

ERAP75 Functions as a Coactivator to Enhance Estrogen Receptor α Transactivation in Prostate Stromal Cells

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BACKGROUND. Estrogen receptor α (ER α) has been reported to be expressed and function in the prostate stromal cells, and numerous evidences indicated that the stromal ER α signal pathway plays critical roles in prostate development and cancer. ER α requires distinct coregulators for efficient transcriptional regulation. The goal of this study is to examine physical and functional interaction between ER α and ERAP75 in the context of prostate stromal cells.

METHOD. Yeast two-hybrid assays were used to screen novel ER α interaction proteins. The interaction between ER α and ERAP75 was confirmed by mammalian two-hybrid, GST pull-down, and co-immunoprecipitation methods. The interaction motif was examined by site-directed mutagenesis. The effect of ERAP75 on ER α transactivation and the expression of ER α target genes were determined by luciferase assay and real-time PCR, respectively.

RESULT. ER α can interact with the C terminus of ERAP75 via its ligand binding domain both in vivo and in vitro. The conserved LXXLL motif within the C terminus of ERAP75 is required for the interaction between ER α and ERAP75. ERAP75 can enhance ER α transactivation in a dose-dependent manner and up-regulate the expression of the endogenous ER α target gene, stromal-derived factor-1 (SDF-1), in the prostate stromal cells.

CONCLUSION. ERAP75 functions as a novel coactivator that can modulate ER α function in the prostate stromal cells. The understanding of the mechanism of ER α transactivation in prostate stromal cells could possibly help in the development of new strategies to control or treat prostate cancer by targeting its transactivation protein complex. *Prostate* 68: 1273–1282, 2008. © 2008 Wiley-Liss, Inc.

KEY WORDS: estrogen receptor coactivator; prostate stromal cells; SCD-1; prostate cancer

INTRODUCTION

Estrogens regulate the growth, differentiation, and development functions in a broad range of human target tissues, including both male and female reproductive systems [1]. The estrogens actions are mediated by the ERs, members of the nuclear receptor (NR) superfamily [2–5], which are encoded by two distinct genes, ER α and ER β . ERs share a common structural architecture with other NR superfamily members and consist of independent but functionally interactive domains: an amino terminal A/B domain containing a ligand-independent transactivation function (AF1), a DNA binding domain (DBD), and a C-terminal ligand binding domain (LBD) containing a ligand-dependent transactivation function (AF2) [6–9]. In the absence of

ligand, the ER protein is found predominantly in the nucleus and squelched by the Hsp90-based chaperone complex [10]. Upon ligand binding, the ERs undergo a conformational change within the LBD of the receptors. This allows receptors to dimerize, to bind to estrogen

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response elements (ERE) and recruit coregulator proteins in order to mediate transcription [11,12]. In addition to this classical ERE dependent mechanism, ERs also can regulate the target genes' expression without directly binding to DNA via interaction with other transcriptional factors (TFs), such as AP-1 and SP-1, to regulate the expression of ovalbumin and cyclin D1 [13,14].

Recent studies have shown that a wide range of ER coregulators can modulate ERs at target promoters [15–19]. The most characterized ER coactivators are members of the p160/steroid receptor coactivator (SRC) family including SRC-1 [20], GRIP/TIF2/NCoA-2/SRC-2 [21,22], and SRC-3/AIB1/pCIP/RAC3 [23–26]. The interaction between ERs and the p160 family is mediated by short amphipathic α -helices, which consist of leucine-rich motifs: LXXLL (where L is leucine and X is any amino acid) [27]. The ligand induced conformation changes in ERs reposition the Helix 12 and form a hydrophobic groove on the receptor surface and interact with the coactivator LXXLL motif [27,28]. The other important group of ER coactivators is the p300 and the highly related CREB-binding protein (CBP) [29,30], which are histone acetyltransferases (HATs) that mediate the acetylation of nucleosomal histones. p300/CBP, as a bridging factor, forms a complex with other coactivators and ERs and co-integrates the signals at distinct steps in ER-mediated transcription activation [31]. Other coactivators have been suggested to function at different steps by directly binding to the basal transcriptional machinery and promoting the assembly of transcription initiation complexes [32,33].

Prostate cancer (PCa) is the second leading cause of cancer deaths in American men. An estimated 27,050 men in the USA will lose their lives because of its malignancy in 2007 [34]. Estrogens have been used for the treatment of PCa since the early 1940s [35]. It is generally believed this action is indirectly mediated at the hypothalamic level to suppress the circulating androgens [36,37]. However, in the early 1960s, a direct action of estrogens via their own receptors in the prostate was proposed by Mangan et al. [36]. Recently, the evidence that ERs expressed in different prostate cell lines, normal prostate, benign prostate hyperplasia (BPH), and PCa, along with the demonstration of the stimulatory or inhibitory effects of estrogens on PCa cells growth, suggested estrogens may exert direct effects on prostate via their own receptors [38–43].

Both ER α and ER β express in the human and rodent prostate tissue [4,5,44–46]. Interestingly, the two ER subtypes exhibit very different expression patterns. In human and rodent prostate, ER α is found mainly in the prostatic stroma, whereas ER β is found mainly in

prostatic epithelium [40,43,47–49]. Recent studies have shown that estrogens, in combination with androgens, play critical roles in prostate carcinogenesis [50,51]. The aberrant estrogen signaling induced by estrogens during prostatic carcinogenesis could be mediated by stromal ER α [52]. To elucidate estrogens action during prostatic carcinogenesis, it is important to understand the ER α -mediated signaling pathway and the proteins which interact with ER α and might influence the ER α transactivation and target gene expression in the prostatic stromal cells.

Using the yeast two-hybrid system, we previously identified ERAP75, Homo sapiens coiled-coil domain containing 62 (CCDC62) protein, as a novel ERs coactivator [53]. Here, we examined the role of ERAP75 as a coactivator for ER α in the prostate stromal cells. Our results showed ERAP75 functions as an ER coactivator to enhance ER α -mediated transactivation and endogenous target genes' expression in the prostate stromal cells. The understanding of the mechanism of ER α transactivation in prostate stromal cells could possibly help in the development of new strategies to control or treat PCa by targeting ER α transactivation protein complex.

MATERIALS AND METHODS

Plasmids

pGBKT7-ER α -LBD (aa 260–595), pM-ER α -LBD, pcDNA3.1.(-)-ER α , ERE-Luc, pGADT7-ERAP75-C, pVP16-ERAP75-C, pVP16-ERAP75, pCDNA3-flag-ERAP75, pSG5-ERAP75, pRev-TRE-ERAP75, pGADT7-SRC-1, pSG5-SRC-1, pGEX-ERAP75, different Glutathione S-transferase (GST)-ERAP75 mutants, and pVP16-ERAP75 mutants have been described previously [53]. All the plasmids, after construction, were verified by sequencing. The expression of plasmids was either confirmed by TNT *in vitro* expression or Western blotting.

Yeast Two-Hybrid System

The yeast two-hybrid assay has been described previously [53]. The pGBKT7-ER α -LBD and pGADT7-ERAP75-C were retransformed into yeast strain AH109. The interaction specificity was further confirmed by liquid β -galactosidase assay.

Antibodies

ERAP75 monoclonal antibody was produced by injecting the purified human ERAP75-carboxyl terminus [338–684 amino acids (aa)] antigen into a mouse, and the antigen purified as previously described [54]. ER α , GAPDH, and β -actin antibodies were purchased

from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-Flag antibody M2 was purchased from Sigma.

Cell Culture and Transfections

COS-1 and WPMY-1 cells were maintained in DMEM media (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Normal human prostate stromal cells (PrSC) were purchased from Cambrex (Clonetics Co. San Diego, CA) and maintained in optimized stromal media (SCGM BulletKit-Clonetics, San Diego, CA). PrSC used in this study were from a Caucasian male of 53 years of age. Transient transfections were done by either SuperFect (Qiagen, Chatsworth, CA) or electroporation. For the luciferase assays, the cells were cultured in corresponding media supplemented with 10% charcoal-stripped FBS (CDFBS) phenol red free media for 24 or 48 hr before transfection. The total amount of transfected DNA was kept constant and normalized by the corresponding empty vectors. After transfection, cells were treated in the presence or absence of 10 nM 17- β -estradiol (E2) for 24 hr. The assay was performed as described previously [53]. For Western blotting assays, exponentially growing COS-1 were resuspended in the cell media with 2% FBS (no antibiotics). Electroporation was performed at 280 mV (Voltage) and 950 μ F (Capacity) using a Gene Pulser II (Biorad, Hercules, CA). The total DNA amounts were 10 μ g and sample volumes were 400 μ L.

In Vitro GST Pull-Down Assays

GST pull-down assays were carried out as described previously [55]. Briefly, [³⁵S]methionine-labeled ER α -LBD and different ERAP75 mutant proteins were expressed using T7 polymerase and the coupled transcription/translation kit (Promega). GST alone and GST fusion proteins were expressed in *Escherichia coli* BL21(DE3) bacterial strain (Stratagene) and purified with glutathione–Sephadex beads as instructed by the manufacturer (Amersham Pharmacia). For in vitro interactions, mixtures of glutathione bead-bound GST fusion proteins and 5 μ l of [³⁵S]methionine-labeled input proteins in 100 μ l of interaction buffer [20 mM Tris/pH 8.0, 60 mM NaCl, 6 mM MgCl₂, 1 mM EDTA, 0.05% Nonidet P-40 (NP-40), 1 mM dithiothreitol (DTT), 8% glycerol, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] were incubated in the presence or absence of 100 nM E2 on a rotating disk at 4°C for 2 hr. After washing with NETN buffer (20 mM Tris/pH 8.0, 100 mM NaCl, 6 mM MgCl₂, 1 mM EDTA, 0.5% NP-40, 1 mM DTT, 8% glycerol, and 1 mM PMSF) four times, the proteins were resuspended in SDS–PAGE loading buffer, and resolved on 10% SDS–PAGE followed by autoradiography.

Co-Immunoprecipitation Assays and Western Blotting

COS-1 cells were seeded on 10-cm-diameter cell culture dishes and electroporated to transfect pcDNA 3.1(-)-ER α with pCDNA3-flag vector or pCDNA3-flag-ERAP75. After 24 hr, the cells were treated with vehicle (0.01% ethanol) or 10 nM E2 for another 24 hr in 10% CDFBS phenol red free media. The cells were then lysed using lysis buffer (1% NP-40, 10% glycerol, 135 mM NaCl, 40 mM Tris/pH 7.4, 1 mM PMSF, 1 mM DTT, and 1X protease inhibitor cocktail; Roche, Indianapolis, IN) in the presence or absence of E2. Lysates were centrifuged and supernatants were precipitated by 2 μ g ERAP75 monoclonal antibody or normal mouse IgG for 4 hr at 4°C with agitation followed by the addition of 40 μ l protein A/G plus agarose for another 2 hr. Lysates were centrifuged and the immunoprecipitates were washed three times with PBS and resolved on a 10% SDS–polyacrylamide gel. The results were analyzed by Western blotting as described previously [56].

RNA Extraction and Real-Time PCR for RNAi Experiment

Total RNA was extracted and purified using Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RT-PCR has been described previously [57]. Briefly, 3 μ g total RNA was subjected to reverse transcription using Superscript III (Invitrogen). Real-time PCR was performed with first strand cDNA, specific gene primer, and SYBR Green PCR Master Mix (Biorad). The PCR cycle was performed as follows: 94°C for 3 min, 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec on an iCycler iQ Multi-color real-time PCR detection system (Biorad). Primer sequences were as follows: SDF-1: sense, 5'-AGTCA-GGTGGTGGCTTAACAG-3'; antisense, 5'-GAGGAG-GTGAAGGCAGTGG-3'; β -actin: sense, 5'-TGTGCC-CATCTACGAGGGGTATGC-3'; antisense, 5'-GGTACATGGTGG TGCCGCCAGACA-3'. Each sample was run in triplicate. Data were analyzed using iCycler iQ software (Biorad).

RESULTS

Ligand-Dependent Interaction of ER α and ERAP75 in Yeast and Mammalian Cells

ER α and ER β have displayed some significant differences in terms of their ligand-binding, transcriptional properties, tissue distribution, and knockout phenotype [1,45,58]. We found that ERAP75 can interact with both ER α and ER β in the yeast two-hybrid assays [53] (data not shown for ER β). The current study will focus on examining the role of ERAP75 on

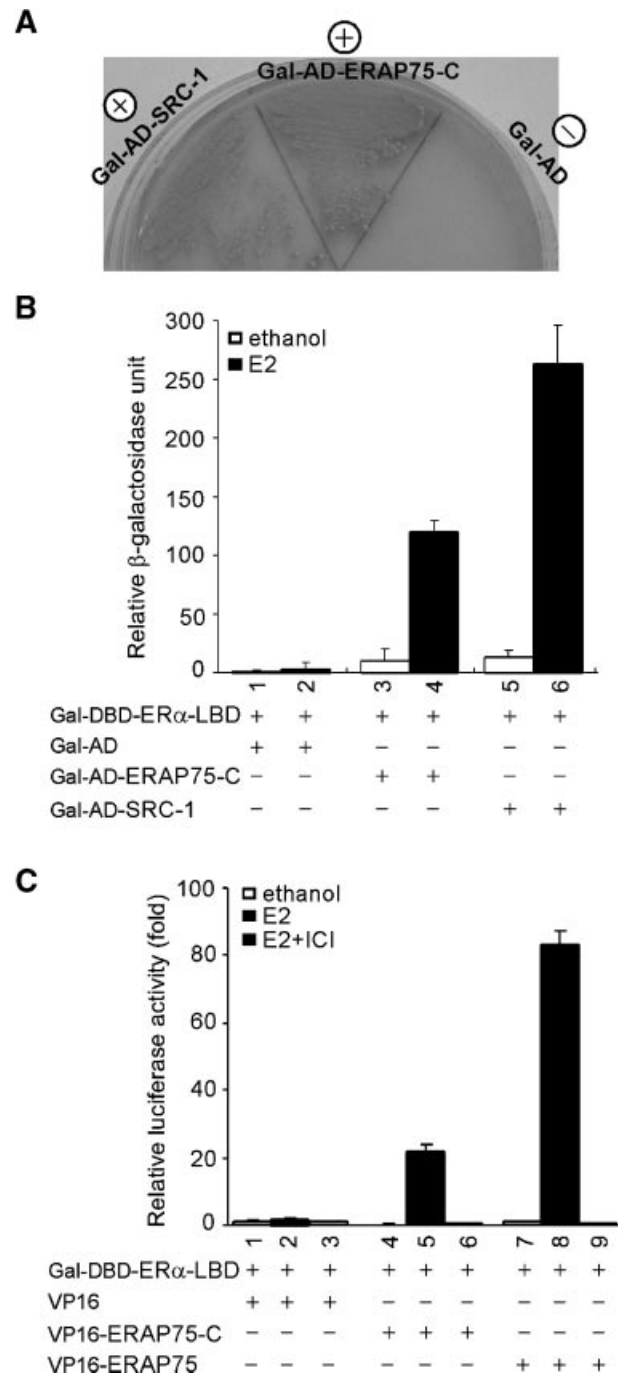
the ER α activity and function in prostate stromal cells. The ERAP75 fragment (ERAP75-C, aa 600–684) from yeast two-hybrid screening lies in the C terminus of ERAP75 and contains two conserved LXXLL motifs (Fig. 4A). Co-transformation of the pGBKT7-ER α -LBD with pGAD7-ERAP75-C or SRC-1 clones into the yeast strain AH109, followed by growth selection and α -galactosidase assay (X- α -gal as substrate), further confirmed the interaction between ER α -LBD and both ERAP75 and SRC-1 (Fig. 1A). Next, we applied the liquid β -galactosidase assays to quantify the interaction between ER α and ERAP75. Constructs containing either ERAP75 or SRC-1 showed a strong interaction with the ER α in a ligand-dependent (E2) manner (Fig. 1B, lane 4 vs. lane 3; lane 6 vs. lane 5).

Interaction Between ERAP75 and ER α and Expression of ERAP75 in the Prostate Stromal Cells

To further confirm the interaction between ERAP75 and ER α from yeast two-hybrid screening, we examined the interaction using both *in vitro* and *in vivo* assays. In mammalian two-hybrid assays, the interaction between Gal-DBD-ER α -LBD and pVP16-ERAP75-C or pVP16-ERAP75 full length (Fig. 1C, lane 5 vs. lane 4; lane 8 vs. lane 7) can be detected in the presence of E2. Furthermore, ICI 182,780, a pure antagonist of E2, abolished the E2-induced interaction between ER α and ERAP75 (Fig. 1C, lane 6 vs. lane 5; lane 9 vs. lane 8). The association of the ERAP75 protein with ER α was also tested by *in vitro* co-immunoprecipitation. The [³⁵S]methionine-labeled ER α -LBD and HA-fused ERAP75 were transcribed and translated *in vitro* separately. After interacting with HA-fused ERAP75, ER α -LBD was co-precipitated by an anti-HA antibody in the presence of E2 (Fig. 2A, lane 3). The interaction between ER α and ERAP75 was also confirmed by *in*

vivo co-immunoprecipitation from protein extracts prepared from COS-1 cells transfected with ER α and ERAP75. We demonstrated that ER α in COS-1 cells could be co-immunoprecipitated with flag-ERAP75 using the anti-flag antibody in the presence of E2 (Fig. 2B, lane 2 vs. lane 1). We also detected the expression of ERAP75 in prostate stromal cells. Western blotting was performed using a mouse monoclonal against human ERAP75 C terminal antigen (aa 338–684; Fig. 2C). The ERAP75/CCDC62 protein was detectable with the moderate expression in

Fig. 1. The interaction of ERAP75 with ER α in the yeast and mammalian cells. **A,B:** The interaction between ERAP75-C and ER α -LBD in the yeast two-hybrid assay. AH109 was co-transformed with Gal-DBD-ER α -LBD and Gal-AD-ERAP75-C growing on the -Leu/-Trp/-His/-Ade selection media supplemented with X- α -gal in the presence of 10 nM E2 (A); or growing the co-transformed yeast in the liquid selection media in the presence or absence of 10 nM E2 and the interaction was quantified by β -galactosidase activity expressed in the yeast cells (B); SRC-1 acted as a positive control. **C:** Interaction between ER α and ERAP75 as examined by mammalian two-hybrid assays. COS-1 cells were transiently transfected with 0.4 μ g of reporter plasmid pG5-Luc and 0.3 μ g VP16-AD-fused ERAP75-C fragment or ERAP75 full-length with or without 0.3 μ g of Gal-DBD-ER α -LBD as shown, after 24 hr transfection, the cells were treated with 10 nM E2, 1 μ M ICI 182,780, or 0.1% ethanol, for another 24 hr prior to lysis. phRL-tk-LUC expression vector was used as an internal control. Results shown here are the means \pm SD for three independent experiments.



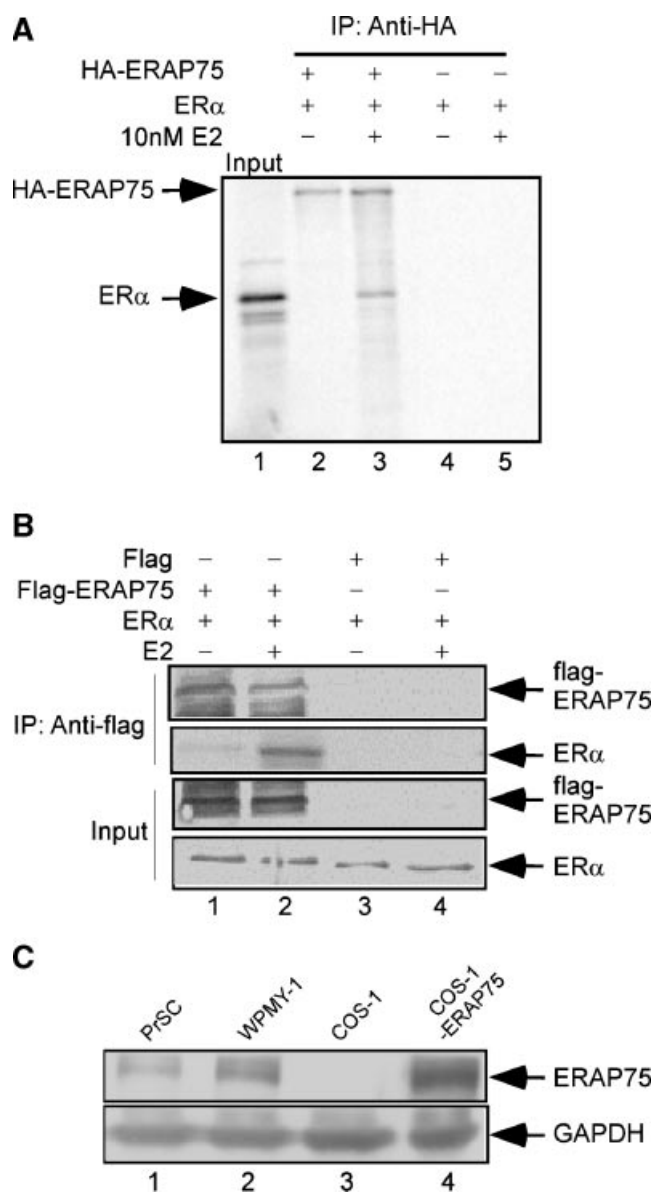


Fig. 2. ERAP75 can interact with ER α both in vitro and in vivo and expression of ERAP75 in the prostate cells. **A:** In vitro co-immunoprecipitation of HA-ERAP75 with ER α proteins. In vitro translated ER α (5 μ l) alone or with 5 μ l of in vitro translated HA-ERAP75 were incubated at 4°C in the presence or absence of 10 nM E2. The proteins were immunoprecipitated with anti-HA antibody and analyzed on a 10% SDS-PAGE gel. In vitro translated ER α protein (0.5 μ l) was loaded into the gel as a synthesis and loading control. **B:** In vivo co-immunoprecipitation of ERAP75 with ER α . Lysates were prepared from COS-1 cells transfected with flag-ERAP75 and ER α and subjected to immunoprecipitation (IP) with an anti-flag antibody followed by anti-ERAP75 or anti-ER α immunoblotting. **C:** Western blotting detection of ERAP75 protein in prostate stromal cell lines. **Lane 1**, PrSC cells; **lane 2**, WPMY-1 cells; **lane 3**, COS-1 cells; **lane 4**, COS-1 cells transiently transfected with pSG5-ERAP75. GAPDH immunoblotting as an indication of equal loading.

WPMY-1 cells, low expression in PrSC cells and undetectable expression in the COS-1 cells, which was consistent with our previous studies [53]. Together, results from mammalian two-hybrid assay, in vitro, and in vivo co-immunoprecipitation assays all demonstrated that ERAP75 could interact with ER α in a ligand-dependent manner, and ERAP75 can be detected in the prostate stromal cell lines.

Domains Involved in the Interaction Between ER α and ERAP75

The yeast two-hybrid assay showed that the C terminus of ERAP75 could interact with ER α -LBD. To further examine whether other domains are involved in the interaction between ER α and ERAP75, we applied the GST pull-down to detect the direct interaction between ER α and ERAP75. To dissect the ERAP75 interaction domain on the ER α , four ER α mutants fused with GST were tested in GST pull-down assays. As shown in Figure 3A, GST-ER α -LBD, but not GST-ER α -N, GST-ER α -DBD-Hinge, or GST-ER α -F domain,

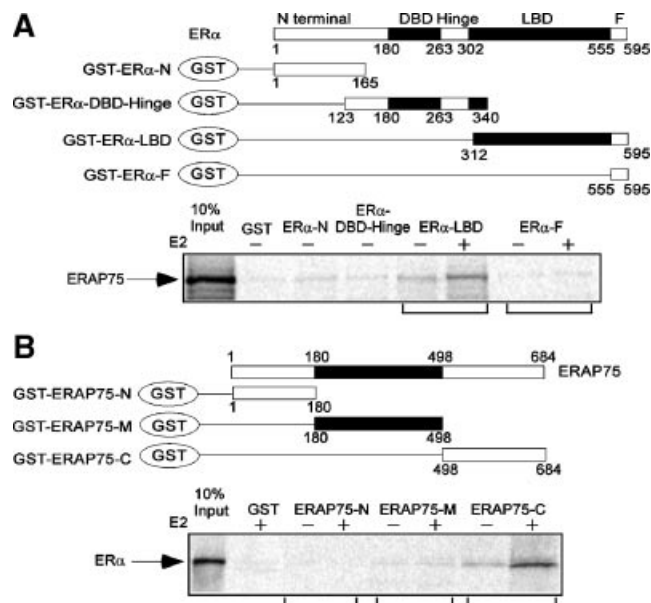


Fig. 3. Mapping the interaction domains between the ER α and ERAP75. **A:** The construction of GST-ER α fragments is illustrated schematically. GST alone and different GST-ER α fusion proteins were bound to glutathione-Sepharose beads and incubated with 5 μ l of [³⁵S]-methionine-labeled ERAP75 in the absence or presence of 100 nM E2. After extensive washing, bead-bound protein complexes were incubated with SDS-gel loading dye and resolved by 10% SDS-PAGE and analyzed by PhosphorImager (Molecular Dynamics; lower panel). **B:** Schematic representation of GST-ERAP75 constructs is illustrated. GST or three GST-ERAP75 fusion proteins were bound to glutathione-Sepharose beads and incubated with 5 μ l of [³⁵S]-methionine-labeled ER α in a pull-down assay (lower panel). The input represents 10% of the [³⁵S]-methionine-labeled proteins used in each pull-down assay.

can interact with ERAP75 in the presence of E2. This indicates that ER α -LBD in the ER α protein is required for the interaction between ER α and ERAP75. On the other hand, three GST-fused ERAP75 mutants: GST-ERAP75-N (aa 1–180, containing the first coiled-coil), GST-ERAP75-M (aa 180–498, containing the second coiled-coil), and GST-ERAP75-C (aa 498–684, containing the LXXLL motif), were used to determine which domain of ERAP75 interacted with the ER α protein. As shown in Figure 3B, GST-ERAP75-C, but not GST-ERAP75-N or -M, was required for binding to ER α in the presence of E2. Therefore, those results are consistent with the studies from yeast two-hybrid screening.

The LXXLL Motif Is Required for the Interaction Between ER α and ERAP75

LXXLL is a conserved motif first identified in the SRC family of coactivators, and is necessary and sufficient to mediate binding of the coactivators to liganded NRs [27]. The individual LXXLL motifs are able and sufficient to bind to hormone receptors, but display preferences for certain receptors [59]. ERAP75 contains two LXXLL motifs. We investigated whether both motifs are required for the interaction with ER α . ERAP75 mutants of the LXXLL motifs were tested in a GST pull-down assay (Fig. 4A). The results showed that if the mutation occurred in the first LXXLL motif, the interaction between ER α and ERAP75 was abolished. In contrast, when the second LXXLL motif of ERAP75 was mutated, there was no effect on its interaction with ER α (Fig. 4B). We also tested the interaction between ERAP75 mutants and ER α in a mammalian two-hybrid assay. Similarly, mutation of the first LXXLL mutant, but not of the second LXXLL mutant, diminished the interaction between ER α and full-length ERAP75 by around 70% (Fig. 4C). The results were consistent and suggested that the first LXXLL motif is critical to mediate the interaction between ERAP75 and ER α .

ERAP75 Enhances the ER α Transactivation Activity

To determine whether the interaction between ERAP75 and ER α affects the ER α transactivation, we used luciferase assays with ERE-luc reporter genes. In the COS-1 cell line, which is a ERAP75 negative cell line [53], E2 treatment led to around a sixfold increase in the ER α transactivation (Fig. 5A, lane 3 vs. 1). Importantly, addition of ERAP75 led to a substantial, ERAP75 dose-dependent increase in E2-induced ER α transactivation in COS-1 cells (Fig. 5A, lanes 4, 5 vs. 3). A similar activation was also observed in the PrSC cells (Fig. 5A, lane 9, 10 vs. 8). These results demonstrate that ERAP75 can function as a coactivator to mediate ERs transcriptional activation.

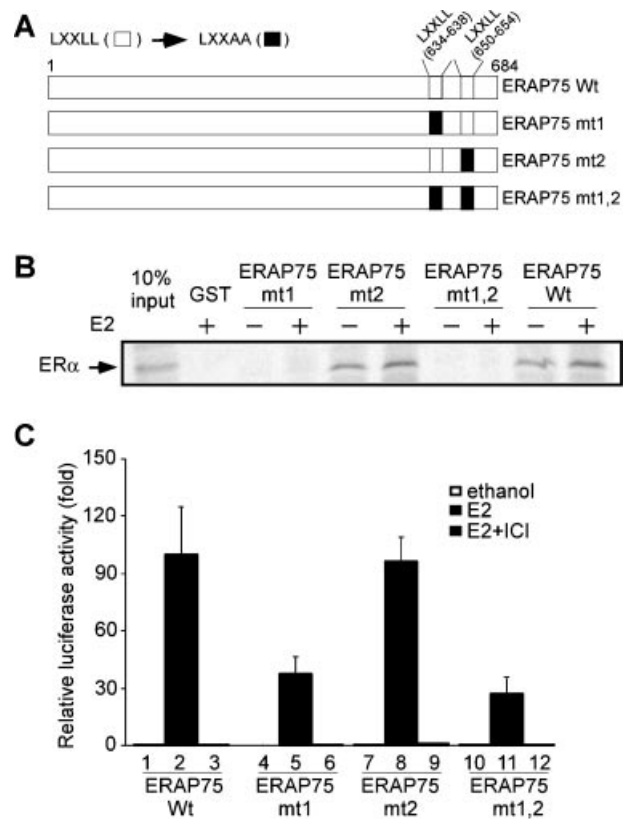


Fig. 4. The conserved LXXLL motif is required for the interaction between ER α and ERAP75. **A:** Schematic diagram of the ERAP75 putative NR boxes and the strategy of mutation. The mutated boxes are in black. All mutants are produced by site-directed mutagenesis. **B:** In vitro interaction between ERAP75 and ER α . The GST alone and the different ERAP75 mutants were purified and incubated with [35 S]methionine-labeled ER α and analyzed in a pull-down assay in the absence or presence of 100 nM E2. **C:** Mammalian two-hybrid interaction between ERAP75 and ER α . COS-1 cells were transiently transfected with 0.4 μ g pG4-Luc, and 0.3 μ g Gal-DBD-ER α -LBD, and 0.3 μ g different VPI6-fused ERAP75 mutants as indicated. The luciferase activity was measured in the absence or presence of 10 nM E2. The luciferase activity observed with wild-type ERAP75 in the presence of E2 was set at 100%.

Increase of ER α Target Gene Expression in PrSC Cells With Overexpression of ERAP75

We studied the consequence of ERAP75 as a coactivator to enhance the ER α transactivation in the PrSC cells. Stromal-derived factor-1 (SDF-1) is a well-studied estrogen target gene and highly expressed in the stromal cells [60]. Using real-time PCR, we showed that the expression of SDF-1 in the PrSC cells was only slightly increased by E2 treatment (Fig. 5B, lane 2 vs. lane 1), which suggested that endogenous ER α transactivation activity is very low in the PrSC, yet, we can detect the expression of ER α in the human and mouse prostate stromal cells, but not the epithelial cells

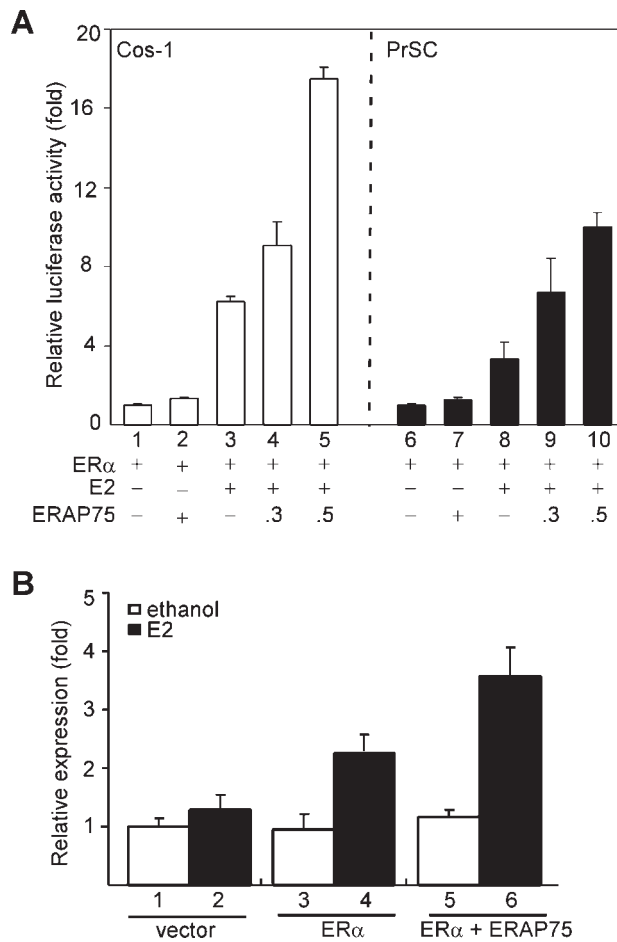


Fig. 5. ERAP75 enhances ER α transactivation and increases the expression of ER α target gene, SDF-1. **A:** COS-1 or PrSC cells were co-transfected with 100 ng of pcDNA3.1(-)-ER α , different doses of pSG5-ERAP75 and the ERE luciferase reporter vectors with 2 ng pRL-tk-Luc used as an internal control for transfection efficiency. Cells were treated with ethanol or 10 nM E2, and were then lysed for luciferase activities. Results shown are the means \pm SD from three independent experiments. **B:** PrSC cells were transfected with pcDNA3.1(-) or pcDNA3.1(-)-ER α with or without pcDNA3-flag-ERAP75 for 24 hr followed by treatment with 10 nM E2 or ethanol for another 24 hr. The cells were harvested and RNA was extracted. Real-time PCR was performed to determine the expression level of SDF-1 mRNA. The β -actin was used as an internal control. The data are presented as mean value \pm SD of triplicate samples.

using immunohistochemical staining (data not shown). Loss of the expression of certain proteins is often seen in the primary cells after being cultured in vitro. PrSC cells were transiently transfected with ER α or ERAP75 or both to detect the expression of ER α target genes. Transfection of ER α into PrSC cells induced a twofold increase of SDF-1 expression (Fig. 5B, lane 4 vs. lane 3). More importantly, co-transfection of ER α and ERAP75 further induced the SDF-1 expression (Fig. 5B, lane 6 vs.

lane 4). Together, these results extended our in vitro studies and demonstrated that the protein-protein interaction between ER α and ERAP75 results in the ER α target gene expression changes.

DISCUSSION

Using the yeast two-hybrid system, we previously identified ERAP75 as a novel ER β coactivator in human PCa cells [53]. Although certain coactivators have shown a preference when transactivating ER α versus ER β [61], here we found that ERAP75 can interact and enhance ER α transactivation in a similar pattern as ER β , which suggests that the interaction between ERAP75 and ERs is highly conserved. However, in terms of the effect of ERAP75 on other NRs, we did find that ERAP75 has very little effect on AR and VDR transactivation, which implicated the relative importance of ERAP75 in mediating different NRs signaling pathways.

Our previous studies also showed that ERAP75 is a nuclear protein and widely expressed in the PCa cell lines, but has low expression in breast cancer cell line, MCF7 [53]. The preferential expression of ERAP75 in the PCa cells prompted us to study the function of ERAP75 in the PCa cells with ER β . We also found that ERAP75 moderately expresses in the normal prostate stromal tissue (data not shown). Since ER α is found mainly in the prostatic stroma, we went further to study whether ERAP75 can affect the ER α function in the context of stromal cells.

The prostate is primarily considered to be an androgen target, but it is also an estrogen target organ. Recent studies have shown that estrogens, in combination with androgens, play critical roles in prostate carcinogenesis [50,51,62,63]. The aberrant estrogen signaling induced by estrogens during prostatic carcinogenesis could be mediated by stromal ER α [52]. Our studies found that ERAP75 can enhance ER α transactivation in the prostatic stromal cells and increase the expression of SDF-1, one of the ER α target genes. SDF-1 has been shown to be overexpressed in human breast and prostate carcinoma-associated fibroblasts [64,65]. SDF-1 could contribute to both prostatic tumor growth and bone metastasis [66,67]. Induction of SDF-1 expression by estrogens/ER α in normal prostatic stromal cells could contribute to the prostate carcinogenesis, and the enhancement of SDF-1 expression by ERAP75 could further promote this process.

In the human tumor specimens, the amplification of coactivators are frequently observed, which may contribute to the pathogenesis of cancer. For example, SRC3/RAC3/AIB1 is often overexpressed in steroid-regulated tumors, especially in breast, ovarian, and prostate cancers [25,68]. ER α gene is transcriptionally

inactivated by DNA methylation in most PCa cell lines and specimens [16,43,69]. However, how the expression of ER α changes in the stromal cells during prostatic carcinogenesis is largely unknown. Whether the expression of ERAP75 is amplified in the prostate stromal tissue and correlates with ER α expression in the prostate stromal cells is also currently unknown. Further studies are needed to determine the functional role of the interaction between ER α and ERAP75 in the prostate stromal cells during prostatic carcinogenesis.

Since estrogen signaling is involved in prostatic hormone carcinogenesis, the tissue distribution of ER α has direct implications as a pharmaceutical target for the treatment of PCa. If the aberrant epithelial signaling induced by estrogens during prostatic carcinogenesis involves indirect action via stromal ER α , the paracrine factors induced by estrogen/ER α , such as SDF-1, and those coactivators, which can enhance ER α transactivation and target genes expression, could be the potential targets for preventing prostatic carcinogenesis. In summary, we found that ERAP75 enhances the ER α transactivation and target genes expression in the prostate stromal cells. The understanding of the mechanism of ER α transactivation in prostate stromal cells could possibly lead to the development of new strategies to control or treat PCa by targeting ER α transactivation protein complex. Further studies may help us to better understand the roles of ER α and ERAP75 in the PCa.

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REFERENCES

- Couse JF, Korach KS. Estrogen receptor null mice: What have we learned and where will they lead us? *Endocr Rev* 1999;20(3):358–417.
- Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P. Human oestrogen receptor cDNA: Sequence, expression and homology to v-erb-A. *Nature* 1986;320(6058):134–139.
- Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J. Sequence and expression of human estrogen receptor complementary DNA. *Science* 1986;231(4742):1150–1154.
- Kuiper GG, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996;93(12):5925–5930.
- Mosselman S, Polman J, Dijkema R. ER beta: Identification and characterization of a novel human estrogen receptor. *FEBS Lett* 1996;392(1):49–53.
- Giguere V, Yang N, Segui P, Evans RM. Identification of a new class of steroid hormone receptors. *Nature* 1988;331(6151):91–94.
- Tsai MJ, O'Malley BW. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 1994;63:451–486.
- Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM. The nuclear receptor superfamily: The second decade. *Cell* 1995;83(6):835–839.
- Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* 1988;240(4854):889–895.
- Catelli MG, Binart N, Jung-Testas I, Renoir JM, Baulieu EE, Feramisco JR, Welch WJ. The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat-shock protein. *EMBO J* 1985;4(12):3131–3135.
- Rosenfeld MG, Glass CK. Coregulator codes of transcriptional regulation by nuclear receptors. *J Biol Chem* 2001;276(40):36865–36868.
- Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. Mechanisms of estrogen action. *Physiol Rev* 2001;81(4):1535–1565.
- Gaub MP, Bellard M, Scheuer I, Chambon P, Sassone-Corsi P. Activation of the ovalbumin gene by the estrogen receptor involves the fos-jun complex. *Cell* 1990;63(6):1267–1276.
- Sabbah M, Courilleau D, Mester J, Redeuilh G. Estrogen induction of the cyclin D1 promoter: Involvement of a cAMP response-like element. *Proc Natl Acad Sci USA* 1999;96(20):11217–11222.
- McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: Cellular and molecular biology. *Endocr Rev* 1999;20(3):321–344.
- Klinge CM. Estrogen receptor interaction with co-activators and co-repressors. *Steroids* 2000;65(5):227–251.
- McKenna NJ, O'Malley BW. Minireview: Nuclear receptor coactivators—An update. *Endocrinology* 2002;143(7):2461–2465.
- Barnes CJ, Vadlamudi RK, Kumar R. Novel estrogen receptor coregulators and signaling molecules in human diseases. *Cell Mol Life Sci* 2004;61(3):281–291.
- Dobrzycka KM, Townson SM, Jiang S, Oesterreich S. Estrogen receptor corepressors—A role in human breast cancer? *Endocr Relat Cancer* 2003;10(4):517–536.
- Onate SA, Tsai SY, Tsai MJ, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 1995;270(5240):1354–1357.
- Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR. GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc Natl Acad Sci USA* 1996;93(10):4948–4952.
- Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H. TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J* 1996;15(14):3667–3675.
- Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* 1997;90(3):569–580.
- Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG. The transcriptional co-activator p/CIP binds

- CBP and mediates nuclear-receptor function. *Nature* 1997; 387(6634):677–684.
25. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 1997;277(5328):965–968.
 26. Suen CS, Berrodin TJ, Mastroeni R, Cheskis BJ, Lyttle CR, Frail DE. A transcriptional coactivator, steroid receptor coactivator-3, selectively augments steroid receptor transcriptional activity. *J Biol Chem* 1998;273(42):27645–27653.
 27. McInerney EM, Rose DW, Flynn SE, Westin S, Mullen TM, Kronen A, Inostroza J, Torchia J, Nolte RT, Assa-Munt N, Milburn MV, Glass CK, Rosenfeld MG. Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. *Genes Dev* 1998;12(21):3357–3368.
 28. Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ, West BL. Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. *Science* 1998;280(5370):1747–1749.
 29. Eckner R, Ewen ME, Newsome D, Gerdes M, DeCaprio JA, Lawrence JB, Livingston DM. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. *Genes Dev* 1994;8(8):869–884.
 30. Kwok RP, Lundblad JR, Chrivia JC, Richards JP, Bachinger HP, Brennan RG, Roberts SG, Green MR, Goodman RH. Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature* 1994;370(6486):223–226.
 31. Glass CK, Rosenfeld MG. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev* 2000; 14(2):121–141.
 32. Freedman LP. Increasing the complexity of coactivation in nuclear receptor signaling. *Cell* 1999;97(1):5–8.
 33. Dotson MR, Yuan CX, Roeder RG, Myers LC, Gustafsson CM, Jiang YW, Li Y, Kornberg RD, Asturias FJ. Structural organization of yeast and mammalian mediator complexes. *Proc Natl Acad Sci USA* 2000;97(26):14307–14310.
 34. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007;57(1):43–66.
 35. Huggins C, Hodges CV. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *J Urol* 2002;168(1):9–12.
 36. Mangan FR, Neal GE, Williams DC. The effects of diethylstilboestrol and castration on the nucleic acid and protein metabolism of rat prostate gland. *Biochem J* 1967;104(3):1075–1081.
 37. Huggins C, Hodges CV. Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA Cancer J Clin* 1972;22(4):232–240.
 38. Brodin J, Skoog L, Ekman P. Immunohistochemistry and biochemistry in detection of androgen, progesterone, and estrogen receptors in benign and malignant human prostatic tissue. *Prostate* 1992;20(4):281–295.
 39. Bonkhoff H, Fixemer T, Hunsicker I, Remberger K. Estrogen receptor expression in prostate cancer and premalignant prostatic lesions. *Am J Pathol* 1999;155(2):641–647.
 40. Royuela M, de Miguel MP, Bethencourt FR, Sanchez-Chapado M, Fraile B, Arenas MI, Paniagua R. Estrogen receptors alpha and beta in the normal, hyperplastic and carcinomatous human prostate. *J Endocrinol* 2001;168(3):447–454.
 41. Carruba G, Pfeffer U, Fecarotta E, Coviello DA, D'Amato E, Lo Castro M, Vidali G, Castagnetta L. Estradiol inhibits growth of hormone-nonresponsive PC3 human prostate cancer cells. *Cancer Res* 1994;54(5):1190–1193.
 42. Castagnetta LA, Carruba G. Human prostate cancer: A direct role for oestrogens. *Ciba Found Symp* 1995;191:269–286; discussion 286–289.
 43. Lau KM, LaSpina M, Long J, Ho SM. Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: Regulation by methylation and involvement in growth regulation. *Cancer Res* 2000;60(12):3175–3182.
 44. Prins GS, Birch L, Couse JF, Choi I, Katzenellenbogen B, Korach KS. Estrogen imprinting of the developing prostate gland is mediated through stromal estrogen receptor alpha: Studies with alphaERKO and betaERKO mice. *Cancer Res* 2001;61(16):6089–6097.
 45. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* 1997;138(3):863–870.
 46. Prins GS, Birch L. Neonatal estrogen exposure up-regulates estrogen receptor expression in the developing and adult rat prostate lobes. *Endocrinology* 1997;138(5):1801–1809.
 47. Leav I, Lau KM, Adams JY, McNeal JE, Taplin ME, Wang J, Singh H, Ho SM. Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. *Am J Pathol* 2001;159(1):79–92.
 48. Chaisiri N, Pierrepoint CG. Examination of the distribution of oestrogen receptor between the stromal and epithelial compartments of the canine prostate. *Prostate* 1980;1(3):357–366.
 49. Ehara H, Koji T, Deguchi T, Yoshii A, Nakano M, Nakane PK, Kawada Y. Expression of estrogen receptor in diseased human prostate assessed by non-radioactive in situ hybridization and immunohistochemistry. *Prostate* 1995;27(6):304–313.
 50. Leav I, Ho SM, Ofner P, Merk FB, Kwan PW, Damassa D. Biochemical alterations in sex hormone-induced hyperplasia and dysplasia of the dorsolateral prostates of Noble rats. *J Natl Cancer Inst* 1988;80(13):1045–1053.
 51. Li X, Nokkala E, Yan W, Streng T, Saarinen N, Warri A, Huhtaniemi I, Santti R, Makela S, Poutanen M. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. *Endocrinology* 2001;142(6): 2435–2442.
 52. Ricke WA, McPherson SJ, Bianco JJ, Cunha GR, Wang Y, Risbridger GP. Prostatic hormonal carcinogenesis is mediated by in situ estrogen production and estrogen receptor alpha signaling. *FASEB J* 2008;22(5): online.
 53. Chen M, Ni J, Muyan M, Lin C-Y, Yeh S. ERAP75/CCDC62 functions as a coactivator to enhance estrogen receptors mediated transactivation and target genes expression in prostate cancer cells. *Endocrinology* 2007. (Submitted).
 54. Ni J, Wen X, Yao J, Chang HC, Yin Y, Zhang M, Xie S, Chen M, Simons B, Chang P, di Sant'Agnes A, Messing EM, Yeh S. Tocopherol-associated protein suppresses prostate cancer cell growth by inhibition of the phosphoinositide 3-kinase pathway. *Cancer Res* 2005;65(21):9807–9816.
 55. Yeh S, Miyamoto H, Nishimura K, Kang H, Ludlow J, Hsiao P, Wang C, Su C, Chang C. Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells. *Biochem Biophys Res Commun* 1998;248(2):361–367.

56. Ni J, Chen M, Zhang Y, Li R, Huang J, Yeh S. Vitamin E succinate inhibits human prostate cancer cell growth via modulating cell cycle regulatory machinery. *Biochem Biophys Res Commun* 2003;300(2):357–363.
57. Zhang M, Altuwaijri S, Yeh S. RRR-alpha-tocopheryl succinate inhibits human prostate cancer cell invasiveness. *Oncogene* 2004;23(17):3080–3088.
58. Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P. Estrogen receptor pathways to AP-1. *J Steroid Biochem Mol Biol* 2000;74(5):311–317.
59. Leers J, Treuter E, Gustafsson JA. Mechanistic principles in NR box-dependent interaction between nuclear hormone receptors and the coactivator TIF2. *Mol Cell Biol* 1998;18(10):6001–6013.
60. Hall JM, Korach KS. Stromal cell-derived factor 1, a novel target of estrogen receptor action, mediates the mitogenic effects of estradiol in ovarian and breast cancer cells. *Mol Endocrinol* 2003;17(5):792–803.
61. Warnmark A, Almlöf T, Leers J, Gustafsson JA, Treuter E. Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERalpha and ERbeta. *J Biol Chem* 2001;276(26):23397–23404.
62. Bianco JJ, Handelsman DJ, Pedersen JS, Risbridger GP. Direct response of the murine prostate gland and seminal vesicles to estradiol. *Endocrinology* 2002;143(12):4922–4933.
63. McPherson SJ, Wang H, Jones ME, Pedersen J, Iismaa TP, Wreford N, Simpson ER, Risbridger GP. Elevated androgens and prolactin in aromatase-deficient mice cause enlargement, but not malignancy, of the prostate gland. *Endocrinology* 2001;142(6):2458–2467.
64. Ao M, Franco OE, Park D, Raman D, Williams K, Hayward SW. Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. *Cancer Res* 2007;67(9):4244–4253.
65. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* 2005;121(3):335–348.
66. Cooper CR, Chay CH, Gendernalik JD, Lee HL, Bhatia J, Taichman RS, McCauley LK, Keller ET, Pienta KJ. Stromal factors involved in prostate carcinoma metastasis to bone. *Cancer* 2003;97 (3 Suppl):739–747.
67. Wang J, Shiozawa Y, Wang J, Wang Y, Jung Y, Pienta KJ, Mehra R, Lober R, Taichman RS. The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. *J Biol Chem* 2008;283(7):4283–4294.
68. Gnanapragasam VJ, Leung HY, Pulimood AS, Neal DE, Robson CN. Expression of RAC 3, a steroid hormone receptor co-activator in prostate cancer. *Br J Cancer* 2001;85(12):1928–1936.
69. Li LC, Chui R, Nakajima K, Oh BR, Au HC, Dahiya R. Frequent methylation of estrogen receptor in prostate cancer: Correlation with tumor progression. *Cancer Res* 2000;60(3):702–706.