CHAPTER 3

Estrogen Regulates the JNK1 Genomic Localization Program to Control Gene Expression and Cell Growth*

*Dr. Nina Heldring, Dr. Matt Gamble, and Adam Diehl contributed to this work by assisting with the expression, ChIP-chip, and bioinformatic analysis, respectively.
Summary

Steroid hormone and MAPK signaling pathways functionally intersect, but the molecular mechanisms of this crosstalk are unclear. Here I demonstrate an extensive and unexpected convergence of the estrogen and JNK1 signaling pathways at the genomic level in breast cancer cells. Estrogen signaling promotes a nearly complete redistribution of JNK1 to estrogen receptor alpha (ERα)-bound promoters, primarily through an ERα tethering pathway. JNK1 functions as a transcriptional coregulator of ERα at many of these promoters in a manner dependent on its kinase activity. The convergence of ERα and JNK1 at target gene promoters regulates estrogen-dependent gene expression outcomes, as well as downstream estrogen-dependent cell growth responses. Analysis of existing gene expression profiles from breast cancer biopsies suggests a role for functional interplay between ERα and JNK1 in the progression and clinical outcome of breast cancers.

Introduction

Diverse signaling pathways regulate a wide variety of cellular processes, including global transcription programs, in normal and disease states. For example, steroid hormones, such as estrogens, act through nuclear receptors to directly regulate the expression of a defined set of target genes (Acevedo and Kraus, 2004; Kininis and Kraus, 2008). In contrast, growth factors act through cytoplasmic membrane receptors to stimulate intracellular signaling pathways, including mitogen activated protein kinase (MAPK) cascades, that indirectly regulate gene expression through a variety of target transcription factors (Turjanski et al., 2007). Although functional crosstalk between steroid hormone and growth factor/MAPK signaling pathways was demonstrated nearly two decades ago in models of steroid hormone-dependent cancer
To explore the convergence of these signaling pathways at target gene promoters, I considered the possibility that Jun N-terminal kinase (JNK1), a MAPK whose expression is upregulated in breast cancers (Figure 3.1), might function as a coregulator of estrogen receptor alpha (ERα) at the promoters of estrogen-regulated genes. Although the traditional view has been that MAPK-mediated phosphorylation events (e.g., phosphorylation of transcription factors) do not occur at the genes that they ultimately regulate, the terminal kinases of various signaling pathways are found in the nucleus under activating conditions (Edmunds and Mahadevan, 2004; Turjanski et al., 2007). In addition, genomic analyses in yeast (Pascual-Ahuir et al., 2006; Pokholok et al., 2006) and gene specific analyses in cultured mammalian cells (Edmunds and Mahadevan, 2004) have shown that some signaling kinases bind to the promoters of genes whose expression they regulate. The aim of this study is to characterize the genomic relationship between ERα and JNK1 in regard to their genomic occupancy and transcriptional outcomes.

**Results**

**JNK1 is recruited to genomic regions after estrogen treatment.**

In order to determine if JNK could be modulated by estrogen (E2) *in vivo*, I performed chromatin immunoprecipitation (ChIP) in MCF-7 cells treated with and without E2 using antibodies for JNK1. I examined the JNK1 occupancy at several genomic regions previously reported by ChIP to bind the ERα (Kininis et al., 2007). Since it was already known that E2 could modulate protein-DNA interactions at these regions, they seemed to be the best candidates for JNK1 ChIP. Indeed, I could detect the presence of JNK1 by ChIP-qPCR at these loci (Figure 3.2A). Interestingly, I noted
Figure 3.1 JNK1 expression is elevated in breast carcinomas.

The relative expression of MAPK8 (i.e., JNK1) from 4 normal breast stroma samples and 51 breast tumor samples is shown. The data were obtained from a larger gene expression analysis (Finak et al., 2008) through the Oncomine database (Rhodes et al., 2004). The Oncomine-reported p-value was $<3.0 \times 10^{-4}$. The values were normalized to an average expression level of 1 for the normal breast samples. Red lines represent the average signal in each category.
that JNK1 occupancy was induced by E2 treatment. The hormone-dependent binding of ERα was also demonstrated for these regions, confirming the previous report (Figure 3.2B). This novel JNK1 result was quite amazing since it implies that the MAP kinase might be an estrogen-dependent cofactor in DNA-bound complexes.

**Estrogen treatment does not affect global JNK1 localization.**

It is known that mitogen activated protein kinases (MAPKs), like JNK1, are regulated by the dual phosphorylation of their Thr-Pro-Tyr motif by upstream MAPK kinases, and that this modification results in the translocation of the MAPK into the nucleus and the activation of its enzymatic activity [for a review, see (Davis, 2000)]. Because glucocorticoids and other steroid hormones have been shown to alter the enzymatic activity and cellular distribution of JNK (Bruna et al., 2003), I wondered if E2 treatment altered the localization or activation status of JNK1 in this system.

To this end, cytoplasmic and nuclear extracts were prepared from MCF-7 cells with and without E2 treatment. Western blotting showed that the nuclear retention of ERα was increased upon hormone treatment demonstrating the effectiveness of estrogen signaling, while GAPDH served as a cytoplasmic control illustrating proper fractionation. Blotting for JNK1 revealed that although it was present in both the cytoplasmic and nuclear extracts, only the phosphorylated form of JNK1 was detected in the nuclear extract (Figure 3.3A), consistent with previous findings (Gupta et al., 1995). The constitutive JNK1 phosphorylation may be the result of HER-2 dependent MAP kinase hyperactivation [described for ERK (Keshamouni et al., 2002; Kurokawa et al., 2000)] or related to the elevated kinase activity associated with breast cancer cell-lines [e.g., AKT (Lin et al., 2005)]. Surprisingly, E2 treatment did not alter the localization of JNK1 or the fraction of activated (phosphorylated) protein. Analysis of MCF-7 cells by immunofluorescence also demonstrated similar levels of JNK1 in the
Figure 3.2 ChIP analysis of JNK1 at ERα-occupied regions.

ChIP-qPCR results demonstrating JNK1 (A) and ERα occupancy (B) at promoters in MCF-7 cells treated with ethanol (U) or E2 (E). Each bar = mean + SEM, n ≥ 3.
cytoplasmic and nuclear compartments (Figure 3.3B).

Although the antibodies used for ChIP analysis were not phosphorylation specific, I believe that the JNK1 complexes identified contain the phosphorylated form of the protein since this is the predominant JNK form in the nucleus. Taken together, I conclude that E2 treatment alters the occupancy of activated JNK1 on DNA without altering the overall nuclear pool of JNK1.

**ChIP-chip identifies global patterns of E2-dependent JNK1 occupancy at promoter regions.**

To obtain a broader understanding of our JNK1 ChIP results I turned to DNA microarrays that allow a more global analysis of ChIP material rather than a gene by gene approach. I used commercially available arrays from Nimblegen that contained approximately 19,000 promoter regions tiled from -2200 to +500 base pairs (bp) relative to each transcription start site. Three biologically independent ChIP samples (representing two different JNK1 antibodies) were hybridized to the arrays and analyzed using strict peak finding criteria. The comparison of JNK1 occupancy before and after E2 treatment revealed 801 promoters that contained significantly higher levels of JNK1 occupancy after E2 treatment (“JNK1-recruited”) and 235 promoters that demonstrated a significant loss of JNK1 occupancy after E2 treatment (“JNK1-released”) (Figure 3.4A). Averaging of peak centered ChIP-chip data across these classes illustrates the distinct patterns JNK1 promoter localization in response to E2 (Figure 3.4B). Gene-specific ChIP-qPCR revealed both a high confirmation rate (~93%) for JNK1 peaks on the array (Figure 3.5) and local features consistent with our ChIP-chip tiling (Figure 3.6). I also noted that the JNK1 peaks were found throughout the promoter regions with no apparent preference for the TSS, as is the case for other DNA-associated factors (i.e., PARP-1) (Krishnakumar et al., 2008).
Figure 3.3 Estrogen treatment does not affect global JNK1 localization.

(A) MCF-7 cells were treated with ethanol (U) or E2 (E) for 45 min., and fractionated into cytoplasmic (Cyto) and nuclear (Nuc) extracts. The extracts were analyzed by Western blotting to determine the subcellular localization of JNK1. Arrows indicate the slower migration of phosphorylated JNK1. ERα and GAPDH were used as fractionation controls.

(B) MCF-7 cells treated as above were subjected to immunostaining with a JNK1 antibody, and visualized by confocal microscopy. A single nucleus in each panel is denoted by a dotted circle. This data is consistent with my subcellular fractionation analysis, which shows no gross change in JNK1 localization upon estrogen treatment.
Figure 3.4 ChIP-chip reveals JNK1 localization patterns at promoter regions.

(A) A heatmap showing JNK1 ChIP-chip signals from MCF-7 cells across target promoters before (U) and after (E) a 45 min. treatment with E2 is shown. The promoters were arrayed from -2200 to +500 bp relative to each TSS (x-axis). Analysis of JNK1 occupancy revealed promoters with more JNK1 after E2 treatment (“Recruited”), less JNK1 after E2 treatment (“Released”), no change in JNK1 after E2 treatment (“Constitutive”), and no JNK1 (“Absent”). The genes in each category (y-axis) are ordered from those with the 5′-most JNK1 peak to those with the 3′-most JNK1 peak. Only 2% of the 17,297 "Absent" genes are represented.

(B) The genes in each category were aligned to the maximal JNK1 ChIP-chip signal. The peak-centered data was then averaged to demonstrate the overall pattern for each category shown in (A).
Figure 3.5 ChIP-qPCR confirms JNK1 ChIP-chip categories.

(A, B) ChIP-qPCR confirmation of "JNK1-recruited" (A) and "JNK1-released" (B) promoters in MCF-7 cells treated with ethanol (U) or E2 (E). Greater than 93% (28/30) of the regions tested by ChIP-qPCR confirmed the ChIP-chip results. CHPT1 and UGT2B15 are false negatives due to the limited number of probes present in their respective peak regions on the ChIP-chip array (due to repeat masking). ChIP-qPCR and visual inspection of their ChIP-chip tiling profiles confirms that they are true "JNK1-recruited genes." Each bar = mean + SEM, n ≥ 3. The red line in each graph represents background JNK1 signal.

(C) ChIP-qPCR to determine JNK1 occupancy at bound (Significant; Sig) and unbound (Background; Bkg) regions as defined by the ChIP-chip experiments demonstrates the validity of the peak calls. Red bars represent the average signal in each category.
**Figure 3.6 ChIP-qPCR analysis is consistent with ChIP-chip tiling features.**

ChIP-chip tiling (*left*) and ChIP-qPCR (*right*) analyses of JNK1 at three promoter regions from MCF-7 cells treated with ethanol (U) or E2 (E) is shown. The average JNK1 ChIP-qPCR signals from peak (gray box) and non-peak ( bracket) regions defined by the ChIP-chip tiling are consistent with the array profiles. For the ChIP-qPCR, each bar = mean + SEM, n ≥ 2.
These results confirmed the previous finding that JNK1 required E2 treatment for occupancy at some loci, but also revealed that E2 treatment could contribute to the removal of JNK1 from other loci. Interestingly, a relatively small number of promoters (29 genes) contained similar levels of JNK1 before and after hormone treatment demonstrating that the majority of identified JNK1 complexes are regulated by E2.

**JNK1-bound promoters are enriched for specific cellular functions.**

I next wanted to determine if the JNK1-recruited and JNK1-released genes were enriched for particular cellular functions. Using Genecodis (Carmona-Saez et al., 2007), I determined that both the “JNK1 recruited” and “JNK1 released” gene sets showed enrichment for specific ontological categories (Table 3.1). For example, the “JNK1 recruited” gene set is enriched in genes encoding components of G-protein-coupled receptor signaling pathways and enzymes that metabolize steroid hormones. The proteins encoded by both of these ontological categories of genes would be expected to affect estrogen signaling responses, either by modulating growth factor signaling (Smith, 1998) or by metabolizing estrogens into less active or alternately active forms (Zhu and Conney, 1998). The “JNK1 released” gene set is enriched in genes encoding several mRNA-binding proteins associated with nuclear splicing. These include SF3B5 [a component of the splicesome complex (Zhou et al., 2002)], DHX38 [an RNA helicase (Schwer and Guthrie, 1991)], and RBM8A [a component of the exon junction core complex (Ballut et al., 2005)]. These genes reinforce the recent link between JNK signaling and the regulation of alternative splicing (Pelisch et al., 2005).
Table 3.1 Gene ontology of JNK1 bound promoters.

| Gene set                      | Ontology                              | p-value  
|-------------------------------|---------------------------------------|----------
| "JNK1-recruited" promoters   | • GPCR signaling pathway              | 9.3 x10^{-4} |
| (801 genes)                  | • Glucuronosyltransferase activity    | 2.5 x10^{-5} |
|                               | • Metabolism of androgens and estrogens | 6.0 x10^{-6} |
| "JNK1-released" promoters    | • Nuclear mRNA splicing via the spliceosome | 1.3 x10^{-5} |
| (235 genes)                  |                                       |           |
| Five random gene sets        | • None                                | <0.001   |

a Ontologies were obtained using Genecodis for the JNK1-recruited and JNK1-released genes. The entire gene list represented on the ChIP-chip array was used as the background reference. Ontological assignments representing less than 5 genes were not considered.

b p-values were determined by Genecodis using Chi-square tests. Randomized gene lists (of equal size to each gene set analyzed) were generated from the genes present on the ChIP-chip array to determine a significance threshold and demonstrate the specificity of ontology assignments.

c Five random gene sets were generated using the programming language R from the total number of genes present on the ChIP-chip array. No gene ontologies were enriched (i.e., all p-values were >0.001) in the random lists using the criteria described above.
Recruited JNK1 peaks correlate with ERα occupancy.

Given the estrogen-dependent alterations in the JNK1 genomic localization program, I tested the possibility that the JNK1 peaks might correspond to sites of ERα binding. My initial investigation of JNK1 localization already demonstrated that JNK1 was recruited to regions where ERα was also recruited. To explore this more globally, I performed ERα ChIP-chip using the same array platform that I used for JNK1. These results show a strong correlation between JNK1 and ERα binding sites in the promoter regions, with about 85% of the JNK1 recruited peaks overlapping an ERα peak (Figure 3.7). ChIP-qPCR assays confirmed that the JNK1 recruited peaks correlated with an E2-induced occupancy of ERα at promoter (Figure 3.8A) and distal enhancer regions (Figure 3.8C). To my surprise, the JNK1 released peaks were not associated with ERα occupancy (Figure 3.8B). These patterns of E2-dependent regulation of JNK1 and ERα binding are clearly evident for specific target promoters where the overlay of JNK1 and ERα peak regions using ChIP-chip tiling illustrates the specificity of their association (Figure 3.9). This suggests that JNK1 and ERα are co-recruited at JNK1 recruited regions. JNK1 seems to be modulated by a different mechanism at JNK1-released regions.

Nuclear E2 signaling is required for JNK1 recruitment.

The induced binding of JNK1 and ERα at promoters suggests that E2 signaling causes the convergence of ERα and MAP kinase pathways at these particular regions. Although it is well-known that E2 is a lipophilic compound, able to diffuse directly into the nucleus to facilitate ER activation, it is also known that E2 can activate cellular processes (e.g., kinase cascades) in the cytoplasm (“extra-nuclear” signaling) mediated by membrane-associated ERs and other E2-activated proteins [reviewed in
Figure 3.7 JNK1 binding correlates with ERα occupancy at target promoters.

(A) Heatmaps showing JNK1 and ERα ChIP-chip signals for the "JNK1 recruited" genes from MCF-7 cells after a 45 min. treatment with E2. The promoters were arrayed from -2200 to +500 bp relative to each TSS (x-axis). The genes in each category (y-axis) are ordered from those with the 5'-most JNK1 peak to those with the 3'-most JNK1 peak. ERα heatmap is ordered according to JNK1 heatmap.

(B) Pearson correlation analysis of the JNK1 and ERα peak data from (A) shown as a scatter-plot.
Figure 3.8 ERα is bound at "JNK1-recruited" regions.

(A, B) ChIP-qPCR analyses of ERα binding at "JNK1-recruited" (A) and "JNK1-released" (B) promoters in MCF-7 cells treated with ethanol (U) or E2 (E) revealed the association of ERα with JNK1 recruited but not JNK1 released peaks. Each bar = mean + SEM, n ≥ 2.

(C) ChIP-qPCR analysis of JNK1 and ERα for three previously identified ERα-bound enhancer regions (Carroll et al., 2005) demonstrating that ERα / JNK1 complexes do not just occur at promoter regions. Each bar = mean + SEM, n ≥ 3.
Figure 3.9 JNK1 recruited peaks have a peak profile similar to ERα.

(A) The average JNK1 and ERα ChIP signals from ChIP-chip (left) and ChIP-qPCR (right) are shown for three "JNK1-recruited" promoters (GREB1, HOXC10, CYP1B1).

(B) Similar analysis as in (A) but for three “JNK1-released” promoters (CEP350, CENPA, RNF167). ChIPs from MCF-7 cells treated with ethanol (U) or E2 (E). The gray box indicates the regions of ChIP-qPCR; x-axis represents number of bp from the TSS. Each bar = mean + SEM, n ≥ 2.
Figure 3.9 (Continued)
(Fu and Simoncini, 2008)). These cascades could ultimately be responsible for the JNK1 recruitment observed in the ChIP assays.

In order to determine the requirement of E2 signaling on JNK1 recruitment, I performed ChIP assays using conjugated E2 derivatives, which can initiate cytoplasmic but not nuclear estrogen signaling pathways (Harrington et al., 2006). Indeed, treatment of MCF-7 cells with these compounds resulted in the rapid phosphorylation of ERK demonstrating that the E2-conjugates, like E2, could initiate a cytoplasmic response (Figure 3.10A). These reagents failed to promote the binding of ERα, as expected, since they can not enter the nucleus (Figure 3.10B). Examination of JNK1 occupancy revealed that, like ERα, the E2-conjugates could not induce the binding of the MAP kinase to these regions (Figure 3.10C). These results suggest a direct role for ERα at the sites of E2-dependent JNK1 recruitment. Although membrane-initiated estrogen signaling may contribute to the hormone-induced binding of ERα and JNK1 to promoters, it is not sufficient to elicit the response alone.

**Bioinformatics links specific transcription factors with JNK1 occupancy.**

Because JNK1 does not bind to DNA directly, its association with DNA must be facilitated by DNA-bound transcription factors (TF). I used a series of bioinformatic analyses to determine which TFs might be facilitating the binding of JNK1 to the promoter regions. First, I used MEME (Multiple Em for Motif Elicitation) and MAST (Motif Alignment and Search Tool) (Bailey et al., 2006) in an unbiased search for DNA sequence motifs enriched in JNK1-bound regions. These results yielded a number of high confidence motifs for both the “JNK1 recruited” and “JNK1 released” peaks, but notably did not include estrogen response elements (EREs) (Figure 3.11). I then used TESS (Transcription Element Search System) to predict the TFs that might bind to these enriched sequences. The TF whose binding site had the highest
Figure 3.10 Nuclear estrogen signaling is required for JNK1 recruitment to target promoters.

(A) MCF-7 cells were treated with ethanol (U), 10 nM E2 (E), 10 nM E2-conjugated BSA (E2-BSA), or 10 nM E2-dendrimer conjugate (EDC) for 10 min., followed by Western blotting for ERK and phosphorylated ERK (P-ERK). The concentrations of the estrogen derivatives were based on the amount of E2 in the conjugates, as previously described (Harrington et al., 2006).

(B,C) The binding of ERα (A) and JNK1 (B) was determined by ChIP-qPCR at three selected promoters after a 45 min. treatment as described in (A). Bars represent the mean ChIP signal relative to the maximal E2 signal for each experiment, n ≥ 3. Error bars = SEM for U, E2-BSA, and EDC.
Figure 3.11 Unbiased bioinformatic analysis using MEME/MAST.

The DNA sequences from JNK1-bound regions (± 250 bp from the center of each JNK1 peak) were analyzed using Multiple Em for Motif Elicitation (MEME) and Motif Alignment and Search Tool (MAST) software (Bailey et al., 2006), as described in the Materials and Methods. The results are divided into "JNK1-recruited" and "JNK1-released" peaks. The p-values for enriched sequences were determined by a Fisher exact test using the base count of motifs within peak regions versus those in JNK1-negative regions. Motif predictions were examined by Transcription Element Search System (TESS) (Schug, 2008) to determine the transcription factor most likely to bind that sequence. "Genes" represents the number of promoters where the enriched sequence falls within 225 bp of the JNK1 peak. The JNK1 ChIP-chip heatmap (JNK1) and the corresponding heatmap for the enriched sequence (Motif) is shown for these promoters. The number of genes and the motif mapping refer to the enriched sequences from MEME (not from mapping TRANSFAC weight matrices; shown in subsequent figures).
<table>
<thead>
<tr>
<th>Motif No.</th>
<th>&quot;JNK1 Recruited&quot;</th>
<th>&quot;JNK1 Released&quot;</th>
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<tbody>
<tr>
<td></td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>1</td>
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<td>$8.8 \times 10^{-6}$</td>
</tr>
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<td>AP-1</td>
<td>AP1</td>
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<td>MEF-2</td>
<td>GATA-1</td>
</tr>
<tr>
<td>3</td>
<td>POU3F2 (OCT7)</td>
<td>POU1F1 (PIT1)</td>
</tr>
<tr>
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<td>Type</td>
<td></td>
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<td>Fos/Jun Family bZIP</td>
<td>Fos/Jun Family bZIP</td>
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<td>2</td>
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<td>MADS Box</td>
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<td>3</td>
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</table>
alignment score with the MEME motif was chosen as the best TF candidate. This analysis identified AP-1, as well as other TFs not previously associated with JNK1 (Figure 3.11). These included the MADS box transcription factors MEF2 and SRF; the POU homeodomain transcription factors (PIT-1) and POU3F3 (OCT7); and the zinc finger transcription factor GATA-1.

Next, I mapped motif probability weight matrices for each of these transcription factors [obtained from TRANSFAC (Wingender et al., 2001)] (Figure 3.12) to the JNK1 peaks. Selected TRANSFAC motifs were mapped across the promoter regions present on the ChIP-chip array using MAST. Alignments with a p-value < 1.5 x 10^{-4} were considered true motif calls, a threshold previously reported (Kininis et al., 2007). Motifs that fell within 375 bp of a JNK1 peak were counted as peak motifs and compared to the number of motifs outside of the peak regions. Although EREs were not identified in the unbiased search, I also included an ERE probability weight matrix in this directed search since I had already determined that ERα was associated with JNK1 recruited peaks. This analysis yielded high confidence sites for all of the matrices searched (Table 3.2 and Figure 3.13A).

To test the validity of the bioinformatics analyses, I determined if the results could be used to make accurate predictions about factor binding. For this analysis, I focused on genomic regions containing high confidence AP-1 motifs (Figure 3.13 B and C). Although “JNK1 recruited” regions showed E2-induced binding of JNK1 and ERα, as expected (Figure 3.13D), only the regions with a high confidence AP-1 motif showed binding of c-Fos, a component of the AP-1 heterodimer. Surprisingly, the binding of c-Fos was also induced by E2 treatment (Figure 3.13D, bottom). Together, these results support the validity of my bioinformatic analyses by demonstrating the recruitment of JNK1 and c-Fos to regions containing predicted AP-1 sites.
Figure 3.12 TRANSFAC motifs used for motif mapping.

An unbiased bioinformatic analysis using MEME and TESS (Figure 3.11) identified several putative transcription factors (TFs) as potential mediators of JNK1 binding. The weight matrices for these factors (obtained from TRANSFAC) are shown with the corresponding TRANSFAC ID or reference [the ERE sequence was previously reported (O'Lone et al., 2004)]. The mapping of these matrices was used to define TF binding sites used in subsequent analysis. Note, no weight matrix was available for PIT-1.
Table 3.2 Directed bioinformatic analysis of JNK1 Peak Regions

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<th>Genes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p-value&lt;sup&gt;c&lt;/sup&gt;</th>
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<td></td>
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<tr>
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<td>V$\text{SGATA1}_\text{04}$</td>
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<td>&lt;1.00 x 10&lt;sup&gt;-300&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
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<tr>
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<td>V$\text{SMEF2}_\text{01}$</td>
<td>Recruited 325</td>
<td>&lt;1.00 x 10&lt;sup&gt;-300&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>Released 77</td>
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<tr>
<td>POU3F2</td>
<td>V$\text{SBRN2}_\text{01}$</td>
<td>Recruited 209</td>
<td>&lt;1.00 x 10&lt;sup&gt;-300&lt;/sup&gt;</td>
</tr>
<tr>
<td>(OCT7)</td>
<td></td>
<td>Released 53</td>
<td>3.60 x 10&lt;sup&gt;-138&lt;/sup&gt;</td>
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<tr>
<td>SRF</td>
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<td></td>
<td></td>
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<td>ER</td>
<td>(O'Lone et al., 2004)</td>
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</table>

Selected TRANSFAC motifs were mapped across the promoter regions present on the ChIP-chip array using MAST. Motifs that fell within 375 bp of a JNK1 peak were counted as peak motifs and compared to the number of motifs outside of the peak regions.

<sup>a</sup> The TRANSFAC IDs (or reference) for the weight matrices used in the analysis are listed. The ERE sequence was previously reported (O'Lone et al., 2004).

<sup>b</sup> "Genes" = the number of promoters that have the given motif within 375 bp of the center of the JNK1 peak.

<sup>c</sup> p-values were determined by Fisher's Exact Tests using the programming language R. They were based on the probability of finding bases that compose the given motif in a JNK1-bound region divided by the probability of finding bases that compose the given motif in JNK1-absent regions.
Figure 3.13 JNK1 peaks contain likely ERα tethering factor motifs.

(A) The results of the targeted search for transcription factor binding sites under the JNK1-recruited regions is summarized (shown in Table 3.2). Motifs and their likely associated binding factors were identified based on an initial unbiased search (Figure 3.11) and were mapped with MAST using position weight matrices from TRANSFAC (Figure 3.12).

(B) The AP-1 motif from TRANSFAC is shown as a position weight matrix.

(C) Heatmaps showing the location of JNK1 binding (by ChIP-chip) and predicted AP-1 binding sites (by MAST) for promoters containing an AP-1 motif within 225 bp of a JNK1-recruited peak.

(D) ChIP-qPCR analyses of c-Fos binding at JNK1- and ERα-recruited regions before (U) and after estrogen (E) treatment. UGT2B15, SPTBN4, TFF1 and GREB1 contain at least one predicted AP-1 motif under the JNK1 peak. PLAC1 contains an ERE sequence and is included (along with BLK44) as a negative control for c-Fos binding. Mean ± SEM, n ≥ 3. Red lines represent background binding levels.
A

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<tr>
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<td>SRF</td>
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B

TRANSFAC call: AP-1

C

D

Relative Enrichment

- JNK1-U
- JNK1-E

Relative Enrichment

- ERα-U
- ERα-E

Relative Enrichment

- cFOS-U
- cFOS-E

120 Promoters

AP-1 motif
JNK1 acts as a coregulator at E2-responsive genes.

Since JNK1 occupancy at genomic loci is affected by E2 signaling I wondered if JNK1 might have a direct role in mediating E2-dependent transcriptional responses. To this end, JNK1-depleted MCF-7 cells were generated by retroviral-mediated gene transfer of a short hairpin RNA (shRNA) sequence targeting JNK1 mRNA. Two different shRNA sequences gave similar levels of JNK1 mRNA and protein depletion (results for one are shown, Figure 3.14A). Control cells harboring an shRNA sequence directed against GFP [described previously (Kim and Rossi, 2003)] were generated in parallel. I examined the E2-dependent expression of target genes in MCF-7 cells using reverse transcription-qPCR (RT-qPCR). Stable short hairpin RNA (shRNA)-mediated knockdown of JNK1 or chemical inhibition of JNK catalytic activity using a JNK-specific ATP competitor, SP600125 (Bennett et al., 2001), inhibited the E2-stimulated (or E2-repressed) expression of some, but not all, estrogen target genes (Figure 3.14 B and C). Although “off-target” effects can occur when using chemical inhibitors in vivo, I believe that the SP600125 effects (Figure 3.14 B and C) represent specific JNK inhibition since they agree so strongly with the JNK1 knock-down data for the same genes. Thus, JNK1 and its kinase activity are required for full E2-dependent regulation of estrogen target genes in MCF-7 cells implicating JNK1 as a hormone-dependent transcriptional coregulator of ERα.

E2-dependent growth of breast cancer cells requires JNK1.

As stated above, JNK1 expression is elevated in breast cancer carcinomas relative to healthy breast tissues. One implication from this correlation would be that elevated JNK1 levels provide a proliferative advantage to hormone-dependent tissues like the breast. JNK1 is required for full E2-responsiveness at target genes, but does it affect other E2-regulated outcomes, such as cell growth? Along these lines, I tested
Figure 3.14 JNK1 activity is required for full estrogen-dependent transcriptional responses.

(A) Analysis of JNK1 mRNA and protein levels in MCF-7 cells stably expressing control (GFP) and JNK1 shRNA.

(B) The transcriptional response of E2-regulated genes from MCF-7 cells treated ± E2 for 3 hrs was determined by RT-qPCR. The effect of JNK1 knockdown (Top) or the effect of the JNK inhibitor SP600125 (SP) (Bottom) on E2-regulated, JNK1 recruited genes is shown. Asterisks represent p-values <0.05 (*) or <0.01 (**) (Student’s t-test) versus E2 control (E; black bars). The E2-regulation of HOXC10 is not affected by JNK1 knock-down or chemical inhibition. Mean ± SEM, n ≥ 3.

(C) E2-dependent down-regulation of gene expression also requires JNK1 activity as the JNK inhibitor impairs E2-dependent repression of target genes. MCF-7 cells treated as in (B) Expression data for GOLGB1 was from 6 hrs E2 treatment. Each bar = mean ± SEM, n ≥ 2. Raw expression data from independent experiments were normalized to ACTB expression; the untreated control condition was set to 100.
Figure 3.14 (Continued)
my JNK1 knock-down line to determine if the loss of JNK1 affected the growth of these hormone stimulated breast cancer cells. Indeed, JNK1 knock-down resulted in the loss of E2-stimulated growth while the GFP knock-down responded to hormone as expected (Figure 3.15). This evidence, along with the role of JNK1 as a hormone-dependent transcriptional coregulator of ERα, suggests a physiological link between estrogen and JNK1 signaling at the genomic level with cell growth outcomes. This link may have relevance for the growth and clinical outcomes of estrogen-dependent breast cancers.

**Discussion**

Collectively, my results characterize the functional interplay between the estrogen and MAPK signaling pathways that has been observed previously (Lange, 2004; Smith, 1998). This association is manifested in an extensive and unexpected molecular crosstalk at the genomic level.

I demonstrated that JNK1 binds to specific sites in the genome. This illustrates the fact that signaling molecules, like MAP kinases, associate with chromatin-bound complexes broadening the understanding of how and where these kinases phosphorylate their substrates. An even more amazing finding was that E2 treatment caused a nearly complete redistribution of the JNK1 promoter localization pattern (97% of the peaks changed) (Figure 3.4). This redistribution was not due to the net movement of JNK1 to or from the nuclear compartment, nor was it due to a net change in the phosphorylation status of JNK1 (Figure 3.3). These facts are in agreement with each other since the phosphorylation of JNK is tightly linked to its nuclear translocation (Gonzalez et al., 1993; Lenormand et al., 1998). Cytoplasmic E2 signaling was not sufficient for the recruitment of JNK1 to E2-induced promoters (Figure 3.10). Together, these data highlight the fact that the E2-dependent, genomic
**Figure 3.15 JNK1 is required for E2-induced proliferation in MCF-7 cells.**

Cells expressing a shRNA construct for GFP or JNK1 were grown for 2 days in E2-free media before being plated at equal densities and treated with ethanol or E2 (100 nM; E).

(A) Cells were counted 2 and 4 days after E2 addition.

(B) The average E2-dependent fold in proliferation for day 4 is shown. Bars equal the mean ± SEM, n ≥ 2.
changes of JNK1 occupancy involve the activated, nuclear pool of JNK1. The E2-independent activation (and thus translocation) of JNK has no doubt played a role in keeping the hormone-dependent crosstalk between these pathways enigmatic to date.

My investigation into the E2-modulated binding of JNK1 at target genes led to the discovery that JNK recruited but not JNK released peaks correlated with ERα binding (Figure 3.7-9). This suggests that at least two modes of hormone signaling influence the genomic occupancy of JNK1. Bioinformatic analyses determined several transcription factors as likely JNK1-tethering factors. It is interesting to note that due to the strong correlation between JNK1 and ERα at JNK1 recruited regions, the TFs associated with these JNK1 complexes are also implicated in mediating ERα complexes. Indeed, ERα exhibits two distinct modes of genomic binding: direct binding to DNA containing ERE sequences and indirect binding or tethering through other DNA-binding transcription factors, such as activator protein-1 (AP-1, a heterodimer of c-Fos and c-Jun or related proteins) (Kushner et al., 2000). My analysis identified E2 recruited JNK1 and ERα complexes at promoters containing EREs, but implied that this was not the major mode of JNK (and ERα) recruitment. EREs represented only ~10 percent of all the sites identified (100 out of 1091 sites for the JNK1-recruited peaks (Table 3.2 and Figure 3.13A). These bioinformatic results, together with the ChIP-chip results described above, suggest that the E2-dependent recruitment of JNK1 occurs primarily, but not exclusively, through an ERα tethering mechanism mediated by diverse types of DNA-binding transcription factors.

I further demonstrated that JNK1 can act as a coregulator of ERα-dependent transcriptional outcomes in a manner that requires its catalytic activity. The genespecific impairment of E2-dependent transcriptional responses by JNK1 knock-down was mirrored in the loss of E2-stimulated growth in the JNK1 shRNA expressing line. It is quite interesting to note that these effects are mediated specifically by JNK1
despite the presence of the highly redundant MAPK, JNK2 (note that JNK2 expression actually increased with JNK1 knock-down) (Figure 3.16). Together, these data suggest that the specific E2-regulated genomic activities of JNK1 can ultimately affect hormone-dependent cellular processes. Indeed, JNK activity is important in tumor growth and development as several studies have demonstrated the role of JNK in Ras-mediated tumoricity [reviewed in (Davis, 2000)] and in oncogene activation [reviewed in (Ip and Davis, 1998)]. Estrogen signaling may play a large role in these JNK-dependent processes since JNK1 expression is elevated in breast carcinomas (Figure 3.1). In addition, the expression of the JNK1 phosphatase, MPK-1, a negative regulator of JNK1 activity, is reduced in high grade malignant breast cancers (Figure 3.17).

These results support a model for the estrogen- and ERα-dependent recruitment of pre-activated JNK1 from the nuclear compartment (i.e., nucleoplasm or chromatin) to the promoters of estrogen target genes. JNK1, in turn, serves a coregulator function required for efficient estrogen-dependent transcription of these genes. This role of JNK1 in the genomic estrogen signaling pathway is supported by JNK1's kinase activity, which likely targets histones or other proteins in the promoter-assembled transcription complexes (claims supported by the work presented in Chapter 4) (Figure 3.18). In sum, my studies have identified a genomic nexus between the estrogen and JNK1 signaling pathways that regulates target gene expression and downstream cell growth responses. Similar genomic systems are likely to integrate the signaling pathways for other steroid hormones and signal-regulated nuclear kinases. Future studies will aim to define the E2-regulated targets of JNK1 and determine the molecular mechanisms of JNK-dependent phosphorylation in mediating E2-regulated outcomes.
Figure 3.16 JNK2 expression is regulated by JNK1.

JNK2 expression in MCF-7 cells stably expressing an shRNA to GFP or JNK1 was determined by RT-qPCR. Raw expression data from independent experiments were normalized to ACTB expression and expressed relative to the GFP shRNA sample. Each bar = mean + SEM, n = 4.
Figure 3.17 Expression of the JNK phosphatase, MKP-1, decreases with breast cancer progression.

The relative expression of MKP-1 across three breast carcinoma grades is shown from five independent studies (Bittner, 2005; Desmedt et al., 2007; Ivshina et al., 2006; Miller et al., 2005; Sotiriou and Desmedt, 2006). The p-values for negative correlation was <0.001 for all five studies. The values were normalized so that the average expression level for the Grade 1 sample from each study was 1. Red bars represent the average signal in each category.
Figure 3.18  JNK1 is an estrogen-dependent coregulator.
A model illustrating the estrogen-dependent regulation of JNK1 localization at promoters, including features described in Chapter 4 (i.e., JNK1 phosphorylation targets), is shown. Activated (phosphorylated) JNK1 is co-recruited with ERα to promoters containing certain transcription factors, such as AP-1. JNK1 then phosphorylates transcriptionally relevant targets, such as H3 or coactivators of ERα, allowing full E2-responsiveness at the given promoter.
Materials and Methods

Cell culture. MCF-7 cells were maintained in MEM with Hank’s salts (Sigma; M1018) supplemented with 5% calf serum, sodium bicarbonate, penicillin/streptomycin, and gentamicin. Prior to all experimental procedures and treatment with control vehicle (ethanol) or E2 (100 nM), the cells were grown for at least 3 days in phenol red-free MEM Eagle modified, with Earle's salts (Sigma; M3024) supplemented with 5% charcoal-dextran calf serum, L-glutamine, sodium bicarbonate, penicillin/streptomycin, and gentamicin. For the JNK inhibition experiments, the cells were treated with 20 µM SP600125 (SP) (BIOMOL) for 10 hrs before treatment with E2.

Antibodies. The antibodies used are as follows: JNK1 (Santa Cruz, sc-474), phosphorylated JNK (Santa Cruz, sc-6254), JNK (Santa Cruz, sc-7345), c-Fos (rabbit polyclonal generated in the Kraus lab), ERα (rabbit polyclonal generated in the Kraus lab), ERK (Santa Cruz, sc-154), phosphorylated ERK (Cell Signaling, 9106L), GAPDH (kindly provided by Eric Alani, Cornell University).

Preparation of cell extracts.

- JNK1 localization: Estrogen-starved MCF-7 cells were treated with ethanol or 100 nM E2 for 45 min., washed with ice-cold PBS, released by scraping, and collected by centrifugation. The cell pellets were resuspended in hypotonic buffer (10 mM Tris-HCl pH 7.9, 10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM sodium vanadate, 4 µg/ml aprotonin, 4 µg/ml leupeptin, 1 mM DTT, 1 mM PMSF), incubated on ice for 10 min., and homogenized by Dounce 40 times with a tight glass pestle. The lysate was centrifuged at 8,000 rpm in a microfuge at 4°C and...
the supernatant was collected as the cytoplasmic fraction. The nuclei were washed twice with hypotonic buffer + 0.1% NP-40 and resuspended in hypertonic buffer (10 mM Tris•HCl pH 7.9, 420 mM KCl, 10% glycerol, 10 mM NaCl, 3 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM sodium vanadate, 4 µg/ml aprotonin, 4 µg/ml leupeptin, 1 mM DTT, 1 mM PMSF). After a 10 minute incubation on ice, the extracted nuclei were pelleted by centrifugation as above and the supernatant was collected as the nuclear fraction. The protein concentration for both fractions was determined by Bradford assays.

- **Detection of Activated ERK:** Estrogen-starved MCF-7 cells were grown for 24 hrs in serum-free medium, followed by a 10 min. treatment with ethanol, 10 nM E2, 10 nM 17β-estradiol 17-hemisuccinate:BSA (E2-BSA) (Steraloids), or 10 nM estrogen-dendrimer conjugate (EDC) (Harrington et al., 2006) (kindly provided by John Katzenellenbogen, University of Illinois, Urbana-Champaign). The cells were washed with ice-cold PBS, released by scraping, and collected by centrifugation. The cell pellets were resuspended in lysis buffer (50 mM Tris•HCl pH 7.9, 500 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 5 mM NaF, 1 mM sodium vanadate, 4 µg/ml aprotonin, 4 µg/ml leupeptin, 1 mM DTT, 1 mM PMSF) and subjected to three freeze-thaw cycles using liquid nitrogen. Lysates were collected after maximum centrifugation in a microfuge at 4°C. Protein concentrations were determined by Bradford assays.

**Immunofluorescence.** Estrogen-starved MCF-7 cells were grown on coverslips and treated with ethanol or 100 nM E2 for 45 min. After a wash with PBS, the cells were crosslinked on the coverslips for 10 min. at room temperature with a formaldehyde solution (3% formaldehyde, 5% sucrose in PBS) and the reaction was stopped by
addition of 125 mM glycine. The cells were rinsed twice with PBS, permeabilized for 15 min. with 0.1% Triton X-100 made in PBS, and blocked for 20 min. with 5% BSA made in PBS. The cells were washed two more times with PBS and incubated for 30 min. with a JNK1 antibody (1:250 dilution with PBS). Afterwards, the cells were washed 3 times with TBST (10 mM Tris•HCl pH 7.9, 150 mM NaCl, 0.05% Tween-20) and incubated with a fluorescein-conjugated secondary antibody (Jackson; 115-095-146) (1:1000 dilution with PBS) for 30 min.. The coverslips were then washed 5 times with TBST, mounted to slides using Vectashield (Vector Laboratories; H-1000), and visualized using a Leica Confocal Microscope System.

Chromatin immunoprecipitation (ChIP). ChIP assays were performed as described previously (Kininis et al., 2007), with minor modifications. The cells were grown to ~80% confluence and treated with ethanol or 100 nM E2 for 45 min. The cells were then crosslinked with 10 mM dimethyl suberimidate•HCl (DMS; Pierce, 20700) for 10 min. at room temperature, followed by 1% formaldehyde for 10 min at 37°C, with subsequent quenching by 125 mM glycine for 5 min. The crosslinked cells were collected by centrifugation, resuspended in lysis buffer [0.5% SDS, 10 mM EDTA, 50 mM Tris•HCl pH 7.9, 1x protease inhibitors (Roche; 1836153)], and sonicated three times for 10 seconds using a Branson Digital Sonifier at 27% power. This resulted in DNA fragments of ~500 bp as determined by agarose gel electrophoresis. Cell debris was removed by centrifugation and the remaining lysate was diluted 10-fold using dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 17 mM Tris•HCl pH 8.0, 167 mM NaCl, 1x protease inhibitors). After a 1 hr pre-clearing step using protein G-agarose beads (Invitrogen; 15920-010), a portion of the lysate was collected as “input” material, while the remaining lysate was incubated overnight with antibodies against JNK1, ERα, c-Fos, or without antibodies as a control. The lysates were then
incubated with protein G-agarose beads for 4 hours to capture the immunoprecipitated complexes. The beads were then washed three times with wash buffer (0.25% NP-40, 0.05% SDS, 2 mM EDTA, 20 mM Tris•HCl pH 8.0, 250 mM NaCl, 2 µg/ml leupeptin and 2 µg/ml aprotinin) and once with TE. The immunoprecipitates were resuspended in elution buffer (1% SDS, 100 mM NaHCO₃) and incubated overnight at 65°C to reverse the crosslinks. The proteins were digested for 45 min. at 37°C with 12.5 µg proteinase K and the DNA was precipitated with ethanol/sodium acetate following an extraction with phenol:chloroform:isoamyl alcohol. The DNA pellets were dissolved in water and analyzed by qPCR. Before averaging, the ChIP values for each region were normalized. Each ChIP experiment was conducted with at least three independent chromatin isolates to ensure reproducibility.

**Ligation-mediated PCR (LM-PCR).** For ChIP-chip analysis, immunoprecipitated genomic DNA was blunted and amplified by LM-PCR as described previously (Krishnakumar et al., 2008). The material was purified following digestion with RNase (Roche) using QIAquick columns (QIAGEN). qPCR on selected regions was used to confirm that the LM-PCR procedure preserved the binding patterns of the initial immunoprecipitated material. The LM-PCR for the ChIP-chip experiments was done using three independent ChIP experiments from cells treated with or without E2.

**ChIP-chip.** After LM-PCR, the immunoprecipitated material was labeled with Cy5 and the reference ("input") material was labeled with Cy3. The labeled samples were combined and hybridized to human HG18 RefSeq Promoter Arrays (Nimblegen; C4226-00-01). Briefly, this array contains ~19,000 well-characterized RefSeq promoters tiled with 50-mer to 75-mer probes every 100 bp. The tiled regions cover ~2200 bp upstream and ~500 bp downstream of each TSS.
ChIP-chip data analysis.

• **Moving window analysis:** Data processing was done essentially as described previously (Krishnakumar et al., 2008) using the statistical programming language R (Team, 2006). All R scripts are available upon request. The pairwise data files supplied by Nimblegen were used to calculate the log2 ratio data for each array. The ratio values were subjected to lowess normalization and the arrays were normalized to each other using equivalent sum of squares scaling. An error model was generated using a 600 bp moving window with 150 bp steps in which both the mean probe log2 ratio and p-value were calculated for each window. The moving window analysis was also performed on a composite fold array that represented the average JNK1 ratio in the presence of E2 divided by the average JNK1 ratio in the absence of E2. All p-values were calculated using a nonparametric Wilcoxon signed-rank test.

• **Definition of significant bound regions:** JNK1-bound regions were defined as the windows containing: (1) positive means in all three biological replicates, (2) at least 5 probes, and (3) p-values <0.05. Constitutive regions were defined as JNK1-bound regions (present in the E2-treated and untreated samples) that did not have a significant p-value (≥0.032) from the composite fold analysis. Recruited regions were defined as JNK1-bound regions (present in the E2-treated samples) that had both a significant p-value (<0.032) and a fold ratio >1. Released regions were defined as JNK1-bound regions (present in the untreated samples) that had both a significant p-value (<0.032) and a fold ratio <1. Of the defined regions, 98% of the recruited regions and 95% of the released regions had an absolute fold change of ≥1.3.

• **Visual representation of the data:** The TSS-anchored heat maps used to visualize the ChIP-chip data were generated with Java Treeview (Saldanha, 2004).
For genes with multiple TSSs, the most 5’ TSS in a given tiled region was used for alignment as +1.

**Bioinformatic analyses.**

- **De novo motif prediction:** Three gene lists were generated for de novo motif predictions: (1) JNK1-recruited, (2) JNK1-released, and (3) JNK1-negative. The recruited and released lists report the 500 bp surrounding the location of the maximum fold change (positive or negative) for the JNK1-recruited and JNK1-released genes, respectively. The JNK1-negative list reports the regions on the array with no significant JNK1 signal. These lists were formulated using the tools on the Galaxy browser (Elnitski et al., 2006) so genomic locations from JNK1-bound regions would not be present in the background regions. Genomic sequences for all regions were obtained from a local mirror of the UCSC genome browser, release HG18. JNK1-negative sequences were extracted in the same manner and used to compute background nucleotide frequencies and 1<sup>st</sup>- through 3<sup>rd</sup>-order Markov background models.

De novo motif detection was carried out using MEME (Multiple Em for Motif Elicitation) (Bailey et al., 2006) on repeat masked sequences, using the 3<sup>rd</sup> order background model. A width range of 6 to 15 nucleotides was specified and any number of sequence occurrences was allowed within peak regions. The top 20 motifs in each peak class were retained for further analysis. Motifs with a Pearson’s correlation coefficient ≥ 0.6 were grouped as similar motifs and were represented by the motif with the greatest MEME score. MAST (Motif Alignment and Search Tool) (Bailey et al., 2006) was used to scan for the locations of all motif instances within both bound and unbound sequences, using a p-value threshold of 1.5 x 10<sup>-4</sup> as previously reported (Kininis et al., 2007). Motifs were accepted as having a potential
association with JNK1 binding only if they were significantly enriched within bound peaks relative to background sequences. Fisher’s exact tests were used to determine enrichments relative to background (heretofore generically referred to as “foreground” and “background” classes) with p-values corrected for multiple testing using the Holm method in R. Contingency tables were constructed based on the number of observed motifs and total number of k-tuples in foreground and background sequences, where k is the width of the motif.

- **Assigning transcription factors to the predicted motifs:** TESS (Transcription Element Search Software) (Schug, 2008) was used to predict the transcription factors that might bind to the enriched sequences from MEME. Position weight matrices for the predicted transcription factors (listed below with their TRANSFAC identification tags) were obtained from the TRANSFAC database (Wingender et al., 2001) and were converted to probability models. Pseudocounts were introduced to avoid over-fitting the motif models, which were based on relatively limited training datasets. The adjusted matrices for the predicted transcription factors were mapped to the JNK1-bound and JNK1-negative regions with MAST using a 6th order Markov model. Fisher's exact tests were used to determine the enrichments for each motif, as described above. In addition, promoters were scanned for the presence of EREs in the same manner and the enrichment calculated. The TRANSFAC transcription factor motifs used for mapping are as follows: AP-1 (M00199), MEF2 (M00006), SRF (M00152), POU3F2/OCT7 (M00145), and GATA1 (M00128). The ER motif was a consensus defined by O'Lone et al., 2004 (O'Lone et al., 2004).

**Gene ontology.** Gene ontology (GO) analyses were performed using Genecodis (Carmona-Saez et al., 2007). JNK1 gene sets (i.e., "JNK1-recruited", "JNK1-
released") were uploaded and compared to the total gene list represented on the ChIP-chip array. Search parameters included the following: (1) lowest GO level, (2) a minimum of 5 genes per category, (3) each category represented by a single GO identifier, false discovery rate (FDR) <0.01. P-values were calculated by Genecodis using Chi-square tests. Ten randomly selected gene lists (5 of similar size to the JNK1-recruited list, 5 of similar size to the JNK1-released list) were analyzed in a similar manner to empirically determine the FDR. No GO terms were reported for these random lists using the criteria above. Uninformative gene categories were not recorded.

**JNK1 knockdown.** JNK1-depleted MCF-7 cells were generated by stable retroviral-mediated gene transfer of a short hairpin RNA (shRNA) sequence specifically targeting the JNK1 mRNA using the pSUPER.retro system under appropriate drug selection (Oligoengine). Two different shRNA sequences, obtained from SuperArray and cloned into the pSUPER vectors using unique EcoRI/XhoI sites, gave similar levels of JNK1 mRNA depletion. The JNK1 target sequences are as follows: 5’-CAGAGAGCTAGTTCTTATGAA-3’ and 5’-CCTACAGAGAGCTAGTTCTTA-3’. Control cells harboring a shRNA sequence directed against GFP were generated in parallel. The GFP target sequence used (5’-GAAGCTGACCCTGAAGTTCATC-3’) was based on previous work (Kang et al., 2001).

**Gene-specific expression analyses.** The expression of endogenous target genes was determined as described previously (Kininis et al., 2007), with minor modifications. MCF-7 cells were grown to ~80% confluence and treated with ethanol or 100 nM E2 for 3 or 6 hours. Cells were washed with cold PBS and the total RNA was collected using TRIZOL (Invitrogen) according to the manufacturer’s specifications. First
strand cDNA synthesis was performed using 2 µg of total RNA, 2 µg oligo(dT), and 600 units of MMLV reverse transcriptase (Promega). The resulting cDNA from each sample was treated with 3 units of RNAsae H (Ambion) for 30 min. at 37°C and then diluted 1:5 with water. E2-treated samples were further diluted 1:10 and analyzed by q PCR using a 96-well DNA Engine Opticon (MJ Research) or a 384-well Prism 7700 (ABI) real-time PCR thermocycler for 45 cycles (95°C for 15 sec, 60°C for 1 min) following an initial 10 min. incubation at 95°C. The fold change in expression of each gene was calculated using a standard curve of diluted cDNA from untreated samples (1:1, 1:10, 1:100) and normalized against the fold change of β-actin, a well-characterized housekeeping gene that I used as an internal control. Independent experiments were scaled in relation to E2 expression levels with error bars representing the SEM.

**Primers for quantitative real-time PCR (qPCR).**

The qPCR primers used for ChIP analyses are as follows:

ACO2 forward    5’- CTTGCACCAGGCCCGTCT -3’
ACO2 reverse    5’- AAGATGTTTTACCCAAGAACAAAT -3’
ACO2distal forward    5’- CTCAGTCCTCTGTATCCTCTG -3’
ACO2distal reverse    5’- CCAAGTTTTGTGATGCAAG -3’
ADORA1 forward    5’- GCCTTGTGTCTGGATGTT -3’
ADORA1 reverse    5’- GCCCTGGACTCTTGATGACAT -3’
Blk4 forward    5’- ATCCTTGATTTGACAT -3’
Blk4 reverse    5’- CTTGCAGGCTCTCTTCTA -3’
Blk42 forward    5’- GGCAGGCAACACACATG -3’
Blk42 reverse    5’- GCCCTGGACACAACTGCAT -3’
Blk44 forward 5’- GGGAAAATATGCAGAAGAAAACG -3’
Blk44 reverse 5’- CATTATTTCAACACCTCTGATGTCCTA -3’
CENPA forward 5’- CCATCTCTGCGTTGCTAAGG -3’
CENPA reverse 5’- GTGCCCTCCAGTCAAAACAC -3’
CEP350 forward 5’- AGTGACAGCAGTGGAAGCAACG -3’
CEP350 reverse 5’- GGCGATTTCGAGAAGCTCAGA -3’
CHPT1 forward 5’- TCTCTGAATCCGCAGTGATG -3’
CHPT1 reverse 5’- TCCCTTTCTGTACGGAGGAA -3’
CYP1B1 forward 5’- CGTGCGGCGCTCGTAAGG -3’
CYP1B1 reverse 5’- AGGTGCCCACGTTTCCATT -3’
FLJ13305 forward 5’- GAAGGAGGGCGGTACATTCT -3’
FLJ13305 reverse 5’- CCAACTCTGCGTTTATTGGA -3’
FLJ31818 forward 5’- ACAGCAGATGSCCTCAAGAA -3’
FLJ31818 reverse 5’- TCCAAATTAAAGGACAGGAGGT -3’
GOLGB1 forward 5’- ATGCCTCCGCTTCCCTCAAG -3’
GOLGB1 reverse 5’- CCACCTCCAGACTTCTGA -3’
GREB1 forward 5’- AGTGAGCACTGGGTACTTCTGA -3’
GREB1 reverse 5’- GGATGTAGTCCATGCTGTCTGCG -3’
HDGF2 forward 5’- CCCGTCCTGCTTCAAGTCCATT -3’
HDGF2 reverse 5’- GAGGTTGGAGCAACAGAGTT -3’
HLA-DMA forward 5’- TTGCACATATAACAGTACCTTCT -3’
HLA-DMA reverse 5’- TATCTCCTCCGCCTTCTCT -3’
HOXC10 forward 5’- AACGGTTTTCCATCAAACTGTTGGA -3’
HOXC10 reverse 5’- AGCAGTCAATCCAGGGAGCATT -3’
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<td>P2RX7</td>
<td>5' - TGGAAAGCTCCAGTCTTGTGA -3'</td>
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<tr>
<td>P2RX7</td>
<td>3' - CACCTTTTTGTCATGTCTTCTTG -5'</td>
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<tr>
<td>PCYT1A</td>
<td>5' - CCCTCGCTGTCACTTACCA -3'</td>
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<tr>
<td>PCYT1A</td>
<td>3' - GTTGCAGGTGTGTCCTATC -5'</td>
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<tr>
<td>PDCD6IP</td>
<td>5' - TTCCTGATACTTTTCCCGTTTACC -3'</td>
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<tr>
<td>PDCD6IP</td>
<td>3' - ACTACTGTGGACGGGCTGCT -5'</td>
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<tr>
<td>PLAC1</td>
<td>5' - TGACAGAAACTCATTCAACAGGAAG -3'</td>
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<tr>
<td>PLAC1</td>
<td>3' - GGCAACAGCAAGCACTACAA -5'</td>
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<tr>
<td>PPM2C</td>
<td>5' - TTGGTGAAACACTAGGGAAGATAAG -3'</td>
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<tr>
<td>PPM2C</td>
<td>3' - GGCATTGGTATTGTCTGTGG -5'</td>
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<tr>
<td>PRUNE</td>
<td>5' - ACATACACATTTGTTTACCGAAGCA -3'</td>
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<tr>
<td>PRUNE</td>
<td>3' - TCCGCAATGTCCTAGCAA -5'</td>
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<tr>
<td>RNF167</td>
<td>5' - CCAGAGGGAGGAGGAGTTTG -3'</td>
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<tr>
<td>RNF167</td>
<td>3' - AGGTTAGCGATGGGAGGACT -5'</td>
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<tr>
<td>SERPINA1</td>
<td>5' - TGGAGGAGGAATGAAGAAAGCA -3'</td>
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<tr>
<td>SERPINA1</td>
<td>3' - AGCAGGACCCCAAATTCTGA -5'</td>
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<tr>
<td>SLC27A2</td>
<td>5' -CACGCCTGCAATATCCTCTTATAAT -3'</td>
<td></td>
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<tr>
<td>SLC27A2</td>
<td>3' - CACGGTTTCTTTAATGCTGATGA -5'</td>
<td></td>
</tr>
<tr>
<td>SPTBN4</td>
<td>5' - GACTACACGTGCCTGACACC -3'</td>
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<tr>
<td>SPTBN4</td>
<td>3' - ACGTCCACACCCCTATCGTA -5'</td>
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</table>
The qPCR primers used for expression analyses are as follows:

ACTB forward 5'- AGCTACGAGCTGCCTGAC -3'
ACTB reverse 5'- AAGGTAGTTTCGTTGAGTC -3'
ANK3 forward 5'- CGCTCCTTCAGTTCGGATAG -3'
ANK3 reverse 5'- TTCCCTTGTGAATGTTAGATGCT -3'
CEP350 forward 5'- AAAGTGGCCTTAGCTTTTTGC -3'
CEP350 reverse 5'- GAAGATGTAAGTTTGTATTTCGCTTGAG -3'
ELOVL2 forward 5'- AGAGGGTGTTGGTTGCTTGAG -3'
ELOVL2 reverse 5'- CAAGGTGAGGATACCCCTG -3'
FAM5C forward 5'- TTTACAGTGCCTTTTGTGGAACAG -3'
FAM5C reverse 5'- TTGTCAGCAAGTTTCATGTGCTGTG -3'
GOLGB1 forward 5'- CATGGGAGGACAGCATCTTC -3'
GOLGB1 reverse 5'- GATCAAGGGCAAAGCAAAAG -3'
GREB1 forward 5'- GCCGTGACAAGAGGTTC -3'
GREB1 reverse 5'- GGGTGAAGGTTGTCAGTTTC -3'
HOXC10 forward 5'- GACACCTCAGATAACGAAAGC -3'
HOXC10 reverse 5'- TTTCTCAATTCCAGCAGTCT -3'
MAPK8 forward 5'- CATCATGAGCAAGCAAGG -3'
MAPK8 reverse 5’- GCTGCGCATACCTATTCCTTG -3’
MAPK9 forward 5’- TCATCCTGGGTATGGGCTAC -3’
MAPK9 reverse 5’- CAATATGGTCAGTGCCTTG -3’
NUAK1 forward 5’- CAGTCACACACGCTGCTTT -3’
PLAC1 forward 5’- CAGTGAGCACAAGCCACAT -3’
PLAC1 reverse 5’- AACCACAGGAAACAGGAAGC -3’
REFERENCES


Lange, C. A. (2004). Making sense of cross-talk between steroid hormone receptors and intracellular signaling pathways: who will have the last word? Mol Endocrinol 18, 269-278.


