Supplemental Materials and Methods:

Plasmids and reagents: DN MAML1 was a gift from Drs. Jon Aster (Brigham and Women's Hospital and Harvard Medical School, Boston, MA) and Warren Pear (University of Pennsylvania, Philadelphia, PA). Notch-1 tagged at the C-terminus with Renilla luciferase was a gift of Dr. Raphael Kopan (Washington University St. Louis, MO). ERα-Renilla luciferase, Cdc2-Renilla Luciferase and GAPDH-Renilla luciferase constructs were generated as following: Plasmid PCS2+mN1FL6MT (Vooijs *et al.*, 2004) was cut with EcoRI and Mlu I. The plasmid backbone was gel-purified and ligated (in frame, upstream from the Renilla luciferase gene) to PCR products coding for ERα, human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Cdc2 (from the first ATG to the last codon preceding the termination codon). Such PCR fragments were obtained using a plasmid containing full length ERα or a lung cDNA library as templates and the following primers: ERα, 5- GCGCGCGAATTCATGACCATGACCCTCCAC-3 and CGCGCGACGCGTGACTGTGGCAGGGAAAC-3;GAPDH, 5–GGGGAATTC ATGGGGAAGGTGAAGGTC-3 and 5-GGGACGCGTCTCCTTGGAGGCCATGTG-3; cdc2, 5-GGGGAATTCATGGAAGATTATACCAAA-3 and 5-GGGACGCGTCATCTT CTTAATCTGATT-3 (underlined sequences represent initiation codons). PCR products were purified from 1% agarose gels, digested with EcoRI and Mlu I and ligated into the plasmid backbone. Recombinant plasmids pERα-RL, pGAPDH-RL and pCDC2-RL were verified by DNA sequencing, and the expression of recombinant proteins was verified by Western blotting with Renilla antibodies. Renilla activity was assayed in transient transfection experiments.

MRK-003 GSI was provided by Dr Peter Strack (Merck Inc.). Hs_ESR1_8_HP Validated siRNA (SI02781401) and scrambled control was from Qiagen. Notch-1 (N-1) siRNA (sc-36095), RBP-Jκ siRNA(h) (sc-38214) and IKKα siRNA (sc-29365) were from Santa Cruz Biotechnology. 17-β estradiol and 4-hydroxytamoxifen (Sigma Aldrich, St. Louis, MO), fulvestrant (ICI182,780) (Tocris Bioscience, Elllisville, MO) were dissolved in ethanol and stored in aliquots at -80°C.

Quantitative ChIP assay: Briefly, MCF7 cells were crosslinked and lysed in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1). Lysates were sonicated and diluted with IP dilution buffer (10mM Tris-HCl, pH 8.1, 150mM NaCl, 1mM EDTA, 0.01% SDS, and 1%TritonX-100). Precleared lysates were incubated with the indicated antibodies overnight. Immune complexes were collected by incubation with protein G plus/protein A Agarose suspension (Calbiochem) for 2 h with gentle rocking. After phenol-chloroform extraction and ethanol precipitation, DNA was resuspended in 30 μl TE buffer. Aliquots (2 μl) were analyzed by quantitative real-time PCR. Products were quantified relative to a standard curve generated from a titration of input chromatin.

Quantitative Renilla Immunoprecipitation: Briefly, cells were transfected with various luciferase constructs. 48 h after transfection, cells were washed and lysed in co-IP buffer (0.2M KCl, 25mM HEPES, pH 7.4, 1% Nonidet P-40, and 0.2mM EGTA) supplemented with a cocktail of protease inhibitors (Roche). Cells lysates in 500 μl co-IP buffer were immunoprecipitated with 2 μg of antibodies or correspondent control IgG for 2 h. Immunocomplexes were recovered after 1 h incubation with protein G plus/protein A

Agarose suspension (Calbiochem). Beads were washed and Renilla luciferase assay was performed as described by the manufacturer (Promega).

Nuclear extraction and Co-immunoprecipitation: MCF7 cells were lysed in Buffer A (110 mM $KC_2H_3O_2$, 15 mM $NaC_2H_3O_2$, 2 mM $MgC_2H_3O_2$, 0.5 mM EGTA and 20 mM HEPES at pH 7.3, 2 mM in dithiothreitol and 50 μg ml^{-1} digitonin). Nuclei were pelleted by centrifugation at 1,500*g* for 10 min and were resuspended in hypotonic buffer (Buffer B, 1 mM HEPES at pH 7.5 and 0.5 mM EDTA supplemented with 0.5% NP-40). Nuclear suspensions were then layered on top of a 10 ml sucrose cushion (100 mM Sucrose and 0.5 mM Tris-HCl at pH 8.5) and centrifuged at 3,500*g* for 15 min at 4 °C. Chromatin pellet were suspended in 0.25 mM EDTA at pH 8.0 and gently sonicated. After sonication, chromatin suspensions were centrifuged twice at high speed for 10 min at 4 °C, and supernatants retained. Precleared lysates were incubated with indicated antibodies overnight in a total volume of 1 ml of NETN buffer (250 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl at pH 8.0 and 0.5% NP-40). Immune complexes were collected by incubation with protein G/A beads for 2 h with gentle rocking.

Bioinformatics studies: We have designed and implemented a computer program called TransPatt (Cheung, unpublished) that identifies transcriptional regulatory site locations, their combinatorial and spatial patterns as well as associated annotated genes from whole genome sequence data. This system includes extraction, transformation, loading, multiple data querying, data mining and statistical analysis operations. The calculation of statistical significance of a pair of sites with a spacing distance is based on permutation

testing principle, i.e. assessing the chance of observing a particular pair of sites with a specific spacing distance against a random genomic background. We simulated random genomic background by shuffling genome sequence data (using the Build 36 UCSC mm8 Mouse Genome data for our mouse studies and the Build 36.1 UCSC hg18 Human Genome data for our human studies) with same nucleotide composition within each chromosome. In our current version of TransPatt, it stores and integrates local instances of external data sources such as GenBank (Benson *et al.*, 2007), RefSeq (Benson *et al.*, 2007), Entrez Gene, Human Genome Browser (HGB) at UCSC (Kent *et al.*, 2002). We applied TransPatt to help reveal transcriptional regulation patterns in terms of specific genomic sequence binding motif combinations between CBF1 and ERE sites, specific spacing distances between the two binding motifs and identity of associated annotated genes. Supplemental Table 1 shows the sequences used for responsive elements.

Xenograft experiments: T47DA18 cells (grown in RPMI containing 10% FCS , NEAA and 6ng/ml insulin) were injected s.c. ($1X10^7$ cells in 200 µl of Matrigel on each side) into the axillary mammary fat pad of 40 ovariectomized 4-6 week- old BALB/c athymic mice. E2 was be admistered via silastic capsules (1.0 cm long containing 25 mg of estradiol) implanted s.c. between the scapulas. When the tumor became palpable (200 mm^2) mice were divided into 4 treatment groups consisting of 10 mice/group: vehicle, tamoxifen (25 mg/kg in 1% carboxymethylcellulose by gavage), GSI (100mg/kg of MRK003 0.5% methylcellulose by gavage), TAM plus GSI (0.5 mg of tamoxifen plus 100 mg/kg of MRK003). Tamoxifen was administered 5 days on and 2 days off whereas GSI was administered 3 days on and 4 days off. After 2 cycles of treatment 2 mice from

each group were sacrificed and tumors were excised and snap frozen in liquid nitrogen. RNA was extracted using RNAeasy kit (Qiagen,Valencia CA) and DNase treatment was performed (DNase I kit, Fermentas). cDNA was syntetized using 250 ng of total RNA and 15 pmole of specific primers in 20 μl reaction using the Superscript III reverse transcriptase kit (Invitrogen). 2.5 μl of cDNA reaction were analysed by real time- PCR as described above. The levels of pS2 mRNA relative to RPL13A mRNA were measured. See Supplemental Figure 14 for primers used.

Crystal violet cell number assay: 2000 cells/well were seeded in a 96 well plate. After 24 hours, medium was removed and fresh media with indicated treatments were added to the plate. Plates were incubated for 72 hours. On the day of the assay, the medium in each well was discarded and 200 μl of a 0.2% crystal violet solution in 2% ethanol was added to each well and incubated at room temperature for 10 minutes. The plate was then washed with deionized water and dried. 200 μl of solvent (0.5% SDS in 50% ethanol) was added to each well and the plate was incubated for one hour to dissolve crystals. Optical density was then measured at 562 nm using a microplate reader (Molecular Devices, CA).

CFSE staining and flow cytometry: MCF-7 cells were labeled with carboxyfluorescein, diacerate succinimidyl ester (CFSE; Molecular Probes, Inc., Eugene, OR). Briefly, cells were resuspended in PBS/0.1% BSA at a concentration of 10^6 cells/ml. CFSE was added to the cells at a final concentration of 10 μM. The cell suspension was incubated with the

dye at 37°C for 10 min. Labeled cells were washed 3 time with cold PBS, then mixed and plated with indicated LTK fibroblasts. MCF-7 cells and the LTK fibroblasts were co-cultured for three days under different medium conditions. After that the cells were harvested and analyzed immediately by a flow cytometer with 488nm excitation (FACS Canto, BD Biosciences).

Supplemental Figure Legends

Figure S1: E_2 induces expression of VEGFα, CD44, cyclin D1, c-MYC and pS2. MCF-7 cells were grown in RPMI containing 10%FBS, or in phenol red-free RPMI containing 10%DCC-FBS (CS) for a total of 3 days. Cells were treated with 5nM E_2 or ethanol control for 4 h, 1μM fulvestrant for 24 h, or the combination prior to harvest. The mRNA levels of target genes were measured by real-time RT-PCR.

Figure S2: The induction of pS2 transcription by Jagged-1 is mediated by Notch-1 and not Notch-2 or Notch-4. A. MCF-7 cells were grown in charcoal-stripped medium for 3 days, and transfected with Notch-1, Notch-2, or Notch-4 siRNA (or scrambled control). Western blots show efficient down-regulation of specific Notch paralogs. B. pS2 Real time RT-PCR from cells transfected with different siRNAs in charcoal stripped medium (as described in A) and co-cultured with LTK cells for 12 hours. Data expressed as relative copy number after normalization to internal control RPL13aR mRNA level. *P ≤0.001.

Figure S3: E_2 or fulvestrant had no effect on Notch-1IC-induced HEY1 expression. HEY-1 real-time RT-PCR. MCF-7 cells were grown in charcoal stripped medium for 3 days prior to harvest. A. 12 h after serum starvation, MCF-7 cells were transfected with Notch-1IC (NIC) or pcDNA vector control. Cells were treated with 5nM E_2 (4 h), 1μM fulvestrant (24 h) or the combination before harvest. Data are expressed as relative fold induction by NIC over pcDNA after normalization to the internal control 18S rRNA. * $P \leq 0.001$. B. MCF-7 cells were treated with E_2 alone or in combination with fulvestrant, and co-cultured with Jagged-1 expressing LTK fibroblasts for 12 h. Data are expressed as relative fold induction by LTK-Jagged-1 over LTK-Parental after normalization to the internal control RPL13a. * $P \leq 0.001$.

Figure S4. Tamoxifen did not affect pS2 or HEY1 induction by Notch-1IC. In all experiments, MCF-7 cells were grown in charcoal stripped medium for a total of 3 days. Cells were transfected with NIC or empty vector pcDNA 48 h prior to harvest. Cells were treated with either 1μM 4-hydroxytamoxifen, 1nM E_2, or the combination, or 1μM fulvestrant 24 h prior to harvest. pS2 (A) or HEY1 (B) mRNA levels were measured by real-time RT-PCR. Data were expressed as relative fold induction by NIC over pcDNA after normalization to internal control 18S rRNA. *$P \leq 0.001$.

Figure S5. The Notch-1-ERα interaction is DNA-dependent. IP-Western with cytoplasmic or nuclear extracts treated with 50μg/mL ethidium bromide for 30 min on ice before immunoprecipitation.

Figure S6. Activation of Notch increases the nuclear localization of ERα. MCF-7 cells were transfected with ER-RL construct and grown in charcoal-stripped medium for 3 days before co-cultured with LTK-Jagged1 (or LTK-Parental) cells for 45 minutes. Nuclear extraction was performed. A. Renilla luciferase activity of cytoplasmic lysates or nuclear extracts was measured. B. Western blot for β-actin, HDAC1 and ERα with MCF-7 or LTK whole cell lysates, and the cytoplasmic (cyto) and nuclear (nuc) fractions after co-culture. Numbers indicate densitometric scanning of ERα bands divided by loading controls. Note that the ratio between nuclear and cytoplasmic ERα increased from 1.39 (2.5/1.8) to 2.7 (3/1.1), consistent with the ER-RL data in panel A.

Figure S7. Human and Mouse CBF1/ERE Motif Combination Distributions. Peaks indicate spacing distances at which motifs occur with significantly non-random frequencies (P-value < 1.0E-303 in all cases) with respect to the specific spacing distance between motifs. The distances were defined as the number of nucleotides between the last nucleotide of the first motif and the first nucleotide of the next motif in a motif pair.

Figure S8. Notch-1 siRNA downregulates a subset of E_2-dependent genes. MCF-7 cells were grown in standard RPMI medium containing 10%FBS, or in phenol red-free RPMI containing 10% DCC-FBS (CS) for a total of 3 days. Cells were transfected with Notch-1 siRNA or scrambled (SCB) control 48 h prior to harvest. A. Western blot showing downregulation of Notch-1 expression by siRNA. Actin was used as loading control. B-F. mRNA levels of target genes were measured by real-time RT-PCR. Data are

expressed as relative copy number after normalization to internal control 18S rRNA. *P ≤0.001.

Figure S9. Notch-1 or IKKα siRNA prevents E_2 from activating cyclin D1 and pS2 transcription. MCF-7 cells were grown in charcoal-stripped medium for a total of 3 days. Cells were transfected with Notch-1 siRNA, IKKα siRNA or scrambled control. 48 h later, cells were treated with 5nM E_2 or ethanol control for 4 h. mRNA levels were measured by real time RT-PCR. Data are expressed as relative copy number after normalization to internal control 18S rRNA.

Figure S10. Notch-1 Knockdown inhibits MCF-7 cells growth. MCF-7 cells were grown in charcoal-stripped medium for total of 3 days. Cells were treated with 1μM Fulvestrant (or Et control), or transfected with Notch-1 siRNA (or scrambled control) on day 0. Cell growth were measured by crystal violet assay for 3 consecutive days. Error bars are standard deviations (n =3).

Figure S11. Notch-1 regulates ERα-targets in T47D:A18 cells in the absence of E2. In all experiments T47D:A18 cells were grown in phenol red-free RPMI containing 10% DCC-FBS for 3 days prior to harvest. (A) A18 cells were co-cultured with mouse fibroblasts expressing Jagged-1 (LTK-JAG1), or vector-transfected controls (LTK-PAR) for 12 h prior to harvest. (B) A18 cells were transfected with Notch-1 siRNA (or the scrambled control). The mRNA levels of VEGFα, CD44, c-MYC, CCND1, pS2, HEY1 and β-Tubulin were measured by real time RT-PCR with validated human-specific

primers. Values are expressed as relative fold induction after internal normalization for RPL13a mRNA.

Figure S12. GSI decreases pS2 levels in T47D:A18 xenograft tumors. T47D:A18 established xenografts receiving menopausal estrogen levels were treated with GSI (MRK003 100 mg/kg orally), tamoxifen (0.5 mg tamoxifen in carboxymethylcellulose suspension) or the combination thereof. Tumors were harvested and mRNA levels were measured by real-timer RT-PCR. Either drug alone inhibited pS2 expression. The combination GSI/tamoxifen caused further downregulation of pS2 mRNA level, and was significantly different not only from controls but also from each individual treatment. * $P \leq 0.001$.

Figure S13. Primers used for real-time RT-PCR or for ChIP-real time PCR.

Supplemental Table 1. Sequences of core NCRE and ERE used for our analysis. Note that EREs were used as tandem sites or half-sites. NCRE, Notch-CSL response element.