

Src promotes estrogen-dependent estrogen receptor α proteolysis in human breast cancer

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Estrogen drives both transcriptional activation and proteolysis of estrogen receptor α (ER α ; encoded by *ESR1*). Here we observed variable and overlapping *ESR1* mRNA levels in 200 ER α -negative and 50 ER α -positive primary breast cancers examined, which suggests important posttranscriptional ER α regulation. Our results indicate that Src cooperates with estrogen to activate ER α proteolysis. Inducible Src stimulated ligand-activated ER α transcriptional activity and reduced ER α $t_{1/2}$. Src and ER α levels were inversely correlated in primary breast cancers. ER α -negative primary breast cancers and cell lines showed increased Src levels and/or activity compared with ER α -positive cancers and cells. ER α $t_{1/2}$ was reduced in ER α -negative cell lines. In both ER α -positive and -negative cell lines, both proteasome and Src inhibitors increased ER α levels. Src inhibition impaired ligand-activated ER α ubiquitylation and increased ER α levels. Src siRNA impaired ligand-activated ER α loss in BT-20 cells. Pretreatment with Src increased ER α ubiquitylation and degradation in vitro. These findings provide what we believe to be a novel link between Src activation and ER α proteolysis and support a model whereby crosstalk between liganded ER α and Src drives ER α transcriptional activity and targets ER α for ubiquitin-dependent proteolysis. Oncogenic Src activation may promote not only proliferation, but also estrogen-activated ER α loss in a subset of ER α -negative breast cancers, altering prognosis and response to therapy.

Introduction

Estrogen regulates the proliferation and development of tissues expressing estrogen receptors (ERs) and is a risk factor for breast cancer development. One-third of new breast cancers lack detectable ER α protein; these ER α ⁻ cancers have a worse prognosis than do ER α ⁺ breast cancers (1). ER α ⁻ breast cancers do not respond to hormone response modifiers like tamoxifen (2) and often show de novo or acquired resistance to chemotherapy (1). While there are 2 forms of ERs, ER α and ER β (3–5), considerably more is known about the role of ER α in human breast cancer, and in this study we investigated ER α exclusively. While estrogen is mitogenic for cultured ER α ⁺ breast cancer lines, ER α ⁻ breast cancer lines proliferate in the absence of estrogen, and ER α ⁻ breast cancers are generally believed to be estrogen independent.

Factors responsible for the ER α ⁻ status of breast cancers remain largely unknown. Deletions, rearrangements, and point mutations in the *ESR1* gene, which encodes ER α , are too uncommon to account for the ER α ⁻ phenotype (6, 7). ER α promoter hypermethylation has been observed in a minority (up to 25%) of ER α ⁻ breast carcinomas (6). In 3 early nonquantitative studies, *ESR1* mRNA was detected in a majority (67%–71%) of 64 primary ER α ⁻ cancers (8–10), indicating posttranscriptional or posttranslational control of ER α levels in human breast cancers. Transcriptional profiling has demonstrated that *ESR1* mRNA is detected but variably

reduced in ER α ⁻ compared with ER α ⁺ cancers (11–13). The distinct gene expression profiles of ER α ⁺ and ER α ⁻ cancers have led to the hypothesis that these 2 tumor groups arise from different cellular origins: ER α ⁻/Her2⁻ tumors are derived from the basal epithelium, while ER α ⁺ cancers have a luminal epithelial origin (14, 15). The results of our present study shed further light on mechanisms regulating ER α levels.

ER α is a 66-kDa nuclear hormone receptor (HR) transcription factor (16). Upon ligand binding, ER α dimerizes and associates with coactivators and chromatin remodeling factors to activate transcription of genes containing estrogen response elements (EREs) (17). ER α contains 2 transcription activation functions, AF-1 and AF-2. AF-1 can be phosphorylated and activated in a ligand-independent manner following growth factor stimulation, while AF-2 is activated by ligand-stimulated changes in ER α conformation (18, 19). The ER α phosphorylation state affects coactivator binding and ER α -DNA binding affinity.

In addition to transcriptional activation, ligand-ER α binding rapidly activates crosstalk with mitogenic signaling kinases (reviewed in refs. 20, 21). Estrogen-ER α binding promotes a rapid and transient interaction of ER α with cellular Src (cSrc), binding to Shc and Ras-MAPK activation (22–25). In some cell types, estrogen stimulates tripartite ER α , cSrc, and PI3K complex formation, leading to PKB/AKT and MAPK activation (26). Signaling kinases activated by liganded ER α not only activate mitogenic cascades, but also phosphorylate the ER α and its coactivators, generating a feed-forward loop that augments ER α transcriptional activity (20, 21, 27).

The ER α can also be phosphorylated and activated in a ligand-independent manner in response to peptide growth factors including IGF-I (28), TGF- α (29), and EGF (30, 31) that activate PKB and MAPK signaling pathways and cause ER α phosphorylation and

Nonstandard abbreviations used: cFBS, charcoal-stripped FBS; CHX, cycloheximide; cSrc, cellular Src; ER, estrogen receptor; ERE, estrogen response element; HR, hormone receptor; LBA, ligand binding assay; PA, ponasterone A; PR, progesterone receptor; QPCR, quantitative real-time RT-PCR; RPPA, reverse phase tissue lysate array.

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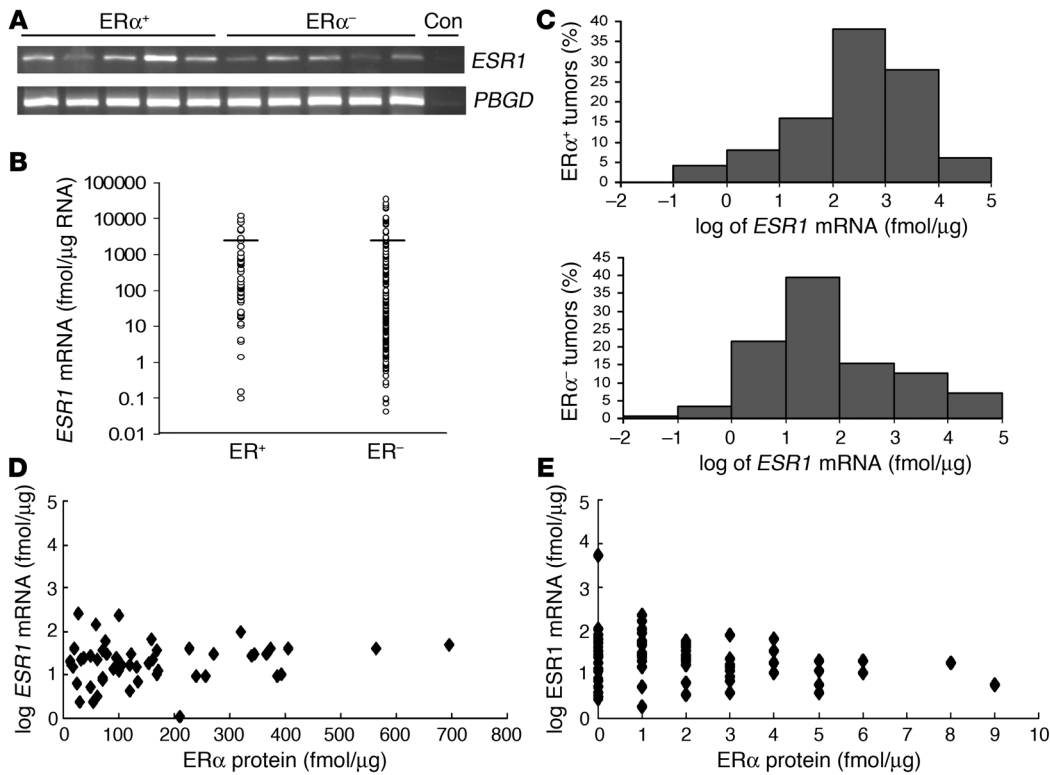


Figure 1 ERα⁻ and ERα⁺ human breast cancers express *ESR1* mRNA. **(A)** PCR of *ESR1* and human *PBGD* from breast tumors. Blots are representative of 50 ERα⁺ and 200 ERα⁻ cancers. Con, control. **(B)** *ESR1* mRNA concentrations. The horizontal line denotes the mean. **(C)** Frequency of *ESR1* mRNA concentrations rounded to the nearest logarithm value. **(D and E)** *ESR1* mRNA (fmol/μg total RNA) plotted versus ERα protein by LBA (fmol/μg cytosolic protein) in the same human breast cancers.

ERα-dependent gene transcription. Phosphorylation of aminoterminal (30, 32, 33) and carboxyterminal (34–36) sites on the ERα increase ERα transcriptional activity.

Estrogen binding to the ERα rapidly stimulates ERα ubiquitylation and proteolysis (37–39). Unliganded ERα is very stable, with a *t*_{1/2} of up to 5 days (37). Upon ligand binding, the ERα *t*_{1/2} drops dramatically, to 3–5 hours (37, 39). The detection of ubiquitinated ERα in vivo in uterine tissue (37) and the finding that proteasome inhibition abrogates estrogen-stimulated ERα loss confirmed an in vivo role for proteasome-mediated ERα degradation in regulating ERα levels (38, 39).

ERα ubiquitination and proteasome activity are intimately linked to ERα-dependent transcriptional activation (40, 41). Ligand binding activates both ERα-dependent transcription and ERα ubiquitination (40). Proteasome inhibitors and mutations that inhibit coactivator binding both abrogate ligand-mediated ERα proteolysis and ERE transcriptional activity (41). Different ligands stimulate ERα proteolysis to different degrees (42), and ubiquitin ligases BRCA1 (43), MDM2 (44), and E6AP (45) can all stimulate estrogen-induced transcriptional activity.

The 60-kDa cSrc tyrosine kinase regulates cellular proliferation and motility as well as tumor metastasis (46). Increased levels and/or activity of cSrc have been observed in primary breast cancers (47), but to our knowledge, an association with ERα levels has not previously been reported. Here we demonstrate that both ERα⁺ and ERα⁻ primary human breast carcinomas expressed *ESR1* mRNA. Crosstalk between liganded ERα and cSrc appeared to promote proteasomal degradation of the ERα. cSrc inhibition impaired ligand-activated ERα ubiquitylation and ERα proteolysis, while Src induction shortened the ERα *t*_{1/2}. Src induction also increased ERα-driven transcription. ERα⁻ breast cancer specimens and cell lines showed elevated cSrc levels and/

or activity compared with ERα⁺ tumors and cell lines, and ERα proteolysis was increased in ERα⁻ cell lines. Src stimulated both ERα ubiquitylation and proteasome-dependent ERα degradation in vitro. These observations and the inverse correlation between cSrc and ERα levels in primary breast cancers suggest that Src may promote cell proliferation by stimulating transcription-coupled ERα degradation in human breast cancers.

Results

ERα⁻ breast cancers express ESR1 mRNA. *ESR1* mRNA expression was quantitated in 200 ERα⁻ and 50 ERα⁺ primary human breast cancers. ERα protein levels were determined in a single clinical reference laboratory by ligand binding assay (LBA). Tumor *ESR1* mRNA was quantitated by quantitative real-time RT-PCR (QPCR). Crossing-point values for each tumor sample were compared to a standard curve generated from serial dilutions of ERα cDNA plasmid (data not shown). Quantitation of housekeeping gene human porphobilinogen deaminase (*PBGD*) expression demonstrated similar mRNA quality in both ERα⁺ and ERα⁻ breast cancers. *ESR1* mRNA was detected in all of 50 ERα⁺ and 200 ERα⁻ cancers (Figure 1A). The *ESR1* mRNA values (Figure 1B) and distribution (Figure 1C) showed considerable variability and overlap in *ESR1* mRNA concentrations in ERα⁺ and ERα⁻ tumors. The mean *ESR1* mRNA concentration was 1.14×10^3 fmol/μg RNA in ERα⁺ cancers (range, 1.02×10^{-1} to 1.19×10^4 fmol/μg RNA) and 1.27×10^3 fmol/μg RNA in ERα⁻ cancers (range, 4.55×10^{-2} to 3.56×10^4 fmol/μg RNA). While the lowest and highest *ESR1* mRNA concentrations were similar and the mean *ESR1* mRNA values did not differ significantly between the ERα⁺ and ERα⁻ cancers (*P* > 0.50), the modal *ESR1* mRNA value in the ERα⁻ tumors was approximately 1 log lower than in the ERα⁺ tumors. When ERα protein concentrations were graphed versus *ESR1* mRNA values, there was no

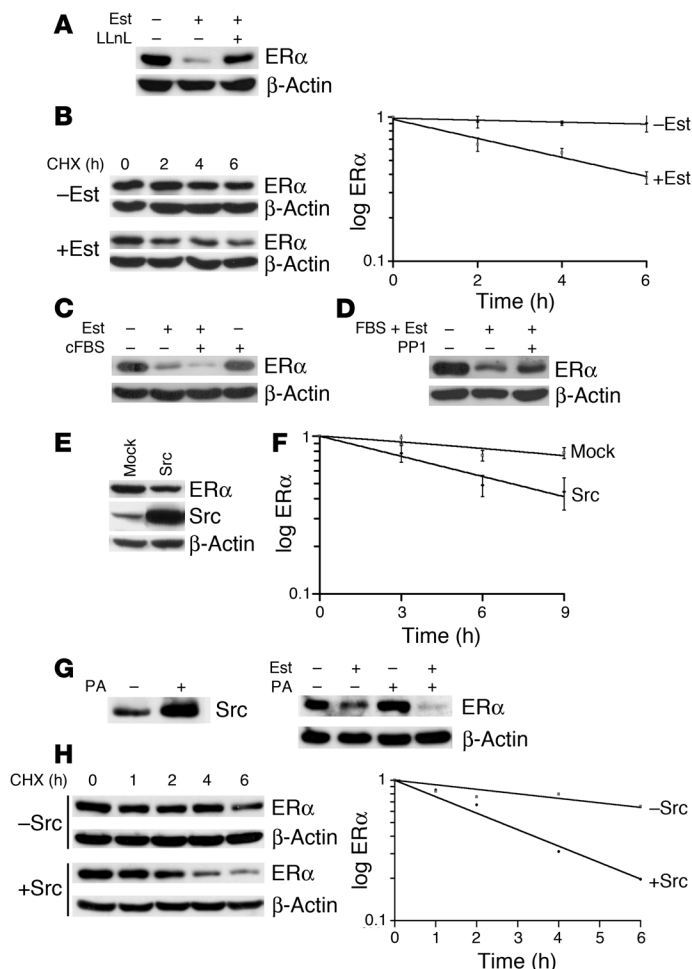


Figure 2

Src promotes estrogen-dependent ER α degradation. (A) ER α before and 6 hours after addition of estradiol (Est) with or without the proteasome inhibitor N-acetyl-Leu-norleucinal (LLnL) to estrogen-depleted MCF-7 cells. Equal loading was confirmed by β -actin. (B) ER α $t_{1/2}$ was assayed by CHX chase in estrogen-depleted cells and at 2, 4, and 6 hours after addition of estradiol. Graph shows results of densitometric analysis of 3 CHX chase experiments (mean \pm SEM). (C) Cells were grown in 0.1% cFBS for 48 hours and then treated with estradiol alone, 5% cFBS plus estradiol, or 5% cFBS alone. ER α and β -actin were assayed 6 hours later. (D) Serum- and estrogen-deprived MCF-7 cells were transferred to 5% FBS plus estradiol with or without added Src inhibitor PP1, and ER α was assayed 6 hours later. (E and F) MCF-7 cells were transfected with PCI-Src Y530F (Src) or empty vector (Mock). After 24 hours, (E) ER α and Src levels were assayed and (F) ER α $t_{1/2}$ was assayed by CHX chase (mean \pm SEM). (G and H) The MCFpINDSrc2 line was estrogen depleted for 72 hours, and Src was induced or not for 24 hours with PA prior to addition of estradiol. (G) Src at time of estradiol addition (left), and ER α and β -actin before and 6 hours after estradiol (Est + or -) was added (right). (H) CHX pulse chase, starting 6 hours after estradiol addition with (+Src) or without (-Src) prior induction of Src by PA.

clear relationship between ER α protein and *ESR1* mRNA levels for either ER α^+ or ER α^- cancers (Figure 1, D and E).

Serum growth factors cooperate with estrogen to activate ER α proteolysis. As a baseline for further study, we showed that addition of β -estradiol, the primary estrogen type in humans, to estrogen-depleted MCF-7 cells stimulated a rapid reduction of ER α protein that was impaired by proteasome inhibition with N-acetyl-Leu-Leu-norleucinal (Figure 2A). The ER α $t_{1/2}$ was greater than 24 hours in estrogen-depleted MCF-7 cells. Within 6 hours of estradiol addition, the ER α $t_{1/2}$ fell to 5 hours (Figure 2B). A significant reduction in ER α $t_{1/2}$ was also noted within 1 hour of ligand addition (data not shown).

Crosstalk between cSrc, PI3K, and receptor tyrosine kinases and liganded ER α leads to ER α phosphorylation and activation of ER α transcriptional activity (21, 28). To determine whether crosstalk between ER α and signaling pathways may also modulate ligand-activated ER α proteolysis, we tested whether addition of growth factors would affect estrogen-stimulated ER α loss. MCF-7 cells were growth factor and estrogen deprived in 0.1% charcoal-stripped FBS (cFBS) for 48 hours. Estradiol together with 5% cFBS reduced ER α levels more rapidly than did estradiol alone (Figure 2C). Growth factor stimulation with 5% cFBS without added estradiol was not sufficient to trigger ER α proteolysis. Thus, serum growth factors may activate signaling kinases to promote estrogen-activated ER α proteolysis.

Src promotes estrogen-stimulated ER α degradation. Liganded ER α binds cSrc leading to cSrc activation (22). Treatment of MCF-7 cells

with the Src inhibitor PP1 caused a dose-dependent accumulation of ER α over 48 hours (data not shown). PP1 also impaired the fall in ER α levels observed when estrogen- and growth factor-starved cells were transferred to serum together with estradiol (Figure 2D). Thus, cSrc may promote ligand-activated ER α proteolysis. Transfection of activated Src (PCI-Src Y530F) reduced ER α levels (Figure 2E). The ER α $t_{1/2}$ fell from 14 hours in asynchronously proliferating MCF-7 cells to 9 hours at 24 hours after Src transfection (Figure 2F).

To assay further the effect of Src on ER α stability, 2 different MCF-7 derivatives were generated to inducibly express activated Src-Y350F. Results of experiments using 1 of these Src inducible MCF-7 lines, designated MCFpINDSrc2, are described below. MCFpINDSrc2 cells were deprived of estradiol and growth factors for 72 hours. Src was induced by treatment with ponasterone A (PA) for 24 hours prior to addition of estradiol (Figure 2G, left). Induction of activated Src did not reduce ER α levels in the absence of estradiol. However, within 6 hours of estradiol addition, ER α levels were markedly lower in Src-induced cells (Figure 2G, right), and ER α $t_{1/2}$ was reduced to 2.6 hours in cells stimulated by estradiol together with Src induction, compared with an ER α $t_{1/2}$ of 8.1 hours in cells treated with estradiol alone (Figure 2H). Treatment with PA alone did not reduce ER α $t_{1/2}$ (data not shown). At 24 hours after Src induction, QPCR showed a modest increase in *ESR1* mRNA expression compared with uninduced cells (data not shown); thus, the more rapid ER α protein loss in estradiol-treated,

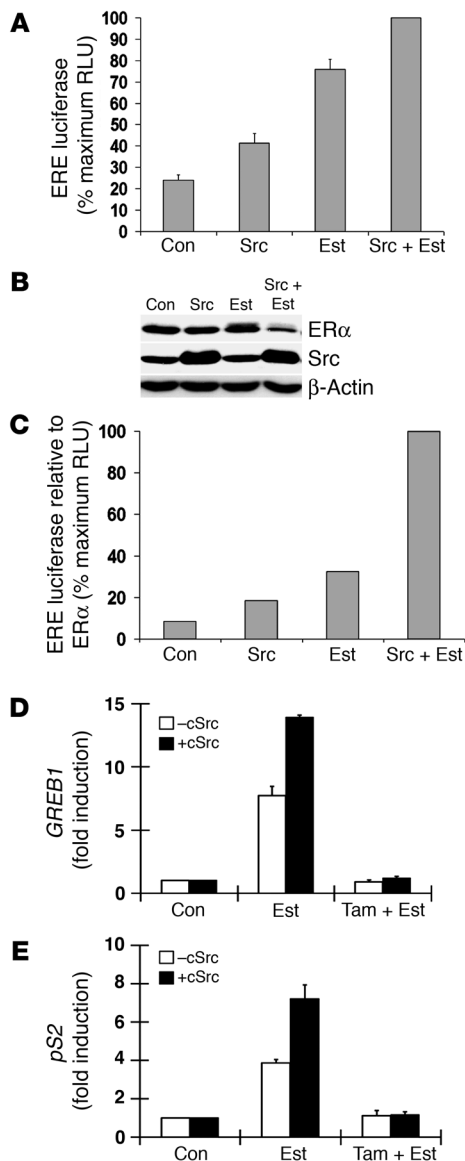


Figure 3

Src promotes estrogen-dependent ER α transcriptional activity. (A–C) MCF-7 was transfected with ERE luciferase reporter and either PCI-Src Y530F or empty vector control and then stimulated with estradiol. Shown are (A) ERE luciferase activity as well as ER α and Src levels (B) before and (C) 4 hours after Src transfection, estradiol addition, or both. (C) Relative ERE luciferase activity corrected for ER α level. (D and E) MCFpINDSrc2 cells were estrogen depleted for 72 hours, and cSrc was induced or not for 24 hours prior to the addition of estradiol (Est) or estradiol plus tamoxifen (Est + Tam). (D and E) QPCR of cellular (D) GREB1 and (E) pS2.

To investigate further if Src affects estrogen-mediated transcription activity of ER α , we used QPCR to quantitate estrogen stimulated expression of cellular ER α target genes, *pS2* and *GREB1* with and without prior Src induction in the MCFpINDSrc2 line. MCFpINDSrc2 cells were estrogen deprived for 3 days, and Src was induced or not within the last 24 hours of starvation. Within 3 hours of estradiol addition, *GREB1* and *pS2* mRNA levels increased by 8- and 4-fold, respectively, compared with 14- and 7-fold higher than baseline when Src was induced prior to estradiol addition. Src induction alone did not activate *GREB1* or *pS2* (data not shown). Neither gene was activated when cells were treated with estradiol together with tamoxifen, with or without Src induction. Thus the effect of Src on these genes was ER α mediated, and Src increased the transcriptional potency of ER α on these ER α target genes (Figure 3, D and E).

MEK and PI3K are not sufficient to promote ligand-mediated ER α proteolysis. MEK inhibition of asynchronous MCF-7 cells with U0126 for 48 hours reduced ER α levels (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI21739DS1). In estrogen-deprived MCF-7 cells, MEK inhibition prior to estradiol addition led to a greater loss of ER α (Supplemental Figure 1B) and a shorter ER α $t_{1/2}$ (data not shown) than with estradiol alone. Thus, in these assay conditions, MEK effectors appear to oppose ligand-stimulated ER α proteolysis.

Treatment with the PI3K inhibitor LY294002 did not affect ER α levels in asynchronous MCF-7 cells (Supplemental Figure 1C). In estrogen-deprived MCF-7 cells, PI3K inhibition prior to estrogen repletion inhibited PKB phosphorylation and cell cycle progression, but did not affect estrogen-mediated ER α loss (Supplemental Figure 1, D and E). Thus, estrogen-stimulated ER α proteolysis does not require PI3K/PKB action or cell cycle entry.

ER α protein levels and stability are reduced in breast cancer lines with activated cSrc. The BT-20 breast cancer line shows both cSrc and EGFR activation, while Her2 and cSrc are activated in MDA-MB-361 (49). *ESR1* mRNA was detected in MCF-7, BT-20, and MDA-MB-361 by nonquantitative RT-PCR (data not shown). Although the BT-20 cell line has been characterized as ER α^- , low but detectable ER α protein was present on ER α immunoprecipitation from 1 mg cell lysate (Figure 4A). cSrc kinase activities were increased (Figure 4B), while the level and $t_{1/2}$ of ER α were reduced in BT-20 and MDA-MB-361 compared with MCF-7 cells (Figure 4C). The calculated ER α $t_{1/2}$ was 14 hours in asynchronous MCF-7, 9 hours in MDA-MB-361, and 5 hours in BT-20 cells.

To further assay effects of Src and proteasome function on ER α levels in ER α^- breast cancer lines, the BT549 line was transfected with ER α to generate the stable line BT549-ER α . Asynchronous BT549-ER α cells had elevated Src activity and the ER α $t_{1/2}$ was 3.9 hours (data not shown). In both ER α^+ (MCF-7 and MDA-MB-361)

Src-induced cells was not due to reduced *ESR1* mRNA expression. Data in a second Src-inducible cell line also confirmed these findings (data not shown). Thus, Src appears to cooperate with estrogen to stimulate ER α proteolysis. Proteasome inhibition reduced the effect of expression of activated Src (data not shown).

Src promotes ligand-activated ER α transcriptional activity. Activation of many transcriptional factors is linked to factor proteolysis (48). Because our data suggested that Src promotes ligand activated ER α proteolysis, we assayed effects of Src on ER α transcriptional activity. In cells grown in the presence of full serum and estradiol, addition of 10^{-8} M estradiol and Src transfection each reproducibly increased ERE luciferase activity within 4 hours, albeit less notably with Src alone (Figure 3A). Estradiol addition together with Src transfection increased ERE luciferase activity beyond that induced by estradiol alone. Src transfection and estradiol stimulation also decreased ER α levels beyond that seen with estradiol alone (Figure 3B). For ERE luciferase activity relative to available ER α (i.e., correcting for the reduced ER α level at 4 hours), Src transfection and estradiol stimulation had more than additive effects (Figure 3C).

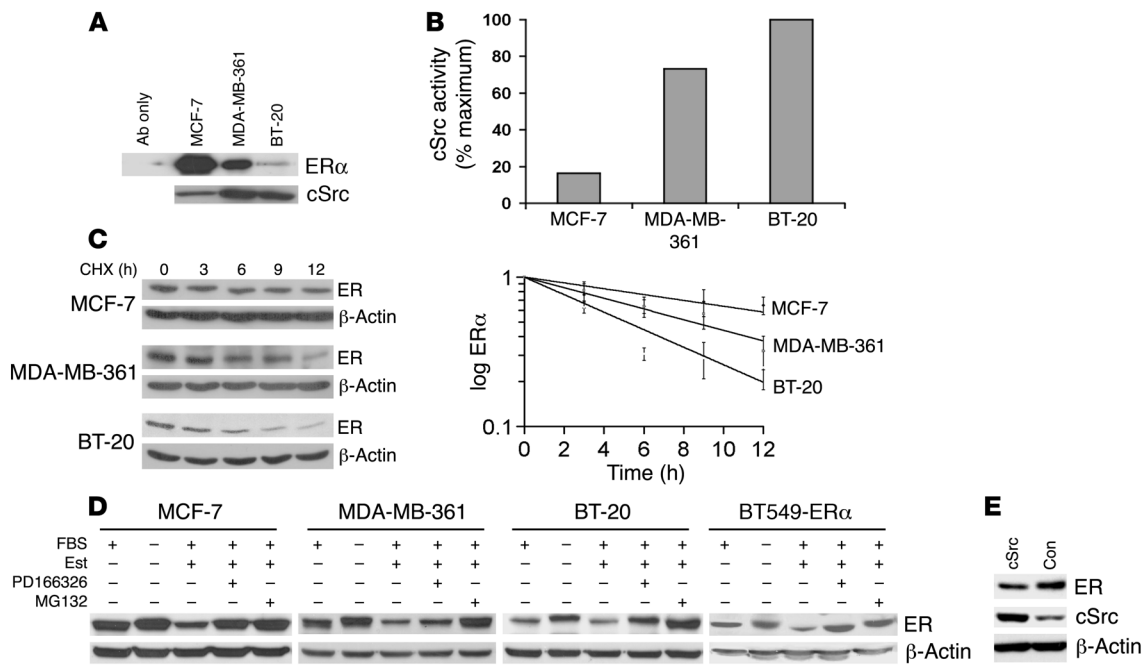


Figure 4

Estrogen regulation of ER α levels in ER α ⁺ and ER α ⁻ breast cancer lines. (A) ER α was detected by immunoblotting ER α precipitates from 1 mg cell lysate of asynchronous ER α ⁺ MCF-7, MDA-MB-361, and ER α ⁻BT-20 cells. (B) cSrc activity in asynchronous MCF-7, MDA-MB-361, and BT-20 cells. (C) ER α $t_{1/2}$ in asynchronous cells as assayed by CHX chase, calculated from 3 independent assays (mean \pm SEM). (D) After 48 hours serum and estrogen deprivation in 0.1% cFBS, MCF-7, MDA-MB-361, BT-20, and BT549-ER α cells were stimulated with estradiol plus 5% FBS with or without prior addition of MG132 or Src inhibitor PD166326, and ER α was assayed 6 hours later. (E) BT-20 cells were transfected with siRNA to cSrc or nonspecific control siRNA (Con) and deprived of estrogen for 48 hours. Cells were then treated with estradiol for 4 hours prior to Western blot analysis of cSrc and ER α levels.

and ER α ⁻ (BT-20 and BT549-ER α) lines, ER α levels increased with estrogen deprivation. Estradiol-stimulated ER α loss was impaired by proteasome inhibition with MG132 and also by Src inhibition with PD166326 (Figure 4D). For BT-20 cells, blots were exposed longer and more protein was loaded than for ER α ⁺ lines.

Because PD166326 inhibited Src and Src family kinases, we further tested the specific role of Src in ER α regulation by transfecting BT-20 cells with either siRNA to Src or control siRNA and then depriving them of estrogen for 48 hours. Reduction of Src expression by Src siRNA impaired estrogen-stimulated ER α loss in BT-20 cells (Figure 4E). Thus Src appears to activate estrogen-stimulated ER α proteolysis in both ER α ⁺ and ER α ⁻ breast lines.

Src inhibition impairs estrogen-stimulated ER α ubiquitylation in vivo. To test the effect of Src inhibition on ligand-driven ER α ubiquitylation, MCF-7 cells were estrogen deprived and then stimulated with estradiol with or without prior addition of the proteasome inhibitor MG132 or the Src inhibitor PP1. The ER α was immunoprecipitated from equal amounts of protein lysate, and complexes were resolved, immunoblotted with anti-ubiquitin antibody, and stripped and reprobed for total ER α . ER α levels were maximal in estrogen-deprived cells. Although ER α levels were reduced 6 hours after estradiol stimulation, detection of ER α ubiquitylation was modestly increased. When estrogen-deprived cells were treated with estradiol and MG132, the ER α protein level remained elevated, and ubiquitylated ER α was readily detected (Figure 5A). In contrast, while Src inhibition with PP1 prevented estrogen-stimulated loss of the ER α protein and maintained high

ER α protein levels, ER α ubiquitylation was minimal (Figure 5A). Thus, Src inhibition impaired ligand-activated ER α ubiquitylation and prevented ligand-mediated loss of ER α .

Src activates ER α ubiquitylation and degradation in vitro. To assay the effect of Src on ER α ubiquitylation and degradation in vitro, recombinant ER α was pretreated with Src kinase or mock treated, after which equal amounts of ER α were reacted with recombinant ubiquitin, ubiquitin-activating enzyme (E1), UbcH7 (E2), and E3 ubiquitin ligase supplied from asynchronous MCF-7 cell lysate. ER α was then precipitated and resolved, and ubiquitylated ER α was detected by immunoblotting with anti-ubiquitin antibody. In these assays, little ER α degradation occurred. Tyrosine phosphorylation of ER α was detected only in Src-treated samples. ER α ubiquitylation was enhanced by pre-treatment of the ER α with Src kinase (Figure 5B).

For ER α degradation, assay conditions were modified as described in Methods. Recombinant ER α was pretreated or not with Src kinase as above and then treated with the E1, E2, and E3 mixture with or without addition of 26S proteasome fraction. ER α degradation was minimal in assays with Src or 26S proteasome alone. When Src-pretreated ER α was incubated with E1, E2, and E3 together with 26S proteasome, ER α was completely degraded (Figure 5C).

cSrc is activated in ER α ⁻ primary breast cancers. cSrc kinase activity was assayed in lysates from 18 ER α ⁻ and 22 ER α ⁺ primary human breast cancers. The ER α status determined at diagnosis by LBA was verified by ER α immunoblotting. Blotting with β -actin verified equal loading and equal protein input in cSrc kinase assays. Elevated cSrc activity was observed in 78% (14 of 18) of ER α ⁻ breast

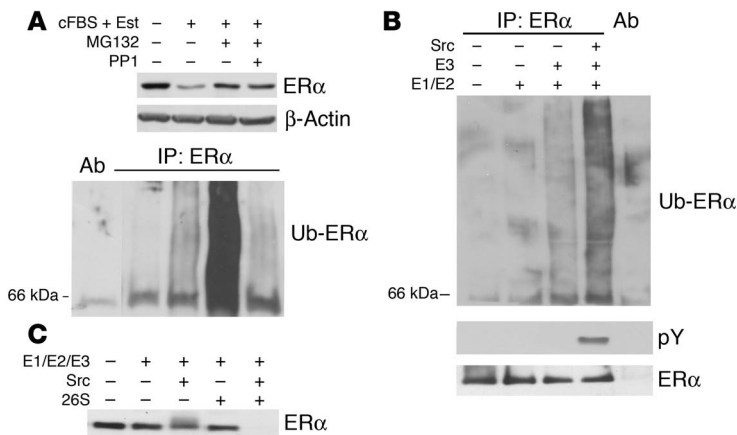


Figure 5 Src stimulates ER α ubiquitylation and degradation in vivo and in vitro. **(A)** Serum- and estrogen-deprived MCF-7 cells were treated with estradiol and 5% cFBS for 6 hours with or without immediate prior addition of MG132 or PP1, and ER α levels were assayed. Equal loading was confirmed by β -actin. ER α was precipitated, ER α complexes were resolved, and ubiquitylated ER α (Ub-ER α) was detected with anti-ubiquitin antibody. **(B)** For in vitro ER α ubiquitylation, recombinant ER α protein was reacted with E1 and E2, with or without E3 and with or without prior treatment of ER α with Src kinase (as described in Methods), for 60 minutes. ER α immunoprecipitates were resolved and blotted with anti-ubiquitin or anti-phosphotyrosine (pY) antibodies. The membrane was stripped and reprobed for ER α . **(C)** In vitro degradation of recombinant ER α used E1, E2, and E3 with or without prior incubation with Src and/or addition of 26S proteasome fraction as described in Methods.

cancers. In contrast, only 18% (4 of 22) of ER α^+ tumors showed Src activity above that of nonspecific antibody controls (Figure 6A).

Src and ER α levels are inversely correlated in primary human breast cancers. To extend these findings, we quantitated ER α and Src protein expression by reverse phase tissue lysate array (RPPA) in 101 primary breast cancers using validated monospecific antibodies previously demonstrated to reflect Western blotting results with multiple tumor samples, providing a high-throughput quantitative analysis (50). Of 98 tumors in which the HR status was known, 68 were classified as positive for ER α and/or progesterone receptor (PR) by immunohistochemistry in pathology evaluation at diagnosis. ER α quantified by RPPA was significantly higher in pathologic ER α^+ breast cancers ($P < 0.001$), as expected. Src protein was significantly higher in pathologic ER α^- and PR $^-$ ($P = 0.03$) than in HR $^+$ tumors (ER α^+ and/or PR $^+$). The distribution of Src values in HR $^+$ and HR $^-$ cancers is shown in Figure 6B. When 68 HR $^+$ tumors were compared with 23 “triple receptor-negative” tumors (ER α^- and PR $^-$ by immunohistochemistry; HER2 $^-$ by FISH), Src levels were highest in triple receptor-negative tumors ($P = 0.02$). In all tumors, quantified ER α and Src expression were inversely correlated ($r = 0.26$, $P = 0.008$; Figure 6C). In the subset of 68 HR $^+$ tumors, there was also a statistically significant inverse correlation between quantified expression of ER α and Src ($r = 0.30$; $P = 0.01$).

Discussion

ER α^- breast cancers have distinct gene expression profiles and are clinically more aggressive than are ER α^+ cancers (12). The present study supports the hypothesis that, at least in a subset of ER α^- breast cancers, Src activation may drive estrogen-dependent ER α proteolysis. *ESR1* gene alterations are too infrequent to explain

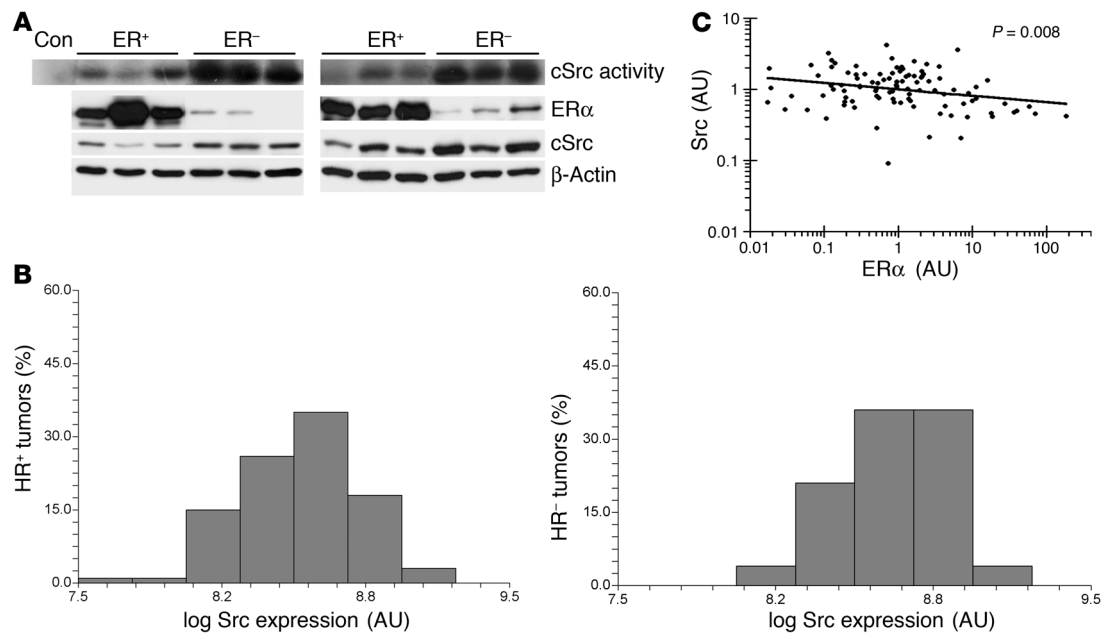
the lack of detectable ER α protein in up to one-third of breast cancers (6, 7). Early studies indicated that as many as 60%–70% of ER α^- tumors express *ESR1* mRNA (8–10). More sensitive QPCR demonstrated *ESR1* mRNA in all of 52 ER α^- primary breast cancers (51). In the present analysis, all of 200 ER α^- breast cancers expressed *ESR1* mRNA, with considerable variability and overlap in values in ER α^+ and ER α^- cancers. While mean *ESR1* mRNA concentrations did not differ significantly between ER α^+ and ER α^- cancers, the modal distribution of *ESR1* mRNA concentrations was lower in ER α^- cancers. This is consistent with results from microarray studies that compared individual tumor *ESR1* mRNA to reference cRNA pooled from ER α^+ and ER α^- tumors (11) or to the average signal from all tumors (12, 13) to reveal lower average *ESR1* gene expression in ER α^- versus ER α^+ cancers. The variability in *ESR1* mRNA levels and the discordance observed between *ESR1* mRNA and protein in both tumor types point to important posttranscriptional controls of ER α levels.

Up to one-third of primary breast cancers show *HER2/erbB-2* amplification, and a similar proportion has increased EGFR expression. Both are strongly associated with ER α^- status (52, 53). Transfection of either *EGFR* or activated *Her2* can reduce ER α levels in MCF-7 cells, and this has been attributed to MAPK activation (54). However, both of these receptors activate Src. In breast cancer cells, cSrc binds phosphorylated Her2 or EGFR, promoting synergistic activation to stimulate breast cancer cell proliferation and survival (49). Indeed, Src inhibitors impair Her2- and EGFR-driven mitogenesis (49, 55). Src is also transiently recruited to and activated by estrogen-bound ER α , leading to MAPK activation (22–25).

Src can phosphorylate ER α in vitro (56, 57). ER α phosphorylation by Src increases its affinity for estrogen (27), and may also affect ER α -coactivator binding and transcriptional activity (58, 59). The present study indicates that Src can drive expression of certain ER α target genes, which suggests the presence of an important feed-forward signaling loop involving estrogen, the ER α , and Src.

Crosstalk between liganded ER α and Src may not only regulate ER α transcriptional activity, but also activate ER α proteolysis. Inhibition of cellular Src impaired estrogen-mediated ER α ubiquitylation and ER α loss. Induced Src expression increased *pS2* and *GREB1* expression and ligand-activated ER α proteolysis. In breast cancer lines, increased Src activity correlated with a shortened ER α $t_{1/2}$. In ER α^+ as well as ER α^- lines, proteasome inhibition increased ER α protein levels. Moreover, in both ER α^+ and ER α^- lines, estrogen withdrawal increased ER α levels and estrogen-stimulated ER α loss was impaired by Src inhibition. Because the Src inhibitor drug used affects not only Src, but other Src family kinases, we tested the effect of specific Src siRNA on ER α levels in the BT-20 line. Downregulation of cSrc expression using siRNA reduced estrogen-stimulated ER α loss in BT-20 cells. While we cannot exclude a possible contribution of other Src family kinases to estrogen-driven ER α proteolysis, this Src siRNA data supports a role for Src itself in this action.

Src kinase assays showed cSrc activation in a majority of primary ER α^- tumors in a relatively small primary tumor set. In a larger group of over 100 primary breast cancers, Src protein levels correlated inversely with ER α in both ER α^+ and ER α^- tumors, as assayed by sensitive RPPA. ER α^- cancers had higher Src levels than did ER α^+ cancers, and this inverse statistical association was stronger

**Figure 6**

Elevation of cSrc activity and/or levels in ER α ⁻ primary breast cancers. **(A)** Cryopreserved breast tumors were lysed, ER α status was verified by Western blot, and cSrc kinase activity was assayed. Equal loading was confirmed by β -actin. Blots are representative of 22 ER α ⁺ and 18 ER α ⁻ tumors. **(B and C)** ER α and Src protein levels were quantitated by RPPA in 101 primary breast cancers as described in Methods. **(B)** Histogram distribution of log Src protein levels (arbitrary units) rounded to nearest log for HR⁺ (ER α ⁺ and/or PR⁺) and HR⁻ (ER α ⁻ and PR⁻) cancers. **(C)** Dot plot of Src and ER α protein values (expressed as log values, arbitrary units) in all cancers ($P = 0.008$).

in the subset of triple-negative compared with ER α ⁺ tumors. These findings are consistent with our recent analysis of over 700 primary cancers in which the ER α ⁻ status correlated significantly ($P < 0.001$) with Src activation as detected by immunohistochemical staining for Y416-phosphorylated Src (A. Arnaout and J.M. Slingerland, unpublished observations). Although our data indicate that Src contributes to ER α regulation in breast cancers, there are clearly a number of tumors with high Src levels that retain ER α protein as well as tumors with low ER α levels that do not have high Src levels or activity. Thus, additional Src-independent mechanisms may regulate ER α protein levels. Tumors with very low *ESR1* mRNA levels may reflect ER α promoter methylation (6).

cSrc appears to promote the ubiquitylation of ER α since cSrc inhibition impaired cellular ER α ubiquitylation and proteolysis in vivo. Moreover, ER α phosphorylation by Src increased both ER α ubiquitylation and 26S proteasome-mediated ER α degradation in vitro. These data support a model in which liganded ER α recruits cSrc or cSrc-dependent kinases, leading to phosphorylation events that facilitate ER α binding to coactivators and/or components of the proteolytic machinery. Ligand- and Src-activated ER α ubiquitylation may be linked to transcriptional activation of a subset of ER α -regulated genes. While our in vitro data support a direct effect, with Src phosphorylation of ER α promoting its ubiquitin-dependent degradation, Src may also have indirect effects to promote ER α degradation. Src or its downstream effectors may also affect ligand-activated ER α coactivator phosphorylation to regulate ER α degradation and transcriptional activity. SRC-3/AIB1 proteolysis accompanies estrogen-stimulated ER α activation (60). How specific Src-dependent ER α and/or coactivator phosphorylation events modulate the profile of coactivator binding, ERE

selection, and ER α proteolysis will require further investigation. A recent report suggests that Src-mediated tyrosine phosphorylation may also regulate androgen receptor function (61).

Signaling pathways that activate many transcription factors, including c-Jun, c-Myc, and E2F-1, also trigger their ubiquitin-dependent degradation (48), thereby limiting transactivator function. Ubiquitylation is required for transcriptional activity of certain transcription factors (48, 62). Transcription factor ubiquitylation may influence coactivator/repressor binding (48), with coactivators subsequently enhancing ubiquitylation of certain transcription factors (63). Ligand-mediated proteolysis regulates turnover of most nuclear receptors (reviewed in ref. 64). Several ER α coactivators are also known to be ubiquitin ligases (43–45) or proteasome subunits (65).

In some models (40, 41), but not all (66, 67), proteasome inhibition decreases estrogen-ER α transcriptional activity despite an increase in ER α abundance. *ESR1* mutations that impair coactivator binding abrogate ligand-stimulated ER α degradation (41). Thus, co-activator binding may regulate not only transcriptional activity but also ligand mediated ER α degradation. ER α cycles on and off the ERE (40, 68). Ligand increases the duration of ER α -ERE binding and modifies ubiquitin ligase binding (40). Proteasome inhibition has been shown to dissociate ubiquitin-bound ER α from ERE motifs and reduce ER α transcriptional activity. Thus, for a subset of ER α -driven genes, ER α ubiquitylation and transcription may be closely linked.

Cell type- and promoter-specific differences affect how ER α proteolysis influences target gene expression (66, 67). In one study, proteasome inhibition increased expression of cellular *pS2* and *CTSD* but decreased *PR* expression (67). In certain promoter



contexts, ligand-activated ER α may escape ubiquitylation and proteasomal degradation and yet remain functional. While proteolytic degradation of the ER α after ERE firing may allow reloading of the promoter, ubiquitylation and proteasomal degradation may potentially serve a more global role in regulating the abundance and overall activity of the ER α . Moreover, constitutive ER α activation could potentially lead to reduced ER α levels as a result of constitutive ER α proteolysis.

ER α phosphorylation by different signaling pathways could theoretically promote recruitment of different coactivators or ubiquitin pathway components, changing both the profile of ER α targets expressed and the rate of ligand-stimulated ER α proteolysis in different tissues. During breast cancer progression, Src activation may alter coactivator binding, shifting ER α transcriptional targets to profiles that promote oncogenic change. The present data do not allow us to estimate the contribution of Src-mediated ER α transcriptional activity and degradation to the overall oncogenic effect of Src in breast cancer. While we speculate that the effects of Src on ER α signaling crosstalk and transcriptional activity may make an important contribution to its oncogenicity, further work is required to tease out the specific contribution to breast carcinogenesis of Src acting on ER α .

ER α ⁻ breast cell lines are considered estrogen insensitive because they do not require estrogen for growth; coupled with the clinical observation that ER α ⁻ breast cancers do not respond to tamoxifen (2), this led to the belief that ER α ⁻ tumors are estrogen independent. Our data raise the concern that at least a subset of ER α ⁻ breast cancers, particularly those with oncogenic Src activation, may indeed be responsive to estrogen *in vivo*. Constitutive ER α proteolysis in at least a subset of ER α ⁻ cancers may not reflect extinguished ER α -dependent transcription, but rather indicate a shift to constitutive activation of different ER α transcriptional targets. The therapeutic implications of this work are potentially very significant and warrant further investigation.

Methods

Breast cancers used for *ESR1* mRNA quantitation. Cryopreserved primary invasive human breast cancers were obtained from the tumor repository of the Sunnybrook Health Sciences Center clinical ER α quantitation reference lab with approval from the Sunnybrook Health Sciences Center Review Ethics Board, Toronto, Ontario, Canada. One expert clinical biochemist performed all ER α cytosolic LBA (69). Concordance of ER α LBA with ER α immunohistochemistry was verified in 40 tumors.

RNA extraction and *ESR1* mRNA quantitation. mRNA was extracted from 300 macrodissected carcinomas (100 g) using TRIzol per the manufacturer's instructions (Molecular Research Center). All RNAs were visualized on ethidium gels. A total of 250 tumor RNA samples with an OD₂₆₀/OD₂₈₀ greater than 1.3 and less than 2.1 from 50 ER α ⁺ cancers (>30 fmol/ μ g protein by LBA) and 200 ER α ⁻ cancers (<10 fmol/ μ g protein by LBA) were analyzed. QPCR of human *PBGD* expression using the LightCycler hPBGD Housekeeping Gene Kit (Roche Applied Science) primer/hybridization mixture demonstrated similar expression and equal RNA quality in both groups (Student's *t* test).

QPCR reactions used the LightCycler System (Roche Applied Science) and the QuantiTect SYBR Green RT-PCR kit (QIAGEN). Primers are listed in Supplemental Methods. A standard curve for *ESR1* mRNA quantitation was generated using serial dilutions of full-length human ER α cDNA plasmid PCMV5hER- α (provided by B. Katzenellenbogen, University of Illinois, Urbana, Illinois, USA). MCF-7 *ESR1* mRNA was quantitated using the PCMV5hER- α plasmid standard curve. MCF-7 mRNA was run as a positive

control in all tumor *ESR1* mRNA QPCR reactions. Tumor *ESR1* mRNA values ranged from 10 fg/ μ l to 1 μ g/ μ l. Melting curve analysis ensured exclusion of primer dimers from each analysis. *ESR1* mRNA concentrations in ER α ⁺ and ER α ⁻ cancers were compared by Student's *t* test.

Sequencing of ER α cDNA PCR product. All tumor ER α PCR products were visualized by gel electrophoresis. For a subset, PCR-amplified ER α cDNA was gel extracted with QIAquick Gel Extraction Kit (QIAGEN), and 10 ng DNA was sequenced using 3.2 pmol of each ER α sequencing primer, Terminator Reaction Mix (ABI Prism dGTP BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit; Applied Biosystems), and the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Cell culture. MCF-7 cells were grown in 5% FBS, and estrogen was depleted in 5% cFBS for 48 hours as described previously (70). For depletion of both growth factors and estrogen, cells were transferred to 0.1% cFBS for 48 hours. The ER α ⁻ BT-20 and BT549 cell lines and the weakly ER α ⁺ MDA-MB-361 cell line (provided by S. Parsons) were grown in DMEM (49). The identity of ER α ⁺ and ER α ⁻ lines was confirmed by karyotyping. To assay effects of growth factors on ER α levels, 10 nM β -estradiol with or without 5% FBS or 5% cFBS alone was added to MCF-7 cells that had been estrogen and growth factor depleted for 48 hours; ER α levels were assayed 1–6 hours later.

Plasmids and transfection. Activated human cSrc vector, PCI-Src Y530F (from D. Fujita, University of Calgary, Calgary, Alberta, Canada) or empty PCI (10 μ g) was transfected into MCF-7 cells using lipofectamine PLUSTM (Invitrogen). BT549 cells were transfected with PCMV5hER- α , and stable lines were cloned.

Construction of MCF-7 lines with inducible Src expression. Src Y530F cDNA was cloned into pIND and transfected into the MCF-7 line with an integrated pVgRXR vector (Invitrogen). Src was induced with 2 μ M PA. MCFpINDSrc2 cells had high Src induction 8–24 hours after induction with PA. This line was estrogen deprived as described above for 72 hours, and 2 μ M PA was added or not for the last 24 hours of estrogen deprivation. Cells were then transferred to 0.1% cFBS, 10 nM estradiol was added for 6 hours, and ER α *t*_{1/2} was assayed by cycloheximide (CHX) chase as described below.

Flow cytometric analysis. BrdU pulse labeling and flow cytometric analysis were performed as described previously (70).

Antibodies. The ER α mAb H222 was supplied by G. Greene (University of Chicago, Chicago, Illinois, USA), ER α antibody HC-20 and anti-ubiquitin antibody P4D1 were from Santa Cruz Biotechnology Inc., and anti-Src mAb GD11 was from Upstate Biotechnology. Antibodies to MAPK, phosphorylated MAPK, total PKB, and activated PKB as well as anti-phosphotyrosine antibody P-tyr-102 were from Cell Signaling; antibody to β -actin was from Sigma-Aldrich.

Immunoblotting and CHX chase. Cells were lysed in ice-cold D/RB buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8.0; 2.5 mM EGTA, pH 8.0; 10% glycerol; 10 mM β -glycerophosphate; 1 mM NaF; 0.1% Tween-20; 1 mM PMSF; 0.1 mM Na₂VO₄; 0.5 mM DTT; and 0.02 mg/ml each of aprotinin, leupepsin, and pepstatin). Protein was quantitated by Bradford protein assay. Western blots used 20–100 μ g protein per lane. The ER α *t*_{1/2} was determined by CHX chase, with addition of 100 μ g CHX considered *t* = 0. Cells were lysed at the times indicated in Figures 2 and 4, and ER α was blotted. ER α protein was quantitated by densitometry from 3 experiments using ImageQuant imaging system (version 5.2; GE Healthcare).

Effects of MEK and PI3K inhibition on ER α stability. To assay effects of MEK or PI3K inhibition on ER α levels, increasing concentrations of UO126 (0.1–10 μ M; Promega) or LY294002 (0.5–8 μ M; Promega) were added to asynchronous MCF-7 cells for 48 hours prior to immunoblotting or flow cytometry. Estrogen- and growth factor-depleted MCF-7 cells were treated with either 10 μ M UO126 or 8 μ M LY294002 for 30 minutes prior



to stimulation with 17 β -estradiol for 6 hours, followed by immunoblotting and flow cytometry.

Cellular Src kinase assays. Cell lines or primary human breast cancers were lysed in ice-cold NP40 lysis buffer (70) with added 0.1 mM Na₂VO₄ and 1 mM EDTA (pH 8.0). Src was precipitated from 200 μ g lysate, and Src kinase was assayed as described previously (71).

ERE luciferase assays. MCF-7 cells were transfected with 500 ng of plasmid bearing 2 tandem ERE (2 \times ERE luc), 50 ng phRL-TK luc, and 100 ng cSrc-Y530F using Lipofectamine Plus (Invitrogen) per the manufacturer's instructions. Cells were treated with 10 μ M PP1 and/or 10 nM estradiol for 4 hours prior to luciferase assays using dual-luciferase reporter assays (Promega) and Beckman Coulter LD400 Luminescence Detector.

QPCR of ER α target genes pS2 and GREB1. MCFpINDSrc2 cells were maintained in 5% cFBS for 2 days before adding 2 μ M PA for 24 hours to induce Src. The cells – with and without Src induction – were then treated with either 10 nM β -estradiol or 100 nM tamoxifen plus estradiol for 3 hours. Total RNA was isolated using TRIzol according to the manufacturer's instructions (Invitrogen). cDNA synthesis was performed with 1 μ g total RNA using iScript cDNA kit (Bio-Rad). QPCR was performed using icyclerIQ PCR detection system (Bio-Rad) with 10 ng cDNA sample in iQ SyberGreen supermix (Bio-Rad). PCR conditions and primers are described in Supplemental Methods.

siRNA-mediated inhibition of cSrc expression. Dharmacon ON-TARGET plus SMARTpool siRNA reagent (Thermo Fisher Scientific) targeting cSrc and siCONTROL Non-Targeting siRNA Pools (Dharmacon; Thermo Fisher Scientific) were transfected into BT-20 cells cultured in media with 5% FBS using Lipofectamine 2000 (Invitrogen) for 4 hours. The cells were then estrogen deprived by transfer to media containing 5% cFBS for 48 hours. Cells were then treated with estradiol or not for 4 hours prior to lysis for analysis of ER α and Src levels by Western blotting.

Detection of ER α ubiquitylation in vivo. MCF-7 cells were starved in 0.1% cFBS for 48 hours and then either maintained in 0.1% cFBS or transferred to 5% cFBS medium with 10 nM estradiol with or without 10 μ M PP1 or 10 μ M MG132. Six hours after estradiol addition, cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 2 mM EDTA, 50 mM NaF, 1% NP40, and 1% SDS; boiled for 10 minutes; and centrifuged for 1 minute at 14,000 g at 22°C. Supernatant protein was quantitated, and ER α and β -actin were assayed by Western blot. To detect ubiquitylated ER α , ER α was immunoprecipitated from 500 μ g lysate, resolved by SDS-PAGE, and transferred to nitrocellulose (Bio-Rad). The membrane was boiled in transfer buffer for 10 minutes and immunoblotted with antibody against ER α or ubiquitin.

In vitro ER α ubiquitylation assay. Ubiquitylation assays used 40 ng recombinant ER α (Sigma-Aldrich), GST-ubiquitin-activating enzyme (E1), GST-ubiquitin-conjugating enzyme UbcH7 (E2), MCF-7 lysate (50 μ g) as E3 source, and an energy regenerating solution (Boston Biochem) in 7.4 mM HEPES (pH 7.4), 5 mM KCl, and 1.5 mM MgCl₂ for 60 minutes at 37°C. Prior to ubiquitylation assays, recombinant ER α was either incubated with 10 ng recombinant Src kinase (Upstate) or mock-treated for 5 minutes at 30°C in 7.4 mM HEPES (pH 7.4), 5 mM KCl, and 1.5 mM MgCl₂. ER α was precipitated, and complexes were resolved and transferred to nitrocellulose (0.45 μ m; Bio-Rad). The membrane was boiled for 10 minutes, and ER α and ubiquitylated ER α were detected as described above.

In vitro ER α degradation assay. ER α degradation assays used E1, E2, and E3 as described above, with the following modifications. To catalyze in vitro degradation of ER α , 50 nM of 26S proteasome fraction (Boston Biochem) was added for 30 minutes at 37°C in 7.4 mM HEPES (pH 7.4), 5 mM KCl, 1.5 mM MgCl₂, and 1 mM DTT. ER α was assayed by Western blot.

RPPA. A total of 101 fresh-frozen primary breast tumors from the University of Texas MD Anderson Cancer Center Breast Tissue Tumor Bank were obtained with approval of the Institutional Review Board of the University of Texas MD Anderson Cancer Center. Tumors were macrodissected and lysed as described previously (50) and boiled in 1% SDS, and protein-rich supernatants were serially diluted manually. A robotic GeneTAC arrayer (Genomic Solutions) created arrays of 6 2-fold serial dilutions for each tumor lysate on nitrocellulose-coated glass slides (FAST Slides; Schleicher & Schuell). Arrayed slides were probed with ER α antibody (NeoMarkers) and Src (Upstate), and the signal was amplified using a Dako-Cytomation catalyzed system. A secondary antibody (anti-rabbit) was used as a starting point for signal amplification. The slides were scanned and each protein in each sample was assigned a relative quantification value in arbitrary units using MicroVigene software (version 2.0; Vigene Tech). All samples were normalized for protein loading as described previously (50, 72). NCSS software (2004 version; NCSS) was used for 2-tailed Student's *t* tests and canonical correlation. ER α and/or PR were assayed by immunohistochemistry in pathology evaluation at diagnosis.

Statistics. Differences in *ESR1* and *PBGD* mRNA, quantitated by QPCR in the fresh frozen ER α ⁺ and ER α ⁻ breast cancer samples, were analyzed statistically using the Student's *t* test. The relationship between Src kinase activity and ER α protein status (positive or negative) in 40 primary breast cancers was determined using the Student's *t* test. For the RPPA analysis, the arbitrary units expressing ER α and Src protein levels were converted to logarithms and the relationship between ER α and Src protein levels was analyzed using 2-tailed Student's *t* tests and canonical correlation. *P* values of less than 0.05 were considered statistically significant.

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