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Genome-wide activity of unliganded estrogen receptor-α in breast cancer cells

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Estrogen receptor-α (ERα) has central role in hormone-dependent breast cancer and its ligand-induced functions have been extensively characterized. However, evidence exists that ERα has functions that are independent of ligands. In the present work, we investigated the binding of ERα to chromatin in the absence of ligands and its functions on gene regulation. We demonstrated that in MCF7 breast cancer cells unliganded ERα binds to more than 4,000 chromatin sites. Unexpectedly, although almost entirely comprised in the larger group of estrogen-induced binding sites, we found that unliganded-ERα binding is specifically linked to genes with developmental functions, compared with estrogen-induced binding. Moreover, we found that siRNA-mediated down-regulation of ERα in absence of estrogen is accompanied by changes in the expression levels of hundreds of coding and noncoding RNAs. Down-regulated mRNAs showed enrichment in genes related to epithelial cell growth and development. Stable ERα down-regulation using shRNA, which caused cell growth arrest, was accompanied by increased H3K27me3 at ERα binding sites. Finally, we found that FOXA1 and AP2γ binding to several sites is decreased upon ERα silencing, suggesting that unliganded ERα participates, together with other factors, in the maintenance of the luminal-specific cistrome in breast cancer cells.

chromatin binding | transcriptome | enhancer | pioneer factors | epigenetics

Estrogen receptor-α (ERα) expression in breast cancer defines the Luminal A phenotype, which represents the subset of tumors that are responsive to endocrine treatments. Spontaneous or experimentally induced (1) loss of ERα elicits growth arrest or epithelial to mesenchymal transition in vitro, whereas estrogen withdrawal from culture media, albeit reducing proliferation rate, has no such effect. These data suggest that loss of ERα does not equal depletion of estrogen. ERα is a DNA-binding, ligand-activated transcription factor, but it can be activated in absence of ligands by diverse mechanisms, especially by phosphorylation through different pathways, including protein kinase A, mitogen-activated protein kinases, and others (ref. 2 and references therein). Ligand-independent activity of ERα was reported by several groups on individual genes (3–5). Genome-wide ERα binding in the absence of estrogen was also described in breast cancer cells acquainted with growing in hormone-depleted media (6–8) and in mouse uterus (9). These data suggest that ERα may have a wide genomic function in breast cancer cells independent of its ligands. Estrogen response in breast cancer cells was extensively characterized in terms of chromatin binding and gene-expression regulation using both cell lines and human tumor biopsies. In vitro models were especially useful because they allowed correlating ERα-binding events, which are rarely located at gene promoters, with gene-expression data (10–13). In these studies, the experimental setting, together with the fact that breast cancer cell lines show a high grade of genomic rearrangements (14), made it difficult to evaluate ERα binding in cells treated with vehicle alone. As a consequence, most authors have dismissed the question of hormone-independent binding as compromised peak calling or as unspecific background (15, 16). However, especially for the clinical problem concerning the response to aromatase inhibitors, identifying possible ERα genomic actions in the absence of ligands would be very relevant. In this work, we first identified bona fide genomic ERα binding sites in the absence of estrogen in breast cancer cells. We then evaluated the effect of ERα silencing on gene transcription and binding of pioneer factors, demonstrating that ERα controls genomic activity by binding to several chromatin sites independently of estrogen exposure. Thus, unliganded ERα may participate, together with other factors, in the definition of the chromatin landscape of hormone-dependent breast cancer cells.

Results

Unliganded ERα Cistrome in MCF7 Cells. To identify ERα binding in the absence of estrogen, MCF7 cells were maintained in hormone-depleted (HD) medium, transfected with control (siCTR) or ERα-specific (siERα) siRNA and subjected to chromatin immunoprecipitation followed by high-throughput sequencing

Significance

Estrogen receptor-α (ERα) is a key protein in breast cancer and treatments targeting ERα are among the most widely used and effective in clinics. Although the role of estrogen-stimulated ERα in breast cancer has been exhaustively described, the functions of ERα in the absence of estrogen is hill-defined. In this work, we show that ERα binds extensively to the genome of breast cancer cells in the absence of estrogen, where it regulates the expression of hundreds of genes endowed with developmental functions. Our data suggest that ERα has a fundamental role in the homeostasis of luminal epithelial cells also when estrogen is ablated physiologically or pharmacologically.

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(ChIP-seq) using antibodies against ERα or IgG as control. Analysis of ERα enrichment over IgG in siCTR conditions evidenced 4,232 unliganded ERα binding sites (apo-ERα binding sites, aERBS) (P < 1e-05). These sites were almost entirely contained in the ERα cistrome reported in MCF7 cells cultured in full medium (FM-ERBS) or after 17β-estradiol (E2) treatment (E2-ERBS) (Fig. 1*A*) (15, 17). Accordingly, aERBS showed genomic distribution similar to estrogen-induced events, with increased prevalence of intergenic location (Fig. S1*A*).

To verify the specificity of the signal, we examined how siERα, which reduced ERα protein level by 80% (Fig. S1*B*), affected these binding events. ChIP signal was strongly reduced upon ERα knockdown (Fig. 1*B* and Fig. S1*C*), confirming that these are bona fide ERBS in the absence of hormone. Comparison of ERα binding enrichment in siCTR over siERα allowed ranking aERBS by significance (Fig. 1*B*) and this unraveled diversity among aERBS. Analysis of top 25% aERBS revealed a higher average number of reads and a full estrogen-response element (fERE) as the most represented motif at peak center (63% fERE-positive, P < 6.2e-58), compared with bottom 25% (27% fERE-positive, P < 3.9e-07). Bottom aERBS presented a half-ERE as the most represented motif (Fig. 1*C*). In addition to this finding, distribution around the peak center of the fERE probability was also different in top and bottom peaks (Fig. 1*D*). Moreover, the calculated theoretical fERE affinity was significantly higher in top aERBS (Fig. 1*E* and Fig. S1*D*).

Given that aERBS overlap extensively with those observed in the presence of E2 (Fig. 1*A*), an important issue is whether aERBS may represent "residual binding" after estrogen deprival. Using ChIP-quantitative PCR (qPCR), we verified that apo-ERα binding to several sites was stable up to 12 d in HD medium (Fig. S1*E*), thus excluding simple estrogen carryover when cells were switched to HD medium. Furthermore, using GREAT analysis (18), we found that aERBS lie close to genes associated with development, cell differentiation, and morphogenesis, whereas E2-ERBS and FM-ERBS, not in common with aERBS, showed enrichment in metabolism, lipid metabolism and biosynthesis terms (Datasets S1 and S2). This difference was clearly shown by semantic analysis of the associated Gene Ontology (GO) terms (19), as shown in Fig. 2*A*. Thus, this result suggests that ERα chromatin binding in absence of hormone has different functions than estrogen-induced binding.

Transcription factor binding sites (TFBS) analysis confirmed that apo-ERα binding is most likely facilitated by cooperating factors, as previously shown for liganded ERα (11, 12, 20). aERBS are frequently accompanied by forkhead box protein A1 (FOXA1/HNF3A), activating enhancer binding protein 2 gamma (AP2γ/TFAP2C), glucocorticoid receptor, and other motifs (Fig. 2*B*, *Left*). Interestingly, predicted TFBS were different in the top 25% vs. bottom 25% aERBS, showing fERE and FOXA1 as the most enriched motifs, respectively (Fig. S1*F*). We then compared TFBS predictions with available ChIP-seq datasets in MCF7 cells (Fig. 2*B*, *Right*). The highest overlap was observed in the case of FOXA1, GATA binding protein 3 (Gata3), nuclear receptor subfamily 2 group F member 2 (NR2F2), and AP2γ (connecting arcs in Fig. 2*B*). Noteworthy, FOXA1 and AP2γ binding in HD medium were among the most overlapped data. As we recently reported for E2-ERBS (21), aERBS overlap significantly with transposable elements of the mammalian interspersed repetitive (MIR) and endogenous retroviral sequence 1 (ERV1) superfamilies (Fig. S1*G*), which have been proposed as tools to coevolve TFBS modules.

To investigate the relevance of the aERBS identified in our study, we performed comparative analyses with ERBS reported in other available datasets (Fig. S1*H* and Dataset S3*A*). First we verified significant overlap with datasets of MCF7 (15, 22), T47D (22), and H3396 cells (23) cultured in HD medium, which was particularly consistent for the top 25% aERBS. We then investigated whether aERBS are conserved in cells adapted to long-term estrogen deprival (LTED cells). This analysis showed extremely variable overlap, from 84.9% in MCF7:2A (7), to almost none (0.32%) in other MCF7-derived LTED cell line (6), suggesting that alternative pathways contribute to adaptation to hormone deprivation. We also observed significant overlap with ERBS reported in hydroxy-tamoxifen (OH-T)–treated MCF7 cells in two studies (11, 17), as well as in OH-T–resistant MCF7-derived clones (Fig. S1 *H* and *I*) (11, 24). Semantic analysis of GO terms comparing ERBS described in OH-T–treated MCF7 cells (15), MCF7:2A LTED cells (7), and aERBS, showed again a clear association of aERBS with developmental terms (Fig. S1*J*). Interestingly, we found that 420 of the aERBS described in our study were present in the set of 484 ERBS identified in human breast tumor samples (24), further emphasizing the role of aERBS in breast tumor cells (Fig. S1*H*). Noteworthy, among those samples, 264 overlapping peaks were in top 25% aERBS, whereas only 27 were in the bottom 25%.

Taken together, these results demonstrate that ERα is bound to chromatin in absence of hormone to sites that represent a functionally significant subset of estrogen-induced binding sites.

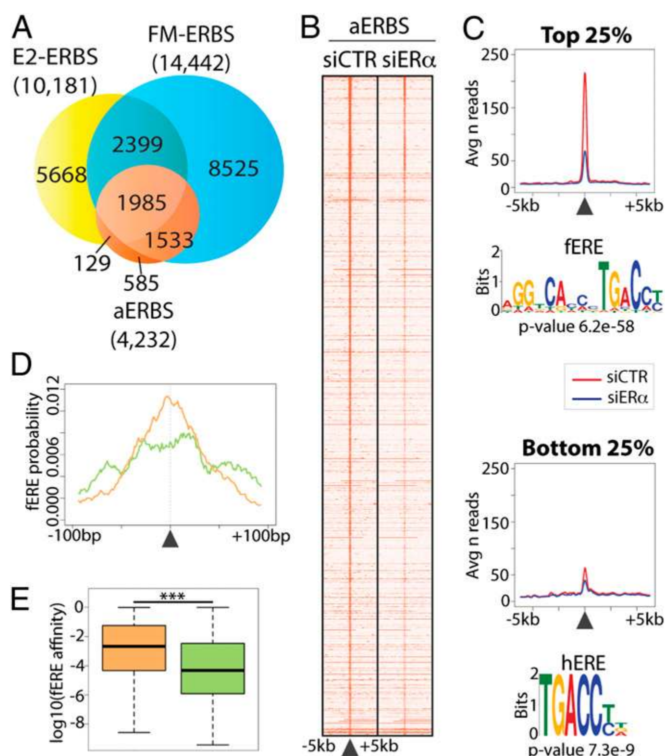

Fig. 1. (*A*) Venn diagram of aERBS, FM-ERBS (17), and in E2-treated MCF7 cells (E2-ERBS) (15). (*B*) Peak intensity heat map of aERBS in a ± 5-Kbp genomic window. The 4,232 aERBS significantly enriched over IgG (siCTR) are ranked on P value versus ERα ChIP-seq in siERα-treated cells. (*C*) Average read counts for top and bottom quartiles in siCTR and siERα peaks. The S-logo indicates the most enriched motif in this quartile. (*D*) Localization probability of a fERE within 200 bp around the peak center of top 25% (orange) and bottom 25% (green). (*E*) Box plot depicting the predicted affinity of fERE as in *D* of the top (orange) and bottom (green) 25% (***P < 0.001; two-tailed unpaired t-test). Black arrow indicates ERα peak center.

aERBS Are Functional Sites. Individual ChIP-qPCR analysis of selected aERBS not only confirmed ERα binding in the absence of estrogen but showed consistent decrease after siERα transfection (Fig. 3*A*, blue bars). To rule out residual estrogenic activity in HD medium, we repeated the experiments in serum-free (SF) medium, showing essentially similar results (Fig. 3*A*, orange bars).

The fact that most aERBS overlap estrogen-stimulated ERBS poses the question of whether these sites are fully occupied by

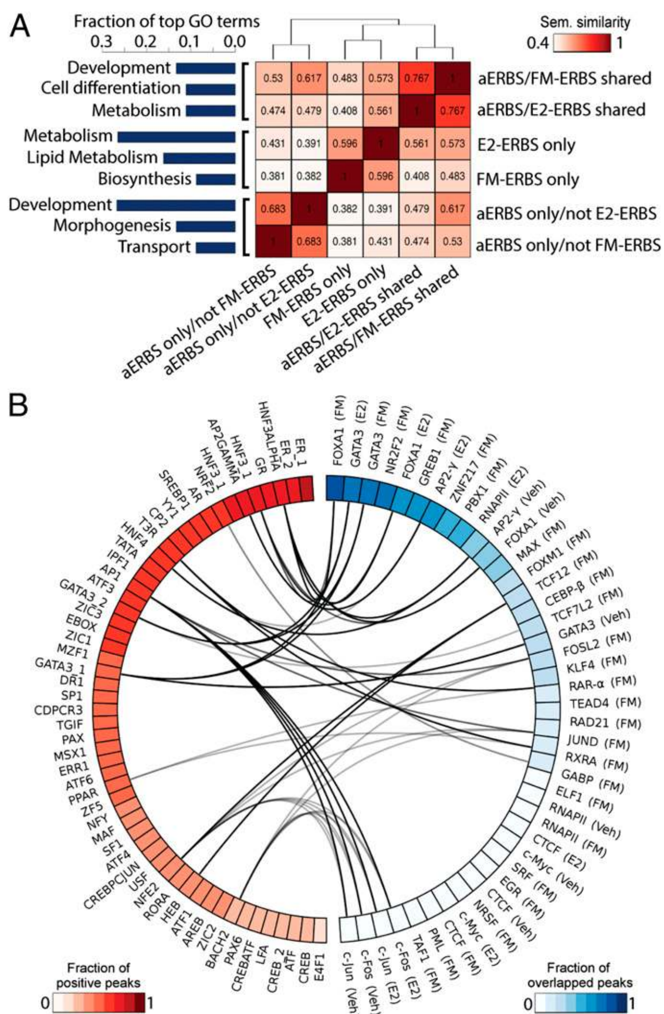

Fig. 2. (*A*) Semantic similarity of GO Biological Process terms enriched by GREAT (18) in aERBS, FM-ERBS (17), or in E2-treated cells (E2-ERBS) (15). The heat map (*Right*) reports the semantic similarity computed between the following subsets: aERBS in common with FM or E2-treated conditions (aERBS/FM-ERBS shared; aERBS/E2-ERBS shared); ERBS detected in FM or E2 only (FM-ERBS only; E2-ERBS only); aERBS not present in FM or E2 (aERBS only/not E2-ERBS and aERBS only/not FM-ERBS). The three most represented GO categories for each cluster are indicated on the left. (*B*) Circos plot of TFBS predictions versus ChIP-seq datasets overlap relative to aERBS. *Left* heat map (red scale): predicted TFBS matrix frequency. *Right* heat map (blue scale): fraction of aERBS overlapped to TFBS reported by ChIP-seq. TFBS and ChIP-seq datasets characterized by the highest similarity are connected by lines of increasing color intensity, proportional to matrix similarity. Veh, untreated; E2, estrogen-treated; FM, full medium.

ERα in the absence of hormone. As shown in Fig. 3*B*, treatment of cells with E2 for 45 min induced a significant increase of ERα binding, confirming that these sites presented a low occupancy in absence of ligands, yet maintained E2-inducibility. We also noted that induction was less pronounced for peaks having a higher basal level (see for example *FKBP4* and *RARA*), as previously reported for the intronic *RARA* binding site (25).

Next, we asked whether ERα down-regulation affects transcription even in the absence of estrogen. Using qRT-PCR, we verified that mRNA expression of five of seven genes containing aERBS was indeed significantly decreased 48 h after siERα transfection. This down-regulation was also reproduced in SF medium (Fig. 3*C*). As expected, E2 treatment caused an increase in mRNA levels up to ninefold (Fig. 3*D* and Fig. S2*A*). However, we observed no correlation between the level of repression after siERα and the induction by E2.

We also examined the effects of silencing ERα to a greater extent by transducing MCF7 cells with an shRNA-expressing vector in different growth conditions: in the absence of hormone (HD) versus serum-containing medium (FM) or versus E2 treatment (E2) (Fig. 3*D* and Fig. S2*A*). The results obtained indicate that ERα shRNA significantly impaired the response to E2 treatment. Of note, the mRNA level obtained in ERα-knockdown cells upon E2 treatment is below the level observed in control cells in HD medium. Taken together, these data indicate that in the absence of hormone ERα binds to regulatory sites, where it maintains basal transcription of its target genes, which can be either stimulated after ligand administration or repressed upon ERα depletion.

To evaluate the genome-wide effects of ERα depletion on the transcriptome of MCF7 cells, we performed polyA+ RNA-seq from cells cultured in absence of estrogen. RNAs were extracted 48 h after transfection of siCTR or siERα. To detect a broad range of variations in RNA levels we combined two complementary strategies (*SI Materials and Methods*). This analysis led to the identification of 912 differentially expressed (DE) genes with at least 1.5-fold change ($P < 0.05$). ERα knockdown in the absence of hormone elicited both decrease (504 genes) and increase (408 genes) of coding and noncoding transcripts (Fig. 4 *A* and *B*). Even though most DE genes were protein coding (727), we found evidence of regulation of lncRNA expression, in particular of lincRNAs (57 genes), antisense transcripts (48 genes), and pseudogenes (54 genes).

To understand whether transcriptional changes observed upon ERα knockdown overlap E2-stimulated genes, we compared our data to seven different public expression datasets from MCF7 cells treated with E2 for 4, 6, and 24 h (Dataset S3*B*). We found that 27.6% of the deregulated genes upon ERα knockdown were E2-regulated genes. Additional comparison with two time-course expression datasets (12, 26) did not increase this ratio (Fig. S2*B*). This analysis showed that ERα silencing causes, on average, a transcriptional effect with an opposite trend compared to E2-induction (Fig. 4*C*), confirming that genes controlled by unliganded ERα are a subset of estrogen-responsive genes. Finally, we examined two public MCF7 datasets measuring the effect of hormone withdrawal for 48–72 h (Dataset S3*B*) and we observed small overlap, though of coherent sign (Fig. 4*C*). In our experimental design, the time required for ERα down-regulation (48 h) accounts for the occurrence of indirect effects, in addition to primary ERα-mediated regulation. Nevertheless, we cannot exclude that some DE genes may represent, in part, an estrogen-independent gene expression response to ERα depletion.

Next, we sought to correlate aERBS with gene regulation. Nineteen percent of down-regulated and 5% of up-regulated genes had an aERBS within 20 kb from the transcription start site (TSS) and this ratio increased to 75% and 25%, respectively, extending the range up to 100 kb. In addition, we compared the distance from the TSS to the nearest aERBS in down-regulated vs. up-regulated genes. This analysis showed that aERBS accumulated significantly closer to down-regulated genes compared to random, whereas up-regulated genes did not (Fig. 4*D* and Fig. S2*C*). Taken together, these observations suggest that down-regulated genes are directly regulated by apo-ERα binding, whereas the up-regulated set may contain secondary responders.

Looking for functions of DE genes, pathway analysis (IngenuitySystems) showed "cell death and survival," "cellular growth and proliferation," and "cellular movement" as the most significant terms (Fig. S2*D*). However, unexpectedly, "interferon signaling" was indicated as the top canonical pathway and several interferon (IFN)-related molecules were predicted as activated upstream regulators (Dataset S4). This double-faced functional aspect became clear when we considered DE genes separately. In fact, all of the immune and IFN-related terms and upstream

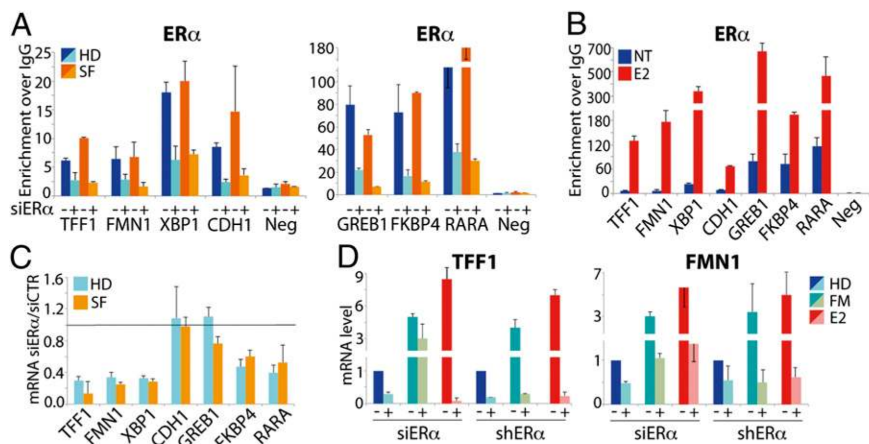

Fig. 3. ChIP-qPCR analysis of ERα target genes (*A*) following siCTR or siERα transfection, in HD and in SF medium; (*B*) after treatment with vehicle (NT) or 10 nM E2 for 45 min (E2). *GAPDH* promoter was used as a negative control region (Neg). (*C*) qRT-PCR mRNA analysis of target genes in HD and SF medium. Values are shown as ratios of relative mRNA level in siERα versus siCTR treated cells. (*D*) qRT-PCR mRNA analysis of *TFF1* and *FMN1* after siERα transfection or shERα transduction in HD, FM, and 10 nM E2 treatment in HD (E2). Error bars represent the SD of three independent biological replicates.

regulators were confined to up-regulated genes. Conversely, down-regulated genes showed cellular growth, survival, proliferation, development, and cell-cycle functions, together with the expected "tamoxifen," "estradiol," and "ESR1", as most scored upstream regulators (Fig. S2 *E–G* and Dataset S4). This function was clear-cut among down- and up-regulated genes and was confirmed by gene-set enrichment analysis (27) (Dataset S5*A*).

Taken together, these results suggest that unliganded ERα controls directly a set of genes related to cell growth and survival and to the maintenance of the epithelial phenotype. Conversely, depletion of apo-ERα induces a stress-like response in the cell that is underpinned by the activation of immune and inflammatory-related genes. This idea was further confirmed by isolating DE genes possessing an aERBS within 100 kbp from the TSS. Noteworthy, functions associated with cell proliferation, death, migration, and invasion were segregated specifically to aERBS proximal genes (Fig. S2 *H* and *I* and Datasets S4 and S5*A*).

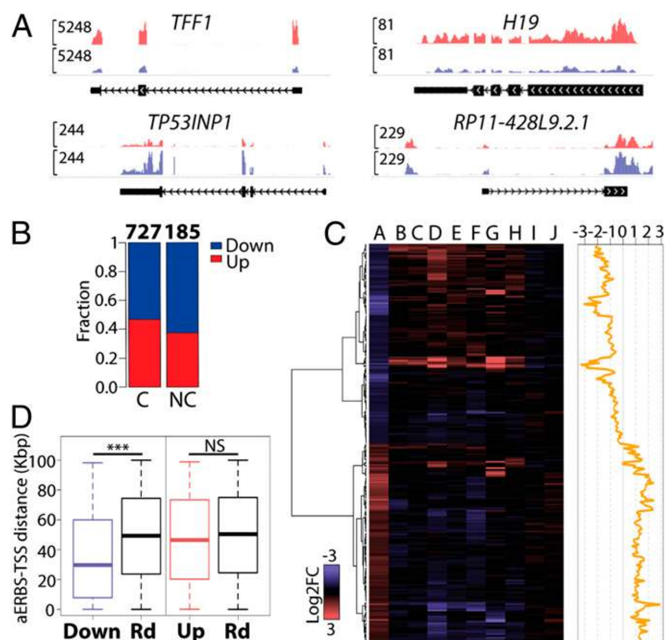

Fig. 4. (*A*) Genome-browser view of examples of DE protein coding (*TFF1* and *TF53INP1*) and noncoding genes (*H19* and *RP11-428L9.2.1*) after siCTR or siERα transfection. Only the main gene isoform is shown. (*B*) Fraction of up-regulated and down-regulated genes upon ERα silencing. C, coding genes; NC, noncoding genes. (*C*) Heat map representation of the overlap between DE genes (column A) and seven microarray gene expression datasets in E2-treated MCF7 cells (columns B–H) and two datasets in MCF7 switched to HD medium for 48 (column I) and 72 h (column J). Details of each dataset are reported in Dataset S3*B*. (*Right*) Relative effect of siERα vs. E2-effect, calculated as siERα Log_2FC minus median Log_2FC of E2-treated datasets. (*D*) Box plot distribution of the distance between the TSS of the DE genes and the closest aERBS center, compared with 1,000 random gene sets. (***$P < 0.001$; two-tailed unpaired *t* test). Down, down-regulated; Up, up-regulated; Rd, random genes.

Unliganded ERα Binding Sites Function in Breast Cancer Cells. To appreciate phenotypic and epigenetic changes induced by ERα depletion, we used MCF7 cells cultured in HD medium and transduced with an shRNA-expressing vector, leading to a stable ERα down-regulation (Fig. S3*A*). Depletion of ERα completely stopped cell growth in HD medium (Fig. 5*A*) and triggered a mesenchymal-like morphology (Fig. 5*B*), as previously reported (1, 5). Decreased apo-ERα binding and mRNA expression of target genes (Fig. 3*D* and Figs. S2*A* and S3*B*) was accompanied by increased level of the Polycomb-dependent histone modification H3K27me3 at ERα binding sites (Fig. 5*C* and Fig. S3*C*), although we were not able to detect the occupancy of Polycomb components by ChIP at these sites. As described above, several TFBS accompany the ERE in aERBS, in particular AP2γ and FOXA1, which are considered pioneer factors and whose binding is a necessary prerequisite for ERα function (11, 20). As expected, we observed that down-regulation of either AP2γ or FOXA1 reduced apo-ERα binding to aERBS, as reported for E2-induced binding (20) (Fig. S3*D*). In contrast to previous reports (11), FOXA1 siRNA, as well as AP2γ siRNA, reduced in part ERα protein level (Fig. S3 *E* and *F*). Contrary to expectations, though, we observed that ERα silencing resulted in marked decrease of FOXA1 and dramatic decrease of AP2γ occupancy in HD medium (Fig. 5 *D* and *E*). AP2γ is an ERα-dependent gene (28) and it is able to stabilize the binding of FOXA1 at colocalized ERBS (20). AP2γ expression decreases as a consequence of ERα silencing (Fig. 5*F*). To exclude the possibility that the decrease of FOXA1 occupancy reflected AP2γ down-regulation, we investigated additional aERBS not possessing AP2γ binding sites. We observed that in this case as well, markedly decreased apo-ERα binding (Fig. 5*G*) was followed by a decrease in FOXA1 occupancy (Fig. 5*H*), despite the absence of AP2γ binding at these sites (Fig. 5*I*).

Taken together, these data demonstrate that unliganded ERα is an essential factor for the maintenance of the luminal epithelial cistrome in unstimulated MCF7 breast cancer cells.

Fig. 5. (*A*) Growth curves of MCF7 cells transduced with control shRNA (black line) or ERα shRNA (gray line) maintained in HD medium. (*B*) Morphological changes occurring in MCF7 cells after ERα silencing. (*C*) ChIP analysis of H3K27me3 at selected aERBS ($*P < 0.05$, $**P < 0.01$; one-tailed paired *t* test). (*D* and *E*) ChIP analysis of FOXA1 (*D*) and AP2γ (*E*) as above. (*F*) Western blot analysis of ERα, FOXA1 and AP2γ proteins. αtub, α-tubulin: loading control. (*G*–*I*) ChIP analysis of ERα (*G*), FOXA1 (*H*), and AP2γ (*I*) at selected aERBS predicted to contain FOXA1 binding but not AP2γ binding sites. Intg1, -2, -3: anonymous intergenic aERBS. For ChIP experiments, *GAPDH* promoter was used as negative control (Neg). For all of the experiments shown (*B*–*I*), MCF7 cells transduced with shCTR or shERα were cultured for three days in HD medium before analysis. Error bars are SD of three independent biological replicates. (Magnification: *B*, 10×.)

Discussion

Previous work has exhaustively described chromatin binding of ERα in the presence of ligands and its effects on gene regulation (10–13, 15). Here, we report an unexpected role of ERα in breast cancer cells in the absence of estrogen. Unliganded ERα binding sites represent a nonrandom subset of the estrogen-induced cistrome, which is connected with developmental functions. Moreover, unliganded ERα contributes to transcriptional activity, because its down-regulation causes changes in gene expression and chromatin modifications. Finally, we show that depletion of ERα in the absence of estrogen leads to a reduced binding of pioneer factors, such as FOXA1 and AP2γ, to shared enhancers. This is an unexpected feature of ERα, whose binding to chromatin is thought to depend hierarchically from pioneer factors binding (29–31).

Genome-wide binding of ERα in the absence of hormones was observed previously in breast cancer (15, 22, 23) and recently in mouse uterus (9). Interestingly, we found that these sites, mostly located at enhancer regions, do not represent a random selection of high-affinity sites. In fact, the functions connected with aERBS neighboring genes are fundamentally related to cell development and differentiation that is strongly reminiscent of the primary action of ERα during development (32). In contrast, the analysis of ERBS specific for the estrogen treatment highlights the role of ERα in metabolic regulation, which is in agreement with the physiological action of estrogen on many tissues. To our knowledge, there was no previous support to differential functions of ERα in breast cancer cells, depending on the presence or absence of hormones.

The analysis of the transcriptome upon depletion of ERα allowed us to unveil the unexpected function of unliganded-ERα as modulator of specific loci. Indeed, we found that siERα down-regulated genes often present an aERBS, whereas those that are up-regulated do not, thus suggesting that the latter might be affected through an indirect mechanism. Our data suggest that unliganded-ERα contribute to enhancer activity, granting basal transcription of neighboring genes that are functionally related to cellular proliferation and development. In contrast, up-regulated genes are linked to different functions, in particular to the IFN/inflammatory response, most likely representing an indirect response to ERα ablation, in line with the protective role of ERα in several tissues (2). Interestingly, a similar signature can be observed also in data obtained during long-term hormone deprivation (8) (Dataset S5*B*). Almost 30% of the siERα-regulated genes in HD medium was present among E2-responsive genes, further supporting the observation that aERBS represent a subset of the ERα-regulated cistrome.

We observed that stable ERα silencing by shRNA transduction induced complete growth arrest. This finding is consistent with the transcriptional program controlled by unliganded ERα. In similar conditions, establishment of cells with a complete epithelial to mesenchymal transition has been reported (1), mirroring the invasive phenotype of breast tumor cells escaping ERα control in vivo. Genes controlled by unliganded ERα are not only related to cell growth, but several functional terms linked to the maintenance of the epithelial phenotype were also present. For example, the MIST1 transcription factor-encoding gene (*BHLHA15*), which is repressed 2.1-fold by siERα, is required to maintain mammary gland differentiation in transgenic mice (33).

One important point in the context of antiestrogen treatments in breast cancer is whether aERBS are conserved in cells acquainted with estrogen deprivation (LTED). When we compared our aERBS dataset with those reported in two previous studies using LTED cell lines, we found that the overlap was almost complete in MCF7:2A (7) but null in the LTED cells reported by Miller et al. (6). Noteworthy, this latter case maintains a proliferative response to estrogen, whereas the former does not. It is conceivable that in the latter case the unliganded ERα cistrome has been widely reprogrammed (6), but ERα activity is maintained on (some) genes linked to the proliferative response. In contrast, in the former case, unliganded ERα is possibly fully activated by other endogenous factors (e.g., coactivator amplification) as a main growth-sustaining axis. Noteworthy, GO terms analysis demonstrated that in LTED ERα is connected to metabolic functions, similarly to E2- or OH-T–induced sites but in contrast to aERBS.

Most aERBS colocalize with other TFBS. Extensive overlap with public ChIP-seq datasets confirmed that these sites are indeed composite enhancers, possibly evolved from extensive spread of transposable elements in mammals (21). Significantly, top factors comprise FOXA1 and AP2γ, known as "pioneer factors" for sex-steroid receptors (i.e., factors that open the chromatin allowing the landing of nuclear receptors on DNA) (29–31). We confirmed that silencing of these factors also reduces unliganded ERα

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occupancy. Unexpectedly, we observed that ERα silencing brought about a reduction of either AP2γ or FOXA1 binding at several loci, although not completely. The full interpretation of these results is complicated by the observation that AP2γ transcription is directly controlled by ERα (28) and its level is reduced in shRNA-transduced cells. However, we detected a reduction of FOXA1 binding also at enhancers that do not contain AP2γ, in contrast to previous data (34). A possible reason for this discrepancy is the use of stable, rather than transient, ERα silencing used in our study.

The data presented here may in part argue against the concept of pioneer factors as primary drivers of nucleosome remodeling, leading to ERα binding. Indeed, the elegant demonstration of progesterone receptor binding to nucleosome PRE, recently published (35), further challenges this view. Our results are consistent with a model where ERα, in the absence of estrogen stimulation, collaborates with other transcription factors to maintain the luminal epithelial enhancer landscape. When one of these factors is suppressed, enhancers progressively collapse, also because of the coordinated decrease of expression of other transcription factors. It is important to note, in this scenario, that a feed-forward loop exists involving AP2γ and ERα, which sustain each other's expression in breast cancer cells (28, 36).

Ligand-independent functions of ERα have been described in several tissues in addition to breast cancer cells (2–5). Results reported here provide a frame to understand why ERα is required to respond to aromatase inhibitors in breast cancer. Luminal epithelial cancer cells are stable and survive until ERα is present. The absence of estrogen keeps these cells growing at a very low rate that is presumably controlled by the host. Loss of ERα exposes the cells to immune control, removes a brake on reprogramming, which results, in vivo, in the emergence of other growth-sustaining pathways.

Materials and Methods

Detailed protocols are provided in *SI Materials and Methods*.

SiRNA and shRNA Interference. Cells were transfected with siERα (Stealth RNAi Invitrogen), siAP2γ (Qiagen), siFOXA1 (Santa Cruz), and siCTR (Invitrogen). Transfection was performed using Lipofectamine 2000 (Invitrogen). MCF7 cells were infected with control shRNA (MISSION shRNA; Sigma-Aldrich) or shRNA against ERα (MISSION shRNA; Sigma-Aldrich) and selected with 2 μg/mL puromycin for 3 d.

ChIP and ChIP-Seq. ChIP was performed as previously described (37, 38). Antibodies and PCR primers used in this assay are reported in *SI Materials and Methods*. For ChIP-seq, library preparation for sequencing was performed starting with 10 ng of immunoprecipitated DNA (GAIIX, Illumina).

RNA-Seq. RNA-seq libraries were prepared from poly(A)+ selected, gel-purified > 200 bp RNA. Sequencing was performed on Illumina HiSeq2000.

Bioinformatic Analysis. Published algorithms were used to analyze ChIP-seq and gene expression datasets. Ingenuity pathway analysis (IngenuitySystems) was used for gene ontological analysis. ChIP-seq and RNA-seq data are accessible in the Gene Expression Omnibus database (accession no. GSE53533).

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