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Reciprocal roles of DBC1 and SIRT1 in regulating estrogen receptor α activity and co-activator synergy

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ABSTRACT

Estrogen receptor α (ERα) plays critical roles in development and progression of breast cancer. Because ERα activity is strictly dependent upon the interaction with coregulators, coregulators are also believed to contribute to breast tumorigenesis. Cell Cycle and Apoptosis Regulator 1 (CCAR1) is an important co-activator for estrogen-induced gene expression and estrogen-dependent growth of breast cancer cells. Here, we identified Deleted in Breast Cancer 1 (DBC1) as a CCAR1 binding protein. DBC1 was recently shown to function as a negative regulator of the NAD-dependent protein deacetylase SIRT1. DBC1 associates directly with ERα and cooperates synergistically with CCAR1 to enhance ERα function. DBC1 is required for estrogen-induced expression of a subset of ERα target genes as well as breast cancer cell proliferation and for estrogen-induced recruitment of ERα to the target promoters in a gene-specific manner. The mechanism of DBC1 action involves inhibition of SIRT1 interaction with ERα and of SIRT1-mediated deacetylation of ERα. SIRT1 also represses the co-activator synergy between DBC1 and CCAR1 by binding to DBC1 and disrupting its interaction with CCAR1. Our results indicate that DBC1 and SIRT1 play reciprocal roles as major regulators of ERα activity, by regulating DNA binding by ERα and by regulating co-activator synergy.

INTRODUCTION

Estrogen receptor α (ERα) is a member of the superfamily of nuclear receptors (NRs), and it functions as a ligand-dependent transcription factor that mediates the diverse biological effects of estrogens, including development, maintenance of female reproductive functions and the etiology of breast cancer (1). NRs consist of a variable N-terminal region, a DNA binding domain (DBD), a hinge region and a conserved ligand binding domain (LBD). ERα and other NRs bind to hormone response elements in their target promoters and regulate the expression of a variety of target genes through the recruitment of co-regulators (co-activators and co-repressors) that mediate local chromatin remodeling as well as communications with the RNA polymerase II (Pol II)-associated basal transcription machinery (2). The p160 co-activators (e.g. SRC-1, GRIP1 and AIB1) interact directly with hormone-activated NRs and serve as protein scaffolds for the assembly of multicomponent co-activator complexes on target promoters. p160 co-activators recruit secondary co-activators, including histone acetyltransferase p300/CBP, histone methyltransferase CARM1 and CoCoA, and act synergistically with secondary co-activators to enhance NR function (2,3). Mediator,

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another multisubunit co-activator complex, is believed to act as a molecular bridge between NRs and Pol II-associated basal transcription machinery (4). Recently, we identified cell cycle and apoptosis regulator 1 (CCAR1) as a CoCoA binding protein (5). CCAR1 interacts with ERα and cooperates synergistically with components of the p160 co-activator complex. CCAR1 is important for estrogen-induced expression of ERα target genes and estrogen-dependent growth of breast cancer cells. CCAR1 associates with components of the Mediator complex and facilitates recruitment of Mediator complex to the promoter of target genes by providing a physical link between p160 co-activator and Mediator complexes. In addition, CCAR1 binds to and cooperates synergistically with β-catenin as a secondary co-activator for LEF1 (6). Thus, CCAR1 is a physiologically relevant part of several transcriptional activation processes.

In addition to co-regulators, post-translational modifications are also crucial for the regulation of NR function such as DNA binding, interaction with co-regulators, stability and subcellular localization (7). For example, ERα is acetylated by p300 at several lysine residues in the hinge region, and the acetylation enhances DNA binding and *trans*-activation activities of ERα (8). Although, a previous report suggested that the acetylation of ERα is reversed by cellular deacetylases, including Trichostatin A (TSA)-sensitive enzymes (classes I and II deacetylases) and nicotinamide-sensitive enzymes (class III deacetylases such as SIRT1), their roles in ERα-mediated transcription is still largely unknown.

To further characterize the mechanism by which CCAR1 contributes to transcriptional activation, we used a biochemical approach to identify CCAR1-associated proteins and found deleted in breast cancer 1 (DBC1; also known as KIAA1967 and p30 DBC) as a CCAR1-interacting protein. DBC1 was originally cloned from a region 8p21 that was homozygously deleted in breast cancer (9,10). Recently, DBC1 has been reported as a negative regulator of SIRT1 (11,12). DBC1 interacts with the catalytic domain of SIRT1, inhibits its deacetylase activity, and then increases acetylation levels of p53, thereby enhancing p53-mediated apoptosis. However, despite the tumor suppressor function of DBC1, several studies reported increased expression of DBC1 in breast carcinoma (13,14), suggesting that DBC1 may participate in the development and progression of breast cancer. In this study, we show that DBC1 associates and cooperates synergistically with CCAR1 to enhance ERα function. DBC1 is required for the expression of a subset of ERα target genes and estrogen-dependent growth of breast cancer cells. SIRT1 deacetylates ERα, causing reduced DNA binding. DBC1 inhibits SIRT1-mediated deacetylation and restores optimal recruitment of ERα to target gene promoters. SIRT1 also represses the co-activator synergy between CCAR1 and DBC1 by competing with CCAR1 for binding to DBC1 and thereby disrupting the DBC1–CCAR1 interaction. Collectively, our results suggest that DBC1 and SIRT1 play reciprocal roles in the regulation of ERα activity and co-activator synergy.

MATERIALS AND METHODS

Isolation of DBC1

HeLa S cells were infected with lentiviruses expressing FLAG-tagged CCAR1 and selected with hygromycin. A stable cell line expressing FLAG-CCAR1 (HeLa S-FLAG-CCAR1) was grown in spinner culture in DMEM supplemented with 10% bovine calf serum. Preparation of nuclear extracts and M2-agarose affinity chromatography were performed as described previously (15). After extensive washings, the bound proteins were eluted using 3× FLAG peptide. The purified CCAR1-associated proteins were identified by mass spectrometry performed at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School.

Protein interaction assays and immunoblot

For GST pull-down assays, HA or V5 epitope-tagged proteins were synthesized *in vitro* by using TNT-Quick coupled transcription/translation system (Promega) and incubated with immobilized GST-fusion proteins. After washing, bound proteins were analyzed by immunoblot with anti-HA or anti-V5 antibody. For co-immunoprecipitation (CoIP) assays, COS-7, 293T or MCF-7 cell extracts were immunoprecipitated by specific antibodies or control IgG and protein G Dynabeads (Invitrogen) as indicated in figure legends.

Acetylation and deacetylation assays

Expression plasmids for GST-ER-V5/His, HA-p300, FLAG-SIRT1 and HA-DBC1 were transfected into 293T cells as indicated in the figure legends. After 36 h of transfection, the cells were treated with 0.5 μM TSA (Sigma) and 100 nM E2 for 12 h and then lysed in FLAG lysis buffer (50 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton-X 100, 0.2% Sarkosyl, 10% glycerol) supplemented with 0.5 μM TSA and protease inhibitor cocktail (Roche). ERα was immunoprecipitated with anti-ERα antibody, and acetylation levels were determined by immunoblot with anti-acetyl lysine antibody. *In vitro* deacetylation assays were performed according to the procedure described previously (11,12). 293 T cells were transiently transfected with FLAG-tagged expression plasmids for SIRT1 and DBC1. Transfected cells were lysed in BC500 buffer (20 mM Tris–HCl pH 7.6, 500 mM NaCl, 0.2 mM EDTA, 0.5% Triton X-100, 10% glycerol and protease inhibitor cocktail), and the cell lysates were immunoprecipitated with anti-FLAG M2 agarose beads. After three washes with BC500, followed by two washes with BC100 (20 mM Tris-HCl pH 7.6, 100 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 10% glycerol and protease inhibitor cocktail), the bound proteins were eluted with 3× FLAG peptide in BC100. FLAG-tagged ERα and HA-tagged p300 were transiently transfected in 293 T cells. After 48 h of transfection, cells were treated with 100 nM E2 and 0.5 μM TSA for 4 h and lysed in FLAG lysis buffer. The whole-cell lysates were immunoprecipitated with anti-FLAG M2 agarose beads, and the beads were washed with FLAG lysis buffer,

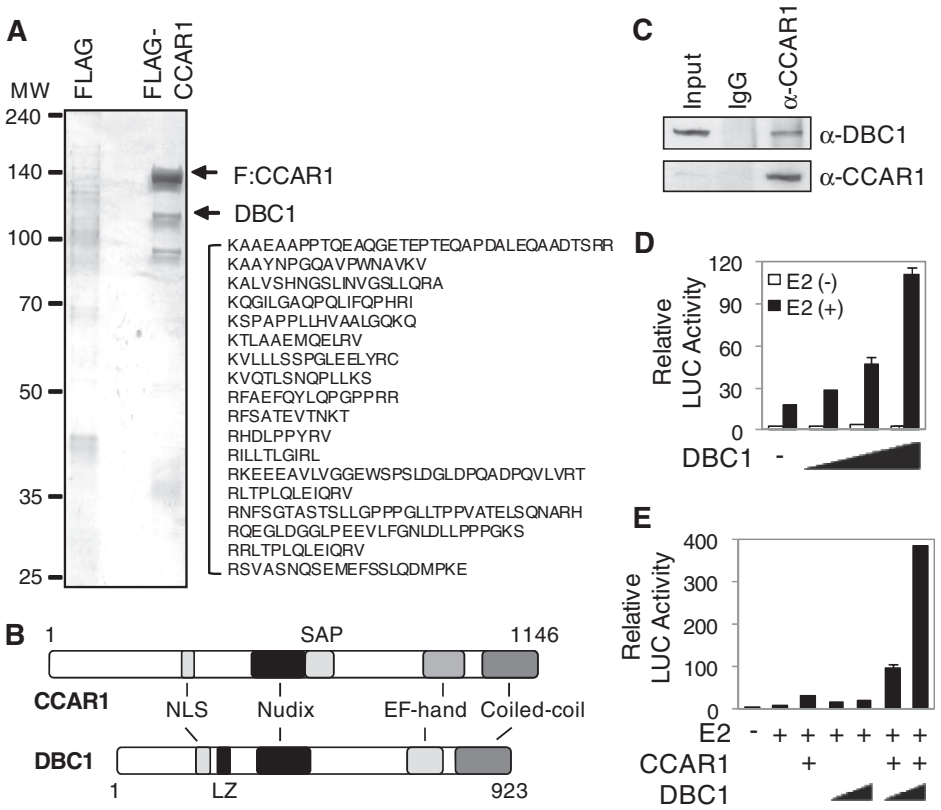

Figure 1. DBC1 interacts with CCAR1 and functions as an ERα co-activator. (**A**) Nuclear extracts from HeLa S cells stably expressing FLAG-CCAR1 were immunoprecipitated with anti-FLAG M2 agarose. Bound proteins were eluted with FLAG peptide, resolved on 4%–12% gradient Bis-Tris gels, silver-stained, and analyzed by mass spectrometry. The identified peptides of DBC1 are listed. (**B**) Schematic representation of DBC1 and CCAR1 structures. NLS, nuclear localization signal; LZ, leucine zipper motif; SAP, SAP domain; Nudix, Nudix hydrolase domain. (**C**) Endogenous interaction between DBC1 and CCAR1. MCF-7 cell lysates were immunoprecipitated with anti-CCAR1 antibody or normal rabbit IgG, followed by immunoblot with the indicated antibodies. (**D**) CV-1 cells were transfected with pHE0 encoding ERα (2 ng) and MMTV(ERE)-LUC reporter in combination with various amounts (200, 400 and 800 ng) of pSG5.HA-DBC1 and grown in medium containing or lacking 100 nM estradiol (E2) before conducting luciferase assays on cell extracts. Data are means ± SD ($n = 3$). (**E**) Synergy between CCAR1 and DBC1. Transient transfections were performed as described in **D**. Expression vectors: pSG5.HA-CCAR1 (200 ng), pSG5.HA-DBC1 (200 and 400 ng). Data are means ± SD ($n = 3$).

followed by two washes with BC100. The bound acetylated ERα was then eluted with 3× FLAG peptide and incubated with purified SIRT1 and DBC1 in SIRT1 reaction buffer (50 mM Tris–HCl pH 8.8, 50 mM NaCl, 4 mM $MgCl_2$, 0.5 mM DTT, 0.02% NP-40, 5% glycerol and 1 mM NAD^+) for 3 h at 30°C. The reactions were analyzed by immunoblot with anti-acetyl lysine antibody.

Plasmids and antibodies, cell culture and transient transfection, MTT and flow cytometric analysis, chromatin immunoprecipitation (ChIP) assays, RNA interference and real-time qRT–PCR, and statistical analysis are explained in detail in Supplementary Data.

RESULTS

Identification of DBC1 as a CCAR1-interacting protein

To identify proteins associated with CCAR1, we generated a HeLa cell line stably expressing FLAG-tagged CCAR1 and purified CCAR1-associated proteins from nuclear extracts using anti-FLAG M2-agarose beads. Several proteins were selectively co-purified with FLAG-CCAR1 but not with the FLAG control. We analyzed these CCAR1-associated proteins by tandem mass spectrometry and identified DBC1 as a CCAR1-associated protein (Figure 1A). Other proteins associated with CCAR1 are listed in Supplementary Table S1. DBC1 shows significant homology to CCAR1 (10,16) and, like CCAR1, has a nuclear localization signal (NLS), a Nudix domain, an EF hand, and a coiled-coil domain (Figure 1B). We next verified the association between endogenous CCAR1 and DBC1 in ER-positive MCF-7 breast cancer cells by CoIP assays. Consistent with the mass spectrometry data, endogenous DBC1 was detected in the immunoprecipitates of endogenous CCAR1 (Figure 1C). Similarly, HA-tagged CCAR1 was co-immunoprecipitated specifically with V5-tagged DBC1 from extracts of transiently transfected COS-7 cells (Supplementary Figure S1A). In addition, *in vitro* GST pull-down assays confirmed the interaction between CCAR1 and DBC1 (Supplementary Figure S1B), suggesting that CCAR1 interacts directly with DBC1.

Since DBC1 shares homology with CCAR1, which was identified as a CoCoA AD binding protein (5), we next asked whether DBC1 might also interact with CoCoA

AD. DBC1 bound to CoCoA *in vitro* and *in vivo* (Supplementary Figure S1C and S1D). In addition, DBC1 specifically interacted with CoCoA AD but not with the central coiled-coil or N-terminal domains of CoCoA (Supplementary Figure S1E). Since a previous study showed that ERα interacts with DBC1 (17), we confirmed the interaction between ERα and DBC1. DBC1 bound to ERα in a hormone-independent manner in GST pull-down assays and in CoIP experiments performed with both overexpressed and endogenous proteins (Supplementary Figure S1F–S1H). Taken together, these findings demonstrate that DBC1 can interact with CCAR1, ERα and CoCoA.

DBC1 functions as a NR co-activator and cooperates synergistically with CCAR1

Since DBC1 interacts with CCAR1 and ERα, we tested whether DBC1 can function as a co-activator for ERα. In reporter gene assays, DBC1 enhanced the hormone-dependent activity of exogenous ERα in a dose-dependent manner (Figure 1D). We also performed similar reporter gene assays with androgen receptor (AR), glucocorticoid receptor (GR), thyroid hormone receptor β1 (TR) and their reporters to test the co-activator activity of DBC1 for other NRs (Supplementary Figure S2A). Consistent with a previous report (18), DBC1 enhanced the hormone-induced AR activity. DBC1 also stimulated the transcriptional activities of GR and TR in a hormone-dependent manner, suggesting that DBC1 functions as a NR co-activator. We next tested whether DBC1 and CCAR1 can function synergistically as co-activators for ERα in reporter gene assays (Figure 1E). As expected, CCAR1 and DBC1 individually enhanced the transcriptional activity of ERα. In addition, co-expression of CCAR1 and DBC1 enhanced ERα function in a synergistic manner. Similarly, DBC1 cooperated synergistically with CCAR1 to enhance endogenous ERα activity in MCF-7 and T47D cells (Supplementary Figure S2B). The transcriptional activities of AR, GR and TR were also synergistically enhanced by co-expression of CCAR1 and DBC1 (Supplementary Figure S2C). Together, these results suggest that DBC1 functions as a co-activator for multiple NRs and cooperates synergistically with CCAR1.

DBC1 is required for E2-induced expression of a subset of ERα target genes

To further assess the functional involvement of DBC1 in ERα-mediated transcription, the expression of DBC1 was reduced by RNA interference (RNAi). To avoid possible off-target effects caused by the DBC1 RNAi, we used two different siRNA sequences that target different regions of DBC1 mRNA (Supplementary Figure S3A). When DBC1 mRNA and protein levels were specifically reduced in MCF-7 cells by siRNA transfection, estradiol (E2)-induced expression of the endogenous pS2 gene was significantly inhibited compared with the results using non-specific (NS) siRNA (Supplementary Figure S3B and Figure 2A). In seven such independent experiments, we found a direct linear relationship between the levels of DBC1 and pS2 mRNAs (Supplementary Figure S3C). Similarly, E2-induced expression of a transiently transfected reporter gene in MCF-7 cells was inhibited by the DBC1-specific siRNA (siDBC1) but not by siNS (Supplementary Figure S3D). As shown in Figure 2A, depletion of DBC1 also repressed the E2-induced expression of GREB1, c-Myc, CTSD and CCND1 genes. Intriguingly, the expression of PgR was not affected under the same conditions, suggesting that DBC1 is required for the expression of a subset of ERα target genes.

To further explore the functional link between CCAR1 and DBC1, the expression of DBC1 and CCAR1 was reduced either individually or in combination using DBC1 siRNA in MCF-7 cells infected with lentivirus expressing a non-specific (shNS) or CCAR1 shRNA (shCCAR1). The protein levels of DBC1 and/or CCAR1 were specifically reduced under these experimental conditions (Figure 2B). As expected, E2-induced levels of pS2 and CTSD mRNAs were reduced by individual knockdown of DBC1 and CCAR1, and their expression was further reduced by simultaneous depletion of DBC1 and CCAR1 (Figure 2C). However, neither single nor double depletion of DBC1 and CCAR1 affected PgR gene expression. Again, these results suggest that DBC1 and CCAR1 cooperate to enhance the expression of a subset, but not all, of ERα target genes.

To assess the potential relevance of our results to the biology of breast cancer cells, we examined the effect of reduced DBC1 levels on E2-stimulated cell proliferation in MCF-7 cells infected with lentivirus expressing a shNS or DBC1 shRNA (shDBC1). E2 treatment-stimulated proliferation of MCF-7/shNS cells, but E2 stimulation of MCF-7 cell growth was attenuated by reduction of DBC1 expression (Figure 2D). To study the potential mechanistic defect underlying the observed effect on cell proliferation, the cell cycle profile was examined. As expected, E2-treatment of MCF-7/shNS cells resulted in an increase in the combined percentage of S-phase and G2/M-phase cells (from 25.7% to 42.94%). In contrast, only 33.93% of E2-stimulated MCF-7/shDBC1 cells were found in S-phase and G2/M-phase (Supplementary Figure S3E). Thus, these results suggest that DBC1 plays a critical role in E2-stimulated proliferation of breast cancer cells and in promoting cell cycle progression.

DBC1 is recruited to selective ERα target gene promoters and is required for optimal recruitment of ERα to the promoters

To examine whether DBC1 is directly involved in ERα-mediated transcription, we performed ChIP assays in MCF-7 cells (Figure 3A). ERα, AIB1, CoCoA and CCAR1 were recruited to the ER binding site associated with the pS2 gene promoter in a hormone-dependent manner, as reported previously (5). E2 treatment also led to the recruitment of DBC1 to the pS2 promoter, whereas the normal IgG control was not affected by E2. A similar recruitment pattern of these proteins was observed on ER binding sites associated with the promoters of other ERα target genes, CTSD and GREB1

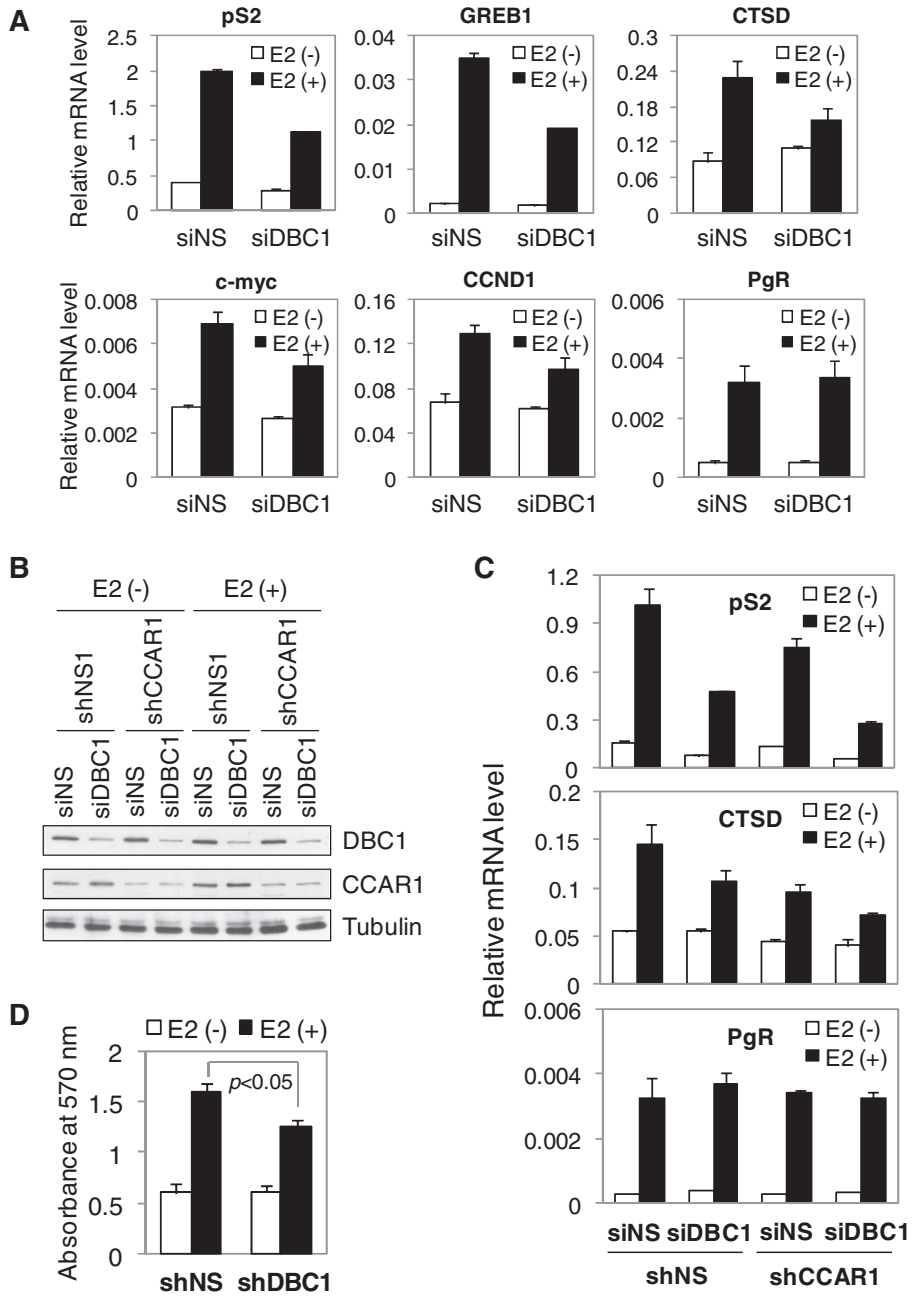

Figure 2. DBC1 is required for ERα function and estrogen-dependent growth of MCF-7 cells. (**A**) MCF-7 cells were transfected with 40 pmol of DBC1 siRNA#1 (siDBC1) or NS siRNA duplex. After 48 h transfection, cells were treated with 100 nM E2 or untreated and harvested after an additional 24 h. Total RNA was examined by real-time qRT–PCR analysis with primers specific for the indicated mRNAs. Results shown were normalized to β-actin mRNA levels and are means ± SD ($n = 3$). (**B** and **C**) MCF-7 cells infected with lentiviruses encoding a NS or CCAR1 shRNA were transfected with siDBC1#1 or siNS and treated or untreated with E2 as in A. Protein levels were monitored by immunoblot using the indicated antibodies. The indicated mRNA levels were determined by real time qRT–PCR as in A. (**D**) MCF-7 cells infected with lentiviruses encoding a NS or DBC1 shRNA were cultured in the absence or presence of 10 nM E2 for 8 days. Cell viability was determined by MTT assay. Data are means ± SD ($n = 6$). *P*-value was determined by Student's *t*-test.

(Supplementary Figure S3F). ChIP experiments with sequential immunoprecipitation (ChIP and ReIP) demonstrated that DBC1 and CCAR1 exist in the same complex on the pS2 promoter (Figure 3B). Thus, E2 induces DBC1–CCAR1 complex formation on the pS2 promoter. However, on ER binding sites associated with the PgR gene, little or no recruitment of CCAR1 and DBC1 was detected under the same condition, whereas ERα, AIB1 and CoCoA were recruited to the ER binding sites associated with the PgR gene (Figure 3A). Only background signals were observed with or without E2 treatment for binding of the same proteins to an irrelevant site 1 kb downstream from the transcription start site of the PgR gene. These results are consistent with the data

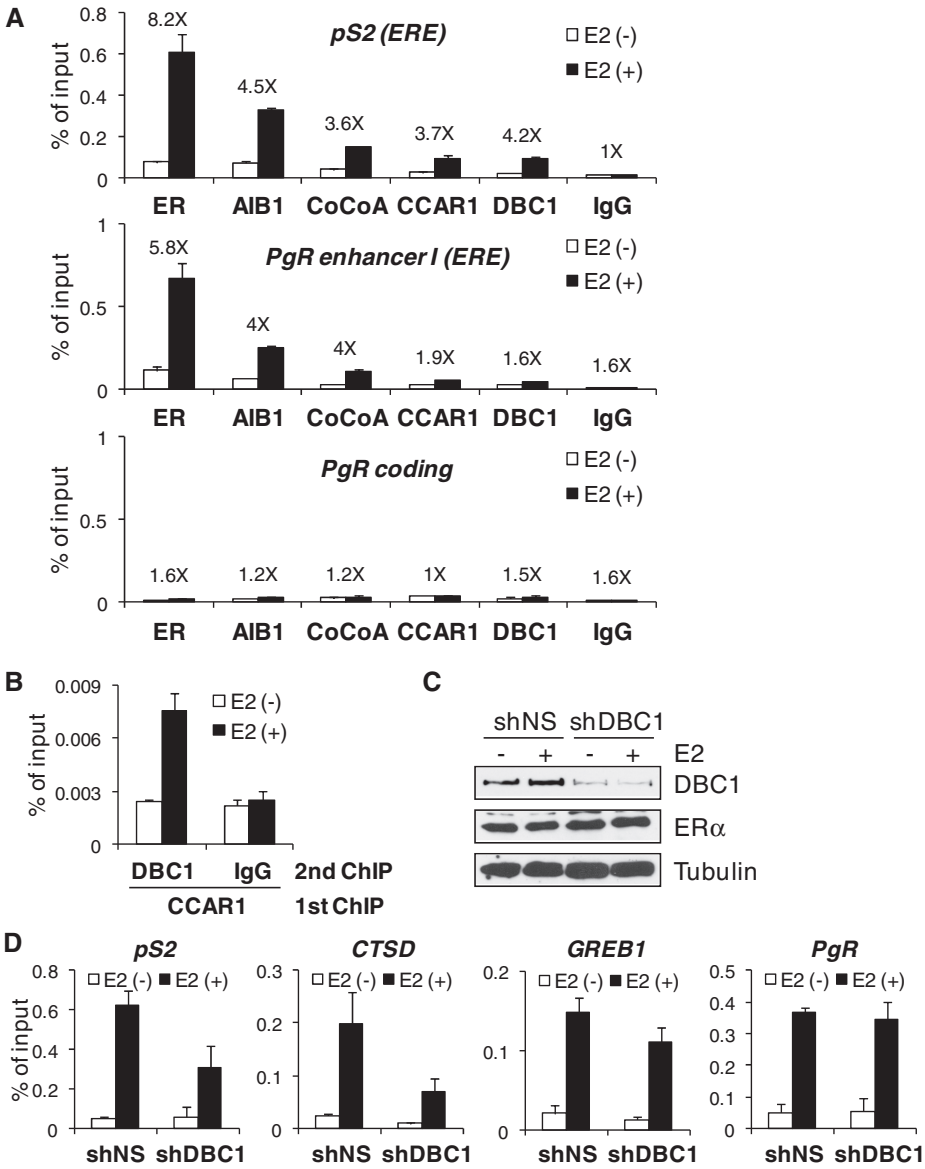

Figure 3. Recruitment of DBC1 to selective ERα target genes and requirement of DBC1 for optimal recruitment of ERα to the target promoters. (**A**) ChIP assay. Crosslinked, sheared chromatin from MCF-7 cells treated with or without 100 nM E2 (45 min) was immunoprecipitated with the indicated antibodies. qPCR analyses were performed using primers specific for the indicated promoters. The results are shown as percentage of input and are means ± SD ($n = 3$). Numbers above the bars indicate fold increase in the promoter occupancy with E2 treatment. (**B**) ChIP and ReIP assays, using the indicated antibodies, were performed as described in A. (**C** and **D**) MCF-7 cells infected with lentiviruses encoding a NS or DBC1 shRNA were treated with 100 nM E2 for 45 min. Protein levels were monitored by immunoblot using the indicated antibodies. ChIP assays using ERα antibody were performed as described in A.

that DBC1 and CCAR1 did not significantly affect PgR gene expression (Figure 2C) and strongly suggest that DBC1 and CCAR1 are directly involved in E2 induction of a specific subset of ERα target gene expression.

To further test the role of DBC1 as an ERα co-activator, we examined the effect of DBC1 depletion on the recruitment of ERα to its target promoters. Reduction of DBC1 levels by shRNA had no measurable effect on the cellular levels of ERα (Figure 3C). However, depletion of DBC1 substantially compromised the E2-dependent recruitment of ERα to the promoters of target genes pS2, CTSD and GREB1 (Figure 3D). Interestingly, DBC1 depletion had no effect on the recruitment of ERα to the PgR enhancer. Thus, DBC1 is required for optimal association of ERα with the target promoters, and ERα occupancy at the PgR enhancer bypasses the requirement of DBC1.

DBC1 inhibits SIRT1-mediated deacetylation of ERα and repression of ERα activity

ERα is acetylated by p300, and the acetylation enhances DNA binding and transactivation activities of ERα (8). SIRT1, an NAD-dependent protein deacetylase, has been shown to deacetylate ERα (8). Furthermore, recent

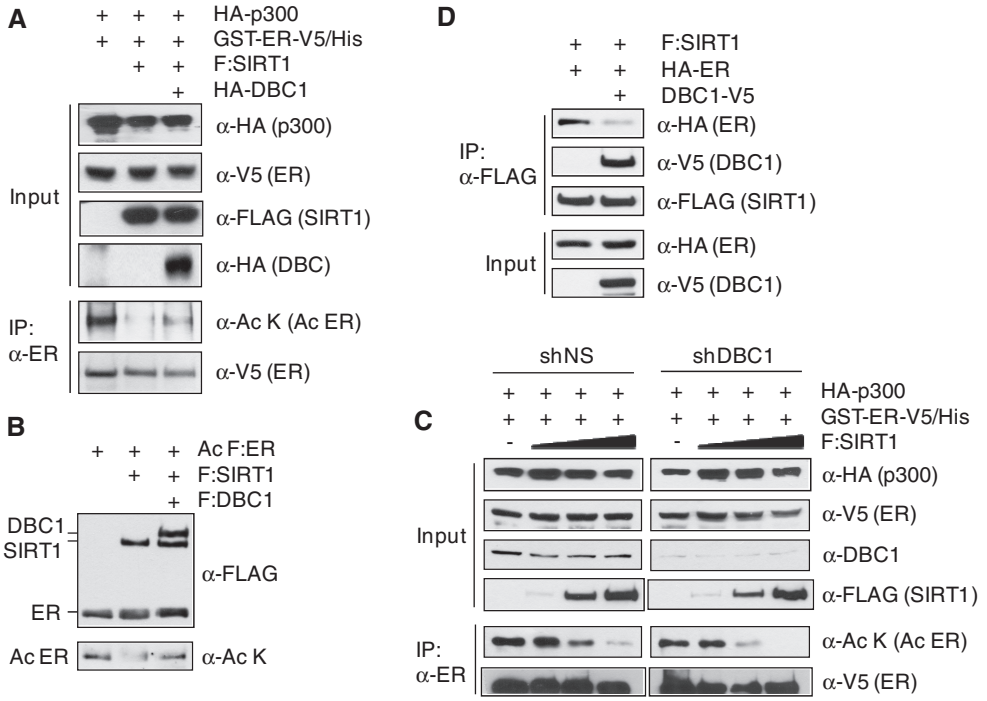

Figure 4. DBC1 inhibits SIRT1-mediated deacetylation of ERα. (**A**) 293 T cells were transfected with expression vectors as indicated. After 36 h transfection, the cells were treated with 0.5 μM TSA (Sigma) and 100 nM E2 for 12 h. Cell extracts were immunoprecipitated with anti-ERα antibody, and levels of acetylated ERα were determined by immunoblot using anti-acetyl lysine antibody. Input and immunoprecipitated proteins were analyzed by immunoblot with the indicated antibodies. (**B**) Acetylated ERα was incubated with purified SIRT1 and DBC1 in the presence of 1 mM NAD+ as indicated. Immunoblots were performed as described in A. (**C**) 293 T cells infected with lentiviruses encoding a NS or DBC1 shRNA were transfected with expression vectors as indicated and treated with TSA and E2 as described in A. Immunoprecipitation and immunoblots were performed as described in A. (**D**) Competition between DBC1 and SIRT1 for ERα binding. 293 T cells were transfected with expression plasmids as indicated and treated with E2 as described in A. FLAG-SIRT1 immunoprecipitates were analyzed by immunoblot using antibodies as indicated.

studies reported that DBC1 binds to SIRT1 and inhibits its deacetylase activity (11,12). We first examined the effect of DBC1 expression on ERα deacetylation by SIRT1 *in vivo*. 293 T cells were transfected with plasmids expressing GST-ER, HA-p300 and FLAG-SIRT1, and acetylated levels of ERα were determined after immunoprecipitation of ERα by immunoblots using anti-acetylated lysine antibodies. As reported previously (8), the expression of p300 strongly increased ERα acetylation, and co-expression of SIRT1 dramatically reduced the acetylated level of ERα (Supplementary Figure S4A and Figure 4A). However, the deacetylation of ERα by SIRT1 was partially reversed by DBC1 expression (Figure 4A). Similar results were observed in deacetylation assays *in vitro* using purified acetylated ERα, SIRT1 and DBC1 (Figure 4B). To further confirm the inhibitory role of DBC1 in SIRT1-mediated deacetylation of ERα, endogenous DBC1 expression was reduced by RNAi. As expected, the levels of acetylated ERα were reduced by SIRT1 expression in a dose-dependent manner, and acetylated ERα became more sensitive to deacetylation by SIRT1 in DBC1-depleted cells (Figure 4C). These results strongly support the role of endogenous DBC1 in inhibiting the deacetylase activity of SIRT1 for ERα. In CoIP experiments, SIRT1 interacted with ERα (Supplementary Figure S4B), and SIRT1 binding to ERα was decreased when DBC1 was co-expressed (Figure 4D). Similar results were observed in GST pull-down assays (Supplementary Figure S4C). These results indicate that DBC1 inhibits SIRT1 activity, at least in part, by blocking the interaction between ERα and SIRT1.

To assess further the roles of DBC1 and SIRT1 in ERα activity, we determined the effects of SIRT1 on the transcriptional activity of ERα. In ERα reporter gene assays, SIRT1, but not SIRT6 or SIRT7 (other nuclear sirtuins), strongly repressed the transcriptional activity of ERα in a dose-dependent manner (Figure 5A). Interestingly, a catalytic mutant of SIRT1 (19), H363Y, also repressed ERα activity, although slightly less efficiently than wild-type SIRT1 (Supplementary Figure S4D). EX-527, a SIRT1-specific inhibitor, partially rescued ERα activity from SIRT1-mediated repression (Figure 5B). Thus the repression of ERα by SIRT1 involves its deacetylase activity but also appears to require other aspects of SIRT1 function, such as protein–protein interactions. DBC1 also blocked the inhibitory effect of SIRT1 on ERα activity in a dose-dependent manner (Figure 5C). In contrast, DBC1ΔLZ (lacking the leucine zipper region, residues 250–257), which fails to bind SIRT1 (11) (Supplementary Figure S4E), failed to rescue the transcriptional activity of ERα (Figure 5C). In addition, the DBC1ΔLZ mutant showed neither co-activator activity nor synergy with CCAR1 (Figure 5D). These results indicate that DBC1 inhibits SIRT1 activity through

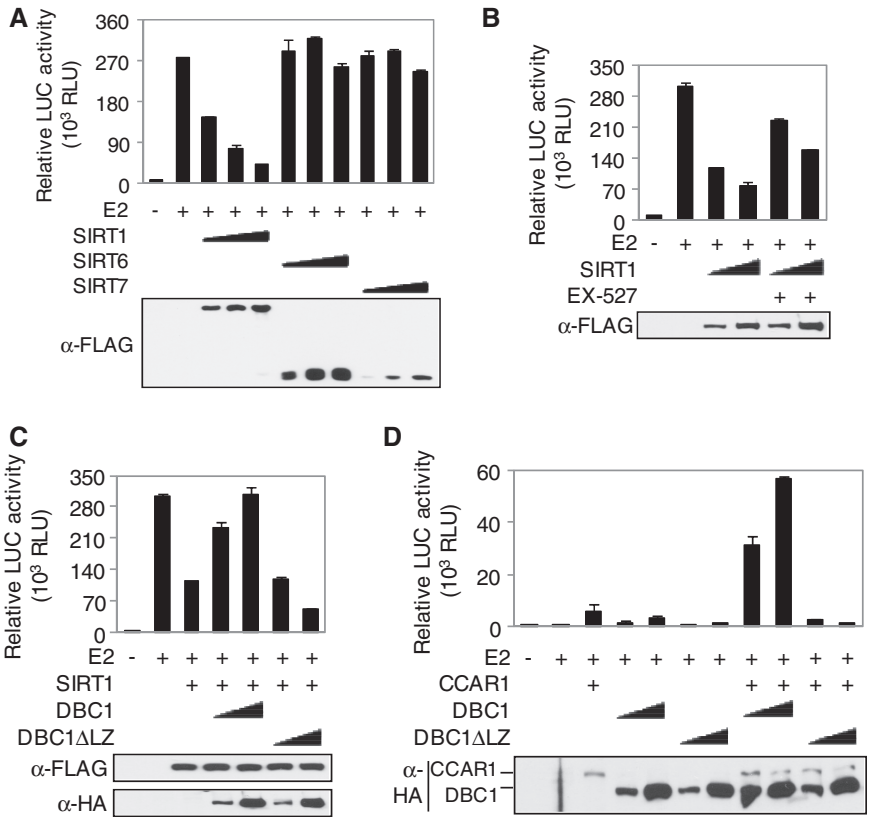

Figure 5. DBC1 reverses SIRT1-mediated repression of ERα. (**A**) CV-1 cells were transfected with pHE0 (2 ng) and MMTV(ERE)-LUC reporter (200 ng) in combination with various amounts (10, 25 and 50 ng) of expression vectors for FLAG-tagged nuclear sirtuins and grown in medium containing or lacking 100 nM E2. Luciferase activity was measured 48 h after transfection. Expression levels of transfected sirtuins were analyzed by immunoblot with anti-FLAG antibody. (**B**) CV-1 cells were transfected with pHE0, MMTV(ERE)-LUC reporter, and FLAG-SIRT1 (25 and 50 ng) as in A and grown in medium containing or lacking 100 nM E2 and 5 μM EX-527 as indicated before measuring luciferase activity. Expression levels of transfected SIRT1 were analyzed by immunoblot with anti-FLAG antibody. (**C**) DBC1 rescues SIRT1-mediated repression of ERα. CV-1 cells were transfected with MMTV(ERE)-LUC reporter and pHE0 as in A in combination with expression vectors for FLAG-SIRT1 (50 ng) and HA-DBC1 or HA-DBC1ΔLZ (300 and 600 ng) as indicated before measuring luciferase activity. Expression levels of transfected SIRT1 and DBC1 were analyzed by immunoblot with anti-FLAG and anti-HA antibody, respectively. (**D**) Transient transfections using MMTV(ERE)-LUC and expression vectors for ERα, FLAG-SIRT1, HA-DBC1 or HA-DBC1ΔLZ and HA-CCAR1 (200 ng) were performed as described in **C**. Expression levels of transfected CCAR1 and DBC1 were analyzed by immunoblot with anti-HA antibody.

direct interaction with SIRT1 and that the DBC1 LZ motif, the SIRT1-binding region, is also required for its co-activator function and the cooperation with CCAR1.

SIRT1 represses the co-activator synergy between DBC1 and CCAR1 by competing with CCAR1 for binding to DBC1

Because the LZ motif of DBC1 is important for its co-activator function and synergy with CCAR1, we next examined the ability of DBC1ΔLZ mutant to interact with CCAR1 (Figure 6A). CCAR1 interacted with DBC1 *in vitro*, but not with DBC1ΔLZ mutant, indicating that the LZ motif of DBC1 is required for the interaction not only with SIRT1 but also with CCAR1. These results led us to further test whether SIRT1 and CCAR1 compete to bind to DBC1. Indeed, expression of SIRT1 (either WT or H363Y mutant) dramatically reduced the interaction between CCAR1 and DBC1 in CoIP experiments (Figure 6B). We next addressed whether the synergy between CCAR1 and DBC1 is regulated by SIRT1. Again, CCAR1 and DBC1 synergistically enhanced the transcriptional activity of ERα in reporter gene assays, but this synergistic cooperation was strongly inhibited by SIRT1 in a deacetylase activity-independent manner (Figure 6C). These results suggest that SIRT1 negatively regulates the synergy between CCAR1 and DBC1 by interfering with the interaction between CCAR1 and DBC1.

DISCUSSION

Transcriptional regulation by DBC1

Transcriptional regulation is a dynamic process involving association and dissociation of transcription factors with gene-specific co-regulators at the target gene promoters and post-translational modifications of components of chromatin and the transcription complexes. In this study, we identified DBC1 as a CCAR1-interacting protein, and our results demonstrate that DBC1 functions as an ERα co-activator. DBC1 enhanced the transcriptional activity of ERα and cooperated synergistically with CCAR1 (Figure 1), and depletion of DBC1 caused

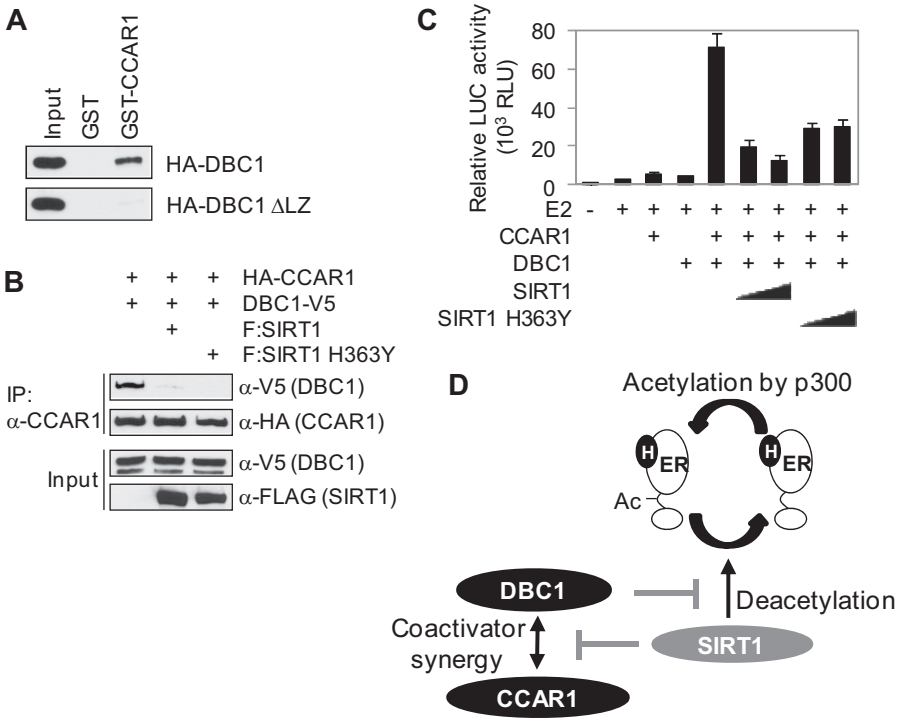

Figure 6. SIRT1 represses the co-activator synergy between DBC1 and CCAR1. (**A**) The LZ motif of DBC1 is required for DBC1-CCAR1 interaction. *In vitro* translated HA-DBC1 (WT or ΔLZ mutant) was incubated with GST or GST-CCAR1, and bound proteins were analyzed by immunoblot with anti-HA antibody. (**B**) SIRT1 blocks the interaction between DBC1 and CCAR1. 293T cells were transfected with expression plasmids as indicated. HA-CCAR1 immunoprecipitates were analyzed by immunoblot using antibodies as indicated. (**C**) Transient transfections using MMTV(ERE)-LUC and expression vectors for ERα, HA-CCAR1, HA-DBC1 and FLAG-SIRT1 (WT or H363Y mutant, 25 and 50 ng) were performed as described in Figure 1E. (**D**) The proposed reciprocal roles of DBC1 and SIRT1 in ERα-mediated transcription and co-activator synergy. DBC1 has a dual function in regulating ERα activity: first as a co-activator by cooperating synergistically with CCAR1, and second as a negative regulator of SIRT1 by inhibiting SIRT1-mediated ERα deacetylation, and thus increasing DNA binding and transcriptional activity of ERα. SIRT1 also has a dual role in regulating ERα activity. In addition to deacetylating ERα, SIRT1 disrupts the interaction between ERα co-activators, DBC1 and CCAR1, by competing with CCAR1 for DBC1 binding.

reduction in E2-induced expression of endogenous ERα target genes (Figure 2A). Furthermore, simultaneous depletion of DBC1 and CCAR1 caused further reduction of ERα target gene expression (Figure 2C), demonstrating the functional importance of the interaction between DBC1 and CCAR1. DBC1 is recruited to the promoters of native ERα target genes (Figure 3A and Supplementary Figure S3F), and depletion of DBC1 protein attenuated hormone-dependent growth and cell cycle progression of breast cancer cells (Figure 2D and Supplementary Figure S3E). These results firmly established DBC1 as an ERα co-activator that plays an important physiological role in ERα function in breast cancer cells. pS2 (also known as trefoil factor 1, TFF1), CTSD (cathepsin D) and GREB1 (gene regulated by estrogen in breast cancer 1) have been shown to promote tumor cell invasion, metastasis and proliferation (20–22). PgR (progesterone receptor), a member of NR superfamily, also plays a critical role in tumor initiation and proliferation (23). Interestingly, unlike p160 co-activators and CoCoA, no significant occupancy of DBC1 and CCAR1 was observed on the estrogen-dependent enhancers of the PgR gene (Figure 3A), which is consistent with the data that neither single nor double depletion of DBC1 and CCAR1 affected PgR gene expression (Figure 2C). These findings suggest that DBC1 and CCAR1 regulate the estrogen-induced expression of a subset, but not all, of ERα target genes. Although the precise mechanism underlying gene-specific recruitment of DBC1 and CCAR1 is not fully understood, one intriguing possibility is that sequences surrounding target ERα binding sites determine DBC1 and CCAR1 recruitment to estrogen-regulated promoters. Recently, cooperating transcription factors such as FoxA1, Oct-1, C/EBPα have been reported to play an important role in regulating ERα-dependent transcription (24). The binding sites of these factors are highly enriched near ERα binding sites, thus it is possible that they may contribute to selective recruitment of co-activators to ERα binding sites. Further study will be needed to define the exact mechanism of gene-specific requirement for DBC1 and CCAR1 in ERα-mediated transcription.

It should be noted that a previous study showed that DBC1 preferentially bound to and modulates the steady-state level of unliganded but not liganded ERα protein (17). However, in this report, we showed that DBC1 interacts with ERα both in the presence and absence of E2 (Supplementary Figure S1F and S1H) and that DBC1 depletion using two different siRNA or shRNA against DBC1 did not affect the level of

either unliganded or liganded ERα (Figure 3C and Supplementary Figure S5). The reason for these conflicting results is currently unknown. However, consistent with our results, recent studies have also shown that DBC1 functions as a co-activator for NRs. DBC1 was identified as an AR-interacting protein through a biochemical approach (18). The interaction between DBC1 and AR enhances the DNA binding activity of AR, thereby enhancing the transcriptional activity of AR. DBC1 was also found in NIF-1 (NRC interacting factor 1) complexes (25). As a component of NIF-1 complex, DBC1 functions as a co-activator for RARα. In other studies, DBC1 was found to be involved in transcriptional repression. DBC1 associates with COUP–TFI complex, stabilizes the interaction between NCoR and COUP-TFI, and thereby contributes to COUP–TFI-mediated transcriptional repression (26). DBC1 was also found to interact with BRCA1 and repress the transcriptional activity of BRCA1 (27). Another study reported that DBC1 suppresses the ligand-dependent transcriptional activation function of ERβ (28). Thus, the roles of DBC1 in transcriptional regulation, positive and negative, are likely to be very complex. It will be interesting to test whether the mechanisms described in our study for DBC1 regulation of ERα (interaction of DBC1 with CCAR1 or with SIRT1, or the inhibition of NR deacetylation by SIRT1) are responsible for the effects of DBC1 on the actions of other NRs described above.

Reciprocal roles of DBC1 and SIRT1 in transcriptional regulation

Acetylation of ERα enhances its DNA binding and transcriptional activity and is reversed by the native cellular deacetylases (8), suggesting reversible acetylation as a modulator of ERα activity. Our study provides evidence for an ERα regulatory pathway controlled reciprocally by SIRT1 and DBC1. We showed that SIRT1 binds and inhibits the transcriptional activity of ERα by regulating acetylation of ERα (Figures 4 and 5). The ability of SIRT1 to deacetylate and repress ERα activity mechanistically resembles the effects of SIRT1 on p53, FOXO, PGC1α and AR (29), suggesting that SIRT1 plays a broad inhibitory role in regulating the activity of various transcription factors. Thus, one mechanism of the co-repressor function of SIRT1 is to deacetylate and thereby inactivate ERα and other transcription factors (Figure 6D). However, and importantly, overexpression of SIRT1 H363Y mutant still effected a significant repression of ERα-mediated transcription (Supplementary Figure S4D), which suggests that SIRT1 may have another mechanism of action in addition to deacetylating ERα. A recent study showed that the deacetylase activity of SIRT1 may not be responsible for all of the functions of SIRT1, suggesting that, in some cases, the interaction of SIRT1 with other proteins may be also important for its function (19). In this regard, the present study showed that another mechanism of the inhibitory function of SIRT1 is to block the interaction between co-activators (Figure 6D). Our work demonstrates that co-activator synergy between DBC1 and CCAR1 can be regulated through a protein–protein interaction (Figure 6). SIRT1 competes with CCAR1 for binding to the LZ motif of DBC1. Consequently, the interaction between DBC1 and CCAR1 is disrupted by SIRT1, which results in repression of their synergy in transcriptional activation by ERα. Thus, our results reveal that SIRT1 is a critical regulator, which negatively regulates physical interaction and functional synergy between DBC1 and CCAR1, and suggest that SIRT1 can regulate ERα function indirectly through disrupting co-activator interaction in addition to acting directly through an ERα deacetylation-dependent mechanism (Figure 6D).

Recent studies identified DBC1 as a regulator of several proteins. DBC1 interacts with and functions as a negative regulator of SIRT1 (11,12), SUV39H1 (30) and HDAC3 (31). As a SIRT1 inhibitor, DBC1 binds to the SIRT1 catalytic domain and inhibits p53 deacetylation by SIRT1, leading to upregulation of p53 function and increase of p53-mediated apoptosis induced by genotoxic stress. DBC1 also binds to catalytic domains of SUV39H1 and HDAC3 and inhibits their histone methyltransferase and deacetylase activities. Thus, DBC1 may be an important regulator of a wide variety of cellular processes including gene expression, chromatin remodeling, cell proliferation, apoptosis and metabolism. Here, we showed several lines of evidence that DBC1 negatively regulates SIRT1-mediated deacetylation and repression of ERα. SIRT1-mediated ERα deacetylation was inhibited by DBC1 overexpression but stimulated by DBC1 knockdown (Figure 4). DBC1 repressed SIRT1 activity through blocking the interaction between SIRT1 and its substrate ERα (Figure 4D and Supplementary Figure S4C), and the SIRT1-mediated repression of ERα was reversed by DBC1 expression (Figure 5C). In addition, our ChIP analyses revealed that DBC1 is required for efficient recruitment of ERα to its target promoters (Figure 3D), probably through inhibiting SIRT1-mediated deacetylation of ERα. These results are consistent with the previous report that acetylation of ERα enhances its DNA binding activity (8). It is also possible that ERα binds to its target DNA more efficiently when complexed with DBC1. Importantly, our previous ChIP analyses demonstrated that the inhibition of CCAR1 recruitment to the pS2 promoter had no observable effect on recruitment of ERα (5). These results indicate that, despite the sequence similarity between DBC1 and CCAR1, these two co-activators likely have distinct functions. In addition, the fact that their cooperation for ERα-mediated transcription is not additive but synergistic suggests that each co-activator makes a distinct contribution to the transcriptional activation process: CCAR1 contributes to transcriptional activation by coordinating actions of p160 and Mediator complexes; DBC1 enhances the DNA binding activity of ERα by regulating the acetylation state of ERα. Consistent with our observations, a recent study reported that DBC1 acts as an AR co-activator and is required for efficient binding of AR to the target promoters (18). It will be interesting to test whether DBC1 enhances AR DNA binding activity by inhibiting SIRT1-mediated deacetylation of AR.

Interestingly, during the preparation of this article, it was reported that over 70% of breast cancer tissues

express a high level of DBC1 and that DBC1 expression is significantly associated with short overall and relapse-free survival (13). These results support our findings that DBC1 plays a critical role in the regulation of ERα and SIRT1 function and hormone-dependent growth of breast cancer cells. However, the same report also showed that DBC1 expression was positively correlated with SIRT1 expression. Although the underlying mechanism and significance for this correlation is currently unknown, it is possible that cells express more SIRT1 to balance or better control the DBC1 function when the DBC1 function becomes higher. Consistent with this idea, SIRT1 expression is transcriptionally induced by E2F1 (32), which is known as one of the ERα target genes (33). Also of note is that resveratrol, a SIRT1 activator, has been shown to inhibit estrogen-dependent growth of breast cancer cells (34). Taken together, with the fact that ERα signaling is oncogenic to the breast, it is likely that upregulation of DBC1 could lead to an enhanced ERα function, thus accelerating the development and progression of breast cancer. In conclusion, our findings support and extend previous observations that DBC1 is a negative regulator of SIRT1, establish reciprocal roles of DBC1 and SIRT1 in regulating ERα function, and provide new insight into the regulatory mechanism of co-activator synergy. It would be interesting to determine whether DBC1 and SIRT1 reciprocally regulate the transcriptional activity of other NRs and co-activator synergy through a similar mechanism.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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