Resistance to anti-estrogen arzoxifene is mediated by overexpression of Cyclin D1
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Abstract

Resistance to tamoxifen treatment occurs in approximately 50% of the ERα positive breast cancer patients. Resistant patients would benefit from treatment with other available anti-estrogens. In this study, we show that overexpression of a regular component of the ERα transcription factor complex, cyclin D1, which occurs in approximately 40% of breast cancer patients, renders cells resistant to a new promising anti-estrogen arzoxifene. Arzoxifene is an effective growth inhibitor of ERα positive breast cancer cells, including tamoxifen resistant tumors. Overexpression of cyclin D1 alters the conformation of ERα in the presence of arzoxifene. In this altered conformation, ERα still recruits RNA polymerase II to an ERE-containing promoter, inducing transcription of an ERα-dependent reporter gene and of endogenous pS2, and promoting arzoxifene-stimulated growth of MCF-7 cells. By this, arzoxifene is converted from an ERα-antagonist into an agonist. This is explained by a stabilization of the ERα/SRC-1 complex in the presence of arzoxifene, only when cyclin D1 is overexpressed. These results indicate that subtle changes in the conformation of ERα upon binding to anti-estrogen are at the basis of resistance to anti-estrogens.

Introduction

Estrogen receptor alpha (ERα) positive breast cancer patients are commonly treated with either aromatase inhibitors or anti-estrogens. The clinically most frequently applied anti-estrogen, tamoxifen, leads in an adjuvant setting to an approximately 50% reduction in recurrence during 10 years of follow-up of ER-positive patients, and to a decrease in mortality by a third. Tamoxifen resistance is, however, still a major clinical problem in the treatment of breast cancer. Various mechanisms may account for insensitivity to tamoxifen, including activation of the mitogen-activated protein kinase (MAPK), protein kinase A (PKA) and p21-activated kinase-1 (PAK1) signaling pathways that show enhanced activity in tamoxifen-resistant breast tumors (1-3). All of these kinase activities directly phosphorylate ERα, resulting in anti-estrogen resistance. In the case of PKA- and MAPK-mediated resistance, tamoxifen binds but then fails to induce the inactive conformation of ERα (1;4). For PKA, we found that its activity alters the orientation between ERα and steroid receptor coactivator-1 (SRC-1) in the presence of tamoxifen, which ultimately leads to RNA polymerase II recruitment and subsequently, to ERα-dependent transactivation instead (5).

Next to phosphorylation events on the ERα, expression levels and/or phosphorylation of cofactors such as SRC-1 (6) and SRC-3 (7;8) are reported to be associated with anti-estrogen resistance or tamoxifen non-responsiveness. Moreover, amplification of 11q13, a gene-rich region on Chromosome 11, that is found in approximately 15% of the primary human breast cancers (9-11), is associated with tamoxifen resistance. Among the genes present in this region are PAK1 and cyclin D1. PAK1 overexpression is correlated with tamoxifen resistance in patients, whereas cyclin D1 overexpression by itself fails to do so (3;12), nor did it enhance cell growth in tissue culture experiments under tamoxifen conditions (13). Cyclin D1 enhances ligand-independent activation of ERα activity (14;15). The cyclin D1 protein is overexpressed in approximately 40% of human breast cancers, yet it does not indicate a poor prognosis in ERα-positive cases (16;17).

New anti-estrogens are continuously generated and tested for clinical application. These anti-estrogens can be subdivided in Selective Estrogen Receptor Modulators (SERMs), such as tamoxifen, and Selective Estrogen Receptor Downregulators (SERDs), such as ICI-182,780 (Fulvestrant), whereas anti-estrogen GW5638 has mixed SERM/SERD properties (18). New compounds may then fall in one of these classes. Outcome to treatment and resistance to anti-estrogens

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are unpredictable parameters. Many factors may affect clinical response to new anti-estrogens, including overexpression of cyclin D1 and/or SRC-1 in possible conjunction with activation of PKA and or MAPK.

In the present study, we found that SRC-1 overexpression did not influence efficacy of anti-estrogens to inhibit ERα. Overexpression of cyclin D1, however, resulted in a conformational arrest of ERα, which indicated an activated ERα, but now in the presence of GW5638 and arzoxifene, two novel anti-estrogens that are at present in phase III clinical testing. In this conformation, the ERα was capable of recruiting RNA Polymerase II and inducing chromatin remodeling required for initiation of transcription. Whereas these events eventually enhanced ERα driven transcription and tumor cell growth by arzoxifene, the breakdown of ERα by GW5638 prevented these long-term effects. Arzoxifene resistance resulted from stabilization of the cyclin D1:ERα/SRC-1 complex. Cyclin D1 overexpression did not affect the capacity of the other anti-estrogens tested to inhibit ERα. Other modifications of ERα were required for induction of resistance by these anti-estrogens. These findings illustrate that estrogen receptor degradation is a dominant factor over a resistance-associated conformation. Moreover, they define the frequently overexpressed cofactor cyclin D1 as a causal factor in resistance to particular anti-estrogens, and show that subtle changes in the conformation of ERα are at the basis of resistance to anti-estrogens.

Results

Overexpression of cyclin D1/SRC-1 induces resistance to anti-estrogens as measured by FRET

We determined the effect of overexpression of cyclin D1 and/or SRC-1 on the ability of a subset of anti-estrogens to inhibit transactivation of ERα. The anti-estrogens used in this study and their structure are depicted in Figure 1. They differ widely in biological effects in vitro and in vivo (19;20). These compounds were tested in U2OS osteosarcoma cells, a highly suitable cell line for testing activation status of ERα and ERβ (1;21), that are themselves devoid of expression of endogenous ER and did not show apparent overexpression of any of the p160 coactivators when related to other cell lines (22-25). As a first indication for the capacity of the tested anti-estrogens to inactivate ERα, we applied a Fluorescence Resonance Energy Transfer (FRET) assay. FRET is the energy transfer from a donor fluorophore to a suitable acceptor. When the two fluorophores come in close enough proximity, typically less than 80 Å, energy can be transferred from the CFP to the YFP fluorophore. This depends on the orientation and distance between the two fluorophores and intramolecular FRET is thus highly sensitive to conformational changes. In our setup, an YFP fluorophore was fused to the N-terminus and a CFP to the C-terminus of ERα, as illustrated in Figure 2A, left.
panel. An inactive conformation of ERα is typically associated with an increase in FRET between the CFP and YFP fluorophores, as we reported previously (1:4). This enabled us to monitor the capacity of anti-estrogens to inactivate ERα and their sensitivity to overexpression of cyclin D1 and SRC-1.

To perform these experiments in a semi-high throughput manner, we applied Fluorescence Lifetime Imaging Microscopy (FLIM), a highly quantitative method for measuring FRET. In FLIM, the lifetime of the donor fluorophore emission is measured which is for the donor CFP typically 2.7 ns (26). This lifetime is reduced when the energy of the donor is transferred to the acceptor fluorophore in the case of FRET (27).

In our assays, cells expressing YFP-ERα-CFP were cocultured with cells only expressing CFP, in order to provide an internal control for the FLIM measurements, Figure 2A, right panel. After addition of anti-estrogen ICI-182,780, the lifetime of CFP in the YFP-ERα-CFP expressing cells was reduced from 2.5 ns to 2.1 ns after treatment, indicating a donor FRET efficiency of 7% prior to treatment and 22% after treatment with ICI-182,780. The same cell was monitored before and after treatment with the ligand, where the FRET efficiency after treatment was related to the efficiency before, which was set at one. The final relative donor FRET efficiency was depicted in Figure 2B. The FRET efficiency prior to treatment was unaltered by co-expression of cyclin D1 or SRC-1 (data not shown).

A significant increase in relative FRET efficiency was found for all anti-estrogens tested, Figure 2B. However, when SRC-1 and cyclin D1 were cotransfected, the relative FRET efficiency after EM-652, arzoxifene and GW5638 addition was significantly reduced. These data were verified using an alternative ratio-based FRET-approach (Supplemental Figure S1), where synergy between cyclin D1/SRC-1 overexpression and PKA activity could be observed, which had also been illustrated previously (1). When FRET was measured in cells overexpressing SRC-1 alone, no ef

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**Figure 2.** Inactivation of ERα by anti-estrogens measured by FRET.

A. left panel: Principle of FRET. Exciting CFP at 430 nm results in an emission from CFP unless energy is transferred to YFP, resulting in yellow light. An increased YFP emission at the expense of CFP emission can occur as the result of a conformational change of ERα affecting the orientation of the two fluorophores. Right panel: A representative image from a FLIM measurement before and after ICI-182,780 addition. FLIM measurements were performed in U2OS cells, transfected with YFP-ERα-CFP (arrowhead). As an internal reference, these cells were cocultured with MelJuSo cells only expressing CFP (asterisk). FLIM images were generated from cells cultured in hormone-depleted (CTS) medium only, or after treatment with 1 μM ICI-182,780 (ICI) prior to data acquisition and visualized with a lookup table with the corresponding lifetimes indicated in the bar.

B. FRET values as determined by FLIM in YFP-ERα-CFP expressing U2OS cells after addition of 1 μM of the indicated anti-estrogen. Donor FRET efficiency (ED) was calculated as ED=1- (lifetime cell of interest/ lifetime reference cell). Pair-wise analysis was performed, where the ED under CTS conditions is set to 1 for each experiment (relative ED), bars indicate mean and standard error of the mean from n=20 cells’ condition. Student T-test was performed, * indicates a statistically significant relative ED reduction as compared with the control value, p < 0.05.
fect of SRC-1 overexpression was found on the relative FRET efficiency for any of the tested anti-estrogens. Cyclin D1 overexpression, however, did result in reduced FRET efficiency for arzoxifene and GW5638, indicating that an elevated cyclin D1 level is sufficient to induce resistance against these two compounds as determined by this biophysical approach.

**Overexpression of cyclin D1 enhances recruitment of RNA Polymerase II to the ERα promoter in the presence of anti-estrogens.**

Our FRET and FLIM results suggested that resistance to anti-estrogens GW5638 and arzoxifene may be due to overexpression of cyclin D1. To determine whether this altered conformation of ERα by cyclin D1 also induces transcription in the presence of these anti-estrogens, we assayed their effects on RNA Polymerase II binding to an artificial prolactin promoter/ enhancer (PRL) array in HeLa cells. These cells contain a DNA array, consisting of a ~200 copies of stably transfected modified PRL (28). The PRL array allowed visualization of a defined DNA structure in the nucleus where ERα and cofactors are recruited to regulate reporter gene mRNA synthesis. Resistance to particular anti-estrogens by overexpression of cyclin D1 may result in anti-estrogen mediated trans-activation of ERα and consequently, to recruitment of RNA polymerase II to the PRL array. In order to ensure cyclin D1 co-expression in ERα expressing cells, five times more cyclin D1 expression vector was transfected than of ERα. This was verified in a separate staining experiment (see supplemental Figure S2), where overexpressed cyclin D1 was recruited to the PRL array structure, colocalizing with ERα. Cells expressing ERα-CFP showed co-localization with RNA-polymerase II at the DNA array when treated

![Figure 3](image)

**Figure 3.** Cyclin D1 overexpression induces RNA polymerase II recruitment on the PRL array and increases array size in the presence of GW5638 and arzoxifene.

Staining for RNA polymerase II signals on the PRL array. PRL array containing HeLa cells were transfected with ERα-CFP alone or cotransfected with cyclin D1. Cells were cultured in hormone depleted (CTS) medium and treated with 1 μM estradiol (E2) or with 1 μM of the indicated anti-estrogen for 2 hours and then fixed and stained for RNA Polymerase II. The data are subdivided in the presence or absence of RNA Polymerase II on the array, and presented in a box plot with the horizontal bar indicating the median array size in μm². The box size is determined by the upper and lower quartiles, the median value of the upper and lower half of the data points (for each experiment n > 10), respectively. Microscopic images of the cells are shown in supplemental Figure S3.
for 2 hours under conditions of charcoal treated serum (CTS; deprived of hormone) and estradiol (E2), as quantified in Figure 3 and shown in supplemental Figure S3. The average DNA array size was 1.9 μm² under CTS conditions, which increased to 2.8 μm² when E2 was added to the cells. This widening of the DNA array structure was previously reported to be associated with increased transcriptional activity (28;29). Overexpression of cyclin D1 did not significantly alter the array size or RNA Polymerase II recruitment under CTS conditions.

Addition of any of the tested anti-estrogens resulted in a loss of RNA Polymerase II from the PRL array structure, and in a marked condensation of the array size (Figure 3 and Figure S3), which is indicative of an inactive ERα (28). Cyclin D1 overexpression did not affect RNA Polymerase II recruitment for cells treated with tamoxifen, ICI-182,780 and EM-652 and the PRL array appeared condensed. However, when cyclin D1 was co-expressed, RNA Polymerase II recruitment was restored for cells treated with GW5638 and arzoxifene. In addition, the array size for both GW5638 and arzoxifene was increased from 0.7 to 2.4 μm² in the presence of exogenous cyclin D1. Our findings demonstrated that cyclin D1 overexpression prevented an increase in the FRET signal for arzoxifene and GW5638 and resulted in a recruitment of RNA Polymerase II and in a subsequent widening of the array structure. All of these features indicate that resistance to GW5638 and arzoxifene can be induced by overexpression of cyclin D1.

Overexpression of cyclin D1 enhances ERα mediated transcription and subsequent growth of MCF-7 cells in the presence of arzoxifene.

To study the direct consequences of GW5638 and arzoxifene-driven, ERα-mediated RNA polymerase II recruitment under conditions of cyclin D1 overexpression as a means of drug resistance, we measured transcription of an integrated DsRed2 reporter gene that is under control of the ER-responsive elements on the PRL array (28) by quantitative RT–PCR (Figure 4B). These cells were transfected with ERα-CFP and treated overnight with the ligand, after which RNA was isolated for RT-PCR analysis, as described in the materials and methods section. Only cells treated with estradiol showed an increase in DsRed2 mRNA levels of 2.7 fold, this in contrast to any of the anti-estrogens tested. DsRed2 mRNA levels increased under hormone deprived conditions 1.8 fold over control CTS values when cyclin D1 was overexpressed, which is consistent with the hormone-independent activation of ERα in cyclin D1 overexpressing cells (15). When cyclin D1 was overexpressed, only cells treated with arzoxifene showed increased DsRed2 mRNA levels (from 0.7 to 1.7 times over control, respectively). No enhanced transcription was observed in GW5638 treated cells, for reasons described below. Not only activation of an exogenous reporter gene, but also transcription of an ERE-dependent endogenous gene was tested, namely pS2 in MCF-7 breast cancer cells. pS2 mRNA levels were enhanced by arzoxifene when cyclin D1 was overexpressed, whereas none of the other anti-estrogens tested did so (Figure 4C). The RT-PCR data for the levels of endogenous pS2 mRNA in MCF-7 cells showed similar results as was observed the for DsRed2 mRNA reporter signals in HeLa cells (Figure 4B). These data indicated that overexpression of cyclin D1 induced resistance to arzoxifene by enhancing expression of endogenous and of exogenously transfectioned, ER-dependent genes. Since transcription efficiency of cyclin D1 mutants was approximately 40-50 % in MCF7 and Hela cells (data not shown), the results obtained in Figure 4, represented underestimates of the effects of these mutants. Although overexpression of cyclin D1 also showed resistance to GW5638 as indicated by FRET and RNA Polymerase II recruitment analysis, this did not result in elevated transcription of ER-dependent genes in the presence of GW5638. GW5638 is, however, known to possess SERD capacities, which will lead to degradation of ERα. The discrepancy between the (short term) FRET and RNA Polymerase II recruitment assays which indicated resistance, and the (long term) absence of DsRed2 and pS2 mRNA induction could be explained by GW5638-associated degradation of ERα that will then prevent specific transcription on the long term. Western blot analysis on ERα–transfected PRL-HeLa cells and MCF-7 cells expressing only endogenous ERα which were treated with the different anti-estrogens with or without additional cyclin D1 overexpression, confirmed the SERD nature of GW5638, Figure 4A. The ERα protein levels in both ERα-transfected HeLa and MCF-7 cells corresponded in a comparable way to the treatment with (anti-)estrogens, where E2 lowered and tamoxifen stabilized the protein level of ERα, as compared with untreated cells. The ERα protein levels for cells treated with GW5638 or ICI-182,780, a widely accepted SERD, were similar and showed that treatment with these compounds leads to degradation of ERα. Treatment with arzoxifene and EM-562 however, stabilized the protein level of ERα similar as for tamoxifen. Cyclin D1 overexpression had no effect on the protein level of ERα in the cells treated with the various anti-estrogens.

In order to show a biological effect of cyclin D1 overexpression on resistance to arzoxifene, we performed a cell growth assay using the MCF-7 breast tumor cell line (Figure 4D), of which the proliferation is dependent on an activated ERα. The MCF-7 cells were
therefore, transfected with various mutants of cyclin D1 or with an empty pcDNA3-YFP vector as a negative control. In comparison to wild type cyclin D1, a cyclin D1 LALA mutant that fails to stabilize ERα/SRC-1 interaction, and a cyclin D1 KE mutant that prevents its binding to cdk (cyclin dependent kinase) (30) were used. The cyclin D1 KE mutant stimulates ERα/SRC-1 dependent cell growth, whereas the cyclin D1 LALA mutant might enhance proliferation, independent of ERα activity (29). Transfection efficiency in these experiments was approximately 50% (data not shown). Therefore, any effect of the cyclin D1 overexpression is an underestimate. The cells were cultured for 10 days in the presence of the anti-estrogens and samples were taken every few days for cell counting. For each ligand, except for arzoxifene, minor effects were observed on the cell growth when cyclin D1 variants were expressed. Cells treated with arzoxifene, however, significantly increased in number when cyclin D1 wild type was transfected. The cells transfected with the cyclin D1 KE mutant, stimulating ERα/SRC-1 dependent proliferation, showed a comparable growth
response. This in contrast to the cells, transfected with cyclin D1 LALA, where the cell growth was increased, but not to the extent of the cyclin D1 variants that are capable to stabilize ERα/SRC-1 interactions. These data showed that the cyclin D1 dependent cell growth of MCF-7 cells was stimulated in the presence of arzoxifene, indicating genuine anti-estrogen resistance to this compound by overexpression of cyclin D1.

Cyclin D1 stabilizes ERα/SRC-1 complex under arzoxifene conditions. Ov ere xpress i on  o f  cy c lin  D 1  apparen tl y  fa- cilitates transcription and cell growth in the presence of arzoxifene, but the molecular mechanism for this effect is unclear. To investigate the mechanism of the arzoxifene-specific transcription under conditions of cyclin D1 overexpression, we investigated ERα/SRC-1 complex formation in the PRL array containing HeLa cells, with or without cyclin D1 overexpression, Figure 5. In nontreated cells, colocalization between ERα and SRC-1 occurred in the absence and presence of exogenous cyclin D1. In cells treated with arzoxifene, however, SRC-1 recruitment to the array was lost. But when cyclin D1 was cotransfected, both SRC-1 and cyclin D1 were recruited to the ERα-containing array structure. These data imply that, whereas arzoxifene induced dissociation of the ER/SRC-1 complex, this interaction was rescued by cyclin D1 overexpression. These results were verified by using a fluorescence recovery after photobleaching (FRAP) approach, presented in Figure S5. These results indicated that overexpression of cyclin D1 mediated resistance to arzoxifene by stabilizing the ERα/SRC-1 complex.

**Discussion**

Ligands of ERα induce specific conformations of the receptor, with alterations beyond the ligand binding domain of ERα. These altered conformations include distinctive orientations of the receptor’s carboxy-terminal transactivation helix (31), and are recognized by particular peptides which bind to distinct ERα-ligand conformations (32). A glimpse of these different conformations is revealed in X-ray crystallography studies of ERα-ligand interactions which are restricted to the AF-2 moiety of the receptor (33). We have demonstrated that these distinct ERα-ligand conformations have far reaching consequences, since they influence resistance to a particular anti-estrogen (1;4). Each anti-estrogen can be characterized by a specific set of phosphorylation sites in ERα that, when being phosphorylated by PKA and/or MAPK, are responsible for resistance to that particular anti-estrogen. These phosphorylations are responsible for an altered conformation of ERα that is measured by intramolecular FRET. In the present study, we showed that overexpression of cyclin D1, here a specific cofactor of ERα, renders ERα resistant to new 3rd generation anti-estrogens, arzoxifene and GW5638. Arzoxifene is an effective growth inhibitor of ERα positive human breast cancer cells, including tamoxifen resistant cells (34;35), and would be an alternative SERM in the clinic next to the broadly applied tamoxifen. We demonstrated that overexpression of cyclin D1, however, alters the conformation of ERα in the presence of arzoxifene (by FRET and FLIM), and showed that ERα in this altered conformation still forms a complex with SRC-1 and recruits RNA polymerase II to an ERE-containing promoter (Figures 2,3 and 5). This then promotes transcription of either endogenous or exogenously transfected, ER-dependent genes and stimulates growth of MCF-7 cells in an arzoxifene dependent manner (Figure 4). Overexpression of cyclin D1 thus converts arzoxifene from an antagonist into an agonist. This feature, among others, may have been causally involved in a significantly shorter progression-free survival and time to treatment failure of arzoxifene-treated patients as compared to tamoxifen in the treatment of locally advanced and metastatic breast cancer (36). However, no material of the breast tumors of these patients was available to study this. In previous studies, we have
overexpression of cyclin D1 is also responsible for the two short term markers of anti-estrogen resistance (a conformational change of the receptor and a recruitment of RNA Polymerase II) when the SERM/SERD compound GW5638 was used, but failed to stimulate GW5638-mediated gene expression and growth of MCF-7 cells. The SERD nature of the mixed antagonist GW5638 induced degradation of ERα, even in the presence of overexpressed cyclin D1, and prevented the late features of anti-estrogen resistance to occur (Figures 4A-D). This is comparable to the resistance found for Fulvestrant, ICI-182,780, where ERα becomes resistant to Fulvestrant when it is phosphorylated by both PKA at Serine 305 and by MAPK at Serine 118 (4), or by phosphorylation of Serine 305 by PKA in conjunction with overexpression of cyclin D1 and SRC-1 (1). This resistance is manifested by an altered ERα conformation, but is not sustained in enhanced Fulvestrant-dependent reporter read-out or proliferation. The two short term features of anti-estrogen resistance (conformational alteration and recruitment of co-factors and RNA polymerase II) are immediate effects upon the ERα-SERD binding, whereas the two long term features (transcription and cell growth) take place during, or after a SERD-mediated degradation of ERα. Apparently, degradation of ERα is not prevented by the modifications that render ERα resistant to SERMs.

Cyclin D1 forms a specific complex with the estrogen receptor that facilitates ligand-independent recruitment of co-activators to the receptor complex (15;37). It is noteworthy that cyclin D1 overexpression does not lead to resistance to tamoxifen under experimental conditions (13), and does not predict response to tamoxifen treatment in breast cancer patients (12;38). Our results confirmed these findings by the absence of a FRET change (Figure 2), RNA polymerase II recruitment (Figure 3), nor responsive gene activation and cell growth (Figure 4) under tamoxifen conditions when cyclin D1 is overexpressed.

Each anti-estrogen apparently binds to ERα in a distinct manner and induces a characteristic inhibitory conformation that is released by compound-specific modifications of the estrogen receptor. For arzoxifene and GW5638, we found in this study that overexpression of cyclin D1 renders ERα resistant to these compounds. Cyclin D1 protein binds to the 338-379 aa region of ERα (39), just adjacent to the hinge region, in which Serine 305, when phosphorylated by PKA, determines resistance to tamoxifen.

Our findings of anti-estrogen specific modifications of ERα that are responsible for resistance to particular anti-estrogens may be of clinical relevance, since they indicate resistance to a particular anti-estrogen in the treatment of breast cancer (40) and could provide guidelines for the development of new anti-estrogens. Both of these may aid in eventually developing personalized endocrine treatment in breast cancer, where different anti-estrogens are subsequently applied in a rationalized manner, instead of in an empirical order as is now common practice (41).

Materials and Methods

Cell culture, transfection, constructs and antibodies

Human osteosarcoma U2OS, MCF-7 and HeLa cells were cultured in DMEM medium in the presence of 10% FCS and standard antibiotics. Cells containing ERα were cultured in phenol red-free DMEM medium containing 5% charcoal-treated serum (CTS; HyClone). For the FRET and FLIM experiments, cells were cultured overnight on 2 cm round glass coverslips. 24 hours prior to analysis, cells were transfected with pcDNA3-YFP-ERα-CFP or cotransfected with cyclin D1 and/or SRC-1, where indicated, using PEI (Polyethyleneimine, Mw 25kDa, Polysciences) (42). 4-OHTamoxifen (Sigma), EM-652 (kindly provided by dr. C. Labrie, University of Quebec, Canada), arzoxifene, GW7604, the active form of GW5638 (43) (kindly provided by GlaxoSmithKline), or ICI-182,780 (Tocris) were added at a final concentration of 1 μM, to ensure saturating ligand concentrations for overexpressed ERα. Forskolin (Sigma) was added 15 min prior to measurements at a final concentration of 10 μM. YFP-ERα-CFP, ERα-CFP, SRC-1, SRC-1(aa623-711)-YFP, SRC-1(aa1051-1240)-YFP and cyclin D1 constructs were generated as described previously (1;5;30). All used constructs and point mutants were sequence verified. Antibodies applied in this study were raised against ERα (Novoceastra), tubulin (Sigma), cyclin D1 (Santa Cruz), RNA polymerase II (8WG16; Covance, Inc.) and GFP (44).

Fluorescence Lifetime Imaging Microscopy (FLIM)

Prior to FLIM experiments, cells on coverslips were mounted in bicarbonate-buffered saline in a heated tissue culture chamber at 37°C under 5% CO2. FLIM experiments were performed on a Leica inverted DMIRE2 microscope equipped with a Lambart Instruments frequency domain lifetime attachment (Leutewegelode, The Netherlands), controlled by the vendors LI FLIM software. CFP was excited at 430nm with ~4mW power using a LED modulated at 40MHz. Emission was collected at 450-490nm using an intensified CCD camera. FLIM measurements were performed in...
U2OS cells, transfected with YFP-ERα-CFP only or cotransfected with cyclin D1 and/or SRC-1 constructs. Calculated CFP lifetimes were referenced to a 1 μM solution of Rhodamine-G6 in medium that was set at 4.11ns lifetime, and internally calibrated using co-cultured CFP containing MelJuSo reference cells for which the lifetime was set to 2.7ns (26). Donor FRET efficiency (E_D) was calculated as E_D=1- (lifetime cell of interest/ lifetime reference cell). Pair wise analysis was performed for each cell prior and after addition of anti-estrogen, where the E_D under CTS conditions is set to 1 for each experiment (relative E_D).

CLSM analysis

For CLSM analysis, prolactin promoter/enhancer (PRL-) array containing HeLa cells (28) were cultured in Dulbecco’s medium containing CTS and were transfected where indicated with ERα-CFP, SRC-1-YFP and/or cyclin D1 by electroporation and subsequently seeded in Dulbecco’s medium without phenol red supplemented with 5% CTS. Cells were treated for 2 hours with 1 μM estradiol, 1 μM of the indicated anti-estrogen or left untreated, to ensure saturating ligand concentrations for overexpressed ERα. Thereafter, cells were fixed with 3.7% formaldehyde in PBS and subsequently stained with antibodies detecting RNA polymerase II (8WG16; Covance, Inc.) or anti-Cyclin D1 (Santa Cruz) and secondary antibodies conjugated to Alexa 647 (Molecular Probes, Leiden, The Netherlands). Images were taken with a Leica TCS SP2 System equipped with a 63 x oil emersion objective. CFP was excited at 458 nm, and emission measured at 460-500 nm. Alexa 647 was excited at 633 nm, emission measured at 645-720 nm. Array size quantification was performed using Leica software, and plotted using proFit (QuantumSoft).

Quantitative RT-PCR

Prolactin promoter/enhancer (PRL-) array containing HeLa cells (28) were transfected with ERα-CFP only or cotransfected with cyclin D1 by electroporation and subsequently cultured in phenol-red free Dulbecco’s medium containing CTS. Immediately after seeding, the cells were treated with 10 nM estradiol or 100 nM anti-estrogen for 16 hours or the cells were left untreated. After exposure to hormones, cells were lysed and RNA was extracted using Trizol (Invitrogen), according to the manufacturer’s protocol. RNA was reverse transcribed using SuperScript(tm) III Reverse Transcriptase (Invitrogen), on which QPCR was performed using CYBR Green (Applied Biosystems), according to the manufacturer’s protocols. The DsRED2 cDNA was amplified with the forward primer 5’ CCAAGCTTCAATAGGCGTCC and the reverse primer 5’ GCCGTCCTCGA AGTTTCA-CA. pS2 cDNA was amplified with the forward primer 5’ CATCGA CGTCCCTCCAGAAGA and the reverse primer 5’ CTCTGGGAATACACCG TGCT. As a control for equal loading, the observed signals were related to β-actin RNA levels, using a forward primer 5’ CCTGGACCCACGACAGA and reverse primer 5’ GGGCCGAGCTCGTCA TACT.

Cell growth assay

MCF-7 cells, grown on 5% CTS serum containing phenol-red free Dulbecco’s medium for 7 days, were electrooporated with cyclin D1 wild type, cyclin D1-L244A/L245A (LALA), cyclin D1-K112E (KE) or pcDNA3-YFP empty vector as control. Immediately after electroporation, 40.000 cells were seeded in per well (24 well plate). Cells were treated with 10 nM E2, 100 nM anti-estrogen or left untreated and ligand-supplemented medium was replaced every 2 days. At 10 hours after seeding, cells were detached from the plate using trypsin and fixed in 3,7 % formaldeyde in PBS in triplicate. Similar samples were taken at 2, 5, 8 and 10 days after transfection. Total number of cells obtained from each well was determined using a Casy model TT cell counter (Schärfe-System GmbH, Germany).

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Supplemental Data

Supplemental Materials and Methods

Ratio-Fluorescence Resonance Energy Transfer (FRET)

Prior to FRET experiments, cells on coverslips were mounted in bicarbonate-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 23 mM NaHCO3, 10 mM glucose and 10 mM HEPES at pH 7.3) in a heated tissue culture chamber at 37°C under 5% CO2. Cells were analyzed on an inverted Zeiss Axiosvert 135 microscope equipped with a dry Achroplan 63x objective. FRET equipment was as described previously (44). CFP was excited at 432 ± 5 nm and emission of YFP was detected with a 527 nm longpass filter and CFP using a 478-527 nm bandpass filter. FRET was expressed as the ratio of YFP to CFP signals. The ratio was arbitrarily set as 1.0 at the onset of the experiment. Changes are expressed as percent deviation from this initial value of 1.0. For data acquisition, Felix software (PTI Inc.) was used. Data were plotted using proFit (QuantumSoft).

Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss in Photobleaching (FLIP)

U2OS cells were cultured on coverslips in CTS containing medium. 24 hours prior to imaging, cells were transfected with ERα-CFP and/ or SRC-1 using PEI. Where indicated, cells were additionally cotransfected with exogenous cyclin D1. Prior to analysis, coverslips were placed in 2 ml bicarbonate-buffered saline and analyzed in a heated tissue culture chamber at 37°C under 5% CO2. Cells were treated with 100 nM arzoxifene or left untreated. Images were acquired on a TCS-SP2 confocal microscope (Leica, Mannheim, Germany), using a 63x oil immersion objective. Fluorescence intensities were measured at the bleach spot (FRAP) and at the far end of the nucleus (FLIP). Zoom fluorescence was detected with a 527 nm longpass filter. FRET equipment was as described previously (44). CFP was excited at 432 ± 5 nm and emission of YFP was detected with a 527 nm longpass filter and CFP using a 478-527 nm bandpass filter. FRET was expressed as the ratio of YFP to CFP signals. The ratio was arbitrarily set as 1.0 at the onset of the experiment. Changes are expressed as percent deviation from this initial value of 1.0. For data acquisition, Felix software (PTI Inc.) was used. Data were plotted using proFit (QuantumSoft).
Figure S1. Inactivation of ERα by anti-estrogens measured by ratio-FRET.

A. Time course of emission of YFP (yellow line) and CFP (blue line) and corresponding ratio of YFP/CFP emission (red line) of one YFP-ERα-CFP containing U2OS cell after addition of 1 μM ICI-182,780, indicated by arrowhead. No effect of the ethanol solvent could be detected.

B. FRET values in YFP-ERα-CFP expressing U2OS cells after addition of 1 μM of the indicated anti-estrogens. Cells were either (+) or not (-) pretreated with forskolin 15 minutes prior to FRET measurements. The FRET values are shown as separate values of the percentage alteration in the FRET ratio, where each square shows one experiment (n > 5 for each condition). The data are presented in a box plot with the horizontal bar indicating the median value. The box size is determined by the upper and lower quartiles, the median value of the upper and lower half of the data points, respectively.

C. FRET values in U2OS cells expressing YFP-ERα-CFP, SRC-1 and cyclin D1 after addition of 10⁻⁶ M of the indicated anti-estrogen. The FRET values are presented as under B.

TAM: 4-OH-tamoxifen; ICI: ICI-182,780 (Fulvestrant); EM: EM-652; GW: GW5638; ARZ: arzoxifene.

* indicates a statistically significant FRET reduction for conditions in both B and C, compared to the non-forskolin treated samples in B, p < 0.05

Figure S2. Cyclin D1 overexpression verified in PRL array containing HeLa cells.

Staining for cyclin D1 and RNA Polymerase II was performed on PRL array containing HeLa cells that were cotransfected with ERα and excess cyclin D1. Cells expressing ERα overexpressed cyclin D1. ERα, cyclin D1 and RNA Polymerase II were all recruited to the array structure, indicated by arrowhead.
Figure S3. Cyclin D1 overexpression induces RNA polymerase II recruitment on the PRL array and increases array size in the presence of GW5638 and arzoxifene.

PRL array containing HeLa cells were transfected with ERα-CFP alone or cotransfected with cyclin D1 and stained for RNA polymerase II. Cells were cultured in hormone depleted (CTS) medium and treated with 1 μM estradiol (E2) or with 1 μM of the indicated anti-estrogen for 2 hours and then fixed and stained for RNA Polymerase II. Arrowheads indicate the PRL array that was analyzed for RNA polymerase II staining. Quantifications are shown in Figure 3.
Figure S4. Overexpression of cyclin D1 wild type and mutants in MCF-7 cells.

Western blot analysis of MCF-7 cells, transfected by electroporation with an empty vector, cyclin D1 wild type, cyclin D1 LALA or cyclin D1 KE. Blots were stained with antibodies detecting cyclin D1 and tubulin, verifying equal overexpression of the applied cyclin D1 variants.

Figure S5. Cyclin D1 stabilizes ERα/SRC-1 complex under arzoxifene conditions

A. An example of FRAP analysis where the determination of T1/2 and Immobile fraction (IF) is illustrated. Underlying calculations are described in the supplemental materials and methods section.

B. FRAP analysis of U2OS cells expressing ERα-CFP and/or SRC-1-YFP, with or without cyclin D1 co-expression. Both t1/2 (top panel) and immobile fraction (bottom panel) are given. Mean and standard deviation for tested conditions is given. t1/2 and immobile fraction of SRC-1-YFP was altered by co-expression of ERα-CFP, suggesting an interaction. Arzoxifene treatment induced a deviation between ERα-CFP and SRC-1-YFP dynamics, thus interaction is lost. When cyclin D1 was overexpressed, SRC-1-YFP t1/2 and immobile fraction were again comparable to those of ERα-CFP under arzoxifene conditions, implying a stabilization of the complex. * indicates a statistically significant differences in between ERα-CFP and SRC-1-YFP (black for controls, red for cotransfected ERα-CFP and SRC-1-YFP) within the same treatment; p< 0.05.