

Molecular basis and dual ligand regulation of tetrameric Estrogen Receptor $\alpha/14-3-3\zeta$ protein complex

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Molecular basis and dual ligand regulation of tetrameric estrogen receptor α /14-3-3 ζ protein complex

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Therapeutic strategies targeting nuclear receptors (NRs) beyond their endogenous ligand binding pocket have gained significant scientific interest driven by a need to circumvent problems associated with drug resistance and pharmacological profile. The hub protein 14-3-3 is an endogenous regulator of various NRs, providing a novel entry point for small molecule modulation of NR activity. Exemplified, 14-3-3 binding to the C-terminal F-domain of the estrogen receptor alpha (ER α), and small molecule stabilization of the ER α /14-3-3 ζ protein complex by the natural product Fusicoccin A (FC-A), was demonstrated to downregulate ER α -mediated breast cancer proliferation. This presents a novel drug discovery approach to target ER α ; however, structural and mechanistic insights into ER α /14-3-3 complex formation are lacking. Here, we provide an in-depth molecular understanding of the ER α /14-3-3 ζ complex by isolating 14-3-3 ζ in complex with an ER α protein construct comprising its ligand-binding domain (LBD) and phosphorylated F-domain. Bacterial co-expression and copurification of the ER α /14-3-3 ζ complex, followed by extensive biophysical and structural characterization, revealed a tetrameric complex between the ER α homodimer and the 14-3-3 ζ homodimer. 14-3-3 ζ binding to ER α , and ER α /14-3-3 ζ complex stabilization by FC-A, appeared to be orthogonal to ER α endogenous agonist (E2) binding, E2-induced conformational changes, and cofactor recruitment. Similarly, the ER α antagonist 4-hydroxytamoxifen inhibited cofactor recruitment to the ER α LBD while ER α was bound to 14-3-3 ζ . Furthermore, stabilization of the ER α /14-3-3 ζ protein complex by FC-A was not influenced by the disease-associated and 4-hydroxytamoxifen resistant ER α -Y537S mutant. Together, these molecular and mechanistic insights provide direction for targeting ER α *via* the ER α /14-3-3 complex as an alternative drug discovery approach.

Nuclear receptors (NRs) are a family of structurally similar, mostly ligand-regulated, transcription factors that control a diverse set of biological processes, including metabolism, cell proliferation, development, and immunity (1, 2). Several small

molecules, including steroid hormones, fatty acids, vitamin D, and thyroid hormones, regulate NR activity by binding the NR's ligand binding domain (LBD) (3, 4). Given their central role in gene regulation, NRs are associated with a broad scope of diseases, including autoimmune diseases, metabolic disorders, and cancer (2, 5, 6). The involvement of NRs in various pathological processes, combined with their ability to bind "drug-like" small molecules, have made NR-targeting therapeutics extensively utilized in the clinic to treat diseases (4, 7). Most NR-targeting drugs antagonize the activity of the protein by orthosteric inhibition of native ligand binding to the LBD (4, 6). For example, 4-hydroxytamoxifen (4-OHT) inhibits ER α activity by displacing its natural ligand estradiol (E2, Fig. 1, A and B) (5, 6). Despite the huge successes of orthosteric NR inhibitors, there is a growing interest in targeting NRs in alternative manners (8–11). Novel therapeutic approaches include ligands that target allosteric sites in the LBD (12–14), NR–DNA interactions (15–17), NR dimerization (18–21), NR-cofactor interfaces (22–25), or induce targeted NR degradation (26–28). Drugging NRs beyond the orthosteric site of the LBD offers entry points into selective NR targeting, fine-tuning of pharmacological effects, targeting of drug-resistant NRs, or addressing orphan NRs that lack an accessible ligand binding pocket (8–10).

An alternative approach to modulate NR activity is *via* their protein–protein interaction (PPI) with the hub protein 14-3-3. These 14-3-3 proteins are known to interact, and thereby regulate, hundreds of different, mainly phosphorylated, protein partners. Within the large interactome of 14-3-3 are many well-known disease-related proteins such as the Raf kinases (cancer), LRRK2 (Parkinson's), and CFTR (cystic fibrosis), which make 14-3-3 proteins highly involved in a larger variety of cellular processes and which makes 14-3-3 protein complexes highly interesting as drug discovery targets (29). Among the 14-3-3 protein partners are various NRs, including the steroid hormone-responsive Androgen Receptor (AR), Glucocorticoid Receptor (GR), and Estrogen Receptor alpha (ER α) (Figs. S1 and S2). Specifically, 14-3-3 binds AR upon phosphorylation of Ser213 in the N-terminal domain (NTD), thereby modulating AR transcriptional activity (30–34). Similarly, 14-3-3 binding enhances GR activity upon binding

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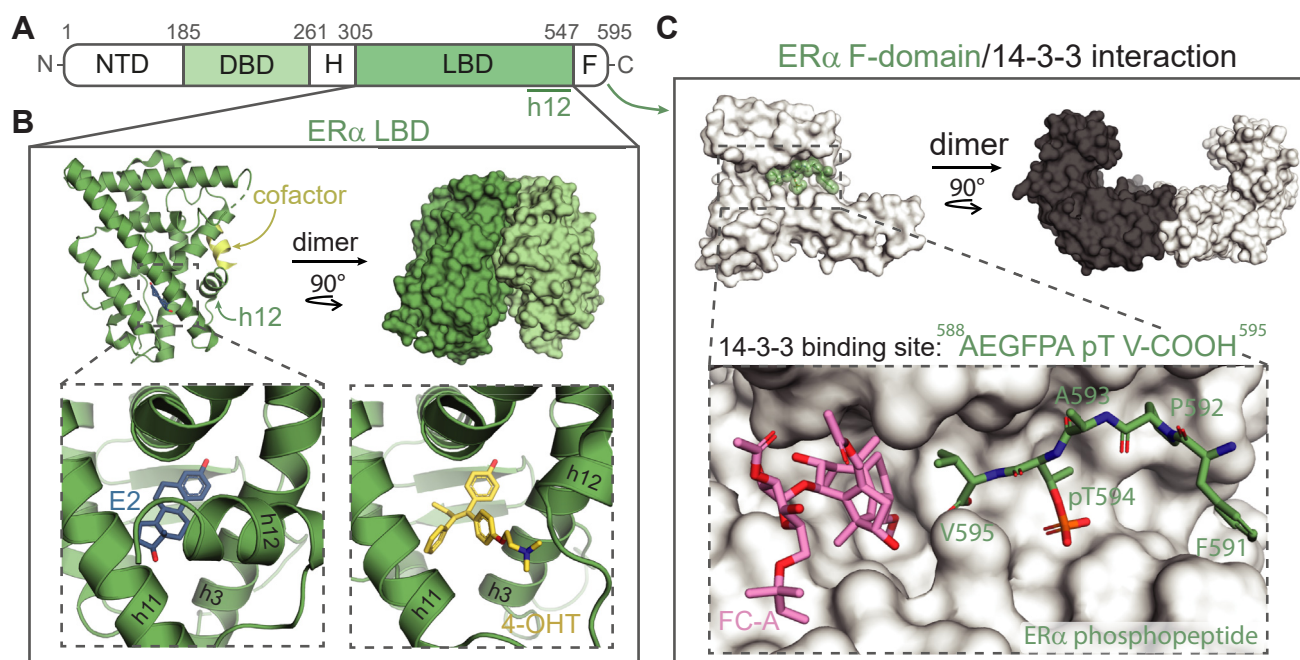


Figure 1. The ER α /14-3-3 protein-protein interaction. *A*, schematic representation of ER α domains, including the N-terminal domain (NTD), DNA-binding domain (DBD), hinge-region (H), ligand-binding domain (LBD), and C-terminal F-domain (F). *B*, ER α LBD crystal structure (monomer, *green cartoon* and dimer, *green surface*). The binding of its endogenous ligand E2 (*blue sticks*) induces the folding of helix 12 (h12) in an active conformation, allowing cofactor binding (*yellow cartoon*). In contrast, antagonist 4-hydroxytamoxifen (4-OHT, *yellow sticks*) inhibits this conformational change and cofactor recruitment, PDB: 5WGD and 3ERT. *C*, crystal structure of 14-3-3 dimer (*gray/white surface*) and co-crystal structure of 14-3-3 σ (*white surface*) with the ER α derived C-terminal phosphopeptide (*green sticks*) and small molecule stabilizer Fusicoccin-A (FC-A, *pink sticks*), PDB: 4JDD.

to pS134 in the NTD or pT524 in the LBD (35–37). In the case of ER α , 14-3-3 binds the phosphorylated penultimate residue T594 in the C-terminal F-domain (Fig. 1C), thereby reducing E2-dependent transcriptional activity (38). Cellular studies have further reported 14-3-3 binding to the DNA-binding domain (DBD) of ER γ , the LBD of PPAR γ , and ER β , TR α , and PXR of which the exact binding sites remain unknown (39–43). These examples illustrate the diverse role that 14-3-3 plays in NR regulation and, by extension, the potential of targeting NR/14-3-3 PPIs.

Initial studies have shown that NR/14-3-3 PPIs can be modulated using small molecules. Specifically, the natural product FC-A or covalently tethered small molecules have been shown to stabilize the ER α /14-3-3 and ER γ /14-3-3 PPIs (Figs. 1C and S3) (38, 44–46). These small molecules, also called molecular glues, increase the binding affinity between the two protein partners (PPI stabilization) by binding in a composite pocket formed at the interface of 14-3-3 and the phosphorylated NR protein. Further, stabilization of the ER α /14-3-3 PPI by FC-A suppresses ER α chromatin binding and subsequent transcriptional activity (38). These results illustrate the potential of the stabilization of the ER α /14-3-3 interaction as an alternative drug discovery entry.

Despite the potential of therapeutic targeting of NR/14-3-3 complexes, little is known about NR/14-3-3 interactions on a molecular level. To date, biochemical and structural studies of NR/14-3-3 complexes have been exclusively performed using short phosphopeptide mimics of the NRs (Figs. 1C and S2) (45–47). Studies of NR/14-3-3 complexes using protein

domains or full-length NRs, such as those performed for other 14-3-3 PPIs (48–51), are needed to gain an enhanced molecular and structural understanding of NR/14-3-3 complex formation. As an example, the use of the entire NR LBD allows studies on the interplay between NR/14-3-3 complex formation and aspects like NR dimerization, ligand binding, and cofactor recruitment. Additionally, small molecule stabilization of full-length NR/14-3-3 complexes can be investigated in the context of clinically relevant NR point mutations which is of high interest (52–55). Molecular insights into NR/14-3-3 PPIs beyond the phospho-binding groove would also provide the potential to identify novel composite binding pockets for small molecule targeting, thereby expanding the number of entry points for novel PPI modulator design and increasing the potential for selectivity (56).

Within this work, we aim to enhance our understanding of 14-3-3 binding to ER α *via in vitro* characterization of the protein complex formed by 14-3-3 ζ and an ER α construct comprising the LBD and phosphorylated F domain. To gain a robust understanding of protein complex formation and the stoichiometry of binding, several biophysical assays were performed, including analytical size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC). In addition, differential scanning fluorimetry (DSF), fluorescence anisotropy (FA), and hydrogen-deuterium exchange (HDX) experiments determined the role of ER α ligands E2 and 4-OHT on ER α /14-3-3 complex formation and identified 14-3-3 binding to the drug-resistant ER α -Y537S mutant. Finally, we show that the ER α /14-3-3 PPI can be stabilized by the natural product

FC-A and that this PPI stabilization can be achieved independently from ER α ligand binding in both wild-type ER α and the ER α -Y537S mutant, thus presenting a potential orthogonal therapeutic strategy for targeting endocrine resistance in breast cancer.

Results

Construct design and protein expression of ER α LBD-F domain

Most biochemical and structural studies on the ER α protein have focused on the ER α LBD, most often ending after helix 12 (~ residue S554) (57, 58). These studies exclude the 42-residue-long, intrinsically disordered, and solvent-exposed C-terminal F-domain (Fig. S4). The ER α F-domain is described to influence ER α dimerization and co-activator binding; however, as one of the least conserved regions among NRs, the structure

and role of the ER α F-domain remain largely unknown (38, 59–61). The ER α F-domain contains a penultimate threonine residue (T594) that upon phosphorylation facilitates the binding of 14-3-3 to ER α (38). To gain mechanistic insights into the combined role of the ER α LBD and F-domain in concert with 14-3-3 binding, an ER α construct was designed comprising both domains (residues 302–595) (Fig. 2A). Since the responsible kinase is not known, native T594 phosphorylation is inaccessible. We thus introduced double-point mutations (F591R/P592R) within the ER α construct, enabling PKA phosphorylation at T594. The two-point mutations make the ER α C-terminal end closely match with the consensus sequence of known PKA substrates (RRXS/TY), thereby increasing the chances of successful phosphorylation of T594 by PKA (62). Fluorescence anisotropy binding studies of 14-3-

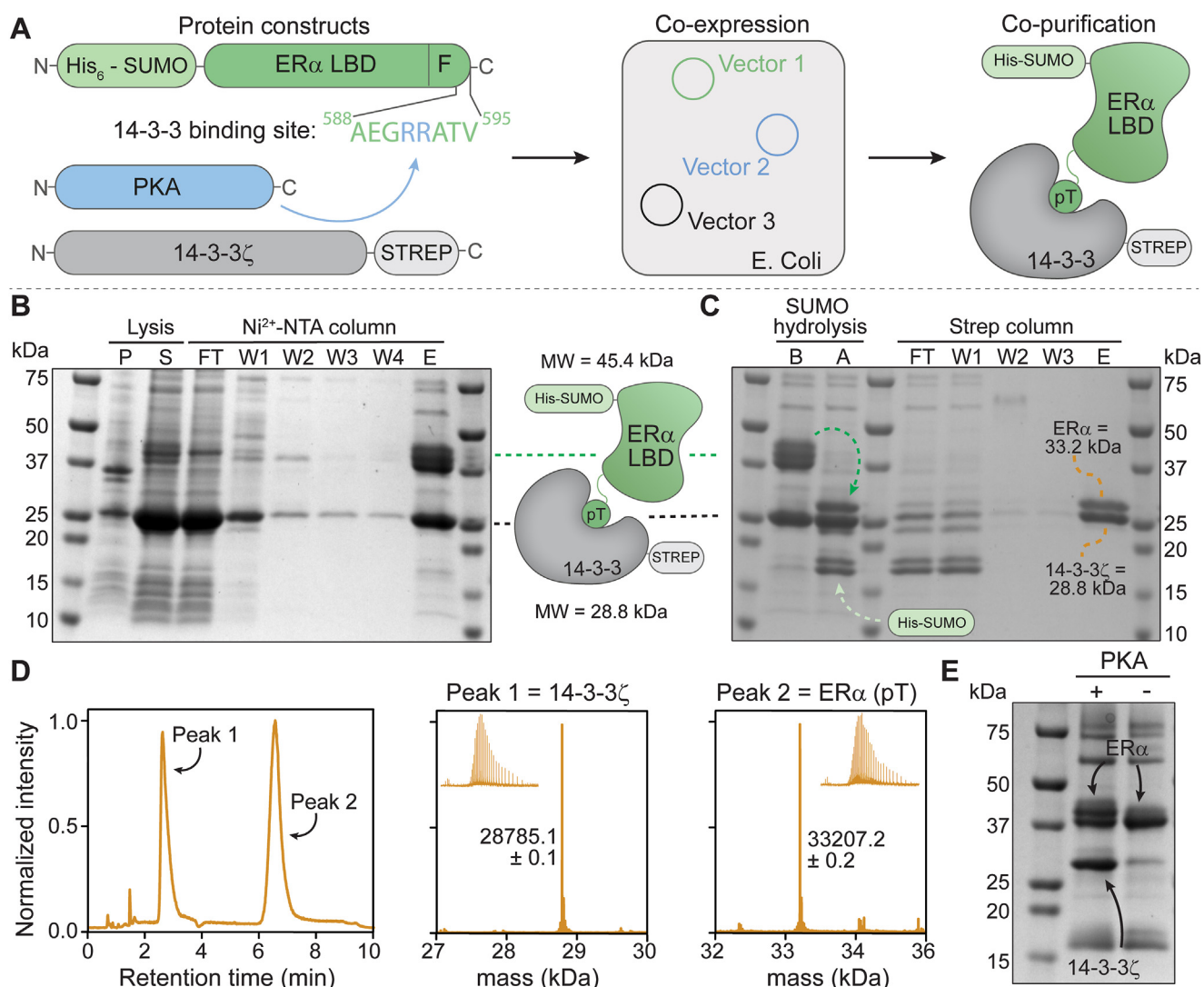


Figure 2. ER α /14-3-3 ζ complex expression and purification. A, schematic representation of ER α /14-3-3 co-expression and co-purification. Three protein constructs (left) were designed for bacterial expression: (1) His-SUMO-ER α LBD-F domains (green) including two mutations to make ER α T594 PKA responsive (blue); (2) PKA (blue); and (3) 14-3-3 ζ -strep (grey). These constructs were co-expressed in the same *E. coli* cells (middle), leading to the formation of the ER α /14-3-3 complex which was co-purified (right). B and C, SDS-PAGE analysis (Coomassie stain) of the Ni-NTA purification, SUMO-cleavage, and strep purification of the ER α /14-3-3 ζ complex. Labels: pellet (P), supernatant (S), flowthrough (FT), wash (W), elution (E), before (B), and after (A). D, QToF-MS analysis of ER α /14-3-3 ζ complex including the LC chromatogram and correlated m/z and mass spectra of both peaks corresponding to 14-3-3 ζ (expected mass: 28785.1 Da) and phosphorylated ER α (expected mass: 33,207.3 Da). E, SDS-PAGE analysis (Coomassie stain) of Ni-NTA purification elution fraction of ER α /14-3-3 ζ co-expression in the presence and absence of PKA.

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3 ζ to peptide mimics of the wild-type (WT) ER α and PKA-responsive ER α sequence confirmed that the PKA-responsive point mutations did not hamper binding of ER α to 14-3-3 ζ (Fig. S5).

The PKA-responsive ER α (ER α (PKA)) construct was expressed and purified from *E. coli*; however, yielded solely in truncated ER α protein (at residues 570 and 572) as apparent from mass spectrometry analysis (Fig. S6). The truncation of the F-domain was addressed by incorporation of a C-terminal strep-tag (ER α (PKA)-strep), resulting in successful expression, purification, and PKA-mediated phosphorylation of ER α (Fig. S7). Notably, the introduction of a C-terminal strep-tag significantly changed the 14-3-3-binding site of ER α . Fluorescence anisotropy binding studies of WT ER α , ER α (PKA), and ER α (PKA)-strep phosphopeptides to 14-3-3 ζ showed a 50-fold reduction in binding affinity upon introduction of the strep-tag to the C-terminal end of ER α peptide (Fig. S8). Moreover, stabilization of the ER α (PKA)-strep phosphopeptide/14-3-3 complex by natural product FC-A was significantly reduced. Cooperativity analysis of the ternary 14-3-3/peptide/FC-A complex using WT ER α and ER α (PKA) peptide sequences elicited cooperativity factors (α) of 245 and 366, respectively, whereas the cooperativity factor of FC-A stabilization of the 14-3-3/ER α (PKA)-strep complex was only 15 (Figs. S8 and S9) (63).

Co-crystal structures of 14-3-3 bound to ER α -phosphopeptides revealed why the strep-tagged ER α peptide showed a reduced FC-A responsiveness (Fig. S10). The C-terminal strep-tag occupied the FC-A binding site within the 14-3-3 binding groove, forcing a structural rearrangement of the ER α peptide upon FC-A binding. This makes stabilization by FC-A less favorable compared to the WT ER α peptide. While the ER α (PKA)-strep construct could be used as a representative protein for ER α itself, the protein is less favorable to study 14-3-3 binding and small molecule PPI stabilization. As such, we shifted our focus to an alternative recombinant protein expression approach to obtain the ER α /14-3-3 protein complex.

Co-expression and co-purification of ER α /14-3-3 ζ complex

To circumvent proteolytic cleavage of ER α or the use of C-terminal purification tags, we used bacterial co-expression of ER α , 14-3-3 ζ , and PKA to obtain the ER α /14-3-3 ζ protein complex. Specifically, N-terminally His-SUMO-tagged ER α (PKA) was expressed together with PKA and strep-tagged 14-3-3 ζ in *E. coli* (Fig. 2A). Co-expression of 14-3-3 ζ and ER α with PKA allowed 14-3-3 to bind *in situ* to the phosphorylated ER α protein shielding the disordered F domain from proteolytic degradation. A similar co-expression approach was previously applied to successfully identify 14-3-3 binding to Ataxin-1 and to obtain a purified Tau/14-3-3 complex (64, 65). Notably, the 14-3-3 protein comprises seven human isoforms which have proven to feature highly similar biochemical and structural features (66, 67). Yeast two-hybrid studies have previously shown that ER α is able to interact with all seven isoforms of 14-3-3 (38). Therefore, we have selected here one 14-3-3 isoform, zeta, as a representative for the class of 14-3-3 proteins. 14-3-3 ζ

was specifically selected as it expresses well in *E. Coli*, because of its relatively high abundance in the human body (68), and because it has been successfully used in similar biochemical studies of larger 14-3-3 protein complexes, such as the BRAF/14-3-3 complex (50, 51, 69).

The ER α /14-3-3 protein complex was co-purified using three subsequent chromatography methods. First, a Ni-NTA column was used to select for His-tagged ER α protein (Fig. 2B). Elution fractions contained both His-tagged ER α and 14-3-3 ζ protein, indicating strong binding of 14-3-3 ζ to ER α , as 14-3-3 did not contain a His-tag itself. After hydrolysis of the N-terminal His-SUMO tag of ER α , the complex was purified with a streptavidin-column, which again showed co-elution of 14-3-3 ζ and ER α (Fig. 2C). The ER α /14-3-3 ζ protein complex was finally purified by SEC which resulted in the elution of a uniform peak that contained both proteins (Fig. S11). High-resolution mass spectrometry (LC-QToF-MS) analysis showed two protein peaks of which the first corresponded to the mass of 14-3-3 ζ and the second to that of the phosphorylated ER α LBD-F protein (Fig. 2D). Notably, truncation of ER α was not observed after co-expression and -purification with 14-3-3 ζ . Quantitative MS experiments showed the approximately equimolar presence of both 14-3-3 ζ and ER α within the purified protein complex sample, indicating a 1:1 binding of the two proteins (Figs. S12 and S13). Furthermore, ER α binding to 14-3-3 proved to be phosphorylation-dependent as co-expression in the absence of PKA did not result in co-elution of 14-3-3 ζ with His-tagged ER α (Fig. 2E).

ER α /14-3-3 bind in a 2:2 stoichiometry

Native PAGE analysis of the purified ER α /14-3-3 ζ complex showed a single band protein complex indicating stable complex formation between ER α and 14-3-3 ζ without the presence of any major excess of either protein (Fig. S14). The addition of a high-affinity competitive 14-3-3 binder (70) disrupted the ER α /14-3-3 ζ complex as apparent by two distinct bands that ran in the native PAGE gel at the same heights as 14-3-3 ζ and ER α individually, testifying to the stable complex formation between ER α and 14-3-3 ζ .

Analytical SEC and sedimentation velocity AUC (SV-AUC) were used as orthogonal approaches to determine the ER α /14-3-3 ζ protein complex size and the stoichiometry of ER α and 14-3-3 ζ binding (Fig. 3, A and B). Analytical SEC results showed a single peak for the individual 14-3-3 ζ (theoretical M_w 29 kDa) and ER α (theoretical M_w 33 kDa) proteins which eluted similar to monomeric BSA (theoretical M_w 66 kDa), indicating that both proteins themselves were present as stable homodimers in solution (\sim 60 kDa), which is in line with reported observations (Fig. 3A) (59, 67, 71, 72). The ER α /14-3-3 ζ complex eluted as a single peak with a clear shift in molecular weight compared to ER α and 14-3-3 ζ individually, approaching the dimeric BSA peak around 132 kDa. Together with the quantitative MS studies, which showed the equimolar presence of both ER α and 14-3-3 ζ in the purified mixture (Figs. S12 and S13), these results indicated the tetrameric complex formation of an ER α and a 14-3-3 ζ homodimer.

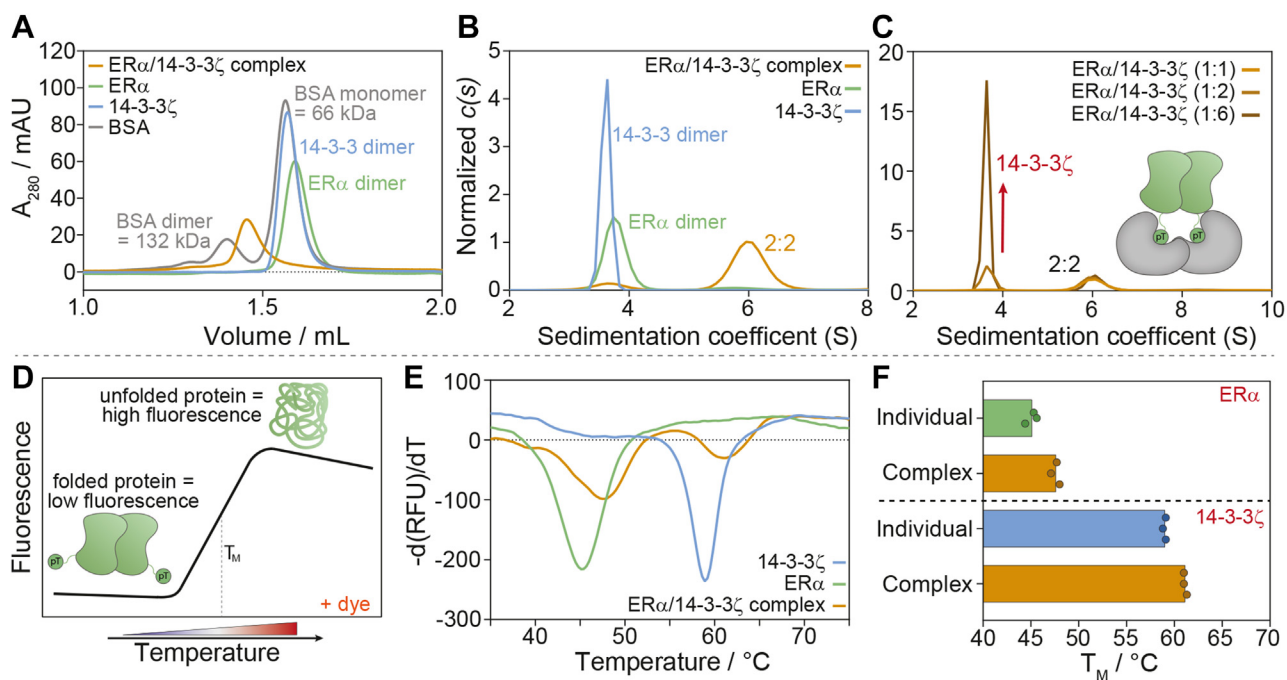


Figure 3. ER α /14-3-3 ζ protein complex characterization. *A*, analytical SEC results of 10 μ M BSA (grey), 10 μ M 14-3-3 ζ (blue), 10 μ M ER α -strep (green), and 20 μ M ER α /14-3-3 ζ protein complex (orange) with peak detection based on the absorbance at 280 nm. *B*, area-normalized $c(s)$ distributions of 10 μ M 14-3-3 ζ (blue), 10 μ M ER α (green), and the 30 μ M ER α /14-3-3 ζ complex (orange). *C*, area-normalized $c(s)$ distributions of 10 μ M ER α /14-3-3 ζ complex (1:1) and 10 μ M complex with the addition of 5 μ M 14-3-3 ζ (1:2) or 25 μ M 14-3-3 ζ (1:6) to compare the effect of different ER α /14-3-3 ζ ratios on complex stoichiometry. *D*, schematic representations of differential scanning fluorimetry (DSF) assay where folded proteins are exposed to increasing temperatures which leads to the protein unfolding and subsequent binding of a ProteoOrange dye, resulting in increased fluorescence. *E*, differential melting curve of 5 μ M 14-3-3 ζ (blue), 5 μ M ER α (green), and 10 μ M ER α /14-3-3 ζ complex (orange). *F*, melting temperatures (T_m) of ER α and 14-3-3 ζ individually and in the complex.

SV-AUC sedimentation coefficient distributions $c(s)$ validated results observed with SEC. ER α and 14-3-3 ζ showed individual peaks with weight-averaged sedimentation coefficients (corrected to 20.0 $^{\circ}$ C and to the density of water), $s_{w(20,w)}$, of 4.0 S and 3.8 S, corresponding to $M_w \sim 55$ kDa and ~ 58 kDa, respectively (theoretical M_w of dimeric ER α is 66 kDa; and of dimeric 14-3-3 ζ is 58 kDa) (Fig. 3B). The ER α /14-3-3 ζ complex showed a main peak with a weight-averaged sedimentation coefficient of 6.3 S, corresponding to a $M_w \sim 124$ kDa, thus supporting the 2:2 stoichiometry of the ER α :14-3-3 ζ complex (72, 73). Notably, a minor peak was observed at peak positions of the 14-3-3 ζ and ER α indicating traces of the non-interacting proteins present in the solution under these conditions.

Dimer-to-dimer binding enhances the ER α /14-3-3 protein complex affinity

Multivalent dimer-to-dimer complex formation was further accessed using a competitive SV-AUC experiment where 14-3-3 ζ was added to ER α /14-3-3 ζ protein complex to obtain various molar ratios of ER α and 14-3-3 ζ . The addition of up to six equivalents of 14-3-3 ζ to ER α resulted only in species representing the ER α /14-3-3 ζ tetramer and the 14-3-3 ζ dimer but did not show any 2:1 14-3-3 ζ :ER α complex formation (Fig. 3C). This result showed that ER α binds to 14-3-3 ζ as a stable dimer. The dimeric state of ER α is hypothesized to cooperatively enhance ER α binding affinity to 14-3-3 ζ , similar

to earlier described multivalent 14-3-3 binders CFTR and LRRK2 (74, 75). The binding of one ER α monomer to the 14-3-3 dimer brings the second ER α monomer in proximity to 14-3-3, thereby increasing the effective molar concentration of ER α to 14-3-3 ζ . Furthermore, dissociation of the protein complex is hypothesized to be slower since two binding interfaces need to dissociate in close succession for 14-3-3 ζ and ER α to dissociate. Therefore, dimer-to-dimer binding of ER α and 14-3-3 ζ is expected to increase the affinity of ER α for 14-3-3 ζ and the stability of the tetrameric protein complex. The dissociation constant K_D of dimeric 14-3-3 ζ and dimeric ER α was estimated using SV-AUC results of the ER α /14-3-3 complex (Fig. S15). Based on the area under the curve, the amount of ER α and 14-3-3 ζ in complex and alone was quantified from which the K_D was calculated using the steady state equilibrium binding equation ($K_D = [A][B]/[AB]$). This calculation provided a $K_D \sim 32 \pm 6$ nM. This affinity is almost 10-fold higher than that of the ER α phosphopeptide binding to 14-3-3, indicating stronger binding of the phosphorylated ER α LBD-F domain protein due to the dimer-to-dimer binding mechanism.

Complex formation increases the thermal stability of both protein partners

The ER α /14-3-3 ζ complex stability was studied using differential scanning fluorimetry (DSF) studies (Fig. 3D). ER α and 14-3-3 ζ alone showed melting temperatures of 45.2 ± 0.8

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deg. C and $59.0 \text{ }^\circ\text{C} \pm 0.2 \text{ deg. C}$, respectively (Fig. 3, E and F). ER α phosphorylation at T594 did not influence its melting temperature (Fig. S16). The ER α /14-3-3 ζ protein complex showed two distinct melting peaks (Fig. 3E), with $\sim 2 \text{ }^\circ\text{C}$ increased thermal stability when compared to the individual protein partners (Fig. 3F). A similar increase in thermal stability for 14-3-3 ζ was observed upon the addition of an ER α phosphopeptide (Fig. S16). These results thus show a mutual stabilizing effect of 14-3-3 ζ and ER α upon tetramer formation.

Mapping of interactions between ER α and 14-3-3 ζ using HDX-MS

Hydrogen-deuterium exchange (HDX-MS) assays were performed on the individual proteins 14-3-3 ζ and ER α , and the ER α /14-3-3 ζ protein complex, to identify which regions within the ER α and 14-3-3 ζ protein were involved in protein complex formation (Figs. 4 and S17–S20). The proteins were incubated in deuterium-containing buffers which were quenched after 20 s, 2 min, 20 min, and 2 h. Proteins were digested using pepsin after which the amount of deuterium of individual peptides was determined using mass spectrometry. HD exchange kinetics of ER α was followed for 248 peptides, covering 98.6% of the protein sequence, and 240 peptides for 14-3-3 ζ which cover 100% of the sequence.

An HD exchange profile was made to visualize the deuteration kinetics of each ER α residue for *apo*-ER α and ER α within the ER α /14-3-3 ζ complex (Figs. 4A and S17). Fast deuterium

exchange kinetics ($>30\%$ deuteration after 20 s) were mainly observed in helix 1 to 2 (H1-2), the beta sheets (B), helix 7 (H7), residues between helix 9 and 10, helix 12 (H12), and the entire F-domain. Residues in H5-6, H8-9, H10, and H11, on the other hand, showed low amounts of deuterium exchange. These results corresponded nicely with the ER α crystal structures where regions with fast deuterium exchange kinetics are typically present in flexible and/or solvent-exposed regions of ER α . Notably, the C-terminal F-domain of ER α has never been crystallized before or studied with alternative structural biology techniques. Within this study, fast HD exchange kinetics were observed within the entire F-domain indicating that the ER α F-domain is most probably unstructured and solvent-exposed, as observed in the predicted AlphaFold structure (76, 77).

14-3-3 ζ binding to ER α decreased the deuteration kinetics of several regions within the ER α protein (Fig. 4, A–C; Figs. S17 and S18). Shielding effects were determined by calculating the difference in HDX between 14-3-3 ζ -bound ER α and ER α by itself, after which this difference profile was visualized on the ER α AlphaFold structure (Fig. 4C and S17). Most pronounced shielding effects were observed in the N-terminal side of H3 (residues 328–354), the beta sheets through H7 (residues 397–420), the C-terminal side of H11 (residues 521–528), and the C-terminal end of the F-domain (residues 583–591) (Fig. 4, A–C; Figs. S17 and S18). Smaller effects were observed in H1-2 (residues 302–327), H8 (residues 421–444), H12, and the F-

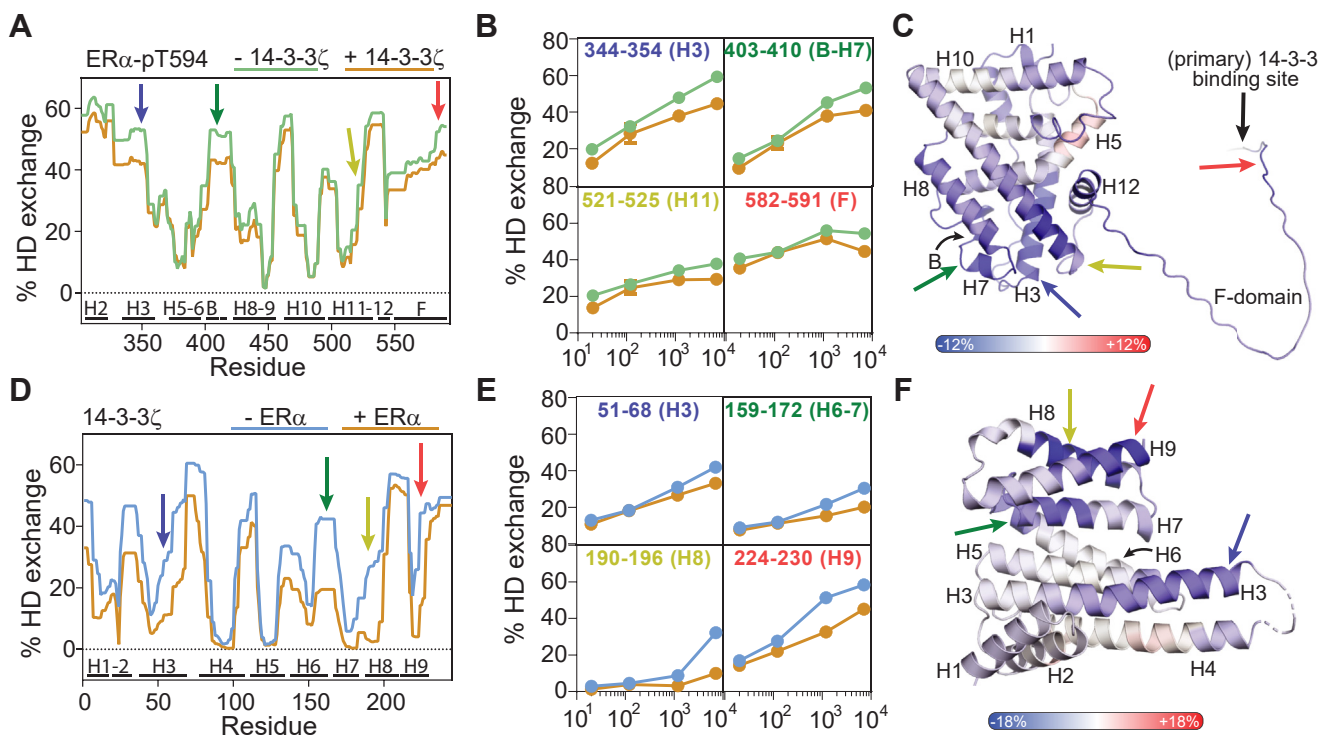


Figure 4. Hydrogen-deuterium exchange (HDX) of ER α /14-3-3 ζ protein complex. A, HDX-MS exchange profile of ER α -pT594 (green) and ER α in ER α /14-3-3 ζ protein complex (orange) after 2 h of HDX. B, time-dependent HDX profile of individual ER α peptides. C, HDX difference profile of ER α -pT594 in the ER α /14-3-3 ζ complex minus ER α -pT594 alone after 2 h incubation which displays the effect of 14-3-3 ζ binding on deuterium exchange in ER α . Shielding effects (less deuterium exchange) are shown in blue and deshielding effects (increase in deuterium exchange) in red. Results are displayed on the predicted ER α AlphaFold structure (LBD-F domains). D, HDX-MS exchange profile of 14-3-3 ζ (blue) and 14-3-3 ζ in ER α /14-3-3 ζ protein complex (orange) after 2 h of HDX. E, time-dependent HDX profile of individual 14-3-3 ζ peptides. F, HDX difference profile of 14-3-3 ζ in the ER α /14-3-3 ζ complex minus 14-3-3 ζ alone after 2 h incubation which displays the effect of ER α binding on deuterium exchange in 14-3-3 ζ . Results are displayed on the 14-3-3 ζ crystal structure, PDB: 6F09.

domain (residues 531–571). H4-6, H9-10, and the N-terminal part of H11 seemed unaffected, although it should be mentioned that these regions showed minor deuteration in the first place. Interestingly, although the 14-3-3 binding groove is known to primarily bind the C-terminal end of the ER α F-domain, multiple regions additional to the F-domain seem to be affected by 14-3-3 binding. Shielding effects were observed for almost all flexible and solvent-exposed regions within ER α , indicating an overall stabilization of the ER α fold upon tetramer formation. Most pronounced effects were clustered on the ‘bottom’ of the ER α LBD structure (H7, H3 N-term, H11 C-term), indicating the proximity of 14-3-3 ζ to this side of ER α , albeit with the dynamic movement of the ER α LBD dimer, facilitated by the long and flexible F-domain, leading to mild shielding effects at all sides of the ER α LBD. Notably, ER α /14-3-3 ζ dimer-to-dimer binding might result in differential binding of one ER α monomer in comparison to the other ER α monomer, potentially further explaining why many regions within the ER α protein are mildly affected upon 14-3-3 ζ binding.

Deuteration kinetics for 14-3-3 ζ in the absence and presence of ER α protein were similarly analyzed (Fig. 4, D–F, Figs. S19 and S20). The regions of high and low exchange rates of 14-3-3 ζ alone corresponded well to known crystal structures and previously published HDX of 14-3-3 (78, 79), with high deuteration in loops between H2-3, H3-4, H4-5, and H8-9, and none to minor amounts of deuteration in H2, H3, H4, H5, H7, and H9. ER α binding to 14-3-3 ζ led to shielding effects on various parts of the 14-3-3 ζ protein, which was most pronounced after 2 h of incubation (Fig. 4, D–F; Figs. S19 and S20). The largest shielding effects were observed within peptides in the C-terminal end of H3 (residues 50–68), H6-7 (residues 154–174), H8 (residues 180–199), and the C-terminus in H9 (residues 217–230). These shielded regions strongly correlated with the 14-3-3 binding groove (H3, H7 and H9) where the ER α C-terminus binds. Interestingly, also H8 on ‘top’ and H6 on the ‘back’ of the 14-3-3 protein were partially shielded, indicating that these 14-3-3 regions are potentially in close proximity to other parts of ER α . In contrast, the ‘base’ of the 14-3-3 protein seems to be less affected by ER α binding, indicating the absence of direct contact with ER α (Fig. S19). Crystallography and HDX studies of other 14-3-3 PPIs typically show similar binding interfaces involving the ‘top’ of 14-3-3 (H8-9), whereas the ‘base’ of 14-3-3 is not involved with the PPI formation (48, 69, 79–81).

14-3-3 ζ can bind the ER α Y537S drug-resistant mutant

Point mutations in the ER α LBD are known to modulate ER α conformation and transcriptional activity. ER α Y537S is one of the most prevalent somatic mutations in patients with breast cancer, typically acquired after antiestrogen treatment (54). Structural and biophysical characterization has shown that the Y537S mutation places H12 in an agonistic, constitutively active, conformation (Fig. S21), causing this ER α mutant to be resistant to antiestrogen treatment (54, 82). Therefore, it is of high interest to study 14-3-3 ζ binding to

ER α -Y537S as it may provide a new approach to modulate the transcriptional activity of the drug-resistant ER α -Y537S mutant. The ER α -Y537S/14-3-3 ζ complex could successfully be co-expressed and co-purified using the aforementioned methodologies for WT ER α /14-3-3 ζ complex purification, indicating that the Y537S point mutation did not impede 14-3-3 binding. SV-AUC confirmed this as similar sedimentation distributions were obtained for the ER α -Y537S/14-3-3 ζ complex and the WT ER α /14-3-3 ζ protein complex (Fig. S22A). Furthermore, DSF studies showed a ~ 2 °C enhancement of thermal stability of both ER α -Y537S and 14-3-3 ζ upon complex formation, similar to WT ER α (Fig. S22, B–D). Interestingly, ER α -Y537S in the absence of 14-3-3 showed melting temperatures of 47.1 °C \pm 0.5 deg. C, which is ~ 2 °C higher than wildtype ER α , indicating higher thermal stability of ER α when mutated. All data together confirmed ER α -Y537S/14-3-3 ζ complex formation, providing an interesting entry point of targeting this mutant *via* its interaction with 14-3-3.

ER α ligand binding is independent of ER α /14-3-3 complex formation

Small molecule ligands play an important role in the regulation of ER α transcriptional activity in both healthy and diseased state, making it highly valuable to study their effects on ER α /14-3-3 complex formation. Therefore, we set out to study the effect of ER α /14-3-3 complex formation on ligand binding to both WT and the Y537S mutant of ER α . Here, we specifically studied the endogenous ER α agonist E2 and therapeutic partial antagonist 4-OHT (Fig. 5A) (83, 84). In both SV-AUC and native PAGE, E2 and 4-OHT did not show any effect on the ER α /14-3-3 protein complex size, apparent by the similar *c(s)* distribution of the ER α /14-3-3 ζ complex in the presence and absence of ligands (Figs. 5B and S23). Furthermore, ligand-dependent DSF studies were performed to determine the effect of ER α ligand binding on the thermal stability of ER α in complex with 14-3-3 ζ . (Fig. 5, C–E; Figs. S24 and S25). The addition of E2 or 4-OHT to both phosphorylated and non-phosphorylated ER α strongly increased the ER α melting temperature by 14 °C and 16 °C, respectively (Figs. 5C and S24). Similarly, E2 or 4-OHT addition to the ER α /14-3-3 ζ complex resulted in a clear shift of the ER α T_M from 47.6 to 60.4 °C and 61.3 °C, respectively (Figs. 5, D–F and S24). Similar effects of E2 and 4-OHT were observed for experiments with the ER α -Y537S/14-3-3 ζ complex (Fig. S25). Overall, the SV-AUC and DSF data indicated that ER α ligands did not disrupt ER α /14-3-3 ζ complex formation and ER α was fully ligand responsive when bound to 14-3-3 ζ .

E2 induces the active conformation of ER α also when bound to 14-3-3

The effect of E2 on the conformation of ER α when bound to 14-3-3 was determined using HDX experiments. The effect of E2 on each ER α residue was determined by subtracting the HDX exchange profiles of ER α with and without ligand. Interestingly, E2 binding seemed to affect similar regions in ER α alone and ER α bound to 14-3-3 ζ (Figs. 6, A–D and S26–

The 14-3-3/ER α -LBD-F-domain complex

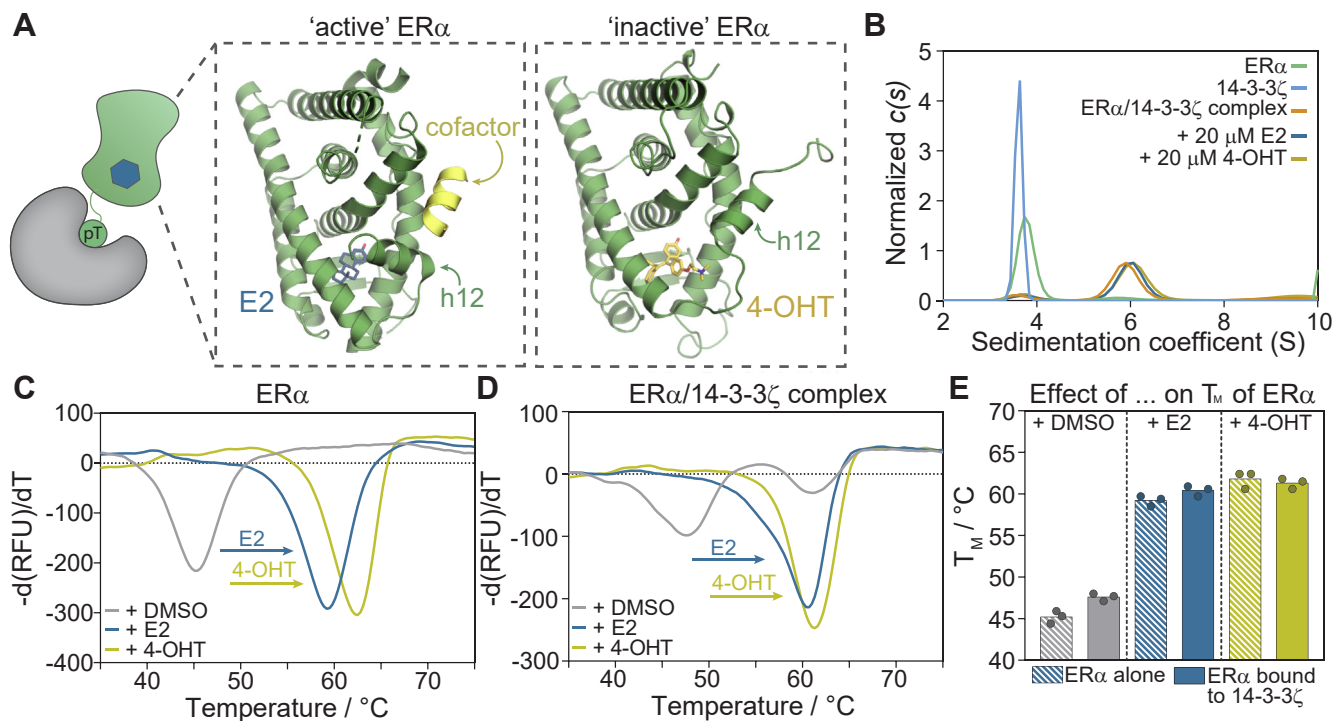


Figure 5. ER α ligand binding to the ER α /14-3-3 ζ complex. **A**, schematic representation of ER α /14-3-3 complex with ER α ligand (blue hexagon) binding in the ER α LBD. Crystal structures of ER α ligands E2 and 4-OHT bound to the ER α ligand binding domain (green cartoon). Ligand binding changes the conformation of helix 12 and allows, in case of E2, to cofactor peptide binding (yellow cartoon), PDB: 5WGD & 3ERT. **B**, area-normalized sedimentation distributions $c(s)$ of 10 μ M 14-3-3 ζ (blue), 10 μ M ER α (green), and 10 μ M ER α /14-3-3 ζ complex (orange). The latter was also analyzed in the presence of 20 μ M E2 (blue) or 20 μ M 4-OHT (yellow). **C** and **D**, differential melting curves of 5 μ M ER α (**C**) or 10 μ M ER α /14-3-3 ζ protein complex (**D**) in the presence of DMSO (negative control), 100 μ M E2 or 100 μ M 4-OHT. **E**, melting temperatures T_M of ER α by itself or when bound to 14-3-3 ζ in the presence of DMSO, E2, or 4-OHT.

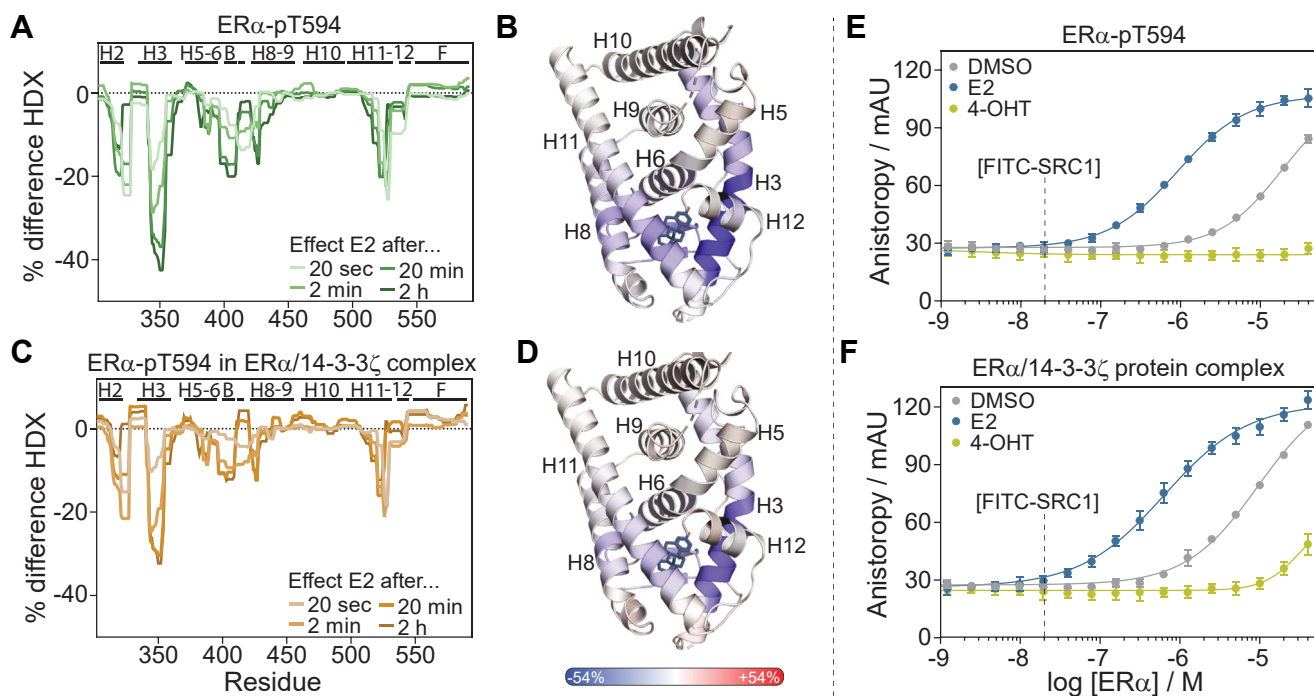


Figure 6. Effect of ER α ligand binding to ER α /14-3-3 ζ complex on ER α conformation and co-factor binding. **A** and **C**, difference HDX exchange profile of ER α -pT594 (**A**) and ER α -pT594 in ER α /14-3-3 ζ protein complex (**C**) upon E2 binding after 20 s, 2 min, 20 min or 2 h of HDX. **B** and **D**, HDX difference profile of ER α -pT594 (**B**) and ER α in ER α /14-3-3 ζ protein complex (**D**) upon addition of E2 ligand after 2 h incubation. The shielding effect (less deuterium exchange) is shown in blue and deshielding effects (increase in deuterium exchange) in red. Results are displayed on the ER α LBD crystal structure, PDB: 5WGD. **E** and **F**, fluorescence anisotropy SRC-1 co-factor recruitment assay where ER α -pT594 or ER α /14-3-3 ζ protein complex was titrated to 20 nM FITC-labeled SRC-1 peptide in the presence of DMSO (negative control), 100 μ M E2 or 100 μ M 4-OHT.

S30). Major shielding effects were observed for parts of H3 (residues 341–361), the beta sheet region with adjacent loops (residues 398–410), and the C-terminal part of H11 (512–527). Smaller effects were also observed in H1-2 (residues 314–320), H6 (residues 380–396), H8 (residues 421–438), the H11 to 12 loop, and H12 (residues 528–541). No E2-induced differences were observed around H5, H9, H10, and the F-domain of ER α . Notably, the overall effects of E2 appeared to be smaller (max. shielding effect \sim 30% instead of 40%) when ER α was bound to 14-3-3 ζ which can be explained by the partial shielding effects that 14-3-3 ζ already has on ER α , making the effect of E2 less pronounced. The affected regions of ER α upon E2 binding correlated well with published ER α -E2 crystal structures (Fig. 6, B and D), where E2 binds in a pocket formed by H3, H6, H8, the beta sheets, and H11 to 12 (84). Interestingly, these results indicated that the ER α F-domain conformation was not significantly affected by E2 binding, which has not been studied on a structural level previously. The similar shielding effects upon E2 binding to ER α alone and the ER α /14-3-3 ζ complex, clearly showed that 14-3-3 ζ does not influence the E2-induced conformational changes in ER α . This suggests that the ER α F-domain is sufficiently long and flexible to accommodate ligand-induced conformational changes to helix 12, without affecting 14-3-3 binding to ER α .

ER α -cofactor binding is permitted when 14-3-3 is bound to ER α

Next, we sought to understand how 14-3-3 influences ER α cofactor recruitment. ER α binding to a cofactor was studied in FA experiments in which ER α was titrated, in the absence and presence of 14-3-3 ζ , against a fluorescein-labeled peptide representing an alpha-helical LXXLL recognition motif from the p160 family member SRC-1/NC α -1 (Fig. 6, E and F; Fig. S31) (59). These ER α -cofactor binding studies were furthermore performed in the presence of agonist ligand E2 or antagonistic ligand 4-OHT. Both non-phosphorylated and phosphorylated ER α showed typical binding curves to the LXXLL peptide ($K_D \sim 20 \mu\text{M}$), which was strongly enhanced upon the addition of E2 ($K_D = 1.0 \pm 0.2 \mu\text{M}$) and abolished in the presence of 4-OHT (Figs. 6E and S31). Titration of the ER α /14-3-3 ζ complex showed similar binding curves as observed for ER α alone. ER α , in complex with 14-3-3 ζ , bound SRC-1 peptide with a K_D of $9.6 \pm 1.1 \mu\text{M}$, and featured a strong E2 responsiveness ($K_D = 0.6 \pm 0.1 \mu\text{M}$) (Figs. 6F and S31). Furthermore, 4-OHT strongly reduced ER α binding to the LXXLL peptide, when ER α was bound to 14-3-3 ζ .

FA assays with the mutated ER α -Y537S construct showed improved cofactor recruitment of *apo* ER α -Y537S in comparison to WT ER α (Fig. S31). *Apo* ER α -Y537S provided a binding affinity for the LXXLL peptide of $1.7 \pm 0.1 \mu\text{M}$, which was almost similar to the amount of cofactor recruitment in the presence of agnostic ligand E2 ($K_D = 0.8 \pm 0.1 \mu\text{M}$). These results align with previously published data where the Y537S mutation resulted in a constitutively active conformation of ER α in the absence of agonistic ligands (54). Furthermore, ER α -Y537S was found to be less sensitive to 4-OHT inhibition

of cofactor recruitment (Fig. S31). Similar to WT ER α , 14-3-3 binding did not significantly influence SRC-1 recruitment to the ER α -Y537S protein (Fig. S31).

ER α /14-3-3 PPI stabilization by FC-A is orthogonal to ER α ligand binding

The natural product FC-A is a known stabilizer of the ER α /14-3-3 ζ PPI. This small molecule binds at the interface of ER α /14-3-3 protein complex (Fig. 7A) and thereby increases the affinity between the binding partners (38, 46). DSF studies were used to determine the effect of FC-A on 14-3-3 ζ , ER α , or the ER α /14-3-3 ζ protein complex, for both WT ER α and ER α -Y537S (Fig. 7B; Figs. S24 and S25). As expected, FC-A had no effect on the T_M of 14-3-3 ζ or ER α alone (Fig. S24) but increased the T_M of the 14-3-3 ζ protein in complex with WT ER α from 61.1 to 67.6 °C (+6.5 °C) (Fig. 7, B and C). Similarly, FC-A was found to stabilize the ER α -Y537S/14-3-3 ζ complex as apparent from the increase 14-3-3 ζ melting temperature from 60.2 to 66.3 °C (+6.1 °C) (Fig. S25). FC-A did not affect the T_M of the ER α protein, in the ER α /14-3-3 ζ complex, indicating a local effect of FC-A, confined to the composite binding pocket. This is in line with the previous observations, where the ER α F-domain acts as a long and flexible linker between the most C-terminal ER α residues binding in the 14-3-3 binding groove, and the globular ER α LBD dimer.

Interestingly, the FC-A-induced increase of 14-3-3 ζ thermal stability was fully orthogonal to E2 or 4-OHT binding to both the WT ER α and the Y537S mutant. In the presence of E2 and 4-OHT, FC-A still increased the melting temperature of 14-3-3 ζ in the ER α /14-3-3 ζ protein complex with +6.4 °C and +6.5 °C, respectively (Figs. 7C and S24). In reverse, the earlier described increase in ER α melting temperature upon the addition of E2 and 4-OHT (Fig. 5, D and E) was not influenced by ER α /14-3-3 ζ stabilization by FC-A (Figs. 7D and S24). E2 and 4-OHT increased the ER α T_M , in the ER α /14-3-3 ζ complex, with +12.8 °C and +13.7 °C, respectively, which was even slightly increased in the presence of FC-A (+14.1 °C E2; +15.4 °C 4-OHT) (Figs. 7D and S24). FC-A thus clearly stabilized the ER α /14-3-3 ζ complex, for both WT ER α and Y537S mutant, and showed to be independent of ER α ligand binding.

Discussion

NR drug discovery approaches have mainly focused on targeting the NR endogenous ligand binding pocket present within the LBD. Despite great successes using this approach, significant interest has developed in alternative manners to modulate NRs. An orthogonal entry for NR modulation is offered by their PPIs with the 14-3-3 protein. However, the highly relevant molecular understanding of these NR/14-3-3 PPIs is often lacking, while this is necessary to identify new entry points for NR drug discovery. Here we studied the NR ER α and its interaction with 14-3-3 on a molecular level. Co-purification of the intact complex of 14-3-3 ζ and the ER α LBD and F domains revealed high-affinity binding between ER α and 14-3-3 ζ *via* the formation of a tetrameric complex between an ER α homodimer and a 14-3-3 ζ homodimer. Furthermore, the

The 14-3-3/ER α -LBD-F-domain complex

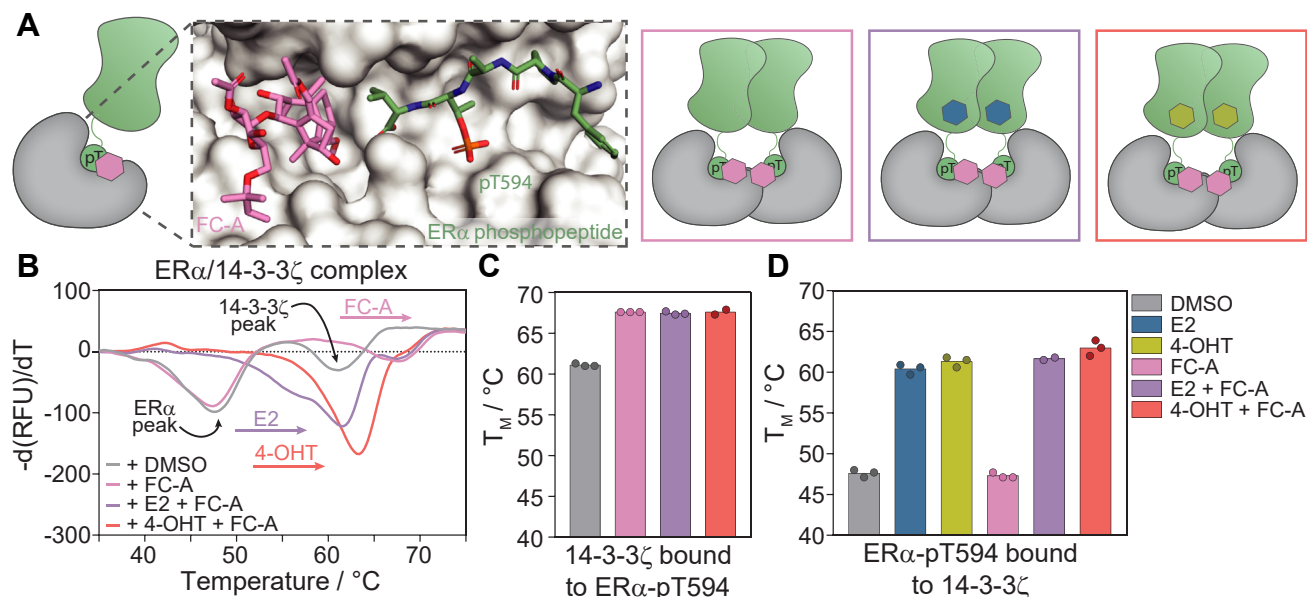


Figure 7. ER α /14-3-3 ζ complex stabilization by FC-A. A, schematic representation of FC-A (pink hexagon) binding to the ER α /14-3-3 protein interface with the co-crystal structure of 14-3-3 α (gray surface) with the Era-derived C-terminal phosphopeptide (green sticks) and the stabilizing molecule Fusicoccin-A (FC-A, pink sticks) and PDB: 4JDD. B, differential melting curve of 10 μ M ER α /14-3-3 ζ protein complex in the presence of DMSO (negative control), 100 μ M FC-A, 100 μ M FC-A and 100 μ M E2 and, 100 μ M FC-A and 100 μ M 4-OHT. C, bar plot of melting temperatures of 14-3-3 ζ in the ER α /14-3-3 complex from differential melting curves in panel b. D, bar plot of melting temperatures of ER α in the ER α /14-3-3 complex from differential melting curves in panel b and Fig. S24.

binding of 14-3-3 ζ to the disease-relevant Y537S-ER α was confirmed, highlighting the possibility of targeting the ER α /14-3-3 ζ PPI as an alternative drug discovery approach for drug-resistant mutants of ER α .

Both agonist (E2) and antagonist (4-OHT) binding to the ER α LBD did not disrupt 14-3-3 binding to ER α , as apparent from SV-AUC and the native PAGE. Furthermore, natural ligand E2 induced activating conformational changes of the ER α LBD and subsequent cofactor peptide recruitment, in a similar fashion for ER α in isolation and ER α in complex with 14-3-3 ζ . Similarly, synthetic ligand 4-OHT showed antagonistic behavior for ER α alone and ER α in complex with 14-3-3 ζ , as observed by reduced cofactor recruitment. Finally, the ER α /14-3-3 ζ PPI stabilization by FC-A was shown to be functional and not impeded, nor dependent, on ER α ligand binding in both wild-type ER α and the Y537S mutated protein.

Combined, these results inform that 14-3-3 ζ binding to ER α , and stabilization of this PPI by FC-A, function independently of conformations, mutations, and liganded state of the ER α LBD. This orthogonality is most likely facilitated by the long and flexible 42-residue F-domain of ER α , accommodating ER α conformations to not affect 14-3-3 binding. Molecular stabilization of the ER α /14-3-3 protein complex with molecular glues like FC-A would therefore be a potential entry point for targeting ER α and its drug-resistant variants. The orthogonality of the molecular events within the ER α would even bode for dual targeting of both the PPI interface and the classical ER α ligand binding pocket. Furthermore, whereas orthosteric drugs such as 4-OHT also show binding to ER β (85) and ERR γ (86), next to ER α , 14-3-3 ζ binding to ER α occurs at the ER α -unique C-terminus, providing the possibility to target ER α in a highly selective manner.

The concept of therapeutic targeting of the ER α /14-3-3 PPI could be envisioned to be translatable to other NR/14-3-3 protein complexes. So far, 14-3-3 has been identified as the binding partner of eight NRs, for which 14-3-3 binds to each NR in a unique manner. The NR/14-3-3 interactions form a potential entry point for targeting 'hard-to-drug' NRs due to, for example, drug resistance or the absence of an orthosteric pocket in the LBD. A potential example is the Androgen Receptor (AR), an established prostate cancer target. Although prostate cancer is initially often successfully targeted with androgen deprivation therapy or AR antagonists such as enzalutamide, drug- and castration-resistant AR mutants or splice variants are often developed within patients with prostate cancer (25, 87, 88). The most prevalent AR splice variant, AR-V7, even lacks the entire LBD while remaining constitutively active, making it extremely challenging to target this drug-resistant variant of AR (87, 88). The binding of 14-3-3 to the NTD of AR, which remains present in the AR splice variants, provides an alternative entry point of targeting AR in drug- and castration-resistant patients with prostate cancer. In all cases, mechanistic and structural insights into the formation of the NR/14-3-3 complex, such as those obtained in this study for the ER α /14-3-3 complex are urgently needed.

Experimental procedures

Protein expression and purification - ER α -strep

A His-Sumo-ER α LBD-F-strep (residues 302–595 in pCDFDuet-1, uniprot P03372) construct was designed which contained three mutations (S305A, F591R, and P592R) to introduce a PKA recognition motif for *in vitro* phosphorylation of T594, and to remove a potential PKA phosphorylation site S305.

pCDFDuet-1-His-SUMO-ER α LBD-F-strep was transformed into NiCo21 (DE3) competent cells using heat stock and were plated on LB-agar plates containing 100 μ g/ml streptomycin. A single colony was selected and cultured overnight in 50 ml LB medium (100 μ g/ml streptomycin) at 37 °C. After overnight incubation, cultures were transferred to 2 l ZYP-5052 medium (100 μ g/ml streptomycin) and incubated at 37 °C, 180 rpm until an OD₆₀₀ of \sim 2 was reached. Protein expression was then induced with 0.4 mM isopropyl- β -D-thiogalactoside (IPTG), and cultures were incubated overnight at 18 °C, 180 rpm. Cells were harvested by centrifugation (8600 rpm, 20 min, 4 °C) and resuspended in lysis buffer (10 ml/g pellet; 20 mM Tris, pH 7.5, 300 mM NaCl, 10 mM imidazole, 5 mM MgCl₂, 5% (v/v) glycerol, 2 mM beta-mercaptoethanol (β ME) containing cComplete EDTA-free Protease Inhibitor Cocktail tablets (1 tablet/100 ml lysate) and Benzonase Nuclease (Milipore, 5 μ l/100 ml). The cell suspension was lysed using an Emulsiflex-C3 homogenizer (Avestin) and the cell lysate was cleared by centrifugation (20,000 rpm, 30 min, 4 °C). Protein-containing supernatant was loaded on 2 \times 5 ml Ni-NTA columns (HisTrap column, Cytiva) at 5 ml/min and 4 °C. Columns were washed with 3 \times 5 CV Wash Buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 25 mM imidazole, 2% (v/v) glycerol, 2 mM β ME) until proteins concentrations dropped below 0.05 mg/ml. His-SUMO-ER α LBD-F-strep was eluted using 2 to 3 CV Elution Buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 500 mM imidazole, 2% (v/v) glycerol, 2 mM β ME). His-SUMO-ER α LBD-F-strep containing elution fractions were subsequently incubated with SUMO hydrolase (1:100) to remove the His-SUMO tag from ER α during o/n dialysis at 4 °C (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 5% (v/v) glycerol, 2 mM β ME). SUMO-cleaved ER α LBD-F-strep was loaded on a 5 ml Streptavidin column (Strep-TactinXT 4Flow cartridge, IBA) at 3 ml/min. Columns were washed with Buffer W (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) collecting 2 CV until protein concentrations dropped below 0.05 mg/ml. ER α LBD-F-strep was eluted using 4 CV Buffer BXT (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 50 mM biotin). Finally, ER α LBD-F-strep was purified by SEC (Superdex 75pg Hiload 16/600) at 1 ml/min and 4 °C using SEC buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM TCEP). Protein-containing fractions were combined and concentrated to >5 mg/ml, aliquoted, and stored at -80 °C. Correct mass and purity of the proteins were confirmed using SDS-PAGE and Q-ToF LC-MS analysis.

Phosphorylated ER α (ER α -pT594-strep) was expressed and purified in a similar manner as described earlier for non-phosphorylated ER α -strep. An *in vitro* phosphorylation was additionally included directly after SUMO cleavage, and before purification on the streptavidin and SEC columns. SUMO-cleaved ER α LBD-F-strep was phosphorylated by incubation with in house-expressed His-PKA-strep (in a 1:50 ratio PKA:substrate) and 500 μ M ATP, for 3 h at 4 °C.

Protein expression and purification–14-3-3 ζ -strep

A 14-3-3zeta-strep (residues 1–245 in pETDuet-1, uniprot P63104) construct was transformed into NiCo21 (DE3) competent cells using heat stock and was plated on LB-agar

plates containing 100 μ g/ml ampicillin. A single colony was selected and cultured overnight in 8 ml LB medium (100 μ g/ml ampicillin) at 37 °C. After overnight incubation, cultures were transferred to 500 ml TB medium (100 μ g/ml ampicillin) and incubated at 37 °C, 180 rpm until an OD₆₀₀ of 0.6 to 1.2 was reached. Protein expression was induced with 0.4 mM IPTG, and cultures were incubated overnight at 18 °C, 180 rpm. Cells were harvested by centrifugation (8600 rpm, 20 min, 4 °C) and resuspended in lysis buffer (10 ml/g pellet; 20 mM Tris, pH 7.5, 300 mM NaCl, 10 mM imidazole, 5 mM MgCl₂, 5% (v/v) glycerol, 2 mM β ME) containing cComplete EDTA-free Protease Inhibitor Cocktail tablets (1 tablet/100 ml lysate) and Benzonase Nuclease (Milipore, 5 μ l/100 ml). The cell suspension was lysed using an Emulsiflex-C3 homogenizer (Avestin), and the cell lysate was cleared by centrifugation (20,000 rpm, 30 min, 4 °C). Protein-containing supernatant was loaded on a 5 ml Streptavidin column (Strep-TactinXT 4Flow cartridge, IBA) at 3 ml/min. Columns were washed with 4 \times 2CV Buffer W (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA). 14-3-3 ζ -strep was eluted using 2 CV Buffer BXT (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 50 mM biotin). Finally, 14-3-3 ζ -strep was purified by SEC (Superdex 75pg Hiload 16/600) at 1 ml/min and 4 °C using SEC buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM TCEP). Protein-containing fractions were combined and concentrated to >5 mg/ml, aliquoted, and stored at -80 °C. Correct mass and purity of the protein were confirmed using SDS-PAGE and Q-ToF LC-MS analysis.

Protein expression and purification—ER α /14-3-3 ζ protein complex

His-SUMO-ER α LBD-F (residues 302–595 in pCDFDuet-1, uniprot P03372) was co-transformed with full-length 14-3-3zeta (residues 1–245 in pETDuet-1, uniprot P63104) and SUMO-PKA (residues 1–351 in pACYC, UniProt P17612) (see all protein sequences in [Supporting information](#)) into NiCo21 (DE3) competent cells using heat stock and were plated on LB-agar plates containing antibiotics for selection: 100 μ g/ml streptomycin, 100 μ g/ml ampicillin and 25 μ g/ml chloramphenicol). A single colony was selected and cultured overnight in 50 ml LB medium (containing the same antibiotics) at 37 °C. After overnight incubation, cultures were transferred to 2 l ZYP-5052 medium (containing the same antibiotics) and incubated at 37 °C, 180 rpm until an OD₆₀₀ of \sim 2 was reached. Protein expression was induced with 0.4 mM IPTG and cultures were incubated overnight at 18°C, 180 rpm. Cells were harvested by centrifugation (8600 rpm, 20 min, 4 °C) and resuspended in lysis buffer (10 ml/g pellet; 20 mM Tris, pH 7.5, 300 mM NaCl, 10 mM imidazole, 5 mM MgCl₂, 5% (v/v) glycerol, 2 mM β ME) containing cComplete EDTA-free Protease Inhibitor Cocktail tablets (1 tablet/100 ml lysate) and Benzonase Nuclease (Milipore, 5 μ l/100 ml). The cell suspension was lysed using an Emulsiflex-C3 homogenizer (Avestin) and the cell lysate was cleared by centrifugation (20,000 rpm, 30 min, 4 °C). Protein-containing supernatant was loaded on 2 \times 5 ml Ni-NTA columns (HisTrap column,

The 14-3-3/ER α -LBD-F-domain complex

Cytiva) at 5 ml/min and 4°C. Columns were washed with Wash Buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 25 mM imidazole, 2% (v/v) glycerol, 2 mM β ME) collecting each time 1 to 3 CV fractions until proteins concentrations dropped below 0.05 mg/ml to ensure removal of all excess 14-3-3. 14-3-3 ζ /ER α complex was then eluted using 2 to 3 CV Elution Buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 500 mM imidazole, 2% (v/v) glycerol, 2 mM β ME). The 14-3-3 ζ -strep/His-SUMO-ER α complex was subsequently incubated with SUMO hydrolase (1:100) to remove the His-SUMO tag from ER α during overnight dialysis at 4 °C (20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 5% (v/v) glycerol, 2 mM β ME). SUMO-cleaved 14-3-3 ζ -strep/ER α complex was loaded on a 5 ml StrepTactin column (Strep-TactinXT 4Flow cartridge, IBA) at 3 ml/min. Columns were washed with Buffer W (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) collecting 2 CV fractions until protein concentrations dropped below 0.05 mg/ml to ensure removal of all excess ER α . 14-3-3 ζ /ER α complex was eluted using 8 CV Buffer BXT (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 50 mM biotin). Finally, the protein complex was purified by SEC (Superdex 75pg Hiloal 16/600) at 1 ml/min and 4 °C using SEC buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM TCEP). Protein-containing fractions were combined and concentrated to >10 mg/ml, aliquoted and stored at -80 °C. Correct mass and purity of the proteins was confirmed using SDS-PAGE and Q-ToF LC-MS analysis.

Analytical SEC

Protein samples were diluted in 20 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM TCEP to a final concentration of 5 to 10 μ M. All analytical SEC experiments were performed on an Agilent 1260 bio-inert HPLC in combination with a Superdex200 increase 3.2/300 column at a flow rate of 0.075 ml/min and 20 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM TCEP as running buffer. Peak detection was performed by absorbance measurements at 280 nm.

Sedimentation-velocity AUC

Protein samples were dialyzed into 20 mM HEPES pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM TCEP before all AUC measurements to obtain the best buffer match between the blank and the sample. Protein samples were diluted to their final concentrations in dialysis buffer and ligands were added where described. Samples were placed into double sector titanium centerpieces with 12-mm optical path length. SV-AUC experiments were performed using a ProteomLabTM XL-I analytical ultracentrifuge (Beckman Coulter) at 20 °C and at 43.000 to 45.000 rev./min rotor speed (An-50 Ti rotor, Beckman Coulter). All sedimentation profiles were collected by absorbance measurements at 280 nm. The calculated distributions were integrated to establish the weight-average sedimentation coefficients corrected to 20 °C and to the density of water ($s_{w(20,w)}$).

QToF-MS quantification

Dilution series of 14-3-3 ζ -strep and ER α -pT594-strep were prepared in MQ (0.1% FA) to final concentrations of 0.025,

0.020, 0.015, 0.010, and 0.005 mg/ml. Furthermore, a 500 \times , 750 \times and 1000 \times dilution of the ER α /14-3-3 ζ protein complex were prepared. The final samples (~100 μ l) were transferred to a 200 μ l LC-MS vial. UPLC-QToF-MS analysis was performed on a Waters (Milford, MA, USA) Acquity I-Class UPLC system coupled to a Waters Xevo G2-XS quadrupole time-of-flight (QToF) mass spectrometer. The devices were controlled by MassLynx Software (version 4.2, Waters). Full scan in positive electrospray ionization (ESI+) mode was used as MS acquisition mode with an acquisition range from 150 to 2000 m/z. A 3 μ m, 100 \times 2.0 mm Polaris 3 C8-A column (Agilent, Middelburg, the Netherlands) was placed inside a column oven at 40 °C and used for chromatographic separation. Flowrate was set at 0.3 ml/min, and a gradient of water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B) was set as follows (all displayed as % v/v): 0.0 to 2.0 min (30% to 39% B), 2.0 to 5.0 min (39% B), 5.0 to 7.5 min (39% to 60% B), 7.5 to 8.0 min (60% B), 8.0 to 8.1 min (60% to 30% B) 8.1 to 10.0 min (30% B). Sample injection volume 2 μ l. Mass Spectrometry settings were set as follows: capillary voltage: 0.80 kV, cone voltage: 40 V, source offset: 80 V, source temperature: 120 °C, desolvation temperature: 450 °C, cone gas: 10 l/h desolvation gas: 1000 l/h.

Data were analyzed using MassLynx software. Chromatograms were background subtracted (polynomial order 1, below curve 40%, tolerance 0.010, flatten edges). The area under the peak was determined using integration in the MassLynx software with a relative area threshold of 10. The obtained area under the curve was then plotted against the protein concentration, after which a linear regression was determined between the five data points. Using the equation of the linear regression, the concentration of 14-3-3 and ER α was determined in each protein complex sample.

To perform mass analysis of the individual peaks deconvolution was performed on m/z spectra of each individual peak. After visual inspection of the m/z spectrum, the spectrum was zoomed to the five most abundant peaks from which the mass spectrum was determined using MaxEnt1 (mass ranges 27–30 kDa or 32–36 kDa; resolution 0.10 Da/channel, Simulated Isotope Pattern with Spectrometer Blur width 0.32–0.38 Da, minimum intensity ratios left 33%, right 33%, iterate to converge). Mass spectra were centered and errors of the deconvolution process were determined.

HDX - peptide mapping

100 pmol of 14-3-3 ζ -strep or ER α (PKA)-strep was mixed in 1:1 (v/v) ratio with 1 M glycine at pH 2.3 and injected on a mixed Pepsin/Nepenthesin-2 acidic protease column. Generated peptides were trapped and desalted by a Micro trap column (Luna Omega 5 μ m Polar C18 100 Å Micro Trap 20 \times 0.3 mm) for 3 min at a flow rate 200 μ l min⁻¹ using isocratic pump delivering 0.4% formic acid in water. Both protease column and trap column were placed in an icebox. After 3 min, peptides were separated on a C18 reversed-phase column (Luna Omega 1.6 μ m Polar C18 100 Å, 100 \times 1.0 mm) with a linear gradient 5 to 35% B in 26 min, where solvent A

was 2% acetonitrile/0.4% formic acid in water and solvent B 95% acetonitrile/5% water/0.4% formic acid. The analytical column was placed in an icebox. TimsToF Pro mass spectrometer (Bruker Daltonics) operating in positive MS/MS mode was used for the detection of peptides. Data were processed by DataAnalysis 5.3 software (Bruker Daltonics). MASCOT search engine was used for the identification of peptides using a database containing the sequence of 14-3-3 ζ or ER α .

HDX

All proteins were dialyzed and diluted into 20 mM Hepes, 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM TCEP pH7.5 to a final concentration of 20 μ M. E2 or DMSO was added to a final concentration of 150 μ M. Hydrogen deuterium exchange was initiated by 10-fold dilution of the proteins under different conditions in a deuterated buffer. Fifty microliter aliquots (100 pmol) were taken after 20 s, 2 min, 20 min and 2 h of incubation in deuterated buffer, quenched by 50 μ l of 1 M glycine, pH 2.3 and snap frozen in liquid nitrogen. Aliquots were quickly thawed and analyzed using the same system as described above. Peptides were separated by linear gradient 10 to 30% B in 18 min. Mass spectrometer was operated in positive MS mode. Spectra of partially deuterated peptides were processed by Data Analysis 5.3 (Bruker Daltonics) and by in-house program DeutEx.

Native PAGE

Samples were prepared in 20 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM TCEP with protein at a final concentration of 2.5 to 5 μ M. Ligands were added at a final concentration of 100 μ M. All samples were 1:1 diluted into native PAGE loading dye (62.5 mM Tris pH 7.1, 75 mM NaCl, 5 mM MgCl₂, 20% glycerol, 0.01% bromophenolblue). after which 12 μ l of each sample was loaded on a 4 to 20% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad). Gels ran at 130 V for 2.5 h at 4 °C in running buffer (25 mM Tris, 192 mM Glycine, pH 8.3). Gels were washed in MilliQ (20 min), stained with Coomassie Brilliant Blue G-250 (Bio-Rad), and destained in MilliQ until bands were clearly visible. Gels were imaged and analyzed with ImageJ.

Differential scanning fluorimetry

Proteins were diluted (in 20 mM Hepes pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 500 μ M TCEP) to obtain 40 μ l samples containing 5 μ M 14-3-3 ζ -strep, 5 μ M ER α -strep or 10 μ M ER α /14-3-3 ζ complex, with either 1% DMSO (negative control) or 100 μ M ligand (E2, 4-OHT, FC-A). All samples additionally contained 10x ProteoOrange dye (Lumiprobe, 5000x stock in DMSO) and were heated from 35 to 79 °C at a rate of 0.3 °C per 15 s in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Fluorescence intensity was determined using excitation 575/30 nm and emission 630/40 nm filters. Based on these melting curves, the (negative) first derivative melting curve was obtained, from which the melting temperature T_M could be determined. Reported T_M

values were obtained from three independent experiments from which the average and standard deviations were determined using excel.

Fluorescence anisotropy

All FA dilution series were prepared in polystyrene (non-binding) low-volume Corning Black Round Bottom 384-well plates (Corning 4514 or 4511). FA measurements were performed directly after plate preparation, using a Tecan Infinite F500 plate reader at room temperature (I_{ex} : 485 \pm 20 nm; I_{em} : 535 \pm 25 nm; mirror: Dichroic 510; flashes: 20; integration time: 50 ms; settle time: 0 ms; gain: 60; and Z-position: calculated from well). Wells containing only fluorescein-labeled peptide were used to set as G-factor at 35 mP. All data were analyzed using GraphPad Prism (7.00) for Windows and fitted using a four-parameter logistic model (4PL) to determine apparent binding affinities (K_D^{app}). All results are based on two independent experiments from which the average and standard deviations were calculated to obtain the final values.

FA - SRC1 cofactor peptide binding studies

ER α (PKA)-strep, ER α (PKA)-pT594-strep, and ER α /14-3-3 ζ protein complex (for WT ER α and Y537S mutant) were titrated in a 2-fold dilution series (starting at 40 μ M ER α) to 20 nM of fluorescein-labeled SRC1 peptide in 20 mM HEPES pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.1% (v/v) Tween20, 0.1% (w/v) BSA. This is done in the presence of DMSO (negative control), 100 μ M E2 or 100 μ M 4-OHT.

FA - ER α peptide mimic binding studies

14-3-3 ζ was titrated in a 2-fold dilution series (starting at 300 μ M 14-3-3 ζ) to 2 nM of fluorescein-labeled peptide (WT-ER α , ER α (PKA), ER α (PKA)-strep) in 20 mM HEPES pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 0.1% (v/v) Tween20, 0.1% (w/v) BSA. This is done in the presence of a 2-fold dilution series of 3'-de-Ac FC-A (0.015–250 μ M); each well contained a final concentration of 0.25% DMSO.

X-ray crystallography data collection and refinement

N-acetylated ER α (PKA)-pT594 and ER α (PKA)-pT594-strep peptides were mixed with 10 mg/ml 14-3-3 $\sigma\Delta$ C protein (truncated after T231 to reduce flexibility) in complexation buffer (20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂ and 20 μ M TCEP) using a final molar stoichiometry of 1:2 or 1:4 protein to peptide. These complexes were used to set up sitting-drop crystallization plates were set up in which each of the four complexation mixtures were combined with 24 crystallization buffers, optimized for 14-3-3 σ crystallization (0.095 M HEPES (pH7.1, 7.3, 7.5, 7.7), 0.19 M CaCl₂, 24 to 29 % (v/v) PEG 400 and 5% (v/v) glycerol). For each combination, a 1:1 mix (both 250 nl) of complexation mixture and crystallization buffer was prepared for crystal growth. Crystals grew within 10 to 14 days at 4 °C. Co-crystals of 14-3-3 ζ with ER α peptides (ER α (PKA)-pT594 and ER α (PKA)-pT594-strep) were

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soaked with 10 mM 3' deAc-FC-A in crystallization buffer and incubated for 3 days at 4 °C.

Both soaked and non-soaked crystals were fished and flash-frozen in liquid nitrogen. X-ray diffraction data were collected at the p11 beamline of PETRA III facility at DESY (Hamburg, Germany) with the following settings: 1440 image, 0.25°/image, 100% transmission, and 0.1 s exposure time. Initial data processing was performed at DESY using XDS after which pre-processed data was taken to further scaling steps, molecular replacement, and refinement.

Data were processed using the CCP4i2 suite (version 7.1.18). XDS-preprocessed data were scaled using AIMLESS. The data were phased with MolRep, using protein data bank (PDB) entry 4J3C as a template. A three-dimensional structure of 3' dAc-FC-A was generated using AceDRG, which was thereafter built in the electron density based on visual inspection Fo-Fc and 2Fo-Fc electron density map. Sequential model building (based on visual inspection Fo-Fc and 2Fo-Fc electron density map) and refinement were performed with Coot and REFMAC, respectively. Finally, alternating cycles of model improvement (based on isotropic b-factors and the standard set of stereo-chemical restraints: covalent bonds, angles, dihedrals, planarities, chiralities, non-bonded) and refinements were performed using Coot and phenix.refine from the Phenix software suite (version 1.20.1-4487). Pymol (version 2.2.3) was used to make the figures in the manuscript. All structures were deposited in the protein data bank (PDB) and obtained IDs: 8C40, 8C42, 8C3Z and 8C43. See [Table S1](#) for x-ray crystallography data statistics.

Data availability

Crystal structures described in this manuscript have been deposited to the PDB. They have the following PDB codes: 8C40, 8C42, 8C3Z, and 8C43.

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare the following competing interest(s): L. B. and C. O. are scientific co-founders of Ambagon Therapeutics. C. O. and E. S. are employees of Ambagon Therapeutics.

Abbreviations—The abbreviations used are: 4-OHT, 4-hydroxytamoxifen; AE, Androgen Receptor; AUC, analytical ultracentrifugation (AUC); DBD, DNA-binding domain; DSF, differential scanning fluorimetry; E2, estradiol; ER α , estrogen receptor alpha; FA, fluorescence anisotropy (FA); FC-A, Fusicoccin A; GR, Glucocorticoid Receptor; HDX, hydrogen-deuterium exchange; LBD, ligand binding domain; NRs, Nuclear receptors; PPI, protein–protein interaction; SEC, size exclusion chromatography; SV-AUC, sedimentation velocity AUC.

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