Ciz1, a Novel DNA-Binding Coactivator of the Estrogen Receptor α, Confers Hypersensitivity to Estrogen Action

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Abstract

The transcriptional activity of the estrogen receptor (ER) is affected by regulatory cofactors, including chromatin-remodeling complexes, coactivators, and corepressors. Coregulators are recruited to target gene promoters through protein-protein interactions with ER and function as linker molecules between the DNA, DNA-binding proteins, and DNA-modifying enzymes. We recently showed that Cip-interacting zinc finger protein 1 (Ciz1) participates in the regulation of the cell cycle in estrogen-stimulated breast cancer cells. Despite the emerging significance of Ciz1 in the biology of breast cancer cells, regulation of endogenous Ciz1 in hormone-responsive cancer cells remains unknown. To shed light on the role of Ciz1 in breast tumorigenesis, we defined the regulation of *Ciz1* by the ER pathway and found that *Ciz1* is an estrogen-responsive gene. We also discovered that Ciz1 protein, a DNA-binding factor, coregulates ER by enhancing ER transactivation activity by promoting the recruitment of the ER complex to the target gene chromatin. In addition, we found that Ciz1 overexpression confers estrogen hypersensitivity to breast cancer cells and promotes the growth rate, anchorage independency, and tumorigenic properties of breast cancer cells. These findings revealed the inherent role of Ciz1, a novel DNA binding and ER coactivator, in amplifying estrogenic responses and promoting breast cancer tumorigenesis. (Cancer Res 2006; 66(22): 11021-9)

Introduction

The development of human breast cancer is promoted by estrogen stimulation of mammary epithelial cell growth. The biological effects of estrogen are mediated by its binding to the structurally and functionally distinct estrogen receptors (ER) α and β. ERα is the major ER in the human mammary epithelium. Estrogen triggers the stimulation of ERα, which interacts directly with estrogen response elements (ERE) in target gene promoters to induce gene transcription (1, 2). ERs also regulate the transcription of ER target genes through nonclassic response sites involving protein-protein interactions with transcription factors such as Sp1, c-Jun, and activator protein-1 (2). Ligand-activated ERα undergoes a conformational change that initiates a series of events leading to the formation and recruitment of multiprotein complexes to the target gene chromatin and, consequently, the transcription of ER-regulated genes. The transcriptional activity of ER is affected by a number of regulatory cofactors, including chromatin-remodeling complexes, coactivators, and corepressors, that are recruited to target gene promoters through protein-protein interactions with ER and function as linker molecules between the DNA, DNA-binding proteins, and DNA-modifying enzymes.

Tumor progression involves the gradual transition of the normal phenotype to the malignant phenotype through a series of cumulative cellular, genetic, and epigenetic alterations. During tumorigenesis, the cellular machineries responsible for the proliferative, migratory, and invasive properties become deregulated, enabling altered cells to grow, survive, and metastasize to distant organs. The progression of hormone-responsive cancers is also characterized by the deregulation of the cell cycle and cytoskeleton signaling. Furthermore, the development of breast cancer is profoundly influenced by the stimulatory action of estrogen. Accordingly, studies on the effects of estrogen on cell proliferation and differentiation have focused primarily on the role of estrogen and its receptors in controlling the entry into, progression through, and exit from the G_1 phase of the cell cycle.

Some of the regulatory components involved in the G_1-S transition are the cyclin D1-cdk4 and cyclin E-cdk2 complexes, which phosphorylate substrates such as pRB. Estrogen induces a significant increase in the phosphorylation of pRB (3–5), thereby allowing the initiation of DNA synthesis (6). Treatment with antiestrogenic agents triggers a decline in cyclin D1 (7), which can be up-regulated again by estrogen treatment owing to increased cyclin D1-cdk4 association and cdk4 activity (8). Another well-recognized estrogen target is the transcription factor c-Myc, which controls a number of cellular pathways. Rapid and direct regulation of c-Myc by estrogen places c-Myc induction among the earliest detectable transcriptional responses to estrogen. Furthermore, functionally inactivating c-Myc with the use of antisense oligonucleotides inhibits estrogen-stimulated breast cancer cell proliferation (9).

A large body of previous work has provided detailed insight about the roles of cell cycle components or coregulatory proteins in transcriptional stimulation by estrogen in breast cancer cells. However, the specific participation of coactivators in cell cycle regulation remains poorly defined. An earlier study from this laboratory revealed a coactivator function of dynein light chain 1 (DLC1) in targeting the ER to the chromatin of estrogen-inducible genes in breast cancer cells (10). More recently, we showed that DLC1 interacts with a novel protein, Cip-interacting zinc finger protein 1 (Ciz1), and participates in the regulation of cell cycle by increasing cdk2 kinase activity and inducing the G1-S transition (11, 12). Despite the emerging significance of Ciz1 in the biology of breast cancer cells, how endogenous Ciz1 is regulated in hormone-responsive cancer cells is unknown. To shed light on the role of Ciz1 in breast tumorigenesis, here we studied the gene regulation of *Ciz1* by the ER pathway and found that *Ciz1* is an

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estrogen-responsive gene. We discovered that Ciz1 protein, a DNA-binding factor, coregulates ER by enhancing ER transactivation activity and recruitment to target gene chromatin, and contributes to hypersensitivity of breast cancer cells to estrogen. In addition, overexpression of Ciz1 promoted the growth-rate, anchorage-independence and tumorigenesis of breast cancer cells.

Materials and Methods

Cell cultures and reagents. Cell lines stably overexpressing Ciz1 were generated by transfecting ZR-75 human breast ductal carcinoma cells (American Type Culture Collection, Manassas, VA) with pcDNA-T7-Ciz1. After 48 hours, the cells were selected in medium containing G418 (500 μg/mL). ZR-75 cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS). MCF-7, T47D, MDA231 (human breast ductal carcinoma cells), and HeLa (human cervical carcinoma) cells were maintained in DMEM/F12 supplemented with 10% FBS. Estrogen was purchased from Sigma-Aldrich (St. Louis, MO). Charcoal-stripped serum was purchased from Gemini Bio-Products (Woodland, CA). Antibodies against cyclin D1 were from Neomarkers (Fremont, CA), those against vinculin were from Sigma-Aldrich, those against ER were from Chemicon International (Temecula, CA), those against T7 tag were from Novagen (San Diego, CA), those against proliferating cell nuclear antigen (PCNA) were from Genetex (San Antonio, TX), and those against Ki-67 were from Invitrogen (Carlsbad, CA). The anti-Ciz1 antibody used here has been characterized previously (13).

Plasmid construction. Glutathione *S*-transferase (GST)-Ciz1 full-length and deletion constructs were generated as described earlier (11). To generate an expression vector containing Myc-tagged Ciz1 (Myc-Ciz1) or a Ciz1 expression vector with T7 tag (T7-Ciz1), *Ciz1* cDNA was amplified by PCR and cloned into pCMV-Myc vector (Clontech, Palo Alto, CA) or pcDNA3.1 vector (Invitrogen), respectively. Expression of Myc-Ciz1 and T7-Ciz1 were verified by immunofluorescence and Western blotting with Myc and T7 epitope monoclonal antibody (mAb), respectively. Luciferase construct for the Ciz1 ERE was generated by amplifying the ERE and flanking regions by PCR and cloning them into pGL3-luciferase vector. ERE-luciferase and pS2-luciferase were described previously (10).

RNA extraction, reverse transcription-PCR, and real-time quantitative PCR. RNA was extracted with Trizol reagent (Invitrogen) according to the instructions of the manufacturer. Reverse transcription-PCR (RT-PCR) was done using the Access RT-PCR kit from Promega (Madison, WI) and 100 ng RNA according to the protocol of the manufacturer. Real-time PCR was done by generating cDNA using the First Strand cDNA synthesis kit (Invitrogen). The Taqman assay with iQ Supermix (Bio-Rad, Hercules, CA) was used for quantitative analysis of *Ciz1* expression and normalized with actin expression using a 6-FAM-TAMRA-labeled probe (Applied Biosystems, Foster City, CA). Primers and probes used for *Ciz1* were forward 5′-ACATATCCACAGGTCCACACAC-3′, reverse 5′-CTGCTCATGGGTC-TGCTCTG-3′, probe (6-FAM) 5′-CACAGCCAAGCGTCCAGCCACAGG-3′ (TAMRA), and those used for actin were forward 5′-TGACTGACTACCT-CATGAAGATCC-3′, reverse 5′-CCTTAATGTCACGCACGATTTCC-3′, probe (6-FAM) 5′-CGGCTACAGCTTCACCACCACGGC-3′(TAMRA). All experiments were done in duplicate. The relative induction of *Ciz1* mRNA was then calculated using the comparative C_T method.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were done as described previously (14). Equal amounts of DNA were used for each sample. For input DNA, 10% of the chromatin solution was put aside before immunoprecipitation. Chromatin solutions were immunoprecipitated at 4°C overnight. PCR analysis was done with primers for the *Ciz1* gene (ERE1 +6,213 and ERE2 +13,111) and for *pS2* gene (*pS2* promoter primers) −463 to −159 or 1 kb upstream of this element to serve as a negative control (*pS2* upstream primers) −1,953 to −1,651; 3 μL of sample DNA; and 1 μL of input DNA. PCR was restricted to 30 cycles. PCR products (228, 172, 304, and 302 bp, respectively) were resolved on a 2% agarose gel and visualized with ethidium bromide. The sequences of the *pS2* promoter primers were 5′-GAATTAGCTTAGGCCTAGACGGAATG-3′ and 5′-AGGATTTGCTGATAGACAGAGACGAC-3′. For the *pS2* upstream primers, the sequences were 5′-CTCCCTCTTCAGGCCTCTCT-3′ and 5′-TTCCCTGGT-GTTGTCAAGTG-3′. For ERE1 in *Ciz1*, 5′-GGAGCCCTTCAACAGGAGAT-3′ and 5′-CATCCTGCTGGAATGGAAGT-3′. For ERE2 in Ciz1, 5′-TTCAATAC-CGCAACCCTCTC-3′ and 5′-GCTGTGTCTGCGGAGGAG-3′.

Reporter gene assay. For reporter gene transient transfections, cells were cultured for 48 hours in minimal essential medium, without phenol red, containing 5% charcoal-stripped serum. Next, 200 ng ERE-luciferase reporter constructs were cotransfected with 500 ng cytomegalovirus (CMV) or Ciz1 plasmid using the Fugene-6 reagent according to the protocol of the manufacturer (Roche, Indianapolis, IN). In the case of assaying the *Ciz1* promoter, 100 ng promoter construct were transfected with 500 ng pcDNA or ER. Twenty-four hours later, the cells were treated with estrogen (10^{-9} mol/L) for 24 hours. The cells were then lysed with passive lysis buffer, and the luciferase assay was done using a kit (Promega). β-Galactosidase activity was used to normalize the transfection. Each transfection was done in six-well plates in triplicate.

GST pull-down assay. *In vitro* transcription and translation of the Ciz1 and ER proteins were done using the TNT transcription/translation system (Promega) in the presence of [^{35}S]methionine. The reaction mixture was diluted 10 times with NP40 lysis buffer (25 mmol/L Tris, NaCl 50 mmol/L, and 1% NP40). The GST pull-down assays were done by incubating equal amounts of GST, GST-Ciz1-domain, GST-ER, or GST-ER deletion proteins immobilized on GST beads (Amersham Pharmacia Biotech, Piscataway, NJ) with *in vitro*-translated recombinant protein. Bound proteins were isolated by incubating the mixture for 2 hours at 4°C and then washed five times with NP40 lysis buffer. The proteins were eluted with a 2× SDS buffer, separated by SDS-PAGE, and visualized by autoradiography.

Cell extracts, immunoblotting, and immunoprecipitation. To prepare the cell extracts, cells were washed thrice with PBS and lysed in buffer [50 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 1% Triton X-100, 1× protease inhibitor mixture (Roche), and 1 mmol/L sodium vanadate] for 30 minutes on ice. Cell lysates containing equal amounts of protein were then resolved on an SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose, probed with the appropriate antibodies, and detected using an enhanced chemiluminescence method. The cell lysates for immunoprecipitation were prepared using NP40 lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 0.5% NP40, 1×protease inhibitor cocktail, and 1 mmol/L sodium vanadate]. Immunoprecipitation was done for 4 hours at 4°C using 1 μg of antibody per milligram of protein. The anti-Ciz1 antibody used in these experiments has been characterized previously (13).

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay with radiolabeled probe was done with commercial double-stranded ERE consensus sequence probes purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The probes were end-labeled using T4-polynucleotide kinase (Invitrogen) and ^{32}P-labeled ATP (Perkin-Elmer, Wellesley, MA). Binding to ERE was visualized by running a 5% acrylamide gel, drying the gel, and visualizing the results by autoradiography.

Cell growth and soft-agar experiments. For the cell growth experiments, equal numbers of ZR-75/Ciz1 and ZR-75/pcDNA cells were plated in triplicate in 24-well plates for 48 hours in phenol red–free DMEM supplemented with 5% charcoal-stripped serum. The cells were then treated with estrogen (10^{-9} mol/L) for 6 days, and their growth rate was measured on days 0, 2, 4, and 6 with a Coulter Counter (Beckman Coulter, Fullerton, CA).

Colony growth assays were done as described previously (14). Briefly, 1 mL solution of 0.5% noble agar in modified essential medium supplemented with 5% charcoal-stripped serum was layered onto 30 × 10-mm tissue culture plates. A total of 1×10^4 cells was mixed with 1 mL of 0.3% agar solution prepared in a similar manner and layered on top of the 0.5% agar layer. Plates were incubated at 37°C in 5% CO_2 for 21 days. When indicated, some cultures were treated with estradiol (E2; 10^{-9} mol/L) or the antiestrogenic agent ICI-182780 (10^{-8} mol/L). The experiment was done in triplicate.

Mouse xenograft studies. For the tumorigenesis studies, 3 days after implantation of a 60-day release estrogen pellet, 4-week-old ovariectomized

female athymic nude mice (Charles River, Wilmington, MA) were bilaterally injected with 5×10^6 cells of either ZR-75/pcDNA or ZR-75/Ciz1 clones into the mammary fat pads, as previously described (15). Tumors were allowed to grow for 5 weeks, and tumor size was measured every 15 days. The animals were divided into four groups. Group 1 mice were injected with ZR-75/pcDNA cells. In group 2 mice, estrogen pellets were implanted 3 days before the ZR-75/pcDNA cells were injected. The mice in groups 3 and 4 were treated similarly, but were injected with ZR-75/Ciz1 cells. All animal procedures were done in compliance with the Institute Animal Care and Use Committee and the NIH Policy on Humane Care and Use of Laboratory Animals.

Immunohistochemical analysis. For immunohistochemical detection of Ki-67, PCNA, and cyclin D1, xenograft sections were deparaffinized with xylene; rehydrated using graded ethanol; incubated in 0.3% H_2O_2 and methanol for 30 minutes to inactivate endogenous peroxidase; boiled for 15 (Ki-67), 20 (PCNA), and 30 (cyclin D1) minutes in 0.01 mol/L citrate buffer; and cooled for 30 minutes at room temperature to expose antigenic epitopes. The sections were incubated with 2% normal goat serum in 1% bovine serum albumin and PBS for 30 minutes and then with anti-Ki-67, anti-PCNA, and anti-cyclin D1 antibodies and incubated overnight at 4°C. The sections were washed thrice with 0.05% Tween in PBS for 10 minutes, incubated with secondary antibody, developed with 3,3′-diaminobenzidine-H_2O_2, and counterstained with Mayer's hematoxylin. Negative controls were done by replacing the primary antibody with the corresponding IgG.

Immunofluorescence and confocal studies. The cellular localization of different proteins was determined by indirect immunofluorescence. Briefly, ZR-75 cells were grown on glass coverslips and fixed with methanol. The cells were incubated first with primary antibodies and then with secondary antibodies conjugated with Alexa-546 (red) and Alexa-488 (green; both from Molecular Probes, Eugene, OR). Topro 3 (blue; Molecular Probes) was used to counterstain the DNA. Cells treated with only the secondary antibodies served as the controls. Confocal scanning analysis was done using an FV300 laser-scanning confocal microscope (Olympus, Olympus America, Inc., Center Valley, PA). Each slide was examined for each stain at three excitation wavelengths (488, 546, and 633 nm), and the data were compared pixel by pixel. Colocalization was indicated by the development of a yellow color.

Results

***Ciz1* is an estrogen-inducible gene.** To investigate the role of Ciz1 in the estrogen signaling pathway in breast cancer cells, we first searched for the potential presence of ERE in the putative *Ciz1* promoter both upstream and downstream of the transcriptional start site of *Ciz1* (Genbank accession no. NM_012127). The fact that we found multiple half-EREs and two palindromic EREs in the first intron of the *Ciz1* gene (Fig. 1*A, top*) raised the possibility that *Ciz1* is an estrogen-inducible gene. To explore this hypothesis, we treated MCF-7 and ZR-75 breast cancer cells with estrogen and found that it rapidly induced the level of *Ciz1* mRNA in both cell lines (Fig. 1*A, bottom*). To validate these finding in a quanititative manner, we did real-time PCR for a panel of estrogen-sensitive and ER-negative breast cancer cell lines. Estrogen induced *Ciz1* in the

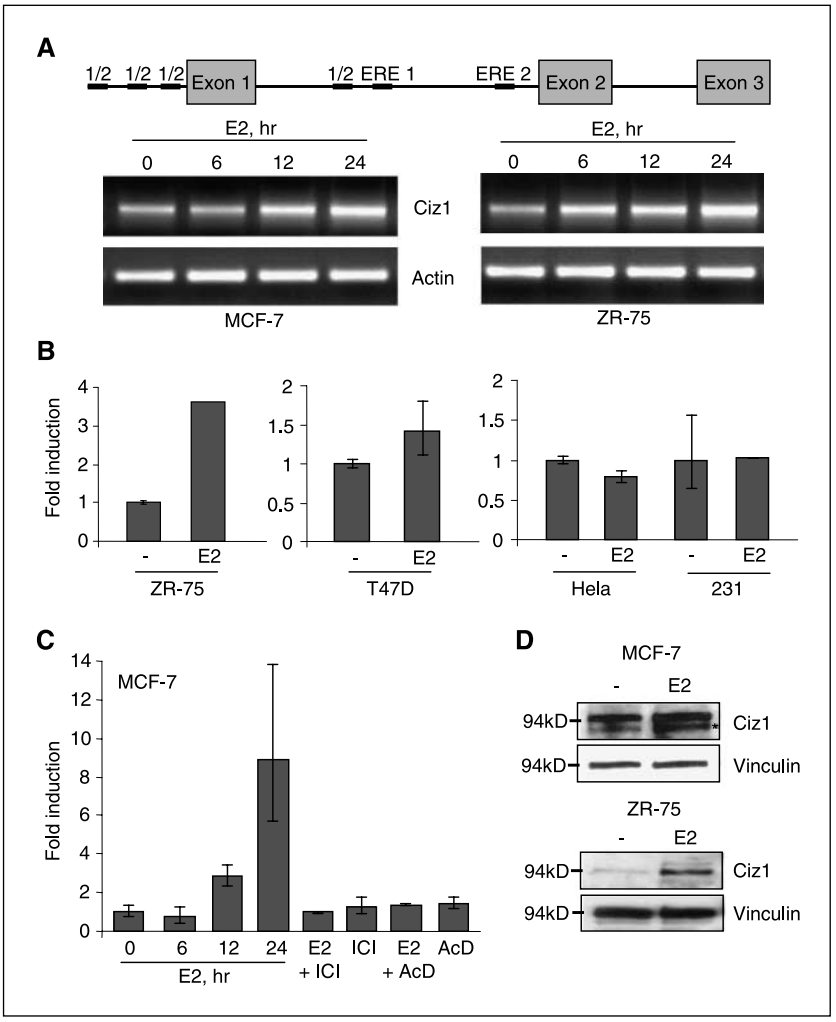

Figure 1. *Ciz1* is an estrogen-inducible gene. *A, top,* map of ERE in *Ciz1* gene chromatin. *A, bottom, B,* and *C, Ciz1* mRNA induction by estrogen. *A, bottom,* MCF-7 and ZR-75 cells were treated with E2 (10^{-9} mol/L) for 6, 12, or 24 hours. *B,* ZR-75, T47D, HeLa, and MDA231 cells were treated with E2 (10^{-9} mol/L) for 24 hours. *C,* induction of *Ciz1* mRNA can be blocked by ICI-182780 and is caused by enhanced transcription of the *Ciz1* gene. MCF-7 cells were treated with E2 (10^{-9} mol/L) for 6, 12, or 24 hours. Pretreatment was 1 hour with ICI-182780 and 4 hours with actinomycin *D*. For (*B*) and (*C*), the relative induction of *Ciz1* mRNA was calculated using the comparative C_T method. All experiments were done in duplicate. *D,* induction of Ciz1 protein levels by estrogen. MCF-7 and ZR-75 cells were treated with E2 (10^{-9} mol/L) for 24 hours.

ER-positive cells, but not in the ER-negative cells (Fig. 1*B*). The specificity of this estrogen induction of *Ciz1* mRNA in an ER-dependent manner was confirmed by using the pure anti-estrogenic agent ICI-182780 (Fig. 1*C*, compare *lanes 4* and *5*). The observed increase in the levels of *Ciz1* mRNA was due to newly synthesized *Ciz1* mRNA, because pretreatment of the cells with actinomycin D, a transcriptional inhibitor, prevented the ability of estrogen to induce the levels of *Ciz1* mRNA (Fig. 1*C*). Estrogen stimulation of MCF-7 and ZR-75 cells was also accompanied by increased levels of Ciz1 protein (Fig. 1*D*).

To define the molecular basis of estrogen stimulation of *Ciz1* expression, we focused on the palindromic EREs present in the *Ciz1* gene chromatin and explored whether ER is recruited to these elements. To this end, we carried out ChIP studies to examine the potential recruitment of ER to two elements (ERE1 +6,213 bases and ERE2 +13,111 bases from the transcriptional start site) in the first intron of *Ciz1*. ER was indeed recruited onto both elements and with a similar cyclic pattern, first at 15 minutes and again at 60 minutes (Fig. 2*A*). An earlier study showed that the first recruitment of ER is nonfunctional but at the second recruitment, ER is recruited as a more potential inducer of transcription and induces gene expression (16). To show the specificity of the association of ER with the *Ciz1* chromatin, we did a PCR of a part of the *pS2* promoter known not to interact with ER (Fig. 2*A, right*). We next did a double ChIP of RNA polymerase II and ER and assessed its association with *Ciz1* chromatin upon estrogen treatment. We found that, indeed, RNA polymerase II and ER are associated with ERE1 and ERE2 regions of the *Ciz1* gene chromatin in a estrogen-dependent manner (Fig. 2*B*). We cloned the *Ciz1* gene fragment that contains the ERE into a pGL2-luciferase reporter system and showed that estrogen stimulation of breast cancer cells was accompanied by increased transcriptional activity of *Ciz1* (Fig. 2*C*). Taken together, these findings suggested that Ciz1 is an estrogen-inducible gene product.

Ciz1 interacts with ER. Earlier studies have shown that many estrogen-inducible gene products, such as PELP1 and DLC1, interact with ER and thus are recruited to the target gene chromatin as a complex with ER (10, 17). For this reason, we searched the protein sequence of Ciz1 for the known nuclear receptor–binding motif LXXLL and discovered one such motif between the first and second zinc finger motifs (Fig. 3*A*). We next carried out a series of GST pull-down studies using *in vitro*–translated ER and Ciz1-deletion GST-fusion proteins. We found no evidence of ER interaction with the LXXLL motif–containing region of Ciz1. Rather, Ciz1 used a region containing the second glutamine-rich region at the NH_2 terminus of the protein to interact with the ER (Fig. 3*A*). Because Ciz1 did not interact with ER via its NR box, we expected to find that Ciz1 interacts with a region or regions other than the AF2 domain of ER. Indeed, Ciz1 did not interact through its AF2 domain of ER; instead, it bound to the DNA-binding domain of ER (Fig. 3*B*).

We next examined the physiologic interaction between Ciz1 and ER in breast cancer cells. ZR-75 breast cancer cells were stimulated with estrogen, the cell lysates were immunoprecipitated with an anti-Ciz1 antibody, and the precipitated material was immunoblotted with antibodies against ER and Ciz1. We found that estrogen stimulation enhances the physical interaction between ER and Ciz1 dramatically (Fig. 3*C*). We also detected distinct colocalization of ER and Ciz1 in the nuclear compartment by scanning confocal microscopy (Fig. 3*D*). These results suggested that ER and Ciz1 interact in a ligand-dependent manner in a physiologic setting.

Ciz1 is a coactivator of ERα. One of the primary cellular actions of ER is the induction of gene transcription via interaction with the ERE in the target genes. Because we found that Ciz1 physically interacts with ER *in vivo* and that Ciz1 is predominantly a nuclear protein, we speculated that Ciz1 is associated with the ER target gene chromatin. Using ChIP–based assays and ZR-75 cells stably expressing T7-Ciz1, we found that T7-Ciz1 interacted with the *pS2* chromatin, which is an estrogen-inducible gene (Fig. 4*A, left*). We also noticed a potentiating effect of T7-Ciz1 expression on the amount of ER recruited to the *pS2* gene, and this effect was further enhanced on estrogen stimulation (Fig. 4*A, left*). The specificity of the binding of Ciz1 to the *pS2* chromatin was determined by using a primer set 1 kb upstream of the ERE in *pS2*, which served as a negative control (Fig. 4*A, middle*). To show the existence of ER and Ciz1 within the same complex, we did a double ChIP assay involving the first ChIP with an anti-T7 mAb to precipitated T7-Ciz1 and a second ChIP with an ER-mAb. We found that, indeed, T7-Ciz1/ER complex is effectively recruited to the pS2 chromatin (Fig. 4*A, right*).

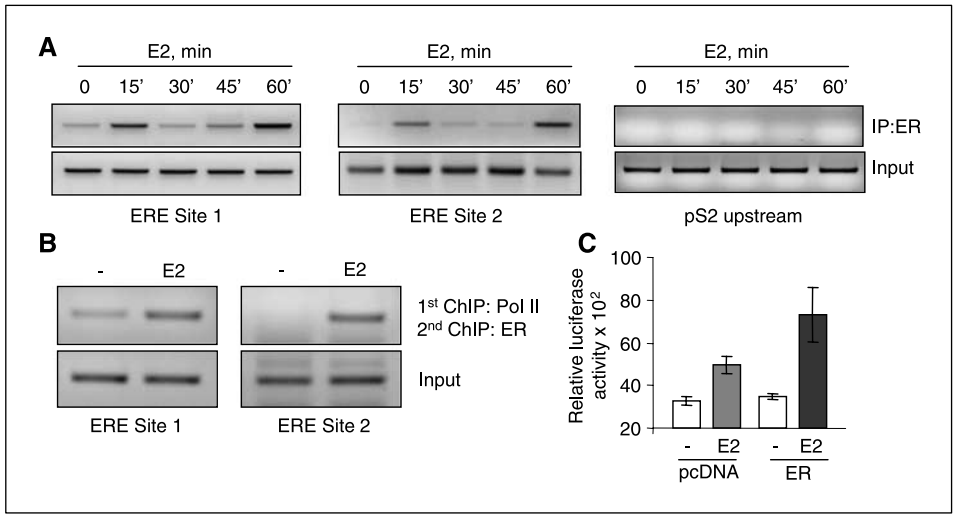

Figure 2. ER is recruited to the ERE in *Ciz1* chromatin and induces gene expression. *A,* ER recruitment to the ERE in *Ciz1* gene. MCF-7 cells were treated with estrogen (10^{-9} mol/L) for 15, 30, 45, or 60 minutes before ER was immunoprecipitated with the associated chromatin. PCR for *Ciz1* was done. *A, right*, PCR for *pS2* upstream of ERE was done as negative control. *B,* Pol II recruitment to the *Ciz1* gene. MCF-7 cells were treated with estrogen (10^{-9} mol/L) for 60 minutes before ER was immunoprecipitated with the associated chromatin and after this Pol II using a Pol II antibody. PCR for *Ciz1* was done. *C,* induction of *Ciz1* promoter luciferase by ER. MCF-7 cells were transfected with pcDNA or ER and with *Ciz1* promoter constructs. Cells were deprived of E2 for 48 hours before E2 (10^{-9} mol/L) was added for 24 hours.

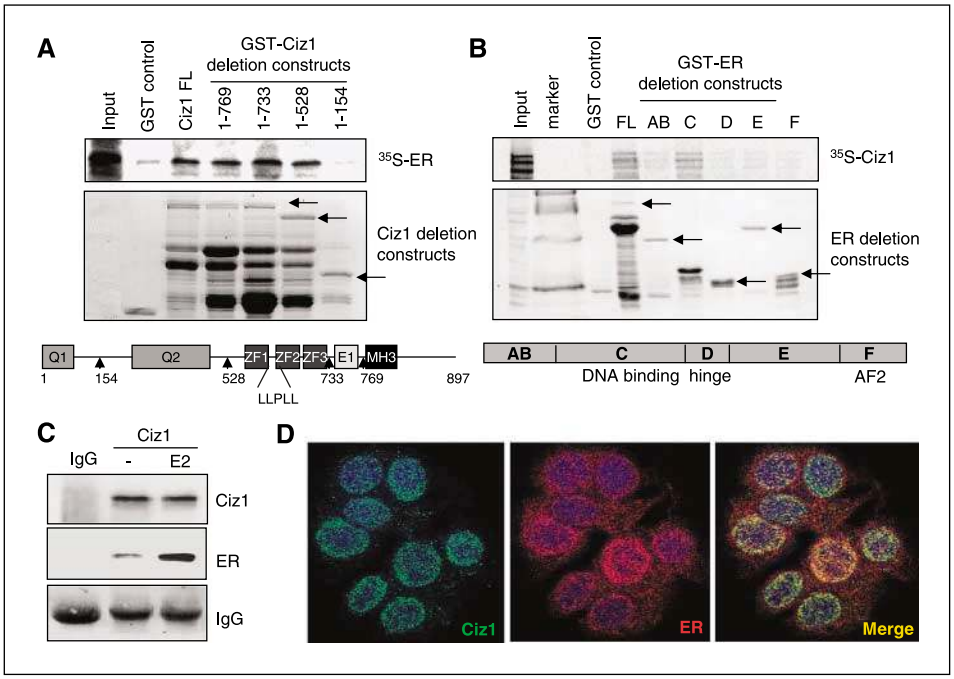

Figure 3. Ciz1 binds ER *in vivo* and directly *in vitro*. *A,* ER interacts with the second glutamine-rich region of Ciz1. GST pull-down assays show the association of GST-Ciz1 deletion constructs with *in vitro*–translated ^{35}S-ER. *Arrows,* deletion protein bands. *B,* Ciz1 interacts with the DNA-binding domain of ER. GST pull-down assays show the association of GST-ER deletion constructs with *in vitro*–translated ^{35}S-Ciz1. *Arrows,* deletion protein bands. *C,* Ciz1-ER interaction is enhanced after estrogen stimulation. ZR-75 cells were treated with E2 (10^{-9} mol/L) for 1 hour before Ciz1 was immunoprecipitated. *D,* Ciz1 (*green*) and ER (*red*) localize in the nucleus. *Yellow,* colocalization.

Because we found that Ciz1 associated with the ERE-containing *pS2* chromatin and potentiated the recruitment of ligand-activated ER to that chromatin, we hypothesized that Ciz1 might act as a coactivator for ER. To test this hypothesis, we did a luciferase assay using a reporter construct containing EREs and found that Ciz1 acts as a coactivator for ER and increased its transactivation activity under both basal and ligand-induced conditions (Fig. 4*B, left*). Consistent with these results, selective knockdown of endogenous Ciz1 by using Ciz1-specific small interfering RNA reduced the transactivation activity of ER (Fig. 4*B, middle*), suggesting an inherent role of Ciz1 in optimal ER responsiveness.

To further validate our discovery that the coactivator function of Ciz1 is due to direct binding of the Ciz1-ER complex to the ERE in a physiologically relevant endogenous promoter, we used a more physiologically relevant endogenous promoter of *pS2* and were still able to observe induction after Ciz1 transfection (Fig. 4*B, right*). We then carried out gel-shift studies using an oligonucleotide containing ERE by using the nuclear extracts from T7-Ciz1–transfected cells. As we expected, a distinct ER-Ciz1 complex formed on the ERE itself that could be supershifted by antibody against ER or Ciz1 (Fig. 4*C*). The above findings suggested that Ciz1 is recruited to the native target gene chromatin. We also showed that Ciz1 overexpression promoted the expression of cyclin D1 (Fig. 4*D, top*).

The fact that Ciz1 expression potentiated the estrogen stimulation of ER transactivation activity, ER recruitment to the target gene chromatin, and expression of ER target gene *cyclin D1* suggested that Ciz1 confers estrogen hypersensitivity to breast cancer cells. To test this notion at a biochemical level, we stimulated the Ciz1-overexpressing and control cells with a serially diluted dose of estrogen over 6 logs (10^{-9}, 10^{-11}, and 10^{-13} mol/L E2) and evaluated the expression of cyclin D1 as an indicator of estrogen sensitivity. Ciz1 expression conferred hypersensitivity to the responsiveness of breast cancer cells to estrogen: Cyclin D1 was induced by a very low dose of estrogen in the Ciz1-overexpressing cells but not in the control cells (Fig. 4*D, bottom*). This noted up-regulation of cyclin D1 may contribute to the Ciz1-mediated stimulation of DNA replication reported previously (13). These results supported the notion that Ciz1 acts as a coactivator of ER and induces hypersensitivity to estrogen in breast cancer cells.

Ciz1 promotes tumorigenic phenotypes in breast cancer cells. We explored the functional consequence of the overexpression of the newly identified coregulator Ciz1. Ciz1 overexpression enhanced the growth rate of ZR-75 cells under both estrogen-depleted and estrogen-supplemented conditions (Fig. 5*A*). When we evaluated the specificity of those results, we observed that the addition of the pure antiestrogenic agent ICI-184280 resulted in complete abrogation of the growth-promoting activity of Ciz1-overexpressing cells (Fig. 5*B*). This finding strengthened the idea that Ciz1 overexpression induces hypersensitivity to estrogen in breast cancer cells. In addition, Ciz1-overexpressing cells grew in an anchorage-independent manner (Fig. 5*C*) that could be completely reverted to the control level on addition of ICI-184280 (Fig. 5*D*). Together, these observations suggested that Ciz1 enhances the tumorigenic phenotype of breast cancer cells in an ER-dependent manner.

Ciz1-overexpressing cells induce tumor formation in nude mice. In general, tumor formation by ER-positive cells, such as ZR-75 cells in immunocompromised mice, depends on continuous estrogenic stimulation by estrogen pellets (15). To examine the effect of Ciz1 overexpression on the tumor-forming ability of breast cancer cells, we examined the ability of ZR-75/pcDNA and ZR-75/Ciz1 cells to form tumors in a xenograft model involving implantation of estrogen pellets in ovariectomized female nude mice. ZR-75 cells are known to form tumors in estrogen-treated mice, which was also observed in our study. Ciz1 overexpression in these ZR-75 cells induced a significantly higher amount of tumors in the mice with estrogen pellet (χ^2; $P = 0.01$; Fig. 6*A*). Ciz1 overexpression conferred tumor-forming ability to ZR-75 cells even without exogenous estrogen treatment, suggesting that deregulation of Ciz1 might be sufficient to promote estrogen sensitivity and tumorigenic phenotypes.

As we expected, the tumors formed by ZR-75/Ciz1 cells were larger than the tumors formed by estrogen-stimulated ZR-75/pcDNA cells (Fig. 6*A, graph*). Thus, Ciz1 seems to have a role in promoting ligand-induced tumorigenic activity of breast cancer cells. To determine whether the larger tumor size was caused by increased cell proliferation, we did immunohistochemical analysis of PCNA and Ki-67, and found higher staining levels in tumors with Ciz1 overexpression, which translates into a higher proliferative index (Fig. 6*B*). To determine whether Ciz1-expressing tumors also exhibit increased levels of ER target genes, such as cyclin D1, we carried out immunohistochemical analysis of cyclin D1 in the tumor samples from all treatment groups. Consistent with the results from our tissue culture studies, tumors that formed in the ZR-75/Ciz1 group, stimulated by estrogen, exhibited clearly enhanced levels of cyclin D1 (Fig. 6*C*). These findings suggested a role of Ciz1 in promoting the estrogenic stimulation of breast cancer cells, thereby leading to the tumor-forming ability of these cells. They also suggest that the reported ability of Ciz1 to promote DNA replication may be partly mediated via up-regulation of cyclin D1.

Discussion

We found that Ciz1 is an estrogen-inducible gene product that facilitates ER recruitment to ER target gene chromatin and ER transactivation activity (Fig. 6*D*). The Ciz1-mediated increased ER transactivation activity was functional, because it was accompanied by increased protein expression of ER target genes, enhanced cell proliferation and soft-agar colony formation, and increased ability of Ciz1-expressing cells to form tumors after estrogen stimulation in mice. Another interesting observation was the complete blockage by the antiestrogenic agent ICI-184280 of the observed growth-promoting advantage of Ciz1-expressing breast cancer cells, because the proliferation, and anchorage-independent

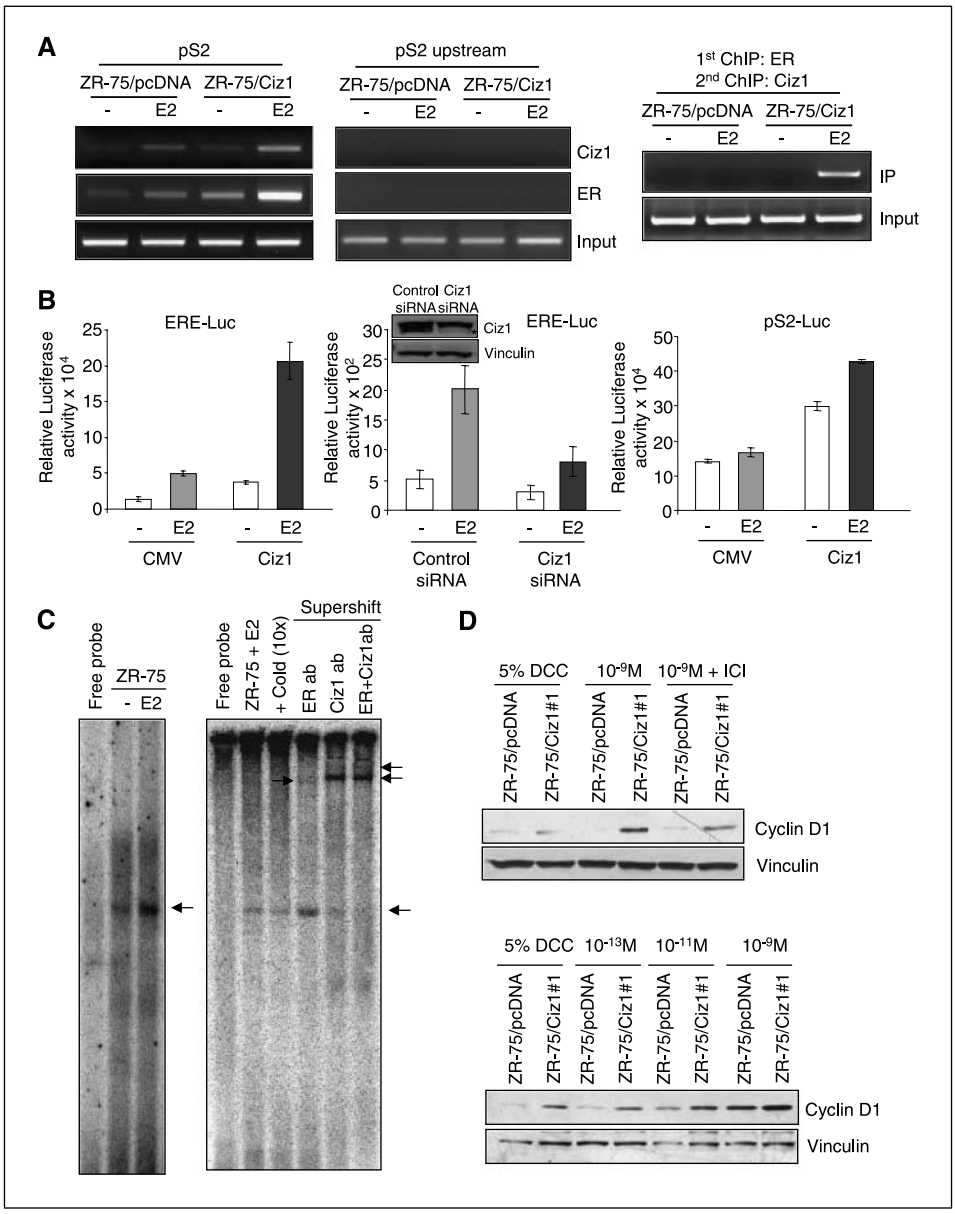

Figure 4. Ciz1 is a coactivator of ER. *A,* Ciz1 is recruited to the ER target gene chromatin and enhances ER recruitment. ZR-75/pcDNA and ZR-75/Ciz1 cells were treated with E2 (10^{-9} mol/L) for 1 hour before cross-linking. Lysates were immunoprecipitated with anti-T7-Ciz1 or anti-ER antibody. PCR was done for ERE in the *pS2* chromatin and 1 kb upstream as a negative control. *B,* Ciz1 affects ER transactivation activity. *Left,* Ciz1 overexpression increases ER transactivation activity. MCF-7 cells were transfected with CMV, Ciz1, and ERE-luciferase for 24 hours and treated with E2 (10^{-9} mol/L) for 24 hours, and then ERE-luciferase activity was measured. *Middle,* Ciz1 knockdown reduces ER transactivation activity. MCF-7 cells were treated with Ciz1-specific small interfering RNA for 48 hours, transfected with ERE-luciferase for 24 hours, and treated with E2 (10^{-9} mol/L) for 24 hours, and then ERE-luciferase activity was measured. *Inset,* knockdown efficiency of Ciz1 by Western blot. *Right,* Ciz1 overexpression increases ER transactivation activity on the endogenous promoter of *pS2*. MCF-7 cells were transfected with CMV, Ciz1, and ERE-luciferase for 24 hours and treated with E2 (10^{-9} mol/L) for 24 hours, and then ERE-luciferase activity was measured. *C,* Ciz1 and ER form a complex on the ERE. ZR-75 cells were treated with E2 (10^{-9} mol/L) for 1 hour before the nuclear extracts were made. Bands were supershifted by adding anti-Ciz1 or anti-ER antibody. *Arrows,* supershift bands. *D,* Ciz1 induces cell hypersensitivity to estrogen. *Top,* Ciz1 overexpression induces cyclin D1 expression. ZR-75/pcDNA and ZR-75/Ciz1 cells were treated with E2 (10^{-9} mol/L) for 24 hours. Cells were pretreated with ICI-182780 for 30 minutes. *Bottom,* Ciz1 induces cyclin D1 expression even with a low dose of E2. ZR-75/pcDNA and ZR-75/Ciz1 cells were treated with 10^{-13}, 10^{-11}, or 10^{-9} mol/L E2 for 24 hours.

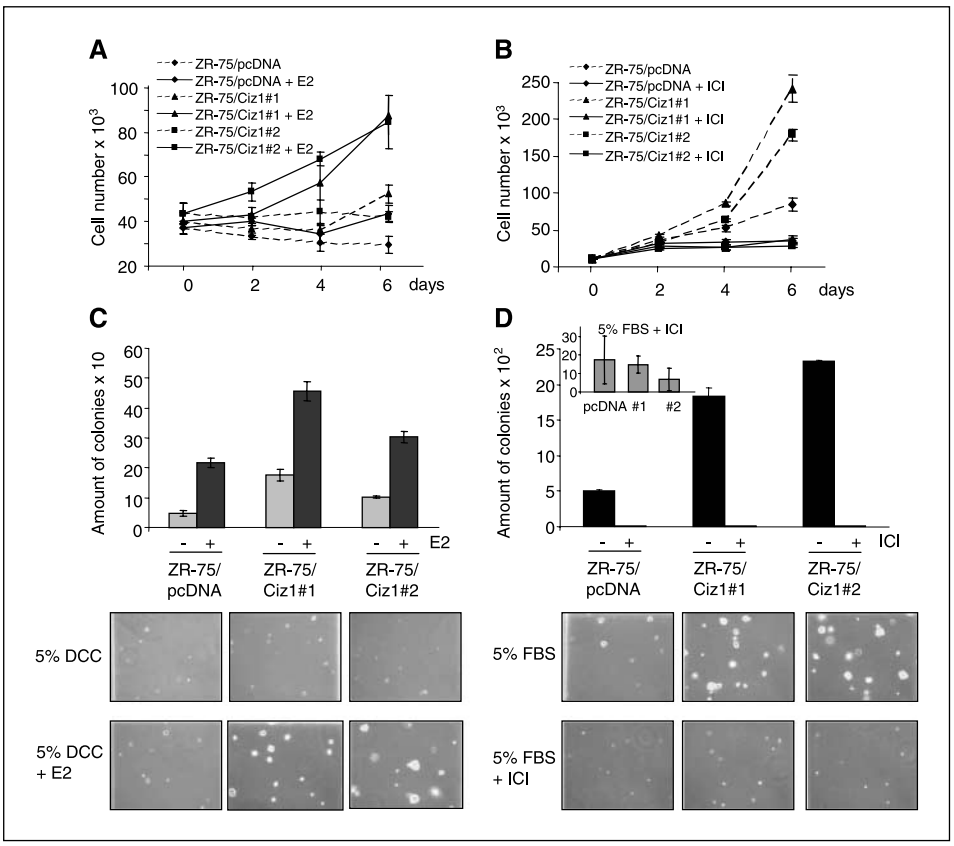

Figure 5. Ciz1 induces a tumorigenic phenotype. *A,* Ciz1 induces cell proliferation. ZR-75/Ciz1 and ZR-75/pcDNA cells were counted on days 0, 2, 4, and 6. *Points,* cell number; *bars,* SD. *B,* the proliferative advantage of Ciz1 overexpression is blocked by ICI-182780. ZR-75/Ciz1 and ZR-75/pcDNA cells were grown in 5% FBS with or without ICI-182780 and were counted on days 0, 2, 4, and 6. *Points,* cell number; *bars,* SD. *C,* Ciz1 overexpression promotes anchorage-independent colony formation. ZR-75/pcDNA and ZR-75/Ciz1 cells were plated in soft agar and stimulated with E2 for 21 days. *Columns,* amount of colonies; *bars,* SD. *D,* colony formation advantage of Ciz1 overexpression is blocked by ICI-182780. ZR-75/pcDNA and ZR-75/Ciz1 cells were plated in soft agar with 5% FBS and treated with ICI-182780. *Columns,* amount of colonies; *bars,* SD.

growth of Ciz1-overexpressing cells, was inhibited by this agent. In the absence of ligand-activated ER (due to antiestrogenic treatment), Ciz1 was unable to induce the transactivation or other downstream functions of ER. These findings imply that the effects of Ciz1 on the proliferation and anchorage-independent growth of breast tumor cells are dependent on ER. The mechanism by which Ciz1 acts as a coactivator for ER is unclear at the moment. Because Ciz1 is known to interact with the DNA element (18) and because Ciz1 binds to the DNA-binding region of ER, we hypothesized that the DNA-binding activity of Ciz1 has an inherent role in ER-modifying activity. The DNA-binding motif of Ciz1 and its interaction with ER might create a better ER docking site on the DNA. We previously discovered a glutamic acid–rich region in the amino acid sequence of Ciz1, a region previously shown to be important in histone binding (17, 19). Ciz1 may play an important role in histone modification near the ER target gene chromatin.

Another notable finding of the present study is that Ciz1 itself is an estrogen-inducible gene and contains ERE motifs. The functional implication of this finding resides in the potential signal amplification role of Ciz1 in maintaining the duration and strength of estrogenic signaling once ER-positive breast cancer cells are momentarily exposed to estrogen. The physiologic relevance of this implication was derived by our experiment involving selective knockdown expression of Ciz1, which resulted in a substantial reduction of the ER responsiveness of breast cancer cells and thus suggested a role of Ciz1 in optimal ER responsiveness.

Although we observed a profound hyperstimulatory effect of Ciz1 in the presence of estrogen, there was a modest but reproducible effect of Ciz1 alone in promoting the growth of the control cells grown in 5% charcoal-stripped serum. This is consistent with the observation that Ciz1 can promote cell cycle progression in other cell types (13). However, because charcoal stripping can substantially, but not completely, deplete estrogen in serum, the modest ER-promoting activity of Ciz1 might have resulted from the residual extremely low levels of estrogen in the medium. The residual low concentration of estrogen was fully capable of inducing cell growth and anchorage-independent growth of Ciz1-expressing cells. In addition, Ciz1-expressing cells were extremely sensitive to the femtomolar concentration of estrogen in that their growth and expression of cyclin D1 increased. Hypersensitivity to estrogen has been observed in patients with breast cancer; this phenomenon is typically known as adaptive hypersensitivity. In hypersensitive breast cancer patients, tumor growth requires premenopausal levels of estrogen (50-600 pg/mL) initially but later needs only 10 to 15 pg/mL of estrogen (20). This hypersensitivity is believed to be a result of adaptive pressure of estrogen depletion over a long period. During this period of deprivation, cells with particular alterations might be selected over the remaining tumor cells, leading to better survival of the hypersensitive cells. In this context, it is important to point out that Ciz1 is located on chromosome band 9q34 and that its amplification has been reported in several cancers (21, 22). However, the relationship of Ciz1 to breast cancer and ER status remains to be investigated.

The finding that Ciz1 functions were effectively blocked by the inclusion of a potent ER antagonist in studies using tissue culture models might be of great interest in therapeutic development because it suggests that although Ciz1 might confer tumorigenic properties to breast cancer cells, these cells remain sensitive to treatment modalities targeting the ER.

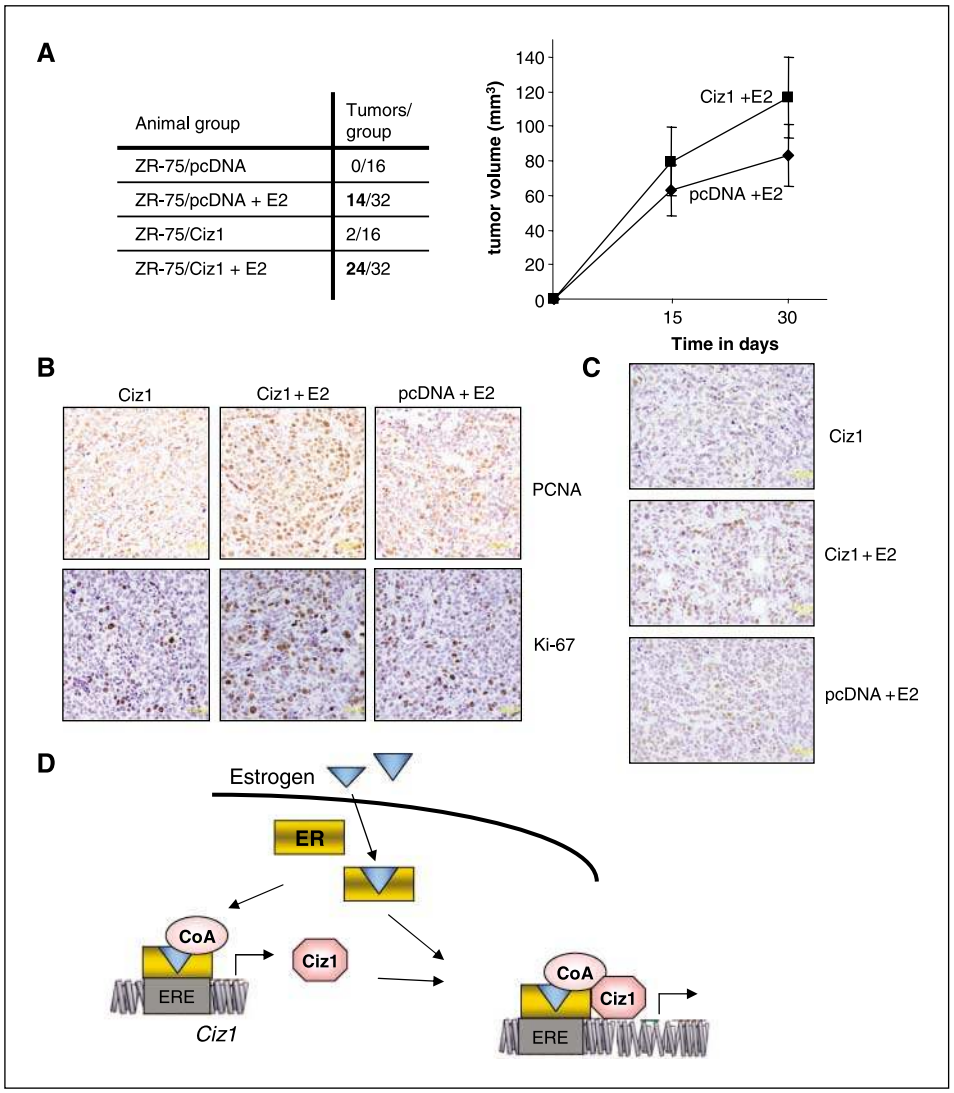

Figure 6. Ciz1-overexpressing cells induce tumors in nude mice. *A,* ZR-75/Ciz1–overexpressing cells have a higher tumor incidence rate than ZR-75/pcDNA cells. ZR-75/pcDNA or ZR-75/Ciz1 cells were injected 2 days after the implantation of the 60-day release estrogen pellets. Mice were monitored for tumor incidence. *A, right,* tumors from ZR-75/Ciz1 cells grow bigger than the ZR-75/pcDNA cells. ZR-75/pcDNA or ZR-75/Ciz1 cells were injected 2 days after the implantation of the 60-day release estrogen pellets. Tumor size was measured every 15 days. *Points,* tumor volume; *bars,* SD. *B,* tumors from ZR-75/Ciz1 cells have a higher level of proliferation then the control tumors. Tumors were dissected, formalin fixed, and paraffin embedded. Sections were stained for PCNA and Ki-67. One representative example. *C,* tumors from Ciz1-overexpressing cells show more cyclin D1 expression than the tumors from the control cells. *D,* working model of the potential role of Ciz1 action in hormone action.

In conclusion, we found that *Ciz1* is an estrogen-responsive gene and that Ciz1 protein coregulates ER by enhancing its transactivation activity and recruitment to target gene chromatin. Ciz1 induces the hypersensitivity of breast cancer cells to estrogen and induces the expression of ER target gene cyclin D1 at a femtomolar dose of estrogen, with likely downstream effects on G_1 progression and DNA replication. Moreover, Ciz1 promotes the proliferation, anchorage-independent growth, and tumorigenesis of breast cancer cells. Taken together, these findings reveal the inherent role of Ciz1 in amplifying estrogenic responses and its role in promoting breast cancer tumorigenesis.

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