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Molecular understanding of tamoxifen resistance in breast cancer

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Citation

Leeuw, R. de. (2012, February 9). *Molecular understanding of tamoxifen resistance in breast cancer*. Retrieved from <https://hdl.handle.net/1887/18458>

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Molecular understanding of tamoxifen resistance in breast cancer

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Omslag ontworpen door: Theophile Suijkerbuijk (www.theophile.nl)

Molecular understanding of tamoxifen resistance in breast cancer

proefschrift

ter verkrijging van de graad van Doctor
aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 9 februari 2012
klokke 11:15 uur

door

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geboren te Breda
in 1983

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Drukkerij: Ipskamp Drukkers

The work described in this thesis was performed in the division of Tumor and Cell Biology of the Netherlands Cancer Institute (NKI-AVL), Amsterdam, The Netherlands. This work was supported by a grant from Top Institute Pharma (TI Pharma). Financial support for the publication of this thesis was provided by the Netherlands Cancer Institute (NKI-AVL).

voor oma



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Scope of this thesis

Estrogen receptor signalling,...

The estrogen receptor is a nuclear hormone receptor that is activated by estrogens. The activated receptor dimerizes and binds to responsive elements on the DNA. Cofactors are recruited to regulate the transcription of genes. Thereby, estrogens can induce cell proliferation.

...breast cancer...

The clinic makes clever use of this characteristic in breast cancer treatment. In about 70% of all breast cancer cases, the tumor expresses the estrogen receptor. Patients diagnosed with this type of breast cancer commonly receive endocrine treatment after surgical removal of the tumor. Endocrine treatment can either target the receptor with competitive drugs (antiestrogens), or prevent formation of natural estrogens by inhibiting the aromatase enzyme (aromatase inhibitors). Because such tumors are dependent on estrogens for their growth, both treatment modalities should result in growth arrest and prevention of disease recurrence.

...and endocrine resistance

Endocrine treatment in breast cancer is often successful, yet a subset of patients develop therapeutic resistance. This thesis focuses on one type of endocrine resistance: the widely used antiestrogen tamoxifen. A number of factors have been attributed to tamoxifen resistance, among which phosphorylation of the estrogen receptor.

A general introduction into the role of phosphorylation of ER α in tamoxifen resistance is given in **Chapter 1**. In this review, biological and clinical data are discussed concerning known and putative phosphorylation sites of ER α . Phosphorylation is the addition of a phosphate group onto a protein, which is a bulky and negatively charged molecule. Phosphorylation can affect the structural conformation of the receptor. This can in turn influence ER function, e.g. by interfering with dimerization or by differential recruitment of coregulators. Kinases and molecular pathways involved in targeting these sites are briefly discussed. Some phosphorylation events are beneficial to tamoxifen response, whereas others may induce resistance.

Chapter 2 addresses a more fundamental question of how different structural domains of the estrogen receptor define its function. This was done by a comparison between the two receptor subtypes: ER α and ER β . Both proteins contain two activation function domains, AF-1 and AF-2, separated by a hinge region. Although both are members of one family and are partially conserved, their differences result in different function. By swapping domains between ER α and ER β , we discovered that the synergy between the AF-1 and AF-2 domains is dependent on the hinge region. Together, the domains determine the quantitative response to estradiol or tamoxifen.

The estrogen receptor requires coregulators to perform its transactivation function on DNA. By differential coregulator recruitment it determines which genes are transcribed and which are blocked. In **Chapter 3** we applied a high-throughput chip containing coregulator motif peptides, to investigate coregulator binding by full-length ER α . This technique enabled us to study both overexpressed and endogenous proteins in lysates from different cell lines.

Moreover, peptide binding was measured in crude lysates from patient tumor tissue.

Phosphorylation of the estrogen receptor can affect its function by altering coregulator recruitment. Therefore, we investigated if protein kinase A (PKA) induced phosphorylation of the ER α Serine 305 residue, which has been associated with tamoxifen resistance, influences coregulator binding. Phosphorylation resulted in enhanced coregulator interactions.

How does phosphorylation of ER α result in a conformational change and differential recruitment of coregulators ultimately lead to cell proliferation, tumor growth and tamoxifen resistance? **Chapter 4** addresses these questions by genome wide analyses of the chromatin binding landscape and gene expression profile of cells expressing Serine 305 phosphorylated ER α . We discovered that one post-translational modification, i.e. ER α Ser305 phosphorylation, can dramatically influence the chromatin binding preferences of the receptor, here showing a strong enrichment at promotor start sites, 3' and 5' UTRs. We defined a 100-gene classifier, and determined distinct targets for the phosphorylated receptor, including MYC, a known oncogene. A differential regulation of the MYC pathway could very well explain resistance to tamoxifen. This suggestion is supported by the significant correlation of the classifier with poor disease outcome in two independent datasets of ER α positive, tamoxifen treated patients.

Finally, **Chapter 5** puts the work presented in this thesis in perspective and provides an outlook for future studies.

CHAPTER 1
review

A role for estrogen receptor phosphorylation
in the resistance to tamoxifen

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International Journal of Breast Cancer 2011, Article ID 232435

A role for estrogen receptor phosphorylation in the resistance to tamoxifen

Renée de Leeuw, Jacques Neefjes, and Rob Michalides

Abstract

About two thirds of all human breast cancer cases are estrogen receptor positive. The drug of first choice for these patients is tamoxifen. However, about half of the recurrences after removal of the primary tumor are or become resistant to this drug. While many mechanisms have been identified for tamoxifen resistance in the lab, at present only a few have been translated to the clinic. This paper highlights the role in tamoxifen resistance of phosphorylation by different kinases on different sites of the estrogen receptor. We will discuss the molecular pathways and kinases that are involved in phosphorylation of ER α and how these affect tamoxifen resistance. Finally, we will elaborate on the clinical translation of these observations and the possibility to predict tamoxifen responses in patient tumor samples before treatment onset. The findings made originally on the bench may translate into a better and personalized treatment of breast cancer patients using an old and safe anticancer drug: tamoxifen.

1. Introduction

Worldwide, some 1.5 million women are diagnosed with breast cancer annually. Approximately, 70% of human breast cancer expresses estrogen receptor alpha (ER α). These tumors are eligible to endocrine therapy. Over the last 30 years, tamoxifen has been the antiestrogen of first choice. However, about half of the recurrences in ER-positive breast cancer do not respond to tamoxifen, which is due to either acquired resistance or to intrinsic insensitivity to tamoxifen [1, 2]. From experimental studies, many different mechanisms have been suggested to explain resistance, including activation of kinase pathways or inactivation of pRb, that render the tumor cell independent of the ER pathway for its proliferation [2]. However, with exception of cErbB2 (neu) overexpression, which mostly, but not exclusively occurs in ER α -negative breast cancer [3, 4], currently none of the resistance mechanisms identified have been translated into clinical implementation.

It is evident that multiple factors are involved in tamoxifen resistance. Therefore, they should be examined together as an integrated set of predictive markers for diagnosis of individual patients. Not only the number of clinically relevant indicators for tamoxifen resistance is unknown, but also the proportion in which a particular marker contributes to resistance in patients is unclear. The relative contribution of these factors should be defined and potentially integrated into a combined set of predictive markers for tamoxifen responses of individual patients.

Tamoxifen stimulates the growth of osteoblasts, while it inhibits ER α -positive breast tumor cells. These two opposing effects of tamoxifen on cell growth can be explained by the fact that tamoxifen is a partial antagonist, acting as an agonist under particular conditions [5]. Tamoxifen resistance is usually due to a direct effect on ER α ; tamoxifen may acquire agonistic properties for transactivation of ER α [6]. Therefore, a molecular understanding of the underlying mechanism of tamoxifen resistance could result in markers that specify how patients will respond to endocrine therapy. The potential translation of these markers into clinical evaluation has to be examined with historical material and ultimately in a prospective study. Identification of markers predicting the antibreast cancer response to tamoxifen would have major clinical implications. Currently, the clinical benefit of tamoxifen is similar to that

of aromatase inhibitors, although the side effects of the drugs markedly differ. Ultimately, by finding predictive markers, responsiveness to tamoxifen can be defined before treatment and patients will only receive tamoxifen if they are likely to benefit from it. And in case of resistance, patients may still respond to another treatment modality, such as aromatase inhibitors or the full antiestrogen antagonist, like fulvestrant, which may still be beneficial [5].

The estrogen receptor superfamily consists of two homologous nuclear receptors: ER α and ER β . ER β is encoded by a different gene, and the two receptors exhibit different transcriptional activities and functions in breast cancer [7]. Because the phosphorylation of ER β and a potential role in tamoxifen resistance have not been well characterised, we will not discuss this estrogen receptor subtype.

In this chapter, we focus on phosphomodifications of ER α in tumor cells that, by themselves, do not affect the female hormone estradiol (E2) dependency of the tumor cells for proliferation, but could affect the response to tamoxifen. We will address the following questions: which phosphorylation sites are identified on ER α ? How do these sites become phosphorylated? Which sites are associated with tamoxifen resistance? How does tamoxifen sensitivity become affected without any effect on E2 dependency? Which molecular pathways upstream or downstream of the phosphorylated ER are involved in this form of tamoxifen resistance? Which clinical data are in support of tamoxifen resistance due to phosphorylated ER α ?

2. Effects of phosphorylation on the structure of ER α , which are relevant for tamoxifen resistance

Phosphorylation of the estrogen receptor may change the 3-dimensional structure of the protein. Unfortunately, thus far no full-length ER α has been crystallised. This complicates characterisation of structural changes upon ligand binding or posttranslational modifications, such as phosphorylation. Furthermore, a conformational change due to phosphorylation could have consequences for the action of estrogens and antiestrogens. X-ray crystallography studies have thus far been performed on the ligand binding domain (LBD) of ER α . Estradiol binds to amino acids Glu353 from helix 3(H3), Arg394 from H5, and to His524 from H11 in the LBD of ER [8], whereas D351 in the LBD is critical for the interaction with the antiestrogen. Specific mutation of D351 into D351Y resulted in a receptor that shows an estrogenic, instead of an antiestrogenic, response to tamoxifen [9].

Coactivators have a common signature motif, LXXLL, with which they can interact with ER α in a hormonedependent manner [10]. Whereas in a nonligand-bound state helix 12 is highly mobile, upon binding of an agonist it takes a more fixed position, stabilising the conformation of ER α . Helix 12 forms a charge clamp with helix 3, creating a hydrophobic groove to which a coactivator can bind. In contrast, crystallography shows that, when an antagonist, such as tamoxifen, binds to the LBD, helix 12 itself occupies the coactivator binding site, rendering ER α inactive [11–13]. Structural changes of ER can influence coregulator binding and hence potentially the response to ligands.

Besides binding to the LBD in the AF-2 domain, coactivators also bind to the AF-1 domain of ER α , in a ligand-independent manner. Phosphorylation of sites within or outside the AF-1 region may affect the AF-1-dependent binding of cofactors as well.

Phosphorylation of particular sites, especially of S118 and S305, affects the binding of coactivators in the presence of tamoxifen [14]. In case of S305, this is due to an altered conformation of ER α , which can be measured by fluorescence resonance energy transfer (FRET) [15]. In the presence of tamoxifen, S305 phosphorylation changes the orientation between ER α and coactivator SRC-1 [14]. This altered orientation renders ER α transcriptionally active in the presence of tamoxifen. An altered conformation of ER α due to phosphorylation

of S305 resulted in a tamoxifen-resistant phenotype of ER α , not only measured by FRET, but also by biological assays [6, 16]. Not only tamoxifen but also arzoxifene is converted from an antagonist into an agonist after the S305 phosphorylation-induced conformational arrest of ER α [16]. These findings strongly suggest that subtle changes in the conformation of ER α upon binding to antiestrogens are at the basis of resistance to antiestrogens. This provides the framework to consider a role for phosphorylation of ER α in resistance to tamoxifen.

3. ER α phosphorylation sites with a putative role in tamoxifen resistance

Several kinase pathways have been associated with tamoxifen resistance, including activation of the protein kinase A (PKA) [17], mitogen-activated protein kinase (MAPK) [18] and p21-activated kinase-1 (PAK-1) signaling pathways [19]. These kinases induce phosphorylation of ER α or of its coregulators. This paper focuses on the phosphorylation sites on ER α that could contribute to an altered response to tamoxifen and on which kinase pathways and upstream activators are involved. A summary of the putative phosphorylation sites in ER α is presented in Figure 1 and Table 1. They are discussed separately below.

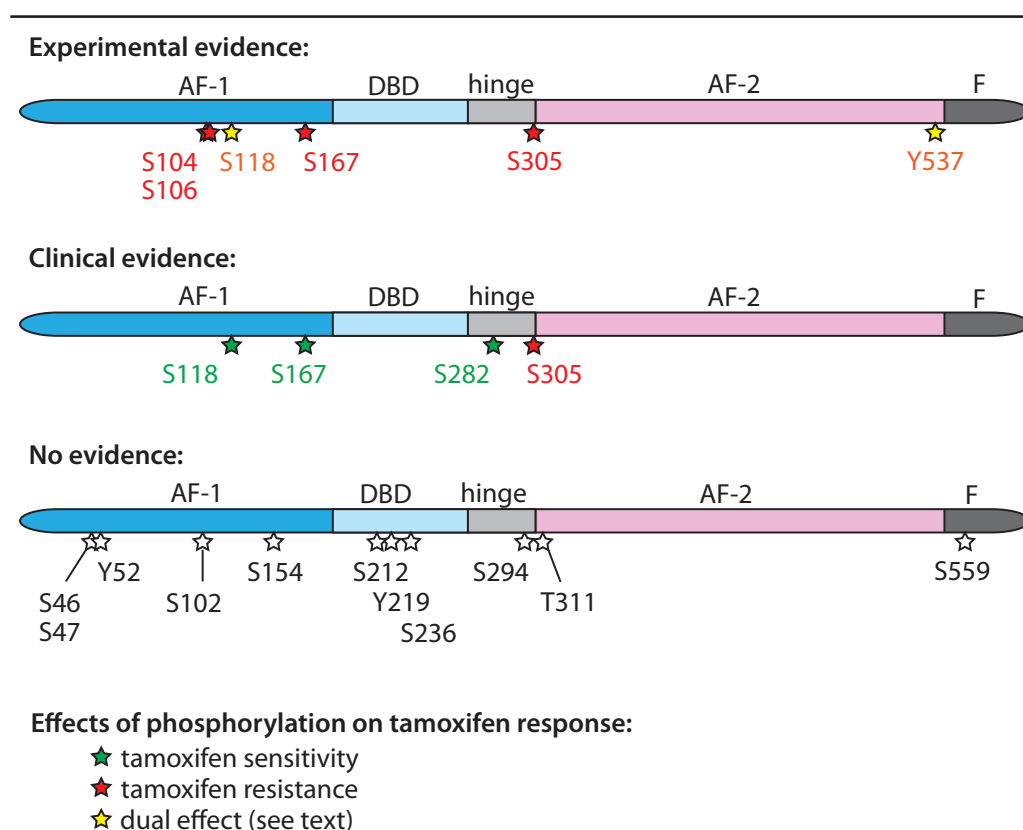


Figure 1: ER α phosphorylation involved in tamoxifen response. From left to right: AF-1 domain, DNA-binding domain (DBD), hinge region, AF-2 domain, and F domain containing helix 12.

3.1. S102/S104/S106.

Serine residues S102, S104, and S106 at the N-terminal AF-1 region of ER α are phosphorylated by glycogen synthase kinase-3 (GSK-3) and by extracellular signal-regulated

kinases 1 and 2 (ERK1/2) and mitogenactivated protein kinase (MAPK) (=MEK1/2) pathways. These modifications lead to ligand-independent transcription of ER α and to an agonistic activity of tamoxifen [22, 23]. S102, a phosphorylation site discovered by mass spectrometry, requires concurrent phosphorylation of S104 [20]. ER α phosphorylation by GSK-3, which also targets S118, stabilizes ER α without ligand and modulates ER α transcriptional activity upon ligand binding. S104 and S106 can also be phosphorylated by the CDK2/cyclinA complex [24]. Cyclin A has been reported as a predictive marker for tamoxifen resistance in breast cancer patients [51].

Phosphosite	Kinases	Tamoxifen	Reference
S46/47	PKC		[22]
Y52	c-Abl		[55]
S102/4/6	GSK-3, ERK1/2 MAPK, CDK2	resistance	[20-23]
S118	CDK2, ERK1/2 MAPK, RAS/MAPK, GSK-3, CDK7, IKK α , mTOR/p70S6K	dual effect	[18, 25-30, 32-35]
S154			[22]
S167	ERK1/2 MAPK, p90RSK, CK2, Akt, mTOR/p70S6K	sensitivity	[25, 30, 33, 35, 37-41]
S212			[22]
Y219	c-Abl		[55]
S236	PKA		[17, 33]
S282	CK2	sensitivity	[22, 42, 56]
S294			[22, 42]
S305	PKA, PAK1?	resistance	[15, 19, 33, 43-47, 51]
T311	RAS/MAPK		[42, 55]
Y537	SrcY kinases	dual effect	[25, 33, 52-54]
S554			[22]
S559	CK2		[22, 56]

Table 1: Putative ER α phosphosites, the kinases that target them, and the effect on tamoxifen response.

3.2. S118.

Serine 118 is one of the most reported phosphorylation sites of ER α . It is targeted by a number of kinase pathways: MAPK, GSK-3, IKK α , CDK7, and mTOR/p70S6K. S118 phosphorylation by MAPK increases binding of coactivator SRC3 [25] and renders ER α hypersensitive to estradiol [26]. Phosphorylated S118 decreases ER α affinity for tamoxifen and reduces binding to DNA, when ER α is tamoxifen bound [25]. In a tamoxifen-resistant cell line obtained by selection after prolonged exposure to tamoxifen, MAPK activity was found to be elevated and S118-P was increased [26]. Upstream, the RAS/MAPK pathway can be activated by IGF stimulation inducing phosphorylation of ER α S118 and resulting in ER α activation and enhanced response to estradiol [18]. Estradiol and EGF can induce the ERK1/2 MAPK pathway, which also leads to S118 phosphorylation of ER α [27]. Estrogen-dependent phosphorylation of S118-P can occur not only through the ERK1/2 MAPK pathway, but also by IKK α [28] and CDK7, a subunit of transcription factor II H [29].

In MCF7 cells, the receptor tyrosine kinase RET mediates ER α phosphorylation at S118 and S167 via the mTOR/p70S6K pathway [30]. Activation of RET leads to estrogen-independent transcriptional activation of ERdependent genes and resistance to tamoxifen, strongly suggesting that RET activity acts through the estrogen receptor. This hypothesis is

supported by a chromatin immunoprecipitation (ChIP) study on ER α and S118 mutants [52]. Phosphorylation of S118 influences the recruitment of coregulators to ER α -regulated genes pS2, c-myc, and cyclin D1 and affects E2-induced gene expression. The nonphosphorylatable S118A mutant has a greater impact on genes regulated through nonclassical mechanisms, such as ER α binding to fos/jun on an AP-1 promoter, than on estrogen responsive elements (ERE).

The clinical relevance of S118 phosphorylation in tamoxifen resistance is still unresolved. On the one hand, S118-P has been associated with a more differentiated phenotype, good prognosis, and better response to tamoxifen [28], which is supported by other studies (see [31], Wigerup et al. unpublished data). Most importantly, these studies reported that the S118 phosphorylation had no effect on progression of disease or survival without tamoxifen treatment [31], thereby emphasizing that S118 phosphorylation is a clear predictive marker for response to tamoxifen in these studies.

On the other hand, S118 phosphorylation was negatively correlated with response to endocrine therapy in patients in other studies [32–34]. Nontreated patients have a better prognosis when they are positive for S118-P in these studies [32, 33]. These results are not easily reconciled with the previously mentioned studies (see [28, 31], Wigerup et al. unpublished data). Besides differences in patient series and tumor types, it is not clear which kinase activities in the tumors are resulting in S118 phosphorylation. In patients, both MAPK and RET expressions are associated with poor response to antihormonal therapy [53]. Activation of the ERK1/2 MAPK pathway apparently results in S118 phosphorylation, but it also induces a bypassing of the ER pathway, thereby rendering tumors hormone-independent.

CDK7-mediated phosphorylation is indicative of an active ER α . Whereas the MAPK mechanism may well be responsible for a worse outcome of disease, irrespective of tamoxifen treatment, the CDK7 mechanism would indicate a proper functioning of ER α , being an adequate target for tamoxifen treatment [33].

3.3. S167.

Serine 167 is phosphorylated by Akt, p90RSK, and mTOR/p70S6K. The latter kinase also phosphorylates S118. Akt is induced by EGF and IGF [35], p90RSK only by EGF stimulation [36]. EGFR overexpression induces S167 phosphorylation, increases binding of ER α to DNA, enhances the binding of coactivator SRC3 to ER α in the presence of E2, and consequently enhances transcription. Moreover, in vitro, S167-P reduces sensitivity to tamoxifen [25, 32]. Other kinases that target S167 include ERK1/2 MAPK [32, 37] and, upon E2 binding of ER α , casein kinase II (CK2) [38]. S167-P does not affect ligand binding [25].

The clinical data of S167 phosphorylation are conflicting. In ER α -positive, tamoxifen-treated patients, activated AKT (pAKT) is associated with high risk for relapse and decreased overall survival [39], which would imply that S167-P is associated with a worse disease outcome. However, it is important to realise that Akt, like ERK1/2 MAPK, has many other targets, which could well bypass the estrogen-receptor-dependent signaling.

Notwithstanding, in a set of 75 primary breast carcinomas of patients with metastatic breast cancer who received first-line endocrine treatment after relapse, those staining high for S167-P relapsed later. The metastases responded well to endocrine treatment and S167-P correlated with longer survival after relapse. This implies that S167-P is a predictive marker for a good response to endocrine therapy [34, 37].

3.4. S282.

Serine 282 resides in the hinge region and, like S167, can be phosphorylated by CK2. Estradiol increases phosphorylation of S282, stabilizes ER, and induces transcriptional activity

[20]. In patients, low levels of S282 phosphorylation are associated with reduced overall survival in ER-positive breast tumors from tamoxifen-treated patients, suggesting that S282 phosphorylation can be predictive for response to tamoxifen [40].

3.5. S305.

Serine 305 resides at the C-terminus of the hinge region that provides a centre of rotation to the total ERα. The region around Ser305 is a multifunctional domain that binds to many coregulatory proteins and is involved in the regulation of activity and stability of ERα [42]. Phosphorylation of Ser 305 occurs by protein kinase A and is associated with resistance to tamoxifen in patients (see [43–45], Wigerup et al. unpublished data). This domain also controls ERα ubiquitination and subsequent proteosomal degradation of ERα, that is influenced by ligands [46]. Different ligands can induce different conformations of ERα and hence affect accessibility to the hinge region for modifying proteins, such as ubiquitin ligases. This implies that ligands can be selective for specific posttranslational modifications.

Within the hinge region, lysines K302/303 are involved in proteasomal degradation of ERα by fulvestrant and are the targets of polyubiquitination. K302 and K303 are both required for monoubiquitination by the BRCA1/BARD1 E3 ligase of E2- or tamoxifen-bound ERα [54]. K303 is also target for acetylation (inhibiting ER activity) and for methylation (stabilizing ER and increasing activity), whereas S305 phosphorylation prevents acetylation of K303 [55], thereby stimulating ER activity. The reverse is also true: a K303R mutation is frequently found in breast cancer, which prevents acetylation and increases phosphorylation of Ser305 by PKA [56]. These findings indicate that the hinge region is affected by various posttranslational modifications that affect structure and functioning of ERα. Some of these modifications and their cross-talk are shown in Figure 2.

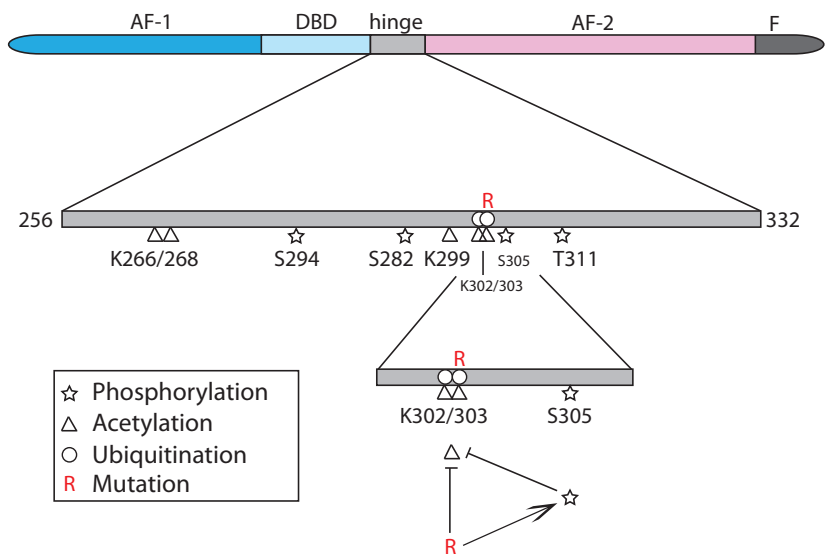


Figure 2: Posttranslational modifications in the ERα hinge region. S305 phosphorylation prevents acetylation of K302/303. The natural K303R mutation blocks K302/303 acetylation and stimulates S305 phosphorylation.

Besides PKA, p21-associated kinase 1 (PAK1) has been suggested as an upstream kinase involved in the phosphorylation of Ser305. PAK1 phosphorylation of ERα S305 can lead

to a secondary event on S118, presumably due to a conformational change of the estrogen receptor [15, 19]. PAK1 overexpression by itself is associated with resistance to tamoxifen in vitro [19] as well as in patients [32, 44, 45, 47]. Notably, in an experimental tamoxifen-resistant setting, tamoxifen induces PAK1, maintaining ER α in the tamoxifen-insensitive state [19]. The evidence that PAK1 phosphorylates S305 [19] was indirect and was not confirmed by a direct inspection of the phosphorylation of ER α Ser305 using specific antibodies or by the introduction of a dominant-active PAK1 into breast cancer cells [44]. Moreover, overexpression of PAK1 was not correlated with S305 phosphorylation in two different studies on breast cancer, indicating that these two events are independent (see [44], Wigerup et al. unpublished data). PKA phosphorylates S305, keeping ER in an active conformation when tamoxifen is bound, which means that it mimics an estrogen-bound ER [15]. This was not observed with overexpression of PAK1 [44].

Clinical studies show that tamoxifen resistance occurred in endocrine-treated patients with detectable S305-P in the primary human breast tumor [44]. Since S305 phosphorylation has no effect on patients that were not endocrine treated, this Ser305P marker appears to be a predictive marker for treatment outcome and not for general disease progression [43]. A combination of PAK-1, phospho-PKA, a marker of activated PKA, and the phosphorylated S305 marker identified approximately 60–70% of all tamoxifen resistant cases in breast cancer. This occurred in series of breast cancer from premenopausal and postmenopausal patients, in early to advanced stages of disease, indicating that the marker is independent of clinical stage of disease and of the hormonal status of the patient (see [31, 43, 45], Wigerup et al. unpublished data).

3.6. Y537.

Tyrosine 537 is phosphorylated by the Src family tyrosine kinases. Phosphorylation of this tyrosine inhibits ER dimerisation and estrogen binding and reduces transcriptional activity of ER α [48]. Tyr 537 is located at the N-terminus of helix 12, and mutation of this Tyr into a nonphosphorylatable alanine facilitates the rotation of helix 12 into an active conformation of ER α in the absence of any ligand [49]. Phosphorylation by activated Src increases affinity for E2 and decreases affinity for tamoxifen [25]. Nonphosphorylatable mutants show ligand independent transactivation, but this is inhibited by tamoxifen [32]. There is no apparent clinical evidence that Y537 phosphorylation influences tamoxifen response in patients. Of note, a naturally, but rarely occurring, Y537 mutation to asparagine (Y537N) in breast cancer metastasis constitutively activates the estrogen receptor by a conformational change of helix 12, which may contribute to breast cancer progression and resistance to endocrine treatment [50].

4. Other ER α phosphorylation sites with no (known) role in tam oxifen resistance

Several other phosphorylation sites of ER α have been found, which have not been associated with tamoxifen, either since tamoxifen was not included in the studies or because the phosphosite has not been included in clinical studies on tamoxifen resistance. These sites are briefly discussed below.

4.1. S46/47.

Ser-46/47 phosphorylation plays a role in ligand-dependent activation of ER α . Mutation of Ser-46/47 or Ser-294 to alanine markedly reduced estradiol-dependent reporter activation. S47 phosphorylation may influence other posttranslational modifications of ER α . S46 is a putative recognition site for protein kinase C and seems to hold a predominant effect

on transcriptional activity, rendering S47 phosphorylation a “bystander” effect [20].

4.2. Y52 and Y219.

Tyrosine 52 and 219 are phosphorylated by c-Abl, a Src-like nonreceptor tyrosine kinase. Y219 phosphorylation affects ER dimerization and DNA binding. This results in enhanced ER α transcriptional activity, both in absence and presence of estradiol. Stabilisation of ER α through c-Abl ultimately leads to proliferation and invasion of breast tumor cells [21].

4.3. S154, S212, S294, and S554.

Serine 154, 212, 294, and 554 are putative ER α phosphorylation sites discovered by mass spectrometry on phosphopeptides [20]. In vitro, an alanine mutation of S294 reduces estradiol-dependent transcription [20], suggesting that S294 phosphorylation is needed for a functional ER. Furthermore, S294 phosphorylation has been detected by immunohistochemistry (IHC) in human breast carcinoma but no significant effect of S294-P on tamoxifen response in terms of recurrence or overall survival has been observed [40]. The biological relevance of the other three serines remains to be tested.

4.4. S236.

Serine 236 is located in the DNA binding domain (DBD). It is phosphorylated by PKA, upon which ER dimerisation and DNA binding in the absence of ligands are lost, rendering ER transcriptionally inactive [32], but both estradiol and tamoxifen can overcome this inhibition [17]. This would imply that S236-P in itself has no effect on tamoxifen sensitivity.

4.5. T311.

Threonine 311 is the only known threonine phosphorylation site on ER α . An active RAS/MAPK pathway stimulates ER α phosphorylation at Thr-311 [21]. Phosphorylation of T311 can be detected by immunohistochemistry, but thus far has not been significantly associated with altered tamoxifen sensitivity in breast cancer patients [40].

4.6. S559.

Serine 559, like Y537, resides in the F domain of the estrogen receptor, in helix 12. This is of particular interest, because the position of helix 12 determines interaction with coactivators and corepressors and regulates response to (ant)agonists. Therefore, S559 phosphorylation can probably influence ER binding to coregulators, such as SRC-1, by changing the position of helix 12 and as a consequence the response to ER ligands. S559 is targeted by CK2 [20]. Phosphorylation inhibits ligand-independent activation of ER α . ER α is phosphorylated at S559 in human breast carcinoma biopsies [41].

5. Main points, side-issues and interrelated affairs

We presented, thus far, the effects of phosphorylation of relevant sites in ER α as single events. Of course, reality is more complex and modifications not only occur on ER α itself, but also take place on the associated cofactors and on targets outside the ER α signaling pathways that could have an effect on ER α -mediated signaling. Three examples below illustrate this point.

- (a) Phosphorylation of CARM1, an arginine methyltransferase, by PKA [57]. Phosphorylation of CARM1 by PKA enhances its interaction with S448 in the LBD of ER α and creates a novel, more firm platform for binding of other cofactors. The net result is

tamoxifen resistance by the buildup of a PKA-specific coactivator complex. Because the arginine methyltransferase CARM1 is involved in methylation of histones H₃ and H₄ that is crucial for transcription to occur, the ER α -phosphoCARM1 complex provides a specific regulatory unit for transcription. Still, additional events are needed for tamoxifen resistance, among which possibly the phosphorylation of ER α S305 by PKA.

(b) PAK1 phosphorylates an alternate, but in breast cancer frequently present, isoform of the SRC3 steroid-receptor cofactor (SRC3-3 δ 4), allowing it to bridge between EGF-R and FAK1 (focal adhesion kinase 1) and thereby activating ERK1/2 MAPK [58]. Activation of this pathway possibly renders breast tumor cells tamoxifen resistant. This provides a novel turn to the role of PAK1 overexpression in breast cancer.

(c) The selective activity of SRC-3 depends on specific phosphorylation of SRC3 [59]. SRC3 has six specific phosphorylation sites targeted by multiple kinases. These phosphorylated sites determine the optimal of SRC3 [59]. SRC3 has six specific phosphorylation sites targeted by multiple kinases. These phosphorylated sites determine the optimal interaction with other transcription factors and are required for different physiological functions.

These three examples demonstrate that there is a complex interrelated network of regulatory circuits influencing ER transcriptional activity and that, by modification of one circuit, other circuits are affected. They also indicate that one particular mode of modification can have multiple effects. Most of the studies have addressed only one significant mode of action, but it is evident that many factors can play a role in the resistance to endocrine treatment.

6. Downstream signaling / gene expression / pathways

How does phosphorylation of ER α affect resistance to tamoxifen? The estrogen receptor is a nuclear receptor, which binds to specific sequences in the DNA and regulates the expression of ER-dependent genes. Phosphorylation of ER α can affect DNA binding, for example, by inhibiting dimerization of the receptor, and can influence ER α activity by changing the binding to coactivators or the orientation of components of the transcription factor complex. Which genes are then affected? In the classical way, an estradiolbound estrogen receptor dimerizes, binds to an estrogen responsive element (ERE), and transcribes the gene that lies within its proximity. The estrogen receptor can also regulate transcription of genes in an indirect manner, by binding to other transcription factors: AP-1, SP-1 [60], or activated NF κ B [61]. When these interactions occur, transcription of the AP-1-, SP-1-, or NF κ B-dependent genes becomes also dependent on ER α . When tamoxifen is bound to ER α , the classical estrogen responsive genes are not expressed but tamoxifen-bound ER α has its own, different transcriptome, most likely generated through the nonclassical pathway [62, 63].

Different kinase pathways can be activated chemically in cells by adding growth factors (EGF or IGF) or cAMP, which induces PKA. This approach was used in a gene expression study on MCF7 breast cancer cells [64]. Kinases were activated, and gene expression profiles were compared in the presence or absence of tamoxifen. Tamoxifen treatment resulted in differential gene expression with either growth factor stimulation or PKA activation. Which of these genes is essential for tamoxifen resistance remains a crucial question.

A more complete, but also more complex, picture arises from microarray analyses performed in tumors of tamoxifentreated ER α -positive breast cancer patients. Frasor et al.

described a set of genes associated with disease recurrence, a subset of which is associated with treatment with tamoxifen [63]. Loi et al. applied gene expression profiling in a similar way. They developed a gene classifier to predict clinical outcome in tamoxifen-treated ER α -positive breast cancer patients. This classifier contains genes involved in invasion (SLIT2 and RECK), anti-inflammatory response (TGFB β 4, PTGER4, C3, and GNG2), and cell cycle regulation [65]. In later studies, this group validated a number of hits by qPCR and hence demonstrated that EZH2 downregulation is associated with a favourable outcome [66] and that downregulation of SIAH2, an E3-ubiquitin ligase, would imply tamoxifen resistance [67]. They also showed that an extracellular matrix cluster of genes (TIMP3, FN1, LOX, and SPARC) is associated with tamoxifen resistance [68]. In any of these studies, it is unclear whether phosphorylation of the estrogen receptor plays a role in tamoxifen resistance in these patients. Looking at multiple genes, instead of only one, could be more informative for treatment outcome. Therefore, Kok et al. compared three gene classifiers [69–71] for tamoxifen. This comparison indicates that a multigene approach would improve the prediction of response to tamoxifen [31].

There is as of yet only one microarray study on tamoxifen-treated ER α -positive human breast cancers that addresses a specific phosphorylation site, S305P, and the effect on gene expression. A pathway analysis highlighted several pathways being affected, including PKA, ERK1/2 MAPK, EGF signaling, CDK regulation, and interferon alpha signaling [44].

7. Discussion

In total, 19 phosphorylation sites have been identified in ER α thus far, as summarized in Figure 1. Phosphorylation of S167, S118, S282, and Y537 is beneficial for tamoxifen response according to experimental and, for S167, S118 and S282, because of reported clinical data. Tamoxifen resistance is likely to occur when S104/S106 or S305 is phosphorylated. The contribution of phosphorylation of other target sites to tamoxifen resistance remains to be determined. Some of these phosphorylation sites have been shown with FRET technology to induce a conformational change of ER α , when exposed to other antiestrogens, such as fulvestrant and raloxifene [6]. Thereby, they may affect the antagonistic behavior of these compounds but the molecular mechanisms remain to be elucidated.

Upstream of ER α , different kinase pathways are involved. Dependent on the pathway and the phosphorylation sites involved, tamoxifen response can be affected either directly through ER α modification or by activation of other signaling pathways. Phosphorylation of S118 is described as an example of this: an activated ERK1/2 MAPK pathway phosphorylates S118 but possibly induces tamoxifen resistance through the ERK1/2 MAPK pathway itself, rather than ER signaling. S118 phosphorylation by the ER-associated CDK7 indicates an activated ER which would imply a beneficial effect on tamoxifen treatment in patients.

EGFR and cErbB2 can also affect ER signaling. Tamoxifen response may be restored by blocking EGFR with gefitinib [3, 72]. In a clinical study, blocking cErbB2 with trastuzumab restores ER α signaling in ER α -positive tumors and improves response to the aromatase inhibitor letrozole [73]. This would suggest a better response to tamoxifen as well.

It is challenging to extrapolate experimental data from ER activation to the clinic and vice versa. In *in vitro* studies, it is feasible to examine differential gene expression after treatment and compare the profile before and after treatment, or in absence or presence of phosphorylation. Translation of this information to the clinic can, however, be troublesome, since adjuvant tamoxifen treatment is started after surgical removal of the primary tumor. The *in vitro* experiments measure gene expression changes associated with acquired resistance, whereas investigation of primary tumors that respond better to treatment highlights genes which play a role in intrinsic resistance to tamoxifen. Because primary tumors have not been

exposed to tamoxifen, endocrine treatment cannot be a selective factor for these resistance markers. They may occur at random during normal tumor development or may well coincide with other tumor progression markers. For example, overexpression of cErbB2 or of EGFR in breast cancer marks worse course of disease not only in ER α -negative, but also in ER α -positive tumors and is a marker for tamoxifen resistance as well [74].

Phosphorylation of Ser305 is a marker for intrinsic resistance to tamoxifen. It is not associated with disease progression in the absence of tamoxifen treatment [44]. It is also a marker for the choice of treatment, since a combination of S305-P, S118-P, and overexpression of SRC-1 or cyclin D1 coactivators dictates resistance to different antiestrogens [14, 15]. Since outgrowth of micrometastases into tamoxifen resistant tumors occurs over longer periods of time (up to 15 years), extra alterations in the micrometastases outgrowths, in addition to the S305 phosphorylation status, potentially influence tamoxifen resistance. Phosphorylation of Ser305, however, was still maintained in the few metastases samples that could be examined [44]. Alternatively, one could study acquired and intrinsic resistance during neoadjuvant treatment with antiestrogens, where patients are treated up to three months prior to the surgical removal of the primary tumor. Hence, samples can be obtained before and after treatment for comparison [75]. In another study by this group, activation of the ERK1/2/MAPK pathway was a major factor associated with acquired resistance to tamoxifen [74].

Phosphorylation of S305 has experimentally been linked to resistance to tamoxifen, because of an altered conformation of ER, where tamoxifen behaves as an agonist in FRET and expression reporter assays [15]. In patients, S305-P was associated with alterations in the PKA pathway that result in stimulation of PKA activity [15, 44]. Also experimental enhancement of PKA activity in breast tumor cells led to proliferation of T47D breast tumor cells in the presence of tamoxifen [15]. It is, however, still possible that S305P is a marker for tamoxifen resistance without any direct involvement. It could merely mark PKA related events that bypass the estrogen receptor and hence induce tamoxifen resistance. The altered orientation of components of the transcription factor complex and the conformational changes in ER α strongly suggest, but are no proof of, a direct involvement. S305-P is, however, one of the few selective markers that predict resistance to tamoxifen in breast cancer patients. Definition of the activation of relevant signaling pathways in the ER α -positive breast tumors (that constitute the bulk of human breast cancers) prior to endocrine treatment is essential for treatment success and will ultimately lead to personalised treatment of breast cancer patients.

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Chapter 2
research article

The hinge region of the human estrogen receptor determines
functional synergy between AF-1 and AF-2 in the quantitative
response to estradiol and tamoxifen

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Journal of Cell Science 2010, Vol. 123, pp1253-1261

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Abstract

Human estrogen receptors α and β (ER α and ER β) greatly differ in their target genes, transcriptional potency and cofactor-binding capacity, and are differentially expressed in various tissues. In classical estrogen response element (ERE)-mediated transactivation, ER β has a markedly reduced activation potential compared with ER α ; the mechanism underlying this difference is unclear. Here, we report that the binding of steroid receptor coactivator-1 (SRC-1) to the AF-1 domain of ER α is essential but not sufficient to facilitate synergy between the AF-1 and AF-2 domains, which is required for a full agonistic response to estradiol (E₂). Complete synergy is achieved through the distinct hinge domain of ER α , which enables combined action of the AF-1 and AF-2 domains. AF-1 of ER β lacks the capacity to interact with SRC-1, which prevents hinge-mediated synergy between AF-1 and AF-2, thereby explaining the reduced E₂-mediated transactivation of ER β . Transactivation of ER β by E₂ requires only the AF-2 domain. A weak agonistic response to tamoxifen occurs for ER α , but not for ER β , and depends on AF-1 and the hinge-region domain of ER α .

Introduction

Estrogen receptors α and β (ER α and ER β) are homologous members of the nuclear receptor superfamily and are encoded by two different genes. They control crucial processes in physiology and are prognostic markers in breast cancer; their expression determines whether or not endocrine treatment is given as an adjuvant therapy (Speirs et al., 2004). On binding of agonistic ligands, the DNA-bound receptor recruits cofactors, which enables the receptor to transmit its regulatory information to the cellular transcription complex, including RNA polymerase II (transactivation). ER α and ER β mediate distinct profiles of gene expression (Chang et al., 2008; Williams et al., 2008). This is due to a combination of differential ligand response (Barkhem et al., 1998; Paech et al., 1997; Paige et al., 1999; Van Den Bemd et al., 1999), distinct interaction with coactivators (Kraichely et al., 2000) and binding to specific estrogen response elements (EREs) (Klinge et al., 2004). Consequently, ER α and ER β exert different effects on the organization, growth and differentiation of various tissues, including colon, uterus, bone, brain and mammary gland (Forster et al., 2004; Helguero et al., 2005; Kudwa et al., 2005; Wada-Hiraike et al., 2006b; Wada-Hiraike et al., 2006a). Transactivation of ERs can occur in two different ways. One is by classical ER transactivation, in which an ER homodimer binds to a palindromic ERE sequence (Klein-Hitpass et al., 1989). The other way is by non-classical transactivation, in which the ER is tethered to other transcription factors, including the Fos-Jun complex, NF- κ B and Sp1, to render transcription of their genes responsive to ER activity (Pearce and Jordan, 2004; Sabbah et al., 1999). ER α and ER β differ in their classical and non-classical transactivation by estradiol (E₂) (Paech et al., 1997; Weatherman et al., 2001). These differences between ER α and ER β are reflected in differences in their amino acid sequences and/or different interactions between the various domains of the receptor. ER α is composed of 595 amino acids, whereas ER β is 530 amino acids. The N-terminal A/B domain (also known as the AF-1 domain) has a ligand-independent transactivation function

and has 17% amino acid homology between the ERs. The highly conserved central C region, with 96% homology, encompasses the DNA-binding domain (DBD). The flexible hinge, or D region, contains nuclear localization signal (NLS) information and links the C-domain to the multifunctional C-terminal E/F domain. The E/F domain (also known as the AF-2 domain) of the ERs shares 53% amino acid homology and contains the ligand-binding domain (LBD) and the ligand-dependent transactivation domain, including the cofactor-binding groove to which cofactors are recruited when the ER becomes activated. For ER α , it has been shown that interaction between the AF domains is essential for effective transactivation (Metivier et al., 2001; Yi et al., 2002a).

Despite similar in vitro ERE-binding capacities and comparable affinities for E2 (Bowers et al., 2000; Yi et al., 2002b), human ER β is considerably weaker than ER α with respect to ERE-dependent transactivation following E2 exposure (Yi et al., 2002b). Also, differences between human ER α and ER β have been reported for the non-classical pathway (Gustafsson, 2000). The molecular mechanism underlying these differences in transcription by the ER subtypes is still unclear.

The precise process of transactivation of genes by ERs is still unresolved. ER can dimerize and bind to its cofactors even when not bound to DNA, which occurs also in the absence of ligands (Carroll et al., 2006; Padron et al., 2007; Zwart et al., 2007a). For proper recruitment of RNA polymerase II, however, the ER needs to be bound to an ERE (Carroll et al., 2006; Sharp et al., 2006; Zwart et al., 2007a). Effective transactivation of ER α requires interaction between the N-terminal and C-terminal regions of ER α (Merot et al., 2004; Metivier et al., 2001), which is accomplished by the binding of ER α to cofactors (Metivier et al., 2001). The antiestrogen tamoxifen inhibits ER α -mediated transactivation by arresting the conformation of ER α such that the groove that interacts with the steroid receptor coactivator-1 (SRC-1) cofactor remains covered by the twelfth a helix of the LBD (Shiau et al., 1998). However, tamoxifen also has weak agonistic activity. This mild agonistic behavior can be enhanced by phosphorylation of serine 305 in the hinge region of ER α by protein kinase A (Michalides et al., 2004).

Here, we investigate the differences between human ER α and ER β with regard to E2-driven transactivation and the agonistic effect of tamoxifen. We show that a specific interaction of the AF-1 domain of ER α with SRC-1 is required, but by itself not sufficient, to induce a maximal response to E2. The maximal response to E2 demands, in addition, the hinge region of ER α . Human ER β lacks an active AF-1 domain. The weak agonistic response to tamoxifen is dependent on the AF-1 domain of ER α , but also involves the hinge region of ER α for optimal transcriptional activity. The AF-1 domain of ER β is not involved in tamoxifen-mediated transactivation, explaining the differential response to the antiestrogen tamoxifen.

Results

Extent of transactivation by ER is dependent on the AF-1 domain

ER α and ER β differ mostly in their N-terminal AF-1 regions, which share 17% sequence homology and differ in size by 41 amino acids. We therefore examined the contribution of the AF-1 domains to optimal E2-driven transactivation. We generated swap mutants, in which AF-1 of ER α (amino acids 1-185) was replaced by the corresponding region of ER β (amino acids 1-144) and vice versa, resulting in ER α AF-1 β and ER β AF-1 α mutants (Fig. 1A). A detailed alignment of both AF-1 domains is shown in supplementary material Fig. S1. All four ER variants were analyzed for the extent of E2-induced transcription using an ERE-luciferase reporter construct with various concentrations of ligand (Fig. 1B). Maximal transactivation of ER α was considerably higher than that of ER β , 47- versus 6-fold induction, respectively. Exchanging the AF-1 region of ER β for that of ER α in ER β AF-1 α greatly affected this response

and increased transactivation from 6- to 16-fold. Exchanging AF-1 of ER α for that of ER β in ER α AF-1 β , however, reduced transactivation from 47- to 6-fold, the level also reached by wild-type ER β . These differences were not due to differences in RNA or protein levels, as has been determined by quantitative PCR (QPCR) analysis, western blot and luciferase reporter assays (supplementary material Fig. S2). The ER β AF-1 domain therefore reduces E2-induced transactivation, whereas the AF-1 domain of ER α is responsible for its enhancement, which is in agreement with a previous report (McInerney et al., 1998). Although exchange of AF-1 of ER β for that of ER α in ER β AF-1 α did increase maximal transactivation approximately threefold when compared with wildtype ER β , the extent of transactivation did not match that of ER α . This suggests that additional features are involved in optimal E2-driven transactivation.

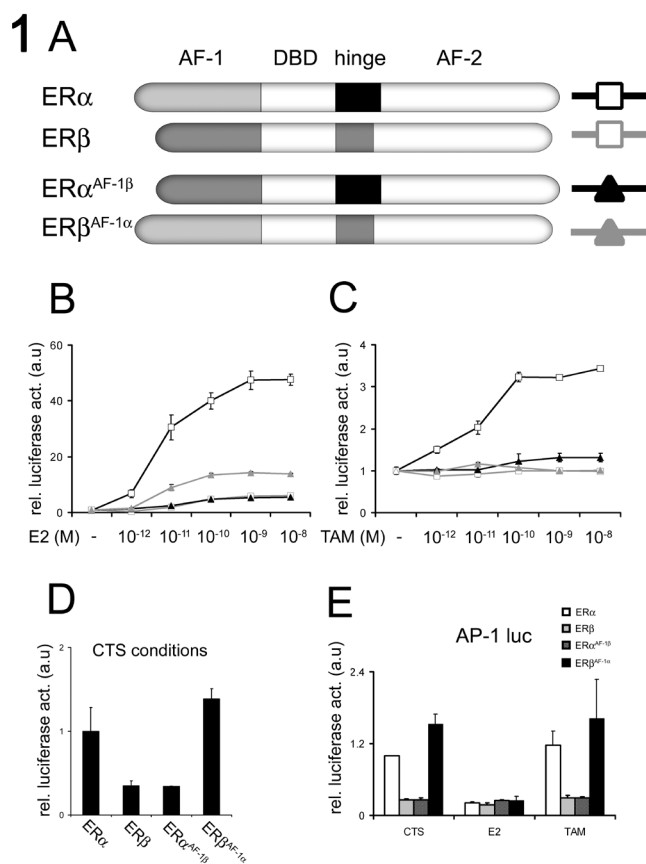


Fig. 1. Extent of transactivation by ER is dependent on the AF-1 domain of ER. (A) Structural overview of ER α , ER β and AF-1 swap mutants. (B) E2-concentration-dependent transcription of an ERE-luciferase reporter. U2OS cells were transfected with ER α , ER β , ER α AF-1 β or ER β AF-1 α (see key in A), co-transfected with the ERE-luciferase reporter gene and Renilla luciferase as the control. Cells were treated with 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} M E2 for 36 hours or left untreated, and relative luciferase activity was measured. A representative experiment out of three independent experiments is shown. Error bars indicate standard deviation from duplicate samples. (C) The same conditions as in B were applied, but with 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} M 4-OH-tamoxifen (TAM). (D) Luciferase signal for the tested ER variants under hormone-depleted conditions. Signals for all ER variants are related that of to

ER α , which is set at 1. A representative experiment out of two independent experiments is shown. Error bars indicate standard deviation from duplicate samples. (E) Non-classical transactivation by ER is influenced by AF-1 of ER α . An AP-1-luciferase reporter assay was performed in U2OS cells, transfected with ER variants, the AP-1-luciferase reporter construct and Renilla luciferase as the control. Cells were treated with 10^{-8} M E2, 10^{-7} M tamoxifen or left untreated for 36 hours. Relative luciferase activity was measured and normalized using the Renilla signal. The relative luciferase signal for all ER variants was related to the CTS (charcoal-treated serum) value of ER α , which was set at 1. A representative experiment out of three independent tests is shown. Error bars indicate standard deviation from triplicate samples.

We then examined whether ligand-independent and tamoxifen associated transactivation are also linked to AF-1 of ER α . The AF-1 of ER α appeared responsible for the minor ligand-independent transactivation of ER α , because exchange of the AF-1 region between the ERs completely switched this ligand-independent behavior (see Fig. 1D). These results indicate that the AF-1 domain of ER α mediates ligand-independent transactivation, whereas such activity is not observed for the AF-1 domain of ER β .

Tamoxifen exhibits a weak agonistic effect on ER α activity (Michalides et al., 2004; Zwart et al., 2007b). We confirmed these data; transactivation by tamoxifen resulted in a 3.5-fold enhancement over hormone-depleted conditions for ER α (see Fig. 1C). Neither ER β nor the two swap mutants responded to tamoxifen, indicating that the weak response of ER α to tamoxifen is mediated by AF-1 of ER α . Because the ER β AF-1 α construct, now containing AF-1 of ER α , did not respond in an agonistic way to tamoxifen, additional features that are not conserved between the two ER isoforms are required for this response (see below).

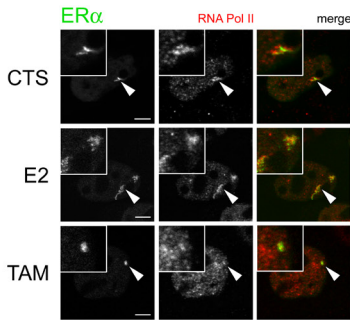
Our results indicated that AF-1 of ER α determines the extent of E2-mediated transactivation in a classical ERE-mediated transactivation assay. To investigate the role of AF-1 in a nonclassical transactivation reaction, we tested ER α , ER β and the AF-1 swap mutants in an AP-1-driven Luciferase assay (Fig. 1E). AP-1-mediated transcription by ER α and ER β AF-1 α could be inhibited through E2 and not by tamoxifen. These features appeared to be AF-1 mediated, because ER β and ER α AF-1 β failed to respond. These data indicate that the discriminatory role of AF-1 between ER α and ER β is not restricted to classical ERE-mediated transcription, but also applies to non-ERE, AP-1-dependent transactivation.

The AF-1 domain of ER α , but not of ER β , binds to SRC-1

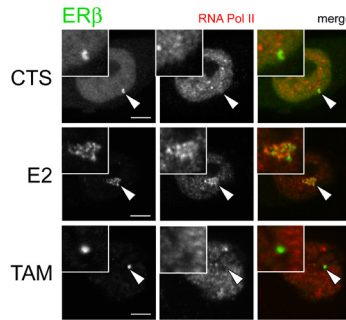
The AF-1 of ER α appeared to be responsible for efficient transactivation of ER α in a ligand-dependent and -independent manner, unlike AF-1 of ER β , which lacks this activity. We next investigated whether this difference in transcriptional capacity correlated with the binding of these domains to the p160 coregulator SRC-1. SRC-1 binds to both the AF-1 and AF-2 domains of ER α ; an interaction that generates a functional synergy between the two transactivation domains (Metivier et al., 2001; Webb et al., 1998). The Q-rich region of SRC-1 binds AF-1 in ER α , whereas the consensus LxxLL-containing middle region of SRC-1 binds to the ligand-dependent AF-2 domain of ER α (Merot et al., 2004).

We visualized these interactions in intact cells on a genuine ERE promoter sequence – the prolactin promoter-enhancer (PRL) region in HeLa cells. These cells contain a DNA array consisting of ~52 copies of stably transfected modified PRL (Sharp et al., 2006). The PRL array allowed visualization of a defined, ERE-containing DNA structure in the nucleus, to which ER and cofactors can be recruited. In addition, the size of this structure correlates with transcriptional activity of the ER-cofactor complex bound to this region (Hatzis and Talianidis, 2002; Sharp et al., 2006; Zwart et al., 2007a). ER α , ER β and both AF-1 swap mutants could recruit RNA polymerase II to the array structure under E2 conditions (see Fig. 2A-D). Under these conditions, the array structures were enlarged, which is associated with transcriptional activity. However, whereas ER α was capable of recruiting RNA polymerase II to the array under hormone-depleted conditions, ER β failed to do so. This difference in RNA polymerase II recruitment was associated with the AF-1 domain of ER α , because swapping this region to ER β sufficed to enable RNA polymerase II recruitment under hormone-deprived conditions. This suggests that the AF-1 domain of ER α possesses ligand-independent transactivation activity, which is absent in ER β , supporting the results of the transactivation studies presented in Fig. 1. We confirmed these interactions using truncation mutants of SRC-1 that bind only AF-1 or AF-2. These truncation mutants of SRC-1 act as dominant-negative mutants over the endogenous coactivators (Zwart et al., 2007a). One truncation mutant of SRC-1 (amino acids 1051-1240),

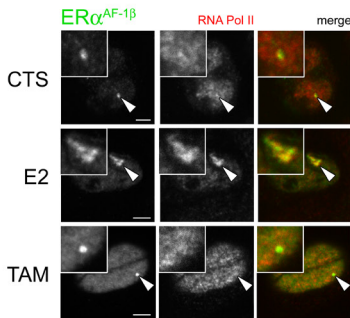
2A



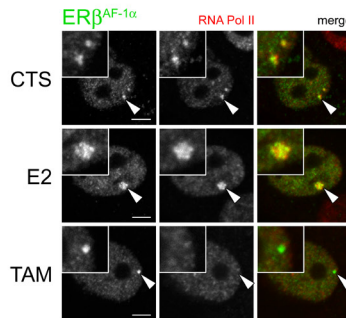
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D



E

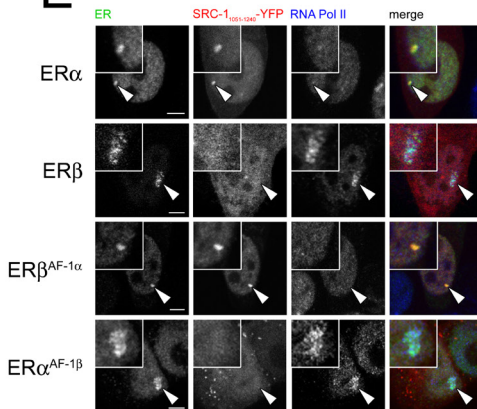


Fig. 2. RNA polymerase II recruitment by ER variants and AF-1-dependent SRC-1 recruitment to PRL array structures. PRL arrays containing HeLa cells were transfected with ER α (A), ER β (B) or AF-1 swap mutants (C,D) and treated with 10–6 M E2, tamoxifen or left untreated (CTS). Cells were fixed and stained with antibodies recognizing ER α or ER β and RNA polymerase II. Arrowheads indicate the array structure, analyzed for the presence of the RNA polymerase II (close-up shown in inset). Scale bars: 5 μ m. (E) PRL arrays containing HeLa cells were transfected with one of the different ER constructs and treated with 10–6 M E2. Cells were then cotransfected with the SRC-1(amino acids 1051-1240)-YFP (AF-1-inhibiting) truncation mutant. Cells were fixed and stained with antibodies recognizing ER α or ER β and RNA polymerase II. Arrowheads indicate the array structure, analyzed for the presence of the SRC-1-YFP fragment and RNA polymerase II (close-up shown in inset). Scale bars: 5 μ m.

which binds to the AF-1 domain of ER α in a yeast two-hybrid assay (Merot et al., 2004), was tested under E2 conditions. In the absence of ER, the SRC-1 truncation mutants were not recruited to the array structure (data not shown). When this SRC-11051-1240 mutant was cotransfected with ER α , the SRC-1 mutant was specifically recruited to the array structure by ER α , preventing recruitment of RNA polymerase II to the now condensed array structure, which is indicative of an inactive promoter region (Fig. 2E). The SRC-11051-1240 mutant was not

recruited to the array by ER β and therefore could not prevent RNA polymerase II accumulation on the array structure. Swapping the AF-1 domains of ER α and ER β resulted in exchange of the effect of the SRC-1 truncation mutant to interfere with accumulation of RNA polymerase II. The results of these inhibition studies indicated that the AF-1 domain of ER α is capable of binding to the Q-rich region of SRC-1, unlike the AF-1 domain of ER β . These data were verified in a co-immunoprecipitation experiment (supplementary material Fig. S3). Blocking AF-2 using a second SRC-1 truncation mutant (amino acids 623-711), which specifically binds to the AF-2 region of ER (Llopis et al., 2000), prevented RNA polymerase II recruitment for all tested ER constructs (supplementary material Fig. S4), resulting in a condensed array.

The results of these binding studies indicate that the AF-2 domains of both ER α and ER β directly interact with SRC-1, whereas only AF-1 of ER α is capable of interacting with the AF-1-binding domain of SRC-1.

We next investigated the functional consequences of the difference in SRC-1 binding between the AF-1 domains of ER α and ER β . We therefore performed an ERE-luciferase reporter assay, in which the function of AF-1, AF-2 or both in each of the ER constructs was inhibited by the different SRC-1 truncation mutants (see Fig. 3). A total of 10 nM E2 was added for all conditions, which resulted in maximal transactivation for each construct (Fig. 1B). The extent of transactivation of ER α was much higher than that of ER β . The response of ER α was significantly reduced by co-transfection of SRC-1 truncation mutants that inhibit either AF-1 or AF-2. This was not the case for ER β , for which only transcriptional inhibition by the AF-2-binding, but not by the AF-1-binding, SRC-1 truncation mutant occurred. Exchanging AF-1 of ER β for that of ER α in ER β AF-1 α rendered this construct dependent on AF-1 for its transcriptional potency.

These results indicate that the extent of transactivation by E2 is largely determined by both the AF-1 and AF-2 domains of ER α . Because the AF-1 domain of ER β lacks the ability to bind to SRC-1, this synergism is absent in ER β . The E2-mediated transactivation of ER β , which is low in comparison to that of ER α , is due to the transactivation capacity of its AF-2 domain. Replacement of AF-1 of ER β by the AF-1 domain of ER α in ER β AF-1 α restored the synergism between both domains, although not to the extent observed with ER α .

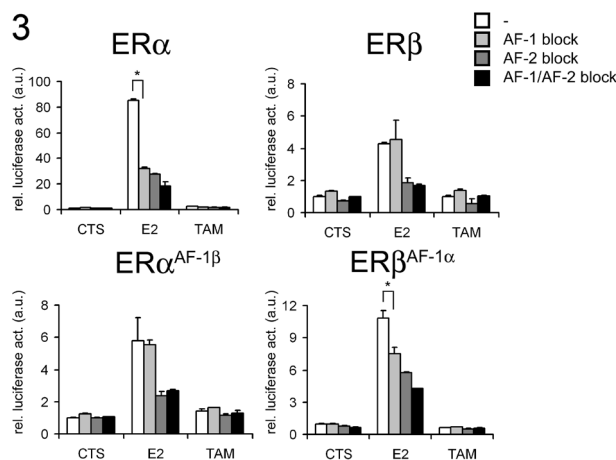


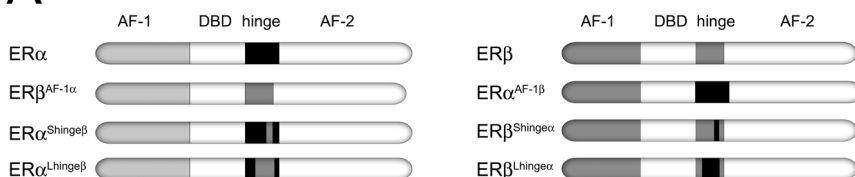
Fig. 3. Functional synergy between AF-1 and AF-2 is ER and AF-1 specific. An ERE-luciferase reporter assay was performed in U2OS cells transfected with the ER variants, the ERE-luciferase reporter construct and Renilla luciferase as a control. Where indicated, cells were co-transfected with SRC-1 amino acids 1051-1240 or SRC-1 amino acids 623-711, inhibiting AF-1 or AF-2, respectively (AF-1 and AF-2 block). Cells were treated with 10 nM E2 or 100 nM tamoxifen or left untreated (CTS) for 36 hours, after which cells were lysed and the luciferase activity was determined.

Representative data from two independent experiments is shown. Error bars indicate standard deviation from duplicate samples. The asterisk indicates a statistically significant reduction of luciferase signal by the AF-1 block, $P < 0.05$.

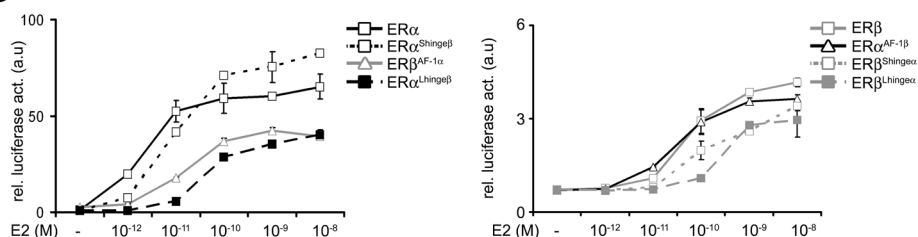
Effect of the hinge region on E2-driven transactivation by ER

To investigate the differences in the extent of transactivation by E2 between wild-type ER α and ER β AF-1 α , we exchanged the hinge region of ER α for that of ER β and vice versa (Fig. 4A), and tested these constructs in an ERE-dependent luciferase reporter assay using various concentrations of E2 (see Fig. 4B). A detailed alignment of the hinge regions of ER α and ER β , as well as the hinge mutants, is shown in supplementary material Fig. S5. Two different hinge swaps were made for each ER subtype: a small swap in which amino acids 294-309 in ER α were replaced with corresponding amino acids 253-262 in ER β and a larger swap in which amino acids 256-332 in ER α were replaced with corresponding amino acids 225-283 in ER β . Upon transfection of these constructs, similar mRNA levels were detected for all of the mutants applied (supplementary material Fig. S2). The small hinge swap, ER α Shinge β , showed a similar response to E2 as wild-type ER α , whereas the large hinge swap, ER α Lhinge β , resulted in decreased transactivation, similar to that of ER β AF-1 α (Fig. 4B). This suggests that the reduced response of the ER β AF-1 α mutant, in which the AF-1 region has been replaced with that of ER α , is due to the composition and/or length of the hinge region. No effect on maximal transactivation of the hingeswap mutants of ER β was observed; both mutants behaved similarly to wild-type ER β or ER α AF-1 β . This might be because of the absence of any AF-1 activity in ER β , preventing hinge-mediated functional synergy between AF-1 and AF-2.

4A



B



C

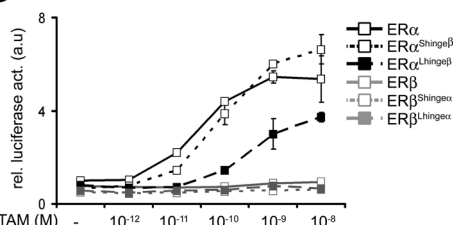


Fig. 4. The hinge region enables complete AF1 and AF2 synergy in ER. (A) Structural overview of ER α , ER β and its hinge-swap mutants used in this experiment. (B) The ERE-luciferase reporter assay was performed in U2OS cells transfected with ER variants, the EREluciferase reporter construct and Renilla luciferase as the control. Cells were treated with 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} ,

10^{-8} M E2 for 36 hours or left untreated (CTS), and relative luciferase activity was measured. A representative experiment out of three independent tests is shown. Error bars indicate standard deviation from duplicate samples. (C) The same conditions as in B are applied, but with 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} M 4-OH-tamoxifen (TAM).

Synergy between AF-1 and AF-2 is also essential for the agonistic behavior of tamoxifen (Zwart et al., 2007a). We therefore studied the role of the hinge region in the synergy involved in tamoxifen response. The tamoxifen response of the ER α large hingeswap mutant was impaired, whereas that of the ER α small hingeswap mutant resembled ER α (Fig. 4C). Tamoxifen did not increase the response of any of the ER β hinge-swap mutants. Interestingly, exchanging the hinge region of ER α for that of ER β in the ER α large hinge-swap mutant, resulted in a tenfold shift in EC₅₀ (effector concentration for half-maximum response) values in the response curves for E2 (Fig. 4B), as well as for tamoxifen (Fig. 4C), indicating that the ligand efficacy is influenced by the hinge region. The EC₅₀ of the ER β hinge mutants was also affected (Fig. 4B). This does not involve AF-1 and AF-2 functional synergy, but is determined by the hinge region alone.

Taken together, these results indicate that full E2-driven transactivation of ER α involves cooperation of the AF-1 and AF-2 domains that is dependent on the length and/or composition of the hinge region. For ER β , E2-mediated transactivation is associated with only its AF-2 domain.

The hinge domain determines tamoxifen- and PKA-induced conformational alterations in ER α and ER β

After tamoxifen binding to ER α , a conformational change is induced in the receptor, which is indicative of ER α inactivation (Michalides et al., 2004; Zwart et al., 2007b; Zwart et al., 2009). This conformational alteration can be monitored by intramolecular fluorescence resonance energy transfer (FRET), in which two fluorophores (typically CFP and YFP) are fused to both termini of the receptor. FRET is the radiationless energy transfer from a donor fluorophore to a suitable acceptor fluorophore, and is highly dependent on the distance between them and their orientation (Förster, 1948), enabling a probe to monitor even subtle conformational changes. In the case of tamoxifen resistance, as induced by protein kinase A (PKA)-mediated phosphorylation of serine 305 close to the hinge region of ER α , this tamoxifen-induced conformational change does not occur (Michalides et al., 2004). To monitor the possible influence of the hinge region on the conformational changes induced by ligand binding, we tested the effect of tamoxifen and PKA activation on the FRET responses of the swap mutants (Fig. 5). We monitored FRET by fluorescent lifetime imaging microscopy (FLIM), in which the lifetime of the donor fluorophore emission is measured. The lifetime of the donor CFP is typically 2.7 ns (Vermeer et al., 2004) and is reduced when the energy of the donor is transferred to the acceptor fluorophore in the case of FRET (Bastiaens and Squire, 1999). Cells expressing YFP-ER-CFP were co-cultured with cells only expressing CFP to provide an internal control for the FLIM measurements (Zwart et al., 2009). As we reported previously for ER α , the FRET signal was increased by treating the cells with tamoxifen, which could be prevented by pre-incubation with the PKA activator forskolin (Michalides et al., 2004). In the case of ER β , no increase in FRET signal was detected after tamoxifen treatment and after forskolin treatment. Combining both treatments, however, did induce a significant increase in FRET efficiency. When the hinge regions of both receptors were swapped, the characteristic responses to tamoxifen and PKA activation were also exchanged between receptors; ER α Lhinge β did not show a large increase in FRET efficiency after tamoxifen or forskolin exposure, whereas combination of these treatments did induce a conformational change. These FRET characteristics of ER α Lhinge β are therefore comparable to those of ER β . ER β Lhinge α showed a response comparable to ER α and tamoxifen now induced a FRET change. The combination of tamoxifen and forskolin treatment did not induce an increase in FRET for the ER β Lhinge α mutant, whereas this was observed for wild-type ER β . Therefore, introducing the hinge of ER α into ER β sufficed to swap the ligand-induced conformational characteristics. The small hinge swaps showed intermediate effects. There were no differences in transcriptional potency, as induced by tamoxifen and forskolin, between the ER hinge-

swap mutants (supplementary material Fig. S6). This indicated that the N- and C-terminal orientations within the ER variants are determined by the non-conserved hinge regions in ER α and ER β . More importantly, this hinge-specific feature appeared to be responsible for the different conformational responses induced by ligand treatment and kinase activities.

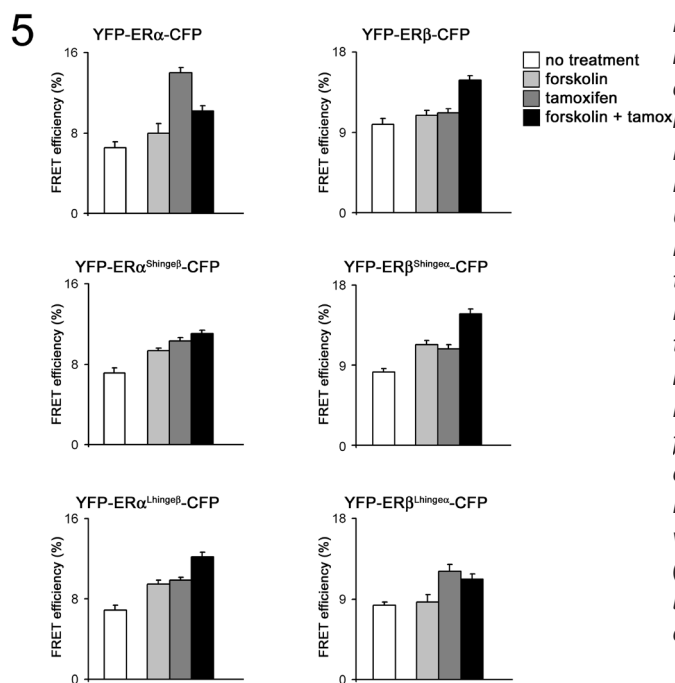


Fig. 5. Tamoxifen and PKA-induced conformational changes of ER and hinge mutants. Intramolecular FRET, as determined by FLIM, was measured in U2OS cells expressing YFP-ER α -CFP, YFP-ER β -CFP or their hinge-swap mutants. FRET was determined prior to ligand addition, after 1 mM tamoxifen addition or PKA activation by 10 mM forskolin, or a combination of both treatments in CTS. Donor FRET efficiency (ED) was calculated as $ED = 1 - (\text{lifetime cell of interest} / \text{lifetime reference cell})$. For each condition, $n > 25$ cells.

Discussion

ER β arose from ER α by a duplication event to different chromosomes approximately 450 million years ago (Kelley and Thackray, 1999). The homology between the receptors is largely maintained in the DNA-binding domains (96%) and ligand-binding domains (53%), which indicates a functional selection pressure. In the present study, we confirmed previous work (Metivier et al., 2001) showing that the AF-1 domain of ER α binds to SRC-1. This binding, in combination with the hinge region of ER α , is responsible for the extent of transactivation by E2 and for ligand-independent transactivation. Both of these functions are lost in ER β , which reflects the largely deviating sequence of AF-1 and the hinge region of ER β compared with ER α . The expression of ER α and ER β is different in various human tissues, ranging from exclusive expression of ER β in the colon and brain to variations in the relative expression of ER α and ER β in other tissues. This variation influences the extent of response to E2. Indeed, ER β has been reported to interfere with E2-driven proliferation of breast cancer cells (Strom et al., 2004), either as homodimers or as heterodimers of ER α and ER β (Li et al., 2004).

Our study confirms a previous report (McInerney et al., 1998) and illustrates that the AF-1 domain of ER α is crucial for enhanced transactivation by E2. This is mediated by a unique feature of AF-1 of ER α , because it directly interacts with the Q-rich region of the SRC-1 cofactor (Metivier et al., 2001), whereas AF-1 of ER β does not. As a consequence, the small E2-driven transactivation and RNA polymerase II recruitment by ER β is solely mediated through its AF-2 activity. We also showed that the orientation between the AF-1 and AF-2 domains, determined by the composition and/or length of the hinge region, affects the final extent of

E2-driven transactivation.

The results of the luciferase reporter studies (Fig. 3) confirmed the co-immunoprecipitation experiments (supplementary material Fig. S3) and in situ localization studies (Fig. 2). These results showed that non-liganded ER α binds to SRC-1 through its AF-1 domain, which is sufficient for RNA polymerase II recruitment. The composition of the AF-1 domain is crucial for binding to SRC-1, whereas the spacing and/or orientation between the AF-1 and AF-2 domains are essential for transactivation of ER α . When the large hinge region of ER α was replaced with that of ER β , the resulting ER α Lhinge β mutant was equally active as the ER β AF-1 α mutant (Fig. 4). This suggests that a combination of AF-1–AF-2 interactions, together with the hinge region, determines the prominent E2-driven transactivation of ER α . This spatial positioning of the AF domains might also be relevant for resistance to particular antagonists, because phosphorylation of serine 305 of ER α within the hinge region by protein kinase A or PAK-1 (Michalides et al., 2004; Rayala et al., 2006) results in resistance to tamoxifen.

Both the length and the composition of the hinge region might affect the capacity for transactivation, because the hinge region of ER α is a target of extensive post-translational modifications that affect the stability and/or activity of the receptor. In particular, the residues in the hinge between K299 and S305 are targeted for acetylation, ubiquitylation, methylation and phosphorylation. These modifications might interfere with one another and affect final activation of ER α (Eakin et al., 2007; Kim et al., 2006; Michalides et al., 2004; Subramanian et al., 2008; Wang et al., 2001). These sequences and corresponding opportunities for modification and modulation of the receptor are lacking in the hinge region of ER β . Indeed, exchanging the hinge regions between ER α and ER β altered the conformation of ER, as induced by tamoxifen and the PKA-activating compound forskolin (Fig. 5).

The antagonist tamoxifen also has weak agonistic activity (32). We showed that the AF-1 domain of ER α enhanced this tamoxifen-mediated transactivation, but only on an ER α background (Fig. 1C). This background involves the proper hinge region, which facilitates interaction between AF-1 and AF-2 and thereby the extent of transactivation for ER α , but not for ER β (Fig. 4C).

We have performed these studies in U2OS osteosarcoma cells; this is a model cell line for testing the direct effects of transactivation of ER α and ER β (Michalides et al., 2004; Stossi et al., 2004), because these cells do not express endogenous ER and allow reconstitution of the ER-cofactor complex. We have focused in this study on the interaction between ER and SRC-1 as a most relevant cofactor of ER. There are three different SRC cofactors, which all belong to the p160 cofactor family (Xu and Li, 2003). They interact with the AF-2 domain of ER α and ER β through their LxxLL motifs (Heery et al., 1997). However, when AF-1 of ER α was blocked using an AF-1-inhibiting fragment of SRC-1 (Fig. 2), no recruitment of RNA polymerase II is observed in the presence of E2. These data showed that this SRC-1 truncation functions in a dominant-negative manner, implying no residual interaction between AF-1 of ER α and SRC-2 or SRC-3 that would otherwise have resulted in recruitment of RNA polymerase II.

It has been reported that AF-1 of ER β binds to SRC-1 when the complex is bound to the SP-1-directed promoter of the TIEG gene in osteoblast cells, enhancing its E2-mediated expression (Hawse et al., 2008). This study and our results indicate that the effects of ER α and ER β are dependent on the promoter region to which they (in)directly bind, as was also suggested by others (Klinge et al., 2004). The results of our study emphasize that, with the 'classical' palindromic ERE-containing promoters, E2- and ligand-independent transactivation are determined by the binding of AF-1 of ER α and SRC-1, which does not occur in ER β . AF-1 of human ER β does not bind to SRC-1, unlike mouse ER β (Tremblay et al., 1999). This corresponds to a difference in transactivation by E2 between human ER α and ER β , whereas there is no such difference between mouse ER α and ER β (Picard et al., 2008). In the mouse,

specific phosphorylation sites in the AF-1 domain of mouse ER β can alter its activity (Tremblay et al., 1999) and stability (Picard et al., 2008).

This difference between human ER α and ER β also emphasizes the importance of domain interactions in ER α . The orientation between the AF-1 and AF-2 domains in ER α is affected by selective anti-estrogens and can be subtly modified by the structure of these anti-estrogens. How the various domains of ER α and cofactors collaborate in breast-tumor growth and in the response to antiestrogens is gradually becoming clear.

Materials and Methods

Cell culture and antibodies

Human osteosarcoma U2OS, MCF-7 and HeLa PRL array cells (Sharp et al., 2006) were cultured in DMEM medium in the presence of 10% FCS and standard antibiotics. Cells containing ER constructs were cultured in phenol-red-free DMEM containing 5% charcoal-treated serum (CTS; HyClone). Antibodies used were raised against ER α (NovoCastra/Cell Signaling Technology), tubulin (Sigma), ER β (Santa Cruz Biotechnology), RNA polymerase II (8WG16; Covance) and GFP (van Ham et al., 1997).

Cloning procedures and oligomers

ER α , ER β , SRC-1 (amino acids 623-711)-YFP and SRC-1 (amino acids 1051-1240)-YFP constructs were generated as described previously (Michalides et al., 2004; Zwart et al., 2007a). The ER α AF-1 β and ER β AF-1 α swap constructs were made by pairwise ligation of four separate PCR fragments in a second PCR reaction. The AF-1 α (amino acids 1-180) and AF-1 β (amino acids 1-144) fragments were made using forward primer (A) 5'AATTGGATCCACCACCATGGCATACCCATACGACGTCCCAGACTACGCTATGACCATGACCCTCCACACC and reverse primer (B) 5'GCGCAGAAGTGAGCATCCTTGGCAGATTCCATAGCC, and forward primer (C) 5'AATTGGATCCACCACCATGGCATACCCATACGACGTCCCAGACTACGCTATGGATATAA AAAACTCACC and reverse primer (D) 5'GCACAGTAGCGAGTCTCCCTCTTTGAACCTGGACC, respectively, introducing a BamHI restriction site and a hemagglutinin tag at the 5' ends, and a ER β or ER α overhang at the 3' ends of the fragments. The ER $\alpha^{\Delta AF-1}$ and ER $\beta^{\Delta AF-1}$ fragments, lacking the AF-1 regions, were made using forward primer (E) 5'GGTCCAGGTTCAAAGAGGGAGACTCGCTACTG TGC and reverse primer (F) 5'TGGGGGATCCTTATCAGACTGTG GCAG GGAAACC, and forward primer (G) 5'GGCTATGGAATCTGCCAAGGATGCTCACTTCTGCGC and reverse primer (H) 5'AATTGGATCCTCACTGAGA CTGTGGGTTCTGG, respectively, introducing a ER β or ER α overhang at the 5' ends, and a stop codon and BamHI restriction site at the 3' ends of the fragments. The AF-1 α and ER $\beta^{\Delta AF-1}$ and AF-1 β and ER $\alpha^{\Delta AF-1}$ fragments were then ligated to each other in a second PCR reaction using forward primer (A) and reverse primer (H), and forward primer (C) and reverse primer (F). These swap constructs were cloned in the dephosphorylated BamHI site of the pcDNA3 vector.

ER α Shinge β , ER α Lhinge β , ER β Shinge α and ER β Lhinge α constructs were made by sequential PCR reactions, ligating ten separate smaller PCR fragments into four different larger fragments containing the chimeric hinge regions. The two PCR fragments used for the construction of ER α Shinge β (a.a. 253-262) were made using forward primer (1) 5'AATTCCGCGGCCGCCCAACGCGCAGG and reverse primer (2) 5'GGCGTCCAGCAGCAGTCCCGCACTCGGGGTGGCCAAAGGTTGGC, and forward primer (3) 5'CCCCGA GTGCGGGAGCTG CTGCTGGACGCCCTGACGGCCGACCAG and reverse primer (4) 5TGGTCTAGAAGGTGGACCTGATCATGGAG. The fragments were then ligated in a second PCR reaction using forward primer (1) and reverse primer (4), introducing a SacII

restriction site at the 5' end and an XbaI site at the 3' end. This construct was then inserted into the corresponding restriction sites in a pcDNA3-ER α vector. The two PCR fragments used for the construction of ER β Shingex (a.a. 294-309) were made using forward primer (5) 5'CCAGATATCACTATGGAGTCTGGTCGTGTG and reverse primer (6) 5GGACAAGGCC AGGCTGTTCTTCTTAGAGCGTTTGATCATGAGCGGGCTCGCGTGGCCGCCACTTCTCTTGGCC, and forward primer (7) 5'AGCCCGTCTCAT GATCA AACGCTCTAAGAAGAAGACAGCCTG GCCTTGT CCCTGAGCCCCGAGCAGCTAGTGCTCACC and reverse primer (8) 5'TGGGAATTCCTTC TACG CATTTCCTCATCC. The fragments were then ligated in a second PCR reaction using forward primer (5) and reverse primer (8), and introducing an EcoRV restriction site at the 5' end and an EcoRI site at the 3' end. This construct was then inserted into the corresponding restriction sites in a pcDNA3-ER β vector.

The three PCR fragments used for the construction of ER α Lhinge β (a.a. 220-283) were made using forward primer (1) and reverse primer (9) 5'CACATCTCTCTCCGTATCCACCTTTCATCATTCCC, forward primer (10) 5'CGGAGAGAGAGATGTGGGTACC and reverse primer (11) 5'GCGGCTGATCAGCACATGGGGC, and forward primer (12) 5'TGTGCTGATCAGCCGCCCTACCAGACCTTCAGTGAAGCTTCG and reverse primer (4). The fragments were then sequentially ligated in a second and third PCR reaction using forward primer (1) and reverse primer (11), and forward primer (1) and reverse primer (4), respectively, introducing a SacII restriction site at the 5' end and an XbaI site at the 3' end. This construct was then inserted into the corresponding restriction sites in a pcDNA3-ER α vector.

The three PCR fragments used for the construction of ER β Lhinge α (a.a. 256-332) were made using forward primer (5) and reverse primer (13) 5'CTCCTCTTCGGTCTTTTCGGGAGCCACACTTCACCATTCCC, forward primer (14) 5'CGAAAA GACCGAAGAGAGGGGAG and reverse primer (15) 5'ATCATACTCGGAA TAGAGTATGGG, and forward primer (16) 5'CTCTATTCCGAGTATGATCCCAGTGCGCCCTTCACCGAGG and reverse primer (8). The fragments were then ligated in a sequential second and third PCR reaction using forward primer (5) and reverse primer (15), and forward primer (5) and reverse primer (8), respectively, introducing an EcoRV restriction site at the 5' end and an EcoRI site at the 3' end. This construct was then inserted into the corresponding restriction sites in a pcDNA3-ER β vector.

For QPCR analysis of ER expression levels, hybrid primers were applied based on the two DNA-binding domains, with forward primer 5'GAGAAGCA TTCAAGGACATAACGAT and reverse primer 5'CCACATTTACCATTCCCAC. As a control for equal loading, the observed ER signals were related to β -actin RNA levels, using a forward primer 5'CCTGGCACCCAGCACAAAT and reverse primer 5'GGGCCGGA CTGTCATACT.

Immunoprecipitation

U2OS cells were transfected with ER α , ER β or ER β AF-1 α in the presence or absence of SRC-1 (amino acids 1051-1240)-YFP using polyethylenimine (PEI). Twenty-four hours after transfection, cells were lysed in 125 mM NaCl, 50 mM HEPES (pH 7.5), 0.1% Nonidet P-40, 10 mM MgCl₂ and protease inhibitor cocktail (Roche) on ice, sonicated and debris removed by centrifugation. The supernatant was used in immunoprecipitation using anti-GFP antibody immobilized on protein A-sepharose beads (Invitrogen) during incubation overnight. Samples were taken from the supernatant for analysis of the total lysate. Beads were extensively washed, boiled and samples were analyzed by western blotting. For detection, antibodies identifying ER α , ER β and GFP were used, and the signal was detected using an ECL detection kit (Amersham).

ERE- and AP-1-dependent luciferase assays

Luciferase assays were performed as described previously (Bindels et al., 2002), transfecting 2 ng of ER and 0.2 mg ERE-tk-firefly luciferase, or 0.2 mg of ER and 0.2 mg AP-1-tk-firefly luciferase using PEI (25 kDa; Polysciences) (Boussif et al., 1995). As a control, 2 ng Simian virus (SV40) Renilla luciferase was used. For specific inhibition of the AF-1 or AF-2 activity of ER α , SRC-1 truncation mutants comprising amino acids 1051-1240-YFP or amino acids 623-711-YFP (Zwart et al., 2007a) were co-transfected at 0.5 and 0.2 mg per well, respectively, and supplemented with pcDNA3 empty vector to equalize the total amount of DNA per well.

Microscopy

RNA polymerase II recruitment was assayed as described before (Zwart et al., 2007a). Where indicated, cells were co-transfected with YFP-tagged SRC-1 fragments comprising amino acids 623-711 or amino acids 1051-1240. Two hours before fixation, cells were treated with 1 mM estradiol, tamoxifen or left untreated. Cells were fixed with 3.7% formaldehyde in PBS, permeabilized for 5 minutes with 0.05% Triton X-100 in PBS at room temperature, and subsequently stained with antibodies detecting ER α , ER β and RNA polymerase II, and secondary antibodies conjugated to AlexaFluor405 and AlexaFluor647 (Molecular Probes). After staining, cells were mounted in Vectashield mounting medium (Vector Laboratories). The specimens were analyzed with confocal laser-scanning microscopes (TCS-SP1, TCS-SP2 or AOBS; Leica) equipped with HCX Plan-Apochromat 63 NA 1.32 and HCX Plan-Apochromat lbd.bl 63NA 1.4 oil-corrected objective lenses (Leica). The acquisition software used was LCS (Leica).

Fluorescence lifetime imaging microscopy

Prior to FLIM experiments, cells on cover slips 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. FLIM experiments were performed on a Leica inverted DMIRE2 microscope equipped with a Lambert Instruments frequency domain lifetime attachment (Leutengewolde), controlled by the vendor's LI FLIM software. CFP was excited at 430 nm with ~4 mW power using an LED modulated at 40 MHz. Emission was collected at 450-490 nm using an intensified CCD camera. FLIM measurements were performed in U2OS cells, transfected with YFP-ER α -CFP, YFP-ER β -CFP or one of the hinge-swap mutants. Calculated CFP lifetimes were referenced to a 1 mM solution of rhodamine-G6 in medium that was set at a 4.11 ns lifetime, and internally calibrated using co-cultured CFP containing MeJuSo reference cells, for which the lifetime was set to 2.7 ns (Vermeer et al., 2004). Donor FRET efficiency (ED) was calculated as $ED=1-(\text{lifetime cell of interest}/\text{lifetime reference cell})$.

Acknowledgements

We thank Lennert Janssen and Cristiane Bentin-Toaldo (The Netherlands Cancer Institute, The Netherlands) for generating the chimeric mutants of ER α and SRC-1, and Aisling Redmond (CR UKCRI, Cambridge, UK) for critically reading the manuscript. This study was supported by grant NKI 2005-3388 of the Dutch Cancer Society and was in part performed within the framework of Top Institute Pharma project: number T3-107.

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Supplementary material

Fig. S1. Sequence alignment of the N termini of ER α and ER β . Highlighted in ER α are the AF-1 transactivation regions (A box 1, A box 2, A box 3) and the small α -helical stretch (in red) that is essential, but not sufficient, for AF1 activity. Essential amino acids in this α -helical sequence are underlined.

Fig. S2. Activity and mRNA and protein levels of ER variants used in this study. (A) U2OS cells were transfected with ER α , ER β or one of the swap mutants, RNA was isolated and mRNA levels of each ER variant were determined by quantitative RT-PCR. mRNA levels were related to the ER α level, which was set at 1. Data are from two independent experiments. Error bars show standard deviations. (B) Luciferase activity was measured in U2OS cells transfected with 2 ng or 10 ng of ER α , ER β or one of the AF-1 swap mutants. Cells were co-transfected with an ERE-luciferase reporter construct and Renilla luciferase reporter to correct for transfection efficiency. Cells were treated for 2 days with tamoxifen (10⁻⁷), E₂ (10⁻⁸) or left untreated (CTS). All data were normalized to the CTS value of 2 ng ER α , which was set at 1. Error bars show standard deviations from triplicate samples. (C,D) Protein levels of ER α , ER β or one of the swap mutants in U2OS cells were determined by western blot, with antibodies raised against ER α or ER β (C). For expression testing of the FRET constructs, an antibody detecting GFP was used (D). Actin signal was used as a loading control.

Fig. S3. The AF-1 domain of ER α but not of ER β binds to SRC-1. U2OS cells were transfected with ER α , ER β or one of the AF-1 swap mutants, and co-transfected with SRC-11051-1240-YFP where indicated. SRC-11051-1240 was immunoprecipitated, after which the co-immunoprecipitated ER was identified using antibodies against ER α and ER β . The arrow indicates the SRC-11051-1240-YFP signal, detected below the IgG background band.

Fig. S4. AF-2-dependent SRC-1 recruitment to the PRL array structures. PRL arrays containing

HeLa cells were transfected with one of the different ER constructs and with the SRC-1623-711-YFP (AF-2 inhibiting) truncation mutant, and subsequently treated with 10⁻⁶ M E2. Arrowheads indicate the array structure, analyzed for the presence of the SRC-1-YFP fragment and RNA polymerase II (close up shown in inset). Scale bar: 5 μm.

Fig. S5. Sequence alignment of the hinge regions of ERα, ERβ and the corresponding hinge swaps. Highlighted in red are the NLS consensus sequences in both ER variants. The described targets of PKA in ERα (S236 and S305) and ERβ (S200) are also indicated.

Fig. S6. PKA activity enhances the tamoxifen response for ERα hinge swaps. Luciferase activity was measured in U2OS cells transfected with ERα, ERβ or one of the hinge-swap mutants. Cells were co-transfected with an ERE-luciferase reporter construct and Renilla luciferase reporter to correct for transfection efficiency. Cells were treated for 2 days with tamoxifen (10⁻⁷ M) or left untreated. The last 16 hours, cells were co-treated with 100 μM 8-Br-cAMP to activate PKA. All data were normalized to the CTS value of ERα, which was set at 1. Error bars show standard deviations from triplicate samples.

Fig S1

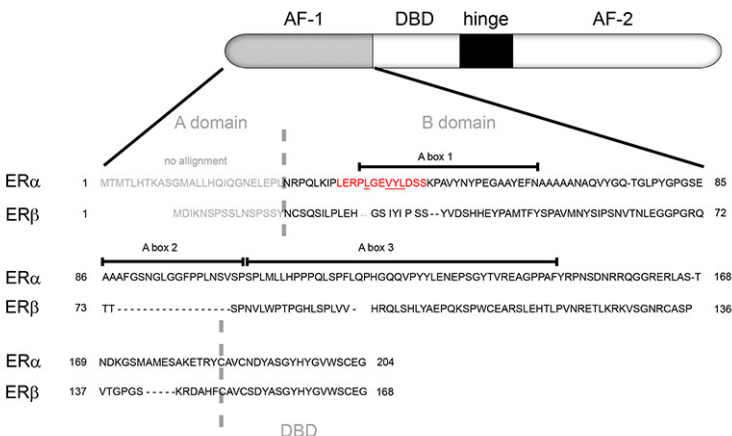


Fig S2

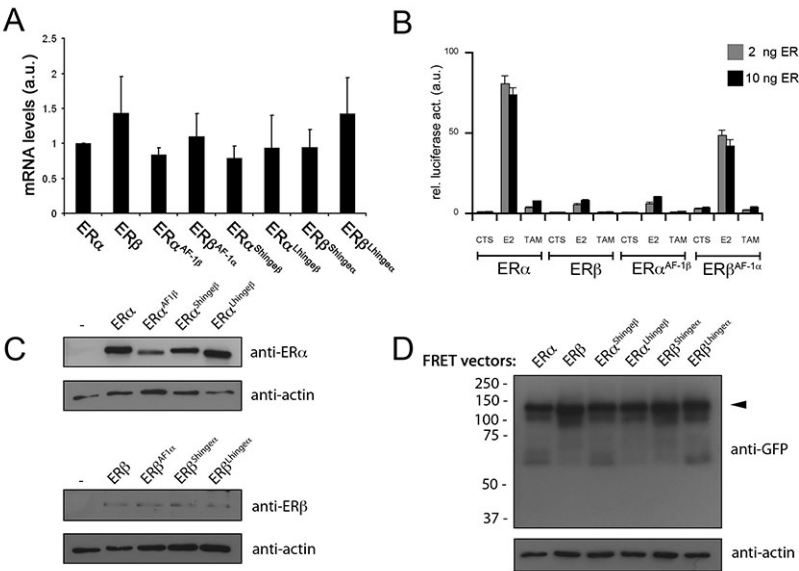


Fig S3

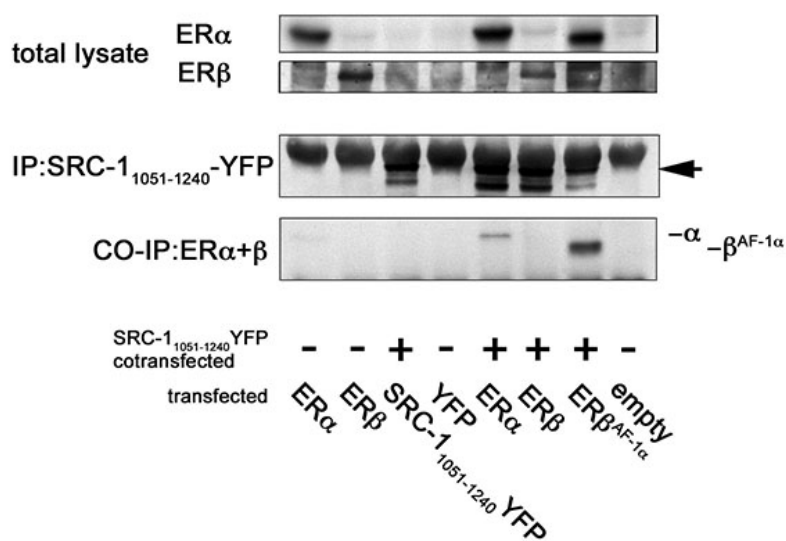


Fig S4

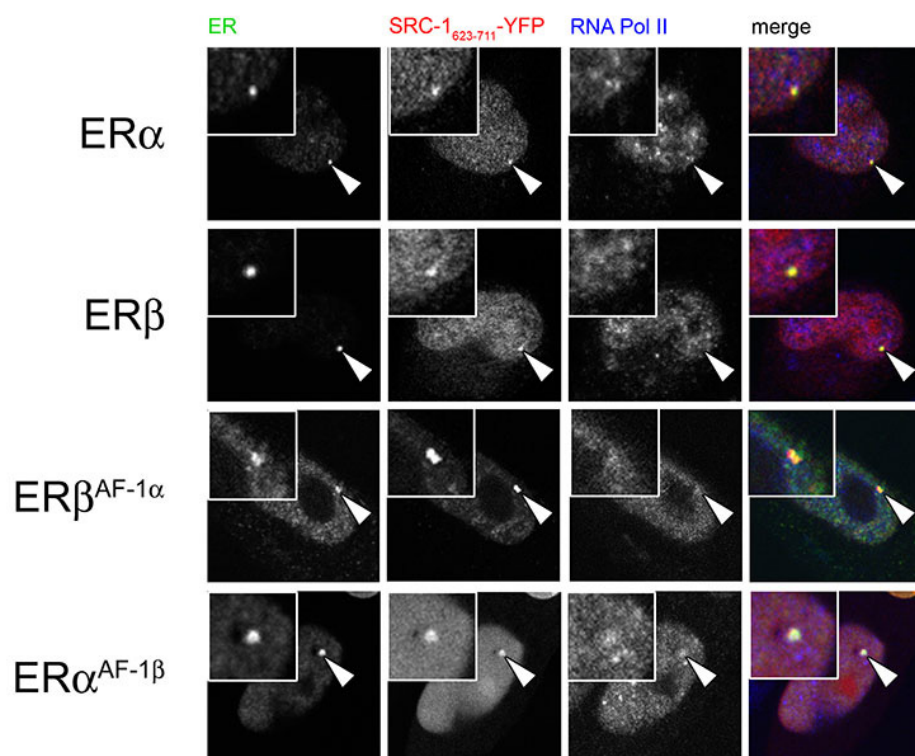


Fig S5

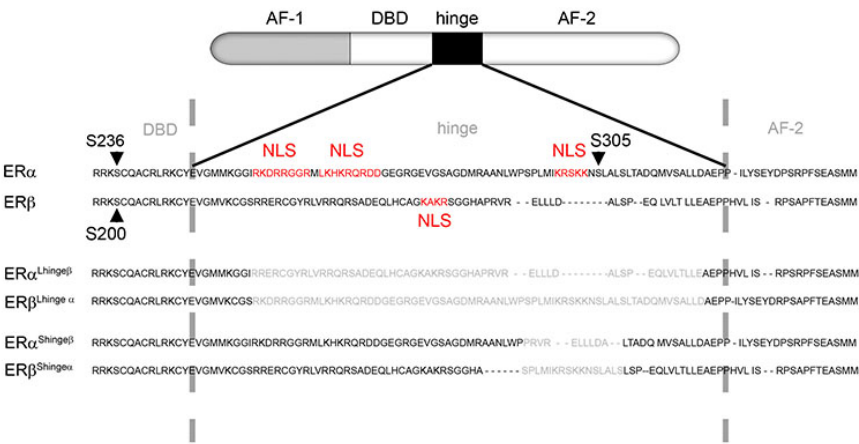
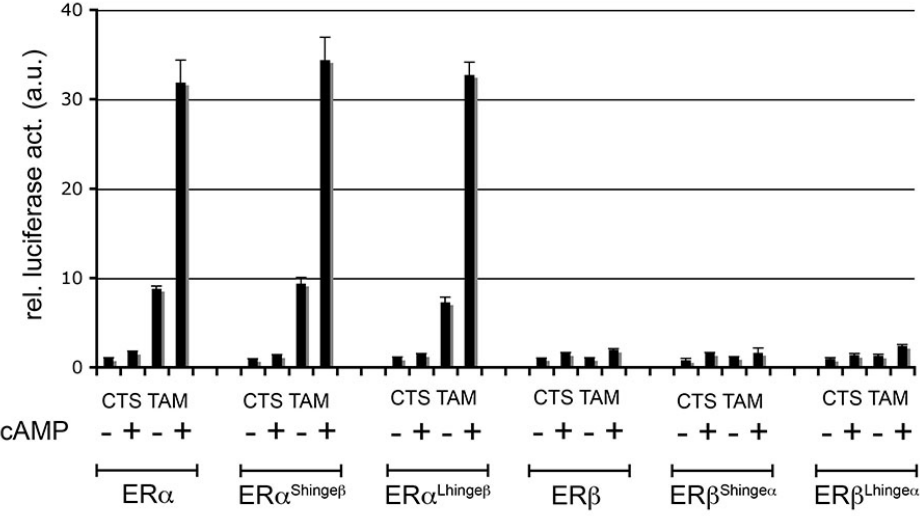


Fig S6



Chapter 3
research article

A high-throughput coregulator peptide array detects estrogen
receptor alpha Serine-305 phosphorylation that is associated with
response to tamoxifen treatment

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Molecular Cancer Therapeutics (in revision)

A high-throughput coregulator peptide array detects estrogen receptor alpha Serine-305 phosphorylation to predict responses to tamoxifen

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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 (ER α). ER α is critical in many breast tumors and is inhibited by antiestrogens such as tamoxifen in cancer therapy. ER α is also modified by acetylation and phosphorylation that affect responses to the anti-estrogens as well as interactions with co-regulators. Phosphorylation of ER α 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 ER α binding in cell and tumor lysates. We show that ER α phosphorylated at Ser305 associates stronger to various coregulator peptides on the chip. This implies that ER α Ser305 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 promotor 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 (ER α) 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 (ER α) 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 ER α -positive breast cancer (17, 18). Endocrine therapy, which aims for inactivation of ER α , uses competitive estrogen antagonists (e.g. tamoxifen) or aromatase inhibitors that block estrogen synthesis.

This prevents the formation of the coactivator binding surface on ER α (19). A group of patients does not respond to endocrine therapy, because ER α remains transcriptionally active despite endocrine treatment. One factor that is associated with resistance to tamoxifen is phosphorylation of ER α Serine 305 (ER α S305-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 ER α 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 ER α 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 ER α , 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 ER α Serine 305 and show that our assay allows detection of phosphorylation-correlated alteration of ER α 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-ER α -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).

Sample preparation

ER α 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. ER α in the samples was quantified using ELISA (Active Motif). Equal amounts of ER α wild-type (ER α 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), ER α Ser305-P (Millipore, #124.9.4.9, (21)) and ER α (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 μ l tumor lysate was incubated for 45 minutes at 30°C in a reaction volume of 5 μ l of lambda protein phosphatase buffer, 5 mM DTT and 30U lambda protein phosphatase (#14-405, Millipore). Treatments were ended by adding 5 μ l of the stopbuffer with HALT protease inhibitor cocktail (PIERCE) and phosphatase inhibitors (100 μ M 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

All assays were performed in a PamStation®-g6 (PSg6) controlled by EvolveHT

software (PamGene International BV, Den Bosch, the Netherlands) (26, 27). Nuclear Receptor PamChip® Arrays (PamGene International BV, Den Bosch, the Netherlands) with immobilized peptides with coregulator derived sequences (Suppl. table 1). Lysate assay mixes contained 10 µl lysate, 4 µg/ml Alexa488-conjugated anti-GFP antibody (#A21311, Invitrogen, Carlsbad, USA) in Tris-buffered Saline (TBS) containing 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, Carlsbad, USA) was prepared as described previously (22, 28) using 4 µg/ml Alexa488-conjugated anti-GST (#A11131, Invitrogen, Carlsbad, USA) 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. 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 (bindingpeptide-mean(bindingall peptides))/stdev(bindingall 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 1, Suppl. 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-liganded 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

Figure 1

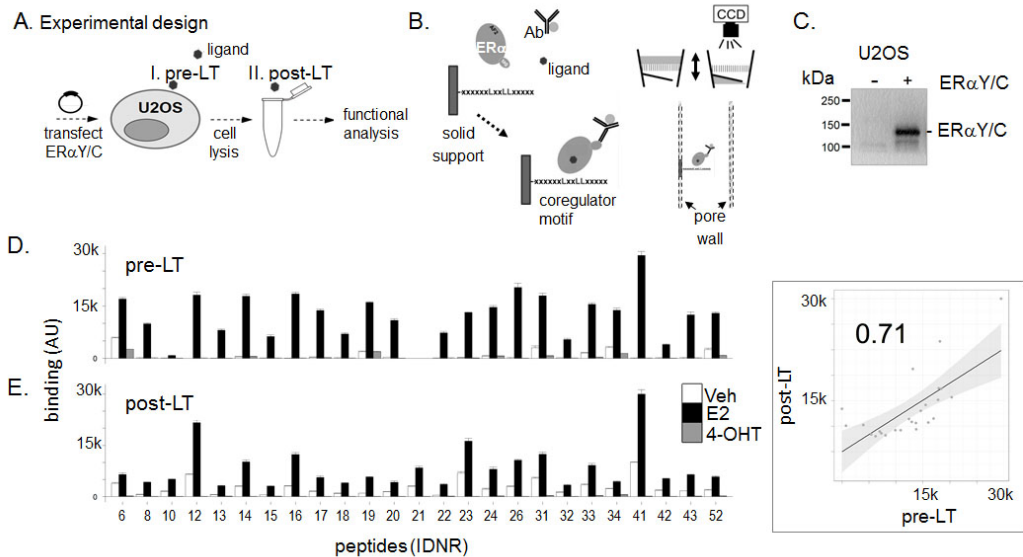


Figure 1. Analysis of coregulator binding of full-length ERα in crude lysates of transfected cells. A. U2OS cells transfected with a YFP-ERα-CFP (ERα Y/C) construct. Transfectants were I: treated with ligand or kept under hormone-free conditions and lysed (Pre-LT), or II: lysed first and ligand was added 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 Suppl. table 1.

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 10⁻⁷M) 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 Suppl. table 1) are shown in figure 2A.

The E2 potency (EC₅₀) for each peptide was derived from these curves. EC₅₀ values for all peptides (Supplementary Table 1, set I) 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

ER α was slightly higher than for the ER α -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 ER α -LBD construct which therefore binds less efficiently to cofactors. The specificity of the interactions is not affected.

Figure 2

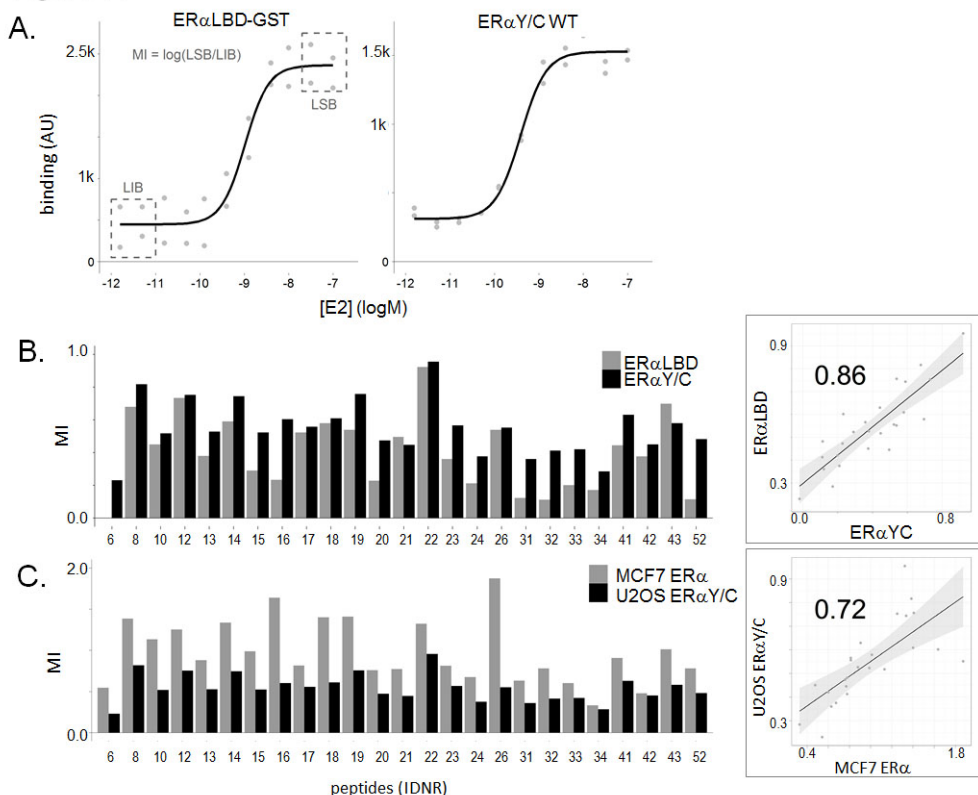


Figure 2. E₂-induced modulation of ER α -coregulator binding: purified ligand-binding domain vs. full-length protein in crude lysates. Recombinant ER α LBD-GST or ER α Y/C lysate was incubated with a concentration range of E₂ (10⁻¹² to 10⁻⁷M), one chip per ligand concentration. Each condition was measured 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 log₁₀ transformed ratio of LSB over LIB. Example of E₂ dose-response curves of the full-length ER α Y/C (left) and ER α -LBD- (right) binding to a NCOA1-derived peptide (IDNR13). B. E₂-induced MI of ER α -LBD and full-length receptor binding to the NR-boxes are shown. Subgraph: Scatter plot and correlation coefficient of E₂- MI of ER α LBD-GST vs. full-length ER α Y/C. C. MI of MCF7 endogenous ER α and U2OS full-length ER α Y/C, subgraph represents correlation. For peptides, see corresponding numbers in Suppl. table 1.

Exogenous versus endogenous ER α in cell lysates

The results of the experiments described thus far indicated that the full-length ER α from transfected cells behaved similarly in this analysis as ER α -LBD fragments that were

generated in vitro. This full length ER α construct contained two GFP variants for detection and this might affect binding efficacy. Therefore, we compared the tagged full-length ER α from transfected U2OS cells with an endogenous ER α from the human breast cancer cell line MCF7. The E2-induced binding of ER α 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 ER α and also is sensitive enough to be used for endogenous ER α protein in lysates.

ER α from ER-positive breast cancer

Since we could detect binding of endogenous ER α in crude lysates to the chip, we decided to test the technology on breast tumor material. We prepared lysates from a primary ER α -positive breast tumor sample. E2 (1 μ M) or no ligand (control) was added 10 minutes prior to loading onto the array. The results showed that ER α from tumor material is still responsive to E2 (figure 3A) and the E2 response (MI) of ER α in these samples resembles that of MCF7 cells (R^2 :0.78, figure 3B).

Figure 3

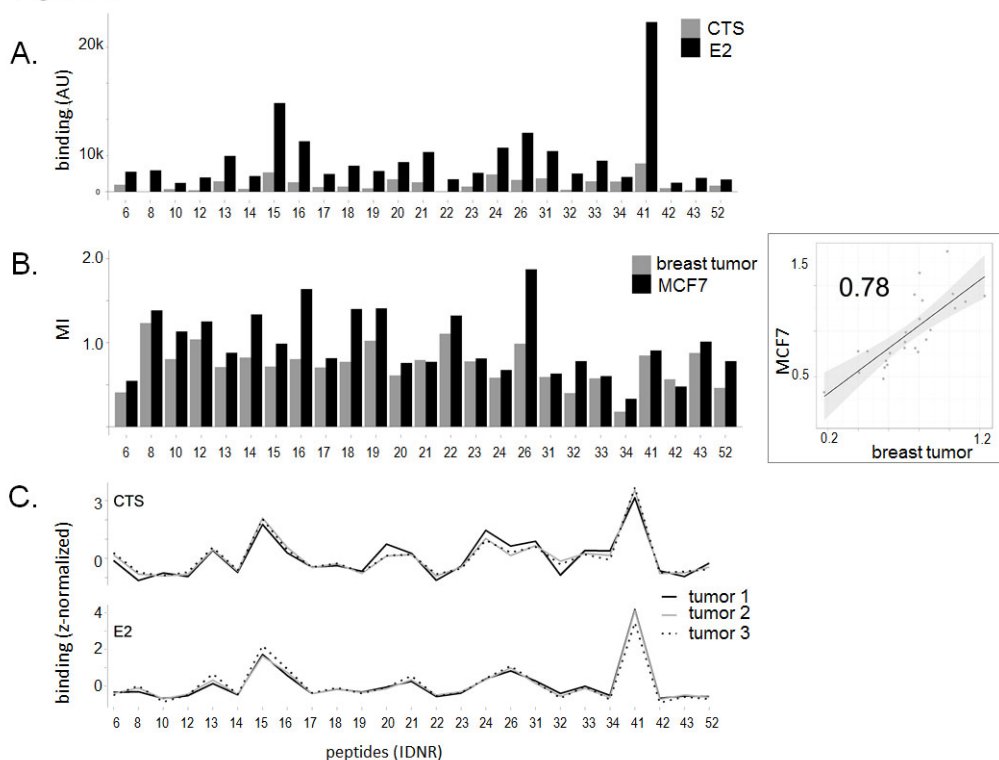


Figure 3. Coregulator binding of ER α from in breast tumors and binding modulation by estradiol. A. Coregulator binding of ER α in a tumor in the absence or presence of ligand (E2). B. E2-induced modulation indices (MI) of MCF7- and breast tumor-derived ER α , subgraph represents correlation. C. Extracts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

Clinical samples generally show heterogeneity. To investigate whether this plays a role in our peptide chip, we compared three ER α -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 ER α from human breast cancer specimens under different ligand conditions.

Functional analysis of ER α Serine305 phosphorylation

Post-translational modifications on ER α 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 ER α or to some other NRs, in addition to set I (Supplementary Table 1, set II). PKA-induced ER α S305-P has been linked to resistance to tamoxifen treatment (20, 21). Under tamoxifen conditions, this phosphorylation affects the conformation of ER α and changes its orientation to NCOA-1 (22). Here we studied the effect of ER α S305-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, Suppl. table 1). The serine305-to-alanine mutant (ER α Y/C 305A) was included as a negative control (Figure 4A). Next, we generated lysates that were incubated with a concentration range of up to 10-8M E2 and analyzed binding to the co-factor peptides on the chip. This resulted in a dose-dependent modulation of ER α binding to each coregulator peptide on the array, as illustrated for the control peptide (IDNR13) in Figure 4B. Wild-type (WT) and mutant ER α (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 ER α is slightly enhanced when compared to 305A mutant receptor. This may be due to some background phosphorylation of wild-type ER α 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 ER α is more substantial (Figure 4C). This has been observed for all peptides on the array and suggests that ER α S305-P increases the ER α 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 ER α Y/C 305A (Figure 4D), which is not completely abolished by the mutation of this site. A plausible candidate is Serine 236 in ER α (33). Therefore, we assessed binding of the ER α 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 ER α 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 ER α sites Ser236 and 305.

The effect of PKA activation on peptide binding was also studied under tamoxifen (4-OHT) conditions. This is illustrated by ER α binding to the same peptide (IDNR13) using a concentration range of up to 10-5M 4-OH-tamoxifen (Figure 5A). Although PKA activation increased the initial binding (LIB) of ER α WT, binding of both ER α and ER α S305A 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 ER α activity by phosphorylation could result in ligand independent activation and/or enhanced responses to E₂, and may result in enhanced residual receptor activity at non-saturating tamoxifen levels.

Figure 4

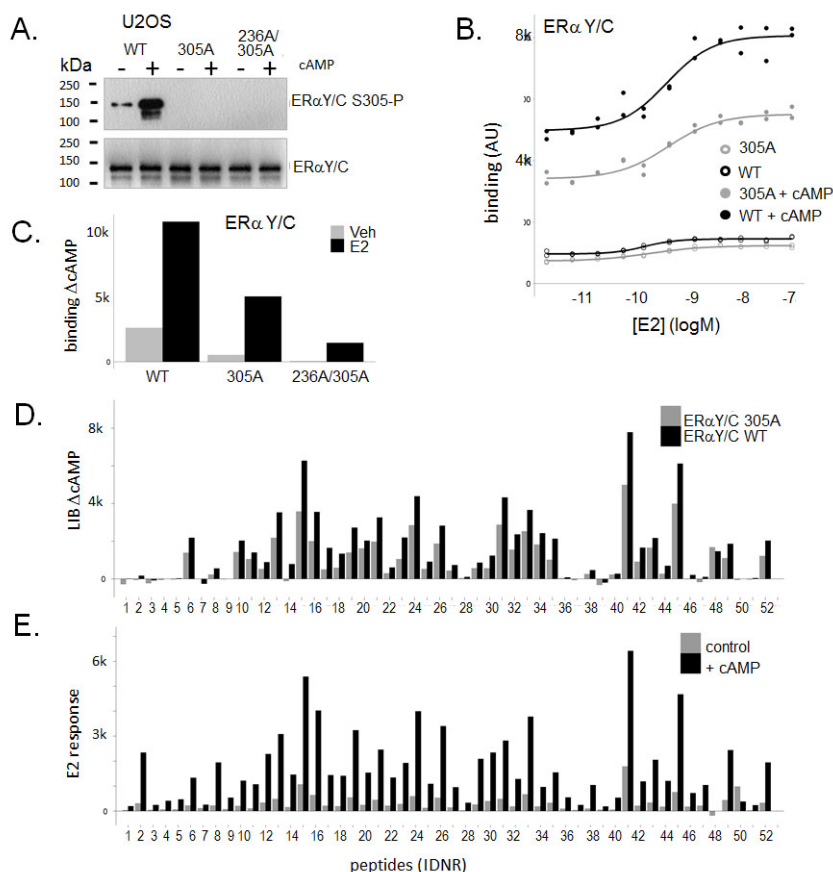


Figure 4. Effect of serine 305 phosphorylation on ER α -coregulator binding. U2OS cells were transfected with wild-type (WT), single (305A) or double (236A-305A) serine mutant full-length ER α tagged with YFP/CFP. Cells were stimulated with (+) or without cAMP to induce PKA-mediated receptor phosphorylation. **A.** Western blot analysis of ER α Ser305 phosphorylation in the three transfectants with or without cAMP stimulation. **B.** Dose dependent 17- β -Estradiol (E₂)-modulated binding of wild-type or ER α Y/C 305A from control or cAMP-stimulated cells with NCOA1_677_700 (IDNR13). **C.** cAMP-induced enhancement of NCOA1_677_700 binding by ER α Y/C WT, ER α Ser305Ala and ER α Ser305Ala/Ser236Ala in the absence or presence of E₂. **D.** MI for cAMP-induced ligand independent binding of WT and 305A-ER α to all peptides. **E.** MI for E₂-induced binding (delta) of WT ER α from control and cAMP-stimulated cells (D and E were calculated using LIB and LSB, see figure 2).

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 ER α under these tamoxifen

conditions. This provides a novel mechanism for the previously reported association between phosphorylated ER α Ser305 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 ER α .

The peptides showing increased ER α -binding represent the classical ER α coactivators SRC-1/2/3 and nuclear receptor protein 1 (NR1p, (34)) (IDNR12-21, Figure 5C). Two peptides that were unaltered are derived from CREB-binding protein (CBP) and p300 (IDNR3-4).

Figure 5

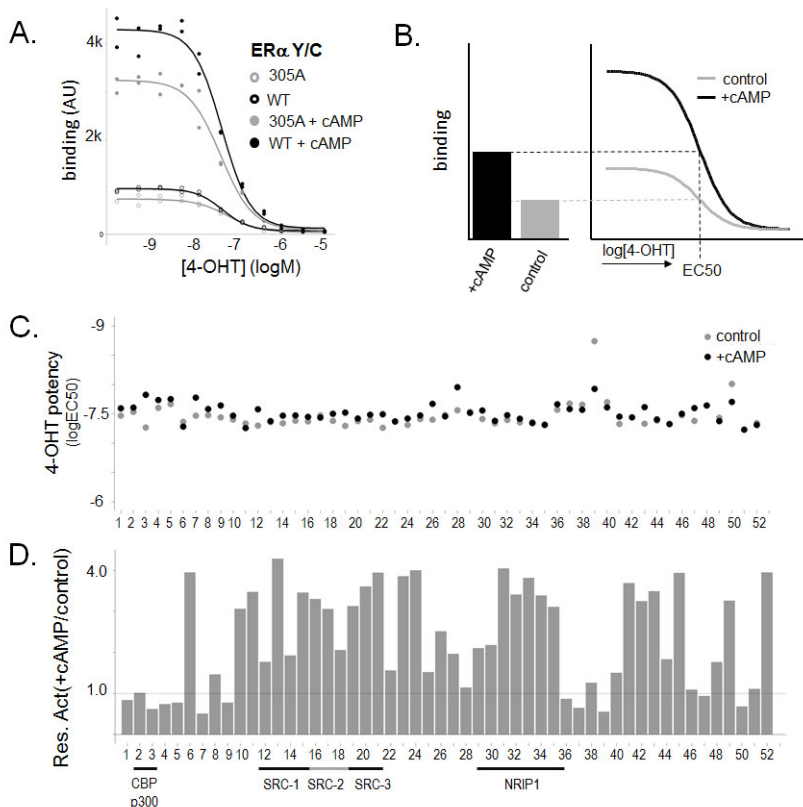


Figure 5. Tamoxifen-induced modulation of ER α -coregulator binding. U2OS cells were transfected with ER α Y/C wild-type (WT) or ER α Ser305A-Y/C (305A). Cells were stimulated with (+) or without 8-Bromo-cAMP (cAMP). A. Dose dependent 4-hydroxy-Tamoxifen (4-OHT)-modulated binding to NCOA1_677_700 (IDNR13) by wild-type or ER α 305A-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 ER α vs. cAMP-stimulated ER α 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.

Does the peptide array predict patient responses to tamoxifen?

We then examined whether Serine 305 phosphorylation is correlated with enhanced coregulator binding of ER α from patient material. We selected two breast tumors (A and B)

by immunohistochemical evaluation (figure 6B). Both tumors stained positive for ER α and tumor B was positive for ER α S305-P, which was confirmed by Western blot (Figure 6C). ER α 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 ER α S305-P positive tumor B (Figure 6C). For functional analysis on the array, sample input was normalized for ER α content measured by ELISA.

Figure 6

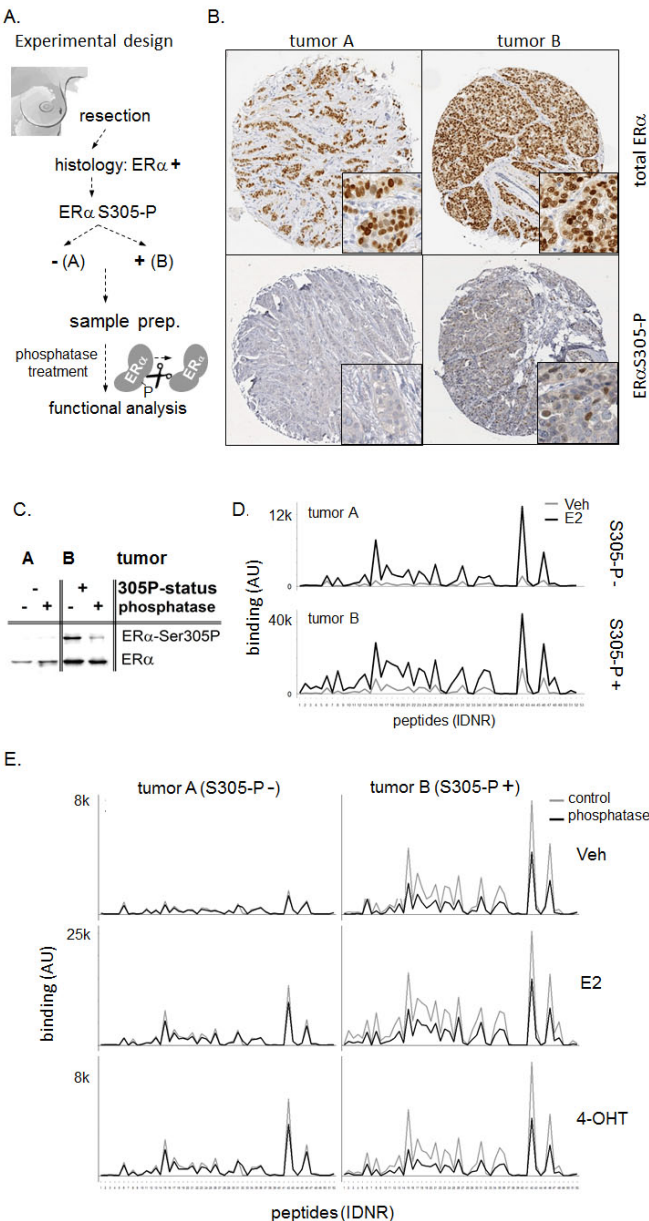


Figure 6. Functional analysis of ER α in crude lysates of two breast tumors. A. Immunohistochemistry and B. Western blot analysis of total ER α and ER α Ser305-P status in the sample of a S305P - (tumor A) or + (tumor B) patient before (-) and after (+) phosphatase treatment of tumor lysates. C. ER α -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) or 4-OHT (EC50).

Comparison of ER α in untreated lysates (control) of both tumors (grey lines in figure 6E), showed enhanced binding in the ER α Ser305-P positive tumor B (right panel), implying increased activity. This was the case for endogenous activity (no ligand added, veh), maximal activity at a saturating dose of estradiol and residual activity at EC₅₀ of tamoxifen. This enhanced activity in 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 ER α in tumor B reduces ligand independent activity (vehicle) as well as the response to E₂ and the residual activity after tamoxifen (EC₅₀) treatment. These data show that serine 305 phosphorylation of ER α , which is associated with tamoxifen resistance, can be measured as increased coregulator binding of ER α in clinical samples. This assay may therefore be applied for response prediction in the clinic, i.e. enhanced ER α activity 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 ER α biology and provides several advantages.

It allows the high throughput assessment of ER α 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 ER α activity in crude cell lysates, thereby avoiding troublesome purification procedures of full-length receptor. Previous studies report peptide binding only to purified fragments of ER α , generally the LBD (27, 28, 31). The analysis of a full-length ER α more closely reflects what happens *in vivo* than the LBD. Moreover, the sensitivity of the assay allows analysis of endogenous ER α 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 ER α . 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 ER α antagonist tamoxifen, the receptor adopts a conformation that reduces binding of coactivators (35), as is also evident from the binding profiles presented in figure 1C/D. Tamoxifen displaced ER α from LxxLL motif-containing peptides on the array (Figure 5A, 1C). However, if the cells develop resistance, tamoxifen fails to block growth. In this case the interaction between ER α 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 ER α 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 ER α 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 ER α 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 ER α .

We applied the assay to functionally analyze one well-described NR post-translational modification: Serine 305 phosphorylation of ER α . which is clinically associated with resistance

to tamoxifen (20, 21, 40). Full-length ER α is essential in studying this kind of resistance to anti-estrogens used in the clinic, because post-translational modifications of ER α outside the LBD domain influence the conformation of ER α and thereby the ability of the cofactor interacting surface to bind cofactors (11, 41). PKA-activation leads to ER α phosphorylation and enhanced receptor binding to the coregulator peptides in a ligand independent manner (Figures 4B and 5A), and could largely be attributed to ER α S305-P (Figure 4C). The underlying mechanism could be the previously reported conformational change of ER α , which would make the cofactor binding groove more accessible for the peptides on the chip (42). Coregulator binding by ER α S305A mutant was still slightly enhanced by PKA activation (Figure 4C), which means that PKA has additional effects on ER α . 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 ER α from binding to its cognate ERE sequence in the DNA, whereas ER α S305-P does not hamper binding to DNA (33, 43).

To extrapolate our findings to the clinic, we selected tumors from two ER α positive breast cancer patients, of which one was ER α Ser305-P positive. This parameter correlated with enhanced receptor activity in the lysate of this tumor. Upon phosphatase treatment to undo ER α phosphorylation, only the receptor from the ER α S305-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 ER α S305-P positive patient had a recurrence of disease after only nine months, in spite of tamoxifen treatment, whereas the ER α S305-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 ER α Ser305-P is associated with resistance to tamoxifen (20, 21, 40). The enhanced binding to coregulators by ER α S305-P provides a likely mechanism for tamoxifen resistance.

Resistance to endocrine treatment is reflected by enhanced receptor activity in a tumor lysate (Figure 6C), so this assay may 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 ER α 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 ER α biology and find underlying mechanisms for drug resistance. Moreover, functional profiling of ER α may prove a valuable tool for clinical purposes, such as prognostics or prediction of treatment response.

Acknowledgements:

RL and DV were in part supported by TI Pharma grant T3-107, MR was supported by a grant of the Netherlands Cancer Foundation.

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IDNR ID	setI	setII	Sequence	Uniprot	GeneID	Protein
1 BL1S1_1_11	NA	y	MLSRLLKEHQA	P78537	2647	BL1S1
2 BRD8_254_276	NA	y	TVAASPAASGAPTSLRLLLEAGPT	Q9H0E9	10902	BRD8
3 CBP_57_80	NA	y	GNLVPDAASKHKQLSELLRGSGS	Q92793	1387	CBP
4 EP300_69_91	NA	y	GMVQDAASKHKQLSELLRSGSSP	Q09472	2033	EP300
5 HAIR_745_767_C755S/C759S	NA	y	EDRAGRGLPSPSLSELLASTAV	Q43593	55806	HAIR
6 IKBB_277_299	y	y	PLGSAMLRPNPILARLLRAHGAP	Q15653	4793	IKBB
7 ILK_131_153	NA	y	KYGEMPVDKAKAPLRELLERAE	Q13418	3611	ILK
8 JHD2C_2054_2076	y	y	PLVSQNNQGGSTLRDLLTTTAKG	Q15652	221037	JHD2C
9 LCOB_40_62	NA	y	TTSPTAATTQNPVLSKLLMADQD	Q96JN0	84458	LCOB
10 MED1_591_614	y	y	HGEDFSKVSQNPILTSLLQITGNG	Q15648	5469	MED1
11 MLL2_4175_4197	NA	y	LLAGPRSEAGHLLQLKLRANKV	Q14686	8085	MLL2
12 NCOA1_620_643	y	y	SDGDSKYQTSKHLVQLLTITAEQ	Q15788	8648	NCOA1
13 NCOA1_677_700	y	y	PSSHSSSLTERHKILHRLLEQSGSPS	Q15788	8648	NCOA1
14 NCOA1_737_759	y	y	ASKKKESKDHQLRLYLDDKDEK	Q15788	8648	NCOA1
15 NCOA1_1421_1441	y	y	TSGPQTPQAQKSLQLQLLLE	Q15788	8648	NCOA1
16 NCOA2_628_651	y	y	QGSRLHDSKGQTKLLQLLTTKSDQ	Q15596	10499	NCOA2
17 NCOA2_677_700	y	y	STHGTSLEKHKILHRLLDQSSSP	Q15596	10499	NCOA2
18 NCOA2_733_755	y	y	EPVSPKKKENALLRYLLDKDDTK	Q15596	10499	NCOA2
19 NCOA3_609_631	y	y	QRGPLESKGHKLLQLLTCSSDD	Q9Y6Q9	8202	NCOA3
20 NCOA3_673_695	y	y	MHGSLLEKHKILHRLLDQSSSP	Q9Y6Q9	8202	NCOA3
21 NCOA3_725_747	y	y	EQLSPKKKENALLRYLLDRDDP	Q9Y6Q9	8202	NCOA3
22 NR0B1_1_23	y	y	MAGENHQWQGSILYNMLMSAKQT	P51843	190	NR0B1
23 NR0B1_68_90_C69S	y	y	FSGKDHPRQGSILYSMLTSKQT	P51843	190	NR0B1
24 NR0B1_136_159	y	y	GEDHPRQGSILYSMLTSKQTHVA	P51843	190	NR0B1
25 NR0B2_9_31_C9S/C11S	NA	y	SPSQGAASRPAILYALLSSSLKA	Q15466	8431	NR0B2
26 NR0B2_106_128	y	y	TFEVAEAPVPSILKKILLEPSS	Q15466	8431	NR0B2
27 NR0B2_201_223_C207S	NA	y	EVLEPWPSPAAQGRRLRVLLTAST	Q15466	8431	NR0B2
28 NRBF2_128_150	NA	y	PEIQGIFDRDPDILLYLLQKQSE	Q96F24	29982	NRBF2
29 NRIP1_120_142	NA	y	VDSVPKGQDSTLLASLLQSFSS	P48552	8204	NRIP1
30 NRIP1_253_275_C263S	NA	y	PATSPKPSVASSQLALLSSEAH	P48552	8204	NRIP1
31 NRIP1_368_390	y	y	RNNIKQAANNLSLLHLKLSQTP	P48552	8204	NRIP1
32 NRIP1_488_510	y	y	KNSKLSNSHQVTLQLLGHKNE	P48552	8204	NRIP1
33 NRIP1_805_831	y	y	PVSPQDFFSKNGLLSRLLRQNDQS	P48552	8204	NRIP1
34 NRIP1_924_946	y	y	RSWARESKSFNVLLKQLLSSENCV	P48552	8204	NRIP1
35 NRIP1_1055_1077	NA	y	EKDSPLRTKTNPILYMLQKGGN	P48552	8204	NRIP1
36 NSD1_894_916	NA	y	SSQNIHPIEPDYKFTLLMMLKD	Q96L73	64324	NSD1
37 PELP1_20_42	NA	y	GTGGLSAVSSGPRRLRLLESVS	Q8IZL8	27043	PELP1
38 PELP1_446_468	NA	y	AGMLQGGASGEALLTHLLSDISP	Q8IZL8	27043	PELP1
39 PNRC1_306_327	NA	y	ENSNQNRLEMAVHLKTLKLVQT	Q12796	10957	PNRC1
40 PPRC1_151_173	NA	y	DSELLVSPREGSSHLKLLTLRT	Q5VV67	23082	PPRC1
41 PRGC1_130_155	y	y	DGTPPPQEAEPSSLKKLLAPANTQ	Q9UBK2	10891	PRGC1
42 PRGC2_146_166	y	y	PAPEVDELSLLQKLLATSYP	Q86YN6	133522	PRGC2
43 PRGC2_338_358	y	y	AEFSILRELLAQDVLCDVSKP	Q86YN6	133522	PRGC2
44 PROX1_57_79	NA	y	SVVQHADGEKSNVLRKLLKRANS	Q92786	5629	PROX1
45 TIF1A_747_769	NA	y	ESRPQANANYRSILTSLLNSSQ	Q15164	8805	TIF1A
46 TIP60_476_498	NA	y	LSEDIVDGERAMLKRLLRIDSK	Q92993	10524	TIP60
47 TREF1_168_190	NA	y	TQSAVMDGAPDSALRQLLSQKPM	Q96PN7	55809	TREF1
48 TREF1_850_872	NA	y	HSLFEAKGDVMMVALEMLLRKPV	Q96PN7	55809	TREF1
49 TRXR1_132_154	NA	y	GHGPTLKYAQEGRLKQLLKMNGP	Q16881	7296	TRXR1
50 WIP1_119_141	NA	y	ESIYIHNIKDKMLKTLTDIPAN	Q5MN29	55062	WIP1
51 WIP1_313_335_C318S	NA	y	GQRNISTLSIQKLPRLLVASSS	Q5MN29	55062	WIP1
52 ZNH3_89_111	y	y	LQNLKNGESATLSLLNPHLR	Q15649	9326	ZNH3

Supplementary table 1. List of coregulator-derived sequences each containing an LxxLL domain (NR box) used in the MARCoNI assay. ID as follows: [coregulator]_[aa start]_[aa end of peptide].

Chapter 4
research article

PKA Phosphorylation redirects Estrogen Receptor to promoters of
a unique gene set to induce tamoxifen resistance

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submitted

PKA Phosphorylation redirects Estrogen Receptor to promoters of a unique gene set to induce tamoxifen resistance

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Abstract

Protein Kinase A-induced estrogen receptor alpha (ER α) phosphorylation at serine residue 305 (ER α S305-P) can induce tamoxifen resistance and growth of breast cancer cells. How this phospho-modification affects ER α specificity and translates into tamoxifen resistance is unclear. Here we show that S305-P modification of ER α reprograms the receptor, redirecting it to new transcriptional start sites, thus modulating the transcriptome. By altering the chromatin binding pattern, Ser305 phosphorylation of ER α translates into a 26-gene expression classifier that identifies breast cancer patients with a poor disease outcome after tamoxifen treatment. MYC-target genes and networks were significantly enriched in this gene classifier that includes a number of selective targets for ER α S305-P. The enhanced expression of MYC increased cell proliferation in the presence of tamoxifen. We demonstrate that activation of the PKA signaling pathway alters the transcriptome by redirecting ER α to new transcriptional start sites, resulting in altered transcription and tamoxifen resistance.

Introduction

Breast cancer is the most frequently diagnosed malignancy among women, with annually around 1.4 million new diagnoses worldwide. Although treatment has strongly improved with the development of adjuvant systemic therapies, still about half a million patients die of the consequences of breast cancer (Ferlay J et al., 2010). The choice of adjuvant treatment is largely based on the pathological subtype of the breast tumor, which can be classified by morphological, molecular and immunohistochemical markers. These subtypes correspond to distinct transcriptional repertoires, which translate in a different aggressiveness and metastatic potential (Sorlie et al., 2001). 75% of all breast tumors are luminal and proliferate dependent on the activity of the estrogen receptor α (ER α). Inhibition of ER α by endocrine therapy is therefore a major treatment modality of these tumors. Endocrine therapy can be subdivided into two treatment modalities; aromatase inhibitors that block synthesis of the hormone oestrogen and anti-oestrogens. Anti-oestrogens (including tamoxifen) compete with natural oestrogens by occupying the hormone-binding site of ER α and either arresting it in the inactive state (Shiau et al., 1998) or inducing degradation of the receptor (Robertson et al., 2004). However, patients can acquire resistance to either type of endocrine therapy. About 25% of the tamoxifen-treated tumors are resistant to this anti-oestrogen, even though the tumor continues expressing ER α (Holm et al., 2009). Consequently, patients unresponsive to tamoxifen may still respond to other anti-oestrogens such as fulvestrant or to aromatase inhibitors (Robertson, 2004). A major step in treatment success would be achieved when responses to endocrine treatment could be predicted on an individualized basis. Detection of ER α S305-P in patient tissues has provided a means of selecting a group of patients resistant to tamoxifen prior to the onset of treatment (Bostner et al., 2010; Holm et al., 2009; Kok et al., 2011). However, full coverage is not achieved and the classification on ER α S305-P signal by IHC has to be improved to identify all patients not responding to endocrine therapy due to PKA activation.

Several causes and contributing factors for inducing tamoxifen resistance have been

described in breast cancer patients or cell models, including upregulation of growth factor receptors (like EGFR (Fan et al., 2007; Massarweh et al., 2008), IGFR and HER2 (Riggins et al., 2007; Shou et al., 2004)), activation of kinases (such as AKT (Kirkegaard et al., 2005), MAPK (Gee et al., 2001; Kato et al., 1995), PKA in combination with PAK1 (Kok et al., 2011; Rayala et al., 2006a; Rayala et al., 2006b), and the resulting phosphorylation status of ER α (de Leeuw et al., 2011; Michalides et al., 2004; Skliris et al., 2010; Skliris et al., 2009).

The effects of phosphorylation by PKA of ER α at Serine residue 305 (ER α S305-P) in the region between the ligand binding domain and the DNA binding domain are understood in molecular detail. Phosphorylation at Ser305 results in a conformational arrest when exposed to tamoxifen (Michalides et al., 2004), which affects recruitment of coregulators (Zwart et al., 2007). Consequently, tamoxifen acts as an agonist of ER α instead of an antagonist, now inducing cell growth of breast cancer cell lines (Dudek and Picard, 2008; Michalides et al., 2004). An antibody detecting ER α S305-P in tumor sections was successful in identifying breast cancer patients with a poor outcome after tamoxifen treatment (Bostner et al., 2010; Holm et al., 2009; Kok et al., 2011), translating observations in tissue culture into clinical patient responses.

Since the oestrogen receptor is a nuclear receptor, and its phosphorylation affects recruitment of coregulators, the chromatin binding landscape and transcriptome may change following this modification. The transcriptome for tamoxifen resistance has been profiled in a tamoxifen resistant breast cancer cell line model. Several kinase pathways including PKA link to tamoxifen resistance (Dudek et al., 2008; Michalides et al., 2004; Miller, 2002). Several classical targets for ER α were differentially regulated including TFF1 (Dudek et al., 2008). PKA activation does not only phosphorylate ER α , but has many targets, including coregulators of the receptor (Wu et al., 2004; Yi et al., 2008). The phospho-status of coregulators can also affect ER α function, thereby indirectly affecting the ER α cistrome and transcriptome (Carascossa et al., 2010; Lupien et al., 2009). Other PKA targets may even bypass the receptor and change the transcriptome independently. Since kinase activity can alter the chromatin-interaction landscape of ER α (Lupien et al., 2010), deciphering a direct connection between ER α S305-P modification and direct targets is essential for understanding tamoxifen resistance. Here, we aim to define the direct target genes of the modified ER α S305-P and test whether that yields predictors for tamoxifen resistance. We determined the resulting transcriptome and performed further bioinformatic analyses to determine a predicting gene signature in patient material. This signature includes unique ER α S305-P induced pathways that explain tamoxifen resistance.

Results

ER α S305 phosphorylation by Protein Kinase A

To study PKA-induced tamoxifen resistance, we used two well-defined and intensely studied breast cancer cell lines MCF7 and MDA-MB134. Both MCF7 and MDA-MB134 express ER α and require oestrogens for growth, which is inhibited by tamoxifen (Michalides et al., 2004; Reiner and Katzenellenbogen, 1986). In MCF7 cells, we activated the PKA pathway by forskolin (Al Dhaheri and Rowan, 2007) (Figure 1A top) and isolated RNA for microarray and qPCR analyses after 4 hours of tamoxifen exposure to probe early transcriptional responses of ER α S305 phosphorylation. Forskolin treatment induces phosphorylation of ER α S305 as detected by a specific antibody (Figure 1B top). While we chemically activated the PKA pathway in MCF7 cells, we decided to confirm results by genetically activating this pathway in MDA-MB134 cells. Here, PKA was activated by silencing the inhibitory subunit of PKA, PKA-R1 α (Figure 1A bottom) (Bossis and Stratakis, 2004), which is also observed in tamoxifen-

resistant patients (Michalides et al., 2004). When PKA-R1 α is silenced, PKA is activated yielding phosphorylation of ER α S305 (Michalides et al., 2004). Silencing PKA-R1 α was confirmed by Western blot analysis (Figure 1B bottom). In addition, increased phosphorylation of PKA substrate CREB confirms that PKA is activated in both cell lines (Figure 1B), yielding an elevated ER α S305-P signal.

Figure 1

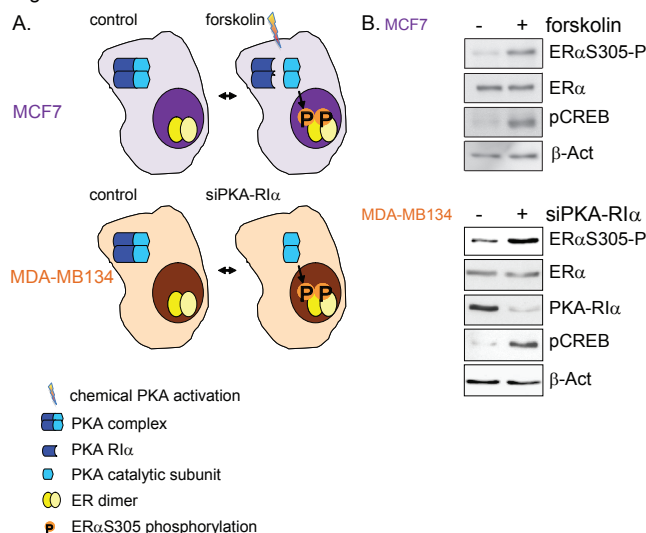


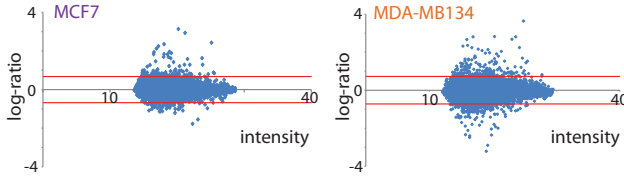
Figure 1: ER α S305 phosphorylation by Protein Kinase A. A. Experimental setup to activate PKA in MCF7 (top) by forskolin stimulation and in MDA-MB134 (bottom) by PKA-R1 α knockdown with lentiviral shRNA. Dissociation (MCF7) or loss (MDA-MB134) of PKA-R1 α liberates the active, catalytic subunit of PKA, leading to ER α S305 phosphorylation. B. Western blot analysis of the model systems. Top: Forskolin treatment of MCF7 cells leads to activated PKA, illustrated by increased p-CREB, and elevated ER α S305 phosphorylation. Bottom: shRNA approach successfully decreases PKA-R1 α level in MDA-MB134 cells, leading to increased p-CREB and ER α S305-P.

A gene signature for tamoxifen resistance after PKA activation

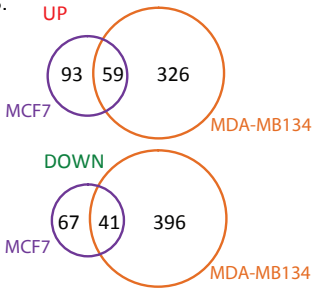
We analyzed the effects of PKA-induced ER α S305 phosphorylation on the transcriptome by expression microarray analyses. Under conditions corresponding to the experiments above (Figure 1), cells were deprived of hormones for three days and subsequently treated with tamoxifen, after which the influence of PKA activation was assessed in both cell lines (Figure 2). PKA was chemically activated in MCF7 cells and genetically in MDA-MB134 cells. Gene expression distribution is illustrated by the log-ratio, for PKA-activated versus non-activated cells, over intensity (RI) dot plots (Figure 2A). In MCF7 cells, we identified 152 upregulated and 108 downregulated genes following PKA activation (260 in total). In MDA-MB134, we find 385 up- and 437 downregulated genes (822 in total). In these gene expression profiles, 59 up- and 41 downregulated genes overlap between MCF7 and MDA-MB134 (Figure 2B). By focusing on the overlap of 100 differentially regulated genes, we eliminated cell line or treatment-specific effects. Among the upregulated hits are two classical targets for estradiol-stimulated oestrogen receptor: TFF1 and XBP1, the latter of which is in the top 5 of differentially regulated genes. The top 10 up- and downregulated hits for MCF7 cells are indicated (Figure 2C), and a subset tested and confirmed by qPCR (Figure 2D). Next, we tested the 100 differentially regulated genes that are shared between the two cell lines and conditions (59 up, 41 down) as a classifier to identify ER α -positive breast cancer patients responding poorly to tamoxifen treatment, using a publicly available dataset (Loi et al., 2007). The gene classifier was found to significantly correlate with poor outcome after tamoxifen treatment ($p=0.019$; hazard ratio=2.5) (Figure 2E top). This classifier was validated in an independent patient series (Buffa et al., 2011), again identifying the patients with a poor outcome after tamoxifen treatment ($p=0.045$; hazard ratio=1.97, Figure 2E bottom).

Figure 2

A. RI plots



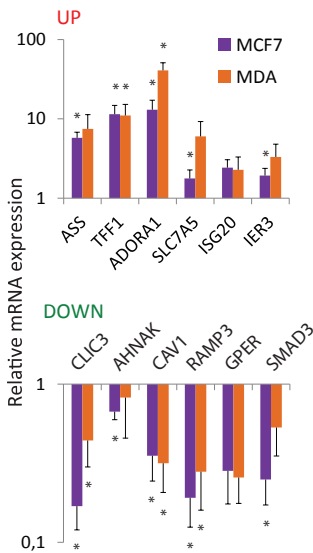
B.



C.

Top 10 hits (MCF7)	
up	down
ASS	CLIC3
TFF1	AHNAK
MUC5AC	CAV1
ADORA1	TACC1
SLC7A5	PMP22
ISG20	RAMP3
CPE	YPEL3
PCP4	NRCAM
SEMA3B	GPER
IER3	SMAD3

D. qPCR



E.

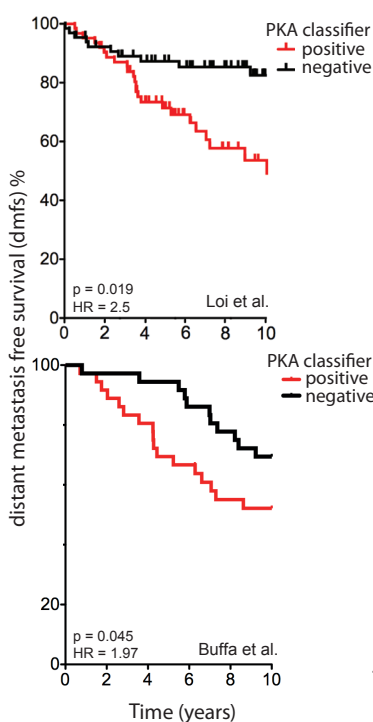


Figure 2: Gene signature for tamoxifen resistance through PKA activation

A. RI-plots of microarray profiles of MCF7 (left) and MDA-MB134 (right). Log-ratio is plotted over intensity. For further analysis, a threshold is used of $>1.5\times$ difference ($\log\text{-ratio} = 0.585$), indicated by the red line, and $p < 0.05$.

B. Venn diagrams of up- (top) and downregulated (bottom) genes show overlap between MCF7 (purple) and MDA-MB134 (orange) gene expression signatures.

C. Table of the top 10 of up- and downregulated genes ranked on MCF7 mRNA expression values. Genes represented in red (up) or green (down) are tested and confirmed by qPCR as illustrated in:

D. qPCR validation of a subset of the top hits. mRNA expression relative to house-keeping gene β -Actin. MA = Microarrays values. Error bars represent standard error of the mean (SEM). * $p < 0.05$ by Student's t-test.

E. Genes shared between MCF7 and MDA-MB134 are combined into a 100-gene signature. This signature was applied as a gene classifier in a disease metastasis free survival analysis. The average gene expression values in $ER\alpha$ positive,

endocrine treated patients selected from Loi et al. (top, (Loi et al., 2007)) and Buffa et al. (bottom, (Buffa et al., 2011)) were calculated and ranked for up- and downregulated genes. Patients were stratified as described in the materials and methods. Patients who have a positive signature for the classifier show significantly worse disease progression (Loi data: $p = 0.019$; hazard ratio(HR)=2.5; Buffa data: $p = 0.045$; HR=1.97).

S305P modification of ER α targets the receptor to promoters

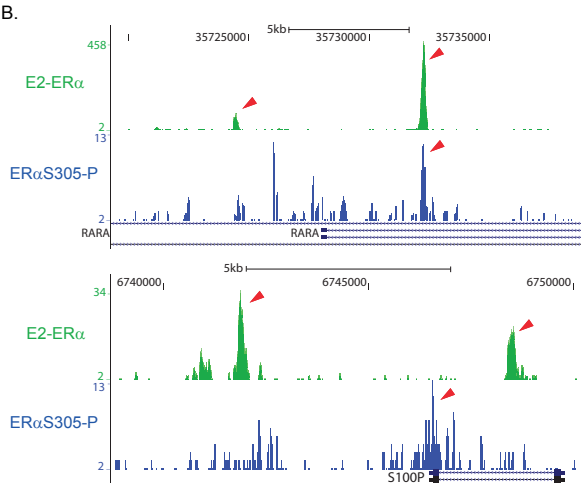
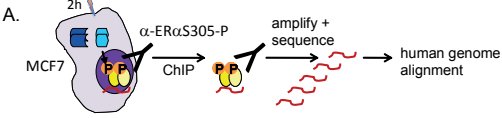
PKA activity results in the phosphorylation of many targets, including ER α . ER α itself has two target sites for PKA, Serine 236 (Chen et al., 1999) and Serine 305, which is predominant and also correlates to tamoxifen resistance (Michalides et al., 2004). To determine the effects of ER α S305 phosphorylation on gene transcription and chromatin deposition, we analyzed the chromatin-binding landscape of ER α S305-P by means of ChIP-seq with a specific monoclonal antibody (Holm et al., 2009). To this purpose, cells were cross-linked, chromatin fragmented and ER α S305-P immunisolated. The co-isolated chromatin fragments were amplified, sequenced and mapped against the human genome reference (Figure 3A) (Schmidt et al., 2009). MCF7 cells were hormone deprived for three days and stimulated with forskolin. Since transcriptional alterations were observed four hours after tamoxifen treatment, the chromatin interaction patterns of ER α S305-P were studied at an earlier time point (two hours of forskolin treatment) as DNA binding precedes transcription. ER α S305-P binding patterns were compared to chromatin interactions of total ER α from asynchronously proliferating MCF7 cells (Robinson et al., 2011) to determine the shared events and unique binding sites for the phosphorylated receptor. ER α S305-P shows 2657 binding events, of which 947 overlap with total ER α (Figure 2C and D). This implies that S305-phosphorylated ER α shares only a subset of the conventional ER α binding sites, but also has its own specific targets sites. Examples of shared and unique sites are shown in Figure 2B. When the binding peaks were annotated, a striking enrichment for ER α S305-P was observed for promoter regions, 3'UTRs and 5'UTRs, whereas total ER α generally prefers distal enhancers (Figure 3E), as was described before (Carroll et al., 2006; Madak-Erdogan et al., 2011). This enrichment on promoter regions is not only observed for the shared interaction sites, but also for unique ER α S305-P peaks. Consequently, DNA motif analyses of ER α and ER α S305-P reveal different motifs concurrent with differences in the transcriptome (Figure 3F, shown are some genes identified in the microarray analyses in Figure 2). Modification of ER α at Ser305 not only affects ER α conformation (Michalides et al., 2004) but also chromatin binding sites, transcription and cellular responses.

Interconnection of genes in the classifier

To understand how phosphorylation of ER α S305 drives differential gene expression, resulting in tamoxifen unresponsiveness of breast tumors, we decided to define the functional networks that are differentially (in)activated due to the modification of ER α . An Ingenuity Pathway Analysis (IPA) of the differentially regulated genes in both conditions was performed, which identifies functional connections from a gene list using literature data. Within the top pathways listed in Figure 4A, classical ER α targets (TFF1, XBP1, CAV1) as well as interacting partners of ER α for non-classical gene transactivation, such as AP-1 and NF κ B (Suppl. Figure S1) were found, illustrating expected ER α -mediated gene expression rather than only PKA activation.

PKA-activation can induce cell growth in tamoxifen-stimulated breast cancer cells (Dudek et al., 2008; Michalides et al., 2004). We therefore focused on the third network, which links genes in the classifier to cell growth and proliferation. This network includes MYC as a central player (Figure 4B). MYC is not a direct hit in our microarray analysis as it was enriched just below the threshold (1.49x) in MCF7 cells. We validated elevated MYC expression in MCF7 cells following forskolin and tamoxifen stimulation by a more quantitative method: qPCR (Figure 4C). PKA activation combined with tamoxifen treatment upregulates MYC expression 1.65-fold, directly coupling ER α S305 phosphorylation to expression of a well-known oncogene involved in tamoxifen resistance (Miller et al., 2011; Musgrove et al., 2008). Of note, the effect of PKA activation on the transcription of the genes tested by qPCR appears to be independent of ligand.

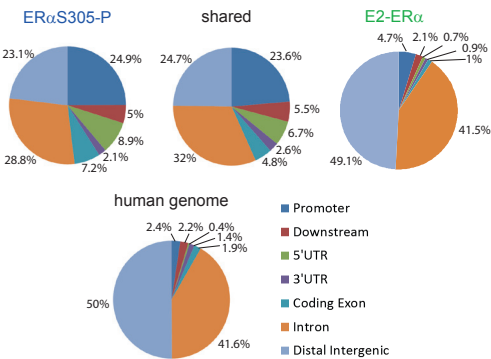
Figure 3



C. binding events



D. Genomic distribution



E. motif analysis

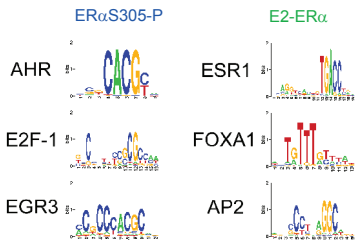


Figure 3: Distinct chromatin binding patterns of ER α S305-P

A. Experimental setup: after 2 hours of forskolin stimulation, ChIPseq is performed using a specific antibody against ER α S305-P.

B. Examples of chromatin interaction to two gene areas for total ER α (green) and S305-phosphorylated ER α (blue). Retinoic acid receptor alpha (RARA) has an ER α binding site within a 20k nucleotide region of the transcription start, to which ER α S305-P also binds. S100P shows binding for both total and phosphorylated ER α , but ER α S305-P prefers the promoter, whereas total ER α binds to a distal enhancer. Arrows denote binding peaks. A 5 kb size marker is indicated.

C. Venn diagram, showing the overlap of ER α S305-P (blue) chromatin binding events versus total ER α (green). Number of shared or unique peaks are indicated. Called peaks were interrogated for overlap and intersected using Galaxy (<http://main.g2.bx.psu.edu/>).

D. Genomic distribution of overall ER α S305-P binding (left), total ER α (right) and sites shared between the two (middle). The genomic distributions of binding sites were analyzed using the cis-regulatory element annotation system (CEAS) (Ji et al., 2006). The genes closest to the binding site on both strands were analyzed. If the binding region is within a gene, CEAS software indicates whether it is in a 5'UTR, a 3'UTR, a coding exon, or an intron. Promoter is defined as 1 kb upstream from RefSeq 5' start. If a binding site is >1 kb away from the RefSeq transcription start site, it is considered distal intergenic. ER α S305-P shows preference for promoter sites.

E. Motif analysis of binding sites for ER α S305-P (blue) and total, estradiol-stimulated ER α (green) show a difference in motif preference. To identify motifs, SeqPos was used (He et al., 2010). SeqPos use the distances from motif positions to the peak summits (center of the regions) to find the most enriched motifs near peak summits, using TRANSFAC.

Figure 4: Interconnection of genes in the classifier

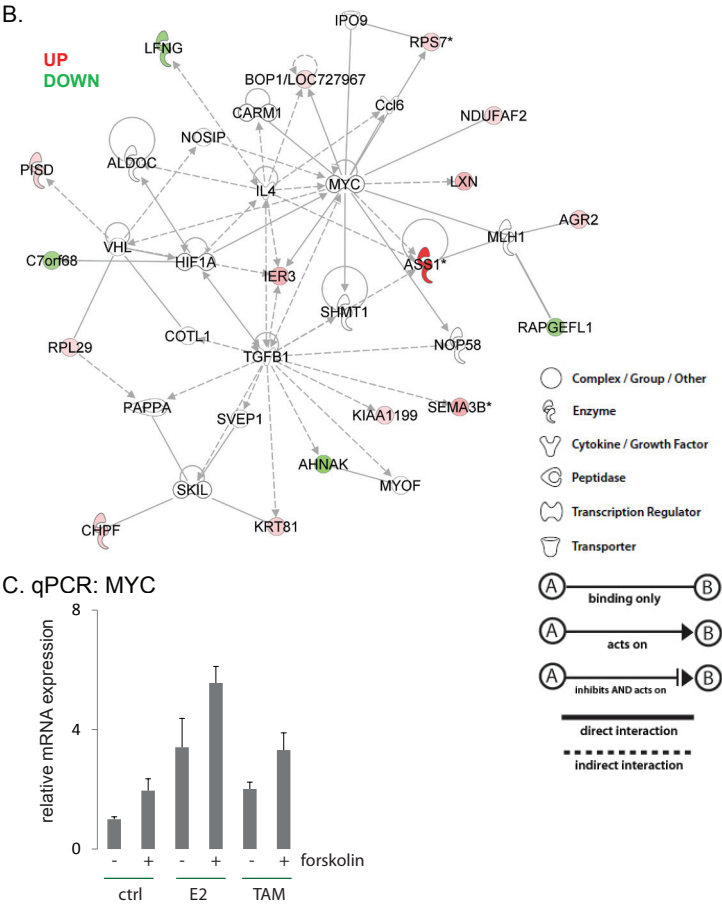
A. Ingenuity pathway analyses reveal direct and indirect links between the genes in our classifier, resulting in the top 5 of significant pathway terms listed in the table and shown in Supplementary Figure 1. Networks are scored based on the number of network eligible genes the list contains. Score is a likeliness parameter.

B. Shown is network 3 as shown in bold in Figure 4A. MYC plays a key role, targeting several of the genes from the classifier. Differential expression in the microarray in Figure 2 is illustrated by a red (up) or green (down) color. The nature of the different hits is indicated on the right.

C. MYC qPCR of MCF7 cells after 4 hours of treatment with estradiol (E2), tamoxifen (TAM) or hormone depleted (ctrl) in presence or absence of forskolin to stimulate PKA. Error bars represent SEM. * $p < 0.05$ by Student's t-test.

Figure 4

A.	Associated Network Functions	Score
1	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry	50
2	Cell Signaling, Nucleic Acid Metabolism, Small Molecule Biochemistry	39
3	Cell Cycle, Cellular Development, Cellular Growth and Proliferation	32
4	Lipid Metabolism, Small Molecule Biochemistry, Cellular Movement	24
5	Cellular Development, Cellular Growth and Proliferation, Tissue Morphology	13



Integration of ERαS305-P chromatin binding with gene expression signatures

Selective ERαS305-P interactions with chromatin should translate into transcriptional differences. To assess these, we integrated the ChIP-seq data with the expression data obtained from the microarray studies. This allowed us to extract the direct targets of ERαS305-P from the bulk of genes that are differentially regulated due to overall PKA activation. Among the 100 hits from our classifier, we defined the genes that had a chromatin binding peak for ERαS305-P within a 20k region from the transcription start site, which indicates direct transcriptional regulation by the receptor (Fullwood et al., 2009). This identified 26 genes as direct targets of ERαS305-P. 14 of these genes were upregulated and 12 downregulated (Figure 5A). Of these direct ERαS305-P targets, nine are distinct from estradiol-stimulated, total ERα, implying

that they are specific for the phosphorylated form. Utilized as a classifier in the previously mentioned patient dataset by Loi et al. (Loi et al., 2007), the 26 ER α S305-P targets result in a significant correlation with poor disease outcome (Figure 5B. $p=0.008$; HR=2.33). Applying the classifier to the Buffa et al. (Buffa et al., 2011) dataset, the number of patients became too small for a reliable analysis.

When separately analyzing the direct ER α S305-P targets versus the non-phosphorylated ER α by qPCR, we observed a ligand independent effect for PKA activated genes that do not have a chromatin binding site for ER α S305-P (Figure 5C). This in contrast to the majority of the tested genes with a proximal ER α S305-P binding site, where tamoxifen exposed an additional agonistic behaviour next to the effects of PKA stimulation alone (Figure 5C).

We then analyzed the new classifier for biological relevance. Seven of the 26 direct targets are functionally connected with MYC, implying that ER α S305-P directly affects the MYC pathway (Figure 5D), rather than upregulating Myc only. Since MYC was upregulated in the PKA-activated, tamoxifen-treated MCF7 cells, we explored whether MYC is a direct target of ER α S305-P. To this end, the proximity of the Myc locus was analyzed for ER α S305-P peaks. We observed a chromatin interaction peak at a distal enhancer that has been recently described for ER α in synergy with AP-1 (Wang et al., 2011) and a second peak at the promoter region of MYC (Figure 5E, arrows denote the peaks). Taken together, we show by ChIP-seq, microarray and qPCR that S305-phosphorylated ER α plays a direct role in MYC transcriptional regulation. Myc upregulation will affect cell growth in response to tamoxifen.

To directly assess the influence of MYC on MCF7 cells proliferation, and the influence of tamoxifen treatment thereon, MYC was transiently overexpressed in MCF7 cells (quantified in Figure 5G). This resulted in a significant increase in cell proliferation both in absence and presence of tamoxifen (Figure 5F), linking the enhanced expression of MYC with cell growth even in presence of this anti-estrogen and thus inducing resistance.

Discussion

Activation of kinase pathways is one of the hallmarks of tumor formation. Most breast cancers are critically dependent on ER α and this nuclear hormone receptor can be modified by a series of kinases, including PKA (Gee et al., 2001; Kato et al., 1995; Kirkegaard et al., 2005; Michalides et al., 2004; Rayala et al., 2006a). PKA phosphorylates ER α at position 305, inducing a conformational arrest of the receptor upon tamoxifen exposure (Michalides et al., 2004). This eventually results in tamoxifen resistance and cell proliferation in response to tamoxifen exposure (Dudek et al., 2008; Michalides et al., 2004). However, the exact mechanism of tamoxifen resistance remains unknown and this is studied here. We show that S305-P modification has a marked effect on the accurate positioning of ER α on transcriptional start sites. This is highly surprising and, in more general terms, suggests that post-translational modifications can have major effects on chromatin binding of transcription factors and thus the transcriptome. In fact, this couples extracellular signalling (in our case by PKA activation) to alterations in transcriptional output by a retargeting of the transcription factor to alternative binding regions.

The phosphorylated receptor displayed an enrichment for DNA motifs that were distinct from that of total ER α in proliferating cells, suggesting that the phosphorylation directly alters the DNA binding capacities of specificity of the receptor. The crystal structure data of the full length RXR:PPAR γ heterodimer shows an alignment of the hinge region of PPAR γ along the DNA (Chandra et al., 2008). This may also occur with the hinge domain of ER α , thereby determining the DNA motif specificity of the receptor.

Figure 5

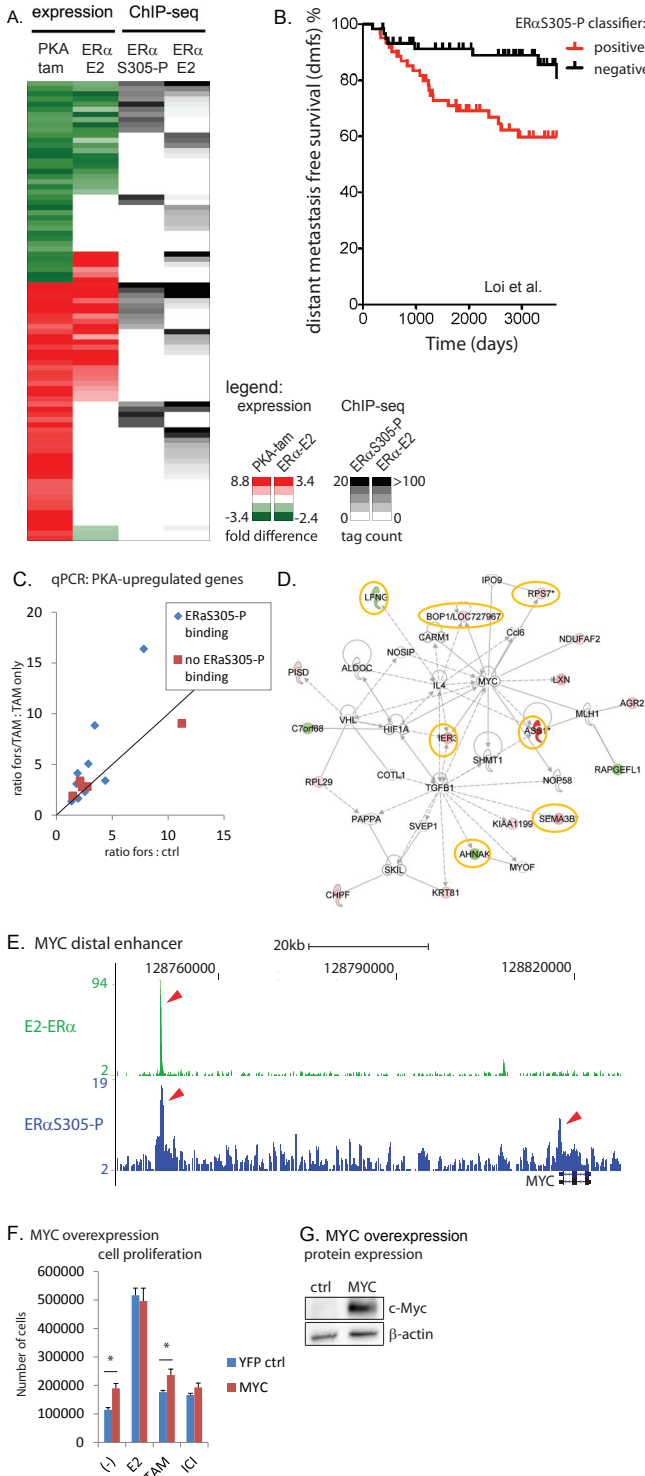


Figure 5: Integration of ERαS305-P chromatin binding and gene expression signatures

A. Hierarchical clustering of the 100 genes in the classifier, based on ERαS305-P chromatin binding, estradiol-induced chromatin interactions and gene expression of total ERα.

B. The 26 ERαS305-P hits were applied as a classifier on the patient dataset derived from Loi et al. (Loi et al., 2007), resulting in a significant correlation with poor disease outcome ($p=0.008$; $HR=2.33$). Analysis was performed as described in Figure 2D.

C. Scatter plot for qPCR data from genes upregulated by PKA activation, derived from the 100-gene classifier and subdivided into two groups: with (blue diamonds) or without (red squares) a proximal binding site for ERαS305-P. Ratio for PKA activation over no activation is plotted for presence (Y-axis) or absence (X-axis) of tamoxifen. Diagonal line represents equal ratios irrespective of tamoxifen.

D. Six (representing 23%) of the direct targets are found in the MYC-related Ingenuity network, highlighted in orange.

E. ERαS305-P selectively targets MYC by binding to a distal enhancer and the promoter. Shown are the reads for ERα and ERαS305-P around the MYC gene and the 20kb marker. Arrows indicate the two peaks.

F. MYC overexpression enhances tamoxifen-specific MCF7 cell growth. Absolute cell numbers of triplicates are counted and plotted for YFP-control (ctrl in blue) and MYC (in red). In triplo, scale bars = standard error of the mean (SEM). A student T-test was performed; $p=0.03$.

G. c-Myc protein expression analysis

of MYC overexpression, compared to control (ctrl) by western blot. Loading control is β-actin.

Any modifications in the hinge domain (including S305 phosphorylation) may, as a consequence, alter the DNA-binding preferences of the receptor. The altered positioning of ER α when phosphorylated at S305 is surprising but unlikely to be dictated by this post-translational modification alone. Most likely, the S305-P modification attracts different co-factors that in assembly alter the chromosome-binding preferences of the receptor. Such effects can be mediated further by phosphorylation of CARM1 (Carascossa et al., 2010) or AIB1 (Yi et al., 2008), but may also be dictated by other factors. ER α binding events are not rigid, but can differ between cell lines (Krum et al., 2008) and the chromatin-binding pattern can be manipulated by growth factor stimulation (Lupien et al., 2010). To our knowledge, this is the first report describing the direct effect of phosphorylation on the chromatin binding landscape of ER α . The distinct and unique patterns as observed here suggest that phosphorylation events on the receptor not only dictate the transcriptional readout (Michalides et al., 2004), the transcript repertoire (Dudek et al., 2008) and cofactor preferences (Zwart et al., 2007), but also determine to what DNA regions the receptor is capable of binding. This yields a complicated view on transcriptional regulation by ER α . As this protein can be modified at different locations by different kinases, different chromatin deposition and thus transcription may be the result. Depending on the activated signalling pathway, a different DNA binding preference of ER α after oestrogen activation or tamoxifen exposure may be the result.

We show here for the S305 modification that distinct transcriptional pathways are generated that can explain cell growth of breast cancer cells in response to tamoxifen. This is visualized in the development of a classifier that allows prediction of patient's responses to tamoxifen treatment. Further analyses show that the MYC pathway in particular can be activated, which may explain the more aggressive behaviour of such tumours.

Our data illustrate that one single post-translational modification can have a major impact on the chromatin interaction patterns and transcriptome of the oestrogen receptor. ER α S305 phosphorylation greatly affects its DNA-binding sites, giving rise to distinct responsive gene signature that includes MYC and its related genes. MYC overexpression overcomes tamoxifen action on cell proliferation and hence, a PKA-induced elevation of MYC would induce resistance to tamoxifen. The plasticity in the chromatin binding patterns of ER α as induced by PKA activation has significant downstream effects that may lie at the very basis of tamoxifen-resistance of breast cancer patients. The genes differentially targeted and transcribed by S305-phosphorylated ER α indeed act as a biologically relevant and understandable classifier for breast cancer patient responses to tamoxifen treatment.

Materials and Methods

Cell culture

MCF7 and MDA-MB134 cells were cultured in DMEM supplemented with 8% FBS and antibiotics (penicillin, streptavidin). To deprive cells of hormones, they were cultured in phenol-red free DMEM with 5% charcoal-treated serum and antibiotics. Cells were stimulated with 10^{-7} M 4-OH-tamoxifen and/or 10 μ M forskolin or vehicle for 4 or 24 hours.

PKA-R1 α knockdown

MDA-MB134 cells were infected with lentivirus containing a shRNA (sequence: GGGGATAACTTCTATGTGA) targeting PKA-R1 α . Infection was performed in DMEM after 2h incubation with polybrene (5 μ g/ml). After infection overnight, DMEM with 8% FBS was refreshed. Selection of infected cells was done with 3 μ g/ml puromycin.

Biochemical analysis

Western blotting was performed according to standard protocols. Antibodies used are anti-ER α (Santa Cruz Biotechnology), anti-ER α S305-P (Millipore/Upstate), anti-b-Actin (Millipore/Chemicon), anti-p-Creb (Cell Signaling Technology), anti-PKA-R1 α (BD Transduction Laboratories) and anti-c-Myc (Santa Cruz Biotechnology). Signals were detected with a Lumi-light Plus detection kit (Roche).

Microarray experiments

Cells were harvested and homogenized in trizol (Invitrogen). RNA was isolated and hybridized on IlluminaWG-6 expression BeadChip (MDA-MB-134) and Human HT-12 v4 Expression BeadChip (MCF7, performed in triplicate). For data extraction, we used no background correction, applied variance stabilizing transformation and robust spline normalization. Data were log-transformed, ratios of the absolute values were calculated. For the RI plots, the log-ratio was plotted over the intensity, calculated from the absolute intensities as follows: $R = \log(\text{PKA-activated} / \text{control})$ and $I = \log(\text{PKA-activated} \times \text{control})$. P-values absolute-value ratios were calculated with a two-tailed paired t-test. Hits were selected on $p < 0.05$ with a ratio threshold of 1.5x.

Bioinformatics patient datasets

We extracted the ER α positive, tamoxifen treated tumors from two published patient datasets (Buffa et al., 2011; Loi et al., 2007). All the expression data were retrieved for the 100 hits in the classifier. The average expression of all the tested genes was calculated, ranked and divided in two groups. Patients were stratified in two groups: 1. upregulated genes in the top 50% and the downregulated genes in the bottom 50%. 2. upregulated genes in the bottom 50% and the downregulated genes in the top 50%. Kaplan-Meijer plots were generated using Prism 5 (Graphpad software). P-values were calculated using a log-ranked Gehan-Breslow-Wilcoxon method.

qPCR

Cells were harvested and homogenized in trizol. RNA isolation for qPCR was performed by a phenol-chloroform extraction. cDNA was made with a Superscript III RT kit (Invitrogen) using the manufacturer's protocols. qPCR was performed with SYBR Green (Applied Biosystems) on a Chromo4 RT detector (Bio-Rad) using standard protocols. Primers (Invitrogen) were designed with primer3 vo.4.0 and are shown in Supplementary table 4.

ChIP-seq

ChIP experiments were performed as described previously (Carroll et al, 2005). The antibody used was anti-ER α S305-P (Millipore/Upstate). ChIP DNA was amplified as described (Schmidt et al., 2009). Sequences were generated by the Illumina GAIIx genome analyzer (using 36-bp reads), processed by the Illumina analysis pipeline version 1.6.1, and aligned to the Human Reference Genome (assembly hg18, NCBI Build36.1, March 2008) using BWA version 0.5.5. Reads were filtered by removing those with a BWA alignment quality score less than 15. For each biological replicate, a corresponding set of input sequence reads of similar size was obtained by random sampling from the full set of input sequence reads. Enriched regions of the genome were identified by comparing the ChIP samples to input samples using the MACS peak caller (Zhang et al., 2008) version 1.3.7.1. All ChIP-seq data was from intersected peaks, shared between two independent replicates.

MYC overexpression and cell proliferation assay

MCF7 cells were cultured in 12-wells plates. After one day of hormone deprivation,

MYC was overexpressed by polyethylenimine (PEI, Polysciences (Boussif et al., 1995)) transfection of a RC-CMV c-Myc vector. A pcDNA-YFP empty vector was used as control. Cells were treated in triplicate with estradiol (10^{-8} M), tamoxifen (10^{-7} M), fulvestrant (ICI, 10^{-7} M) or control for 7 days. After trypsinization, cells were counted with a CAsYton cell counter (Casy Technology).

Acknowledgements

We thank the Central Microarray Facility of the Netherlands Cancer Institute for processing the microarray samples. RL is in part supported by Top Institute Pharma. WZ is supported by a KWF Dutch Cancer Society Fellowship.

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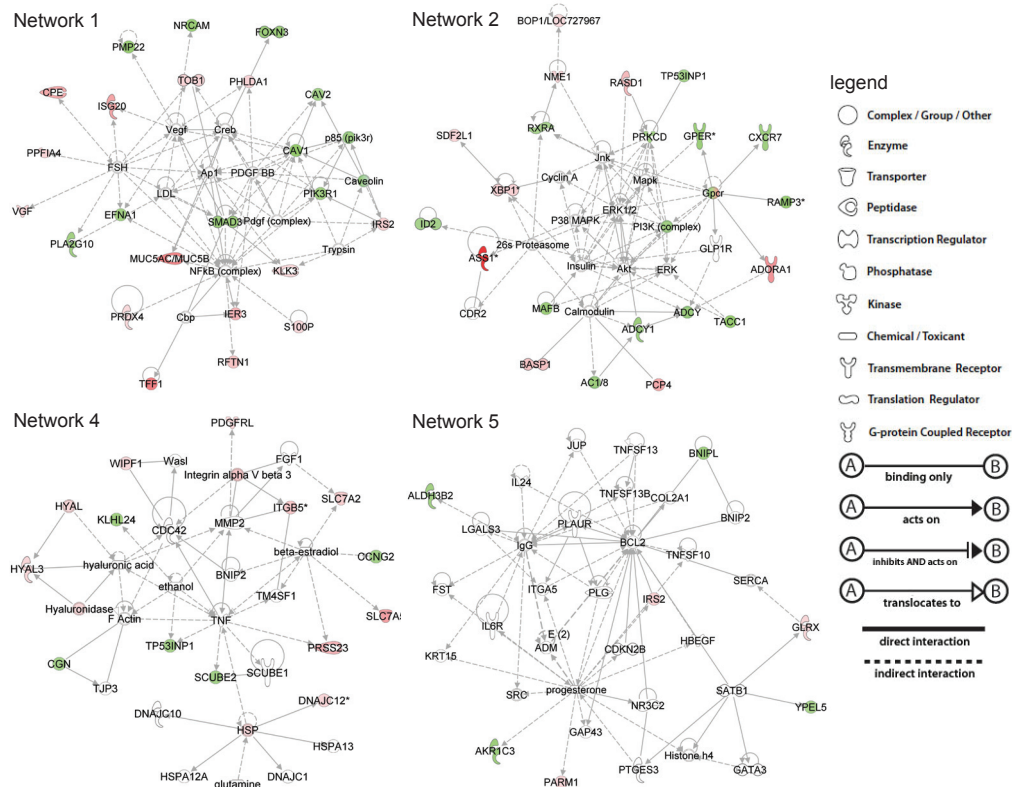
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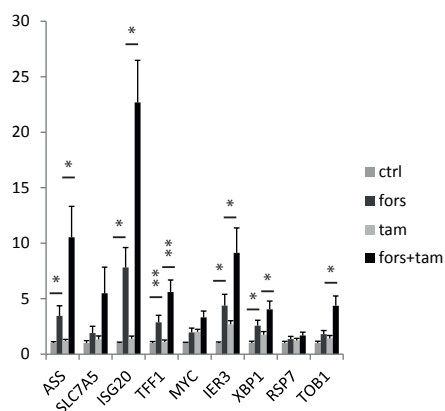
Supplementary figure S1



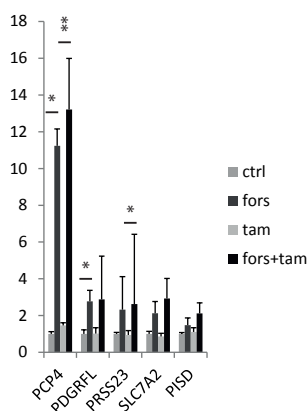
Supplementary Figure S1: Networks derived from the Ingenuity analysis presented in Figure 4A

Supplementary Figure S2 - qPCR data for Figure 5C

A. genes with ER α S305-P binding



B. genes without ER α S305-P binding



Supplementary Figure S2: qPCR data for Figure 5C, in triplo, scale bars = SEM, * $p < 0.05$.

Supplementary Table 1 - Gene expression profile for PKA-activation in MCF7

	Symbol	Accession	Ratio		Symbol	Accession	Ratio
1	CLIC3	NM_004669.2	0.293	76	CRIP2	NM_001312.2	0.629
2	TXNIP	NM_006472.2 NM_006472.1	0.341	77	CCNG2	NM_004354.1	0.629
3	EFHD1	NM_025202.2	0.433	78	SCUBE2	NM_020974.1	0.632
4	CALML5	NM_017422.3	0.454	79	HMG2	NM_002129.2	0.634
5	ATPEV1B1	NM_001692.3 NM_001692.2	0.457	80	TP53NP1	NM_033285.2	0.638
6	MB	NM_005368.2	0.460	81	CAV2	NM_001233.3	0.638
7	LOC643431	XM_928128.1	0.471	82	LMTK3	XM_936372.2 XM_936372.1	0.639
8	FGD3	NM_033086.2 NM_033086.1	0.472	83	NEURL1B	NM_00114265.1	0.639
9	HSPBL2	NR_024392.1	0.492	84	HIG2C7orf68	NM_013332.3 NM_013332.1	0.640
10	ANXA9	NM_005368.1	0.494	85	NCOR2	NM_006312.3	0.641
11	AHNAK	NM_001620.1	0.499	86	CXXC5	NM_016463.5	0.642
12	ABCA1	NM_005502.2	0.500	87	STMN3	NM_015894.2	0.645
13	LYPD1	NM_144586.5 NM_144586.3	0.504	88	CGN	NM_020770.1	0.648
14	CAV1	NM_001753.3	0.508	89	LOC647954	XR_018676.1 XM_938141.1	0.649
15	KRT18P13	XM_001726959.1	0.510	90	ID2	NM_002166.4	0.650
16	TACC1	NM_006283.1	0.514	91	MARCKSL1	NM_023009.4	0.651
17	PMP22	NM_153321.1 NM_153322.1	0.522	92	VEGFB	NM_003377.3 XM_938483.1	0.652
18	RAMP3	NM_005856.2	0.524	93	LOC644743	XR_016703.1 XM_932346.1	0.652
19	YPEL3	NM_031477.4 NM_031477.3	0.531	94	HIST3H2A	NM_033445.2	0.652
20	LOC100134073	XM_001718050.1	0.532	95	LOC643873	XR_039149.1	0.655
21	MB	NM_005368.2	0.535	96	LOC728661	XM_001717735.1	0.656
22	LOC399965	XR_036921.1	0.538	97	GADD45B	NM_015675.2 NM_015675.1	0.656
23	NRCAM	NM_005010.3	0.546	98	TEAD2	NM_003598.1	0.657
24	TP53NP1	NM_033285.2	0.550	99	RXRA	NM_002957.3	0.658
25	GPFR/GPR30	NM_001039966.1 NM_001031682.1	0.551	100	YPEL5	NM_016061.1	0.659
26	COL18A1	NM_130445.2 NM_030582.2	0.553	101	LOC200810 ALG1L	NM_001015050.1	0.659
27	SMAD3	NM_005902.3	0.554	102	ZMYND8	NM_183048.1	0.660
28	MAFB	NM_005461.3	0.555	103	AMOT	NM_133265.2	0.660
29	CHES1	NM_005197.2	0.558	104	SMI1	NM_005068.2	0.661
30	KRT8P9	XR_017231.2	0.559	105	MXD4	NM_006454.2	0.662
31	AKR1C3	NM_003739.4	0.561	106	SNHG7	NR_003672.2	0.662
32	MAP3K1	NM_005921.1 XM_042066.10	0.564	107	ZNF342 ZNF296	NM_145288.1	0.664
33	ADCY1	NM_021116.1	0.567	108	SAMD11	NM_152486.2	0.664
34	RAMP3	NM_005856.1 NM_005856.2	0.571	109	P4HA2	NM_001017973.1 NM_001017974.1	1.501
35	ST6GALNAC2	NM_006456.1	0.571	110	MYBPH	NM_004997.2	1.502
36	KRT18P28	XR_017689.1	0.574	111	SLC35B1	NM_005827.1	1.502
37	TRAF3P2	NM_147686.1 NM_147200.1	0.576	112	SPOCK2	NM_014767.1	1.505
38	CXCR7 CMKOR1	NM_020311.2 NM_020311.1	0.578	113	ULBP2	NM_025217.2	1.509
39	GPR30 GPR	NM_001039966.1	0.579	114	PGD	NM_002631.2	1.511
40	RAPGEFL1	NM_016339.2 NM_016339.1	0.579	115	TMED3	NM_007364.2	1.512
41	LFNG	NM_001040167.1 NM_002304.1	0.579	116	LOC728969	XM_001132928.1	1.513
42	HS.278265	XM_168073	0.580	117	RPS6KA2	NM_001006932.1 NM_021135.4	1.516
43	KRT18P17	XR_037953.1	0.582	118	BOP1	NM_015201.3	1.516
44	LOC149501	XR_017100.2	0.583	119	RPS24	NM_001026.3	1.517
45	C6orf141	NM_153344.1	0.584	120	RRS1	NM_015169.3	1.517
46	BNIP1	NM_138278.2 NM_138278.1	0.584	121	ST3GAL1	NM_003033.2 NM_173344.1	1.519
47	FAM63A ANXA9	NM_018379.3 NM_005368.1	0.585	122	TMED10P	NR_002807.1	1.523
48	RARA	NM_000964.2 NM_001024809.2	0.587	123	EVA1 MPZL2	NM_144765.1	1.523
49	CAMK2N1	NM_018584.5 NM_018584.4	0.589	124	SLC39A6	NM_012319.2	1.525
50	KLHL24	NM_017844.3	0.589	125	DST	NM_001723.4 NM_001723.3	1.525
51	NRCAM	NM_005010.3 NM_005010.2	0.592	126	MAGED1	NM_001005332.1 NM_001005333.1	1.526
52	TSPAN9	NM_006875.3	0.594	127	CIRH1A	NM_032830.1	1.526
53	ALDH3B2	NM_001031615.1 NM_000695.3	0.594	128	NHP2	NM_017838.3	1.527
54	PIK3R1	NM_181523.1 NM_181504.2	0.594	129	ELP2 STATIP1	NM_018255.1	1.532
55	FAM46C	NM_017709.3 NM_017709.2	0.594	130	MRPL24	NM_145729.1 NM_024540.2	1.537
56	HMG2	NM_002129.2	0.597	131	C7orf40	NR_003697.1	1.538
57	IGFBP5	NM_000599.2	0.597	132	PPP2R2A	NM_002717.2	1.540
58	EFNA1	NM_004428.2	0.597	133	IGF1R	NM_000875.2	1.544
59	EGR1	NM_001964.2	0.598	134	ALDH6A1	NM_005589.2	1.544
60	BMP5	NM_021073.2	0.599	135	FAM108C1 LOC58489	NM_021214.1 XM_051862.7	1.546
61	PLA2G10	NM_003561.1	0.601	136	HS.543887	CD640673	1.548
62	SDX4	NM_017420.3 NM_017420.2	0.607	137	GAB2	NM_080491.1	1.549
63	YPEL2	NM_001005404.3	0.608	138	PRDX4	NM_006406.1	1.550
64	ATAD4 PRR15L	NM_024320.2	0.609	139	RG522	NM_015668.2	1.551
65	UGDH	NM_003359.2	0.609	140	LOC92755	XR_016140.2	1.553
66	PPP1R3C	NM_005398.4 NM_005398.3	0.613	141	CTSL1	NM_001912.3 NM_145918.2	1.557
67	ABCA2	NM_212533.2 NM_001606.3	0.613	142	NDUFA12L miminit NDUFAF2	NM_174889.3 NM_174889.2	1.558
68	LOC642817	XR_018564.1 XM_926703.1	0.616	143	S100P	NM_005980.2	1.560
69	PRKCD	NM_006254.3 NM_212539.1	0.616	144	SVIL	NM_003174.3 NM_003174.2	1.562
70	MED30 THRAP6	NM_008051.1	0.620	145	FADS1	NM_013402.3	1.562
71	CDC2L6	NM_015076.3	0.621	146	TFPI	NM_006287.4 NM_001032281.1	1.565
72	KRT8	NM_002273.2	0.624	147	RPL29	NM_000992.2	1.565
73	IGFBP5	NM_000599.2	0.625	148	PAPSS2	NM_004670.3	1.567
74	TNK2	NM_005781.4	0.625	149	EVA1 MPZL2	NM_144765.1	1.568
75	SEC14L5	NM_014692.1 XM_940295.1	0.625	150	SEC11C SEC11L3	NM_033280.2 NM_033280.1	1.571

Supplementary Table 1 - continued

Symbol	Accession	Ratio	Symbol	Accession	Ratio
151 NOLA2JHP2	NM_001034833.1NM_017838.3	1,577	206 CGA	NM_000735.2	1,765
152 CARD10	NM_014550.3	1,581	207 PAM	NM_138821.1	1,779
153 PRPS1	NM_002764.2	1,583	208 ALDH3B1	NM_000694.2NM_001030010.1	1,787
154 MAGED1	NM_006986.3	1,585	209 RHPN2	NM_033103.3	1,787
155 CACNB2	NM_201572.1	1,589	210 HS.579631	BU536065	1,787
156 GNL3	NM_206826.1	1,591	211 DCAMK1 DCLK1	NM_004734.2	1,788
157 KLK3	NM_001030050.1	1,595	212 METTL1	NM_005371.5	1,790
158 BCAS1	NM_003657.1	1,597	213 XBP1	NM_001079539.1	1,795
159 PRDX1	NM_181696.1	1,599	214 INHA	NM_002191.2	1,833
160 CRELD2	NM_024324.2	1,602	215 LSM10	NM_032881.1	1,833
161 KIAA1199	NM_018689.1	1,606	216 SEMA3B	NM_001005914.1	1,838
162 YIF1A	NM_020470.1	1,609	217 XBP1	NM_005080.2	1,847
163 DNAJC12	NM_201262.1	1,609	218 KCNK6	NM_004823.1	1,866
164 METTL1	NM_005371.4NM_005371.3	1,615	219 WISP2	NM_033881.2	1,896
165 PAWR	NM_002583.2	1,623	220 KRT81 KRT8B1	NM_002281.2	1,897
166 WIPF1	NM_001077269.1	1,635	221 AGR2	NM_006408.2	1,907
167 TGFB1	NM_000358.1	1,636	222 ASCC1	NM_015947.2	1,935
168 LONRF2	NM_198461.3NM_198461.1	1,637	223 TFF3	NM_003226.2	1,937
169 C11orf75 FN5	NM_020179.1	1,639	224 ITGB5	NM_002213.3NM_944693.1	1,953
170 LOC493869	NM_001008397.1	1,640	225 ID3	NM_002167.2	1,954
171 LRRC50	NM_178452.3	1,641	226 IRS2	NM_003749.2	1,962
172 RPP40	NM_006638.2	1,644	227 EVA1 MPZL2	NM_005797.2	1,972
173 KIAA1324	NM_020775.2	1,652	228 VTCN1	NM_024626.2NM_024626.1	1,979
174 GLRX	NM_002064.1	1,657	229 SLC7A2	NM_001008539.2NM_003046.2	1,984
175 HYAL3	NM_003549.2	1,659	230 LOC100132635	XM_001717880.1	1,989
176 SLC47A1 FLJ10847	NM_018242.2	1,661	231 AVP1	NM_021732.1	1,991
177 SNF1LK SIK1	NM_173354.3NM_173354.2	1,661	232 FKBP11	NM_016594.1	2,002
178 DNAJC12	NM_021800.2	1,663	233 TUBB3	NM_006086.2	2,059
179 CUTL1	NM_181552.1	1,676	234 CHPF	NM_024536.4	2,073
180 GDF15	NM_004864.1	1,679	235 C10TNF6	NM_031910.3	2,278
181 PDGFRL	NM_006207.1	1,680	236 LOC730167	XM_001726158.1	2,317
182 NUCB2	NM_005013.2NM_005013.1	1,681	237 ITGB5	NM_002213.3	2,318
183 SRPRB	NM_021203.2	1,687	238 RFTN1 RAFTLIN	NM_015150.1	2,408
184 GNL3	NM_014366.4	1,691	239 LOC100134134	XM_001720850.1	2,438
185 PPIA4	NM_015053.1	1,692	240 FAM46A	NM_017633.2NM_017633.1	2,440
186 DNAJC12	NM_021800.2	1,694	241 BASP1	NM_006317.3	2,441
187 MGST1	NM_145792.1	1,694	242 LRRC54 TSKU	NM_015516.3	2,682
188 DKFZP564O0823 PA	NM_015393.2	1,694	243 RASD1	NM_016084.3	2,756
189 STC2	NM_003714.2	1,698	244 LXN	NM_020169.2	2,991
190 LOC730274	XM_001125976.1 BM724647	1,698	245 PRSS23	NM_007173.4NM_007173.3	3,071
191 RPS7	NM_001011.3	1,702	246 IER3	NM_003897.3NM_052815.1	3,091
192 RABEPK	NM_005833.2	1,703	247 SEMA3B	NM_001005914.1	3,224
193 SDF2L1	NM_022044.2	1,703	248 KRT13	NM_002274.3NM_002274.2	3,225
194 PISD	NM_014338.3	1,706	249 PCP4	NM_006198.2	3,542
195 TXNDC5	NM_030810.2	1,711	250 CPE	NM_001873.1	3,557
196 TMEM147 NIFE14	NM_032635.2	1,721	251 LOC341230	XR_018617.1	3,563
197 NOL6	NM_139235.3	1,721	252 ISG20	NM_002201.4	3,703
198 NME1	NM_000269.2NM_198175.1	1,735	253 LOC392871	XR_018049.2	3,830
199 TOB1	NM_005749.2	1,740	254 SLC7A5	NM_003486.5	3,886
200 VGF	NM_003378.2	1,745	255 ADORA1	NM_000874.1	4,362
201 SLC39A6	NM_012319.3NM_012319.2	1,746	256 LOC340274	XR_017256.2	4,741
202 SLC26A2	NM_000112.3NM_000112.2	1,758	257 MUC5AC	XM_001134429.1NM_495860.2	5,106
203 MAOA	NM_000240.2	1,761	258 TFF1	NM_003225.2	5,373
204 PHLDA1	NM_007350.3NM_007350.2	1,762	259 ASS1 ASS	NM_054012.2NM_054012.3	7,646
205 RPS7	NM_001011.3	1,762	260 ASS1 ASS	NM_000050.4NM_000050.3	8,758

Supplementary Table 2 - Gene expression profile for PKA-activation in MDA-MB134

Symbol	Accession	Ratio	Symbol	Accession	Ratio
1 PRKAR1A	NM_002734.3	0.108	14 LOC284379	NR_002938.1	0.289
2 PRKAR1A	NM_212472.1NM_002734.3	0.135	15 PGM5	NM_021965.3	0.309
3 UGT1A6	NM_001072.3NM_001072.2	0.179	16 TMEPAI	NM_199169.1	0.317
4 UGT1A6	NM_205862.1	0.189	17 KCNMB4	NM_014505.4	0.344
5 TMEM46	NM_001007538.1	0.196	18 ANXA8	NM_001630.1NM_001630.2	0.365
6 FBN2	NM_001999.3	0.198	19 DLL1	NM_005618.3NM_005618.2	0.369
7 KRT6A	NM_005554.3	0.232	20 ADFP	NM_001122.2	0.370
8 KCNJ8	NM_004982.2	0.243	21 EPHA4	NM_004438.3	0.374
9 TMEPAI	NM_199169.1	0.251	22 C14orf78	XM_001132404.1NM_290629.6	0.375
10 CAV1	NM_001753.3	0.264	23 REG4	NM_032044.2	0.375
11 PGM5	NM_021965.3NM_936702.1	0.270	24 TMSL8	NM_021992.2	0.376
12 ANXA1	NM_000700.1	0.280	25 CLDN9	NM_020982.2	0.377
13 MYO1B	NM_012223.2	0.289	26 PKP1	NM_001005337.1	0.382

Supplementary Table 2 - continued

	Symbol	Accession	Ratio		Symbol	Accession	Ratio
27	KRT80LOC144501	NM_182507.2NM_182507.1	0.384	102	PLSCR3	NM_020360.2	0.524
28	GNMB	NM_001005340.1	0.387	103	TMEM18	NM_152834.2	0.524
29	UPK1A	NM_007000.2	0.387	104	LOC643389	XM_926721.1	0.526
30	NUAK1	NM_014840.2	0.389	105	MEX3BIRKHD3	NM_032246.3	0.526
31	HS.510367	AK124402	0.407	106	MYH9	NM_002473.3	0.528
32	KRT17	NM_000422.1	0.410	107	ADM	NM_001124.1	0.529
33	LOC653506	XM_927769.1	0.414	108	MBNL2	NM_207304.1	0.529
34	MG4677	NM_052871.3	0.414	109	FAM20C	NM_020223.2NM_020223.1	0.530
35	METRNL	XM_941466.2XM_941466.1	0.420	110	LRRN2LRRN5	NM_201630.1NM_006338.2	0.531
36	HS.25318	AF131784	0.420	111	LMTK3	XM_936372.2XM_936372.1	0.531
37	CAV2	NM_001233.3	0.427	112	TGFB1	NM_000358.1	0.533
38	RCN1	NM_002901.1	0.430	113	GSN	NM_198252.2NM_198252.1	0.534
39	SPRY1	NM_005841.1	0.435	114	HS.576106	AK097377	0.534
40	SAMD11	NM_152486.2	0.446	115	DBN1	NM_004395.2NM_080881.1	0.535
41	CXCR7	NM_001047841.1	0.446	116	GABBR2	NM_005458.5	0.536
42	CDH3	NM_001793.3	0.450	117	UBE2L6	NM_004223.3	0.536
43	LOC731895	XM_001133620.1IL10374	0.451	118	COBLL1	NM_014900.3	0.536
44	LOC57228	NM_001031628.1	0.452	119	HIST1H2BH	NM_003524.2	0.536
45	KLHL24	NM_017644.3	0.453	120	C14orf119	NR_002937.1	0.538
46	LOC728643	NR_003277.1	0.453	121	ALDH3B2	NM_001031615.1NM_000695.3	0.538
47	JUN	NM_002228.3	0.454	122	SPANXA1	NM_013453.2	0.539
48	CLIC3	NM_004669.2	0.455	123	TP53NP1	NM_033285.2	0.544
49	COL5A1	NM_000093.3NM_000093.2	0.458	124	RPL22	NM_000963.3	0.545
50	CXCR7CMKOR1	NM_020311.2NM_020311.1	0.459	125	EGLN3	NM_022073.3NM_022073.2	0.545
51	LARP6	NM_197958.1	0.460	126	BEX2	NM_032621.2	0.546
52	PHF3	NM_015153.1	0.460	127	APOBEC3B	NM_004900.3	0.547
53	LOC144501	NM_182507.1	0.462	128	C1orf106	NM_018265.1	0.547
54	HS.569718	BX115170	0.462	129	C4orf19FLJ11017	NM_018302.1	0.548
55	ALDH3A1	NM_000691.3	0.463	130	MYO1B	NM_012223.2	0.549
56	SCUBE2	NM_020974.1	0.465	131	CA12	NM_001218.3	0.549
57	TNS3	NM_022748.10NM_022748.9	0.465	132	PCM1	NM_006197.3NM_006197.2	0.549
58	ZNF91	NM_003430.2NM_003430.1	0.472	133	PP1R15A	NM_014330.2	0.549
59	SH3BGR1	NM_003022.1	0.472	134	KYNU	NM_003937.2	0.551
60	ANKRD1	NM_014391.2	0.472	135	SPOCK1SPOCK	NM_004598.3NM_004598.2	0.552
61	NEDD9	NM_006403.2	0.474	136	NFX	NM_002501.2	0.552
62	SMAD3	NM_005902.3	0.474	137	LOC643985	XM_927235.1	0.552
63	LOC730525SPRY1	XM_001126202.1NM_199327.1	0.475	138	GPR30	NM_001039966.1	0.552
64	P2RX2	NM_170682.2	0.475	139	EFNB3	NM_001406.3	0.552
65	PTGER4	NM_000958.2	0.476	140	SOC2	NM_003877.3	0.553
66	ARL4AARL4	NM_001037164.1NM_005738.2	0.477	141	ACTG1	NM_001614.2	0.553
67	BTG1	NM_001731.1	0.478	142	TPP3CGI-38	NM_015964.2	0.553
68	LOC57228	NM_001033873.1NM_001031628.1	0.480	143	GABARAPL1	NM_031412.2	0.554
69	EYA2	NM_172112.1	0.480	144	HMOX1	NM_002133.1	0.554
70	GRB14	NM_004490.2	0.480	145	RPL14	NM_001034996.1NM_003973.2	0.555
71	CXCL12	NM_199168.2	0.483	146	SLC24A3	NM_020689.3	0.556
72	DKK1	NM_012242.2	0.486	147	PRKCD	NM_006254.3NM_212539.1	0.557
73	FAM43A	NM_153690.4	0.491	148	LOC652683LOC653571	XM_942283.2XM_928153.1	0.557
74	BMP4	NM_130851.1NM_001202.2	0.492	149	TSPAN3	NM_005724.4	0.557
75	LOC653110	XM_926584.1	0.493	150	CDH26	NM_021810.3	0.558
76	TSPAN9	NM_006675.3	0.495	151	COL4A5	NM_000495.3NM_033381.1	0.558
77	TACC1	NM_006283.1	0.496	152	SLC35C1	NM_018389.3	0.560
78	EML1	NM_004434.2	0.497	153	BTG2	NM_006763.2	0.560
79	KLK11	NM_144947.1NM_006853.2	0.497	154	SEMA4B	NM_198925.1NM_020210.2	0.561
80	DKK1	NM_012242.2	0.501	155	LARP6	NM_018357.2	0.561
81	CD44	NM_001001391.1NM_001001390.1	0.501	156	YPEL5	NM_016061.1	0.562
82	PPP2R2B	NM_181676.1	0.502	157	TSC22D3	NM_198057.2NM_004089.3	0.563
83	MFGE8	NM_005928.1	0.503	158	FZD8	NM_031866.1	0.565
84	FAM46B	NM_052943.2	0.504	159	LOC645436	XM_928473.1	0.566
85	PGM5	NM_021965.3	0.507	160	DDIT4	NM_019058.2	0.567
86	RB1CC1	NM_001083617.1NM_014781.3	0.507	161	ADAM2	NM_001464.3	0.567
87	ARL4AARL4	NM_005738.3NM_005738.2	0.508	162	BCAS1	NM_003657.1	0.568
88	ANKRD50	NM_020337.1	0.511	163	MBNL2	NM_144778.2	0.568
89	KIAA0513	NM_014732.2	0.511	164	PCM1	NM_006197.2	0.569
90	SPRY1	NM_199327.1NM_005841.1	0.511	165	SH3BP4	NM_014521.2NM_014521.1	0.569
91	CTSH	NM_004390.2	0.511	166	CRTAP	NM_006371.3	0.569
92	BCAS3	NM_017679.2	0.515	167	SPTAN1	NM_003127.1	0.571
93	TIGA1	NM_053000.1	0.515	168	DDIT3	NM_004083.4	0.573
94	C7orf41E1b1	NM_152793.2NM_152793.1	0.517	169	PLXDC2	NM_032812.7	0.573
95	MAFB	NM_005461.3	0.517	170	ITGB4	NM_001005619.1	0.574
96	FLJ30058	NM_144967.2	0.518	171	ZFP36L1	NM_004926.2	0.574
97	NUAK1	NM_014840.2	0.519	172	LTB4DH	NM_012212.2	0.574
98	AKR1C3	NM_003739.4	0.520	173	SLC31A2	NM_001860.2NM_001860.1	0.575
99	GPX2	NM_002063.2	0.521	174	NCK2	NM_001004720.1NM_003581.2	0.578
100	C6orf48	NM_016947.1	0.523	175	ZNF33BZNF11B	NM_006955.1	0.578
101	SMAD6	NM_005585.3NM_005585.2	0.524	176	HIG2	NM_013332.3NM_013332.1	0.578

Supplemental Table 2 - continued

Symbol	Accession	Ratio	Symbol	Accession	Ratio
177 PMP22	NM_153321.1 NM_153322.1	0.579	252 BMF	NM_001003943.1	0.613
178 ERMAP	NM_001017922.1	0.579	253 GFRA3	NM_001496.3	0.613
179 TLE4	NM_007005.3	0.580	254 ANXA2P1	NR_001562.1	0.614
180 ANK3	NM_001149.2	0.581	255 PPP1R13B	NM_015316.2 NM_015316.1	0.614
181 PALM	NM_002579.2 NM_002579.1	0.581	256 MTMR11	NM_181873.2 NM_181873.1	0.614
182 CA5B	NM_007220.3 NM_007220.2	0.581	257 RPL32	NM_001007074.1 NM_000994.3	0.614
183 LOC648210 LOC647727	XR_018923.1 XM_936790.1	0.581	258 DYRK1B	NM_006484.1	0.615
184 C9orf61	NM_004816.2	0.582	259 LOC137107	XM_940214.1 XM_070233.3	0.615
185 TGFBI3	NM_003239.1	0.582	260 VPS37D	NM_001077621.1 XM_376631.3	0.616
186 LOC653344	XM_933085.1	0.582	261 TCEA2	NM_198723.1 NM_003195.4	0.616
187 PLA2G10	NM_003561.1	0.583	262 GIMAP2	NM_015660.2	0.617
188 CAV2	NM_001233.3	0.585	263 RRAS	NM_006270.3 NM_006270.2	0.618
189 KTN1	NM_004986.2	0.586	264 SP110	NM_004510.2	0.618
190 PRICKLE2	NM_198859.2 NM_198859.1	0.587	265 ANKRD35	NM_144698.2	0.619
191 DKFZP564J102	NM_015398.2 NM_015398.1	0.587	266 TMEM106B	NM_018374.2	0.619
192 LAMB3	NM_000228.2	0.587	267 PIM1	NM_002648.2	0.619
193 CDKN2B	NM_078487.2	0.588	268 CHES1	NM_005197.2	0.619
194 CHMP4B	NM_176812.3	0.588	269 FAM83B C6orf143	NM_001010872.1	0.620
195 BMF	NM_003503.3 NM_001003942.1	0.588	270 HS.531457	AK094914	0.621
196 SIPA1L2	NM_020808.3 NM_020808.1	0.588	271 IGFBP3	NM_000598.4 NM_001013398.1	0.621
197 SLC41A3	NM_001008485.1	0.589	272 FLJ13236	NM_024902.2	0.621
198 ARL16	NM_001040025.1 XM_290777.4	0.589	273 CDK7	NM_001799.2	0.622
199 TMEM18	NM_152834.2	0.590	274 ZNF721	NM_133474.2 NM_133474.1	0.622
200 RWDD2A RWDD2	NM_033411.2	0.591	275 RASAL1	NM_004658.1	0.622
201 SPATA7	NM_001040428.2 NM_018418.1	0.591	276 NRCAM	NM_005010.3	0.622
202 PCDH10	NM_032961.1	0.591	277 AMY1A	NM_004038.3	0.622
203 PALLD	NM_016081.3 NM_016081.2	0.593	278 GALT	NM_000153.2 NM_000153.1	0.622
204 EFNA1	NM_004428.2	0.593	279 RARRES2	NM_002889.2	0.623
205 EML1	NM_004434.2	0.593	280 KLHL5	NM_001007075.1	0.623
206 ID2	NM_002166.4	0.593	281 AKR1C4 LOC648517	NM_001818.2 XM_940680.1	0.623
207 PPAP2B	NM_003713.3	0.594	282 TAF1C	NM_139353.1	0.624
208 RAMP3	NM_005856.1 NM_005856.2	0.594	283 PTPRF	NM_130440.1	0.624
209 SPANXA1	NM_013453.2	0.594	284 C1orf51	NM_144697.2	0.624
210 C9orf3	NM_032823.3	0.594	285 ABCC3	NM_003786.2 NM_020037.1	0.624
211 KRT6C KRT6E	NM_173086.3 NM_173086.2	0.594	286 MALL	NM_005434.3	0.624
212 ID1	NM_181353.1	0.595	287 CLPTM1	NM_001294.1	0.626
213 SOX3	NM_005634.2	0.595	288 LOC645385	XM_928427.1	0.627
214 SH3GL3	NM_003027.2	0.595	289 CACNB3	NM_000725.2	0.627
215 FGFR3	NM_022965.1 NM_00142.2	0.595	290 FAM50B	NM_012135.1	0.627
216 FLJ25476	NM_152493.2	0.595	291 STAT4	NM_003151.2	0.629
217 ALCAM	NM_001627.2	0.597	292 ITGB4	NM_001005731.1	0.629
218 JOSD3	NM_024116.2	0.597	293 METTL7A	NM_014033.3 NM_014033.2	0.629
219 C21orf34	NM_001005734.1 NM_001005732.1	0.598	294 BIK	NM_001197.3	0.630
220 C3orf10	NM_018462.4	0.598	295 PLXNB2	NM_012401.2 XM_371474.3	0.630
221 MGC39900	NM_194324.1	0.599	296 CCNB1 P1	NM_182849.1 NM_021178.2	0.630
222 KRT23	NM_015515.3	0.599	297 SCIN	NM_033128.1	0.630
223 CTHRC1	NM_138455.2	0.599	298 DYNC1H1	NM_001376.2	0.630
224 ID4	NM_001546.2	0.600	299 ZNF581	NM_016535.3	0.631
225 EFNA1	NM_004428.2	0.601	300 CDC23	NM_199342.1	0.631
226 LETMD1	NM_001024668.1	0.602	301 PTPRF	NM_002840.3	0.631
227 EVL	NM_016337.2	0.602	302 RDH16	NM_003708.3 NM_003708.2	0.631
228 TNF2	NM_012461.1	0.603	303 LOC642817	XR_018564.1 XM_926703.1	0.631
229 PPL	NM_002705.4 NM_002705.3	0.603	304 CDH26	NM_177980.1 NM_021810.3	0.631
230 TTC3	NM_003316.3	0.604	305 LOC388275	XM_928429.1	0.632
231 CXCL12	NM_000609.4	0.604	306 LOC643008	XM_942845.2 NM_928053.1	0.632
232 FLJ21839	NM_021831.5	0.604	307 C6orf141	NM_153344.1	0.632
233 HTRA1	NM_002775.3	0.604	308 FLJ20152	NM_001034850.1	0.632
234 HS.576735	B258188	0.605	309 YPEL2	NM_001005404.3	0.633
235 RHOC	NM_001042678.1 NM_175744.3	0.606	310 FAM110B C8orf72	NM_147189.2 NM_147189.1	0.633
236 C9orf19	NM_022343.2	0.606	311 PIK3IP1 GMC17330	NM_052880.3	0.633
237 ID2	NM_002166.4	0.606	312 TNFRSF21	NM_014452.3	0.633
238 CXCL16	NM_022059.1	0.606	313 PHLD1	NM_015157.1	0.633
239 MGC39900	XM_936687.1	0.606	314 CYP11B1	NM_000104.2	0.633
240 PCDH10	NM_020815.1	0.606	315 HBP1	NM_012257.3	0.633
241 SUSL4	NM_001037175.1	0.607	316 RAMP3	NM_005856.2	0.634
242 C4orf14	NM_032313.2	0.607	317 ANXA8	NM_001630.2	0.634
243 BRD8	NM_183359.1	0.609	318 KYNL	NM_001032998.1 NM_003937.2	0.634
244 NR2F2	NM_021005.2	0.610	319 API2	NM_003916.3	0.634
245 NUDT11	NM_018159.2	0.610	320 ST3GAL1	NM_003033.2 NM_173344.1	0.634
246 GRM4	NM_000841.1	0.610	321 RXRA	NM_002957.3	0.635
247 NOTCH3	NM_000435.1	0.610	322 CAPN9	NM_016452.1	0.636
248 HCFC1R1	NM_001002018.1 NM_001002017.1	0.611	323 MRPL10	NM_145255.2	0.636
249 PALM	NM_002579.2	0.611	324 EDN2	NM_001956.2	0.637
250 GPR68	NM_003485.3	0.612	325 GPER GPR30	NM_001039966.1 NM_001031682.1	0.637
251 BNIP1	NM_138278.2 NM_138278.1	0.613	326 LOC143543	NR_002197.1	0.637

Supplementary Table 2 - continued

	Symbol	Accession	Ratio		Symbol	Accession	Ratio
327	HSPA12A	NM_025015.2	0.637	402	LOC348262	NM_207368.3 NM_207368.1	0.657
328	RAB40B	NM_006822.1	0.637	403	LSM14A	NM_015578.1	0.657
329	DNAL4	NM_005740.2	0.638	404	C6orf166	NM_018064.2	0.658
330	SH3PX3	NM_153271.1	0.638	405	C1orf115	NM_024709.4 NM_024709.2	0.658
331	PRICKLE1	NM_153026.1	0.639	406	LFNG	NM_001040167.1 NM_002304.1	0.658
332	KIAA1600	NM_020940.2	0.640	407	SYCP2	NM_014258.2	0.659
333	METRNL	XM_941466.2 NM_001004431.1	0.640	408	SRI	NM_003130.2	0.659
334	C6orf48	NM_001040437.1	0.640	409	TSC22D3	NM_004089.3	0.659
335	FLJ10986	NM_018291.2	0.641	410	SCARA3	NM_016240.2	0.660
336	FLJ41603	NM_001001669.2 NM_001001669.1	0.641	411	CAV1	NM_001753.3	0.660
337	LOC730256 LOC440667	XM_001134398.1 XM_498810.2	0.641	412	CAMSAP1L1	NM_203459.1	0.660
338	SCNN1A	NM_001038.4	0.641	413	CCNG2	NM_004354.1	0.661
339	TROVE2	NM_001042369.1	0.641	414	FAM10A4	NR_002183.1	0.661
340	CGN	NM_020770.1	0.641	415	C20orf108	NM_080821.2 NM_080821.1	0.662
341	USP41	XM_036729.5 XM_036729.4	0.641	416	CIRBP	NM_001280.1	0.662
342	MGC42367	NM_207362.1	0.642	417	MOV10	NM_020963.2 NM_020963.1	0.662
343	SMC3 CSP66	NM_005445.3	0.642	418	FLJ22795	NM_025084.1	0.662
344	C11orf74 LOC119710	NM_138787.2	0.642	419	ZNF358	NM_018083.4 NM_018083.3	0.663
345	IFITM3	NM_021034.2 NM_021034.1	0.642	420	CKAP2	NM_018204.2	0.663
346	AHNAK	NM_001620.1	0.643	421	NAP1L1	NM_139207.1	0.663
347	ARL2BP	NM_012106.3	0.643	422	DP58	NM_001004441.1	0.664
348	BNIP3L	NM_004331.2	0.644	423	AXUD1	NM_033027.2	0.664
349	LOC642934	XM_942991.2 XM_931177.1	0.644	424	ZBTB46 BTBD4	NM_025224.2	0.664
350	NPNT	NM_001033047.1	0.644	425	YPEL3	NM_031477.4 NM_031477.3	0.664
351	SORBS3	NM_005775.3	0.644	426	DYRK1B	NM_004714.1	0.664
352	TMEM101	NM_032376.2	0.644	427	CGO18	NM_001079691.1	0.664
353	SLC44A2	NM_020428.2	0.644	428	EXOC3 SEC6L1	NM_007277.4 NM_007277.3	0.664
354	MYH9	NM_002473.3	0.644	429	ZYX	NM_003461.4	0.665
355	SHRM	NM_020859.1	0.644	430	IDH2	NM_002168.2	0.665
356	SEPT5 GP1BB	NM_002688.4 NM_000407.3	0.645	431	BAPX1	NM_001189.2	0.665
357	KCNF1	NM_002236.4	0.645	432	LOC390466	XM_372521.1	0.666
358	PLCD3	NM_133373.3 NM_133373.2	0.646	433	MGC39900	NM_194324.1 XM_936687.1	0.666
359	SCD5	NM_001037582.1	0.646	434	LPHN1	NM_014921.3	0.666
360	PKR3R1	NM_181523.1 NM_181504.2	0.646	435	KIAA0430 LKAP	NM_014647.2 NM_014647.1	0.666
361	EEF1D	NM_002378.2	0.646	436	SH3GLB2	NM_020145.2	0.667
362	BCAS1	NM_003657.1	0.646	437	FAM123A	NM_199138.1	0.667
363	HCFC1R1	NM_001002018.1	0.646	438	CPT2	NM_000098.1	1.502
364	RPL32	NM_001007073.1	0.647	439	YRDC	NM_024640.3	1.502
365	RAB6IP1	NM_015213.2	0.647	440	CXCR4	NM_001008540.1	1.503
366	IGBP1	NM_001551.2	0.647	441	NOP5 NOP58	NM_015934.3	1.504
367	FZD1	NM_003505.1	0.648	442	ICAM3	NM_002162.2	1.505
368	CALCOCO1	NM_020898.1	0.648	443	APBP1	NM_001018160.1 NM_003905.3	1.506
369	TMEM37	NM_183240.2 NM_183240.1	0.648	444	SNRPA1	NM_003090.2	1.506
370	METRNL	XM_941466.2 XM_941466.1	0.648	445	RFESD	NM_173362.2	1.506
371	FSCN1	NM_003088.2	0.648	446	BXDC1	NM_032194.1	1.507
372	JARID1B	NM_006618.3	0.649	447	GOLSYN FLJ20366	NM_001099743.1 NM_017786.2	1.508
373	LETMD1	NM_015416.3 NM_001024668.1	0.649	448	HSCB	NM_172002.3	1.508
374	AKR1C2	NM_001354.4	0.649	449	CHUK	NM_001278.3	1.508
375	TRIM33	NM_015906.3 NM_033020.2	0.649	450	ETFB	NM_001985.2	1.509
376	C14orf45	NM_025057.1	0.649	451	RAB10	NM_016131.3 NM_016131.2	1.510
377	CCDC83 MGC34732	NM_173556.3 NM_173556.2	0.650	452	RAPH1	NM_013589.1	1.510
378	POLM	NM_013284.1	0.650	453	RBM39	NM_184234.1	1.512
379	BNIP3L	NM_004331.2	0.650	454	HYAL3	NM_003549.2	1.512
380	PGRMC2	NM_006320.1	0.651	455	HRSP12	NM_005836.2	1.513
381	ADCY1	NM_021116.1	0.651	456	HSPCAL3	XM_934529.1	1.513
382	PLLPTM4SF11	NM_015993.1	0.651	457	CACYBP	NM_014412.2	1.514
383	COPS2	NM_004236.2 NM_004236.1	0.652	458	PPFIA4	NM_015053.1	1.514
384	LOC730746 LOC645001	XR_015514.1 XM_928068.1	0.652	459	LOC26010 DNAPT6	NM_001100422.1 NM_015535.1	1.514
385	NP1P	NM_006985.1	0.652	460	C20orf24	NM_018840.2	1.515
386	HNRPUL1	NM_144732.1	0.653	461	POLE2	NM_002692.2	1.516
387	RBIMX	NM_002139.2	0.653	462	PGAM1	NM_002629.2	1.517
388	TMEM43	NM_024334.1	0.653	463	HLA-DRB6	NR_001298.1	1.518
389	MFAF2	NM_017459.1	0.654	464	PDXP	NM_020315.4 NM_020315.3	1.518
390	C15orf38	NM_182616.1	0.654	465	PRODH	NM_016335.2	1.519
391	GBP2	NM_004120.3	0.654	466	TMEM38B	NM_018112.1	1.519
392	EIF4B	NM_001417.4 NM_001417.2	0.654	467	CYP51A1	NM_000786.2	1.520
393	RAPGEFL1	NM_016339.2 NM_016339.1	0.654	468	SGK3	NM_001033578.1 NM_170709.1	1.520
394	LOC440341	XM_932044.1	0.654	469	COMTD1	NM_144589.2	1.521
395	CCNB1P1	NM_182851.1	0.655	470	LOC389541	NM_001008395.2 NM_001008395.1	1.522
396	LYPD3	NM_014400.2 NM_014400.1	0.655	471	DCUN1D5	NM_032299.2	1.522
397	RPS23	NM_001025.4	0.655	472	RPP25	NM_017793.2 NM_017793.1	1.524
398	SPSB3	NM_080861.3	0.656	473	GREB1	NM_014668.3 NM_014668.2	1.524
399	ANXA2	NM_001002857.1	0.656	474	SLC47A1 FLJ10847	NM_018242.2	1.525
400	CLIP2	NM_032421.2	0.657	475	TTC13	NM_024525.2 NM_939087.1	1.525
401	NAP1L1	NM_139207.1	0.657	476	PGK1	NM_000291.2	1.527

Supplementary Table 2 - continued

	Symbol	Accession	Ratio		Symbol	Accession	Ratio
477	NUCB2	NM_005013.2 NM_005013.1	1,528	552	ACLY	NM_198830.1	1,584
478	IRS2	NM_003749.2	1,528	553	CORO2A	NM_003389.2 NM_052820.2	1,585
479	HPSE	NM_006665.3	1,528	554	ELOVL6	NM_024090.1	1,585
480	RTN4	NM_007008.2	1,528	555	REEP1	NM_022912.1	1,586
481	LARP2	NM_032239.2	1,530	556	LOC644869	XM_932470.1	1,587
482	KCTD3	NM_016121.3	1,531	557	ADORA1	NM_000674.2	1,589
483	ABAT	NM_000663.3 NM_020686.3	1,531	558	BR3BP	NM_000626.5 XM_941876.1	1,592
484	LOC653354	XM_927053.2 XM_927053.1	1,532	559	SEC24D	NM_014822.1	1,593
485	NFIA	NM_005595.1	1,532	560	KLHL2	NM_007246.2	1,593
486	PGAM5 MGC5352	XM_001126125.1 XM_945649.1	1,532	561	FABP5	NM_001444.1	1,594
487	WIPF1	NM_001077269.1	1,533	562	SLC39A8	NM_022154.5	1,597
488	NOLC1	NM_004741.1	1,533	563	MFS3D	NM_138431.1	1,598
489	ABAT	NM_000663.3	1,534	564	FABP5	NM_001444.1	1,598
490	NAV2	NM_145117.3	1,534	565	ANKRD15	NM_015158.2 NM_153186.3	1,599
491	PDE8A	NM_173454.1 NM_173455.1	1,534	566	PGM1	NM_002633.2	1,601
492	PKM2	NM_182471.1	1,534	567	HNMT	NM_001024074.1	1,602
493	FGFR1	NM_021923.3 NM_001004358.1	1,535	568	GCLM	NM_002061.2	1,602
494	RPS7	NM_001011.3	1,535	569	SLC20A1	NM_005415.3	1,604
495	HLA-DRB3	NM_022555.3	1,535	570	PPFIB1	NM_003622.2 NM_177444.1	1,604
496	TLC1	NM_138463.2	1,535	571	CD320	NM_016579.2	1,605
497	FLJ38482	NM_001100389.1 NM_152681.1	1,536	572	STAMBPL1	NM_020799.2	1,606
498	C12orf60	NM_175874.2	1,536	573	C16orf74 MGC17624	NM_206967.1	1,607
499	ISG20L1	NM_022767.2	1,536	574	CROT	NM_021151.2	1,611
500	PNKD	NM_022572.2	1,537	575	G6PD	NM_000402.3	1,611
501	DNAJC10	NM_018981.1	1,537	576	TUBG1	NM_001070.3	1,612
502	ATP2A3	NM_174955.1	1,538	577	ST6GALNAC4	NM_175039.3	1,612
503	CTSC	NM_001814.2	1,539	578	SELM	NM_000430.2	1,614
504	DHCR24	NM_014762.3 NM_014762.2	1,539	579	STARD10	NM_006645.2 NM_006645.1	1,616
505	ODC1	NM_002539.1	1,539	580	ZNF703	XM_001129663.1 NM_025069.1	1,616
506	RORC	NM_00101523.1	1,539	581	SLC39A4	NM_017767.1	1,616
507	PAPD5	NM_001040285.1 NM_022447.1	1,540	582	TDG	NM_003211.3	1,617
508	PGAM4 LOC643025	NM_001029891.2 XM_926168.1	1,540	583	SYK	NM_003177.3	1,620
509	C8orf53	NM_032334.1	1,541	584	LOC648852	XM_940430.1	1,621
510	DUSP5	NM_004419.3	1,542	585	CATSPER2	NM_172097.1	1,621
511	ICT1	NM_001545.1	1,543	586	NOL5A	NM_006392.2	1,625
512	TMEM26	NM_178505.5 NM_178505.4	1,544	587	MST4 MASK	NM_016542.3 NM_016542.2	1,626
513	ITGB5	NM_002213.3 XM_944693.1	1,544	588	RPL8	NM_033301.1	1,628
514	HS.132305	AK125960	1,546	589	DHCR7	NM_001360.2	1,628
515	NME1	NM_000269.2	1,547	590	ITGAE	NM_002208.4 NM_002208.3	1,628
516	MLSTD1	NM_018099.3	1,547	591	BATF	NM_006399.2	1,630
517	PTPLAD1	NM_016395.2 NM_016395.1	1,547	592	C6orf66	NM_014165.1	1,632
518	UGT2B10	NM_001075.3 NM_001075.2	1,547	593	AMMECR1	NM_015365.2 NM_001025580.1	1,634
519	SOLE	NM_003129.3	1,549	594	PKM2	NM_182470.1	1,640
520	PYCR2	NM_013328.2	1,550	595	RORC	NM_001001523.1	1,640
521	NOL1	NM_001033714.1 NM_006170.2	1,551	596	LDLRAD3	NM_174902.2	1,642
522	PDHA1	NM_000284.1	1,551	597	HS6ST2	NM_001077188.1	1,642
523	RPS7	NM_001011.3	1,551	598	LYAR	NM_017816.1	1,643
524	NBPF4	NM_152488.1	1,551	599	MAD1L1	NM_001013836.1	1,645
525	UGCG1	NM_020120.2	1,553	600	SLC27A2	NM_003645.2	1,646
526	DDX21	NM_004728.2	1,553	601	GTPBP3	NM_032620.1	1,646
527	CXorf38	NM_144970.1	1,554	602	HS.580797	BF996074	1,646
528	PDE8A	NM_002605.2	1,554	603	PLA2G3	NM_015715.3 NM_015715.2	1,647
529	ENPP1	NM_006208.1	1,555	604	TARBP1	NM_005646.3 NM_005646.2	1,648
530	JPH1	NM_020647.2	1,556	605	LOC653381 SORD	XR_017364.1 XM_939180.1	1,648
531	CCDC25	NM_018246.2 NM_001031708.1	1,559	606	FAM119A	NM_145280.3	1,649
532	POLR3K	NM_016310.2	1,559	607	FAM108B1 C9orf77	NM_001025780.1	1,650
533	C6orf192	NM_052831.2	1,560	608	CHPF	NM_024536.4	1,650
534	RPS15	NM_001018.3	1,560	609	FAM63A ANXA9	NM_018379.3 NM_003568.1	1,653
535	DPP9	NM_139159.3	1,562	610	PCYOX1	NM_016297.2	1,654
536	MLKL	NM_152649.1	1,565	611	DUSP16	NM_030640.1	1,656
537	LOC652595	XM_942117.1	1,566	612	HS6ST2	NM_147175.3 NM_147175.2	1,659
538	CREB1	NM_004379.2	1,567	613	ITGB5	NM_002213.3	1,659
539	GABPB2	NM_016655.3 NM_181427.2	1,568	614	PSMA3	NM_152132.1	1,660
540	DNAJC12	NM_021800.2	1,574	615	ZNF462	NM_021224.4 NM_021224.3	1,660
541	VGF	NM_003378.2	1,574	616	PAK1P1	NM_017906.2	1,661
542	ERIC1	NM_001031711.1	1,574	617	BR3BP	NM_000626.5	1,664
543	PCYOX1	NM_016297.2	1,575	618	PON2	NM_000305.2	1,665
544	LOC729148 LOC389850	XM_001129792.1 XM_372205.4	1,575	619	LOC654174	XM_940438.2 XM_940438.1	1,665
545	C16orf14 MGC15416	NM_138418.2	1,576	620	MCM4	NM_005914.2	1,667
546	PSMD12	XM_001134072.1 XM_946047.1	1,577	621	LOC653377	XM_929420.1	1,667
547	MSJ2	NM_138962.2	1,582	622	ST3GAL4	NM_006278.1	1,669
548	AMD1	NM_001634.4	1,582	623	ID2B	NM_152361.1	1,669
549	TAX1BP1	NM_001079864.1	1,582	624	SNX28	NM_014035.1	1,672
550	HSD17B12	NM_016142.2 NM_016142.1	1,582	625	C12orf24	NM_013300.1	1,672
551	PGM3	NM_015599.1	1,582	626	XBP1	NM_001079539.1	1,674

Supplementary Table 2 - continued

	Symbol	Accession	Ratio		Symbol	Accession	Ratio
627	LOC100008589(CDC14B)	NR_003287.1 NM_003671.2	1,674	702	SOX21	NM_007084.2	1,863
628	UNG	NM_003362.2 NM_080911.1	1,676	703	ZMAT3	NM_152240.1	1,863
629	CRYZ	NM_001889.2	1,679	704	PCP4	NM_006198.2	1,863
630	TPD52L1	NM_001003396.1	1,679	705	PON2	NM_000305.2	1,867
631	ZNF14	NM_021030.2	1,682	706	PDGFR	NM_006207.1	1,873
632	CYCSL1	NR_001561.1	1,684	707	MAP3K5	NM_005923.3	1,873
633	ANXA9	NM_003568.1	1,687	708	NME1	NM_198175.1	1,879
634	HMGCS1	NM_002130.6 NM_002130.4	1,688	709	HLA-DRB1	NM_002124.1	1,883
635	KCTD3	NM_016121.3	1,689	710	C3orf34	NM_032898.2	1,883
636	DNAJA1	NM_001539.2	1,689	711	TMEM167 MG23909	NM_174909.3 NM_174909.1	1,886
637	RPL36A	NM_021029.4	1,690	712	MCM10	NM_018518.3	1,893
638	RFTN1 RAFTLIN	NM_015150.1	1,690	713	HIST1H4H	NM_003543.3	1,893
639	GGH	NM_003878.1	1,690	714	IGSF1	NM_205833.1	1,894
640	MRPL42	NM_172177.1	1,697	715	HPN	NM_002151.1	1,899
641	P2RX4	NM_002560.2 NM_175568.1	1,700	716	STRAP	NM_007178.3 NM_007178.2	1,902
642	CNTD2 FLJ13265	NM_024877.1	1,700	717	PPARG	NM_015869.4 NM_138712.3	1,903
643	GRIPAP1	NM_207672.1	1,701	718	FAM46A	NM_017633.2 NM_017633.1	1,907
644	EIF5	NM_001969.3	1,702	719	ST6GALNAC4	NM_175039.3 NM_175040.1	1,907
645	DNAJC12	NM_021800.2	1,703	720	IGFBP4	NM_001552.2	1,908
646	IDH1	NM_004508.2	1,703	721	HIGD1A	NM_001099668.1 NM_014056.1	1,920
647	FUT8	NM_178156.1 NM_178154.1	1,707	722	ISG20	NM_002201.4	1,921
648	LOC400768	XM_378883.2	1,709	723	CTSD	NM_001909.3	1,931
649	ZNF652	NM_014897.1	1,709	724	HIGD1A	NM_014056.1	1,934
650	LXN	NM_020169.2	1,710	725	FAM108C1 LOC58489	NM_021214.1 XM_051862.7	1,936
651	G6PD	NM_000402.3 NM_000402.2	1,713	726	SPINK4	NM_014471.1	1,936
652	LOC151579	XM_045290.6 XM_045290.5	1,725	727	PRDX4	NM_006406.1	1,952
653	MAOA	NM_000240.2	1,730	728	ALPP	NM_001632.3 NM_001632.2	1,953
654	SMPDL3A	NM_006714.2	1,731	729	HSD17B12	NM_016142.1	1,955
655	SRP19	NM_003135.1	1,736	730	LOC387934	XM_937508.2 XM_937508.1	1,958
656	VRK2	NM_006296.3	1,736	731	DNAJC12	NM_201262.1	1,972
657	SYT13	NM_020826.1	1,740	732	SC4MOL	NM_006745.3	1,993
658	RPL29	NM_000992.2	1,748	733	RAXL1	NM_032753.2	2,012
659	FGF18	NM_003862.1 NM_033649.1	1,751	734	AGR2	NM_006408.2	2,017
660	MRPL17	NM_022061.2	1,759	735	NME1	NM_000269.2 NM_198175.1	2,040
661	NETO2	NM_018092.3	1,761	736	FHL2	NM_201557.2 NM_001450.3	2,040
662	LRAP	NM_022350.1	1,763	737	HS.163752	AA204695	2,040
663	XBP1	NM_005080.2	1,763	738	NDUFA12L mitin	NM_174889.3 NM_174889.2	2,043
664	RDH11	NM_016026.2	1,763	739	AKAP1	NM_003488.3 NM_003488.2	2,048
665	S100Z	NM_130772.1	1,766	740	GLRX	NM_002064.1	2,063
666	GARS	NM_002047.2 NM_002047.1	1,770	741	B4GALT1	NM_001497.2	2,074
667	MGAT4A	NM_012214.2 NM_012214.1	1,773	742	ITIH2	NM_002216.2	2,077
668	IFI6 G1P3	NM_022873.2 NM_002038.2	1,775	743	HS.543887	CD640673	2,079
669	AMD1	NM_001303059.1	1,776	744	LY6E	NM_002346.1	2,089
670	F12	NM_000505.3 NM_000505.2	1,781	745	UGT2B17	NM_001077.2	2,090
671	C14orf58	NM_017791.1	1,782	746	RBM24	NM_153020.1	2,091
672	SEMA3E	NM_012431.1	1,783	747	ELOVL6	NM_024090.1	2,097
673	UGT2B17	NM_001077.2	1,785	748	PTGES	NM_004878.3	2,097
674	RPL29	NM_000992.2	1,785	749	HS.168950	BC036496	2,109
675	MAPK13	NM_002754.3	1,794	750	STC1	NM_003155.2	2,111
676	PKM2	NM_002654.3	1,795	751	RDH11	NM_016026.2	2,116
677	C9orf32	NM_014064.2	1,796	752	LRP8	NM_017522.3 NM_033300.2	2,133
678	SLC9A3R1	NM_004252.2 NM_004252.1	1,797	753	MSI2	NM_170721.1	2,155
679	FRK	NM_002031.2	1,798	754	CHPT1	NM_020244.2	2,161
680	RET	NM_020630.4 NM_020630.3	1,802	755	FLJ20273	NM_019027.1	2,174
681	SDF2L1	NM_022044.2	1,803	756	PHLDA1	NM_007350.3 NM_007350.2	2,174
682	SEMA3B	NM_001005914.1	1,806	757	FHL2	NM_201555.1	2,185
683	BAMBI	NM_012342.2	1,807	758	C4orf18	NM_016613.4	2,201
684	MRPL13	NM_014078.4	1,808	759	RBM24	NM_153020.1	2,214
685	GPR172A	NM_024531.3	1,809	760	TDP1	NM_018319.3	2,218
686	ANKRD33	NM_182608.2	1,812	761	ARMCX6	NM_019007.3	2,236
687	BOP1	NM_015201.3	1,816	762	PTHLH	NM_198965.1	2,257
688	RASD1	NM_016084.3	1,820	763	SC4MOL	NM_001017369.1 NM_006745.3	2,276
689	RHOA	NM_019034.2	1,823	764	GOLPH2	NM_177937.1 NM_016548.2	2,292
690	GPR37	NM_005302.2	1,825	765	SCD	NM_005063.4	2,314
691	UGT2B15	NM_001076.2	1,826	766	HS3ST3A1	NM_006042.1	2,317
692	MOSC1	NM_022746.2	1,828	767	CHPT1	NM_020244.2	2,352
693	CCBE1	NM_133459.1	1,828	768	KRT81 KRT8B1	NM_002281.2	2,354
694	CLDN7	NM_001307.3	1,833	769	BCYRN1	NR_001568.1	2,355
695	ARMCX6	NM_019007.3	1,838	770	CCRN4L	NM_012118.2	2,358
696	S100P	NM_005980.2	1,839	771	RET	NM_020975.4 NM_020630.3	2,379
697	CTPS	NM_001905.1	1,841	772	TFF2	NM_005423.3	2,416
698	C8orf51	NM_138408.2	1,845	773	SHS2D	NM_032855.2	2,419
699	PDE8A	NM_002605.2	1,850	774	KLK3	NM_001030050.1	2,449
700	ZNF682	NM_033196.1	1,850	775	ANKRD40	NM_052855.2	2,471
701	HIGD1A LOC643940	NM_001099668.1 XM_927199.1	1,863	776	KIAA1199	NM_018689.1	2,496

Supplementary Table 2 - continued

	Symbol	Accession	Ratio		Symbol	Accession	Ratio
777	MGST2	NM_002413.3	2,539	800	MGP	NM_000900.2	3,233
778	RGS22	NM_015668.2	2,565	801	SERPINA1	NM_001002235.1	3,267
779	FAM102B	NM_001010883.1	2,569	802	M160	NM_174941.3	3,319
780	RDX	NM_002906.3	2,588	803	ASS1/ASS	NM_000050.4/NM_000050.3	3,471
781	MSI2	NM_138962.2	2,590	804	SERPINA5	NM_000624.3	3,481
782	TRQ1	NR_000037.1	2,632	805	IER3	NM_003897.3/NM_052815.1	3,769
783	LOC387882	NM_207376.1	2,656	806	SLC7A2	NM_001008539.2/NM_003046.2	3,880
784	DKFZP564O0823	NM_015393.2	2,685	807	RHOB/TB3	NM_014899.3/NM_014899.2	3,927
785	MUC5AC	XM_001134429.1/XM_495860.2	2,694	808	PRSS23	NM_007173.4/NM_007173.3	4,128
786	BASP1	NM_006317.3	2,726	809	SLC7A5	NM_003486.5	4,248
787	C4orf18	NM_016613.5/NM_016613.4	2,741	810	SERPINA3	NM_001085.4	4,399
788	PISD	NM_014338.3	2,757	811	CEACAM6	NM_002483.3	4,481
789	S100A16	NM_080388.1	2,779	812	PKIB	NM_0032471.4	4,727
790	KIAA1324	NM_020775.2	2,827	813	MGP	NM_000900.2	4,744
791	GLA	NM_000169.2/NM_000169.1	2,889	814	ADORA1	NM_000674.1	5,006
792	TOB1	NM_005749.2	2,895	815	NAV2	NM_145117.3	5,140
793	HS.579631	BU536065	2,898	816	AOX1	NM_001159.3	6,385
794	IGSF1	NM_001555.2	2,909	817	MSMB	NM_138634.1	6,434
795	RERG	NM_032918.1	3,018	818	SGCG	NM_000231.1	7,013
796	SERPINA1	NM_001002236.1	3,033	819	MSMB	NM_002443.2	7,017
797	FJX1	NM_014344.2	3,114	820	MSMB	NM_002443.2	7,103
798	SEMA3B	NM_001005914.1	3,122	821	TFF1	NM_003225.2	12,485
799	ASS	NM_054012.2	3,163	822	CPE	NM_001873.1	16,949

Supplementary Table 3 - Upregulated genes shared between MCF7 and MDA-MB134

Symbol	Accession	Gene expression (ratio)		ChIPseq (absolute intensity)	
		MCF7	MDA-MB134	S305-P	E2/ERa
1 ASS1/ASS	NM_000050.4/NM_000050.3	8,758	3,471	10	10
2 ASS	NM_054012.2	7,646	3,163	10	10
3 TFF1	NM_003225.2	5,373	12,485	14	146
4 MUC5AC	XM_001134429.1/XM_495860.2	5,106	2,694	0	9
5 ADORA1	NM_000674.1	4,362	5,006	0	27
6 SLC7A5	NM_003486.5	3,886	4,248	11	0
7 ISG20	NM_002201.4	3,703	1,921	8	16
8 CPE	NM_001873.1	3,557	16,949	0	0
9 PCP4	NM_006198.2	3,542	1,863	0	0
10 SEMA3B	NM_001005914.1	3,224	3,122	13	68
11 IER3	NM_003897.3/NM_052815.1	3,091	3,769	20	274
12 PRSS23	NM_007173.4/NM_007173.3	3,071	4,128	0	11
13 LXN	NM_020169.2	2,991	1,710	0	0
14 RASD1	NM_016084.3	2,756	1,820	0	0
15 BASP1	NM_006317.3	2,441	2,726	0	22
16 FAM46A	NM_017633.2/NM_017633.1	2,440	1,907	0	0
17 RFTN1/RAFTLIN	NM_015150.1	2,408	1,690	0	6
18 ITGB5	NM_002213.3	2,318	1,659	0	17
19 CHPF	NM_024536.4	2,073	1,650	0	11
20 SLC7A2	NM_001008539.2/NM_003046.2	1,984	3,880	0	31
21 IRS2	NM_003749.2	1,962	1,528	13	0
22 ITGB5	NM_002213.3/XM_944693.1	1,953	1,544	0	17
23 AGR2	NM_006408.2	1,907	2,017	0	0
24 KRT81/KRTHB1	NM_002281.2	1,897	2,354	0	6
25 XBP1	NM_005080.2	1,847	1,763	10	294
26 SEMA3B	NM_001005914.1	1,838	1,806	13	68
27 XBP1	NM_001079539.1	1,795	1,674	10	294
28 HS.579631	BU536065	1,787	2,898	N/A	N/A
29 RPS7	NM_001011.3	1,762	1,551	9	0
30 PHLDA1	NM_007350.3/NM_007350.2	1,762	2,174	0	0
31 MAOA	NM_000240.2	1,761	1,730	0	0
32 VGF	NM_003378.2	1,745	1,574	8	0
33 TOB1	NM_005749.2	1,740	2,895	17	0
34 NME1	NM_000269.2/NM_198175.1	1,735	2,040	0	0
35 PISD	NM_014338.3	1,706	2,757	0	13
36 SDF2L1	NM_022044.2	1,703	1,803	0	0
37 RPS7	NM_001011.3	1,702	1,535	9	0
38 DKFZP564O0823	NM_015393.2	1,694	2,685	0	23
39 DNAJC12	NM_021800.2	1,694	1,703	0	41
40 PPFIA4	NM_015053.1	1,692	1,514	0	81
41 NUCB2	NM_005013.2/NM_005013.1	1,681	1,528	0	27
42 PDGFR	NM_006207.1	1,680	1,873	0	0
43 DNAJC12	NM_021800.2	1,663	1,574	0	41
44 SLC47A1/FLJ10847	NM_018242.2	1,661	1,525	0	86
45 HYAL3	NM_003549.2	1,659	1,512	0	0
46 GLRX	NM_002064.1	1,657	2,063	0	0
47 KIAA1324	NM_020775.2	1,652	2,827	0	0
48 WIPF1	NM_001077269.1	1,635	1,533	0	118
49 DNAJC12	NM_201262.1	1,609	1,972	0	41
50 KIAA1199	NM_018689.1	1,606	2,496	0	0
51 KLK3	NM_001030050.1	1,595	2,449	0	0
52 RPL29	NM_000992.2	1,565	1,748	0	0
53 S100P	NM_005980.2	1,560	1,839	13	0
54 NDUFA12L/mimitin	NM_174889.3/NM_174889.2	1,558	2,043	0	0
55 RGS22	NM_015668.2	1,551	2,565	0	0
56 PRDX4	NM_006406.1	1,550	1,952	0	0
57 HS.543887	CD640673	1,548	2,079	N/A	N/A
58 FAM108C1/LOC58489	NM_021214.1/XM_051862.7	1,546	1,936	8	108
59 BOP1	NM_015201.3	1,516	1,816	6	0

Supplementary Table 4 - Downregulated genes shared between MCF7 and MDA-MB134

	Symbol	Accession	Gene expression (ratio)		ChIPseq (absolute intensity)	
			MCF7	MDA-MB134	S305-P	E2/ERa
1	CLIC3	NM_004669.2	0.293	0.455	0	38
2	AHNK	NM_001620.1	0.499	0.643	0	0
3	CAV1	NM_001753.3	0.508	0.264	0	0
4	TACC1	NM_006283.1	0.514	0.496	0	8
5	PMP22	NM_153321.1 NM_153322.1	0.522	0.579	0	12
6	RAMP3	NM_005856.2	0.524	0.634	0	0
7	YPEL3	NM_031477.4 NM_031477.3	0.531	0.664	17	8
8	NRCAM	NM_005010.3	0.546	0.622	0	0
9	GPER GPR30	NM_001039966.1 NM_001031682.1	0.551	0.637	10	31
10	SMAD3	NM_005902.3	0.554	0.474	0	58
11	MAFB	NM_005461.3	0.555	0.517	12	0
12	CHES1	NM_005197.2	0.558	0.619	0	0
13	AKR1C3	NM_003739.4	0.561	0.520	0	25
14	ADCY1	NM_021116.1	0.567	0.651	0	37
15	RAMP3	NM_005856.1 NM_005856.2	0.571	0.594	0	0
16	CXCR7 CMKOR1	NM_020311.2 NM_020311.1	0.578	0.459	0	47
17	GPER GPR30	NM_001039966.1	0.579	0.552	10	31
18	RAPGEFL1	NM_016339.2 NM_016339.1	0.579	0.654	0	168
19	LFNG	NM_001040167.1 NM_002304.1	0.579	0.658	11	5
20	C6orf141	NM_153344.1	0.584	0.632	0	10
21	BNIP1	NM_138278.2 NM_138278.1	0.584	0.613	0	0
22	KLHL24	NM_017644.3	0.589	0.453	0	0
23	TSPAN9	NM_006675.3	0.594	0.495	0	0
24	ALDH3B2	NM_001031615.1 NM_000695.3	0.594	0.538	16	6
25	PIK3R1	NM_181523.1 NM_181504.2	0.594	0.646	0	0
26	EFNA1	NM_004428.2	0.597	0.601	9	12
27	PLA2G10	NM_003561.1	0.601	0.583	9	49
28	YPEL2	NM_001005404.3	0.608	0.633	0	63
29	LOC542817	XR_018564.1 XM_926703.1	0.616	0.631	N/A	N/A
30	PRKCD	NM_006254.3 NM_212539.1	0.616	0.557	0	25
31	CCNG2	NM_004354.1	0.629	0.661	11	0
32	SCUBE2	NM_020974.1	0.632	0.465	0	28
33	TP53INP1	NM_033285.2	0.638	0.544	9	4
34	CAV2	NM_001233.3	0.638	0.427	0	0
35	LMTK3	XM_936372.2 XM_936372.1	0.639	0.531	0	0
36	HIG2	NM_013332.3 NM_013332.1	0.640	0.578	0	19
37	CGN	NM_020770.1	0.648	0.641	10	0
38	ID2	NM_002166.4	0.650	0.593	13	0
39	RXRA	NM_002957.3	0.658	0.635	12	190
40	YPEL5	NM_016061.1	0.659	0.562	0	0
41	SAMD11	NM_152486.2	0.664	0.446	0	0

Chapter 5

Summary & discussion

Summary & discussion

About 70% of all breast cancers express the estrogen receptor and are dependent on estrogens for tumor cell proliferation. These patients commonly receive endocrine therapy, which includes two different classes of drugs: aromatase inhibitors and antiestrogens. These two classes have different mechanisms of action to establish the same effect: blockage of estrogen-dependent growth of tumor cells. Aromatase inhibitors prevent estrogen synthesis, hence depriving the receptor of natural ligand. Antiestrogens block the estrogen receptor by binding to the ligand binding pocket, thereby competing with the natural ligand and inactivating the receptor. This thesis focuses on the antiestrogen most applied in the clinic, tamoxifen, with which many patients have been treated successfully (1). However, not everyone benefits from this form of endocrine treatment and develop a recurrence (2, 3). To understand resistance to tamoxifen in breast cancer patients, there is a need to explore the underlying molecular biology and translate this knowledge into biological and clinical relevance.

Because tamoxifen acts directly on the estrogen receptor, it is imperative to study its function and the alteration thereof when drug resistance occurs. In Chapter 2, we set out to explore the different subdomains the receptor consists of and how these domains regulate processes upon ligand binding. The estrogen receptor family consists of two members, estrogen receptor alpha (ER α) and beta (ER β). Estrogen receptor positivity in breast cancer generally refers to the ER α subtype. ER α and ER β differ in classical and non-classical gene transactivation and cofactor-binding capacity (4-6). We compared ER α and ER β to better understand the individual contributions of distinct domains of the receptor on ligand responses. Between the two receptors, the DNA binding domain is highly conserved (93%) and can interact with the classical estrogen responsive element (ERE) on the chromatin to induce gene expression. The ligand binding domain is only 53% conserved between the two members of this family, which may explain that subsets of ligands exist that are specific for either for ER α or ER β (7). This does however not explain the fact that ER α and ER β can respond differently to shared ligands such as tamoxifen and estradiol (8). In Chapter 2, we demonstrate that these differences are determined by the activation function domain 1 (AF-1) and hinge region that are largely dissimilar between ER α and ER β . We swapped these domains between ER α and ER β and thereby affected estrogen receptor structure, as demonstrated by intramolecular FRET changes. Swapping the hinge or AF-1 domain at least partially switches ER subtype specific behavior in terms of the ER α distinct AF-1 binding of coregulator SRC-1, recruitment of the transcription machinery RNA polymerase II (i.e. before or after ligand binding), subsequently influencing gene transactivation on an estrogen responsive element (ERE). It also switches the effect on gene transactivation in a reporter assay with a non-classical AP-1 motif. The hinge and AF-1 domains appear to act in synergy with AF-2 to determine estrogen receptor function. Nonetheless, some questions remain. The ultimate control for this mechanism that has not been addressed is a combined swap of the AF-1 and the hinge region, or alternatively the AF-2 domain alone, which in essence would be the same. The expected outcome of this experiment would be a complete swap in estrogen receptor function for ligands shared between ER α and ER β , but not for distinct ligands: ER α needs both the active AF-1 and the longer flexible hinge for its full E2-induced response. Swapping only a small region of the hinge is not sufficient, even though this is a region in ER α that is prone to post-translational modifications and thereby a central node for estrogen receptor function. These post-translational modifications, including S305 phosphorylation, affect ER α function, but do not influence ER β function upon swapping. Still, a conformational change is detected with intramolecular fluorescence resonance energy transfer (FRET). This technique allows assessment of the orientation of the AF-1 and AF-2 domain, by tagging the estrogen receptor with a donor fluorophore on one terminus and

an acceptor on the other. When the conformation changes and the two ends come closer together or alter their orientation, the donor may transfer energy to the acceptor fluorophore, which consequently emits light (9). Although FRET detects a conformational change with the small hinge swap, the lack of effects on transcription could be due to the shorter, more rigid hinge of ER β , leaving less room for proper positioning of AF-1 and AF-2 to recruit coregulators and bind chromatin. This means that in this case, FRET is not indicative of downstream gene transactivation.

The ratio between ER α and ER β may be of relevance in how breast cancer responds to endocrine treatment: ER β has been described to block estrogen dependent growth (10). When the estrogen receptor binds a ligand, it dimerizes, enters the nucleus and binds to the chromatin to induce gene expression. It not only forms homodimers (ER α :ER α and ER β :ER β), but also heterodimers (ER α :ER β) (11). This could imply a different function, and indeed, both ER β homodimers and ER α :ER β interfere with E2-driven proliferation of breast cancer cells. Because the DNA-binding domain is 96% conserved, the chromatin binding landscape should be the same, unless other regions play an additional role in estrogen receptor positioning. The crystal structure of a more distant ER family member, the heterodimer PPAR:RXR reveals that the C-terminal helix of PPAR positions itself along the DNA, suggests a role in its chromatin interaction (12). This could mean that the C-terminus of the estrogen receptor may also play a synergetic role with its DBD in chromatin binding. Nonetheless, one report describes that the vast majority of chromatin binding sites of ER β are shared with ER α (13). It might still be interesting to compare the chromatin binding landscape of ER heterodimers and homodimers by re-ChIP-seq experiments to test whether they have distinct chromatin binding specificity. However, the dissimilarities in behavior of the two estrogen receptor subtypes do not seem to be caused by differential DNA interactions. More likely, the differences in AF-1 domain and hinge region influence the structural conformation of the receptor and thereby determine which coregulators are recruited.

We addressed the recruitment of coregulators by ER α in Chapter 3. This is the first report in which a high-throughput approach for coregulator binding to full length, endogenous estrogen receptor is applied. This approach consists of an array with coregulator peptides containing a motif for nuclear receptor binding: the LxxLL motif. We found that the peptide binding profile of full length, estrogen-bound receptor on the array highly resembles the profile that was observed for the ligand binding domain alone. Moreover, tagged, overexpressed or endogenous protein behaves very similar, suggesting that these differences have no substantial effect on coregulator peptide binding and estrogen receptor functionality. Measurements for endogenous protein were performed in MCF7 cells, whereas overexpression was done in U2OS cells that do not endogenously express the estrogen receptor. The difference in cell context did not affect the peptide binding profile. As expected, ligands have an impact on the cofactor interaction: estradiol induces stronger binding of coregulators, whereas tamoxifen reduces this interaction. After having established the applicability of this peptide array for testing estrogen receptor function, we addressed its application as a system to monitor tamoxifen resistance. One mode of tamoxifen resistance is ER α phosphorylation at Serine 305 (S305), as induced by protein kinase A (PKA) (14-16). S305 phosphorylation induces a structural change of ER α , altering the orientation between the receptor and its coactivators SRC-1, allowing SRC-1 to bind not only to the AF-1 but also the AF-2 domain of ER α . Consequently, the transcription machinery becomes active when tamoxifen is bound (17). The PKA-induced conformational change of ER α potentially affects orientation and strength of binding to other coregulators, affecting DNA interaction and induction of transcription of responsive genes, which ultimately results in an increased cell proliferation. Hence, tamoxifen will act as an agonist when ER α S305 is phosphorylated (18). On the peptide array, we also detected stronger coregulator

interactions with a tamoxifen-bound, S305 phosphorylated estrogen receptor, indicating a more active ER α in a tamoxifen resistant setting.

We did not observe obvious coregulator specific differences. Estradiol treatment or S305 phosphorylation by PKA seems to elevate coregulator binding for all peptides that already interact with ER α ligand, rather than specific ones. Moreover, the conformational change upon ligand binding or phosphorylation of the receptor does not induce new interactions with other peptides. This implies that the conformation as such does not determine binding to distinct coregulator motifs, but other factors appear to determine specificity. In order to achieve specificity, a full length coregulator may be required. ER α is known to interact with SRC-1 with both the AF-1 and AF-2 domains. The AF-2 domain binds an LxxLL motif, whereas the AF-1 domain interacts with a Q-rich region on SRC-1 (17). This second interaction could not be detected in our peptide assay, because the peptides only include the LxxLL(-like) motifs and not the Q-rich region. Since this interaction may be essential for specificity in coregulator recruitment, the overall, rather than peptide specific, increase in binding may simply be due to more efficient exposure of the AF-2 coregulator binding surface of ER α upon estradiol-binding or phosphorylation at S305.

Nonetheless, the stronger binding to the LxxLL-coactivator motifs is indicative of a more active ER α . It is striking that we can detect this effect with one single post-translational modification: S305 phosphorylation. There is no need to isolate the receptor and in fact using cell lysates avoids an intricate and troublesome process of isolation, with the risk of loss of phosphorylation. Even in tumor cell lysates, we were able to detect an increased ER α function, when S305 is phosphorylated as validated by immunohistochemistry. It is important to realize that such a biochemical assay is likely to expose a lower-detection threshold, which still needs to be determined. As a direct consequence, this approach may not prove useful for tumors with low staining and few S305-P positive cells as any positive signal will be diluted by the normal tumor. What could aid in improving detection is extracting tumor cells from surrounding stroma and normal cells, which would concentrate the (phosphorylated) estrogen receptor in the sample. Such technical hurdles should be addressed in a more extensive study to assess the applicability of this assay in a clinical setting.

A stronger overall binding of ER α from patient material to the peptide array would imply a higher basal activity of the estrogen receptor, which could be an indication of resistance to tamoxifen. The coregulator peptide array, detecting ER α function, may be of added value in predicting tamoxifen response in patient material. Stronger binding of the estrogen receptor implies that it is phosphorylated, thereby resistant to the drug. This assay, however, needs further testing for clinical application.

Thus far, we have shown that ER α S305 phosphorylation elevates coregulator binding to ER α when associated to tamoxifen. In the clinic, tamoxifen resistance caused by PKA-induced phosphorylation of ER α S305 is correlated with poor outcome in estrogen-receptor positive, tamoxifen-treated breast cancer patients (14-16). What was still unresolved is how a phosphorylated receptor, possibly by inducing better coregulator binding, ultimately leads to tumor cell proliferation. In Chapter 4, we addressed this gap in understanding of this form of tamoxifen resistance by an integrative analysis of the chromatin binding landscape and the resulting transcriptome from ER α phosphorylated by PKA. This single post-translational modification has a striking effect on the chromatin binding landscape of the estrogen receptor: whereas generally ER α prefers distal enhancers, ER α S305-P is redirected to promoter regions, 5' and 3' UTRs. This is the first report demonstrating that one single post-translational modification of ER α can have major impact on the chromatin binding landscape. With this novel finding, it is imperative to interrogate other post-translationally modifiable sites for ER α , e.g. by acetylation (19), and compare the results to our findings for ER α S305-P to define

the consequences of the 'ER α modification landscape'.

The transcriptome of ER α S305-P with tamoxifen was studied by PKA-activation in breast cancer cells. MCF7 is a commonly used model breast cancer cell line, which we treated with a chemical (forskolin) to induce PKA activity. To reduce false positives and cell line specific artefacts, we applied a knockdown approach on the regulatory subunit of PKA, PKA-R1 α (20), in another breast cancer cell line (MDA-MB134). This may, however, create false negatives instead, e.g. by an already activated PKA pathway even before experimental induction. Moreover, this approach highlights stable effects, rather than immediate early targets after PKA-activation. Nonetheless, we obtained a set of 100 genes, which could serve as a classifier in ER α positive, tamoxifen treated breast cancer. Integration of the PKA-induced 100-gene classifier with the chromatin binding landscape of ER α S305-P to extract the direct targets for the modified receptor resulted in a list of 26 genes. These 26 targets showed a significant correlation with poor disease outcome, suggesting that phosphorylation of ER α plays an essential role in tamoxifen response and is not just a bystander effect.

Studying the 100-gene classifier in more detail, we discovered that MYC-related genes were enriched, some of which were identified as direct ER α S305-P targets. In addition, the oncogene MYC itself was upregulated. Chapter 4 thus provides a potential functional link between ER α phosphorylation and the resulting tamoxifen resistance in breast cancer. This is in line with literature correlating the Myc-pathway with tamoxifen resistance in breast cancer (21, 22). Moreover, MYC had been described as a direct estrogen-regulated target for ER α and hence playing a key role in proliferation of breast cancer cells (22). Here we demonstrate a direct link between PKA-induced phosphorylated ER α and MYC in terms of chromatin binding landscape, transcriptome and cell proliferation. Taken together, and summarized in Figure 1, our findings span the entire range from estrogen receptor phosphorylation to tamoxifen resistance in molecular terms.

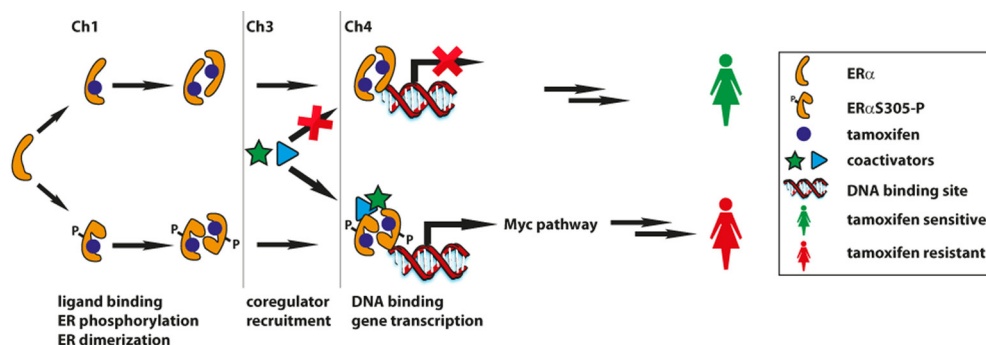


Figure 1: Schematic summary of Chapter 1, 3 and 4 of this thesis. Estrogen receptor alpha (ER α) binds tamoxifen and dimerizes. Depending on the ER α S305 phosphorylation (Ch1) status the structural conformation of the receptor is different, which influences the coregulator recruitment (Ch3). Non-phosphorylated ER α still binds to the DNA, as the normal estradiol-bound receptor would, but does not induce transcription and thereby blocks tumor cell proliferation in the breast cancer patient. ER α S305-P shows enhanced binding of coregulators in the presence of tamoxifen, resulting in an enhanced responsive gene activity, including MYC and MYC-related genes, which induces cell proliferation and ultimately renders the tumor resistant to tamoxifen.

Not only do our data provide an understanding into the molecular mechanism of tamoxifen resistance through PKA-induced ER α S305 phosphorylation, but also show that the effects on gene expression, although dependent on ER α S305 phosphorylation, are not tamoxifen specific. Notably, several genes with a proximal binding site for ER α S305 appear to

show an additional effect for activated PKA in presence of tamoxifen, as compared to hormone-depleted conditions, but the molecular mechanism needs further analysis. The presence of a PKA-induced effect in absence of tamoxifen implies that the genes in the classifier could already be differentially regulated in breast tumors before tamoxifen treatment. The fact that our classifier works in two independent patient datasets supports this hypothesis. After all, the gene expression data are derived from the primary tumors that have not been exposed to tamoxifen. It could therefore prove to be a useful classifier for predicting tamoxifen responses in patients before the onset of treatment. A qPCR assay could be developed that assesses expression of selected genes, as well as chromatin fragments that are distinctly targeted by ER α S305-P. This assay would serve as a prediction tool for tamoxifen responses, in combination with the previously reported ER α S305-phospho antibody (18). The immunohistochemical staining with this antibody, although successful in a clinical setting, already shows a correlation with poor disease outcome when only one nucleus in a tumor slice scores positive (14-16). One nucleus in a tumor can be easily missed, but an additional gene classifier may be more sensitive to infrequent ER α S305-P tumor cells in tissue and could reduce false negatives. A solid method to detect such cells would avoid unnecessary tamoxifen treatment of patients who will not benefit from this drug. In addition, the classifier would not be sensitive to dephosphorylation events occurring during tissue preparation for immunohistochemistry. An optimal signature could include downregulation of PKA-R1 α , which has been correlated with PKA activity (and ER α S305-P) resulting in tamoxifen resistance (18), as well as MYC and the MYC-related 26-gene classifier presented in Chapter 4. Combining different approaches should optimize prediction of tamoxifen resistance in breast cancer patients by ER α S305 phosphorylation and present a way towards personalized medicine for patient treatment with effective compounds on rational grounds.

Efforts are already made to set up a prospective clinical trial for tamoxifen resistance by PKA-induced ER α S305 phosphorylation. Patients will be stratified based on immunohistochemical staining of tumor tissue with the ER α S305-P recognizing antibody. As mentioned earlier, determination of this form of resistance could be fine-tuned by combining IHC with gene expression profiling. And since microarrays are routinely performed on breast cancer, a gene classifier could easily be integrated in such a clinical trial. When a tumor would stain negative for ER α S305-P, the patient would be selected to receive tamoxifen as adjuvant treatment after tumor excision, because she is likely to benefit. Patients staining positive for this phosphorylation will not receive tamoxifen, but would receive aromatase inhibitors instead. Although the optimal setup would be to administer no drugs to the latter group of patients, it would be unethical to deny patients any treatment. In a control group, patients will be randomized, receiving tamoxifen or aromatase inhibitors, irrespective of their ER α S305-P status. We expect that this prospective study will tell us that we can indeed predict (a subset of) tamoxifen resistant cases by defining ER α S305-P positivity in breast tumors.

In summary, phosphorylation of ER α at Serine 305 has major impact on estrogen receptor conformation and coregulator binding. One single post-translational modification of this receptor, as induced by PKA, redirects its binding positions on the chromatin, which affects the transcriptome and thereby induces resistance to tamoxifen. An essential question along these lines is: are there treatment alternatives for patients who are defined as non-responders to tamoxifen? Previous reports suggest that selective estrogen receptor downregulators (SERD), such as fulvestrant, can be effective against cancer cells that have a PKA-induced tamoxifen resistance (23, 24). The effect of PKA activation leading to ER α S305 phosphorylation on the transcriptome and cell proliferation appears to be independent of ligand (tamoxifen, estradiol or no ligand). It would be interesting to test whether a SERD, such as fulvestrant, by degrading ER α can inhibit the ER-induced MYC pathway and block cell growth. Another

hypothesis that should not be ruled out is that a patient who is resistant to one therapeutic may become sensitized to another drug, which would normally be less effective in this type of breast cancer. Any form of cancer might become resistant to one therapeutic compound, bypassing the targeted molecular pathway and switching to other pathways to evade cell and tumor death. These other pathways in turn could be targetable by other drugs, rendering the tumor responsive to therapeutics it would normally not respond to. Prostate cancer provides a representative example: whereas chemotherapy in the form of taxanes is generally ineffective in prostate cancer, a subset of castrate resistant patients suddenly becomes more sensitive to this type of treatment (25, 26). A differential regulation of cell cycle pathways lies at the basis of this sensitization. In breast cancer, triple negative tumors have been predicted to respond much better to chemotherapy than luminal (ER+) breast cancer (27). Along these lines, it would be interesting to test whether endocrine resistant tumors, or a subgroup thereof, are sensitized to chemotherapy compared to endocrine sensitive breast tumors.

A challenge for the future would be to map the ER α chromatin binding landscape and transcriptome for other post-translational modifications on ER α and to correlate this with therapeutic responses to antiestrogens or aromatase inhibitors. Data could be integrated to develop new tools that predict response of each individual patient to a range of drugs and thereby truly personalize breast cancer treatment. If we can predetermine upon diagnosis to which therapy a patient will respond best, we can avoid unnecessary treatment with drugs that are not beneficial, and hence also their potential adverse side-effects. Moreover, cancer will have less chance to progress by defining the right treatment upfront, instead of the current approach, which is largely based on trial and error. Personalization of cancer treatment to the individual patient will improve chances of disease-free survival and quality of life of cancer patients and this thesis describes how to reach this.

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Nederlandse samenvatting

List of publications

Curriculum vitae

Nederlandse samenvatting & discussie

Wereldwijd worden er jaarlijks 1,4 miljoen nieuwe diagnoses gesteld van borstkanker en komen bijna een half miljoen borstkankerpatiënten te overlijden aan de gevolgen van borstkanker. Ongeveer 70% van alle gevallen van borstkanker bevatten de oestrogeenreceptor en deze tumoren hebben oestrogenen nodig om te groeien. Patiënten met dit type borstkanker worden geopereerd en krijgen vaak bestraling. Daarnaast krijgen deze patiënten een extra behandeling om de kans op uitgroeiende metastasen te verkleinen. Dit wordt adjuvante endocrine (of hormonale) therapie genoemd. Deze hormonale therapie, welke onder te verdelen is in twee subklassen van medicijnen: aromataseremmers en anti-oestrogenen. Deze twee subklassen verschillen in werkingsmechanismen, maar bereiken hetzelfde effect: het blokkeren van de oestrogeenafhankelijke groei van tumorcellen. Aromataseremmers voorkomen de synthese van oestrogenen door het verantwoordelijke enzym, aromatase, te remmen en ontnemen de oestrogeenreceptor zodanig het natuurlijke ligand (hormoon). Omdat de oestrogeen receptor niet geactiveerd kan worden door het natuurlijke ligand, resulteert dit in een remming van tumorgroei. Anti-oestrogenen blokkeren de oestrogeenreceptor door te binden in de ligandbindende holte, waar normaal gesproken het oestrogeen zou binden. Door deze competitie met het natuurlijke ligand om dezelfde bindingsplek kan de oestrogeen receptor niet meer binden aan co-activatoreiwitten, die essentieel zijn voor de vorming van een actief transcriptie complex. Het gevolg van deze remming is dat tumorgroei ook geremd wordt.

Dit proefschrift gaat over het anti-oestrogeen dat het meest wordt toegepast in de kliniek, namelijk "tamoxifen", waarmee veel patiënten reeds succesvol zijn behandeld. Echter, niet iedereen heeft baat bij deze vorm van hormoontherapie en zien onverhoopt de kanker terugkeren. Het is essentieel om de onderliggende moleculaire biologie van tamoxifen resistentie bij borstkanker te bestuderen, opdat we deze kunnen vertalen naar relevante toepassingen voor de kliniek, zoals een assay om respons op tamoxifen te kunnen voorspellen vóór behandeling.

Omdat tamoxifen direct op de oestrogeenreceptor werkt, is het belangrijk om de functie van dit eiwit te onderzoeken en om te bepalen wat hiermee gebeurt bij resistentie tegen het medicijn. In Hoofdstuk 2 hebben we de verschillende subdomeinen waaruit de receptor bestaat bestudeerd, daarbij de vraag stellend welke rol(len) deze domeinen spelen in de functie van de oestrogeenreceptor na het binden van een ligand. De familie van oestrogeenreceptoren bestaat uit twee leden: oestrogeenreceptor alfa ($ER\alpha$) en beta ($ER\beta$). Als een borsttumor positief is voor de oestrogeen receptor, wordt normaliter gerefereerd naar het alfa subtype. $ER\alpha$ and $ER\beta$ zijn allebei nucleaire receptoren, die na het binden van een hormoon aan het DNA binden en bepaalde genen aan kunnen zetten, ook wel gentransactivatie genoemd. Ze verschillen echter zowel in klassieke gentransactivatie, waarbij de oestrogeenreceptor direct aan het DNA bindt, als in de non-klassieke gentransactivatie, waarbij ze samenwerken met andere transcriptiefactoren. Tevens vertonen ze verschil in bindingscapaciteit met cofactoren, hulpeiwitten die oestrogeenreceptor nodig heeft voor gentransactivatie. We hebben $ER\alpha$ and $ER\beta$ vergeleken om de individuele bijdragen van de verschillende domeinen te onderscheiden in hoe de receptor reageert op een ligand. Tussen de twee oestrogeenreceptoren is het DNA-bindende domein (DBD) het meest geconserveerd (96%). Dit domein kan een interactie aangaan met een oestrogeen responsief element (ERE) op het DNA, zodat de receptor vervolgens de expressie van een gen induceren. Het ligand bindende domein van beide receptoren is slechts voor 53% geconserveerd, wat zou kunnen verklaren dat er specifieke liganden bestaan voor óf $ER\alpha$ óf $ER\beta$. Dit verklaart echter niet waardoor $ER\alpha$ en $ER\beta$ juist geheel anders kunnen reageren op één en hetzelfde ligand, zoals

het geval is voor tamoxifen en oestradiol. In Hoofdstuk 2 laten we zien dat een groot deel van deze verschillen worden bepaald door het activatie functie domein 1 (AF-1) en het scharnier (hinge) domein, welke grotendeels verschillend zijn tussen ER α and ER β . We hebben deze domeinen uitgewisseld tussen ER α and ER β , wat de eiwitstructuur beïnvloedde, geobserveerd als intramoleculaire fluorescence resonance energy transfer (FRET) veranderingen. Het vervuilen van het scharnier of het AF-1 domein wisselde in ieder geval deels het specifieke gedrag van de oestrogenreceptor subtypes: de ER α -kenmerkende interactie van het AF-1 domain met de coregulator SRC-1 en het recruterende van de transcriptie machinerie RNA polymerase II (bv. vóór of na ligand binding), waardoor gentransactivatie op het ERE wordt beïnvloed. Tevens wordt het effect op gentransactivatie uitgewisseld in een reporter assay met een niet-klassiek AP-1 motief. Als de oestrogenreceptor indirect via andere factoren aan dit motief bindt, wordt een eenvoudig te meten reporter gen overgeschreven. Het scharnier en AF-1 domain lijken in synergie te werken met het activatie functie 2 (AF-2) domein en daarmee de functie van de oestrogenreceptor te bepalen. Desalniettemin blijven sommigen vragen onbeantwoord. De ultieme controle voor dit moleculaire mechanisme die nog niet uitgevoerd is, is een gecombineerde uitwisseling van zowel het AF-1 en scharnier domain, of als alternatief alleen het AF-2 domain, wat in essentie op hetzelfde neerkomt. De verwachte uitkomst van deze proef zou een algehele uitwisseling zijn van oestrogenreceptorfunctie voor de liganden die zowel ER α and ER β kunnen binden. Maar niet voor subtype-specifieke liganden: ER α heeft zowel het actieve AF-1 domain als het langere, flexibele scharnier nodig om tot zijn volledige oestradiol response te kunnen komen. Het ruilen van een klein deel van het scharnier blijkt onvoldoende, ook al is dit deel in ER α hoogst gevoelig voor post-translationele modificaties en daarmee een centraal knooppunt voor de functie van de receptor. Deze post-translationele modificaties, waaronder de fosforylering van het aminozuur Serine 305, beïnvloeden ER α functie, maar niet die van ER β na het uitwisselen van dit eiwitdeel. Toch detecteren we een verandering in structurele conformatie van het eiwit met FRET. Met deze techniek kunnen we de orientatie van het AF-1 en AF-2 domein bestuderen door aan de twee uiteinden van oestrogenreceptor een verschillend kleurlabel te hangen: een donor fluorofoor (blauw) en een acceptor (geel). Als het donor label met een laser belicht wordt, zendt deze blauw licht uit. Als de acceptor echter dichtbij de donor komt door een verandering van eiwitstructuur, kan de donor zijn energie overdragen aan de acceptor, die daardoor geel licht af geeft ten koste van het blauwe signaal. Als de vouwing van het eiwit verandert, kunnen de labels dicht bij of verder van elkaar komen te staan. Een verandering in FRET is zo een maat voor verandering in receptorvouwing en op deze wijze hebben wij een verandering in eiwitvouwing gedetecteerd bij de uitwisseling van het kleine stukje scharnier domein. Ondanks dat we verschillen meetten met FRET, bleek dit zich niet te vertalen in verschillen op gentranscriptie. Dit zou kunnen komen doordat de hinge van ER β korter is en minder flexibel dan die van ER α , wat minder ruimte laat om de AF-1 en AF-2 op een juiste manier te plaatsen om coregulators te kunnen werven en chromatine te binden. In dit geval blijkt FRET niet indicatief voor downstream gentransactivatie.

De verhouding tussen ER α and ER β zou bepalend kunnen zijn voor het success van endocriene therapie bij borstkanker: ER β zou oestrogenafhankelijke groei tegengaan. Na het binden van een ligand dimeriseert de oestrogenreceptor en bindt aan het DNA om genexpressie te induceren. Het maakt niet alleen dimeren met zichzelf, c.q. homodimeren (ER α :ER α and ER β :ER β), maar ook met het andere subtype, heterodimeren (ER α :ER β). Aangezien ER α and ER β verschillen in functie, zou dit kunnen betekenen dat ook de dimeren verschillen in functie. Dit blijkt inderdaad het geval: zowel ER β homodimeren and ER α :ER β gaan oestradiolafhankelijke groei van borstkankercellen tegen in een experimentele opzet. Aangezien het DNA-bindende domein tussen beide oestrogenreceptoren voor 96%

geconserveerd is, zou je verwachten dat beide subtype receptoren op dezelfde plaatsen aan het chromatine kunnen binden, en daarmee hetzelfde “chromatine-bindingslandschap” hebben. Het zou echter kunnen dat andere eiwitdomeinen buiten het DNA-bindend domein ook een rol spelen in het positioneren van de oestrogeenreceptor op het DNA. Een suggestie hiervoor is beschreven voor een andere nucleaire receptor, PPAR, dat heterodimeriseert met RXR (PPAR:RXR). Het kristalstructuur laat zien dat het C-terminale helix-uiteinde van PPAR langs het DNA strekt. Dit zou kunnen betekenen dat de C-terminale helix van de oestrogeenreceptor een vergelijkbare synergetische rol samen met het DNA-bindende domein speelt in chromatine interactie. Al beschrijft één verhaal dat het chromatine-bindingslandschap overwegend gelijk is tussen ER α en ER β , waarbij ook overlappend genexpressie profiel te verwachten is, zou het toch interessant kunnen zijn om alle DNA-interacties in kaart te brengen van de ER hetero- en homodimeren middels re-ChIP-seq experimenten om te testen of ze chromatine-bindingsspecificiteit vertonen. Met deze techniek worden chromatinefragmenten in twee stappen geïsoleerd: eerst met een antilichaam tegen de ene receptor (c.q. ER α) en vervolgens met een antilichaam tegen het tweede (ER β), opdat slechts de DNA-fragmenten overblijven waaraan het heterodimeer bindt. Echter, de oorzaak van de functionele verschillen tussen de twee oestrogeenreceptoren lijkt niet te liggen in differentiële DNA interacties, aangezien het DNA-bindende domein zeer geconserveerd is. Waarschijnlijker is dat de variaties in AF-1 en scharnierdomein de structurele conformatie van de receptor veranderen en zo de binding van coregulatoreiwitten beïnvloeden.

Het recruteren van coregulatoren door de oestrogeenreceptor hebben we bekeken in Hoofdstuk 3. Dit is het eerste verhaal, waarin een high-throughput benadering wordt toegepast om binding van coregulatoren aan een volledige, endogene oestrogeenreceptor te meten. Deze benadering bestaat uit een platform (array), waarop peptidefragmenten van coregulatoreiwitten zijn bevestigd. Deze peptides bevatten een motief dat herkend en gebonden wordt door nucleaire receptoren: het LxxLL motief. In interactiestudies tussen coregulatoreiwitten en de oestrogeenreceptor wordt vaak alleen het ligandbindende domein tot overexpressie gebracht. In onze benadering resulteerde een volledige oestrogeen receptor, na behandeling met oestradiol, in een peptidebindingsprofiel wat hoogst vergelijkbaar was met dat van het ligandbindende domein alleen. Ook blijken verschillen door grote labels, zoals de eerder genoemde gele en blauwe fluoroforen, exogene overexpressie of juist endogene oestrogeenreceptor verwaarloosbaar voor peptide binding op deze array. Zelfs de celcontext blijkt van weinig invloed op het peptidebindingsprofiel, blijkend uit verwaarloosbare verschillen tussen endogeen eiwit in de MCF7 borstkankercellijn en overexpressie in de U2OS botkankercellijn. Zoals verwacht hebben liganden impact op cofactorinteracties: oestradiol versterkt binding met coregulatoren, terwijl tamoxifen deze interacties juist verzwakt. Na het testen van de toepasbaarheid van de peptide array voor oestrogeenreceptorfunctie, vroegen we ons af of we hiermee ook tamoxifenresistentie zouden kunnen meten. Een vorm van tamoxifen resistentie werkt via de fosforylering van de oestrogenreceptor op het Serine 305 (S305) residue door proteïne kinase A (PKA). Deze fosforylering leidt tot een structurele verandering van ER α , waarbij de oriëntatie tussen de receptor en de coactivator SRC-1 verandert, zodat SRC-1 niet alleen aan het AF-1, maar ook aan het AF-2 domain van ER α bindt. Dientengevolge wordt het transcriptiecomplex actief, als ER α tamoxifen heeft gebonden. De PKA-geïnduceerde conformationele verandering van ER α is mogelijk ook van invloed op de oriëntatie en bindingscapaciteit van andere coregulatoren, met als gevolg tevens de DNA interacties en genexpressie, wat uiteindelijk kan leiden tot meer celgroei. Op die manier kan tamoxifen werken als agonist, in plaats van antagonist, als ER α S305 gefosforyleerd is. Zo observeerden we op de peptide array inderdaad sterkere coregulatorbindingen bij tamoxifengebonden, S305 gefosforyleerde oestrogenreceptor, wat een meer actieve receptor

impliceert in deze tamoxifenresistente setting.

We detecteerden geen duidelijke coregulatorspecifieke veranderingen. Behandeling van de cellen met oestradiol of stimulatie van PKA, waardoor S305 gefosforyleerd wordt, resulteerden in een algehele verhoging van coregulatorbinding voor alle peptiden, die voor stimulatie al enige interactie vertoonden met ER α ligand. Ook veroorzaakte de verandering van receptorconformatie na ligandbinding geen nieuwe interacties met andere peptiden. Dit suggereert dat de conformatie op zichzelf niet verantwoordelijk is voor specificiteit in interacties met coregulatormotieven, maar dat er andere factoren hierbij een rol lijken te spelen. Mogelijkerwijs is het volledige coregulator eiwit nodig om specificiteit te verkrijgen. We weten bijvoorbeeld dat ER α een interactie aangaat met SRC-1 middels zowel het AF-1, als het AF-2 domain. Het AF-2 domein bindt het klassieke LxxLL motief, maar het AF-1 herkent een aminozuurregio van SRC-1 die rijk is aan glutaminen (Q-rijk domein). Deze tweede interactie kan niet gemeten worden in de peptide array, omdat de peptides alleen LxxLL(-achtige) motieven bevatten, en geen Q-rijk domein. Bij het ontbreken van een interactie tussen het ER α -AF-1 domein het coregulator-Q-rijk domein, welke specificiteit zou kunnen bepalen, kan het LxxLL-motief vrij binden na oestradiolbinding meer ruimte begrijp ik niet,,,,, of S305-fosforylering aan het bindingsoppervlak van het AF-2 domein, zonder sterische hinder van de rest van de coregulator. Dit kan wellicht de algeheel versterkte binding op de array zou kunnen verklaren.

Dat neemt niet weg dat de versterkte binding aan LxxLL-coactivator motieven een indicatie is voor verhoogde activiteit van de oestrogenreceptor. Het is opmerkelijk dat we dit effect zelfs kunnen meten met een enkele post-translationele modificatie: S305-fosforylering (S305-P). De receptor hoeft niet opgezuiverd te worden uit cellen, wat nogal problematisch kan zijn en waarbij een fosforylering makkelijk verloren kan gaan. Zelfs in tumorlysaten is een verhoogde ER α functie te detecteren, wanneer S305 gefosforyleerd is, wat in parallel bepaald is volgens immunohistochemische analyse. Wel zal een biochemische analyse zoals deze een detectielimiet hebben, waaronder het effect van S305-P niet meer te meten is, maar deze moet nog bepaald worden. Dientengevolge zal deze benadering niet toepasbaar blijken op tumoren met een zeer lage, doch positieve kleuring voor ER α S305-P of als slechts enkele cellen in de hele tumor positief zijn. In dat geval wordt het positieve S305-P signal teveel verdund door de rest van de tumor. Extraheren van tumorcellen uit het omliggende stroma en de normale (niet-tumor) cellen zou de detectie kunnen verbeteren, doordat zo de (gefosforyleerde) oestrogenreceptor geconcentreerd wordt in het tumormonster. Om deze array te kunnen gaan toepassen in een klinische setting, moet eerst dit type technische vragen beantwoord worden in een uitgebreidere studie.

Een sterkere binding van ER α uit patiëntenmateriaal aan de peptide array suggereert een hogere basale activiteit van de oestrogenreceptor, welke tamoxifenresistentie zou kunnen verklaren. De coregulator peptide array, die ER α meet, zou van toegevoegde waarde kunnen zijn in het voorspellen van hoe een patiënt zal reageren op tamoxifenbehandeling. Sterkere coregulatorbinding impliceert dat de oestrogenreceptor gefosforyleerd is en dus resistent zal zijn tegen tamoxifen.

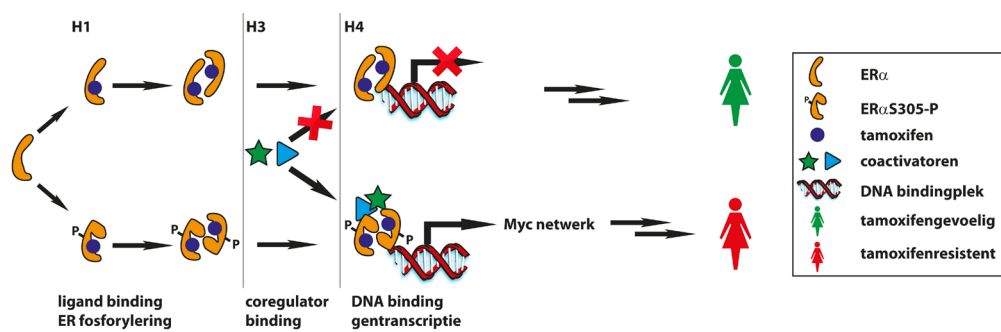
In de kliniek is fosforylering op ER α S305 gecorreleerd met slechter ziekteverloop in oestrogenreceptor-positieve borstkanker bij patienten die met tamoxifen behandeld zijn. Een tot nog toe onbeantwoorde vraag is hoe de gefosforyleerde receptor, mogelijk via betere binding met coregulatoren, uiteindelijk leidt tot de groei van tumorcellen. In Hoofdstuk 4 hebben wij deze vraag benaderd middels een geïntegreerde analyse van het chromatinebindingslandschap en het genexpressieprofiel, of transcriptoom, over het volledige genoom van PKA-gefosforyleerd ER α . Deze enkele modificatie heeft een opvallend effect op chromatinebinding: normaliter bindt ER α het liefst aan motieven op afstand van genen (distal

enhancers), maar ER α S305-P prefereert juist promotor regionen, 5' en 3' onvertaalde regionen (UTRs) op het DNA, die zich precies aan het begin of eind van genen bevinden. Dit is het eerste verslag dat aantoonde dat een enkele post-translationale modificatie van ER α grote impact kan hebben op het chromatinebindingslandschap van de receptor. Na deze nieuwe ontdekking is de vraag onvermijdelijk wat andere post-translationale modificaties van ER α voor effect hebben, b.v. fosforylering op andere plekken van de receptor of acetylering. En is dit effect vergelijkbaar met wat wij vinden bij ER α S305-P?

Het transcriptoom is in Hoofdstuk 4 bekeken door PKA-activatie in borstkankercellen. MCF7 is een veelgebruikte borstkankercellijn, die we gestimuleerd hebben met een chemische stof (forskolin) om PKA te activeren. Om het aantal vals-positieve hits te reduceren hebben we daarnaast een benadering toegepast op een andere borstkankercellijn (MDA-MB134), waarbij het regulerende subeiwit (PKA-R1 α), dat de activiteit van PKA remt, verlaagd wordt. Hierdoor kunnen echter meer vals-negatieve hits ontstaan, b.v. doordat het PKA signaaltransductienetwerk reeds verhoogde activiteit vertoont vóór het experiment gestart wordt. Bovendien laat deze benadering juist stabiele, lange-termijn effecten zien van PKA-activatie, en niet zozeer de vroege, tijdelijke effecten. Deze experimentele opzet leverde een set van 100 genen op, die als classifier kan worden toegepast op ER α positieve, tamoxifenbehandelde borstkanker patiënten. Integratie van deze 100 genen met het chromatinebindingslandschap van ER α S305-P om de directe targets voor deze gemodificeerde receptor eruit te extraheren, resulteerde in een lijst van 26 genen. Deze 26 targets vertonen een significante correlatie met slecht ziekteverloop, wat impliceert dat de fosforylering van ER α een essentiële rol speelt in hoe de patiënt reageert op tamoxifen, en dat deze modificatie niet enkel een toevallig neveneffect is van PKA-activatie.

Toen we de 100-genen classifier meer in detail bekeken, ontdekten we dat MYC-gerelateerde genen verrijkt waren in deze set. MYC is een bekend oncogen, wat cel- en tumorgroei bevordert. Sommige van deze genen waren geïdentificeerd als directe ER α S305-P targets, omdat de gefosforyleerde receptor op het chromatine in de buurt van deze genen bindt. Bovendien was het oncogen MYC zelf ook verhoogd in expressie, zij het net beneden de arbitraire drempelwaarde. Hiermee presenteert Hoofdstuk 4 een mogelijke verklaring hoe ER α fosforylering kan leiden tot resistentie tegen tamoxifen bij borstkanker, wat verder gaat dan enkel een correlatie tussen ER α S305-P en resistentie. Dit past in wat reeds beschreven is in de literatuur: MYC-signalering is gecorreleerd met tamoxifenresistentie bij borstkanker. Tevens wordt MYC-genexpressie geactiveerd door ER α en speelt zo een sleutelrol in de groei van borstkankercellen. Hier tonen wij met chromatinebindings-, genexpressie- en celgroei-experimenten een directe link tussen door PKA gefosforyleerde ER α en MYC. Samenvattend spannen onze bevindingen over het hele traject van oestrogenreceptormodificatie tot en met tamoxifenresistentie bij borstkankerpatiënten.

Onze data verklaren niet alleen het moleculaire mechanisme van tamoxifenresistentie door PKA-geïnduceerde ER α S305-fosforylering, maar laten ook zien dat PKA ook effect heeft op genexpressie in afwezigheid van tamoxifen. Wel lijken sommige genen met een chromatinebindingsplek voor ER α S305-P een extra effect te vertonen met tamoxifen, bovenop wat geactiveerd PKA veroorzaakt bij afwezigheid van hormoon, maar het moleculaire mechanisme dat dit veroorzaakt moet nog opgehelderd worden. Het effect van PKA-activatie in afwezigheid van tamoxifen suggereert dat de genen in de classifier al differentieel gereguleerd zouden kunnen zijn in borsttumoren vóór behandeling met tamoxifen. Het feit dat de classifier werkt in twee onafhankelijke patientendatasets steunt deze hypothese, aangezien de genexpressiedata zijn gegenereerd op primair tumormateriaal wat niet is blootgesteld aan tamoxifen. Deze genclassifier zou dus van toegevoegde waarde kunnen blijken bij het voorspellen van tamoxifenrespons bij patiënten, voordat de behandeling gestart wordt.



Figuur 1: Schematische samenvatting van Hoofdstuk 1,3 en 4 van dit proefschrift. Oestrogenreceptor alpha (ER α) bindt tamoxifen en dimeriseert. De ER α S305 fosforyleringsstatus (H1) bepaalt de structurele conformatie van de receptor, wat de binding van coregulatoreiwitten beïnvloedt (H3). Niet-gefosforyleerd ER α kan nog steeds aan het DNA binden, zoals een normale, oestradiolgebonden receptor, maar induceert geen transcriptie en zo wordt tumorcelgroei geblokkeerd. ER α S305-P bindt sterker aan coregulatoren in aanwezigheid van tamoxifen dan een niet gefosforyleerde receptor, wat resulteert in verhoogde expressie van specifieke genen (H4). Deze genen zijn onder andere MYC en MYC-gerelateerde genen, die celgroei stimuleren en zo de tumor resistent maken tegen tamoxifen.

Een qPCR test kan worden ontwikkeld om de expressie van geselecteerde genen te meten, gecombineerd met chromatinefragmenten voor specifieke targets van ER α S305-P. Deze test zou dan kunnen worden toegepast als predictietool voor tamoxifenrespons, in combinatie met het eerder beschreven ER α S305-phospho antilichaam. De immunohistochemische kleuring met dit antilichaam, hoewel succesvol in een klinische setting, correleert al met slechter ziekteverloop als slechts één celkern positief kleurt in een tumorplakje onder de microscoop. Een enkele celkern kan makkelijk over het hoofd worden gezien, maar een goede genclassifier kan wellicht meer detecteren als de kleuring niet sterk genoeg is, alsmede vals-negatieven eruit filteren. Een goede methode om ER α S305-P positieve tumorcellen te detecteren zou onnodige tamoxifenbehandeling van patiënten die hier geen baat bij zullen hebben kunnen voorkomen. Bovendien zou een genclassifier, in tegenstelling tot de kleuring, geen last hebben van defosforylering van de receptor, wat kan gebeuren bij weefselpreparatie voor immunohistochemie. De optimale genexpressieclassifier zou verlaagd PKA-R1 α bevatten, wat al gecorreleerd is met PKA-activatie, ER α S305-P tamoxifenresistentie bij borstkankerpatiënten, evenals MYC en de MYC-gerelateerde 26-genen set uit Hoofdstuk 4. Door verschillende benaderingen te combineren kunnen betere voorspellingen gedaan worden over tamoxifenresistentie bij borstkankerpatiënten, veroorzaakt door ER α S305 fosforylering. Dit is een belangrijke stap naar rationele, persoonsgebonden behandeling van patiënten met het juiste, effectieve medicijn.

Er worden momenteel stappen gezet om een prospectieve, klinische studie te starten naar tamoxifenresistentie door PKA-geïnduceerde ER α S305-fosforylering. Patiënten zullen in twee groepen verdeeld worden op basis van de immunohistochemische kleuring van tumormateriaal met het antilichaam dat ER α S305-P herkent. Zoals eerder aangegeven zou de bepaling van deze vorm van resistentie verbeterd kunnen worden door de kleuring te combineren met een selectief genexpressieprofiel. Aangezien het in kaart brengen van het transcriptoom middels microarrays een routinebepaling is bij borstkanker, zou een genclassifier gemakkelijk geïntegreerd kunnen worden in een dergelijke klinische studie. Als een tumor negatief kleurt voor ER α S305-P, krijgt de patient tamoxifen voorgeschreven als adjuvante behandeling na het verwijderen van de tumor, omdat ze er waarschijnlijk baat bij gaat hebben. Patiënten die positief zijn voor deze fosforylering zullen niet behandeld worden met tamoxifen, maar krijgen daarvoor

in de plaats aromataseremmers, die mogelijk wel werken. De ideale opzet van deze studie zou zijn om de tweede groep een placebo te geven, maar het is onethisch om deze patiënten enige behandeling te weigeren. In een controlegroep zullen patiënten gerandomiseerd worden, waarbij ze tamoxifen of aromataseremmers ontvangen ongeacht hun ER α S305-P status. We verwachten met deze prospectieve studie aan te tonen dat (een deel van de gevallen van) tamoxifenresistentie te voorspellen is aan de hand van ER α S305-P positiviteit in borsttumoren.

Samenvattend heeft fosforylering van ER α op het Serine 305 aminozuur impact op oestrogeenreceptor-eiwitvouwing en coregulatorbinding. Eén enkele post-translationele modificatie, geïnduceerd door PKA, veroorzaakt een relocalisatie van deze receptor op het chromatine, wat het transcriptoom verandert en daarmee uiteindelijk ook de respons op tamoxifenbehandeling bij een patiënt. Een belangrijke vraag die hierop volgt is: zijn er alternatieven voor de behandeling van patiënten die gedefinieerd worden als non-responders op tamoxifen? Eerdere publicaties suggereren dat selectieve oestrogeenreceptor downregulators (SERD), zoals fulvestrant, effectief de oestrogeenreceptoractivatie in kankercellen zouden kunnen remmen, die PKA-geïnduceerde resistentie vertonen tegen tamoxifen. Het effect van PKA activatie, wat leidt tot ER α S305 fosforylering, op het transcriptoom en celgroei lijkt niet geheel tamoxifenspecifiek te zijn, echter voor een deel van de directe gentargets lijkt tamoxifen een additief effect te hebben. Het zou interessant kunnen zijn om te testen of een SERD, zoals fulvestrant, door ER α af te breken tevens het ER-geïnduceerde MYC netwerk kan blokkeren met als gevolg ook de celgroei kan remmen. Een andere hypothese die niet over het hoofd gezien mag worden is dat een patiënt die resistent is tegen één behandeling gevoeliger kan worden voor een andere, welke normaal gesproken minder effectief zou zijn bij dit type borstkanker. Kanker, ongeacht welke vorm, zou resistent kunnen worden tegen een bepaalde therapie door het ontstaan van een zijspoor langs het moleculaire netwerk waarop de therapie gericht is. Zo kunnen de tumorcellen hun groei afhankelijk maken van andere netwerken en daarmee cel- en tumorafsterving vermijden. Op deze andere netwerken kan echter weer mogelijk worden ingegrepen worden door andere medicijnen, waardoor de tumor ineens reageert op een behandeling die normaal gesproken niet (goed) werkt. Prostaatkanker is een goed voorbeeld: chemotherapie in de vorm van taxanen is normaal gesproken niet effectief in prostaatkanker, maar een subgroup van castratieresistente patiënten wordt juist meer gevoelig voor dit type therapie. Aan de basis van deze nieuwe gevoeligheid ligt een verandering van regulatie in celcyclusnetwerken. Bij borstkanker wordt voorspeld dat triple negatieve type borstkanker veel beter zullen reageren op chemotherapie dan lumbinale (ER-positieve) borstkanker. Het zou dan ook interessant kunnen zijn om te testen of ER-positieve tumoren, die resistent zijn tegen hormoontherapie, gevoeliger geworden zijn voor chemotherapie, in vergelijking met de niet-resistente tumoren.

Een uitdaging voor de toekomst is om het hele chromatinebindingslandschap en transcriptoom voor ER α in kaart te brengen voor andere post-translationele modificaties op de receptor en deze te correleren met het wel of niet aanslaan van antioestrogenen en aromataseremmers bij patiënten. Het is belangrijk om deze data te integreren, opdat nieuwe tools ontwikkeld kunnen worden voor het voorspellen van response van iedere, individuele patiënt op verschillende medicijnen en op die manier werkelijk de behandeling tegen borstkanker te kunnen personaliseren. Als we bij diagnose direct kunnen bepalen bij welke therapie een patiënt het meeste baat zal hebben, kunnen we onnodige behandeling met medicijnen die niet werken voorkomen en daarmee ook eventuele akelige bijwerkingen. Tot nu toe wordt de bepaling veelal op basis van trial-and-error bepaald. Door vooraf de juiste behandeling te bepalen, zal kanker minder kans krijgen om te ontwikkelen tot een agressief stadium. Patiëntgerichte behandeling van kanker zal de kans op ziektevrije overleving en de kwaliteit van leven van de kankerpatiënt verbeteren en dit proefschrift beschrijft hoe dit doel mogelijk bereikt kan worden.

List of publications

A Role for Estrogen Receptor Phosphorylation in the Resistance to Tamoxifen

Renée de Leeuw, Jacques Neefjes, Rob Michalides

International Journal of Breast Cancer 2011, Article ID 232435

The hinge region of the human estrogen receptor determines functional synergy between AF-1 and AF-2 in the quantitative response to estradiol and tamoxifen

Renée de Leeuw*, Wilbert Zwart*, Mariska Rondaij, Jacques Neefjes, Michael A. Mancini and Rob Michalides

Journal of Cell Science 2010, Vol.123, pp1253-1261

A high-throughput coregulator peptide array detects estrogen receptor alpha Serine-305 phosphorylation that is associated with response to tamoxifen treatment

Renée de Leeuw*, René Houtman*, Mariska Rondaij*, Diana Melchers, Desiree Verwoerd, Rob Ruijtenbeek, John Martens, Jacques Neefjes, Rob Michalides

Molecular Cancer Therapeutics (in revision)

PKA Phosphorylation redirects oestrogen receptor to promoters of a unique gene set to induce tamoxifen resistance

Renée de Leeuw, Cristiane Bentin Toaldo, Sander Canisius, Jacques Neefjes, Rob Michalides, Wilbert Zwart

Submitted

Curriculum vitae

Renée de Leeuw werd 6 mei 1983 geboren te Breda. Zij genoot haar Gymnasium (VWO) opleiding aan het Stedelijk Gymnasium Breda in 2001 en begon vervolgens haar studie Moleculaire Wetenschappen in Wageningen. In 2004 behaalde zij haar BSc Molecular Sciences en vervolgde haar opleiding in Wageningen met de Master Biomedical Research. Zij liep stage op de afdeling Toxicologie bij Dr. Ivonne Rietjens en Dr. Tinka Murk, waar zij de biobeschikbaarheid van xeno-oestrogenen voor de foetus onderzocht. Haar tweede stage liep zij bij Dr. Jan Hoeijmakers met Dr. Ambra-Giglia Mari en Drs. Wim Vermeulen als begeleiders aan het Erasmus MC te Rotterdam. Daar keek zij naar XPB, een eiwit in het TFIIH transcriptiefactor-complex en van belang voor nucleotide excisie reparatie (NER) van DNA. Een derde stage werd gewijd aan Nurr1, een orphan nucleaire receptor, waarvoor geen ligand bindende pocket of cofactor bindingsplek bekend is. Dit onderzoek voerde zij uit bij Organon Pharmaceuticals onder begeleiding van Drs. Arie Visser. Daarmee voltooide zij tevens haar MSc Biomedical Research in 2006 en verkreeg haar ingenieurstitel (ir.).

In 2006 begon Renée haar promotieonderzoek in de groep van Dr. Rob Michalides op de afdeling Tumorbioologie aan het Nederlands Kanker Instituut te Amsterdam en werd aan het einde begeleid door Prof. Dr. Jacques Neefjes en Dr. Wilbert Zwart. Hier voerde zij haar translationele onderzoek uit, dat gericht was op moleculair en cellulair begrip van tamoxifen resistentie, met name door fosforylering van het serine 305 aminozuur van de oestrogeenreceptor, en de vertaling van ontdekkingen naar de kliniek. Betrokkenheid bij de kliniek blijkt tevens uit vrijwilligerswerk in het Antoni van Leeuwenhoek ziekenhuis.

Na de verdediging van haar proefschrift gaat Renée werken in het lab van Dr. Karen Knudsen aan de Thomas Jefferson University, Kimmel Cancer Center in Philadelphia (VS). Daar verruilt zij de oestrogeen receptor voor de mannelijke equivalent, de androgeenreceptor, en gaat onderzoek doen naar verbetering van therapie voor castratie resistente prostaatkanker.

