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Holding the balance; the equilibrium between ER α -activation, epigenetic alterations and chromatin integrity

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Chapter 3

Posttranslational modification of ERα -part 2

Interaction of 14-3-3 proteins with the estrogen receptor alpha F domain provides a drug target interface

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Abstract

Estrogen receptor alpha (ER α) is involved in numerous physiological and pathological processes, including breast cancer. Breast cancer therapy is therefore currently directed at inhibiting the transcriptional potency of ER α , either by blocking estrogen production through aromatase inhibitors or anti-estrogens that compete for hormone binding. Due to resistance, new treatment modalities are needed and as ER α dimerization is essential for its activity, interference with receptor dimerization offers a new opportunity to exploit in drug design. Here we describe a unique mechanism of how ER α dimerization is negatively controlled by interaction with 14-3-3 proteins at the extreme C terminus of the receptor. Moreover, the small-molecule fusicoccin (FC) stabilizes this ER α /14-3-3 interaction. CocrySTALLIZATION of the trimeric ER α /14-3-3/FC complex provides the structural basis for this stabilization and shows the importance of phosphorylation of the penultimate Threonine (ER α -T594) for high-affinity interaction. We confirm that T594 is a distinct ER α phosphorylation site in the breast cancer cell line MCF-7 using a phospho-T594-specific antibody and by mass spectrometry. In line with its ER α /14-3-3 interaction stabilizing effect, fusicoccin reduces the estradiol-stimulated ER α dimerization, inhibits ER α /chromatin interactions and downstream gene expression, resulting in decreased cell proliferation. Herewith, a unique functional phosphosite and an alternative regulation mechanism of ER α are provided, together with a small molecule that selectively targets this ER α /14-3-3 interface.

Introduction

The estrogen receptor alpha (ERα) is a ligand-dependent transcription factor and the driving force of cell proliferation in 75% of all breast cancers. Current therapeutic strategies to treat these tumors rely on selective ER modulators (SERMs), like tamoxifen (TAM) (1) or aromatase inhibitors (AIs) that block estradiol synthesis (2). Although the benefits of treating hormone-sensitive breast cancers with SERMs and AIs are evident, resistance to treatment is commonly observed (3, 4). To overcome resistance, selective ERα down-regulators (SERDs) can for instance be applied that inhibit ERα signaling through receptor degradation (5, 6). Approaches that target the ERα/DNA or ERα/co-factor interactions are explored as well (5, 7), but other essential steps in the ERα activation cascade are currently unexploited in drug design, also due to a lack of molecular understanding of the processes at hand.

One such step that is crucial for many aspects of ERα functioning is ligand-driven receptor dimerization (8, 9). 17β-Estradiol (E2) association with the ERα ligand binding domain (LBD) drives large conformational changes (10) resulting in ERα dissociation from chaperones (11, 12), unmasking of domains for receptor dimerization, and DNA binding (13, 14). Whereas the LBD contains the main dimerization domain (15), the extreme C-terminal domain of the receptor (F domain) imposes a restraint on dimerization (15, 16), although the regulation of this remains fully elusive. The F domain is a relatively understudied part of the receptor and due to its flexibility, no structural information has been available until now (16). Analysis of F-domain truncation mutants point to an important role for the last few amino acids in receptor dimerization and transactivation activity (17).

Recently, we reported that the diterpene glucoside fusicoccin (FC), a product of the fungus *Phomopsis amygdali* (18), induces apoptosis in a number of cancer cell lines, in synergy with the cytokine IFN alpha (IFNα) (19). In plants, the molecular mechanism of FC's action is highly specific through a unique stabilization of the interaction of 14-3-3 proteins and the C terminus of plasma membrane proton ATPases, with a key role for the penultimate (phosphorylated) Thr of the ATPase (20–22). 14-3-3 Proteins are a family of adapter proteins conserved in all eukaryotic organisms, with key positions in vital cellular processes as well as pathogenesis, like neurodegeneration and tumor development (23, 24). The sequence homology of the extreme C terminus of the plant ATPase and human ERα and the observed effect of FC on the growth of ERα positive breast tumor cells led us to explore the effect of FC on ERα function in these cells.

We show here that ER α interacts with 14-3-3 proteins, with a key role for the penultimate Threonine of ER α (T594). Mutation of T594 strongly enhances the estradiol-dependent ER α dimerization and transactivation. As shown by cocrystallization, binding of the T594 phosphorylated ER α C terminus in the 14-3-3 binding groove leaves a cavity that can be filled by the FC molecule. We confirm that T594 is a distinct ER α phosphorylation site in the breast cancer cell line MCF-7 using a phospho-T594-specific antibody and by mass spectrometry. Furthermore, FC has a negative effect on ER α /chromatin interactions, E2-dependent gene transcription, and cell growth. With this, we provide an alternative ER α regulating mechanism, involving the ER α F domain and provide a unique druggable interface between ER α and 14-3-3 proteins, together with a small molecule (FC) that functions as a proof of principle, which highlights the potential druggability of this protein/protein interaction surface for alternative therapeutics design in breast cancer.

Results

ER α F Domain Interacts with 14-3-3 Proteins.

Sequence alignment of the ER α F domain from a wide range of animals, from human to frog, shows a high degree of variation in amino acid composition, with the exception of the last two amino acids, which are invariably Thr,Val or Thr,Ile (TV or TI) (**Fig. 1A**). This conservation of the ER α C terminus points to a conserved function of the tip and in view of the analogy with the plant ATPase C-terminal tip (Fig. S1A), which is involved in 14-3-3 interactions (22, 25), we performed a yeast-two hybrid (Y2H) assay with the C-terminal half of ER α (ER α -LBD302–595) against all seven human 14-3-3 isoforms. Yeast growth is observed with all 14-3-3 isoforms on triple drop-out plates (**Fig. 1B**), providing evidence for direct physical interaction between these proteins. The penultimate T594 of ER α is essential for 14-3-3 interaction because cells transformed with ER α T594A did not grow (**Fig. 1B**). Helix 12, which is directly N terminal to the F domain, undergoes dramatic conformational changes upon ligand binding (26) and this will most likely change the position of the F domain as well. To test whether ligand binding renders the F domain more accessible for interaction with 14-3-3 proteins, a yeast two-hybrid (β -galactosidase, β -gal) assay was performed to quantitatively assess the ER α /14-3-3 interaction. Both E2 and 4-hydroxytamoxifen (4OH-TAM) strongly enhance the ER α -LBD/14-3-3 θ interaction and again ER α -LBDT594A does not interact with 14-3-3 θ (**Fig. 1C**). Similar results

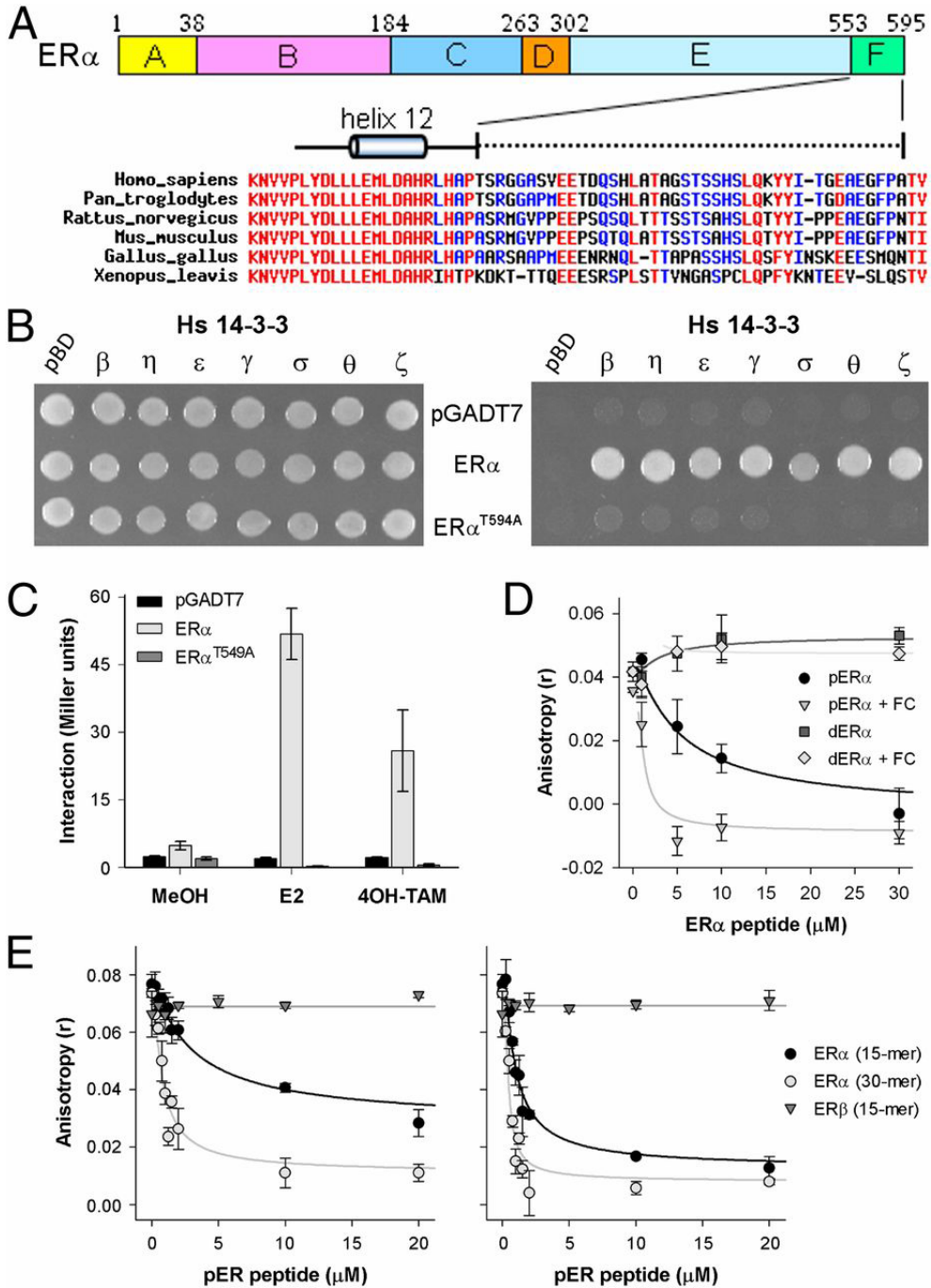


Figure 1: Interaction of ER α and 14-3-3 depends on T594 phosphorylation and is enhanced by FC.

(A) Overview of ER α , with the F domain highlighted and the alignment of the ER α /F domain from various species. (B) ER α -LBD and ER α -LBDT594A interaction with all seven human 14-3-3 isoforms in yeast, tested for colony growth (Left; DDO) and for interaction (Right; TDO). ER α -LBD interacts with all seven human 14-3-3 isoforms, whereas no interaction is observed for ER α -LBDT594A. (C) The 14-3-3 θ interactions with ER α -LBD and ER α -LBDT594A with ER α ligands ($n = 3$, $\pm$ SD) (Fig. S1 B and C). (D) Interaction between 14-3-3 θ and the C-terminal (de)-phospho-ER α peptide, as measured by fluorescence anisotropy, with (open symbols) or without (closed symbols) FC ($n = 2$, $\pm$ SD) (Fig. S1D and Table S1). (E) Comparison of the interaction of 14-3-3 ζ with a short (15 aa) or long (30 aa) C-terminal pER α peptide as well as a short (15 aa) C-terminal pER β peptide with (Right) or without (Left) FC ($n = 2$, $\pm$ SD).

have been obtained with other 14-3-3 isoforms as well as full-length ER α (Fig. S1 B and C), which shows that (ant)agonist binding to the receptor increases the accessibility of the F domain for 14-3-3 interaction. Using a competitive fluorescence anisotropy 14-3-3 assay, we tested if T594 phosphorylation and FC influence the affinity of the ER α F domain for 14-3-3 proteins (27). The ER α F-domain peptide, last 15 amino acids, revealed two aspects of interaction: phosphorylation of T594 is essential for interaction (in support of the Y2H results) and the presence of FC increases the apparent affinity of the peptide 5- to 16-fold, depending on the 14-3-3 isoform used (**Fig. 1D** and Fig. S1D and Table S1). Although the ER β protein contains a penultimate serine residue that can be phosphorylated, no interaction with 14-3-3 protein is observed for the phosphorylated ER β F-domain peptide with or without FC, indicating ER isoform specificity (**Fig. 1E**). Furthermore, a longer ER α F domain phosphopeptide (30 amino acids) is still responsive to FC, while having a higher affinity for 14-3-3 proteins, which suggests that the F domain has multiple points of contact with the 14-3-3 protein (**Fig. 1E**).

Crystal Structure of the Trimeric Complex.

The structural basis for the effects described above was elucidated by co-crystallization of the 15-aa F-domain phosphopeptide (pER α), 14-3-3 σ and FC. First, the peptide was crystallized with the 14-3-3 protein. Crystals were obtained within 5–7 d and could directly be flash cooled and diffracted to 2.02 Å. The 14-3-3 protein displayed the typical, W-like shaped dimer with both

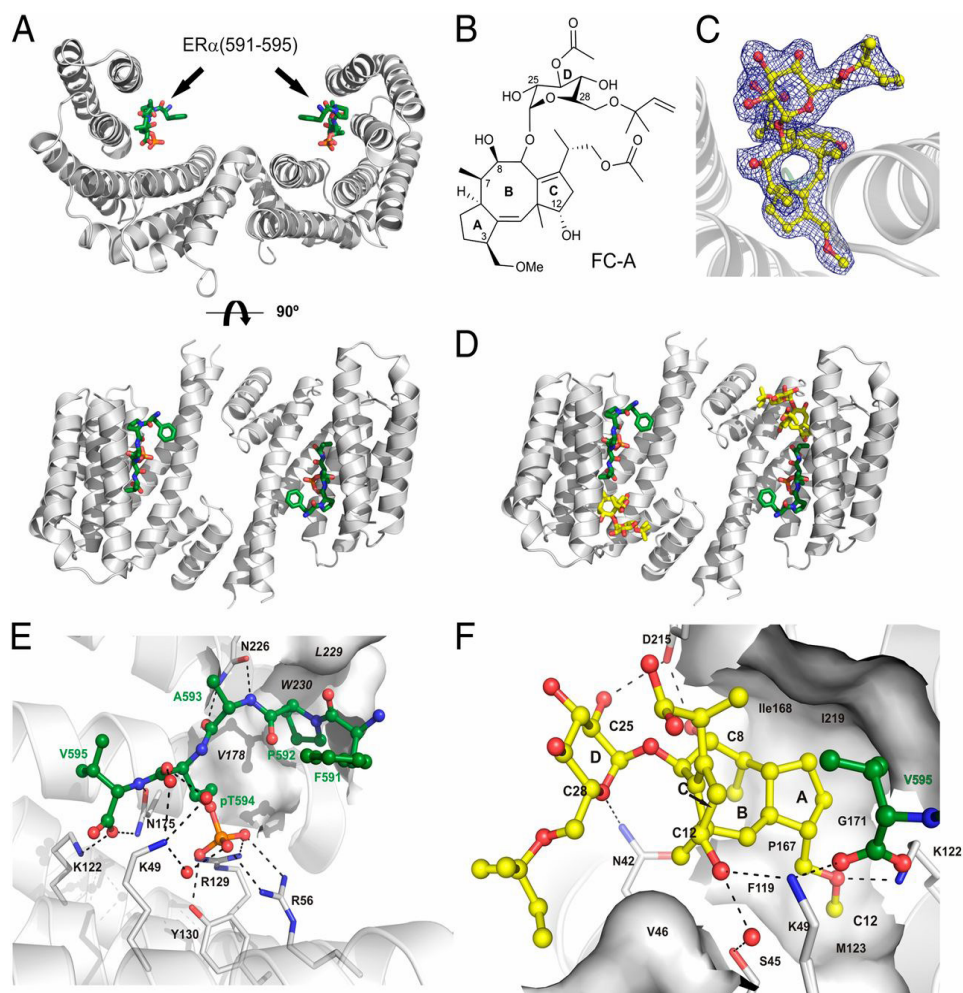


Figure 2: CocrySTALLIZATION of 14-3-3, the phospho-ER α peptide, and fusicoccin. (A) Overview of 14-3-3 σ dimer (gray) complexed with phospho-ER α peptide (green). (B) Structure of fusicoccin A (FC). (C) Electron density map (2Fo-Fc, contoured at 1 σ) of fusicoccin (yellow) bound to 14-3-3/pER α complex. (D) Overview of 14-3-3 dimer (gray) complexed with phospho-ER α peptide (green) and FC (yellow). (E) pER α (green) interaction with 14-3-3 σ (gray). (F) Fusicoccin (yellow) interaction with 14-3-3 σ (gray) and pER α peptide (green). Polar interactions: dashed lines, 14-3-3 residues for interaction, black; hydrophobic 14-3-3 interaction surfaces, white; and water molecules conferring polar interactions, red.

monomers accommodating one ER α peptide (**Fig. 2A** and Fig. S2A). The peptide shows an elongated conformation and is mainly bound by polar contacts with coordination of the phosphate moiety of pT594 by 14-3-3's K49, R56, R129 and Y130. To determine how FC (**Fig. 2B**) acts on this protein complex, we soaked binary 14-3-3 σ /pER α crystals with FC. Clear additional electron density for the FC molecule could be determined (**Fig. 2C**), allowing the unambiguous spatial determination of the binding mode. One FC molecule is coordinated by each 14-3-3 monomer sitting right next to the C terminus of the pER α peptide (**Fig. 2D** and Fig. S2B). Here, FC is contacting both protein partners thereby filling a gap in the interface of 14-3-3 and pER α (**Fig. 2E and F** and Fig. S2C). Binding of FC to the binary 14-3-3 σ /pER α complex seems to be mainly driven by entropic effects and shape complementarity. FC covers 147.1 Å² of solvent-exposed surface in the complex and dislocates at least 19 water molecules. Because the free, protein-unbound form of FC (28) is very similar to the structure of FC observed in our ternary complex, also the entropy penalty upon binding of FC is expected to be rather low (see also Table S2).

ER α C Terminus Controls Receptor Dimerization.

To examine the capacity of FC to bind endogenous 14-3-3 and ER α , an affinity pull-down with FC beads (FC was coupled covalently to magnetic hydrazide beads after changing the vinyl group into a reactive aldehyde) was performed in a lysate prepared from MCF-7 cells. The FC beads were first functionally tested (Fig. S3 A and B). Subsequently, a pull-down with MCF-7 cell lysate was performed and this shows that both endogenous 14-3-3 and ER α bind specifically to the FC beads (**Fig. 3A**). In a reverse pull-down experiment with recombinant ER α -LBD as bait, FC also enhanced the binding of 14-3-3 proteins to ER α (**Fig. 3B**). Next, we addressed the question how 14-3-3 protein interaction affects ER α function. In view of the reported function of the F domain in receptor dimerization (16), we tested whether 14-3-3 binding and FC interfere with receptor dimerization. A Y2H β -gal assay with ER α -LBD or ER α -LBDT594A confirmed that the F-domain C terminus controls receptor dimerization (**Fig. 3C**), as reported before (17). Strikingly, the T594A mutation, which annihilates the 14-3-3 interaction (**Fig. 1 B and C**), strongly enhances (ant)agonist-driven receptor dimerization. Similar results have been obtained with full-length ER α or ER α T594A (Fig. S3C). To test whether FC affects ER α dimerization in human cells as well, two N-terminally tagged ER α constructs, HA-ER α and GFP-ER α , were expressed in

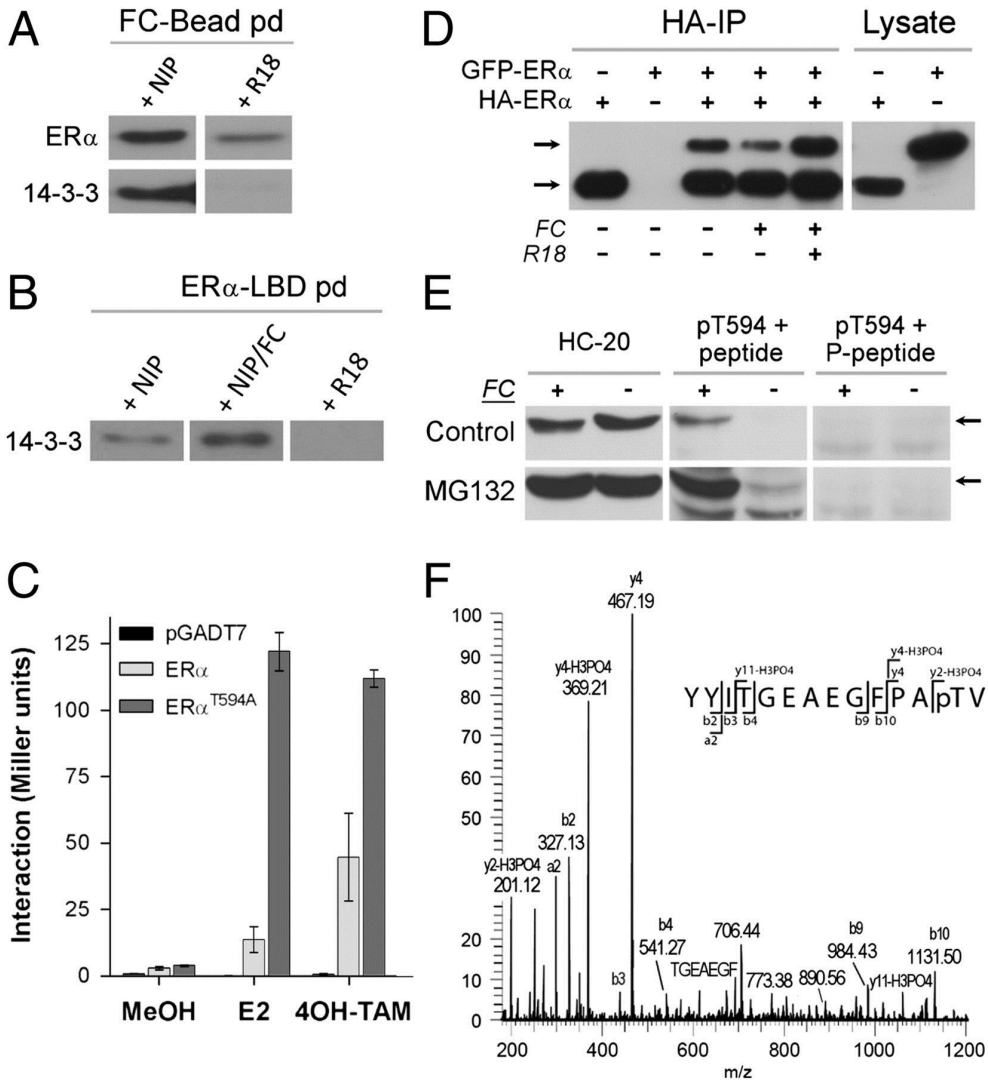


Fig. 3: 14-3-3 Interaction with ER α affects ER α dimerization and T594 is phosphorylated in MCF-7 cells.

(A) Pull-down with FC-coated beads isolates endogenous ER α and 14-3-3 (Western blot) from MCF-7 cell lysate; NIP, noninteracting peptide; R18, 14-3-3 blocking peptide (see also Fig. S3 A and B). (B) Endogenous 14-3-3 binding to recombinant ER α -LBD in the presence of NIP, NIP+FC, and R18. (C) Yeast two-hybrid assay with ER α -LBD/ER α -LBD, and ER α -LBDT594A/ER α -LBDT594A showing enhanced dimerization of the ER α mutant (E2, 17 β -estradiol; 4OH-TAM, tamoxifen (*n*

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= 3, $\pm$ SD) (Fig. S3C). (D) Western blot analysis (HC-20 antibody) of HA-ER α IP from HEK293 cells expressing HA-ER α and GFP-ER α ; cells treated with FC (10 μ M) show reduced dimerization, whereas R18 added to the cell lysate enhances the dimerization. (E) Western blot analysis with the HC-20 and pT594 antibodies of cell lysate from MCF-7 cells treated with combinations of the proteasome inhibitor MG132 (5 μ M) and/or FC (30 μ M). The pT594 antibody was used in the presence of the nonphosphorylated (Center) and the T594 phosphorylated ER α peptide (Right). (F) Mass-spectrometry analysis of the C-terminal ER α peptide purified from a trypsin digested MCF-7 cells lysate with the pT594 antibody. Shown is the tandem MS (MS2) spectrum of the C-terminal ER α tryptic peptide showing modification by phosphorylation at threonine 594 (Fig. S5).

HEK293 cells. Immunoprecipitation (IP) of HA-ER α shows receptor dimerization: besides HA-ER α also GFP-ER α is present in the IP (**Fig. 3D**, lane 3). Cells treated with FC show less GFP-ER α in the IP, whereas inclusion of the 14-3-3 competing R18 peptide in the cell lysate during the IP strongly enhances dimerization. These results are in line with the Y2H results and suggest that interaction of 14-3-3 proteins at the ER α C terminus has a negative effect on receptor dimerization.

T594 Is a Distinct ER α Phosphosite.

Thus far, experimental evidence for ER α -T594 phosphorylation has not been described in the literature. However, all evidence shown above indicates that T594 phosphorylation is essential for creating a high-affinity 14-3-3 binding site at the ER α C terminus. To demonstrate endogenous ER α -T594 phosphorylation, we generated an antibody that specifically recognizes the phosphorylated T594 residue. Specificity of the pT594 antibody is demonstrated with a dot blot (Fig. S4A) and Western blotting of cell lysate of HEK293 cells expressing ER α , ER α -T594A, and ER α - Δ 4 (Fig. S4B). Next, we did Western blots using the ER α common antibody (HC-20) and the pT594 antibody on cell lysate from MCF-7 cells that were treated without or with FC for 24 h (**Fig. 3E**). Whereas control cells do not show a band recognized by the pT594 antibody, cells treated with FC clearly show a band, which disappears when the antibody is blocked with its antigen, the pT594 peptide. When cells are also treated with the proteasome inhibitor MG132, phosphorylated ER α is already detectable without FC treatment (**Fig. 3E**) and with FC the effect on T594 phosphorylation is even more prominent. To confirm that T594 is a genuine phosphoresidue, we digested the FC/MG132 MCF-7 cell lysate

with trypsin and used the pT594 antibody to IP the C-terminal ER α phosphopeptide. Mass-spectrometry analysis of this fraction identified the C-terminal ER α peptide (14 aa) with T594 phosphorylated (**Fig. 3F** and **Fig. S5**). We conclude that T594 is a phosphorylated residue in MCF-7 cells and that FC “protects” the T594 phosphosite resulting in increased phosphorylation.

FC Reduces Genome-Wide Chromatin Interactions of ER α .

Because ER α interacts with DNA as a dimer, we expected that an FC/14-3-3-induced reduction of receptor dimerization would prevent ER α /DNA interactions. Chromatin immunoprecipitation (ChIP) was performed for ER α , and the receptor/chromatin interaction for two well-described ER α binding events [nuclear receptor-interacting protein 1 (NRIP1) and X-box binding protein 1 (XBP1)] (29) was studied. For both ER α binding sites, FC significantly reduced the chromatin interaction of ER α as well as its coactivator amplified in breast cancer 1 (AIB1) (**Fig. 4A**). To assess the effect of FC on ER α /chromatin associations on a genome-wide scale, ChIP was followed by high-throughput sequencing (ChIP-seq). Again, FC decreased ER α /chromatin interactions, and peak intensities were decreased by FC treatment (**Fig. 4B**). Under control conditions, 26,987 ER α binding events were found on a genome-wide scale (**Fig. 4C**). This number of binding events was greatly diminished by FC treatment, where 16,829 ER α sites were found. The sites shared under both control and FC conditions (“shared regions”) were the strongest ER α binding events, which were significantly lowered in intensity by FC treatment (**Fig. 4D** and quantified in **Fig. 4E**). The less strong ER α binding sites were unique for the control conditions (“control unique”) and lost due to an FC-induced decrease of peak intensity beyond the detection threshold of the peak-calling algorithm. Consequently, the number of ER α peaks decreased upon FC treatment (**Fig. 4C**). No selectivity was observed for the type of ER α interaction that was lost (monomer versus dimer) based on DNA motif analysis or whether they were mediated by direct ER α /DNA binding or through specificity protein 1 (SP1) or complexes of the transcription factors Fos and Jun (Fos/Jun) (**Fig. S6**). These results show that FC-mediated loss of ER α /chromatin interaction is highly effective, nonselective for the mode of ER α chromatin interactions as based on DNA motif analysis, and occurs genome-wide.

Fusicoccin Reduces ER α Transactivation and Cell Growth.

Next, the biological consequences of FC-induced ER α /14-3-3 stabilization and reduced ER α /chromatin interactions were investigated. First, the influ-

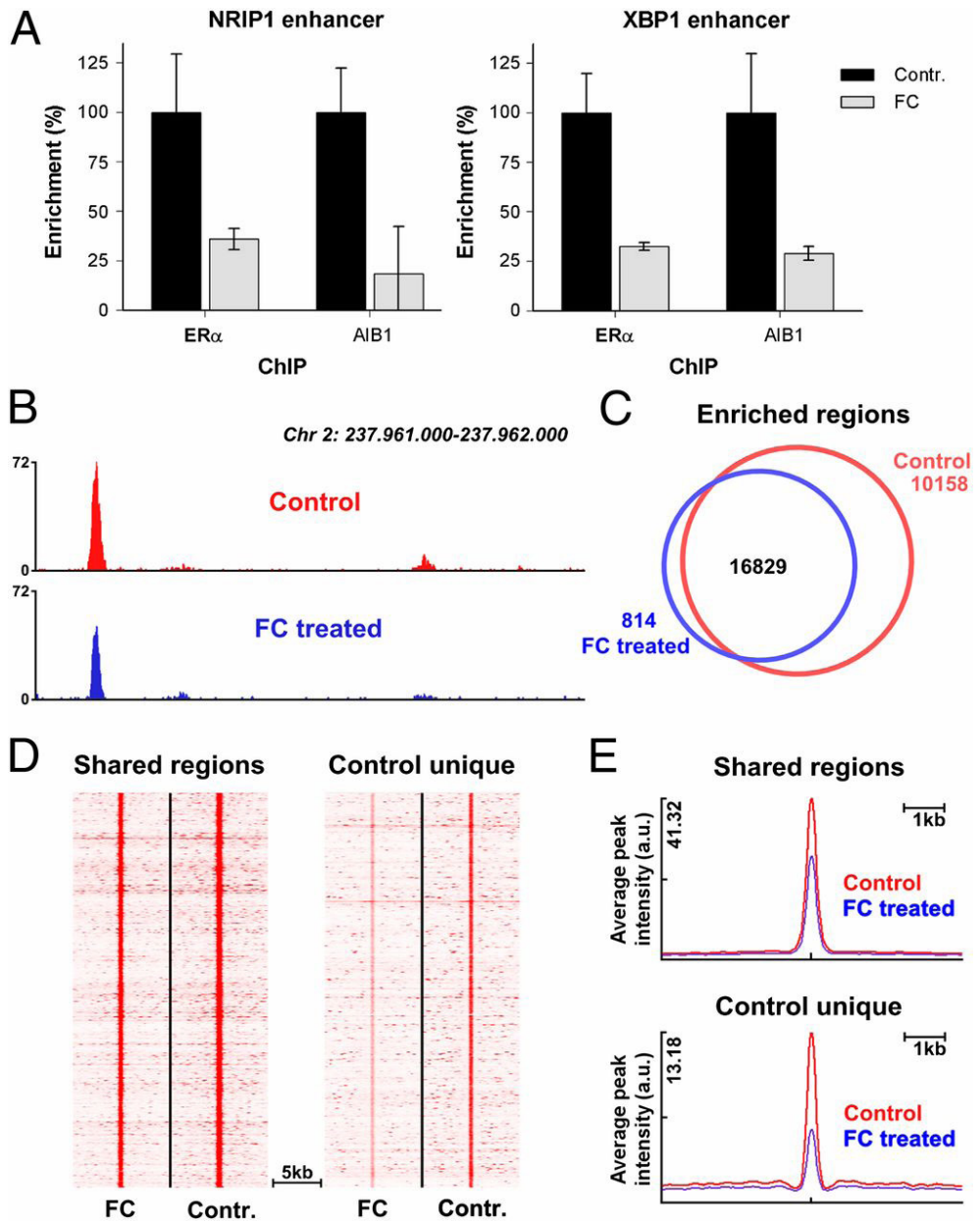


Fig. 4: FC reduces genome-wide chromatin/ER α interactions.

(A) qPCR of NRIP1 and XBP1 enhancer elements after ChIP for ER α and AIB1 in the absence or presence of 10 μ M FC ($n = 3$, $\pm$ SD). (B) Genome browser snapshot, illustrating decrease of ER α /chromatin interaction after FC treatment. Genomic

coordinates and tag count are indicated. (C) Venn diagram showing ER α binding events in absence (red) and presence (blue) of FC. (D) Heatmap visualizing intensity of ER α binding events in FC and control-treated cells at regions found under both conditions (shared; Left) and sites that are lost after FC treatment (control unique; Right) (E) Average peak intensity of ER α binding sites as visualized in D.

ence of FC on ER α transcriptional activity was tested, as well as the role of the F-domain C-terminal tip therein. To rule out any influence of endogenous receptor, we made use of ER α -negative human osteosarcoma cell line U2OS, a well-annotated model system for ER α action (30). ER α -mediated estrogen response element-luciferase reporter (ERE-luc) expression was measured in U2OS cells cotransfected with ER α wild type (ER α -WT) and two C-terminal mutants: ER α -T594A and ER α - Δ 4, a construct lacking the last four amino acids. ER α -T594A may still exhibit partial FC sensitivity, because studies on the FC target in plants (the H⁺-ATPase) have shown that interaction of the nonphosphorylated H⁺-ATPase and 14-3-3 proteins do occur, provided that FC is present (31, 32). ER α - Δ 4 should be FC insensitive because the amino acids that line the 14-3-3 groove and contact the FC molecule (**Fig. 2**) are missing. As shown in **Fig. 5A**, FC significantly reduces the ER α -WT transcriptional activity in a dose-dependent manner, with an inhibition of more than 60% at 1 nM E2. The transcriptional activity of ER α - Δ 4 is indeed unaffected by FC and is much higher than that of ER α -WT (note that the scale of the y axis is different). Cells transfected with ER α -T594A also show enhanced transcriptional activity compared with ER α -WT in the absence of FC and, as expected, the transcriptional activity shows some FC sensitivity, albeit less than that of ER α -WT. These experiments illustrate that the ER α C terminus is essential for regulating ER α activity and for the inhibitory effect of FC thereon. Furthermore, FC does not affect the transcriptional activity of ER β (**Fig. 5B**), indicating that the 14-3-3/FC interaction is indeed isoform specific (see also Fig. 1E).

To further determine the effect of FC on endogenous ER α -mediated gene transcription, we analyzed transcript levels of a number of E2-dependent genes in the absence and presence of FC. As shown in **Fig. 5C**, FC treatment significantly reduced E2-mediated transcription of these genes. In line with these data, FC treatment significantly inhibited E2-induced cell proliferation in a dose-dependent manner (), and this effect on proliferation was not apoptosis related (Fig. S7).

Cumulatively, we have shown a unique mode of ER α inhibition in-

Posttranslational modification of ER α - part 2

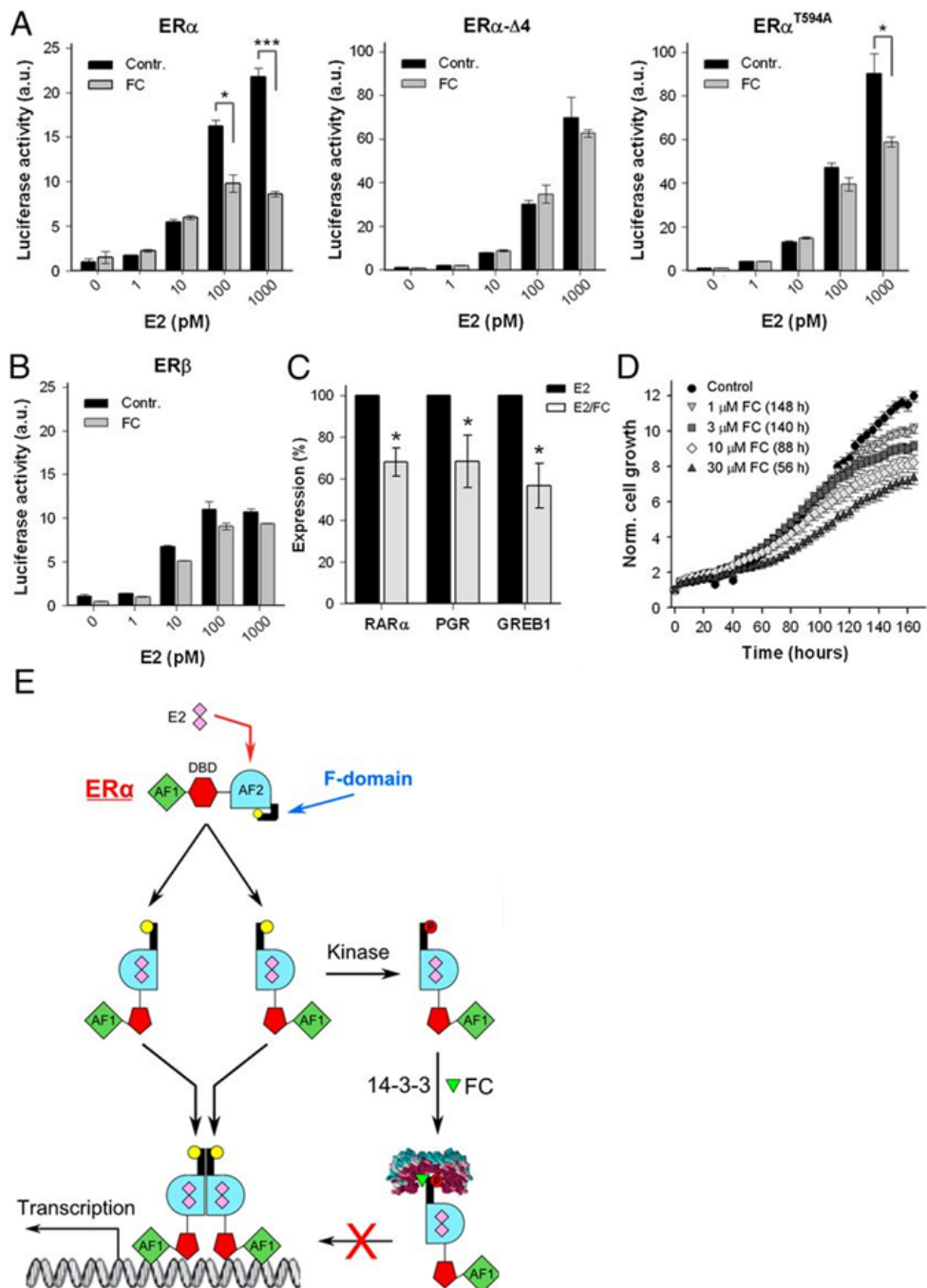


Fig. 5: Effect of fusicoccin on ER α gene activation and cell growth.

(A) Normalized transactivation activity (ERE-luciferase assay) of ER α , ER $\alpha\Delta 4$ (lacking the last four amino acids), and ER α T594A, for various E2 concentrations in the presence and absence of 10 μ M FC ($n = 3$, $\pm$ SD * $P < 0.05$; *** $P < 0.001$). (B) Same as A, now analyzing the normalized ER β transactivation ($n = 2$, $\pm$ SD). (C) qPCR expression analysis of ER α regulated genes [progesterone receptor (PGR), retinoid acid receptor alpha (RAR α), and gene regulated by estrogen in breast cancer 1 (GREB1)] in hormone-deprived MCF-7 cells treated with E2 (10 nM) or E2/FC (10 μ M) ($n = 7$, $\pm$ SEM, * $P < 0.05$). (D) E2-induced MCF-7 cell proliferation is inhibited by FC. Time to reach significant ($P < 0.05$) inhibition is indicated in parentheses ($n = 12$, $\pm$ SEM) (Fig. S6). (E) Model showing ER α activation, the function of 14-3-3, and FC on the F domain and receptor activation. Ligand binding (E2) drives conformational changes that displace the F domain, which enables receptor dimerization and transcriptional activation. Displacement of the F domain also renders the C-terminal tip (yellow) accessible for phosphorylation of T594 (red). Subsequent 14-3-3 binding, and stabilization by FC, keeps the receptor in a monomeric state, thereby reducing DNA interaction, gene transcription, and cell growth.

volving the newly identified phosphorylated T594 residue, which operates through the interaction of the ER α F-domain tip with 14-3-3 proteins. Stabilizing this ER α /14-3-3 interaction through small molecule inhibitors like FC suffices in functionally reducing ER α /DNA interactions, gene transcription, and cell proliferation.

Discussion

Blocking ER α functioning is the major treatment modality in luminal breast cancer (33–35). Most efforts to modulate the ER α activity have focused on a single pocket buried in the ER α protein, where agonists, antagonists, and selective modulators interact with ER α : the ligand-binding pocket. Because treatment resistance is commonly observed, focus is shifting toward the identification of small-molecule inhibitors that target sites outside this ligand-binding pocket, like the coactivator-binding groove, allosteric sites in the LBD, and the interface for DNA contact (34, 36). Receptor dimerization is an essential step in the cascade of events through which ER α modulates gene expression and therefore any changes that alter ER α dimerization will have profound effects on ER α function. After binding of ligand, ER α monomers undergo dramatic conformational changes exposing sequences required for dimerization and evidence has been presented that the carboxy terminal

F domain imparts internal restraint on ER dimerization (17, 37). Notably, mutations in the last few amino acids of the F domain somehow relieve the restraint on dimerization imposed by the F domain and enhance transcriptional activity (17). Understanding the molecular mechanism that gives the F-domain C terminus control over ER α dimerization will provide new tools to interfere with the ligand-driven dimerization process and thus ligand-dependent ER α activation in ER α -positive tumor cells.

In this report we demonstrate that the ER α F-domain C terminus contains a mode-III binding motif for 14-3-3 proteins (38) and moreover, that the ER α /14-3-3 interface can be targeted by the small-molecule FC. The effect of FC described here is unique among all known small molecules that modulate the ER α activity, as it targets a unique protein–protein interaction interface and stabilizes rather than disturbs an ER α /macromolecule interaction. This mode of FC action, which can be described as a “molecular glue,” has been well documented for the plant ATPase/14-3-3 interaction (21, 22) and this study shows that the compound/substrate interactions, as well as their functional consequences, are conserved across species.

At the molecular level, the Y2H, fluorescence anisotropy and cocrystallization studies all point to an alternative mechanism of ER α regulation, where 14-3-3 proteins interact with the very C terminus of the ER α F domain, with a key role for phosphorylation of the penultimate T594. We uniquely demonstrate that T594 is an *in vivo* phosphosite in the breast cancer cell line MCF-7. Because phosphorylated ER α accumulates in cells where proteosomal degradation is inhibited, we hypothesize that the T594 phosphorylated ER α is a short-lived intermediate in the cycle of receptor activation/degradation. This may be the reason why phosphorylation of T594 has gone unnoticed thus far. FC clearly enhances the level of T594 phosphorylation, probably because the phosphosite is shielded from phosphatase activity by an increase in affinity for 14-3-3 proteins, a well-known effect described for the FC target in plants, the H⁺-ATPase (39, 40). This mode-III 14-3-3 interaction provides the framework for a model where the ER α C terminus negatively affects receptor dimerization, consistent with previously published work (17), through interaction with 14-3-3 proteins, as shown here. At the cellular level, the (FC stabilized) interaction between ER α and 14-3-3s negatively affects receptor/DNA interactions, the transactivation activity and ER α -dependent cell growth. Furthermore, this interaction can be targeted by small molecules, like FC, and FC is receptor specific as it only targets ER α without affecting ER β , which is a positive feature in view of the antiproliferative role described

for ER β (41, 42).

Taken together, our results establish an alternative and selective mode of ER α regulation (Fig. 5E), where the receptor's F domain becomes amenable for interaction with 14-3-3 proteins after ligand binding. FC is a small molecule ligand that specifically modulates the interaction surface between ER α and its regulatory 14-3-3 protein, albeit at a relatively low affinity. Therefore, this small molecule and related fusicoccans (43) may provide the very basis for the development of an entirely unique class of antiestrogenic compounds in the treatment of breast cancer.

Materials and Methods

Human ER α (WT or T594A point mutant) and/or 14-3-3 proteins were transfected in yeast cells, using the lithium acetate method (44), to analyze their interaction or study ER α dimerization in a Y2H assay. Double dropout plates (DDOs) were used to check for colony viability and triple dropout plates (TDOs) to test for interaction. The interaction in the presence of various ligands was quantified with a yeast two-hybrid β -galactosidase assay as described before (44).

Competitive anisotropy measurements were performed with ER α peptides consisting of the last 15 (short) or 30 (long) amino acids of ER α , with T594 being phosphorylated (pER α) or dephosphorylated (dER α). In this, the peptides need to compete with the carboxyfluorescein labeled SWpTY peptide (FAM-SWpTY, where pT indicates phosphorylated Threonine) for 14-3-3 binding as described before (27).

In pull-down assays, with GST-ER α -LBD or FC-coated beads, MCF-7 lysate was mixed with a noninteracting peptide (NIP) or the 14-3-3 interacting R18 peptide. The associated endogenous ER α and/or 14-3-3 proteins were subsequently visualized by Western blotting.

ER α activity was measured with an ERE-Luc assay in transfected U2OS cells, using the Dual-Luciferase Reporter Assay (Promega). MCF-7 cell growth and apoptosis induction, treated with FC or methanol and E2, was measured on the IncuCyte FLR (Essen BioScience), using a CellPlayer 96-Well Kinetic Caspase-3/7 apoptosis assay kit. Cell confluence and apoptosis was determined by analyses of phase-contrast/fluorescent images using an algorithm from Confluence v1.5 in combination with IncuCyte software.

For the identification of the phosphorylated C-terminal ER α peptide, cell lysate from MG132/FC-treated MCF-7 cells was trypsin digested and the pT594 antibody was used to IP the phosphopeptide. MS/MS spectra of the

eluted peptides were acquired with a Q Exactive mass spectrometer (ThermoScientific).

ChIPs were performed as described previously (29). Sequences were generated on the Illumina HisEq. 2000 and aligned to the human reference genome. Tools used for enriched region analyses, motif analyses, data snapshots, and heatmap generation are described in SI Materials and Methods. For gene expression analyses equal amounts of cDNA from (un)treated MCF-7 cells were analyzed with SYBR Green (Applied Biosystems) and an MJ Opticon Monitor (BioRad). Data were analyzed with qgene96.

The complex of 14-3-3 σ Δ c (amino acids 1–231) and the short pER α peptide was crystallized using the hanging-drop method. The structure was solved by molecular replacement using Protein Data Bank (PDB) ID: 3P1N as template. The ternary complex was produced by soaking fusaric acid into the binary crystals. Details are described in SI Materials and Methods. The structures of the 14-3-3 σ Δ c/pER α (4JC3) and the 14-3-3 σ Δ c/pER α /FC (4JDD) complexes have been deposited in the PDB.

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The authors declare no conflict of interest.

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Supplementary information

Fig. S1. C-terminal ER α F domain tip interacts with 14-3-3 proteins.

(A) Alignment of the last 52 amino acids of human ER α and the H⁺-ATPase of *Nicotiana glauca* (PMA2) and *Arabidopsis thaliana* (AHA2). The three amino acids most important for the FC/14-3-3/receptor complex formation are indicated (↓). (B) Yeast two-hybrid assay; ligands stimulate the interaction of various 14-3-3 isoforms with WT ER α -LBD, but not ER α -LBDT594A. (C) Quantification of the interaction of 14-3-3 θ with full-length WT ER α and ER α T594A, in the presence of ER α ligands. (D) Analysis of the interaction between the short (15 aa) phosphorylated C-terminal ER α peptide (pER α) and all human 14-3-3 isoforms and 14-3-3 σ -AC. Curves are fitted as described in SI Material and Methods.

Fig. S2. CocrySTALLIZATION of 14-3-3 and the pER α peptide and soaked fusicoccin.

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(A) Stereoview of the pER α peptide (green) in the 2Fo-Fc density map (contoured at 1σ) occupying half of the amphipathic 14-3-3 binding groove (white surface). (B) Stereoview of the pER α peptide (green) and fusicoccin (yellow) in the 2Fo-Fc density map (contoured at 1σ) occupying the amphipathic full binding groove of a 14-3-3 σ monomer (white). (C) Detailed view of the interaction of ER α (green) and fusicoccin (yellow) with 14-3-3 σ (white). Polar interactions are indicated by dashed lines and 14-3-3 residues implicated in these interactions are labeled in black. Hydrophobic 14-3-3 interaction surface is depicted as white solid surface. Red spheres are water molecules conferring polar interactions.

Fig. S3. Functional test on FC beads and 14-3-3 interaction with full-length ER α affects dimerization.

(A) Specifically bound endogenous 14-3-3 proteins can only be eluted from FC-coupled beads (FC beads) with the 14-3-3 interacting R18 peptide, when bound in the presence of a C-terminal peptide derived from the plant H⁺-ATPase (YpTV) and not with a noninteracting peptide (NIP). (B) 14-3-3 Western blot on the pull-down samples of A confirms the specificity of the 14-3-3 interaction to the FC-coupled beads. (C) Yeast two-hybrid assay for ER α dimerization: The full-length-ER α /ER α -LBD and full length-ER α T594A/ER α -LBDT594A dimerize in the presence of ER α ligands.

Fig. S4. The pT594-antibody is specific for the T594-phosphorylated ER α C-terminal epitope.

(A) Dot blot with different amounts of nonphosphorylated (peptide: KYIITGEAEGFPATV) and phosphorylated peptide (P-peptide: KYIITGEAEGFPATP V) spotted. The blot was probed with the pT594-ER α antibody. (B) Western blot with HC-20 antibody and pT594-ER α antibody on cell lysate of HEK293 cells expressing wild-type ER α , ER α -T594A, and ER α - $\Delta 4$; cells were grown with FC (10 μ M) for 24 h. Whereas the HC-20 antibody shows that ER α is expressed in all three transfected cells, the pT594-ER α antibody only recognizes a band in wild-type ER α transfected cells. This demonstrates that the pT594-ER α antibody is specific for the T594 phosphorylated ER α protein.

Fig. S5. Mass-spectrometry analysis of the C-terminal ER α peptide shows phosphorylation of the penultimate threonine, T594.

(A) Extracted ion chromatogram of synthetic ER α C-terminal tryptic phosphopeptide YYITGEAEGFPAPTV phosphorylated at T594. The extraction window is m/z 799.31–38. (B) The corresponding peptide from MCF-7 digest, after IP is shown. In A and B coelution at 89–90 min is observed. (C) Accurate intact mass of the peptide, corresponding to the $[M+2H]^2+$ monoisotopic peak at m/z 799.3447, for the synthetic and MCF7-derived phosphopeptide, respectively. (D) See C. (E) MS/MS spectrum of synthetic YYITGEAEGFPAPTV. The absence of basic residues (protein C terminus) and presence of acidic residues, as well as a phosphate

group, result in a poor MS/MS spectrum. However, the y2-H3PO4 (m/z 201.1234), y4 (m/z 467.1901), and y4-H3PO4 (m/z 369.2132) ions conclusively localize the phosphate group to T594. (F) Corresponding MS/MS spectrum for the MCF-7-derived phosphopeptide. The fragment ions b2, a2, y2-H3PO4, y4-H3PO4, and y4 ions are detected in similar intensity ratios as observed for the synthetic phosphopeptide identifying the MCF-7-derived phosphopeptide and localizing the site of phosphorylation to T594.

Fig. S6. ER α /DNA binding mode is not selectively altered by FC treatment.

(A) Top three motifs for the shared (Left) and control unique (Right) ER α binding events. (B) Average peak intensity of shared (Left) and control unique (Right) ER α binding events mediated directly by ER α or through specificity protein 1 (SP1) and complexes of the transcription factors Fos and Jun (Fos/Jun) as identified through motif scanning of ER α -binding sites, in the absence (red) or presence (blue) of FC.

Fig. S7. Fusicoccin does not induce apoptosis in E2-treated MCF-7 cells.

E2-induced MCF-7 apoptosis was assessed over time in the presence of various FC concentrations (30 μ M FC shown here), by analyzing the CellPlayer 96-Well Kinetic Caspase-3/7 Apoptosis assay kit. For all FC concentrations, no significant apoptosis induction was observed.

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Fig. S1

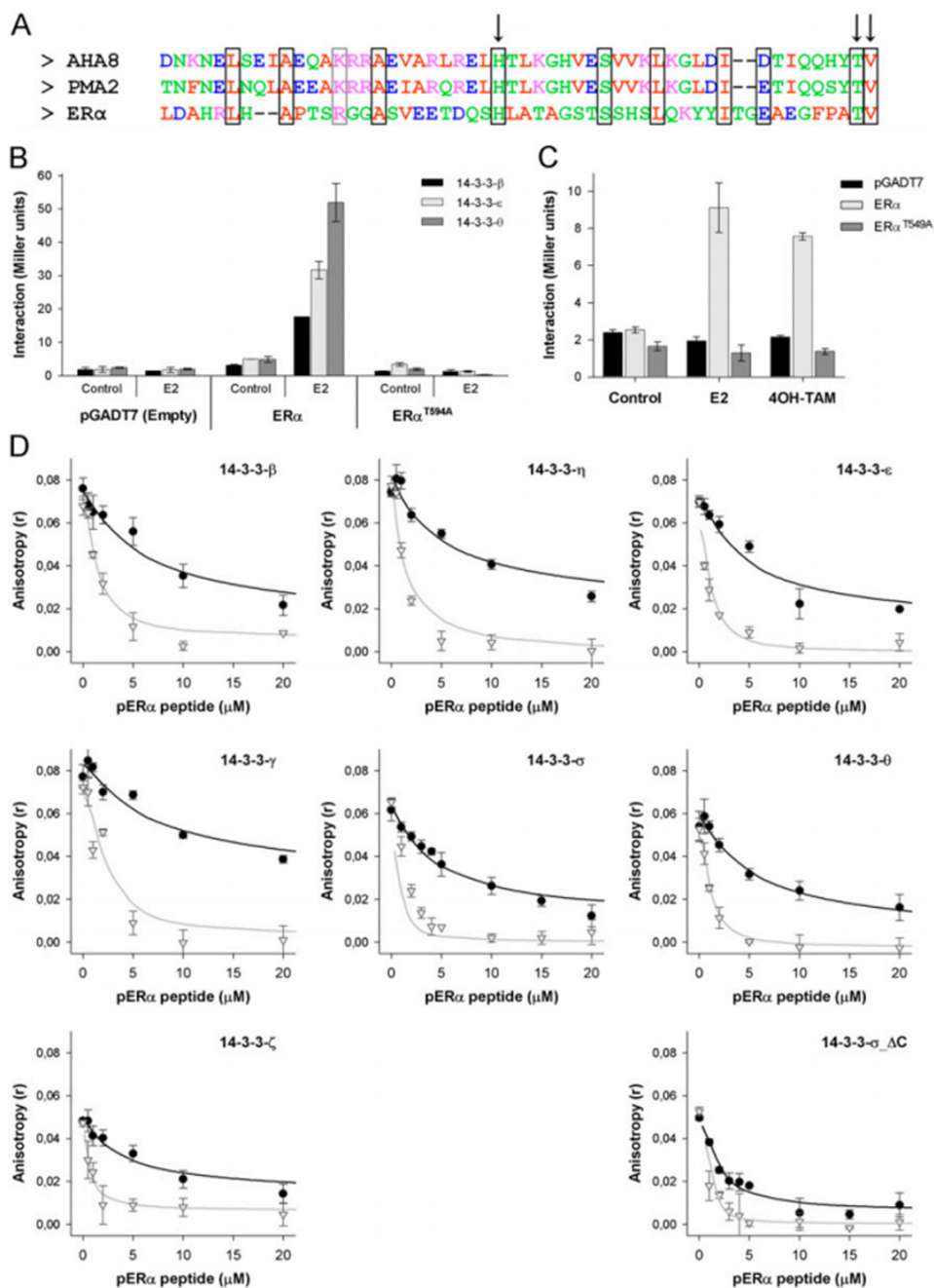


Fig. S2

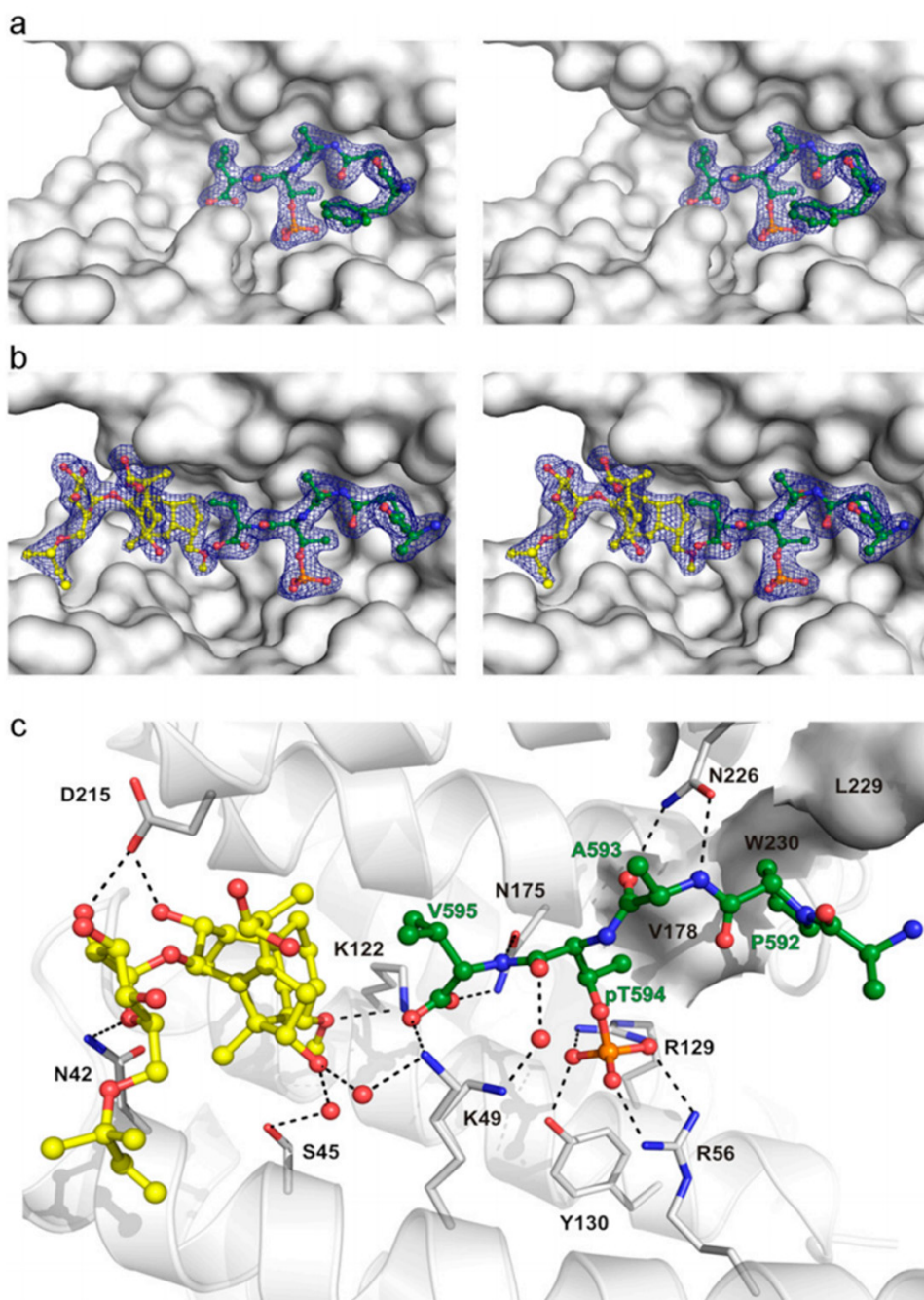


Fig. S3

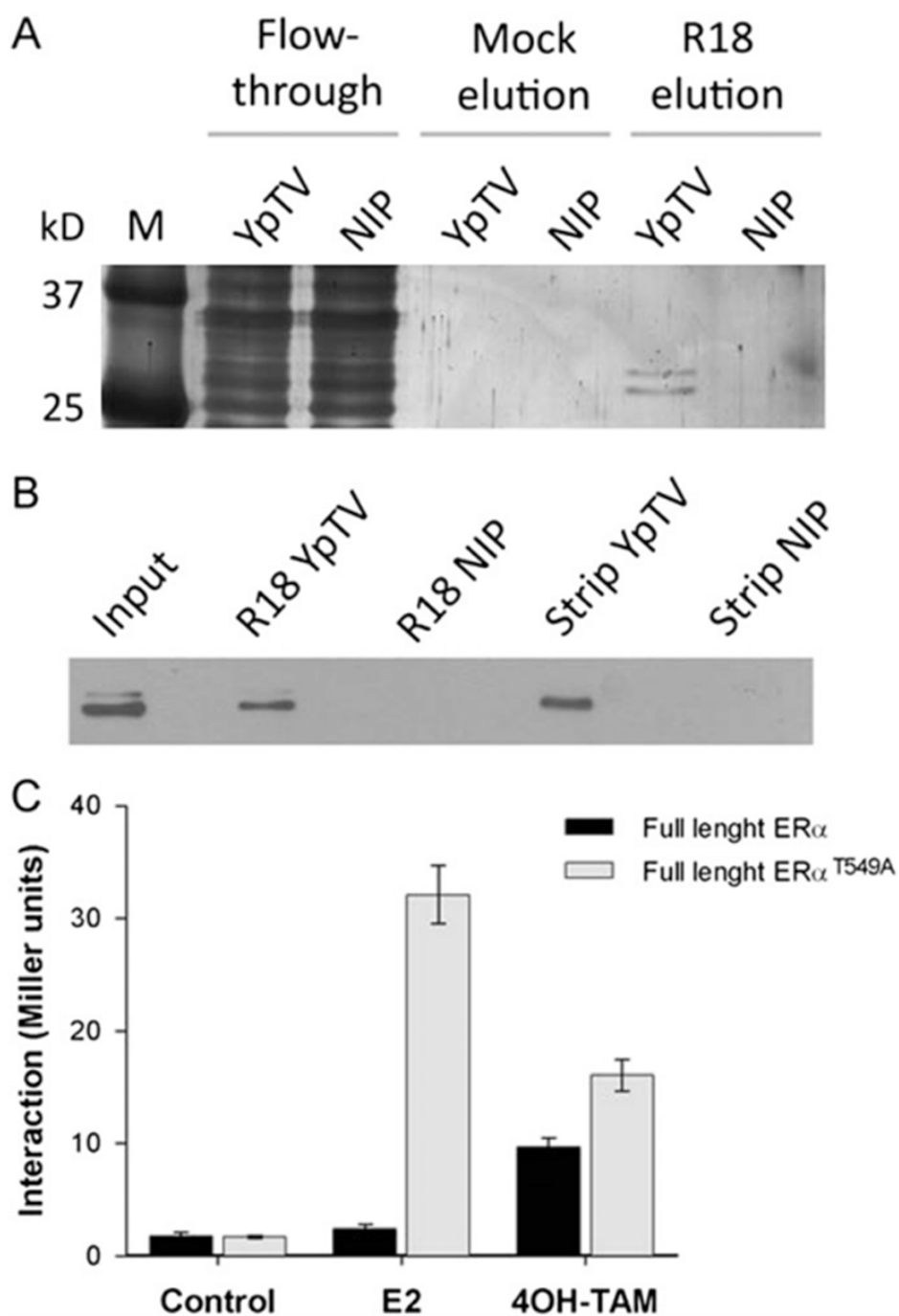


Fig. S4

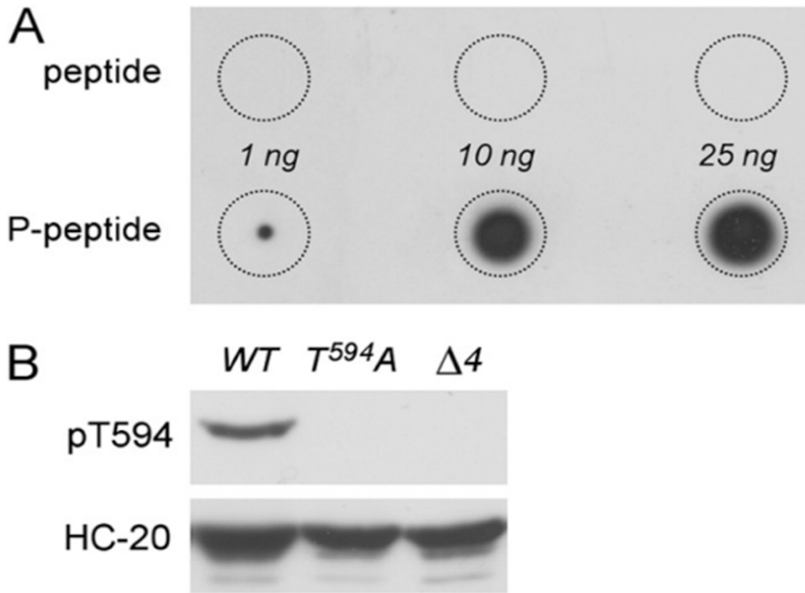
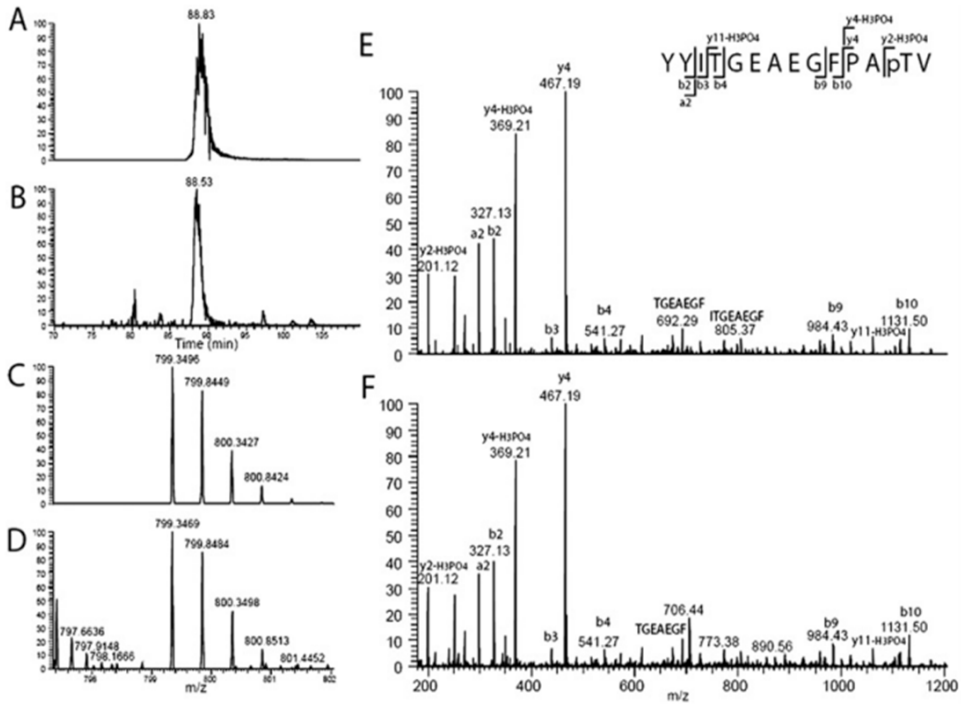


Fig. S5



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Fig. S6

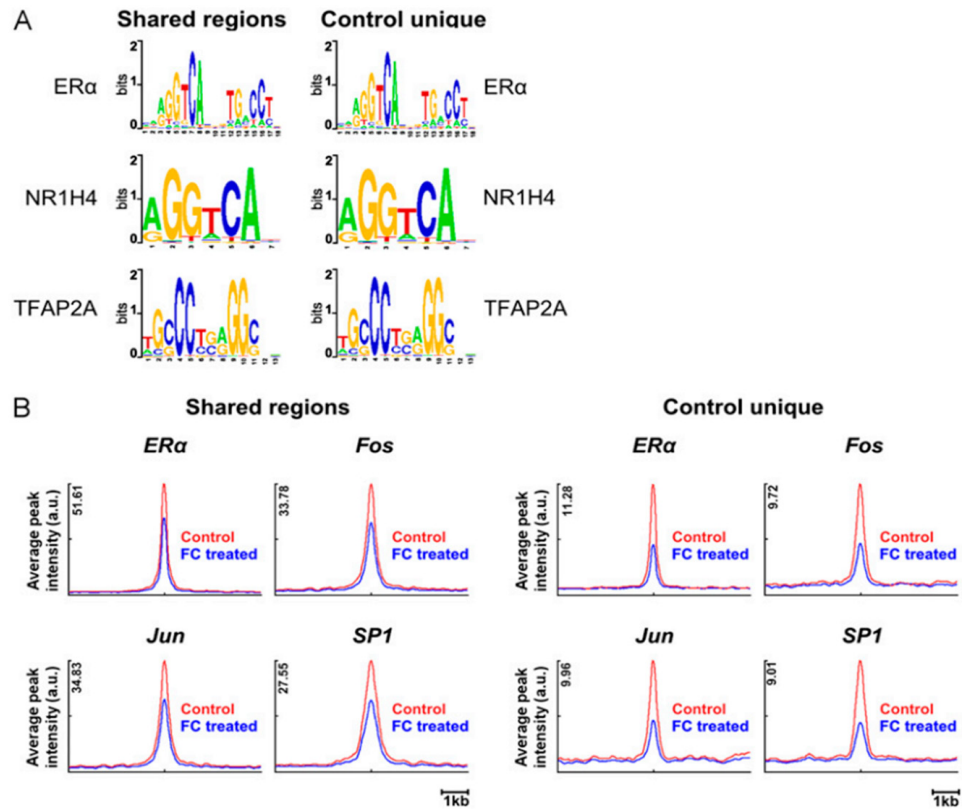


Fig. S7

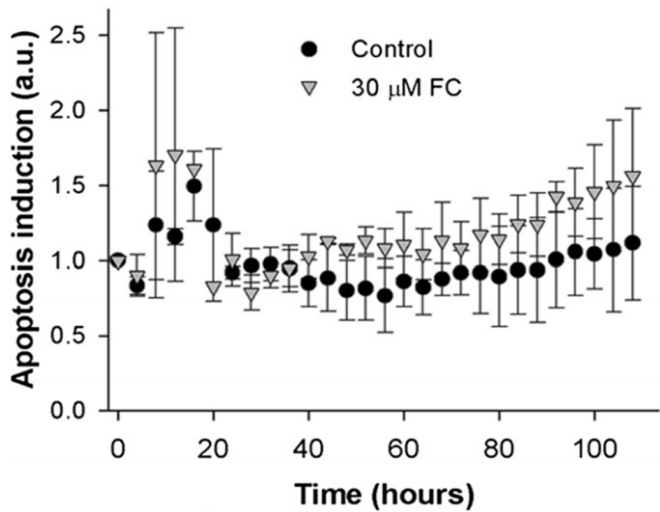


Table S1. FC enhances the affinity of the short pERα peptide for all 14-3-3 isoforms

K_d , μM , $\pm\text{SEM}$	Control	10 μM FC	Affinity increase
β	0.99 (0.29)	0.11 (0.03)	9.1
η	0.94 (0.32)	0.19 (0.06)	4.9
ε	0.69 (0.21)	0.07 (0.04)	10.4
γ	1.09 (0.36)	0.15 (0.11)	7.1
σ	1.61 (0.32)	0.10 (0.03)	16.4
θ	0.90 (0.20)	0.06 (0.01)	15.1
ζ	0.57 (0.18)	0.05 (0.02)	10.9
$\sigma\text{-}\Delta\text{C}$	0.33 (0.07)	0.07 (0.05)	5

Interaction affinity (K_d) of the short pERα peptide for all 14-3-3 isoforms, as calculated from the curves in Fig. S1D. Values in parenthesis are $\pm$ SEM.

Table S2. Data collection and refinement statistics for 4JC3 and 4JDD

	4JC3	4JDD
Data collection		
Space group	C222 ₁	C222 ₁
Cell dimensions		
A, b, c, Å	82.2, 112.49, 62.45	82.49, 111.43, 62.24
A, β , γ , °	90, 90, 90	90, 90, 90
Resolution, Å	19.52–2.05 (2.1–2.05)	19.80–2.1 (2.15–2.0)
R_{meas} , %	8.4 (34.9)	12.0 (51.0)
$I/\sigma I$	24.16 (6.85)	17.79 (4.77)
Completeness, %	99.3	100.0
Redundancy	14.94 (14.7)	15.1 (14.9)
Refinement		
Resolution, Å	19.5–2.05 (2.1–2.05)	19.80–2.1 (2.15–2.0)
No. reflections	141777	135424
Unique reflections	18518	17090
$R_{\text{work}}/R_{\text{free}}$	0.1778/0.235	0.1924/0.2316
No. atoms		
Protein	1850	1847
Ligand	42	42
		(FUSICOCCIN 48)
Solvent	177	101
B factors, Å ²		
Protein	14.3	19.70
Ligand	23.7	26.78
		(FUSICOCCIN 23.14)
Water	19.01	21.0
R.m.s. deviations		
Bond lengths, Å	0.019	0.020
Bond angles, °	1.767	1.992
Ramachandran plot		
Favored, %	98.7	97.9
Allowed, %	1.7	2.1
Generously allowed, %	0	0
Disallowed, %	0	0

Values in parenthesis are for the highest resolution shell.

