



Published in final edited form as:

J Neuroendocrinol. 2015 December ; 27(12): 861–871. doi:10.1111/jne.12326.

Phosphorylation alters Oestrogen Receptor β -mediated transcription in neurons

Elena Pinceti, Cody L. Shults, Yathindar S. Rao, Natasha N. Mott, and Toni R. Pak
Loyola University Chicago Stritch School of Medicine, Department of Cell and molecular Physiology, Maywood IL 60153

Abstract

Nuclear steroid hormone receptors are ubiquitously expressed transcription factors whose activity can be altered by post-translational modifications, such as phosphorylation. The consequences of post-translational modifications have been described for several members of the nuclear steroid hormone receptor superfamily, however little is known about the effects of oestrogen receptor beta (ER β) phosphorylation in the brain. Moreover, to our knowledge the presence of phosphorylated ER β has not been detected in the brain of any species to date. Oestrogen receptor β is highly expressed in several regions of the brain and *in vitro* studies have demonstrated that it can be phosphorylated at two serine residues (S87 and S105) in the N-terminal AF-1 region. The goal of this study was to determine whether phosphorylated ER β is detectable in the hippocampus of aged female rats, and to determine the functional consequences of ER β S87 and S105 phosphorylation on transcriptional activity in neuronal cells. First, we used a novel PhosTagTM approach to detect phosphorylated forms of ER β in the dorsal hippocampus of aged female rats. The data demonstrated several abundant forms of phosphorylated ER β in the dorsal hippocampus, suggesting that this post translational modification might be an important regulator of ER β function. To assess the functional consequences of ER β phosphorylation in neuronal cells, we created phospho-mimetic (S87E, S105E) and phospho-null (S87A, S105A) ER β receptors that were transiently transfected in a hippocampal-derived cell line. Collectively our results showed that phosphorylation of S87 and S105 altered both ligand-independent and ligand-dependent ER β transcriptional regulation. Overall these data demonstrate that phosphorylated forms of ER β are present in the brain of aged female rats and that phosphorylation of ER β could differentially alter ER β -mediated gene expression.

Keywords

Oestrogen Receptor β ; phosphorylation; hippocampus

INTRODUCTION

Nuclear steroid receptors are master regulators of a broad range of physiological processes through their actions as ligand-activated transcription factors. Oestrogen receptors (ER α and

ER β) are members of this receptor superfamily and their cognate endogenous ligand is 17 β -estradiol (E2), which is the major circulating form of oestrogen in premenopausal women. ER α and ER β are widely expressed in a variety of tissues, although ER α is particularly abundant in breast, uterus and ovary, due to its primary role in mediating the reproductive-related effects of oestrogens. By contrast, ER β is highly expressed in many non-reproductive tissues such as nervous, cardiovascular, skeletal, gastric, and adipose tissues, and has been implicated in mediating oestrogens effects on anxiety, mood, and memory, as well as numerous other physiological processes [1–6]. Our basic understanding of ER α structure, function, and signalling pathways has significantly advanced in recent years, however similar aspects of ER β function, especially in non-reproductive tissue/cell types, remain unclear. The overall goal of these studies was to define the functional consequences of post-translational modifications to ER β , namely site-specific phosphorylation, on its ability to transcriptionally activate gene promoter activity in neuronal cells.

Post-translational modifications (PTMs) such as phosphorylation, ubiquitination, acetylation, and sumoylation have been identified for several nuclear steroid receptors, including ERs [7–11]. These modifications have the potential to alter all aspects of ER function including ligand binding, dimerization, protein:protein interactions, DNA binding and, ultimately, alter ER-mediated transcription. Although several kinase consensus sites have been predicted for ER β , only a few have been experimentally confirmed [12]. Specifically, the serine residues S87 and S105 located in the N-terminal domain of ER β are highly conserved among the mouse, rat, and human, suggesting that ER β phosphorylation at these sites could be a common regulatory mechanism across species [13]. The N-terminal domain of ER β exhibits greater than 80% homology across species and the specific amino acid residues flanking S87 and S105 are highly conserved [14]. By contrast, the N-terminal domain of ER β has relatively low homology with ER α , which could contribute to the divergent actions of the two receptors. These S87 and S105 sites are targets of MAPKs (Mitogen Activated Protein Kinases) and the specific MAPKs, P38 and ERK, have been shown to phosphorylate human and mouse ER β *in vitro* [13, 15]. Functionally, phosphorylation of these sites increased recruitment of the coregulatory protein SRC-1 (steroid receptor coactivator-1), while coincidentally increasing transcriptional activation at an oestrogen response element (ERE) [15].

Phosphorylation of ER β has been studied primarily *in vitro* using breast tumour cell models. The only reports of detection of phosphorylation of ER β *in vivo* come from immunohistochemistry analysis of human breast cancer tissue using a human-specific antibody generated against the phosphorylated S105 ER β [13]. Therefore, our first goal in these studies was to determine whether ER β is phosphorylated *in vivo* in the brain of female rats. Using PhosTagTM Acrylamide we were able to detect several phosphorylated species of ER β in the dorsal hippocampus of aged (18 mo. old) female rats. This, to our knowledge, is the first report of phosphorylated ER β detection in the brain of any species.

Previous work by our laboratory showed that p38 kinase inhibition altered ER β -dependent activation of ERE and AP-1 (activator protein-1) promoter activity in neurons [16]. However p38 kinase inhibitors are broad-spectrum inhibitors and can affect multiple signalling pathways in the cell, thereby making it unclear whether ER β was a direct target of

phosphorylation by p38 in those studies. Furthermore ER β phosphorylation states were not determined. Therefore, in these studies we created phospho-mutants of ER β to directly assess the consequences of ER β phosphorylation on its transcriptional activity in neuronal cells.

We hypothesized that phosphorylation of ER β at specific sites, S87 and S105, would alter ER β mediated gene regulation in neuronal cells, both directly at a canonical ERE site and also indirectly through protein:protein interactions at an AP-1 site. Collectively, our results demonstrate that phosphorylation of S87 and S105 altered both oestrogen-independent and oestrogen-dependent ER β mediated transcriptional regulation at ERE and AP-1 sites in neuronal cells. Taken together, these data suggest that altered kinase activity in the brain, as occurs during aging, has the potential to alter the downstream expression of ER β gene targets resulting in fundamental changes in brain function.

METHODS

Animals

18 month old Fisher 344 female rats were obtained from the National Institutes of Aging (NIA) aged rat colony (Charles River Laboratories, Wilmington, MA) and allowed to acclimate for 7 days. Following acclimation, animals were ovariectomized (OVX) as previously reported [17] and allowed to recover for 1 week. Surgeries were performed under vaporized isoflurane anaesthesia. Post-operation, animals were singly housed and provided with acetaminophen analgesic (122.7 mg/kg) in tap water for 3 days. At 1 week post-OVX, animals were administered a subcutaneous injection of 2.5 μ g/kg of 17 β -estradiol (E2) (Sigma, St. Louis MO) dissolved in sesame oil (N=10) or oil alone (vehicle, N=10) once a day for 3 consecutive days. This dose results in plasma E2 concentrations of 60–80 pg/ml as described previously [17, 18]. Animals were euthanized 24 hours after the last injection, brains rapidly removed, flash frozen and then sectioned at 200 μ m using a freezing microtome. The dorsal hippocampus (–2.30 to –4.16 relative to bregma) was microdissected using a Palkovit's brain punch tool (Stoelting, Inc., Woodale, IL) according to "*The Rat Brain in Stereotaxic coordinates*" [19]. All measures were taken to minimize pain and suffering and animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Loyola University Chicago, permit number 2009018.

Protein Isolation

Total protein was isolated from the dorsal hippocampus and hypothalamus using T-Per reagent (ThermoFisher Scientific, Rockford IL) containing an added protease and phosphatase inhibitor (ThermoFisher Scientific, Rockford IL). Protein concentration was measured using BCA assay kit according to manufacturer's instructions (ThermoFisher Scientific, Rockford IL).

PhosTag™ SDS PAGE

50 μ g of dorsal hippocampus protein was run on precast PhosTag™ Acrylamide 12.5% Acrylamide gels, (Wako Pure Chemical Industries, Osaka, Japan). The gel was then transferred on a PVDF membrane (Promega, Madison WI), blocked for 1 hour with 5%

BSA, then incubated with the oestrogen receptor β antibody H150 (epitope: 1–150 fragment of hER β , N-terminal domain) (Santa Cruz, sc-8974, Dallas TX) at a 1:250 dilution in 5% BSA TBST overnight. Blots were washed twice with TBST for 10 minutes prior to application of 1:5000 goat α -rabbit-HRP (Santa Cruz, sc-2004, Dallas TX) in 5% BSA TBST. Blots were washed twice with TBST for 10 minutes and imaged on the Bio-rad Chemidoc XRS+ imager (Bio-rad, Hercules, CA) using ECL Chemiluminescent substrate (Pierce Scientific, Rockford IL). Densitometry was performed using ImageLab software. Antibody specificity was confirmed with parallel Western Blots and PhosTagTM blots using ER α H-184 antibody (Santa Cruz, sc-7207, 1:1000 dilution; data not shown).

Alkaline Phosphatase treatment

Specificity of phosphorylated proteins were confirmed by treating 50 μ g of dorsal hippocampus protein (vehicle-treated animals) with 0, 30 or 60 units of alkaline phosphatase (Roche, Basel, Switzerland) for 2 hours at 37°C to dephosphorylate all phosphorylated proteins.

Cell culture

The mouse hippocampal-derived cell line HT-22 (generously provided by Dr. David Schubert, Scripps Institute, San Diego, CA) was maintained in DMEM (Corning, Tewksbury, MA) containing 4.5% glucose and L-glutamine supplemented with 1x nonessential amino acids (Corning, Tewksbury, MA) and 10% foetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). Cells were used at 70–80% confluency for all experiments.

Hormone treatments

Cells at 70–80% confluency were rinsed with 1x PBS and then media replaced with phenol red-free DMEM plus 10% charcoal-stripped FBS (Atlanta Biologicals, Norcross, GA) at least 36 hours prior to hormone treatments in order to remove all exogenous hormone sources. 17 β -estradiol (Sigma, St. Louis, MO) and 4-OH Tamoxifen (Sigma, St. Louis, MO) were diluted in molecular grade ethanol (EtOH) (Sigma, St. Louis, MO) and used at a final concentration of 100 nM as described previously [16].

Expression vectors and reporter constructs

Plasmid expression vector (pcDNA 3.0; Invitrogen, Carlsbad, CA) containing inserts for rER- β 1 was provided by Dr. Tom Brown (Pfizer Corp., Cambridge, MA) and has been extensively characterized [20]. The ERE-tk-luciferase reporter construct (generously donated by Dr. Paul Budworth, Case Western Reserve University, Cleveland, OH) contains two repeats of the consensus vitellogenin ERE sequence upstream of the minimal thymidine kinase promoter-firefly luciferase (2xERE-tk-luc) in pGL2-basic plasmid (Promega, Madison, WI). The AP-1-tk-luciferase reporter construct (generously provided by Dr. Colin Clay, Colorado State University, Fort Collins, CO) contains three repeats of the AP-1 sequence into pGL2-basic plasmid. The renilla luciferase pGL4 reporter construct (Promega, Madison, WI) was used as an internal control for transfection efficiency.

Site directed mutagenesis

The pcDNA3.0 plasmid expression vector (Invitrogen, Carlsbad, CA) containing a cDNA insert coding rat ER β 1 was mutated using the Quick Change II XL site-directed mutagenesis kit (Agilent, Santa Clara, CA) to create the phospho-mutants (see Table 1). Primers were designed using the QuickChange primer design available from the Agilent website and point mutations were inserted following manufacturer's instructions. Vectors were validated by DNA sequencing (ACGT, Inc, Wheeling, IL) to confirm successful site directed mutagenesis.

Transient Transfections

HT-22 cells were plated at a density of 20000 cells/well in 96-well plates for 48 h before transfection. Transfections were carried out using Fugene6 (Roche, Basel, Switzerland) or Fugene9 (Roche, Basel, Switzerland) according to manufacturer's instructions. Twenty-four hours after transfection, cells were washed with 1x PBS and incubated with dextran charcoal-stripped media containing hormone treatment or vehicle (EtOH) for 15 h and then lysed for luciferase assays. Transfection efficiency and expression was verified prior to luciferase experiments using GFP-tagged constructs (data not shown).

Luciferase assays

Following lysis, control reporter (Renilla Luciferase) and reporter (Firefly Luciferase) activities were measured using the Dual-Luciferase Reporter Assay system (DLR; Promega, Madison, WI). Relative light units (RLU) were detected using a Biotek Synergy HT plate reader (Biotek, Winooski, VT) with automatic dual injector system and represented as a ratio of Firefly/Renilla Luciferase RLU. All experiments were conducted with 6 replicates for each condition in each 96 well plate and each assay was repeated in 4 or more independent experiments.

Statistics

Two way ANOVA was performed to determine statistical significance and interaction between the groups followed by Tukey post hoc test for comparisons between mutants and wild type vector. Significance was set at P value < 0.05. All transfection data are represented as the mean percent change in fLUC/rLUC compared to vehicle-treated cells transfected with empty vector \pm SEM.

RESULTS

Phosphorylated species of ER β are present in the dorsal hippocampus of aged female rats

The detection of phosphorylated ER β in the brain *in vivo* has not been previously demonstrated, likely due to its relatively low expression and the lack of commercially available phospho-specific antibodies targeting rER β . A powerful tool that has recently emerged is PhosTagTM, a phosphate-binding tag that slows the migration of phosphorylated protein during the electrophoretic run on polyacrylamide gels [21]. Use of PhosTagTM acrylamide results in the detection of several bands when analysed by Western Blot using specific antibodies for the protein of interest, and each band represents the target protein

with a different degree of phosphorylation. For instance, higher bands indicate that the target protein contains several phosphate groups, which result in a slower migration through the gel and is visualized as a higher band shift. To verify the specificity of the phosphorylated bands detected, protein samples can be treated with alkaline phosphatase (AP), which will dephosphorylate the protein and result in the absence of detectable phosphorylated bands on the Western blot. The only band detected will be a lower band representing the non-phosphorylated protein of interest.

We tested whether phosphorylated ER β is present in the dorsal hippocampus of aged female rats to provide rationale for assessing the functional consequences of phosphorylated ER β in neuronal cells. Oestradiol (E2) is known to have direct effects on the dorsal hippocampus, a brain region involved in cognition and memory formation and a region where ER β is more highly expressed than ER α [22, 23]. Because E2 therapy is often prescribed to ameliorate the negative cognitive issues that accompany menopause, we used a model of surgically-induced menopause followed by acute E2 treatment to test whether 1) phosphorylated ER β is present in the brain, and 2) E2 treatment alters ER β phosphorylation levels. Aged Fisher 344 rats (18 mo. old) underwent ovariectomy (OVX) followed by acute E2 or vehicle administration for 3 days (see Methods). Fig. 1 shows a representative PhosTag™ SDS-PAGE blot probed for ER β with the ER β specific antibody. Phosphorylated ER β was detected in the brains of both the vehicle and E2-treated animals (Fig. 1, arrow). Following AP treatment this same band disappeared, indicating that the band that shifted was specific for phosphorylated ER β . AP treatment did not diminish the intensity of the lower band, which represent unphosphorylated ER β , however the higher band is very dark indicating that a large amount of ER β in the aged female dorsal hippocampus was phosphorylated to some degree (Fig. 1).

ER β phosphorylation alters ligand dependent and ligand independent activation of ERE-mediated transcription in neuronal cells

To study the functional effects of phosphorylation of ER β in neurons, we used site-directed mutagenesis to mutate S87 and S105 into alanine (A), a residue that cannot be phosphorylated, or glutamic acid (E), a residue that resembles a phosphorylated serine because of its similar negative charge and molecular bulk [24]. The expression vector constructs used in the transient transfection analysis are listed in Table 1. ER β acts as a transcription factor *in cis* by directly binding to DNA at consensus ERE sequences and activating downstream gene transcription. To test whether phosphorylation of ER β alters its ability to regulate ERE-mediated transcription, we transiently co-transfected wild type (WT)-ER β or one of the mutants listed in Table 1 with the reporter construct ERE-tk-luc in a hippocampal-derived cell line (HT-22).

First we analysed the ERE-mediated promoter activity for each of the phospho-mutants with a single site mutated. A two-factor ANOVA analysis revealed that there was a statistically significant interaction between plasmid and treatment, demonstrating that the effect of E2 on ERE-mediated promoter activity depends on whether ER β is phosphorylated at serine 87 and/or 105 (Fig. 2A; F (5,74)= 9.790, p<0.001). Consistent with previous studies, WT-ER β tended to increase ERE-dependent transcription in the absence of ligand (approx. 200%

increase) and E2 treatment increased it to a much greater statistically significant extent (approx. 550% increase, Fig. 2A) [20]. We then analysed the ERE-mediated promoter activity for each of the phospho-mutant receptors. Mutation of ER β at serine 87 had differential effects on ERE-mediated promoter activity depending on whether it was phospho-null or phospho-mimetic. First, mutation to alanine (87A, phospho-null) increased ERE-mediated promoter activity to a similar extent as WT-ER β in the absence of E2, whereas E2 treatment significantly increased ERE-mediated activity to a much greater extent. These results suggest that phosphorylation at this site hinders the E2-dependent activation of the receptor. Interestingly, the opposite effect was observed for mutations at the serine 105 site. In that case, the phospho-null mutation (105A) was not different from WT-ER β . By stark contrast, the phospho-mimetic (105E) increased not only the E2-independent (approx. 400%), but also the E2-dependent, activation of ER β .

The previous experiments demonstrated the effects of a single amino acid manipulation on ERE- and AP-1-mediated transcription. In those experiments, the phosphorylation status of the opposing site was unknown and entirely dependent on the endogenous kinase activity in the cell. Therefore, in this next series of experiments we tested whether simultaneous phosphorylation (i.e. S87E + S105E (EE)) or complete absence of phosphorylation (i.e. S87A and S105A (AA)) at both serine residues could further alter ER β regulation. In addition, we also tested the effects on ERE-mediated transcription when one site was phosphorylated, but not the other (i.e. S87A + S105E (AE); S87E + S105A (EA)). Each of the double mutant vectors (see table 1) were transiently co-transfected with an ERE-tk-luc reporter construct in HT-22 cells as described before.

A two-factor ANOVA analysis revealed a statistically significant interaction between plasmid and treatment, once again indicating that E2 regulation of promoter activity depends on the phosphorylation status of both ER β S87 and S105 residues (Fig. 4, $F(5,78) = 5.271$, $p < 0.001$). Figure 2B demonstrates the effects of the double mutants on ERE-mediated promoter activity. All of the double mutants increased ERE-mediated transcription in the presence of E2, although the increase was greater when the two serine residues had an opposite phosphorylation status (AE, EA, Fig. 2B). Moreover, the AE mutation increased ERE-mediated activity by more than 200% in the absence of ligand.

Overall, these data demonstrate that phosphorylation of ER β at both S87 and S105 has important functional consequences on its ability to transcriptionally activate ERE-mediated promoters in neurons.

ER β phosphorylation alters ligand dependent and ligand independent activation of AP-1 dependent transcription in neuronal cells

Estrogen receptor β can also act as a transcription factor *in trans* by tethering other transcription factors, thereby regulating a larger subset of genes whose promoters might lack a consensus ERE. For instance, ER β -mediated regulation at AP-1 sites requires ER β interaction with transcription factors of the Fos and Jun families. Similar to our observed results on ERE-mediated promoter activity, a two-factor ANOVA analysis revealed that there was a statistically significant interaction between plasmid and treatment (Fig. 3A; $F(5, 59) = 6.046$, $p < 0.001$). First, WT-ER β had a constitutive (ligand independent) inhibition of

AP-1-mediated promoter activity (approx. 50% decrease, Fig. 3A), which showed a trend towards a decrease following E2 treatment. These results are consistent with our previous reports of ER β repression of AP-1-mediated promoter activity in neuronal cells [16, 20]. Surprisingly, however, mutation of S87 or S105 to any form (phospho-null or phospho-mimetic) completely abolished the ligand independent inhibition of AP-1-mediated transcriptional regulation, yet the E2-induced reduction was preserved. These results suggest that S87 and S105 are critical residues mediating the ligand independent actions of ER β at an AP-1 site.

We next tested whether simultaneous phosphorylation (i.e. S87E + S105E (EE)) or complete absence of phosphorylation (i.e. S87A and S105A (AA)) at both serine residues could further alter ER β regulation at an AP-1 site. The regulation of AP-1-mediated transcription by double ER β mutants is shown in Figure 3B. Surprisingly, the ligand independent inhibition of AP-1 was restored when both S87 and S105 were concurrently mutated, while the single mutation of those same sites abolished the constitutive inhibition of AP-1 regulation by WT-ER β (Fig. 3B). Moreover, E2 significantly inhibited the constitutive repression to a greater extent when S105 was mutated to a phospho-mimetic (E) regardless of the phosphorylation status of S87 (Fig. 3B; $F(5,70) = 3.741, p < 0.005$).

Overall, we show that ER β regulation of AP-1 dependent transcription is fundamentally altered by single, but not double, mutation of S87 and S105.

Phosphorylation of ER β alters Tamoxifen effects on ERE and AP-1 transcription in neuronal cells

Next, we tested whether phosphorylation of ER β alters the effects of tamoxifen (TAM), a known selective ER modulator (SERM). TAM can be both agonistic and antagonistic, depending on cell type, ER subtype, and promoter response element [25–29]. We have previously shown that TAM abrogates the constitutive activation of ERE in HT-22 neuronal cells, and this was also true for some of the phospho-mutants tested (Fig. 4) [20]. Similar to the previously described experimental results, a two-factor ANOVA revealed a significant interaction between the two factors, plasmid type and TAM treatment ($F(5, 71) = 12.978, p < 0.001$). Specifically, TAM treatment completely eliminated the constitutive ligand-independent activation (i.e. vehicle-treated) of ERE-mediated transcription (Fig. 4A). Further, phosphorylation of S105 (105E) increased constitutive activity to the greatest extent (>400%), and also increased TAM dependent activation of ERE-mediated transcription compared to wild type ER β (Fig. 4A). These results suggest that there would be differential activation of ERE regulated genes following TAM treatment when S105 is phosphorylated.

Our previous studies showed that TAM abolished the observed ligand independent inhibition of AP-1 activity by ER β . The current studies confirm that TAM does abolish the ligand independent inhibition of AP-1 activity for the WT-ER β (Fig. 4B). However, mutation of S87 or S105 alone abolished the ligand independent repression of AP-1-mediated transcription (see Figs. 3, 4B vehicle), and treatment with TAM did not have any additional effects.

DISCUSSION

The overall objective of these studies was to determine the consequences of ER β phosphorylation on its functional capacity to act as a transcription factor at known promoter enhancer sites in neurons. Further, we hypothesized that phosphorylated ER β would be detectable in the brain of aged animals, due to the potential alterations in kinase activity that can occur with aging [30–34]. Our data demonstrated the novel finding that phosphorylated ER β is not only present in the brain of aged females, it is likely the major form of ER β expressed in the dorsal hippocampus and phosphorylation tended to increase following E2 treatment. These data provide strong evidence for the physiological relevance of our functional *in vitro* data, which together demonstrated that phosphorylation of ER β at specific serine residues altered its ability to activate and/or repress promoter activity in neurons. Collectively, these data suggest that age-related changes in hormonal milieu and cellular kinase activity could impact the expression of ER β -regulated genes, such as those mediating stress, anxiety, and cognitive function.

Post-translational modifications of nuclear steroid receptors have been widely investigated and these modifications are known to regulate their signalling abilities [7, 10–12]. Structurally, ER β is similar to other members of the nuclear steroid receptor superfamily. The N-terminal A and B domains are collectively defined as the AF-1 (Activation Function-1, or N-terminal transactivation) domain, which is a highly variable domain amongst the steroid receptor family and is fundamental for binding coregulatory proteins that assist in transcriptional activation or repression [16, 20]. Phosphorylation of mouse ER β at the N-terminal serine 106 and 124 has previously been shown to mediate ligand independent recruitment of the coregulatory protein SRC-1 (steroid receptor coactivator 1) and alter its subsequent transcriptional activity at an ERE in COS-1 cells [15]. In those studies, overexpression of SRC-1 significantly increased ligand independent activation at an ERE site. This effect was dependent on the MAPK phosphorylation of S106 and S124, as the double phospho-null mutation (S106A/S124A) completely abolished the SRC-1-induced activation at an ERE. However, that study did not evaluate the effects of S106A or S124A in the presence of ligand (E2), or the transcriptional activity at an ERE resulting from a S106/S124 phospho-mimetic (i.e. mutation to glutamic acid). In our studies the single mutation of S87A significantly increased E2-induced transcription at an ERE, but there was no effect in the absence of E2. Moreover, there was no difference in ERE-mediated transcription when S87 and S105 were both phospho-null (87A/105A) compared to WT-ER β . The discrepancies between our results and the previously reported could be due to the overexpression of SRC-1 in those studies. In addition, it is possible that there is a differential endogenous expression of SRC-1 in neurons or that ERs interact with different set of proteins depending on cell-type, resulting in altered signalling.

A similar study evaluated the effects of phosphorylated human ER β at S105 in cancer cell lines [13]. In that study, phosphorylation of hER β at S105 inhibited breast cancer cell invasion and migration *in vitro*, suggesting that phosphorylation of ER β at S105 mediates the anti-proliferative action of ER β in the breast. [13, 15]. Moreover, phosphorylated S105 ER β has been detected both in benign and invasive breast cancer tissue samples using a human-specific phosphoS105 antibody; however no studies to date have detected the

presence of phosphorylated ER β in the brain. Our results using neuronal cells showed that the phospho-mimetic S105E significantly increased both ligand independent and ligand dependent transcriptional activation at an ERE compared to WT-ER β , whereas conversely, the opposite phospho-null mutation of S105A had no effect. The LBD of ER β lies between residues 223–457, suggesting that phosphorylation of S87 or S105 is unlikely to alter ligand binding. However, cross talk between the N-terminal and C-terminal domain has been demonstrated, suggesting that altered ligand binding is a possible mechanism for phosphorylation-mediated changes in transcriptional activity [35].

It is well accepted that ER β has lower transactivation ability at an ERE site compared to ER α and our data suggest that this discrepancy could be partly explained by the phosphorylation status of ER β in the N-terminal AF-1 domain [25]. For instance, phosphorylation of the N-terminal domain could alter the ability of ER β to bind consensus ERE sites, thereby allowing preferential binding of ER α to those same sites. This concept is supported by the observation that various types of post translational modifications have been shown to alter DNA binding in other contexts. For example, acetylation of ER α at two lysine residues in the DBD enhanced its ability to bind to an ERE [36]. By contrast, sumoylation of oestrogen-related receptor alpha (ERR α) at two sites in the N-terminal domain did not affect its DNA binding ability, yet still altered its transcriptional activity [37]. Our results add to existing evidence supporting the idea that post translational modifications in nuclear receptors at regions distant to the DBD alters their ability to modulate transcription, and possible alterations in DNA binding affinity cannot be ruled out as a potential explanation for the observed increase in ERE-mediated promoter activity.

Perhaps the most interesting results from this study were the effects of ER β phosphorylation at an AP-1 site. We have previously shown that ER β exerts strong transcriptional repression at an AP-1 site in neuronal cells in the absence of ligand and the results presented here indicate that this might depend on phosphorylation of the N-terminal domain [16, 20]. The single mutation of either S87 or S105 completely abolished ligand independent inhibition of AP-1 mediated transcription compared to WT-ER β . However, AP-1 mediated transcription was equally repressed with WT-ER β as it was with double mutations of AA, EE, or AE. Notably, only the 87E/105A mutation showed a significant difference from the other mutants, suggesting that S87 phosphorylation status is the critical serine residue for mediating ER β -induced repression at an AP-1 site. The precise dynamics of p38 and ERK phosphorylation of ER β are unknown and the folding of singly phosphorylated ER β could render the other site inaccessible to the kinase. Nevertheless, our data suggest that a single phosphorylation change in either S87 or S105 abrogates the ligand independent inhibition of AP-1, whereas no effect is observed due to concurrent phosphorylation of both sites. This could be explained by altered ER β protein-protein interactions with Jun/Fos proteins or with other co-regulatory proteins recruited to the AP-1 complex, such as p160, based on evidence that phosphorylation alters coregulatory protein recruitment to ER β [15]. The next step will be to determine which of these sites are phosphorylated *in vivo* and the precise molecular environment that facilitates those phosphorylation changes.

Recently, Vivar and colleagues identified 3 classes of ER β target genes and discovered that the majority of genes (453) fell into the Class I category, which were genes regulated by

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unliganded ER β [38]. Further, the Class I genes showed a high enrichment for AP-1 binding sites, demonstrating that ligand independent repression of AP-1-mediated promoter activity is a common mechanism of ER β signalling. Similarly, Zhao et al. showed that over 60% of ER β interacting regions in MCF-7 breast cancer cells contain an AP-1 site [39]. Together these studies underscore the significance of our findings that ER β ligand independent activity at an AP-1 site is abolished when the N-terminal domain is phosphorylated. Aging in women results in dramatic declines in circulating oestrogens raising the possibility that the ligand-independent actions of ER β could play a prominent role in regulating gene transcription in the aging brain.

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Our studies were limited to the investigation of just one type of post-translational modification (phosphorylation) at specific serine residues. However, there is evidence that other post-translational modifications of ER β could also be present, such as acetylation and sumoylation, and these modifications can create cross talk between different residues of the receptor. For example, phosphorylation at S305 on ER α inhibits subsequent acetylation at K303 leading to enhanced transcriptional regulation [40]. Moreover, phosphorylation of S94 and S106 on mER β leads to recruitment of ubiquinating enzymes to the AF-1 domain resulting in enhanced degradation of the receptor [41]. Future studies are required to determine the precise mechanisms and consequences resulting from post-translational modifications acting in concert to modulate overall ER β function. Our *in vitro* studies were conducted using rat ER β expression vectors in a mouse-derived neuronal cell line. Given the high degree of homology between rat and mouse ER β , we expect that similar results would be obtained using rat neuronal cell lines or primary neurons derived from either species [14]. However, it is important to consider these results in this context as both brain-region and species-specific effects of ER β have been observed [42, 43].

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Tamoxifen (TAM) has been described as a partial antagonist due to its cell-type specific effects [27, 29, 44–47]. Relevant to these studies, TAM action in the brain has been shown to block the neuroprotective effects of E2, confirming that the beneficial effects of E2 are mediated by classical oestrogen receptors [1]. In addition, TAM can activate numerous second messenger signalling pathways, including MAPK, thereby potentially phosphorylating and altering ER β transcriptional activity [48]. Our results demonstrated that TAM worked as an antagonist of both the WT and phosphorylated forms of ER β by preventing the ligand independent increase in ERE-mediated transcription. Consistent with our previous studies, TAM also blocked the ligand independent repression of WT-ER β at an AP-1 site. The ligand binding domain (LBD) of ER α and ER β is more conserved than other domains and X-Ray crystallography studies have shown that both receptors bind several types of selective estrogen receptor modulators (SERMs), including TAM [49]. The structure of TAM complexed with the ER α or ER β LBD have been resolved and TAM:LBD binding resulted in conformational changes that inhibited subsequent binding of coactivator proteins, pointing to a clear mechanism for TAM antagonism [50, 51].

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In summary, our results show that phosphorylation of ER β alters its function in neuronal cells both in a ligand dependent and independent manner, and that ER β is phosphorylated *in vivo* in the hippocampus. Our work highlights the importance of further understanding the effects of post-translational modifications on nuclear steroid receptors, as these

modifications fundamentally alter their function as transcription factors and could result in clinically important physiological changes.

Acknowledgments

Support for this work was provided by NIH R01AG033605.

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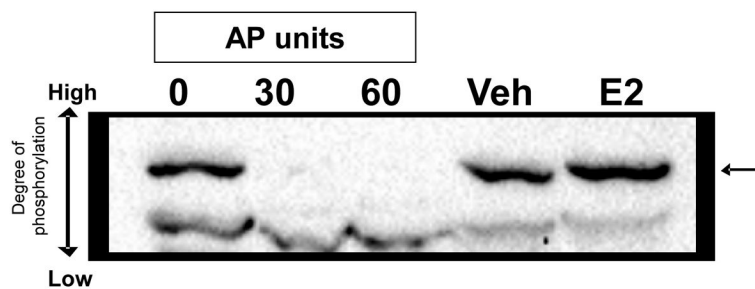


Figure 1. Expression of phosphorylated ER β in the dorsal hippocampus of aged female rats
Protein isolated from the dorsal hippocampus (50 μ g) was resolved using Phos-TagTM SDS-PAGE electrophoresis and probed for ER β with the ER β specific antibody (SC-150). Phos-TagTM binds phosphate groups and increased protein phosphorylation retards gel migration. Alkaline phosphatase (AP) treatment removes phosphate groups allowing the protein to migrate further through gel. Aliquots of the same dorsal hippocampus protein sample were treated with different amounts of AP. Arrow indicates phosphorylated ER β . Samples not treated with AP show upper band, indicating presence of phosphorylated ER β that is abrogated in samples treated with either 30 or 60 units of AP.

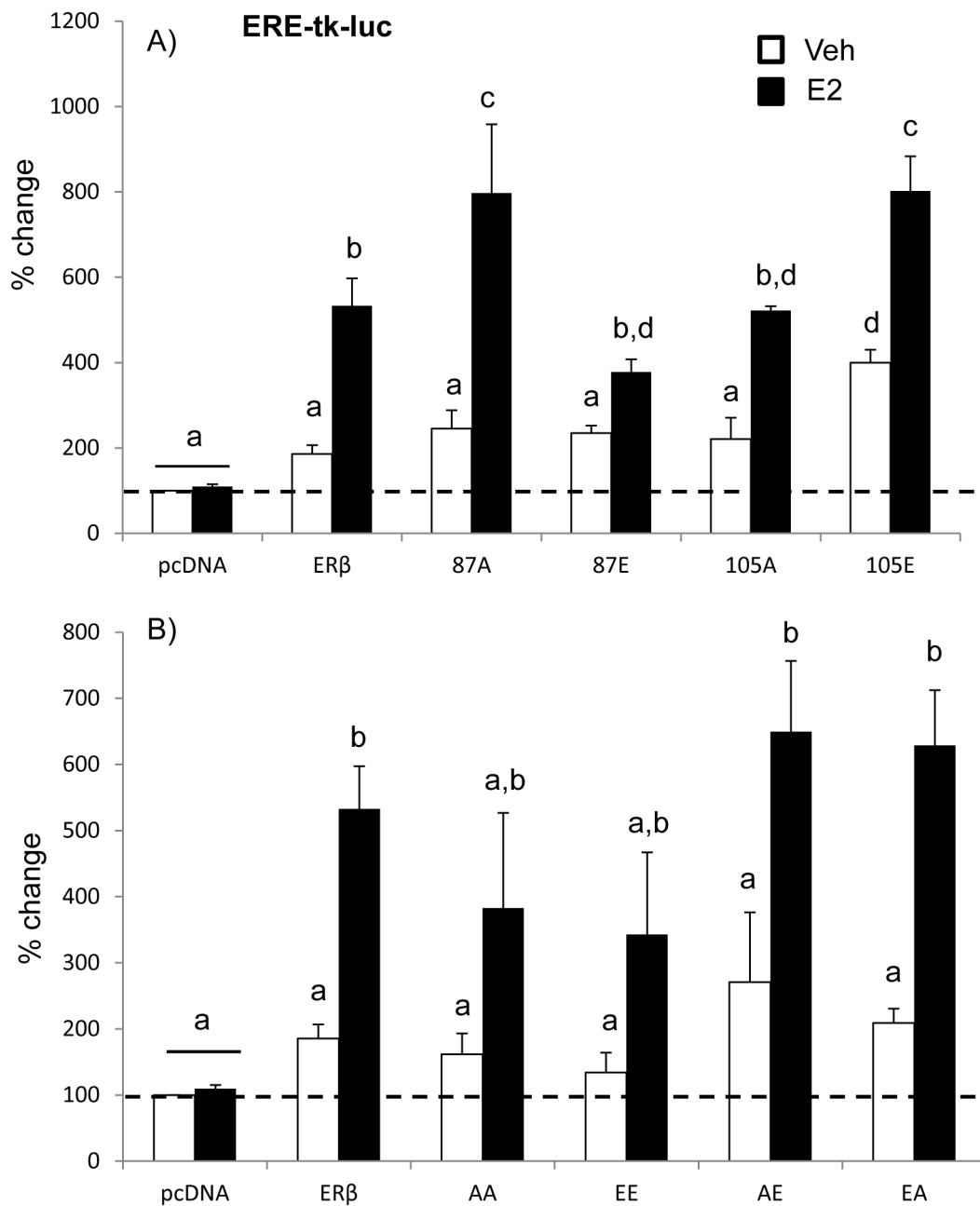


Figure 2. Effects of ER β phosphorylation on ER β -mediated promoter activity at an ERE site Hippocampal-derived (HT-22) cell lines were transiently co-transfected with an ERE-tk-Luciferase reporter construct and the wild type ER β or (A) single phospho-mutant ER β expression vector (S87A, S87E, S105A, S105E) or (B) double phospho-mutant ER β expression vector (S87A+S105A; S87E+S105E; S87A+S105E, S87E+S105A) (B). Cells were treated with 100 nM E2 or vehicle (0.01% ethanol) for 15 hours. Data are expressed as the mean percent change compared to empty vector control \pm SEM. Different letters denote statistically significant differences as calculated with two-way ANOVA and Tukey post-hoc analysis ($p < 0.05$).

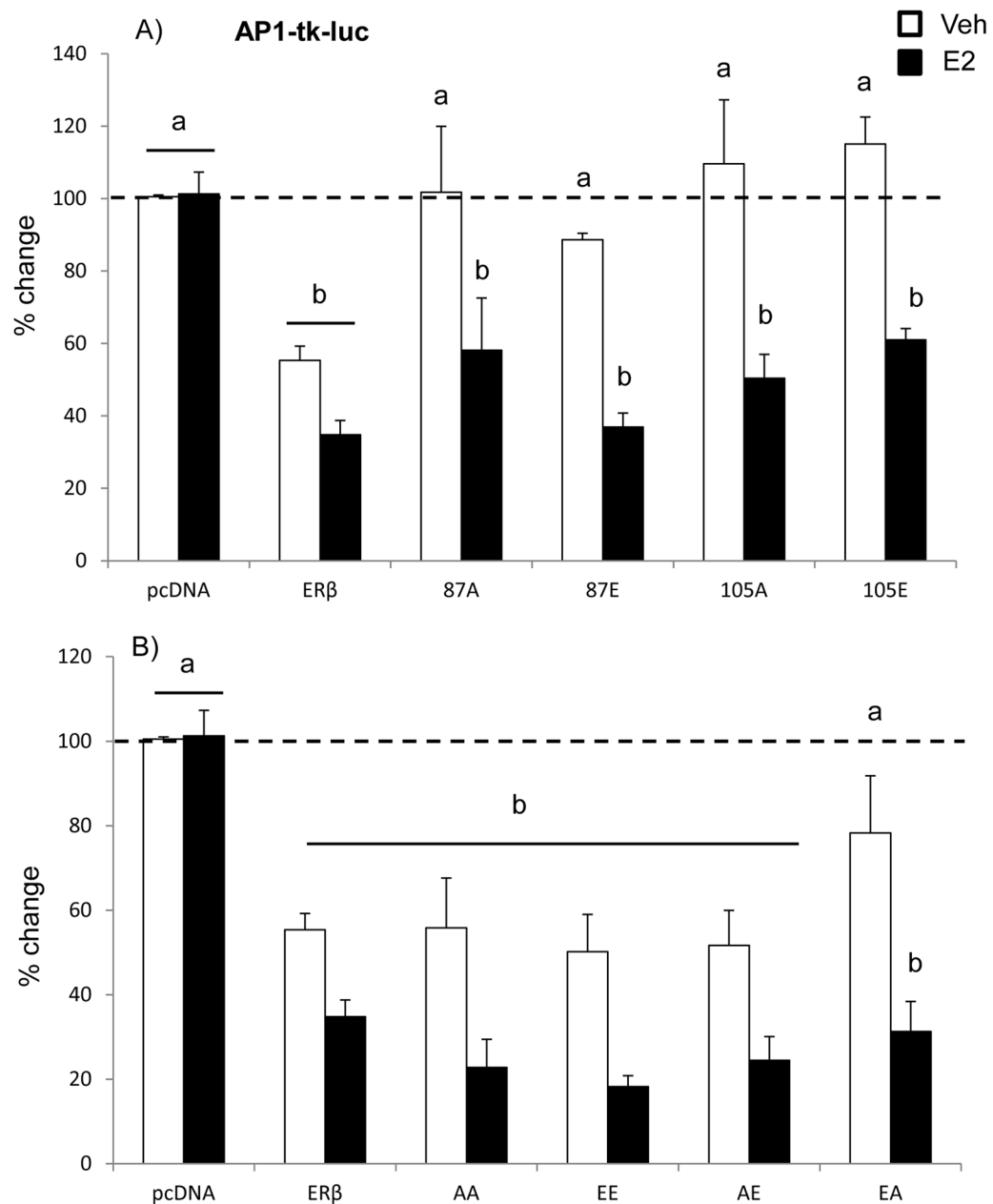


Figure 3. Effects of ER β phosphorylation on ER β -mediated promoter activity at an AP-1 site
 Hippocampal-derived (HT-22) cell lines were transiently co-transfected with an AP-1-tk-Luciferase reporter construct and the wild type ER β or (A) single phospho-mutant ER β expression vector (S87A, S87E, S105A, S105E) or (B) double phospho-mutant ER β expression vector (S87A+S105A; S87E+S105E; S87A+S105E, S87E+S105A) (B). Cells were treated with 100 nM E2 or vehicle (0.01% ethanol) for 15 hours. Data are expressed as the mean percent change compared to empty vector control \pm SEM. Different letters denote statistically significant differences as calculated with two-way ANOVA and Tukey post-hoc analysis ($p < 0.05$).

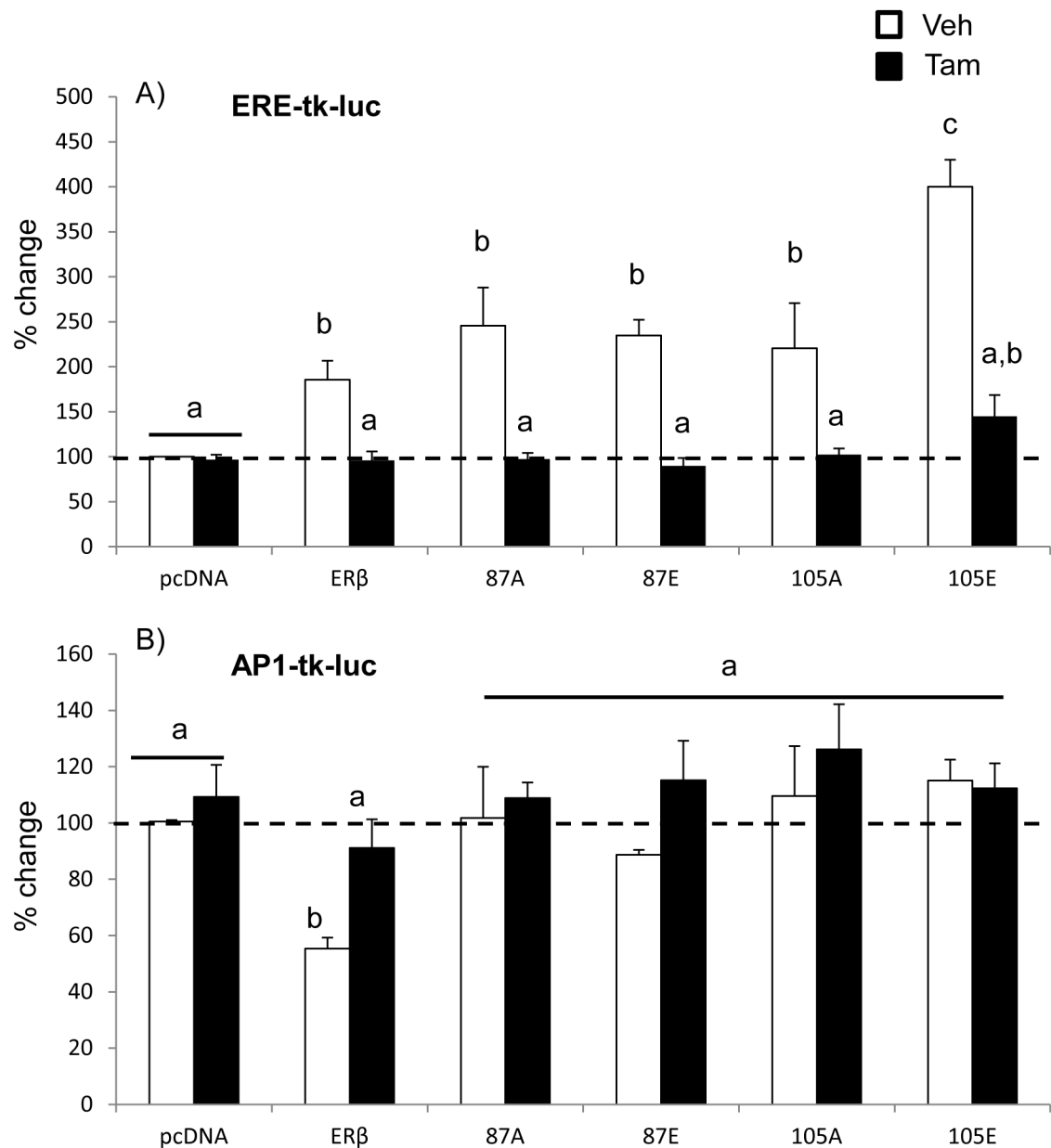


Figure 4. Effects of tamoxifen treatment on ERβ or ERβ mutant-mediated promoter activity at an ERE or AP-1 site

Hippocampal-derived (HT-22) cell lines were transiently co-transfected with an (A) ERE-tk-Luciferase or (B) AP-1-tk-Luciferase reporter construct and the wild type ERβ or phospho-mutant ERβ expression vector (S87A, S87E, S105A, S105E). 24 hours following transfection, cells were treated with 100 nM 4-OH-tamoxifen (TAM) or vehicle (0.01% ethanol) for 15 hours. Transfection efficiency was normalized using a second renilla luciferase reporter construct (rLUC) in all experiments. Data are expressed as the mean percent change in fLUC/rLUC compared to empty vector control \pm SEM taken from 4 independent experiments with 6 replicates/experiment. Different letters denote statistically

significant differences as calculated with two-way ANOVA and Tukey post-hoc analysis ($p < 0.05$).

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Table 1

List of ER β expression vectors.

Vector name	Characteristics
ERβ	Wild type ER β
87E	Phospho mimetic at S87
87A	Phospho null at S87
105E	Phospho mimetic at S105
105A	Phospho null at S105
AA	Phospho null at S87 and S105
EE	Phospho mimetic at S87 and S105
AE	Phospho null at S87, phospho mimetic at S105
EA	Phospho mimetic at S87, phospho null at S105

Schematic summary of reporter assay results. Arrows indicate direction of change in relation to wild type ER β . In grey are highlighted the instances of differences between each mutant compared to wild type ER β .

Table 2

	ERE (DNA binding)		API (Prot-Prot interaction)	
	constitutive	E2 dependent	constitutive	E2 dependent
wtER β	↑	↑↑	↓	↓↓
S87A	↑	↑↑↑	-	↓↓
S87E	↑	↑↑	-	↓↓
S105A	↑	↑↑	-	↓↓
S105E	↑↑	↑↑↑	-	↓