SUPPLEMENTAL MATERIALS

Estrogen Regulates the JNK1 Genomic Localization Program in Breast Cancer Cells to Control Gene Expression and Cell Growth

by Sun *et al*., 2012

Contents

1) Supplemental Materials and Methods

Cell culture and treatments. MCF-7 cells, kindly provided by Dr. Benita Katzenellenbogen (University of Illinois, Urbana-Champaign), were maintained in MEM with Hank's salts (Sigma; M1018) supplemented with 5% calf serum, sodium bicarbonate, HEPES, penicillin/streptomycin, and gentamicin. Prior to all experimental procedures and treatments, the cells were grown for at least 3 days in phenol red-free MEM Eagle medium with Earle's salts (Sigma; M3024) supplemented with 5% charcoal-dextran-treated calf serum, L-glutamine, sodium bicarbonate, HEPES, penicillin/streptomycin, and gentamicin. Adherent HeLa cells were purchased from the ATCC and HeLa-ERα cells were kindly provided by Dr. David Shapiro (University of Illinois, Urbana-Champaign) (1). They were maintained in DMEM/F12 (Sigma, D2906) supplemented with 10% charcoal-dextran stripped calf serum, penicillin/streptomycin, and gentamicin as described previously (2). ZR75T cells were kindly provided by Dr. Khandan Keyomarsi (MD Anderson Cancer Center, Houston). They were maintained in α-MEM (Mediatech; MT15012CV) supplemented with 10% fetal bovine serum, 1 μg/mL insulin, 1 μg/mL hydrocortisone, 12.5 ng/mL EGF, HEPES, non-essential amino acids, L-glutamine and sodium pyruvate. Prior to all experimental procedures and treatments, the cells were grown for at least 3 days in phenol-red free RPMI-1640 (Sigma; R7509) supplemented with 10% charcoal-dextran-treated fetal bovine serum, 1 μg/mL insulin, 1 μg/mL hydrocortisone, HEPES, L-glutamine, penicillin/streptomycin, and gentamicin..

The cells were treated with control vehicle (ethanol) or E2 (100 nM) for the times specified below or in the figure legends. For the JNK inhibition experiments, the MCF-7 cells were pre-treated with control vehicle (DMSO) or 20 μM SP600125 (SP; Biomol) for 1 hour before treatment with E2.

Antibodies. The antibodies used were as follows: JNK1/3 (Santa Cruz, sc-474; note that JNK3 is not expressed in MCF-7 cells, so we have unambiguous identification of JNK1 using this antibody), pan-JNK (Santa Cruz, sc-7345), phosphorylated pan-JNK (Santa Cruz, sc-6254), ERα (rabbit polyclonal generated in the Kraus lab), c-Fos (rabbit polyclonal generated in the Kraus lab), and GAPDH (kindly provided by Eric Alani, Cornell University).

JNK1 subcellular 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 $MgCl_2$, 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 resulting lysates were centrifuged at 8,000 rpm in a microfuge at 4°C and the supernatants were collected as the cytoplasmic fractions. The nuclei were washed twice with hypotonic buffer containing 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 min. incubation on ice, the extracted nuclei were pelleted by centrifugation as above and the supernatants were collected as the nuclear fractions. The protein concentrations of the fractions were determined by Bradford assays. The extracts were subjected to immunoblotting using antibodies to JNK1, phosphorylated JNK1, ERα, and GAPDH.

Immunofluorescent staining of cells for JNK1. 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 the addition of 125 mM glycine. The cells were rinsed twice with PBS, permeablized 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 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 on slides using Vectashield (Vector Laboratories; H-1000), and visualized using a Leica Confocal Microscope System.

Chromatin immunoprecipitation (ChIP). ChIP assays for JNK1, ERα, and c-Fos were performed using a ChIP protocol described previously (3), 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 hour pre-clearing step using a 1:1 mixture of protein A beads (Upstate; 16-125) and 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 A and protein G-agarose beads for 4 hours to capture the immunoprecipiated 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 $NaHCO_3$) 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 using a set of gene-specific primers, as described below. Each ChIP experiment was conducted with at least three independent chromatin isolates to ensure reproducibility.

ChIP-reChIP. Following the primary ChIP, JNK1- and ERα-precipitated complexes were eluted with 10 mM DTT twice for 20 min at 37°C. Eluates were diluted 20 times in ChIP dilution buffer, incubated with a second antibody at 4°C overnight, followed by the addition of the protein-A/G-agarose bead mixture. After this secondary ChIP, washing, elution, reversal of the crosslinks and analysis by qPCR were carried out as described for the standard ChIP protocol described above.

ChIP-chip. Ligation-mediated PCR (LM-PCR), labeling, and hybridization to arrays was carried out as described here.

• ***LM-PCR.*** JNK1 and ERα immunoprecipitated genomic DNA from the standard ChIP protocol described above was blunted and amplified by LM-PCR as described previously (4). 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.

• ***Labeling and hybridization.*** 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. Data processing was done essentially as described previously (4) using the statistical programming language R (5). All R scripts are available upon request. The ChIP-chip data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE13200 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13200).

• ***Moving window analysis.*** The pairwise data files from the ChIP-chip analyses were used to calculate the $\log_2$ ratio data for each array. The ratio values were subjected to lowess normalization (6) and the arrays were normalized to each other using equivalent sum of squares scaling. The normalized individual replicates were averaged to generate composite arrays, which were used for subsequent analysis. An error model was generated using a 1000 bp moving window with 250 bp steps in which both the mean probe $\log_2$ ratio and p-value from a nonparametric Wilcoxon signed-rank test were calculated for each window.

• ***Definition of significant bound regions.*** JNK1- and ERα-bound regions were defined as the middle one of three consecutive windows containing (1) mean probe $\log_2$ ratios larger than 2 standard deviations from the average value of all windows and the middle window is the highest of the three, (2) at least 6 probes, and (3) p-values < 0.016. In the case of JNK1, a window fold change list was calculated from the composite arrays by dividing the average JNK1 ratio of each window in the presence of E2 by the corresponding average JNK1 ratio in the absence of E2. Constitutive regions were defined as JNK1-bound regions (present in either the E2-treated or the untreated samples) that did not pass a threshold window fold change ratio (> 1.7 or < -1.7). Recruited regions were defined as JNK1-bound regions that had a fold change ratio > 1.7. Released regions were defined as JNK1-bound regions that had a fold change ratio < -1.7.

• ***Definition of overlapping peaks.*** For calling overlaping peaks of JNK1 and ERα, we defined a region ±750 bp from the center of the JNK1 peak. If this region had any overlap with an ERα peak, the peaks were said to be "overlapping."

• ***Visual representation of the data.*** The TSS-anchored heat maps used to visualize the ChIP-chip data were generated with Java Treeview (7). For genes with multiple TSS the most 5' TSS was used.

Knockdown of ERα and JNK1 in MCF-7 cells. Transient RNAi-mediated knockdown of ERα using siRNAs and stable RNAi-mediated knockdown of JNK1 using shRNAs was performed as described here.

• ***Transient knockdown of ERα and JNK1.*** Transient depletion of ERα or JNK1 in MCF-7 or ZR75T cells was achieved by transfection of small interfering RNAs (siRNAs). siRNA duplexes specifically targeting ERα or JNK1 (SMARTpool) and nontargeting control siRNAs were purchased from Dharmacon and transfected using Dharmafect following the manufacturer's instructions. Knockdown was verified by immunoblotting.

• ***Stable knockdown of JNK1.*** 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 described previously (8). Knockdown was verified by immunoblotting and RT-qPCR.

Gene-specific expression analyses by RT-qPCR. The expression of endogenous target genes was determined by reverse transcription-quantitative PCR (RT-qPCR), as described previously (3), with minor modifications. MCF-7 cells were grown to ~80% confluence and treated with ethanol or 100 nM E2 for 3 hours. The cells were washed with ice 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 RNAse H (Ambion) for 30 min. at 37°C and then diluted 1:5 with water. The cDNA samples were analyzed by qPCR using a set of gene-specific primers, as described below. Each experiment was conducted with at least three independent RNA isolates to ensure reproducibility.

Quantitative PCR (qPCR). qPCR analysis of ChIP and cDNA samples was performed as described here using the primer sets listed below. Each experiment was performed a minimum of three times to ensure reproducibility.

• ***ChIP-qPCR.*** For qPCR analyses of the ChIP and re-ChIP material, 5 μl out of 100 μl of ChIP DNA, 1x SYBR green PCR master mix, and 250 nM each of forward and reverse primers were used in 40 cycles of amplification (95°C for 15 seconds and 60°C for 2 minutes) using a 384-well Lightcycler 480 (Roche) real-time PCR thermocycler after an initial 10 minute incubation at 95°C. Melting curve analysis was performed to ensure that only the targeted amplicon was amplified. Mock IP (i.e. nonspecific ChIP) values were subtracted from the IP values to generate the values shown in the graphs.

• ***RT-qPCR.*** The cDNA samples from the expression analyses described above were analyzed using a 384-well Lightcycler 480 (Roche) 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 pooled and serial

diluted cDNA from all samples and normalized against the fold change of β-actin, a well-characterized housekeeping gene that we used as an internal control.

Cell proliferation assays. Estrogen-deprived MCF-7 cells with stable knockdown of JNK1, or controls cells stably transfected with an shRNA targeting GFP, were plated at a density of 1 x 10^5 per well of a 6-well dish. After attachment, the cells were treated with E2 for a 10-day time course. At two-day intervals, the cells were collected, stained with trypan blue, and counted. Estrogen-deprived ZR75T cells were plated at a density of 1.5 x 10^5 cells per well of a 6-well dish. Twenty-four hours prior to the start of E2 treatment, siRNA-mediated JNK1 knockdown was performed with an appropriate matching control. The number of cells in each condition was counted every day during a three-day treatment time course. Given the considerable differences in the proliferation rates of the MCF-7 and ZR75T cells, we matched the densities of the cells, not the day of growth or E2 treatment, when comparing the effects of JNK1 knockdown.

Bioinformatic analyses. Motif predictions and transcription factor assignments were performed as described here.

• ***De novo motif predictions.*** De novo motif predictions were performed on a set of promoters where significant JNK1 binding in the E2-treated condition (either induced or constitutive) was evident. We examined a region of 1000 bp surrounding the JNK1 peaks, with a JNK1-negative background list that reports regions on the array with no significant JNK1 signal. The lists were formulated using the tools on the Galaxy browser (9) 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. The JNK1-negative list was used to compute background nucleotide frequencies and 1st- through 3rd-order Markov background models.

De novo motif detection was carried out using MEME (Multiple Em for Motif Elicitation) (10) on repeat-masked sequences, using the 3rd 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) (10) 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^{-4} as previously reported (3). 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) (11) was used to predict the transcription factors that might bind to the enriched sequences from MEME. Position weight matrices for the predicted transcription factors were obtained from the TRANSFAC database (12) 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 was calculated.

Gene ontology (GO) analyses. Gene ontologies were obtained using Genecodis (13) for the "All JNK1-bound", "JNK1-released", "JNK1-constitutive", and "JNK1-recruited" gene sets. The entire gene list represented on the ChIP-chip array was used as the background reference. GO terms representing less than 5 genes were not considered. 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. Five random gene sets were generated using the programming language R from the total number of genes present on the ChIP-chip array. No GO terms were enriched (i.e., all p-values were >0.001) in the random lists using the criteria described above.

In vitro kinase assays. Kinase reactions were conducted using recombinant JNK1 purified from *E. coli* using an activated MAP kinase purification system kindly provided by Dr. Melanie Cobb (University of Texas Southwestern Medical Center at Dallas) and described previously (14) with two major modifications: (1) the human JNK1 cDNA (JNK1α1) was cloned in to replace the rat JNK2 cDNA sequence and (2) the final cation exchange purification was omitted. Purified activated JNK1 (300 nM) was incubated with various substrates for 30 min. at 30°C in kinase buffer (25 mM HEPES pH 7.5, 10 mM magnesium acetate, 50 μM ATP, 2 μCi γ-^{32}P-ATP). The labeled proteins were resolved using SDS-polyacrylamide gel electrophoresis, the gels were dried on filter paper, and the ^{32}P signal was detected using a phosphorimager system. The substrates tested were as follows: core histones from HeLa cells (140 ng), salt-dialyzed mononucleosomes with an AP-1 binding site containing approximately 140 ng of HeLa cell core histones assembled as described (15), FLAG-tagged SRC1 (160 nM) purified as described (16), FLAG-tagged ERα (160 nM) and 6xHis-tagged p300 (160 nM) purified as described (17), and c-Fos/c-Jun dimers (300 nm) purified as described (18).

Primers for qPCR. The following oligonucleotide primers were used for the ChIP-qPCR and RT-qPCR assays.

• ChIP-qPCR:

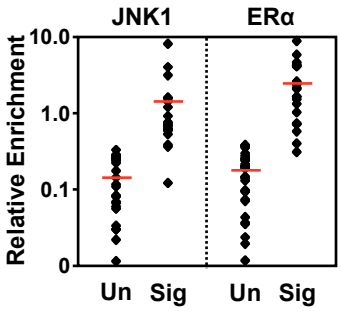
ACO2 forward	5'- CTTGCACCAGGCCCGTCT-3'
ACO2 reverse	5'- AAGATGTTTTACCCAAGAACAAAT - 3'
Blk44 forward	5'- GGGAAAATATGCAGAAGAAAACGA -3'
Blk44 reverse	5'- CATTTATTCAACACCTCTGATGTCCTA -3'
CYP1B1 forward	5'- CGTGCGGCCTCGATTG -3'
CYP1B1 reverse	5'- AGGTGCCCACGTTTCCATT -3'
GREB1 forward	5'- AGTGTGGCAACTGGGTCATTCTGA -3'
GREB1 reverse	5'- GGTATGATTCATCATTGTCTGCTGCG -3'
PCYT1A forward	5'- CCCTCGCTGTCACTTACCA -3'
PCYT1A reverse	5'- GTTGCAGGTGTGTGCCTATC -3'
PLAC1 forward	5'- TGACAGAACTCATTCACAGGAAG -3'
PLAC1 reverse	5'- GGCAACAGCAAGCACTACAA -3'

SPTBN4 forward	5’- GACTACACGTGCGTGACACC -3’
SPTBN4 reverse	5’- ACGTCCCACACCCTATCGTA -3’
TFF1 forward	5’- ATAACATTTGCCTAAGGAGGCCCG -3’
TFF1 reverse	5’- TCAGCCAAGATGACCTCACCACAT -3’
UGT2B15 forward	5’- TGAACTGTACACACTAATTGGTGAGTCA -3’
UGT2B15 reverse	5’- TCGTGGTGCAAGTAATGTCTTCTAA -3’

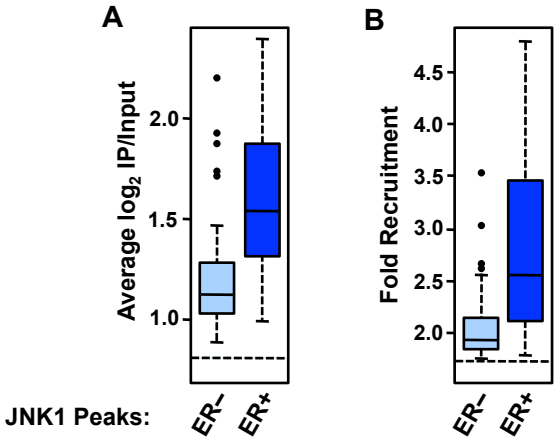
• RT-qPCR:

ACTB forward	5’- AGCTACGAGCTGCCTGAC -3
ACTB reverse	5’- AAGGTAGTTTCGTGGATGC -3’
GREB1 forward	5’- GCCGTTGACAAGAGGTTC -3’
GREB1 reverse	5’- GGGTTGAGTGGTCAGTTTC -3’
ELOVL2 forward	5’- AGAGGGTGGTTCATGTTGGA -3’
ELOVL2 reverse	5’- CAAGGTGAGGATACCCCTGA -3’
HOXC10 forward	5’- GACACCTCGGATAACGAAGC -3’
HOXC10 reverse	5’- TTTCTCCAATTCCAGCGTCT -3’
PLAC1 forward	5’- CAGTGAGCACAAAGCCACAT -3’
PLAC1 reverse	5’- AACCACAGGAAACAGGAAGC -3’

2) Supplemental Figures

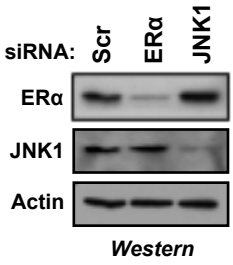


Supplemental Fig. S1. Confirmation of JNK1 and ERα peaks from the ChIP-chip analysis by ChIP-qPCR.
ChIP-qPCR was performed to determine JNK1 *(left)* and ERα *(right)* occupancy at significantly bound (Significant; Sig) and unbound (Un) regions as defined by the ChIP-chip analyses shown in Figs. 1 and 2. The relative enrichment of each qPCR amplicon tested is shown in the cluster plot, grouped by whether or not the region of the amplicon is bound by JNK1 or ERα based on ChIP-chip peak definition. Red bars represent the average signal in each group. Significant JNK1- and ERα-bound regions from the ChIP-chip analyses show enrichment of factor binding compared to the unbound regions in the ChIP-qPCR experiments, confirming the ChIP-chip analysis.

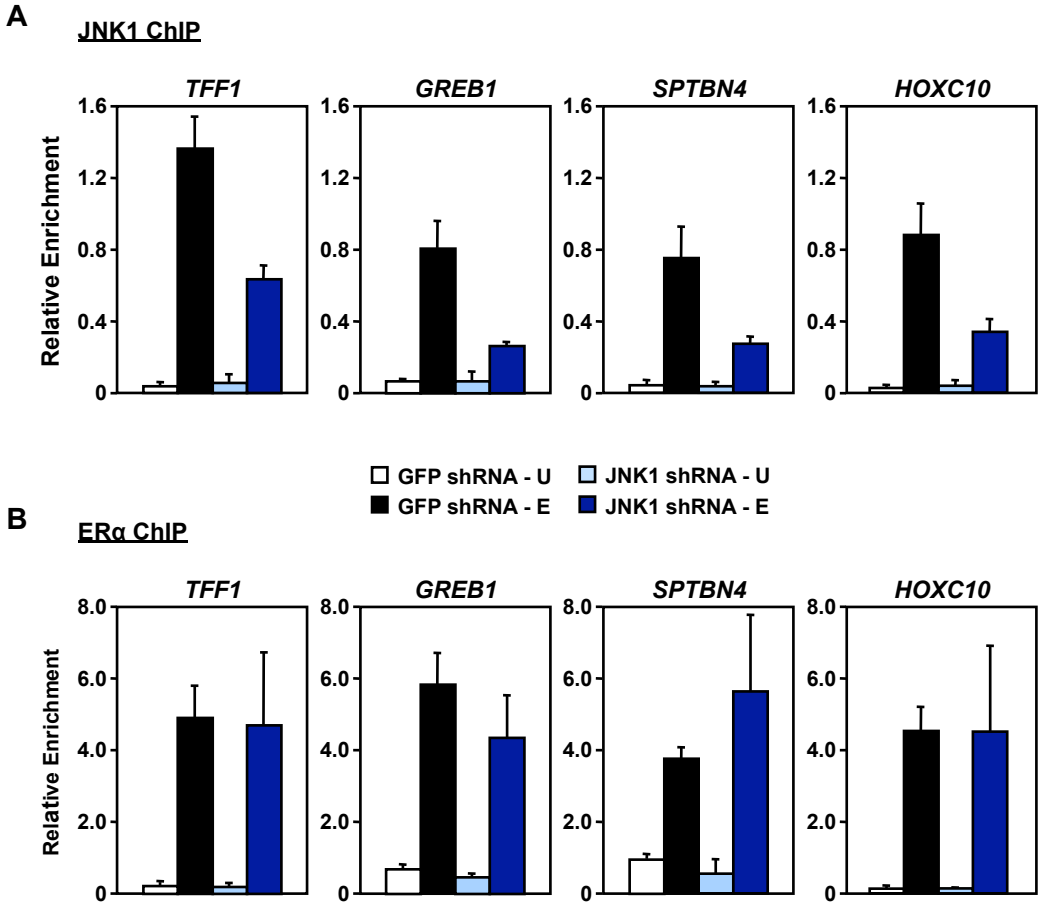


Supplemental Fig. S2. ERα-positive, E2-recruited JNK1 peaks, as a group, have stronger ChIP signals and show greater fold recruitment than ERα-negative, E2-recruited JNK1 peaks.

Box plots showing the $\log_2$ IP/input **(A)** and the fold recruitment upon E2 treatment **(B)** calculated for each window that shows statistically significant enrichment of E2-induced JNK1 binding. The samples were grouped by whether or not ERα was also absent (ER–; *left bars*) present (ER–; *right bars*) in the vicinity of the JNK1-bound window. The dotted horizontal line in each plot indicates the threshold used in determining JNK1 peaks above background (A) and E2-induction (B), respectively.

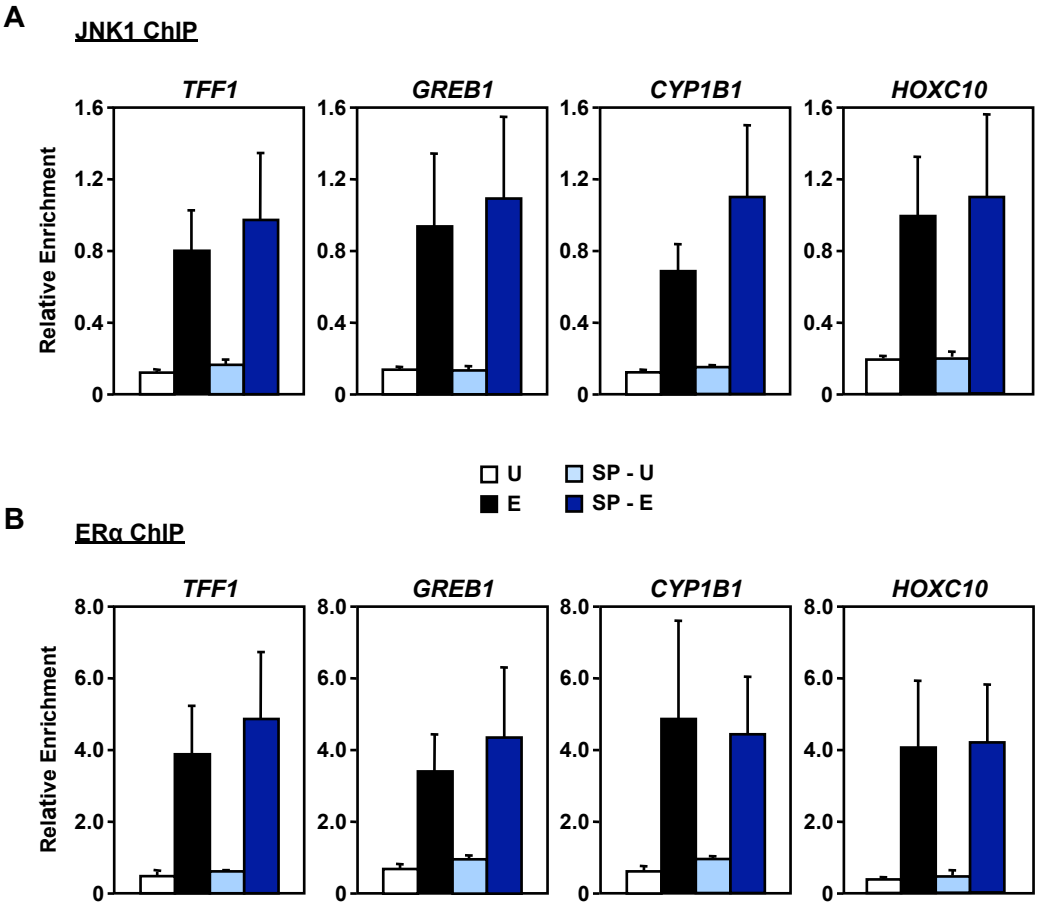


Supplemental Fig. S3. Knockdown of ERα does not affect the total cellular levels of JNK1, and vice versa.
ERα and JNK1 were transiently knocked down in MCF-7 cells by siRNA-mediated gene silencing. A pool of scrambled siRNAs ("Scr") was used as a control. Analysis of ERα *(top)* and JNK1 *(middle)* protein levels by Western blotting was performed 3 days (72 hours) after siRNA transfection. β-actin was used as a loading control *(bottom)*.

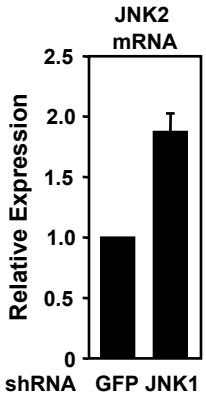


Supplemental Fig. S4. Reduction in JNK1 occupancy at target gene promoters in JNK1 knockdown cells occurs without an appreciable effect on ERα binding.

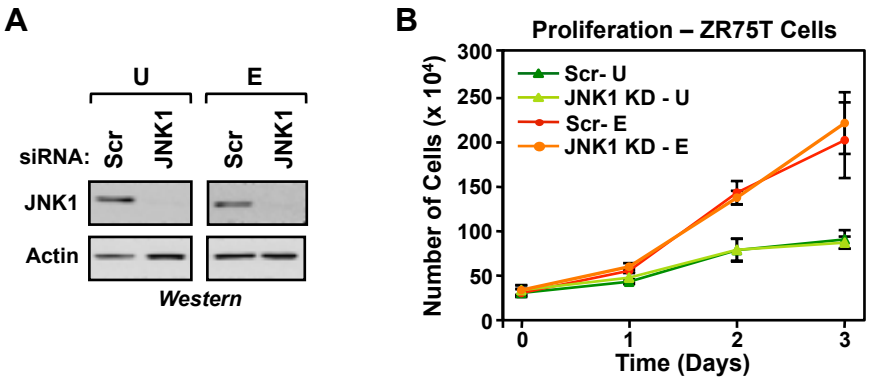
JNK1 was stably knocked down in MCF-7 cells by retroviral-mediated delivery of an shRNA targeting JNK1 into the cells, followed by drug selection. Knockdown of JNK1 mRNA and protein was confirmed as shown in Fig. 6A. ChIP-qPCR for (A) JNK1 or (B) ERα was performed in JNK1 knockdown and control (GFP shRNA) cell lines in the absence (U) and presence (E) of E2 treatment for the four "JNK1-recruited" gene promoters shown (*TFF1*, *GREB1*, *SPTBN4*, and *HOXC10*). Each bar represents the mean + SEM, n = 3. The results demonstrate a reduction of E2-induced JNK1 binding at gene promoters upon JNK1 knockdown, as expected.



Supplemental Fig. S5. Treatment of MCF-7 cells with the JNK inhibitor SP600125 does not have an appreciable effect on the occupancy of JNK1 or ERα at target gene promoters. The occupancy of (A) JNK1 and (B) ERα at target gene promoters in MCF-7 cells in the absence or presence of the JNK inhibitor SP600125 (SP) for 2 hours, before a 3-hour treatment with vehicle (U) or E2 (E) was determined by ChIP-qPCR for the four "JNK1-recruited" gene promoters shown (*TFF1*, *GREB1*, *CYP1B1*, and *HOXC10*). Each bar represents the mean + SEM, n = 3.

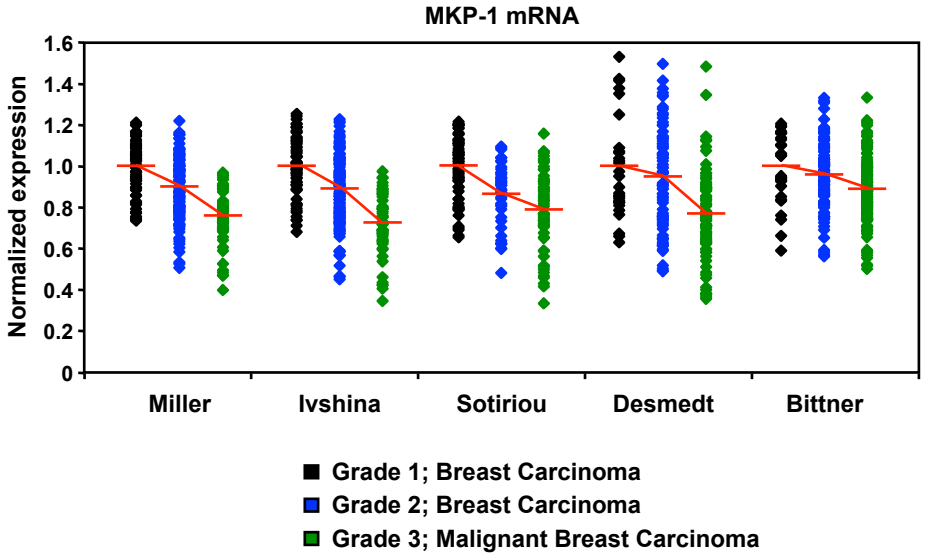


Supplemental Fig. S6. Knockdown of JNK1 mRNA in MCF-7 cells increases JNK2 mRNA. JNK1 was stably knocked down in MCF-7 cells by retroviral-mediated delivery of an shRNA targeting JNK1 into the cells, followed by drug selection. JNK2 mRNA levels were determined by RT-qPCR. Each bar represents the mean + SEM, n = 3.

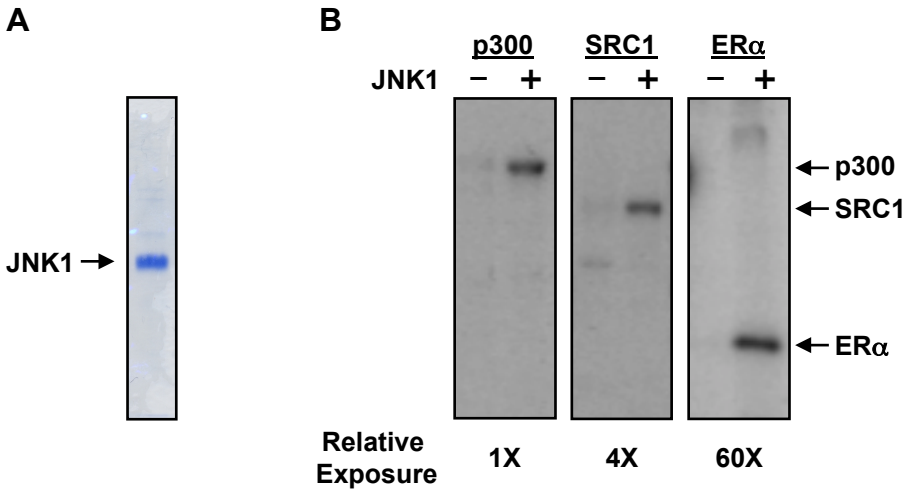


Supplemental Fig. S7. Knockdown of JNK1 does not affect estrogen-dependent proliferation in ZR75T cells.
(A) JNK1 was transiently knocked down in ZR75T cells by using transfection of siRNAs. A pool of scrambled siRNA ("Scr") was used as a control. Analysis of JNK1 protein levels by Western blotting 3 days after siRNA transfection [2 days in the absence (U) or presence (E) of E2]. β-actin was used as a loading control.
(B) Analysis of E2-dependent cell proliferation in JNK1 knockdown ZR75T cells grown for 3 days in the absence (U) or presence (E) of E2. siRNA-mediated JNK1 was performed 24 hours prior to the start of E2 treatment. The number of cells in each condition was counted every day over the course of a three-day E2 treatment. Each point represents the mean ± SEM, n = 3.

Note: Given the considerable differences in the proliferation rates of the MCF-7 and ZR75T cells, we matched the densities of the cells, not the day of growth or E2 treatment, when comparing the effects of JNK1 knockdown.



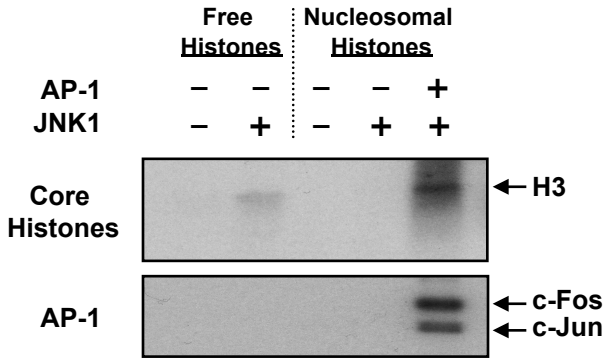
Supplemental Fig. S8. Expression of the JNK phosphatase, MKP-1, decreases with breast cancer progression.
Data obtained from the Oncomine database were plotted as shown. The relative expression of MKP-1 across three breast carcinoma grades is shown from five independent studies (indicated by the last names of the first authors). The p-values for negative correlation were <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.



C

JNK Binding Motif ++xxxxTΦxΦ		
1635	RRxxxxTMxM	p300
1013	RxKxxxxTMxV	SRC1
	None	ERα

Supplemental Fig. S9. JNK1 phosphorylates ERα coactivators in vitro.
(A) Activated recombinant JNK1 was expressed in bacteria, purified as described previously (14), and analyzed by SDS-PAGE with staining using Coomassie Brilliant Blue.
(B) In vitro kinase assays using recombinant JNK1. JNK1 was incubated with ERα, p300, or SRC1 in the presence of ^{32}P-ATP, and phosphorylation of the target proteins was detected by autoradiography. p300 was strongly phosphorylated by JNK1 (note the relative exposure times for each autoradiogram).
(C) p300 and SRC-1, but not ERα, contain a putative JNK interaction domain, similar to the JNK-interacting sequence in c-Jun. Key: + = basic amino acid; x = any amino acid; T = Threonine; Φ = hydrophobic amino acid; R = Arginine; K = Lysine; M = Methionine; V = Valine. Numbers represent the first amino acid position in the motif.



Supplemental Fig. S10. JNK1 phosphorylates nucleosomal histone H3 in vitro.
Kinase reactions were performed using recombinant JNK1, as shown in Fig. S3A. JNK1 was incubated with core histones or an equivalent amount of core histones assembled into mononucleosomes containing an AP-1 binding site by salt dialysis. The nucleosome reactions were performed in the absence or presence of recombinant AP-1 (c-Fos/c-Jun heterodimers) to target JNK1 specifically to the nucleosome. ^{32}P phosphorylation of histones, c-Fos, and c-Jun was detected by autoradiography. Nucleosomal H3 is a target of JNK1 enzymatic activity when JNK1 is specifically targeted to the nucleosome.

3) Supplemental Tables

Supplemental Table S1. Gene ontology analysis of JNK1-bound promoters.

Gene set	Ontology[a]	p-value[b]
All JNK1-bound promoters	• Response to stimulus	1.99×10^{-12}
	• Signal transduction	3.02×10^{-10}
	• GPCR signaling pathway	1.64×10^{-8}
	• RNA splicing	3.22×10^{-8}
JNK1-released promoters	• Regulation of transcription	7.67×10^{-4}
JNK1-constitutive promoters	• Response to stimulus	5.44×10^{-11}
	• GPCR signaling pathway	1.07×10^{-8}
	• Signal transduction	1.51×10^{-6}
	• RNA splicing	8.30×10^{-6}
JNK1-recruited promoters	• Regulation of transcription, DNA-dependent	3.65×10^{-7}
	• Response to drugs	1.22×10^{-6}
	• Signal transduction	1.71×10^{-5}
	• Metabolism of androgens and estrogens	6.0×10^{-6}
Five random gene sets[c]	• None	<0.001

[a] Ontologies were obtained using Genecodis for the (1) All JNK1-bound, (2) JNK1-released, (3) JNK1-constitutive, and (4) JNK1-recruited genes. The entire gene list represented on the ChIP-chip array was used as the background reference. GO terms 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 GO terms were enriched (i.e., all p-values were >0.001) in the random lists using the criteria described above.

Supplemental Table S2. Overlap of JNK1 peaks with ERα peaks.

	Number of JNK1 Peaks	Number of JNK1 Peaks Overlapping an ERα Peak	Percent of JNK1 Peaks Overlapping an ERα Peak
All JNK1 Peaks	508	74	15
JNK1-Released	35	0	0
JNK1-Constitutive	367	35	9.5
JNK1-Recruited	106	39	37

The peaks calls are based on ChIP-chip using a RefSeq promoter arrays spanning approximately -2 kb to +0.5 kb relative to the transcription start site.

Supplemental Table S3. Unbiased motif analysis of JNK1 peaks.

Sequence[a, b]	No. of sites[c] (No. of windows)[d]	p-value[e]	TESS call[f]	ID[g]
JNK1 - E peaks				
GCCTGTAAGTCCCAGC	50 (38)	3.24E-06	Bcd	Q00016
AGGCTGAGGCAGGAG	50 (36)	1.09E-07	AP-1	-
TGTTGCCCAGGCTGG	50 (40)	1.50E-12	½ ERE	R04883
GAGGTTGCAGTGAGC	47 (43)	1.16E-08	–	-
GCCACCACGCCCGGC	50 (36)	1.27E-03	Sp1	R01702
TAGAGACGGGGTTTC	50 (38)	8.02E-20	SIF	R02244
CTTGAGCCCAGGAGT	50 (37)	1.13E-10	LBP-1	I00191
GATCGTGCCACTGCA	38 (35)	7.24E-04	NF-1/L	R01322
CACCTCAGCCTCCCA	19 (18)	8.10E-07	Sp1	R02245
TGAATGAATAAATA	44 (38)	7.70E-82	POU1F1a	R00623
GCACAGCTTCCCTGC	19 (10)	3.96E-28	Sp1	R08166
CTCGAACTCCTGACC	22 (22)	3.71E-15	AP-1	R00368
			½ ERE	I00276
JNK1-recruited				
GCCTGTAATCCCAGC	43 (27)	1.74E-14	Bcd	Q00016
CTGCCTCAGCCTCCC	50 (23)	9.99E-16	Sp1	R02245
TGTTGCCCAGGCTGG	43 (25)	3.06E-10	½ ERE	R04883
GCCACCACGCCCGGC	36 (22)	1.24E-04	Sp1	R01702
GAAACCCCGTCTCTA	32 (23)	3.06E-10	SIF	R02244
GAGGATCACTTGAGC	47 (31)	7.36E-11	IRF-2	R00917
CAGTGAGCTGAGATC	22 (20)	6.12E-10	Zeste	R04948
AGTGCAGTGGC	18 (17)	3.88E-16	Sp1	R01021

[a] Unbiased search for DNA sequence motifs enriched under the JNK1-bound regions using MEME. De novo motif predictions were performed for: (1) all promoters showing JNK1 binding at in the E2-treated condition ("JNK1 - E peaks") or (2) promoters showing JNK1 recruitment upon E2 treatment ("JNK1-recruited"). The gene lists were formulated using the tools on the Galaxy browser (9) so genomic locations from JNK1-bound regions would not be present in the background regions. De novo motif detection was carried out using MEME (10) on repeat-masked sequences. MAST (Motif Alignment and Search Tool) (10) was used to scan for the locations of all motif instances within both bound and unbound sequences, using a p-value threshold of 1.5×10^{-4} (10). Fisher's exact tests were used to determine enrichments relative to background, with p-values corrected for multiple testing using the Holm method in R.

[b] The sequences are listed 5' to 3'.

[c] Number of times each motif listed was identified in the promoter regions of the genes in each category (i.e., "JNK1 - E peaks" or "JNK1-recruited").

(continued on the next page)

Supplemental Table S2. ***(continued)***

[d] The number of peak-containing windows in which the sequence is found. Note that two or more sites may be in one peak-containing window.

[e] p-value generated by a Fisher exact test.

[f] The transcription factor binding site associated with the sequence, as called by TESS (11). TESS was used to predict the transcription factors that might bind to the enriched sequences from MEME. Position weight matrices for the predicted transcription factors were obtained from the TRANSFAC database (12). 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.

[g] The ID from TESS, IMD (information matrix database) ID for each transcription factor.

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