Serine-305 phosphorylation modulates estrogen receptor alpha binding to a coregulator peptide array, with potential application in predicting responses to tamoxifen

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Running title: Coregulator fingerprint for tamoxifen resistance

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Abstract

With current techniques it remains a challenge to assess coregulator binding of nuclear receptors, e.g. the estrogen receptor alpha (ERα). ERα is critical in many breast tumors and is inhibited by anti-estrogens such as tamoxifen in cancer therapy. ERα is also modified by acetylation and phosphorylation that affect responses to the anti-estrogens as well as interactions with co-regulators. Phosphorylation of ERα at Ser305 is one of the mechanisms causing tamoxifen resistance. Detection of resistance in patient samples would greatly facilitate clinical decisions on treatment, where such patients would receive other treatments such as aromatase inhibitors or fulvestrant. Here we describe a coregulator peptide array that can be used for high throughput analysis of full-length estrogen receptor binding. The peptide chip can detect ERα binding in cell and tumor lysates. We show that ERα phosphorylated at Ser305 associates stronger to various coregulator peptides on the chip. This implies that ERαSer305 phosphorylation increases estrogen receptor function. As this is also detected in a breast tumor sample of a tamoxifen insensitive patient, the peptide array as described here may be applicable to detect tamoxifen resistance in breast tumor samples at an early stage of disease and contribute to personalized medicine.
Introduction

Nuclear receptors (NRs) regulate gene expression levels by gene promoter binding and by recruitment of coregulators (1). Modulation of NR activity is usually quantitatively analyzed by assaying target gene transcription or downstream events. These parameters are however the net result of the NR interaction with a wide range of individual coregulators. Thus far, studying nuclear receptor interactions with coregulators in a more global manner has been a challenge. Conventional methods providing NR-coregulator interaction data are intermolecular FRET, Y2H, phage display and colocalization studies in fluorescence microscopy (2-4). These techniques generally allow the study of one receptor-coregulator pair while nuclear receptors usually have multiple coregulators to choose from and the biological outcome of activation of the receptors then depends on the different coregulators expressed and their relative affinity to the nuclear receptor. Here we apply a peptide array that can assess interaction of full length estrogen receptor alpha (ERα) with a set of coregulators, in a high throughput manner. Apart from the ligand, post-translational modifications (PTM) also play a major role in NR transactivation and can yield differential response to ligands (5-13). It is therefore likely that PTMs can also play a role in coregulator recruitment.

The most widely studied group of estrogen receptor alpha (ERα) coregulators includes the p160 protein family, consisting of three members: NCOA1 (SRC-1), NCOA2 (SRC-2) and NCOA3 (SRC-3) (14-16), which have specific effects (14). Moreover, NCOA3 (AIB1) gene amplification and elevated expression was discovered in a subset of ERα-positive breast cancer (17, 18). Endocrine therapy, which aims for inactivation of ERα, uses competitive estrogen antagonists (e.g. tamoxifen) or aromatase inhibitors that block estrogen synthesis. This prevents the formation of the coactivator binding surface on
ERα (19). A group of patients does not respond to endocrine therapy, because ERα remains transcriptionally active despite endocrine treatment. One factor that is associated with resistance to tamoxifen is phosphorylation of ERα Serine 305 (ERαS305-P) by protein kinase A (PKA) (11, 20, 21). This post-translational modification affects receptor function by a conformational change that alters binding to NCOA-1 (22). Since ERα transcriptional activity is defined by interaction of the receptor with a multitude of different coregulators, we set out to develop a high throughput assay to functionally analyze the interaction of ERα and its Ser305-P modified form with a broader panel of coregulators. In this study, we applied an array on which a set of peptides representing coregulator NR-box sequences are immobilized (23). This format allows for high throughput in vitro functional analysis of ERα, i.e. coregulator interaction, and modulation by ligand and receptor phosphorylation. We do detect differences in binding upon one single post-translational modification: phosphorylation of ERα Serine 305 and show that our assay allows detection of phosphorylation-correlated alteration of ERα activity in breast tumor lysates as a first step towards developing a test to detect resistance to anti-estrogens such as tamoxifen.
Materials and Methods

DNA constructs, cell culturing and transfections

pcDNA3-YFP-ERα-CFP transfected human U2OS osteosarcoma cells and human MCF-7 breast cancer cells were cultured in DMEM medium in the presence of 10% FCS and standard antibiotics. Cells were transferred to phenol red-free DMEM containing 5% charcoal treated serum (CTS, Hyclone) prior to the addition of ligands (for MCF7 cells) in order to omit the estrogenic effect of phenol red and serum-estrogens as described previously (11). MCF7 cells were derived from the Netherlands Cancer Institute cryostore bank. It expresses ERα and this expression was constantly verified by WB, CHIP and functional assays during the study. U2OS cells were used as recipient cells for ERα constructs and frequently new stocks obtained from the Netherlands Cancer Institute cryostore bank were cultured for transfection experiments. Absence of ERα expression in the untransfected U2OS cell lines was frequently tested during the study.

Sample preparation

ERα transfected U2OS cells or MCF7 cells, 48 hours after transfection, were harvested in 1 ml TNRL01-lysisbuffer (24) using a cell scraper. Cells were subsequently lysed by sonification using a Branson sonifier for 10 pulses at output 50% and duty cycle 5. Lysates were aliquoted and flash frozen in liquid nitrogen. ERα in the samples was quantified using ELISA (Acive Motif). Equal amounts of ERα wild-type (ERα WT) were used in further experiments. Deep-frozen breast tumor samples were immediately pulverized using a dismembrator and processed in TNRL01-lysisbuffer as described.
previously (24). Equal amounts of protein were separated by SDS-PAGE, blotted and the filter was probed with antibodies against GFP (25), ERαSer305-P (Millipore, #124.9.4.9, (21)) and ERα (HC20, Santa Cruz). Human actin (I19, Santa Cruz) was used as the loading control. Signals were detected using an ECL detection kit (Amersham). Experiments were repeated at least twice, with reproducible results.

Phosphatase treatment of tumor lysates

Per array, 5 µl tumor lysate was incubated for 45 minutes at 30°C in a reaction volume of 5µl of lambda protein phosphatase buffer, 5 mM DTT and 30U lambda protein phosphatase (#14-405, Millipore). Treatments were ended by adding 5µl of the stopbuffer with HALT protease inhibitor cocktail (PIERCE) and phosphatase inhibitors (100µM NaF, 80mM B-glycerophosphate and 2 mM orthovanadate). Subsequently, samples were directly supplemented with the MARCoNI assay mix and analysed (30).

Micro Array assay for Real-time analysis of Coregulator-Nuclear receptor Interaction: MARCoNI.

All assays were performed in a PamStation®-96 (PS96) controlled by EvolveHT software (PamGene International BV, Den Bosch, the Netherlands) (26, 27). Nuclear Receptor PamChip® Arrays (23) (PamGene International BV, Den Bosch, the Netherlands) with immobilized peptides with coregulator derived sequences (Supplementary Table 1) were used. Lysate assay mixes contained 10 µl lysate with 4 µg/ml Alexa488-conjugated anti-GFP (#A21311, Invitrogen) for ERαY/C transfectants; or anti-ERα (D547, Santa Cruz) and goat-anti-rat (Bet-A110-109F-15, Axxora) for untagged (endogenous) ERα, in Tris-buffered Saline (TBS) with 0.05% Tween 20, 0.2% BSA, 50 µM DTT, 2% DMSO
(with or without E2). The assay mix with ERα LBD-GST (#PV4543; Invitrogen) was prepared as described previously (22, 28) using 4 μg/ml Alexa488-conjugated anti-GST (#A11131) as detecting antibody. Mixes were prepared and stored on ice in a master 96 well plate until use. All incubations were performed at 20°C, applying a sample rate of 2 cycles per minute. The initial blocking was performed by incubating the arrays for 20 cycles with 25 μl blocking buffer (TBS with 1% BSA, 0.01%, Tween-20 and 0.3% skimmed milk (Oxoid)). After removal of the blocking buffer by aspiration, each array was incubated with 25 μl of assay mix for 80 cycles, washed with 25 μl TBS and finally (cycle 102) a tiff format image was obtained by the CCD camera. Binding of untagged ERα in lysates was detected by additional 5 min incubation with FITC-conjugated mouse-anti-goat (SC2356, Santa Cruz) and washing as described. Image analysis consisting of automated spot finding and quantification was performed using BioNavigator software (PamGene International BV, Den Bosch, the Netherlands). In short, the boundaries of a spot are determined and the median fluorescent signal was quantified within the spot (signal) as well as that in a defined area surrounding it (background). The signal-minus-background value was subsequently used as the quantitative parameter of binding.

Ligand dose-responses were analyzed using the drc package in R (version 2.12.0, www.r-project.org). Sigmoidal dose-response curves (DRC) were fitted using a four-parameter logistic model and delivered values for potency (EC50) and for efficacy (signal difference between the bottom and top value of the curve).

Z-score normalization, or standard score, which converts the absolute value (binding) of a data point in a population (profile) into the distance of that data point to the mean of
the population in units of the standard deviation of that population, was calculated as

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\frac{\text{binding}_{\text{peptide}} - \text{mean(binding all peptides)}}{\text{stdev(binding all peptides))}}.
\]
Results

Co-factor binding profile of full-length ERα

We generated a peptide array containing a series of peptides representing co-regulator binding sites (set I, Supplementary Table 1). This series of peptides included most of the known cofactors interacting with ERα. To test whether ERα was able to bind to these peptides, we produced lysates from U2OS cells expressing fluorescently tagged ERα (ERαY/C, figure 1C). First, we analyzed the binding profile of ERα, treating the cells with different ligands prior to lysis (pre-lysis ligand treatment, pre-LT). The results showed a binding pattern for ERα to the coregulator motifs when stimulated with E2, whereas hardly any binding was observed for non-ligated or tamoxifen-bound ERα (figure 1D).

Next, we investigated whether addition of ligands could still modulate the binding of ERα to coregulators after lysis (post-LT). Treating the samples before or after lysing the cells with E2 resulted in a similar binding profile (figure 1D/E). This indicates that the full-length receptor stays functional in terms of ligand binding and coregulator recruitment in crude lysates as well. This allowed us to consider one crude lysate in different treatment strategies followed by functional analysis of ERα on the array.

Full-length ERα versus ERα-LBD

Isolating full-length nuclear receptors is time consuming and difficult (29, 30). In our experiments we used crude lysates, instead of purified proteins. Another, more common approach is using the ligand binding domain (ERα-LBD) (27, 31). We therefore compared the E2-response of the ERα-LBD with that of the full-length protein in lysates of ERαY/C transfectants. Both protein preparations were incubated at different concentrations of E2 (ranging from $10^{-12}$ to $10^{-7}$M) 10 minutes before loading of the
samples onto the array. Curves for all peptides were visually inspected. Full receptor saturation with ligand was achieved for both proteins, reflected by a binding plateau for all peptides at the high-end of the ligand concentration range. As an example, E2 binding curves of ERαY/C and ERα-LBD to a control peptide (NCOA1-677-700: IDNR 13 in Supplementary Table 1) are shown in figure 2A.

The E2 potency (EC50) for each peptide (set 1, Supplementary Table 1) was derived from these curves. EC50 values for all peptides were in the low nM range for both ERα and ERα-LBD (data not shown) and corresponded with published data (32). Ligand-independent binding (LIB) and ligand-saturated binding (LSB) were calculated for each peptide on the array from the signal at the two lowest and two highest E2 concentrations respectively, each performed in duplicate arrays. E2-induced modulation of ERα binding to each peptide is represented as the modulation index (MI), which is the log-10 transformed ratio of the LSB over LIB (figure 2B/C).

The MI values of purified ERα-LBD protein were similar to those of the cell lysates with full-length ERα (figure 2B). The coefficient of correlation (R²) between the E2-induced binding profiles of both proteins was 0.86 (figure 2B scatterplot). The overall E2-MI for full-length ERα was slightly higher than for the ERα-LBD fragment, suggesting more efficient binding to coregulators. As shown previously, coregulator binding does not require, but is enhanced by the presence of the AF-1 domain (22, 32). The AF-1 domain is absent in the ERα-LBD construct which therefore binds less efficiently to cofactors. The specificity of the interactions is not affected.

Exogenous versus endogenous ERα in cell lysates
The results of the experiments described thus far indicated that the full-length ERα from transfected cells behaved similarly in this analysis as ERα-LBD fragments that were generated \textit{in vitro}. This full length ERα construct contained two GFP variants for detection and this might affect binding efficacy. Therefore, we compared the tagged full-length ERα from transfected U2OS cells with an endogenous ERα from the human breast cancer cell line MCF7. The E2-induced binding of ERα in transfected U2OS and in MCF cells was highly similar ($R^2:0.72$), although the absolute signal was slightly enhanced in the latter sample (Figure 2C). This suggests that the YFP and CPF tags do not affect receptor function and that the assay does not require overexpression of ERα and also is sensitive enough to be used for endogenous ERα protein in lysates.

\textit{ERα from ER-positive breast cancer}

Since we could detect binding of endogenous ERα in crude lysates to the chip, we decided to test the technology on breast tumor material. We prepared lysates from a primary ERα-positive breast tumor sample. E2 (1 µM) or no ligand (control) was added 10 minutes prior to loading onto the array. The results showed that ERα from tumor material is still responsive to E2 (figure 3A) and the E2 response (MI) of ERα in these samples resembles that of MCF7 cells ($R^2:0.78$, figure 3B). No signals were detected when using a lysate prepared from ERα negative breast tumor tissue, which demonstrates that the signals in this assay are selective for ERα binding to peptides on the array (figure S1, Supplement).

Clinical samples generally show heterogeneity. To investigate whether this plays a role in our peptide chip, we compared three ERα-positive breast tumors. Absolute differences in signal between the three tumor samples were observed. The signals were adjusted by z-score normalization, which enables a direct comparison of the profiles.
Addition of E2 modulated the binding profiles of all tumors. Moreover, the endogenous (CTS) binding profiles as well as under saturating E2 concentration were highly similar between tumors (Figure 3C). These data indicate that the analysis of human tumors and cell lines by the cofactor peptide array is consistent and reproducible and thereby suitable for identifying cofactor binding to ERα from human breast cancer specimens under different ligand conditions.

*Functional analysis of ERα Serine305 phosphorylation*

Post-translational modifications on ERα and other nuclear receptors have been shown to influence coregulator binding, but this has been demonstrated only on single coregulators (22). We investigated whether the effect of such modifications could be monitored in our high-throughput assay. From this point, we included more peptides from cofactors binding to ERα or to some other NRs, in addition to set I (Supplementary Table 1, set II).

PKA-induced ERαS305-P has been linked to resistance to tamoxifen treatment (20, 21). Under tamoxifen conditions, this phosphorylation affects the conformation of ERα and changes its orientation to NCOA-1 (22). Here we studied the effect of ERαS305-P on the binding of cofactors in transfected cells and in breast tumor specimens. We therefore applied a next generation array with an extended set of 52 coregulator peptides (set II, Supplementary Table 1). The serine305-to-alanine mutant (ERαY/C305A) was included as a negative control (Figure 4A). Next, we generated lysates that were incubated with a concentration range of up to $10^{-8}$ M E2 and analyzed binding to the co-factor peptides on the chip. This resulted in a dose-dependent modulation of ERα binding to each coregulator peptide on the array, as illustrated for the control peptide (IDNR13) in Figure 4B. Wild-type (WT) and mutant ERα (305A) from control and 8-Br-cAMP-
stimulated cells were responsive to E2. From unstimulated cells, the ligand-independent binding (LIB, left extreme of the curves) of wild-type ERα is slightly enhanced when compared to 305A mutant receptor. This may be due to some background phosphorylation of wild-type ERα at this residue in unstimulated cells (figure 4A). When 8-Br-cAMP activates PKA, both LIB and E2-saturated binding (LSB) to this peptide were elevated. This was observed for both the wild-type receptor and 305A mutant, but the effect on wild-type ERα is more substantial (Figure 4C). This has been observed for all peptides on the array and suggests that ERαS305-P increases the ERα binding affinities in absence of ligand (figure 4D) and amplifies the E2-induced modulation of binding (figure 4E) to various coregulators.

Of note, additional phosphorylation sites may play a role in the residual cAMP effect seen in the ERαY/C 305A (Figure 4D), which is not completely abolished by the mutation of this site. A plausible candidate is Serine 236 in ERα (33). Therefore, we assessed binding of the ERα double mutant (236A/305A), in which none of the two known PKA target sites could become phosphorylated, to the control peptide. The PKA-enhanced binding to ERα WT was partially reduced by the single Ser305Ala mutation, but almost completely abrogated in the double mutant (Fig 4C). This indicates that the PKA-associated effect on binding to this peptide is mainly due to phosphorylation of ERα sites Ser236 and 305.

The effect of PKA activation on peptide binding was also studied under tamoxifen (4-OHT) conditions. This is illustrated by ERα binding to the same peptide (IDNR13) using a concentration range of up to 10^{-5}M 4-OH-tamoxifen (Figure 5A). Although PKA activation increased the initial binding (LIB) of ERα WT, binding of both ERα and ERαS305A were strongly diminished at saturating tamoxifen concentrations. The
potency (EC50) of 4-OH-tamoxifen was not affected and largely similar for the various motifs on the chip (figure 5C). This implies that enhanced ERα-coregulator binding through phosphorylation could result in ligand-independent binding and/or enhanced responses to E2, and may result in enhanced residual receptor binding at non-saturating tamoxifen levels.

To illustrate this, we calculated the binding ratio for each peptide of 8-Br-cAMP-treated over control samples at a non-saturating EC50 concentration of 4-OH-tamoxifen (Figure 5B). The results demonstrated that PKA-mediated phosphorylation enhances residual binding (ratio>1) to the majority of the peptides, suggesting a generally more active ERα under these tamoxifen conditions. This provides a novel mechanism for the previously reported association between phosphorylated ERαSer305 and resistance to tamoxifen in breast tumors. It implies that patients treated at suboptimal doses of tamoxifen may get tumors escaping tamoxifen control by the residual activity of the phosphorylated ERα.

The peptides showing increased ERα-binding represent the classical ERα coactivators SRC-1/2/3 and nuclear receptor protein 1 (NRIP1, (34)) (IDNR12-21, Figure 5D). Two peptides that were unaltered are derived from CREB-binding protein (CBP) and p300 (IDNR3-4).

*Does the peptide array predict patient responses to tamoxifen?*

We then examined whether Serine 305 phosphorylation is correlated with enhanced coregulator binding of ERα from patient material. We selected two breast tumors (A and B) by immunohistochemical evaluation (figure 6B). Both tumors stained positive for ERα and tumor B was positive for ERαS305-P, which was confirmed by Western blot
(Figure 6C). For functional analysis on the array, sample input was normalized for ERα content measured by ELISA. ERα in the lysates of both these tumors is still responsive to addition of E2, showing increased peptide binding on the array (Figure 6D). The role of S305 phosphorylation on receptor activity was assessed by dephosphorylation of the receptor in the lysates by addition of lambda phosphatase. This treatment largely reduced the level of phosphorylation in the ERαS305-P positive tumor B (Figure 6C).

Comparison of ERα in untreated lysates (control) of both tumors (grey lines in figure 6E), showed enhanced binding in the ERαSer305-P positive tumor B (right panel). This was the case for endogenous binding (no ligand added, veh), but also maximal binding at a saturating dose of E2 and even residual binding where the latter condition was antagonized by a suboptimal (subsaturating, EC50) dose of tamoxifen. This enhanced coregulator binding of ERα from tumor B was strongly reduced by phosphatase treatment of the lysate (black lines), whereas the binding levels of the unphosphorylated receptor from tumor A (left panel) were unaffected. Dephosphorylation of ERα in tumor B reduces endogenous binding activity (vehicle) as well as the response to E2 and the residual binding after tamoxifen (EC50) treatment. These data show that serine 305 phosphorylation of ERα, which is associated with tamoxifen resistance, can be measured as increased coregulator binding of ERα in clinical samples. This assay may therefore be applied for response prediction in the clinic, i.e. enhanced ERα binding affinity could be a parameter for drug resistance.
Discussion

The application of the peptide array assay as described in this study adds a useful tool to gain insight into ERα biology and provides several advantages.

It allows the high throughput assessment of ERα binding to multiple NR-boxes. The receptor is still functional after sample preparation, reflected by modulation of coregulator interaction by reference ligands added on the array. In contrast to reporter and growth assays, it directly measures nuclear receptor function instead of its downstream events. The array requires minimal sample volumes, which is an advantage when sample volume is limiting, e.g. from patient material. We measure ERα activity in crude cell lysates, thereby avoiding troublesome purification procedures of full-length receptor. Previous studies report peptide binding only to purified fragments of ERα, generally the LBD (27, 28, 31). The analysis of a full-length ERα more closely reflects what happens in vivo than the LBD. Moreover, the sensitivity of the assay allows analysis of endogenous ERα from cells and even from tumor material. The binding profiles are minimally influenced by cellular components, which might potentially block the peptides by aspecific binding or compete for the coregulator binding pocket of ERα. The minor differences we observed in overall binding could be due to differences in sample preparation, e.g. isolation of LBD versus crude lysate, the use of different antibodies or the presence of tags (CFP/YFP).

In the presence of ERα antagonist tamoxifen, the receptor adopts a conformation that reduces binding of coactivators (35), as is also evident from the binding profiles presented in figure 1D and E and the DRC in figure 5A, where tamoxifen displaced ERα from LxxLL motif-containing peptides on the array. However, if the cells develop resistance, tamoxifen fails to block growth. In this case the interaction between ERα and
coactivators, such as NCOA-1 can take place even in presence of tamoxifen (22). This is also what we observed upon PKA activation (Figure 4). We saw differences on peptides derived from coactivators of ERα that interact directly with the receptor: NCOA-1/2/3 and NRIP1. In contrast, no effect was observed on a couple of coactivators that can act indirectly on ERα transactivation (CBP and p300) (36, 37).

Tamoxifen regulates a specific set of genes distinct from E2, suggesting recruitment of another transcription machinery with different coregulators (38, 39). However, we did not observe specific differences in the binding to LxxLL motifs from directly interacting coregulators of ERα as a result of the different treatments. Specific effects may require full-length coregulators, which contain multiple NR-boxes and potentially interact with both the AF-1 and AF-2 domains of ERα.

We applied the assay to functionally analyze one well-described NR post-translational modification: Serine 305 phosphorylation of ERα, which is clinically associated with resistance to tamoxifen (20, 21, 40). Full-length ERα is essential in studying this kind of resistance to anti-estrogens used in the clinic, because post-translational modifications of ERα outside the LBD domain influence the conformation of ERα and thereby the ability of the cofactor interacting surface to bind cofactors (11, 41). PKA-activation leads to ERα phosphorylation and enhanced receptor binding to the coregulator peptides in a ligand independent manner (Figures 4B and 5A), and could largely be attributed to ERαS305-P (Figure 4C). The underlying mechanism could be the previously reported conformational change of ERα, which would make the cofactor binding groove more accessible for the peptides on the chip (42). Coregulator binding by ERαS305A mutant was still slightly enhanced by PKA activation (Figure 4C), which means that PKA has additional effects on ERα. These effects include a previously reported phosphorylation
of Ser236 (33). The contribution of phosphorylated Ser236 to enhanced transcriptional activity may, however, be limited, since it prevents ERα from binding to its cognate ERE sequence in the DNA, whereas ERαS305-P does not hamper binding to DNA (33, 43).

To extrapolate our findings to the clinic, we selected tumors from two ERα positive breast cancer patients, of which one was ERαSer305-P positive. This parameter was correlated with enhanced cofactor binding by receptor in the lysate of this tumor. Upon phosphatase treatment to undo ERα phosphorylation, only the receptor from the ERαS305-P positive tumor lost a substantial part of its binding capacity. Although this is only a proof of principle on two tumor samples, it is striking that the ERαS305-P positive patient had a recurrence of disease after only nine months, in spite of tamoxifen treatment, whereas the ERαS305-P negative patient survived without recurrence in the follow-up time of 13.5 years. These current findings in the tumors support previous reports that PKA-mediated ERαSer305-P is associated with resistance to tamoxifen (20, 21, 40). The enhanced binding to coregulators by ERαS305-P provides a likely mechanism for tamoxifen resistance.

**Endogenous tumor ERα from a patient with resistance towards endocrine treatment also displayed phosphorylation-dependent enhancement of coregulator binding on the array (figure 6C). This assay may therefore prove** a valuable tool in the future for therapy response prediction at an early stage of disease, *i.e.* immediately after tumor resection and before start of adjuvant therapy. Testing the effect on ERα activity *ex vivo* (e.g. by phosphatase treatment of the patient sample) provides clues to the mechanisms of the resistance and may add to personalized medicine for breast cancer, *e.g.* by application of combination therapy with a kinase inhibitor.
In summary, here we present an assay that provides insight in ERα biology and find underlying mechanisms for drug resistance. Moreover, functional profiling of ERα may prove a valuable tool for clinical purposes, such as prognostics or prediction of treatment response.
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Figure Legends

Figure 1. Analysis of coregulator binding of full-length ERα in crude lysates of transfected cells. A. U2OS cells transfected with a YFP-ERα-CFP (ERα Y/C) construct. Transfectants were I: treated with ligand or kept under hormone-free conditions and lysed (Pre-LT), or II: lysed first and ligand was added to the lysate (post-LT), finally ERα in the samples was subjected to functional analysis by MARCoNI. B. Schematic overview of peptide array technology. C. Western blot analysis of ERαY/C expression in transfected U2OS cells detected with anti-GFP. D. Peptide binding (in arbitrary units of fluorescent signal, detected with anti-GFP-ALEXA488) of ERα Y/C in samples obtained by pre-LT or E. post-LT to a set of 25 immobilized coregulator-derived LxxLL motives, in response to 17-β-estradiol (E2), 4-hydroxy-tamoxifen (4-OHT) or absence of ligand (Veh). For peptides, see corresponding numbers in Supplementary Table 1 (set I).

Figure 2. E2-induced modulation of ERα-coregulator binding: purified ligand-binding domain vs. full-length protein in crude lysates. Recombinant ERαLBD-GST or ERαY/C lysate was incubated with a concentration range of E2 (10^-12 to 10^-7M), one chip per ligand concentration. Each condition was measured in duplicate (2 arrays) and receptor binding was visualized using ALEXA488-conjugated anti-GST or anti-GFP respectively. A. The E2 EC50 values were calculated from the resulting dose-response curves. E2-induced modulation of the binding to each peptide was indicated by the modulation index (MI) which is the log-10 transformed ratio of LSB over LIB. Example of E2 dose-response curves of the full-length ERαY/C (left) and ERα-LBD- (right) binding to a NCOA1-derived peptide (IDNR13). B. E2-
induced MI of ERα-LBD and full-length receptor binding to the NR-boxes are shown. Subgraph: Scatter plot and correlation coefficient of E2- MI of ERαLBD-GST vs. full-length ERαY/C. C. MI of MCF7 endogenous ERα and U2OS full-length ERαY/C, subgraph represents correlation. For peptides, see corresponding numbers in Supplementary Table 1 (set I).

Figure 3. Coregulator binding of ERα from in breast tumors and binding modulation by estradiol. A. Coregulator binding of ERα in a tumor in the absence or presence of ligand (E2). B. E2-induced modulation indices (MI) of MCF7- and breast tumor-derived ERα, subgraph represents correlation. C. Extracts from three different ERα-positive breast tumors and MCF7 were incubated in the presence of E2 or vehicle. ERα binding was visualized with anti-ERα and FITC-labeled secondary antibody. Binding profiles were normalized using z-score transformation, \( (x-x_{\text{mean}})/sd(x) \) for direct comparison between the three tumors. For peptides, see corresponding numbers in Supplementary Table 1 (set I).

Figure 4. Effect of serine 305 phosphorylation on ERα-coregulator binding. U2OS cells were transfected with wild-type (WT), single (305A) or double (236A-305A) serine mutant full-length ERα tagged with YFP/CFP. Cells were stimulated with (+) or without cAMP to induce PKA-mediated receptor phosphorylation. A. Western blot analysis of ERαSer305 phosphorylation in the three transfectants with or without cAMP stimulation. B. Dose dependent 17-β-Estradiol (E2)–modulated binding of...
wild-type or ERαY/C 305A from control or cAMP-stimulated cells with NCOA1_677_700 (IDNR13). C. cAMP-induced enhancement of NCOA1_677_700 binding by ERαY/C WT, ERαSer305Ala and ERαSer305Ala/Ser236Ala in the absence or presence of E2. D. MI for cAMP-induced ligand independent binding of WT and S305A-ERα to all peptides. E. MI for E2-induced binding (delta) of WT ERα from control and cAMP-stimulated cells (D and E were calculated using LIB and LSB, see figure 2). For peptides, see corresponding numbers in Supplementary Table 1 (set II).

Figure 5. Tamoxifen-induced modulation of ERα-coregulator binding. U2OS cells were transfected with ERαY/C wild-type (WT) or ERαSer305A-Y/C (305A). Cells were stimulated with (+) or without 8-Bromo-cAMP (cAMP). A. Dose dependent 4-hydroxy-Tamoxifen (4-OHT)–modulated binding to NCOA1_677_700 (IDNR13) by wild-type or ERα305A-Y/C in vehicle or cAMP-stimulated cells. B. Binding ratio of 8-Br-cAMP-treated over control samples at a non-saturating EC50 concentration of 4-OH-tamoxifen. C. Dose-response curve for binding of wild-type ERα vs. cAMP-stimulated ERα to coregulator peptides in the presence of 4-OH-tamoxifen. D. Binding ratio of cAMP (+) vs. vehicle stimulated cells in the presence of 4-OHT at EC50. For peptides, see corresponding numbers in Supplementary Table 1 (set II).

Figure 6. Functional analysis of ERα in crude lysates of two breast tumors. A. Immunohistochemistry and B. Western blot analysis of total ERα and ERαSer305-P
status in the sample of a S305P - (tumor A) or + (tumor B) patient before (-) and after (+) phosphatase treatment of tumor lysates. C. ERα-coregulator binding profiles of tumor A and B after treating lysates without (vehicle) or with (E2) 17-β-estradiol. D. Coregulator binding in samples from S305P - or + patients, untreated (grey) or after phosphatase treatment (black). In the absence (vehicle) or presence of E2 (saturating concentration) only (E2) of with 4-OHT (EC50). Note that differences in range of Y-axis between graphs. For peptides, see corresponding numbers in Supplementary Table 1 (set II). E. ERα-coregulator binding profiles of tumor A and B before and after treating lysates with phosphatase, under hormone-depleted conditions or in presence of 17-β-estradiol (E2) or 4OH-tamoxifen (TAM).
Figure 1

A. Experimental design

I. pre-LT
   - U2OS
   - transfected with ERα/Y/C
   - ligand
   - cell lysis

II. post-LT
   - functional analysis

B. Ab
   - ligand
   - ERα
   - solid support
   - coregulator motif
   - pore wall

C. U2OS
   - ERαY/C
   - kDa

D. binding (AU)

pre-LT

E. binding (AU)

post-LT

peptides (IDNR)

- Veh
- E2
- 4-OHT

Graph showing binding levels before and after treatment.

E2

0.71

30k

15k

30k

15k

pre-LT

post-LT
ERα

305P-status phosphatase
ERα-Ser305P
ERα

D. binding (AU)

12k tumor A

Veh E2

40k tumor B

S305-P -

S305-P +

peptides (IDNR)

- tumor A (S305-P -)

- control

- phosphatase

- Veh

- E2

- 4-OHT

- tumor B (S305-P +)

peptides (IDNR)
Molecular Cancer Therapeutics

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