites (FDR 1%) (Figure S3). Interestingly, the genomic distribution of FoxA1-binding sites was reminiscent of that of ERα (Carroll et al., 2005; Lin et al., 2007). Indeed, the majority of the sites (96.9%) were found distant from the proximal 1 kilobase (kb) promoter regions of genes (Figure S4B). Accordingly, this distribution contrasted with that of RNA polymerase II (RNA PolII) (Carroll et al., 2005), which is found primarily at proximal promoters (Figure S4C). Comparing the FoxA1 and ERα cistromes revealed a highly significant overlap with ~50%–60% ERα-binding sites occurring on FoxA1 occupied sites (Figures 1A, S5A, and S5B). To determine the functional significance of this co-binding, we subsequently determined the distribution of FoxA1- and ERα-binding sites with regards to E2-regulated genes in MCF7 cells (Carroll et al., 2006). Hence, we compared the fraction of E2-regulated versus -nonregulated genes in MCF7 cells with at least one binding site specific to ERα or FoxA1 or shared by the two factors (as defined in Figure S5) within 20 kb of their transcription start site (TSS). Importantly, E2-upregulated genes were significantly enriched compared to nonregulated genes near sites of overlapping ERα/FoxA1 recruitment (Figure 1B). Strikingly, this was also the case for E2-downregulated genes (Figure 1B). These results demonstrate that genes having enhancers within 20 kb of the TSS that bind both ERα and FoxA1 together compared to ERα or FoxA1 separately are much more likely to be regulated in response to E2 treatment in breast cancer cells. A role for FoxA1 in E2-downregulated genes independently of its association with ERα was also revealed through the enrichment for this category of genes near

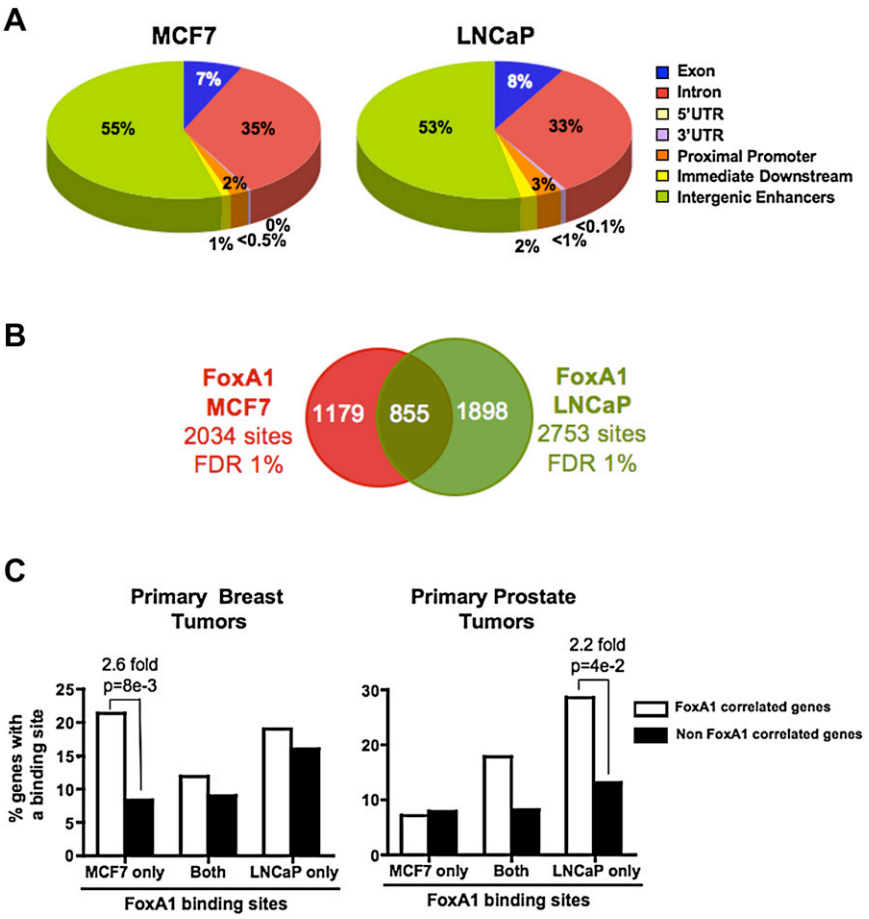

Figure 2. Cell Type-Specific Recruitment of FoxA1 Correlates with Differential Gene Expression Patterns

(A) *cis*-regulatory element annotation system (CEAS) (Ji et al., 2006) was used to determine the distribution of FoxA1-binding regions identified within chromosomes 8, 11, and 12 in MCF7 and LNCaP cells regarding known genes.

(B) Overlap analysis at FDR1% showing the number of FoxA1-binding sites specific to MCF7 or LNCaP or shared between the two cell lines.

(C) Correlation between cell type-specific or shared FoxA1-binding sites and genes coexpressed with FoxA1 in primary breast (Wang et al., 2005) or prostate (S.R. Setlur, K.D. Mertz, Y. Hoshida, F. Demichelis, M.L., S. Perner, A. Sboner, Y. Pawitan, O. Andren, L.A. Johnson, et al. unpublished data) tumors. The occurrence of FoxA1-binding sites within 20 kb of the TSS of FoxA1 coexpressed genes was compared to that of non-coexpressed genes. Fold change is presented for instances where significant differences are observed.

sites recruiting FoxA1 only (Figure 1B). In fact, FoxA1 silencing in MCF7 cells reduced the basal expression of these genes to levels equivalent to the reduction seen after E2 treatment (Figures S6A and S6B). This is most likely a consequence of FoxA1's role in allowing for the basal activity of enhancers for those genes (Figures S6C and S6D). These data indicate that FoxA1 controls the E2 response in breast cancer cells through a combination of mechanisms consisting of maintaining the basal expression of genes repressed following hormone treatment and allowing for the induction of E2-upregulated genes through a direct collaboration with ERα. Interestingly, genes with FoxA1-binding sites within 20 kb of their TSS also had a greater chance to be expressed together with FoxA1 and ERα in primary breast tumors pointing to the biological relevance of the FoxA1 cistrome beyond the MCF7 cell line (Figures 1C, S7, and S8).

FoxA1 Cell Type-Specific Activity Depends on Differential Recruitment to Chromatin

Having shown that FoxA1 recruitment to the chromatin within the MCF7 cell line was correlated with the regulation of the transcriptional program specific to ERα-positive breast tumors, we investigated how FoxA1 binding to the chromatin relates to its cell-specific functions. This was accomplished by comparing the FoxA1 cistromes originating from cell types of different lineages, namely the MCF7 breast cancer and LNCaP prostate cancer cell lines. Through genomic-scale studies performed across the nonrepetitive regions of human chromosomes 8, 11, and 12 using ChIP-chip assays, we identified over 2000 high-confidence sites of FoxA1 recruitment (FDR 1%) in both cell lines. As in MCF7 cells, these sites were predominantly found at enhancer positions in LNCaP cells (Figures 2A and S9). Importantly, comparison of the FoxA1 partial cistromes in these two cell lines revealed both a significant number of shared sites and an even greater number of cell type-specific regions (Figure 2B). Indeed, comparisons of the datasets using various cut-offs indicated that the overlap did not exceed 55% and 40% of the MCF7- and LNCaP-binding sites, respectively (Figures S10A–S10C). Therefore, of all sites identified in both cell lines (3932 sites total), over 65% of them correspond to regions of cell type-specific recruitment (886 sites specific to MCF7 cells and 1654 sites specific to LNCaP cells). The accuracy of these predictions was validated by ChIP-qPCR experiments (Figure S10D). Hence, on a genomic scale the majority of FoxA1 recruitment sites within the chromatin of two distinct cellular lineages are cell type specific. These results strongly suggested that FoxA1 might regulate differential transcriptional programs as a result of its cell type-specific recruitment pattern in MCF7 and LNCaP cells.

We next investigated the association of FoxA1-binding sites unique to MCF7 or LNCaP, or sites shared between the two cell lines, with genes coexpressed with *FoxA1* in primary breast or prostate tumors. This revealed a significant enrichment of genes coexpressed with *FoxA1* in primary breast tumors over non-coexpressed genes near FoxA1-specific binding sites unique to MCF7 breast cancer cells (Figures 2C and S11) (van de Vijver et al., 2002; Wang et al., 2005). Reciprocally, genes coexpressed with *FoxA1* in primary prostate tumors were significantly enriched

over non-coexpressed genes near FoxA1-binding sites unique to LNCaP prostate cancer cells (Figure 2C) (S.R. Setlur, K.D. Mertz, Y. Hoshida, F. Demichelis, M.L., S. Perner, A. Sboner, Y. Pawitan, O. Andren, L.A. Johnson, et al., unpublished data). Altogether, these results demonstrate that differential recruitment is the primary mechanism responsible for the differential function of FoxA1 in these two different cell lineages.

FoxA1 Alternatively Collaborates with ERα or AR at Cell-Specific Enhancers

In order to further characterize the functional mechanisms involved in FoxA1 regulation of the breast and prostate cancer-specific transcriptional programs, we monitored the transcription factor binding motifs enriched within the common FoxA1 recruitment sites, as well as those unique to each cell line. As expected, the Forkhead motif (FKHR) was enriched in all three subsets of FoxA1-binding regions (Figure 3A). Conversely, we found that the recognition motifs for the nuclear receptors ERα (ERE and ERE half-site) and AR (ARE and ARE half-site) were specifically enriched in FoxA1-binding sites unique to MCF7 or to LNCaP cells, respectively (Figure 3A). This suggested that the differential FoxA1 recruitment between MCF7 and LNCaP was correlated with cell-specific transcriptional collaborations with ERα or AR. This hypothesis was tested by comparing the FoxA1 cistrome on chromosomes 8, 11, and 12 from both cell lines to that of AR in LNCaP cells (Q.W. and M.B., unpublished data) and to that of ERα in MCF7 cells (Carroll et al., 2006). Interestingly, as was the case for ERα, we found that more than half of AR-binding sites in LNCaP cells occurred on sites where FoxA1 was also present (Figure 3B). These data strongly suggest that the functional relationship between FoxA1 and AR previously demonstrated at a few model genes (Gao et al., 2003) in fact extends to a large fraction of regions used by this nuclear receptor. Accordingly, FoxA1 silencing modulated the transcriptional response to dihydroxytestosterone (DHT) of several studied target genes (Figure S12). Importantly, the majority of FoxA1-binding sites overlapping with ERα were sites specific to MCF7 cells, while the majority of FoxA1-binding sites overlapping with AR were sites specific to LNCaP cells (Figure 3B). These data suggest that the cell type-specific recruitment of FoxA1 to the chromatin is linked to breast and prostate cancer transcriptional programs through specific collaborations with ERα in breast cells and AR in prostate cells. Indeed, these nuclear receptors are known to be master regulators of the behavior of a large subset of breast and prostate tumors through transmission of estrogenic and androgenic signals. Hence, we investigated the association of the different classes of sites with genes regulated by E2 in MCF7 cells or those regulated by DHT in LNCaP cells (Carroll et al., 2006; Wang et al., 2007). Only genes regulated by E2 were significantly enriched over nonregulated genes near ERα sites overlapping with FoxA1 in MCF7 cells (Figure 3C). In contrast, genes regulated by DHT were specifically significantly enriched over nonregulated genes near AR sites overlapping with FoxA1 in LNCaP cells (Figure 3C). Importantly, E2 or DHT regulated genes were mostly associated with the cell type-specific FoxA1-binding sites overlapping with ERα or AR and not those common to both cell lines (100% for AR/FoxA1 sites and 70% for ERα/FoxA1 sites). Overall, these data clearly implicate a role for FoxA1 in the regulation of breast- and prostate-specific transcriptional programs through cell-specific recruitment and subsequent differential collaboration with the sex steroid nuclear receptors ERα and AR.

Differential recruitment to the chromatin extends to other transcription factors present in both MCF7 and LNCaP cells. Indeed, AP-1, whose recognition motif was enriched within the FoxA1-binding sites from MCF7 and LNCaP cells (Figure S13A), was found to be corecruited together with FoxA1 at a subset of its cell-specific binding sites (Figure S13B). Hence, these data demonstrate that cell-specific recruitment also extends to ubiquitously expressed transcription factors such as AP-1 and suggest that this differential recruitment could also play an important role in its well-known cell-lineage differential activities (Jochum et al., 2001).

A Cell Type-Specific Histone Signature Correlates with Differential FoxA1 Recruitment

The functional importance of FoxA1 cell-specific recruitment described above raises the question as to how FoxA1 is able to bind to distinct regions within the genome of the MCF7 and LNCaP cells. Accordingly, we first considered the possibility that the sequence recognized by FoxA1 could be different between the two cell lines. However, de novo motif analysis revealed that the Forkhead factor recognition sequence enriched within the FoxA1-binding sites did not show any significant difference between shared and cell-specific binding regions though it varied somewhat from the previously established consensus motif (Figure 4A). Therefore, we investigated whether the differential FoxA1 binding could rather be linked to specific epigenetic modifications. First, we looked at several repressive histone marks (Bernstein et al., 2007; Kouzarides, 2007) and found that H3K9me2 was more highly enriched on sites not recruiting FoxA1 in both cell lines although not exclusively found on sites not recruiting FoxA1 (Figures 4B, 4C, and S14A). We then sought to determine if FoxA1 recruitment was on the other hand associated with the presence of active histone marks. Recently, a genomic-scale study demonstrated the occurrence of mono- (me1) and dimethylation (me2) of H3K4 at active enhancers (Heintzman et al., 2007). Analyzing the presence of these specific histone modifications at the FoxA1 recruitment sites revealed significant enrichment for H3K4me1 and me2 in a cell type-specific manner (Figures 4D–4G). Indeed, in MCF7 cells, FoxA1-binding sites unique to MCF7 cells as well as sites common to both cell lines were significantly mono- and dimethylated on H3K4 compared to the LNCaP unique FoxA1-binding sites (Figures 4D and 4F). On the other hand, in LNCaP cells, the LNCaP-specific FoxA1-binding sites together with the common sites were significantly enriched for these histone modifications compared to MCF7-specific sites (Figures 4E–4G). To confirm this correlation between H3K4 methylation and FoxA1 occupancy on a genomic scale we performed a ChIP-chip analysis of H3K4me2 levels in MCF7 cells across chromosomes 8, 11, and 12. These data revealed that on a genomic scale levels of H3K4me2 in MCF7 cells were indeed significantly greater on MCF7-specific or shared FoxA1 recruitment sites than on LNCaP-specific ones (Figure 4H). H3K4me2 levels were also significantly higher on regions with FoxA1 recognition motifs bound by FoxA1 compared

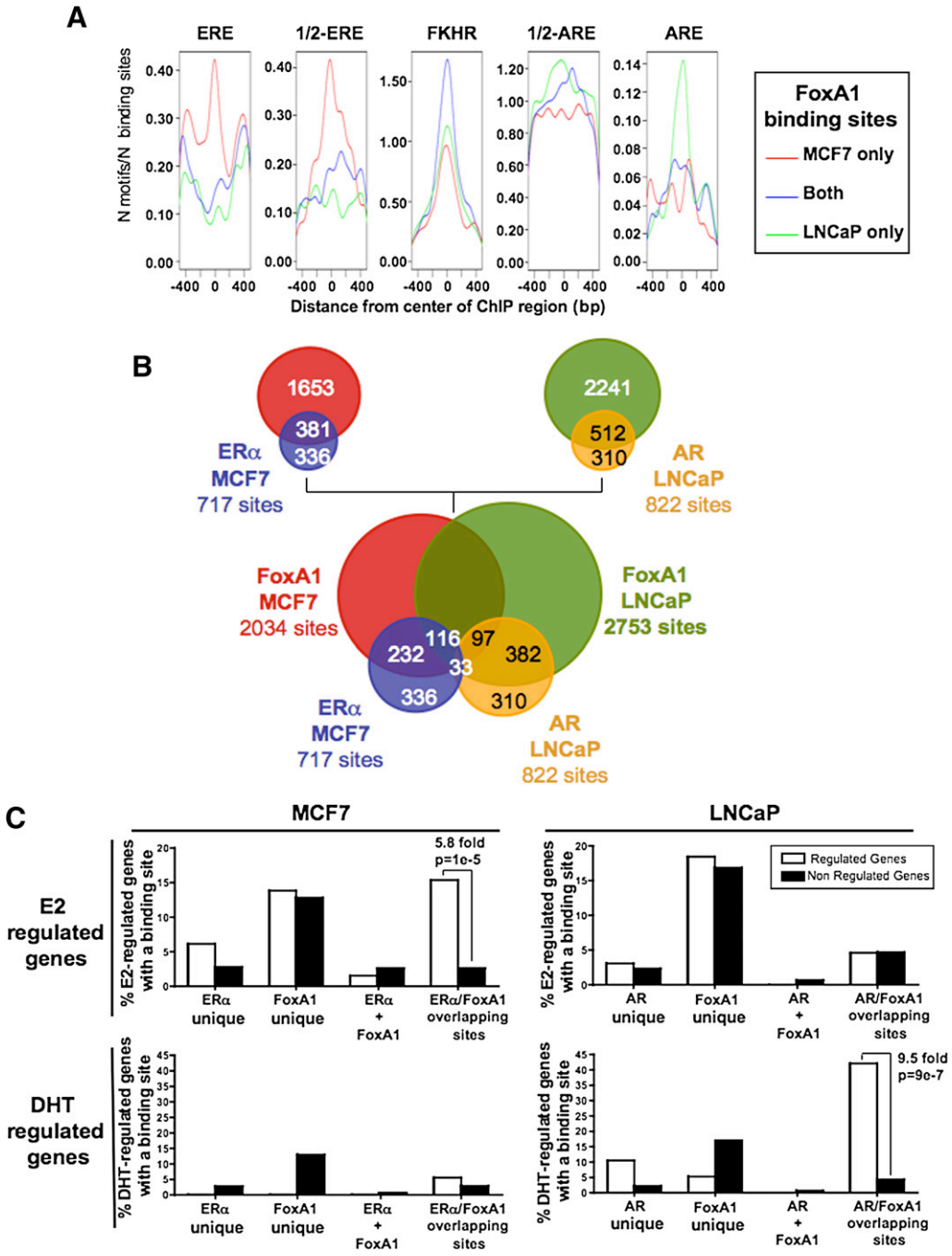

Figure 3. FoxA1 Cell Type-Specific Binding Sites Also Recruit Nuclear Receptors ERα or AR and Correlate with Regulation of Sex Steroid Signaling in Breast and Prostate Cancer Cells

(A) Enrichment for the ERE, ERE half-site, FKHR, ARE, and ARE half-site in the center of the binding sites specific to MCF7 cells (MCF7-only) or LNCaP cells (LNCaP-only) or shared between the two cell lines (Both). The occurrence of the motifs (N motifs) was normalized to the number of sites in each subset (N binding sites).

(B) Venn diagrams depicting the overlap between FoxA1 (red) and ERα (blue) binding sites from MCF7 cells together with FoxA1 (green) and AR (orange) binding sites from LNCaP cells.

(C) Correlation between E2 or DHT regulated genes and binding sites for FoxA1 and ERα in MCF7 cells or for FoxA1 and AR in LNCaP cells. Analyses were performed as in Figure 1B using hormone-regulated or -nonregulated genes from chromosomes 8, 11, and 12. Fold change is presented for instances where significant differences are observed between regulated and nonregulated genes.

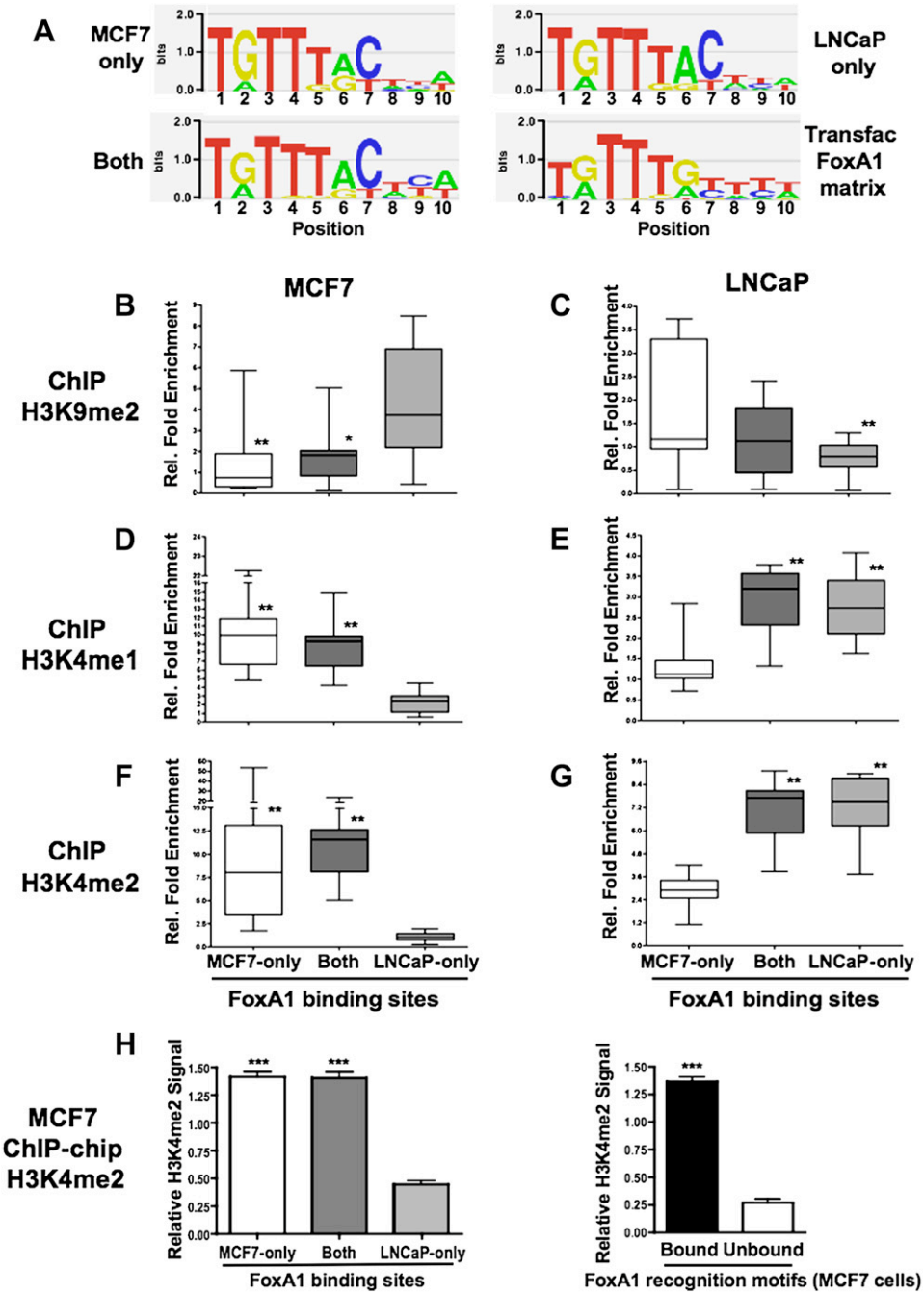

Figure 4. Methylation Pattern of Histone H3 Lysine 4 Correlates with Cell Type-Specific FoxA1 Recruitment

(A) De novo determination of the sequence recognized by FoxA1 within its cell type-specific or shared binding sites. Logos show the consensus sequences of the enriched Forkhead motifs found by de novo analyses within the FoxA1-binding sites specific to MCF7 (MCF7-only) or LNCaP (LNCaP-only) cells or common to the two cell lines (Both) in comparison to the Transfac FoxA1 matrix (http://www.gene-regulation.com/pub/databases.html#transfac).

(B–G) Levels of H3K9me2 (B and C), H3K4me1 (D and E), and H3K4me2 (F and G) on FoxA1 recruitment sites specific to MCF7 cells (MCF7-only) or LNCaP cells (LNCaP-only) or shared between the two cell lines (Both) were determined by ChIP-qPCR. Box plots were generated from data obtained from three independent experiment testing 11 sites specific to MCF7 cells, 12 to LNCaP cells, and 8 common to both cell types. Statistical analyses of the difference between the non-cell type-specific sites and the other sites are presented, *: $p \leq 0.05$ and **: $p \leq 0.01$. Whiskers correspond to the largest and smallest nonoutlier values from each dataset.

(H) ChIP-chip analyses of H3K4me2 levels across chromosomes 8, 11, and 12 in MCF7 cells. Two independent ChIP-chip experiments were combined and analyzed using the MAT algorithm. The signals given by the probes localized in the 200 bp central regions of the FoxA1-binding sites unique to MCF7 (MCF7-only) or LNCaP (LNCaP-only) or shared (Both) by the two cell lines were compared (left graph). Similarly, H3K4me2 levels at 200 bp regions containing the FoxA1 recognition motif bound by FoxA1 were compared to randomly selected FoxA1-unbound FoxA1 recognition motif-containing regions (right graph). Means ± SEM of H3K4me2 levels given by MAT are shown as well as statistically significant differences with *** corresponding to $p \leq 0.001$.

to an equivalent number of randomly selected unbound regions with FoxA1 recognition motifs in MCF7 cells (Figure 4H). Importantly as less than 3.7% of sites harboring FoxA1 recognition motifs actually recruit FoxA1 in MCF7 cells (Figure S14C), these data derived from the analysis of thousands of sites reveal a strong correlation between the presence of H3K4me2 and FoxA1 binding. Of the FoxA1 recruitment sites tested, as expected, very few demonstrated enrichment for H3K4me3 in accordance with the predominant occurrence of this modification at promoters rather than enhancers (Heintzman et al., 2007) (Figure S14B). Overall, these results suggest a link between FoxA1 recruitment with the presence of H3K4me1 and me2.

FoxA1 Is Required for Chromatin Remodeling but Not for H3K4 Methylation

In MCF7 cells, H3K4me1 and me2 are detected at enhancers prior to E2 stimulation and ERα binding, reminiscent of FoxA1 recruitment (Figure S15). Accordingly, ERα silencing in these cells did not dramatically affect H3K4 methylation levels or FoxA1 recruitment at most sites where these two factors are recruited (Figures 5A and S16). Moreover, the vast majority (~80%) of FoxA1 sites specific to MCF7 cells do not recruit ERα (Figure 3B). Hence, while we cannot entirely rule out a potential role for ERα in stabilizing FoxA1 binding at a small subset of sites, these results suggest that in general cell-specific FoxA1 recruitment occurs independently of ERα action in MCF7 cells. This raises the issue of whether H3K4me1 and me2 are required for FoxA1 recruitment or are induced as a result of FoxA1 binding to the chromatin. This question was first addressed by investigating whether FoxA1 silencing would affect H3K4 methylation, chromatin remodeling, or both in MCF7 and LNCaP cells. Consistent with its cell type-specific recruitment, FoxA1 silencing impacted the DNase I sensitivity only at those sites to which it was recruited (Figure 5B). Under these conditions, however, these sites did not in general show a significant reduction in the levels of H3K4me1 or me2 in either MCF7 or LNCaP cells (Figure 5C). In fact, a significant increase in H3K4me1 was detectable at most sites tested in LNCaP cells. Similarly, levels of H3K9me2 were unaffected by FoxA1 silencing (Figure S17). Overall, these data do not favor a model whereby FoxA1 recruitment leads to the induction of these modifications but rather suggest an important contribution of FoxA1 in opening genomic regions marked by H3K4me1 and me2. Accordingly, even though FoxA1 silencing did not modulate H3K4 methylation levels at enhancers (Figure 5D), it affected the transcriptional regulation of their target genes (Figures 5E and S18). Considering that H3K4me2 is typically associated with gene transcription (Bernstein et al., 2005), these results highlight the critical interplay between the pioneer factor FoxA1 and H3K4me2 at enhancers for efficient gene regulation.

Reduction of H3K4 Methylation Impairs Cell Type-Specific FoxA1 Recruitment

To establish the capacity of H3K4 mono- or dimethylation to define the cell type-specific recruitment of FoxA1, we overexpressed the H3K4me1 and me2 specific demethylase KDM1 (also known as LSD1/BHC110) in MCF7 cells and established its impact on FoxA1 recruitment (Shi et al., 2004). Under these conditions, H3K4me1 was slightly reduced (Figure S19A) and H3K4me2 was significantly lowered on FoxA1-binding sites (Figure 6A). The level of H3K9me2 remained unchanged at these sites (Figure 6C). Although FoxA1 protein levels were unaffected by KDM1 overexpression (Figure 6D), its recruitment to the chromatin was significantly impaired (Figure 6B). Importantly, no global alteration in ChIP efficiency was observed upon KDM1 overexpression (Figures S20B and S20C). Hence, these results suggest that H3K4me2 is required to define the cell type-specific regions competent for recruitment of FoxA1. The correlation between the presence of histone marks and FoxA1, ERα, or AR recruitment is shown for specific examples of hormone-regulated genes (Figure 6E).

DISCUSSION

Networks of transcription factors are known to be at the center of cell type-specific transcriptional programs that characterize different cell lineages (Olson, 2006; Schrem et al., 2002). However, how a particular transcription factor manages to regulate gene expression in a cell type-specific fashion within the context of different transcription factor networks is still poorly understood. In particular, it is still elusive how a pioneer factor, such as FoxA1, that is able to bind condensed chromatin structures in vitro can mediate differential gene regulation in vivo (Cirillo et al., 2002; Eeckhoute et al., 2006). Here, we show that FoxA1 differential transcriptional activities in breast and prostate cells relies primarily on its differential recruitment to the chromatin and alternative collaboration with the lineage-specific factors ERα or AR at cell-specific enhancers (Figures 6E, 7, and S21). These findings indicate that alternative transcriptional programs depend both on the orchestrated expression of a particular set of collaborating transcription factors together with their ability to bind cell-specific enhancer elements in the vicinity of their target genes. Alternatively, other transcription factor networks may primarily target gene promoters (Bieda et al., 2006; Geles et al., 2006). This may allow for a tight regulation of gene expression both at basal levels and in response to stimuli through combined activities of promoter- and enhancer-bound regulatory complexes (Hatzis and Talianidis, 2002; Marr et al., 2006). Importantly, we found that even ubiquitous transcription factors, such as AP-1, show differential recruitment to cell type-specific enhancers. Combined with other recent studies (So et al., 2007), this suggests that cell-specific binding to the chromatin represents a general mechanism for differential transcription factor regulatory activities. Cell-specific recruitment of AP-1 to FoxA1 sites could have important functional implications in breast cells especially for E2 downregulated genes where FoxA1-binding sites are enriched for AP-1 and Sp1 motifs ($p \leq 0.05$) that can tether ERα to mediate gene repression (Carroll et al., 2006; Stossi et al., 2006). Other important candidates for a global role in control of sex steroid signaling through collaborations with FoxA1 and ERα or AR include GATA family members (Eeckhoute et al., 2007; Wang et al., 2007), c-*myc* (Cheng et al., 2006), and NFIC (Eeckhoute et al., 2006).

The occurrence of specific histone modifications at *cis*-regulatory elements commonly characterizes transcriptionally active or inactive regions (Bernstein et al., 2007; Kouzarides, 2007). Recently, the balance between the presence of active or repressive

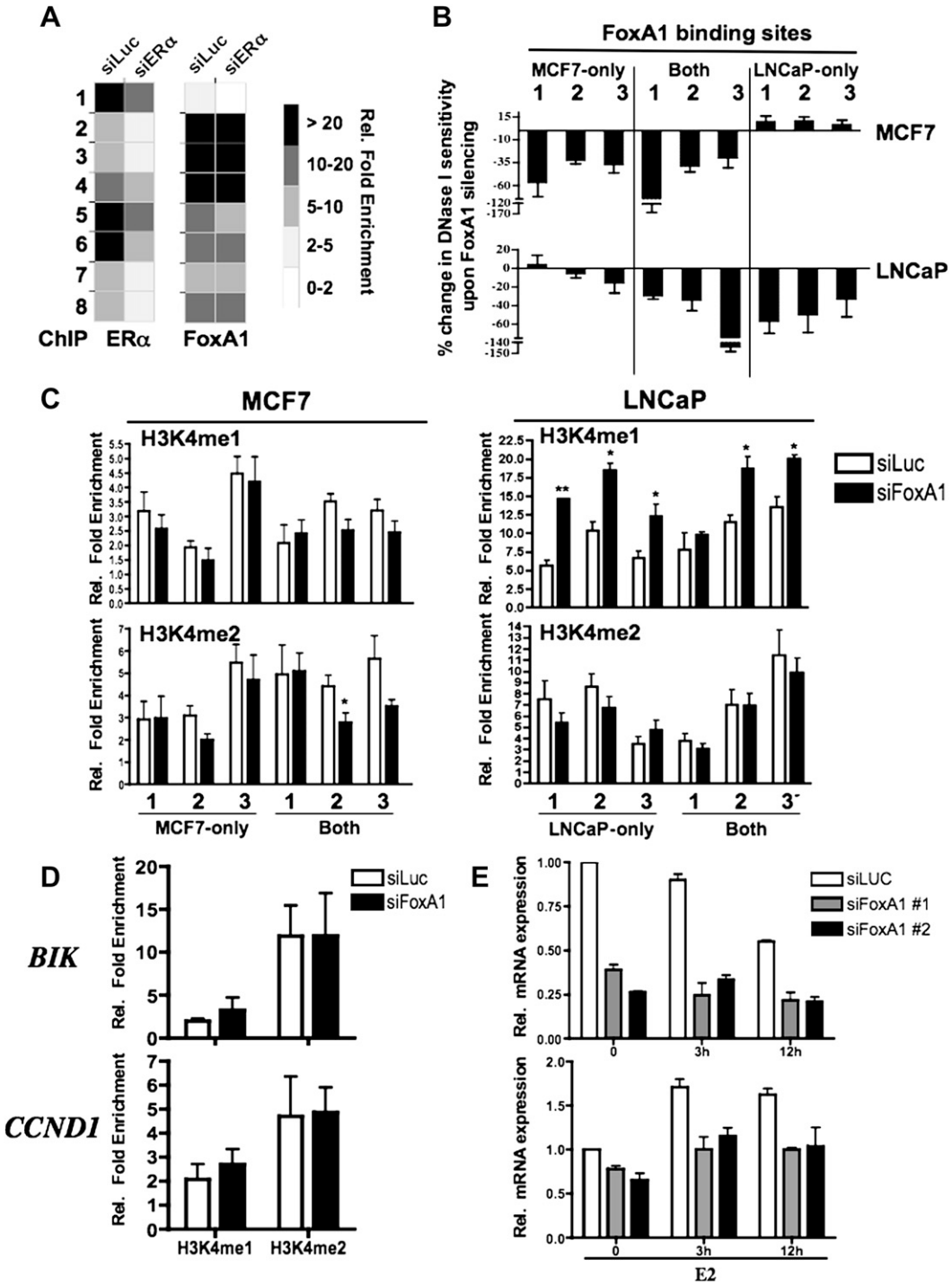

Figure 5. FoxA1 Silencing Decreases Chromatin Accessibility of Enhancers but Not H3K4 Methylation Levels

(A) Effect of ERα silencing on FoxA1 recruitment. Eight sites recruiting both ERα and FoxA1 in MCF7 cells were used to monitor the effect of ERα silencing on ERα and FoxA1 recruitment by ChIP-qPCR. Reduction in ERα protein levels by siERα was also demonstrated by western blot (Figure S16A).

(B) DNaseI sensitivity assays were performed in both MCF7 and LNCaP cells, and the percent change triggered by FoxA1 silencing from at least three independent experiments is reported. Data are means ± standard deviation (SD).

(C) Effect of FoxA1 silencing on the levels of H3K4me1 and me2 at binding sites used in the DNaseI sensitivity assays in both MCF7 and LNCaP cells from three experiments is presented, *: $p \leq 0.05$ and **: $p \leq 0.01$. Data are means ± SD.

(D and E) Presence of H3K4me1/2 at enhancer is not sufficient for transcriptional regulation of *BIK* and *CCND1* in MCF7 cells. H3K4me1/2 levels at FoxA1 recruiting enhancers localized within or nearby FoxA1 target genes were determined by ChIP-qPCR in MCF7 cells transfected with siLuc or siFoxA1 (D). Even though FoxA1 silencing did not modulate the levels of H3K4 methylation, the expression of the target genes was significantly reduced (E). Data are means ± SD.

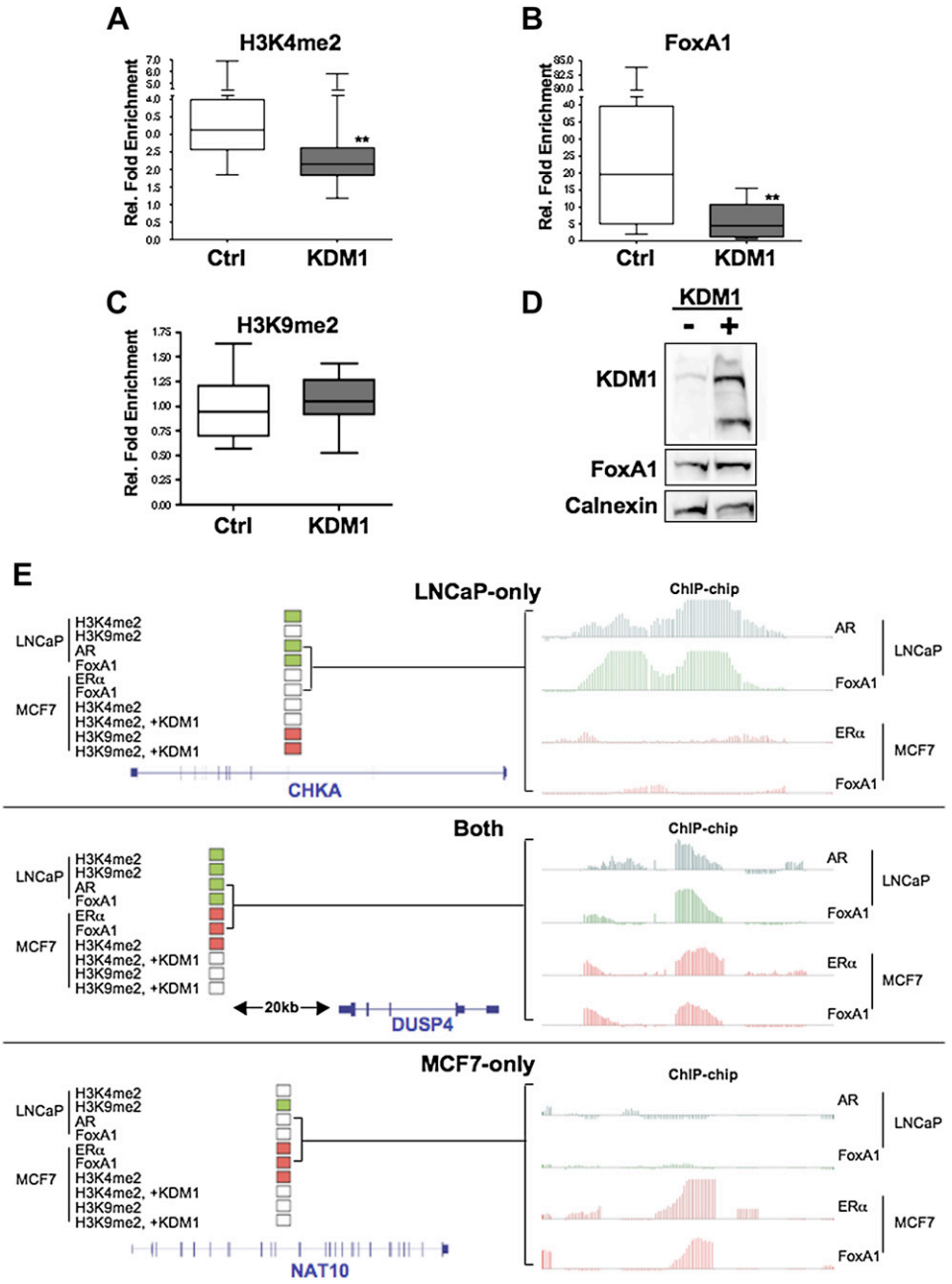

Figure 6. Role of H3K4me2 in FoxA1 Recruitment to the Chromatin

(A–C) Effect of KDM1 overexpression on H3K4 methylation (A), FoxA1 recruitment (B), and H3K9 methylation (C). H3K4me2 and H3K9me2 levels as well as FoxA1 recruitment were determined in control or KDM1-overexpressing cells by ChIP-qPCR. Box plots were generated from data obtained for 16 sites. Results from one representative experiment are presented with the statistical analyses of the difference between control and KDM1-overexpressing cells, **: $p \leq 0.01$. Whiskers correspond to the largest and smallest nonoutlier values from each dataset.

(D) Western blots showing KDM1, FoxA1, and Calnexin (Control) levels in MCF7 cells transfected with an empty control plasmid or a plasmid coding for KDM1.

(E) Specific examples of genes regulated by E2, DHT, or both hormones. One gene specifically regulated by E2 in MCF7 cells (MCF7-only), by DHT in LNCaP cells (LNCaP-only), and by both hormones in MCF7 and LNCaP cells, respectively (both), is shown. E2- and DHT-regulated genes were identified using expression array analyses performed in MCF7 and LNCaP cells, respectively. Significantly regulated genes were determined using a t test and a p value cut-off of 5×10^{-3}. ERα-, AR-, and FoxA1-binding sites from ChIP-chip are indicated together with the occurrence of histone modifications derived from ChIP-qPCR at these sites. Enrichment for the various factors is presented by green and red blocks in LNCaP and MCF7 cells, respectively. White blocks indicate the absence of enrichment for the ChIPed factors or a decrease of more than 2-fold for histone marks in MCF7 cells following KDM1 overexpression. A 4 kb wide view of the probe signals obtained by ChIP-chip for FoxA1, ERα, and AR at the analyzed binding sites is also shown. Complete probe signal across the three genes selected is presented in Figure S21.

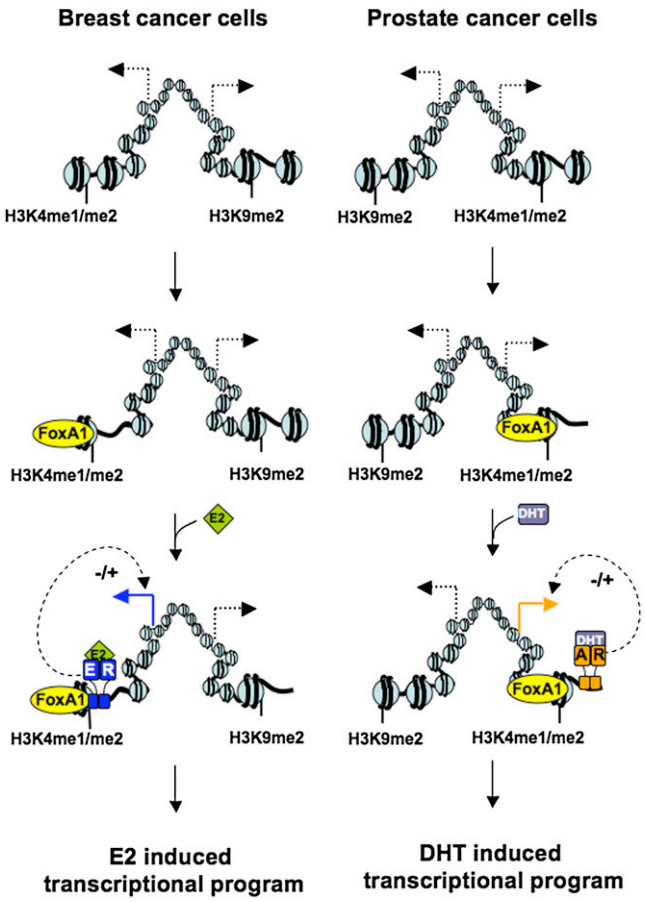

Figure 7. Model of the Cell Type-Specific Interplay between the Epigenetic Signature and FoxA1 for the Establishment of Lineage-Specific Transcriptional Programs

Schematic representation of how FoxA1 recruitment occurs primarily on H3K9me2-poor but H3K4me1/2-rich regions. H3K4me1/2 could guide FoxA1 cell type-specific recruitment through direct physical interactions. FoxA1 regulation of differential transcriptional programs is subsequently achieved through transcriptional collaborations with cell type-specific (ERα and AR) as well as ubiquitously expressed (AP-1) transcription factors.

histone modifications (trimethylation of H3K4 and H3K27) has been shown to correlate with promoter activity (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007). Here, we show that the cell type-specific activity of enhancers correlates with the presence of the positive mark H3K4me2, previously shown to be distributed in a cell type-specific manner (Bernstein et al., 2005), while inactive enhancers lack H3K4me2 and harbor higher levels of the repressive mark H3K9me2. Interestingly, even though FoxA1 silencing does not modulate levels of H3K4 and K9 methylation at enhancers (Figures 5 and S17), it is required for their activity and consequently for their target gene transcriptional regulation (Figures 5, S6, and S18). Therefore, H3K4me1/2 appear to correlate with competent enhancers but not necessarily with transcriptional activation of target genes that requires factors such as FoxA1 to activate the functionality of these enhancers.

The capacity of FoxA1 to bind unique binding sites in reconstituted chromatin has been studied extensively in vitro (Cirillo et al., 1998, 2002; Sekiya and Zaret, 2007). Under these conditions, no histone modifications appear to be required for FoxA1 recruitment. However, our results demonstrate that in vivo FoxA1 actually occupies only a very small fraction of all its potential recognition motifs found in the genome (less than 3.7%). Moreover, this limited number of occupied sites is significantly different between two different cell types. Therefore, although FoxA1 can act as a pioneer factor able to bind to condensed chromatin, we show here that in vivo its pioneer function is limited to a small subset of sites that are largely cell type specific. Our data further define on a genomic scale the chromatin components involved in directing FoxA1 recruitment to this subset of its potential binding sites. Indeed, our results point to an important role of active and repressive histone marks, notably H3K4me2 and H3K9me2, respectively, in guiding FoxA1 recruitment. These data indicate that a better understanding of cell-lineage transcriptional commitment will require the study of how these marks are established and how they regulate recruitment of pioneer transcription factors such as FoxA1. Altogether, our data reveal an additional layer of complexity in the regulation of FoxA1 recruitment to chromatin in vivo that goes beyond the mere presence of its recognition motif. Indeed, FoxA1 translates an epigenetic signature into functional cell type-specific enhancers leading to the establishment of cell type-specific transcriptional programs.

EXPERIMENTAL PROCEDURES

ChIP-chip and ChIP-qPCR

ChIP-chip experiments using Affymetrix Human Tiling 2.0R Array Set were performed as previously described (Carroll et al., 2005, 2006). For each ChIP-chip experiment, at least three independent assays were performed. Analyses were performed using MAT (Johnson et al., 2006), whose probe mapping had been updated to the latest human genomic sequence (Hg18). We used statistical FDR as cut-off in those analyses. All ChIP-chip data used in this study can be accessed at http://research.dfci.harvard.edu/brownlab/datasets/. ChIP-qPCR experiments were performed as in Carroll et al. (2005). Statistical analyses were performed using Student's t test comparison for unpaired data. Primer sequences can be found in Table S1.

Antibodies used for ChIP experiments were FoxA1 (Ab5089 and Ab23738 from Abcam, FOX1 from CeMines), ERα (Ab-10 from Neomarkers, HC-20 from Santa Cruz), pan-jun (D from Santa Cruz), pan-fos (K-25 from Santa Cruz) (Schwartz et al., 2007), AR (N20 from Santa Cruz), H3K4me1, me2, me3, H3K9me1, me2, me3, H4K20me1, me2, me3 (Ab8895, Ab7766, Ab8580, Ab9045, Ab1220, Ab8898, Ab9051, Ab9052, and Ab9053, respectively, from Abcam) (Mikkelsen et al., 2007; Barski et al., 2007), H3K27me1, me2, me3 (07-448, 07-449, and 07-452 from Upstate Biotechnology Inc.) (Barski et al., 2007; Mikkelsen et al., 2007; Vakoc et al., 2006), RNA PolII (H-224 from Santa Cruz and Ab5408 from Abcam), H3 (Ab1791 from Abcam), and AcH4 (from Cell Signaling).

Genomic Distribution and Binding Site Overlap

Genomic distribution of binding sites identified by ChIP-chip was performed using *cis*-regulatory element annotation system (CEAS) (Ji et al., 2006). Two binding sites were considered to overlap as long as they had one base pair in common. The average size of the ChIP-chip regions being 1 kb, this means that the center of the two binding sites had to be in average within 1 kb of each other to be considered overlapping.

Transcription Factor Recognition Motif Enrichment Analysis

Known DNA motifs that are enriched relative to the center of ChIP-chip sites were identified using the following statistic. All sites were trimmed or expanded to 600 bp centered at the middle point of the identified ChIP-enriched regions. All subsequences within the trimmed regions were scored by a TRANSFAC

motif (Matys et al., 2006) and the genomic background sequence composition to identify hits above certain relative entropy cutoff t. Letting x_i, a value between 0 and 1, denote the relative location of motif hit i on the ChIP regions (0 and 1 representing the center and edge of a ChIP region, respectively), out of N total motif hits, we define a z score, $z = \sum_{i=1toN}(x_i - 0.5)/\sqrt{N/12}$ to assess the positional bias of a motif toward the centers of the regions. Different integer cutoffs $t \geq 3$ were tested for each motif, and the cutoff resulting in the highest z was selected. This statistic is based on the assumptions that insignificant DNA motifs will be uniformly distributed across the ChIP regions and the null distribution of $\sum x_i$ can be estimated as the N-fold convolution of uniform density functions. In Figure 3A, a Gaussian kernel was used to smooth the curves in case too few motif hits appeared at particular positions.

Association of Trends in Gene Expression with Transcription Factor Binding Sites

Gene expression data were normalized and summarized using RMA (Irizarry et al., 2003) and updated RefSeq probeset definitions (Dai et al., 2005). Where multiple transcripts were associated with a single gene expression index the transcript with the TSS closest to a ChIP-enriched region was selected. "Differentially expressed" genes were denoted as those genes with a t test p value $\leq 10^{-3}$. Genes "close" to a ChIP region were defined as those having such a region within 20 kb of the TSS. Fisher's exact test was used to assess the statistical significance of the association between close genes and differentially expressed genes.

De Novo Motif Search

De novo motif searches were performed on sequences ±100 bp from the centers of FoxA1 ChIP regions in MCF7 cells or LNCaP cells by using LeitMotif (J. Song and X.S.L., unpublished data), a modified MDscan (Liu et al., 2002) with ninth-order Markov dependency for the genome background. Motif logos were generated by enoLOGOS (Workman et al., 2005).

RNA Interference

FoxA1 was silenced using the following small-interfering RNA duplexes: siFoxA1 #1 sense 5′-GAGAGAAAAAAUCAACAGC-3′; antisense 5′-GCUGUU GAUUUUUUCUCUC-3′ (Carroll et al., 2005; Eeckhoute et al., 2006) and siFoxA1 #2 5′-GGACUUCAAGGCAUACGAAUU-3′; 5′-UUCGUAUGCCUUGA AGUCCUU-3′ (Figure S17). SMARTpool siRNA directed against ERα was purchased from Dharmacon. Small-interfering RNA against Luciferase was used as a negative control (Carroll et al., 2005).

DNase I Hypersensitivity Assays

DNase I hypersensitivity assays were performed as in Eeckhoute et al. (2006).

KDM1 Overexpression Experiments

A total of 15 μg of pCMX-KDM1 construct or the control empty vector were transfected in MCF7 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 76 hr of expression, cells were processed for ChIP-qPCR as previously described.

Real-Time RT-PCR

RNA was isolated from MCF7 and LNCaP cells using RNeasy mini kit (QIAGEN), with on-column DNase treatment to remove contaminating genomic DNA. Real-time reverse transcription-PCR (RT-PCR) was done as in Keeton and Brown (2005). Primers used in RT-qPCR are listed in Table S2.

Western Blots

Western blots were processed as described in Lupien et al. (2007) using antibodies against KDM1 kindly provided by R. Schule (Universitäts-Frauenklinik und Zentrum für Klinische Forschung, Freiburg, Germany), FoxA1 (Abcam), and Calnexin (Stressgen Biotechnologies).

SUPPLEMENTAL DATA

Supplemental Data include twenty-one figures, two tables, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/132/6/958/DC1/.

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Coactivator Function Defines the Active Estrogen Receptor Alpha Cistrome▽†

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Proper activation of transcriptional networks in complex organisms is central to the response to stimuli. We demonstrate that the selective activation of a subset of the estrogen receptor alpha (ERα) cistrome in MCF7 breast cancer cells provides specificity to the estradiol (E2) response. ERα-specific enhancers that are subject to E2-induced coactivator-associated arginine methyltransferase 1 (CARM1) action are critical to E2-stimulated gene expression. This is true for both FoxA1-dependent and independent enhancers. In contrast, a subset of E2-suppressed genes are controlled by FoxA1-independent ERα binding sites. Nonetheless, these are sites of E2-induced CARM1 activity. In addition, the MCF7 RNA polymerase II cistrome reveals preferential occupancy of E2-regulated promoters prior to stimulation. Interestingly, E2-suppressed genes tend to lie in otherwise silent genomic regions. Together, our results suggest that the transcriptional response to E2 in breast cancer cells is dependent on the interplay between polymerase II pre-occupied promoters and the subset of the ERα cistrome associated with coactivation.

The transcriptional response to estrogen in numerous tissues, including mammary gland, bone, and uterine tissues, and in diseases such as breast cancer is dependent on estrogen receptor alpha (ERα). Genome-wide positional analyses defining the set of *cis*-regulatory elements recruiting ERα, known as its cistrome, in breast cancer cells have revealed its predominant recruitment to enhancers as opposed to promoter regions (6, 7, 37, 39). As for many other transcription factors, genomic recruitment of ERα is restricted to a small proportion of its putative binding sites (<4.4%) offering a primary means of defining the response to estradiol (E2) (5, 7, 37). Similarly, the promoter predominant Pol II recruitment in breast cancer cells is restricted to a subset of promoters upon E2 stimulation (7, 32, 33, 35). Epigenetic modifications are central to the lineage-specific recruitment at enhancers and promoter regions. Indeed, promoters of activated genes harbor trimethylated histone H3 lysine 4 (H3K4me3) favoring the recruitment of chromatin remodeling enzymes and histone acetylases (1, 18, 42, 52, 56, 58). In contrast, promoters associated with transcriptional repression harbor trimethylated H3K27 (H3K27me3) (1, 3, 36, 42). Similarly, functional enhancers are associated with mono- and dimethylation of H3K4 (H3K4me1, me2) restricting the recruitment and the chromatin remodeling activity of the pioneer factor FoxA1, required for ERα binding, in a lineage-dependent manner, while levels of H3K9me2 are elevated on nonfunctional enhancers (15, 25, 40).

Despite these epigenetic constraints, RNA polymerase II (Pol II) and ERα together are recruited to more than 9,000 independent high-confidence (false discovery rate [FDR], 1%) sites across the genome of breast cancer cells upon E2 stimulation (7). Studies limited to a small number of ERα target sites have implicated coactivators, such as the coactivator associated arginine methyltransferase 1 (CARM1), in the E2 response (22). As they are recruited to ERα binding sites, coactivators allow for a series of posttranslational modifications on histones and other coactivator proteins in order to facilitate chromatin remodeling and cycling of the transcriptional unit essential for the E2 response (41, 57). In the case of CARM1, this involves dimethylation of arginine residues on histone H3 as well as on the coactivator AIB1 (8, 48). In addition, recent studies in *Drosophila* have revealed the dominant presence of poised Pol II at promoters of genes involved in the response to stimuli and developmental signals (47, 69). In the present study, we investigated the impact of CARM1 coactivator's activity on ERα binding sites and of Pol II at promoters in the transcriptional response to E2 through genome-wide positional analyses in human breast cancer cells.

MATERIALS AND METHODS

ChIP-microarray preparation. Cells were hormone deprived for 3 days in phenol red-free medium (Invitrogen) supplemented with 10% charcoal dextran-treated fetal bovine serum. Cells were stimulated with the estrogen 17β-estradiol (10^{-8} M) for 45 min and cross-linked by using 1% formaldehyde. Samples were sonicated (Fisher Sonic Desmembrator, model 500) and immunoprecipitated, as previously described (40), using an antibody against histone H3 arginine 17

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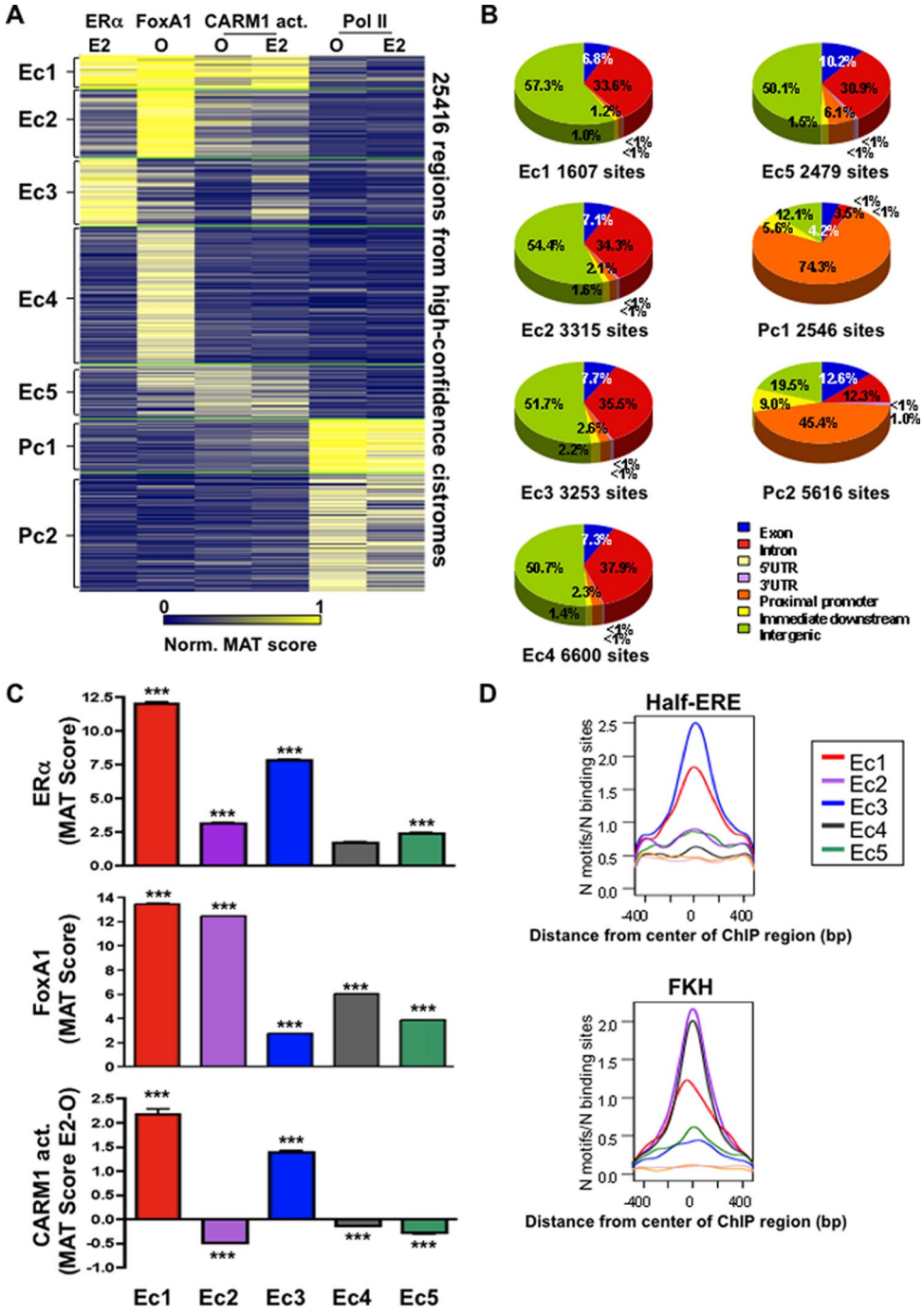

FIG. 1. Establishing classes of enhancer-rich clusters under E2 treatment. (A) Cluster analysis according to the binding activity for the transcription factor ERα, the pioneer factor FoxA1, the mark of CARM1 activity (an antibody raised against dimethylation of arginine 17 on histone H3), and Pol II across the 25,416 high-confidence regions recruiting at least one factor from all analyzed cistromes established through unbiased genome-wide ChIP-on-ChIP in MCF7 breast cancer cells (E2, E2 treated for 45 min; O, vehicle treated). (B) Genomic distribution of binding sites found in each cluster with regard to the TSS of known genes using the *cis*-regulatory element annotation system (28). (C) Average MAT scores of ERα and FoxA1 and the difference in CARM1 activity between E2-treated and control MCF7 cells in each cluster. The average MAT score signal for ERα or FoxA1 for the various clusters significantly different from a 1.5 average MAT score is presented. Similarly, the average change in CARM1 activity MAT score significantly different from 1 between E2- and vehicle-treated cells is presented. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. (D) Half-ERE and Forkhead (FKH) motif enrichment in sites from each cluster.

dimethylated (H3R17me2; Upstate Biotechnology, 07-214) and Pol II (Abcam, 4H8; Santa Cruz Biotechnology, H-224). Purified samples were labeled as previously described (6). The microarrays used were Affymetrix GeneChip Human Tiling 2.0R Array Sets. Genome-wide chromatin immunoprecipitation (ChIP)-on-ChIP analysis was conducted by using a model-based analysis of tiling-arrays program (MAT) (30). All ChIP-on-ChIP data used in the present study can be accessed at http://research.dfci.harvard.edu/brownlab/datasets/.

Cluster analysis. We generated a set of genomic intervals derived from the union of all high-confidence sites associated with either ERα, FoxA1, or CARM1 activity or Pol II. Next, we assigned the score to each interval for each factor as

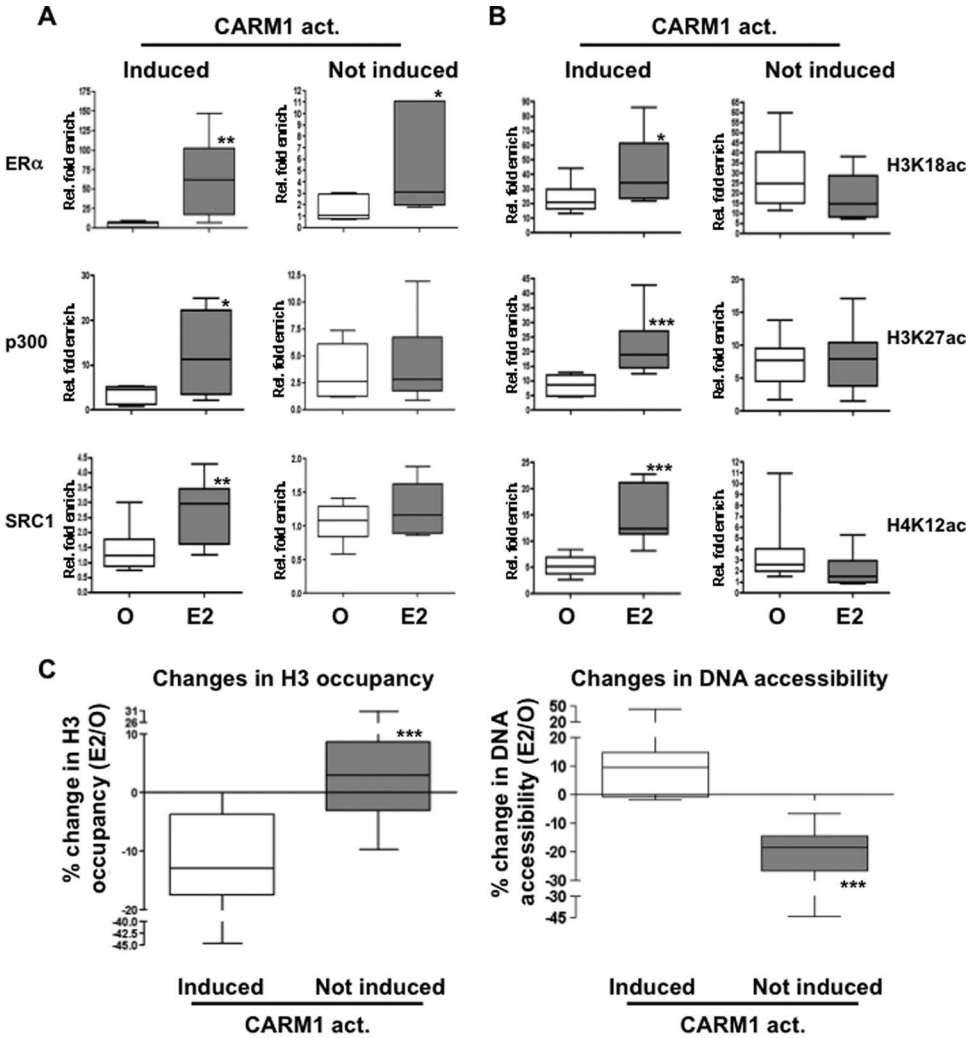

FIG. 2. E2-induced CARM1 activity at ERα sites associates with activating events. (A) Level of recruitment for the coactivators p300 and SRC1 under vehicle (O) or E2 treatment established by ChIP-qPCR on eight ERα sites associated and eight not associated with CARM1 activation in MCF7 breast cancer cells. (B) Levels of histone modifications, namely, H3K18ac, H3K27ac, and H4K12ac, were determined as in panel A. (C) Impact of E2 treatment on nucleosome density. The changes in occupancy of the core histone H3 were determined by ChIP-qPCR as in panel A. Alterations to the DNA accessibility were determined by using FAIRE (21). The results are derived from a minimum of two independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

the maximum MAT score falling within the interval for the given factor. For each factor MAT scores were trimmed at the 2.5 and 97.5 percentiles and scaled to lie between 0 and 1. Genomic regions were clustered by using *k*-means clustering.

Sequence analysis. Genome-wide distribution as well as sequence conservation analysis of H3R17me2 chip-on-chip was determined by using *cis*-element annotation systems (28). Enriched motifs within clusters as well as the associations with gene expression were analyzed as described previously (40).

ChIP assays. At 2 to 3 days before induction, MCF-7 cells were seeded in phenol red-free Dulbecco modified Eagle medium supplemented with 10% charcoal-dextran-treated fetal bovine serum (Omega Scientific, Inc., Tarzana, CA), 2 mM L-glutamine, and 100 U of penicillin-streptomycin/ml at a density of 5×10^6 cells per 150-mm plates. Cells were subsequently induced with 10^{-8} M E2 for 45 min. ChIP experiments were then performed as described previously (16). Antibodies to ERα (Lab Vision, Ab-10; Santa Cruz Biotechnology, sc-543), H3R17me2 (Upstate Biotechnology, 07-214), H3K18ac (Upstate Biotechnology, 07-354), H3K27ac (Upstate Biotechnology, 07-360), H4K12ac (Upstate Biotechnology, 07-595), H3 (Abcam, ab1791), p300 (Santa Cruz Biotechnology, sc-585), and SRC1 (Santa Cruz Biotechnology, sc-8995) were used for this assay. Purified DNA was used in quantitative PCR (qPCR) analysis. The primers used in this analysis are listed in Table S1 in the supplemental material. Immunoprecipitated DNA amounts were normalized to inputs and are expressed as the relative enrichment.

FAIRE analysis. Formaldehyde-assisted isolation of regulatory elements (FAIRE) was performed as described in reference 21. The primers used in this analysis are listed in Table S1 in the supplemental material.

RESULTS

Distinct enhancer-rich clusters characterize genomic ERα recruitment. In order to better characterize the impact of coactivator action on the ERα cistrome upon E2 stimulation, we have established the relative level of CARM1 activity across the genome of MCF7 breast cancer cells. This was achieved through ChIP studies combined with whole-genome tiling-path microarrays (ChIP-on-ChIP) using an antibody that recognizes exclusively sites of CARM1-dependent arginine methylation, including histone H3 dimethylated on arginine 17 (H3R17me2)

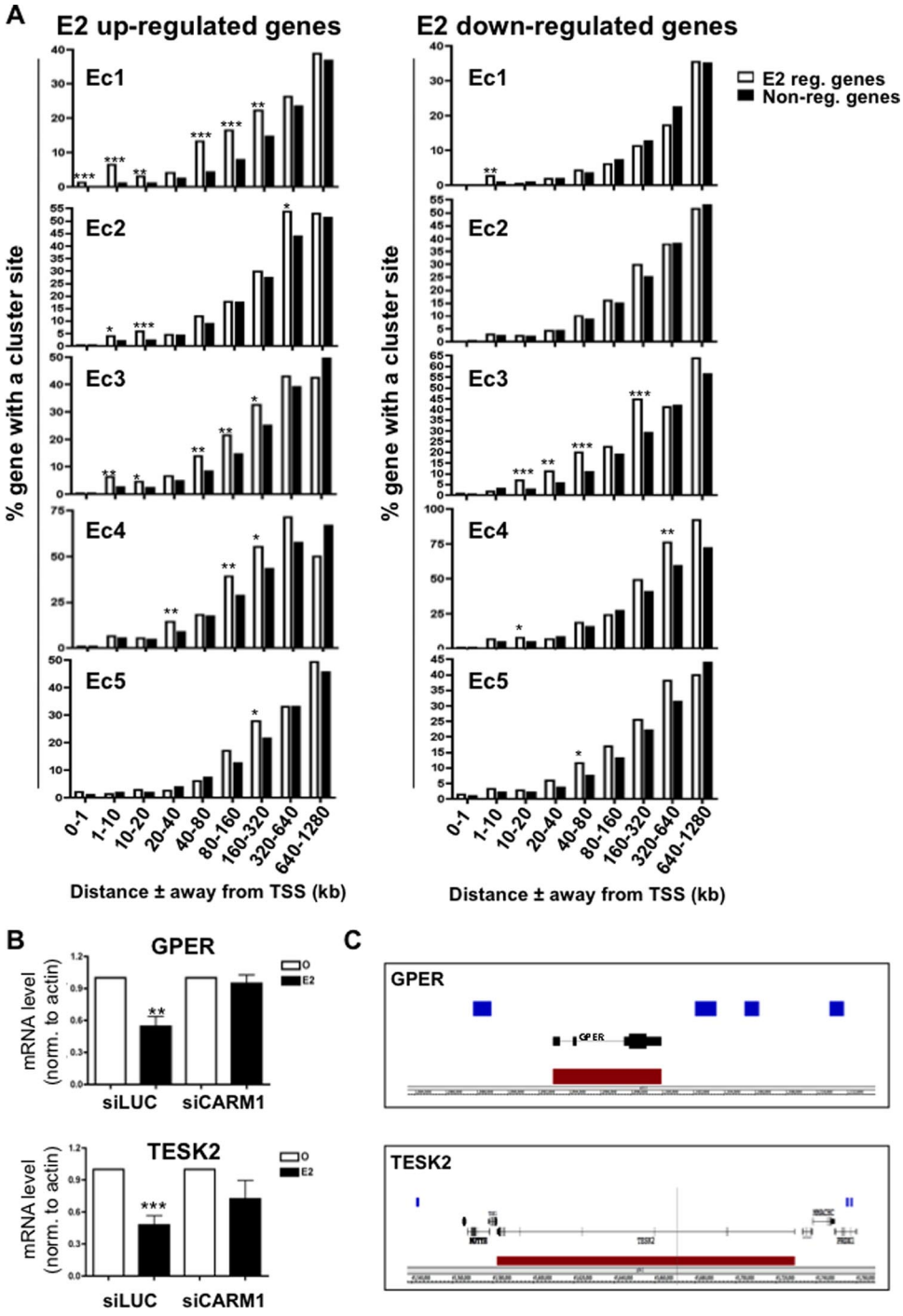

FIG. 3. Clusters associated with CARM1 activation drive the response under E2 treatment. (A) Proportion of E2 upregulated genes compared to nonregulated genes with at least one binding site from a specific cluster within increasing window distances from their TSS in MCF7 cells. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. (B) GPER and TESK2 expression after CARM1 silencing in MCF7 was determined by reverse transcription-qPCR and revealed the requirement for CARM1 in the E2-induced repression of GPER and TESK2. siLUC was used as a control. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. (C) Enrichment of Ec3 cluster sites (blue blocks) near GPER and TESK2 E2-downregulated target genes (red blocks).

and the CARM1-dependent arginine methylation of AIB1 (see Fig. S1 and S2 in the supplemental material) (9, 48, 67). More than 4,088 and 4,461 high-confidence sites were identified before and after E2 stimulation, respectively (FDR, 6%) (see Fig. S1A and B in the supplemental material). Interestingly, CARM1 activity was found predominantly (94.1%) at regions far from known promoters (see Fig. S1C in the supplemental material). The Pol II cistrome was also determined in the

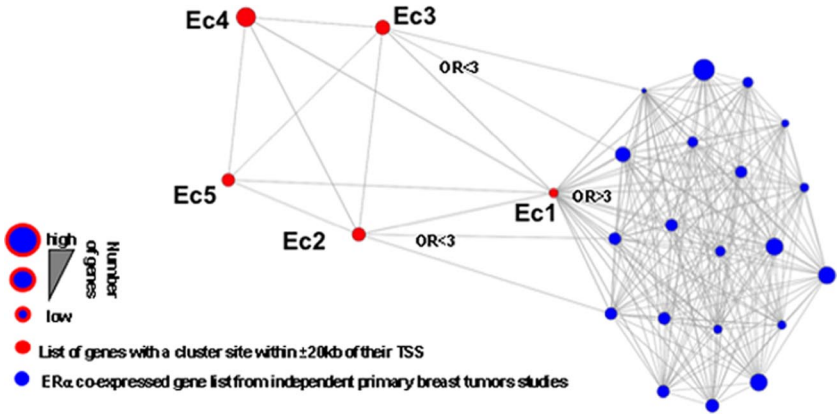

FIG. 4. ERα-positive primary breast tumor expression profile relates to clusters associated with CARM1 activation. Relationship between cluster-associated gene list (genes with a binding site from a given cluster within 20 kb of their TSS) and genes overexpressed in ERα-positive primary breast tumors (the top 1, 5, or 10% overexpressed genes from primary breast tumors were included in the analysis). Twenty independently defined ERα-positive primary breast tumor overexpressing gene signatures (blue) were compared using an Oncomine Concepts Map to the five enhancer clusters (red) derived gene lists. Odds ratios (OR) are presented when clusters are significantly associated with independent primary breast cancer overexpression gene signatures ($P \leq 6e^{-6}$).

absence of E2 to address the role of promoter-associated factors in this system (see Fig. S3 in the supplemental material). As anticipated, of the 7,420 high-confidence sites (FDR, 5%) recruiting Pol II, 55.4% were recruited within 1 kb upstream of annotated transcription start sites (TSS) (see Fig. S3A to C in the supplemental material).

To establish the contribution of CARM1 activation and Pol II recruitment to E2 signaling, we combined our newly derived cistromes with previously published cistromes for FoxA1 in the presence or absence of E2, as well as ERα and Pol II in E2-treated MCF7 cells (7, 40). We first established the binding activity as determined by MAT score (29) for all factors across the 25,416 high-confidence regions recruiting at least one of these factors in MCF7 cells. The use of *k*-means clustering revealed five enhancer-rich clusters and two promoter-rich clusters (Fig. 1A to C). Interestingly, each cluster consisted of sites demonstrating high sequence conservation across vertebrate species (see Fig. S4 in the supplemental material). ERα was most significantly recruited after E2 stimulation to clusters Ec1 (23% of the 5782 ERα high-confidence sites) and Ec3 (47% of the 5782 ERα high-confidence sites), with <2.6% of the high-confidence sites found at promoters (Fig. 1A and C). The previously reported sites of FoxA1 recruitment favoring ERα binding were found predominantly in cluster Ec1 but not Ec3 (Fig. 1A and C). Correspondingly, both cluster Ec1 and Ec3 were highly enriched for the ERE half-site motif, while the Forkhead motif was only enriched in cluster Ec1 (Fig. 1D). In addition, both clusters demonstrated E2-induced CARM1 activity as measured by the MAT score (Fig. 1A and C and see Fig. S5 in the supplemental material). Cluster Ec2 consisted of sites found at <2.1% of promoters where FoxA1 was strongly recruited but where ERα had low binding activity (Fig. 1A to C and see Fig. S5 in the supplemental material). Accordingly, the Forkhead motif was enriched in this cluster, while the ERE half-site motif was not significantly enriched (Fig. 1D). In addition, CARM1 activity was not induced on sites from this cluster following E2 stimulation (Fig. 1A and C and see Fig. S5 in the supplemental material). Finally, sites from the enhancer-rich clusters Ec4 and Ec5, with <6.1% of sites at promoters, did not demonstrate strong ERα recruitment. However, sites from cluster Ec4 but not Ec5 associated with FoxA1 binding. In addition, ligand-independent CARM1 activity was associated with cluster Ec5 independently of E2 stimulation (Fig. 1A and C and see Fig. S5 in the supplemental material). Globally, these data reveal that various classes of regulatory elements are established under E2 stimulation, and those associated with ERα and FoxA1 recruitment, as well as CARM1 activity, are found predominantly in enhancer regions across the genome.

E2-induced CARM1 activity associates with coactivator recruitment, histone modifications, and chromatin opening. A common feature of sites from clusters Ec1 and Ec3 predominantly involved in the E2-mediated regulation of gene expression is their association with the induction of CARM1 activity after E2 treatment (Fig. 1A and C). In order to better characterize the active state of these enhancer regions, we investigated the level of coactivator recruitment and histone modifications after E2 treatment. Sites recruiting ERα and associated with CARM1 activity significantly recruited other coactivators, such as p300 and SRC1, under E2 stimulation (Fig. 2A and see Fig. S6 in the supplemental material). Similarly, histone modifications, such as acetylation of lysine 18 or 27 on histone H3 (H3K18ac, H3K27ac), as well as on lysine 12 of histone H4 (H4K12ac), were significantly induced by E2 on these same sites (Fig. 2B). ERα binding sites not associated with the induction of CARM1 activity did not demonstrate any significant induction of coactivator recruitment or histone modification under E2 treatment (Fig. 2A and B). It is noteworthy that ERα binding sites undergoing coactivator recruitment and histone modifications after E2 treatment also associated with E2-induced chromatin opening measured by histone H3 density or extractability by FAIRE (21) (Fig. 2C). Considering that 30% of ERα binding sites are not associated with clusters Ec1 or Ec3 typified by E2-inducted CARM1 activity, our results reveal that the specific transcriptional response to E2 is in part de-

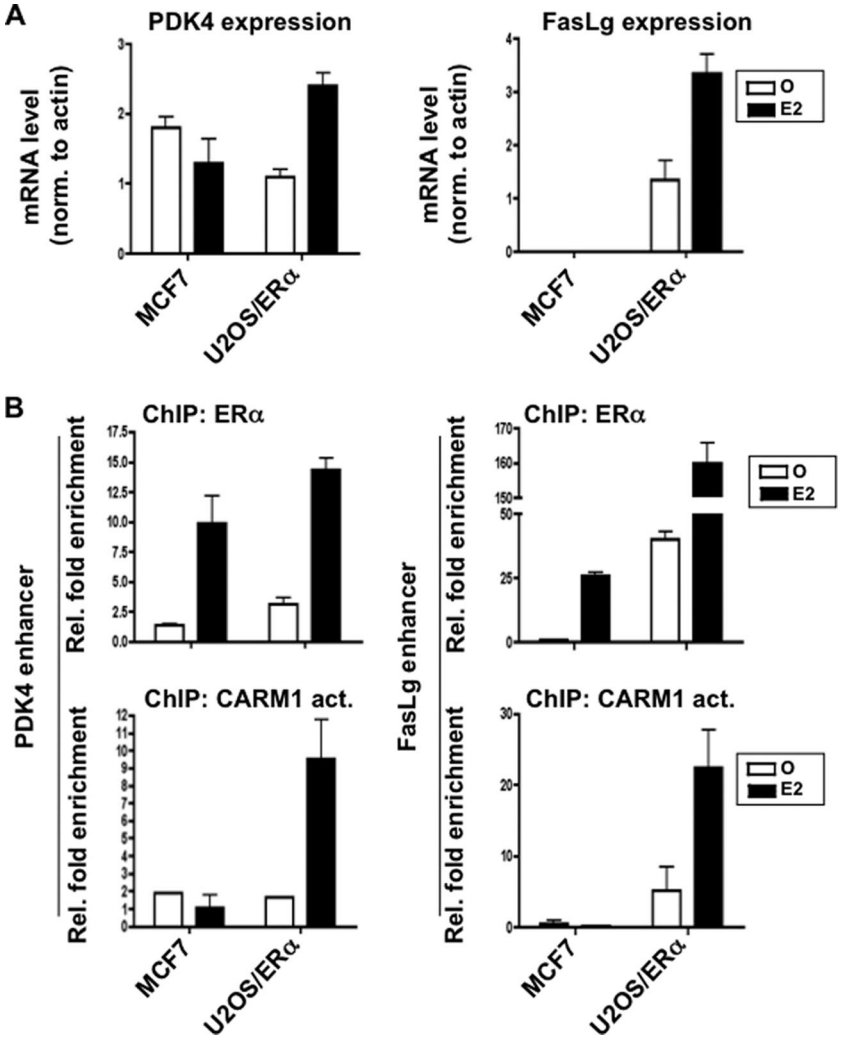

FIG. 5. Cell-type specific coactivation of ERα binding sites associates with the transcriptional response. (A) Relative expression of PDK4 and FasL genes after E2 treatment for 3 h in MCF7 breast cancer and U2OS/ERα cells. (B) Relative enrichment of ERα and CARM1 activity established by ChIP in both MCF7 and U2OS/ERα cells after E2 treatment at the PDK4 and FasL enhancers.

pendent on the selective activation of a fraction of sites recruiting ERα.

CARM1 activation on ERα binding sites drives the transcriptional response to E2. In order to address the role of the various enhancer-rich clusters in gene regulation, we established the proportion of genes regulated after 3 h of E2 treatment versus nonregulated genes with at least one binding site from a particular cluster within increasing window distances in kilobases from the TSS. This revealed a significant enrichment of E2 upregulated genes over nonregulated genes with regard to sites from clusters Ec1, Ec2, and Ec3 from various window distances from the TSS, as far as 160 to 320 kb for both Ec1 and Ec3 (Fig. 3A). Hence, our results suggest that the subset of the ERα cistrome subject to CARM1 activation upon E2 treatment, whether strongly or weakly associated with FoxA1 binding, is responsible for E2-mediated gene induction. Thus, the previously suggested role for CARM1 in mediating the E2 response (19, 67) is due to its activity at enhancer regions defined by a specific subset of the ERα cistrome. Interestingly, genes downregulated after E2 stimulation were significantly enriched over nonregulated genes near sites primarily from cluster Ec3 that could be as far away as 160 to 320 kb (Fig. 3A). Accordingly, silencing CARM1 (see Fig. S2A in the supplemental material) significantly prevented the E2-mediated repression of GPER and TESK2 (Fig. 3B and C). Hence, this finding suggests a predominant role for ERα sites associated weakly or not at all with FoxA1 and undergoing ligand-dependent CARM1 activity in E2-mediated gene downregulation.

In order to address the physiological relevance of the different clusters, we compared the list of genes with a binding site from a particular cluster within 20 kb of their TSS to the top genes coexpressed with ERα in primary breast tumors from 20 independent studies (4, 10, 13, 20, 23, 26, 27, 43, 44, 50, 51, 54, 55, 60–62, 64–66, 68, 70). Remarkably, genes coexpressed with ERα defined in 19 out of the 20 independent studies were highly associated (odds ratio ≥ 3) with sites from Ec1 within 20 kb of their TSS (Fig. 4). Less significant association (odds ratio between 2 and 3) between ERα coexpressed genes and sites

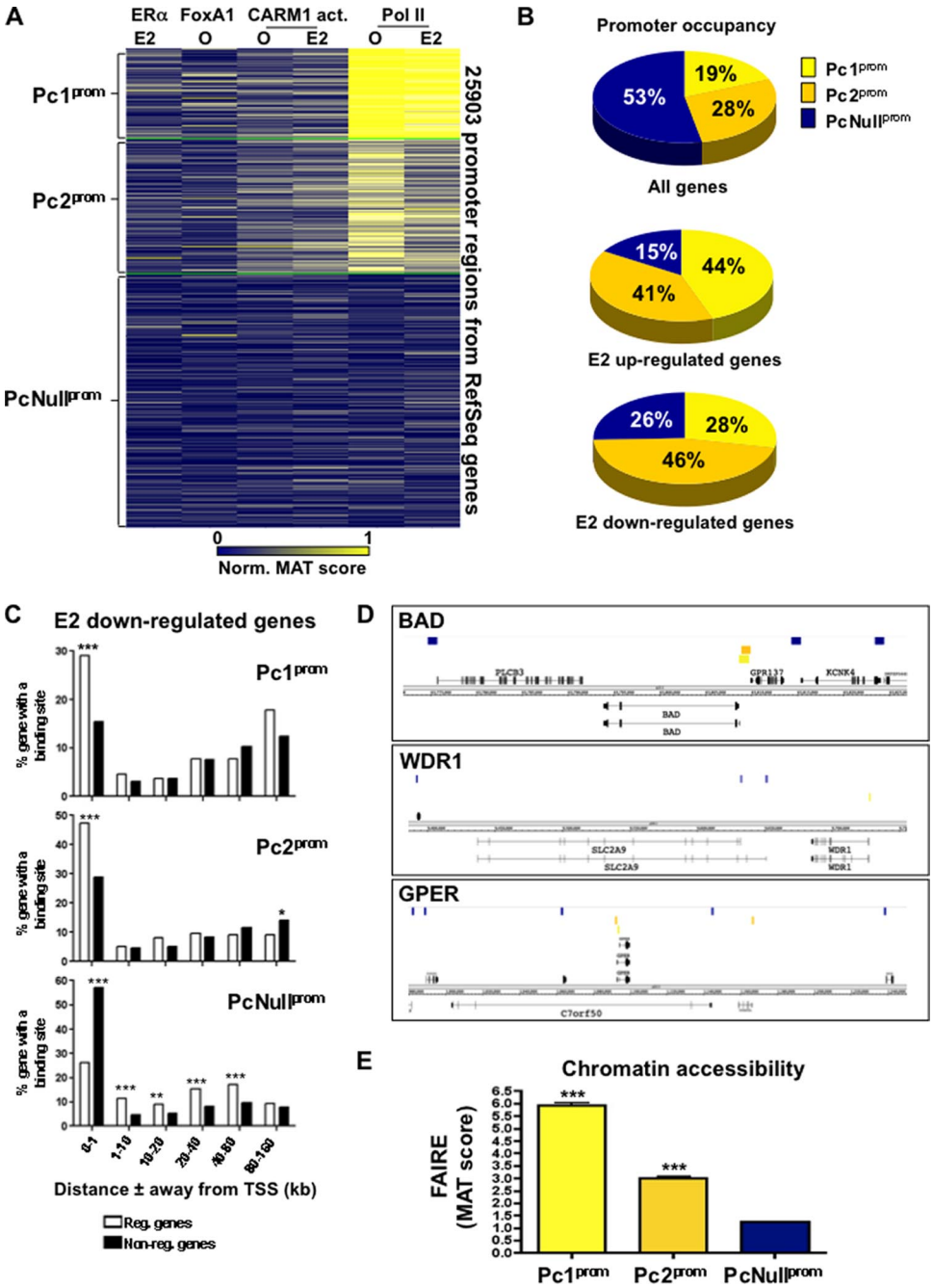

FIG. 6. Pol II occupied promoter of E2 target genes prior to stimulation. (A) Cluster analysis performed as described for Fig. 1A across the 25,903 promoter regions associated with the RefSeq genes. (B) Proportions of all, E2-upregulated, and downregulated genes with a promoter typical of clusters Pc1^{prom}, Pc2^{prom}, or PcNull^{prom}. (C) Enrichment of E2-downregulated versus nonregulated genes with at least one promoter of the Pc1^{prom}, Pc2^{prom}, or PcNull^{prom} cluster within increasing window distances from the genes' TSS. (D) Specific examples of E2 downregulated genes surrounded by gene with Pol II deprived promoters. Sites from cluster Pc1^{prom} (orange), Pc2^{prom} (pink), or PcNull^{prom} (dark blue) are presented with respect to E2-downregulated genes (red block). (E) Relative chromatin accessibilities of promoters from cluster Pc1^{prom}, Pc2^{prom}, or PcNull^{prom} measured by FAIRE-ChIP. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

from Ec2 and Ec3 was also detected in 2 out of the 20 independent expression profiles from primary breast tumors (Fig. 4). Hence, these results further support the predominant regulatory role of sites from cluster Ec1 and less significantly from clusters Ec2 and Ec3 in the establishment of the phenotype of ERα-positive breast cancers.

Further evidence for the association between sites of ERα recruitment and their activation to mediate transcriptional program originates from the comparison of the MCF7 breast cancer and U2OS osteosarcoma cell lines. Indeed, E2 treatment in both cell lines allows for the recruitment of ERα to a number of common sites (34). Interestingly, the transcriptional

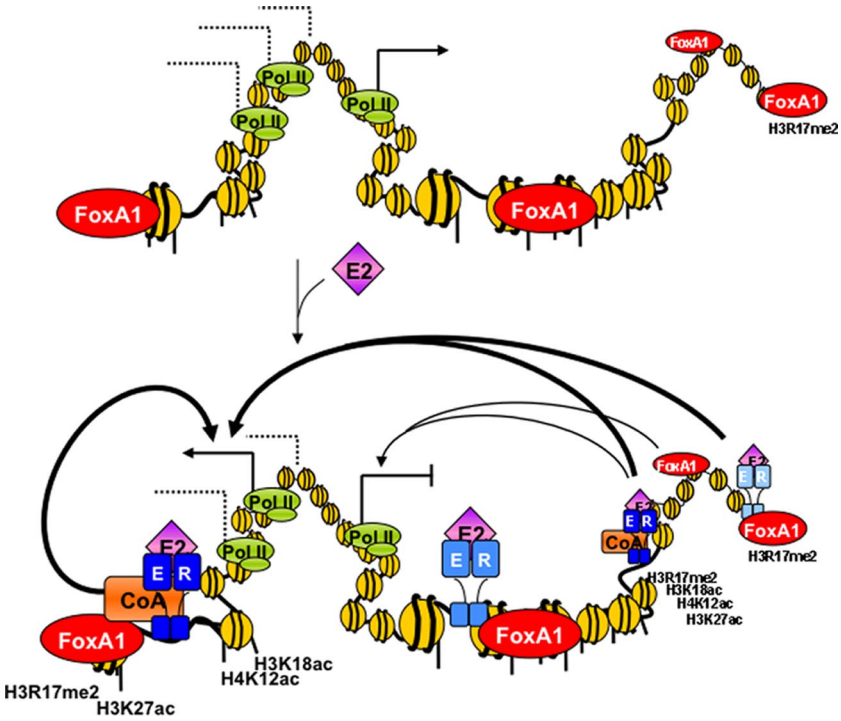

FIG. 7. Model for the selection of functional and active enhancer sites in response to estrogen stimulation in breast cancer cells. A schematic representation of the transcriptional response to E2 stimulation in breast cancer cells is shown. The functional association between ERα recruiting sites undergoing coactivator (CoA) recruitment/activation and histone modifications with transcriptional regulation of the gene harboring Pol II at their promoters both prior to and after E2 stimulation is depicted. Sites of ERα recruitment not associated with these secondary events do not significantly impact E2-induced regulation of gene expression.

program is cell line specific (34). For instance, PDK4 and FasL are two U2OS-specific E2-induced genes (Fig. 5A). Although ERα gets recruited to the PDK4 and FasL enhancers in both cell lines, they are coactivated only in U2OS cells (Fig. 5B). These results suggest that ERα recruitment associates with coactivation in order to mediate gene expression.

Pol II occupies the promoter of E2 regulated genes prior to stimulation. To address how different components of the transcriptional response to E2 signaling impact promoter activity in breast cancer cells, we performed *k*-means clustering on the 25,903 RefSeq gene promoters as described for Fig. 1A. Three distinct clusters could be defined (Fig. 6A). The first promoter cluster ($Pc1^{prom}$) consisting of 4,846 sites revealed strong Pol II recruitment both prior to and after E2 stimulation and no significant recruitment of ERα, FoxA1 nor evidence of CARM1 activity (Fig. 6A). Similarly, the 7,316 promoters found in the second cluster ($Pc2^{prom}$) were specifically enriched for Pol II both prior to and after E2 stimulation, albeit at lower levels than on sites from $Pc1^{prom}$ (Fig. 6A). Finally, 13,741 promoters ($PcNull^{prom}$) in MCF7 cells were not significantly associated with the recruitment of Pol II, ERα, FoxA1, or CARM1 activity (Fig. 6A). Interestingly, more than 47% of promoters were associated with sites from either $Pc1^{prom}$ or $Pc2^{prom}$; hence, with Pol II occupied promoters prior to E2 stimulation (Fig. 6A and B and see Fig. S7A in the supplemental material). Strikingly, more than 85% of the E2 upregulated and 74% of E2 downregulated genes had a promoter typical of either cluster $Pc1^{prom}$ or $Pc2^{prom}$ (Fig. 6B and see Fig. S7A in the supplemental material). Furthermore, downregulated genes were typically found in the regions of Pol II unoccupied promoters (Fig. 6C and D). In agreement with this, the promoters of genes surrounding downregulated genes were found in condensed chromatin measured by FAIRE (Fig. 6E) (17). This suggests that stimulus-dependent gene regulation is predominantly dependent on receptive promoters as defined by the presence of Pol II prior to stimulation and on the state of promoter occupancy in neighboring genes.

DISCUSSION

The selective utilization of enhancer regions and promoters is central to the establishment of lineage-specific transcriptional programs and stimuli specific responses. Exploiting the E2 signaling pathway, we have combined the cistromes from different components of a transcriptional response, namely, the pioneer factor FoxA1, the transcription factor ERα, a marker of the activity of the coactivator CARM1, and Pol II. Our results reveal that FoxA1-dependent and -independent ERα sites coactivated upon E2 treatment are predominantly driving the response to E2 in breast cancer cells (Fig. 7). Hence, the specific transcriptional program associated with E2 stimulation is not only dependent on the restricted genomic recruitment of ERα but also on the activation of a selected number of binding sites associated with coactivator recruitment and histone modifications. This is in agreement with the central role of coactivators in the response to E2 in both cell lines and mouse models (19, 46, 59, 67). Interestingly, our results also reveal an association between ERα binding sites

displaying E2-induced CARM1 activity and gene repression. As we previously suggested, squelching and/or displacement of transcriptional units with greater regulatory capacities could account for this association (7). In addition, we demonstrate on a genome-wide scale that enhancers located as far as 160 to 320 kb from the TSS of the E2 target genes mediate the transcriptional response. This is in accordance with previous studies revealing the intrachromosomal interactions required for optimal transcriptional response upon E2 stimulation in MCF7 cells (6, 12). Considering the commonality of such long-range interactions between promoters and enhancers (38, 49, 63), defining how these are established on a genome-wide scale is of fundamental importance.

Furthermore, we show that Pol II promoter occupancy is typical of E2 responsive genes in MCF7 breast cancer cells that are both up- and downregulated. This is reminiscent of studies in *Drosophila* revealing the contribution of stalled or poised Pol II at the promoter of genes involved in the response to stimuli and developmental signals (47, 69). It also parallels previous work revealing Pol II at the promoter of unexpressed genes (2, 24, 31–33, 53). It is consistent with the concept of Pol II foci in the nucleus known as transcription factories that remain intact in the absence of transcription (45). In fact, postrecruitment regulation of Pol II was recently revealed to be central for the rapid signaling response to estrogen (33). Interestingly, we identified a difference between the genomic environments of E2 up- versus downregulated genes. Indeed, although Pol II typically occupies the promoter of E2 downregulated genes, the promoters of surrounding genes tend to be deprived of Pol II. Hence, the transcriptional response is dependent on the presence of a receptive promoter typified by Pol II occupancy prior to stimulation.

Globally, our results reveal that the specificity of the transcriptional response to E2 stimulation is dependent on the interplay between receptive promoters occupied by Pol II prior to stimulation and subclasses of ERα enhancers associated with E2-induced coactivator activity. Considering the unique expression profiles associated with ERα activation under distinct stimuli (11, 14), it remains to be established whether distinct subclasses of the ERα cistrome will be involved in these responses as well.

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REVIEW

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Cistromics of hormone-dependent cancer

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Abstract

Alterations in transcription programs are a fundamental feature of cancer. Nuclear receptors, such as the estrogen receptor alpha (ERα) and androgen receptors (ARs), are central in this process as they can directly impact gene expression through interaction with the chromatin and subsequent association with coregulators and the transcriptional machinery. Unbiased genome-wide investigations have demonstrated the predominant recruitment of both ERα and AR to distant (non-promoter)-regulatory elements. Furthermore, these studies revealed a clear relationship between sites of transcription factor recruitment and gene regulation. Indeed, expression profiles from AR-positive primary prostate tumors and cell lines directly relate to the AR cistrome in prostate cancer cells, while the ERα cistrome in breast cancer cells relates to expression profiles from ERα-positive primary breast tumors. Additionally, cell-type-specific ERα cistromes are linked to lineage-specific estrogen-induced expression profiles in different cell types, for example osteosarcoma and breast cancer cells. The pioneer factor forkhead box A1 (FoxA1/HNF3α) plays a central role in AR and ERα signaling. It is recruited in a lineage-specific manner translating the epigenetic signature consisting of mono- and dimethylated histone H3 on lysine 4 (H3K4me1/me2) into functional regulatory elements. Hence, through the interplay between the pioneer factor, namely FoxA1, and epigenetic events, the transcriptional potential of a given cell lineage is predefined. Since this directly impacts signaling through nuclear receptors, these discoveries should significantly impact the development of novel therapeutic strategies directed against multiple types of cancer.

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Characteristics of nuclear receptor cistromes

Gene expression profiling of cancer has led to important new insights both in terms of classification and outcome. This is exemplified in breast cancer through studies where different subtypes of primary breast tumors have been identified (Sorlie *et al*. 2001, 2003), which also correspond to different disease/treatment outcomes (Sorlie *et al*. 2003). Similarly, prostate cancer development associates with transcriptional programs distinct from normal tissues (Welsh *et al*. 2001, Lapointe *et al*. 2004, Yu *et al*. 2004). Therefore, understanding the mechanisms that lead to these altered expression profiles is fundamental to the development of effective therapeutic intervention against cancers.

Nuclear receptors are central to the development of both breast and prostate cancer. The estrogen receptor alpha (ERα) is a fundamental feature of more than two-thirds of breast cancers (Sorlie *et al*. 2001, 2003), while prostate cancer is highly dependent on the actions of the androgen receptor (AR; Heinlein & Chang 2004). Both ERα and AR are ligand-dependent transcription factors recruited directly to the chromatin through the estrogen-responsive elements (EREs) and androgen-responsive elements (AREs) respectively. They are also indirectly recruited to other genomic regions through a tethering mechanism involving other transcription factors such as AP-1 and Sp1 (Sanchez *et al*. 2002). Through their interplay with coregulators, ERα and AR regulate the expression of genes central to breast and prostate cancer development, including *CCND1*, *E2F1*, *Myc* as well as *TMPRSS2*, and *PSA* respectively (Prall *et al*. 1998, Balk *et al*. 2003, Demichelis & Rubin 2007, Stender *et al*. 2007, Frietze *et al*. 2008, Setlur *et al*. 2008).

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Recent technological advancements have allowed the mapping of the regulatory regions recruiting either directly or through tethering mechanisms these receptors on a genome-wide scale defining their cistromes (Lupien *et al*. 2008). Indeed, upon stimulation ERα and AR are recruited to 1000 of sites in breast and prostate cancer cell lines respectively (Carroll *et al*. 2005, 2006, Lin *et al*. 2007, Wang *et al*. 2007, Hua *et al*. 2008, Hurtado *et al*. 2008, Liu *et al*. 2008, Lupien *et al*. 2008). These unbiased genome-wide studies have revealed the preferential recruitment of both ERα and AR with non-promoter-regulatory elements. This pattern of promoter-distant recruitment is also typical of other transcription factors in various systems such as forkhead box A1 (FoxA1), RelA (p65), NRSF, SRA, GABP, and many more (Carroll *et al*. 2006, Johnson *et al*. 2007, Lim *et al*. 2007, Lupien *et al*. 2008, Valouev *et al*. 2008). This contrasts with the distribution of other transcription factors such as E2F family members that are primarily recruited to promoters (Bieda *et al*. 2006, Xu *et al*. 2007). This highlights the complexity of the transcriptional response in higher order eukaryotes as regulatory elements can be found hundreds of kilobases (kb) away from their target genes, but still be brought into promoter close proximity through chromosome looping (Dekker 2008). In fact, this process has been previously reported on a limited subset of ERα- and AR-binding sites for regions up to 140 kb away from the target gene (Carroll *et al*. 2005, Deschenes *et al*. 2007, Wang *et al*. 2007). Hence, a key milestone in transcriptional biology will consist of establishing the sum of all chromosome loops guiding transcriptional responses on a genome-wide scale.

From nuclear receptor cistrome to transcription

The comparison of nuclear receptor cistromes and hormone-regulated expression profiles reveals a clear relationship between the two. Indeed, genes over-expressed in ERα-positive primary breast tumors as well as estrogen target genes in breast cancer cell lines are preferentially surrounded by estrogen-induced ERα-binding sites found in this same system (Carroll *et al*. 2006, Lupien *et al*. 2008, Fig. 1). Similarly, MCF7 cells overexpressing AKT induce a unique ERα cistrome that relates to the AKT-dependent expression profile (Bhat-Nakshatri *et al*. 2008, Fig. 1). This is also observed in osteosarcoma where the estrogen-induced ERα cistrome, distinct from the ERα cistrome in breast cancer cells, directly relates to the estrogen-induced expression profile in osteosarcoma (Krum *et al*. 2008, Fig. 1). Furthermore, ERα-binding sites cluster around these regulated genes (Krum *et al*. 2008). Similarly, the AR cistrome in androgen-dependent prostate cancer cells relates to the androgen-induced transcriptional program in these same cells as well as to the expression profile from primary prostate tumors (Wang *et al*. 2007, 2009, Lupien *et al*. 2008, Fig. 2). Furthermore, as prostate cancer cells become castration resistant following androgen-deprivation therapy, they acquire an altered expression program accompanied by a related novel AR cistrome (Wang *et al*. 2009). Hence, the capacity to establish specific cistromes under distinct activation and in different lineages is central to the implementation of transcriptional programs that define the nature of cellular identity.

Coregulators central to nuclear receptor cistromes

Through sequence analysis of regulatory regions recruiting either ERα or AR, conserved networks of regulatory factors have been defined (Carroll *et al*. 2005, 2006, Laganiere *et al*. 2005, Green & Carroll 2007, Wang *et al*. 2007, Hurtado *et al*. 2008). Noteworthy, the GATA, OCT, PAX, NKX, and LEF motifs are significantly enriched near the center of ERα- and/or AR-binding sites. GATA3 recognizing the GATA motif was revealed to be part of a positive cross-regulatory loop with ERα in breast cancer cells required for the estrogen-mediated cell proliferation (Eeckhoute *et al*. 2007). In prostate cancer cells, GATA2 also recognizing the GATA motif was found to interact with AR and potentiates its regulation of target genes (Perez-Stable *et al*. 2000, Wang *et al*. 2007). Similarly, Oct-1 recognizing the OCT motif was shown to physically interact with AR and its expression is required for AR-mediated transcriptional regulation in prostate cancer cells (Gonzalez & Robins 2001, Wang *et al*. 2007). Furthermore, Oct-1 is co-recruited with ERα in breast cancer cells regulating key target genes, namely *CCND1* (Cicatiello *et al*. 2004, Carroll *et al*. 2006). More recently, PAX2 co-recruitment with ERα to the *ERBB2*-regulatory element has revealed its central role as a transcriptional repressor required for inhibition of *ERBB2* expression in breast cancer cells (Hurtado *et al*. 2008). Accordingly, loss of PAX2 recruitment allowed for *ERBB2* expression in the presence of the anti-estrogen tamoxifen conferring anti-estrogen-resistant-like properties to normally anti-estrogen-sensitive breast cancer cells (Hurtado *et al*. 2008). Other factors such as LEF-1 and Nkx3-1 whose DNA recognition motif is enriched

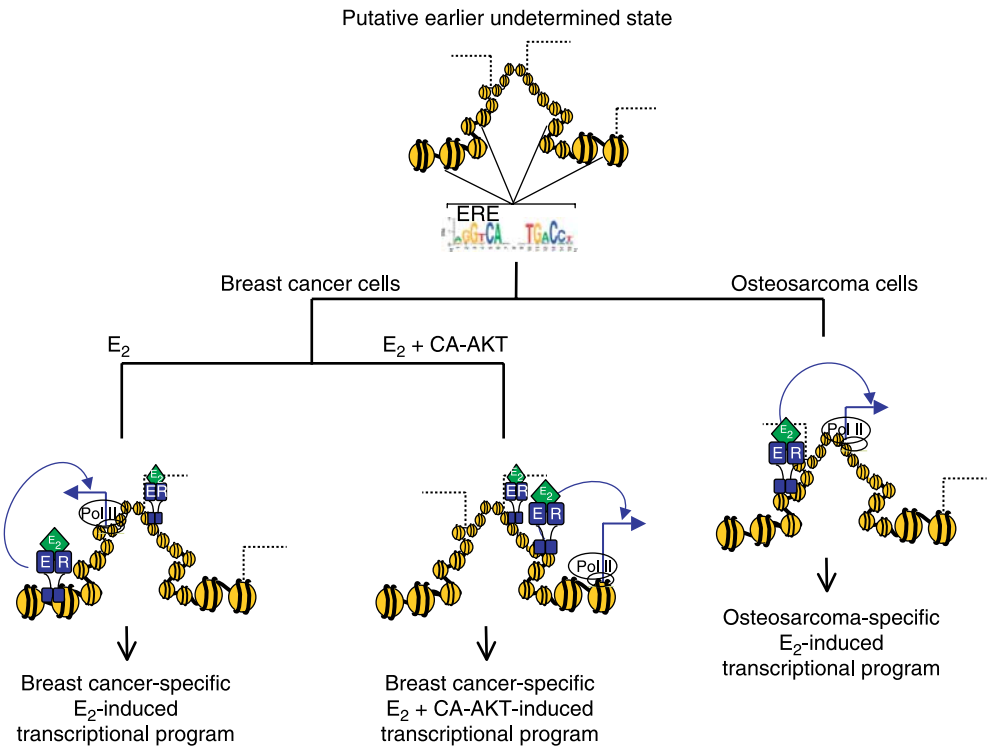

Figure 1 Lineage- and stimuli-specific transcriptional programs are dependent on differential recruitment of ERα. Thousands of putative ERα-binding sites are found across the human genome. This includes over 60 000 estrogen-responsive elements (EREs) and a number of regions recruiting ERα through a tethering mechanism. However, lineage-specific ERα recruitment, as reported between breast and osteosarcoma cancer cell lines, is central to the unique transcriptional program generated in each cell type following estrogen (E_2) treatment. Similarly, the transcriptional program activated through the PI3K/AKT pathway in MCF7 cells expressing a constitutively active AKT (CA-AKT) is dependent on a unique ERα recruitment pattern.

in ERα-binding sites in breast cancer cells behave in a distinct manner. Indeed, instead of being co-recruited with ERα following estrogen stimulation, these transcription factors are bound at the basal state and block ERα recruitment abrogating estrogen growth-promoting properties (Holmes *et al*. 2008). Since LEF-1 and Nkx3-1 can associate with the histone deacetylase HDAC1, increased chromatin condensation is thought to be fundamental to block ERα recruitment (Holmes *et al*. 2008). Therefore, by defining the cistromes of ERα and AR, the intricate interplay between transcription factors and their network of coregulatory proteins taking place at the chromatin is gradually being revealed.

Pioneer factors as mediator of lineage-specific transcriptional programs

The FKH motif is an additional motif highly enriched in both ERα and AR cistromes (Carroll *et al*. 2005, 2006, Laganiere *et al*. 2005, Wang *et al*. 2007). The forkhead family member FoxA1 (HNF3α) is a key partner for ERα and AR transcriptional activity in breast and prostate cancer respectively, recognizing the FKH motif. It was first characterized as a pioneer factor in liver tissue (Gualdi *et al*. 1996, Cirillo *et al*. 1998, Bossard & Zaret 2000). More recently, its ATP-independent chromatin-remodeling activity, distinguishing it from the classical SWI/SNF complex, has shown to be central for ERα recruitment in breast cancer cells (Carroll *et al*. 2005, Laganiere *et al*. 2005, Eeckhoute *et al*. 2006), while it was found to physically interact with AR in prostate cancer cells (Gao *et al*. 2003, Wang *et al*. 2007). Present on the chromatin at the basal state, FoxA1 is found at more than 60% of ERα- and AR-binding sites driving the transcriptional response in breast and prostate cancer cells respectively (Lupien *et al*. 2008). In fact, through its chromatin-remodeling activity, FoxA1 allows for the opening of specific genomic regions in the absence of hormone (Carroll *et al*. 2005, Laganiere *et al*. 2005, Eeckhoute *et al*. 2006). Hence, under hormonal stimulation, ERα and AR are recruited to FoxA1 sites harboring permissive sequences such as EREs and AREs (Lupien *et al*. 2008, Fig. 3). In accordance with its predominant role in ERα signaling, FoxA1 is

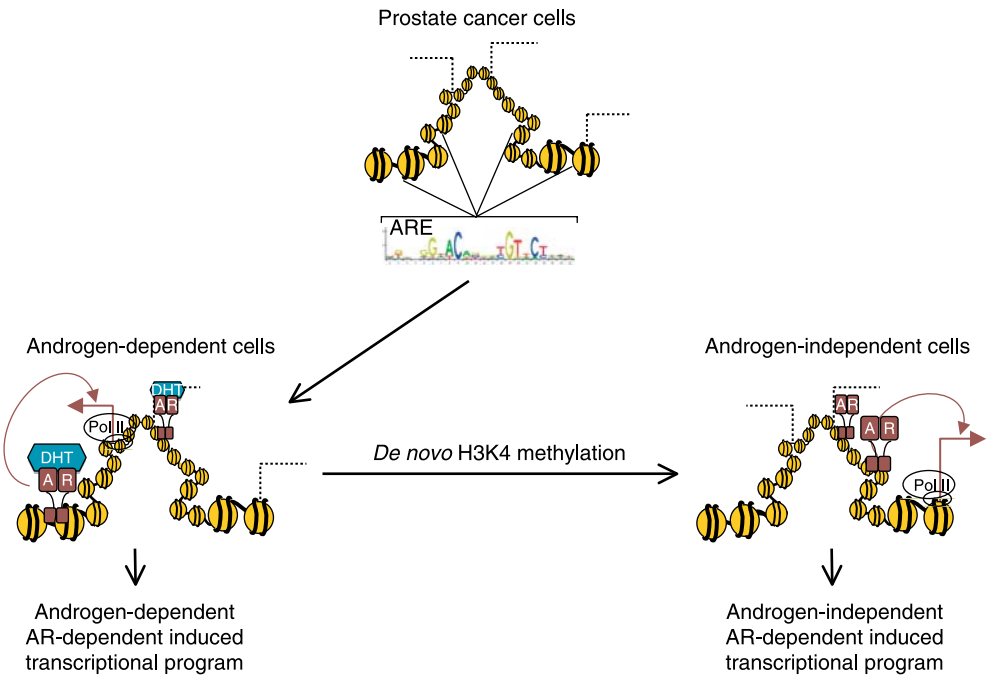

Figure 2 Chromatin architecture reprogramming in castration-resistant prostate cancer cells. Similar to ERα in breast cancer cell lines, AR is recruited to a fraction of its putative binding sites in androgen-dependent prostate cancer cells. In castration-resistant prostate cancer cells, the genome-wide AR recruitment pattern is altered. This is dependent on the reprogramming of the chromatin architecture typified by *de novo* methylation of lysine 4 on histone H3 (H3K4me).

typically highly expressed in ERα-positive primary breast tumors and is an important marker of breast cancer subtype and prognosis (Habashy *et al*. 2008, Thorat *et al*. 2008). FoxA1 is also highly expressed in prostate cancer where it is believed to contribute to the establishment of specific gene expression programs (Mirosevich *et al*. 2006). However, the comparison of FoxA1 cistromes between breast and prostate cancer cells reveals its cell-type-specific recruitment (Lupien *et al*. 2008). Indeed, less than 40% of FoxA1-binding sites are shared between these two cell lines supporting the notion that FoxA1 is recruited in a lineage-specific fashion. Importantly, because FoxA1 guides the recruitment of transcription factors, such as ERα and AR, lineage-specific transcriptional programs dependent on these transcription factors are directly affected by FoxA1′s cell-type-specific recruitment (Fig. 3).

Epigenetic signatures define lineage-specific functional regulatory elements

The requirement for lineage-specific recruitment of the pioneer factor FoxA1 highlights the importance of understanding how such differential recruitment takes place. Recently, specific epigenetic signatures distinguishing non-promoter from promoter-regulatory elements have been reported (Santos-Rosa *et al*. 2002, Ng *et al*. 2003, Schneider *et al*. 2004, Schubeler *et al*. 2004, Bernstein *et al*. 2005, Pokholok *et al*. 2005, Heintzman *et al*. 2007). This signature, characterized by different methylation states (mono-, di-, or tri-) of lysine 4 on histone H3 (H3K4me1, me2, or me3), was also found associated with distinct chromatin regions permissive for transgene expression (Yan & Boyd 2006). Specifically, H3K4me1 and H3K4me2 were associated with non-promoter-regulatory elements, while H3K4me3 was found at promoter regions (Heintzman *et al*. 2007). Furthermore, regions enriched in H3K4me2 or me3 associate with DNase I hypersensitivity, a marker of active-regulatory regions (Xi *et al*. 2007). Therefore, this supports the notion that H3K4me2 and H3K4me3 are specific for functional non-promoter and promoter-regulatory elements respectively.

In agreement, the cell-type-specific cistromes for the pioneer factor FoxA1 in breast and prostate cancer cells are dependent on H3K4me1/me2 distribution (Lupien *et al*. 2008). In reality, H3K4me1/me2 defines in a lineage-specific manner through which regulatory elements are able to recruit FoxA1 (Fig. 3). Indeed, removal of this epigenetic signature through over-expression of the lysine demethylase KDM1/LSD1

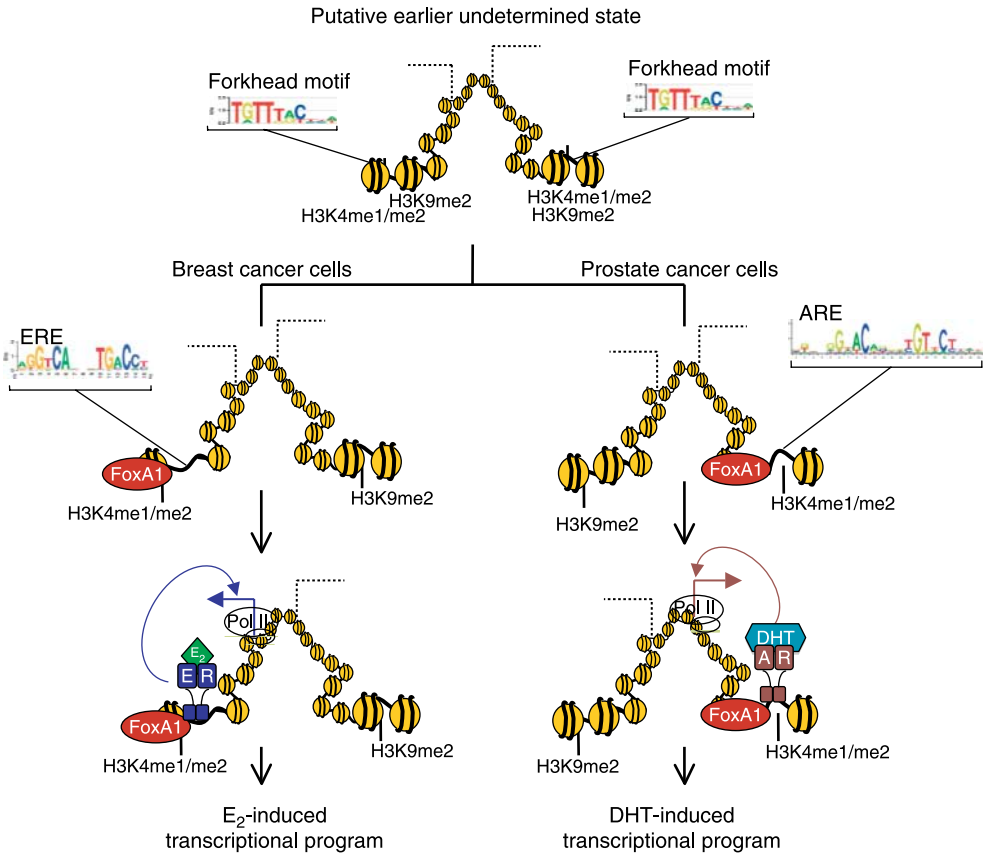

Figure 3 Lineage-specific distribution of histone H3 methylation on lysine 4 guides FoxA1 recruitment. Differentiated cells are characterized by a unique distribution of epigenetic marks. FoxA1 will specifically be recruited to genomic regions harboring the forkhead motif (FKH) marked by H3K4me1/me2. Through its chromatin-remodeling activity, neighboring chromatin will be further opened and accessible to other transcription factors such as ERα and AR in breast and prostate cancer cells respectively. By contrast, FKH regions lacking H3K4me1/me2 will typically associate with H3K9me2 and be deprived of FoxA1 recruitment. Hence, the lineage-specific distribution of H3K4me1/me2 and H3K9me2 guides FoxA1 binding, which in turn restricts the recruitment of other transcription factors.

prevents FoxA1 recruitment (Lupien *et al*. 2008, Wang *et al*. 2009). This signature is also characteristic of the ERα cistrome not overlapping with FoxA1 (M L and M B unpublished data). In fact, lineage-specific ERα cistromes correlate with the H3K4me1/me2 distribution. Indeed, ERα-binding sites specific to breast cancer or osteosarcoma cells relate to the unique distribution of H3K4me1/me2 in these respective cell lines, regardless of FoxA1 status (Krum *et al*. 2008). Similarly, the recruitment of AR to novel sites in castration-resistant prostate cancer cells is dependent on the *de novo* H3K4 mono- and dimethylation (Wang *et al*. 2009). Removal of H3K4 mono- and dimethylation through KDM1 overexpression in this model also suppressed FoxA1 and AR recruitment (Wang *et al*. 2009).

Similar to the role of FoxA1 as a pioneer factor that translates the H3K4me1/me2 signature at non-promoter-regulatory elements, specific chromatin-remodeling components are recruited to H3K4me3-marked promoters. The ATP-dependent chromatin-remodeling enzyme CHD1 and the ATPase SNF2H are recruited to H3K4-methylated promoters (Santos-Rosa *et al*. 2003, Flanagan *et al*. 2005, Sims *et al*. 2005). This suggests that epigenetic marks are insufficient for transcription factor recruitment. Therefore, it appears that the interplay between epigenetic marks and chromatin-remodeling factors is required to open chromatin in specific genomic locations to guide transcription factor recruitment both at promoter and non-promoter-regulatory elements.

Understanding how the epigenetic signatures, such as the methylated-H3K4-based signature, are established in the course of normal and disease development is of central interest. To date, up to ten histone methyltransferases specific to H3K4 have been characterized and a growing number of histone demethylases are being identified (Christensen *et al*. 2007, Eissenberg *et al*. 2007, Iwase *et al*. 2007,

Klose *et al*. 2007, Lee *et al*. 2007, Ruthenburg *et al*. 2007, Seward *et al*. 2007, Shi & Whetstine 2007, Tahiliani *et al*. 2007, Yamane *et al*. 2007). Therefore, the methylation state of H3K4 appears to be under tight regulation. Previous reports have indicated that the distribution of H3K4me2 is established early during differentiation (Chambeyron & Bickmore 2004). Similarly, work on promoter regions has revealed a progression from a broad to a more restricted distribution for H3K4me3 in the course of differentiation (Guenther *et al*. 2007, Mikkelsen *et al*. 2007). Interestingly, the bivalent state of histone modifications found at promoters relates to the transitional state of poised to active or repressed promoters. Indeed, in the course of differentiation, promoters harboring both H3K4me3 and H3K27me3 are enriched for H3K4me3 when the associated genes are expressed and for H3K27me3 when the genes are silenced (Azuara *et al*. 2006, Bernstein *et al*. 2006, Guenther *et al*. 2007, Mikkelsen *et al*. 2007). Although there is no clear indication of a similar signature at non-promoter-regulatory elements, H3K9me2 has recently been found enriched at inaccessible promoter-distant regulatory elements (Lupien *et al*. 2008). Furthermore, H3K4me1/me2 and H3K9me2 occurred together on more condensed FoxA1-binding sites as defined by DNaseI hypersensitivity and Formaldehyde-Assisted Isolation of Regulatory Elements, an additional method that delineates DNA accessibility (Giresi *et al*. 2007, Eeckhoute *et al*. 2009). Therefore, additional studies are needed to reveal fundamental components of the role played by chromatin structure in transcriptional regulation as well as the contribution transcriptional events may have on epigenetic components.

Conclusion

The wealth of information derived from cistrome-based studies is already revealing core concepts of transcriptional regulation. Recently, a predictive model based on the ERα and FoxA1 cistromes from breast cancer cells as well as the cistrome of the insulator protein CCCTC-binding factor (CTCF) has predicted up to 70% of estrogen-regulated genes (Chan & Song 2008). Pursuing this analysis to coregulatory factors and epigenetic components should reveal intricate mechanisms fine tuning the actions of ERα and AR. Considering the increasingly recognized role of epigenetic components in cancer development and progression, a better understanding of their function in transcriptional regulation will prove fundamental in the elaboration of novel therapeutic strategies to breast, prostate, and other cancers.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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