

A Novel Function of the Fe65 Neuronal Adaptor in Estrogen Receptor Action in Breast Cancer Cells*

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Background: Fe65 is a neuronal adaptor with essential roles in neuronal cells.

Results: Fe65 regulates promoter recruitments of the estrogen receptor α and growth response of breast cancers to estrogens and tamoxifen.

Conclusion: Fe65 is a positive regulator of estrogen actions in breast cancer cells.

Significance: The studies define a novel role for a neuronal adaptor in estrogen actions in breast cancer cells.

Fe65 is a multidomain adaptor with established functions in neuronal cells and neurodegeneration diseases. It binds to the C terminus of the A β amyloid precursor protein and is involved in regulating gene transcription. The present studies show that Fe65 is expressed in breast cancer (BCa) cells and acts as an ER α transcriptional coregulator that is recruited by 17 β -estradiol to the promoters of estrogen target genes. Deletion analyses mapped the ER α binding domain to the phosphotyrosine binding domain 2 (PTB2). Ectopic Fe65 increased the transcriptional activity of the ER α in a PTB2-dependent manner in reporter assays. Fe65 knockdown decreased, whereas its stable expression increased the transcriptional activity of endogenous ER α in BCa cells and the ability of estrogens to stimulate target gene expression, ER α , and coactivator recruitment to target gene promoters and cell growth. Furthermore, Fe65 expression decreased the antagonistic activity of tamoxifen (TAM), suggesting a role for Fe65 in TAM resistance. Overall, the studies define a novel role for the neuronal adaptor in estrogen actions in BCa cells.

Estrogens are the female sex steroid hormone with established roles in reproduction and development as well as the biology and pathogenesis of many tissues including those of the central nervous, the cardiovascular, and the skeletal systems, etc. (1, 2). The best defined estrogen target tissues include the mammary gland of which both the normal development and epithelial tumorigenesis are subjected to estrogen control. Due to the retained sensitivity of the majority of human BCa to estrogens, the inhibition of estrogen action at the tumor with synthetic antagonists such as TAM² has been the preferred

therapeutic treatment for decades (3, 4). The effects of estrogens and antiestrogens on mammary epithelial and cancer cells are predominantly mediated through the ER α that belongs to the steroid/thyroid nuclear receptor superfamily of ligand-regulated transcription factors (5, 6). The ER α contains an N-terminal A/B region, a DNA binding domain composed of two C₂C₂ zinc fingers, a hinge region, a ligand binding domain, and an F tail (5). It forms a homodimer and binds to estrogen response elements (ERE) to control target gene expression. In addition, the ER α also regulates target gene expression through “tethering” to other transcription factors (7, 8). Estrogens bind to the ER α and recruit coactivators (9) to induce the expression of growth-promoting genes. Estrogen antagonists, on the other hand, induce a distinct conformation suitable for the binding of corepressor complexes, thereby shutting off gene transcription (10, 11). TAM, which aims to block the ER α action, has mixed agonist/antagonist activity and may either stimulate or inhibit ER α activity, depending on the tissue and gene context (12). The use of TAM has significantly benefitted women with ER α -positive BCa, but the benefit has been limited by the issue of resistance.

Besides mammary gland development and tumorigenesis, another known target organ for estrogens is the brain. Estrogens have been shown to be neuroprotective, and their decrease after menopause is believed to contribute to the development of neurodegenerative diseases such as Alzheimer disease. APP plays important roles in the pathogenesis of Alzheimer disease, and recent studies have shown that its C-terminal fragment produced after the cleavage by γ -secretase, namely APPct or AICD, forms a multimeric complex with the nuclear adaptor protein Fe65 and stimulates transcription (13–15). Published studies have shown that 17 β -estradiol inhibits the transcriptional activity of the APPct complex and impairs the ability of the complex to induce neuroblastoma cell apoptosis (16), providing a mechanism explaining the neuronal protective effects of estrogens. Both *in vitro* and *in vivo* immunological analyses

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² The abbreviations used are: TAM, tamoxifen; ERE, estrogen response element; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide;

E2, 17 β -estradiol; IB, immunoblotting; IP, immunoprecipitation; IHC, immunohistochemistry; TMA, breast tissue microarray; BCa, breast cancer; APP, amyloid precursor protein; VDR, vitamin D receptor; AR, androgen receptor.

Fe65 and Breast Cancer

have revealed that the ER α forms a complex with full-length APP or APPct and that the complex formation occurs between endogenous proteins in mouse brains, which is increased in transgenic mice expressing mutant presenilin 1 and APP (16). Mechanistic investigations have found that the functional interaction between the ER α and APP is indirectly mediated through Fe65, identifying it as a novel ER α interacting protein (16).

Fe65 is a multidomain adaptor protein containing an undefined N terminus, a group II tryptophan-tryptophan (WW) domain in the middle, and two C-terminal PTB domains, namely PTB1 and PTB2 (17). Through PTB2, it forms a multimeric complex with APP or APPct to stimulate transcription through the recruitment of the transcription factor CP2/LSF/LBP1 and the histone acetyl transferase Tip60 (13–15) to PTB1 as well as the nucleosome assembly factor SET to the WW domain (18). The PTB1 domain also interacts with two cell surface lipoprotein receptors, the low density lipoprotein receptor-related protein (19) and ApoEr2 (20), forming trimeric complexes with APP and establishing a biological linkage between APP and the lipoprotein receptors. Besides SET, the WW domain also binds to Mena (21), through which Fe65 regulates actin cytoskeleton, cell motility, and neuronal growth cone formation (22, 23).

There are two Fe65 isoforms produced by the alternative splicing of a 6-bp mini-exon encoding Arg-Glu dipeptide inserted in the PTB1 domain. The isoform with this mini-exon is expressed exclusively in neurons, whereas the isoform lacking the dipeptide exists in non-neuronal cells (24). Besides its neuronal functions in APP processing and Alzheimer disease biology, Fe65 has been reported to regulate other essential cellular functions such as DNA damage repair that goes beyond neuronal cells. Fe65 null mice are more sensitive to DNA damages induced by etoposide and ionizing radiations (25). Studies with Fe65 null mouse embryonic fibroblasts concluded that Fe65 was required for the efficient repair of DNA double-strand breaks, a function dependent on its interaction with Tip60 and APPct (26, 27). However, functions of Fe65 in non-neuronal cells are largely undefined, and nothing is known about its involvement in estrogen actions in BCa.

In the present study we demonstrate for the first time that Fe65 is expressed in mammary epithelial cells and that its expression is increased in BCa cells and human breast tumor samples. Fe65 is recruited by estrogens to the promoters of estrogen target genes in BCa cells and potentiates the recruitment of the ER α and its coactivators to the promoters. It increases the agonistic activity of 17 β -estradiol and decreases the antagonistic activity of TAM. The studies define Fe65 as a positive ER α regulator that increases the growth of human BCa cells and contributes to TAM resistance.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—17 β -Estradiol, anti-FLAG affinity gels, and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) were purchased from Sigma. Fetal bovine serum (FBS), charcoal stripped FBS, and Lipofectamine 2000 were from Invitrogen. Anti-hemagglutinin (anti-HA.11) antibody was obtained from Covance (Princeton, NJ). Anti-Fe65,

anti-c-Myc, anti-cyclin D1 were from Cell Signaling (Boston, MA). Anti-APPct was from Calbiochem. The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): anti-ER α (F10), anti-Fe65 (H-260), anti- β -actin (AC-15), anti-HSP60 (H-1), HDAC1 (H-11), anti-Tip60 (N-17), anti-histone H1 (N-16). Fe65 (siFe65) (sequence 5'-CUACGU-AGCUCGUGAUAAAG-3'), siER α (sequence 5'-GCCAGCAG-GUGCCCUACUA-3'), and scrambled control (siCtrl) siRNA oligonucleotides were synthesized by Dharmacon/Thermo Scientific (Waltham, MA). The ECL Western blotting substrates were from Thermo Scientific. Luciferase assay substrates were from Promega Corp. (Madison, WI). Chip assay kit (EZ-ChipTM) was from Millipore (Billerica, MA). Breast invasive ductal carcinoma tissue array slides were purchased from US Biomax Inc. (Rockville, MD), and their usage was in compliance with policies of the institutional review board at University of South Florida.

To construct tagged Fe65, cDNA of the non-neuronal Fe65 (Thermo Scientific) was amplified by PCR using forward (GCGGGATCCATGTCTGTTCCATCATCACTG) and reverse (GAGGTCGACTCATGGGGTATGGGCCCC) primers. Myc-Fe65 was constructed by cloning the amplified cDNA into the Bam H1 and Sal1 of p-CMV-3Tag-2a-Myc plasmid (Agilent Technologies, Santa Clara, CA). To construct the expression plasmids of HA-Fe65 and deletion constructs, Fe65 cDNA or fragments were amplified by PCR using the Myc-Fe65 as the template, digested with BamH1 and Sal1, and ligated into pCMV-HA plasmid generated by replacing the FLAG tag with HA of pCMV-3Tag-1A (Agilent Technologies). The primers used for the constructions of Fe65 and its deletion mutants are as follows: full-length: GCGGGATCCATGTCTGTTCCATCATCACTGAGC (forward) and GAGGTCGACTCATGGGGTATGGGCCCCAGCCG (reverse); N-terminal 128 amino acid deletion (dN128): GCGGGATCCATGAACCGAGGCCTACGAGGACCT (forward) and GAGGTCGACTCATGGGGTATGGGCCCCAGCCG (reverse); N-terminal 242 amino acid deletion (dN242): GCGGGATCCATGTTCTGGAACCCCAACGCCTTC (forward) and GAGGTCGACTCATGGGGTATGGGCCCCAGCCG (reverse); WW deletion (dWW): TTCACCGGTCAAGAGGAGTCCCAGCTCAC (forward) and CTTACCGGTGTCGGAATCCGTCTCGAAG (reverse); PTB1 deletion (dPTB1): CCCAAGAGGAGGAGAAGTGCTTGGTAAATGGACT (forward) AGTCCATTTACCAAGCACTTCTCCTCCTCTTGGG (reverse); PTB2 deletion (dPTB2): CTTGTGGATGTCCCTTTCCAATCCCAGGCCTC (forward) and GAGGCCTGGGATTGGAAAGGGACATCCACAAG (reverse); C-terminal 182 amino acid deletion (dC182): GCGGGATCCATGTCTGTTCCATCATCACTGAGC (forward) GAGGTCGACTCATTGGAAAGGACATCCACAAG (reverse); C-terminal 42 amino acid deletion (dC42): GCGGGATCCATGTCTGTTCCATCATCACTGAGC (forward) and GAGGTCGACACGGGCATCCAGACACTTCTGGTA (reverse).

FLAG-VDR was generated by sub-cloning VDR cDNA in-frame with 3 \times FLAG epitope in the pCMV vector. FLAG-ER α plasmid was a gift from Dr. Muyan (28). pGEX-ER α (297–595) was provided by Dr. Corbo (29), and AIB1 was provided by Dr. Meltzer (30). FLAG-AR (31), pLEN β gal (16, 32), EREe1bLuc

(16, 32), ARE₂e1bLuc (31), and p23luc (33) plasmids had been used in previous studies.

Cell Culture—MCF-7, T47D, MDA-MB-231, and MDA-MB-361 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. BT474 and ZR75-1 cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. MCF-10A cells were maintained in DMEM/F-12 medium containing 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% FBS. For all experiments involving estrogen and androgen treatment, cells were starved in phenol red-free medium containing 3% stripped FBS for 3 days to remove steroids in the assay system, and all steroid treatments were carried out under the same steroid-free conditions.

Transfections, Fe65 Knockdown, and Reporter Assays—For reporter assays, cells were steroid-starved for 2 days in DMEM containing 3% charcoal stripped FBS, and transfections were performed with Lipofectamine 2000. One day post transfections, cells were treated with vehicle (ethanol (EtOH)) or 17β-estradiol (E2) for 48 h. Cellular extracts were prepared by directly adding lysis buffer containing 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1, 2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, and 0.2% Triton X-100. Luciferase and β-galactosidase (β-gal) activity was determined as previously described (32, 34).

For Fe65 knockdown, cells were transfected with Fe65 (siFe65) or control (siCtrl) siRNA, and 24 h post-transfection, Fe65 knockdown was verified by immunoblotting (IB). For Fe65 stable expression, MCF-7 cells were transfected with Myc-Fe65 or empty vectors, and stable clones were selected in the presence of 1 μg/ml puromycin. Individual clones were isolated, and Fe65 expression was confirmed by IB. For ERα target gene expression, transfected cells were estrogen-starved for 3 days and treated with EtOH or E2 for 2 h, and the expression of selective estrogen target genes was measured by IB and quantified by ImageJ (National Institutes of Health, rsb.info.nih.gov).

GST Pulldown Assays—pGEX-ERα(297–595) in which GST is fused with ERα ligand binding domain (29) was transformed into the BL21(DE3) *Escherichia coli* strain. Transformed bacteria were cultured at 37 °C until the optical density at 600 nm reached 0.8. The culturing was continued for 4 h in the presence of 0.5 mM isopropylthiogalactopyranoside. The bacteria were then harvested and lysed by sonication in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 6 mM MgCl₂, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. GST fusion proteins were purified using glutathione-Sepharose 4B beads (GE Healthcare). Then 50 μg of bead-bound GST-ERα were incubated overnight at 4 °C with extracts of 293T cells transfected with Fe65 constructs and washed 3 times with the lysis buffer containing 0.5% Nonidet P-40. Fe65 protein bound to the beads was released by boiling in sample buffer, resolved in an 8% SDS-polyacrylamide gel (SDS-PAGE), and detected by IB analyses.

Real Time RT-PCR—Total RNA was extracted from cells using TRIzol reagent and cDNA template synthesized from 2

μg of RNA using Oligo(dT)_{12–18} primer and Superscript II reverse transcriptase. Taqman probes (Invitrogen) were used to measure human cyclin D (assay ID number Hs00277039_m1), c-Myc (assay ID number Hs00153408_m1), and GAPDH (assay ID number Hs02758991_g1) mRNA expression. PCR reactions were performed in a 20-μl mixture containing 150 ng of cDNA, 10 μl of 2× TaqMan PCR master mixes, and 1 μl of corresponding TaqMan probes. The reactions were run as described (35) on the ABI Prism 7900 Fast Real-time PCR system in triplicate as follows: 95 °C for 10 min, 45 cycles of a 15-s denaturing at 95 °C, and 1 min annealing at 60 °C. Cyclin D and c-Myc mRNA expression levels were normalized with cognate GAPDH by subtracting the cycle threshold (Ct) value of GAPDH from Ct value of the target genes to produce a ΔCt. The -fold of induction over vehicle control was calculated based on the formula $2^{-[\Delta Ct (17\beta\text{-estradiol}) - \Delta Ct (\text{vehicle})]}$.

Subcellular Fractionation—Subcellular fractionation was carried out as previously described (36) with minor modifications. Briefly, cell pellets were resuspended in lysis buffer containing 10 mM HEPES-NaOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol and incubated on ice for 15 min. To 400 μl of the suspension solution, 12.5 μl of 10% (v/v) Nonidet P-40 was added followed by agitation for 10 s and centrifugation at 13,000 rpm for 1 min. The supernatant was collected as cytoplasmic proteins, and the pellet (crude nuclear fraction) was washed 3 times with lysis buffer containing 0.625% (v/v) Nonidet P-40 and resuspended in extraction buffer containing 10 mM HEPES-NaOH (pH 7.9), 400 mM NaCl, 1 mM EDTA, and 1 mM EGTA. The suspension was incubated on ice for 30 min with a brief vortex every 5 min followed by centrifugation for 5 min at 13,000 rpm. The supernatant was then collected as nuclear proteins.

Immunological Analyses—For immunoprecipitations (IPs), cells suspended in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 1 mM PMSF, and protease inhibitor mixture were lysed by sonication for 6 s with two repeats and incubation on ice for 10 min. After centrifugation, cellular extracts were incubated with primary antibodies overnight at 4 °C and with beads for 2 h. The beads were washed with cold lysis buffer three times and boiled in SDS-PAGE sample buffer to release the precipitates.

For IB analyses, precipitates or cellular extracts were separated in SDS-PAGE, transferred to a nitrocellulose membrane, and probed with cognate antibodies. Horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence reagents (Thermo Scientific, Waltham, MA) were used to detect proteins.

For chromatin IP (ChIP) assays, cells were plated in DMEM medium containing 10% FBS. One day after plating, the medium was changed to DMEM containing 3% charcoal stripped FBS for estrogen deprivation. For T47D cells, siRNA transfections were performed with Lipofectamine 2000 24 h after estrogen depletion. Then cells were cultured in DMEM medium containing 3% stripped FBS for another 48 h before estrogen treatment. For MCF-7 stable clones, cells were estrogen-starved for 72 h and treated with ethanol or 1×10^{-7} M 17β-estradiol for 45 min before cross-linking with 1% formaldehyde. All subsequent steps followed the company's instruc-

tions (EMD Millipore, Billerica, MA). ChIP assay primers for cathepsin D, c-Myc, and IGF-1 had been described previously (37). The primers for EBAG9 were ATGTGTCTGCCCTTCGC-CGT (forward) and TTTGGAGGCTGCGTGCTTT (reverse).

For ER α and Fe65 immunofluorescence staining and imaging, T47D cells in chamber slides were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, permeabilized for 10 min with PBS containing 0.25% Triton X-100, and incubated in PBS containing 0.2% Tween 20 and 1% BSA for 30 min to block nonspecific binding. Then fixed cells were incubated overnight at 4 °C with primary antibodies in the same buffer in a humidified chamber, washed with PBS 3 times, and then incubated with secondary antibodies for 1 h at room temperature in the dark. After three more washes, the slides were mounted in medium with DAPI, and the coverslip was sealed with nail polish. Images were observed and captured under a Leica TCS SP2 confocal microscope in the USF Imaging Core.

Fe65 Immunohistochemistry (IHC) and Breast Tissue Microarray (TMA) Analyses—Fe65 expression was assessed by IHC of breast TMA slides containing paraffin sections from 70 cases of breast invasive ductal carcinomas and 2 cases of normal and 3 cases of adjacent breast tissue with duplicate cores per case. The TMA slides were deparaffinized before the antigen retrieval procedure was performed for 30 min in 10 mM citrate (pH 6.0). Endogenous peroxidase activity was quenched by incubating the slides with 3% H₂O₂ for 20 min. Then the slides were incubated overnight at 4 °C with primary antibody against Fe65 (Santa Cruz, H-260, SC33155, 1:50 dilution) in a humidified chamber and after 3 washes with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at a 1:200 dilution for 1 h at room temperature. For detection, peroxidase-based Elite ABC (avidin/biotin complex) kit (Vector Laboratories) was used together with liquid 3,3'-diaminobenzidine, substrate and color development was monitored under the microscope. The slides were briefly counterstained in hematoxylin, dehydrated, cleared, and mounted in Permount medium (Fisher).

For automated quantitation of Fe65 IBC signals, the slides were scanned using the Aperio™ ScanScope XT system (Vista, CA) with a 200 \times /0.8 NA objective lens with a 2 \times doubler (0.265 μ m/pixel) at a rate of 10 min/slide via Basler tri-linear-array detection. Whole slide images (.svs) were loaded into Tissue Studio v3.0 (Definiens, Munich, Germany) and segmented into individual TMA cores using the TMA mapping functionality. Individual cells were identified using hematoxylin thresholding (0.2) and an IHC threshold (0.5). The typical size of the nuclei was set to be 60 μ m², and the cells were grown (cell simulation at 2 μ m) in every direction. The IHC classification was divided into negative, weak (<0.56), moderate, and strong (>0.64) staining for each cell and compiled into a summary data sheet using Microsoft Excel 2010. Percentages of positive nuclei were a sum of the percentage of the nuclei that were weak, moderate, and strong for Fe65. Statistical analyses were performed with Student's *t* test.

MTT Assays—Cells were seeded into 96-well plates at a density of 2 \times 10³ cells per well, estrogen-starved for 3 days, and treated with ethanol, 1 \times 10⁻⁸ M E2, or 10⁻⁷ M TAM for various times. MTT reagent was added to each well to give a final con-

centration of 0.5 mg/ml and incubated for 3 h. After removing the medium, 100 μ l of DMSO was added, and the absorption at 595 nm was determined with a MRX microplate reader (DYNEX Technologies, Chantilly, VA).

RESULTS

Fe65 Is a Positive ER α Transcriptional Regulator—Published studies have shown that estrogens regulate the transcriptional activity of APPct through a Fe65-ER α protein complex detected both *in vitro* and in the mouse brain (16). The complex formation makes it possible for Fe65 to regulate ER α activity. To test this idea, the ER α and Fe65 were ectopically expressed in HeLa cells, and ER α transcriptional activity was measured with the co-transfected ERE1bluc luciferase reporter gene. As shown in Fig. 1A, ectopic Fe65 expression in HeLa cells increased ER α activity induced by E2 in a dose-dependent manner. The increase was statistically significant and about half of what was induced by AIB1, a *bona fide* ER coactivator. In similar assays ectopic APPct expression did not increase ER α activity either in the absence (Fig. 1B) or presence (Fig. 1C) of Fe65. IB analyses (Fig. 1, right panels) showed that the ER α , Fe65, and APPct proteins were expressed in a manner dependent on the amounts of plasmids used in the transfections and that ER α protein expression was not significantly altered by Fe65. The data suggest that Fe65 increased ER α activity per molecule. The analyses identify Fe65 as a positive ER α regulator that stimulates ER α activity in a manner independent of its complex formation with APPct.

In similar reporter assays (Fig. 2, left panels), Fe65 exhibited little effects on the transcriptional activity of the androgen (Fig. 2B) and vitamin D (Fig. 2C) receptors. The lack of an effect on the androgen and vitamin D receptors was not due to insufficient Fe65 expression because in parallel analyses (Fig. 2A) the same ectopic Fe65 expression increased ER α activity to levels comparable with that presented in Fig. 1, whereas IB analyses revealed a comparable amount of Fe65 protein expression (Fig. 2, right panels). These analyses show that Fe65 may be an ER α -selective transcriptional coactivator.

Fe65 Binds to the ER α and Stimulates Its Transcriptional Activity through the PTB2 Domain—Previous studies defined the Fe65 binding domain to the C-terminal ligand binding domain of the ER α (16). To define the Fe65 domain mediating its interaction with the ER α , Fe65 mutants lacking defined domains were generated (Fig. 3A), and their ability to bind to the ER α was assessed in co-IP analyses (Fig. 3B) and GST pull-down assays (Fig. 3C). In cells co-transfected with FLAG-ER α with either Myc- or HA-tagged full-length Fe65, Fe65 was detected in anti-FLAG precipitates together with the ER α . No Fe65 was detected in the precipitates when cells were transfected with Myc-Fe65 and the control vector, showing that the presence of Fe65 in the FLAG precipitates accurately measured the interaction between the ER α and Fe65. Deletion of Fe65 regions containing PTB2 eliminated its ability to co-precipitate with FLAG-ER α , whereas the deletion of other regions had little effects, showing that the interaction was mediated through PTB2. This conclusion was reaffirmed by subsequent GST pull-down assays (Fig. 3C), in which GST-ER α -C terminus (GST-ER-C) containing the ligand binding domain precipitated all

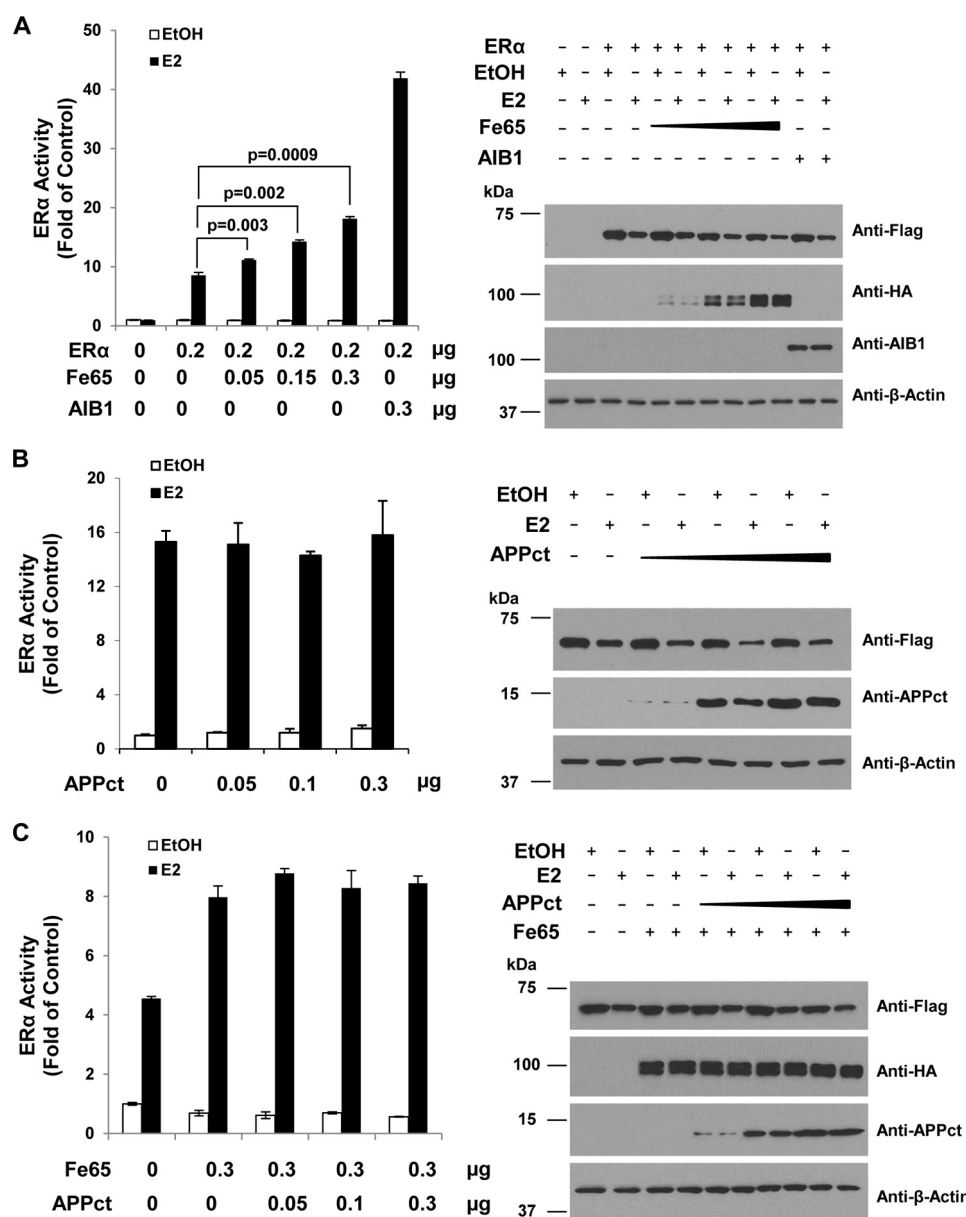


FIGURE 1. **Fe65 potentiates ER α activation by 17 β -estradiol.** *A*, HeLa cells were plated in 12-well plates and transfected with 0.1 μ g of EREe1bluc, 0.1 μ g of pLEN β gal, 0.2 μ g of FLAG-ER α , and indicated amounts of HA-Fe65 or 0.3 μ g of AIB1 and treated with vehicle (EtOH) or 10^{-8} M E2 for 48 h. Luciferase activity was determined and normalized with β -gal activity. ER α activity was calculated by dividing the normalized luciferase activity with the activity of control cells transfected with empty vector and treated with EtOH. The levels of ER α , Fe65, and AIB1 expression were determined by Western blot with indicated antibodies. *B*, HeLa cells were transfected with ER α expression and reporter vectors as in *panel A* but with the indicated amounts of APPct. Cells were treated and ER α activity, and levels of protein expression were similarly determined. *C*, HeLa cells were transfected with ER α expression and reporter vectors as in *panel A* but together with indicated amounts of Fe65 and APPct. Cells were treated, and ER α activity and levels of protein expression were similarly determined. For all reporter assays, each data point represents analyses in triplicate performed in parallel, and each experiment was repeated three times. The error bars represent S.D. Statistical analyses were performed with Student's *t* test ($n = 9$).

tested Fe65 fragments except the one lacking PTB2. Overall, the co-precipitation analyses suggest that the interaction is mediated through direct interaction between Fe65 PTB2 domain and ER α ligand binding domain.

In previous studies co-precipitation and immunofluorescence staining analyses revealed a complex formation between endogenous ER α and Fe65 in the mouse brain (16). Similar analyses were performed to test whether the complex formation occurred with endogenous proteins in BCa cells. As shown in Fig. 3D, anti-ER α , not control IgG, co-precipitated Fe65 together with the ER α in T47D cells, demonstrating that the

complex formation also occurred between endogenous proteins in BCa cells. Similarly, confocal imaging revealed the presence of Fe65 in both the cytoplasm and the nucleus of T47D cells as well as its co-localization with the ER α (Fig. 3E). In the absence of E2 the co-localization was mainly detected in the cytoplasm, particularly areas around the plasma membrane. After E2 treatment the co-localization became predominantly nuclear accompanied with ER α nuclear localization. Fe65 knockdown significantly decreased the red fluorescence signal detected by the Fe65 antibody, showing the specificity of the staining to endogenous Fe65 (Fig. 3E). The data show that the

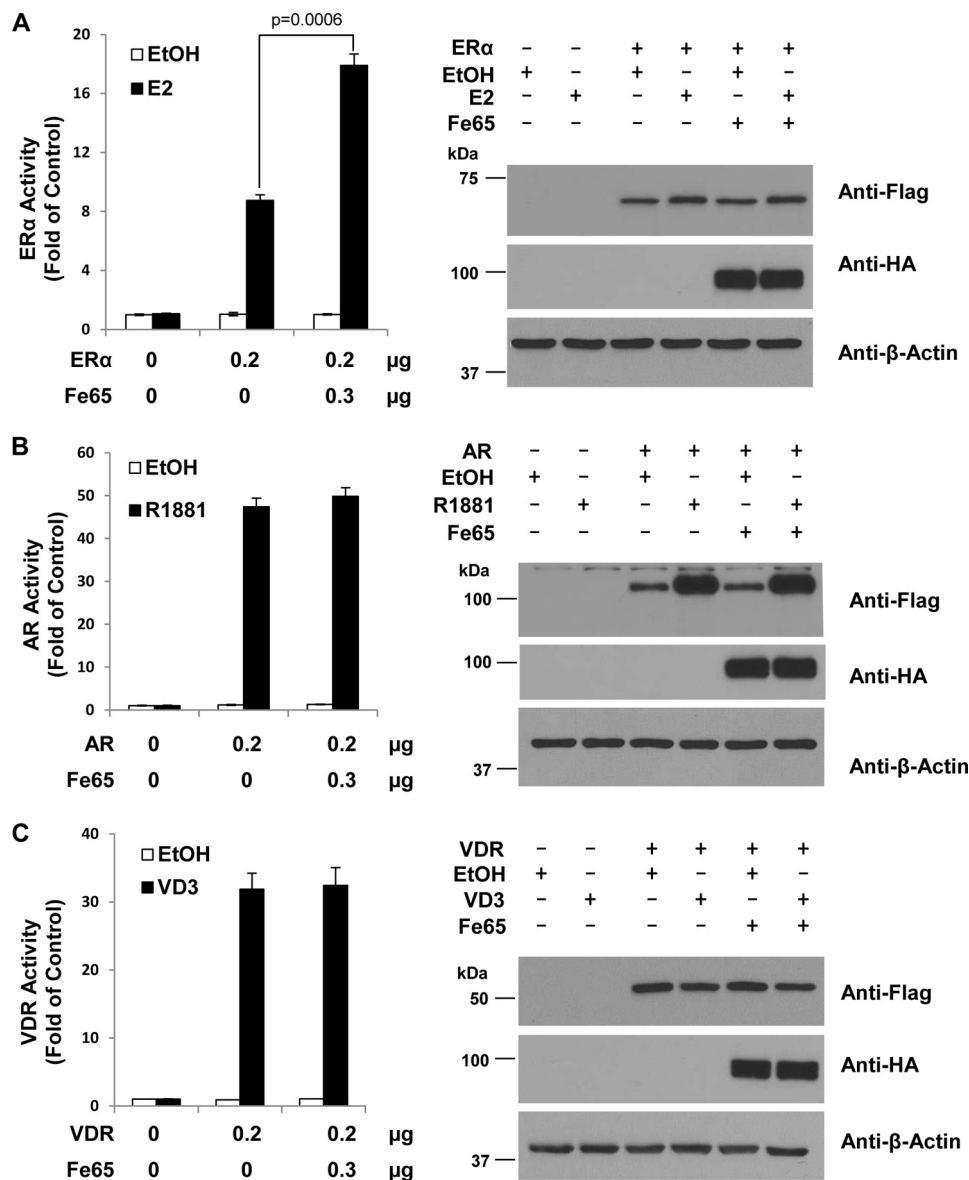


FIGURE 2. **Fe65 selectively increases ERα transcriptional activity.** A, HeLa cells were transfected with 0.1 μg EREe1bluc, 0.1 μg of pLENβgal, and indicated amounts of FLAG-ERα and HA-Fe65. Cells were treated, and ERα activity and the levels of protein expression were determined with the indicated antibodies as in Fig. 1A. B, HeLa cells were transfected with 0.1 μg of AREe1bluc, 0.1 μg of pLENβgal, and the indicated amounts of FLAG-AR and HA-Fe65. Cells were treated with EtOH or 10⁻⁸ M R1881 and AR activity, and the levels of protein expression were determined with the indicated antibodies as in Fig. 1A. C, HeLa cells were transfected with 0.1 μg of p23luc reporter, 0.1 μg of pLENβgal, and the indicated amounts of FLAG-VDR and HA-Fe65. Cells were treated with EtOH or 10⁻⁸ M 1α,25-dihydroxyvitamin D₃ (VD3), and VDR activity and levels of protein expression were determined with the indicated antibodies as in Fig. 1A.

co-localization was mainly determined by ERα localization in the cells.

Consistent with the mapping analyses, reporter assays showed that the PTB2 domain is essential for Fe65 to potentiate ERα transcriptional activation. As shown in Fig. 4A, although full-length Fe65 increased ERα activity in a dose-dependent manner, the PTB2 deletion mutant did not change ERα activity in parallel analyses. IB analyses showed that the PTB2 deletion mutant was expressed in levels comparable with full-length protein (Fig. 4B), ruling out the possibility that the lack of an effect of the PTB2 mutant was due to the insufficient protein expression. Levels of the ERα protein expression were not significantly altered by the full-length Fe65 or the PTB2 deletion mutant (Fig. 4B), showing that the reporter analyses reflected the effect of Fe65 on the specific activity of the ERα per mole-

cule. The reporter analyses together with the domain mapping allow us to conclude that Fe65 binds to the ERα and potentiates its activity through its PTB2 domain.

Fe65 Is Expressed in Mammary Epithelial Cells, and the Expression Increased in BCa Cells and Breast Tumor Tissues—The ERα plays an important role in BCa formation and mediates the mitogenic activity of estrogens in stimulating BCa growth. The interaction between the ERα and Fe65 suggests a possible role for Fe65 in estrogen actions in BCa. Consistent with this idea, Fe65 was detected in the non-tumorigenic MCF-10A human breast epithelial cells, and its expression was found to be increased in human BCa cells as well as two mouse mammary tumor cells: WT145 and VDR-KO (Fig. 5A). The increased expression was detected in both ERα-negative and positive cells with the exception of MCF-7. Subcellular frac-

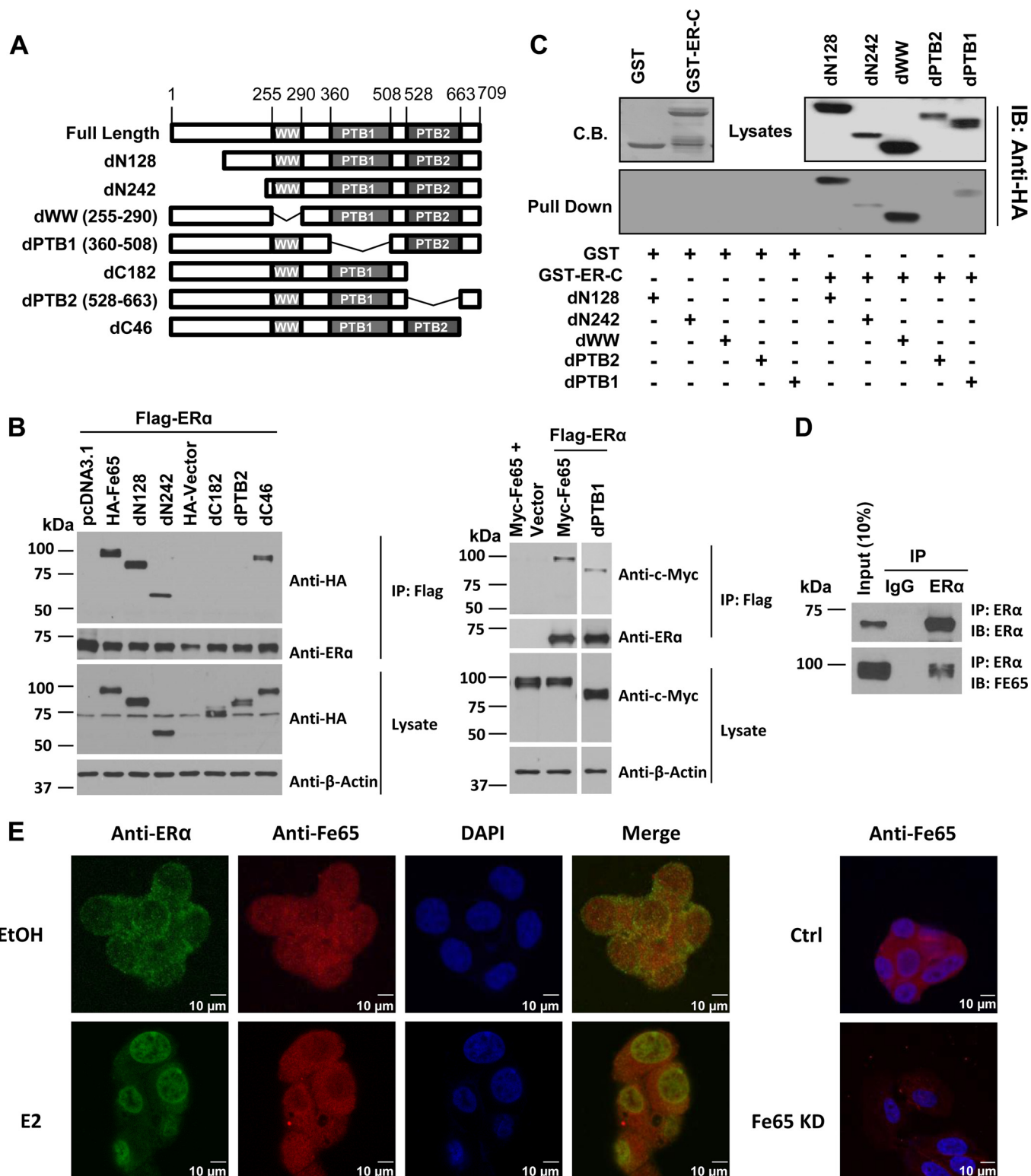


FIGURE 3. Fe65 interacts with the ER α through its PTB2 domain, and the interaction occurs with endogenous proteins in BCa cells. *A*, schematic representations of the domain structure of full-length Fe65 and its deletion mutants. Numbers of amino acids of full-length Fe65 and its domains are marked. *B*, 293T cells were transfected with 1.5 μ g of FLAG-ER α and Myc-tagged (*left panels*) or HA-tagged (*right panels*) Fe65 mutants as indicated. Cellular extracts were subjected to IP analyses followed by IB with indicated antibodies. *C*, GST and GST-ER α -C proteins produced in and purified from bacteria were incubated with lysates of cells transfected with HA-tagged Fe65 deletion mutants. GST pull-down assays were performed, and proteins in the precipitates were detected by IB with anti-HA antibody (*lower panels*). Coomassie blue (C.B.) staining was included to show that comparable amounts of GST and GST-ER-C were used in the pull-down assays. IB analyses of the lysates were also included to show the relative amounts of the Fe65 deletion mutants used in the pull-down assays. *D*, whole cell lysates of T47D cells were subjected to IP analyses with anti-ER α antibody followed by IB analyses with ER α and Fe65 antibodies as indicated. *E*, T47D cells were treated with EtOH or 10^{-7} M E2 for 1 h and subjected to DAPI (*blue*) and immunofluorescence staining with anti-ER α (*green*) and anti-Fe65 (*red*) antibodies. Images were captured under a confocal fluorescence microscope to show Fe65 and ER α location and co-localization. Images of MDA-MB-361 cells stably expressing control (*Ctrl*) or Fe65 (Fe65 KD) shRNA were included as a quality control for Fe65 staining (*right panels*).

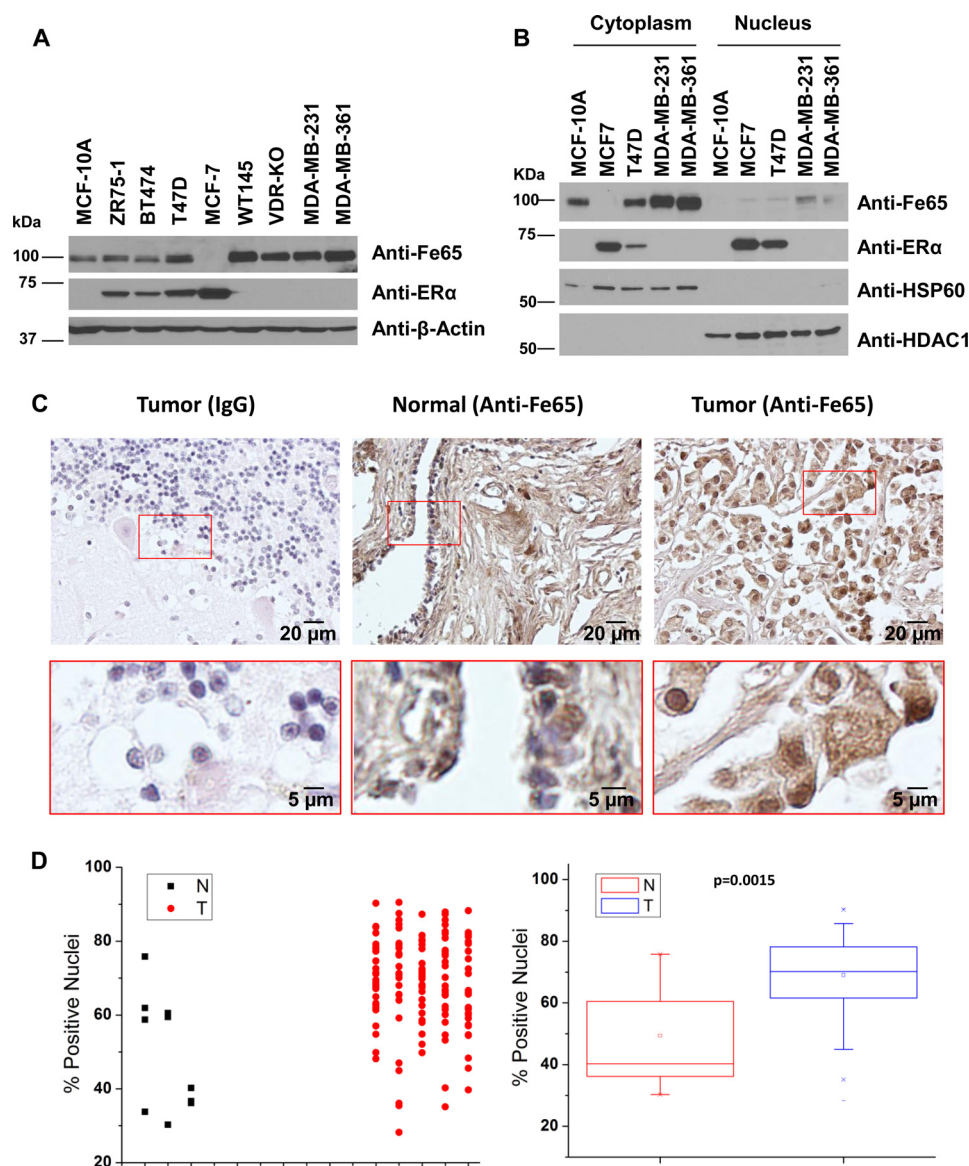


FIGURE 5. Fe65 is expressed in breast epithelial cells, and the expression is increased in BCa cells and breast tumor tissue samples. *A*, whole cell lysates of non-tumorigenic breast epithelial (MCF-10A) and BCa cells were subjected to IB analyses with the indicated antibodies. β -actin blot was included to show even loading. *B*, cytoplasmic and nuclear extracts were subjected to IB analyses with indicated antibodies. Hsp60 and HDAC1 blots were included to show the separation of cytoplasmic and nuclear extracts. *C*, representative images of normal breast and tumor tissue sections that were immunohistochemically stained with control IgG or anti-Fe65 as indicated. Pictures in the lower panels are enlarged (4 \times) versions of the marked areas of those in the top panels to show the bluish nuclei of normal ductal epithelial cells (middle panel), which indicated weak Fe65 signals, and the strong nuclear staining of breast tumor cells (right panels). *D*, Fe65 expression was assessed by IHC staining and automated quantification of TMA slides containing 140 cores of breast ductal carcinomas (T) and 10 cores of normal and adjacent breast tissues (N). The dot plot shows the distribution of nuclear Fe65 signals, whereas the tube plot compares the range and average nuclear Fe65 levels between N and T groups.

ment of the ER α and AIB1 to target gene promoters (Fig. 8B). More importantly, the ability of TAM to block E2-induced ER α and AIB1 recruitments to the promoters was significantly suppressed by Fe65 stable expression, suggesting a potential role for Fe65 in the development of TAM resistance.

The increased ER α and AIB1 recruitment to target gene promoters might be easily explained if Fe65 promoted estrogen-induced ER α nuclear localization. To test this, cellular distribution of the ER α and Fe65 was analyzed with nuclear and cytosolic extracts prepared from control and Fe65 stable clones of MCF-7 cells. As shown in Fig. 8C, the stable Fe65 expression resulted in a significant increase of its presence in both the cytosol and the nucleus but did not alter ER α localization to the

nucleus induced by E2, showing a lack of Fe65 effect on ER α nuclear localization. Overall, the data suggest that the positive Fe65 effect on ER α recruitments to target gene promoters is likely due to a role of Fe65 in facilitating the ER α to interact with chromatin.

To test whether the recruitment of Fe65 to the target gene promoters by E2 depended on the ER α , CHIP assays were performed in T47D cells transfected with control or ER α siRNA (Fig. 9). As expected, ER α siRNA decreased ER α protein expression (Fig. 9A) as well as the amounts of the ER α and AIB1 coactivator on the target gene promoters (Fig. 9B, lower panels). The receptor siRNA did not alter the overall levels of Fe65 protein in the cells (Fig. 9A) but decreased its basal and E2-induced

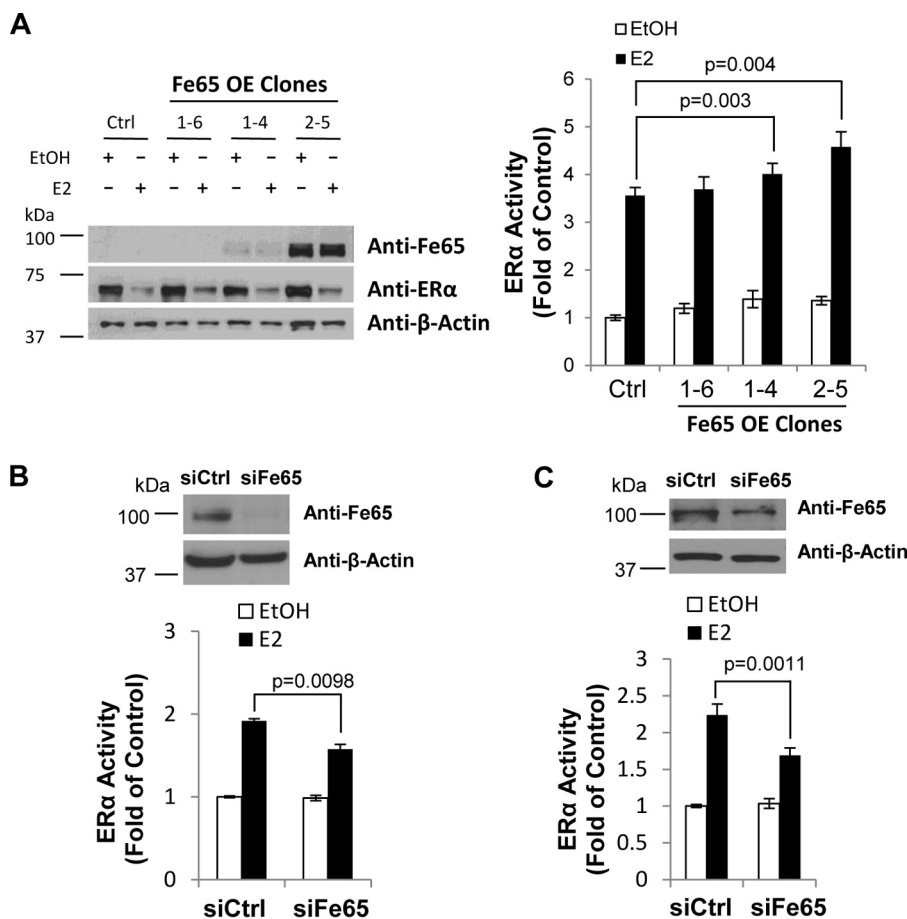


FIGURE 6. **Fe65 potentiates estrogen stimulation of endogenous ERα activity in BCa cells.** A, MCF-7 cells stably transfected with empty vector (*Ctrl*) or Fe65 (*Fe65 OE Clones*) were transiently transfected with 0.3 μg of EREe1luc and pLENβGal. B and C, T47D (*panel B*) and BT474 (*panel C*) cells were transfected with control (*siCtrl*) or Fe65 (*siFe65*) siRNA and 24 h later re-transfected with 0.3 μg of EREe1luc and pLENβGal. Transfected cells were treated with E2, and luciferase activity was determined and normalized as in Fig. 1A. Fe65 overexpression and knockdown as well as its effect on ERα protein expression were monitored by IB analyses with cognate antibodies as indicated.

recruitment to the promoters of all tested estrogen target genes (Fig. 9B, top panels). The analyses show that Fe65 recruitment to estrogen target gene promoters depends on the ERα. Overall, the ChIP analyses support the existence of a mutual positive regulation between the ERα and Fe65 on their recruitment to estrogen target gene promoters.

Fe65 Promotes Estrogen-induced BCa Cell Growth and Suppresses the Antagonistic Activity of TAM—Given the fact that ERα activity and the expression of c-Myc and cyclin D1 are often associated with cell growth in BCa cells, the positive effects of Fe65 on ERα activity and target gene expression are expected to be translated into a positive effect on BCa cell growth and its stimulation by estrogens. Indeed, our cell growth analyses showed that Fe65 knockdown significantly decreased the ability of E2 to stimulate the growth of T47D (Fig. 10A) and ZR75-1 (Fig. 10B) cells. Consistently, the ability of E2 to stimulate MCF-7 cell growth was significantly stronger in Fe65 stable clones than controls (Fig. 10C), and the increase in estrogen stimulation was positively correlated with Fe65 expression levels.

Consistent with the ChIP assay data presented in Fig. 8B, the ability of TAM to suppress E2-induced cell growth was significantly reduced by stable Fe65 expression (Fig. 10D). In the control clone, TAM suppressed E2-induced cell growth by ~81.6%,

whereas in the Fe65 overexpression clone, it was reduced to 61.7%. The data demonstrate that Fe65 promotes estrogen-induced cell growth and reduces the antiestrogenic activity of TAM in suppressing the estrogen stimulation.

DISCUSSION

Fe65 has been well studied in neuronal cells and in the pathogenesis of Alzheimer disease for its functions in mediating the trafficking and processing of APP as well as its participation in transcriptional regulation (38). Up to now there are essentially no reports about the function of Fe65 in cancer cells. The present studies are the first to clearly establish a role for Fe65 in BCa cells. Multiple lines of evidences are presented to support the novel role of Fe65 as a positive ERα regulator that potentiates estrogen stimulation of BCa cell growth. First, Fe65 bound to the ERα through the PTB2 domain and increased ERα activity in reporter assays in a PTB2-dependent manner. Second, Fe65 expression was detected in breast epithelial cells, and its expression was found to be increased in human BCa cells and breast tumor tissue samples. Third, E2 recruited Fe65 to the promoters of natural estrogen target genes. Finally, Fe65 stable expression increased and its knockdown decreased the activity of endogenous ERα in BCa cells as well as estrogen-induced target gene expression, recruitment of the ERα and coactivators to

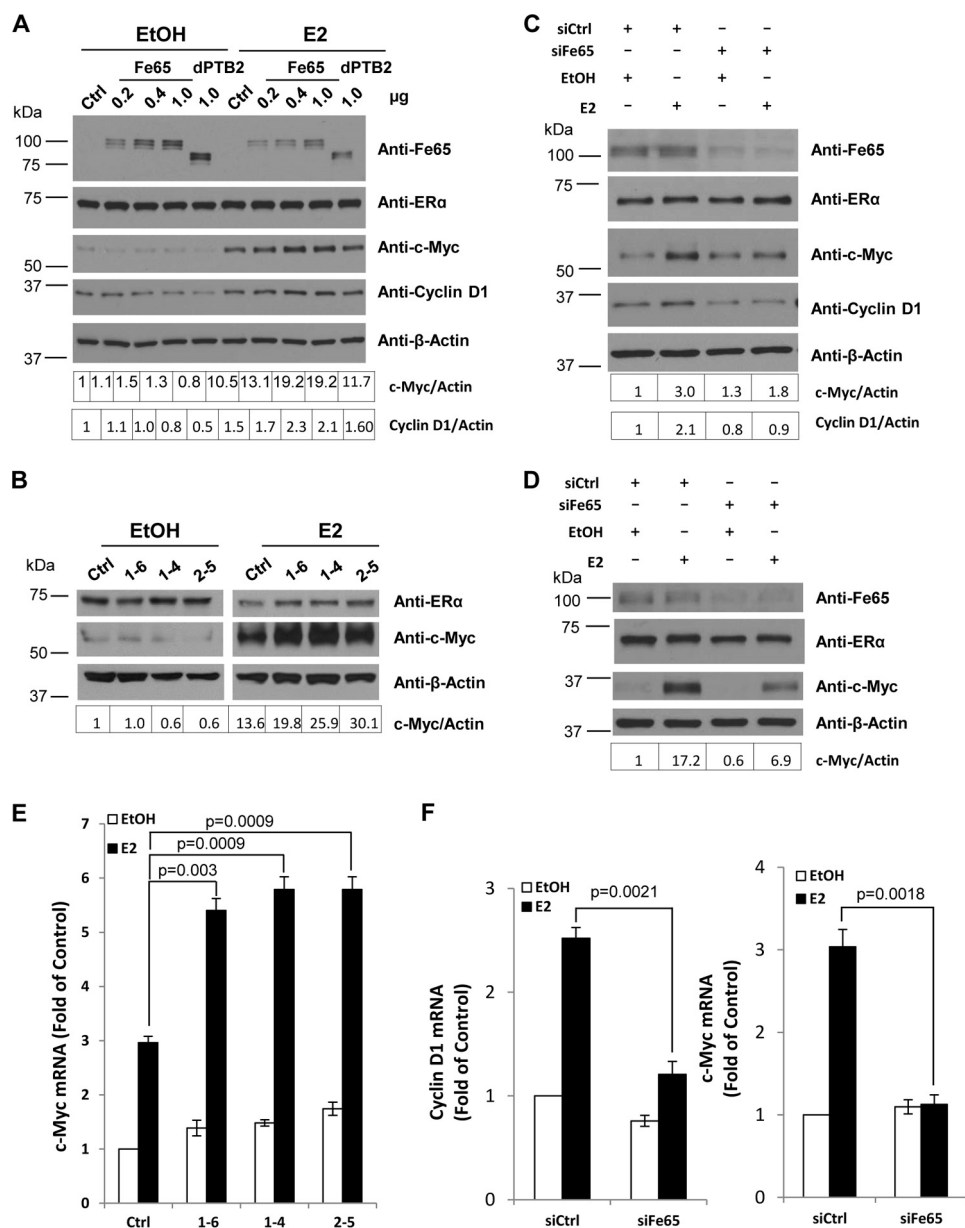


FIGURE 7. Fe65 potentiates the estrogen stimulation of ERα target gene expression in BCa cells. *A*, MCF-7 cells were transfected with empty vector (*Ctrl*), Fe65, or PTB2 deletion mutant and treated with EtOH or 10^{-8} M E2 for 2 h. Cellular extracts were subjected to IB analyses with the indicated antibodies. The expression levels of cyclin D1 and c-Myc were quantified with ImageJ and normalized with cognate β-actin signals. The normalized signals were divided by the signal of control cells that were transfected with empty vector or scrambled siRNA and treated with EtOH and are shown at the bottom of the blots. *B*, MCF-7 cells stably transfected with empty vector or Fe65 were treated, and levels of protein expression determined as in panel *A*. *C* and *D*, T47D (panel *C*) and BT474 (panel *D*) cells transiently transfected with siCtrl or siFe65 were treated, and levels of protein expression determined as in panel *A*. *E*, MCF-7 cells stably transfected with empty vector or Fe65 were treated as in panel *A* but for 1 h. Total RNA was extracted and subjected to real time RT-PCR analyses. The levels of c-Myc mRNA were normalized with GAPDH and are expressed as -fold of the EtOH control. *F*, T47D cells transiently transfected with siCtrl or siFe65 were treated as in panel *A* but for 1 h. Total RNA was extracted and subjected to real time RT-PCR. The levels of cyclin D1 and c-Myc mRNA were normalized with GAPDH and are expressed as -fold of the EtOH controls.

target gene promoters, and cell growth. Together with studies published previously (16), the present studies extend Fe65 actions from neuronal to BCa cells as presented in Fig. 10E. In neuronal cells, Fe65 brings the ERα to APPct transcriptional complex, allowing estrogens to protect neuronal cells from apoptosis induced by toxicity associated with APP cleavage. In BCa cells, Fe65 binds to the ERα and potentiates its chromatin binding at target gene promoters, which permits Fe65 to act as a stimulator of BCa cell growth. Although it remains to be determined, the increased Fe65 expression in breast tumor tis-

sue samples suggests that the findings about Fe65 in BCa cell lines will most likely reflect an *in vivo* role for this neuronal adaptor in breast tumorigenesis.

Tip60 is a histone acetyl transferase that binds to PTB1 domain of Fe65 (13). It was also reported to be a coactivator for the ERα (39). It is a legitimate question whether the positive effect of Fe65 on the ERα involves Tip60. Our studies have yielded conflicting data regarding the role of Tip60 in ERα activation by Fe65. In ChIP assays, estrogens recruited Tip60 to ERα target gene promoters, which were enhanced by Fe65

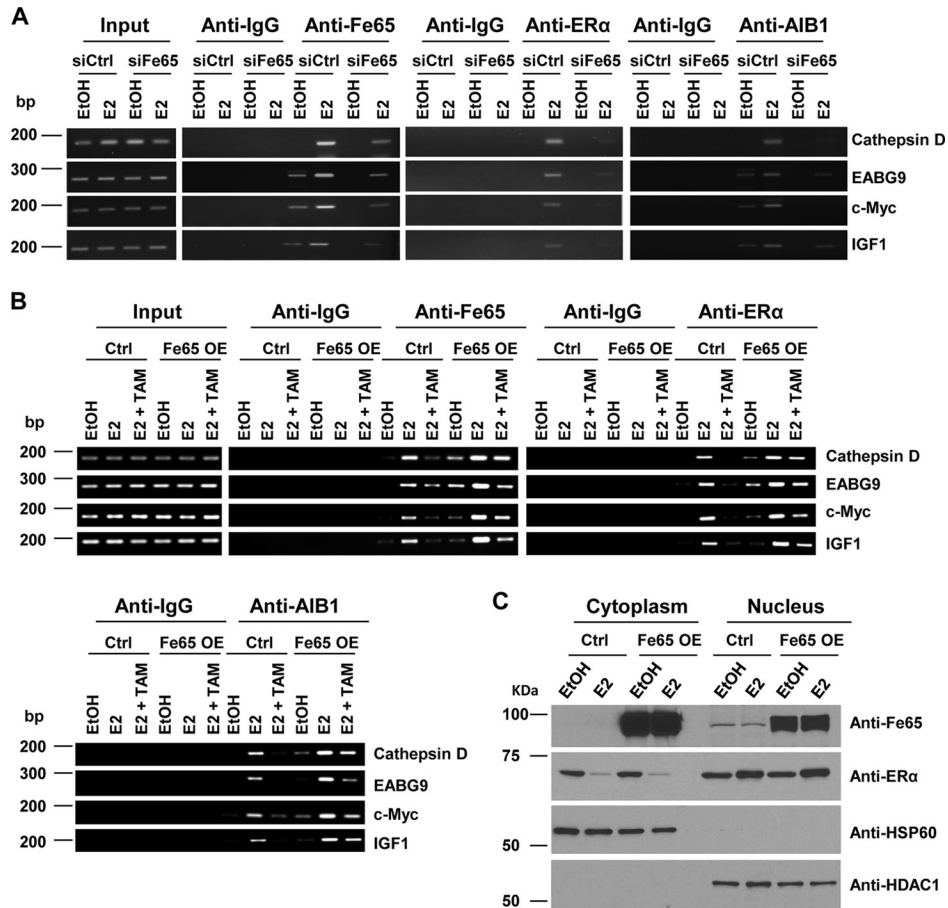


FIGURE 8. **Fe65 is recruited to ERα target gene promoters by estrogens and required for optimal recruitment of the ERα and its coactivators.** A, T47D cells were transfected with siCtrl or siFe65 and treated with EtOH or 10^{-7} M E2 for 45 min. ChIP assays were performed with the indicated antibodies. B, MCF-7 cells stably transfected with empty vector or Fe65 were treated with EtOH, 10^{-7} M E2, or 10^{-7} M E2 plus 10^{-7} M TAM (E2 + TAM) for 45 min. ChIP assays were performed with the indicated antibodies. C, MCF-7 cells stably transfected with empty vector or Fe65 were treated with EtOH or 10^{-8} M E2 for 1 h. Cytoplasm and nucleus proteins were subjected to IB analyses with the indicated antibodies. Hsp60 and HDAC1 blots were included to show the separation of cytoplasmic and nuclear extracts.

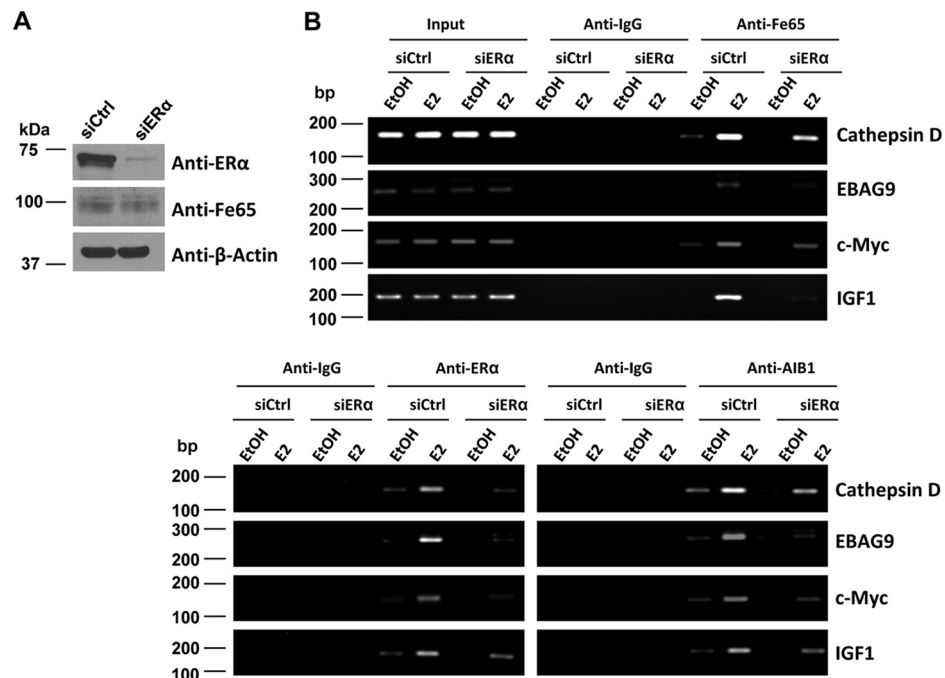


FIGURE 9. **Fe65 recruitment to estrogen target gene promoters is ERα-dependent.** A, T47D cells were transfected with siCtrl or siERα. Cellular extracts were subjected to IB analyses with indicated antibodies. B, T47D cells were transfected with siCtrl or siERα and treated with EtOH or 10^{-7} M E2 for 45 min. ChIP assays were performed with indicated antibodies.

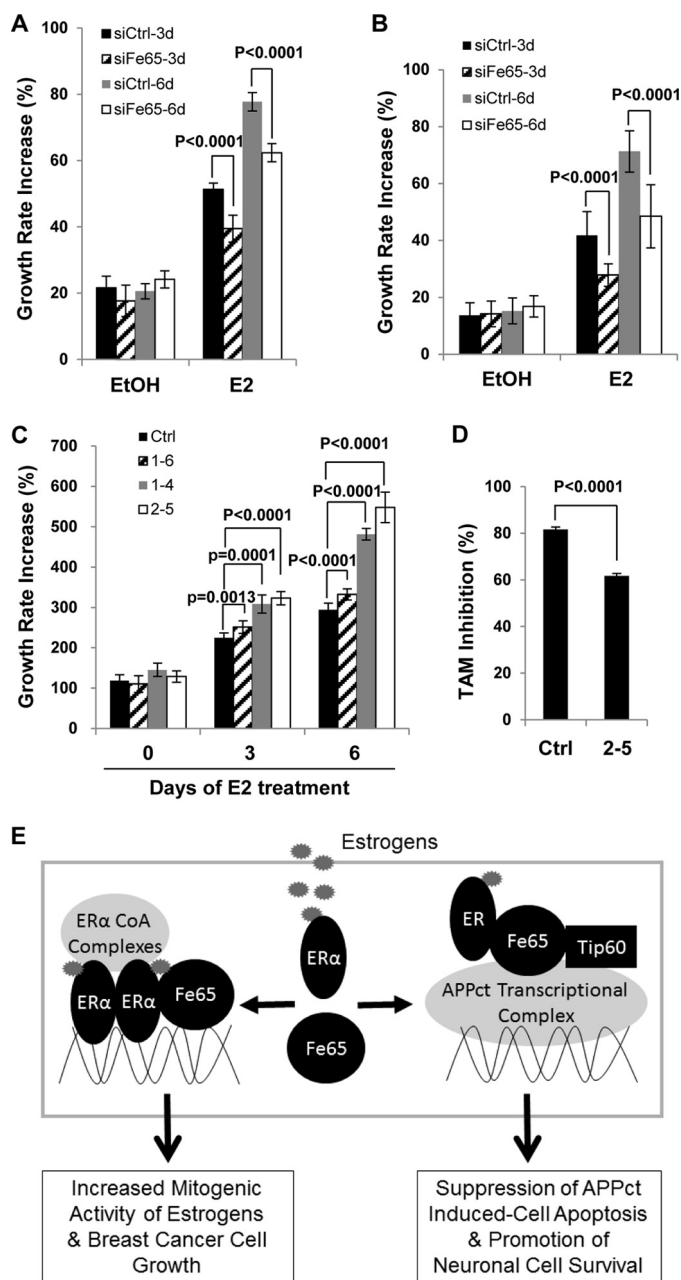


FIGURE 10. Fe65 promotes estrogen-induced BCa cell growth and suppresses the antagonistic activity of TAM. T47D (A) and ZR75-1 (B) cells were estrogen-starved and transfected with siCtrl or siFe65 and 48 h later re-transfected for another time. 24 h post the second transfection, the cells were plated in 96-well plates and treated with EtOH or 10^{-8} M E2 for 3 and 6 days. Cell growth was determined in MTT assays. The increase in cell growth rate was calculated by subtracting from and dividing A_{595} values with A_{595} of control cells (time zero). C, control MCF-7 and Fe65 OE clone (2-5) were estrogen-starved for 72 h and treated with 10^{-8} M E2 for 0, 3, and 6 days. E2 treatment was started at different times to allow the growth of all treated cells being measured in MTT assays at the same time. For example, for 0 day treatment, the cells were treated with EtOH for 6 days. The increase in cell growth rate was calculated as in panels A and B. D, control MCF-7 and Fe65 OE clone (2-5) were estrogen-starved for 72 h and treated with either EtOH, 10^{-7} M E2 or 10^{-7} M E2 plus 1μ M TAM for 3 days. Cell growth was determined in MTT assays. Percentages of TAM inhibition were calculated by subtracting from the E2-induced increase in growth rate (over day 0) with that induced by E2 plus TAM followed by division with E2-induced increase in growth rate. For all growth analyses, each data point represents 18 samples analyzed in parallel. Each experiment was repeated three times. *p* values were calculated by Student's *t* test. The error bars in all growth analyses represent the S.D. E, a model describing the role of Fe65 in estrogen actions in neurons and BCa cells. See the first paragraph of "Discussion" for details.

overexpression (data not shown). The data are consistent with the fact that Tip60 is a common interacting protein for Fe65 and ER α . However, in reporter gene analyses, ectopic expression of Tip60, instead of synergizing with Fe65 in stimulating ER α activity as it would be expected for a coactivator, actually decreased the ability of Fe65 to activate the ER α (data not shown). The reporter data are consistent with the fact that APPct did not increase ER α activity or potentiate the positive effect of Fe65 on the ER α (Fig. 1). Tip60 has been shown to be a tumor suppressor of which the expression, particularly the expression in the nucleus, is decreased in breast tumors (40). Interestingly, it has been shown that estrogen-induced c-Myc and cyclin D1 mRNA expression was not affected by Tip60 depletion in MCF-7 cells (41) even though several non-growth related ER α target genes were altered, suggesting a selective involvement of Tip60 in ER α action in BCa cells.

Our data suggest that APPct is not involved in ER α activation by Fe65 (Fig. 1). Questions remain of what signals regulate Fe65 nuclear localization in BCa cells and its positive effect on the ER α . Under overexpression conditions, Fe65-ER α complex formation appeared to be constitutive and not altered by estrogen treatments (data not shown). However, CHIP assays revealed that Fe65 binding to estrogen target gene promoters was induced by E2 (Figs. 8 and 9). The analyses suggest that estrogens are part of the signals operating in BCa cells to regulate the actions of Fe65-ER α complex in the nucleus. Fe65 overexpression increased both cytoplasmic and nuclear Fe65 levels in MCF-7 cells (Fig. 8C), showing that a simple way of promoting nuclear Fe65-ER α activity is to increase overall Fe65 expression, which naturally occurs in breast tumors as suggested by data in Fig. 5, C and D. In addition to APP cleavage, Fe65 phosphorylation had been shown to induce Fe65 nuclear localization (42). Fe65 has also been reported to interact with the C terminus of Notch1 (43). It would be interesting to find out whether these signaling pathways known to operate in BCa cells regulate ER α activation through Fe65.

Although the present studies have focused on the effect of Fe65 on ER α actions, it is important to point out that Fe65 may have a much broad impact on BCa biology beyond estrogen stimulation of cell growth. For example, Fe65 has been described to be required for efficient repair of DNA double-strand breaks, a function that depends on its interaction with Tip60 and APPct (26, 27). There are also reports showing that the phosphorylated H2A.X is higher in Fe65 null cells than in the wild type under genotoxic damages (25) and that the phosphorylation somehow depends on Fe65 accumulation in the nucleus (44-46). Thus, through Fe65-ER α complex formation, estrogens may regulate DNA damage repair in breast epithelial cells. Furthermore, the WW domain of Fe65 is known to bind to Mena (21), through which it regulates the actin cytoskeleton, cell motility, and neuronal growth cone formation (22, 23). Fe65 thus may also control BCa invasion through Mena and allow estrogens to regulate BCa invasion through the Fe65-ER α complex. In this regard it is important to point out that significant amounts of Fe65 protein were detected in the cytoplasm of BCa cells and that Mena splice isoforms regulate BCa invasion (47), which is apparently a cytoplasmic activity mediated through actin filaments.

In summary, the present studies have extended the functions of the Fe65 neuronal adaptor protein to BCa cells and defined a positive role for Fe65 in estrogen actions and BCa cell growth. The existence of Fe65-ER α complex in BCa cells increases the complexity of the mechanisms underlying estrogen actions in BCa cells. For the reason that Fe65 is a multidomain protein that interacts with a variety of proteins with diverse functions, its interaction with the ER α may change the receptor activity both quantitatively and qualitatively by allowing more complex regulation patterns. Our finding that Fe65 expression suppressed the antiestrogenic activity of TAM implicates that Fe65 may be a new molecular target for BCa intervention and that targeted disruption of the ER α -Fe65 interaction may represent a new strategy to overcome TAM resistance.

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