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Negative regulation of ERα by a novel protein CAC1 through association with histone demethylase LSD1

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ABSTRACT

ERα, a critical transcriptional factor for breast cancer proliferation, is regulated by a complex binding repertoire that includes coactivators and corepressors. Here, we identified a novel class of ERα coregulator called CAC1. The CoRNR box of CAC1 was required for the binding to and inactivation of ERα. CAC1 also associated with histone demethylase LSD1 and suppressed LSD1-enhanced ERα activity. CAC1 impaired recruitment of ERα and LSD1 to the ERα-responsive promoter, leading to greater H3K9me3 accumulation. This effect was reversed by CAC1 depletion. Finally, CAC1 increased paclitaxel-induced cell death in ERα-positive MCF7 cells, which are paclitaxel-resistant. Overall, our study provides the first evidence that CAC1, associated with LSD1, functions as an ERα corepressor, implicating a potential antitumor target in ERα-positive breast cancer.

Structured summary of protein interactions:
ER-alpha physically interacts with **CAC1** by anti tag coimmunoprecipitation (View Interaction: 1, 2, 3)
LSD1 physically interacts with **CAC1** by anti tag coimmunoprecipitation (View interaction)
CAC1 binds to **ER-alpha** by pull down (View interaction)
CAC1 and **ER-alpha** colocalize by fluorescence microscopy (View interaction)

1. Introduction

Estrogen receptor alpha (ERα) belongs to the nuclear hormone receptor (NR) superfamily and mediates the effects of the steroid hormone estrogen 17β-estradiol (E2) as a ligand-dependent transcription factor [1]. ERα is involved in a diverse range of physiological processes in various tissues, including growth, differentiation, and reproduction, and plays an important role in the development and progression of breast cancer [2]. Two ER subtypes, ERα and ERβ, have been identified. The subtypes are encoded by different genes and regulate the expression of different target genes [3]. However, they possess a modular structure common to most NRs consisting of an N-terminal, ligand-independent transcriptional activation function 1 (AF-1), a conserved DNA-binding domain (DBD)-containing zinc-finger motif, a hinge region, and a C-terminal ligand-binding domain that overlaps with a second transcriptional activation function 2 (AF-2) [4]. E2-bound ERα forms homodimers and binds to estrogen response elements (EREs) in the promoter region of target genes and regulates gene expression by recruiting various coregulators, such as general transcription factors, coactivators, corepressors, and histone modifiers [5].

Coactivators are required for ERα transcriptional activation, including steroid receptor coactivator 1–3 (SRC1–3; a p160 family member), CREB-binding protein (CBP)/p300 and PCAF [histone acetyltransferases (HATs)], and coactivator-associated arginine methyltransferase 1 (CARM1) [6]. NR corepressors [e.g., nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT)] usually bind receptors in the absence of ligand and actively repress target gene transcription by recruiting large repressor complexes, including histone deacetylases (HDACs) and nucleosome remodeling complex NURD [7,8]. Different from other NRs, antagonist-loaded ERα binds to target gene EREs and recruits NCoR and SMRT corepressor complexes for transcriptional repression [9,10]. While the ERα coactivator molecular mechanism is well known, the role of ligand-independent corepressors other than NCoR and SMRT remains unclear.

Abbreviations: CAC1, CDK2-associated cullin; ERα, estrogen receptor alpha; LSD1, lysine specific demethylase 1; GST, glutathione S-transferase; β-gal, β-galactosidase; GFP, green fluorescent protein; ERE, ER-responsive element

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Here, we identified a novel ERα-interacting protein, CDK2-associated cullin (CAC1), as a ligand-independent ERα corepressor. In this study, we demonstrated that CAC1 directly binds to ERα and suppresses ERα-mediated transcriptional activity in the absence or presence of E2. CAC1 also interacted with LSD1 and alleviated the ERα coactivator function. Moreover, we showed that CAC1 and LSD1 reciprocally modulate paclitaxel-mediated cell death in ERα-positive, paclitaxel-resistant MCF7 cells. These data suggest that CAC1 negatively regulates ERα activity and provides a beneficial means of chemotherapy for paclitaxel-resistant breast cancers.

2. Materials and methods

2.1. Plasmids and cloning

All cDNAs were constructed according to standard methods and verified by sequencing. CAC1 cDNA full-length and deletion mutants were amplified by PCR and subcloned into suitable vectors [Myc-tagged pcDNA3, pEGFP-C3 (BD Biosciences), pGEX4T-1 (GE Healthcare)].

2.2. Cell lines and cell culture

H1299 cells were incubated in RPMI-1640 medium, and MCF7 cells were incubated in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and an antibiotic–antimycotic mix (all from Invitrogen) in a 5% CO_2 atmosphere at 37 °C. For E2 (Sigma) treatment, FBS was pretreated with charcoal.

2.3. Glutathione S-transferase (GST) pull-down assay

GST-fused CAC1 protein was expressed in *E. coli* and purified on glutathione–Sepharose beads (GE Healthcare) by standard methods. ERα protein was translated in vitro using pSG5-ERα and a TNT® rabbit reticulocyte system (Promega). GST-CAC1 (2 μg) was mixed with 10 μl of in vitro-translated ERα protein. Bound proteins were detected by Western blotting (WB) using an anti-ERα antibody (Santa Cruz Biotechnology).

2.4. Immunofluorescence microscopy

The experimental procedures were carried out as described previously [11]. H1299 cells were seeded on culture chamber and transfected with GFP-ERα and Myc-tagged CAC1. Cells were visualized by immunofluorescence microscopy (Carl Zeiss).

2.5. Transient transfection and luciferase reporter assay

H1299 or MCF7 cells were seeded in a 6-well culture plate and transiently transfected with ERE(3×)-luciferase reporter and SV40-driven-galactosidase (β-gal) expression vector. Depending on the experimental conditions, ERα, CAC1, or LSD1 expression vector was co-transfected using Lipofectamine with Plus Reagent (Invitrogen). Luciferase activity was measured with a luminometer according to the manufacturer's instructions (Promega) and normalized to β-gal activity.

2.6. Immunoprecipitation (IP) and WB

IP and WB were performed as previously described [12]. Briefly, lysates from transfected cells were incubated overnight at 4 °C with a 1:200 dilution of the indicated antibodies. After 2 h of incubation at 4 °C with A/G-agarose beads (Santa Cruz Biotechnology), immune complexes were washed, released from the beads by boiling, and then analyzed by WB using the indicated antibodies. For WB, proteins were separated by electrophoresis on 8–10% sodium

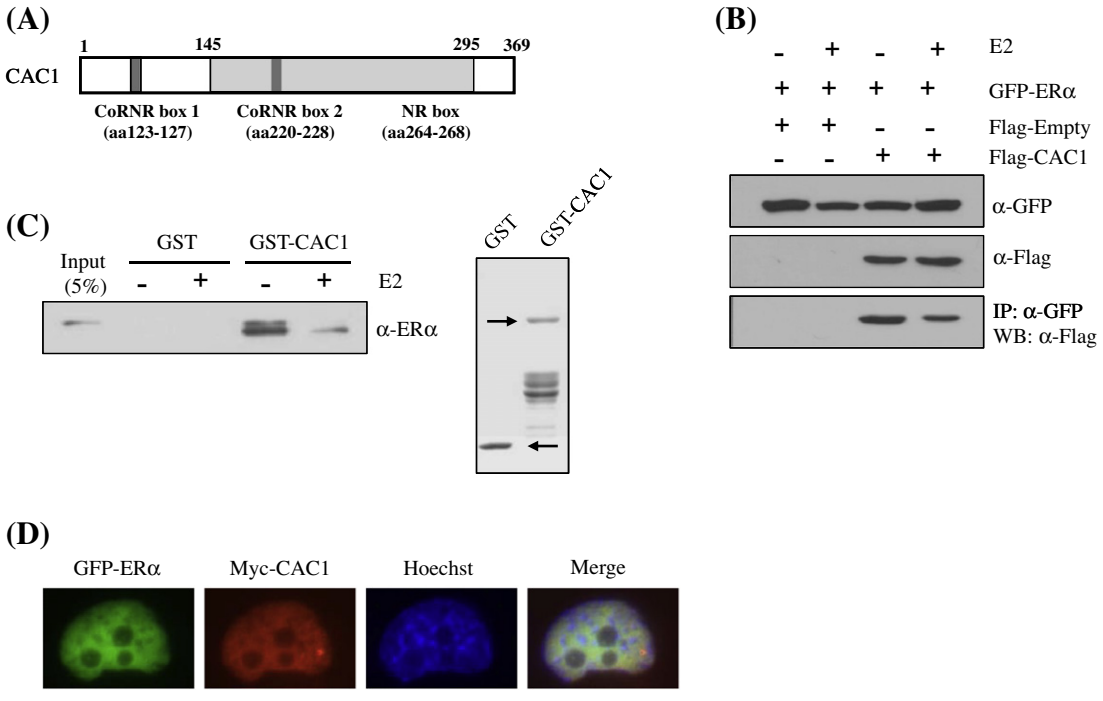

Fig. 1. CAC1 interacts with ERα. (A) Structural features of CAC1. CoRNR box, corepressor binding motif (L/IXXI/VI); NR box, coactivator binding motif (LXXLL); Cullin, homologous Cullin domain (amino acid residues 145–295). (B) Immunoprecipitation (IP) assay. H1299 cells were co-transfected with GFP-fused ERα and Flag-empty or Flag-tagged CAC1 in the absence or presence of 100 nM E2. Lysates were subjected to IP using anti-GFP, and bound protein was observed by Western blotting (WB) with an anti-Flag antibody. (C) GST-pull down assay. In vitro translated ERα was incubated with GST or GST-CAC1 (shown by arrows in right panel) in the absence or presence of 1 μM E2. The bound proteins were examined by subsequent WB using anti-ERα antibody. (D) Immunofluorescence microscopy. H1299 cells were transfected with GFP-ERα and Myc-CAC1. CAC1 cellular location was determined using rhodamine-conjugated anti-mouse IgG. Nuclei were visualized using Hoechst staining.

dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with the following primary antibodies (ERα, sc-7207; GFP, sc-8334; β-actin, sc-47778, Santa Cruz Biotechnology; Flag M2, F3165, Sigma). The blots were then incubated with peroxidase-conjugated mouse or rabbit IgG secondary antibodies (Santa Cruz Biotechnology). The protein bands were detected with an enhanced chemiluminescence (ECL) system (GE Healthcare).

2.7. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

The experimental procedures were performed as previously described [13]. Total RNA from MCF7 cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA (5 μg) was reverse-transcribed using Superscript II reverse transcriptase (RT; Invitrogen) and random oligo (dT) primers (Invitrogen). RT products were amplified using the following primer pair for the *TFF1* coding sequence (133 bp): forward, 5′-CCA TGGAGAACAAGGTGATCTG-3′ and reverse, 5′-GTGACACCAGGAAAA CCACAATT-3′, and analyzed by the iQ™ SYBR Green Supermix and Icycler CFX96 Real-Time PCR detection system (Bio-Rad). All gene expression levels were normalized using *GAPDH* as an internal standard in each well. Fold-expression was defined as a fold increase relative to controls.

2.8. Chromatin immunoprecipitation (ChIP)

ChIP analysis was conducted as previously described [13]. MCF7 cells were transfected with a Flag-empty or a Flag-CAC1 vector and treated with or without 1 μM E2 for 30 min. Cross-linked protein–chromatin complexes were recovered by IP with the indicated antibodies, and the cross-linking was reversed according to Upstate's protocol (Millipore). DNA pellets were recovered and analyzed by PCR using a primer pair directed at the *TFF1* promoter region: forward, 5′-GGCCATCTCTCACTATGAATC-3′ and reverse, 5′-GGCAGGCTCTGTTTGCTTAAA-3′.

2.9. RNA interference (RNAi)

The CAC1 shRNA duplex sequences were as follows: sense, 5′-GATCCATGTGTATGCCAGCAGCACTTCAAGAGAGTGCTGCTGGCATAC ACATTTTTTTGGAAA-3′ and antisense, 5′-AGCTTTTCCAAAAAAATG TGTATGCCAGCAGCACTCTC TTGAAGTGCTGCTGGCATACACATG-3′.

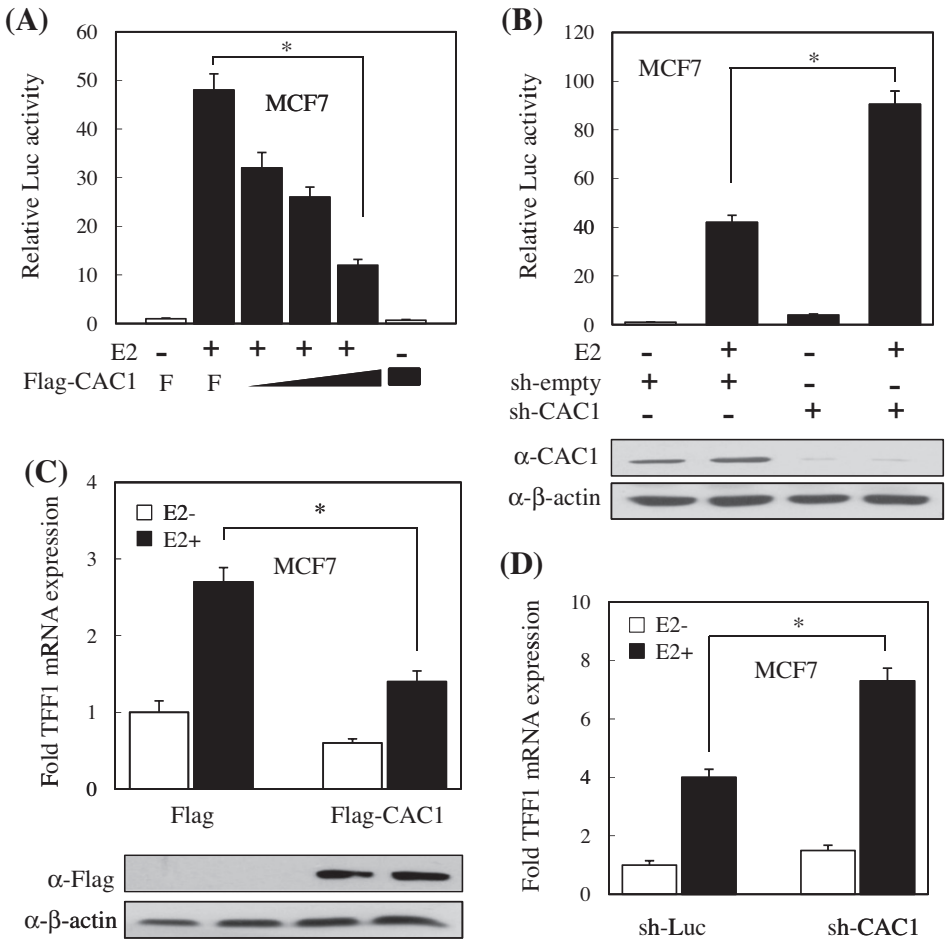

Fig. 2. CAC1 is an ERα corepressor. (A, B) Effect of CAC1 overexpression (A) or depletion (B) on ERα-driven luciferase activity. MCF7 cells were transiently transfected with an ERE-luciferase reporter and increasing amounts of Flag-CAC1 (0, 0.5, 1, or 1.5 μg) in the presence of 100 nM E2. Flag-empty (F) was used for control. Either a sh-empty (control; 2 μg) or a sh-CAC1 (2 μg) expression vector was transfected into MCF7 cells. CAC1 knockdown was monitored by WB using an anti-CAC1 antibody (B, bottom panel). Transfected cell extracts were analyzed by luciferase (Luc) activity assays. Relative Luc activity is shown as the mean ± S.D. of three independent experiments after normalization to β-galactosidase (gal) activity. (C, D) Effect of CAC1 over-expression (C) or depletion (D) on the expression of the endogenous ERα target gene, *TFF1*. MCF7 cells were transfected with Flag-empty, Flag-CAC1, sh-Luc, or sh-CAC1 (2 μg) in the absence or presence of 100 nM E2. Total RNA was extracted, reverse-transcribed, and analyzed by real-time PCR. Fold-increases in *TFF1* mRNA expression were normalized to *GAPDH* RNA expression levels. Data represent the mean ± S.D. of three independent experiments. In all figures, *P* values are less than 0.01 (*).

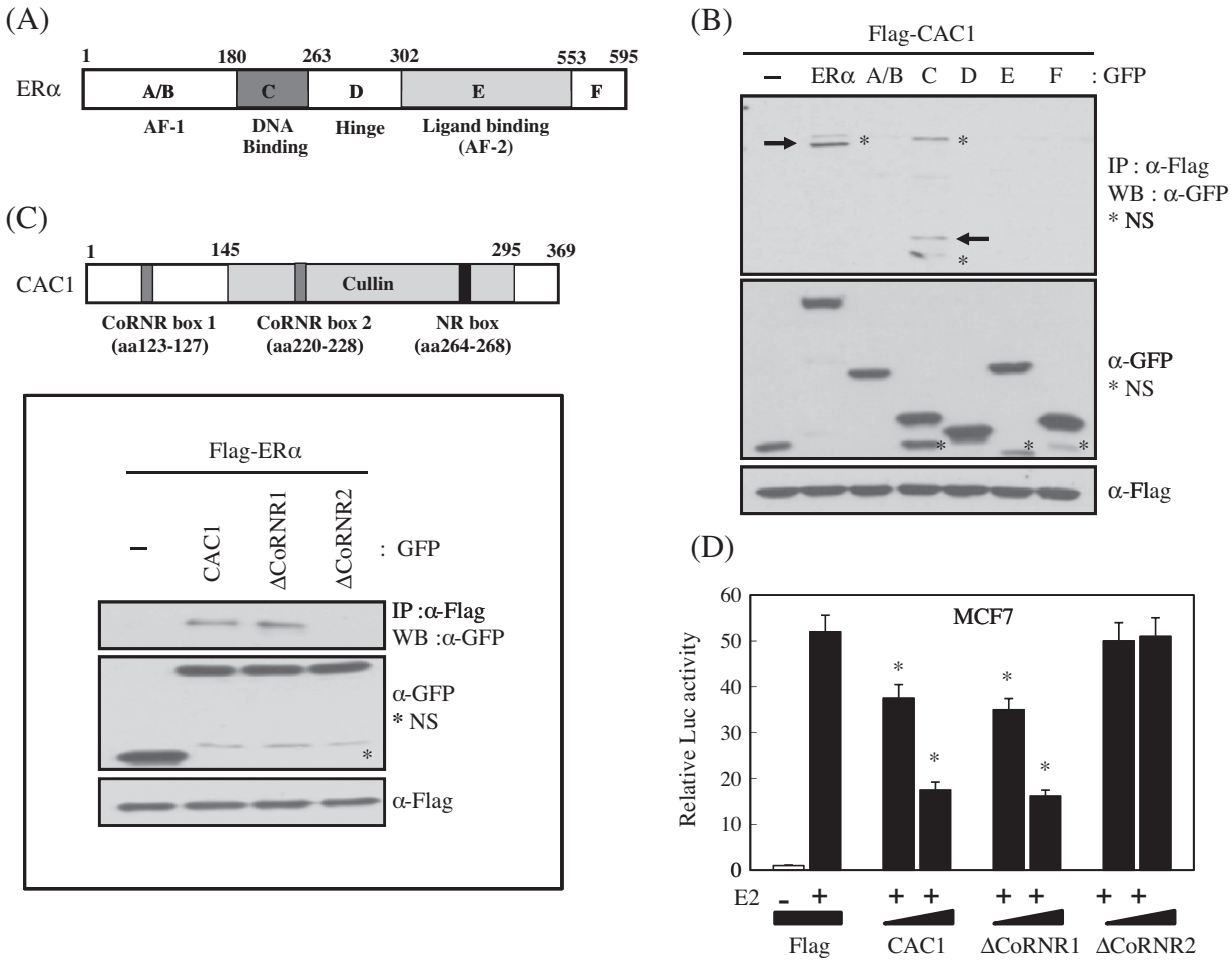

Fig. 3. Mapping of the domain responsible for the interaction between CAC1 and ERα. (A) Schematic representation of ERα and its functional domains. (B) Mapping of ERα domain responsible for CAC1 binding. H1299 cells were transfected with Flag-CAC1 and GFP-ERα full-length or each deletion mutant. Cell extracts were subjected to IP with anti-Flag antibody and precipitated protein was detected by WB using an anti-GFP antibody. NS (*) stands for non-specific protein band. (C) Identification of the CAC1 region responsible for ERα binding. Schematic representations of CAC1 and its putative motifs are shown. H1299 cells were transfected with Flag-ERα and GFP-CAC1 wild-type or two mutants with a deletion in the CoRNR motif. Interaction was monitored by IP with an anti-Flag antibody and WB using an anti-GFP antibody. (D) Effect of CAC1 CoRNR box on ERα repression. MCF7 cells were transiently transfected with an ERE-luciferase reporter and increasing amounts of Flag-CAC1 wild-type or Flag-CAC1 with a deletion in CoRNR box 1 or 2 (0.5 or 1.0 μg) in the presence of 100 nM E2. Data represent the mean ± S.D. of three independent experiments. $P < 0.01$ (*).

The shRNA transfection was performed with Lipofectamine 2000 (Gibco-BRL) according to the manufacturer's instructions. CAC1 depletion was verified by WB using an anti-CAC1 antibody (Peptron).

2.10. Trypan blue exclusion assay

The trypan blue exclusion assay was carried out as previously described [14]. MCF7 cells were transfected with a CAC1 and/or LSD1 expression vector and were treated with 50 nM paclitaxel (Sigma) for 36 h. Cells were treated with 0.4% trypan blue and the number of cells (viable, unstained and non-viable, blue) was counted.

3. Results and discussion

3.1. CAC1 interacts with ERα in a ligand-independent manner

It has been reported that CAC1 is a CDK2-assocaited cullin [15], but its biological role is unclear. CAC1 sequence analysis revealed that it contains two putative motifs: LMNVI and LQSIVPLFI, called the CoRNR box (L/IXXI/VI), and LMPLL, called the NR box (LXXLL) (Fig. 1A). These motifs, responsible for NR interaction, are present in corepressors and coactivators, respectively [16,17]. As CAC1 is highly expressed in ERα-positive MCF7 breast cancer cells [15], we examined whether CAC1 physically interacts with ERα. GFP-tagged ERα was co-transfected with Flag empty or Flag-tagged CAC1 in H1299 cells. IP with an anti-GFP antibody and subsequent WB with an anti-Flag antibody indicated that CAC1 interacts with ERα in the absence of the ligand E2 (Fig. 1B), whereas CAC1 binding to ERα was diminished in the presence of E2. The E2-dependent reduced interaction was further confirmed by a GST pull-down assay in vitro (Fig. 1C). To further substantiate the interaction, we explored the subcellular distribution of CAC1 and ERα in H1299 cells. Immunofluorescence microscopy revealed that Myc-CAC1 and GFP-ERα co-localized in the nucleus (Fig. 1D). Overall, these results demonstrate that CAC1 physically associates with ERα in a ligand-independent manner. This mode of CAC1 interaction is distinct from not only ERα coactivators that bind in the presence of ligand, but also from ERα corepressors (e.g., NCoR and SMRT) that interact with antagonist-bound ERα. Indeed, the CAC1-ERα interaction was greatly impaired in the presence of ERα antagonist, tamoxifen

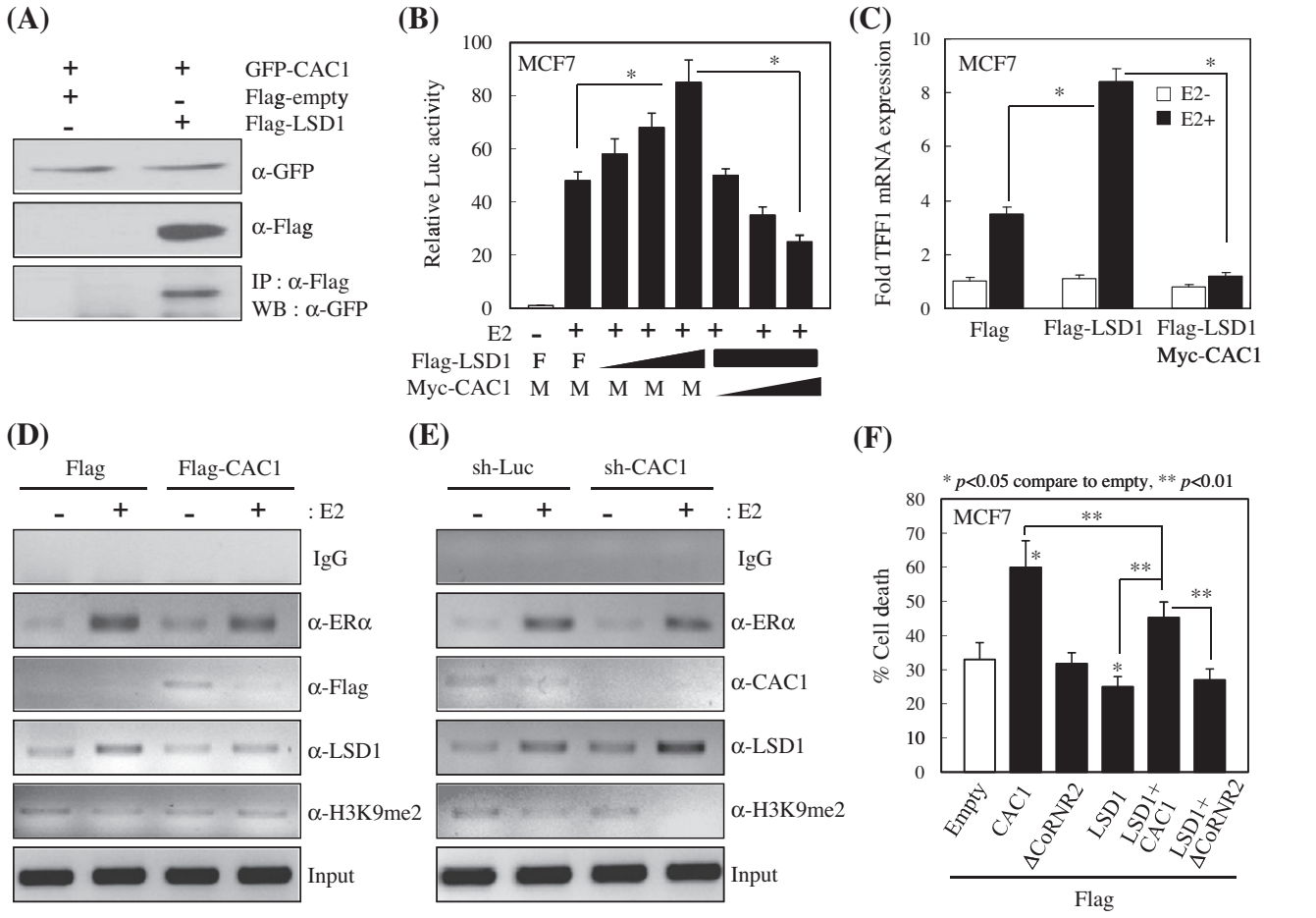

Fig. 4. CAC1 suppresses LSD1-enhanced ERα activity. (A) Interaction between CAC1 and LSD1. H1299 cells were co-transfected with Flag-LSD1 and GFP-empty or GFP-CAC1. Interaction was determined by IP with an anti-Flag antibody and subsequent WB using an anti-GFP antibody. (B) Effect of CAC1 on LSD1-enhanced luciferase activity mediated by ERα. MCF7 cells were co-transfected with increasing amounts of LSD1 or CAC1 (0, 0.5, 1, or 1.5 μg) together with an ERE-luciferase reporter in the presence of 100 nM E2. Flag-empty (F) and Myc-empty (M) were used for controls. (C) Effect of CAC1 on the expression of the LSD1-induced ERα target gene, *TFF1*. MCF7 cells were transfected with Flag-empty, Flag-LSD1, or Flag-LSD1 plus Myc-CAC1 (1 μg) in the absence or presence of 100 nM E2. (D, E) Effect of CAC1 over-expression (D) or knockdown (E) on ERα and LSD1 binding to ER-responsive chromatin DNA. MCF7 cells were transfected as indicated and treated with 100 nM E2 for 30 min. ChIP assays were performed using the indicated antibodies and a primer pair that covered the *TFF1* promoter ERE. (F) Effect of CAC1 on paclitaxel-induced cell death. CAC1, CAC1 lacking CoRNR box 2, and LSD1 were over-expressed in MCF7 cells. Transfected cells were then treated with 50 nM paclitaxel. Cell death was determined by the trypan blue exclusion assay. Data shown in (B), (C), and (F) represent the mean ± S.D. of triplicate experiments. $P < 0.05$ (*) or 0.01 (**).

(Supplementary Fig. 1). Therefore, we assume that CAC1 is the first identified ligand-independent ERα-binding partner, which is distinct from known ERα coregulators.

3.2. CAC1 represses ERα transcriptional activity

To determine the functional relevance of the interaction between CAC1 and ERα, we monitored the effect of CAC1 on ERα transcriptional activity. ERα-positive MCF7 cells were co-transfected with an ERE-luciferase reporter and CAC1 in the presence of E2. As shown in Fig. 2A, CAC1 greatly repressed ERα-mediated transcriptional activity in a concentration-dependent manner. In contrast, CAC1 depletion by shRNA treatment resulted in markedly increased ERα transcriptional activity (Fig. 2B). Consistent with our interaction data, CAC1 knockdown also increased ERα activity in the absence of E2. Next, we analyzed the effect of CAC1 on the endogenous expression of the ERα target, *TFF1* (*pS2*), in MCF7 cells using real-time RT-PCR. The E2-induced *TFF1* gene mRNA expression was significantly reduced by CAC1 overexpression (Fig. 2C) and augmented following CAC1 knockdown (Fig. 2D). We also observed a similar effect of CAC1 on the expression of another ERα target gene, *Cathepsin D* (Supplementary Fig. 2). These data, together with our binding data, suggest that CAC1 is a novel type of ERα corepressor.

3.3. CAC1 CoRNR box is required for ERα inactivation

To map the domain responsible for ERα and CAC1 interaction, we first constructed ERα deletion mutants fused to GFP (Fig. 3A). H1299 cells were co-transfected with Flag-CAC1 and GFP or GFP-ERα deletion mutants. Subsequent IP assay indicated that the ERα DBD was responsible for CAC1 binding (Fig. 3B). Furthermore, we generated truncated CAC1 mutants, which contained three putative motifs (CoRNR1, CoRNR2, and NR box; Fig. 3C). Initial IP analysis revealed that the CAC1 fragment covering CoRNR1 and CoRNR2 (amino acids 1–260) was sufficient, but the fragment including the NR box (amino acids 261–369) was not required for ERα binding (data not shown). Therefore, further IP assays were performed to determine whether the CoRNR box was required for the interaction. As shown in Fig. 3C, CAC1 lacking CoRNR1 remained bound to ERα, whereas the CoRNR2 mutant did not bind ERα, suggesting that CAC1 CoRNR2 is critical for its interaction with ERα. Subsequently, the role of CoRNR2 in ERα inactivation was investigated using an ERE-luciferase reporter. Data shown in

Fig. 3D indicated that CAC1 CoRNR2 but not CoRNR1, was required to suppress ERα transcriptional activity. Overall, our data suggest that CAC1 represses ERα activity by associating through the CoRNR2 box.

3.4. CAC1 inhibits LSD1-enhanced ERα activity

Histone demethylase LSD1, associated with ERα, permits E2-dependent gene activation by removing a methyl group from methylated H3K9 (H3K9me), a repressive histone mark [18]. To investigate the possibility that CAC1 is involved in LSD1-mediated activation of ERα, we first addressed whether CAC1 interacts with LSD1. Co-transfection followed by IP demonstrated the interaction between CAC1 and LSD1 (Fig. 4A). In addition, a luciferase assay showed that CAC1 strongly suppressed LSD1-enhanced transcriptional activity of ERα (Fig. 4B). This result was confirmed by real-time RT-PCR using *TFF1* gene (Fig. 4C). To explore the molecular mechanism underlying the reciprocal roles of CAC1 and LSD1 in ERα activation, we performed ChIP analysis using the ER-responsive *TFF1* promoter under conditions of CAC1 overexpression or depletion in MCF7 cells. The E2-dependent binding of ERα and LSD1 to the promoter diminished, whereas H3K9 dimethylation (H3K9me2) increased due to CAC1 overexpression (Fig. 4D). As expected, CAC1 bound to the *TFF1* promoter in the absence of E2 and the occupancy decreased in the presence of E2. In contrast, CAC1 knockdown increased the binding occupancies of ERα and LSD1, leading to significantly lower H3K9me2 accumulation (Fig. 4E). These results were further quantitated by real-time PCR under overexpression and knockdown conditions of CAC1 (Supplementary Fig. 3). No enrichments of ERα, CAC1, and LSD1 were detected at the distal region of TFF1 promoter (Supplementary Fig. 4). In addition, a similar binding or enrichment pattern was observed when the promoter of GREB1, another ERα target gene, was used as the PCR template (Supplementary Fig. 5). Taken together, these data suggest that CAC1 inhibits LSD1 binding to the ERα target promoter and increases the level of repressive histone mark H3K9me2, resulting in suppressed ERα activity.

3.5. CAC1 sensitizes paclitaxel-induced cell death

Data from clinical trials or retrospective analyses indicate that ER status might affect the efficacy of chemotherapy [19]. Specifically, ERα-expressing MCF7 breast cancer cells are highly resistant to paclitaxel compared to ERα-negative breast cancer cells [20]. Thus, we determined the effect of CAC1 on paclitaxel resistance in MCF7 cells by measuring paclitaxel-induced cell death. As shown in Fig. 4F, CAC1 overexpression increased paclitaxel-induced cell death, while the ERα-binding defective CAC1 mutant (ΔCoRNR) was unaffected. In addition, LSD1 overexpression augmented paclitaxel resistance compared to control cells. Again, co-transfection of LSD1 and CAC1 rescued paclitaxel sensitivity to the level of control cells, whereas CAC1 mutant ΔCoRNR was defective for this rescue, highlighting the requirement of CAC1 binding to ERα for paclitaxel sensitivity. These data support that CAC1, as an ERα corepressor, may be important in breast cancer therapy by increasing paclitaxel sensitivity.

In summary, we identified a novel class of ERα corepressor called CAC1. Our data indicated that CAC1 interacts with the ERα DBD and inhibits ERα transcriptional activity. The CoRNR box of CAC1 is critical for ERα binding and inactivation. Interestingly, CAC1 also binds to histone demethylase LSD1, a known ERα coactivator, and represses LSD1-enhanced ERα activity. This repression may result from the reduced recruitment of LSD1 to the ERα-responsive gene promoter by LSD1–CAC1 interaction. Finally, we observed that CAC1 increases paclitaxel sensitivity in MCF7 cells, which is paclitaxel resistant, due to ERα activation. From our data, we speculate that CAC1, a novel class of ERα corepressor, may be a useful target in breast cancer therapy by its increasing paclitaxel sensitivity.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.10.054.

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