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ERR-10: a new repressor in transcriptional signaling activation of estrogen receptor-α☆

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Abstract Estrogen receptor-alpha (ER-α) is a nuclear transcriptional factor that is part of the nuclear receptor superfamily. In this study, we isolated and identified a new L*XX*LL-containing protein that interacts with the ER-α via a yeast two-hybrid assay. We have termed this protein estrogen receptor repressor-10 (ERR-10). The ERR-10 cDNA is predicted to encode a polypeptide of 94 amino acids, with a molecular mass of about 10 kDa. Although the ERR-10 mRNA transcript is expressed in a wide range of normal human tissues, higher expression levels are found in endocrinal tissues relative to other tissues. We have demonstrated, through immunoprecipitation, Western blot and GST pull-down assays, that ERR-10 associates with ER-α. Moreover, ERR-10 decreased 17β-estrodial-induced activation of ER-α transcriptional activity in transient transfection assays of mammalian cells. The ERR-10 N-terminus, which resembles two L*XX*LL motifs, is essential for ER-α binding and repression activity. Estrogen modulation of estrogen-responsive gene expression was markedly blocked by ERR-10. These results suggest that ERR-10 is a novel mediator in ER transcriptional activation.

Keywords: ERR-10; Estrogen receptor; Transcription; L*XX*LL; Co-repressor; Breast cancer

1. Introduction

Estrogen influences gene expression and affects cellular phenotypic changes by diffusing into the cell and binding to specific estrogen receptors (ER, α and β). ERs belong to the nuclear receptor (NR) superfamily. NRs share common structural and functional features, including acting as transcriptional factors when bound by their appropriate ligands [1–5]. The classical ER (now called ER-α or ER) consists of 595 amino acids and exhibits a modular structure comprised of several domains labeled A–F, with three functional domains [6,7]: an N-terminal region, containing a constitutive activation function (AF-1); a central, highly conserved DNA-binding domain (DBD); and a C-terminal ligand-binding domain (LBD), containing a dimerization and a ligand-transactivation function (AF-2). The AF-1 domain is hormone-independent, whereas the AF-2 domain is hormone-dependent [8–10]. Both domains are required for maximal ER transcriptional activity; however, each is capable of functioning independently [8–10]. Like other activation domains, the AFs of ER are important targets for basal transcriptional factors and cellular proteins that function as co-regulators. In the absence of a ligand, the ER exists in a transcriptionally inactive state, associated with heat-shock proteins and other cellular chaperones. Upon activation by hormone binding, the ER-α receptor undergoes a conformational change of the LBD and dissociates from the heat-shock proteins, allowing the receptor to dimerize and interact specifically with a *cis*-acting DNA sequence called the estrogen response element (ERE) within regulatory regions of target genes to activate or repress transcription [3,11,12], and references therein.

Many of the tissue-specific effects of ER are dependent on the cellular pool of co-regulators and other nuclear proteins that influence ER transcriptional activities [13–16], and references therein. These proteins function as signaling intermediates between ER and the general transcriptional machinery to increase ER transcriptional activity (as co-activators), reduce its transcriptional activity (as co-repressors), or act as protein adaptors for other diverse transcriptional signaling proteins.

Functional and structural studies have elucidated that the precise mechanisms of the interaction between several different co-regulators and the ligand-inducible activation domain of ER take place through short leucine-rich sequences known as L*XX*LL motifs, or NR-Boxes (Table 1) [17–33]. For example, the L*XX*LL motifs present in co-activators PERC and in co-repressors DAX-1 are regarded as essential

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Abbreviations: ER, estrogen receptor; NR, nuclear receptor; AF, activation function; DBD, DNA-binding domain; LBD, ligand-binding domain; ERR-10, estrogen receptor repressor-10; FCS, fetal calf serum; ERE, estrogen response element; wt, wild type; mut, mutant; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; bp, base pair(s); IVT, in vitro transcribed and translated; efp, estrogen-responsive finger protein; S.E., standard error

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Table 1
Amino acid sequence of the NR-interaction box (NR-Box, L*XX*LL) in different ER co-activators and co-repressors

Gene	L*XX*LL motif (aa)	Role in ER Activity	Ref.
CIA	S**LINLL**AD (386–393)	Activator	[17]
DAX-1	I**LYSLL**TS (145–152)	Repressor	[18]
DRIP	A**LRHLL**FL (470–477)	Activator	[19]
	T**LKSLL**RP (559–566)		
	P**LFRLL**TK (720–727)		
	K**LFDLL**YP (397–404)		
	P**LKGLL**PY (251–258)		
	I**LHTLL**EM (599–596)		
	S**LETLL**DH (783–790)		
GRIP1	K**LLQLL**TT (640–647)	Activator	[20]
	I**LHRLL**QD (689–696)		
	L**LRYLL**DK (744–751)		
	Q**LGRLL**PN (877–884)		
LZIP	D**LLALL**EE (12–19)	Activator	[21]
	D**LLSLL**SP (52–59)		
p300	K**LSELL**RS (80–87)	Activator	[22]
	Q**LVLLL**HA (341–348)		
	A**LQNLL**RT (2050–1057)		
P/CAF	Y**LFGLL**RK (189–196)	Activator	[23]
PELP1	C**LLSLL**YG (28–35)	Activator	[24]
	V**LRDLL**RY (72–79)		
	H**LPGLL**TS (94–101)		
	L**LTSLL**GL (98–105)		
	E**LHSLL**AS (181–188)		
	S**LHTLL**GA (188–195)		
	P**LRLLL**LP (281–289)		
	L**LTHLL**SD (376–383)		
	E**LYCLL**LA (496–503)		
	L**LALLL**AP (501–508)		
PERC	L**LQKLL**LA (155–162)	Activator	[25]
	I**LRELL**AQ (342–349)		
PGC-1	L**LKKLL**LA (143–147)	Activator	[26]
PRIP	L**LVNLL**QS (891–898)	Activator	[27]
	S**LSQLL**DN (1495–1502)		
RAP250	L**LVNLL**QS (886–893)	Activator	[28]
	S**LSQLL**DN (1490–1497)		
RIP140	Y**LEGLL**MH (20–27)	Activator	[29]
	L**LASLL**QS (132–139)		
	H**LKTLL**KK (184–191)		
	Q**LALLL**SS (265–272)		
	L**LLHLL**KS (379–386)		
	L**LLLLL**GH (499–506)		
	V**LQLLL**GN (712–719)		
	L**LSRLL**RQ (818–825)		
	V**LKQLL**LS (935–972)		
R-MGMT	V**LWKLL**KV (95–105)	Repressor	[30]
SHP-1	I**LYALL**TS (19–26)	Repressor	[18]
SRC-1	K**LVQLL**TT (252–260)	Activator	[31,32]
	I**LHRLL**QE (309–318)		
	L**LRYLL**DK (368–375)		
	Q**LDELL**CP (532–539)		
	L**LQQLL**TE (1054–1061)		
TRAP220	I**LTSLL**QI (603–610)	Activator	[33]
	M**LMNLL**KD (644–651)		
TRBP	L**LVNLL**QS (886–893)	Activator	[19]
	L**LSQLL**DN (1490–1497)		

CIA, co-activator independent of AF-2 function; DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X chromosome, gene 1; DRIP, vitamin D receptor interacting protein; GRIP1, glucocorticoid receptor interacting protein 1; PELP1, proline-, glutamic acid-, and leucine-rich protein-1; LZIP, leucine zipper protein; PERC, PGC-1 related estrogen receptor co-activator; P/CAF, p300/CBP-associated factor; PGC-1, peroxisome proliferator-activated receptor-γ co-activator-1; PRIP, peroxisome proliferator-activated receptor-interacting protein (also referred to as MNAR, or modulator of nongenomic activity of ER); RAP250, Nuclear receptor-activating protein, 250 kDa; RIP140, receptor-interacting protein 140 (also named Nrip1, nuclear-receptor-interacting protein 1); R-MGMT, O(6)-methylguanine-DNA methyltransferase; SHP-1, Src homology phosphatase-1; SRC-1, steroid-receptor co-activator 1; TRAP220, thyroid hormone receptor-associated protein, 220 kDa; TRBP, TAR RNA- binding protein.

for modulating interaction with ER. In this study, we identify and characterize a new protein, named estrogen receptor repressor-10 (ERR-10), which associates with ER-α and attenuates ER-α transcriptional activity via two L*XX*LL motifs.

2. Materials and methods

2.1. Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC) and maintained as monolayer cultures in

RPMI-1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mg/l glutamine, 0.1 mg/l streptomycin, and 1000 U/l penicillin G (Gibco), at 37 °C in a humidified atmosphere of 5% CO_2 in air. Phenol red-free medium and dextran-coated charcoal-treated FCS were used in experiments to assess the impact of E2.

2.2. Expression plasmids and reporters

All vectors were generated using standard cloning procedures and verified by restriction enzyme analysis and DNA sequencing. The wild-type expression vector pCMV-ER-α (ER-α) was used to express ER-α. The ERE2-TK-LUC reporter, composed of the vitellogenin A2 ERE controlling a minimal thymidine kinase promoter (TK81) and luciferase in a pGL2 plasmid, has been described in our previous studies [34–37]. The wild-type ERR-10 (wt-ERR-10) expression plasmid was created by cloning the full-length ERR-10 cDNA into a mammalian pCMV-Tag2B expression vector (Stratagene), which allows for the expression of proteins with an N-terminal FLAG sequence. L*XX*LL-mutated ERR-10 (ERR-10-Box1mut, ERR-10-Box2mut, and ERR-10-Box1/2mut) expression plasmids were created by replacing leucines in the critical +4 and +5 positions of the L*XX*LL motifs with alanines (e.g., L*XX*LL → L*XX*AA) in wt-ERR-10 cDNA. Gal4-LUC, gal4-ER-α, glutathione *S*-transferase (GST)-ER-α, and GST-ER-α mutant (mut) constructs have been described in previous studies [34–37]. The CAT reporter vectors B1ERE-CAT, pS2ERE-CAT, and OTERE-CAT, which contain the B1ERE sequence CAGTCACTGGTACCC, pS2ERE sequence AGGTCACGGTGGCCA, OTERE sequence CGGTCACGGTCACCT, and Cyclin D1 promoter vector (CD1-LUC), were kindly provided by Richard G Pestell (Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC).

2.3. ERR-10 antibody

Bacterially expressed GST-ERR-10 (amino acids 15–85) was purified by affinity chomatography on glutathione–Sepharose 4B (Amersham Pharmacia Biotech) and eluted in a buffer containing 10 mM reduced glutathione in 50 mM Tris–HCl (pH 8.0). Polyclonal sera were raised against purified GST-ERR-10 in rabbits and affinity-purified.

2.4. Yeast two-hybrid screen

To isolate cDNA-encoding proteins that interact with ER-α, a yeast two-hybrid screening was carried out using the LBD and AF-2 domain of ER-α linked to the rat DBD of gal4 (gal4-ER-LBD/AF-2) as a bait. The protocol for the Benton and Davis method [38] for cDNA library screening was followed. After several rounds of screening with a human brain cDNA library (Clontech) in yeast strain CG1945, a pure positive 1910-base pair (bp) clone was identified and sequenced. Both strands of the isolated cDNA clones were sequenced by dideoxynucleotide-chain termination methods, using a primer walking strategy.

2.5. Northern blot assay

The filter for Northern hybridization (Clontech) was hybridized to the 446-bp ERR-10 cDNA probe, which had been purified from agarose gel using the QIAEX II gel extraction kit (Qiagen) and labeled with [α-^{32}P]dCTP (Amersham) by the random-prime method (Rediprime, Amersham Pharmacia Biotech). The Clontech Multiple Tissue Northern Blots contained 2 μg of oligo(dT)-purified mRNA from various normal human tissues. Hybridization, washing, and X-ray film exposure were performed according to the manufacturer's recommended protocol (Clontech), as described in previous studies [34]. After stripping, the same filter was hybridized to the β-actin probe as a control.

2.6. GST pull-down assay

GST pull-down assays were performed as described in previous studies [35–37]. GST fusion proteins were amplified from *Escherichia coli* strain BL21 grown in Luria–Bertani medium at 37 °C to mid-log phase and induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside for another 4 h at 30 °C. Bacteria were harvested by centrifugation and lysed by sonication in phosphate-buffered saline. The lysates were spun at $10\,000 \times g$ for 30 min at 4 °C and the supernatant was mixed with 50 μl of glutathione–agarose beads (Gibco) for 2 h at 4 °C. Beads were collected by centrifugation and washed thee times in ice-cold phosphate-buffered saline. GST fusion proteins were incubated in a binding buffer (10 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), 0.2% Nonidet P-40, 1 mM dithiothreitol, and 3% bovine serum albumin) with complete protease inhibitors (Roche Molecular Biochemicals). Beads with the pure GST fusion proteins were stored at 4 °C. Aliquots of the beads were boiled in 2× SDS loading buffer, separated by electrophoresis though a 4–20% polyacrylamide gel, and blotted with an anti-GST antibody (Santa Cruz) to analyze for bound proteins. Binding assays were carried out in 0.5 ml of the reaction buffer containing GST fusion protein (30 μg) with 30 μl of IVT [^{35}S]methionine-labeled protein (Promega). After overnight incubation, the beads were collected by centrifugation, washed five times in the binding buffer, and boiled in 2× SDS loading buffer. Bound proteins were separated by electrophoresis though a pre-made 4–20% polyacrylamide gel. After electrophoresis, the gel was fixed for 30 min in fixative solution containing 30% methanol and 10% acetic acid, completely washed in water for 30 min, incubated in 1 M sodium salicylate for 20 min, dried, and exposed to film at −80 °C overnight.

2.7. Immunoprecipitations (IP) and Western blotting (WB)

The in vivo association of ERR-10 and ER-α was achieved by IP–WB, as described in previous studies [35–37]. IP with human 293T cells was performed using cells cultured in 6-well plates 24 h prior to transfection. The cells were transfected with a mammalian pCMV-Tag2B-ERR-10 expression vector by lipofectAMINE 2000 (Invitrogen) with 20 μg of total DNA. Cells were harvested 48 h post-transfection, then lysed in 1 ml of Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, and protease inhibitors). Cell lysates were clarified by centrifugation at $18\,000 \times g$ for 15 min. ER-α IP from MCF-7 cells was performed using 2 μg of affinity-purified anti-ER-α polyclonal antibody (H222, H226, or a combination of H222 and H226, Santa Cruz). 2 μg of normal mouse IgG antibody was used as a negative IP control. ERR-10 was immunoprecipitated with 2 μg of anti-FLAG antibody (mouse monoclonal, M2, Sigma) overnight at 4 °C with 30 μl of a 50% slurry of protein G–agarose (Roche Molecular Biochemicals). Immune complexes were collected by low-speed centrifugation, washed three times in 1% Nonidet P-40 lysis buffer, and boiled in 2× SDS loading buffer. Denatured proteins were separated by SDS–polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon (Millipore Corp.), which was blocked in 5% non-fat milk, 150 mM NaCl, 10 mM Tris (pH 8.0), and 0.05% Tween. Immunoblots were performed with anti-ERR-10 antisera or with an anti-ER-α polyclonal antibody at 1 μg/ml (H-184, Santa Cruz Biotechnology, CA) and developed by enhanced chemiluminescence (Amersham Pharmacia Biotech). For WB assays of estrogen response genes mediated by ERR-10, a polyclonal anti-estrogen-responsive finger protein (efp) antibody (C-20), polyclonal anti-cathepsin D (C-20) and monoclonal anti-cyclin D1 (HD-11) were purchased from Santa Cruz Biotechnology, CA.

2.8. Analysis of transcriptional activity

Transcriptional activity was assessed as described in previous studies [34–37]. One day prior to transfection, cells were seeded at a density of 4×10^3 cells/well in 24-well tissue culture dishes. Cells were then transfected with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's recommendations. Transfections were performed with a constant mixture of 2 μg of the indicated DNA (see figure legend). 250 ng cytomegalovirus (CMV)β-gal (CLONTECH Laboratories) for each transfection was used to normalize each sample for transfection efficiency. The masses of the plasmids are indicated in the figure legends. The transfected cells were then subject to 17β-estrodial (E2) treatments (10 nM final concentration; Sigma) for 24 h prior to lysis. For luciferase activity, 40 μl of cell lysate was assayed for luciferase activity, in accordance with the manufacturer's recommendations (Dual Luciferase, Promega). For the determination of CAT activity, cell lysates were mixed with acetyl CoA and [^{14}C]chloramphenicol and post-incubated for 1 h at 37 °C. Acetylated chloramphenicol and non-acetylated chloramphenicol were separated on Sil G TLC plates and finally quantitated on a Molecular Dynamics Phosphorimager with ImageQuant software (Molecular Dynamics). Student's *t* tests were used to determine whether statistical differences between ethanol and E2-treated groups existed.

Table 2
Primers used for RT-PCR

Gene	Primer pairs
ERR-10	5′-CCC CAG AGT GAT GGC AGA CAA-3′ (forward)
	5′-TTT GGG GAC TTG AGA TGT TTT G-3′ (backward)
Efp	5′-GTG CGG CCA CAA CTT CTG CG-3′ (forward)
	5′-CTT TCA CGG CGG CCT CCT T-3′ (backward)
E2IG4	5′-CCT GAC TCG GGT GGA TTG TAG-3′ (forward)
	5′-GAG AAG GCA GTG GGT GAG ATG C-3′ (backward)
Cathepsin-D	5′-GGG GCT CTG TGG AGG ACC TGA T-3′ (forward)
	5′-AGA GGC TGA CGA CGC TGA CTG G-3′ (backward)
Cyclin D1	5′-GCT GCT CCT GGT GAA CAA GC-3′ (forward)
	5′-TTC AAT GAA ATC GTG CGG G-3′ (backward)
β-Actin	5′-GTC AAC GGA TTT GGT CTG TAT T-3′ (forward)
	5′-AGT CTT CTG GGT GGC AGT GAT-3′ (backward)

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was carried out as described in previous studies [35–37]. cDNA was synthesized from 2 μg of total RNA in a 30-μl reaction mixture containing 5× reverse transcriptase reaction buffer (Life Technologies, Inc., Gaithersburg, MD), 200 μM dNTP, 100 μM solution of primers, 50 units of RNasin (Promega, Madison, WI), 10 mM dithiothreitol, and 100 units of reverse transcriptase (Life Technologies, Inc.). The mixture was incubated at 37 °C for 60 min, heated to 95 °C for 10 min, and then chilled on ice. PCR was carried out in a 50-μl volume containing 10–20 ng of cDNA, chelating buffer (Perkin–Elmer Cetus, Norwalk, CT), 20 μM dNTP mixture, 1.5 units of Taq DNA polymerase (Perkin–Elmer Cetus), and 0.5 μM of each primer (shown in Table 2). To ensure that the RNA was of sufficient purity to undergo RT-PCR, a PCR assay using primers specific for the β-actin cDNA was also performed for each sample. The PCR products were electrophoresed on a 5% nondenaturing polyacrylamide gel. The gel was then dried and exposed to an imaging plate, and the radioactivity was determined using Bioimage Analyzer (Bas1000; Fuji, Kanagawa, Japan).

2.10. Statistical analysis

The statistical analysis was performed using the two-tail Student's *t* test.

3. Results and discussion

3.1. A new ER-interacting protein identified via yeast two-hybrid screen

In an effort to identify ER partner proteins, we cloned the AF-2 domain of ER-α into the DBD of gal4 (Clontech). The resulting vector, gal4-ER-LBD/AF-2, was used as a bait for screening a human brain cDNA library in yeast strain CG1945. While sequencing one of the 41 $His^+/\beta\text{-}gal^+$ clones, we identified a single positive 284-bp sequence (clone 22). This cDNA encodes the predicted 94-amino acid-long protein with a calculated mass of 10 kDa that is homologous to the human gene *NAG-7* (GenBank Accession No.: AF086709) (Fig. 1A). The biological functions of the *NAG-7* gene are unknown to date; however, its expression is downregulated in 26% (5/19 cases) of human nasopharyngeal carcinoma cases [39]. In vitro translation produced a protein of approximately 10 kDa, consistent with the expected size (Fig. 1B). After plasmid isolation, we re-transformed CG1945 with clone 22 alone, with the original bait (gal4-ER-LBD/AF-2), or with one of several control plasmids. We observed positive β-galactosidase expression only with the combination of clone 22 and gal4-ER-LBD/AF-2 (Table 3). The protein was designated ERR-10 to signify its function as a co-repressor of ER-α transcriptional signaling, as well as its size in kilodaltons. The human tissue specificity and the mRNA transcript size of ERR-10 were evaluated through Northern blot analysis (Fig. 1C). Northern hybridization of the ERR-10 cDNA to mRNA from eight different organs showed a 284-bp band in thymus, prostate, testes, colon, and ovary, with minimal expression in spleen, small intestine, and peripheral blood leukocytes.

3.2. In vitro interaction of ERR-10 with ER-α

The interaction between ERR-10 and ER-α was confirmed by a GST pull-down assay. GST fusion proteins containing the full-length ER-α were constructed, expressed in *E. coli*, and purified on glutathione–agarose beads as described previously [35–37]. As shown in Fig. 2B, the GST-wt-ER-α fusion proteins captured IVT [^{35}S]methionine-labeled wt-ERR-10. GST alone did not capture radiolabeled wt-ERR-10. The presence of a ligand resulted in a slight increase in ERR-10–ER-α binding. We also performed the reciprocal experiment with affinity-purified GST-ERR-10. GST-ERR-10 bound to glutathione–agarose beads incubated with radiolabeled ER-α. GST-ERR-10 captured radiolabeled ER-α (Fig. 2C). These results indicate that the new binding protein ERR-10 interacts directly with ER-α.

To identify the ER-α binding domain, we also generated two deletion mutant constructs of ERR-10. The first deletion mutant, ERR-10-delN (amino acid 46–94), lacks the N-terminus and the second deletion mutant, ERR-10-delC (amino acid 1–46), lacks the C-terminus (Fig. 2A). ERR-10-delC exhibited appreciable binding to GST-wt-ER-α in both the presence and absence of E2, whereas ERR-10-delN failed to bind to GST-wt-ER-α under either circumstance (Fig. 2B). Clearly, the N-terminus of ERR-10 is required for ERR-10–ER-α interaction.

Analysis of the ERR-10 amino acid sequences revealed that the N-terminus contains two signature L*XX*LL sequences (LPHLL, residues 25–29, termed ERR-10-Box1 and LIWLL, residues 39–43, termed ERR-10-Box2) resembling the NR-boxes (Fig. 2A). Furthermore, the two L*XX*LL motifs local within the ERR-10-delC bound to the ER-α (Fig. 2B). The presence of L*XX*LL motifs, which are required for the interaction of several different co-regulators with ER (Table 1), motivated us to assess whether the interaction of ERR-10 and ER-α was in fact mediated by these L*XX*LL motifs. To accomplish this, we generated three ERR-10 constructs expressing L*XX*LL mutations: two single mutants (ERR-10-Box1mut and ERR-10-Box2mut) and one double L*XX*LL mutant (ERR-10-Box1/2mut), in which the leucines in the critical +4 and +5 positions of L*XX*LL motifs were replaced with alanines (Fig. 2A). As illustrated in Fig. 2D, both ERR-10-Box1mut and ERR-10-Box2mut retained the ability to interact with ER-α, albeit in a significantly less efficient manner relative to wt-ERR-10. The ERR-10 with double mutations in both L*XX*LL motifs, i.e., ERR-10-Box1/2mut, failed to exhibit any interaction with ER-α. These results suggest that both L*XX*LL motifs of ERR-10 must be intact for optimal interaction with ER-α.

To determine the binding domain within the ER-α protein, we performed GST pull-down assays with various GST-ER-α mutants, all of which have been described in previous studies [35–37] (Fig. 3A). As shown in Fig. 3B, IVT-wt-ERR-10 bound to GST-wt-ER-α, GST-ER 2085-595, and GST-ER 373-595, but failed to bind either GST-ER 1-253 or GST-ER 1-353. These results indicate that ERR-10 interacts with ER-α via binding to the E/F domain of ER-α, which contains the LBD.

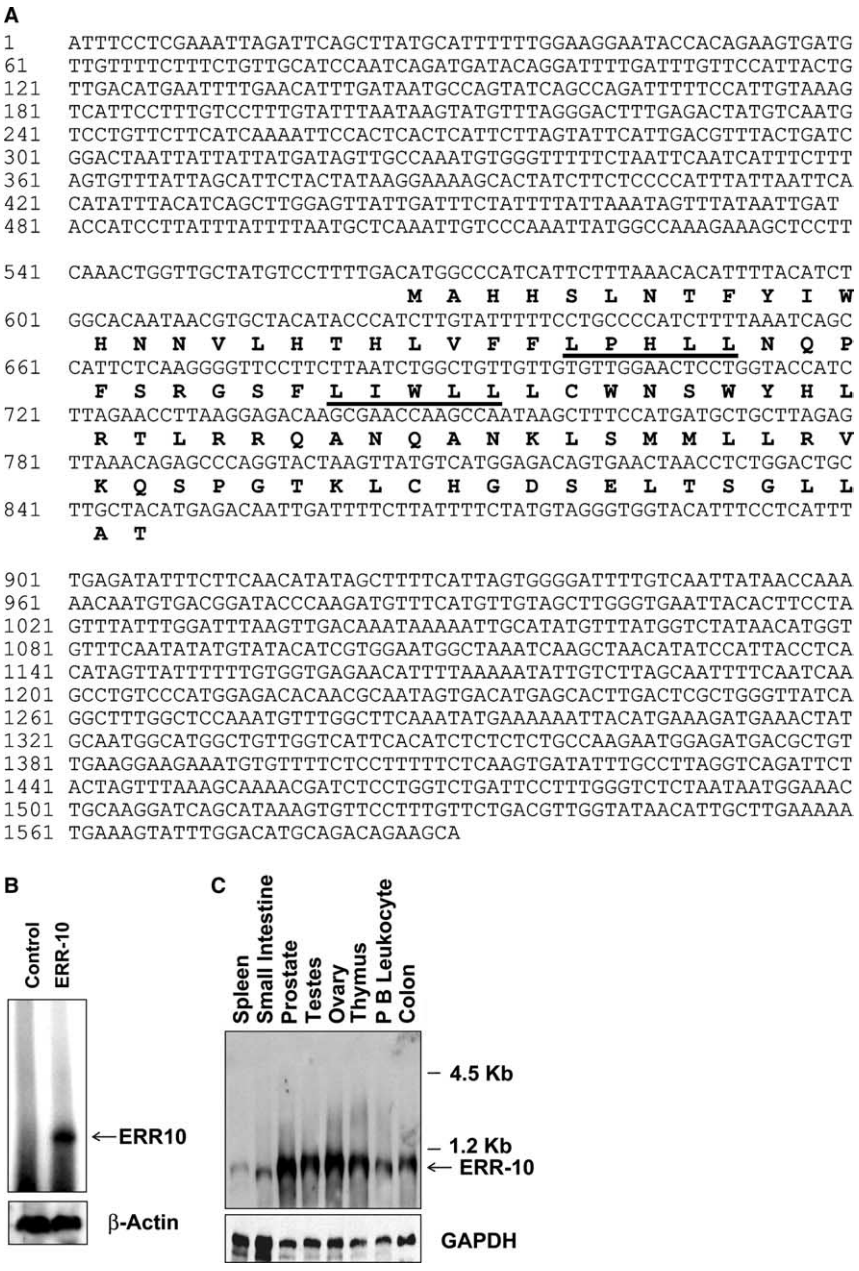

Fig. 1. Nucleotide sequence and amino acid sequence of ERR-10 and ERR-10 expression in various human tissues. (A) cDNA and amino acid sequences of human ERR-10. The sequence of ERR-10 was confirmed by dideoxy method from positive clones isolated through a yeast two-hybrid system. cDNA sequences are numbered on the left. As shown, alignment of NR boxes found in ERR-10 is underlined. (B) In vitro translation of ERR-10 cDNA. The full-length human ERR-10 cDNA was cloned, transcribed, and translated in vitro using rabbit reticulocyte lysate with [^{35}S]methionine. Radiolabeled protein was fractionated on a 20% SDS–polyacrylamide electrophoresis gel and visualized using autoradiography. The cDNA encoded a ~0.9-kb mRNA in all samples tested (top). A β-actin mRNA probe was used to control for mRNA integrity (bottom). Equal loading was also assessed via ethidium bromide staining (not shown). (C) Expression of ERR-10 mRNA in various human tissues. Multiple human tissues (Northern blot II, Clontech) were hybridized with ^{32}P-labeled DNA probes prepared from the original 284 bp ERR-10 cDNA. The same filter was stripped and hybridized with GAPDH probe to control for mRNA integrity.

3.3. *In vivo association of ERR-10 with ER-α in mammalian cells*

To determine whether ERR-10 could physically associate with ER-α in cells, immunoprecipitations were first performed in human 293T cells that had been transiently transfected with the full-length ERR-10 in a Flag-tagged vector (pCMV-Tag2B-wt-ERR-10) and the wt-ER-α in a pCMV vector. Total cell extracts were incubated with the anti-Flag antibody for the ERR-10 IP and the ER-α protein was co-immunoprecipitated with ERR-10-Flag (Fig. 4A, *lane 3*). Similarly, ERR-10 was identified by immunoblot analysis using ERR-10 antisera and determined to be a 10-kDa species present in the ER-α immunoprecipitates when total cell extracts from transfected 293T cells were incubated with the anti-ER-α antibody

Table 3
Yeast two-hybrid interaction of ERR-10 with ER-α

GAD-ERR-10	Gal-LBD/AF2	
	ER-α	
	−E2	+E2
GAD	−	−
GAD-ERR-10	+	+
GAD-ERR-10-Box1	+	+
GAD-ERR-10-Box2	+	+
GAD-ERR-10-Box1/2	−	−

ERR-10 and ER-α interaction was measured as growth on plates without histidine in the presence (+E2) and absence (−E2) of estrogen four days after plating. + represents growth; − represents no growth.

(Fig. 4B, *lane 3*). Thus, the co-precipitation of ERR-10 with ER-α occurred when both proteins were overexpressed in 293T cell lysates. The most important indication of the physiological significance of the ERR-10–ER-α interaction was the visible formation of an endogenous complex between ERR-10 and ER-α. Subconfluent ER-positive MCF-7 breast cancer cells were cultured in phenol red-free medium containing dextran-coated, charcoal-treated FCS ± 10 nM E2 for 4 h, and then subjected to immunoprecipitation with an amino-terminal A/B region-specific antibody against the ER-α, H226, followed by IB with ERR-10 antisera. ERR-10 appeared in the ER-α immunoprecipitates (Fig. 4C, *lane 3*); this association only became more apparent after the cells were treated with E2 (Fig. 4C, *lane 4*). However, we observed that H222, a monoclonal antibody directed against the carboxyl-terminal-specific antibody of ER-α, was unable to co-precipitate ERR-10 (Fig. 4C, *lane 5*), even in the presence of E2 (Fig. 4C, *lane 6*). In fact, the H222 antibody may have blocked the interaction between ERR-10 and ER-α, since immunoprecipitation with a combination of H222 and H226 resulted in a reduction of co-precipitating ERR-10 (Fig. 5C, *lanes 7 and 8)*, supporting the theory that an anti-ER-α monoclonal antibody directed against the LBD/AF-2 region of ER-α can block the interaction between ERR-10 and ER-α.

3.4. ERR-10 represses ER-α transcriptional activation in a ligand-dependent manner

The interaction between ERR-10 and ER-α prompted us to investigate whether ERR-10 is capable of modulating the effects of ER-α transcriptional activation on the activity of estrogen-responsive promoters. This was accomplished using transient transfection assays. Two ER-positive breast cancer cell lines, MCF-7 and T-47D, were transiently transfected with an equal amount of pCMV-Tag2B-ERR-10 and ERE-TK-LUC reporter expression vectors in the presence and absence of E2 (10 nM, 24 h). The luciferase activity was then measured. The left panel of Fig. 5A shows increased expression levels of the ERR-10 protein in the cells transfected with the pCMV-Tag2B-ERR-10 expression vector. Enforced expression of ERR-10 did not affect basal ER-α expression levels (data not

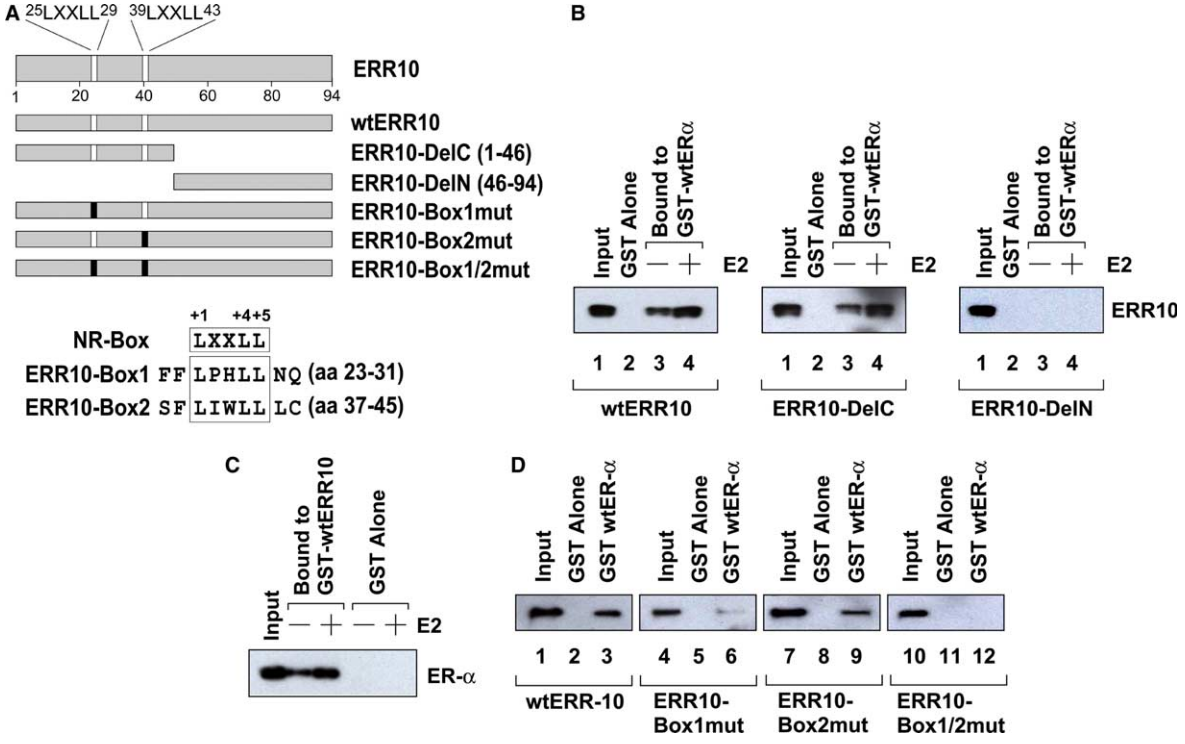

Fig. 2. Interaction of ERR-10 and ER-α. (A) Schematic structure of ERR-10, the ERR-10 deletion mutants, and the L*XX*LL point mutants used in the in vitro GST pull-down assays and the location of putative NR boxes. *Open boxes* indicate consensus L*XX*LL motifs and *black boxes* indicate variants with leucine +4 and +5 substitutions with alanine. (B) Full-length wt-ERR-10 (1–94), ERR-10 with a C-terminus deletion (ERR-10-DelC), and ERR-10 with an N-terminus deletion (ERR-10-DelN) in a mammalian pCMV-Tag2B expression vector were each translated in vitro and incubated with GST-wt-ER-α fusion proteins immunobilized on GST beads without hormone (−) or with 10 nM E2 (+) as indicated. (C) GST-ERR-10 was used as a capture reagent with a radiolabeled IVT-wt-ER-α. (D) ^{35}S-labeled wt-ERR-10, ERR-10-Box1mut, ERR-10-Box2mut, and ERR-10-Box1/2mut were each reacted to GST-ER-α in the presence of 10 nM E2. A control assay was performed simultaneously to evaluate the non-specific binding of IVT-ERR-10 or IVT-wt-ER-α to the GST alone. No binding was observed when using GST alone. *Input* represents 10% of the IVT protein.

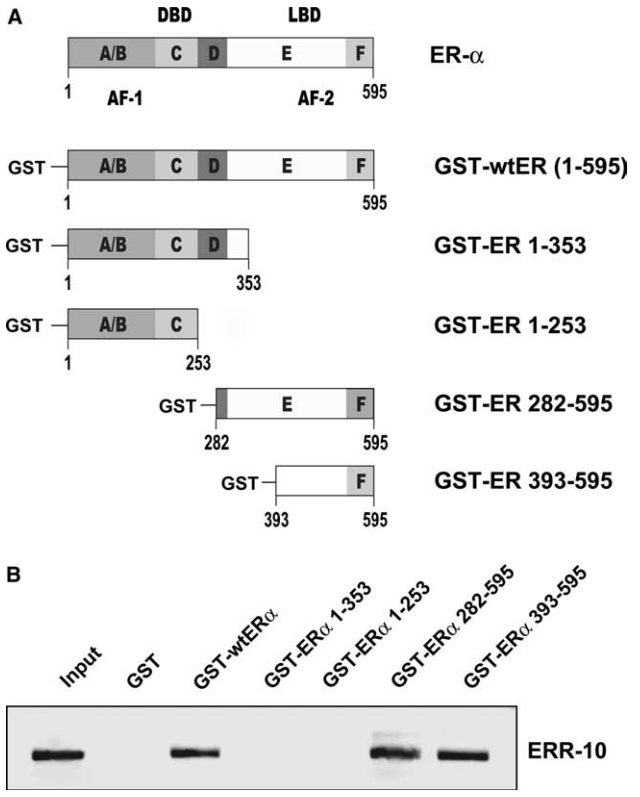

Fig. 3. The C-terminus of ER-α is required for ERR-10 binding. (A) Schematic diagram of ER-α and the ER-α deletion mutations used in the in vitro GST pull-down assays. The A/B domain at the N-terminus contains the AF-1 site where other transcription factors interact. The C/D domain contains the two-zinc finger structure that binds to DNA and the E/F domain contains the ligand-binding pocket, as well as the AF-2 domain. (B) The wt-ER-α and the ER-α mutants indicated were each fused to GST, immobilized on GST beads, and reacted with IVT-ERR-10 in the presence of 10 nM E2. No binding was observed when using GST alone. *Input* represents 10% of the amount of labeled IVT-ERR-10 protein.

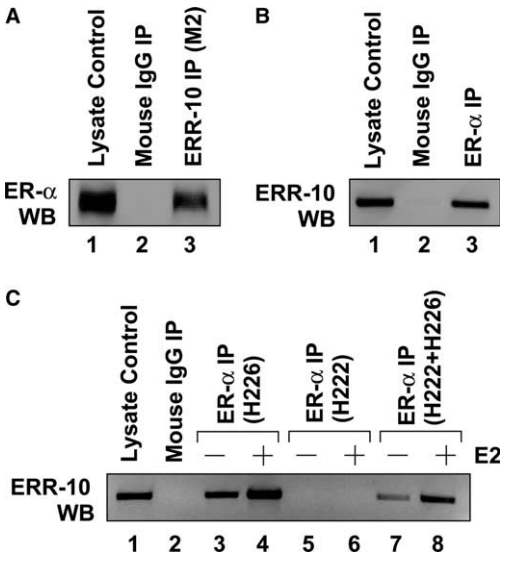

Fig. 4. In vivo association of ERR-10 with ER-α. Human 293T cells were transiently transfected with pCMV-Tag2B-ERR-10 and pCMV-wt-ER-α vectors. The ERR-10 transigene protein was immunoprecipitated with an anti-flag-antibody M2 (A); the ER-α transigene protein was immunoprecipitated with an anti-ER-α N-terminus antibody H226 (B). (C) The endogenous ER-α protein from MCF-7 cells was cultured for 4 h and immunoprecipitated with an anti-ER-α N-terminus antibody H226, an anti-ER-α C-terminus antibody H222 or an antibody mix of H222 and H226. The immunoprecipitates were resolved by 4–20% SDS–PAGE, electroblotted to PVDF membrane, and visualized by WB analysis, using anti-ER-α antibody H-184 or anti-ERR-10 sera. This was followed by visualization with an ECL detection kit, as described in previous studies [35–37].

shown). The endogenous ER-α-driven ERE-TK-LUC activity was significantly activated by 24 h treatment with 10 nM E2 (an about 100-fold increase, set at 100%) compared to when in the absence of E2 (Fig. 5A, right panel). However, E2 did not cause any stimulation of ERE-deficient TK-LUC activity. Co-transfection of ERR-10 significantly reduced E2-stimulated ER-α transcriptional activity ($P < 0.01$), but ERR-10 did not affect the basal transcriptional activity of ER-α in the absence of E2. These results suggest that the effect of ERR-10 on ER-α signaling is ligand-dependent. The transfection with the "empty" pCMV-Tag2B control vector has little or no effect on ERE-TK-LUC reporter activation. Furthermore, the extent to which ER-α transcriptional activation is repressed by ERR-10 is dependent upon the amount of ERR-10 transfected, i.e., in a dose-dependent manner (Fig. 5B). For example, 0.07, 0.4, and 1.0 μg of ERR-10 vector exhibited about 50% ($P < 0.05$), 80% ($P < 0.01$), and 85% ($P < 0.01$) reductions in E2-induced transcriptional activity in both MCF-7 and T-47D cell lines.

When two ER-negative human breast cancer cell lines, MDA-MB-231 and MDA-MB-468, were co-transfected with an ER-α expression vector, an ERE_2-TK-LUC promoter vector, and a pCMV-Tag2B-ERR-10 expression vector, similar results to ERR-10-induced ER-α signaling inhibition were observed (Fig. 5C, right panel). Again, co-transfection of the "empty" pCMV-Tag2B control plasmid did not affect ER-α activity. The ER-α and ERR-10 expressions in the MDA-MB-231 cell line were determined by WB analysis, as illustrated in the left panel of Fig. 5C. Similar inhibitory, ERR-10-induced effects on ER-α transcriptional activity were observed in other cell lines, including human cervical cancer Caski and HeLa cells, human prostate cancer Du-145 cells, and mouse NIH3T3 cells, as shown in Fig. 5D. The suppression of ER-α transcription by ERR-10 does not seem to be a merely general effect upon transcription, as transcription mediated by the early promoter SV40 was not influenced by the expression of ERR-10, as demonstrated in MCF-7 and MDA-MB-231 cells (data not shown). These results clearly indicate that ERR-10 is a specific repressor for inhibiting ER-α transcriptional activity.

We also determined the effects of ERR-10 on estrogen activation of naturally occurring estrogen-responsive promoters driven by ER-α by using different EREs, including the *Xenopus laevis* B1 ERE2, the human pS2 ERE, and the human oxytocin ERE. As shown in Fig. 5E by CAT assays, the activation of these ER-α-driven ERE promoters by E2 was suppressed by ERR-10 to different extents. These data further suggest that ERE-10 is a potent modulator in the ER-α signaling transduction pathway.

3.5. The N-terminus and LXXLL motifs of ERR-10 are required for inhibiting ER-α-mediated transcription

To determine which portion(s) of the ERR-10 protein are responsible for inhibiting ER-α signaling activation, MCF-7

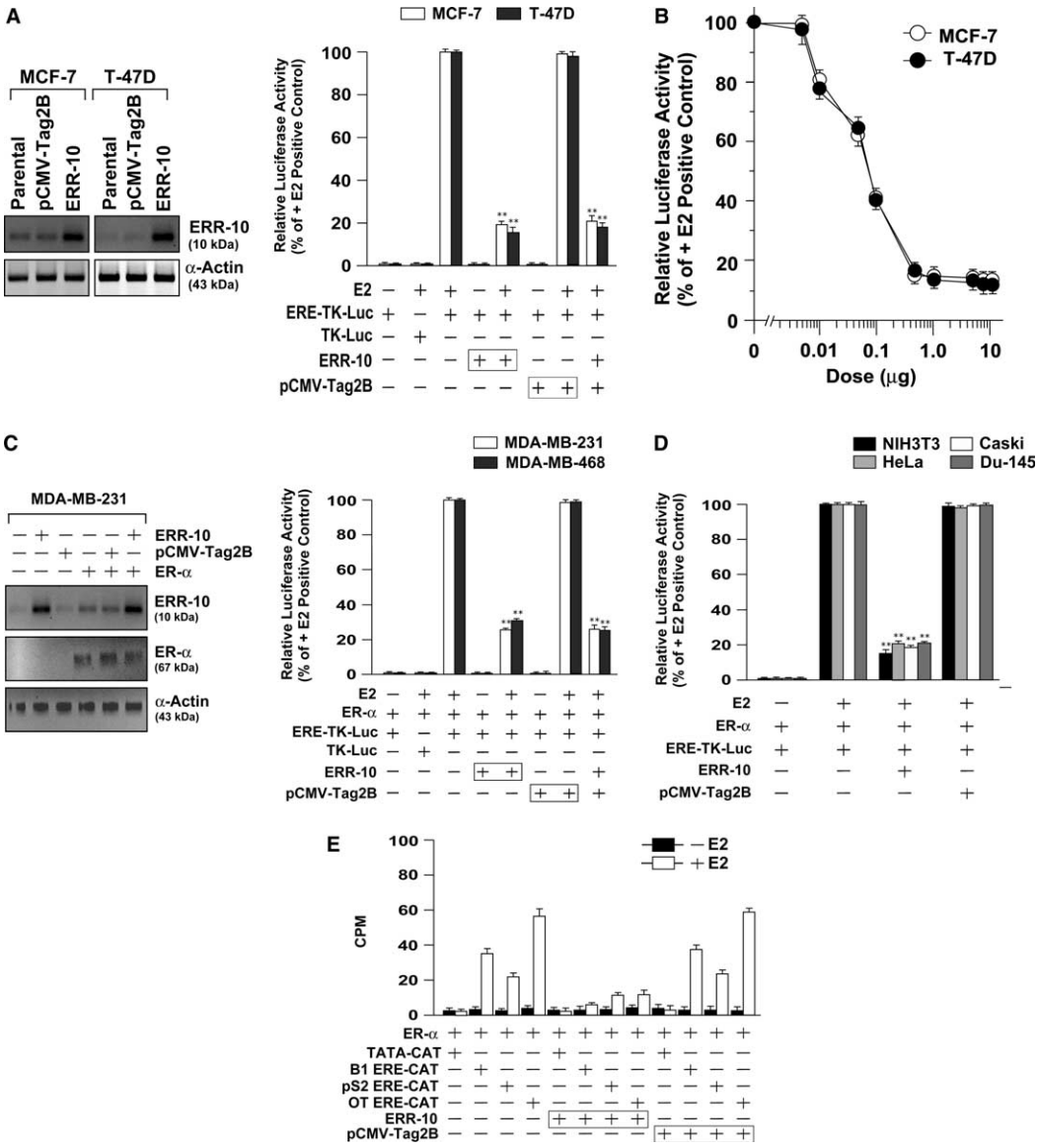

Fig. 5. Inhibition of ligand activation of ER-α transcriptional activity by ERR-10. (A) ERR-10 inhibits ER-α-mediated ERE promoter activity in a ligand-dependent fashion. MCF-7 and T-47D cells were cultured in 24-well tissue culture dishes and co-transfected with equal amounts (0.25 μg/well) of the indicated vectors overnight, incubated in medium ± 10 nM E2 for 24 h and then harvested for assay of luciferase activity (right panel). The ERR-10 transigene was monitored by WB in parallel to the luciferase assay (left panel). (B) ERR-10 decreases ER-α-mediated ERE promoter activity in a dose-dependent manner. Increasing increments (0–10 μg each well/24 well dishes) of ERR-10 plasmid were co-transfected with a 0.25 μg/well of ERE-TK-LUC vector into MCF-7 and T-47D cells as indicated based on the same protocol in (A). (C,D) ERR-10 inhibition of ER-α signaling in other cell lines. Breast cancer MDA-MB-231 and MDA-MB-468 cells, human cervical cancer HeLa and Caski cells, human prostate cancer Du-145 cells and murine fibroblastic NIH-3T3 cells were co-transfected overnight with equal amounts (0.25 μg) of pCMV-ER-α, ERE2-TK-LUC and pCMV-Tag2B-ERR-10 or "empty" pCMV-Tag2B (as a negative control), incubated in medium ± E2 (10 nM) for 24 h and then harvested for assay of luciferase activity. Expressions of the ERR-10 and ER-α transigenes in transfected MDA-MB-231 cells were determined by WB (left panel, C). Luciferase activity in the presence of E2 is set arbitrarily to 100%. Luciferase values are means ± standard errors (S.E.s) from three or four independent experiments. (E) DU-145 cells were co-transfected with an ER expression vector, a pCMV-Tag2B-ERR-10 expression plasmid, and a CAT reporter plasmid containing a TATA sequence alone (−) or in combination with a B1, pS2, or OT ERE. Cells were then treated with ± 10 nM E2 for 24 hr after transfection. CAT activity values are means ± S.E.s from three separate experiments. The statistical significances were analyzed by two-tail Student's *t* test. $*P < 0.05$; $**P < 0.01$.

cells were first transfected with an ERE-TK-LUC promoter vector and either a wt-ERR-10, ERR-10-delC, or ERR-10-delN vector. As shown in Fig. 6B, E2 activation of ER-α-driven transcriptional activity was significantly diminished by the presence of wt-ERR-10. The C-terminal deletion mutant ERR-10-delC inhibited ER-α-mediated transcription in a manner similar to wt-ERR-10. However, the N-terminal deletion mutant ERR-10-delN failed to have any effect upon ER-α-mediated transcriptional activation. Neither wt-ERR-10 nor the two deletion mutants affected the basal level of ERE-LUC promoter activity in the absence of ligand (Fig. 5A and C). Thus, consistent with the binding data above (Fig. 2A and D), the N-terminal region of ERR-10 is also crucial for the ERR-10-induced inhibition of ER-α transcriptional activation.

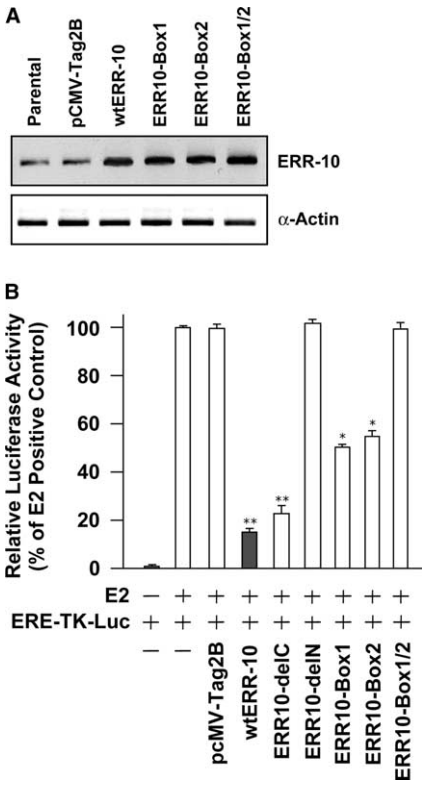

Fig. 6. L*XX*LL motifs were required for ERR-10 to inhibit ER-α signaling. MCF-7 cells cultured in 24-well tissue culture dishes were transiently transfected overnight with equal amounts (25 μg/well), indicated vectors by lipofectAMINE 2000. The transfected cells were harvested for WB to monitor ERR-10 expression (A), and either left untreated or treated with E2 (10 nM) and subjected to luciferase activity assays after 24 h (B). Luciferase activity in the presence of E2 is set arbitrarily to 100%. The data represent the average of at least four independent experiments with standard deviations shown as error bars. The statistical significances were analyzed by two-tail Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$.

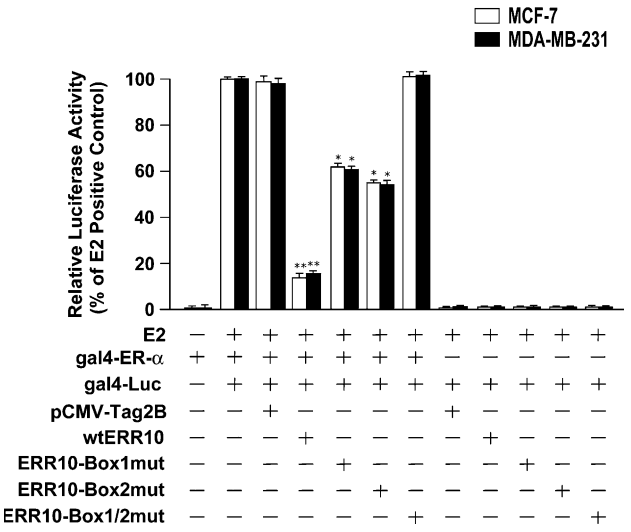

Fig. 7. AF-2 dependent repression of ER-mediated transcription by ERR-10. Cells were transfected overnight with indicated vectors using lipofectAMINE 2000, either left untreated or treated with E2 (10 nM), harvested after 24 h, and subject to luciferase activity assays. Luciferase activity in the presence of E2 is set arbitrarily to 100%. The data represent the average of at least four independent experiments with standard deviations shown as error bars. The statistical significances were analyzed by two-tail Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$.

ERR-10-Box1mut and ERR-10-Box2mut appeared to inhibit ER-α signaling less effectively than did the wt-ERR-10. However, ERR-10-Box1/2mut completely lost the ability to attenuate ER-α signaling activation. The proteins of these L*XX*LL mutant species by transient transfection were produced at levels comparable to wt-ERR-10, as determined by WB analysis (Fig. 6A). These results indicate that the decrease in ER-α mediated transcription by ERR-10 requires a direct interaction between ERR-10 and ER-α.

3.6. *ERR-10 repression of ER-mediated transcription in an AF-2-dependent manner*

The data shown in Figs. 3B and 4C suggest that the binding region of ER-α for ERR-10 is located at the N-terminus. In order to exclude the possibility that ERR-10 interferes with ER transactivation by preventing ER/DNA interaction, we performed gal4-ER-α/gal4-LUC assays, which entailed activating a luciferase reporter gene with gal4 DNA-binding sites in the presence of E2. In both MCF-7 and MAD-MB-231 cell lines, co-transfection of wt-ERR-10, but not the "empty" pCMV-Tag2B control vector, significantly attenuated the E2-activated gal4-ER/gal4-LUC reporter activity (by approximately 85%, Fig. 7), suggesting that ERR-10 may function, at least in part, by targeting the AF-2. ERR-10-Box1/2mut containing mutations in both L*XX*LL motifs was markedly defective in its ability to modulate gal4-ER-α transactivation; however, ERR-10-Box1mut and ERR-10-Box2mut were both able to inhibit gal4-ER-α transactivation, albeit less effectively than wild-type ERR-10, showing ~40% and ~45% reduction rates in inhibition ability, respectively. In the absence of gal4-ER-α, neither wt-ERR-10 nor mutant ERR-10 had any effect on gal4-LUC activity. Thus, increased expression of ERR-10 specifically reduces the activity of gal4-ER-α, but this effect is diminished or disrupted by mutations in either of the two L*XX*LL motifs.

3.7. *ERR-10 inhibition of E2-stimulated expression of estrogen-responsive genes*

To further confirm ERR-10 co-regulator activity and rule out any potential artifact effect from the luciferase assay, we performed RT-PCR assays to investigate the effects of ERR-10 on expression of several estrogen-response genes in ER-positive MCF-7 cells. The RT-PCR data presented in Fig. 8 indicate that E2 increases the expression of a panel of estrogen-response genes, including E2IG4, efp, cathepsin-D, and cyclin D1 at mRNA levels. However, the E2-stimulated expression of these genes was blocked or reduced by ERR-10 to different degrees. For example, the E2-increased expression of E2IG4 mRNA was completely blocked by ERR-10, reduced to below even basal levels. In contrast, ERR-10 had little or no effect on the basal mRNA levels of these genes in the absence of hormone (data not shown). Consistent with the mRNA results, it was also found that ERR-10 inhibited accumulation of these estrogen-response genes at the post-transcript levels, as evidenced by WB assays (Fig. 8C). In addition, we found that ERR-10

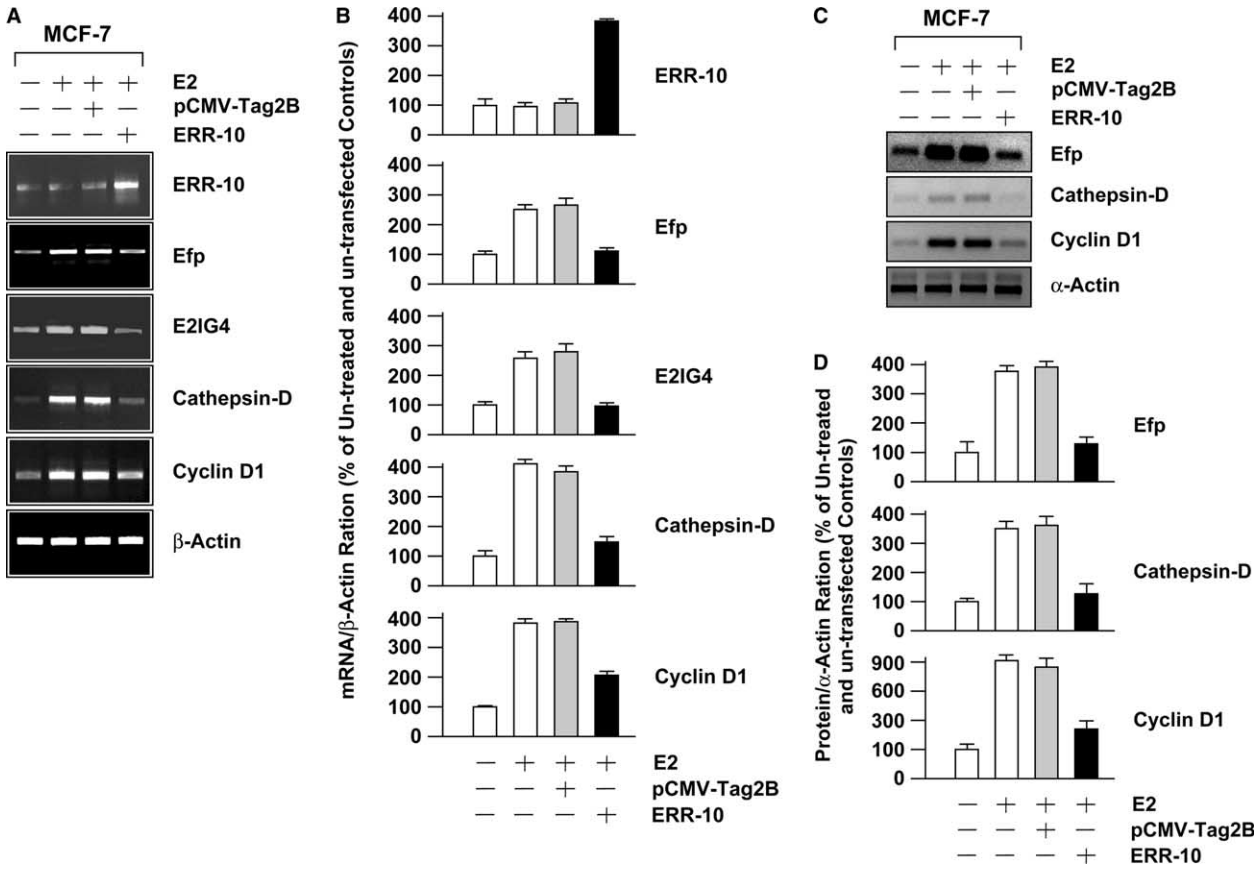

Fig. 8. ERR-10 inhibition of estrogen-mediated expression of estrogen responsive genes. MCF-7 cells were transiently transfected with pCMV-Tag2B-ERR-10 or "empty" pCMV-Tag2B (as a control) overnight, incubated with E2 (10 nM) for 24 h and then harvested for RT-PCR assays (A) and for WB assays (C). Data shown are from a representative experiment that was repeated three times. The densitometric quantitation of mRNA relative to β-actin and protein relative to α-actin is shown (B,D).

overexpression repressed estrogen stimulation of cyclin D1 promoter compared to the control "empty" vector, as shown by luciferase assays (Fig. 9). Taken together, these results clearly indicate that ERR-10 is a potent co-regulator for E2-dependent activation of ER-α transcriptional signaling.

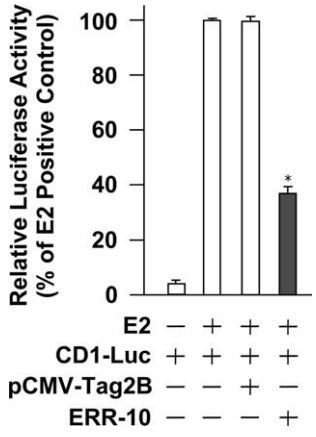

Fig. 9. ERR-10 inhibition of estrogen-dependent cyclin D1 promoter. Du-145 cell were transiently transfected with indicated vectors and post-incubated with and without 10 nM E2 for 24 h. The cells were finally harvested for luciferase activity as described in Section 2. The data represent the average of three independent experiments with standard deviations shown as error bars. The statistical significances were analyzed by two-tail Student's t test. $^{*}P < 0.01$.

In conclusion, we have presented compelling data regarding several properties of a new ER-α-interacting protein, ERR-10, which we discovered through yeast two-hybrid assay. The interaction between ERR-10 and ER-α was confirmed via a GST pull-down assay using both ERR-10 and ER-α capture reagents and by co-immunoprecipitation of the endogenous proteins. Furthermore, two discrete regions of the two proteins are responsible for the interaction: the N-terminus of ERR-10 and the C-terminus region containing the LBD of ER-α. The interaction between ERR-10 and ER-α may be direct, since two L*XX*LL motifs (which are usually present in NR co-activators) in the N-terminus of ERR-10 were required for an interaction between the ERR-10 and ER-α.

Forced expression of ERR-10 significantly decreased endogenous and exogenous ER-α-driven ERE luciferase activity in a ligand- and dose-dependent fashion. In addition, ERR-10 attenuated luciferase activity when the gal4-ER-α was used as transcriptional activator on a luciferase reporter containing gal4 DNA-binding sites. However, ERR-10 mutants containing mutations in both L*XX*LL motifs exhibited a partial or complete loss in ER-α transcriptional activity inhibition. These data indicate that ERR-10 can function as a transcriptional co-repressor for ER-α. Because ERR-10 is an L*XX*LL

motif-containing protein, whether the ERR-10 functions as a general co-regulator in NR transcriptional activity is currently being investigated in our laboratory. Whether the ERR-10–ER interaction plays a role in estrogen-related diseases such as breast cancer needs to be explored in further detail.

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