Estrogen Induces c-myc Gene Expression via an Upstream Enhancer Activated by the Estrogen Receptor and the AP-1 Transcription Factor

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c-myc oncogene is implicated in tumorigenesis of many cancers, including breast cancer. Although c-myc is a well-known estrogen-induced gene, its promoter has no estrogen-response element, and the underlying mechanism by which estrogen induces its expression remains obscure. Recent genome-wide studies by us and others suggested that distant elements may mediate estrogen induction of gene expression. In this study, we investigated the molecular mechanism by which estrogen induces c-myc expression with a focus on these distal elements. Estrogen rapidly induced c-myc expression in estrogen receptor (ER)-positive breast cancer cells. Although estrogen had little effect on c-myc proximal promoter activity, it did stimulate the activity of a luciferase reporter containing a distal 67-kb enhancer. Estrogen induction of this luciferase reporter was dependent upon both a half-estrogen response element and an activator protein 1 (AP-1) site within this enhancer, which are conserved across 11 different mammalian species. Small interfering RNA experiments and chromatin immunoprecipitation assays demonstrated the necessity of ER and AP-1 cross talk for estrogen to induce c-myc expression. TAM67, the AP-1 dominant negative, partially inhibited estrogen induction of c-myc expression and suppressed estrogen-induced cell cycle progression. Together, these results demonstrate a novel pathway of estrogen regulation of gene expression by cooperation between ER and AP-1 at the distal enhancer element and that AP-1 is involved in estrogen induction of the c-myc oncogene. These results solve the long-standing question in the field of endocrinology of how estrogen induces c-myc expression. (Molecular Endocrinology 25: 1527–1538, 2011)

Estrogen plays a vital role in the development and progression of breast cancer, which is the most frequently diagnosed cancer as well as the second leading cause of cancer deaths in women of industrialized countries (1). Estrogen receptors (ER), members of the nuclear receptor superfamily, mediate most of the effects of estrogen in breast cancer. Estrogen stimulation of its genomic target gene expression is complex and involves different mechanisms (2–4), including both

the classical pathway (5) and the transcription factor cross talk (or tethering) pathway (6–8). However, many estrogen-regulated genes do not have consensus estrogen-response elements (ERE) or estrogen-related transcription factor binding sites in their promoters. For these genes, estrogen may regulate gene expression through other mechanisms.

Recently, we and others have identified genome-wide ER binding sites using chromatin immunoprecipitation
(ChIP) methods coupled with tiling microarray (9) and cloning-sequencing (10) strategies. These data show that many ER-binding sites are located in distal intergenic regions rather than in promoter regions. The set of ER-binding sites across the genome defined in these studies provided novel insights into the function of ER and established a new resource for understanding estrogenic action in breast cancer and other estrogen-related diseases.

In our previous studies, we systematically identified a network of transcription factors that may modulate ER function (9). The screening identified activator protein 1 (AP-1) as a cooperating factor for ER (9). We also studied ER and AP-1 transcription factor cross talk and identified a panel of estrogen-induced and AP-1-dependent genes (11) using an MCF-7 Tet-Off cell line expressing an inducible AP-1 dominant negative (TAM67) (12–14). Most of the identified ER and AP-1 comodulated genes are important regulators of cell proliferation, cell cycle, and cell motility and adhesion (11).

One of the identified estrogen-induced and AP-1-dependent genes is c-myc, an oncogene that encodes a nuclear transcription factor. The cMyc protein regulates the expression of a variety of target genes (15), to control cell cycle progression, apoptosis, and cellular transformation (16). Deregulated expression of c-myc is observed in many cancers and is associated with poor prognosis (17–19). c-myc is overexpressed in approximately 20%–30% of breast cancers (20). Decreasing cMyc level in breast cancer cells could significantly inhibit mammary tumor growth (21).

c-myc is a well-known estrogen-regulated oncogene (22, 23), and its induction stimulates cell cycle progression and proliferation. cMyc has also been shown to interact with ER and modulate estrogen-mediated signaling (24). However, unlike other estrogen-dependent genes [e.g. trefoil factor 1 (TFF1/pS2)], the mechanism by which c-myc is regulated, remains a mystery (25). Many studies have been conducted over the last 20 yr to investigate the underlying molecular regulatory mechanism by which estrogen regulates c-myc expression (17, 25–30); however, the precise mechanism has never been determined and remains obscure. A putative estrogen-responsive region was found to be located in the 116-bp region of the c-myc gene between +25 and +141, which does not contain a canonical ERE (28). These investigators proposed a mechanism of ER trans-activation in the c-myc gene promoter, suggesting that activation of the c-myc gene by estrogen requires binding of some yet unidentified ER-associated proteins to ER at the c-myc proximal promoter. However, it remains unclear which cis-acting elements and their cognate trans-acting factors are involved. Thus, the molecular mechanism by which estrogen induces c-myc gene expression is still not known and remains a challenging question in the field.

In this study, we investigated the molecular mechanism by which estrogen induces c-myc expression. Based on our previous findings that AP-1 is a cooperating factor for ER (9) and c-myc is an ER- and AP-1-dependent gene (11), we hypothesized that estrogen induces c-myc expression through an ER/AP-1 cross talk mechanism. We found that the c-myc proximal promoter is not sensitive to estrogen, but that a distal enhancer element present within the 67-kb region upstream of the transcriptional start site of the c-myc gene is an estrogen-activated enhancer. We also identified the specific transcription factors binding to this enhancer region that cooperate with ER to induce c-myc expression. The present study demonstrates a novel molecular mechanism that estrogen can regulate c-myc gene expression through cooperation between ER and AP-1 proteins at a distal enhancer element. These results solve the longstanding question of how estrogen regulates the expression of this important oncogene. This study provides novel insights into the function of estrogen and ER in breast cancer and other estrogen-related diseases.

Results

**c-myc is an estrogen-dependent gene in breast cancer**

We first examined the effect of estrogen on c-myc gene expression in MCF-7 breast cancer cells. As shown in Fig. 1A, 17β-estradiol (E2) induced c-myc expression robustly. The induction of c-myc mRNA was prominent as early as 30 min and remained elevated at least 6 h after E2 treatment. These kinetics indicate a primary transcriptional regulatory mechanism. Next we tested the effect of actinomycin D (ActD) and cycloheximide (CHX) on E2 induction of c-myc expression. As expected, ActD, but not CHX, was able to block E2 induction of c-myc expression (Fig. 1B), indicating that estrogen induction of c-myc expression requires new RNA transcription but does not require new protein synthesis. To study ER dependency of c-myc expression, we pretreated MCF-7 cells with ICI 182.780 (ICI) before E2 treatment. ICI completely blocked E2 induction of c-myc expression, indicating this induction is dependent on ER (Fig. 1C). We also examined the effect of estrogen on c-myc expression in other different breast cancer cell lines. The results showed that E2 induced c-myc expression in ER-positive breast cancer cell lines (MCF-7, T47D, MDA-MB-361, and BT474) but had no effect on c-myc expression in
ER-negative breast cancer cell lines (MDA-MB-231 and MDA-MB-468) (Fig. 1D).

**Effect of AP-1 on estrogen induction of c-myc expression**

We next examined the effect of 12-O-tetradecanoylphorbol-13-acetate (TPA), a potent protein kinase C pathway inducer that activates the AP-1 transcription factor. MCF-7 cells were treated with E2, TPA, or a combination of both. As shown in Fig. 2A, TPA induced c-myc expression, and TPA also enhanced E2-induced c-myc expression even further, showing an additive effect. Because TPA is an AP-1 inducer, this result indicates that ER and AP-1 may cooperate to increase c-myc expression. To further evaluate the role of AP-1 blockade in estrogen induction of c-myc expression, an AP-1 dominant negative TAM67 was used to block AP-1-regulated pathways (12, 13). MCF-7 TAM67 Tet-Off cells were generated by stably transfecting the Tet repressor and TAM67 genes into wild-type MCF-7 cells (14). As shown in Fig. 2, B and C, doxycycline (DOX)-induced TAM67 expression partially blocked c-myc expression in estrogen-treated cells but had no effect on c-myc expression in vehicle-treated cells, indicating that AP-1 blockade can interfere with estrogen induction of c-myc gene expression and that AP-1 proteins cooperate with ER to induce c-myc expression in response to estrogen.

**Role of AP-1 and c-myc on estrogen-induced cell cycle progression**

Our previous results showed that AP-1 blockade inhibited multiple signal transduction pathways, altered the expression of cell cycle-regulatory proteins, and affected cell proliferation and colony-forming efficiency of breast cells (14, 31, 32). To further define the relative importance of AP-1 on estrogen-regulated downstream events, we performed cell cycle analysis using MCF-7 TAM67 Tet-Off cells treated with or without estrogen after culturing with or without DOX. As shown in Fig. 3A, DOX-induced TAM67 expression partially blocked estrogen-induced cell cycle progression, resulting in reduced cell number in S phase as compared with the control groups of either vehicle- or estrogen-treated cells. To investigate the role of c-myc on estrogen-regulated cell cycle progression, we performed cell cycle analysis after knockdown of c-myc expression in wild-type MCF-7 cells. As shown in Fig. 3, B and C, knockdown of c-myc expression greatly suppressed estrogen-induced cell cycle progression, suggesting that estrogen-regulated cell cycle progression is transmitted, in large part, through c-myc. These results demonstrated the functional importance of the ER/AP-1/c-myc axis in regulating cell cycle progression of breast cancer cells.

**The c-myc proximal promoter is not responsive to estrogen**

We next investigated the molecular mechanism by which estrogen regulates c-myc expression. Because estrogen may regulate target gene expression by binding to ERE or by tethering with other transcription factors at gene promoter regions, we investigated whether c-myc proximal promoter is inducible by estrogen treatment. Using bioinformatics analysis, we found several potential half-EREs, AP-1 sites, and other transcription factor-
binding sites in the c-myc proximal promoter region (Fig. 4A). We then cloned the c-myc proximal promoter fragment (2.3 kb) into promoterless luciferase plasmid and found that the proximal promoter was not responsive to estrogen treatment (Fig. 4B). We also generated various lengths of c-myc proximal promoter luciferase reporter plasmids. None of the shorter fragments showed any response to estrogen (Fig. 4B). The 225-bp promoter fragment provided strong basal activity, whereas truncation of the promoter to 27 bp lost more than 80% of the transcriptional activity. These results suggested that the 225-bp promoter is the basal core promoter; this core promoter was chosen for the following experiments.

**A distal enhancer confers estrogen responsiveness to c-myc**

Recently, we and others have identified genome-wide ER-binding sites by using ChIP-chip (9) and ChIP-seq (10) strategies. These data showed that many ER-binding sites are located in intergenic rather than proximal promoter regions. Because c-myc proximal promoter is not responsive to estrogen, we examined c-myc gene structure in this global genomic context and found a distal ER-binding region (~1.2 kb in length) located at about 67 kb upstream of the transcriptional start site of the c-myc gene (Fig. 4C). This region has been identified in two independent genome-wide studies conducted by us and others (9, 10) and therefore may contain a functional ER-binding site. We cloned this 1.2-kb DNA fragment and inserted it into the c-myc promoter luciferase plasmid. Estrogen stimulated the luciferase activity of this c-myc enhancer-promoter-luciferase construct. These results demonstrate that this 1.2-kb fragment functions as an enhancer and contributes to estrogen responsiveness (Fig. 4D).

**A half-ERE and an AP-1 site are both critical for estrogen induction of c-myc expression**

Because this distal enhancer enables estrogen induction of the c-myc promoter, we next investigated which cis-acting elements and trans-acting factors are required for this estrogen induction. We analyzed the transcription factor-binding sites in the enhancer region and identified four potential half-EREs and four potential AP-1-binding sites (Fig. 5A). To determine which specific elements within the enhancer region are required for estrogen inducibility, we generated a series of deletion and mutation constructs and tested whether they retained estrogen responsiveness. Deletion or mutation of the two consensus AP-1 sites, one of the two nonconsensus AP-1 sites (upstream) and three of the four half-EREs (upstream) had little effect on estrogen-induced transcriptional activity (Fig. 5B). By contrast, mutation of the second nonconsensus AP-1 site (67,312 bp from the transcriptional start site) significantly decreased estrogen induction of the luciferase activity, and mutation of the fourth half-ERE (67,280 bp from the transcriptional start site) abolished estrogen responsiveness (Fig. 5B). Furthermore, comparison of the enhancer sequences in 11 mammalian species revealed an evolutionarily conserved pattern of the identified functional half-ERE and the nonconsensus AP-1 site, but not other nonfunctional half-ERE and AP-1 sites, across a variety of species (Fig. 5C and Supplemental Fig.
These results demonstrate that the half-ERE and the AP-1 site within the enhancer region are both critical for estrogen induction of c-myc expression.

Effect of AP-1 family member proteins on estrogen induced c-myc expression

To further determine which specific AP-1 proteins are involved in estrogen induction of c-myc gene expression, we transfected small interfering RNA (siRNA) into MCF-7 cells to specifically silence ER or each AP-1 member. These siRNA can suppress the expression of target proteins by at least 70% (Fig. 6A). The siRNA against the luciferase gene does not have any target in the human transcriptome and was therefore used as a control. As shown in Fig. 6B, the siRNA against ER almost totally abolished E2-induced c-myc expression. By comparison, the siRNA against most AP-1 members (cJun, JunB, cFos, Fra1, Fra2) did not reduce E2-induced c-myc expression; however, the siRNA against JunD or FosB resulted in about 40% reduction of E2-induced c-myc expression. We also performed double knockdown of JunD and FosB and observed a similar level of reduction (data not shown). This level of reduction is similar to that seen with AP-1 blockade induced by TAM67 (see Fig. 2B). These results demonstrate that ER is absolutely necessary for estrogen-regulated c-myc expression and that JunD and FosB are also required for maximal estrogen induction of c-myc expression.

ER, AP-1 proteins, and CREB-binding protein (CBP)/p300 are recruited to the enhancer

We next performed ChIP experiments to identify the specific proteins recruited to the c-myc enhancer and promoter elements. Immunoprecipitated DNA was analyzed by quantitative PCR (qPCR) using primers for c-myc enhancer and proximal promoter (Fig. 7A). Primers for pS2 gene promoter and primers for c-myc exon 2 were used as positive (data not shown) and negative controls, respectively (Fig. 7A). As shown in Fig. 7B, ER, JunD, and FosB were recruited to the enhancer rather than the promoter region when the cells were treated with E2. In addition, we performed ChIP experiments after siRNA knockdown of ER, JunD, and FosB. The results showed that knockdown of these genes inhibited the recruitment of RNA polymerase II (Pol II) to the c-myc promoter region (Fig. 7C). These results demonstrated that ER and AP-1 proteins JunD and FosB cooperate to induce c-myc gene expression by binding to the distal enhancer region and recruiting Pol II to the c-myc proximal promoter.

Specificity protein 1 (Sp1) is not involved in estrogen induction of c-myc expression

Because previous studies suggested that Sp1 might bind to the c-myc promoter (33, 34), we also examined the effect
of Sp1 knockdown on estrogen-induced c-myc expression. The results show that Sp1 knockdown slightly decreased c-myc expression, but the fold induction by estrogen remained similar to that of the siRNA targeting luciferase (siLuc) used as a control (Supplemental Fig. 2). Together, these data indicate that Sp1 may not be involved in estrogen induction of c-myc expression.

Discussion

In the present study, we investigated the role of AP-1 in regulating estrogen induction of c-myc gene expression. We found that ER and AP-1 cooperate to induce c-myc gene expression and that a distal enhancer region located 67 kb upstream of the transcriptional start site of the c-myc gene is capable of conferring estrogen responsiveness. We also demonstrated that a half-ERE and an adjacent AP-1 site located within this distal enhancer serve as two critical elements for estrogen induction of c-myc expression. siRNA and ChIP assays show that the recruitment of ER and AP-1 proteins to this distal enhancer element is required for estrogen induction of c-myc expression. Based on our results, we propose a novel molecular mechanism by which ER and AP-1 cooperate to regulate c-myc expression (Fig. 8). In this model, ER and AP-1 members JunD and FosB are not bound to the distal enhancer region in the absence of estrogen, and c-myc is expressed at a low level. When estrogen is added to the cells, estrogen-liganded ER translocates to the nucleus, and the dimeric ER complex weakly binds to the half-ERE. This entire complex is stabilized by the interaction of ER with AP-1 proteins (JunD and FosB) binding to the AP-1 element. Then the ER-JunD-FosB complex functions as a transactivator, interacting with the c-myc promoter region and recruiting Pol II and ultimately inducing c-myc gene expression.

c-myc oncogene has been regarded as a typical estrogen-induced gene in breast cancer cells (22, 23). Whereas the molecular regulation of most other important estrogen-regulated genes in breast cancer (e.g. TFF1/pS2) has been elucidated, c-myc, by contrast, is famous for its mysterious and complex mode of regulation (25). The exact mechanism by which estrogen induces the expression of this important oncogene remained an unsolved problem in the field for many years (25). In a previous study, a putative estrogen-responsive region was found to be located in the proximal promoter region of the c-myc gene (28). However, reporter gene expression was analyzed 36–42 h after E2 treatment in their study (28). We also observed that c-myc proximal promoter could be regulated after 48 h of estrogen treatment (data not shown). However, this mechanism cannot explain the rapid induc-
tion of c-myc expression seen in cells using the endogenous c-myc promoter. Therefore, this late induction may be due to other newly synthesized proteins and may not be relevant to the rapid primary regulation of c-myc by estrogen that is observed in physiological conditions. It has also been suggested that activation of the c-myc gene by estrogen requires binding of other proteins to ER. Previously, some investigators found Sp1-like proteins binding to c-myc proximal promoter region (33, 34). However, the specific transcription factors that contribute to estrogen responsiveness have not been well characterized. Before the study reported here, it was unclear which cis-acting elements and their cognate trans-acting factors were necessary for estrogen induction of c-myc expression.

The genome-wide studies by us and others (9, 10) have shown that many ER-binding sites are located in distal intergenic regions rather than in promoter regions. We systematically identified AP-1 as a cooperating factor for ER that may modulate estrogen receptor function (9). We also identified c-myc as an estrogen-induced and AP-1-dependent gene using a cell model with inducible AP-1 dominant negative expression (11). These studies provided novel insights into understanding the estrogen-signaling pathway in breast cancer and thus enabled us to further investigate the molecular mechanism of estrogen induction of c-myc gene expression. The study presented here shows that ER and AP-1 cooperate to regulate c-myc gene expression via an upstream enhancer element containing a half-ERE and an AP-1 site.

The study of the role of AP-1 and ER/AP-1 cross talk in breast cancer is an emerging area, and the molecular mechanisms of ER/AP-1 regulation of target gene expression are not completely understood. Some previous studies show that ER may act as a coactivator to enhance the activity of AP-1 to regulate the expression of target genes, such as matrix metalloproteinase-1, fibronectin-1, and cyclin D1 (6, 35–37). Another model of ER/AP-1 co-modulation of target gene expression is through the interactions between distinct ERE- and AP-1-binding sites,
with estrogen inducing transcriptional synergy between the ERE and an adjacent AP-1 site in the pS2 promoter (38). It is important to recognize that ER and AP-1 can cooperate to regulate target gene expression through more complex mechanisms. There is also a growing body of literature supporting estrogen regulation of gene expression through a nonclassical (or tethering) pathway, in which ER cooperates with other transcription factors such as AP-1 (6, 35–37), Sp1 (7), or nuclear-factor-κ-B (8, 39) to activate gene expression. In addition, the cross talk between AP-1 and nuclear receptors is not limited to the ER (40). Glucocorticoid receptor and AP-1 can antagonize each other’s activities by a mechanism that does not require DNA interaction, but involves direct protein-protein interactions (43). Recently, we found that IGF-binding protein 6 is up-regulated by the cooperative action of retinoic acid receptors and AP-1 through a complex response element in the first intron of the IGF-binding protein 6 gene (44). There have also been reports of cross talk between AP-1 and thyroid hormone receptors (45) or with progesterone receptors (46). All these studies suggested that cross talk between nuclear receptors and AP-1 transcription factor is a general mechanism of hormonal modulation of target gene expression.

In summary, the study presented here demonstrates that estrogen induction of c-myc gene expression is via an upstream enhancer activated by ER and AP-1 transcription factor. This study solves the longstanding question of how estrogen regulates c-myc expression in breast cancer. The identified pathway provides novel insights into the function of ER and offers potential targets for the prevention and treatment of breast cancer and other estrogen-related diseases in the future.

**Materials and Methods**

**Cells**

MCF-7, T47D, BT474, MDA-MB-361, MDA-MB-231, and MDA-MB-468 breast cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). For experiments, cells were switched to 10% charcoal-stripped fetal calf serum (CSS, Invitrogen, Carlsbad, CA) and cultured for at least 48 h before estrogen treatment. The production and use of MCF-7 TAM67 Tet-Off cells, which were stably transfected with the Tet repressor and TAM67 (an AP-1 dominant negative) (12, 13), has been described previously (14). The MCF-7 TAM67 Tet-Off cells were routinely cultured in 10% FBS plus 1 μg/ml DOX. For experiments, these cells were switched into 10% TetSafe FBS (HyClone Laboratories, Inc., Logan, UT) and cultured for at least 5 d to induce TAM67 expression, as described previously (31).

**Drugs and treatment**

The chemicals E2, TPA, ICI, ActD, and CHX were dissolved in ethanol or dimethyl sulfoxide. For experiments, cells were seeded into six-well plates in 10% CSS and allowed to attach overnight. Vehicle or E2 was added the next day with fresh 10% CSS. The final concentrations of E2, TPA, ICI, ActD, and CHX were 1 nmol/liter, 100 nmol/liter, 1 μmol/liter, 1 μg/ml, and 10 μg/ml, respectively.
FIG. 7. ChIP assay. A, Schematic representation of the primers used to amplify the c-myc enhancer, promoter, or exon 2 regions. B, Recruitment of ER, AP-1, p300/CBP, and Pol II to c-myc enhancer, promoter, or exon 2 regions. Hormone-depleted MCF-7 cells were treated with vehicle or E2 for 30 min and then fixed with formaldehyde. Data were presented as relative amount of immunoprecipitated DNA normalized to input as measured by q-PCR assay. Primers for the pS2 gene promoter were used as a positive control (data not shown). C, Recruitment of Pol II to the c-myc promoter region after knockdown of ER, JunD, or FosB. Hormone-depleted MCF-7 cells were transfected for 36 h with siRNA against ER, JunD, and FosB after which cells were treated with vehicle or E2 for 30 min and fixed with formaldehyde. Data were presented as relative amount of immunoprecipitated DNA normalized to input as measured by q-PCR assay. Asterisk denotes $P < 0.05$ compared with siLuc control.
The hu-myc plasmid containing the c-myc promoter fragment was provided by Dr. Hua Xiao (47). The promoterless pGL4.10 plasmid (Promega Corp., Madison, WI) served as the cloning backbone for the luciferase reporter plasmids used in this study. The c-myc promoter region (from −2320 bp to +211 bp) was digested from the hu-myc plasmid with HindIII and cloned into the pGL4.10 plasmid (Promega). A series of truncations, which contain 1297, 225, and 27 bp of the c-myc promoter, were generated by PCR. The distal 67 kb upstream enhancer region was cloned by PCR from human genomic DNA and inserted into the c-myc promoter luciferase construct. The primers are listed in Supplemental Table 1. A series of deletions and mutations were generated by site-directed mutagenesis. Half-ERE TGACC were mutated to TGctC. AP-1 sites TGANTCA were mutated to TGcNcCA. All constructs were verified by sequencing.

Luciferase assay
Hormone-depleted MCF-7 cells were transfected overnight with plasmids. On the next day, cells were treated with vehicle or E2 for 6 h and then lysed in Passive Lysing Buffer (Promega). Luciferase activity was measured using a commercially available kit (Promega). Each luciferase assay was performed in triplicate, and the result was confirmed in two independent experiments.

RNA extraction and quantitative RT-PCR (q-RT-PCR)
Total RNA was isolated from cells using RNeasy Mini Kit (QIAGEN, Valencia, CA) and reverse transcribed. Real-time quantitative PCR was carried out on an ABI 7500 System (Applied Biosystems, Foster City, CA). The relative gene expression was determined using the comparative Ct method and normalized to the housekeeping gene cyclophilin as described previously (48). The primers and probes are listed in Supplemental Table 1. Each q-RT-PCR assay was performed in triplicate, and the result was confirmed in two independent experiments.

siRNA experiments
Hormone-depleted MCF-7 cells were seeded into six-well plates. On the next day, chemically synthesized siRNA (Sigma-Aldrich Corp., St. Louis, MO) were transfected into the cells. After 36 h, cells were treated with vehicle or E2 for 1 h, and RNA was extracted using RNeasy Mini Kit (QIAGEN).

Western blot
Total protein was extracted from cells using radioimmunoprecipitation assay buffer containing protease inhibitors (Roche, Basel, Switzerland). Protein was resolved by SDS-PAGE and transferred to nitrocellulose membrane. After incubating sequentially with primary and secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the blots were developed using SuperSignal West Pico kit (Pierce Chemical Co., Rockford, IL) and exposed to film. Each Western blot experiment was performed in triplicate and a representative result is shown.

Chromatin immunoprecipitation (ChIP)
ChIP experiments were performed according to the published protocols with minor modifications (22, 23). Hormone-depleted MCF-7 cells were grown in 15-cm dishes. After 30 min treatment with vehicle or E2, cells were washed twice with PBS and cross-linked with 1% formaldehyde in PBS. The cross-linking reaction was stopped 10 min later by washing with PBS containing glycine (final concentration, 0.1 mol/liter). The cells were scraped and lysed. Sonicated chromatin was then immunoprecipitated with antibodies as indicated (Santa Cruz Biotechnology, Inc.). Immunoprecipitated fragments were purified using QiaQuick columns (QIAGEN) and used for templates for qPCR. The primers and probes are listed in Supplemental Table 1. The recruitment was presented as the ratio of qPCR result of immunoprecipitated fragments normalized to input. Each qPCR assay was performed in triplicate, and the result was confirmed in two independent experiments.
Cell cycle analysis

Hormone-depleted MCF-7 cells were treated with vehicle or E2 for 16 h. Then cells were harvested by trypsinization and fixed in 95% ethanol at room temperature. Fixed cells were stored in 95% ethanol at 4°C for future use. At 30 min before analysis, cells were stained in PBS containing 50 μg/ml propidium iodide (Sigma) and 100 μg/ml ribonuclease (Sigma). Flow cytometry was performed using BD FACS Calibur flow cytometer and Cell Quest Pro software (BD Biosciences, San Jose, CA). Data were analyzed using ModFit LT 3.2.1 (Verity Software House, Topsham, ME). Each cell cycle analysis was performed in triplicate, and a representative result is shown.

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