Supplemental Material - Kininis *et al.* (2009)

"Post-recruitment Regulation of RNA Polymerase II Directs Rapid Signaling Responses at the Promoters of Estrogen Target Genes"

List of Supplemental Material

1) Supplemental Materials and Methods..2

2) Supplemental Figures 6

3) Supplemental Reference 13

1. Supplemental Materials and Methods

ChIP-qPCR

The sequences of the primers used for the ChIP-qPCR analyses are as follows:

CASC5 TSS forward	TCACCTAGGCGGTTAGATTTG
CASC5 TSS reverse	CAGACTTGTTTGGGGTCCA
CASC5 +1 kb forward	TTTCACTTCCGTCGAATCCT
CASC5 +1 kb reverse	CCCCTCTAGGTTTGGGAATC
CEBPB TSS forward	GGCCGCCCTTATAAATAACC
CEBPB TSS reverse	CGGCTCTGACTCGCTAAAGT
CEBPB +1 kbb forward	CAGCGACGAGTACAAGATCC
CEBPB +1 kbb reverse	AGCTGCTCCACCTTCTTCTG
CSH1 TSS forward	TAAAAAGGGCCCACAAGAGA
CSH1 TSS reverse	GATTTTAGGGGCGCTTACCT
CSH1 +1 kb forward	GGAGCTGGTCTCCAGCATAG
CSH1 +1 kb reverse	GTTGGAGGGTGTCGGAATAG
CYP1B1 TSS forward	CGTGCGGCCTCGATTG
CYP1B1 TSS reverse	AGGTGCCCACGTTTCCATT
CYP1B1 +1 kb forward	CTCCGTCCCCATCCCAAT
CYP1B1 +1 kb reverse	TGCCCTCACTGGAAGCTTTAAC
ETV3 TSS forward	TCTGCTGAGGATGGAAGTGA
ETV3 TSS reverse	CGGATCCAACCCAGACAC
ETV3 +1 kb forward	TTAAGAGTCCAGGGCAGCTT
ETV3 +1 kb reverse	GGAGCTTCCTCTTCCACCTT
GREB1 TSS forward	GCCAAATGGAAGAAGGACAG
GREB1 TSS reverse	ACCACCTACCTCCAGTCACC
GREB1 +1 kb forward	CCAGATCAACAGGCTCACC
GREB1 +1 kb reverse	AACCAACCCAATCACTGAGC
HNRPA1 TSS forward	AAAGAGAGGGCGAAGGTAGG
HNRPA1 TSS reverse	GGCAGGGTGAAGAGAGACTTT
HNRPA1 +1 kb forward	GCTCACGGACTGTGTGGTAA
HNRPA1 +1 kb reverse	TCAAAGCAGGACGTTCCTTT
MYC TSS forward	AGGCTTGGCGGGAAAAAG
MYC TSS reverse	CGCTGGAATTACTACAGCGAGTT
MYC downstream forward	GGACCCGCTTCTCTGAAAGG
MYC downstream reverse	GACTTCGGTGCTTACCTGGTTT
NTNG1 TSS forward	CGCACAGGTCTCGAGGTAG
NTNG1 TSS reverse	GCCAGCAAGTGGAAGGATAG
NTNG1 +1 kb forward	AAGGCGAGAAGTGTCAGAGG
NTNG1 +1 kb reverse	TGCCTCTCCCTCCAAAAATA
PGAM2 TSS forward	TCCTCTGTGGTCCCTGCT
PGAM2 TSS reverse	TCCCCTTTTCACTCAGCTCT
PGAM2 +1 kb forward	CATTGTCAAGCACCTGGAAG
PGAM2 +1 kb reverse	GCTTCTCAGCCCTCCTTTTT
PMAIP1 TSS forward	ACTGGACAAAAGCGTGGTCT
PMAIP1 TSS reverse	CGGAGATGCCAACTACACAC
PMAIP1 +1 kb forward	TGTGACCATGCCTAAGTTGG

PMAIP1 +1 kb reverse	AAAGCTTTTCCATCCCACA
RPS18 TSS forward	CGTCACTTCCGCTCTCTCTT
RPS18 TSS reverse	GACGTTTCCAGAACCCTGAC
RPS18 +1 kb forward	CCAGATCACCTTGACTGCTG
RPS18 +1 kb reverse	ATTCGGGTGAGCAACCATAC
SIAH2 TSS forward	CCTGAAGTTGCCTTTCTCGT
SIAH2 TSS reverse	TTGTCTTTTCTGGGTTGAGGA
SIAH2 +1 kb forward	TGGCATTTGCTGATCGTTAG
SIAH2 +1 kb reverse	CTCCTCAATCCCCACACAGT
TFF1 TSS forward	CCTGGATTAAGGTCAGGTTGGA
TFF1 TSS reverse	TCTTGGCTGAGGGATCTGAGA
TFF1 +1 kb forward	GAGTCAAAGCCACCTCTCTCATG
TFF1 +1 kb reverse	CCACTTGACAACACCAGGGAAT
TTC26 TSS forward	GGGTTACCGTGGAGACAGAA
TTC26 TSS reverse	ACCATCTTGACGTCGGTCTT
TTC26 +1 kb forward	AATCAGAATTGGACCATACAGC
TTC26 +1 kb reverse	TCTTCTTTCTTTTGTCAGTGTGC

Gene Expression qPCR Analyses

The sequences of the primers used for the real-time PCR analyses were:

ACTB forward	AGCTACGAGCTGCCTGAC
ACTB reverse	AAGGTAGTTTCGTGGATGC
CASC5 forward	TGTACCAAAGGTTTAAGTAATAGGACA
CASC5 reverse	GGGAAAATGGCTAATGTTCTTG
CEBPB forward	TTTCGAAGTTGATGCAATCG
CEBPB reverse	CAACAAGCCCGTAGGAACAT
CSH1 forward	TACGGGCTGCTCTACTGCTT
CSH1 reverse	ATGCTACTCGGGCACCTAGA
CYP1B1 forward	AACGTACCGGCCACTATC
CYP1B1 reverse	CACGACCTGATCCAATTC
ETV3 forward	CCCCACATTCTCTCTTGATG
ETV3 reverse	TGCTCAGATAAGGGGAACTACTG
GREB1 forward	GTGGTAGCCGAGTGGACAAT
GREB1 reverse	ATTTGTTTCCAGCCCTCCTT
HNRPA1 forward	TTCCAGCAGCAGCAGTAGC
HNRPA1 reverse	GTGCTTGGCTGAGTTCACAA
HOXC5 forward	GACCCCAGCAAGTGGTCCTA
HOXC5 reverse	GGGTCAGGAGGGCACAGA
KRT13 forward	ACGCCAAGATGATTGGTTTC
KRT13 reverse	CGACCAGAGGCATTAGAGGT
MDM2 forward	CATTGTCCATGGCAAAACAG
MDM2 reverse	CATACTGGGCAGGGCTTATT
MYC forward	GGATTTTTTTCGGGTAGTGGAA
MYC reverse	TCCTGTTGGTGAAGCTAACGTT
PMAIP1 forward	GTTTTTGCCGAAGATTACCG
PMAIP2 reverse	CAATGTGCTGAGTTGGCACT
RPS18 forward	GGATGGAAAATACAGCCAGGT
RPS18 reverse	AGAAGTGACGCAGCCCTCTA

SIAH2 forward	GTTCGATTCATGACGGTGTG
SIAH2 reverse	AAAACAAGGCAGTCGCTGTT
STC2 forward	TAACAGGCTCCCAGCTGAAT
STC2 reverse	CAGGTTGCCTTGAGGAAGAG
TBP forward	AGACCATTGCACTTCGTG
TBP reverse	AAATCAGTGCCGTGGTTC
TTC26 forward	GAAGCACAGGTAACACCCAAG
TTC26 reverse	CTAGGACGCTGGCGCTATT
TFF1 forward	TGCTTCTATCCTAATACCATCG
TFF1 reverse	AGATCCCTGCAGAAGTGTC

Nuclear Run-on Assays

The isolation of nuclei, nuclear run-on reactions, and hybridization to membrane-bound oligonucleotides were carried out largely as described previously (1), with some modifications.

Isolation of nuclei: Nuclei were isolated from MCF-7 cells grown in 15 cm diameter plates (~1 x 10^7 cells at ~80% confluence) as follows. The cells were washed on the plate three times with ice cold PBS. The cells were then collected from the plate and lysis was initiated in 10 ml of ice cold lysis buffer (10 mM Tris•HCl pH 7.5, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.5% NP-40, 10% glycerol, 2 units/ml SUPERase-In RNase inhibitor from Ambion). The cells were then transferred to a 15 ml conical tube and centrifuged for 10 min at 900 x *g* at 4°C in a clinical centrifuge. The resulting cell pellets were resuspended in 1 ml of lysis buffer by pipetting up and down to complete the cell lysis and release the nuclei. The volume was brought to 10 ml and the nuclei were washed, pelleted, resuspended in 1 ml of freezing buffer (50 mM Tris•Cl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), frozen in liq N_2, and stored at -80°C.

Nuclear run-on reactions: For the nuclear run-on reactions, ~3.5 x 10^7 MCF-7 cell nuclei (100 µl of the aforementioned preparations) were mixed with an equal volume of reaction buffer (10 mM Tris•HCl pH 8.0; 5 mM $MgCl_2$; 1 mM DTT; 300 mM KCl; 20 units of SUPERase-In; 1% sarkosyl; 500 µM each of ATP, GTP, and UTP; 10 µM CTP; and 1.0 µM α-^{32}P-CTP 3000 Ci/mmole). The reactions were incubated for 5 min at 30°C, followed by the addition of 23 µl of 10x DNAse I buffer (supplied by the manufacturer), and 10 µl RNase free DNase I (Promega). Proteins were digested by the addition of an equal volume of Buffer S (20 mM Tris•HCl pH 7.4, 2% SDS, 10mM EDTA, 200 µg/ml Proteinase K from Invitrogen), followed by incubation at 55°C for 1 hour. The RNA was extracted twice with acid phenol:chloroform (5:1) and once with chloroform, and then precipitated at a final concentration of 300 mM NaCl with 3 volumes of -20°C ethanol. The RNA pellet was washed in 75% ethanol and then dissolved in 20 µl of DEPC-treated water. The RNA was base hydrolyzed by the addition of 5 µl of 1 M NaOH with incubation on ice for 10 min. The reaction was neutralized by the addition of 25 µl of 1M Tris•HCl pH 6.8. The reaction was then run twice through a Bio-Gel P-30 RNAse-free spin column (BioRad) according to the manufacturer's instructions. Before proceeding with the hybridization to the membrane, DNA was further removed by another digestion with RNase-free DNase I for 10 min at 37°C and the reaction was stopped by the addition of 10 mM EDTA.

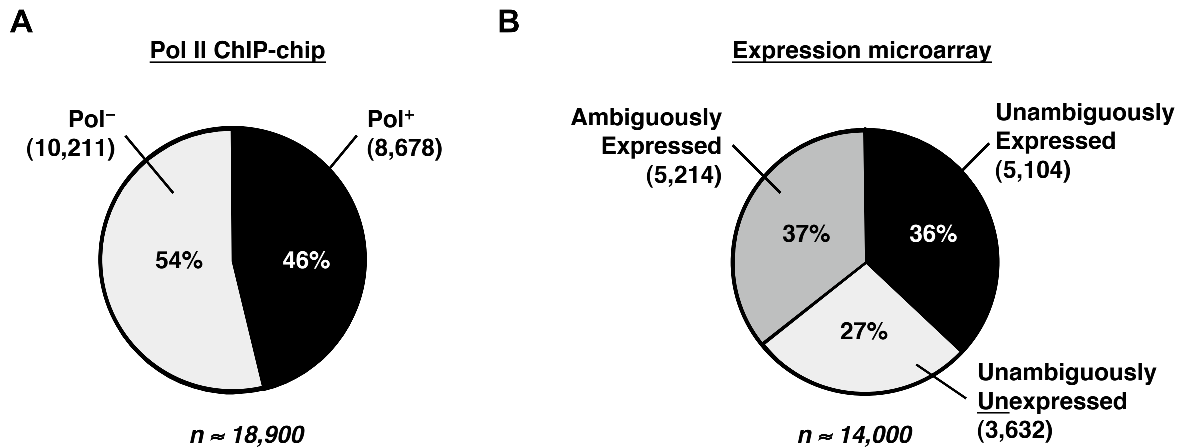
Probe immobilization and hybridization: Single-stranded oligonucleotides (500 picomoles) were dT-tailed with 10 units of terminal transferase in 1x NEB buffer 4, 0.25 mM $CoCl_2$, and 500 µM TTP in a 25 µl reaction. These reaction conditions add a homopolymer tail of ~10 to 20 bases. The reaction was stopped and the oligonucleotides were denatured by the addition of 75 µl of 0.4 M KOH, 10 mM EDTA with incubation at 65°C for 5 min. The

oligonucleotides were spotted onto positively charged nylon membrane (GeneScreen Plus, Pall) using a slot blotting apparatus (Minifold I, Whatman), fixed to the membrane by UV irradiation at 120 mJ/cm^2, and dried at 80°C for 1 hour. Prior to hybridization, the membranes were rehydrated in water for 5 min, followed by a 5 min incubation with 2x SSPE. Prehybridization was carried out overnight in 500 μl/cm^2 of Church buffer at 68°C in hybridization bottles. Nuclear run-on RNA was denatured by heating to 95°C for 5 min, quenched on ice for 2 min, and then added to the membranes in 100 μl/cm^2 of Church buffer. Hybridization was carried out for 24 hours. The membranes were washed once at 58°C in 2x SSPE, 0.5% SDS for 20 minutes, and once 58°C in 0.5x SSPE, 0.5% SDS for 20 minutes, followed by a brief rinse in 2x SSPE. The membranes were exposed to phosphorimaging screens (Molecular Dynamics) and the digital images were visualized on a Typhoon 9400 variable mode imager.

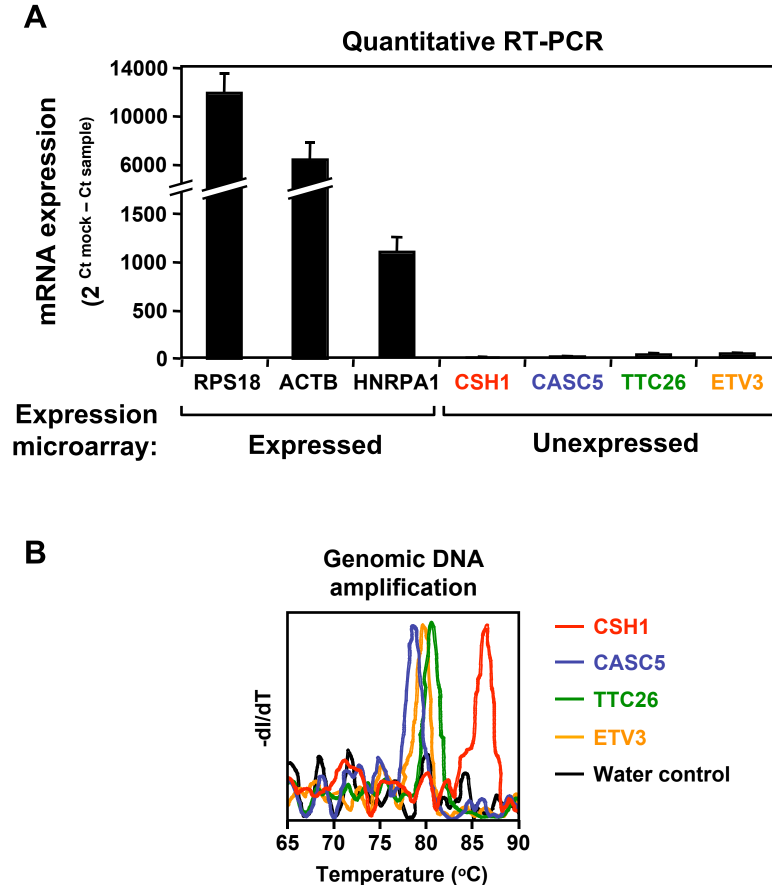
The oligonucleotides used were as follows:

MYC-P1	GCCGTCCAGACCCTCGCATTATAAAGGGCCGGTGGGCGGAGATTAGCGAGAGAGG
MYC-P2	TCTAAGCAGCTGCAAGGAGAGCCTTTCAGAGAAGCGGGTCCTGGCAGCGGCGGGGAAGTG
MYC-P3	ACCAAGGGTAGCAGCTGTTCTGGAACCGCCCAGAGCCCCGCTCCTCGCAGTTCCTC
MYC-P4	GGCTGCAAACATGGGCAGTCTAAGGGGAAGGGATGGGAGGAAACGCTAAAGCCCA
SIAH2-P1	AAGAGGGCGAAGCGGCCACCCGCGTTCTGTGTTCGGAGTCAGGACGAGAAGCATTG
SIAH2-P2	CCTGCTCGGGCTGCGGGCCGGGCCTGCGCCAGTGAGCATGCCTGGCTCGCTGCAGC
SIAH2-P3	GTGCGCAGAGCCCTGGCCCACCCGCTTCTGGAACAGCCGCTGACGCAAGCCCCGG
SIAH2-P4	CAACCAAAGGTGAGGGACACACACCCCAGCTTTATACAACTCACACACATGCACA
TFF1-P1	CCCGAGTCAGGGATGAGAGGCCGCCCGAGCCCCGGATTTTATAGGGCAGGCTCTGTTTGC
TFF1-P2	CCAGCATGGACACCAGGACCAGGGCGCAGATCACCTTGTTCTCCATGGTGGCCATTGCC
TFF1-P3	CCTAACACAGGAAAACAGCCCCGACTGAAGGCAGCCCCCTCCTCGTCGCACTTCT
TFF1-P4	GGACATGAGAGGGAGACGTGGTCCTCACATCCTGATGTGCAAACATTACGCTCAGGGAAA
KRT13-P1	GGCCGAGGACTGTGGCTCTTCCCCAGAGTGGACACCTTTTATACACCTCCATAGGGGCTG
KRT13-P2	ACAAACCGAGTTGAACAGGTAGAGACACCACGGCCTCCTCCCAGCTGGCAAGAGC
KRT13-P3	TCTGCTCCAACAGGCTGGTATCACAGCACAACAGACAGGGCTCAACAAAGGGCACC
KRT13-P4	TTCTGGAGAGTGTTTCCAGGTCCCACAGTGGAGGACTGTGTCCAGTTGCAGGGGAGGGCT

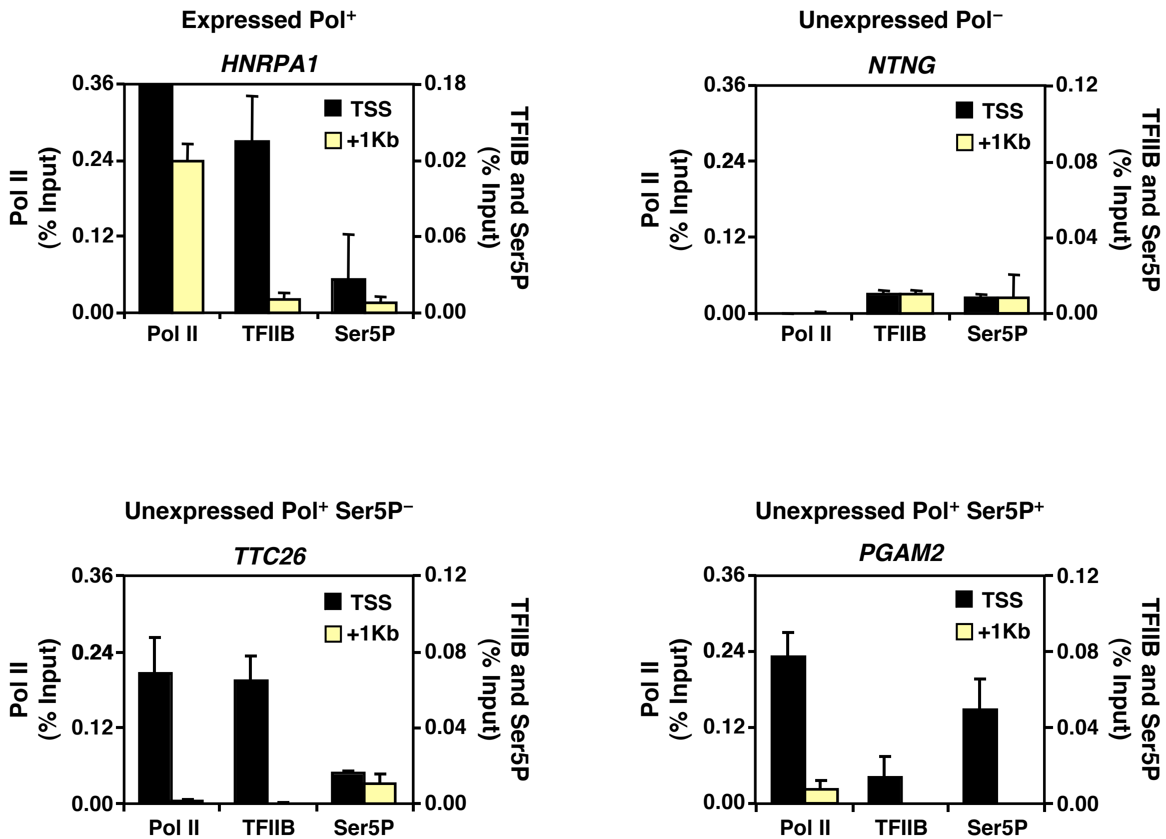
2. Supplemental Figures



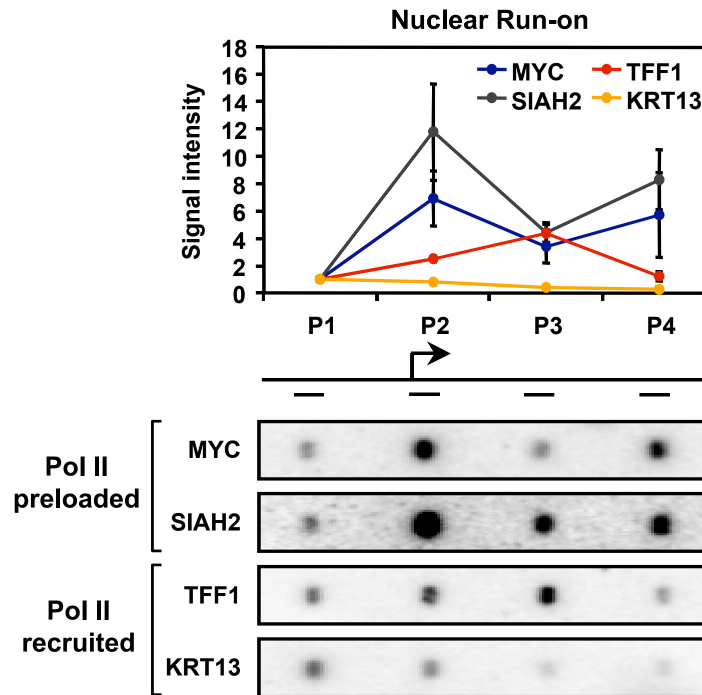
Supplemental Figure 1. Genome-wide analysis of Pol II binding and gene expression.
(A) Pol II ChIP-chip revealed that about half of the promoters analyzed (8,678 out of ~18,900) were bound by Pol II in MCF-7 cells grown under basal estrogen-free growth conditions.
(B) Gene expression microarray analysis defined 5,104 unambiguously expressed and 3,632 unambiguously unexpressed genes (see Methods for details on the criteria used). About 37% of the genes analyzed (5,214 out of ~14,000) had ambiguous expression status and were eliminated from further analyses presented in Fig. 1.



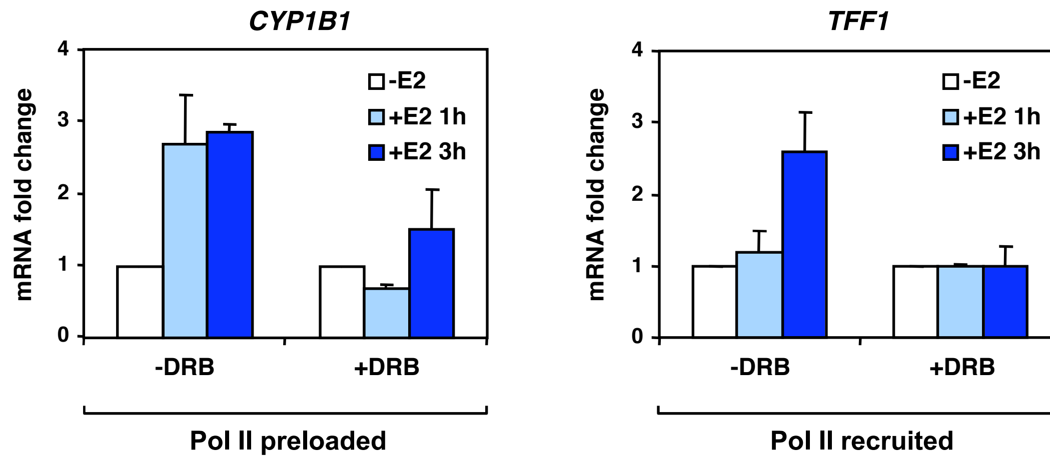
Supplemental Figure 2. Gene expression microarray results are consistent with quantitative RT-PCR data.
(A) Gene-specific analysis of mRNA expression for representative "expressed" and "unexpressed" genes from expression microarray analyses. Gene expression was quantified as fold mRNA expression of each gene over mock control ($2^{Ctmock-Ctsample}$). Each bar represents the mean plus SEM, n = 4.
(B) Melting curves of genomic DNA amplification by the same primers as used in (A). All primers efficiently amplify the genomic DNA indicating that a lack of a signal in (A) is not due to failed primer sets.



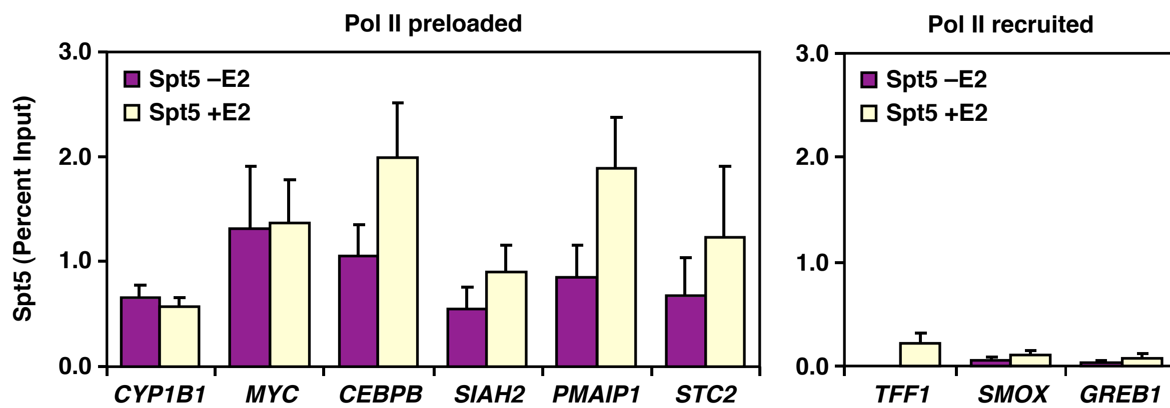
Supplemental Figure 3. Analysis of Pol II, TFIIB, and Ser5-phosphorylated Pol II CTD binding at expressed and unexpressed genes.
Gene-specific ChIP analyses of Pol II, TFIIB, and Ser5P binding at TSSs (black) and downstream regions (+1 kb) (yellow) for a set of representative genes. Each bar represents the mean plus the SEM from at least three independent experiments.



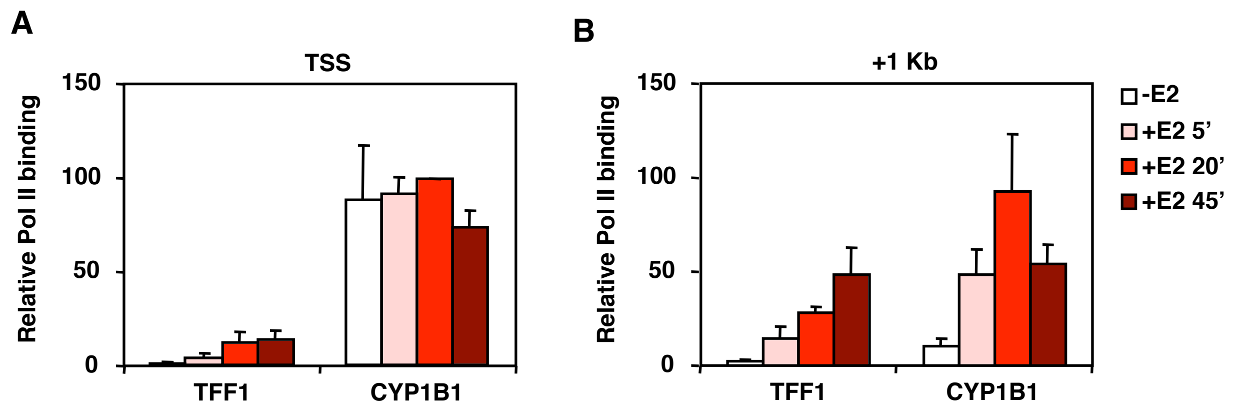
Supplemental Figure 4. Nuclear Run-on analysis of representative E2-stimulated Pol II preloaded and Pol II recruited genes prior to E2 treatment.
Gene-specific nuclear run-on analysis of Pol II preloaded (MYC and SIAH2) and Pol II recruited (TFF1 and KRT13) genes in the absence of E2. Quantification of results shows that the Pol II preloaded genes contain significantly more transcriptionally-poised Pol II at the TSS, compared to Pol II recruited genes. Each data point represents the mean ± SEM from at least three independent experiments.



Supplemental Figure 5. Inhibition of Cdk9 kinase activity by DRB blocks the E2-dependent induction of representative Pol II preloaded and Pol II recruited genes.
Gene-specific analysis of mRNA expression for representative Pol II preloaded (*CYP1B1*) and Pol II recruited (*TFF1*) genes in MCF-7 cells before or after E2 treatment for 1, 3, and 6 hours. Fold change is shown relative to the "–E2" condition for each gene. Each bar represents the mean plus SEM, n ≥ 3.



Supplemental Figure 6. DSIF binds at the promoters of E2-stimulated genes with preloaded Pol II, but not with recruited Pol II, prior to E2 treatment.
Gene-specific ChIP analysis of SPT5 binding at promoters of representative E2-stimulated genes ± E2 (45 min.). Each bar represents the mean plus the SEM from at least three independent experiments.



Supplemental Figure 7. Kinetics of Pol II binding for the *TFF1* ("Pol II recruited") and *CYP1B1* ("Pol II preloaded") genes.
ChIP analysis of Pol II binding at the TSS **(A)** and +1 Kb **(B)** regions of TFF1 (Pol II recruited) and CYP1B1 (Pol II preloaded) genes before and after 5, 20 and 45 min. treatments with E2. The data from two independent experiments were scaled so that the Pol II signal at the CYP1B1 TSS after 20 min. of E2 treatment was set to 100 for each experiment. Each bar represents the mean + SEM.

3. Supplemental Reference

1. **Eick, D., F. Kohlhuber, D. A. Wolf, and L. J. Strobl.** 1994. Activation of pausing RNA polymerases by nuclear run-on experiments. Anal Biochem **218:**347-51.