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Chromosome-Wide Mapping of Estrogen Receptor Binding Reveals Long-Range Regulation Requiring the Forkhead Protein FoxA1

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Summary

Estrogen plays an essential physiologic role in reproduction and a pathologic one in breast cancer. The completion of the human genome has allowed the identification of the expressed regions of protein-coding genes; however, little is known concerning the organization of their *cis*-regulatory elements. We have mapped the association of the estrogen receptor (ER) with the complete nonrepetitive sequence of human chromosomes 21 and 22 by combining chromatin immunoprecipitation (ChIP) with tiled microarrays. ER binds selectively to a limited number of sites, the majority of which are distant from the transcription start sites of regulated genes. The unbiased sequence interrogation of the genuine chromatin binding sites suggests that direct ER binding requires the presence of Forkhead factor binding in close proximity. Furthermore, knockdown of *FoxA1* expression blocks the association of ER with chromatin and estrogen-induced gene expression demonstrating the necessity of FoxA1 in mediating an estrogen response in breast cancer cells.

Introduction

Estrogen is an essential regulator of female development and reproductive function and has been implicated as a causal factor in breast and endometrial cancers. Estrogen-regulated gene expression is mediated by the action of two members of the nuclear receptor family, ERα and ERβ, with ERα being dominant in both breast epithelial cells and in breast cancer. Significant progress has been made over the past decade in defining the complex interactions between chromatin and an array of factors involved in ER-mediated gene expression (Halachmi et al., 1994; Metivier et al., 2003; Shang and Brown, 2002; Shang et al., 2000), including the cyclic association of ER, p160 coactivators (such as AIB-1), histone acetyl transferases (HAT), and chromatin modifying molecules, such as p300/CBP and p/CAF, with target promoters in an ordered temporal fashion (Metivier et al., 2003; Shang et al., 2000).

In addition, a number of recent strategies including gene expression profiling on microarrays have identified potential ER target genes in human breast cancer cells and only a few *cis*-elements targeted directly by ER have been identified to date. For example, estrogen responsive elements (ERE) have been identified within the 1 kb 5′-proximal region of the estrogen-regulated genes *TFF-1 (pS2)*, *EBAG9*, and *Cathepsin D* (Augereau et al., 1994; Berry et al., 1989; Ikeda et al., 2000), and the proximal promoters of target genes that lack EREs, including *c-Myc* and *IGF-I*, contain AP-1 and Sp-1 sites that appear essential for transcription in in vitro reporter assays (Dubik and Shiu, 1992; Umayahara et al., 1994). Few, if any regulatory elements at significant distances from the mRNA start sites of target genes have been shown to be directly targeted by ER, and computation approaches to identify novel ER binding domains have focused primarily on gene proximal regions (Bajic and Seah, 2003; Bourdeau et al., 2004). However, more progress has been made in studies of *β-globin* gene regulation which has contributed to our understanding of general mechanisms of transcriptional regulation and has shown that locus control regions (LCR) up to 25 kb from the gene are capable of enhancing gene transcription (recently reviewed in Bulger et al. [2002]). In this study, we have undertaken an unbiased approach to identify all regulatory regions that may play a role in ER-mediated transcription by combining chromatin immunoprecipitation (ChIP) analyses of in vivo ER-chromatin complexes with Affymetrix tiled oligonucleotide microarrays that cover the entire nonrepetitive sequences of chromosomes 21 and 22, including, importantly, all the intergenic regions. Most previous ChIP-microarray studies have focused primarily on promoter regions (Odom et al., 2004) or CpG islands, which represent promoter-rich sequences (Weinmann et al., 2002). The tiled arrays used here are composed of 25 bp probes located at 35 nucleotide resolution (Cawley et al., 2004; Kapranov et al., 2002) and permit the opportunity to interrogate previously unexplored regions of chromosomal DNA. The 780 characterized or predicted genes on chromosomes 21 and 22 represent about 2% of the total number of genes (Kapranov et al., 2002) and thus provide a representative model for

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the unbiased identification of ER-mediated gene regulation paradigms.

Here we find a discrete number of ER binding sites across chromosomes 21 and 22, almost all of which are in nonpromoter proximal regions. We explored underlying biological patterns within the list of genuine chromatin-interacting domains and identified common motifs highly enriched in these regions. Using this information, we prove that the distal ER binding sites are discrete chromatin regions involved in transcriptional regulation and that a Forkhead protein, at these sites, is required for activity by ER.

Results

ER Occupies a Limited Number of Binding Sites on Chromosomes 21 and 22

Estrogen-dependent MCF-7 breast cancer cells were deprived of hormones and stimulated with estrogen or vehicle for 45 min, a time we have previously shown to have maximal recruitment of ER to the promoters of several known gene targets, including *Cathepsin D* and *TFF-1* (Shang et al., 2000). Following ChIP, ER-associated DNA was amplified using nonbiased conditions, labeled, and hybridized to the tiled microarrays. Relative confidence prediction scores were generated by quantile normalization across each probe followed by an analysis using a two-state Hidden Markov model (Rabiner, 1989). These scores included both probe intensity and width of probe cluster. Triplicate experiments eliminated stochastic false positives, after which peaks that reproducibly appeared at least twice in the three replicates were included. Real-time PCR primers were designed against numerous peaks in the list, and directed ER ChIP was conducted to identify the boundary between the true ER binding peaks (>1.5-fold enrichment over input) and the false positives (data not shown) and generate the final list of 57 estrogen-stimulated ER binding sites within 32 discrete clusters (Figures 1A and 1B and see the Supplemental Raw Data in the Supplemental Data available with this article online).

As one example of the validity of this method, the localization of ER to the proximal promoter 400 bp region of the estrogen-regulated gene, *TFF-1*, was observed. A functional ERE had been previously mapped to the region 393 to 405 bp upstream from the transcription start site of *TFF-1* (Berry et al., 1989). Furthermore, a region 10.5 kb upstream of the *TFF-1* transcription initiation site (Figure 1A) was also found to be bound by ER. Interestingly, an estrogen-inducible DNase I hypersensitive site has been previously mapped 10.5 kb upstream from the *TFF-1* start site (Giamarchi et al., 1999), though the region had not been further characterized. Our data now define these regions as authentic ER binding sites.

Within the small list of 57 ER binding sites, we observed 32 ER binding clusters, some of which were proximal to genes previously implicated as estrogen targets, including the transcription factor *XBP-1, DSCAM-1*, and the nuclear receptor coregulator *NRIP-1* (Cavailles et al., 1995; Pedram et al., 2002; Wang et al., 2004). Binding sites were also observed within 200 kb from genes not previously implicated as estrogen targets, including *SOD-1*, a superoxide dismutase gene involved in scavenging oxygen-free radicals (Beckman et al., 1993; Singh et al., 1998) and implicated in tamoxifen-resistant progression in MCF-7 xenografts (Schiff et al., 2000). None of these genes recruited ER to a proximal 5′ promoter region, but possessed divergent patterns of association. The *XBP-1* gene, recruited ER to three distinct and discrete regions 13.2 kb to 22.9 kb upstream of the transcription start site (Figure 1B). *DSCAM-1* contained a clustering of ten intronic ER binding sites, more than 0.5 Mb from the transcription initiation site. *NRIP-1* contained six ER binding sites in a region of chromosome 21 well known for its scarcity of genes (Katsanis et al., 1998). 5′ RACE was performed on *NRIP-1* to determine the exact location of the transcription start site and the distance between the ER binding sites and the genuine transcriptional start site. Sequencing of the 5′ terminus of the *NRIP-1* transcript after estrogen stimulation revealed the presence of two previously missed exons for *NRIP-1*, 74.96 kb and 97.39 kb from the previously annotated gene start site (data not shown). Therefore, the ER binding domains exist 107 to 144 kb from the genuine transcription start site of *NRIP-1*. The locations of all binding sites in relation to genes can be found in Table S1.

The ER binding sites adjacent to *TFF-1*, *XBP-1*, *SOD-1*, *NRIP-1*, and *DSCAM-1* were validated by ER ChIP and standard PCR (Figures 2A–2E). Also, quantitative PCR was performed on each of these sites after ER ChIP (Figure 2F), confirming these putative in vivo binding sites as genuine ER binding sites. To test whether these discrete ER recruitment regions were unique to estrogen action in MCF-7 cells, we performed ER ChIP and directed real-time PCR against the same sites in T47-D breast cancer cells. These data confirmed that the majority of the sites identified in MCF-7 cells were also regions of estrogen-dependent ER binding in a second ER-positive breast cancer cell line (data not shown), highlighting the conservation of specific ER-chromatin association sites.

A Significant Number of ER Binding Sites Reside Adjacent to Estrogen Gene Targets

Estrogen-mediated transcript changes were identified by converting RNA from vehicle or estrogen-stimulated MCF-7 cells into double-stranded cDNA and hybridizing to the chromosome 21 and 22 tiled microarrays. Thirty-five genes (4.4% of all genes) appeared to be transcribed, after which real-time primers were made against all these transcripts and quantitative RT-PCR showed that 12 transcripts on chromosomes 21 and 22 were estrogen induced (Table 1). Eleven of these twelve genes had ER binding clusters within 200 kb. The only estrogen-regulated gene that did not have an adjacent ER binding cluster was *ATP5J*. *TFF-1*, *XBP-1*, and *NRIP-1* were in the small list of 1.5% of genes upregulated following estrogen stimulation (Supplemental Raw Data). *DSCAM-1* and *SOD-1* were not upregulated by estrogen stimulation at the 3 hr time point assessed but were transcribed after 6 hr of estrogen stimulation, as determined by RT-PCR (Figure S2). This delay between ER association and transcription of *DSCAM-1* and

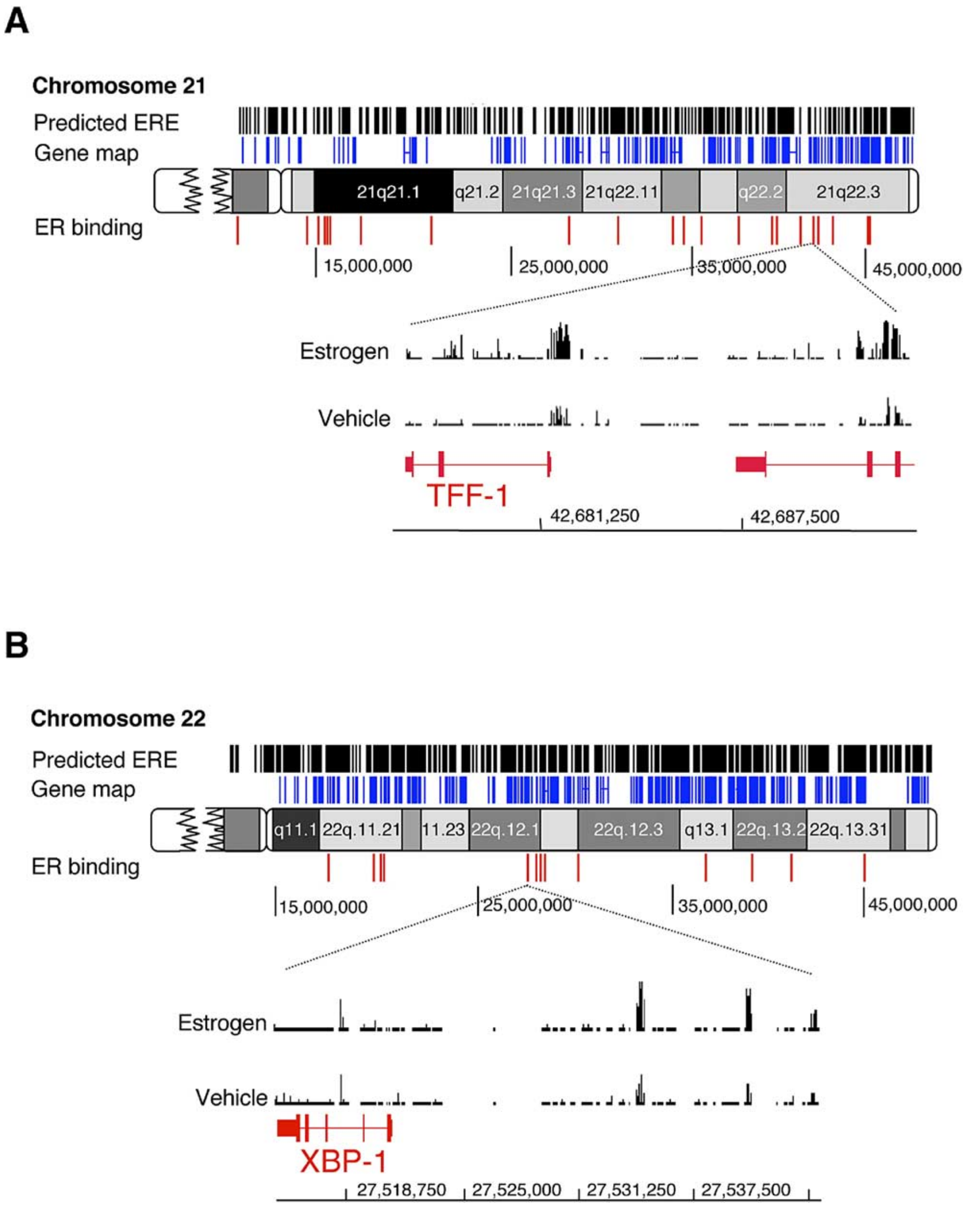

Figure 1. Map of ER Binding Sites on Chromosomes 21 and 22 after Estrogen Stimulation

The visual representation of ER binding sites on chromosomes 21 (A) and 22 (B) are shown. Gene locations are shown in blue bars. Gene locations are based on the April 2003 genome freeze in the UCSC browser using Genbank RefSeq positions. Predicted EREs are shown as black bars and ER binding sites are shown as red bars.

(A) An expanded view of the *TFF-1* gene region is shown as signal difference between ER ChIP and Input DNA for both the estrogen- and vehicle-treated cells. The *TFF-1* gene is shown in its genuine 3′-5′ orientation. The gene adjacent to *TFF-1* is not an estrogen target.

(B) Expanded view of the *XBP-1* gene region on chromosome 22. The *XBP-1* gene is shown in its genuine 3′-5′ orientation.

SOD-1 may be a consequence of a requirement for subsequent modification of the receptor complex or the requirement for the production of other factors involved in ER action but not necessarily part of an ER complex. Regardless of the mechanism for the transcriptional delay, it now appears that early and at least some delayed estrogen-regulated genes recruit the receptor with the same kinetics. This implies that events subsequent to ER binding are responsible for timing the initiation of transcription of these delayed targets.

Distal ER Binding Domains Function as Transcriptional Enhancers

The significant sequence distance between many of the ER binding sites and the putative target gene complicates their functional validation. However, we explored the possibility that these ER binding sites may recruit components indicative of transcriptional activation. RNA PolII ChIP followed by real-time PCR was performed on a subset of the putative regulatory regions adjacent to *TFF-1*, *XBP-1*, *DSCAM-1*, *NRIP-1*, and

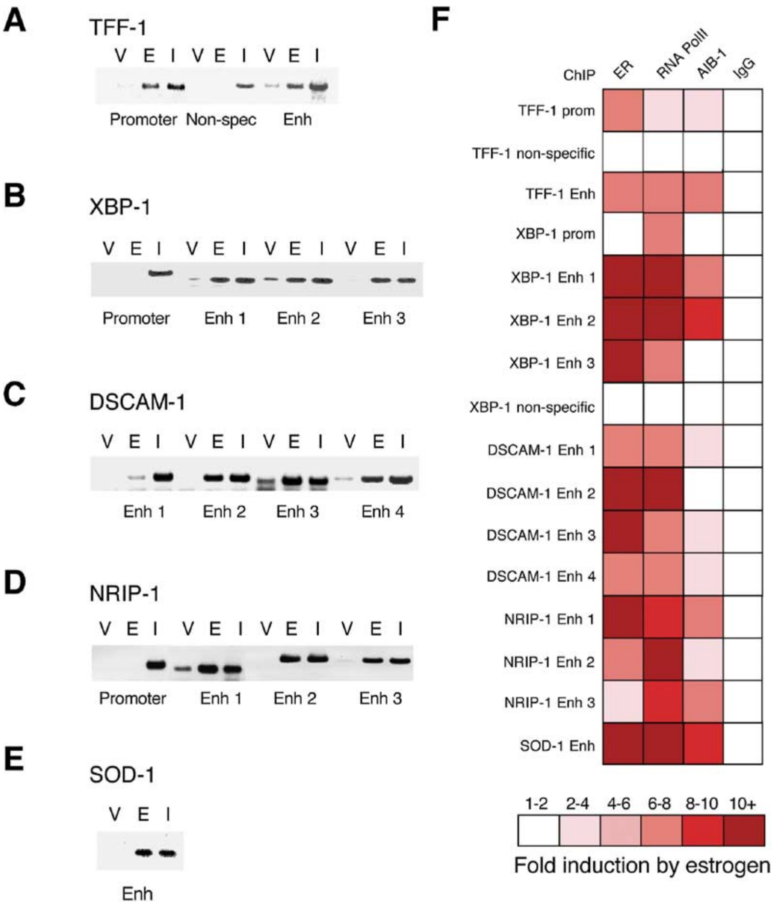

Figure 2. Validation of the In Vivo Binding of the Transcription Complex to Regulatory Regions

ChIP of ER and standard PCR of sites adjacent to *TFF-1* (A), *XBP-1* (B), *DSCAM-1* (C), *NRIP-1* (D), and *SOD-1* (E). *TFF-1* nonspecific and *XBP-1* promoter primers were included as negative controls. The lanes are vehicle (V), estrogen (E), and Input (I).

(F) ChIP of ER, RNA PolII, AIB-1, or IgG control and real-time PCR of binding regions. The data are estrogen-mediated fold enrichment compared to vehicle (ethanol) control. The color intensity reflects the fold change as described in the legend. *TFF-1* nonspecific and *XBP-1* nonspecific primers were included as negative controls. The data are the average of three replicates ± SD.

SOD-1 genes. Interestingly, RNA PolII association was seen with all of these sites in an estrogen-dependent manner (Figure 2F). Furthermore, ChIP of AIB-1, an oncogenic ER coactivator (Kuang et al., 2004; Torres-Arzayus et al., 2004), confirmed that AIB-1 is also present on all of these "regulatory" sites following estrogen exposure (Figure 2F). As negative controls, primers were designed against the intergenic region between the *TFF-1* promoter and enhancer and against a region 7 kb from *XBP-1* enhancer 3. Neither ER nor any of the other factors were found associated with these control regions. In addition, we examined the promoter of *XBP-1*. Although ER protein association was not observed at the *XBP-1* promoter, RNA PolII was found enriched at this site supporting the hypothesis that *XBP-1* is transcriptionally activated by ER.

To explore the possibility that the distal enhancer regions not only function as sites of protein recruitment but physically play a role during transcription of the adjacent gene, we performed a chromosome capture assay (Dekker et al., 2002) to assess whether promoter and enhancer sequences were components of the same chromatin regions. Hormone-depleted MCF-7 cells were stimulated with vehicle or estrogen, and the fixed chromatin was digested with a specific restriction enzyme (BtgI), followed by ER ChIP and ligation. After ligation, the ligated chromatin mix was washed and the crosslinking was reversed. One primer in the *TFF-1* promoter and one primer in the *TFF-1* enhancer were used to PCR potentially ligated fragments of DNA (Horike et al., 2005). As seen in Figure 3A, *TFF-1* promoter and enhancer DNA was ligated together only in the presence of estrogen, confirming that estrogen-mediated transcription of *TFF-1* involves direct physical interaction between the enhancer and promoter. No interaction was seen in the no-digestion control or no-ligation control. We performed the same experiment using the BsmI restriction enzyme that cuts the genuine *NRIP-1* promoter (as determined by 5′ RACE) and enhancer 3 region. Remarkably, after ligation, we were able to PCR a 1 kb fragment that corresponded to the ligated promoter-enhancer regions using one promoter-specific and one enhancer-specific primer (Figure 3B). This estrogen-dependent interaction of the distal (144 kb) ER binding site with the promoter of the *NRIP-1* gene confirms the authenticity of these distal sites as transcriptional regulatory domains.

The finding that RNA PolII is recruited to the majority of ER binding sites, even those removed from known transcription sites, led us to investigate the possibility that these binding sites can function as genuine enhancers. To this end, we cloned 23 ER sites (40% of all ER binding sites) into a pGL-3 luciferase vector containing an SV40 promoter and transfected these vectors into hormone-depleted MCF-7 cells which where subsequently treated with estrogen or vehicle control. pGL-3 empty vector was used as a negative control, and transfections were normalized with pRL null. Al-

Table 1. List of ER Binding Site Clusters and Relative Locations to Putative Gene Targets

Cluster Number	Number of Binding Sites	Start	Stop	Closest Regulated Gene
1	1	21: 10048850	10049271	
2	1	14600251	14600737	
3	1	15171656	15172273	
4	6	15467150	15738864	NRIP-1
5	1	17422343	17422868	
6	1	21532885	21533421	
7	1	29151881	29152882	
8	1	31821967	31822715	SOD-1
9	2	35021165	35027898	
10	1	35510057	35510719	
11	2	36480740	36487032	
12	1	38635468	38636783	
13	10	40363341	40675801	DSCAM-1
14	1	41911683	41912284	
15	1	42005946	42006169	PRDM15
16	2	42680784	42691725	TFF-1
17	1	42830736	42831350	
18	1	43564518	43565261	NDUFV3
19	2	45606461	45663897	
20	1	45790004	45790654	Col18A1
21	2	22: 17159455	17194014	
22	1	19566341	19566809	
23	3	19822950	19945255	
24	3	27534171	27543908	XBP-1
25	1	28106122	28107112	AP1B1
26	1	28237489	28238464	
27	1	28519139	28520023	
28	2	30300284	30307434	PISD
29	2	37030766	37033295	
30	1	39371665	39372232	
31	1	41361325	41361720	Predicted
32	1	45100090	45100552	

The 32 transcriptional clusters are shown, with the start and stop locations of the ER binding sites.

most 75% of the ER binding domains contained estrogen-induced enhancer characteristics in an in vitro transcription model (Figure 3C), supporting the hypothesis that the distal binding sites play transcriptional regulatory roles.

ER Binding Sites Are Conserved Across Species

To identify if the ER binding sites are conserved between human and mouse genomes, we assessed the identity in sequence in a window of 6 kb from the center of all 57 ER binding sites. This conservation was mapped within a 500 bp window at a single nucleotide resolution and confirms a strong conservation at the center of the ER binding site and the 500 bp on either side of the middle of the peak (Figure 4A). However, conservation decreased to background levels at a distance of 1 kb or more from the center of the ER binding sites. This supports the hypothesis that the discrete ER binding sites we see in MCF-7 cells are conserved between species and likely play a more general role in ER action in other cellular systems.

A Screen for Common Sequences Enriched in Genuine ER Binding Regions Suggests the Importance of Forkhead Factors in Estrogen Action

An unbiased search for common sequence motifs (Liu et al., 2002) within the 57 individual ER binding sites on chromosomes 21 and 22 revealed the significant recurrence of two motifs. A consensus 15 base sequence identical to the canonical ERE was present in 49% of all the ER binding sites on chromosomes 21 and 22 (Figure 4B; Klinge, 2001). The likelihood of an ERE occurring in one of the ER binding sites was significantly increased when compared to all of chromosomes 21 and 22 ($p = 1.33E-15$). In the ER binding sites lacking a canonical ERE, a majority were found to contain one or more ERE half-sites, and the occurrence of ERE half-sites was also nonrandom ($p = 2.16E-14$). To confirm that our failure to find ER binding at other EREs (5500 predicted EREs on chromosomes 21 and 22, as listed in Figures 1A and 1B) was not due to the insensitivity of our ChIP-microarray technique, we performed ChIP for ER followed by PCR for several randomly selected, predicted but nonfunctional perfect EREs on chromosomes 21 and 22. No ER association was found at any of these sites (data not shown).

We next determined whether DNA sequences other than the classical ERE were found at the ER binding sites by analyzing the bound sequences for conserved motifs after removing the EREs. This analysis revealed the presence of a Forkhead factor binding site in 54% of the 57 ER binding regions (Figure 4B), a finding that would only occur by chance with a probability of $p = 1.23E-8$. Forkhead binding motifs were found in 56% of the ER binding regions that contain a canonical ERE. Using the consensus Forkhead motif recurring within these regions (Figure 4B), we determined the probability of this motif residing within predicted ERE regions that are not bound by ER in vivo (18.45%). This significant enrichment of a Forkhead motif within ER binding regions ($p = 3.78E-7$) suggested the presence of adjacent Forkhead motifs may play a role in determining ER binding. The finding that the largest category of sites contains both an ERE and a Forkhead motif (47.4%) strongly suggests a functional interaction (Figure 4C).

Forkhead Proteins Play a Combinatorial and Essential Role in ER Binding and ER-Mediated Gene Transcription

A combinatorial interaction between Forkhead and ER pathways has been previously suggested for a small number of specific genes. HNF-3α (FoxA1) Forkhead binding domains within the promoter of the estrogen-regulated genes *TFF-1* (Beck et al., 1999) and Vitellogenin B1 (Robyr et al., 2000) have been shown to be important for gene transcription, and they have been shown to interact directly with ER in yeast two-hybrid experiments (Schuur et al., 2001). The function of Forkhead proteins can be regulated by their nuclear-cytoplasmic distribution depending on their phosphorylation (Brunet et al., 1999; Kops et al., 1999). We therefore determined that FoxA1 localized to the nucleus before and after estrogen stimulation of MCF-7 cells (data not shown).

We next determined whether FoxA1 was recruited along with ER to the ER binding domains. Directed ChIP of FoxA1 followed by real-time PCR of all 57 ER binding regions on chromosomes 21 and 22 revealed a high degree of concordance between regions that recruit ER and FoxA1. Approximately 48% of all of the ER

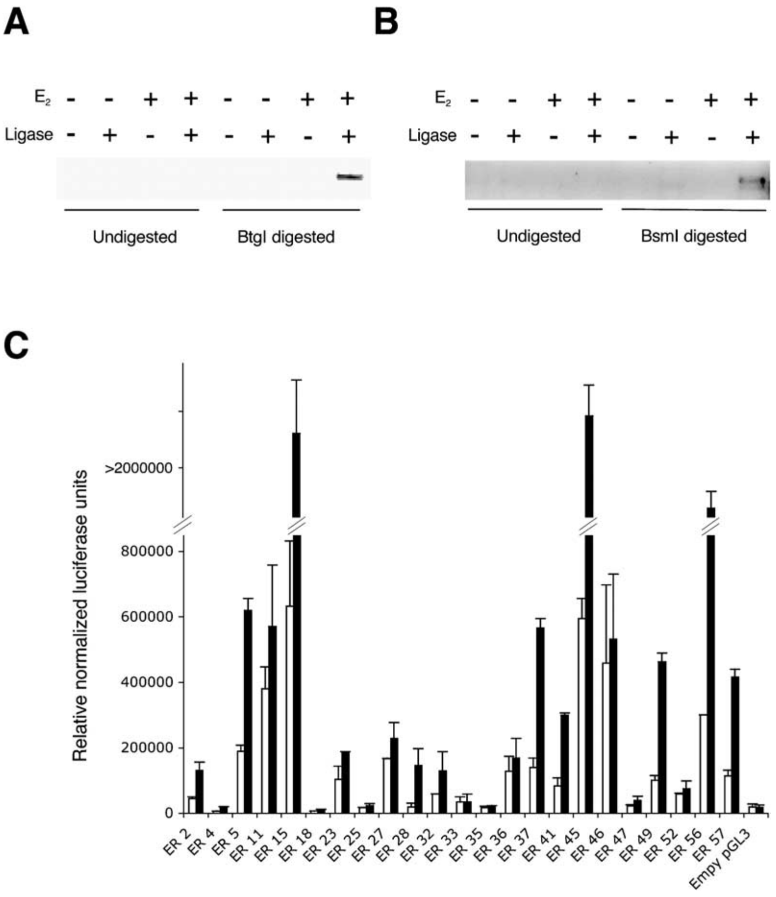

Figure 3. Interaction of Promoter-Enhancer Domains and Transcriptional Activity of Enhancer Regions

(A) Chromosome capture assay was performed after digesting fixed chromatin from vehicle- or estrogen-treated cells with the BtgI restriction enzyme. Primers flanking the *TFF-1* promoter and enhancer were used to amplify DNA after ligation. Undigested controls and no ligase controls were included.

(B) Chromatin was digested with BsmI, and one primer flanking the *NRIP-1* promoter and one in enhancer 3 region were used to amplify a specific product after ligation.

(C) ER binding sites were cloned into the pGL-3 promoter vector and transfected into hormone-depleted MCF-7 cells, after which vehicle (open bars) or estrogen (solid bars) was added. Empty pGL3-promoter vector was used as a negative control. Cotransfection of pRL null Renilla vector was included as a normalizing control. The data are the average of three replicates ± SD.

binding domains showed FoxA1 interaction, although the pattern of recruitment differed from site to site (Figure S3). A majority of the regions containing FoxA1 did so in the absence of estrogen, but FoxA1 binding was decreased following estrogen stimulation. This was the case for *NRIP-1* enhancer 1, *DSCAM-1* enhancer 1, and *TFF-1* promoter (Figure 5A). FoxA1 association with *XBP-1* enhancer 2 was clearly observed but was not diminished after estrogen addition (Figure 5A). All of these ER binding sites contained a Forkhead motif and an ERE or ERE half-site (Figure 5B). FoxA1 was not seen to bind to *XBP-1* enhancer 3, which lacks a Forkhead motif (Figure 5). However, several regions containing Forkhead motifs did not recruit FoxA1, and several ER binding domains that lacked Forkhead motifs did bind FoxA1. This complex interplay between FoxA1, ER, and binding sites within chromatin likely involves adjacent regions to the ER binding sites and may involve other proteins. Despite this, it is clear that a significant proportion of ER binding sites, especially those adjacent to actively transcribed genes, contain FoxA1 prior to estrogen stimulation and ER recruitment to the same regions.

To determine the importance of FoxA1 in mediating ER association with chromatin, we developed siRNA to the 3′UTR of *FoxA1* mRNA. Specific targeted knockdown of FoxA1 protein was achieved (Figure 6A), without changes in control protein or ER protein levels (data not shown). A luciferase siRNA (siLuc) was used as a negative control. MCF-7 cells were deprived of hormones for 24 hr and siLuc, or siRNA to *FoxA1*, was transfected for 6 hr, after which hormone-depleted media was added for a further 48 hr and cells were stimulated with estrogen or vehicle. ER ChIP and real-time PCR of a number of previously validated binding sites was performed. The decrease in *FoxA1* completely impeded the ability of ER to bind to *TFF-1* promoter, *XBP-1* enhancer 1, and *NRIP-1* enhancer 2 (Figure 6B), as well as *DSCAM-1* enhancer 1 (data not shown). No changes were observed on the *XBP-1* promoter, which functioned as a negative control (Figure 6B).

Since the targeted knockdown of FoxA1 inhibited the ability of ER to associate with in vivo ER binding sites, we assessed the effect of Forkhead downregulation on estrogen-mediated transcription. After siLuc or siFoxA1 transfection, cells were stimulated with estrogen or vehicle for 6 hr and mRNA changes in all 12 estrogen target genes on chromosomes 21 and 22 were assessed. The estrogen-induced increases in all 12 estrogen targets were abolished when FoxA1 was downregulated (Figure 6C), but no changes were observed in *GAPDH* control mRNA levels. The essential role for the FoxA1 Forkhead protein during transcription of all estrogen target genes on chromosomes 21 and 22 confirms a general requirement of FoxA1 for ER transcription.

Discussion

A complete picture of ER-mediated gene activation has begun to emerge in recent years, with a coordinated

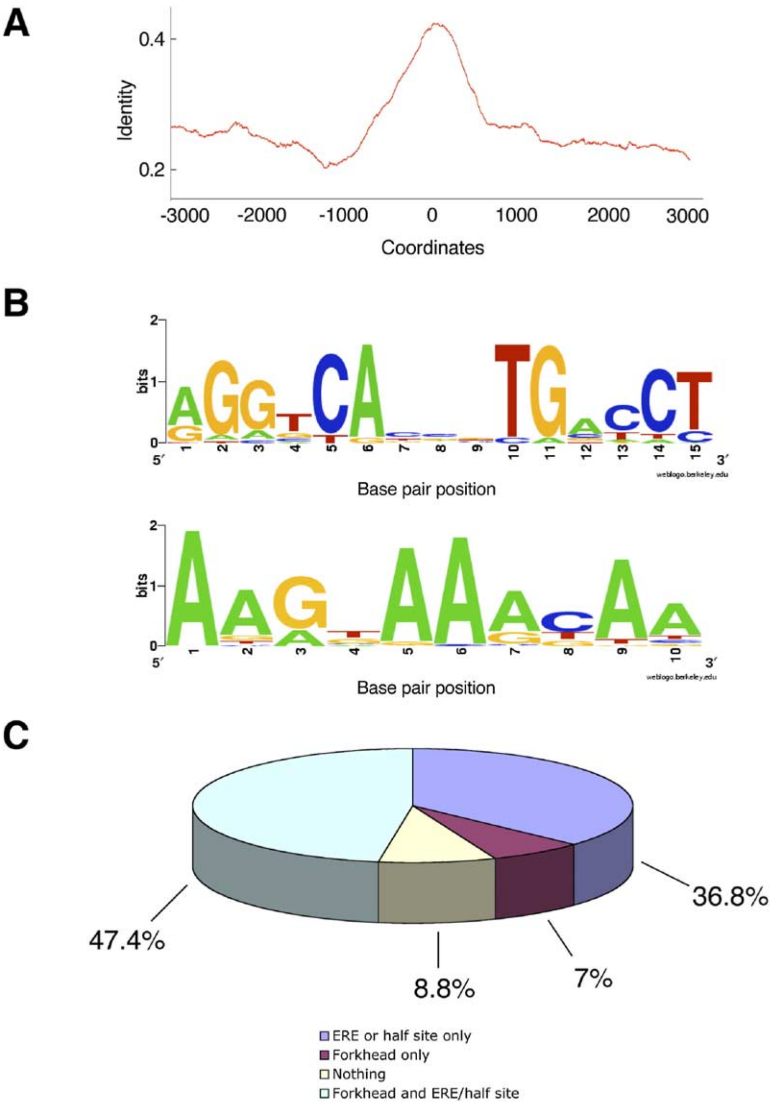

Figure 4. Conservation of ER Binding Sites and Presence of Enriched Motifs

(A) Sequence homology of ER binding sites and surrounding sequence between human and mouse genomes. The center of ER peaks is designated coordinate 0.

(B) An unbiased motif screen of all the ER binding sites on chromosomes 21 and 22 revealed the presence of two enriched motifs, an ERE and a Forkhead binding motif, both of which are visually represented in WebLogo (http://weblogo.berkeley.edu).

(C) The occurrence of ERE or ERE half-sites and Forkhead sites within the 57 ER binding sites on chromosomes 21 and 22.

and timely cycling of receptor, nuclear coactivators, chromatin remodelling proteins, and the transcription machinery on and off target promoters (Metivier et al., 2003; Shang et al., 2000). However, these studies oversimplify the problem by focusing on the promoter proximal region of one or two target genes and largely ignore the remaining chromosomal sequence. Here, we have interrogated the association of ER across entire chromosomes, including intergenic regions that contain potential *cis*-regulatory domains. These ChIP-microarray experiments demonstrate the ability to identify genuine in vivo ER protein binding sites in previously unexplored regions of the genome. Interestingly, while a few of the ER binding sites were found directly adjacent to ER target genes, most were found at significant distances including several >100 kb removed from transcription start sites. Of the 57 ER binding sites (within 32 potential transcriptional regulatory clusters), only a very small number of proximal promoters recruited ER, despite the fact that the other genes were estrogen induced. The presence of multiple components of the transcriptional machinery at distal sites combined with the ability of chromosome conformation capture assays to demonstrate that these distant sites are physically associated with promoter-proximal regions suggests that they play an important role in estrogen-mediated regulation.

A significant volume of work has focused on identifying essential domains within the proximal promoters of known estrogen regulated genes (Dubik and Shiu, 1992; Petz et al., 2002; Porter et al., 1996; Teng et al., 1992; Umayahara et al., 1994; Vyhlidal et al., 2000; Weisz and Rosales, 1990). The conclusions drawn from this large volume of data implicate a number of motifs, including Sp1, AP-1, and GC-rich regions as important *cis*-regulatory domains in ER-mediated transcription. However, our data demonstrate ER regulatory sites at distances several orders of magnitude greater than was focused on in the past, suggesting that they may function in ways analogous to the β-*globin* LCR (Sawado et al., 2003).

Nonbiased motif scanning of the genuine in vivo ER binding sites identified a canonical ERE in the majority of ER binding sites that represented only 1.5% of EREs

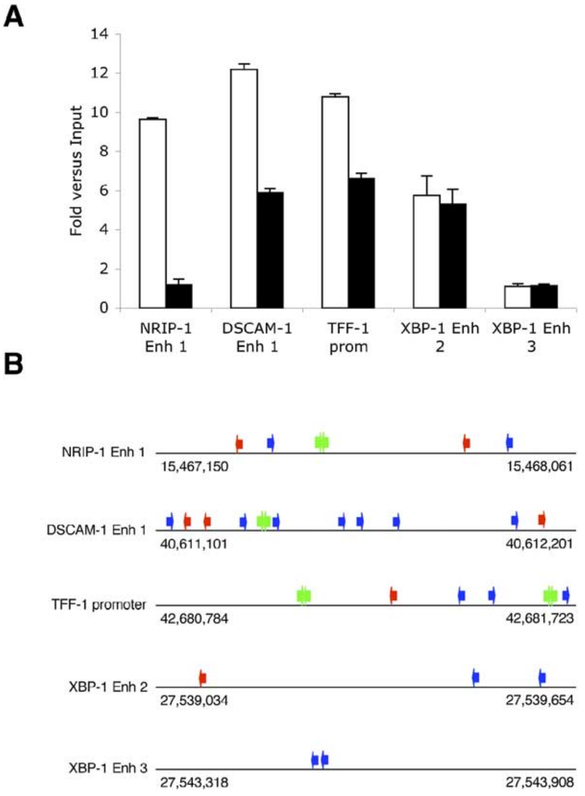

Figure 5. Recruitment of Forkhead Protein to ER Binding Domains

(A) ChIP of FoxA1 followed by real-time PCR of *NRIP-1* enhancer 1, *DSCAM-1* enhancer 1, *TFF-1* promoter, and *XBP-1* enhancer 2. *XBP-1* enhancer 3 is included as a control which does not recruit FoxA1. Data are shown as fold change versus input and are the average of three replicates ± SD. Open bars are vehicle treated and solid bars are estrogen treated.

(B) Schematic diagram showing the relative location of ERE motifs (inverted green arrows), ERE half-sites (blue arrows), and Forkhead motifs (red arrows). Chromosome nucleotide locations are given.

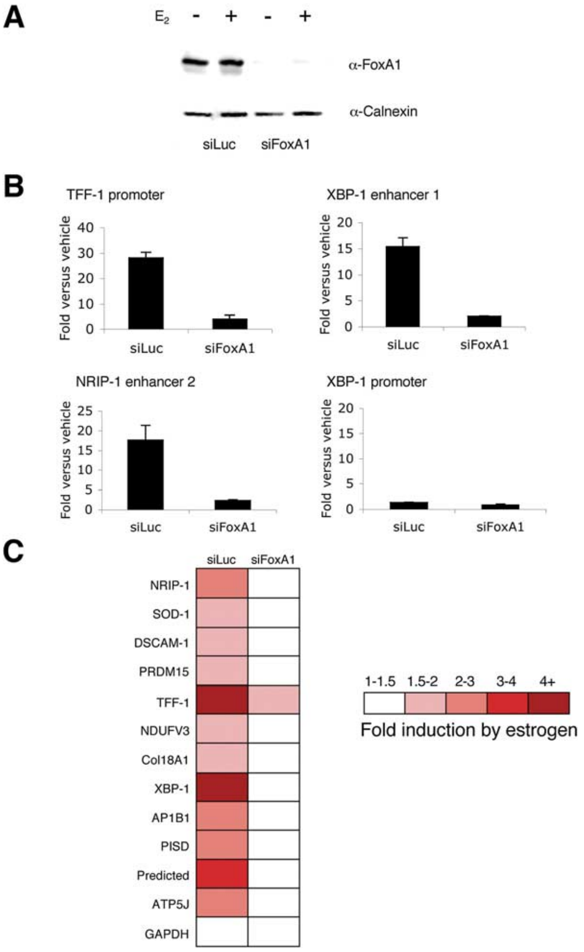

Figure 6. Specific Targeted Knockdown of FoxA1 and the Effects on Estrogen-Mediated Transcription

(A) siRNA to FoxA1 was transfected into hormone-depleted MCF-7 cells, and changes in FoxA1 protein levels were determined after vehicle or estrogen treatment. SiLuc was used as a transfection control and Calnexin was used as a loading control.

(B) ER ChIP was performed after vehicle or estrogen treatment of siLuc or siFoxA1 transfected cells and real-time PCR was conducted on *TFF-1* promoter, *XBP-1* enhancer 1, *NRIP-1* enhancer 2, as well as *XBP-1* promoter as a negative control. The data are fold enrichment over vehicle-treated.

(C) Changes in mRNA levels of all estrogen-regulated genes on chromosomes 21 and 22 after siLuc or siFoxA1. The data are estrogen-mediated fold enrichment compared to vehicle (ethanol) control and are the average of three separate replicates ± SD. The color intensity reflects the fold change as described in the legend.

predicted by bioinformatics alone. Previous approaches for motif identification involved computational-based methods for identifying response elements, after which gene proximal sites are included as potential binding domains (Bajic and Seah, 2003; Bourdeau et al., 2004). The current data suggest that while ER binding involves interaction with consensus ERE motifs, the presence of such motifs is insufficient to dictate receptor-chromatin association. Furthermore, the exclusion of response elements further than several kilobases from transcription start sites eliminates distal regulatory regions that may be the primary receptor-chromatin interaction sites.

Since the presence of an ERE alone is insufficient to define an authentic ER regulatory site, we searched for other conserved sequences and found that Forkhead factor binding sites are present near authentic EREs significantly more frequently than those that do not bind ER. We showed that a Forkhead factor (FoxA1) binding was essential for ER-chromatin interactions and subsequent expression of estrogen gene targets. A link between *ER* and *FoxA1* has previously been shown, with their expression correlated in breast cancer cell lines (Lacroix and Leclercq, 2004). FoxA1 protein can bind condensed chromatin via its winged-helix DNA binding domains that mimic histone linker proteins (Cirillo et al., 2002; Cirillo et al., 1998). Unlike histone proteins however, FoxA1 does not contain the amino acid composition to condense chromatin and it therefore is thought to promote euchromatic conditions. As such, it is possible that the presence of FoxA1 identifies specific regions within chromatin to facilitate the association of the ER transcription complex. Our data suggest that FoxA1 is present on the chromatin at a number of regions, after which ER can associate with these specific sites. Downregulation of FoxA1 inhibits the ability of ER to associate with its binding sites, confirming the requirement for Forkhead-directed association of ER with chromatin, despite the fact that these sites contain sufficient information, in the form of an ERE, for ER docking. This, combined with a recent investigation showing that FoxA1 can directly modulate chromatin in the MMTV promoter and can positively enhance transcription by the glucocorticoid receptor (Holmqvist et al., 2005), supports a general model for FoxA1 involvement in nuclear receptor transcription.

We have taken an unbiased approach to identify regions of chromatin, both promoter proximal and intergenic sequences, which are involved in ER-mediated transcriptional activity. We find a limited number of bona fide ER binding sites on chromosomes 21 and 22, with a significant enrichment of canonical ERE palindromes and half-sites within the binding sites. Moreover, the presence of Forkhead binding motifs and the subsequent identification of a functional role for the Forkhead protein FoxA1 in estrogen signaling exemplifies the power of this approach to identify important regulatory domains within the vast regions of unexplored sequence of the human genome.

Experimental Procedures

Chromatin Immunoprecipitation (ChIP)-Microarray Preparation

ChIP was performed as previously described (Shang et al., 2000), with the following modifications. Two micrograms of antibody was prebound for a minimum of 4 hr to protein A and protein G Dynal magnetic beads (Dynal Biotech, Norway) and washed three times with ice-cold PBS plus 5% BSA and then added to the diluted chromatin and immunoprecipitated overnight. The magnetic bead-chromatin complexes were collected and washed six times in RIPA buffer (50 mM HEPES [pH 7.6], 1 mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 0.5 M LiCl). Elution of the DNA from the beads was as previously described (Shang et al., 2000). Antibodies used were as follows: ERα (Ab-10) from Neomarkers (Lab Vision, United Kingdom), ERα (HC-20), RNA PolII (H-224), AIB-1/RAC3 (C-20), HNF-3α/FoxA1 (H-120), mouse IgG (sc-2025), and rabbit IgG (sc-2027) from Santa Cruz (Santa Cruz Biotechnologies, California). Ligation-Mediated PCR was performed as previously described (Ren et al., 2002). Labeling was performed as previously described (Kapranov et al., 2002). Microarrays used were Affymetrix Genechip chromosome 21/22 tiling set P/N 900545.

Data Analysis

1,054,325 probe pairs were mapped to chromosomes 21 and 22 according to the NCBIv33 GTRANS Libraries provided by Affymetrix. (PM-MM) value was recorded for each probe pair, and a probe pair was removed if either PM or MM was flagged as outlier by the Affymetrix GCOS software. The samples (three ER+ ChIP and three genomic inputs) were normalized by quantile normalization (Bolstad et al., 2003) based on a combined 76 ChIP experiments obtained from public domain and Dana-Farber Cancer Institute. The behavior of every probe pair *i*, assumed to be $N(\mu_i, \sigma_i^2)$, was estimated from the 76 normalized experiments. A two-state (ChIP-enriched state and nonenriched state) Hidden Markov Model with the following parameters was applied to each sample to estimate the probability of ChIP enrichment at each probe pair location:

> Transition probabilities: 300/1,054,325 for transition to a different state,
> 1 − (300/1,054,325) for staying in the same state.
> Emission probabilities: $N(\mu_i, \sigma_i^2)$ for nonenriched hidden state,
> $N(\mu_i + 2\sigma_i, (1.5\sigma_i)^2)$ for enriched hidden state.

To combine the results from the six samples, an enrichment score was calculated as the average enrichment probability in the three ER+ ChIP samples subtracted by the average enrichment probability in the three genomic input samples. Since the tiling array has one 25-mer probe in every 35 bp of nonrepeat regions, the coverage of every probe was extended by 10 bp on both ends. An enriched regions is defined as run of probes with enrichment score >50% and covering at least 125 bp. Each enriched region can tolerate up to two neighboring probes with enrichment score between [10%, 50%]. If two neighboring probes are more than 210 bp apart, the enriched region is broken into two separate blocks. A summary enrichment score was obtain for each enriched region, which is the enrichment score summation for all the probes in the region divided by the square root of the number of probes in the region. This summary enrichment score represents the relative confidence of a predicted enriched region.

Sequence Analysis

The genomic DNA of every ChIP-enriched region was retrieved from UCSC genome browser and ranked by the summary enrichment score. MDscan algorithm (Liu et al., 2002) was applied to the sequences to find enriched sequence pattern that is the putative estrogen receptor binding motif. To find a motif of width w, MDscan first enumerates each w-mer in the highest ranking sequences and collects other w-mers similar to it in these sequences to construct a candidate motif as a probability matrix. A semi-Bayes scoring function was used to remove low-scoring candidate motifs and refine the rest by checking all w-mers in all the ChIP-enriched sequences. A high-scoring motif (with similar consensus) consistently reported multiple times at different motif widths indicates a strong prediction.

We expanded all 57 of the ER binding sites equally in each direction to have a length of 6 kb. The human-mouse conservation score of each nucleotide in the expanded binding region is defined as the average sequence identity (# matched nucleotides − # indels)/500 of a 500-mer window centered at the nucleotide. The human (hg15) /mouse (mm3) BLASTZ (Schwartz et al., 2003) genome alignments were downloaded from http://genome.ucsc.edu.

Real-Time PCR

Primers were selected using Primer Express (Applied Biosystems). Five microliters of precipitated and purified DNA was subjected to PCR using the Applied Biosystems SYBR Green Mastermix. Relative DNA quantities were measured using the PicoGreen system (Molecular Probes, Oregon). All primer sequences and locations are listed in Table S2.

Double-Stranded cDNA Synthesis

Total RNA was converted to double stranded cDNA according to the Invitrogen Superscript double-stranded cDNA synthesis manufacturer's instructions. The RNA was primed with 250 ng oligo(dT) (Invitrogen) and 25 ng random hexamers (Gibco). cDNA was fragmented and labeled as described above.

5′RACE

5′ RACE was performed according to the manufacturer's instructions (Invitrogen). The primers sequences used were as follows: *NRIP-1* RT primer (5′-TGCCTGATGCATTAGTAATCC-3′), *NRIP-1* nested primer 1 (5′-GAGCCAAGCTCTTCTCCATGAGTCATGTTC-3′), and *NRIP-1* nested primer 2 (5′-ACCTTCCATCGCAATCAGAGA GAGACGTACTG-3′). The PCR product was cloned and sequenced by standard methods.

Chromosome Capture Assay

Fixed chromatin was digested overnight with specific restriction enzymes after which ER ChIP was set up as described above. After overnight ChIP, the beads were precipitated and resuspended in ligation buffer (NEB, Massachusetts) and overnight ligation was performed. The beads were collected, washed, and the formaldehyde crosslinking was reversed as described above. Primers used to amplify annealed fragments were as described in Table S2.

Luciferase Enhancer Activity

ER binding sites were amplified by PCR and cloned into the pGL-3-promoter vector (Promega). Hormone-depleted MCF-7 cells were transfected with each of the ER binding domain vectors with Lipofectamine 2000 (Invitrogen), and total protein lysate was harvested after estrogen or ethanol addition for 24 hr. Transfections were normalized by the cotransfection of the pRL null renilla luciferase vector and renilla and firefly luciferase activity was assessed using the dual luciferase kit (Promega).

Western Blotting

SDS-PAGE was performed as previously described (Carroll et al., 2000). Antibodies used were FoxA1/HNF-3α (ab5089), from AbCam (Cambridge, United Kingdom) and Calnexin (H-70) from Santa Cruz (California).

Short Interfering (si) RNA

A 21 bp siRNA was designed against the FoxA1 transcript and synthesized by Dharmacon (Lafayette, Colorado). siRNA was transfected using Lipofectamine 2000 (Invitrogen). The siRNA sequences used were as follows: *siFoxA1* sense 5′-GAGAGAAAAAA UCAACAGC-3′ and antisense 5′-GCUGUUGAUUUUUUCUCUC-3′; siLuc sense 5′-CACUUACGCUGAGUACUUCGA-3′ and antisense 5′-UCGAAGUACUCAGCGUAAGUG-3′.

Supplemental Data

Supplemental Data include four figures, two tables, and raw data files and can be found with this article online at http://www.cell.com/cgi/content/full/122/1/33/DC1/.

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