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Differential regulation of Native Estrogen Receptor Regulatory Elements by Estradiol, Tamoxifen, and Raloxifene

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Abbreviations:; E_2, estradiol; ER, estrogen receptor; ERE, estrogen response element; SERM, selective estrogen receptor modulator; SRC, steroid receptor coactivator; GRIP1, glucocorticoid interacting protein 1; ChIP, chromatin immunoprecipitation, NKG2E, killer cell lectin-like receptor; RT-PCR, real-time polymerase chain reaction, cat eye syndrome chromosome region candidate 6 (CECR6), naked cuticule homolog (NKD), spermatogenesis associated 13 (SPATA13), lethal giant larvae homolog 2, (LLGL2), H19, imprinted maternally expressed untranslated mRNA (H19), killer cell lectin-like receptor subfamily C (NKG2)

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ABSTRACT

Estrogen receptors (ER) regulate gene transcription by interacting with regulatory elements. Most information regarding how ER activates genes has come from studies using a small set of target genes or simple consensus sequences such as ERE, AP-1 and Sp1 elements. However, these elements cannot explain the differences in gene regulation patterns and clinical effects observed with E_2 and SERMs. To obtain a greater understanding of how E_2 and SERMs differentially regulate genes it is necessary to investigate their action on a more comprehensive set of native regulatory elements derived from ER target genes. Here we used chromatin immunoprecipitation-cloning and sequencing (ChIP-CS) to isolate 173 regulatory elements associated with ERα. Most elements were found in the introns (38%) and regions greater than 10 kB upstream of the transcription intiation site (38%). 24% of the elements were found in the proximal promoter region (<10 kB). Only 11% of the elements contained a classical ERE. 23% of the elements did not have any known response elements, including one derived from the *naked cuticle homolog* gene, which was associated with the recruitment of p160 coactivators. Transfection studies found that 80% of the 173 elements were regulated by E_2, raloxifene or tamoxifen with ERα or ERβ. Tamoxifen was more effective than raloxifene at activating the elements with ERα, whereas raloxifene was superior with ERβ. Our findings demonstrate that E_2, tamoxifen and raloxifene differentially regulate native ER regulatory elements isolated by ChIP with ERα and ERβ.

INTRODUCTION

Drugs that interact with estrogen receptors are commonly used to treat numerous reproductive conditions. Estrogens are mainly used for contraception and to treat a variety of menopausal symptoms, such as hot flashes and urogenital atrophy. However, the use of estrogens for menopausal symptoms has been severely curtailed after the results of the Women's Health Initiative trial found that the risks of hormone therapy exceed the benefits (1). The selective estrogen receptor modulators (SERMs) differ from estrogens in that they exhibit both agonistic and antagonistic properties. Tamoxifen is the prototypic SERM that acts as an antagonist in the breast and an agonist in bone and uterus (2). Therefore, tamoxifen is used to treat and prevent ER-positive breast tumors (3). Raloxifene behaves similarly to tamoxifen in the breast and bone (4, 5), but does not elicit an agonistic action in the uterus (6). Raloxifene is hence approved for the prevention and treatment of osteoporosis. While the SERMs have some exciting properties, a major limitation is that they do not prevent hot flashes. Furthermore, the results of clinical trials with SERMs are showing that both drugs can produce serious adverse effects (7). These observations clearly indicate that it essential to develop a new generation of safer drugs that target ERs. To achieve this goal, a greater understanding of how estrogens and SERMs regulate genes that mediate their beneficial and adverse effects is needed.

The execution of the biological effects of estrogens and SERMs is mediated by two ERs: ERα and ERβ. Once the ligand binds to these receptors it induces a conformational change (8) that allows the receptor to interact with a regulatory element in target genes. While the estrogen response element (ERE) is considered to be the major regulatory element in genes regulated by ERs (9), alternative elements, such as AP-1 (10) and Sp1 (11) are required for E_2 and SERMs to regulate the full spectrum of genes. When an estrogen bound ER associates with a regulatory element it recruits distinct classes of proteins, including p160s coactivators, mediator complex proteins and CBP/p300 in a sequential and cyclical manner to activate gene transcription (12-14). On the contrary, SERMs act as antagonists by recruiting corepressor proteins, such as N-CoR, that block the expression of the target gene (15). The mechanism of the agonist

action that leads to transcriptional activation by SERMs is poorly understood, but is thought to be mediated via the recruitment of coactivators to the AF-1 domain of ERs (16, 17).

While the ERE and alternative elements are important mediators of ER regulation of genes, the pharmacology of E_2 and SERMs on target genes cannot be understood solely by the known regulatory elements (18-20). For example, it is not yet known how E_2 distinctly regulates genes with ERα and ERβ (18, 21, 22) and how some genes regulated by tamoxifen are different from those regulated by raloxifene (18, 23). These observations indicate that in addition to the known regulatory elements, other types of elements exist in ER target genes. Identifying new regulatory elements in ER target genes is a critical step in the elucidation of how estrogens and SERMs induce the therapeutic and adverse effects observed in clinical trials. Previously, the standard way to identify ER regulatory elements was to clone the promoter region and map the element with a series of deletion and point mutants upstream of a reporter gene. Recently, ER binding sites were identified by using a combination of chromatin immunoprecipitation (ChIP) and tiling arrays that contained chromosomes 21 and 22 (24) or the whole genome (25). ChIP also has been combined with sequencing to identify ER elements in MCF-7 cells (26). Here, we used a chromatin immunoprecipitation-cloning and sequencing (ChIP-CS) strategy to isolate genome-wide ER response elements from target genes in U2OS bone cells. Our goal was to assess the functional properties of a large set of ER binding elements in response to different ligands. We isolated and tested 173 ER regulatory elements for regulation by E_2, raloxifene and tamoxifen with both ERα and ERβ.

RESULTS

ChIP-Cloning and Sequencing Identifies ERα Binding Elements

U2OS cells that express a stably transfected FLAG-ERα were treated for 2 h with E_2 and then were cross-linked with formaldehyde. After shearing the chromatin, the ERα-bound DNA fragments were isolated using an antibody against the FLAG epitope. DNA fragments were then further purified with an antibody against ERα. Isolated DNA fragments were cloned into plasmids and 192 were randomly selected and sequenced. Bioinformatics was used to match the sequences in the ChIP-CS library to genes by performing a BLAST search against the human genome. Of the clones sequenced, 173 contained inserts, which ranged in size from 200 to 500 base pairs. The chromosomal identifier and sequence of the 173 elements is shown in Supplementary Table 1. The location and name of each element relative to the nearest gene is shown in Tables 1 and 2.

Many Elements do not Contain an ERE or other Known ER Regulatory Elements.

We found that 38% of the regulatory elements were located in introns (Table 1). 24% of the regulatory elements were found less than 10 kB upstream region of the transcription start site (TSS), whereas the remaining 38% of the elements were located beyond 10 kB of the TSS (Table 2). A histogram shows the distance of the elements from the TSS (Figure 1). To explore if our ChIP-CS library contains authentic regulatory elements, we searched ER responsive gene databases as well as our microarray data (18) for known ER target genes. We found that 10% of the genes in our ChIP-CS library were reported to be activated by E_2 or SERMs (Table 1 and 2).

Many Elements do not Contain an ERE or other Known ER Regulatory Elements.

We searched multiple databases, including TRANSFAC and Dragon ERE finder to

identify potential regulatory elements in the ChIP-CS library. Only 11% of the inserts contained classical EREs (Table 1 and 2). AP-1, Sp1, FOXA1 or NFκB sites were present in 41%, 18%, 24% and 33% of the inserts, respectively (Table 1 and 2). Surprisingly, 23% of the inserts did not have an ERE or one of the other alternative elements. These data support our hypothesis that additional types of regulatory elements exist in ER target genes.

E_2 recruits ERα and p160 coactivators to elements derived from the ChIP library in U2OS-ERα cells

We selected elements from six genes to determine if ERα binds to those elements in the native genes in U2OS-ERα cells. In these cells, the native ERα is driven by a tetracycline-inducible CMV promoter that allows for titration of the ERs to approximate physiological levels (18). ChIP shows that E_2 recruited ERα to the *cat eye syndrome chromosome region candidate 6* (*CECR6), spermatogenesis associated 13 (SPATA13), naked cuticule homolog* (*NKD*), *lethal giant larvae homolog 2, (LLGL2), H19, imprinted maternally expressed untranslated mRNA (H19),* and *killer cell lectin-like receptor subfamily C* (*NKG2) genes* (Fig. 2). E_2 activates genes by recruiting p160 coactivators (14, 27), SRC1, SRC2 (TIF2, GRIP1, and NCoA-2) and SRC3 (pCIP, RAC3, AIB1, and ACTR) to the AF-2 surface of ER (28-30). Using ChIP, we examined whether E_2 recruits p160 coactivators to the six genes. E_2 produced a time-dependent recruitment of p160 coactivators to the six genes with maximal recruitment occurring at 1-2 hours (Fig. 3). However, distinct coactivators were recruited to the genes. All three coactivators were recruited to the *CECR6* (Fig. 3A) and *H19* (Fig. 3E) genes, whereas SRC-2 and SRC-3 were recruited to *NKD* (Fig. 3C) and *NKG2* (Fig. 3F) genes. SRC-1 and SRC-2 were recruited to *LLGL2* (Fig. 3D), whereas only SRC-3 was recruited to the *SPATA13* (Fig. 3B) gene.

E_2 activates and recruits ERβ and p160 coactivators to the *CECR6* and *NKD* Genes in U2OS-ERβ cells

The regulation of *CECR6* and *NKD* genes by E_2 was also examined in the U2OS-ERβ cells. U2OS-ERβ cells were treated for increasing time with E_2 and the levels of *CECR6* and *NKD* mRNA were measured by real-time PCR. E_2 produced a maximal activation of the *CECR6* gene in 6 h in both cells types (Fig. 4A and B). The activation of the genes was associated with the recruitment of ERβ to the genes (Fig. 4C and D). In contrast to the ERα cells, ChIP found that only SRC-2 was recruited by E_2 to the *CECR6* (Fig. 4E) gene, whereas SRC-1 and SRC-2 were recruited to the *NKD* (Fig. 4E) gene in the U2OS-ERβ cells. Maximal recruitment of the coactivators was observed after 2 h (Fig. 4E and F). These findings demonstrate that both ERα and ERβ bind to the CECR6 and NKD elements, but they recruit different p160 coactivators with E_2.

Tamoxifen Is More Effective at Regulating the Elements than Raloxifene with ERα, whereas Raloxifene Is Superior with ERβ

The ChIP library represents a powerful set of regulatory elements to study the differential regulation by E_2, tamoxifen and raloxifene. The 173 elements were cloned upstream of the minimal tk promoter and then transfected into wild-type U2OS cells with an ERα or ERβ expression vector. Cells were then treated with E_2, tamoxifen or raloxifene and luciferase activity was measured. We found that 80% of the elements were regulated by at least one of the ligands with ERα or ERβ (Table 3). Many elements that were located outside the promoter region were regulated by the ligands (Table 3). Several regulated elements were located over 500 kB from the transcription initiation site of the nearest gene. We also found that different elements derived from the same gene were differentially regulated by E_2, tamoxifen and raloxifene (Table 3, squares). Some elements were differentially regulated by E_2, tamoxifen or raloxifene. Surprisingly

however, we found that even though the library was derived from cells expressing ERα and treated with E_2, more elements were regulated by SERMs than E_2. Moreover, E_2, tamoxifen and raloxifene activated more elements in the presence of ERβ compared to ERα (Table 3). We also found that more elements were activated by tamoxifen, compared to raloxifene, in the presence of ERα, whereas raloxifene was more effective then tamoxifen at activating the elements in the presence of ERβ (Table 4).

To compare the effects of the ligands on mRNA expression to the transfection results, we examined whether 35 genes randomly selected were regulated by E_2 and SERMs in the previously characterized, tetracycline-inducible U2OS-ERα cells (18). Cells were treated for various times with E_2, tamoxifen or raloxifene and mRNA levels were determined by quantitative real time PCR (qPCR). Similar to the transfection assays several genes were regulated only by tamoxifen or raloxifene, even though the library was derived from cells treated with E_2. Out of the 35 genes examined, several showed good correlation with transfection assays. A similar pattern of regulation with transfection assays and qPCR were found with the *NKG2E, CECR6* and *NKD* genes (Table 5). The *EMI domain containing 2* and *calbindin 2,29kDa* genes were activated with E_2 by qPCR, but E_2 did not activate the elements in transfection studies. On the contrary, elements from the *oligodendrocyte lineage transcription factor 2, fem-1 homolog b, protein tyrosine phosphatase, receptor type, M*, and *astrotactin* genes were activated by SERMs in transfection assays, but were not activated by qPCR.

DISCUSSION

Microarray data from our laboratory (18) showed that E_2 and SERMs exhibit a complex pattern of gene regulation that can not be explained by the pharmacology of known ER responsive elements such as ERE, AP-1 and Sp1. This data raises the possibility that many ER target genes contain unknown regulatory elements. While microarrays easily identify ER target genes (19, 21, 31) they do not provide any information on the location or nature of the regulatory elements in the target gene. Thus, new genome-wide approaches are essential to rapidly isolate regulatory elements in target genes to understand how E_2 and SERMs exert tissue-specific clinical effects and produce the complex gene expression profiles observed with microarrays.

ChIP-chip is a powerful strategy that has been used to identify elements in target genes for ERα (25, 35-37). This approach is valuable to identify and annotate different classes of ER elements and to identify transcription factors that interact with ER binding sites (25). Here we used a ChIP-CS strategy to rapidly isolate and characterize ER regulatory elements. We isolated 173 ER binding elements which were tested for functional activity in response to E_2 and SERMs with ERα and ERβ.

We found that most elements were derived from introns and regions beyond 10 kb of the transcription start site. Only 24% of the elements were found within 10 kB of the TSS. ChIP-chip studies also showed that many ER regulatory elements are located at a distance far from the TSS (24, 25). It also has been shown that 63% of the glucocorticoid response elements are found beyond 10 kb of the TSS (39). These results demonstrate that the proximal promoter region only contains a small subset of response elements for steroid receptors.

Only 11% of the genes derived from the ChIP-CS library had an ERE despite using a fairly low stringency for detection. Although the ERE is considered to be the major response element in target genes, these results suggest that most ER target genes are regulated by response elements other than the classical ERE. 41% of the elements in the ChIP-CS library contained an AP-1 site, whereas 18% of the genes contained Sp1 elements. The most surprising finding was that 23% of the clones from the ChIP library did not contain an ERE or any of the known alternative elements. However, our transfection studies showed that 70% of the elements without a classical ERE, AP-1, Sp1,

FOXA1 or NFκB element were regulated by E_2 or SERMs, demonstrating that many regulatory sequences from the ChIP library contain unknown elements. This notion was supported by the data showing that E_2 activated and recruited p160s coactivators to the *NKD* gene, which did not have any known regulatory element.

A major advantage of having numerous elements from the ChIP library is that they can be examined for activity with different ligands and ERs. Most of the 173 elements were regulated by E_2, tamoxifen or raloxifene with ERα or ERβ using transfection assays, demonstrating that the library contained genuine regulatory elements. By testing a large number of elements, we were able to discover several surprising and interesting observations. We found that more elements were regulated by SERMs, even though ChIP was done with E_2 treated cells. One explanation for this finding is that E_2 regulation might require other factors that are lost during shearing of the chromatin. Genome-wide tiling arrays showed that many ER binding sites are associated with transcription factor elements, such as FOX1A, AP-1, Oct and C/EBP, which are important for regulation by E_2 (25). Our findings suggest that the E_2-ER complex binds to the elements and is recognized by the ChIP antibody. However, some elements are probably functionally inactive because the ER is unable to interact with factors that are absent from the ChIP element and required for transcriptional regulation by E_2. Alternatively, it is conceivable that the antibody, which we used for ChIP, selectively recognizes an ER conformation that is inactive or inhibitory when bound to E_2. Other antibodies, such as the one used in the tiling arrays (25), might recognize an ER conformation that is capable detecting elements activated by E_2. Finally, it is likely that some of the ER binding sites might be silent in U2OS cells and do not function as regulatory elements in response to E_2. Some of these inactive elements might be regulated by E_2 in other cell types.

Another important finding from the functional studies of the ChIP library was that the magnitude of activation of most elements by tamoxifen was greater with ERα, whereas raloxifene was more effective at activating the elements in the presence of ERβ. The reason for the differences in activation with the two receptor subtypes is unclear. Tamoxifen binds equally to ERα and ERβ (40), whereas raloxifene binds with about 2-fold greater affinity to ERβ than ERα (41). These results demonstrate that differences in binding do not account for the difference in the activity of tamoxifen and raloxifene with ERs. Structural studies found that tamoxifen and raloxifene produce different conformations of ERα with raloxifene causing a greater increase in the mobility of helix 12 of the LBD (30). The different conformations elicited by tamoxifen and raloxifene might lead to a differential binding of ERs to the regulatory elements or the recruitment of different coregulators by ERα and ERβ at the elements. We previously showed that tamoxifen and raloxifene activate the *NKG2E* gene by recruiting different coactivators (42). We also found that tamoxifen activated the *CECR6* and *NKD* genes from the ChIP library by recruiting coactivators (data not show). In contrast, raloxifene did not activate these genes or recruit coactivators. These results indicate that the SERMs produce different conformations in ER, which leads to the differential recruitment of coactivators. The observation that the agonist activity of SERMs is mediated by coactivators is consistent with the findings that coactivators can interact with the activation fuction-1 (AF-1) in the A/B domain (16, 17).

One of the more surprising findings was the lack of correlation between the gene expression data and transfection studies. Previous studies selected a few elements derived from ChIP to correlate gene expression with function in response to E_2 with transfection studies (25, 26). To avoid any potential bias in selecting elements to examine, we cloned all 173 elements from the library upstream of tk-Luciferase. Only few of the 35 genes that were tested for expression data by PCR showed an exact correlation with transfection assays. There are several possible explanations for these findings. First, the element from the ChIP library was assigned to

the nearest gene. However, it is possible that the element actually regulates one of the other three adjacent genes, rather than the nearest gene. Second, because of compact folding of chromatin the regulatory element isolated from the ChIP library could actually be in close proximity to distant target genes. It is well recognized that enhancers can operate at far distances (43). For example, the E2 enhancer region interacts with distant Sp1 transcription factors by forming stable DNA loop that can be visualized by electron microscopy (44). These findings suggest that the elements from the ChIP library might bypass adjacent genes and cause regulation of distant ER target genes. Third, it is likely that some regulatory elements function differently in the context of native genes. Genes also contain silencers, which are elements that bind repressor proteins that repress the activation of the gene. Thus, some elements from the ChIP library that are functional when isolated and inserted upstream of the tk promoter might be silenced by other factors that interact with the native gene. This might be an important mechanism for tissue-specific gene expression if the factors are differentially expressed in different cell types. The results from our qPCR and luciferase data suggests that there is a high probability that the element actually regulates the assigned gene only when there is good correlation between ER binding by ChIP, mRNA expression and transfection data.

Our results suggest that many ER target genes are regulated by elements other than ERE, AP-1, Sp1, FOXA1 and NFκB. We did extensive bioinformatic analysis of the elements to identify other motifs that might be involved in regulation by ER. However, we were unable to discover any new motifs present in majority of elements. This might be due to the fact that the ChIP library contained too few genes to detect new motifs. However, our detailed analysis of one element from the ChIP library (42), suggests it will be extremely difficult to detect single motifs even with many more elements. We found that the *NKG2E* gene contains a complex composite element (45), which is comprised of four elements, (AP-1, HSF-2, C/EBPβ and a variant ERE) that cooperate to regulate the gene by E_2 and SERMs (42). The results with the NKG2E regulatory element and our ChIP library showing that different elements even within the same gene are differentially regulated by E_2, tamoxifen and raloxifene, suggest that many ER targets are regulated by a combination of cooperative elements rather than a single element, such as ERE, AP-1 and Sp1. The tiling arrays suggest that ER cooperates with a preferred set of transcription factors (25). However, our data suggests that many different combinations of factors interact with ER, since we were unable detect any consistent combination of elements in the ChIP library. Furthermore, it is also likely that the factors that ER interacts with to regulate genes varies in different cell types. This notion is consistent with our observation that some of the ChIP library elements were regulated differently by E_2 and SERMs in various cell types (data not shown).

The data from our ChIP library and tiling arrays clearly indicate that most ER target genes are regulated by ER interacting with a combination of multiple different transcription factors, rather than binding exclusively to an ERE. Identifying combinatorial regulatory elements will likely require detail mapping of many elements with functional studies. The isolation of regulatory elements from native genes with techniques, such as ChIP-CS is a major step towards understanding how ERα and ERβ regulate different genes in response to diverse ligands, and how E_2 and SERMs produce unique clinical effects. However, our findings also suggest that while ChIP-chip and ChIP-CS can identify ER binding sites and regulatory elements it can be difficult to identify the exact gene that is regulated by the element, even when the element is in close proximity or within a gene. A major challenge will be to develop strategies to accurately assign the proper gene that is regulated by the elements identified with tiling arrays or sequencing.

MATERIALS AND METHODS

Preparation of Stable Cell Lines

U2OS cells stably expressing Flag-tagged ERα were prepared by transfecting wild type U2OS cells with pcDNA 6/V5-His vector containing Flag-ERα. Cells were selected and maintained using 10 μg/ml blasticidin. Tetracycline-inducible U2OS-ERα cells were prepared, characterized and maintained as previously described (18).

Chromatin Immunoprecipitation Cloning and Sequencing (ChIP-CS)

U2OS-Flag-ERα cells were treated with 10 nM E_2 for 2 h, and then cross-linked, washed, collected and lysed as previously described (18, 46). Nuclei were then resuspended in FLAG protein immunoprecipitation buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100) and sonicated. The supernatant fraction was collected and incubated with anti-flag M2 agarose beads (Sigma-Aldrich) for 2 h at 4°C. After the beads were washed (0.05 M Tris HCl, pH 7.4, 0.15 M NaCl) the proteins were eluted using 150 ng/μl 3X FLAG peptide (Sigma-Aldrich) in 0.05 M Tris HCl, pH 7.4, 0.15 M NaCl. Eluted proteins were then incubated with anti-ERα (DAKO) coated magnetic beads (Dynabeads M-280) for 2 h. Beads were washed with PBS containing 1% BSA and cross-linking of DNA/proteins was reversed with 1% SDS, 0.1 M $NaHCO_3$ at 65°C. DNA fragments were purified, (QIAquick PCR Purification Kit, Qiagen), blunt ended, cloned and plasmids were isolated and sequenced (Lark technologies).

Plasmids, Transfections, and Luciferase Assays

Inserts from the plasmids were cloned upstream of -32 to +45 thymidine kinase luciferase (Tk-Luc). Transfections of the various vectors into U2OS cells were carried out by electroporation (47). Cells were assayed for luciferase activity according to manufacture's protocol (Promega).

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted and then treated with DNAse using the Aurum Total RNA Mini Kit (BioRad). Reverse transcription reactions were performed using the iScript cDNA Synthesis Kit with 1 μg of total RNA as previously described (48). Quantitative real-time PCR (qPCR) was performed with a BioRad iCycler Thermal Cycler System using iQ SYBR Green Supermix (BioRad). Mean ± S.E.M. was calculated using Prism curve-fitting program (GraphPad Software). The primers used for qPCR are listed in Supplementary Table 2.

Chromatin Immunoprecipitation (ChIP)

Following treatments, cells were cross-linked, washed, collected and lysed as described above. Immunoprecipitations were performed overnight at 4°C with anti-ERα (HC-20), (Santa Cruz Biotechnology), anti-SRC-1 (1135) (Upstate Biotechnology), anti-SRC-2 (ab9261) and anti-SRC-3 (ab2782), (Abcam). DNA fragments were purified (QIAquick PCR Purification Kit, Qiagen) and PCR-amplified. The primers used for Chip are listed in Supplementary Table 2.

Bioinformatics

To find putative ER target genes, the 192 sequences from the ChIP library were mapped to the human genome (HG 35.1 reference assembly) using BLAST with a threshold 1e-30. A set of 173 sequences which had significant hits was returned with four genes each: the nearest gene on each strand in each of the forward and reverse directions. Putative target genes were selected according to their positions relative to the hits. The computational programs MATCH (49) and Dragon ERE finder (Bajic et al., 2003) were used to scan the 173 sequences for putative EREs, AP-1, SP1, foxA1 and NFκB. These

two programs search for sites by scoring their similarity to experimentally verified EREs. When applying MATCH, two ERE position weight matrices, one from the TRANSFAC (Matys et al., 2003) database (matrix ID: V $ER_Q6), and the other one built from 25 EREs known in literature (O'Lone et al., 2004) were used. In these cases the threshold for declaring a putative ERE was set to minimize the sum of the false positives and false negatives. When applying the Dragon ERE finder, the default threshold for obtaining 0.83 sensitivity was used.

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Figure Legends

Fig. 1. Distances of fragments from the nearest gene in the ChIP-CS library are widely distributed.
Distances of each fragment to the nearest TSS (Tables 1, 2) were plotted on a histogram. The Y-axis represents the number of the clones, whereas the X-axis represents the distance of the clone to the nearest TSS.

Fig. 2. ERα interacts with elements from the ChIP-CS library.
Tetracycline-inducible U2OS-ERα cells were treated with 10 nM E_2 for 3h and ChIP was performed using antibodies to ERα. Immunoprecipitated chromatin fragments were PCR amplified and quantitated by real-time PCR using primers for the *CECR6, SPATA13, NKD, LLGL2, H19, and NKG2* elements.

Fig. 3. E_2 recruits p160 coactivators to genes from the ChIP-CS library.
Tetracycline-inducible U2OS-ERα cells were treated with 10 nM E_2 for increasing times and ChIP was performed using antibodies to the p160 coactivators, SRC-1, SRC-2 or SRC-3. Immunoprecipitated chromatin fragments were PCR amplified and quantitated by real-time PCR using primers for the *CECR6, SPATA13, NKD, LLGL2, H19 and, NKG2* elements (A-F, respectively).

Fig. 4. ERβ activates the *CECR6* and *NKD* genes and recruits p160 coactivators.
Tetracycline-inducible U2OS-ERβ cells were treated with 10 nM E_2 for increasing times. *CECR6* (A) or *NKD* (B) mRNA was measured by qPCR. Each data point is the average of triplicate determinations ± S.E.M. The asterisk represents a significant difference from control ($P<0.05$). ChIP assays were performed using antibodies to ERβ (C, D) and the p160 coactivators, SRC-1, SRC-2 or SRC-3 (E, F). Immunoprecipitated chromatin fragments were PCR amplified and quantitated by real-time PCR using primers for the *CECR6* (C, E) and *NKD* (D, F) elements.

Table 1

Gene Description	Location	Accesssion	ERE	AP-1	SP1	NFκB	FOXA1	Gene Description	Location	Accesssion	ERE	AP-1	SP1	NFκB	FOXA
DnaJ (Hsp40) homolog, subfamily C, member 6	19284	AB007942						adaptor-related protein complex 3 delta 1 subunit	2933	AC005545					
protein tyrosine phosphatase, receptor type, M **	376229	BC040543						astrotactin	133783	AB006627					
cytoplasmic polyadenylation element bp 4	29026	AB051460						NY-REN-41 antigen	1650	AF155108					+
similar to Keratin, type I cytoskeletal 18	3550	XM_497554			+			lipase, hepatic **	21702	AF037404		+	+		+
protocadherin 11 X-linked	348316	AB026187	+	+	+	+	+	FLJ42117 protein	43282	AK124111				+	
sema domain, seven thrombospondin repeats	217329	AC004615						fibroblast growth factor 12	11780	AK125307		+			
solute carrier family 37 member 2	435	AK074100				+		chromosome 10 open reading frame 112	226328	AL590378					
phosphodiesterase 4B **	143115	BC036108		+				putative nuclear protein ORF1-FL49	17774	AJ245877		+	+	+	
CUB and Sushi multiple domains 1	779937	AA889055				+		glutamate receptor, ionotropic, kainate 4	19998	S67803					
calcium channel L type, alpha 1B subunit	74357	M94172			+			inhibitor of kappa light polypeptide gene enhancer	5571	AF062089		+			
RNA binding motif single stranded interacting protein	3451	AF023259		+	+	+	+	chromosome 14 open reading frame 171	31955	AC007375					
amyotrophic lateral sclerosis 2	346514	AB053321		+				protein tyrosine phosphatase receptor type f	43512	BC034046				+	
cat eye syndrome chromosome region candidate 6	10	AF307451	+	+			+	chromosome 20 open reading frame 44	12409	AF173893					
solute carrier family 19 member 1	9602	AF004354	+			+		hypothetical protein LOC285692	586	BC031253					
tumor necrosis factor receptor member 19	34535	BC047321						adenosine deaminase, RNA-specific, B1	29950	AF001042				+	
ryanodine receptor 3	75898	AB001025			+			plasminogen-like	1645	AK124365		+		+	
hypothetical protein FLJ30707	8470	AK055269	+					similar to ankyrin repeat domain 30A	29389	XM_291770		+		+	
l(3)mbt-like 2	3653	AJ305226	+	+				Rab6-interacting protein 2	68743	BC037377					
glutamate receptor, ionotropic, kainate 2	142077	AJ252246		+				guanine nucleotide binding protein gamma 7	51326	AB010414		+			
ATP-binding cassette, sub-family A member 8	78	AB020629						p21 activated kinase 7	2122	AB033090	+			+	
hemicentin	217203	AF156100			+			spectrin repeat containing, nuclear envelope 2	9450	AB023228		+		+	
family with sequence similarity 13 member C1	43164	BC036453						lethal giant larve homolog 2	1991	AK025401	+				
EMI domain containing 2	6846	AJ416091	+			+		ATP synthase, H+ transporting	1856	AF088071			+		
zinc and ring finger 3	20847	AB051436		+				chromosome 2 open reading frame 13	28872	BC030711		+			+
similar to CG7467-PA	6095	XR_000268						similar to hypothetical protein DKFZp586O0120.1	39692	XM_496490		+	+		+
glycogen synthase kinase 3 beta	71512	BC000251		+				START domain containing 9	68175	AB037721			+	+	+
gamma-aminobutyric acid receptor rho 2	15479	M86868		+				KIAA1944 protein	293645	AB061814				+	
hypothetical protein FLJ22222	10514	AK025875		+				spermatogenesis associated 13	10701	AK055770					
ankyrin repeat and SOCS box-containing 17	4339	AF403035	+	+			+	hypothetical protein FLJ22301	1798	AK025954				+	+
naked cuticule homolog NKD	7659	AB062886					+	chromosome 10 open reading frame 11	364330	AF267860				+	
similar to IFIT1; Interferon, alpha-inducible protein	3368	XM_497244		+				calcium channel voltage-dependent gamma	13500	AF096322			+		
RAB31, member RAS oncogene family **	47283	AF183421		+				similar to Ribosome biogenesis protein BMS1 homolog	14205	XM_210011					+
phosphodiesterase 8B	434	AB085824			+	+									
								Total transcription factor sites			**9**	**24**	**13**	**19**	**12**

Table 1. ERα binding sites located in introns of target genes. The nearest gene associated with the ERα binding element from the ChIP library was identified by the human genome. The presence of ERE, AP-1, Sp1, NFκB and FOXA1 binding sites in clones were determined by bioinformatics using programs described in Materials and Methods. **Previously reported estrogen regulated gene.

Table 2

Gene description	Location	Accession #	ERE	AP-1	SP1	NFκB	FOXA1	Gene description	Location	Accession #	ERE	AP-1	SP1	NFκB	FOXA
tRNA alanine	80	GeneID:4553			+			receptor-interacting factor 1	23245	AK001826		+			
single-stranded DNA binding protein 2 **	103	AA013030			+	+		chemokine orphan receptor 1 **	26349	AF030297					
u2 small nuclear ribonucleoprotein A	112	AC002056				+	+	myeloid/lymphoid or mixed-lineage leukemia	26492	AK057058	+			+	
NADH dehydrogenase 6	154&884	AY063322						NYD-SP12 protein	26758	AF345909					
tRNA serine 1	266	GeneID:4574			+			family with sequence similarity 34, member A	27290	BC034621		+			
calbindin 2, 29kDa **	345	BC015484		+				phosphodiesterase 4D interacting protein	29581	AB007923					
hypothetical protein FLJ35827	347	AK093146		+		+	+	similar to Ig kappa chain V region	30659&49	XM_372941	+	+		+	
similar to olfactory receptor, family 7, subfamily A, 17	952	XM_497067				+		similar to ADP,ATP carrier protein	36373	XM_498140		+		+	
THAP domain containing 8	965	BC072416					+	hypothetical gene supported by BC040860	38885	XM_498698		+			+
retinal outer segment membrane protein 1	1020	BC008100		+		+	+	hypothetical protein LOC389072	40610	BC020812					
olfactory receptor, family 7	1121	AC064843				+		LOC441580	40920	XM_499193	+	+	+	+	+
killer cell lectin-like receptor subfamily C,3 **	1484&310	AF027164		+			+	cytidine monophosphate	41193	AF271388		+			
neuronal PAS domain protein 2	1328	AK026791				+		similar to ATP-dependent DNA helicase II	41928	XM_372262				+	+
Snf2-related CBP activator protein	1551	AB002307				+		SNAP25-interacting protein	44729	AB051471		+			
hypothetical LOC401038	1940	XM_379163					+	similar to eukaryotic translation elongation factor 1	48771	XM_497272					+
ATPase, Ca++ transporting, ubiquitous	2147	AF458229						similar to putative	50229	XM_210400		+			+
FLJ46688 protein	2759	AK128530					+	tankyrase TRF1-interacting ankyrin	51841	AF082556		+	+	+	
KIAA0240	3021	AL833540		+	+	+		FAT tumor suppressor homolog 1 **	52081	D88798					
solute carrier family 4	3590	AB018282						hypothetical protein MGC26143	52535	BC014590					
hypothetical gene supported by BC031617	3808	BC031617				+		olfactory receptor family 4 subfamily C	52688	AC126345				+	
fem-1 homolog b	3952	AB007856				+		similar to 33 kDa protein	55690	XM_498064		+			
oligodendrocyte lineage transcription factor 2	4109	AF221520			+			likely ortholog of mouse limb-bud and heart gene	56452	AF110224					
polycystic kidney and hepatic disease 1	4442	AF480064	+	+	+	+		ubiquinol-cytochrome c reductase	57627	AC007786	+	+			+
complement component 1	4508	BC008983				+		glutamate receptor ionotropic, delta 1	61700	AB033046					
similar to zinc finger protein 92	4551	XM_066859	+	+				hypothetical gene supported by AK094765	63164	XM_498861		+		+	
similar to salivary proline-rich protein	4645	XM_497733		+		+	+	solute carrier family 8 member 1	65007	AC007281		+			+
similar to nudix type motif 4 isoform beta	4749	BT020109		+				similar to FLJ10378 protein isoform 2	67385	GeneID:442109		+			
similar to seven transmembrane helix receptor	5219	XM_499490			+	+		inter-alpha inhibitor H5	70933	AB075833	+			+	+
similar to DEAD box polypeptide 10	5508	GeneID:401533						similar to SPCPB16A4.07c	81779	XM_498163		+	+		
cAMP responsive element binding protein 3-like 2	5741	AJ549092		+		+		forkhead box L1	83326	AF315075				+	
sorting nexin family member 27	6195	AB007957	+	+			+	hypothetical protein FLJ10305	89737	AK001167		+			
H19, imprinted maternally untranslated mRNA **	6488	AC004556			+			ribosomal protein L18 pseudogene 1	90890	AL512359				+	
immunoglobulin superfamily receptor	6490	AF329490		+				similar to immunoglobulin heavy-chain-2 light-chain	95986	XM_372543					+
hornerin	7029	BR000036		+			+	SH2 domain containing 4B	98724	NM_207372				+	
similar to TFIIH basal transcription factor	7206	GeneID:3427	+				+	hypothetical protein LOC283432	120703	BC037211		+			
transducin-like enhancer of split 2	7496	BC017364			+			eukaryotic translation elongation factor 1 epsilon 1	122468	AB011079		+			
progesterone receptor **	8931	AF016381					+	hypothetical LOC285307	123535	XM_211837				+	
hypothetical protein FLJ90586	9024	AK075067		+				family with sequence similarity 10, member A3	149575	NG_004762					
ubiquitin-conjugating enzyme E2N	9084	AK098233						peptidylglycine alpha-amidating monooxygenase **	150061	AB095007		+			
methylcrotonoyl-Coenzyme A carboxylase 1	9317	BC004187			+	+	+	hypothetical protein FLJ13197	160477	AK023259		+	+		
similar to Ribosome biogenesis protein BMS1 homolog	11530	XM_372108		+				glycine receptor, alpha 1	187921	BC074980		+		+	
syntaxin binding protein 6	12475	AF161505		+		+	+	7SL cytoplasmic, pseudogene 2	196320	NG_002426					
discoidin, CUB and LCCL domain containing 2 **	12949	AB073146				+	+	similar to Calcyclin binding protein	204123	AK093425					
adrenergic, beta, receptor kinase 2	13589	AK055687		+		+	+	similar to Rpl7a protein	205123	XM_498041		+			
polymerase II polypeptide D	14534	BC002958						cadherin 18 type 2	246876	BC031051				+	+
platelet-activating factor receptor **	14597	AY275466			+			similar to RIKEN cDNA 5730421E18 gene	298531	AK094007				+	
similar to galectin-related inter-fiber protein	15092	XM_499395		+	+			neuron navigator 3 **	307930	AB023155					
U56B small nuclear	17308	AC005994						roundabout, axon guidance receptor	312713	AB046788		+			+
coiled-coil domain containing 5	18850	AK097403		+		+		CUB and Sushi multiple domains 3	357892	AB067481					
UDP glycosyltransferase 2 family polypeptide	19256	AF179879		+	+			ribosomal protein L10-like	542861	AB063608	+	+	+		
FLJ45721 protein	20310	AK127623		+				similar to chromosome 9 ORF 140	579108	BC048267				+	+
Rho guanine nucleotide exchange factor 3	21857	AF249744		+			+	hypothetical LOC389202	606000	XM_371690					
COMM domain containing 3	22486	AY542159													
								Total transcription factor sites			**10**	**47**	**18**	**38**	**29**

Table 2. ERα binding sites located in the upstream region of target genes. The nearest gene associated with the ERα binding element from the ChIP library was identified by the human genome. The presence of ERE, AP-1, Sp1, NFκB and FOX1A binding sites in clones were determined by bioinformatics using programs described in Materials and Methods. **Previously reported estrogen regulated gene.

Gene Name	ERα E2	ERα T	ERα R	ERβ E2	ERβ T	ERβ R
tRNA alanine	0.9	1.5	1.3	0.5	2.2	5.3
single-stranded DNA binding protein 2	0.9	1.3	1.2	0.5	2.4	3.1
u2 small nuclear ribonucleoprotein A	0.8	1.1	3.8	1.0	2.9	7.3
NADH dehydrogenase 6	0.9	2.0	1.5	0.6	3.4	7.2
NADH dehydrogenase 6	0.6	1.4	1.4	1.1	1.5	1.8
tRNA serine 1	1.1	1.3	1.1	0.8	1.8	8.0
calbindin 2,29kDa	1.3	2.2	3.9	0.5	1.5	1.5
hypotetical protein FLJ35827	1.0	1.5	1.2	0.6	2.6	4.1
THAP domain containing 8	1.0	1.3	1.6	0.8	1.1	1.7
retinal outer segment membrane protein 1	0.7	1.1	1.2	1.0	2.0	3.2
olfactory receptor, family 7	3.6	2.8	3.9	0.9	2.5	5.5
killer cell lectin-like receptor subfamily C	32.0	4.5	3.0	10.0	0.1	0.1
killer cell lectin-like receptor subfamily C	1.1	1.2	1.1	0.6	3.9	5.5
neuronal PAS domain protein 2	2.5	4.3	2.6	0.8	2.2	3.4
Snf2-related CBP activator protein	0.8	1.6	1.4	0.1	1.2	1.9
hypothetical LOC401038	0.5	1.5	1.3	0.3	6.4	9.0
ATPase, Ca++ transporting, ubiquitous	1.4	2.2	2.8	0.4	3.4	5.5
FLJ46688 protein	0.6	1.3	1.5	0.7	2.6	3.3
KIAA0240	0.5	2.7	2.3	1.0	1.0	1.2
solute carrier family 4	0.8	1.5	0.7	0.7	3.9	6.0
hypothetical gene supported by BC031617	0.8	2.0	1.4	0.4	3.3	4.4
fem-1 homolog b	1.2	2.2	2.1	1.4	1.2	1.7
oligodendrocyte lineage transcription factor 2	2.3	3.7	2.9	0.7	2.7	4.7
polycystic kidney and hepatic disease 1	0.7	1.5	1.5	1.2	1.0	1.0
complement component 1	1.1	1.7	1.3	0.8	0.7	0.8
similar to zinc finger protein 92	0.6	1.2	1.3	1.2	2.5	4.0
similar to salivary proline-rich protein	1.1	2.9	3.1	0.3	1.7	2.7
similar to nudix type motif 4 isoform beta	1.1	2.0	1.6	0.8	2.9	5.9
similar to seven transmembrane helix receptor	1.1	2.0	1.7	0.6	3.7	5.7
similar to DEAD box polypeptide 10	1.5	0.9	1.1	0.7	2.4	6.3
cAMP responsive element binding protein 3-like 2	0.8	1.3	1.5	0.7	2.9	6.2
sorting nexin family member 27	1.2	3.1	3.6	0.9	2.0	5.6
H19, imprinted maternally expressed untranslated mRNA	0.9	2.5	2.1	0.8	1.2	1.2
immunoglobulin superfamoly receptor	0.9	1.7	1.5	0.8	1.0	1.2
Hornerin	1.0	1.5	1.5	1.7	2.0	8.4
similar to TFIIH basal transcription factor	7.1	2.2	2.0	2.9	1.5	2.5
transducing like enhancer of split 2	0.8	2.1	2.0	0.9	3.2	12.1
progesterone receptor	1.1	1.0	1.3	0.8	2.0	6.5
hypotetical protein FLJ90586	0.9	1.4	1.4	1.2	4.1	9.4
ubiquitin conjugated enzyme E2N	0.9	1.4	1.3	0.8	1.6	2.1
mtylcortonyl coenzyme A carboxylase 1	0.8	1.7	1.8	0.9	1.0	1.5
similar to Ribosome biogenesis protein BMS1 homolog	1.0	1.6	1.5	0.5	1.4	1.4
syntaxin binding protein 6	1.0	1.3	1.0	0.7	2.1	3.3
discoidin, CUB and LCCL domain containing 2	0.9	3.8	2.6	1.1	1.4	1.1
adrenergic beta receptor kinase 2	0.9	1.3	1.3	0.5	0.9	1.0
polymerase II polypeptide D	0.8	1.9	1.7	1.2	2.5	3.2
platelet-activating factor receptor	1.3	1.3	1.3	0.7	1.0	0.9

Gene Name	ERα E2	ERα T	ERα R	ERβ E2	ERβ T	ERβ R
similar to immunoglobulin heavy-chain-2 light-chain	0.7	1.1	1.1	0.8	3.3	5.9
SH2 domain containing 4B	1.0	2.0	1.2	0.7	1.4	1.5
hypothetical protein LOC283432	0.9	1.9	1.6	0.9	1.8	2.5
eukaryotic translation elongation factor 1 epsilon 1	0.9	1.2	1.5	2.1	4.0	12.6
hypothetical LOC285307	0.8	2.0	1.7	1.3	2.1	2.7
hypothetical LOC285307	0.9	0.8	1.3	0.7	2.6	5.6
family with sequence similarity 10, member A3	0.9	2.0	1.5	1.5	1.4	1.3
peptidylglycine alpha-amidating monooxygenase	0.5	1.2	1.6	1.2	4.5	7.0
hypothetical protein FLJ13197	0.7	0.7	0.7	0.8	0.7	0.8
glycine receptor, alpha 1	1.1	2.8	2.0	1.3	1.3	1.3
7SL, cytoplasmic, pseudogene 2	0.7	1.4	1.6	1.2	1.3	0.9
similar to Calcyclin binding protein	0.9	0.8	0.9	1.0	0.8	0.9
similar to Rpl7a protein	0.9	2.1	1.6	1.8	3.0	3.0
cadherin 18, type 2	0.8	1.2	1.4	0.7	2.3	3.4
similar to RIKEN cDNA 5730421E18 gene	1.0	0.9	0.8	1.3	3.4	4.9
neuron navigator 3	0.9	1.3	1.3	2.3	6.9	4.8
roundabout, axon guidance receptor	0.9	1.9	2.2	0.4	2.9	2.8
CUB and Sushi multiple domains 3	0.6	1.3	1.5	0.7	2.4	5.2
ribosomal protein L10-like	0.5	2.6	1.6	2.7	11.8	47.0
similar to chromosome 9 open reading frame 140	0.9	1.6	1.1	0.5	2.8	3.1
hypothetical LOC389202	1.3	2.3	1.7	1.1	1.4	3.3
DnaJ (Hsp40) homolog, subfamily C, member 6	0.7	1.1	1.2	0.6	1.3	5.9
protein tyrosine phosphatase, receptor type, M	1.5	2.1	3.2	2.2	6.0	4.5
cytoplasmic polyadenylation element bp 4	0.4	0.4	0.5	0.3	3.3	4.4
similar to Keratin, type I cytoskeletal 18	0.8	2.0	2.7	0.3	4.7	8.0
protocadherin 11 X-linked	1.0	1.7	1.3	1.1	1.1	1.2
sema domain, seven trombospondin repeats	0.8	0.8	1.0	1.2	1.2	1.1
solute carrier family 37 member 2	0.9	1.6	2.0	0.9	8.4	9.7
phosphodiesterase 4B	1.0	2.0	1.4	1.2	2.1	2.6
CUB and sushi multiple domains1	0.8	1.7	1.9	1.0	1.7	4.0
calcium channel L-type alpha 1B subunit	0.8	0.7	0.8	0.7	2.9	4.4
RNA binding motif single strand interacting protein	1.3	1.0	1.1	1.5	1.5	1.1
amyotrophic lateral sclerosis 2	0.8	1.5	1.5	0.8	3.4	7.3
cat eye syndrome chromosome region candidate 6	70.2	18.9	8.1	15.8	1.4	2.4
solute carrier family 19 member 1	0.7	1.7	1.7	3.4	1.4	2.0
tumor necrosis factor receptor member 19	0.5	0.9	0.8	1.1	1.3	0.6
ryanodine receptor 3	1.2	2.3	3.1	0.6	3.0	4.5
hypotetical protein FLJ30707	1.1	0.9	0.7	0.8	0.9	0.8
l (3) mbt like 2	0.9	1.3	1.0	1.0	2.1	3.3
glutamate receptor ionotrophic kainate 2	0.8	1.8	1.6	0.5	3.0	6.3
ATP-binding cassette, sub-family A member 8	1.6	2.2	1.3	1.0	1.2	1.3
hemicentin	1.0	2.0	1.5	0.8	2.8	5.1
family with sequence similarity 13 member C1	0.8	0.6	0.8	0.9	1.1	1.4
EMI domain containing 2	1.9	2.1	3.5	1.0	1.6	1.6
zinc and ring finger 3	0.7	1.1	0.9	1.1	3.7	4.5
similar to CG7467-PA	1.0	2.5	2.1	0.4	2.6	3.6
glycogen synthase kinase 3 beta	0.8	1.4	1.2	0.8	1.0	0.6

similar to galectin-related inter-fiber protein	0.7	2.0	1.7	0.4	3.4	6.9
U56B small nuclear	0.9	1.8	1.6	0.8	1.3	1.3
coiled-coil domain containing 5	1.1	1.3	1.4	0.7	1.1	0.8
UDP glycosyltransferase 2 family, polypeptide	0.9	1.3	1.4	1.2	2.9	9.2
FLJ45721 protein	0.3	0.7	0.7	0.6	3.4	4.0
rho guanine nucleotide exchange factor 3	1.1	1.6	1.7	1.6	1.9	1.2
COMM domain containing 3	1.0	1.5	1.3	1.2	1.1	1.4
receptor-interacting factor 1	1.4	2.4	1.8	1.2	4.0	8.9
chemokine orphan receptor 1	1.1	7.4	2.9	0.9	4.3	7.2
myeloid/lymphoid or mixed-lineage leukemia	0.6	0.8	1.0	0.7	1.3	1.4
NYD-SP-12 protein	0.6	1.6	1.5	1.3	0.9	1.0
family with sequence similarity 34, member A	0.7	1.0	1.4	0.3	1.5	1.8
phosphodiesterase 4D interacting protein	0.9	0.9	1.0	0.6	4.5	6.2
similar to Ig kappa chain V region	0.6	1.0	0.9	0.5	3.1	4.5
similar to Ig kappa chain V region	1.0	0.9	1.3	1.1	2.5	3.3
similar to ADP,ATP carrier protein	4.6	9.4	4.5	0.6	4.3	6.1
hypothetical gene supported by BC040860	0.4	1.1	1.1	0.8	4.8	6.5
hypothetical protein LOC389072	0.5	1.4	1.1	1.0	1.7	2.9
LOC441580	1.0	2.6	2.0	0.4	2.4	3.9
cytidine monophosphate	0.8	1.1	0.9	0.5	3.0	3.5
similar to ATP-dependent DNA helicase II	0.9	0.9	0.7	1.2	3.2	7.0
SNAP25-interacting protein	0.6	1.7	1.6	0.6	3.0	4.8
similar to eukaryotic translation elongation factor 1	1.6	3.9	2.4	1.0	1.8	3.9
similar to putative	0.8	1.2	1.1	0.7	2.3	5.0
tankyrase, TRF1-interacting ankyrin	0.5	1.6	1.3	1.2	2.4	5.5
FAT tumor suppressor homolog 1	0.8	1.4	1.2	0.6	1.1	1.8
hypothetical protein MGC26143	0.5	0.8	1.1	0.6	3.5	5.1
olfactory receptor, family 4, subfamily C	0.8	1.4	1.5	1.2	2.1	2.9
similar to 33 kDa protein	1.1	1.1	1.0	0.7	1.0	1.7
likely ortholog of mouse limb-bud and heart gene	1.6	2.7	1.8	1.0	2.3	2.5
ubiquinol-cytochrome c reductase	0.7	1.3	1.1	0.9	1.7	3.3
glutamate receptor, ionotropic, delta 1	1.2	1.5	1.7	0.9	1.8	3.2
hypothetical gene supported by AK094765	0.6	1.1	1.4	0.6	2.1	2.4
solute carrier family 8 member1	1.0	1.9	2.0	1.0	1.9	4.1
similar to FLJ10378 protein isoform 2	1.2	1.3	1.3	0.6	2.3	2.7
inter-alpha inhibitor H5	1.0	1.4	1.3	0.9	1.0	1.4
similar to SPCPB16A4.07c	1.1	2.2	1.5	1.0	2.3	2.7
forkhead box L1	0.9	1.6	1.5	0.7	1.7	1.3
hypothetical protein FLJ10305	0.9	1.7	1.4	0.4	3.5	5.8
ribosomal protein L18 pseudogene 1	0.9	1.4	1.2	0.9	1.1	1.3
gamma aminobutyric acid receptor rho2	1.3	2.3	2.1	0.8	1.3	1.8
hypotetical protein FLJ22222	0.8	1.6	1.3	0.6	4.0	5.5
ankyrin repeat and SOCX box-containing 17	24.6	2.5	1.5	0.3	4.1	5.1
naked cuticule homolog	3.5	2.5	1.5	2.4	2.3	2.1
similar to IFIT1; Interferon, alpha-inducible protein	1.1	2.0	1.6	0.7	5.2	11.3
RAB31, member RAS oncogene family	1.0	2.2	1.6	1.1	2.2	2.9
phosphodiesterase 8B	0.8	1.4	1.8	2.5	4.1	4.1
adaptor related protein complex 3 delta 1 subunit	1.0	2.7	2.7	0.8	1.2	1.5
astrotactin	1.5	2.4	2.0	1.0	1.8	2.9
NY-REN-41 antigen	0.7	1.0	1.0	0.6	3.1	4.3
lipase hepatic	0.5	1.3	1.7	0.4	5.6	11.6
FLJ42117 protein	0.6	1.2	1.5	0.5	3.9	5.7
fibroblast growth factor 12	0.6	1.2	1.3	0.4	1.8	2.5
chromosome 10 open reading frame 112	0.7	1.1	1.2	1.0	1.8	1.5
putative nuclear protein ORF1-FL49	1.0	2.1	1.4	0.5	3.6	5.6
glutamate receptor ionotrophic kainate 4	0.7	1.4	1.4	1.2	1.1	1.1
inhibitor of appa light polypeptide gene enhancer	1.0	1.4	2.1	0.6	2.9	6.4
chromosome 14 open reading frame 171	0.9	2.6	2.0	0.4	2.9	6.6
protein tyrosine phosphatase, receptor type, F	0.8	1.2	1.2	0.8	2.0	3.4
chromosome 20 open reading frame 44	1.1	2.2	2.2	0.6	3.9	7.0
hypothetical protein LOC285692	0.9	1.6	1.6	0.4	2.5	2.9
adenosine deaminase RNA specific B1	0.8	0.9	0.6	0.6	3.8	4.7
plasminogen like	0.7	1.6	1.4	1.0	1.7	2.0
similar to ankyrin repeat domain 30A	1.0	2.0	1.7	1.1	1.3	2.4
Rab6-interacting protein 2	0.6	1.7	1.9	1.0	1.1	1.9
guanine nucleotide binding protein (G protein), gamma 7	0.6	1.4	1.3	0.9	0.6	0.6
p21activated kinase 7	0.9	1.6	1.3	0.6	5.4	6.0
spectrin repeat containing, nuclear envelope 2	0.7	0.8	0.9	0.8	1.2	1.6
lethal giant larve homolog 2	0.7	1.2	1.4	1.0	1.2	1.5
ATP synthase, H+ transporting	1.7	1.9	1.4	0.6	2.1	1.9
chromosome 2 open reading frame 13	0.7	1.2	1.6	0.6	1.9	2.7
similar to hypothetical protein DKFZp586O0120.1	0.6	1.4	1.4	0.3	2.4	3.0
START domain containing 9	1.4	1.9	1.3	0.7	1.9	3.4
KIAA1944 protein	0.9	1.3	1.4	1.7	0.4	1.2
spermatogenesis associated 13	0.6	1.3	1.3	0.7	1.6	3.3
hypothetical protein FLJ22301	0.9	1.4	1.4	1.0	1.6	2.5
chromosome 10 open reading frame 11	0.6	0.9	1.1	0.5	4.1	6.4
calcium channel, voltage-dependent, gamma	1.2	2.1	1.7	1.0	2.5	7.7
similar to Ribosome biogenesis protein BMS1 homolog	0.7	1.4	1.5	0.6	0.8	1.8

Table 3. Effects of E_2 and SERMs on the Elements in the ChIP library.

Wild-type U2OS cells were transfected with the different elements from the ChIP library, located upstream of the minimal tk promoter, and an ERα or ERβ expression vector. Cells were then treated with 10 nM E_2, 1 μM tamoxifen or 1 μM raloxifene for 18 hours and luciferase assays were performed. Squares surround different elements located adjacent to one gene. Each number represents the mean of three measurements. The S.E.M. was less than 10%.

	Number of genes regulated by 2 fold or >
ERα	
Estradiol	19
Tamoxifen	51
Raloxifene	34
Tamoxifen > Raloxifene	13
Raloxifene > Tamoxifen	6
ERβ	
Estradiol	38
Tamoxifen	94
Raloxifene	116
Tamoxifen > Raloxifene	3
Raloxifene > Tamoxifen	75

Table 4. Summary of the Genes Activated by Estradiol, Tamoxifen and Raloxifene. The numbers listed are for genes activated with the drug by at least 2-fold. For the genes listed for tamoxifen vs raloxifene the magnitude of activation by one of the drugs ERα and ERβ was at least 1.5-fold greater.

Table 5

	Estradiol				Tamoxifen				Raloxifene			
	LUC.	PCR			LUC.	PCR			LUC.	PCR		
killer cell lectin-like receptor subfamily C	32.0	20.6 ± 9.7	43.5 ± 6.7	86.1 ±11.2	4.5	5.6 ±1.2	8.2 ±7.8	17.3 ±14.6	3.0	1.8 ±0.7	4.1 ±1.6	7.0 ±3.0
cat eye syndrome chromosome region candid	70.2	29.9 ±2.2	39.4 ±2.4	13.0 ±1.0	18.9	2.5 ±0.4	2.6 ±0.8	4.0 ±0.4	8.1	0.9 ±0.1	1.1 ±0.0	1.3 ±1.0
H19, imprinted maternally expressed untransl	0.9	10.4 ± 4.6	11.2 ± 0.9	12.7 ± 2.2	2.5	1.7 ±0.4	2.4 ±0.4	1.9 ±0.2	2.1	0.9 ±0.2	1.1 ±0.4	0.9 ±0.2
EMI domain containing 2	1.9	4.2 ± 1.2	3.6 ± 1.6	3.4 ± 2.6	2.1	1.4 ±0.3	1.8 ±0.5	1.4 ±0.1	3.5	1.6 ±0.5	2.1 ±1.4	1.8 ±1.2
retinal outer segment membrane protein 1	0.7	3.2 ± 2.2	2.4 ± 0.1	8.8 ± 7.1	1.1	1.9 ±0.2	1.8 ±0.4	1.7 ±0.3	1.2	0.7 ±0.4	4.4 ±1.0	2.5 ±1.8
naked cuticule homolog	3.5	2.5 ± 0.2	2.3 ± 0.3	2.1 ± 0.2	2.5	2.6 ±0.9	2.2 ±0.1	1.9 ±0.3	1.5	1.1 ±0.1	1.3 ±0.3	1.1 ±0.1
solute carrier family 4	0.8	2.2 ± 0.0	3.2 ± 0.9	3.5 ± 0.8	1.5	0.7 ±0.2	2.4 ±0.1	2.7 ±0.6	0.7	0.9 ±0.2	1.0 ±0.1	1.0 ±0.1
spermatogenesis associated 13	0.6	2.8 ± 0.5	2.5 ± 0.5	1.7 ± 0.4	1.3	2.5 ±0.9	3.2 ±0.7	3.5 ±1.1	1.3	1.8 ±0.7	2.2 ±0.5	2.4 ±0.6
calcium channel L-type alpha 1B subunit	0.8	1.4 ± 0.0	1.9 ± 0.3	3.7 ± 0.5	0.7	1.1 ±0.0	1.3 ±0.2	1.2 ±0.1	0.8	0.8 ±0.2	0.9 ±0.1	1.1 ±0.2
cytoplasmic polyadenylation element bp 4	0.4	1.9 ± 0.3	2.1 ± 0.0	2.1 ± 0.4	0.4	1.3 ±0.2	1.1 ±0.1	1.7 ±1.0	0.5	1.0 ±0.4	1.0 ±0.1	1.0 ±0.1
glutamate receptor ionotrophic kainate 4	0.7	1.0 ± 0.4	1.0 ± 0.1	2.8 ± 0.0	1.4	0.3 ±0.0	0.1 ±0.0	1.2 ±0.6	1.4	0.4 ±0.0	0.4 ±0.1	0.7 ±0.4
calbindin 2,29kDa	1.3	1.8 ± 0.2	2.4 ± 0.1	2.7 ± 0.0	2.2	1.0 ±0.0	1.6 ±0.3	1.3 ±0.2	3.9	1.0 ±0.3	2.4 ±0.1	2.3 ±0.0
UDP glycosyltransferase 2 family, polypeptide	0.9	1.0 ± 0.2	1.4 ± 0.4	2.9 ± 0.3	1.3	1.0 ±0.0	1.1 ±0.0	1.3 ±0.1	1.4	1.3 ±0.5	0.9 ±0.5	1.7 ±0.7
chromosome 10 open reading frame 11	0.6	1.0 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	0.9	0.8 ±0.1	0.8 ±0.3	0.4 ±0.1	1.1	0.8 ±0.2	0.8 ±0.2	0.6 ±0.1
solute carrier family 19 member 1	0.7	1.2 ± 0.3	0.8 ± 0.2	0.8 ± 0.0	1.7	0.9 ±0.1	0.8 ±0.2	0.5 ±0.0	1.7	1.1 ±0.1	1.2 ±0.3	1.1 ±0.3
eukaryotic translation elongation factor 1 epsil	0.9	0.8 ± 0.1	0.6 ± 0.0	0.9 ± 0.3	1.2	0.7 ±0.1	0.7 ±0.1	0.5 ±0.0	1.5	0.6 ±0.3	0.7 ±0.4	1.0 ±0.1
protocadherin 11 X-linked	1.0	1.1 ± 0.1	0.9 ± 0.4	0.5 ± 0.0	1.7	1.1 ±0.1	1.7 ±0.2	1.7 ±0.3	1.3	0.8 ±0.2	0.9 ±0.3	0.7 ±0.1
START domain containing 9	1.4	0.5 ± 0.0	0.5 ± 0.2	0.5 ± 0.1	1.9	0.9 ±0.1	0.9 ±0.0	0.9 ±0.1	1.3	0.6 ±0.2	0.9 ±0.0	0.9 ±0.1
single-stranded DNA binding protein 2	0.9	0.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.0	1.3	0.6 ±0.1	0.7 ±0.1	0.5 ±0.1	1.2	0.7 ±0.0	1.2 ±0.4	0.9 ±0.2
putative nuclear protein ORF1-FL49	1.0	0.5 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	2.1	0.9 ±0.1	1.0 ±0.2	0.8 ±0.1	1.4	0.7 ±0.0	1.2 ±0.6	1.1 ±0.3
l (3) mbt like 2	0.9	0.9 ± 0.2	0.5 ± 0.1	0.5 ± 0.0	1.3	0.8 ±0.1	0.8 ±0.1	0.8 ±0.1	1.0	0.9 ±0.1	1.3 ±0.3	1.1 ±0.3
plasminogen like	0.7	1.2 ± 0.2	0.6 ± 0.2	0.4 ± 0.1	1.6	1.2 ±0.1	1.0 ±0.2	0.9 ±0.2	1.4	0.8 ±0.2	0.9 ±0.1	1.2 ±0.4
RNA binding motif single strand interacting pr	1.3	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	1.0	0.6 ±0.2	0.4 ±0.2	0.4 ±0.1	1.1	0.7 ±0.1	0.7 ±0.1	0.8 ±0.2
glutamate receptor ionotrophic kainate 2	0.8	0.7 ± 0.5	0.1 ± 0.1	0.2 ± 0.0	1.8	1.0 ±0.2	1.9 ±0.5	1.6 ±0.6	1.6	0.9 ±0.1	0.8 ±0.1	1.0 ±0.3
CUB and sushi multiple domains1	0.8	1.0 ± 0.1	1.0 ± 0.0	1.3 ± 0.0	1.7	0.8 ±0.2	1.0 ±0.3	1.0 ±0.5	1.9	0.8 ±0.1	0.9 ±0.4	1.1 ±0.1
fibroblast growth factor 12	0.6	0.8 ± 0.0	0.9 ± 0.2	1.1 ± 0.0	1.2	0.9 ±0.2	1.0 ±0.2	1.1 ±0.1	1.3	0.9 ±0.0	1.1 ±0.4	0.9 ±0.2
adenosine deaminase RNA specific B1	0.8	1.6 ± 0.2	1.1 ± 0.4	1.3 ± 0.5	0.9	0.8 ±0.1	0.7 ±0.1	0.7 ±0.1	0.6	0.7 ±0.3	1.1 ±0.1	0.9 ±0.2
protein tyrosine phosphatase, receptor type, N	1.5	1.1 ± 0.2	1.2 ± 0.3	1.3 ± 0.5	2.1	1.2 ±0.1	1.8 ±0.1	1.7 ±0.2	3.2	0.8 ±0.1	1.2 ±0.5	1.0 ±0.2
COMM domain containing 3	1.0	1.3 ± 0.3	1.1 ± 0.3	1.2 ± 0.4	1.5	1.0 ±0.3	1.3 ±0.4	1.6 ±0.3	1.3	0.8 ±0.2	0.8 ±0.2	1.1 ±0.2
phosphodiesterase 8B	0.8	1.4 ± 0.2	1.3 ± 0.3	1.2 ± 0.2	1.4	0.8 ±0.3	0.8 ±0.3	1.1 ±0.2	1.8	0.7 ±0.1	1.4 ±0.5	0.8 ±0.2
astrotactin	1.5	0.7 ± 0.0	0.8 ± 0.0	1.1 ± 0.1	2.4	1.0 ±0.2	1.0 ±0.3	0.9 ±0.1	2.0	0.9 ±0.1	1.1 ±0.4	1.0 ±0.2
fem-1 homolog b	1.2	1.0 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	2.2	0.8 ±0.0	1.1 ±0.0	0.7 ±0.1	2.1	0.7 ±0.1	1.2 ±0.3	1.1 ±0.1
hypotetical protein FLJ30707	1.1	0.6 ± 0.2	0.6 ± 0.0	0.8 ± 0.2	0.9	0.9 ±0.1	1.0 ±0.1	0.8 ±0.2	0.7	1.0 ±0.2	1.0 ±0.1	0.9 ±0.1
cAMP responsive element binding protein 3-lil	0.8	0.8 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	1.3	0.8 ±0.2	1.2 ±0.3	1.0 ±0.2	1.5	1.1 ±0.0	1.2 ±0.3	1.3 ±0.1
oligodendrocyte lineage transcription factor 2	2.3	0.7 ± 0.2	0.8 ± 0.2	0.9 ± 0.4	3.7	1.0 ±0.1	1.0 ±0.1	0.8 ±0.0	2.9	1.0 ±0.1	1.3 ±0.2	1.2 ±0.0
	18	6	12	24	18	6	12	24	18	6	12	24
						Time of Treatment (h)						

Table 5. Comparison of the regulation of selected elements and genes by E_2 and SERMs. Luciferase activity of the elements was measured by transfection studies. The expression of the corresponding gene was measured by real-time PCR after 6, 12 and 24h of treatments with the ligands in U2OS-ERα cells. Each data point is the average of triplicate determinations ± S.E.M.

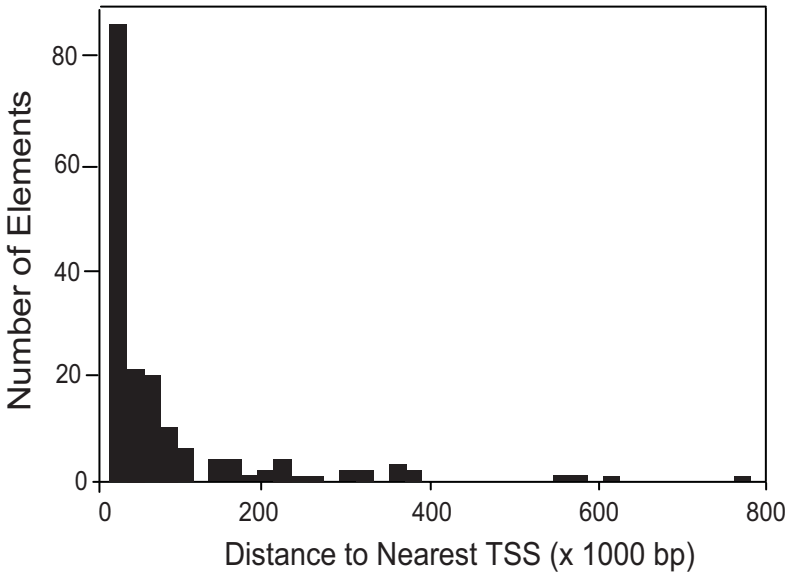
Number of Elements
80
60
40
20
0
0
200
400
600
800
Distance to Nearest TSS (x 1000 bp)

Figure 1

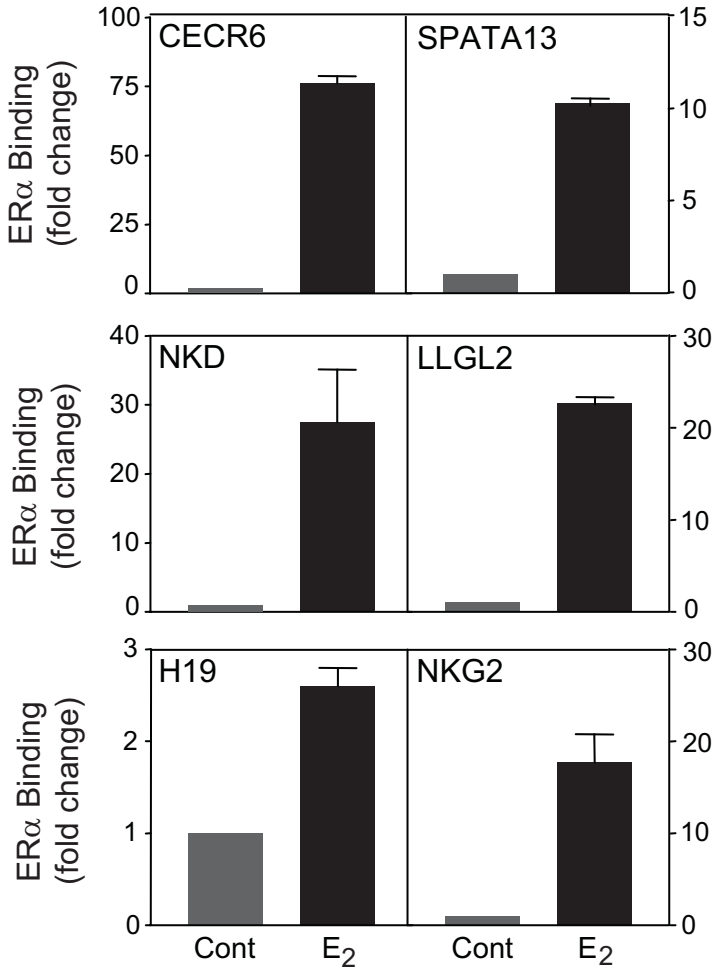
CECR6
SPATA13
NKD
LLGL2
H19
NKG2
ERα Binding (fold change)
ERα Binding (fold change)
ERα Binding (fold change)
100
75
50
25
0
15
10
5
0
40
30
20
10
0
30
20
10
0
3
2
1
0
30
20
10
0
Cont
E2
Cont
E2

Figure 2

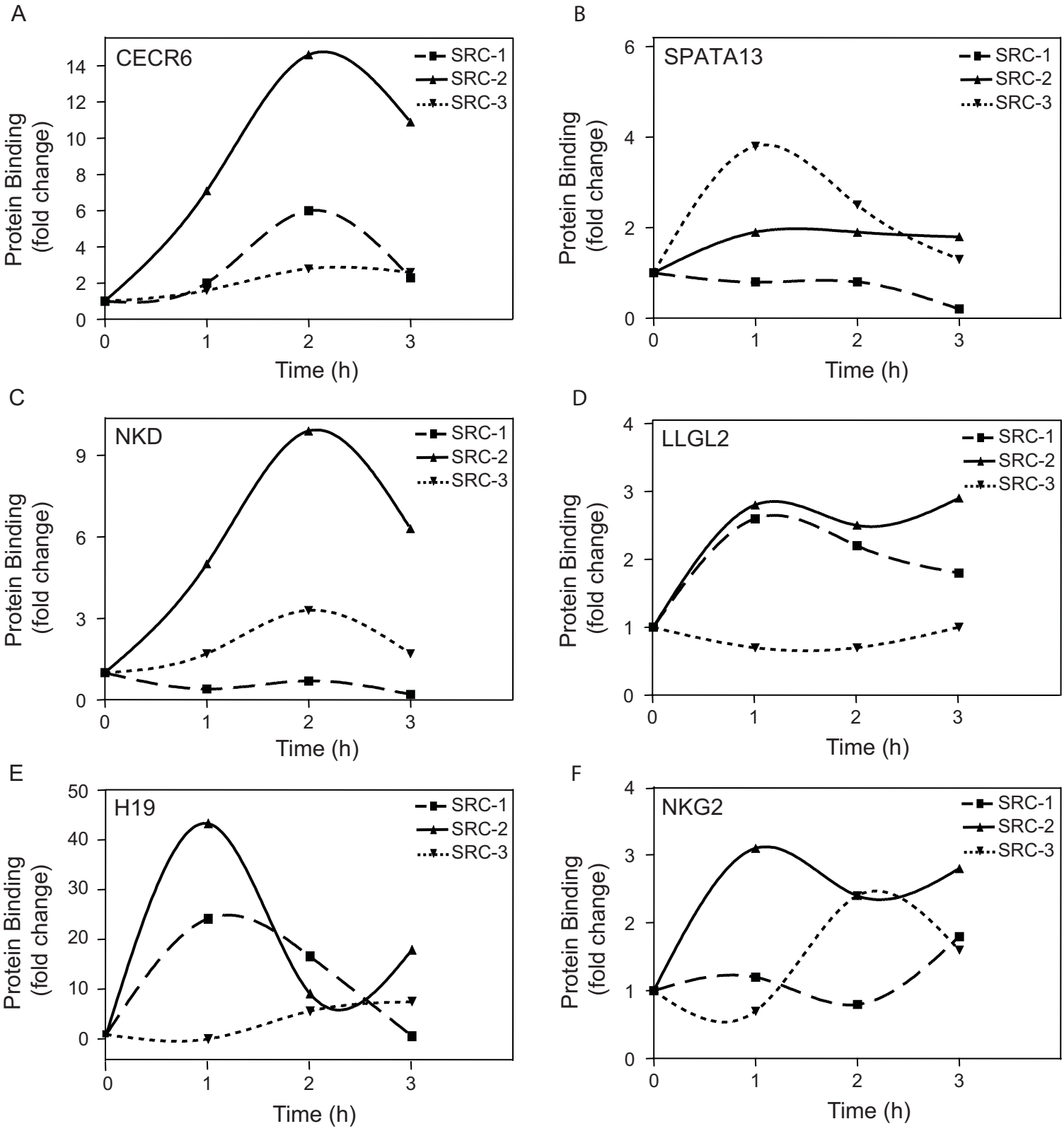
A
CECR6
SRC-1
SRC-2
SRC-3
Protein Binding (fold change)
Time (h)
B
SPATA13
C
NKD
D
LLGL2
E
H19
F
NKG2

Figure 3

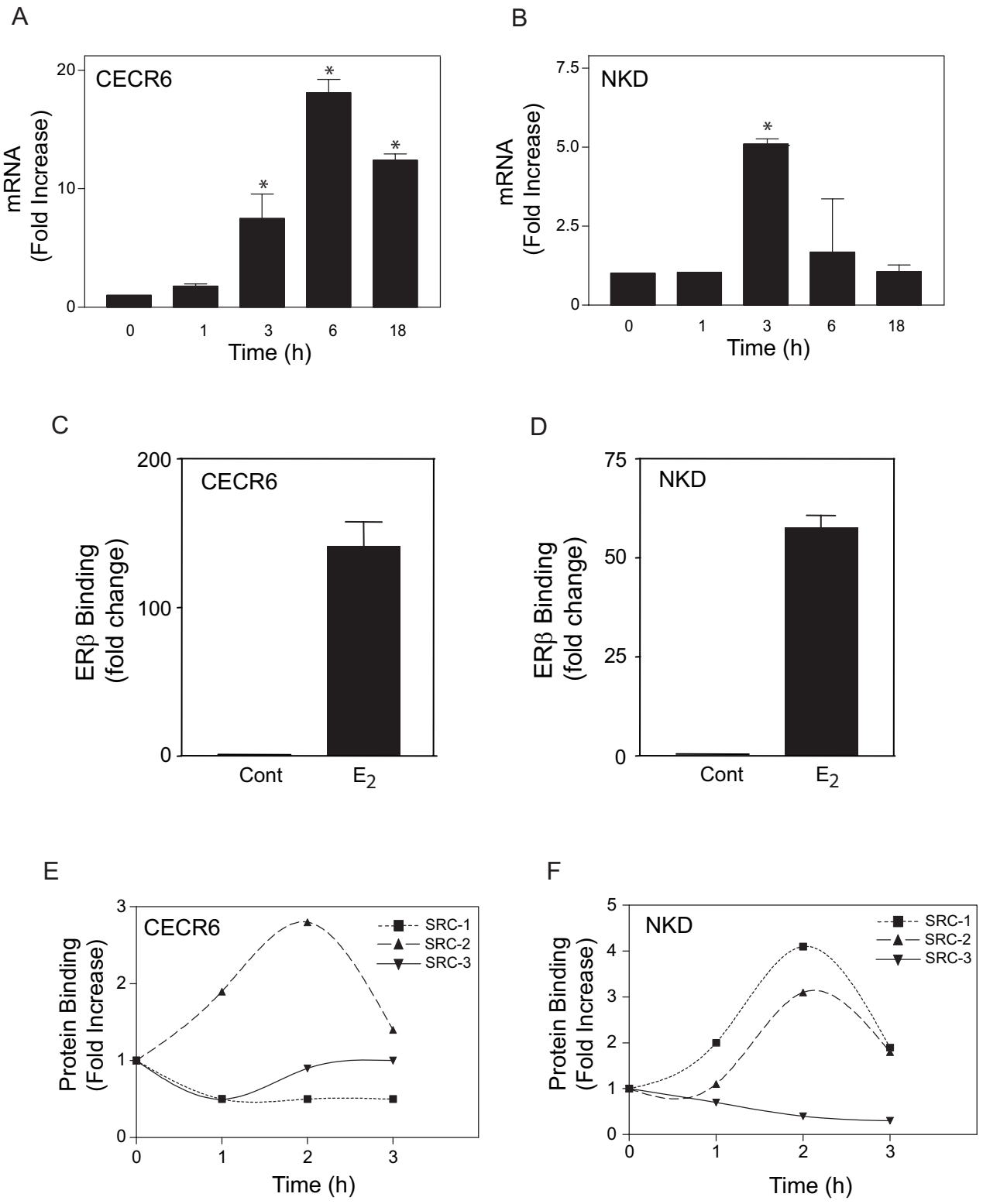
A
CECR6
mRNA (Fold Increase)
20
10
0
*
*
*
0
1
3
6
18
Time (h)
B
NKD
mRNA (Fold Increase)
7.5
5.0
2.5
0
*
0
1
3
6
18
Time (h)
C
CECR6
ERβ Binding (fold change)
200
100
0
Cont
E2
D
NKD
ERβ Binding (fold change)
75
50
25
0
Cont
E2
E
CECR6
Protein Binding (Fold Increase)
3
2
1
0
SRC-1
SRC-2
SRC-3
0
1
2
3
Time (h)
F
NKD
Protein Binding (Fold Increase)
5
4
3
2
1
0
SRC-1
SRC-2
SRC-3
0
1
2
3
Time (h)

Figure 4