

# Nkx3-1 and LEF-1 Function as Transcriptional Inhibitors of Estrogen Receptor Activity

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## Abstract

**Estrogen receptor (ER)-associated cofactors and cooperating transcription factors are one of the primary components determining transcriptional activity of estrogen target genes and may constitute potential therapeutic targets. Recent mapping of ER-binding sites on a genome-wide scale has provided insight into novel cooperating factors based on the enrichment of transcription factor motifs within the ER-binding sites. We have used the ER-binding sites in combination with sequence conservation to identify the statistical enrichment of Nkx and LEF motifs. We find that Nkx3-1 and LEF-1 bind to several ER *cis*-regulatory elements *in vivo*, but they both function as transcriptional repressors of estrogen signaling. We show that Nkx3-1 and LEF-1 can inhibit ER binding to chromatin, suggesting competition for common chromatin-binding regions. These data provide insight into the role of Nkx3-1 and LEF-1 as potential regulators of the hormone response in breast cancer. [Cancer Res 2008;68(18):7380–5]**

## Introduction

Estrogen receptor (ER) transcription is a fundamental process in the regulation of breast cancer cell division and tumor progression. The molecular mechanisms of ER-mediated gene induction have focused primarily on promoter proximal regions of target genes (1–4) but have provided significant information about the multi-protein complexes involved in estrogen-stimulated gene transcription (5, 6). The role of cooperating transcription factors, such as AP-1, Sp-1, and cAMP proteins, in assisting ER transcription was derived from reporter assay experiments, showing that the binding motifs for these specific transcription factors occur in the promoters of estrogen target genes of interest and are required for transcription (2, 3). More recent *in vivo* chromatin immunoprecipitation (ChIP) analyses on endogenous promoter regions have illuminated the proteins that assist in ER association with DNA and modulate chromatin structure.

A large number of factors are involved in regulating ER transcription at promoter regions, although recent chromosome-wide and genome-wide location analyses have revealed the role of distal *cis*-regulatory elements (7, 8). The first genome-wide map of ER binding revealed a total of 3,665 binding sites (using a relatively stringent statistical cutoff) and ~10,600 sites at a lower cutoff, in line with the computational prediction of between 5,000 and 10,000

(9). More recent promoter analysis of ER-binding sites suggests that the genuine number of ER-binding sites may be higher than the first prediction (10). Our genome-wide ChIP-chip analysis (8) and others (11, 12) estimate that only approximately 3% to 5% of ER binding occurs at promoter proximal regions, and as such, the distal enhancer regions are likely to dictate the significant majority of transcriptional regulation (13).

Analysis of motifs that are statistically enriched within the sequence of distal ER *cis*-elements revealed the enrichment of forkhead motifs (7). Subsequent analyses showed that the forkhead protein, FoxA1, was essential for tethering ER to the chromatin (7, 8) and is involved in enhancing the localized chromatin structure at *cis*-regulatory regions of target genes, including cyclin D1 (14). Roles for AP-1, Oct1, and C/EBP factors in ER activity were also shown based on the enrichment of their binding motifs (8, 15, 16), in many cases within binding sites that were conserved between species.

The recent release of genome sequence information for species that diverged from human before rodents provides a powerful tool for identification of important regulatory regions that maintained sequence fidelity during longer periods of evolution. A combination of the conservation information from 16 species (17), with our 3,665 ER-binding sites, provides insight into novel regulatory pathways of ER signaling. We find enriched Nkx and TCF/LEF motifs and on further investigation determine that Nkx and LEF proteins act as repressors by competing with ER for binding to common regions within the genome.

## Materials and Methods

**Motif screen.** The coordinates of 3,665 ER ChIP-enriched regions published in Carroll et al. (8) were converted to the Human Genome Assembly version (HG18, March 2006), and the regions were uniformly resized to 800 bp symmetrically with respect to the center. The phastCons (17) conservation scores, which are computed by aligning 16 vertebrate genomes with HG18, were then obtained. All basepairs with phastCons scores <0.7 were then masked, and the resulting regions were scanned using all motif matrices from TRANSFAC and JASPAR. All motifs can be found in Supplementary Data.<sup>6</sup> The Nkx and LEF-1 motifs were found to be significantly enriched compared with the genomic background, which was chosen to be the promoters of nondifferentially expressed genes on treatment with estrogen. Using cutoffs higher than 0.7 for phastCons scores yielded very similar results.

**Cell culture.** MCF-7 breast cancer cell lines were maintained and treated as previously described (7).

**Small interfering RNA.** MCF-7 cells were transfected with small interfering RNA (siRNA) or control siLuciferase (siLuc) as previously described (7). The siRNA sequences are as follows: *siNkx3-1*, 5'-GCUAUAAGACUAAGCGAAAUU-3' (sense) and 5'-UUUCGUUAGUCUUAUAGCUU-3'

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-08-0133

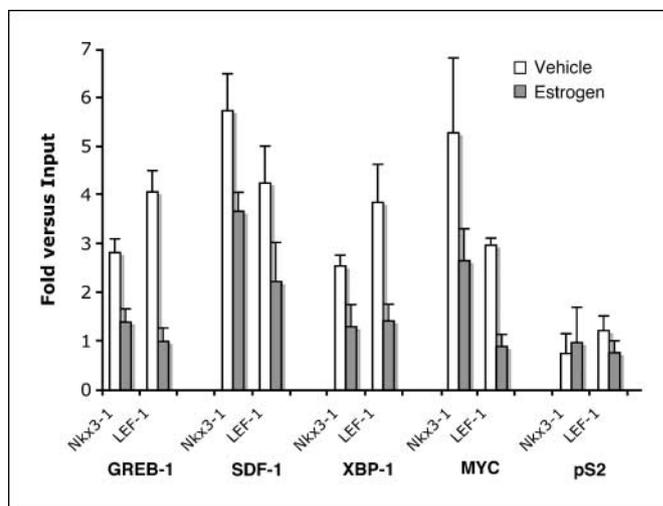
<sup>6</sup> <http://weblogo.berkeley.edu/>

(antisense); *siLEF-1*, 5'-GAACGAGUCUGAAAUCAUCU-3' (sense) and 5'-GAUGAUUCAGACUCGUUCU-3' (antisense).

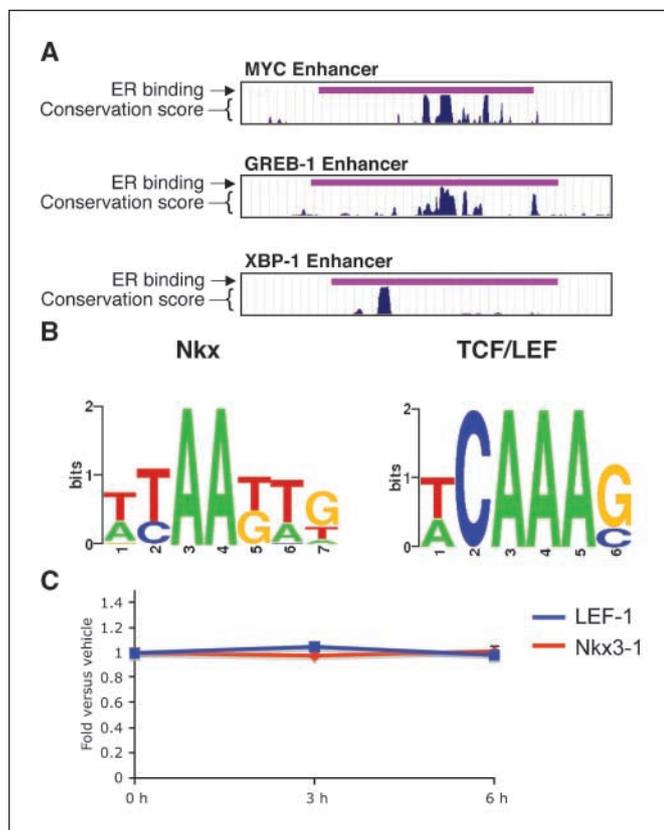
**Overexpression experiments.** pcDNA3-Nkx3-1-expressing mouse Nkx3-1 (a kind gift from Professor Michael Shen, Department of Pediatrics, University of Medicine and Dentistry of New Jersey, Newark, NJ) and pCG-LEF-1 (a kind gift from Dr. Rudolf Grosschedl, University of Munich, Munich, Germany) were transfected into MCF-7 cells that had been deprived of hormones as previously described (7). Lipofectamine 2000 was used for transfections and pcDNA3.1 was included as a control. Estrogen stimulation of cells was performed as previously described (7).

**Western blotting.** Western blots were performed as previously described (18). Antibodies used were Nkx3-1, LEF-1, and ER from Santa Cruz Biotechnology and  $\beta$ -actin from Abcam.

**ChIP and re-ChIP.** ChIP was performed as previously described (7). Re-ChIP experiments were conducted as previously described (6). Antibodies used were Nkx3-1, LEF-1, ER, and histone deacetylase 1 (HDAC1) from Santa Cruz Biotechnology. The primers used for real-time PCR were the following: GREB-1 enhancer 3, 5'-GAAGGGCAGAGCTGATAACG-3' (forward) and 5'-GACCCAGTGGCCACACTTTT-3' (reverse); SDF-1 enhancer, 5'-AGGCATCACAATGCAAATCA-3' (forward) and 5'-AGGCTGGTGAGATGCTGAGT-3' (reverse); XBP-1 enhancer 1, 5'-ATACTTGGCAGCCTGTGACC-3' (forward) and 5'-GGTCCACAAAGCAGAAAA-3' (reverse); MYC enhancer, 5'-ACTCTGCAGTCCAGACAAA-3'



**Figure 2.** Association of Nkx3-1 and LEF-1 with ER-binding sites. ChIP of Nkx3-1 and LEF-1 followed by real-time PCR of ER-binding sites adjacent to *GREB-1*, *SDF-1*, *XBP-1*, *MYC*, and *pS2/TF-1* genes. The data are shown as the fold enrichment compared with input. *White columns*, vehicle-treated cells; *gray columns*, estrogen-treated cells.



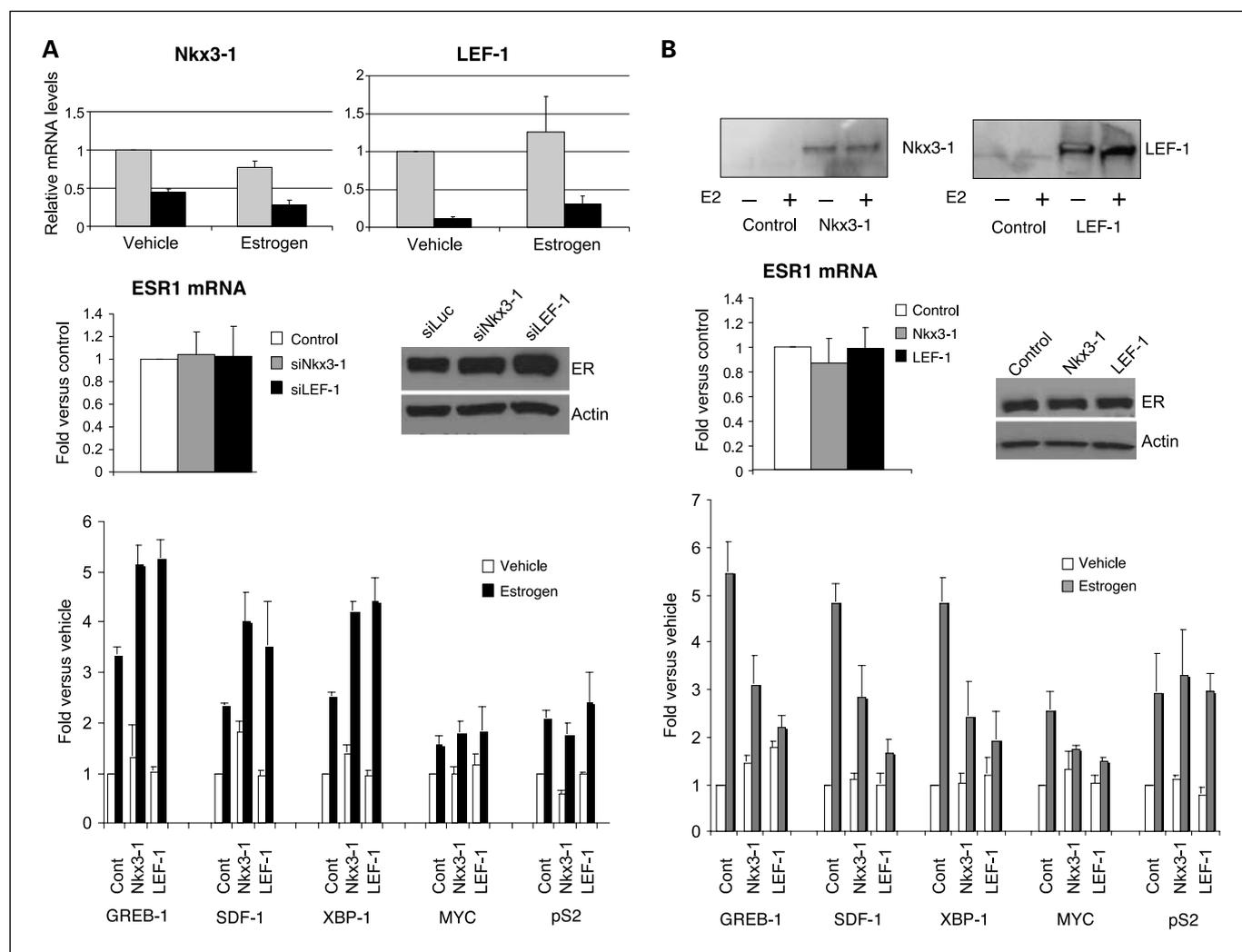
**Figure 1.** Enriched motifs within ER-binding sites. *A*, conservation between the human genome sequence and 15 other species across the ER-binding sites observed in MCF-7 cells revealed hyperconserved regions within the binding sites. Examples shown are ER-binding sites adjacent to *MYC*, *GREB-1*, and *XBP-1* genes. *Purple bars*, ER-binding sites; *blue peaks*, graphical representations of the conservation scores across the entire ER-binding site. *B*, a screen of enriched transcription factor-binding motifs within these hyperconserved regions of all ER-binding sites across the human genome revealed an enrichment of Nkx and TCF/LEF motifs (all motifs can be found in Supplementary Data 1). *C*, mRNA levels of Nkx3-1 and LEF-1 were assessed after estrogen treatment. The data are shown as the fold versus vehicle. *Points*, average of three independent experiments; *bars*, SD.

(forward) and 5'-TGGAAACCACATTTTGGTCA-3' (reverse); and pS2/TF-1 enhancer, 5'-GAGGTGTCTTGGCCACTGTT-3' (forward) and 5'-GACTCCACTGTCTCGAAGC-3' (reverse). To determine enrichment, we normalize both the ChIP DNA and total genomic DNA (input) to nonspecific control regions of the genome. We subsequently normalize the enrichment after ChIP to input levels.

**Reverse transcription-PCR.** Quantitative reverse transcription-PCR (RT-PCR) was performed as previously described (7). The primers used were as follows: SDF-1, 5'-TGAGATGCTTGACGTTGGCT-3' (forward) and 5'-CCTGAGTACAGATGCCCATG-3' (reverse); XBP-1, 5'-GCGCC-TCACGCACCTG-3' (forward) and 5'-GCTGCTACTCTGTTTTTCAG-TTCC-3' (reverse); MYC, 5'-GCCACGTCTCCACATCAG-3' (forward) and 5'-TCTTGGCAGCAGGATAGTCCTT-3' (reverse); GREB-1, 5'-CAAAGAA-TAACCTGTTGGCCCTGC-3' (forward) and 5'-GACATGCCTGCGC-TCTACACTTA-3' (reverse; ref. 19); pS2, 5'-GTGTCACGCCCTCCAGT-3' (forward) and 5'-GGACCCACGACCGTG-3' (reverse); Nkx3-1, 5'-GCCAA-GAACCTCAAGCTCAC-3' (forward) and 5'-AGAAGGCCTCTTTTCAGG-3' (reverse); LEF-1, 5'-ACAGATCACCCACCTCTTG-3' (forward) and 5'-TGA-TGGGAAAACCTGGACAT-3' (reverse); and ESRI, 5'-TGATTGGTCTCCTGTCGGC-3' (forward) and 5'-CATGCCCTTACACATT-TTCC-3' (reverse).

## Results

We analyzed our ER-binding data from previous ER ChIP-on-chip experiments using the sequence information of 16 species and have found discrete elements within the ER-binding sites that are conserved in a broader biological context. A screen of motifs that occurred within these acute regions of hyperconservation across all ER-binding sites (Fig. 1*A* represents examples of these hyperconserved regions) revealed the enrichment of binding sites for Nkx transcription factors (TNAAGTG) that were overrepresented by 1.89-fold ( $P < 1 \times 10^{-27}$ ) and LEF/TCF transcription factor motifs (TCAAAG) that were enriched 2.11-fold over expected frequency ( $P < 1 \times 10^{-33}$ ; Fig. 1*B*). All enriched motifs are in Supplementary Data 1. Nkx motifs had never been shown to be enriched within the ER-binding sites in any of the previous analyses and were only found when focusing on the conserved regions within the ER-binding sites. On average, these motifs were located



**Figure 3.** Regulation of Nkx3-1 and LEF-1 expression can influence estrogen-mediated gene transcription. *A*, siRNA to Nkx3-1 and LEF-1 or siLuc as control was transfected into hormone-depleted MCF-7 cells and mRNA levels of Nkx3-1 and LEF-1 were assessed. *Gray columns*, control-transfected cells; *black columns*, transfected cells. ER (*ESR1*) mRNA and protein levels were assessed after siNkx3-1 or siLEF-1 transfection. Following siRNA inhibition of either Nkx3-1 or LEF-1, total RNA was collected after vehicle (*white columns*) or estrogen (*black columns*) and mRNA levels of the estrogen-regulated gene targets *GREB-1*, *SDF-1*, *XBP-1*, *MYC*, and *pS2/TF-1* were assessed by quantitative RT-PCR. *Columns*, average of three individual replicates; *bars*, SD. *B*, overexpression of Nkx3-1 or LEF-1. Following overexpression of Nkx3-1 or LEF-1, or pcDNA3.1 as a control, either vehicle (*white columns*) or estrogen (*gray columns*) was added for 4 h and mRNA levels of the estrogen-regulated gene targets *GREB-1*, *SDF-1*, *XBP-1*, *MYC*, and *pS2/TF-1* were assessed by quantitative RT-PCR.

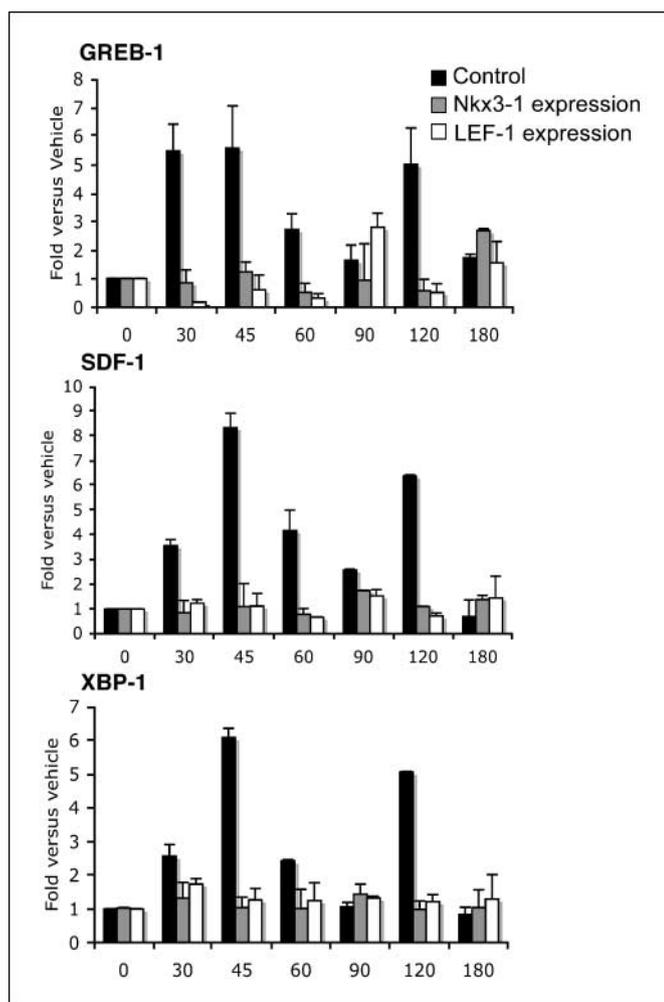
within 130 bp of an estrogen-responsive element. Other motifs enriched in conserved regions include forkhead, GATA, Oct, and AP-1 motifs, all of which have been previously shown to be involved as binding sites for ER-associated cooperating factors (7, 8, 14, 20).

Little is known about the interactions between the Nkx homeodomain proteins and ER, although Nkx3-1 is an androgen-regulated gene (21) and is a putative regulator of prostate cancer (22, 23). A link between TCF/LEF-1 and mammary biology was established several years ago when it was shown that mice lacking LEF-1 fail to develop mammary glands (24). LEF-1 is a downstream target of the Wnt signaling pathway and is an ER-interacting transcription factor *in vitro* (25). Given these pieces of evidence, we focused on characterizing the interactions between Nkx3-1 and LEF-1 with the ER pathway. We confirmed that neither Nkx3-1 nor LEF-1 is an estrogen-regulated gene (Fig. 1C).

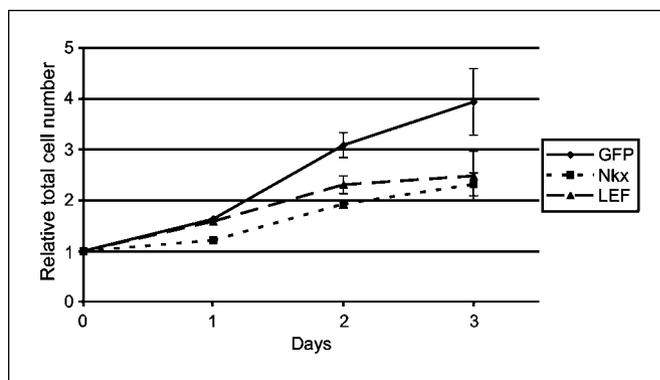
To determine if Nkx3-1 and LEF-1 played a role at the distal ER *cis*-regulatory elements, we performed ChIP of Nkx3-1 and LEF-1 followed by real-time PCR of several previously identified ER-binding sites, as determined by genome-wide ER ChIP-chip experiments (8). These sites included *cis*-regulatory regions adjacent to *GREB-1*, *SDF-1*, *XBP-1*, and *MYC*, all of which are estrogen up-regulated genes and almost all of which were chosen due to their critical roles in estrogen signaling in either breast cancer cell lines or primary breast cancers (26–29). The real-time PCR data suggest that Nkx3-1 binds to a majority of these assessed ER-binding sites, but unlike most other cofactors (5, 6), it is associated with the chromatin before estrogen signaling and dissociates after estrogen treatment (Fig. 2). Surprisingly, LEF-1 binding is also robust on most of the assessed sites before estrogen treatment, although similar to Nkx3-1, occupancy decreases after

estrogen treatment. No binding of Nkx3-1 or LEF-1 was observed on the *pS2/TFF-1* promoter, which has functioned as the archetypal ER target site in previous studies (5, 6). Therefore, Nkx3-1 and LEF-1 seem to associate *in vivo* with the ER *cis*-regulatory regions, although they are not universally observed at all ER-binding sites, similar to what has been previously seen for other ER cooperating factors (8).

To investigate what role, if any, Nkx3-1 and LEF-1 have on ER-mediated transcription, we designed siRNA that specifically targeted either Nkx3-1 or LEF-1. These siRNAs were transfected in growth-arrested MCF-7s that had been deprived of hormones for 3 days. siLuc was included as a control. Basal levels of Nkx3-1 and LEF-1 were insufficient even in the absence of siRNA to detect by Western blot, and as such, we confirmed the specific silencing of Nkx3-1 and LEF-1 by assessing changes in mRNA. These data confirmed that Nkx3-1 and LEF-1 could be specifically inhibited using siRNAs (Fig. 3A). No significant changes were observed in ER mRNA or protein levels (Fig. 3A), although ER binding was generally increased in the presence of siNkx3-1 or siLEF-1



**Figure 4.** Overexpression of Nkx3-1 or LEF-1 inhibits ER binding. Control plasmid or expression plasmids for Nkx3-1 or LEF-1 were transiently transfected into hormone-depleted MCF-7 cells and estrogen was added for increasing time periods. ER ChIP was performed on the ER-binding sites adjacent to the *GREB-1*, *SDF-1*, and *XBP-1* genes. The data are fold enrichment relative to time 0 h after normalization to input.



**Figure 5.** Estrogen-mediated growth is perturbed by expression of Nkx3-1 or LEF-1. MCF-7 cells were transfected with Nkx3-1 or LEF-1 constructs or control vector, after which total cell number was determined. The data are a representative experiment of three independent replicates.

(Supplementary Data 2). We specifically silenced either Nkx3-1 or LEF-1, after which we stimulated MCF-7 cells with estrogen for 4 h and collected total RNA. We subsequently assessed changes in GREB-1, SDF-1, XBP-1, and MYC mRNA levels, all of which were shown to have adjacent *cis*-regulatory elements that were bound by Nkx3-1 and LEF-1. SDF-1, XBP-1, and GREB-1 gene transcription were all marginally increased by estrogen, in the absence of Nkx3-1 or LEF-1 (Fig. 3A), suggesting a potential role for Nkx3-1 and LEF-1 as repressors of estrogen action. *pS2/TFF-1* transcript levels were not appreciably altered by siRNA to Nkx3-1 or LEF-1.

To confirm the findings that both Nkx3-1 and LEF-1 may function as transcriptional repressors, we transiently transfected constructs expressing either Nkx3-1, LEF-1, or a control vector (pcDNA) in growth-arrested MCF-7 cells. Vehicle or estrogen stimulation was performed for 4 h and Western blot analysis was conducted for Nkx3-1 and LEF-1 protein levels (Fig. 3B). ER mRNA and protein levels were not altered by expression of Nkx3-1 or LEF-1 (Fig. 3B). Nkx3-1 and LEF-1 binding was confirmed by ChIP (Supplementary Data 3). After Nkx3-1 or LEF-1 expression, we assessed transcript levels of GREB-1, SDF-1, XBP-1, and MYC and found all four genes were repressed (Fig. 3B). No changes were observed in *pS2/TFF-1* levels, confirming that Nkx3-1 and LEF-1 do not compete for ER binding to the *pS2/TFF-1* promoter. These data support the hypothesis that Nkx3-1 and LEF-1 can function as repressors of ER activity on a subset of estrogen target genes.

To determine if overexpression of Nkx3-1 or LEF-1 had direct effects on ER loading on the chromatin, we expressed Nkx3-1 or LEF-1 constructs or a control vector and performed ER ChIP over a time course of estrogen stimulation (5, 6). In control-transfected cells, ER cycles on the chromatin at 45 min and off by 90 min, with predictable kinetics (Fig. 4A; refs. 5, 30). We observe this on all three ER-binding sites tested (i.e., the GREB-1, SDF-1, and XBP-1 enhancers). However, when we express either Nkx3-1 or LEF-1, we significantly inhibit ER binding to all three enhancer regions at all the time points (Fig. 4B) but not at the *pS2* promoter (Supplementary Data 4).

To determine if Nkx3-1 and LEF-1 were functioning in a repressive manner at the ER-binding sites by competing with ER for loading onto the chromatin, we performed coimmunoprecipitation experiments and could not show any interactions between ER and Nkx3-1 or LEF-1 (data not shown). Furthermore, we performed re-ChIP experiments, where ER ChIP was performed

followed by "release" of the chromatin, and re-ChIP with Nkx3-1 or LEF-1. We did not, under any circumstances, find interactions between ER and Nkx3-1 or LEF-1. However, we also performed re-ChIP of HDAC1, a marker of heterochromatin, followed by Nkx3-1 or LEF-1 ChIP. Real-time PCR of the ER-binding sites adjacent to XBP-1 and SDF-1 confirmed that in vehicle-treated cells, Nkx3-1 and LEF-1 cooccupy the chromatin with HDAC1 in a repressive manner, but this interaction decreases to background levels after estrogen treatment (Supplementary Data 5).

The data suggest that both Nkx3-1 and LEF-1 function in a transcriptionally repressive manner but that they simply compete with ER for binding to the same regulatory domains and that estrogen addition results in dissociation of Nkx3-1 and LEF-1 in favor of ER binding. To determine if this competition for binding can influence the cellular response to estrogen, we overexpressed Nkx3-1 and LEF-1 and assessed for changes in total cell number. Control-transfected cells proliferated as expected, but expression of either Nkx3-1 or LEF-1 blunted the proliferative response, suggesting that elevated Nkx3-1 or LEF-1 can block ER binding (Fig. 4) and proliferative activity (Fig. 5).

## Discussion

This study shows for the first time that Nkx3-1 and LEF-1 can function as direct chromatin-interacting proteins that have repressive properties on estrogen-mediated gene transcription. This discovery was borne of a computational screen for enriched motifs hidden within discrete hyperconserved regions in recently defined ER *cis*-regulatory elements, as determined by ChIP-on-chip analyses. A recent computational approach to finding transcription factor modules, or hotspots, relies on the presence of multiple transcription factor-binding motifs occurring in sequence that is conserved between species (31) and finds more than 118,000 module across the human genome. This program has widespread applicability (32) and will prove to be a useful tool, especially given the fact that it takes into account both genic and intergenic regions, both of which are likely to constitute transcription factor-binding regions (8, 33, 34).

Nkx3-1 has been hypothesized to be a nuclear receptor-interacting factor and has been suggested to be a prostate-specific factor that is regulated by androgens (21) via androgen-responsive elements in the regulatory region of the *Nkx3-1* gene (35). Several pieces of evidence suggest that it functions in a repressive manner. Nkx3-1 has been shown in *in vitro* experiments to possess transcriptional repressor properties from a reporter construct containing multiple Nkx-binding domains (36). Furthermore, loss of Nkx3-1 in mice leads to prostatic intraepithelial neoplasia

(22, 23) and the genomic regions encompassing the *Nkx3-1* gene are thought to be lost in prostate cancers (37), supporting its role as a putative repressor of hormone-driven tumors. We now show that Nkx3-1 can function as an ER repressor in breast cancer cells and that the mechanism of this repression is direct competition with ER for binding to regions within the genome containing adjacent motifs.

TCF/LEF-1 is a downstream regulator of the Wnt signaling pathway, which has been shown to correlate with ER status in tumors and, interestingly, has been shown to inhibit tumor cell invasion in Matrigel assays when overexpressed (38). Furthermore, *Lef-1* knockout mice fail to develop mammary glands (24), confirming a role for LEF-1 in mammary cells and breast cancer. Another investigation suggested that LEF-1 and ER have a protranscriptional interaction (25), although this previous study focused on *in vitro* assays, such as gel shifts, and used a rat mammary epithelial cell line, possibly explaining the differences observed with our data. Interestingly, expression of an NH<sub>2</sub> terminus mutant of LEF-1, ablated its ability to regulate  $\beta$ -catenin pathway, results in increased tumor formation in mice (39), supporting the data that LEF-1 may function to suppress transcription and tumor formation.

Our data suggest that both Nkx3-1 and LEF-1 can modulate ER activity, but this is not due to recruitment as ER cooperating factors but due to competition for binding to DNA. Nkx3-1 and LEF-1 did not interact with ER when tested, but Nkx3-1 and LEF-1 could associate with HDAC1 at the ER-binding sites, suggestive of a repressive role. Estrogen stimulation induces recruitment of ER to the chromatin, but elevated levels of Nkx3-1 or LEF-1 can out-compete ER for binding. This was shown to directly influence the ability of estrogen-ER to induce cell growth. As such, stoichiometric balances between the Nkx and LEF pathways may impinge on ER activity by directly competing for binding to *cis*-regulatory elements, potentially during development as well as in hormone-responsive cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 1/11/2008; revised 6/24/2008; accepted 7/16/2008.

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We thank Dr. Antonio Hurtado for help with some experiments, Dr. Danielle Carroll for discussions, Professor Michael Shen for the pcDNA3-Nkx3-1-expressing mouse Nkx3-1, Dr. Rudolf Grosschedl for the pCG-LEF-1 construct, The University of Cambridge, Cancer Research UK, and Hutchison Whampoa Limited.

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