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CITED2 modulates estrogen receptor transcriptional activity in breast cancer cells

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ABSTRACT

Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2) is a member of the CITED family of non-DNA binding transcriptional co-activators of the p300/CBP-mediated transcription complex. Previously, we identified CITED2 as being overexpressed in human breast tumors relative to normal mammary epithelium. Upon further investigation within the estrogen receptor (ER)-positive subset of these breast tumor samples, we found that CITED2 mRNA expression was elevated in those associated with poor survival. In light of this observation, we investigated the effect of elevated CITED2 levels on ER function. While ectopic overexpression of CITED2 in three ER-positive breast cancer cell lines (MCF-7, T47D, and CAMA-1) did not alter cell proliferation in complete media, growth was markedly enhanced in the absence of exogenous estrogen. Correspondingly, cells overexpressing CITED2 demonstrated reduced sensitivity to the growth inhibitory effects of the selective estrogen receptor modulator, 4-hydroxytamoxifen. Subsequent studies revealed that basal ER transcriptional activity was elevated in CITED2-overexpressing cells and was further increased upon the addition of estrogen. Similarly, basal and estrogen-induced expression of the ER-regulated genes trefoil factor 1 (TFF1) and progesterone receptor (PGR) was higher in cells overexpressing CITED2. Concordant with this observation, ChIP analysis revealed higher basal levels of CITED2 localized to the TFF-1 and PGR promoters in cells with ectopic overexpression of CITED2, and these levels were elevated further in response to estrogen stimulation. Taken together, these data indicate that CITED2 functions as a transcriptional co-activator of ER in breast cancer cells and that its increased expression in tumors may result in estrogen-independent ER activation, thereby reducing estrogen dependence and response to anti-estrogen therapy.

1. Introduction

CITED2 is a non-DNA binding transcriptional co-regulator, known to modulate the activity of transcription factors including p300/CBP [1], transcription factor AP2 (TFAP2) [2], LIM homeobox 2 (Lhx2) [3], peroxisome proliferator-activated receptor-α (PPARα) [4], and Smad2/3 [5]. In addition, evidence documenting the potential of CITED family members to function as co-activators of the estrogen receptor (ER) has been reported [6]. While CITED2 is perhaps best known for its role in development [7–10], it has also been reported to play a role in cancer. In addition to studies in the areas of skin [11], colon [12], and lung [13] cancers, we previously identified CITED2 as being overexpressed in breast cancer relative to normal mammary epithelium, and provided evidence of a potential role for CITED2 in the establishment of breast-bone metastasis [14].

Approximately 75% of breast cancers diagnosed are ER-positive and are thus candidates for treatment with hormone therapy. The two classes of anti-estrogen therapy include selective estrogen receptor modulators (*e.g. Tamoxifen*), which block the interaction of estrogen with ER [15], and aromatase inhibitors (*e.g. Letrozole*), which block the production of estrogen [16]. Despite the documented benefits of anti-estrogen therapy, some patients fail to respond from the outset and many of those that are initially responsive develop resistance over time [17]. Although the precise molecular mechanisms responsible for anti-estrogen resistance are currently unclear, evidence supporting several contributing factors has been described such as loss of ER expression [18,19], alterations in signal transduction [20], and modified levels of ER transcriptional co-regulators [21]. Here, we provide evidence that CITED2 functions as an ER co-activator in breast cancer cells and that overexpression of CITED2 enhances transcriptional activity of ER in a ligand-independent fashion. These data indicate that CI-

Abbreviations: CITED2, Cbp/p300-interacting transactivator with Glu/Asp-rich carboxyterminal domain 2; ER, estrogen receptor; TFF1, trefoil factor 1; PGR, progesterone receptor; TFAP2, transcription factor AP2; Lhx2, LIM homeobox 2; PPARα, peroxisome proliferator-activated receptor-α.

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TED2 overexpression in breast cancer could promote tumor progression and may contribute to the development of resistance to anti-estrogen therapy.

2. Materials and methods

2.1. Cell lines and tissues

The breast cancer cell lines MCF-7, T47D, and CAMA-1 were obtained from American Type Culture Collection (Rockville, MD) and cultured according to conditions specified. CITED2 cDNA was cloned into the retroviral expression vector, pBabe-puro (Addgene plasmid 1764). Breast cancer cell lines were infected with pBabe-puro or pBabe-puro-CITED2 and stable pools were selected in the presence of 1 μg/ml puromycin (Sigma–Aldrich) for one week. For experiments assessing the effect of estrogen withdrawl/stimulation, cells were cultured in phenol-red free media containing 5% charcoal-stripped serum (CSS) for 48 h and subsequently treated with or without 1 nM 17β-estradiol (Sigma–Aldrich). For experiments examining the effect of ER inhibition, cells were cultured in complete media and subsequently treated with or without 1 μM tamoxifen (Sigma–Aldrich). Normal mammary epithelium samples, kindly provided by Dr. Saraswati Sukumar (Johns Hopkins University School of Medicine, Baltimore, MD), were prepared from reduction mammoplasty specimens of women with no breast abnormalities. Normal and tumor tissues were obtained from the Surgical Pathology Division of the Johns Hopkins Hospital following the approval of the institutional review board (IRB) of the Johns Hopkins University School of Medicine. For all specimens, required written informed patient consents were obtained as approved by the IRB.

2.2. Quantitative (q)RT-PCR

Total RNA was extracted using Trizol (Invitrogen) and cDNA was generated by reverse transcription. 25 μl reactions contained 1× SYBR Green Reaction Mix (Applied Biosystems), 1 μl cDNA, and 100 nm of each primer: CITED2 (sense) 5′-ACCATCACCCTGCCCACC-3′, (antisense) 5′-CGTAGTGTATGTGCTCGCCCA-3′; TFF1 (sense) 5′-CATGGAGAACAAGGTGATC-3′, (antisense) 5′-ATTAGGATAGAAGCACCAGG-3′; PGR (sense) 5′-GAACCAGATGTGATCTATGC-3′ (anti-sense) 5′-AGCTTGACAAACTCCTGTGG-3′; GAPDH (sense) 5′-GTCAGTGGTGGACCTGACCT-3′, (antisense) 5′-TGCTGTAGCCAAATTCGTTG-3′. qRT-PCR parameters were: 1 cycle (95 °C for 3 min) and 40 cycles (95 °C for 30 s, 61.9 °C for 30 s, and 72 °C for 45 s). Amplification of GAPDH was used as an internal control. Relative expression between samples was calculated by the comparative C_T method.

2.3. Western analysis

Total protein was extracted from cell lines using lysis buffer consisting of 15% glycerol, 5% SDS and 250 mM Tris–HCl, pH 6.7. Equal amounts of protein were resolved using 10% SDS–PAGE. Protein was transferred to ECL nitrocellulose membranes (Amersham) and probed with anti-CITED2 (Novus Biologicals) and β-actin (Sigma–Aldrich) antibody. Membranes were then incubated with horseradish peroxidase-conjugated antibody against rabbit IgG (Amersham) and binding was revealed by chemiluminescence (Amersham).

2.4. Proliferation assay

Cell Proliferation was determined by direct cell count or MTS assay. For direct cell count, cells were incubated in a 6-well plate. At the desired time points, cells were trypsinized and counted using a hemocytometer. Cell viability was determined by trypan blue dye (0.4%) exclusion. For MTS assay, cells were incubated in a 96-well plate. At the desired time points, MTS (0.2 mg/ml) (Promega) and phenazine ethosulfate (30 μM) (Sigma–Aldrich) were added to each well and plates were incubated at 37 °C for 2 h. The conversion of MTS to formazan by metabolically viable cells was monitored using a 96-well microtiter plate reader at 490 nm (Bio-Rad).

2.5. Reporter assay

Cells were cultured in phenol-red free media containing 5% CSS and transiently transfected (TransIT-LT1, Mirus Bio Corporation) with an ERE-luciferase reporter construct along with the renilla luciferase reporter phRL-CMV (Promega) for the determination of transfection efficiency. Twenty-four hours following transfection, cells were treated with or without 1 nM 17β-estradiol and reporter activity was determined by luciferase assay per manufacturer instructions (Promega).

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using the SimpleChIP Kit (Cell Signaling Technology, Inc.) per manufacturer instructions. DNA/protein complexes were immunoprecipitated using antibodies against CITED2 (Novus Biologicals) while provided anti-Histone H3 and normal rabbit IgG antibodies were used as positive and negative controls, respectively. For PCR amplification of TFF1 and PGR promoter regions, the following primer sequences were utilized: TFF1 (sense) 5′-CACCCCGTGAGCCACTGT-3′, (antisense) 5′-CTGCAGAAGTGAT TCATAGTGAGAGAT-3′; PGR (sense) 5′-CCATCCCAAAGAACCTGC-

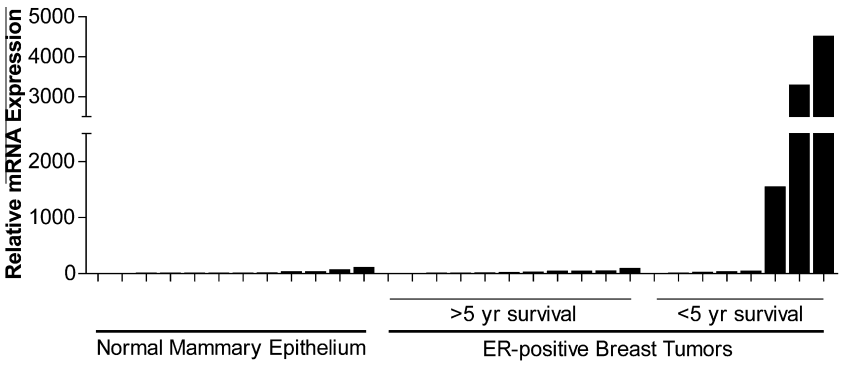

Fig. 1. Expression of CITED2 in ER-positive breast tumors. CITED2 mRNA expression was determined by qRT-PCR in human normal mammary epithelium and ER-positive breast tumor samples from patients surviving greater than ($n = 11$) and less than ($n = 8$) 5 years from the time of diagnosis. Data are representative of three independent experiments performed in triplicate.

TA-3′, (anti-sense) 5′-ACACGCGCAAATACAACAAG-3′. PCR products were quantified by densitometry.

2.7. Statistical analysis

Differences in cell proliferation, transcriptional activity, and PCR amplification between each experimental group were compared by unpaired Student's *t*-test. *p*-values below 0.05 were considered significant. For all figures, (*) denotes $p < 0.05$, (**) denotes $p < 0.01$, and (***) denotes $p < 0.001$.

3. Results

3.1. Overexpression of CITED2 promotes estrogen-independent cell growth

In a previous study, we presented evidence that the transcriptional co-regulator CITED2 was overexpressed in human breast tumors relative to normal mammary epithelium [14]. Considering the potential role of the CITED family as co-activators of ER [6], we next investigated the expression pattern of CITED2 within the ER-positive subset of these breast tumors. By qRT-PCR analysis, CITED2 mRNA expression was significantly ($p < 0.05$) increased in 37.5% (3/8) of tumors from patients surviving less than 5 years from the time of diagnosis relative to that observed in normal mammary epithelium, while no increase was observed (0/11) in tumors from those surviving greater than 5 years (Fig. 1). This finding indicates a potential association between CITED2 expression levels and survival within the ER-positive patient subgroup. Given this observation, we subsequently examined whether overexpression of CITED2 affects ER-mediated cell growth. To accomplish this task, we first generated ER-positive breast cancer cells that stably express elevated levels of CITED2 or vector alone (EV) using the human breast cancer cell lines MCF-7, T47D, and CAMA-1 (Fig. 2A). Next, we assessed the estrogen-induced growth response in these cell lines by MTS assay (Fig. 2B–E). While ectopic overexpression of CITED2 did not alter cell proliferation when cultured in complete media (Fig. 2B) or in estrogen-depleted media supplemented with exogenous estrogen (data not shown), cell growth was significantly enhanced when cultured in estrogen-depleted media alone (Fig. 2C–E). Correspondingly, cells overexpressing CITED2 were

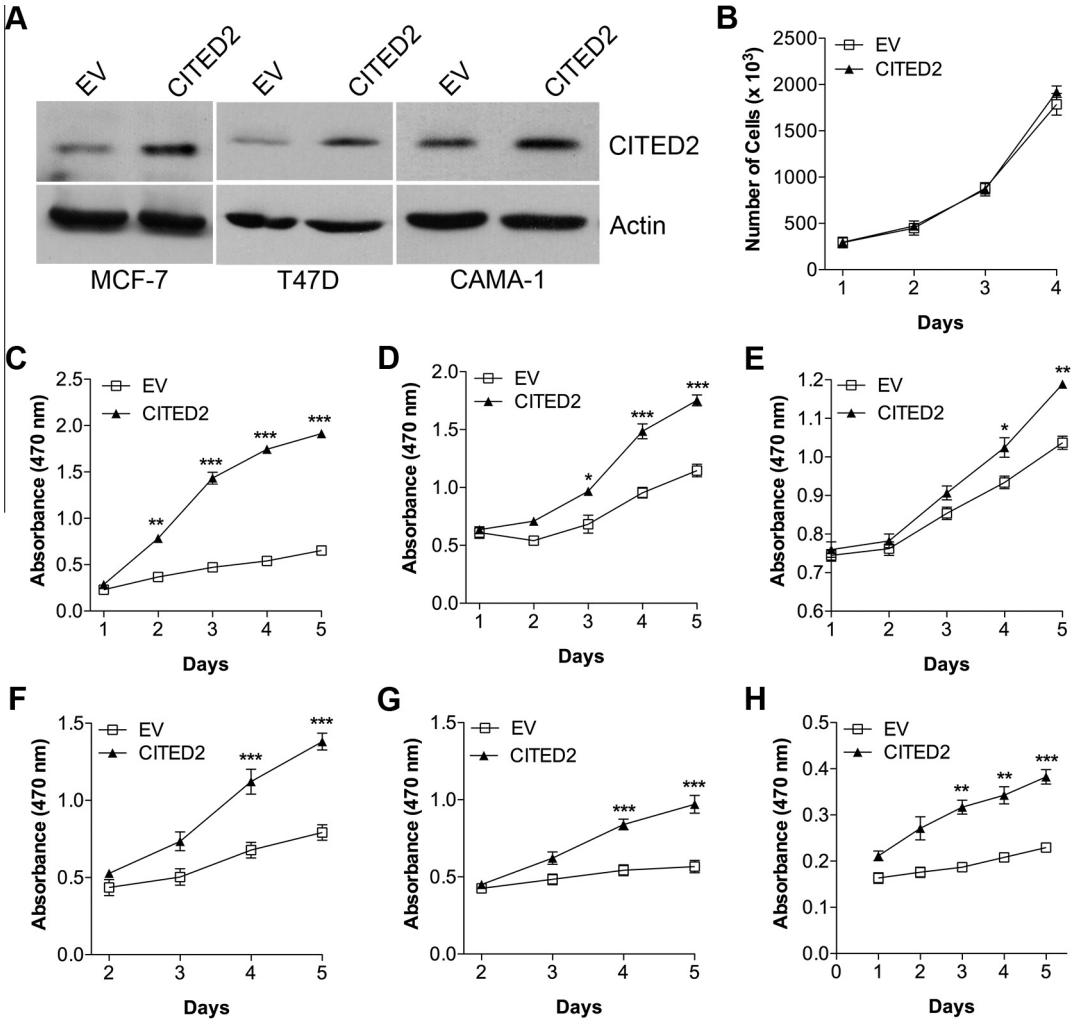

Fig. 2. Effect of CITED2 on breast cancer cell proliferation. (A) ER-positive breast cancer cell lines stably overexpressing CITED2 or vector alone (EV) were generated. Protein expression was determined by Western analysis performed on equal amounts of protein from total cell lysates. (B) Cells were cultured in complete media and cell proliferation was determined by direct cell count. Results for MCF-7 are shown. Similar results were obtained for T47D and CAMA-1 cell lines. (C–E) MCF-7 (C), T47D (D), and CAMA-1 (E) cells were cultured in phenol-red free media supplemented with 5% CSS and cell proliferation was determined by MTS assay. (F–H) MCF-7 (F), T47D (G), and CAMA-1 (H) cells were cultured in complete media and subsequently treated with tamoxifen (1 μM). Cell proliferation was determined by MTS assay. Data are representative of three independent experiments performed in triplicate and expressed as the mean ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

significantly less sensitive to the growth inhibitory effects of the selective estrogen receptor modulator 4-hydroxytamoxifen (tamoxifen), as compared to EV cells (Fig. 2F–H). Taken together, these findings suggest that while overexpression of CITED2 did not affect estrogen-dependent cell growth, it may contribute to estrogen-independence.

3.2. Overexpression of CITED2 induces ER transcriptional activity and expression of ER target genes in the absence of estrogen

Considering ER co-activators such as SRC-1 have been reported to induce ligand-independent receptor activity [21], we next investigated the effect of CITED2 overexpression on ER transcriptional activity. Cells with stable overexpression of CITED2 or vector alone were transiently transfected with an estrogen response element (ERE)-luciferase reporter construct and transcriptional activity was assessed in the absence and presence of exogenous estrogen by luciferase assay. Interestingly, cells overexpressing CITED2 demonstrated a two to fourfold increase in ER transcriptional activity in the absence of exogenous estrogen relative to EV cells, which was further increased upon estrogen addition (Fig. 3A–C). To determine whether this observed increase in ER transcriptional activity translated to gene expression, we next examined the mRNA expression of several ER-regulated genes. Cells overexpressing CITED2 or vector alone were cultured in the absence or presence of exogenous estrogen and mRNA expression was assessed by qRT-PCR. Corresponding with the increase in ER transcriptional activity, mRNA levels of the ER-regulated genes TFF1 and PGR were elevated in CITED2 overexpressing cells relative to EV cells in the absence of estrogen, and were further increased upon estrogen stimulation (Fig. 3D–I). To examine whether CITED2 was acting at the promoter of these genes we conducted ChIP analysis. Consistent with the ligand-independent increase in TFF1 and PGR mRNA expression observed, elevated levels of CITED2 were detected at both the TFF1 and PGR promoters in cells overexpressing CITED2 in the absence of estrogen (Fig. 4). In addition, localization of CITED2 to these promoters was induced in both cells overexpressing CITED2 and EV following estrogen stimulation.

4. Discussion

In summary, upon overexpression of CITED2 in three independent breast cancer cell lines we observed increased activation of an ERE-driven promoter, elevated ER target gene expression, and

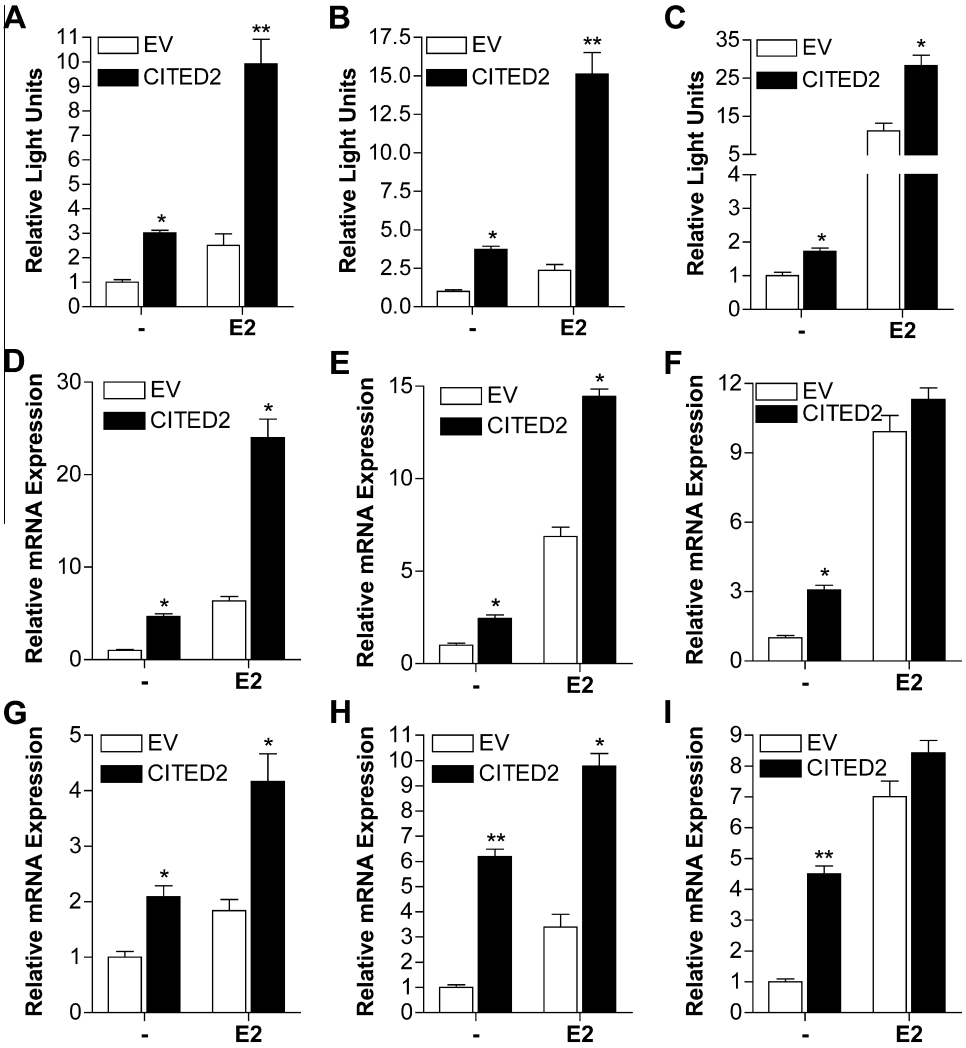

Fig. 3. Effect of CITED2 on ER transcriptional activity and target gene expression. For ER transcriptional activity: MCF-7 (A), T47D (B), and CAMA-1 (C) cells were cultured in phenol-red free media containing 5% CSS, transfected with ERE-luciferase and control reporter constructs, and treated with or without 1 nM estrogen (E2). Transcriptional activity was determined by luciferase assay. For ER target gene expression: MCF-7 (D, G), T47D (E, H), and CAMA-1 (F, I) cells were cultured in phenol-red free media containing 5% CSS and subsequently treated with or without 1 nM estrogen (E2). TFF1 (D–F) and PGR (G–I) mRNA expression was determined by qRT-PCR. Data are representative of three independent experiments performed in triplicate and expressed as the mean ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$.

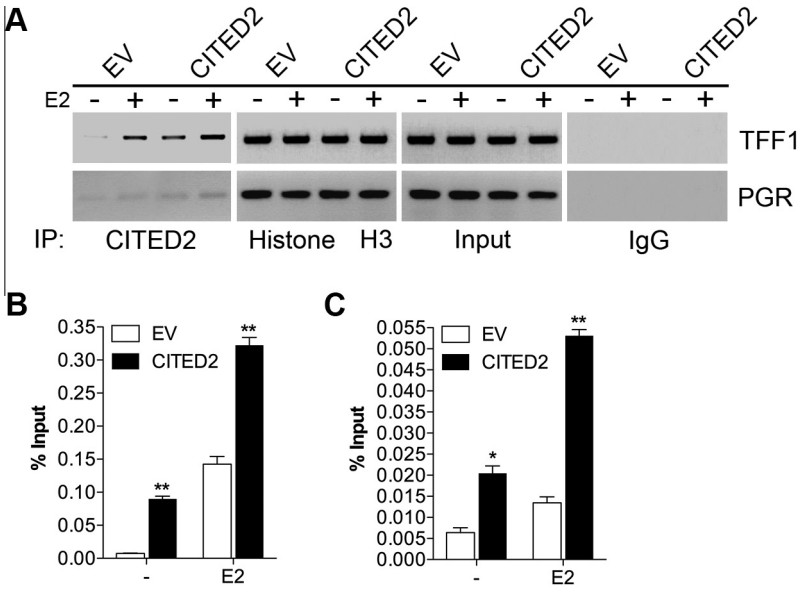

Fig. 4. Localization of CITED2 to ER target gene promoters. T47D cells were cultured in phenol-red free media containing 5% CSS and subsequently treated with or without 1 nM estrogen (E2). Localization of CITED2 to the TFF1 and PGR promoters was determined by ChIP assay using anti-CITED2 antibody. PCR amplification (A) was quantified by densitometry for TFF1 (B) and PGR (C), and reported as a percentage of input chromatin. Data are representative of two independent experiments performed in duplicate and expressed as the mean ± s.e.m. $^{*}p < 0.05$, $^{**}p < 0.01$.

localization of CITED2 to ER target gene promoters in both the presence and absence of exogenous estrogen. These data suggest that CITED2 functions as a transcriptional co-activator of ER that can stimulate transcriptional activity independent of ligand when expressed at elevated levels. Further, CITED2 overexpression promoted *in vitro* cell growth in the absence of estrogen and in the presence of tamoxifen. This finding is consistent with the inverse correlation observed between CITED2 expression and overall survival in breast tumors, and indicates that elevated CITED2 levels may contribute to estrogen-independent tumor growth and resistance to anti-estrogen therapy.

In accordance with our study, it is interesting to note that a prior study by Van Agthoven et al. reported CITED2 as a putative breast cancer anti-estrogen resistance gene [22]. Using an insertion mutagenesis technique, the authors found that CITED2 became overexpressed upon nearby integration of a retrovirus causing tamoxifen resistance in the ZR-75-1 breast cancer cell line, although the authors did not investigate the functional contribution of CITED2 toward this phenotype. It should also be noted that in contrast to our study and their own findings in the ZR-75-1 cell line, a separate report by Van Agthoven et al. indicated that CITED2 mRNA expression was associated with clinical benefit of tamoxifen treatment and metastasis-free survival [23]. Contrary to this report, however, a recent study by Yang et al. identified CITED2 as a member of a gene set that is negatively correlated with metastasis-free survival [24]. While the reason for the disparate results concerning the prognostic value of CITED2 is unclear, the study of CITED2 mRNA levels in clinical samples may be limited by tumor heterogeneity, since CITED2 is ubiquitously expressed. In addition, since transcription factors are often regulated post-translationally, measurement of CITED2 protein may be more informative. Taken together, these data indicate that further examination of the prognostic value of CITED2 in breast cancer is needed.

While the mechanism by which CITED2 facilitates ligand-independent ER activation remains unknown, given that co-activator proteins typically function as multi-protein complexes, it will be critical to determine the proteins with which CITED2 interacts under these conditions. A potentially key interaction is that with Cbp-p300, a known binding partner of CITED2 having documented ability to interact with the A/B domain of ER, thereby increasing its ligand-independent activity [25]. Also of interest is the effect of CITED2 on ER phosphorylation at serine$^{104/106/118}$, reported to influence the interaction of co-activators with the A/B domain of ER [25], as well as any effect on known repressors of ligand-independent ER transcription such as the histone deacetylase SIRT1 [26]. Such studies will not only further our understanding of ligand-independent ER activation, but may contribute toward the advancement of breast cancer prognostication and treatment.

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